DEVELOPMENT OF MITIGATION STRATEGIES TOWARD PREVENTATIVE POSTURES IN FOOD DEFENSE

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ABSTRACT

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Food defense is a multidisciplinary study. The body of this work is divided into a case control study on terrorism organizations and the development of a food based testing platform for detection of food based terrorism agents. To begin, few terrorism studies have explored the factors that distinguish groups that will use Chemical, Biological, Radiological or Nuclear (CBRN) terrorism involving the food supply. Ethno-separatist ideology, increased cultural embeddedness within the global culture, increased connectedness to other organizations and democratic regime type all show significant results as predictors of food based CBRN attacks by an organization ($p = 0.1$). Food defense requires the means to efficiently screen large volumes of food for microbial pathogens. Even rapid detection methods often require lengthy enrichment steps, making them impractical for this application. There is a great need for rapid, sensitive, specific, and inexpensive methods for extracting and concentrating microbial pathogens from food. A carbohydrate coated screen printed carbon electrode (D-FSPCE) was evaluated as a sensitive platform for multiplex evaluation of food samples extracted by immuno-magnetic separation (IMS) with electrically active magnetic nano-particles (EAMNPs). These nanoparticles provide the selectivity of the biosensor through their attached monoclonal antibody (Mab) while the carbohydrate coated chips provide the nonselective, shelf stable electrical detection platform. When combined, the D-FSPCE + Mab-EAMNP, using cyclic voltammetry for an electrical readout, are named the M^3 Biosensor. In this body of work, methodology was optimized for *Escherichia coli* O157:H7. The analytical specificity of the 40 minute IMS method was improved over previous protocols by addition of sodium chloride and a higher

concentration of antibodies (1.0 mg/mL) during the conjugation of antibodies onto MNPs. EAMNP concentrations of 1.0 mg/mL and 0.5 mg/mL provided optimal analytical sensitivity and analytical specificity as potential concentrations for the evaluation of food substances with no statistical difference between them. Antibody-conjugated EAMNPs show no decline in performance up to 149 days after conjugation with a capture efficiency of 92% all the way down to 5 CFU/mL; equivilant to a widely used commercial IMS methodolgy. The EAMNP portion of the M^3 Biosensor can also capture and detect bacterial cells down to 1-4 MPN/mL from 200 mL of whole fluid milk in 1 hour, without pre-enrichment. The extraction protocol's inclusivity within strain is 94% and exclusivity outside the *E. coli* O157 family is 87%. The second half of the M^3 Biosensor is the D-FSPCE and statistically significant qualitative (presence/absence) differentiation ($p = 0.0015$, $n = 188$) can be performed from broth from 100 CFU/mL to 1.0 $*10^8$ CFU/mL. Additionally, 39 organisms of 10 bacterial genera in both gram stain groupings all attached to the carbohydrate coated D-FSPCE, allowing for this platform to be used with many other organisms. M^3 Biosensor has electrical chemical detection with statistically significant differences as low as 5 CFU/mL and a signal to noise ratio of 2:1, in broth. A linear range of 5 CFU/mL to 1.0 $*10^8$ CFU/mL for both IMS analysis and CV analysis is excellent performance through over 200 repeat analyses at a cost for one sample of ~ \$0.43. The M^3 Biosensor can be used to detect bacterial contamination in broth without a pre-enrichment and is an inexpensive, field stable platform, with excellent multiplex capabilities in a wide variety of detection modalities.

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TABLE OF CONTENTS

LIST OF TABLES xi

LIST OF FIGURES xiv

LIST OF EQUATIONS xxiv

CHAPTER 1 Food Defense: An Introduction to the Principles Necessary to Move From Reaction to Prevention for Use of Food CBRN Terrorism Agents 1
Introduction 2
Part I 2
Victim 9
The Perpetrator 12
Capable Guardian 19
Access control 20
Surveillance – LEI: 22
Surveillance – monitoring food 27
Responses to Food CBRN terrorism 30
Part II. 31
REFERENCES 42

CHAPTER 2: CBRN Terrorism: A Critical Quantitative Analysis of Group Components Necessary to Predict Use of Food CBRN Terrorism Agents 50
Abstract 51
Introduction 52
Background 54
Organizational capacity 58
Strategic connectivity 62
Motivational 63
Data and Methodology 66
Organizational capacity factors 69
Strategic connectivity factor 71
Motivational factors 71
Statistical evaluation 74
Results 75
Discussion 80
Conclusions and future direction 83
REFERENCES 86

CHAPTER 3: Optimization of Electrically Active Magnetic Nanoparticles as Accurate and Efficient Microbial Extraction Tools 89
Abstract 90

Introduction 91
Experimental Design 94
Materials and Methods 96
EAMNP production 96
EAMNP Antibody Conjugation 97
Immuno-magnetic Separation (IMS) and Plating of Bacteria 99
Statistical Analysis 102
Results 103
Hypothesis 1a: Effect of sodium chloride addition during conjugation 103
Hypothesis 1b: Effect of Antibody Concentration during Conjugation 104
Hypothesis 1c: Effect of Mab-EAMNP Concentration during IMS 106
Hypothesis 2: Effect of Age of Mab-EAMNP Solution during IMS 107
Discussion 109
Conclusions and Limitations 112
REFERENCES 115

CHAPTER 4: Culture Age on Evaluation of Electrically Active Magnetic Nanoparticles as Accurate and Efficient Microbial Extraction Tools 119
Abstract 120
Introduction 121
Experimental design 124
Materials and Methods 124
EAMNP Production 125
EAMNP Antibody Conjugation 126
Immuno-magnetic Separation (IMS) and Plating of Bacteria 128
Statistical Analysis 130
Results 131
Discussion 133
Conclusions and Limitations 134
Appendix 4.A: Bacterial Growth Curves with Varying Inoculums 137
REFERENCES 144

CHAPTER 5: Evaluation of the Limits of Detection for Electrically Active Magnetic Nanoparticles as Accurate and Efficient Microbial Extraction Tools 146
Abstract 147
Introduction 148
Experimental Design 150
Materials and Methods 150
EAMNP Production 151
EAMNP Antibody Conjugation 152
Immuno-magnetic Separation (IMS) and Plating of Bacteria 154
Results 157
Discussion 161
Conclusions and Limitations 163
REFERENCES 166

CHAPTER 6: Inclusivity and Exclusivity Evaluation of Electrically Active Magnetic Nanoparticles as Accurate and Efficient Microbial Extraction Tools 169
Abstract 170
Introduction 171
Experimental design 172
Materials and Methods 173
EAMNP Production 174
EAMNP Antibody Conjugation 176
Immuno-magnetic Separation (IMS) and Plating of Bacteria 177
Western Blot 179
Statistical analysis 180
Results 181
Discussion 185
Conclusions and Limitations 188
Appendix 6.A: Western Blot Gel Analysis of Selected Antibodies against the Inclusivity and Exclusivity Panel of Bacteria 191
Appendix 6.B: Detailed information on bacteria in the Inclusivity and Exclusivity Panel ... 210
Appendix 6.B (cont'd) 211
Appendix 6.B (cont'd) 212
Appendix 6.B (cont'd) 213
Appendix 6.B (cont'd) 214
REFERENCES 216

CHAPTER 7: Development of a Universal, Shelf Stable, Electrochemical Detection Platform for use in the M^3 Biosensor 218
Abstract 219
Introduction 220
Experimental design 223
Materials and methods: 225
EAMNP Production 225
EAMNP Antibody Conjugation 226
Immuno-magnetic Separation (IMS) and Plating of Bacteria 227
Synthesis of aminated α D + Mannose 229
Functionalization of the SPCE 232
Cyclic Voltammetry evaluation of D-FSPCEs: 234
Statistical Analysis 236
Results 236
Hypothesis 1 236
Hypothesis 2 238
Hypothesis 3 242
Discussion 244
Appendix 7.A: NMR spectra of Animated D + Mannose production 247
REFERENCES 258

CHAPTER 8: Validation of the M^3 Biosensor in Broth 260
Abstract 261

Introduction 262
Experimental design 265
Materials and Methods 266
EAMNP Production 267
EAMNP Antibody Conjugation 268
Immuno-magnetic Separation (IMS) and Plating of Bacteria 269
Synthesis of aminated α D + Mannose 271
Functionalization of the SPCE 272
Cyclic Voltammetry evaluation of D-F SPCEs: 274
Statistical Analysis 277
Results 277
Hypothesis 1: 277
Hypothesis 2 285
Discussion 286
Conclusions and Limitations 289
REFERENCES 292

CHAPTER 9: Proof of Concept for the M^3 Biosensor in Whole Fluid Milk 294
Abstract 295
Introduction 296
Experimental Design 298
Materials and methods 299
Bacterial culture preparation 299
Milk Sample preparation 300
EAMNP production 302
EAMNP Antibody Conjugation 302
EAMNP Immuno-magnetic Separation (IMS) in Milk 304
Enumeration and confirmation of extracted bacteria 308
Synthesis of aminated α D + Mannose 310
Functionalization of the SPCE 311
Cyclic Voltammetry evaluation of D-F SPCEs: 313
FDA BAM Screening for serotype *E. coli* O157:H7 from foods – Gold Standard 314
Most Probable Number (MPN) quantization of spike rate 315
Confirmation Wellcolux® Agglutination kits 316
Confirmation JBAIDS 316
Confirmation QPCR and 2% agarose gel MSU 319
Quality control testing 320
Statistical analysis 322
Results 322
Experiment 1 323
Experiment 2 328
Quality control 329
QPCR Results experiment # 2 333
Experiment #3 344
Overall cumulative results of the M^3 Biosensor 347

Discussion 348
Experiment # 1 348
Experiment #2 350
Experiment #3 355
Compiled Results of Experiments 1-3. 355
REFERENCES 358

LIST OF TABLES

Table 1.1: Receptor based classification of biosensor systems. 36

Table 1.2: Material based classification of biosensor systems. 37

Table 1.3: Signal based classification of biosensor systems..... 37

Table 2.1**:** Organizations that used CBRN terrorism in food 1992–2005 67

Table 2.2: Organizations that use/pursued CBRN terrorism, non-food 1992–2005..... 68

Table 2.3: Demographics and summary statistics of the variables in each model. 72

Table 2.3 (cont'd)..... 73

Table 2.4: Model 1. Univariate and Multivariable analysis for Model 1: Food CBRN organizations verse all other active terrorist organizations in the time frame and Model 2: Food CBRN organizations verse other non-CBRN terrorist organizations. (* denotes models with 1 degree of freedom, ** denotes models with 2 degree of freedom). 75

Table 2.4 (cont'd)..... 76

Table 2.4 (cont'd)..... 77

Table 2.5: Final Binary logistical regression results of Models 1 and 2. (*denotes models with 1 degree of freedom, ** denotes models with 2 degree of freedom)..... 79

Table 2.5: (cont'd) 80

Table 6.1: O157 Agglutination reaction 182

Table 6.2: Results of Reveal 1.0 and Reveal 2.0 testing..... 183

Table 6.3: MLS Mab; Inclusivity and exclusivity by culture or Western blot of IMS separated *E. coli* O157:H7 versus non-*E. coli* O157:H7..... 184

Table 6.4: KPL Mab; Inclusivity and exclusivity by culture or Western blot of IMS separated *E. coli* O157:H7 versus non-*E. coli* O157:H7..... 184

Table 6.5: MLS Mab; Inclusivity and exclusivity by culture or Western blot of IMS separated *E. coli* O157 versus non-*E. coli* O157..... 185

Table 6.6: KPL Mab; Inclusivity and exclusivity by culture or Western blot of IMS separated *E. coli* O157 versus non-*E. coli* O157..... 185

Table 6.A.1: Lane identification key for Gel #1 in Figure 6.A.1. MLS 12-22-10 A..... 191

Table 6.A.2: Lane identification key for Gel #2 in Figure 6.A.2. MLS 12-22-10 B..... 192

Table 6.A.3: Lane identification key for Gel #2 in Figure 6.A.3. MLS 01-05-11 A..... 193

Table 6.A.4: Lane identification key for Gel #4 in Figure 6.A.4. MLS 01-05-11 B..... 194

Table 6.A.5: Lane identification key for Gel #5 in Figure 6.A.5. MLS 05-10-11 A..... 195

Table 6.A.6: Lane identification key for Gel #6 in Figure 6.A.6. MLS 05-10-11 B..... 196

Table 6.A.7: Lane identification key for Gel #7 in Figure 6.A.7. MLS 5-11-11 B..... 197

Table 6.A.8: Lane identification key for Gel #8 in Figure 6.A.8. MLS 5-12-11 A..... 198

Table 6.A.9: Lane identification key for Gel #9 in Figure 6.A.9. MLS 5-19-11 A..... 199

Table 6.A.10: Lane identification key for Gel #10 in Figure 6.A.10. MLS 6-9-11 A..... 200

Table 6.A.11: Lane identification key for Gel #11 in Figure 6.A.11. 201

Table 6.A.12: Lane identification key for Gel #12 in Figure 6.A.12. 202

Table 6.A.13: Lane identification key for Gel #13 in Figure A.13. 203

Table 6.A.14: Lane identification key for Gel #14 in Figure 6.A.14. 204

Table 6.A.15: Lane identification key for Gel #15 in Figure 6.A.15. 205

Table 6.A.16: Lane identification key for Gel #16 in Figure 6.A.16. 206

Table 6.A.17: Lane identification key for Gel #17 in Figure 6.A.17. 207

Table 6.A.18: Lane identification key for Gel #18 in Figure 6.A.18. 208

Table 6.A.19: Lane identification key for Gel #19 in Figure 6.A.19. 209

Table 7.1: Con A conjugated FITC evaluation of the biological activity of aminated D+ mannose on Mab-EAMNP captured *E. coli* O157:H7 Sakai strain..... 238

Table 8.1: Average resistance using cyclic voltammetry on M^3 Biosensor on captured *E. coli* O157:H7 Sakai, in broth. Descriptive statistics and independent T tests for blank verses each category of bacterial concentration. Italic show statistically significant comparisons. 282

Table 9.1: Lane designations for the two agarose gels in figure 9.12. 335

LIST OF FIGURES

Figure 1.1: Operation Risk Management Matrix. (Spink, 2009) For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation. 7

Figure 3.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes..... 100

Figure 3.2: Scanning electron micrograph of (a) an individual EAMNP with diameter of approximately 1.3 μm, and (b) Mab-EAMNP bound to an *E. coli* O157:H7 cell. 103

Figure 3.3: Mean concentration ($\log_{10}$ of CFU/mL) of each bacterial culture captured in IMS, using Mab-EAMNPs made with and without the addition of sodium chloride. Statistical comparisons were made within numbered groups (1-3), and letters (a or b) indicate significant differences ($\alpha = 0.05$, n = 178; p = 0.029). Zero counts were estimated to facilitate analysis. (Setterington, Cloutier, Ochoa, Cloutier, Alocilja, 2010)..... 104

Figure 3.4. Mean concentration ($\log_{10}$ of CFU/mL) of each bacterial culture captured in IMS, using Mab-EAMNPs made with 1.0 mg/mL antibody and with 0.5 mg/mL antibody. Statistical comparisons were made within numbered groups (1-3), and letters (a or b) indicate significant differences ($\alpha = 0.05$, n =178, p = 0.018). Zero counts were estimated to facilitate analysis. (Setterington et al., 2010)..... 105

Figure 3.5: Mean concentration ($\log_{10}$ of CFU/mL) of each bacterial culture captured in IMS, using Mab-EAMNPs, at concentrations of 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL. Statistical comparisons were made within numbered groups (1-3), and letters (a or b) indicate significant differences ($\alpha = 0.05$). Zero counts were estimated to facilitate analysis. (Setterington et al., 2010)..... 107

Figure 3.6: Capture efficiency ($\log_{10}$ of CFU/mL captured/ $\log_{10}$ of CFU/mL present) of each *E. coli* O157:H7 Sakai strain captured in IMS, using Mab-EAMNPs, at varying days from conjugation stored at refrigerated temperatures from 1-5 days (n = 37); 6-130 days (n = 6); 130 to 149 days (n = 12). Data is limited to bacterial concentrations in the linear range of consistent capture. There was no statistical difference between any of the three groups..... 109

Figure 4.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes..... 129

Figure 4.2*: E. coli* O157:H7 Sakai strain growth by hour of a 1 mL inoculum into 10 mL TSB. 131

Figure 4.3*: E. coli* O157:H7, Sakai strain, capture efficiency comparison by age of start culture. Statistical comparisons were made between groups and are shown in figure 4.4. 132

Figure 4.4*: E. coli* O157:H7, Sakai strain, mean capture efficiency comparison by age of start culture. Statistical comparisons were made between groups as a one way anova with a one tailed Dunnet's t-test. Groups were labeled (b) if they were statistically less than groups labeled (a). (3-10 hours vs. 11-18 hours, p=0.001; 3-10 hours vs. 19-24 hours, p = 0.014; $\alpha = 0.05$)..... 133

Figure 4.A.1: *E. coli* O157:H7 Sakai strain growth by hour of a 100 µL inoculum into 10 mL TSB. 137

Figure 4.A.2: *E. coli* O157:H7 Sakai strain growth by hour of a single colony inoculum into 10 mL TSB..... 137

Figure 4.A.3: *E. coli* O157:H7 Sakai strain growth by hour of a loop of ice crystal inoculum into 10 mL TSB..... 138

Figure 4.A.4: *E. coli* O55:H7 growth by hour of a 1 mL inoculum into 10 mL TSB. 138

Figure 4.A.5: *E. coli* 055:H7 growth by hour of a 100 µL inoculum into 10 mL TSB. 139

Figure 4.A.6: *E. coli* 055:H7 growth by hour of a single colony inoculum into 10 mL TSB. ... 139

Figure 4.A.7: *E. coli* 055:H7 growth by hour of a loop of ice crystal inoculum into 10 mL TSB. 140

Figure 4.A.8: *Shigella boydii* growth by hour of a 1 mL inoculum into 10 mL TSB. 140

Figure 4.A.9: *Shigella boydii* growth by hour of a 100 µL inoculum into 10 mL TSB. 141

Figure 4.A.10: *Shigella boydii* growth by hour of a single colony inoculum of 10 mL TSB. ... 141

Figure 4.A.11: *Shigella boydii* growth by hour of a loop of ice crystal inoculum into 10 mL TSB. 142

Figure 5.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes..... 155

Figure 5.2: Scanning electron micrograph of (a) an individual EAMNP with diameter of approximately 1.3 µm, and (b) Mab-EAMNP bound to an *E. coli* O157:H7 cell. 157

Figure 5.7: Capture Efficiency versus the $\log_{10}$ CFU/mLviable cells present in the start culture throughout the range of challenge concentrations. The culture age was held constant for these evaluations at 4-10 hours. 158

Figure 5.8: Capture Efficiency versus the $\log_{10}$ CFU/mL viable cells present in the start culture highlighting and expanding the lower range. Demonstrating the lower limists of detection of the EAMNPs extration modality of LOD = 1 CFU/mL and LOD_{50} = 6-9 CFU/mL. The culture age was not held constant for these evaluations. 159

Figure 5.9: The $\log_{10}$ CFU/mL of captured cells versus the $\log_{10}$ CFU/mL viable cells present in the start culture by culture evaluation, demonstrating the upper limit of detection for the EAMNP extraction of $1.0 * 10^9$ CFU/mL. The culture age was not held constant for these evaluations. 160

Figure 5.10: The average capture efficiency was consistently between 88% and 100% for the middle range of bacterial concentrations. This demostrates that the the functional range of consistent capture is between 6 CFU/mL and $1.0 * 10^9$ CFU/mL. Bacterial start composition concentrations were not held standard for this evaluation. 161

Figure 6.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes..... 178

Figure 6.A.1: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #1. MLS 12-22-10 A. 191

Figure 6.A.2: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #2. MLS 12-22-10 B 192

Figure 6.A.3: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #3. MLS 01-05-11 A 193

Figure 6.A.4: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #4. MLS 01-05-11 B 194

Figure 6.A.5: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #5. MLS 05-10-11 A 195

Figure 6.A.6: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #6. MLS 05-10-11 B. 196

Figure 6.A.7: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #7. MLS 5-11-11 B. 197

Figure 6.A.8: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #8. MLS 5-12-11 A. 198

Figure 6.A.9: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #9. MLS 5-19-11 A. 199

Figure 6.A.10: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #10. MLS 6-9-11 A. 200

Figure 6.A.11: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #11. KPL 5-11-11 A. 201

Figure 6.A.12: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #12. KPL 5-18-2011 A. 202

Figure 6.A.13: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #13. KPL 5-18-11 B 203

Figure 6.A.14: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #14. KPL 5-19-11. 204

Figure 6.A.15: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #15. KPL 5-23-2011 A. 205

Figure 6.A.16: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #16. KPL 5-23-2011 B. 206

Figure 6.A.17: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #17. KPL 6-2-11 A. 207

Figure 6.A.18: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #18. KPL 6-2-11 B. 208

Figure 6.A.19: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #19 KPL 6-9-11 B. 209

Figure 7.1: α-D-mannose was protected using acetic anhydride in pyridine with catalytic DMAP to achieve α-D-mannose pentaacetate. α-D-mannose pentaacetate was reacted with 3-chloropropanol and BF_3•OEt_2 in DCM overnight to give compound 1 shown in figure 7.1. Compound 2 was achieved by reacting compound 1 with sodium azide in DMF at 60°C overnight (El-Boubbou et al. 2007, Joosten, et al., 2004, Teumelsan & Huang, 2007). De-protection of the acetate groups was performed with freshly prepared sodium methoxide and produced compound 3. Upon reduction of azide under hydrogenation condition, the final aminated α-D-mannose, compound 4, was formed; IPAC chemical name: 3-α-Aminopropyl-D-mannopyranoside. 231

Figure 7.2: Schematic of the bare screen-printed carbon electrode (SPCE) sensor chip the detection is performed on, the circuit. 233

Figure 7.3: Layered schematic of the structure in the novel aminated D+ mannose functionalized screen printed carbon electrode (D-FSPCE) biosensor chip. The modifications are made to the center circle of the carbon electrode and HCL is used to connect the Ag/AgCl electrode during cyclic voltammetry. 234

Figure 7.4: Successive electron microscope evaluations of acridine orange and acridine orange stained *E. coli* O157:H7 Sakai strain on varying SPCEs through a 445nm long pass filter 40X lens. 1: Acridine orange on SPCE (Non specific stain attachment) 2: Plain SPCE – no block (Non specific cell attachment) 3: glutaraldehyde coated SPCE (Non specific cell attachment) 4: Blocked glutaraldehyde coated SPCE - w/o carbohydrate and 5: Blocked D-FSPCE w/carbohydrate..... 237

Figure 7.5: Blank D-FSPCE versus 29 bacterial organisms stained with acridine orange and captured on D-FSPCEs measured in Relative Fluorescence Units. (RFU) 240

Figure 7.6: Scanning electron micrographs of the carbohydrate coating on the surface of the D-FSPCE and four individual bacterial solutions present after incubation and rinsing. 1 and 2: Carbohydrate coating on D-FSPCE 3: *Bacillus anthracis* Sterne strain on D-FSPCE 4: *Vibrio fischeri on D-FSPCE.* 5: *E. coli* O157:H7 on D-FSPCE..... 241

Figure 7.7: A scanning electron micrograph of the carbohydrate coating on the surface of a D-FSPCE and a mixed solution of 4 bacteria present after incubation and rinsing..... 242

Figure 7.8: The average resistance change by $\log_{10}$ CFU/mL with a magnetic field under the D-FSPCE..... 243

Figure 7.9**:** Qualitative evaluation of the M^3 biosensor for *E. coli* O157:H7. 244

Figure 7.A.1: ^{1}H-NMR spectra of step one for the D+mannose pentacetate production. In this step the Ac groups are added to all the carbons of the 6 carbon ring. This is start compound B in figure 7.1 (500 MHz, $CDCl_3$). 247

Figure 7.A.2: ^{13}C-NMR spectra of step one for the D+mannose pentacetate production. In this step the Ac groups are added to all the carbons of the 6 carbon ring. This is start compound B in figure 7.1 (500 MHz, $CDCl_3$). 248

Figure 7.A.3: ^{1}H-NMR spectra of step one for the D+mannose pentacetate production, substitution on the anomeric carbon with a chloride linker. This is compound #1 in figure 7.1 (500 MHz, $CDCl_3$)..... 249

Figure 7.A.4: ^{13}C-NMR NMR spectra of step one for the D+mannose pentacetate production, substitution on the anomeric carbon with a chloride linker. This is compound #1 in figure 7.1 (500 MHz, $CDCl_3$)..... 250

Figure 7.A.5: ^{1}H-NMR spectra of the third step for the D+mannose pentacetate production. In this step the chloride group is substituted with a N_3 group. This is compound #2 in figure 7.1 (500 MHz, $CDCl_3$)..... 251

Figure 7.A.6: ^{13}C-NMR spectra of the third step for the D+mannose pentacetate production. In this step the chloride group is substituted with a N_3 group. This is compound #2 in figure 7.1 (500 MHz, $CDCl_3$)..... 252

Figure 7.A.7: ^{1}H-NMR spectra of the third step for the D+mannose pentacetate production. In this step the Ac groups are removed. This is compound #3 in figure 7.1 (500 MHz, D_2O). 253

Figure 7.A.8: ^{13}C-NMR spectra of the third step for the D+mannose pentacetate production. In this step the Ac groups are removed. This is compound #3 in figure 7.1 (500 MHz, D_2O). 254

Figure 7.A.9: ^{1}H -NMR of the final step for the D+mannose pentacetate production. In this step the N_3 group is reduced to an NH_2. This is compound #4 in figure 7.1 (500 MHz, CD_3OD). 255

Figure 7.A.10: ^{13}C-NMR of the final step for the D+mannose pentacetate production. In this step the N_3 group is reduced to an NH_2. This is compound #4 in figure 7.1 (500 MHz, CD_3OD). 256

Figure 8.1: Schematic of the bare screen-printed carbon electrode (SPCE) sensor chip the detection is performed on, the circuit. 273

Figure 8.2: Layered schematic of the structure in the novel aminated D+ mannose functionalized screen-printed carbon electrode (D-FSPCE) biosensor chip. 274

Figure 8.3: A quantitative dose based evaluation of capture efficiency vs IMS challanged concentrations of the M^3 Biosensor extracted and captured *E. coli* O157:H7 Sakai strain, in broth. Capture efficiency is steady from 10 cells to 1 * 10^8 CFU/mL. with an overall mean value of 92% and an overall median value of 93%. All 213 sample cultures used

were in the near optimum range of 3 to 10 hours after innoculation before serial dilution and testing. 278

Figure 8.4: A quantitative dose based evaluation of mean capture efficiency vs IMS challanged concentrations of the M^3 Biosensor extracted and captured *E. coli* O157:H7 Sakai strain, in broth. Capture efficiency is steady from 10 cells to $1 * 10^8$ CFU/mL. with an overall mean value of 92% and an overall median value of 93%. All 213 sample cultures used were in the near optimum range of 3 to 10 hours after innoculation before serial dilution and testing. 279

Figure 8.5: A quantitative dose based evaluation of average resistance vs $\log_{10}$ CFU/mL challenge concentrations on M^3 Biosensor captured bacteria, in broth. NOTE: "0 cells" (red) are concentrations that were at the limit of detection and although innoculated, did not grow cells. "1-100 cells" (orange) are below the consistent culture range reccommended by the FDA BAM (2009). All 257 sample cultures used were in the near optimum range of 3 to 10 hours after innoculation before serial dilution and testing.... 280

Figure 8.6: A quantitative dose based evaluation of Δ Q (mC) vs $\log_{10}$ CFU/mL challenge concentrations on M^3 Biosensor captured bacteria, in broth. All 257 sample cultures used were in the near optimum range of 3 to 10 hours after innoculation before serial dilution and testing. 281

Figure 8.7: The percent of tests that would be considered positive when different cut points were selected in μΩ, by culture verified concentration challenged onto the M^3 Biosensor. The cut points selected correspond to the mean, median and an arbitrarily higher average resistance value of the blank category dataset. As the cut value decreases the percent considered positive at each concentration increases including the blank. 284

Figure 8.8: Electrochemical signal to noise ratio verses culture verified concentration challenged onto the M^3 Biosensor platform. Signal by culture concentration grouping was averaged and compared to the average of all the blank runs..... 285

Figure 8.9: A quantitative evaluation of of the capture efficiency of IMS beads for bacterial concentration ranges of 10^2 and 10^7 CFU/mL. Dynabeads® extracted and captured *E. coli* O157:H7 Sakai strain, in broth and EAMNP extracted and captured *E. coli* O157:H7 Sakai strain, in broth are compared . Capture efficiency of the commercial IMS technology had a median value of 95% compared to 93% for EAMNPs. These were not significantly different. 286

Figure 9.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes..... 306

Figure 9.2: The lab designed, portable, 200 mL bottle rotator used for the 40 minute rotation as the EAMNPs capture the bacteria. 306

Figure 9.3: The lab designed, portable, 200 mL bottle rotator used for the 40 minute rotation as the EAMNPs capture the bacteria in its compacted transport formation. 307

Figure 9.4: The lab designed, 200 mL to 50 mL magnetic separator used for the wash and concentration steps of the IMS with EAMNPs in whole fluid milk. 307

Figure 9.5: Schematic of the bare screen-printed carbon electrode (SPCE) sensor chip the electrochemical detection is performed on, the circuit. 312

Figure 9.6: Layered schematic of the structure in the novel aminated D+ mannose functionalized screen-printed carbon electrode (D-FSPCE) biosensor chip. 312

Figure 9.7: Milk Run # 1, portion 1-4 melt curve results on JBAIDS with the *E. coli* O157 test, 29 MPN/mL. This graph demonstrates the temperature difference at the melt curve for the kit positive control DNA (65°C) and the spiked milk positives from the *E. coli* O157:H7, Sakai strain (62°C). 325

Figure 9.8: Milk Run # 3, portion 1-7 qualitative results on JBAIDS with the *E. coli* O157 test, 10 MPN/mL. 326

Figure 9.9**:** Experiment #1 by portion. Spiked bulk tank milk at three concentrations evaluated by 20 portions in the FDA BAM enrichment and culture verses 20 portions without enrichment and IMS separation with Mab-EAMNPs. The IMS separated portions were split and evaluated by M^3 Biosensor, culture and PCR. 327

Figure 9.10: Experiment #1, by run. The percent positive samples by comparison methods, by run. The M^3 Biosensor matched the FDA gold standard 100% with all three runs showing at least 5 positive verified by PCR. The AOAC PTM standards only require 5 of the 20 for either method for the run to be called positive. 328

Figure 9.11 Conductivity of different EAMNPs. (a) Experiment #2 EAMNPs, (b) old EAMNPs, (c) non-magnetic Polyaniline (d) New EAMNPs. 329

Figure 9.12: (a) TEM Original EAMNPs, (b) old EAMNPs, (c) Experiment #2 EAMNPs, (d) New EAMNPs, (e) an EAMNP captured *E. coli* O157:H7cell. 331

Figure 9.13: JBAIDS results for Milk run #9, portions 3-20 and the corresponding controls. The over dilution of the PCR primers decreased detection sensitivity, despite this at least five of the samples were confirmed positive by the JBAIDS at a milk spike concentration of 51 MPN/mL. 332

Figure 9.14: JBAIDS results in percent positive for each concentration. The over dilution of the PCR primers decreased detection sensitivity. 333

Figure 9.15: Practice 2% agarose gel on dilutions of a pure culture. Table 9.2 shows the lane designations..... 334

Figure 9.17: QPCR results from practice run with mixed primer sets..... 337

Figure 9.18: 2% agarose gel on QPCR product. FDA positive (+) samples were spiked with *E. coli* O157:H7 Spinach strain with a pGFPuv plasmid. Broth was a serial dilution of ethanol extracted DNA from *E. coli* O157:H7 Sakai strain broth cultures. 338

Figure 9.19: Run #17 Portion 1-12 QPCR Dissociation curves. 339

Figure 9.20: Run #17 Portion 1-12 QPCR Dissociation curves. 340

Figure 9.21: Large Gel # 2 of QPCR product. The 100 bp ladder is on either end of each of 4 rows. Positive bands are at 417 bp for STX 1 (narrow melt peak at 72^{o}C) and 263 bp (wide melt peak at 71.5^{o}C) (Bellin, et al, 2001). The remaining gel images are available in Appendix II. 341

Figure 9.22: Experiment #2 EAMNP extraction results, by portion. Spiked bulk tank milk at varying concentrations evaluated by 20 portions in the FDA BAM enrichment and culture verses 20 portions without enrichment and IMS separation with Mab-EAMNPs. The IMS separated portions were split and evaluated by M^{3} Biosensor, culture and PCR when possible. 342

Figure 9.23: Experiment #2, by run. The percent positive samples by comparison methods for EAMNP extraction from whole fluid milk. 343

Figure 9.24: Experiment #3 EAMNP extraction results, by portion. Spiked bulk tank milk at varying concentrations evaluated by 20 portions in the FDA BAM enrichment and culture verses 20 portions without enrichment and IMS separation with Mab-EAMNPs. The IMS separated portions were split and evaluated by M^{3} Biosensor, culture and PCR when possible 344

Figure 9.25: Experiment #3, by run. The percent positive samples by comparison methods for EAMNP extraction from whole fluid milk. 345

Figure 9.26: Experiment #3, by run. The percent positive samples by comparison methods for the M^{3} Biosensor from whole fluid milk..... 346

Figure 9.27: Complied results of all milk trials of the EAMNP extraction part of the M^3 Biosensor, by portion. Any one of the detection techniques positive made the portion positive. 347

Figure 9.28: Compiled results of all whole fluid milk trials of the EAMNP extraction part of the M^3 Biosensor, by run. Detection by any one detection technique (JBAIDS, Culture, Q-PCR) was considered a verified positive sample by the M^3 system. 348

LIST OF EQUATIONS

Equation 3.1: Estimate of CFU/mL for plates with no growth of bacteria. d = dilution factor plated..... 101

Equation 7.1: Ohm's Law used to calculate the electronic behavior in the circuit of each point taken during the cyclic voltammogram..... 235

Equation 7.2: Solving of Ohm's Law to calculate the resistance at each point on the cyclic voltammogram. 235

Equation 7.3: Total average resistance across all points on three consecutive cyclic voltammograms..... 236

Equation 8.1: Ohm's Law used to calculate the electronic behavior in the circuit of each point taken during the cyclic voltammogram..... 275

Equation 8.2: Solving of Ohm's Law to calculate the resistance at each point on the cyclic voltammogram. 275

Equation 8.3: Total average resistance across all points on three consecutive cyclic voltammograms..... 276

Equation 8.4: The change in the rate of current transfer across the circuit at each point taken during the cyclic voltammogram. 276

Equation 8.5: Solving the change in the rate of current transfer across the circuit for the unknown Δ QT..... 276

Equation 8.6: Total average change of rate of current transfer (ΔQ) across three consecutive cyclic voltammograms. 276

Equation 8.7: The calculation for capture efficiency of the EAMNPs in Milk. Equivalent to the spiked levels of available bacteria divided by the amount of bacteria captured after IMS. 277

Equation 9.1: Calculation of the amount of pure bacterial spike volume necessary to approximate a certain bacterial concentration in a bulk tank of whole fluid milk. 305

Equation 9.2: Ohm's Law used to calculate the electronic behavior in the circuit of each point taken during the cyclic voltammogram..... 313

Equation 9.3: Solving of Ohm's Law to calculate the resistance at each point on the cyclic voltammogram. 313

Equation 9.4: Total average resistance across all points on three consecutive cyclic voltammograms..... 314

Equation 9.5: The calculation of the volume of bacterial pure culture to place in the bulk tank to obtain the targeted concentration per mL of contamination. 315

CHAPTER 1 Food Defense: An Introduction to the Principles Necessary to Move From Reaction to Prevention for Use of Food CBRN Terrorism Agents

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Introduction

Food defense is a multidisciplinary study involving disciplines as broad as food safety, supply chain management, and packaging to criminal justice concepts like crime prevention, terrorism studies and emergency management to name just a few. The study of food defense would necessarily encompass a diverse set of concepts that make merging them into one thesis difficult. While the connections are valid, the flow between the disciplines is difficult. To help alleviate this disconnect, this introduction is divided into two parts. Part 1 introduces the concepts of food defense and the criminal justice concepts relevant to it. Part 2 introduces the hard science concepts necessary to develop and test a potential food testing platform for field based environments. The two make up the diverse research this thesis represents.

Part I

The terrorist attacks on the United States of America on September 11, 2001 (9-11) forever changed the face of our country. The New York skyline will never be the same and neither will our collective perspective on the threats that can affect our way of life. Never before had there been a successful terrorist attack of that magnitude on American soil, and American society has changed. To that end, the terrorists were successful. Community emergency preparedness and critical infrastructure protection were concepts that were not really discussed; now they are common in multiple public and private disciplines. This thesis will discuss one of the emerging areas of concern, food defense.

Critical infrastructure protection is not a new concept. The Clinton Administration enacted the 1998 Presidential Decision Directive 63 (PDD 63). It listed specific portions of the American economy and society that were considered critical to continue functioning in the event of an emergency. These were considered highest national priority to protect from disruption.

Agriculture, food, and water supplies were not on that list. Post 9-11, the need and urgency to protect our critical infrastructures was heightened and the Bush Administration enacted Homeland Security Presidential Directive 7 (HSPD-7) and HSPD-9, *Defense of US Agriculture and Food* (Moteff & Parfomak, 2004). This was the first time agriculture and our food and water supply were formally considered critical to our economy and society. As such, the programs and initiatives at the national, state or local levels were not in place to provide that protection. To be frank, the concept of "defense" of these areas was so new, where to start was the real problem.

Food defense, food protection, and food safety are separate concepts that are all interrelated. Food defense (securing food sources against malicious biological attack), food protection (prevention of food fraud), and food safety (identifying and eradicating contamination from natural sources) (Spink, 2009b; CENS, 2008) are growing increasingly relevant as the global nature of the food supply has several inherent difficulties to its monitoring. The United States' food safety programs that protect from natural or accidental contamination are the best in the world. Having personally inspected food production facilities in other countries, nothing compares to the quality that our country's companies are producing in the food safety area. Having stated that, the hundreds of product recalls in the United States every year indicate room for improvement. Food protection is the brand, product, and supply chain protection from counterfeiting, tampering and diversion. Food defense is the protection of our food and water supply from intentional contamination, usually on a widespread basis (Spink, 2009b; Spink & Moyer, 2011).

If defense of our food and water supply was new and nonexistent, the existence of emergency response to an attack on such was even less developed. More work has been done in

agro-terrorism than food based terrorism. In this related concept, agro-terrorism, the Knowles', et al. report from 2005 highlights some aspects of the missing infrastructure from the law enforcement perspective. Knowles, et al. (2005) produced a report on law enforcement's role in an agro-terrorism event. In that report, the lack of coordination and ability to respond to an agro-terrorism event in our livestock population was discussed. The end result was a lack of knowledge on law enforcement's side as to who the stakeholders were and where to get the expert knowledge when needed. Additionally, the report found a lack of resources available to sustain a long term lockdown and investigation of the large geographical area inherent in any agricultural commodity. These findings are applicable to food defense as well. The report highlighted the need for new partnerships with health officials, especially the veterinary community (Knowles et al, 2005). Law enforcement, emergency management and even health care responders are all in the same situation concerning lack of communication among the stakeholders of any terrorism event. The veterinary community's lack of participation was particularly concerning considering all but one of the Centers for Disease Control's (CDC) A-list pathogens are zoonotic or transmissible from animals to people (NIAID, 2009). To assist the law enforcement, scientific, health care and emergency management communities the National Center for Food Protection and Defense (NCFPD) was officially launched as a Homeland Security Center of Excellence in July 2004. The organization was developed as a "multidisciplinary and action-oriented research consortium" (NCFPD website). NCFPD addresses the vulnerability of the nation's food system to attack through intentional contamination with chemical, biological, radiological or nuclear agents (CBRN) with a comprehensive, farm-to-table view of the food system, encompassing all aspects from primary

production through transportation and food processing to retail and food service. Its primary goals include:

- Significant improvement in supply chain security, preparedness, and resiliency
- Development of rapid and accurate methods to detect incidents of contamination and to identify specific agent(s) involved
- Application of strategies to reduce the risk of food-borne illness due to intentional contamination in the food supply chain
- Development of tools to facilitate recovery from contamination incidents and resumption of safe food system operations
- Rapid mobilization and delivery of appropriate and credible risk communication messages to the public
- Delivery of high quality education and training programs to develop a cadre of professionals equipped to deal with future threats to the food system.

This thesis work was partially supported by the NCFPD and seeks to advance several of the NCFPD primary goals, especially security, detection and risk communication.

The real risk of intentional threat related to food is a hotly debated topic at the national level. Some have the tendency to downplay the risk citing the lack of historical evidence of attack, inability of terrorists to effectively produce viable CBRN terrorism agents on a large enough scale to be a major problem, and the difficulty of dispersion via food or water verses aerosol or explosive (Leitenberg, 2002 & 2005; Ackerman, 2009; Parachini, 2003 & 2010; Ozonoff, 2002). Others highlight the lack of adequate defenses and preparation, the ease of reproduction of some agents, the severe economic and social effects of even the rumor of

contaminated food and the ingenuity of the terrorists themselves as reasons to move now before a large scale attack occurs (Atlas, 1999; Enemark, 2005; Garrett, 2001; Henderson, 1999; Lawler, 2001). Monke (2004) discussed the potential risk and available response to an agro-terrorism event. He concluded the risk was unlikely but catastrophic in results and our systems are unprepared. Dalziel (2009) examined verifiable incidences of malicious contamination of food along the "farm to fork" continuum. His conclusions were that such incidences are very unlikely and that terrorists are unprepared to mount a large scale CBRN terrorism event in both laboratory methods and intent.

Also varying is the perspective of different segments of the economy. The public health concerns are more often related to agents that can cause death and serious injury whereas the military is concerned with ineffective fighting forces related to even mild to moderate illness without the need for death; agriculture is concerned with agents that destroy animals or plants and thus cause economic impact. Full agreement on the level of risk, need for preparation and the method of preparation is unlikely to occur. Most involved in the field seem to agree on the unlikely or low designation for frequency; but catastrophic or very high for severity for consequences in a risk analysis of the issue. Using the risk matrix presented to packaging professionals by Spink (2009a), shown in figure 1.1, the low probability (or unlikely) and high severity (or catastrophic) category is a medium risk threat. Medium risk threats require some form of risk mitigation. Many of the risk mitigation procedures available or under development will benefit food defense, food safety and food protection; allowing risk mitigation resources invested to cover all three areas (Knowles et al., 2005). This is especially true if risk mitigation procedures are accomplished through public private partnerships to spread the responsibility and cost across both sectors (Jones, Kowalk & Miller, 2000).

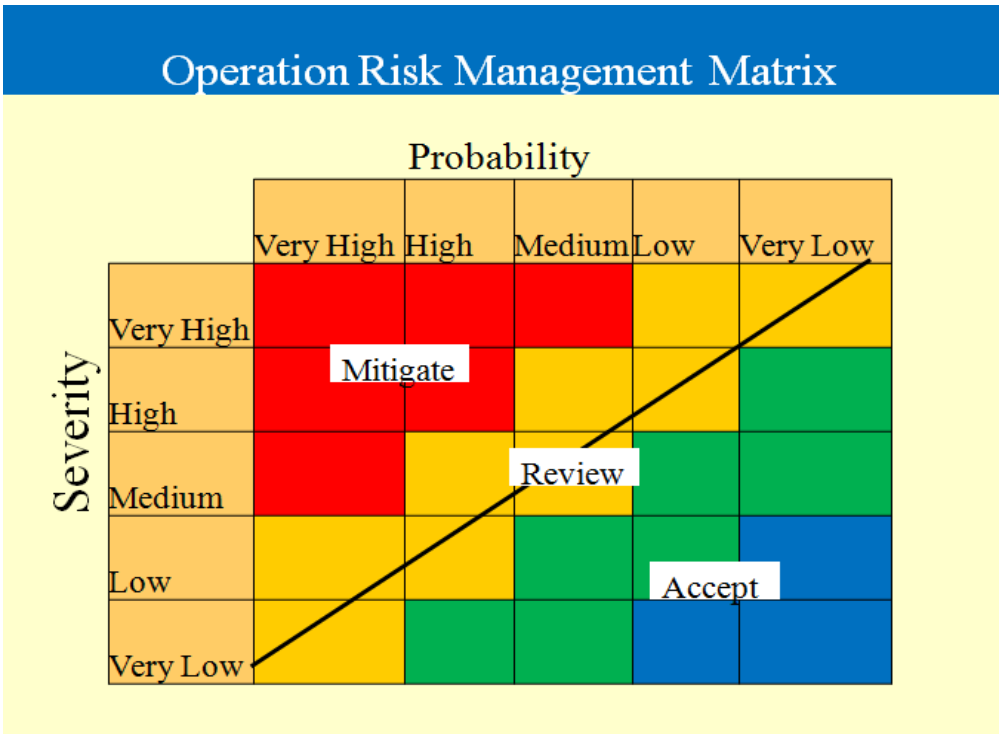

Figure 1.1: Operation Risk Management Matrix. (Spink, 2009) For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

The knowledge base about the current threat of food CBRN terrorism in the United States is nebulous at best. In an age where anything is being considered possible, realistic evaluation of current threat levels requires evaluation of historical precedence of the act, feasibility in current systems for the act, perpetrator capabilities and motivations to pull off the act and the effects of an act. To facilitate understanding of the risk mitigation possibilities, a criminal justice and behavioral science concept is employed called the chemistry of the crime (Clarke, 1997). It is illustrated by the crime triangle. The crime triangle is a concept where a crime occurs only if three minimal elements are present in any point in time: "a likely offender, a suitable target, and the absence of a capable guardian against crime". These are called the chemistry of the crime (Felson & Clarke, 1998). It was adapted from the fire triangle where a fire does not occur without oxygen, fuel and heat.

How likely an event is must include discussions of those three interacting agents; therefore how likely (will it happen?) an event is can be differentiated from just the plausibility (can it happen?) of an event. Subsequently, those same topics are used to develop recommendations as to what needs to be discovered to develop effective assessment tools and to determine the capacity of terrorist organizations to commit a CBRN terrorism attack in the United States. They are also used to develop adequate mitigation and response efforts. If we consider the consequences to a food CBRN attack to be catastrophic, the mitigation strategy should be preventative in nature. In order to move from a reaction based posture to a preventative posture all three legs of the crime triangle need to be addressed.

In order to develop strategies to most effectively assess the capacity of terrorist organizations to commit a food based CBRN terrorism attack, we need to understand the who, what, when and where of potential food based CBRN attacks.

- Who: Who are the major organizations likely to perform a CBRN attack? What are their primary characteristics and demographics? Knowing this can narrow the field of terrorist organizations necessary to monitor with surveillance by law enforcement.
- What: What types of food based CBRN attack are most likely by organism and by matrix? What points in the supply chain are weak or unmonitored, where are the critical control points to focus attention on?
- When: When an attack might occur can be narrowed by studying past trends on the adaptation of the terrorist groups to certain stimuli, we need to know what the trigger events there are to move an organization toward CBRN terrorism agent use, for those organizations most likely to do so, to further improve our ability to monitor with surveillance. Organizations preparing to or in the act of designing and implementing a

CBRN event in the food supply or elsewhere generally have second set of characteristic trigger events that when monitored for can give clues to impending activity. We need to know what those trigger events are to improve our reaction time.

- Where: Where our vulnerabilities are, especially those that cannot be controlled must be identified by performing realistic risk assessments of food commodities and agents to allow mitigation strategies to be used at the most effective point in the supply chain.

All of these questions can be related to all three legs of the crime triangle. In order to discuss them, each leg of the crime triangle will be examined separately.

Victim

There are two victims of concern in any terrorist event. No crime can occur without a victim who is incapable of protecting themselves from a targeted attack, actual victims. The second type of victim is a victim of fear. Victims of fear are those that are affected in their daily lives by the fear of being attacked. No matter how hard performing a CBRN food attack is, terrorists will get tremendous reaction if they can mount a successful attack. The media attention and public reaction present when "naturally occurring" food borne and other disease outbreaks occur feeds the fear victim status that terrorist groups seek to capitalize on (Rothe & Muzzatti, 2004). As an example, I will use the peanut butter associated *Salmonella* outbreak in 2008-9. The world coverage of an American food safety issue was astounding. The outbreak was on every TV news show for the entire fall and spring. Panic caused avoidance of any food containing peanut butter and economic impacts that companies have still not fully recovered from (Cook, 2009). The global nature of the food supply, the invisibility of CBRN agents, and the lack of available medical treatment for many all add to the fear engendered by the threat of biowarfare no matter what the vehicle of dissemination (Zach, Doyle, Bier, Cxuprynski, 2012).

The media, terrorist propaganda and terrorism all affect the fear based victim status of a population. A discussion of the relationship between media, propaganda and terrorism includes many public interest and policy implications (Roth, Tsay, Pullman & Gray, 2008; White, 2001). The interwoven agendas of terrorists, media, public opinion and public policy relate to each other through terrorists' acts and the propaganda about them. Propaganda, according to the American Heritage Dictionary (1985) is: "The systematic propagation of a given doctrine or allegations reflecting its views and interests." Propaganda is the medium of international and national communication.

Terrorists need the media to send their message. The media needs terrorists to create compelling stories (Chermak & Gruenewald, 2006; Roth, et. al., 2008; White, 2001). The media is not only a news reporting agency. They are interrelated private enterprise organizations affected by market share and profit margins, just like every other business. Their market share is viewership and associated ratings. Their profit margin is related to advertising dollars spent for every dollar in overhead. Competition in the news entails being the first to get to a story. This is analogous to the hook to grab readers and moviegoers to make them interested and emotionally involved in the story. Terrorists use this "hook concept" to develop shocking, interest grabbing terror tactics to gain attention. Once they have the story, the media outlet must create the drama to hold the audience. If this were a play or a book, the drama is fashioned to meet the storyline of the book or movie. The terrorists knowingly create this. If there is not enough drama as the world awaits the terrorist's demands being met, the media outlet must create it. They do this by searching for any new information or human interest stories often times leading right where the terrorist wants them to go, to publicize their problem for the world to see. Bruce Hoffman's description of this dynamic is enlightening, stating that 1/3 of the stories around a terrorist

incident are human interest and <1/2 of those 1/3 are related to the work anyone is doing to resolve the issue (Hoffman, 2006). Theater requires climax and anticlimax to complete the story. Terrorists create this with killing or releasing their captives as dramatically as possible and then allowing exclusive interviews after the fact to further promote their cause and mitigate the negative image the violence they just perpetrated brings. The exclusive interviews with terrorist leaders Abi Abbas in 1996 and Osama bin Laden in 1997 both occurred after terrorist attacks and allowed further exposure to the "message" of the terrorist organization (Hoffman, 2006; Perl, 1997). As stated in Perl's 1997 Congressional Research Service article, terrorists need publicity, unedited if possible, to alert the world to their plight and demands. Terrorists used propaganda messages to influence public opinion through terror and fear. Media needs fantastic stories to sell air time. The two feed off each other.

The apparent fascination of the American public with homicide, terrorism, death and destruction is supported in quantitative studies like those of Chermak and Gruenewald (2006) in "The Media's Coverage of Domestic Terrorism". In this article the authors develop a predictive model to determine which terrorists events will be covered by the media. Death, destruction and domestic incidents of such had the highest frequency of terrorism reporting. Food CBRN terrorism surely fits this description. The media, as discussed above, is driven by ratings; ratings equal public willingness and desire to watch. Raphael Perl (1997) does an excellent job summarizing the relationship between the media, public policy and response. A summary of the pertinent points is included below. Politicians are elected officials who depend on public trust. Public opinion affects policy if not drives it. Public pressure on politicians to help the poor victims involved no matter what the concessions becomes apparent when the human interest stories are what the media promotes. This was evident in the decision by the Reagan

administration to convince Israel to trade captured terrorists in exchange for American hostages in the 80s.

When the media is capable of immediate wide spread dissemination of information that is more for entertainment than information. This speed of information causes politicians to come under pressure to solve dilemmas now, at the cost of considerable debate and investigation into the appropriate response. Sort of like the hero in television dramas. Unfortunately, the half hour start to finish television dramas that go through hook, drama, climax and anticlimax are not real life but they influence the public opinion of how quickly these things should be resolved. An example of this rushed decision making was made apparent in the decision to invade Iraq to investigate weapons of mass destruction rumors (Goodnight, 2010). It was also apparent in the decision by Clinton to send in troops to Somalia after Blackhawk down. In the rush to preempt criticism he made a decision not supported by most Americans. Public policy and public opinion feed off each other. The method of cross over between the two sets of relationships is propaganda. The American public and international media make awesome victims. This thesis does not deal directly with the victim because it is not a factor easily affected in the move from reaction to preventative posture.

The Perpetrator

The perpetrator is the group or individual that would carry out an attack. Crenshaw (1981) established several short term instigators for use of terrorism as a strategy. Most notably those goals are impatience, weakness, recognition, tyranny, discredit of the opponent and even oppression. Traditional tactics (i.e. bombing and guns), will prevail until such a time as the terrorists goals begin to stagnate. With that stagnation, new forms of terrorism will be explored to move the cause forward (Crenshaw, 1981).

CBRN terrorism is one of those choices. CBRN terrorism in food is an attractive tactic for a terrorist group because it is a rapid way to spread terror and the recognition of the terrorist's cause all over the world; carried by the global food supply. The results go beyond illnesses and death (Rothe, & Muzzatti, 2004). The global food supply, increases in the probability of CBRN use and the increased probability of non military, non high profile targets being used by terrorists all lend credence to the need for preparation to prevent this unlikely, but catastrophic threat.

Food in any country in the 21st century comes from all over the world. Everyone eats and drinks and cannot survive without it. In fact, research by Dusslier in 2009 supports food as a cultural evaluation tool and a social modifier highlighting its importance in society as more than just sustenance. Foods are processed and shipped further and faster than ever before (Zach et al., 2012). United States Senate Hearings (1998) concluded that 33% of all fruit comes from outside of U.S. and 12% of all vegetables are imported. Imported food is the most difficult to regulate and evaluate as well because international agreement is difficult at best and US inspection under foreign sovereignty makes enforcement difficult.

The global food supply itself has inherent weaknesses in its supply chain ranging from smuggling and grey market trade to counterfeit (Roth et al., 2008). All are viable methods available to adulterate and return to the supply chain contaminated food. Many of the illicit food trade practices are already being used to fund terrorist and organized crime syndicates (Hutchison & O'Malley, 2007). If they are using the illicit food trade practices to raise money, they have access to adulterate the food and return it to the food supply chain outside the traditional food safety protective network.

As discussed under the victim leg of the crime triangle, the media is a force multiplier for terrorist attacks in general and this is no different for food based CBRN. The media attention

focused on any natural food borne disease outbreak is tremendous. A good example of this media reaction is the peanut butter outbreak of *Salmonella* in 2008/2009. The world coverage of an American food safety issue was astounding. The outbreak was on every TV news show for the entire fall and spring. Panic caused avoidance of any food containing peanut butter and economic impacts that companies have still not fully recovered from (Cook, 2009). If a food based CBRN attack could be performed, the same media attention would multiply the affects of it. This outbreak also served to highlight the vulnerabilities in the food safety network of the US. The peanut plant that caused all the trouble was inspected and passed by the FDA or the Georgia Department of Agriculture every year (Minor, 2009).

The use of food or even any CBRN tactic is not easy or there would be many more cases of it to study. According to the WMD database, there are 1,100 cases of CBRN use by non-state actors compared to 98,000 cases of terrorism in the global terrorism database (GTD). The most daunting deterrence to use of CBRN terrorism is the *technical knowledge* necessary. Knowledge of food and the microbes in food, the equipment to propagate the microbes, the volume of organisms necessary to propagate a widespread attack and access to the organism with the appropriate dissemination platform are all large deterrents. Having stated that, it is worthy to note that scientific knowledge across the world is increasing and access to it via the internet is tremendous (Garrett, 2001). This may lead to increased availability of the necessary knowledge to put together a food based CBRN attack.

Given the advantages to using CBRN terrorism in the food supply as a tactic for a terrorist group and the global food supply's vulnerability to it, it makes sense to discuss the probability of it occurring despite the difficulty. Mohtadi and Murshid (2009) do just that. The number of CBRN terrorist incidents used in their evaluations was 448 over a 53 year period.

These counts were used in trend analysis that showed terrorist attacks on non military targets increasing while high profile targets are decreasing. They also show the dramatic increase in CBRN incidents over the last decades from zero in the 1960s to forty in the 2000-2005 era. The end result was a probability model predicting continued increasing frequency and decreasing recurrence period of both catastrophic terrorist attacks and CBRN terrorist attacks (Mohtadi & Murshid, 2009).

Most food based attacks listed in terrorism incident lists, or anywhere else, are on a very small scale such as individual attacks on coworkers, relatives or employer's interests (Dalziel, 2009). Those attacks are difficult to find and examine because many never make the news or are considered criminal matters and do not make terrorism act lists (Chermak & Gruenewald, 2006; Dalziel, 2009). They also only lend themselves to case studies at an individual level because many are unclaimed by organizations (Asal, Ackerman & Rethemeyer, 2012). Historically, there has never been a large scale CBRN attack in the food supply anywhere in the world, by terrorists or otherwise (Dalziel, 2009). Of the mostly small scale the attacks; the premier is the Oregon Rajneesh use of *Salmonella* on food (Dalziel, 2009). For the precedence of low scale, single perpetrator acts to change to large scale acts two things are required. The first is solid evidence that terrorist groups have biological weapons. The second is that they are attempting to use them or are developing dissemination techniques.

Logically, if terrorists already have CBRN weapons, attacks or incidences of unusual disease or increases in usual disease incidence should be increasing and federal intelligence about production facilities and supply chains should be increasing. Terrorist organizations are likely to start small and work their way up with proven success principles for this mode of attack (Crenshaw, 1981). It follows then that examination of past CBRN incidents no matter how small

would be a valuable tool to predict future activity. Many state actors have CBRN weapons capability, either from past programs or illegal covert existing programs. There is little evidence that the state actors that do have CBRN agents will share them with terrorists. First, there is no evidence of use by state actors of their CBRN terrorism agents (Garrett, 2001). Second, the public and international outcry against any state that does deploy its CBRN terrorism agents would be just as devastating politically and economically to the host state as the agent was on the attacked state (Meselson, 1999). Third, terrorist groups have a documented tendency to attack their own populations to a greater extent than other populations (Lafree, Yang, & Crenshaw, 2009). States are unlikely to provide uncontrollable agents (once released) to an unstable political group that could be used against them in their own state. This is supported in a quantitative study of CBRN terrorist organizational factors from 2012; state sponsorship of a terrorist group had a negative impact on the use of CBRN tactics (Asal et al.). Because of the Biological Weapons Convention (BWC) treaty, the Chemical Weapons Convention (CWC) treaty and the Nuclear Non-Proliferation Treaty (NPT), state actors who are cheating and have active CBRN programs are hiding them from the world, not just terrorists. Public knowledge of their state possession of them would be devastating to their economic, political and social influence in this global economy. Since there is no indication of increased incidence of unusual disease and federal intelligence of production facilities, terrorists probably do not have existing CBRN capabilities now. There are document incidents of CBRN use on a small scale by terrorist organizations, criminal organizations and individuals. So even if they do not have the capability currently, these incidences also support the conclusion that they are making efforts to develop those capabilities on a larger scale.

Terrorists probably do not currently have existing CBRN agents purchased or stolen. Can they develop CBRN weapons on their own? If they can or are developing them, readily available items would be the easiest to procure (Dalzeil, 2009); suspected and investigated instances would find supporting evidence of biologic weapons presence or all equipment necessary to produce CBRN weapons with or without the actual presence of the agents; and terrorist cells will have increasingly better scientifically trained individuals and documentation than found in the past (Leitenberg, 2005). In 28 case studies by three authors (Leitenberg 2002 & 2005; Paranchini 2003; Dalzeil, 2009) it was demonstrated that for only 2 events did the terrorist organization manufacture or culture their CBRN agent. The Rajneesh group cultured their *Salmonella* and several right winged groups in the U.S. have produced ricin from ground castor beans. No other known terrorist group is known to have *cultured* any pathogen (Leitenberg, 2005). Aum Shinrikyo, an apocalyptic religious cult in Japan had many scientists, sufficient funds, and 10 years of uninterrupted time for development and still failed at all 10 attempts at a biological attack (Parachini, 2003). The Sarin gas they did produce was used in a fairly small scale attack and was not distributed in food. In January 2003, British authorities arrested six men suspected of producing ricin in their north London apartment. The London group was captured with 22 castor beans and a coffee grinder. Investigations showed no identification of ricin in the apartment or on the equipment (Leitenberg, 2005). Subsequent testing of the "recipes" present in the apartment produced only enough ricin to kill one person if injected directly into the target; cause gastrointestinal upset if ingested and have no effect if deployed on the skin. In the spring of 2003 Jordanian officials apprehended and prevented a bombing attempt on the US Embassy in Jordan. The foiled plots involved a suspected involvement of sodium cyanide. The group's plans to use 20 tons of explosives to deliver it

would have destroyed the cyanide, and no cyanide was ever found (Leitenberg, 2005; Cordesman, 2005)

In Afghanistan, al-Qaeda, with a safe haven to work for years, intelligence gathering has revealed evidence that there was interest in CBRN weapons for asymmetrical warfare (Leitenberg, 2005). There was minimal equipment available and no cultures or dissemination techniques for CBRN agents (Leitenberg, 2005). Anthrax is endemic in Afghanistan, but al-Qaeda was unable to successfully isolate Anthrax spores from naturally occurring animal anthrax cases or carcasses of animals that succumbed to anthrax. The evidence from the Kermal and Tarmac Farm al-Qaeda training camps in Afghanistan had no ricin, no biological equipment other than an autoclave, no biological cultures and rudimentary paper knowledge. Captured computer hard drives link to a PhD level scientist attempting to procure anthrax for al-Qaeda's intended CBRN weapons program, but there was no evidence of PhD level scientists working there. Paperwork found showed detailed lists of equipment needed, program requirements and lab layouts, but no actual lab. No publically available information is available to support continued or increased knowledge or procurement by al-Qaeda after 2001 when many operatives were captured (Leitenberg, 2005). All references were in English not Arabic. More than 10% of the Guantanamo Bay Cuba detainees have advanced degrees and still no viable organisms were produced.

The outlying case is the 2001 Anthrax cases in the U.S. which is yet unsolved and unassigned (Leitenberg, 2002 & 2005; Paranchini, 2003; Dalzeil, 2009). Until more information is available on this case it remains as a potential terrorist attack. Excluding the Anthrax case, two successful medium scale CBRN terrorism attacks in 100 years (only one in food), almost 20 years apart with different agents does not support the theory that terrorists have CBRN weapons

at this time. The conclusion that terrorists have the ability to develop or obtain CBRN weapons, *at this time* is questionable. Therefore, neither of the two basic requirements are met for terrorists to have CBRN capabilities. Solid evidence that terrorist groups have biological weapons or that they are attempting to use them does not exist. To date it is unlikely that they will develop them in the near future. As mentioned earlier, the results of a terrorist attack on the food supply would be catastrophic even with the low probability. This creates a medium risk in the operational risk matrix shown in figure 1.1 and requires some form of mitigation. Also, experts in terrorism and CBRN terrorism agree that the existing minor but successful attacks could encourage those with the desire to use CBRN agents in any dissemination medium; including food (Carus, 2002; Chalk, 2004; Atlas, 1999). If the probability of CBRN terrorist attacks in general is increasing, scientific knowledge is increasing and the vulnerable, global nature of the food supply is increasing, then a good viable model for prediction of which groups will use this unconventional strategy is necessary. Chapter two of this thesis seeks to develop the knowledge of who by examining the organizational factors that exist among past perpetrators of CBRN incidents using food as a vehicle compared to other organizations that do not use CBRN at all and other organizations that use CBRN with other dissemination vehicles.

Capable Guardian

A capable guardian is someone or a system that would prevent access to the victim. In the case of an individual house or a company gates, lights, security guards and alarm systems would qualify. When discussing what we know about CBRN terrorism in the food supply, it is helpful to examine what methods or procedures exist to prevent it. The current protective postures and protectors that exist, their presence, and effectiveness as well as the hurdles to the food supply as an effective dissemination medium all become components of a capable guardian.

Protective factors of the system include access control, surveillance, and inherent hurdles to CBRN terrorism as a tactic, and adequate containment and response.

Access control

One component of a capable guardian is the prevention of access to the target (Felson & Clarke, 1998). Knowledge of food and the microbes in food, the equipment to propagate the microbes, the volume of organisms necessary to propagate a widespread attack and access to the organism and the appropriate dissemination platform are all large deterrents and forms of access control (Mohtadi & Murshid, 2009). Food itself has inherent hurdles to large scale contamination. The chemicals, natural and synthetic, compounds and bulk make extraction and detection of agents difficult but also make proliferation from a point source to multiple exposure points difficult as well. Dilution and agent death are significant protectors (Adams & Moss, 2008). To overcome this hurdle, terrorist groups would have to strike multiple targets simultaneously or consecutively over a tight timeline in different food matrices to be effective in a large scale attack. The bulk of food and the non uniform distributions of organisms in food make consistent attack exposures difficult to predict and therefore difficult to cause widespread death and injury of the kind terrorists look for. Fat and other biological components of food have inhibitors for chemical and biological growth and sustainment without the existence of temperature abuse or inadequate cooking on the consumer's end. This distribution hurdle makes the consistency and predictability of each attack's results and thus the terrorist's risk assessment for success more difficult. These combined natural access control issues increase the knowledge level required for the capability component of using CBRN terrorism in the food supply as a terrorist tactic. Many food based evaluations of agent behavior in different food matrices are not known to the general scientific community and relate to the issues of diagnostic testing. As this

science matures, the information necessary to propagate an effective food borne CBRN terrorism event could be pieced together. This will allow future attacks to be more likely (Garrett, 2001). Physical Access control is another form of protection or adequate guardianship. That physical access comes not only in the functional form of guards and gates, but signage, lighting, personnel and visitor evaluation and monitoring and recording and alarm devices. The criminal justice literature contains many articles discussing such controls to prevent any crime. (Crowe & Zahm, 1994) They apply to CBRN terrorism no differently. These physical controls are minimal at the farm level of the farm to fork continuum (Cupp, Walker & Hillison, 2004) with programs like Agroguard attempting to tighten them (Knowles et al., 2005). Agro-Guard is a public private partnership to teach law enforcement and agro-terrorism issues to local farmers. It includes signage to identify premises as aware and vigilant. This could be expanded to include food processing facilities who face many of the security issues farms do and more specific food CBRN terrorism issues. The sign identifying the premise as aware and vigilant makes a target less attractive to a potential terrorist (Knowles et al., 2005). Passive/active systems that fit with something the target audience already does increases acceptance of a change. What we know about CBRN terrorism in the food supply is that these physical controls during transportation, storage processing and display/serving are very weak with the biggest weakness being awareness that they are necessary (author's personal experience). This area is the most expensive to harden and logistically impossible for the vast farming and animal husbandry industries around the world (Knowles et al., 2005). Awareness campaigns like Agroguard and increasing requirements for food defense and Hazard Analysis Critical Control Point (HACCP) control plans that are governmental and consumer driven will improve this. Access control is a critical piece of prevention success.

Surveillance – LEI:

The first aspect of surveillance and the presence of a capable guardian for preventing and even responding to a CBRN terrorism event in the food supply will require the use of Law Enforcement Intelligence (LEI)**.** One important consideration that has been duly noted in the nightly news, literature reviews and the interviews for this report is the lack of funding. Funding for law enforcement in general is in jeopardy. This is evidenced by the decreasing in patrol teams for rural areas in Michigan during 2009 and 2010 (WLNS, 2010; Grand Rapids Press, 2009). Nationally the situation is no better (Police Executive Research, 2009). Any recommendations must consider that additional spending is not likely to happen in an environment of budget cutting, but ignoring the potential for CBRN terrorism is a poor solution.

Law enforcement intelligence to prevent CBRN terrorism involves operational and strategic intelligence. Operational intelligence is the evaluation of data to make decisions on daily activity or response to situations (Carter, 2004). Strategic intelligence is examining patterns and trends to manage asset allocation and policy (Carter, 2004). In its 2005 report, the International Association of Chiefs of Police (IACP) states that "state, tribal, and local law enforcement officers are situated to identify, investigate, and apprehend suspected terrorists. Accordingly, local law enforcement is the cornerstone of any successful crime or terrorism prevention effort." (p. 4). Law enforcement intelligence capabilities in all but the largest cities like New York and Los Angeles are limited or absent (Knowles et al., 2005). The volume of raw data available by local patrolmen is immense, but raw data by itself does no good without analysis to make it into a definable picture and create operational and tactical intelligence; intelligence used in criminal investigations; to prevent terrorist attack (Carter, 2004). In the limited budget environment, analysts and intelligence units are unlikely to be funded when there

are insufficient funds to maintain patrolmen. Analysts already exist at the Joint Terrorism Task Forces (JTTFs) (Carter, 2004). The JTTF intelligence analysts need improved communication channels for information to flow back and forth from them to the local level. This use of existing analysts is a potential solution to the lack of funding available for the task of raw intelligence analysis.

The 9-11 commission report shows that the intelligence sharing in this country, despite policies to require it, is not happening (9-11 Commission report, 2004). Local patrolmen and local police departments, the key to effective counterterrorism, do not feel an integral connection to the counterterrorism effort. In the three interviews conducted for this analysis, (retired FBI, local police officer & a criminal justice professor) all three agreed the architecture for intelligence sharing is there, but the functional use is not. (Martinez, D; Brown, M.; Chermak, S.; personal communication, 2010) The JTTF and the Regional Intelligence Fusion Centers were an attempt to solve this dilemma. Automated Trusted Information Exchange (ATIX) and Joint Regional Information Exchange System (JRIES) databases were also designed for sharing of intelligence information (Knowles et al., 2005). As of August 2005, JRIES joint use by the United States Department of Homeland Security (USDHS) and law enforcement was abandoned due to policy disagreements (GCN, 2005). There was general agreement that unclassified information moves to the local law enforcement level from the federal law enforcement level fairly well, but little information moves up from local level to federal level.

Several proposed solutions to agro-terrorism prevention by law enforcement were presented by Knowles et al. in 2005 that can be jointly used for CBRN terrorism, especially in the food supply. The difference between the two involves the intent of the perpetrator and the target. CBRN terrorism in the food supply is aimed at injuring or killing people with a CBRN

agent using the food as a dissemination vehicle. Agro-terrorism is the use of CBRN agents to affect animals and subsequently people via shared pathogens called zoonotics. Agroterrorism can also be the use of CBRN agents to infect animals and subsequently to affect the economy using the animal as the dissemination vehicle. Agro-terrorism and food bioterrorism are much intertwined. Most discussions of agroterrrorism have focused on Foot and Mouth Disease (not a zoonotic) and the economic impact of its introduction. Many articles focus on response not prevention. (Atlas, 1999; Chalk, 2004; Cupp et al. 2004) According to Officer Brown, a local police officer, any training he has received in his career for terrorism has been focused on response, not prevention (personal communication, November 12, 2010).

The Knowles, et al. (2005) article's recommendations that are applicable to prevention of CBRN terrorism in the food supply are Agro-Guard, discussed under access control, and Smuggled-food Interdiction Teams (SFIT). Development of information sources concerning terrorist threats to agriculture; establishing interaction between local and federal intelligence networks; development of working relationships with the agricultural industry; and development of training programs to teach local law enforcement officers what to look for are some more applicable recommendations. The SFIT have law enforcement personnel and USDA inspectors who conduct investigation and seizure of illegal food products. Smuggled, counterfeit, and adulterated food are some of the easiest ways to introduce a pathogen into the food supply. Information sources and working relationships with the industry, agriculture or food processing, can be developed on a day to day basis by patrolmen in their daily rounds, if they are trained that it is necessary. Information sharing is harder. Various forms of databases and sharing techniques have been developed, but their use is minimal (Knowles et al., 2005).

Intelligence gathering is similar to the diagnostic screening tests used in medicine for the selection of potential positive cases of a disease. To develop a good screening test, it must be sensitive, specific, inexpensive, easy to use and acceptable to current systems (Hennekens & Buring, 1987). A good intelligence gathering system is a screening test for potential terrorist activity. According to Dr. Chermak (2012), only 21% of the existing right-wing terrorist groups are violent actors, with only 9% being repeat offenders. Limiting intensive surveillance to those violent groups could narrow the cost and logistics of that surveillance, and provide potentially actionable intelligence. Local law enforcement officers have a good idea, especially in small towns, of who the most violent organizations are.

Local law enforcement, as stated earlier, sees the suspicious activity. Every day patrolmen file activities reports with their command, mostly to ensure productivity (Brown, personal communication, November 12, 2010). Suspicious events, when they check out areas and buildings, when, where and why they patrolled are all recorded electronically, but they go nowhere from there. Those reports are a form of searchable raw intelligence. Two methods to use this exist: passive automated surveillance for key words and active search capability for other key words. Millions of literature and internet search engines exist, the technology could be harnessed to collect strategic intelligence much the same way that electronic chatter is monitored at the FBI level now, without changing the daily routine of officers on the road. JTTF personnel could monitor this database and use key words to direct reports to appropriate analysts by subject key words. This is a very sensitive method, but the workload by volume can be passively monitored with fewer key words to increase the specificity of what has to be searched by hand. If this database for each state was tied into the National Crime Information Center (NCIC) at the FBI, the extent of raw local data available for analysts already employed with the security

clearance to access more information would allow for a more complete picture. For example, if this database had been operational before 9-11, the repeat complaint in several states about disruptive Middle Eastern men taking flight courses and the chatter of an attack involving planes could have been tied together faster (9-11 Commission report, 2004). Tying Agro-Guard in, local farmers and food processors report to the local police and the local police place this on their daily reports that are updated in the database either at the same time as they turn it in or separately, would capture even more relevant information.

A second form of missed intelligence is the questioning of captured group members for other crimes for information regarding local activities of gangs and terrorist groups. That information could also be put on the daily report format database or sent directly to NCTC or the JTTFs. Existing data bases like the Kansas Law Enforcement Intelligence Network (KsLEIN) and the Federal Bureau of Investigation's Law Enforcement On-Line (LEO) exist but they are user driven and reception to using them has been luke warm, with only 64% of Kansas sheriffs stating they even had access, with no data on use (Knowles et al., 2005). This may be due to compatibility between systems, but the reason is not discussed in the article. A passive/active system that fits with something they already do may increase acceptance. The information collected is not specific enough to be considered dossiers and the reports are already collected and archived now.

The Knowles' report on agro-terrorism and law enforcement (2005) has extensive evaluations of training programs available on LEI training. Key to getting acceptance of additional training is to offer it within basic job training or getting the person responsible for scheduling training to buy into its necessity. Topics necessary include aspects of CBRN terrorism; trigger events to indicate an intent to carry off any terrorist attack and knowledge of

the necessary assets to successfully carry off a bioterrorist attack. These topics should be included in the basic training of all officers and offered in many different formats to existing officers, preferably incorporated into their current training programs. Without systemic improvements in LEI at the local and state level and increased communications between all LEI our ability to predict and prevent CBRN terrorism in the food supply is limited.

The current economic crunch and budget cuts necessitate the need to incorporate counterterrorism measures as seamlessly as possible into the current operational environments of law enforcement agencies. Current recommendations proposed here include: implementation of Agro-Guard with the entire food supply chain and adding CBRN terrorism aspects to it; creating and deploying SFIT teams, especially for border crossing states and entry ports; Training officers to use daily routes and community interaction, including questioning of non terrorist arrests and interrogations to gain local terrorist and gang related intelligence; and adding basic CBRN terrorism and agro-terrorism prevention components to basic and continuing officer training will integrate local police officers into the intelligence process to combat CBRN terrorism.

Developing a method to use daily activity reports from patrol officers to create a passive and active raw intelligence database to assist analyst already in place at the JTTFs is imperative for improved input from the local level into the federal level CBRN terrorism analysis process. Information from Chapter 2 of this thesis on the organizational factors of potential CBRN terrorist perpetrators will contribute to the information to narrow the field of monitoring.

Surveillance – monitoring food

The fourth aspect of being a capable guardian against food based CBRN terrorism is monitoring the food itself. Food defense, food protection and food safety are growing

increasingly relevant as the global nature of the food supply has several inherent difficulties to its monitoring. First, the widespread, international supply chain makes inspecting all the food impossible. Second, it is impossible to test all the food or nothing would remain to eat. Both of these concepts need to be discussed before possible solutions can become apparent. The current military and civilian posture in food safety is based on sanitary inspections of facilities with periodic random sample and outbreak testing. This is not based on principles for food defense. This inspection based approach leaves an incredibly soft target for attack by terrorist entities bent on harming our troops and our population, especially overseas (CSPI, 2007). Food and Drug Administration (FDA) inspections have dropped by 81% since 1972 and 47% between 2003 and 2006 (CSPI, 2007). Even with the new Food Safety Modernization Act (FSMA), the highest risk plants will only be inspected every three years by the FDA (Olsson, Weeda, Bode, 2010; Sjerven, 2012). FDA in the United States inspects less than 1% of the imported food supply before consumption and less than 0.2% of the imported food has laboratory analysis at all (CSPI, 2007). Sanitary inspection and LEI are not enough alone to move from a reactionary to a prevention based posture.

What about testing? One of the common themes surrounding food safety is the comment "you cannot test your way to food safety" (personal experience). Testing is only a component of a comprehensive food safety or food defense system. Organisms and toxins that cause food borne illness are not uniformly distributed in food samples, they don't behave the same way in different food matrices (Blackburn & McCarthy, 2000; Durso & Keen, 2007; Fitzmaurice, Duffy, Kilbride, Sheridan, Carroll & Maher, 2004; Fung, 2008) and a large part of contamination and proliferation of organisms and toxins occur outside the farm to fork continuum areas that can be regulated (Hutchison, 2007; Roth et al., 2008; Zach et al., 2012). For example the smuggling,

grey-market and counterfeit trade are areas where food can be contaminated and returned to the food supply outside the normal supply chain interception points for contamination. In order to facilitate discussion, assume that diagnostic testing was the only form of control we have, at each level of the farm to fork continuum. Assume this diagnostic test was the cheapest, most sensitive and specific in existence and only showed viable organisms and active whole toxin, the "perfect" test. Starting at the farm, with a perfect test, the only way to ensure that not one leaf of lettuce or one tomato or one cow left the farm with any pathogenic bacteria on them would be to grow all food producing plants and animals in a sterile lab chamber. Even then, symbiotic bacteria are required for digestion, skin health and many other as yet discovered uses. They would be killed off too with any procedure to remove the pathogenic ones. Plus, you would have to test every leaf of lettuce, leaving nothing to eat (Roth et al., 2008).

How about the processing plant? Starting with vegetables; tomatoes are picked, packed, transported and arrive in a plant to make sauces. The tomatoes are washed because they are grown in a field with organisms and toxins, but one assumes the wash water is clean. Even after the wash the wash water is tested and the results are clean Adam's and Moss, in their book on Food Microbiology, find that organisms can be inside the plant not just on the surface (2008). This is consistent with other research that finds that bacteria and toxins as living organisms or products of living organisms move into and attach to mammalian and plant cells (Erickson et al., 2010). No amount of washing or disinfection removes them. Living organisms adapt and the surviving organisms can change in subsequent generations to avoid the wash step as well (Adams & Moss, 2008). For those that adapt, or are out of reach of the wash or disinfectant step there is little competition when the wash step removes all competing bacteria.

There currently is no perfect test. The testing of a sample at any level (so we leave something to eat) by chance alone may not select the area, unit or component that is responsible for the contamination due to the non homogeneous distribution that bacteria and toxins tend to exhibit in food matrices (Roth et al., 2008). Testing or inspecting everything or removing all microbial involvement is not possible. A good test is only a part of a layered strategy of defense against food borne illness. Good sanitation, good confinement procedures, good testing at appropriate points with statistical value, temperature and humidity control at all points and separation are all layered components of food safety along the whole continuum.

Responses to Food CBRN terrorism

The unlikely or low designation for frequency but catastrophic or very high for severity of consequences for a food CBRN attack fit into the medium risk threat category demonstrated in figure 1.1. Medium risk threats require some form of risk mitigation. Mitigation procedures can be designed to deal with each leg of the crime triangle: victim, perpetrator and capable guardian. The victim leg of the crime triangle is difficult to mitigate but could be helped with education and adequate regulatory oversight to help people feel less vulnerable. Mitigation strategies for the perpetrator leg of the crime triangle include improving surveillance intelligence, predictive trigger event knowledge and improving governmental counterterrorism responses. A strong component to the ability of terrorist groups to develop the capability to use CBRN terrorism agents in the food supply, or elsewhere, is a permissive environment to develop them. (Parachini, 2003) Therefore, counterterrorism against this threat necessarily involves eliminating those environments by sanctions against state sponsors and assistance to weak states unable to control the terrorist organization. In the case of domestic terrorism, local law enforcement training to monitor the local groups as well as increased monitoring of the activities of these

domestic terrorist groups to detect patterns of preparation by FBI assets will assist in removal of permissive environments here at home (Chermak, Freilich & Shemtob, 2009). Miller (2007) concludes that no single category of policies is successful at dealing with terrorists, especially nationalist/separatist terrorists (the groups designated as ethnic separatists for this thesis). According to successful historical responses to ethnic separatist terrorism, three of five most effective methods are concessions, legal reform and restriction or combinations of those three. Violence and conciliation, the other two, resulted in dismal failure. For reactionary terrorists legal reforms alone have worked best in historical evaluation and for religious terrorists restriction work the best (Miller, 2007). For the capable guardian, food surveillance leg of the crime triangle, methods of prevention include developing duel use technologies that assist with every day threats and terrorism, like increasing detection capabilities of pathogenic microbial populations in food and strengthening the border control and inspection criteria for food imports. Regardless of whether another terrorist attack in the U.S. occurs in the near future or not this medium risk threat of food CBRN attack warrants the effort and attention of the scientific and governmental community. Food defense as a discipline needs to be developed and expanded to provide experts in a diverse, multidisciplinary field to assist in creating mitigation strategies that fit a unique perspective on protecting our food supply from intentional contamination.

Part II.

Hardening a target is a military term used to designate the attempts to present a less attractive target to those who wish to harm whatever you are trying to protect. Suggestions have been made in part I on other non enforcement hardening of targets that will also further direct the expenditures to places that have greater cost effectiveness and broader application for more pervasive threats. None the less, the medium risk of CBRN terrorism in the food supply requires

mitigation strategies. One potential area for improvement that broad application is improved testing of the food supply. In a letter to the United States Department of Agriculture, Food Safety and Inspection Service (USDA FSIS) in 2008 from a group of the meatpacker's associations states the industry's dislike of hold and test policies (Wenther et al., 2008). All five of the concerns these packers had dealt with the speed of testing and the scope of the hold status (Wenther et al., 2008). Regardless of the argument on testing alone being a solid food safety/defense policy or not, improving the speed and decreasing the cost of a diagnostic testing refutes all five arguments presented by the meat and poultry packing industry. Less cost allows more samples and a stronger statistical probability of safety on a wider portion of the produced food. Faster, if effective, testing allows for less hold time and less economic and spoilage concerns. While testing alone is not the answer, it provides a less biased evaluation of individual food safety and defense than sanitary inspection and environmental testing alone. Ultimately, companies are responsible for their own products and must protect their own brands. They cannot depend completely on government inspectors or third-party auditors to ensure authenticity and safety of materials and products (Zach et al., 2012). Decreasing the cost of a first line evaluation of food, should allow a food company to test a greater percentage of their product, protecting their bottom line in preventing recalls and their brand reputation in the market. Moving the first line testing of food to the farm will allow both regulatory agencies and supply chain managers to find problems earlier before combination at the production or packing plant, benefiting both food safety and food defense. Moving effective first line testing in food to the field allows military food safety professionals or even restaurant managers the ability to test more of the food at the level of the consumer, where most of the historical CBRN attacks have occurred no matter who the perpetrator was (Daizel, 2009).

The search for an efficient and effective field based test to allow monitoring of a greater percentage of the food supply at an affordable cost by the military, government or any food company is underway. The largest hurdles to developing such a test include food matrices, low contamination levels, low infective doses, competitive non pathogenic organisms, and field portability (Fung, 2008; Ge & Meng, 2009). Before going into detail about food specific tests, it is helpful to discuss the requirements for a field based diagnostic test at all. The World Health Organization (WHO) Sexually Transmitted Diseases Diagnostics Initiative uses the term 'ASSURED tests' to describe the ideal characteristics of a diagnostic test for a resource limited setting (Mabey, Peeling, Ustianowski, & Perkins, 2004). The following criteria are listed:

1. Affordable by those at risk of infection.
2. Sensitive (few false-negatives).
3. Specific (few false-positives).
4. User-friendly (simple to perform and requiring minimal training).
5. Rapid (to enable treatment at first visit)
6. Robust (does not require refrigerated storage).
7. Equipment-free.
8. Delivered to those who need it.

Mabey et al. also states that developing a portable, field ready diagnostic test that matches all eight criteria is very difficult, but should not prevent the development of a useful test in the interim (2004). There are multiple recent review articles that discuss the same criteria for food microbiological testing from the perspective of the microbiologist, the research perspective and the from the perspective of a food production manager, respectively (Fung, 2008; Ge & Meng, 2009; Jasson, Jacxsens, Luning, Rajkovic & Uyttendaele, 2010). In these review articles,

each of these professions support the need for many of the same criteria in food as the ASSURED process proposes for diagnostics. The largest hurdles to developing such a test include food matrices, low contamination levels, low infective doses, competitive non pathogenic organisms, and field portability.

Focusing on food defense, food itself is an impediment to testing just as it is an impediment to dispersion from the terrorist's perspective. The chemicals, natural and synthetic, compounds and bulk make extraction and detection of agents difficult. Fat and other biological components of food have inhibitors for reagents used in test kits and the sheer obstruction the particulate matter causes to extraction of chemicals or biological agents limits the utility of the equipment currently used for other sample types on the market (Adams & Moss, 2008; Fung, 2008; Ge & Meng, 2009; Jasson et al., 2010). Chemicals and biological agents also are not uniformly distributed in a sample of food, compounding the consistent extraction especially at low levels. Low levels of contamination are thus hard to extract and detect but given the right conditions can grow exponentially in the food itself (Adams & Moss, 2008; Fung, 2008; Ge & Meng, 2009; Jasson et al., 2010). The concentration of bacteria necessary to cause illness for some organisms is very small. For example, *E. coli* O157:H7, is a prominent food safety concern for food product evaluation. Its propensity to cause debilitation and loss of productivity as well as severe renal complications called Hemolytic Uremic Syndrome (HUS) make it a prominent List B CBRN terrorism pathogen (NIAID, 2009) and it has a very low median infective dose of 23 CFU (FSIS, 2001). It is increasingly the cause of outbreaks in multiple meat and non-meat food matrices (Scallan et al., 2011). In vivo, the pathogenic organisms are selected by environmental conditions and grow without competition. Overnight enrichment is required for all current approved testing protocols to overcome this low level of contamination

issue. Overnight enrichment, if non-selective, tends to multiply all the organisms present including the non pathogenic organisms. This leads to more difficulty in detecting the low levels of pathogenic organisms present due to natural competition (Adams & Moss, 2008; Fung, 2008; Ge & Meng, 2009; Jasson et al., 2010).

Food defense necessitates the ability to test food in resource limited settings. The majority of the food based incidents have occurred at the consumer level (Dalziel, 2009). Produce and other ready to eat products have no bacterial kill step from farm to consumer and mixed field loads spread contamination over a wider area (Erikson et al., 2010). To take detection as far forward on the battlefield and in the farm field as possible, the chosen test must be able to be stored with limited refrigeration, work in a dirty, wet environment with a battery or small generator (Jasson et al., 2010). It should be small, portable and light. Ideally for a military environment, but really any food company environment, the least amount of technical expertise and upkeep is necessary. These are the same as the ASSURED criteria show for diagnostic testing in resource limited settings (Mabey et al, 2004). There are no food based detection systems that fit this description on the market. Although sensitive, most of the currently available instrumentation does not work in a field environment (Jasson et al., 2010). Response to and prevention of these events are inadequate to stop terrorists without adequate, faster detection and surveillance. An intentional attack using a low level of contamination would be invisible to the current systems for up to 12 to 24 hours. In 12 to 24 hours the food is already eaten and the food safety system is involved in outbreak response with its associated recalls (Adams & Moss, 2008, Zach et al., 2012). In the field, with a deployed military population, it is imperative that fast, accurate detection and screening be possible. The offensive strategy of removing a unit from their mission with a food borne illness so other terrorist or insurgent activity can be

accomplished is a valid mission sustainment issue that must be considered by combatant commanders. In reality, this is not much different from the goals of some terrorist organizations for the civilian population. The goal of the Rajneesh attack on the salad bar in 1984 was to prevent people from voting in the election (Leitenberg, 2005).

Biosensors provide a unique potential solution to the requirements of an ideal test. Some excellent general overviews of different biosensors are covered in Nayak, Kotian, Marathe & Chakravortty (2009) and Lazcka, Del Campo, Mu˜noz (2007). The basic design of a biosensor includes a biological recognition element held on a capture platform combined with a signal generation and a signal receptor. There are seven types of biological recognition classifications of biosensors. These are shown in tables 1.1 to 1.3. In addition, there are two more means to categorize biosensors. One scheme is based on the materials or chemistry, as to what material you are using and the other scheme is based on the engineer or signal generation, as to how you see the product of capture.

Table 1.1: Receptor based classification of biosensor systems.

Receptor based classification
1. Enzyme based capture (glucometer)
2. DNA based capture (PCRs)
3. Antibody based capture (ELISAs)
4. Aptamer based capture
5. Molecular Imprinted Polymer (MIP) capture
6. Carbohydrate based capture
7. Bacteriophage based capture

Table 1.2: Material based classification of biosensor systems.

Material based classification
1. Silicon
2. Nano tube
3. Nano particles
4. Nano film
5. Gold and other metals
6. Polymers

Table 1.3: Signal based classification of biosensor systems.

Signal based classification
1. Conductometric (electrical)
2. Spectroscopic (optical)
3. Magnetic (atomic spin, proximity)
4. Piezoelectric (mass, impact, acoustics)
5. Potentiometric (Electrochemical)
6. Basoelectric (changes in mass, temp etc)

These components combine to create the unique biosensors seen in the market for different real time evaluations. The shear diversity of this relatively new field leads to a myriad of combinations to solve any diagnostic hurdle. The glucometer is the most famous biosensor on the market Theavnot, Toth, Durst & Wilson, 1999). A glucometer uses an enzyme to detect glucose in the blood and reports a signal in the form of an optical spectroscopic reading in the

machine to determine the level of glucose and convert it to a number the user sees on the screen. Enzymes and MIPs are efficient for chemicals but enzymes are not sufficiently specific for microbiological applications (Nayak et al., 2009) and MIPs are too specific for the biological variation of microbiological applications (Whitcombe, Alexander & Vulfson, 2000). MIPs are similar to an antibody only made out of plastic material. Aptamers are similar to a DNA primer map, only made from inert materials (Torres-Chavolla & Alocilja, 2009). Carbohydrates are stable, but specificity is an issue as many are used by many different bacteria and viruses in their in vivo attachment to mammalian cells (Abraham, Sun, Dale & Beachey, 1988). DNA and aptamer based sensors are highly environment sensitive, especially to small changes in the concentrations of the reagents (Hoorfar, et al., 2003; Ge & Meng, 2009). The same DNA sample in a two different thermocyclers for PCR will show different results as to the presence of the targeted DNA sequence (Hoorfar, et al., 2003) and aptamer science has had trouble with reproducibility from lab to lab (Torres-Chavolla & Alocilja, 2009). Antibodies are only as specific as the epitope they are developed to and are highly dependent on concentration of target (Jasson et al., 2010). Each of these analyte receptors have limits and positive aspects (Jasson et al., 2010). Different combinations of receptor, material and signal generation technologies are used to try and overcome the problems with the chemistry of each material and the physical requirements of each signal reporter as well as matrix, environment and logistical considerations of the food itself (Arora, Sindhu, Dilbaghi & Chaudhury, 2011; Nayak et al., 2009).

Food, with its unique challenges, is an much needed medium for biosensor development. Food based biosensor technology lags behind other diagnostics in the advancement of biosensor technology of any kind (Fung, 2008). Excellent food based biosensor overviews are available in Arora, et al., 2011; Ivnitski, Abdel-Hamid, Atanasov, Wilkins & Stricker, 2000; Leonard et al.,

2003; Wang, 2006; and Wei, Bailey, Andrew & Ryhanen, 2009. Fung states that the minimum requirement for food microbiology pathogen detection is 1 viable CFU in 25 grams of food (2008). The most glaring problem in the literature is the need for pre-enrichment to reach that level of analytical sensitivity. Pre-enrichment adds time, cost and amplification of non target bacteria, as discussed in part I of this introduction.

One potential solution to the problem of low level contamination is serial screening. Serial screening is a method of diagnostic testing that uses a highly sensitive pre-screen to collect a population of test subjects with a higher percentage of true positives followed by a more specific secondary screen (Hennekens & Buring, 1987). Serial screening such as this is common in public health applications and is formatted such that the first test has high sensitivity and low cost with the second test having increased specificity and typically greater cost than the first test in the series. The target for an ideal first line test would be 99 – 100% test sensitivity and 90% or greater test specificity (Hennekens & Buring, 1987). The overall objective of this research is to develop a biosensor to meet the needs of a field based food testing environment designing this biosensor as a first line screening test. To do so the three components of a biosensor extraction, immobilization and detection of the proposed biosensor will all be challenged using the AOAC Performance test methods validation protocol (AOAC PTM, 2009). In the following chapters, an analytically sensitive extraction protocol combined with a non selective carbohydrate coated platform using electrochemical detection will be developed and challenged in a validation study using broth and then milk as a food matrix.

The move to prevention based postures in the response to CBRN terrorism in food or any other vehicle requires the examination of all three legs of the crime triangle. This thesis proposes changes to improve the capable guardian leg of the crime triangle with adequate food

based testing techniques and to the evaluation of the perpetrator leg of the crime triangle in narrowing the field of organizations that food CBRN would be a viable tactical option. Both will advance the knowledge base of their respective fields of study to allow a more effective protective posture.

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CHAPTER 2: CBRN Terrorism: A Critical Quantitative Analysis of Group Components Necessary to Predict Use of Food CBRN Terrorism Agents

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Abstract

Few studies have explored the factors that distinguish groups that will use Chemical, Biological, Radiological or Nuclear (CBRN) terrorism involving the food supply. We examine three categories of factors on CBRN terrorist groups who use the food supply as a vehicle: (1) organizational capacity, (2) strategic connectivity and (3) motivational factors. Ethno-separatist ideology and increased cultural embeddedness within the global culture both show significant results as predictors of food based CBRN attacks by an organization in both models ($p = 0.1$). When the organizations that performed or attempted a CBRN attack in other vehicles are removed, increased degree of connectedness to other organizations and democratic regime type become significant predictors of food based CBRN attacks. By identifying factors that distinguish food CBRN user organizations from non-food CBRN user organizations, this study helps us better understand characteristics of CBRN terrorism involving the food supply in the United States.

Introduction

The terrorist attacks on the United States of America on September 11, 2001 (9-11) forever changed the face of our country. The New York skyline will never be the same and neither will our collective perspective on the threats that can affect our way of life. Never before was there a successful terrorist attack of that magnitude on American soil, and American society has changed. To that end, the terrorists were successful. Community emergency preparedness and critical infrastructure protection were concepts that were not really discussed; now they are common in multiple public and private disciplines. Among those critical infrastructures is our nation's food supply. The Bush Administration enacted Homeland Security Presidential Directive 7 (HSPD-7) and HSPD-9, *Defense of US Agriculture and Food* in 2004 (Moteff & Parfomak, 2004). This was the first time agriculture and our food and water supply were formally considered critical to our economy and society. As such, the programs and initiatives at the national, state or local levels were not in place to provide that protection. To be frank, the concept of "defense" of these areas was so new, where to start was the real problem. Chemical, Biological, Radiological or Nuclear (CBRN) terrorism has captured the attention of thousands of food safety practitioners, industry planners and government policy makers since 9-11 opened our eyes that we could be hit by terrorism at home. Key to all good food safety and defense programs, in fact emergency management programs in general, is risk assessment.

Current thought on CBRN terrorism in food borders on panic at the seemingly rampant unpreparedness of our nation's food supply to natural recurrent contaminations let alone intentional ones. Billions of dollars in research is invested by public and private enterprises every year to develop methods to strengthen our detection, response, mitigation and recovery capabilities. Still the knowledge base about the current threat of CBRN, let alone food based

CBRN is nebulous at best. In an age where anything is being considered possible, realistic evaluation of current threat level is required. The crime triangle is a criminal justice concept involving interactions between a perpetrator, a victim and absence of a capable guardian for a crime to occur, called the chemistry of the crime (Felson & Clarke, 1998). How likely an event is, must include discussions of those three interacting agents to differentiate likelihood (whether a event will happen) from plausibility (whether an event could happen). Subsequently, those same topics are used to develop recommendations to what needs to be discovered or considered, to develop effective assessment tools that determine the capacity of terrorist organizations to commit a CBRN attack. This research seeks to evaluate, at the organizational level, those factors of the perpetrator leg of the crime triangle that affect the decision to use CBRN in the food supply. With this information, realistic risk evaluations will be easier to perform and law enforcement intelligence assets can be better focused. This quantitative evaluation of the existing historical record of CBRN terrorism using food as a vehicle will examine organizations that pursued/used CBRN with food vehicle verses organizations that performed other non CBRN terrorist attacks combined with organizations that performed other CBRN terrorist attacks during the time frame 1992 to 2005. The goal is to determine if the available data can show if the same organizational factors that were significant to the whole of CBRN using organizations holds up to those choosing food as a vehicle. To do so, an expanded version of the dataset designed and used by Drs. Victor Asal, Gary Ackerman & Karl Rethemeyer for their paper, "Connections can be toxic: Terrorist Organizational Factors and the Pursuit of CBRN Weapons" (2012), is examined.

Background

A key problem with terrorism study is the lack of an accepted, inclusive, international definition. This question has academics, politicians, media and public opinion at odds with each other. Studying, countering and avoiding terrorism require a definition to develop policy for counter measures. Terrorism can be defined as an ineluctably political, violent and designed to have psychological repercussions criminal acts, being used to fight for political power or to maintain political status quo by a non state entities (Hutchison, 2006). With such a definition, terrorism is defined by the group who is using the tactics. If that is the case, an international definition will never be agreed upon. With or without an accepted definition of terrorism, research into this phenomenon must continue. The Rules of War delineate certain prohibited forms of dealing with opponents, in particular: massacres of noncombatants, and taking of hostages for extortion, blackmail and intimidation; assassination of unarmed people; torture of prisoners; and disappearances (Schmid, 2004). Terrorist groups may attack military targets, but their deliberate selection of civilian targets is what defines their organization as a terrorist group. Terrorism includes violent reactions to a power differential. According to Crenshaw's *The Causes of Terrorism* (1981), in order for enough unrest to occur to incite terrorism, there has to be a power differential. Repression of alternate viewpoints and opponents or the shirking of oppressive pressure on a group is required for terrorism to manifest itself. The Irish Republican Army (IRA) in the 1920's is an example of a group using terrorist tactics to shirk oppressive pressure. The al Qaeda network believes it is shirking the oppressive pressure of the infidels on their religion and way of life. Non violent extremist or revolutionists are not terrorists until they use violence as the means to an end. For instance, The Humane Society of America is not a terrorist group and does not condone violent behavior, but their counterparts the Animal

Liberation Front (ALF) and People for the Ethical Treatment of Animals (PETA) do support and perpetrate violent dramatic events. ALF and PETA have bombed laboratories and harassed scientists by blowing up their cars. CBRN terrorism in any form fits into refusal to follow the Rules of War. The definition used for this document is the one used by the Monterey WMD Terrorism Database: "Terrorism is violence, or the threat of violence, calculated to create an atmosphere of fear and alarm. These acts are designed to coerce others into actions they would not otherwise undertake, or refrain from actions they desire to take…" (2012).

Almost all discussions of CBRN events in our food supply center around case studies like the 1984 Oregon cult attack on salad bars with *Salmonella* and documents identified in the training camps of Afghanistan. These documents seemed to be brainstorming sessions on CBRN terrorism agents and modes of dissemination. Curiously, they are in English. The Oregon cult attack is the only large scale identified CBRN terrorism event in food propagated by a terrorist organization in history. None the less, these two incidents combined with the intelligence mistakes prior to 9-11 sparked a frenzy of concern and spending to prevent or at minimum detect this nebulous threat. Missing from all of this is solid analysis of the risk itself and from what sector of the multiple terrorist types that risk is likely to come from. This project seeks to investigate which of these terrorist organizations has a higher risk of being the group to pull off a large scale CBRN event using food as a vehicle, should such a thing be possible. It will also evaluate several other organizational factors necessary. CBRN terrorism is not as easy as bombs and guns are. There must be some degree of knowledge of organisms, microbiology and food stuffs beyond high school biology to effectively design and carry out such an attack.

CBRN terrorism in food is an attractive tactic for a terrorist group. It is an easy way to spread terror and the recognition of the terrorist's cause all over the world, carried by the global

food supply. The results go way beyond anyone they make sick or kill (Rothe, & Muzzatti, 2004). Food in any country in the 21st century comes from all over the world. Everyone eats and drinks and cannot survive without it. In fact research by Dusselier in 2009 supports food as a cultural evaluation tool and a social modifier highlighting its importance in society as more than just sustenance. Foods are processed and shipped further and faster than ever before (Zach, Doyle, Bier, Cxuprynski, 2012). Senate hearings in May of 1998 concluded that 33% of all fruit comes from outside of U.S. and 12% of all vegetables are imported. The media attention focused on any natural food borne disease outbreak borders on hysteria. An example is the peanut butter associated *Salmonella* outbreak in 2008/2009. The world coverage of an American food safety issue was astounding. The outbreak was on every TV news show for the entire fall and spring. Panic caused avoidance of any food containing peanut butter, and economic impacts on companies producing peanut butter were significant. This outbreak also served to highlight the vulnerabilities in the food safety network of the US. This peanut plant that caused all the trouble was inspected by the FDA or the Georgia Department of Agriculture every year (Minor, 2009). The global food supply has inherent weaknesses in its supply chain ranging from smuggling and grey market trade to counterfeit (Roth, Tsay, Pullman, & Gray, 2008). All are viable methods available to adulterate and return to the supply chain contaminated food. Many of the illicit food trade practices are already being used to fund terrorist and organized crime syndicates (Hutchison & O'Malley, 2007). If they are using the illicit food trade practices to raise money, they have access to adulterate the food and return it to the food supply outside the traditional food safety protective network.

Given the advantages to using CBRN terrorism in the food supply as a tactic for a terrorist group and the global food supply's vulnerability to it, it makes sense to discuss the

probability of it occurring despite the difficulty. Mohtadi and Murshid (2009) do just that. The number of CBRN terrorist incidents used in their evaluations was 448 over a 53 year period. These counts were used in trend analysis that showed terrorist attacks on soft targets increasing while high profile targets are decreasing. They also show the dramatic increase in CBRN incidents over the last decades from zero in the 1960s to pushing forty in the 2000-2005 era. The end result was a probability model predicting continued increasing frequency and decreasing recurrence period in both catastrophic terrorist attacks and CBRN terrorist attacks (Mohtadi & Murshid, 2009). If probability of CBRN terrorist attacks in general is increasing, scientific knowledge is increasing and the vulnerable, global nature of the food supply is increasing, a good viable model for prediction of which groups will use this unconventional strategy is necessary to even begin a viable risk assessment for informed mitigation to occur.

Asal, et al., (2012) does an excellent job summarizing the combined literature base of CBRN terrorism study. They state that there is a disproportionate number of studies that focus on attainment potential of CBRN agents and response to the consequences of such. We have found the same from agro-terrorism to law enforcement intelligence training (Monke, 2004, Knowles et al., 2005). Lafree and Freilich (2012) found the same in the general terrorism literature stating that less than 3% of studies use any statistical evaluation. The general consensus of the small number of articles that deal with the motivation of a group or individual conclude that many types of organizations have considered CBRN, the motivational incentives cover a wide range and that the technical knowledge diffusion of the 21st century are adding to the ease of CBRN use. As Asal and his colleagues (2012) have noted, examining the useful predictors of the decision to pursue CBRN is more difficult with available data.

Most food based attacks listed in terrorism incident lists, or anywhere else, are on a very small scale such as individual attacks on coworkers, relatives or employer's interests (Dalziel, 2009). Those attacks are difficult to find and examine because many never make the news or are considered criminal matters and do not make terrorism act lists (Chermak & Gruenewald, 2006; Dalziel, 2009). They also only lend themselves to case studies at an individual level because many are unclaimed by organizations (Asal et al., 2012). They also cannot be examined here because they are not usually members of an organization. This work focuses on organizational factors. The organizational factors important to food CBRN can be divided into categories. The categories chosen for this analysis are (1) organizational capacity, (2) strategic connectivity and (3) motivational factors.

Organizational capacity

Crenshaw (1981) established several short term goals for use of a terrorist strategy. Most notably those goals are impatience, weakness, recognition, tyranny, discredit of the opponent and oppression. For CBRN terrorism to be chosen, the short term and long term goals of the terrorist organization are not being met. Traditional tactics, bombing and guns, will prevail until such a time as the terrorists goals begin to stagnate. With that stagnation, new forms of terrorism will be explored to move the cause forward. In a study of 13 far-right hate groups it was found that the age of the organization was positively and significantly related to the number of ideologically motivated homicide events (Caspi, 2010). Ganor promotes a typology of activity based on the level of motivation and the level of operational capability of the organization (2008). Any terrorist group must have sufficient motivation and operational capability to cross the terrorist activity threshold. If one is high and the other low, no terrorist activity can occur (Ganor, 2008). The operational capacity of an organization, as with most organizations increases with size and

age and experience. Therefore, the *size*, *age,* and *experience* of an organization will affect the potential for use of CBRN using food as a vehicle.

Parachini's analysis of CBRN cases supported the conclusions that "The mindset of leadership, opportunity, and technical capacity are the factors that most significantly influence a group's propensity to seek to acquire and to use unconventional weapons." (Parachini, 2003, p. 7). The mindset of the leader refers to their obsession with a particular type of weapon; as with Aum Shinrikyo's sarin attack. Shoko Asahara was obsessed with poison to the exclusion of other forms of violence. Leadership mindset deals also with religious or social constraints on the terrorist group from their support base. Leadership mindset is a fluid component, difficult to evaluate over time. While it may be a significant factor, the available data does not allow its evaluation to go beyond case studies at this time. Post, Ruby and Shaw (2002) propose that evaluation of the psychology of the leader requires more specific information than is usually available in the open literature. Opportunity requires the uninterrupted time to develop and propagate sufficient operational capability to deliver enough of an impact to outweigh the negative effects of CBRN use on the national scale and outweigh the proven effectiveness of conventional weapons. Opportunity may also refer to opportunistic availability in time and place like procuring weapons grade CBRN by accident or provided by a state sponsor. *State sponsorship* and *territorial control* and *host state regime type* are measureable opportunity variables at the organizational level. *Territorial control* allows for time and space for development. Remote unnoticed locations should be a positive influence on the decision to use CBRN in food as that is where the food is grown, harvested and shipped. It is also the area of least control and patrol by law enforcement (Knowles et al., 2005). The lower ability to patrol and control those areas in any country should positively impact the time and freedom to work on

development of CBRN capabilities. *State sponsorship* could allow access to greater resources and technical expertise as well as provide the unfettered area to work as Al Qaeda had in Sudan and Afghanistan. It has been proposed that *host state regime type* greatly affects the ability of terrorists to move around and go unnoticed, with democratic regimes being the easiest to operate in due to their focus on individual freedom (Schmid, 2004). Host state regime type could affect the ability of a terrorist organization to use CBRN.

Ganor from the operational perspective and Parachini from the case study perspective both point to the most daunting deterrence to use of CBRN terrorism, the technical knowledge necessary to build the operational capability. Knowledge of food and the microbes in food, the equipment to propagate the microbes, the volume of organisms necessary to pull off a widespread attack and access to the organism with the appropriate dissemination platform are all large deterrents. Evaluating capability of organizations requires evaluation of their membership, which is secretive in most cases. Post et al. (2002) find the internal workings of a group to be consistently significant on the prediction of violence across all ideological categories of terrorism. Group members with science based higher education might be necessary for CBRN terrorism to occur. Members with military backgrounds might have received CBRN related training. Terrorist groups from countries that lack solid scientific backgrounds among the recruiting population will be unable to plan, coordinate and carry out a food CBRN terrorism event. Being unable to measure actual membership education or military background at this time, the proxy for this analysis is the *technical knowledge of the host country* the terrorist group resides in.

Another predictor of the use of CBRN terrorism as a tactic is domestic verses international. In domestic terrorism, the perpetrators, victims and audience are all from the

country the terrorists are a part of. It is the most frequent form of terrorism, but gets the least amount of attention because countries are expected to handle it themselves. Domestic terrorist attacks outnumber international 7:1 regardless of the typology or target (Lafree, Yang & Crenshaw , 2009). Lafree proposes several reasons for this including supply chain, preference to work at home (logistical knowledge) and expense. The volatility and difficulty of movement for CBRN terrorism agents as well as the domestic operational preference of all groups will ensure that domestic CBRN terrorism is the more important group of the two for a threat assessment. Although domestic based terrorism is hypothesized to be the predominate threat for CBRN terrorism in food or otherwise, many international organizations have branches and recruitment within other countries and large Diaspora populations as well. Therefore, the actual country of origin is not included in this analysis.

The organizational capacity factors under evaluation include *size, age, experience*, *state sponsorship*, *territorial control, host state regime type,* and *technical knowledge of the host country.* For all of these factors food based CBRN is no different than other forms of CBRN and our results should follow those of Asal in the larger comparison study. Asal et al. (2012) found no impact for state sponsorship of the terrorist organization in the larger CBRN organizational evaluation. We hypothesize that state sponsorship will also show no impact on the use of food based CBRN. Host state regime type is not specific to food based CBRN and therefore we hypothesize it will repeat the same trend seen by Asal's group. That is no impact. It has been hypothesized that host state level of technological development would lead to a positive impact on the decision to employ CBRN, but the author's qualitative research shows, recruitment can occur anywhere in the world for locally based attacks. Locally based attacks are statistically more common and for food based CBRN the easier to attack. Thus the host state's technological

advancement is irrelevant to food based CBRN. We hypothesize that our analysis of food based CBRN will follow that of Asal's; no significant effect.

Strategic connectivity

Terrorist also use methods to multiply the effect of the terrorist attack called force multipliers (White, 2001). Force multipliers could be any format that enhances the ability of the terrorist group to advance its agenda beyond its real capabilities. A direct force multiplier of CBRN terrorism against the food supply is that food and water are used by everyone. There is no need to kill or even actually perform the act to get the desired fear and avoidance creating an economic impact worldwide, death just enhances the effect. CBRN terrorism events in the food supply are also difficult to detect early and distinguish from naturally occurring outbreaks. This also multiplies the effect by increasing the fear and anxiety of the target population. Media is a powerful force multiplier. The media react to naturally occurring food safety outbreaks with zealous coverage. They also react to select terrorist attacks around the world with the same focused zeal (Rothe & Muzatti, 2004). The combination of the two would dominate coverage and ensure worldwide fear, withdrawal and economic disruption. *Host state level of embeddedness* in the global economy is a measure of access both to the global food supply and to the global media as force multipliers. This study uses the number of McDonald's restaurants in the host state as a proxy for embeddedness in global culture.

Organizational structure of the terrorist group is important to the discussion of CBRN terrorism in the food supply. A highly paramilitary hierarchical organization would not be conducive to the ability to work undetected long enough to develop CBRN terrorism agents and have the technical knowledge necessary for unconventional tactics. On the other hand, a larger, loosely associated organization with leaderless resistance nodes and a central ideological center

node that develops and conveys strategic influence would be perfect. Strategic development occurs at the central node where finances and talent pools are largest and is communicated to the loose nodes with a planned start date via the internet. Horowitz (2010) and Caspi (2010) both found strategic ties to increase the spread of the suicide bombing terrorist tactics and homicide activity by hate groups, respectively. Therefore strategic ties may be essential for the type of shared knowledge and scope of reach needed to carry out a successful CBRN attack in food. The delayed reaction of CBRN terrorism agents in food allows a staggered start approach to be very effective. Such a staggered start would make diagnosis of a CBRN terrorist attack against the food supply even more difficult to attribute to terrorism verses natural outbreak (Jackson, 2006). Therefore, *interconnectedness* between organizations will likely have a large effect on the use of CBRN in the food supply and is another factor that could overcome the technical difficulty.

The strategic connectivity factors under evaluation include host country embeddedness in global culture, the degree of connectedness to other organizations, and the centrality of that connectedness to other organizations. As discussed under the attraction of food based CBRN attacks, the global food supply is an advantage to a terrorist planning a food based attack and we hypothesize that global embeddeness will positively impact the use of CBRN in food. This follows what the Asal group found in the larger CBRN organization population. Both degree of connectedness and centrality of connectedness are hypothesized to have a significant positive effect on the use of CBRN in the food supply.

Motivational

Many authors have attempted to divide the diverse spectrum of terrorist groups into categories to make evaluation easier. Crenshaw (1981) uses a motivational typology of reformist, anarchist, reactionist, revolutionist, nationalist and separatist. Byman (1998) considers

nationalist/separatist groups to be ethnic terrorists. These methods are used to attempt to understand, prevent and counter the groups in question. Not all forms of terrorism lend themselves to CBRN terrorism tactics, local or transnational. Reformists seek to halt social activities they are against like anti abortion groups or animal rights groups. These single focus groups may be terrorists, but widespread death and economic damage rarely advances their causes. They often target buildings and vehicles, usually trying to ensure no people get hurt. Although anti abortion groups have killed doctors who perform abortions, the large scale mass destruction tactics are not normal for these groups. They also place a high value on life, including the human animal life. The use of CBRN terrorism agents in food would be unusual for this ideological group. Reactionists are reacting to a social counter group trying to prevent changes to the status quo. The Ulster Defence Association in Northern Ireland is one of those. Since reactionists are part of the status quo, it seems unlikely that widespread use of CBRN terrorism agents would promote their cause either. Their tactics involve reaction to another terrorist group that threatens their state, not to society at large.

Revolutionaries, nationalists, minority or ethnic separatists and anarchists or millenarian groups each pose a greater risk for use of CBRN terrorism agents in food. Revolutionaries want to change the social order of an existing state. Nationalists want autonomy from an occupying force, minority or ethnic/religious separatists want their own state with autonomy and usually ethnic or religious purity and anarchists or millenarian groups envision anarchy or the end of the world with them as the divinely chosen survivors (Crenshaw, 1981; Ganor, 2008; Byman, 1998, Ranstorp, 1996). Nationalists and Revolutionaries have the use of terrorism to meet distinct political goals in tandem with those that oppose them, consistent between them. They may use any terrorist tactic to obtain those goals, but they are not generally mass casualty, wipe out as

many as possible oriented events. Political pressure, international recognition and support, fear and unrest in the target population yes, widespread death and destruction, no. Once released, CBRN terrorism agents as well as other CBRN agents are hard to control and keep from affecting the entire country, not just the targets. These two groups like the reactionists and reformists need public opinion to sway toward them with sympathy for their cause. Society as a whole has a revulsion to CBRN agents which decreases the likelihood for their use by groups merely seeking dramatic attention gaining activities (Garret, 2001).

Ethnic or religious separatists and anarchist or millenarian terrorists are a different story. These two groups contain deep seated racism, end of times apocalyptic religious belief, or a tendency toward anarchy as a social organization. Religious separatists and millenarian groups consider their audience the divine (Crenshaw, 2000; Ranstorp, 1996). Ethnic or religious separatists condone genocide of the opposing race/religion or at minimum homogenization of their own (Byman, 1998; Ranstorp, 1996). Anarchists seek destruction of all social order. All remove the social and political constraints for the use of CBRN, by removing the need for international attention and sympathy. Al Qaeda is a religious separatist organization seeking destruction of the infidels and creation of a homogeneous Muslim world. Aum Shinrikyo was a religious/millenarian cult that believed the end times could be hastened by a CBRN attack that they were supposed to perpetrate, to bring about a new world they would lead (Parachini, 2003; Ranstorp, 1996). Right winged white supremacy groups like the National Alliance believe the Jews have hijacked our society and must be violently opposed in order to bring about a homogeneous white society (Chermak, Freilich, Shemtob, 2009; Ranstorp, 1996). Ethnic/religious separatists and anarchist/millenarian groups or combinations thereof are the most likely to be the perpetrators of a CBRN attack on the food supply.

The motivational factors under evaluation in this study include those organizations containing *religion*, containing *ethno-separatists*, those containing *religion with ethno-separatists* and those containing *leftist* organizations. Here is where the most significant differences are expected from the results obtained in the larger Asal study. The qualitative analysis of organization typology discussed above proposes that organizations including ethnic/religious separatist ideology or anarchist/millenarian ideology or combinations thereof would be more likely to for food based CBRN attacks. Asal's quantitative comparisons of all CBRN attacks in the timeframe conducted by organizations with available data failed to show a correlation with religious ideology. We hypothesize that the lack of correlation between CBRN terrorism and religion is due to the common inclusion of religion in a group's ideology in order to appeal to their constituent group. This inclusion, but in an ancillary position may dilute the effect of religion as a motivating factor for CBRN use. More importantly, the distinction made earlier in this work of the separatist nature of the religion or ethnic group is an important confounder that may have masked the significance of religion on the predictability of CBRN use especially in food.

Data and Methodology

Making use of an expanded version of the dataset from Asal, Ackerman and Rethemeyer, (2012), food based CBRN attacks will be evaluated against other terrorist attacks during the time period of 1992 to 2005. This dataset was developed out of the Monterey Weapons of Mass Destruction (WMD) Terrorism Database and the comparison dataset for non CBRN terrorist incidences is developed out of the Memorial Institute for the Prevention of Terrorism (MIPT) Terrorism Knowledge Base (TKB) merged with the National Consortium for the Study of Terrorism and Responses to Terrorism (START) Global Terrorism Database (GTD). The

dependant variable for this study is organizations that used CBRN in food. It is measured as a dichotomous variable, 1 = organizations that used food as a vehicle and performed a CBRN, 0 = organizations that performed other terrorist attack. The dependent variable allows us to compare characteristics of organizations that performed a CBRN attack with food vehicles to organizations that attack using other means. Of the 396 qualifying organizations that propagated attacks during the 1992 – 2005 timeframe, only 5 of those used food as a vehicle. These are listed in table 2.1. When the 21 qualifying organizations that propagated other CBRN attacks in the data set are removed (Table 2.2), there are 375 organizations left to compare to the same five food CBRN organizations for model 2. The purpose for removing the 21 organizations that used other vehicles for CBRN attacks from the control group is to determine if there are organizational factors common to all organizations that use CBRN that may be hidden when they are considered as part of the control group of non food CBRN terrorist organizations.

Table 2.1**:** Organizations that used CBRN terrorism in food 1992–2005

Organization	Age (yrs)	Size (members)	Home
al-Aqsa Martyrs Brigades	6	1-99	Israel
al-Qaeda in the Land of the Two Rivers	30	1000-9999	Iraq
Animal Liberation Front (ALF)	2	1-99	U. S.
Liberation Tigers of Tamil Eelam (LTTE)	4	100-9999	Sri Lanka
Riyad us-Saliheyn Martyrs' Brigade-Chechen rebels	30	100-999	Russia (Chechnya)

Table 2.2: Organizations that use/pursued CBRN terrorism, non-food 1992–2005.

Organization	Age (yrs)	Size (members)	Home
al-Qaeda	19	1000 -9999	Pakistan-Afghanistan
Ansar al-Islam	5	100-999	Iraq
Armed Islamic Group	14	100-999	Algeria
Babbar Khalsa International (BKI)	28	100-999	India
Basque Fatherland and Freedom (ETA)	47	100-999	Spain
East Turkistan Liberation Organization	4	100-999	China
Hamas	19	>10000	Israel (Gaza/West Bank)
Hezbollah	24	>10000	Lebanon
Jaish-e-Mohammad (JeM)	6	100-999	Pakistan
Jamiat ul-Mujahedin (JuM)	16	100-999	Pakistan
Jemaah Islamiya (JI)	13	100-999	Southeast Asia
Kurdistan Workers' Party (PKK)	32	1000 -9999	Turkey
Lashkar-e-Jhangvi (LeJ)	10	100-999	Pakistan
Lashkar-e-Taiba (LeT)	6	100-999	Pakistan
Mujahedin-e-Khalq (MeK)	43	100-999	Iraq
Palestinian Islamic Jihad (PIJ)	29	100-999	Israel (Gaza/West Bank)
Real Irish Republican Army (RIRA)	8	100-999	UK (N. Ireland)
Revolutionary Armed Forces of Colombia (FARC)	42	>10000	Colombia
Tawhid and Jihad	9	1 - 99	Iraq
UNITA	40	>10000	Angola

The independent variables in this study are organizational factors of the two categories of organizations, those that used food CBRN for attacks and those that do not. They are divided into three major categories organizational capacity factors, strategic capacity factors and motivational factors. Each continuous variable had to be divided into approximately equal categories to be able to meet the statistical requirements of the multivariable logistical regression.

Organizational capacity factors

Under the category of organizational capacity, there are seven factors evaluated size, age, experience, state sponsorship, host state regime type, technological advancement and territorial control. The size of the organization was broken into categories based on the population size of the membership directly involved in the organization. The categories are 1-1000 and >1000. The organization's age is designated by the integer number of years the organization was active from the year of founding through 2005. The ages were evaluated in blocks of 0-5 years, 5- 10 and >10 years old. The average age of all the organizations was 11 years old with a range from 1 to 87. The organization's terrorism experience was determined by the number of attacks of any kind each organization performed between 1992 and 2005. Experienced organizations had >4 attacks and inexperienced organizations conducted <3 attacks. Approximately 25% of the organizations in the dataset had greater than 4 attacks attributed to them. State sponsorship was defined as the existence of financial transfers from a state to the organization. Those organizations that had any financial transfers were considered sponsored in a dichotomous variable evaluation of state sponsorship, 8 % were state sponsored. Host state regime type was measured from the POLITY2 database which varied from strongly democratic at 10 to strongly

autocratic at -10. The dataset available was coded present or absent for the autocratic or democratic and those with no code were moved to the moderate category. A final categorical variable was developed using moderate regime type as the reference category. When divided, 9.6% were autocratic regime type, 17.7% were moderate and 72.7% were democratic regime types. The host state embeddedness in global culture is a continuous variable measured by the number of McDonalds restaurants present in the host country in the year 1998. This data is obtained from the McDonalds Corporation's corporate reports. It was divided into categories of < 100, 101 to 1000 and > 1000 with <100 being the reference category. The mean number of McDonalds restaurant's in the home states of the organizations in the dataset was 463 with a range from 0 to 13,732. Sixty six percent were less than 100 restaurants. The organizations' host states were numerically coded variables to account for the home state the terrorist organizations claimed in their literature. Seventy one countries are represented in this dataset with Greece having the highest number of attacks at 72 followed by Iraq and India at 31 and 30 respectively. Territorial control was determined by the amount of uninhibited territorial control the organization has within their home state, not the locality of the controlled territory as far as rural or urban. Only 10.9% of the organizations held territory. The host state's technological advancement was measured by the energy use per person in the host country's population. It was also divided into a categories of <1, 1-5 and >5 with a mean of 0.73. Fifty percent of the countries in the dataset used <1 units of energy per person with the other categories containing 27% and 22% respectively. Table 2.3 summarizes all of the variables per model with the exception of the country of origin as there were too many individual countries to include in the table. Categorical variables with three categories used the first category as the reference group for the multivariable logistical regression analysis.

Strategic connectivity factor:

Centrality of embeddedness with other organizations represents a conglomeration of the TKB's related groups section using a six code system modified into a sociomatrix. Bonacich (1972) developed a sociomatrix approach to measure the centrality of an organization's connections and is described in detail for this application in Asal's paper (2012). This variable was dependent on the centrality of the node being measured and the centrality of the nodes it is connected to, called an eigenvector score. The more centrally connected the group was, the higher the score. This variable was divided into the categories of <1, 1-10 and >11 with a mean of 0.29. Of the eigenvector scores, 80% were <1 whereas 12% were from 1-10 and 9% >11. The second measure of strategic connectivity is the degree of connectedness to other organizations. It was a direct measure of the number of other organizations the organization in question worked with or associated with. It also used a conglomeration of the TKB's related groups section using the same six code system. The more willing a group is to work with other organizations the higher the number of those associations. This factor was divided into categories of 0-2, 3-8 and >8. Percentage wise 81% were in the first category followed by 18% and 1%. The descriptive results are included in table 2.3. Both strategic connectivity variables used the first category as the reference group for the multivariable logistical regression analysis.

Motivational factors

The organizational ideological variables are religious, ethno-separatists, religion combined with ethno-separatists and leftist groups with no religious or ethno-separatist component. Religion was assigned a yes if any degree of religious ideology was present. Each organization could be coded as more than one ideological component after religion. Religion combined with ethno-separatism is a variable to capture those organizations with both

ideological components. Of these dichotomous variables 29% contained religion, 39% contained ethnoseperatist ideology, 16% contained both and 24% were solely leftist ideology. Table 2.3 contains the demographics by model.

Table 2.3: Demographics and summary statistics of the variables in each model.

Model	**1**	n=395		**2**	n =375	
Dependent Variable: Used food CBRN organizations		no	yes		no	yes
		390	5		370	5
Independent Variables:						
Size	<1000	335	3		321	3
	>1000	55	2		49	2
Experience	≤ 3 attacks	294	0		291	0
	> 4 attacks	96	5		79	5
State sponsorship	no	359	4		344	4
	yes	31	1		26	1
Territorial control	no	348	4		335	4
	yes	42	1		35	1
Host state regime	Moderate	22	2		64	2
	Autocratic	83	1		33	1
	Democratic	285	2		273	2
Age	0-5	169	2		167	2
	5-10	105	1		100	1
	>10	116	2		103	2

Table 2.3 (cont'd)

Model	**1**	n=395		2	n =375	
Technical knowledge	<2	194	2		179	2
	2-4	109	1		105	1
	>4	87	2		86	2
Embeddedness in global culture	0-100	260	2		246	2
	101-1999	120	2		114	2
	>2000	10	1		10	1
Degree of connectedness	0-2	318	3		311	3
	3-8	68	1		58	1
	>8	4	1		1	1
Centrality of connectedness	<1	311	2		304	2
	1-10	47	1		44	1
	>11	32	2		22	2
Religious	no	276	3		269	3
	yes	114	2		101	2
Ethno-separatists	no	241	1		234	1
	yes	149	4		136	4
Religion & ethno-separatists	no	330	2		319	2
	yes	60	3		51	3
Leftist	no	296	5		279	5
	yes	94	0		91	0

Statistical evaluation

The likelihood that a terrorist organization would take part in a food based CBRN attack was evaluated using a multivariable logistic regression IBM SPSS Software. All variables of interest were initially assessed against the logs odds through univariable analyses against the log odds of conducting a food CBRN attack. Any variable with a p-value < 0.25 was assessed for significance in the multivariable model. Stepwise selection (forward selection: alpha = 0.05; backward selection: alpha = 0.1) was used to determine the final model and variables that were kept if their significance was <0.1 and run in the unadjusted multivariate model. Confounding was evaluated by assessing the difference in beta coefficients in the adjusted and unadjusted models. Variables were determined to be confounders if their presence resulted in a $\geq 15\%$ difference in the beta coefficients of the significant variables and they were significant in the univariable analysis. Interactions of importance to the researcher were assessed with an $\alpha = 0.1$. Continuous variables in the final model were the assessed against the assumption of linearity with the logit by plotting the median of the quartiles against the predicted logit via simple linear regression at an alpha =0.05. If the continuous variables were not found to be linear, they were converted to categorical variables, attempting to keep the categories somewhat even. Each categorical variable had a reference group assigned. The reference group is designated as the first category in table 2.3. Finally, the Hosmer-Lemeshow Goodness of Fit test was used to evaluate the fit of the model to the data at an alpha = 0.05. The entire analysis was repeated removing the 21 organizations listed in the Asal et al. study that used or pursued CBRN in other vehicles from the non food CBRN organization list (2012).

Results

During the evaluation it was noted that all the continuous variables did not meet the assumption of linearity. Therefore they were all converted to categorical variables. The univariate analysis results are shown in Table 2.4.

Table 2.4: Model 1. Univariate and Multivariable analysis for Model 1: Food CBRN organizations verse all other active terrorist organizations in the time frame and Model 2: Food CBRN organizations verse other non-CBRN terrorist organizations. (* denotes models with 1 degree of freedom, ** denotes models with 2 degree of freedom).

	Model 1		Model 2	
Variable	Univariate analysis: p > 0.25	Multivariable model p > 0.1	Univariate analysis: p > 0.25	Multivariable model p > 0.1
Organizational capacity				
Size dichotomous *	0.013	0.859	0.111	0.530
Experience *	0.994		0.994	
State sponsorship *	0.348		0.292	
Territorial control *	0.519		0.441	
Regime type **	0.257	0.541	0.112	.104
(1) *	0.009	0.284	0.539	.802
(2) *	0.06	0.430	0.038	.059
Age **	0.874		0.831	
(1) *	0.709		0.632	
(2) *	0.630		0.590	

Table 2.4 (cont'd)

	Model 1		Model 2	
Variable	Univariate analysis: $p < 0.25$	Multivariable model $p < 0.1$	Univariate analysis: $p < 0.25$	Multivariable model $p < 0.1$
Technical knowledge **	0.656		0.689	
(1) *	0.426		0.467	
(2) *	0.456		0.469	
Strategic connectivity				
Embeddedness in global culture **	0.129	0.024	0.140	0.053
(1) *	0.043	0.006	0.048	0.016
(2) *	0.158	0.023	0.170	0.036
Degree of connectedness **	0.033	0.345	0.005	0.076
(1) *	0.009	0.222	0.001	0.023
(2) *	0.005	0.148	0.034	0.064
Centrality of connectedness **	0.082	0.306	0.037	0.460
(1) *	0.025	0.126	0.010	0.432
(2) *	0.387	0.466	0.268	0.869

Table 2.4 (cont'd)

	Model 1	Model 2		Model 1
Variable	Univariate analysis: $p < 0.25$	Multivariable model $p < 0.1$	Univariate analysis: $p < 0.25$	Multivariable model $p < 0.1$
Motivational				
Religious *	0.603		0.533	
Ethno-separatists *	0.096	0.104	0.086	0.101
Religion & ethno-separatists *	0.159	0.353	0.123	0.357
Leftist *	0.997		0.997	
Constants		0.315		0.057

In model 1, we study food CBRN organizations against all other organizations and find that most of the variables are not significant. In fact only two were significant, ethno-separatist with a p value of 0.104 and embeddedness in global culture with a p value of 0.024. In model 2, we study food CBRN organizations against all other organizations that did not use or attempt CBRN in any vehicle and find that most of the variables are not significant. Four variables were significant in model 2: ethno-separatists, and increased embeddedness in global culture, democratic regime type and increased degree of connectedness with p values of 0.101, 0.053, 0.104 and 0.076 respectively.

Confounders were identified and added back to the models. There were no confounders. Variables for which interactions were evaluated were degree-eigenvector, religion-ethno religion, religion-eigenvector, religion-degree, technological advancement-cultural

embeddedness, and technological advancement-democratic regime. No interactions were significant. The Hosmer-Lemeshow Goodness of Fit tests for both final models showed a good fit for all variables. Since there were no confounders for either model, there is no table for the unadjusted model. Table 2.5 shows the adjusted model results.

Table 2.5: Final Binary logistical regression results of Models 1 and 2. (*denotes models with 1 degree of freedom, ** denotes models with 2 degree of freedom)

Adjusted		B	S.E.	Wald	Sig	Exp(B)	90% C.I. for EXP(B)	
Model 1								
*	Ethno-separatists	2.152	1.199	3.224	0.073	8.604	1.198	61.795
**	Embeddedness in global culture			5.296	.071			
*	(1)	-3.136	1.371	5.228	0.022	.043	.005	0.415
*	(2)	-2.58	1.395	3.422	0.064	.076	.008	0.751
	Constant	-3.033	1.27	5.703	0.017	.048		
Model 2								
*	Ethno-separatists	2.292	1.453	2.488	.115	9.894	.906	107.993
**	Degree of connectedness			8.426	.015			
*	(1)	-8.701	3.025	8.273	.004	.000	.000	.024
*	(2)	-5.032	2.418	4.330	.037	.007	.000	.348
**	Regime type			6.473	.039			
*	(1)	.744	2.228	.112	.738	2.105	.054	82.153
*	(2)	4.680	1.844	6.441	.011	107.758	5.191	2237.035

Table 2.5: (cont'd)

Adjusted		B	S.E.	Wald	Sig	Exp(B)	90% C.I. for EXP(B)	
Model 2								
**	Embeddedness in global culture			7.714	.021			
*	(1)	-7.113	2.569	7.668	.006	.001	.000	.056
*	(2)	-6.066	2.490	5.935	.015	.002	.000	.139
	Constant	5.595	3.087	3.286	.070	269.177		

As shown in Table 2.5, having an ethno-separatist ideology and greater amounts of cultural embeddedness with the global culture both show significant results as predictors of food based CBRN attacks by an organization in both models ($p = 0.1$). When the organizations that performed or attempted a CBRN attack in other vehicles are removed, greater degree of connectedness to other organizations and democratic regime type in the home country become significant predictors of food based CBRN attacks.

Discussion

As presented earlier in this study a group level analysis is warranted to assist in focusing the law enforcement and intelligence efforts on prevention. Unfortunately, 49% of global terrorism database (GTD) incidents are not claimed by a group (Lafree et al., 2009). The same could be said for the WMD database. This combined with the large percentage of individual to small group incidents in food based CBRN, make adequate numbers of cases to evaluate at the organizational level difficult. This is a component of this study. According to Hennekens and Buring, (1987) the probability of missing a factor that is significant increases the smaller the

number of cases to evaluate. None the less, significant factors were identified in both models. In model 1, the controls, those that did not perform a CBRN attack in food, are combined with organizations that performed or attempted other CBRN terrorist attacks. This combination of the control group to include other CBRN using/pursuing organizations may dilute out the significance of some organizational factors, therefore, any factors that are significant warrant further examination in a study including more cases and excluding the other CBRN organizations from the analysis.

Under the organizational capacity variables in the evaluation, organizational size, age, experience, state sponsorship, territorial control and technical knowledge in both models followed the results of the Asal group showing no significant impact on the decision to use CBRN in food. We hypothesized that size, age and experience would have a positive influence on the decision to use CBRN in food. Although our results model those of the Asal study, the small number of cases may play a role in the inability to visualize a difference in these variables. What was interesting is the significant effect that host state regime type showed in model 2 of this analysis. Contrary to the Asal study that found no difference in regime type, democratic regime type does seem to increase the decision to use CBRN in food when compared to those not using CBRN at all. This may be due to the less restricted movement in democratic countries allowing easier shipping and access to biological organisms in more pure forms or the uninterrupted time in our less monitored society to develop the capacity and plan for a food based CBRN attack. We hypothesized that due to recruitment and Diaspora populations that technical knowledge would show no significant effect. The analysis supported this hypothesis as it did with state sponsorship and both results mirrored those in the larger Asal study.

The strategic connectivity variables host country embeddedness in global culture, the degree of connectedness to other organizations and centrality of connectedness to other organizations; all three were predicted to positively impact the use of CBRN in food. This follows what the Asal group found in the larger CBRN organization population. Both degree of connectedness and host country embeddedness in global culture showed a positive and significant impact on the use of CBRN in food when compared to groups not using or attempting CBRN. Centrality of connectedness showed no significant effect. This could be related to the number of the cases, but potentially the centrality of connectedness is not as important as the degree of connections. For food based CBRN, it is we hypothesized that increased connections to each other and the global culture would decrease the difficulty of propogating a CBRN attack in food by first allowing access to it via the global food chain, second allowing coordinated movement of agent, supplies and technical knowledge and third providing the ability to attack multiple sites at one time in the global food chain. Multiple sites would confound public health officials investigating the outbreaks and mimic natural infections delaying the mobilization of the terrorist investigation assets in any country. The centrality of those connections would be less advantageous from those perspectives.

As for the motivational variables, the only published result from the Asal study was the lack of affect of religion on CBRN users. Our analysis supports this lack of affect. Although the Asal study does not report the results of the other motivational types, ethno-separatists were the only subset showing a significant result in both models. Due to the size constraints muting any ability to see significant affects, this fact has a higher probability of being real and should be investigated further.

The qualitative analysis of organization typology discussed above proposes that organizations including ethnic/religious separatist ideology or anarchist/millenarian ideology or combinations thereof would be more likely to use food based CBRN attacks. We hypothesized the separatist nature of the religion or ethnic group is an important confounder that may have masked the significance of religion on the predictability of CBRN use especially in food. These results support the consideration that the separatist nature has an impact on the use of CBRN at least in a food vehicle.

Conclusions and future direction

No one type of terrorist group is the only group capable of using CBRN terrorism in our food supply at any one point in time. Nor can any country completely prevent this phenomenon from occurring. Attempts to predict the group demographics that will assist in mitigating this risk are valid and as terrorism study expands its knowledge base, that prediction will become easier. For now a defensible general profile has been developed in this study to aid in counterterrorism policy and law enforcement focus. Further study applying this profile to individual groups within the terrorism arena is necessary and advisable to test its strength and predictive value. Hennekens & Buring, (1987) promote that the power of a study to visualize a significant difference is strongest at a ratio of 4:1 cases to controls. While no case is made that there is a loss of power with a greater ratio, the ratios used in this model are around 60:1. Using a random selection of control organizations when the study is repeated with more case organizations might improve the power of the study.

We would like to examine other organizational factors such as regional locality (rural, urban or isolation), leadership type and organizational structure, domestic nature of past attacks among the food CBRN attacks, and membership type like military or scientific, that might also

be important predictors. This study was only able to perform the analysis that the database allowed, but coding of the listed factors will allow continued refinement of the scope of organizations necessary to monitor closely for CBRN activity in the food supply. For now ethnic separatist organizations with lots of connections to other organizations, based in a democratic country with lots of McDonalds restaurants begins to narrow that field.

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CHAPTER 3: Optimization of Electrically Active Magnetic Nanoparticles as Accurate and Efficient Microbial Extraction Tools

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Abstract

Food defense requires the means to efficiently screen large volumes of food for microbial pathogens. Even rapid detection methods often require lengthy enrichment steps, making them impractical for this application. There is a great need for rapid, sensitive, specific, and inexpensive methods for extracting and concentrating microbial pathogens from food. In this study, an immuno-magnetic separation (IMS) methodology was developed for *Escherichia coli* O157:H7, using electrically active magnetic nano-particles (EAMNPs). The analytical specificity of the IMS method was evaluated against *Escherichia coli* O55:H7 and *Shigella boydii*, and was improved over previous protocols by addition of sodium chloride during the conjugation of antibodies onto MNPs. The analytical sensitivity of the IMS method was greatest when a high concentration of antibodies (1.0 mg/mL) was present during conjugation. EAMNP concentrations of 1.0 mg/mL and 0.5 mg/mL provided optimal analytical sensitivity and analytical specificity. The entire IMS procedure requires only 40 minutes, and antibody-conjugated MNPs show no decline in performance up to 149 days after conjugation. This analytically sensitive and specific extraction protocol has excellent longevity and shows promise as an effective extraction for multiple electrochemical biosensor applications.

Introduction

Food-borne microbial pathogens comprise one of the single largest threats to maintaining a safe food supply. Food defense (securing food sources against malicious biological attack) and food safety (identifying and eradicating contamination from natural sources) (Spink, 2009) are growing increasingly relevant, as foods are processed and shipped further and faster than ever before (Zach, Doyle, Bier & Cxuprynski, 2012). Standard overnight culture methods for identifying microbial pathogens are no longer adequate, as the speed and breadth of food movement demands rapid, sensitive, specific, and economical means of extracting and detecting pathogens from food sources. Food and Drug Administration (FDA) inspections have dropped by 81% since 1972 and 47% between 2003 and 2006 (CSPI, 2007). Even with the new Food Safety Modernization Act (FSMA) the highest risk plants will only be inspected every three years by the FDA (Olsson, Weeda, Bode, 2010; Sjerven, 2012). FDA in the United States inspects less than 1% of the imported food supply before consumption and less than 0.2% of the imported food has laboratory analysis at all (CSPI, 2007). Ultimately, companies are responsible for their own products and must protect their own brands. They cannot depend completely on government inspectors or third-party auditors to ensure authenticity and safety of materials and products (Zach et al., 2012). Decreasing the cost of a first line evaluation of food, should allow a food company to test a greater percentage of their product, protecting their bottom line in preventing recalls and their brand reputation in the market. Moving the first line testing of food to the farm and field will allow both regulatory agencies and supply chain managers to find problems earlier before combination at the production or packing plant, benefiting both food safety and food defense.

The objective of this research was to develop an immuno-magnetic separation (IMS) methodology for food borne pathogens that is analytically sensitive and specific, highly inclusive and exclusive as well as inexpensive. Analytical sensitivity and specificity is the ability to isolate target cells with high efficiency, throughout the range of potential concentrations. Inclusivity and exclusivity are the ability to microbiologically discriminate against non-target cells yet include all versions of target cells. Maintaining an inexpensive cost element facilitates increasing the volume of food tested. IMS is a rapid method for extracting and concentrating a target analyte from its sample matrix. This is imperative due to the high level of interference the matrix of a food has on any diagnostic test (Ge & Meng, 2009). IMS has been paired with a wide variety of biosensors for rapid detection of bacterial pathogens (Cheng et al., 2009; Gehring & Tu, 2005; Gehring, Brewster, Irwin, Tu, & VanHouten, 1999; Jaffrezic-Renault, Martelett, Chevolot & Cloarec, 2007; Maalouf, Hassen, Fournier-Wirth, Coste & Jaffrezic-Renault , 2008; Perez, Mascini, Tothill & Turner, 1998; Ruan, Wang & Li, 2002; Tu, Golden, Cooke, Paoli & Gehring, 2005; Varshney & Li, 2007; Varshney, Srinivasan & Tung, 2007; Varshney, Yang, Su & Li, 2005; Yang & Li, 2006). In IMS, micro- or nano-meter scale magnetic particles are immuno-functionalized with antibody, incubated with the sample to bind target cells, and separated from the sample matrix through application of a magnetic field. The magnetic particle-bound target can then be washed and concentrated removing the matrix interference. The possibility of concentrating target cells prior to detection can eliminate the need for time-consuming pre-enrichment steps with a greater real time analytical sensitivity. In comparison to centrifugation, filtration, or capture of target on an immuno-functionalized surface, the IMS is simpler, and generally results in higher capture efficiency due to the greater surface area available for target binding (Cheng et al., 2009; Jiang et al., 2011). This is especially true of

nano sized particles. The surface chemistry of nano sized particles such as surface tension, magnetization and sheer volume of surface area improve the amount of functionalized space for reaction to occur and thus improve the capture ability and longevity of the resultant IMS particles (Cheng et al., 2009; Jiang et al., 2011).

Escherichia coli O157:H7, a type of entero-hemorrhagic E. coli (EHEC), was chosen as the target strain for this study because it is a common and highly infective food- and water-borne pathogen, with a median infectious dose of 23 colony forming units (CFU) (FSIS, 2001). The standard method of identifying *E. coli* O157:H7 from unknown samples is through enrichment in selective media, followed by growth on differential agar. These are identified phenotypically and serologically and toxigenically characterized by PCR, a process lasting several days. The standard method is able to detect <1 CFU/g in foods (FDA, 2009). The IMS method presented here could be applied to extraction and concentration of *E. coli* O157:H7 from food samples, eliminating the standard method's overnight enrichment step. By pairing IMS with PCR or nearly any other rapid detection method, negative or presumptive positive results could be obtained in a few hours or less. The development and application of electrically active magnetic nano-particles (EAMNPs) for IMS has been previously reported by this laboratory (Pal, Setterington & Alocilja, 2008; Pal & Alocilja, 2009). The EAMNPs consist of an iron oxide core with a polyaniline coating which enables them to not only extract target cells, but also to function as the signal transducer in certain electrical detection platforms. The reported method was effective in isolating target cells from pure culture and food matrices with reasonable analytical sensitivity, but when challenged with non-target organisms, it demonstrated inadequate analytical specificity. Adjustment of the environment of the MNPs during conjugation of the antibody probe was hypothesized to correct this problem. Some results from

this portion of the study have been published in the International Journal of Food Safety, Nutrition and Public Health – Food Defence edition (Setterington, Cloutier, Ochoa, Cloutier, Alocilja, 2010).

Experimental Design

To optimize the use of EAMNPs to extract and concentrate microbial targets the following hypothesis was proposed: Mab-EAMNPs can selectively extract and concentrate 1.0 to $1.0*10^9$ CFU/mL of *E. coli* O157:H7 in broth samples. This hypothesis was subdivided into four distinct sub-hypotheses. These sub-hypotheses were developed using the previously reported methodology (Pal et al., 2008, Pal & Alocilja, 2009) as a starting point with the goal of developing a new IMS methodology for *E. coli* O157:H7 that has both analytical sensitivity and specificity. It was hypothesized that the analytical sensitivity and specificity of the IMS methodology is affected by:

1a. The addition of sodium chloride to a concentration of about 0.14 M during conjugation of antibodies onto MNPs.

1b. The concentration of antibodies present during conjugation of antibodies onto MNPs.

1c. The concentration of Mab-EAMNPs present during IMS.

2. The number of days elapsed since conjugation of antibodies onto EAMNPs affect the capture evaluation by culture after IMS.

In order to test the four hypotheses stated above, four factors (sodium chloride addition, antibody concentration, Mab-EAMNP concentration, and age of the Mab-EAMNP solution) were evaluated in terms of their effect on the analytical sensitivity and specificity of the proposed IMS methodology. Therefore, every experiment was applied to three different bacterial species individually: *E. coli* O157:H7 (target species), *E. coli* O55:H7 and *Shigella*

boydii (both non-target species). *E. coli* O55:H7 is another EHEC serotype, closely related to *E. coli* O157:H7. *S. boydii* bears less genotypic and phenotypic similarity to the target organism, but it is a commonly encountered food borne pathogen, and also produces shiga-toxin like *E. coli* O157:H7. The non-target organisms chosen for this study correspond with the recommendations made by the AOAC Task Force on Best Practices in Microbiological Methodology (AOAC, 2006).

To test Hypothesis 1a, Mab-EAMNPs made with the addition of sodium chloride were compared to those made without sodium chloride. (In either case, the initial concentration of antibody was 1.0 mg/mL). Both with and without sodium chloride, three concentrations (1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL) Mab-EAMNPs were used to perform IMS.

To test Hypothesis 1b, Mab-EAMNPs made with an initial antibody concentration of 1.0 mg/mL were compared to those made with an initial antibody concentration of 0.5 mg/mL. (In either case, sodium chloride was added during conjugation). With both 1.0 mg/mL antibody and 0.5 mg/mL antibody, three concentrations (1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL) of Mab-EAMNP were used to perform IMS.

To test Hypothesis 1c, Mab-EAMNPs were made with the addition of sodium chloride and with an initial antibody concentration of 1.0 mg/mL. Each of the four concentrations (1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL) of Mab-MNPs was used to perform IMS.

To test Hypothesis 2, Mab-EAMNPs were made with the addition of sodium chloride, and with initial antibody concentrations of both 1.0 mg/mL and 0.5 mg/mL. Two concentrations (1.0 mg/mL and 0.5 mg/mL) of Mab-EAMNPs were used to perform IMS at various points from 0 to 150 days after conjugation.

Materials and Methods

E. coli O157:H7 strains, *E. coli* non H7 strains and non *E. coli* bacterial strains were obtained from the STEC Center collection at Michigan State University (MSU) (Shannon Manning, MPH, PhD), the Nano-Biosensors Laboratory at MSU (Evangelyn Alocilja, PhD), Neogen Inc. Research and Development, Lansing, Michigan (Jennifer Rice, DVM, PhD) and the University of Georgia, Center for Food Safety (Dr. Michael Doyle, PhD). From frozen purified culture stocks (stored at -80° C), colonies were isolated by streak-plate method on trypticase soy agar (BD Biosciences, MD) plates. A single colony was used to inoculate a vial of tryptic soy broth (BD Biosciences, MD) and grown overnight at 37° C. A 1 mL aliquot of the liquid culture was transferred to a new vial of broth and stored at 37° C for up to 6 days. This culture was used to inoculate a new vial of broth with 1 mL of inoculum 10 to 24 h before each experiment to produce fresh bacterial cells which were serially diluted in 0.1% (w/v) peptone water (Fluka-Biochemika, Switzerland) prior to their use in the IMS procedure. Viable cells were enumerated by microbial plating on MacConkey agar with sorbitol (SMAC) (BD Biosciences, MD or Neogen Inc., MI), according to standard rules for plate counting (FDA BAM, 2009). Optical Density at 600 nanometers (OD 600) spectrophotometer readings (BIO-RAD Smartspec 3000, Hercules, CA) were taken from each culture before use as compared to blank Trypticase Soy Broth (TSB). Three readings were taken and averaged together.

EAMNP production

Ferric chloride hexahydrate (EMD Chemicals, Bedford, MA), sodium acetate (CCI Chemicals, Vernon, CA), sodium acrylate, sodium chloride (NaCl), ethylene glycol, ethylenediamine, hydrochloric acid, aniline, iron (III) oxide nanopowder, ammonium persulfate,

methanol, and diethyl ether were used as received from Sigma Aldrich (St. Louis, MO) in the synthesis of the EAMNPs. EAMNPs were synthesized by polymerization and acid doping of aniline monomer around gamma iron (III) oxide (γ-Fe_2O_3) nano-particles, using a slightly modified published procedure (Pal et al., 2008). Briefly, 0.650 g of iron (III) oxide nanopowder were dispersed in 50 mL of 1 M HCl, 10 mL of deionized water and 0.4 mL of aniline monomer by sonication in an ice bath for 1 hour. A volume of 20 mL of 0.2 M ammonium persulfate (as oxidant) was added drop-wise to the above solution under continuous magnetic stirring. Color change from rust brown to dark green indicated formation of electrically-active (green) polyaniline over the smaller (brown) γ-Fe_2O_3 nano-particles. The solution was stirred for 2 hours in an ice bath and was filtered through a qualitative grade filter (2.5 μm pore size, Ahlstrom, grade 601). The supernatant thus obtained was successively filtered through a nitrocellulose membrane filter (1.2 μm pore size, Millipore) followed by washings with 10 mL each of 1M HCl, 10% (v/v) methanol, and diethyl ether. The particles were dried overnight at room temperature under vacuum. The particles ranged in size from 1.2 to 2.5 μm, and displayed a room temperature saturation magnetization of 30 emu/g.

EAMNP Antibody Conjugation

Nano-particles were immune-functionalized with monoclonal anti-*E. coli* O157:H7 antibodies obtained from Meridian Life Science, Inc. (Saco, ME). Polysorbate-20 (Tween-20), Triton X-100, phosphate buffered saline (PBS), Trizma base, casein, and sodium phosphate (dibasic and monobasic) were used in the IMS procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as

follows: PBS buffer (10mM PBS, pH 7.4), wash buffer (10mM PBS, pH 7.4, with 0.05% Tween-20 or 0.05% Triton-X100), phosphate buffer (100mM sodium phosphate, pH 7.4), blocking buffer (100mM Tris–HCl buffer, pH 7.6, with 0.01% w/v casein). Magnetic separations were performed with a commercial magnetic separator (Promega Corporation, Madison, WI). Hybridization of biological materials was carried out at room temperature with rotation on a tube rotisserie (Labquake, Thermo Scientific, MA). Scanning electron micrographs were acquired using field-emission scanning electron microscopy (JOEL 7500F, acceleration voltage of 5 kV). A superconducting quantum interference device magnetometer (Quantum design MPMS SQUID) was used for magnetic characterization of EAMNPs. Mab-conjugation of the EAMNPs was carried out by physical adsorption of antibodies onto the polyaniline surface. Electrostatic interactions between the negatively charged constant (Fc) portion of the antibodies and the positively charged polyaniline surface are thought to play a role in adsorption and orientation of the biomolecules onto the EAMNPs (Pal and Alocilja, 2009). Successful conjugation of antibodies onto EAMNPs was confirmed by measuring the quantity of antibody in the post-hybridization supernatant with a commercial fluorescence-based protein quantification kit. The measured protein concentration in the supernatant was significantly lower than the concentration of antibodies initially added to the MNPs (data not shown), indicating that antibodies were retained on the MNPs during hybridization.

EAMNPs were conjugated with monoclonal antibodies at an initial EAMNP concentration of 10 mg/mL (1% solid). Two different initial concentrations of monoclonal antibody were used during conjugation: 1.0 mg/mL and 0.5 mg/mL. Conjugation of antibodies onto EAMNPs was performed both with and without the addition of sodium chloride. A 100 µL aliquot of monoclonal, anti-*E. coli* O157:H7 antibody (suspended in 0.1 M phosphate buffer)

was added to EAMNPs suspended in PBS, yielding a final antibody concentration of either 1.0 mg/mL or 0.5 mg/mL. The mixture was hybridized on a rotisserie-style rotator for 1 hour at room temperature, with 25 µL of 10X PBS being added after the first 5 min of hybridization, to increase the Sodium chloride content of the suspension to approximately 0.14 M. (For select experiments, the 10X PBS was omitted). Following hybridization, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer (0.1M tris buffer with 0.01% casein) for 5 min. Again the conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer, this time for 1 hour with rotation. Finally, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 2.5 mL of 0.1 M phosphate buffered saline (PBS). The final concentration of EAMNPs in each solution was 1.0 mg/mL. Immuno-conjugated EAMNPs (Mab-EAMNPs) were stored at 4° C. Prior to experimental use, Mab-EAMNPs were either magnetically separated and concentrated or further diluted in 0.1 M PBS, in order to obtain solutions of Mab-EAMNPs at the following concentrations: 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL EAMNPs.

Immuno-magnetic Separation (IMS) and Plating of Bacteria

Every experiment was applied to three different bacterial species individually: *E. coli* O157:H7 Sakai strain (target species), *E. coli* O55:H7 and *Shigella boydii* (non-target species). *S. boydii* bears less genotypic and phenotypic similarity to the target organism, but it is a commonly encountered food borne pathogen, and also produces shiga-toxin like *E. coli* O157:H7. The standard positive control used was *E. coli* O157:H7 2006 Spinach strain, pGFPuv. Its ability to fluoresce green in the presence of UV light provided confirmation that the

positive samples were not cross contaminated from the positive control. The non-target organisms chosen for this study correspond with the recommendations made by the AOAC Task Force on Best Practices in Microbiological Methodology (AOAC, 2006). Serial dilutions of each bacterium were independently prepared in 0.1% (w/v) peptone water, along with subsequent negative, positive and blank controls. Three or four of the pure dilutions of each bacteria were plated (100-mL aliquots) on sorbitol MacConkey agar (SMAC) and incubated at 37° C overnight. For IMS, 50 mL of Mab-EAMNPs and 50 mL of the appropriate bacterial dilution were combined with 400 mL of 0.01 M PBS (pH 7.4), and hybridized with rotation at room temperature for 30 minutes. After hybridization, the cell-Mab-EAMNP complexes were magnetically separated and the supernatant removed. Complexes were washed twice in wash buffer (0.01 M PBS containing 0.05% Triton-X100), and finally re-suspended in 0.5 mL of 0.01 M PBS. The IMS procedure required 40 min, and is depicted in figure 3.1.

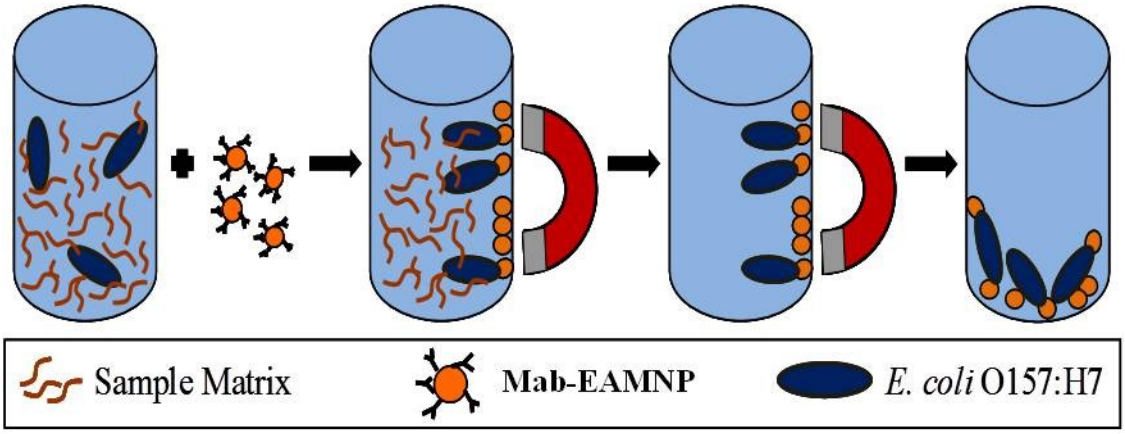

Figure 3.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes.

A 100-mL aliquot was placed on SMAC and incubated at 37° C overnight. The number of colony-forming units (CFU) in the 100-mL aliquot was determined by manually counting the colonies on each plate. For every experimental case (i.e., particular combination of Mab-EAMNP concentration, and bacteria), a minimum of two bacterial dilutions underwent IMS and were plated. In most cases a full spectrum of dilutions from 10^{-1} to 10^{-9} were run as independent units. For the lower dilutions from 10^{-1} to 10^{-5} the final IMS solution was diluted from 5 to 1 time respectively to obtain countable plates. For dilutions from the 10^{-8} and 10^{-9} series, all 500 µL present were plated.

Calculation of bacterial cell concentrations in both pure and IMS separated samples were carried out according to rules provided by the United States Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM, 2009). Plate counts between 25 and 250 colonies were used to calculate what the *original* cell concentrations were in CFU/mL. If all plate counts for a given case fell outside of this range, estimates were made according to FDA rules. Any plate count of zero was therefore estimated using equation 3.1.

Equation 3.1: Estimate of CFU/mL for plates with no growth of bacteria. d = dilution factor plated

$$1.0 * d = \frac{\text{CFU}}{\text{mL}}$$

This leads to an increase estimate of the captured values for those samples where nothing grew and greatly increases the perceived capture of the negative control organisms. This was done to facilitate the statistical analysis comparing the capture of the three organisms and avoid

zero measurements. This should not be considered the analytical specificity or analytical sensitivity limits of the extraction.

Statistical Analysis

The calculated concentrations of cells captured by IMS (in CFU/mL) were converted to their $\log_{10}$ values. The $\log_{10}$ conversion also normalizes the distribution (Mettler & Tholen, 2007). Statistical analysis was performed using SPSS software (Armonk, NY). Missing values were computed with hot-deck imputation or excluded analysis by analysis. Independent, two-tailed T-tests were used to compare experiments in which sodium chloride was added during conjugation, to experiments in which sodium chloride was omitted. Similarly, experiments in which the antibody concentration was 1.0 mg/mL were compared to experiments in which the antibody concentration was 0.5 mg/mL. All experimental results were included for these two analyses. The results presented were <u>not</u> controlled for initial bacterial culture composition and all calculations were estimated if no growth occurred.

Subsequent analysis was performed using both one-way ANOVA and independent two-tailed T-tests, to evaluate the effect of Mab-EAMNP concentration. This analysis included only the results of experiments which had the 1.0 mg/mL antibody concentration and the addition of Sodium chloride during conjugation. (In the previous analyses, these parameters were statistically determined to result in better overall IMS performance). Analyses which showed abnormal data distributions were re-evaluated with Kruskal-Wallis or Mann-Whitney U tests as needed. The results presented were <u>not</u> controlled for initial bacterial concentration off the growth curves. All analyses were calculated with 95% confidence intervals ($\alpha = 0.05$).

Results

Immuno-magnetic capture of *E. coli* O157:H7 cells were quantified by plate counts, but capture was also visually confirmed by scanning electron microscopy (SEM). Figure 3.2 shows SEM images of (a) an individual EAMNP with diameter of approximately 1.3 μm, and (b) a Mab-EAMNP bound to an *E. coli* O157:H7 cell, after washing twice to remove non-specifically bound cells.

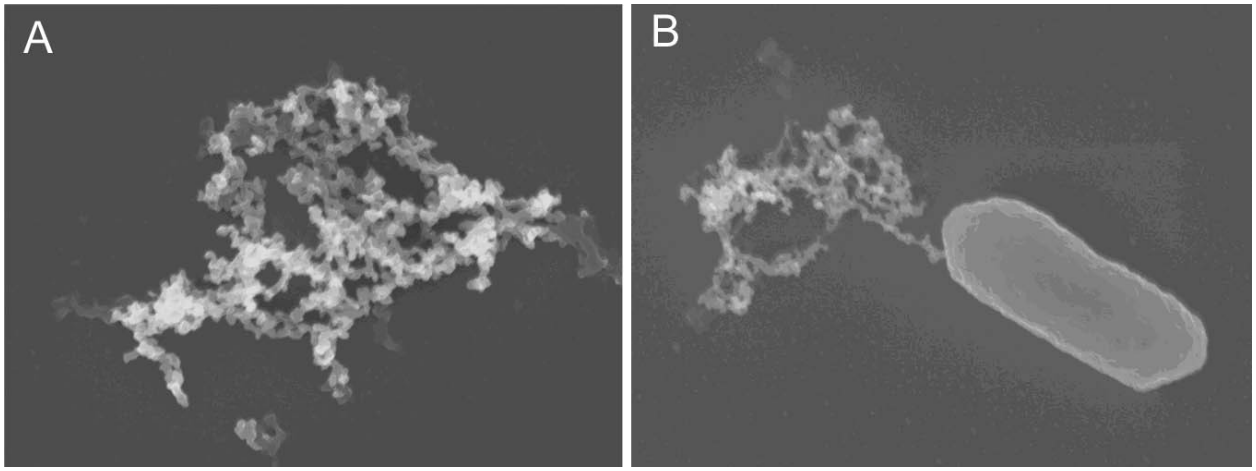

Figure 3.2: Scanning electron micrograph of (a) an individual EAMNP with diameter of approximately 1.3 μm, and (b) Mab-EAMNP bound to an *E. coli* O157:H7 cell.

Hypothesis 1a: Effect of sodium chloride addition during conjugation

Two-tailed independent T-tests performed on the mean concentrations of captured cells ($\log_{10}$ of CFU/mL) for all three bacteria in the initial study showed that the addition of sodium chloride (compared with omitting sodium chloride) causes a significant decrease in capture of the negative control *S. boydii* ($n = 178$; $p = 0.029$), with no significant effect on the capture of the target *E. coli* O157:H7 or the other negative control *E. coli* O55:H7. The addition of 0.14 M sodium chloride during conjugation of antibodies onto EAMNPs increases the specificity at all Mab-EAMNP concentrations evaluated, and has no effect on sensitivity. (Figure 3.3)

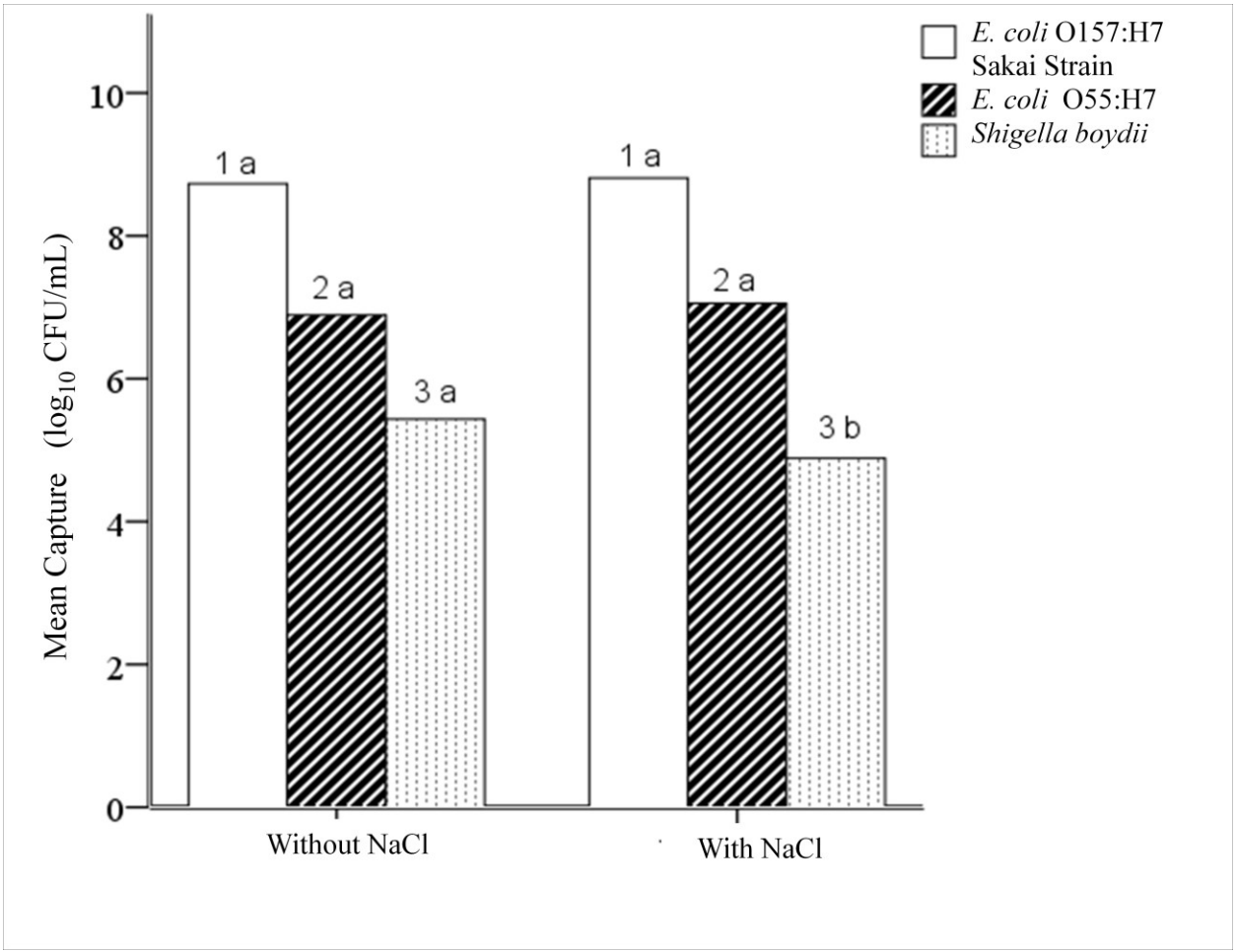

Figure 3.3: Mean concentration (log_{10} of CFU/mL) of each bacterial culture captured in IMS, using Mab-EAMNPs made with and without the addition of sodium chloride. Statistical comparisons were made within numbered groups (1-3), and letters (a or b) indicate significant differences ($\alpha = 0.05$, n = 178; p = 0.029). Zero counts were estimated to facilitate analysis. (Setterington, Cloutier, Ochoa, Cloutier, Alocilja, 2010)

Hypothesis 1b: Effect of Antibody Concentration during Conjugation

Two-tailed independent T-tests performed on the mean concentrations of captured cells (log_{10} of CFU/mL) for all three bacteria showed that the higher antibody concentration (1.0 mg/mL) caused a significant increase in capture of the target *E. coli* O157:H7 (n = 178; p = 0.018), with no significant effect on the capture of the negative control microorganisms. The

higher antibody concentration (1.0 mg/mL) during conjugation increases the analytical sensitivity of EAMNPs at all Mab-EAMNP concentrations evaluated, and has no effect on analytical specificity. (Figure 3.4)

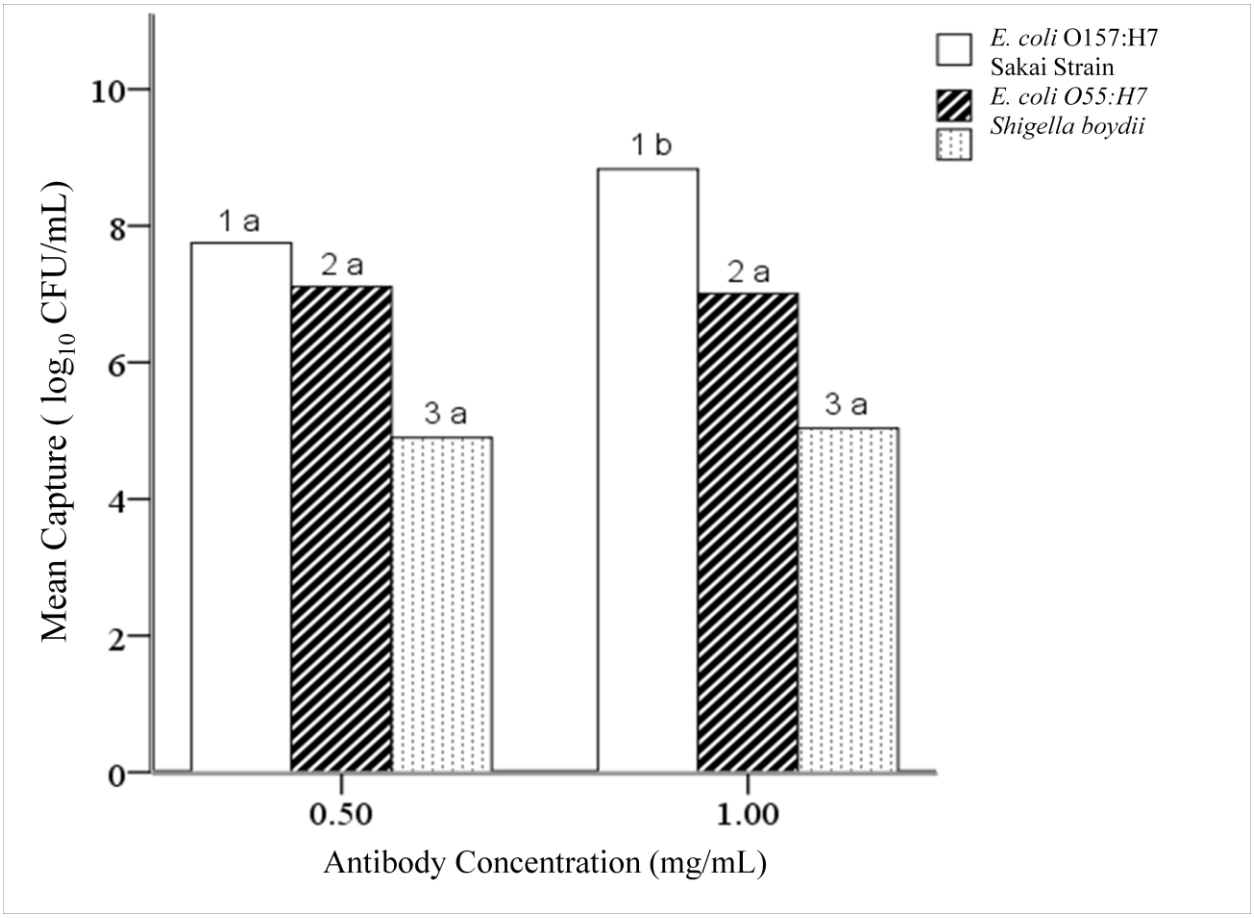

Figure 3.4. Mean concentration (log_{10} of CFU/mL) of each bacterial culture captured in IMS, using Mab-EAMNPs made with 1.0 mg/mL antibody and with 0.5 mg/mL antibody. Statistical comparisons were made within numbered groups (1-3), and letters (a or b) indicate significant differences ($\alpha = 0.05$, n =178, $p = 0.018$). Zero counts were estimated to facilitate analysis. (Setterington et al., 2010).

Hypothesis 1c: Effect of Mab-EAMNP Concentration during IMS

One-way ANOVA was performed on the mean concentrations of captured cells ($\log_{10}$ of CFU/mL) for all three bacteria, separated according to Mab-EAMNP concentration. No significant difference in the capture of the target *E. coli* O157:H7 Sakai was observed at any Mab-EAMNP concentration with this test (LDS and Bonferroni pairwise comparison). However, the ANOVA homogeneity of variance test showed non-normal distributions for various bacteria. To account for the non-normality observed in the ANOVA, independent T-tests were also performed for all three bacteria, and these did show some significant differences in medians, with the nonparametric comparison (using the Kruskal-Wallis test for median and distribution, or the Mann-Whitney two-sample comparison). From these statistical analyses, the following conclusions were drawn:

EAMNPs at both 1.5 mg/mL and 0.1 mg/mL are less analytically specific than EAMNPs at either 1.0 mg/mL or 0.5 mg/mL. Despite the small number of data points (n=5) for EAMNPs at 1.5 mg/mL, this concentration is more analytically sensitive than any other concentration of EAMNPs. Based on these statistical results, null hypothesis 1c is rejected. The concentration of Mab-EAMNPs present during IMS has an effect on both analytical sensitivity and specificity (Figure 3.5). In most cases where the Mab-EAMNP concentration had a significant effect on bacterial capture, concentrations of 1.0 mg/mL and 0.5 mg/mL provide the optimal analytical sensitivity and specificity.

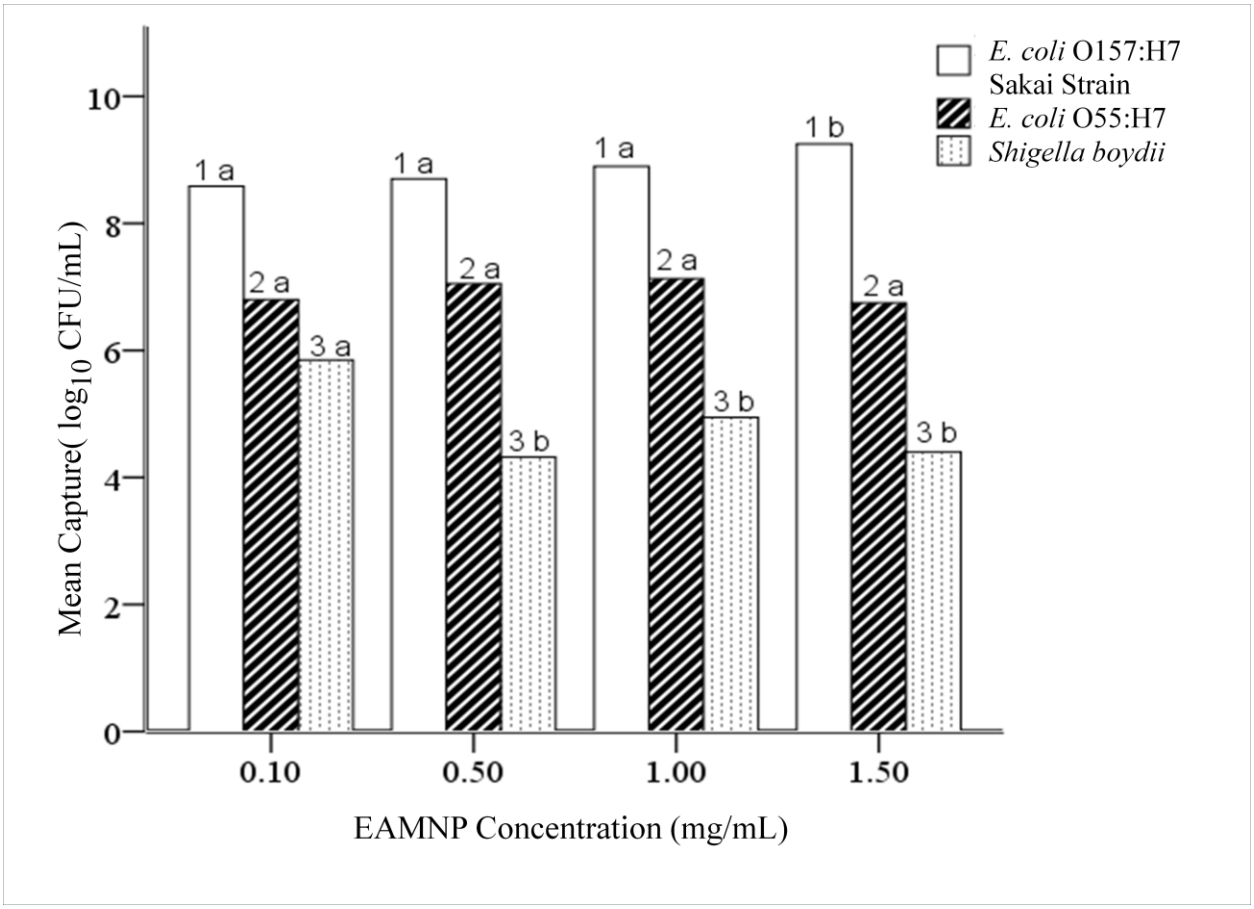

Figure 3.5: Mean concentration (log_{10} of CFU/mL) of each bacterial culture captured in IMS, using Mab-EAMNPs, at concentrations of 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL. Statistical comparisons were made within numbered groups (1-3), and letters (a or b) indicate significant differences ($\alpha = 0.05$). Zero counts were estimated to facilitate analysis. (Setterington et al., 2010)

Hypothesis 2: Effect of Age of Mab-EAMNP Solution during IMS

Longevity of the Mab-EAMNP solutions was also evaluated by one-way ANOVA and independent two-tailed T-tests. With the previously reported method of conjugating antibodies onto EAMNPs (Pal et al., 2008, Pal & Alocilja, 2009), long term storage of Mab-EAMNP solutions (at 4° C) resulted in poorer IMS performance. This observation led to Hypothesis 2, that the number of days elapsed since conjugation of antibodies onto EAMNPs

will affect the analytical sensitivity and specificity of IMS. One-way ANOVA and independent two-tailed T-tests were performed on the mean concentration of captured cells ($\log_{10}$ of CFU/mL) for all three bacteria, comparing the experimental results obtained from Mab-EAMNP solutions ranging in age from 0 days to 149 days. Regardless of which statistical test was applied, no significant difference was observed in IMS capture of any of the three bacteria. Based on these statistical results, null hypothesis 2 is retained. Days elapsed since conjugation of antibodies onto MNPs (stored at 4° C), from 0 to 149 days, has no effect on analytical sensitivity or specificity.

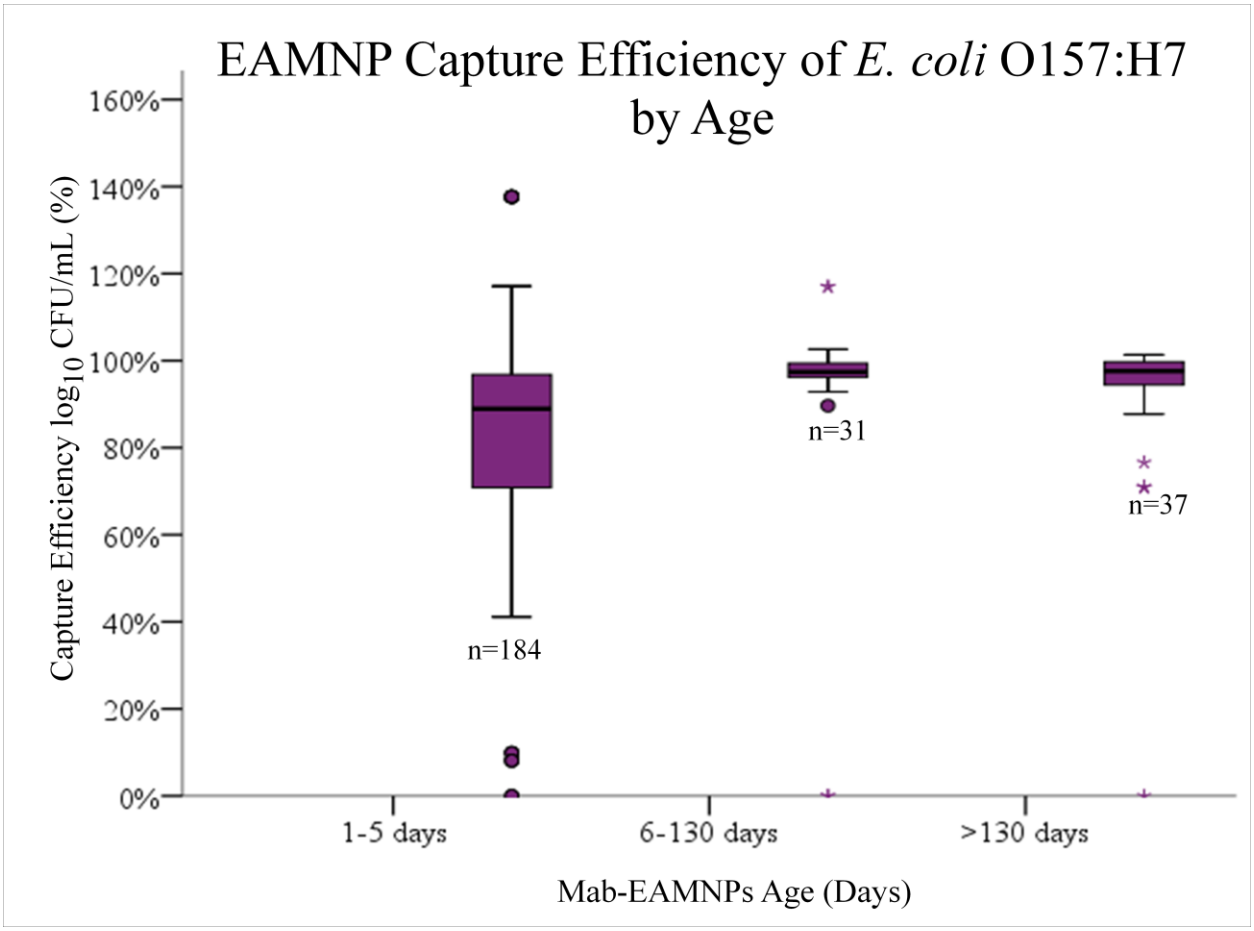

Figure 3.6: Capture efficiency (log_{10} of CFU/mL captured/ log_{10} of CFU/mL present) of each *E. coli* O157:H7 Sakai strain captured in IMS, using Mab-EAMNPs, at varying days from conjugation stored at refrigerated temperatures from 1-5 days (n = 37); 6-130 days (n = 6); 130 to 149 days (n = 12). Data is limited to bacterial concentrations in the linear range of consistent capture. There was no statistical difference between any of the three groups.

Discussion

By changing several portions of the conjugation step for the EAMNPs, the new IMS methodology reported here was able to isolate *E. coli* O157:H7 Sakai strain with excellent analytical sensitivity, and discriminates against *E. coli* O55:H7 and *Shigella boydii.* Additionally,

this methodology requires a smaller volume of EAMNPs per extraction, and results in an EAMNP-antibody conjugate with a much longer storage life, as compared to our previous method. Both of these improvements contribute to a lower overall cost of the IMS assay. Over fifty different independent runs in duplicate or triplicate were accomplished at all concentrations from 1-2 CFU/mL to $1.0x10^{10}$ CFU/mL.

Conjugation of antibodies onto EAMNPs was carried out in phosphate buffer at pH 7.4. A slightly basic pH such as this is recommended for optimal adsorption of the Fc (constant) portion of the antibody (Bangs Laboratories, 2008a), which positions the Fab (antigen-binding) portion outward for maximum target-binding capacity. Also, it has been reported that the addition of sodium chloride at or near physiological concentration (about 0.15 M) increases adsorption efficiency of antibodies onto microspheres (Bangs Laboratories, 2008b). This was the foundation for hypothesis 1a, that the addition of sodium chloride during conjugation will affect the analytical sensitivity and specificity of IMS. Its improvement is possibly due to the more physiologic conditions mimicking the antibody's primary functional environment. Addition of sodium chloride during conjugation is a simple and inexpensive procedural change able to enhance IMS performance for any application.

During conjugation of antibodies onto EAMNPs, EAMNPs were present at a concentration of 10 mg/mL, or 1% solids. The solution volume was kept small (250 µL, until post-conjugation dilution) in order to increase the speed and frequency of interactions between antibodies and EAMNPs during conjugation. Monoclonal antibody was added at relatively high concentrations of 1.0 mg/mL or 0.5 mg/mL during conjugation. Bangs Laboratories recommends 3-10 times antibody concentration needed to create a monolayer (calculated amount) to ensure favorable stoichiometry for helping the Fc region to adsorb first (Bangs,

2008a). This was the foundation for hypothesis 1b, that the concentration of antibodies present during conjugation will affect the analytical sensitivity and specificity of IMS. The antibody concentration changes in the conjugation protocol did have a statistically significant improvement in the analytical sensitivity of the extraction as determined by culture (Figure 3.4). This may be due to competition. The more antibodies present, the more likely the resultant orientation of the antibody is Fc portion down since it is the smallest in diameter. Although consumption of more antibodies increases the cost of the assay, it is worthwhile for some IMS applications. Since the infectious dose of *E. coli* O157:H7 has a median of 23 CFU (FSIS, 2001) high analytical sensitivity is a critical feature in any IMS assay for this organism. However, if IMS is being applied to a pathogen like *Bacillus cereus*, with an infectious dose greater than $1.0 * 10^6$ *cells* (FDA, 2009), then decreasing the cost of the assay would likely be of greater value than increasing the analytical sensitivity, and a lower antibody concentration may be ideal.

With the objective of developing an IMS methodology that is analytically sensitive and specific, but also practical and cost-effective, the concentration of Mab-EAMNPs employed in IMS was identified as an important parameter to be optimized. This concern led to Hypothesis 1c, that the concentration of Mab-EAMNPs present during IMS will affect the analytical sensitivity and specificity of IMS. The concentration of Mab-EAMNPs present during IMS had a significant effect on bacterial capture, concentrations of 1.0 mg/mL and 0.5 mg/mL provide the optimal analytical sensitivity and specificity. These findings offer the experimenter some flexibility in tailoring the IMS methodology to suit a particular application, depending on whether analytical sensitivity or specificity is of greater concern. Also, a very low Mab-EAMNP concentration (such as 0.1 mg/mL) could be employed to drastically decrease the cost of the

assay in cases where neither analytical sensitivity nor specificity must be optimal (for example, high-throughput yes/no screening of food products, with tolerance levels greater than zero).

The EAMNPs were shown to have excellent longevity, with no decline in performance up to 149 days after conjugation. This data includes data taken with no control over the age of the culture used as a testing solution. This provides the operator much more flexibility in reaction time if the conjugate can be made ahead and used when needed. It also allows the resultant biosensor evaluation of time to result to exclude the time needed to conjugate the EAMNPs and only measure the time for the actual IMS.

Conclusions and Limitations

This cumulative total of 450 repetitive broth challenges yielded statistically significant extraction and culture detection at all concentrations, with appropriate negative, positive and blank controls included. The entire IMS procedure requires only 40 min. The experiments designed and executed in this study provided conclusive results, allowing the initial hypotheses to be either rejected or retained. The concentration of EAMNP-Mab during use can be decreased compared to commercially available IMS methods and initially reported methods using this IMS without significant changes to reported analytical sensitivity and specificity. Limitations of this extraction method include the fact that both viable and non-viable cells are extracted with this methodology. Further studies are designed and being implemented to evaluate the Mab-EAMNP to determine the reaction kinetics of non-viable verses viable cells on the antibody target region in broth cultures. Limits of detection, inclusivity and exclusivity of microbial families and biosensor platform experiments are necessary before validation trials of the whole biosensor can proceed. The ultimate goal of this extraction is to be able to multiplex many EAMNPs with different Mab targets to allow multiplexing. Future multiplexing with multiple EAMNP and

multiple bacterial targets could have interactions between the EAMNPs or between the mixed antibodies. Certain matrices may remove the Mab from the surface of the EAMNPs and make their use in that matrix impossible. The largest drawback to this method is the need for refrigeration of the Mab-EAMNPs. When field based technologies are discussed, shelf stable reagents are an advantage.

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CHAPTER 4: Culture Age on Evaluation of Electrically Active Magnetic Nanoparticles as Accurate and Efficient Microbial Extraction Tools

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Abstract

A potential confounding factor in the development and evaluation of biosensors is the diverse nature of the disciplines involved. Biosensor technology involves electrochemistry, microbiology, chemical synthesis, and engineering, among many other disciplines. Biological systems, due to non homogeneous distribution, are already imprecise compared to other systems, especially food based systems. Inadequate knowledge of the techniques to moderate this leads to ineffective evaluation strategies and potentially halting the pursuit of excellent technology that was merely poorly evaluated. This research was undertaken to evaluate the effect culture age had on the capture efficiency of the electrically active magnetic nanoparticles (EAMNP) using culture as the evaluation tool. The age of culture used for IMS over all the experiments was 6 to 18 hours. Ideal culture age range for evaluating biosensors is 4 to 10 hours according to the growth curve for *E. coli* O157:H7 in tripticase soy broth. This is supported by the statistically significant difference between organisms in groups from 3-10 hours old compared to those grouped from 11-18 and >19 hours old ($\alpha = 0.05$, $p = 0.001$ and $p = 0.014$ respectively). The two older categories were no different from each other. The capture efficiency in all biosensor analysis will vary less than when culture of only viable cells is the diagnostic tool. This allows a true evaluation of the consistency and accuracy of the method, less hindered by the variation in the ability to culture the organism.

Introduction

IMS is a rapid method for extracting and concentrating a target analyte from its sample matrix. This is imperative due to the high level of interference the matrix of a food has on any diagnostic test (Ge & Meng, 2009). IMS has been paired with a wide variety of biosensors for rapid detection of bacterial pathogens (Cheng et al., 2009; Gehring & Tu, 2005; Gehring, Brewster, Irwin, Tu, & VanHouten, 1999; Jaffrezic-Renault, Martelett, Chevolot & Cloarec, 2007; Maalouf, Hassen, Fournier-Wirth, Coste & Jaffrezic-Renault , 2008; Perez, Mascini, Tothill & Turner, 1998; Ruan, Wang & Li, 2002; Tu, Golden, Cooke, Paoli & Gehring, 2005; Varshney & Li, 2007; Varshney, Srinivasan & Tung, 2007; Varshney, Yang, Su & Li, 2005; Yang & Li, 2006). In IMS, micro- or nano-meter scale magnetic particles are immuno-functionalized with antibody, incubated with the sample to bind target cells, and separated from the sample matrix through application of a magnetic field. The magnetic particle-bound target can then be washed and concentrated removing the matrix interference. The possibility of concentrating target cells prior to detection can eliminate the need for time-consuming pre-enrichment steps with a greater real time analytical sensitivity. In comparison to centrifugation, filtration, or capture of target on an immuno-functionalized surface, the IMS is simpler, and generally results in higher capture efficiency due to the greater surface area available for target binding (Cheng et al., 2009; Jiang et al., 2011). This is especially true of nano sized particles. The surface chemistry of nano sized particles such as surface tension, magnetization and sheer volume of surface area improve the amount of functionalized space for reaction to occur and thus improve the capture ability and longevity of the resultant IMS particles (Cheng et al., 2009; Jiang et al., 2011).

A potential confounding factor in the development and evaluation of biosensors is the diverse nature of the disciplines involved (Nayak, Kotian, Marathe & Chakravortty (2009) and Lazcka, Del Campo, Mu˜noz (2007). Biosensor technology involves electrochemistry, microbiology, chemical synthesis, and engineering, among many other disciplines. There are few people who are well versed in multiple disciplines. This creates difficulties, for example, when an electrical engineer attempts to produce and challenge a microbiological based biosensor, especially a food based biosensor. Biological systems, due to non-homogeneous distribution, are already imprecise compared to other systems, especially food based systems (Adams & Moss, 2008). Inadequate knowledge of the techniques to moderate this leads to ineffective evaluation strategies and potentially halting the pursuit of excellent technology that was merely poorly evaluated. Theavnot (1999) points out that the rapid growth of biosensors has lead to "lack of rigor" (pg 2335) in their published performance criteria. Concepts like the $\log_{10}$ conversion of colony forming units per milliliter (CFU/mL) (Mettler & Tholen, 2007), plate counting (FDA BAM, 2009), and most probable number (MPN) calculations (USDA FSIS MLG, 2010) are not used consistently in the biosensors literature. International standards for microbiological tests in food exist from the International Standards Organization (ISO) (ISO website, 2011). Organizations like the American Association of Occupational Chemists (AOAC) produce detailed recommendations on analytical expectations of microbiological testing requirements (AOAC, 2006). The field of biosensor technology is improving and expanding every year (Lazcka, et al., 2007). Addition of these concepts may allow better evaluation of the existing technology and prevent the discard of valuable diagnostic tools because of lack of rigor in their evaluation.

Antibody based extraction methodology relies on phenotypic recognition of the bacterium. Antibodies cannot differentiate viable cells from non-viable cells and will often perform differently in lag versus log versus stationary growth phases of the bacteria. This could be due to differences in antigen expression during these stages and can be altered by growth conditions and growth media (Blackburn & McCarthy, 2000; Durso & Keen, 2007; Fitzmaurice, Duffy, Kilbride, Sheridan, Carroll & Maher, 2004; Fung, 2008). While natural contaminations are by nature not controlled, adequate optimization and validation of an extraction methodology and comparison of data from day to day requires minimum variation in all parameters except those being examined. To evaluate a biosensor critically, control over all parameters other than the one under evaluation is a necessity. The objective of the author's body of research was to develop an immuno-magnetic separation (IMS) methodology for food borne pathogens that is analytically sensitive and specific, highly inclusive and exclusive as well as inexpensive. In our initial evaluation of the electrically active magnetic nanoparticles (EAMNP), the error seen in the capture results was too high to allow evaluations of low levels of contamination (Setterington, Cloutier, Ochoa, Cloutier, Alocilja, 2010). Microbiological culture was used as the verification tool for a positive sample as well as to evaluate the efficiency of the extraction method via capture efficiency calculations. One of the hypothesized reasons for the error seen was the starting culture itself. Since the EAMNPs capture viable and non-viable cells, yet the microbiological culture evaluation tool only evaluates viable cells, it seemed prudent to examine the start culture for use as early after the log phase of rapid growth and phenotypic change but before a higher percentage of non-viable cells could skew the resultant capture efficiency data. The evaluated IMS technique extracts cells and cell components due to the binding ability of the antibodies to specific bacterial phenotypic proteins that are present in viable or non-viable cells.

Culture can only evaluate viable, undamaged cells. Non-viable cells, cells with no chance of capture due to probe overload and cells we have no chance to capture due to changing phenotypic expression in the log phase all add to the variability of the resulting data. The goal was to only use cultures that were out of the rapidly changing log phase of growth, below the known limit of detection of the extraction protocol and mostly viable cells as our challenge solution to limit the variability. In order to facilitate this, the following hypothesis was developed and tested.

Experimental design

Hypothesis 1: The concentration and percentage of non-viable bacterial cells in the start culture results in decreased biosensor capture efficiency estimates as a result of non-growth of non-viable cells on culture media.

To test the above hypothesis, detailed growth curves of the chosen strain of *E. coli* O157:H7, 2006 Japanese Sakai outbreak (Sakai), were developed by recording the time and volume of transfer and an OD600 on each culture. Some of the resultant cultures were IMS extracted with EAMNPs and cultured. Capture efficiencies were calculated to compare categories of culture ages. Matching experiments were performed on the control organisms used in the laboratory.

Materials and Methods

E. coli O157:H7 strains, *E. coli* non-H7 strains and non-*E. coli* bacterial strains were obtained from the STEC Center collection at Michigan State University (MSU) (Shannon Manning, MPH, PhD), the Nano-Biosensors Laboratory at MSU (Evangelyn Alocilja, PhD), Neogen Inc. Research and Development, Lansing, Michigan (Jennifer Rice, DVM, PhD) and the

University of Georgia, Center for Food Safety (Dr. Michael Doyle, PhD). From frozen purified culture stocks (stored at -80° C), colonies were isolated by streak-plate method on trypticase soy agar (BD Biosciences, MD) plates. A single colony was used to inoculate a vial of tryptic soy broth (BD Biosciences, MD) and grown overnight at 37° C. A 1 mL aliquot of the liquid culture was transferred to a new vial of broth and stored at 37° C for up to 6 days. This culture was used to inoculate a new vial of broth with 1 mL of inoculum 10 to 24 h before each experiment to produce fresh bacterial cells which were serially diluted in 0.1% (w/v) peptone water (Fluka-Biochemika, Switzerland) prior to their use in the IMS procedure. Varying volumes of stationary broth cultures, colony to broth and freezer stock to broth were inoculated into TSB for the growth curve analysis. Viable cells were enumerated by microbial plating on MacConkey agar with sorbitol (SMAC) (BD Biosciences, MD or Neogen Inc., MI), according to standard rules for plate counting (FDA BAM, 2009). Optical Density at 600 nanometers (OD 600) spectrophotometer readings (BIO-RAD Smartspec 3000, Hercules, CA) were taken from each culture before use as compared to blank Trypticase Soy Broth (TSB). Three readings were taken and averaged together.

EAMNP Production

Ferric chloride hexahydrate (EMD Chemicals, Bedford, MA), sodium acetate (CCI Chemicals, Vernon, CA), sodium acrylate, sodium chloride (NaCl), ethylene glycol, ethylenediamine, hydrochloric acid, aniline, iron (III) oxide nanopowder, ammonium persulfate, methanol, and diethyl ether were used as received from Sigma Aldrich (St. Louis, MO) in the synthesis of the EAMNPs. EAMNPs were synthesized by polymerization and acid doping of aniline monomer around gamma iron (III) oxide (γ-Fe_2O_3) nano-particles, using a slightly

modified published procedure (Pal, Setterington, Alocilja, 2008). Briefly, 0.650 g of iron (III) oxide nanopowder were dispersed in 50 mL of 1 M HCl, 10 mL of deionized water and 0.4 mL of aniline monomer by sonication in an ice bath for 1 hour. A volume of 20 mL of 0.2 M ammonium persulfate (as oxidant) was added drop-wise to the above solution under continuous magnetic stirring. Color change from rust brown to dark green indicated formation of electrically-active (green) polyaniline over the smaller (brown) γ-Fe_2O_3 nano-particles. The solution was stirred for 2 hours in an ice bath and was filtered through a qualitative grade filter (2.5 μm pore size, Ahlstrom, grade 601). The supernatant thus obtained was successively filtered through a nitrocellulose membrane filter (1.2 μm pore size, Millipore) followed by washings with 10 mL each of 1M HCl, 10% (v/v) methanol, and diethyl ether. The particles were dried overnight at room temperature under vacuum. The particles ranged in size from 1.2 to 2.5 μm, and displayed a room temperature saturation magnetization of 30 emu/g.

EAMNP Antibody Conjugation

Nano-particles were immune-functionalized with monoclonal anti-*E. coli* O157:H7 antibodies obtained from Meridian Life Science, Inc. (Saco, ME). Polysorbate-20 (Tween-20), Triton X-100, phosphate buffered saline (PBS), Trizma base, casein, and sodium phosphate (dibasic and monobasic) were used in the IMS procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as follows: PBS buffer (10 mM PBS, pH 7.4), wash buffer (10 mM PBS, pH 7.4, with 0.05% Tween-20 or 0.05% Triton-X100), phosphate buffer (100 mM sodium phosphate, pH 7.4), blocking buffer (100 mM Tris–HCl buffer, pH 7.6, with 0.01% w/v casein). Magnetic

separations were performed with a commercial magnetic separator (Promega Corporation, Madison, WI). Hybridization of biological materials was carried out at room temperature with rotation on a tube rotisserie (Labquake, Thermo Scientific, MA). Scanning electron micrographs were acquired using field-emission scanning electron microscopy (JOEL 7500F, acceleration voltage of 5 kV). A superconducting quantum interference device magnetometer (Quantum design MPMS SQUID) was used for magnetic characterization of EAMNPs.

Mab-conjugation of the EAMNPs was carried out by physical adsorption of antibodies onto the polyaniline surface. Electrostatic interactions between the negatively charged constant (Fc) portion of the antibodies and the positively charged polyaniline surface are thought to play a role in adsorption and orientation of the biomolecules onto the EAMNPs (Pal and Alocilja, 2009). Successful conjugation of antibodies onto EAMNPs was confirmed by measuring the quantity of antibody in the post-hybridization supernatant with a commercial fluorescence-based protein quantification kit. The measured protein concentration in the supernatant was significantly lower than the concentration of antibodies initially added to the MNPs (data not shown), indicating that antibodies were retained on the MNPs during hybridization. EAMNPs were conjugated with monoclonal antibodies at an initial EAMNP concentration of 10 mg/mL (1% solid). A 100 µL aliquot of monoclonal, anti-*E. coli* O157:H7 antibody (suspended in 0.1 M phosphate buffer) was added to EAMNPs suspended in PBS, yielding a final antibody concentration of 1.0 mg/mL. The mixture was hybridized on a rotisserie-style rotator for 1 hour at room temperature, with 25 µL of 10X PBS being added after the first 5 min of hybridization, to increase the sodium chloride content of the suspension to approximately 0.14 M. Following hybridization, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer (0.1 M tris buffer with

0.01% casein) for 5 min. Again the conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 μL of blocking buffer, this time for 1 hour with rotation. Finally, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 2.5 mL of 0.1 M phosphate buffered saline (PBS). The final concentration of EAMNPs in each solution was 1.0 mg/mL. Immuno-conjugated EAMNPs (Mab-EAMNPs) were stored at 4° C. Prior to experimental use, Mab-EAMNPs were or further diluted in 0.1 M PBS, in order to obtain solutions at 0.5 mg/mL EAMNPs.

Immuno-magnetic Separation (IMS) and Plating of Bacteria

Every experiment was applied to three different bacterial species individually: *E. coli* O157:H7 Sakai strain, *E. coli* O157:H7 2006 Spinach strain, pGFPuv (target species), *Shigella boydii* (non-target species). *S. boydii* bears less genotypic and phenotypic similarity to the target organism, but it is a commonly encountered food borne pathogen, and also produces shiga-toxin like *E. coli* O157:H7. The standard positive control used was *E. coli* O157:H7 2006 Spinach strain, pGFPuv. Its ability to fluoresce green in the presence of UV light provided confirmation that the positive samples were not cross contaminated from the positive control. The non-target organisms chosen for this study correspond with the recommendations made by the AOAC Task Force on Best Practices in Microbiological Methodology (AOAC, 2006). Serial dilutions of each bacterium were independently prepared in 0.1% (w/v) peptone water, along with subsequent negative, positive and blank controls. Three or four of the pure dilutions of each bacteria were plated (100-mL aliquots) on sorbitol MacConkey agar (SMAC) and incubated at 37° C overnight. For IMS, 50 mL of Mab-EAMNPs and 50 mL of the appropriate bacterial dilution were combined with 400 mL of 0.01 M PBS (pH 7.4), and hybridized with rotation at

room temperature for 30 minutes. After hybridization, the cell-Mab-EAMNP complexes were magnetically separated and the supernatant removed. Complexes were washed twice in wash buffer (0.01 M PBS containing 0.05% Triton-X100), and finally re-suspended in 0.5 mL of 0.01 M PBS. The IMS procedure required 40 min, and is depicted in figure 4.1.

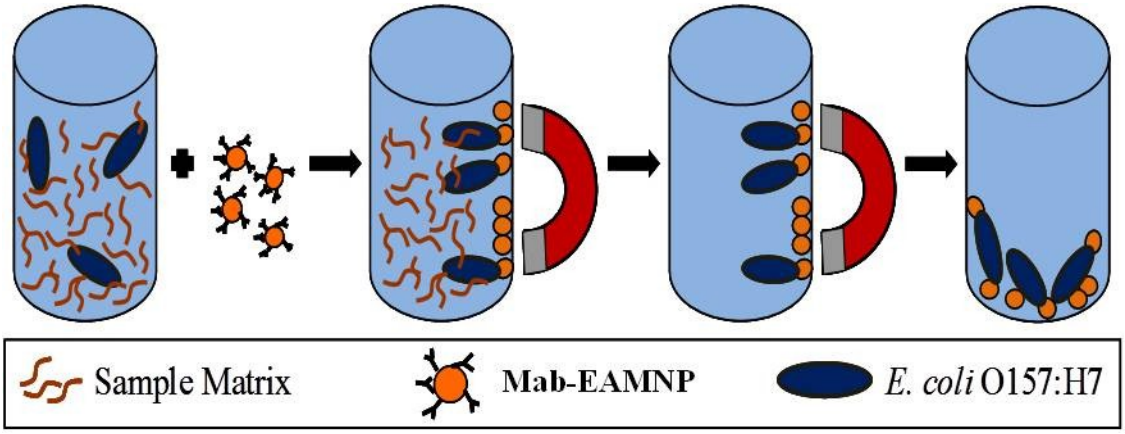

Figure 4.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes.

A 100-mL aliquot was placed on SMAC and incubated at 37° C overnight. The number of colony-forming units (CFU) in the 100-mL aliquot was determined by manually counting the colonies on each plate. For every experimental case (i.e., particular combination of Mab-EAMNP concentration, and bacteria), a minimum of two bacterial dilutions underwent IMS and were plated. In most cases a full spectrum of dilutions from 10^{-1} to 10^{-9} were run as independent units. For the lower dilutions from 10^{-1} to 10^{-5} the final IMS solution was diluted from 5 to 1 time respectively to obtain countable plates. For dilutions from the 10^{-8} and 10^{-9} series, all 500 µL present were plated.

Calculation of bacterial cell concentrations in both pure and IMS separated samples were carried out according to rules provided by the United States Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM, 2009). In addition, separate runs were performed where the final IMS solution was all plated to determine if any cells were collected at all, instead of the standard 100 µL. The CFU/mL calculations were adjusted accordingly to accommodate the change in plated dilution factor.

Capture efficiency as defined by the amount captured divided by the amount present in the original sample was calculated for each sample at each concentration for *E. coli* O157:H7, and each negative and positive control. Capture efficiency was calculated by conversion of CFU/mL to $\log_{10}$ CFU/mL when above 10 CFU/mL. The $\log_{10}$ conversion also normalizes the distribution (Mettler & Tholen, 2007). When calculating capture efficiency at the lower concentrations the log transformation is not performed since with a base 10 the result would be zero. Actual CFU/mL was used in this range to calculate capture efficiency.

Statistical Analysis

Statistical analysis was performed using SPSS software (Armonk, NY). Missing values were excluded analysis by analysis. Data was collected by hour and the resultant capture efficiencies grouped into categories of 3-10 hours, 11-18 hours and 19-24 hours. Subsequent analysis was performed using one way anova evaluation with a post hoc comparison using dunnet's t-tests using the 3-10 hour category as the control to evaluate the effect of age on Mab-EAMNP extraction. All analyses were calculated with 95% confidence intervals ($\alpha = 0.05$).

Results

Accurate growth curves for the bacterial strains we were using were developed under our laboratory conditions and equipment. Cumulative data from all extraction runs done over an 8 month period were tabulated and separated by time in hours and by inoculation volume. Figure 4.2 shows the resultant growth curve for *E. coli* O157:H7 Sakai strain, for a 1 mL transfer aliquot. Identical curves for other inoculums of *E. coli* O157:H7 and all growth curves for *E. coli* O55:H7 and *Shigella boydii* are available in appendix 4.A.

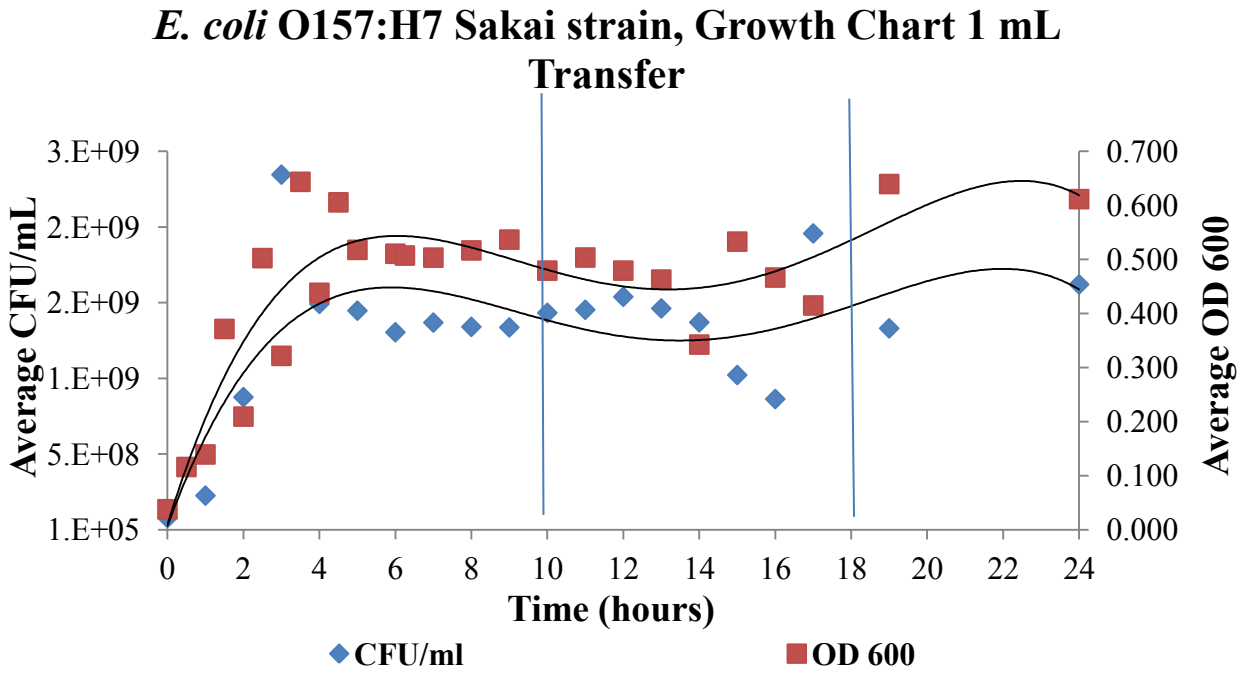

Figure 4.2*:* *E. coli* O157:H7 Sakai strain growth by hour of a 1 mL inoculum into 10 mL TSB.

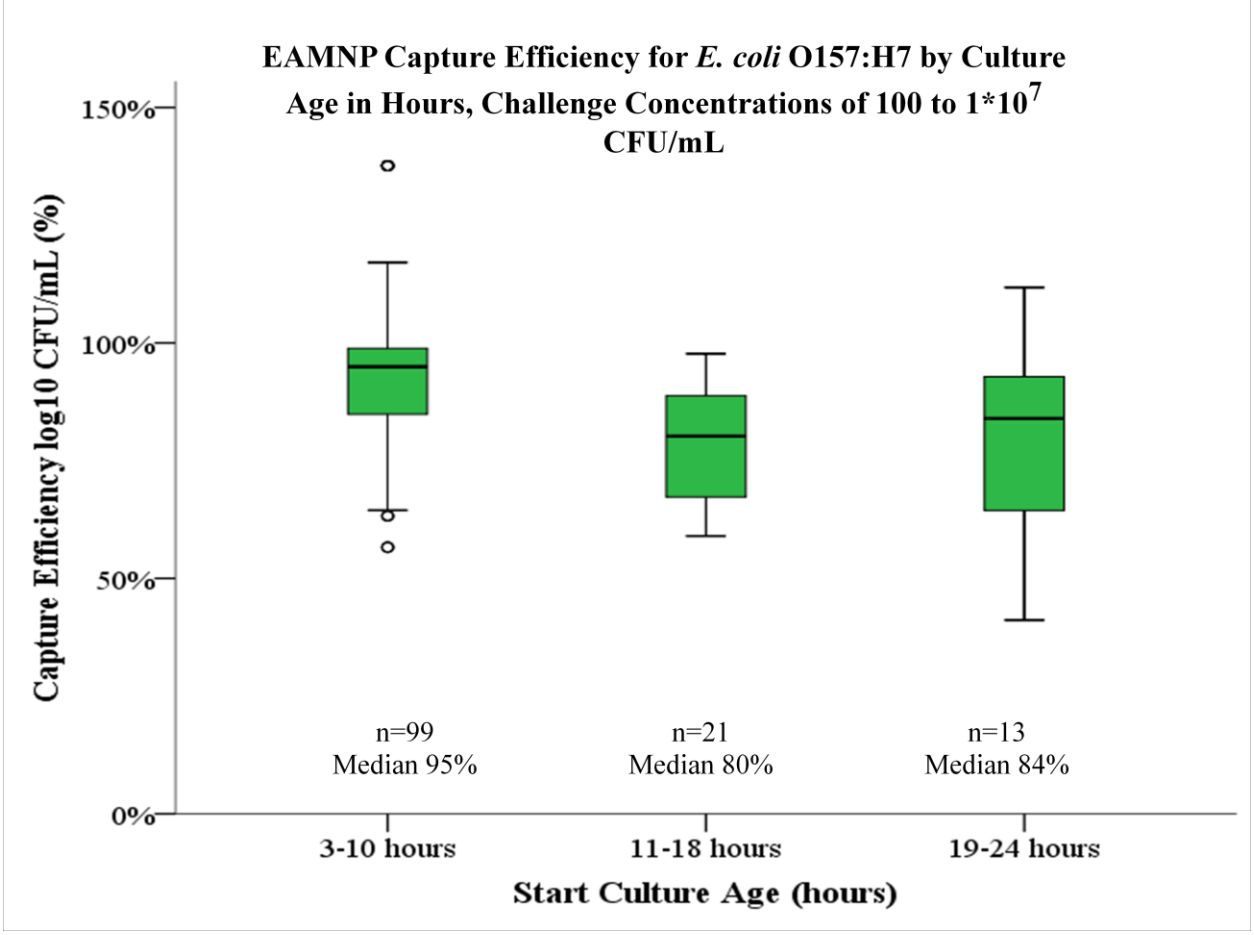

Figure 4.3*: E. coli* O157:H7, Sakai strain, capture efficiency comparison by age of start culture. Statistical comparisons were made between groups and are shown in figure 4.4.

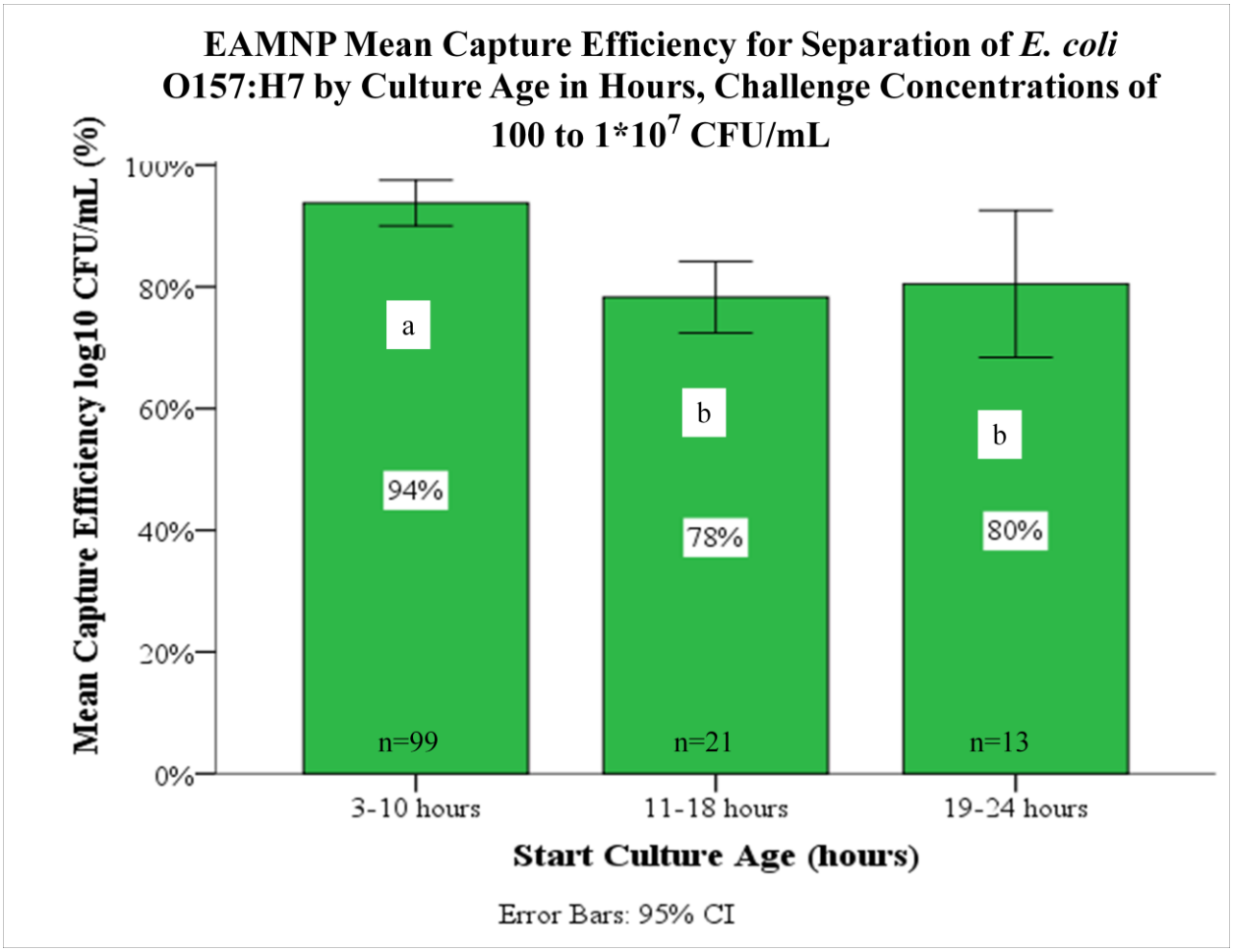

Figure 4.4*: E. coli* O157:H7, Sakai strain, mean capture efficiency comparison by age of start culture. Statistical comparisons were made between groups as a one way anova with a one tailed Dunnet's t-test. Groups were labeled (b) if they were statistically less than groups labeled (a). (3-10 hours vs. 11-18 hours, p=0.001; 3-10 hours vs. 19-24 hours, p = 0.014; $\alpha = 0.05$)

Discussion

As our attempts to hold the cell culture constant were continuing while data was being collected, the data in this report represents data generated with differing culture conditions, ages and concentrations of viable and non-viable cells. The primary age of culture used for IMS over all the experiments was 6 to 18 hours old. Ideal time frames for the age of the culture are from 4 to 10 hours according the growth curve for a 1 mL in to 10 mL inoculums. This is supported by

the statistically significant difference between organisms blocked from 3-10 hours old and those blocked from 11-18 and >19 hours old. The two older categories were no different from each other. The capture efficiency in all biosensor analysis will vary less then when culture of only viable cells is the diagnostic tool. This allows a true evaluation of the consistency and accuracy of the method, less hindered by the variation in the ability to culture the organism. This also has ramifications on the electrochemistry results of a biosensor. Inaccurate known challenge concentrations lead to inaccurate classification on the electrochemical platform and a perceived variability of results by concentration of bacterial cells. Since one of the goals of effective biosensor evaluation is to evaluate the range and differentiation of the linear range results by cell concentrations, this could make a difference in the decision to move a biosensor from concept to validation studies (Thevenot, 1999). Inaccurate known challenge concentrations add to the variability we see in the data for biosensors we create in the author's laboratory. The OD600 spectrophotometer evaluations as compared to the cultured CFU/mL and the age of the inoculums assist in both explaining early data variation and facilitate decisions on logistics for future data collection.

Conclusions and Limitations

This cumulative total of 323 repetitive broth challenges yielded statistically significant extraction and culture detection by capture efficiency. Monitoring the age of the inoculating culture during initial challenges of the EAMNP IMS methodology will allow future studies to better control the unintentional error not related to technology under evaluation. When culture is used as the gold standard, test sensitivity calculations will be depressed due to increasing the false positive fractions on those with non-viable or viable and non-cultureable bacteria in the system. Test sensitivity calculations are already a problem in the range of bacterial

concentrations that are below the culture limit of detection, around 100 CFU/mL (AOAC, 2006). This is mostly due to the non-homogenous nature of bacteria in solution. The probability of pipeting the exact fraction the few bacteria reside in decreases as the culture becomes more dilute, increasing the degree of error.

Limitations of this extraction method include the fact that both viable and non-viable cells are extracted with this methodology. Further studies are designed and being implemented to evaluate the Mab-EAMNP to determine the reaction kinetics of non-viable verses viable cells on the antibody target region in broth cultures. Limits of detection, inclusivity and exclusivity of microbial families and biosensor platform experiments are necessary before validation trials of the whole biosensor can proceed. The ultimate goal of this extraction is to be able to multiplex many EAMNPs with different Mab targets to allow multiplexing. Future multiplexing with multiple EAMNP and multiple bacterial targets could have interactions between the EAMNPs or between the mixed antibodies. Certain matrices may remove the Mab from the surface of the EAMNPs and make their use in that matrix impossible. The for field based diagnostics largest drawback to this method is the need for refrigeration of the Mab-EAMNPs. When field based technologies are discussed, shelf stable reagents are an advantage.

Appendices Chapter 4

Appendix 4.A: Bacterial Growth Curves with Varying Inoculums

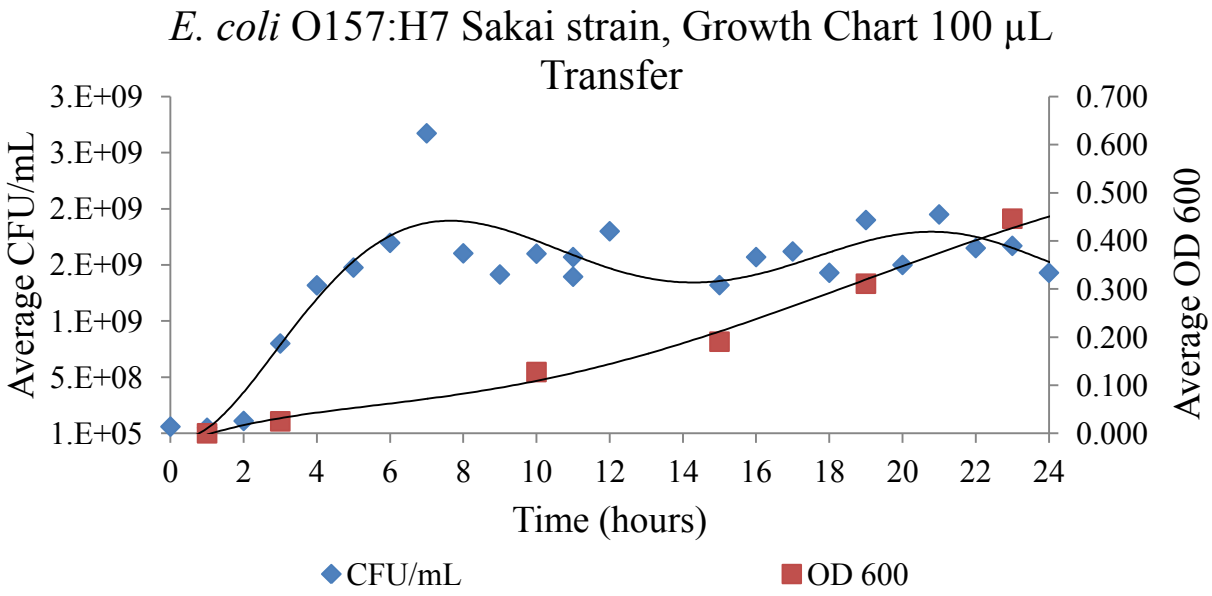

Figure 4.A.1: *E. coli* O157:H7 Sakai strain growth by hour of a 100 µl inoculum into 10 mL TSB.

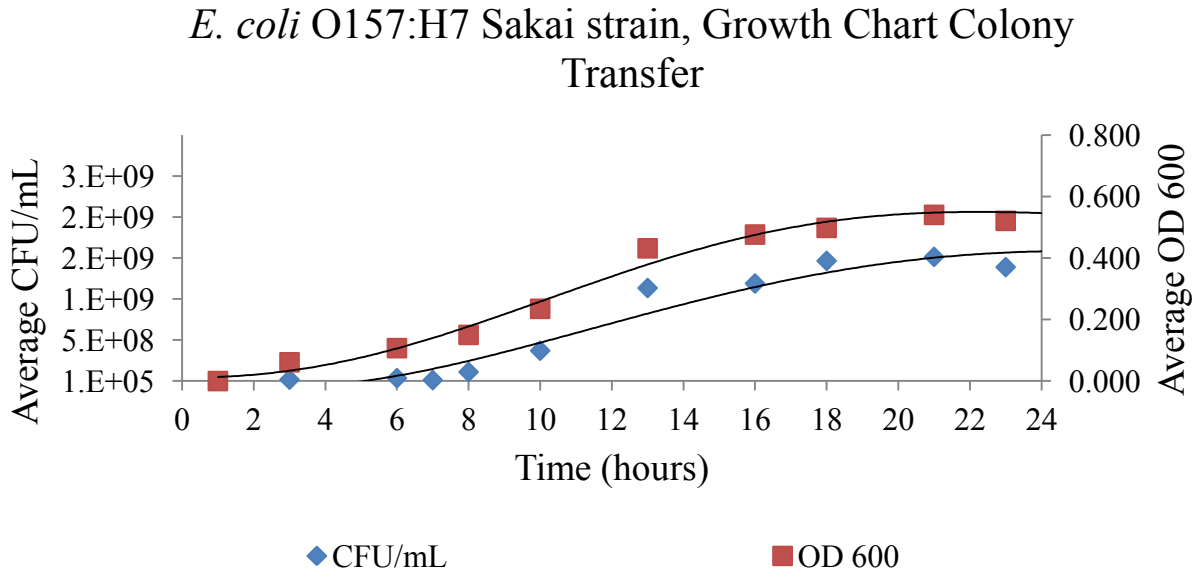

Figure 4.A.2: *E. coli* O157:H7 Sakai strain growth by hour of a single colony inoculum into 10 mL TSB.

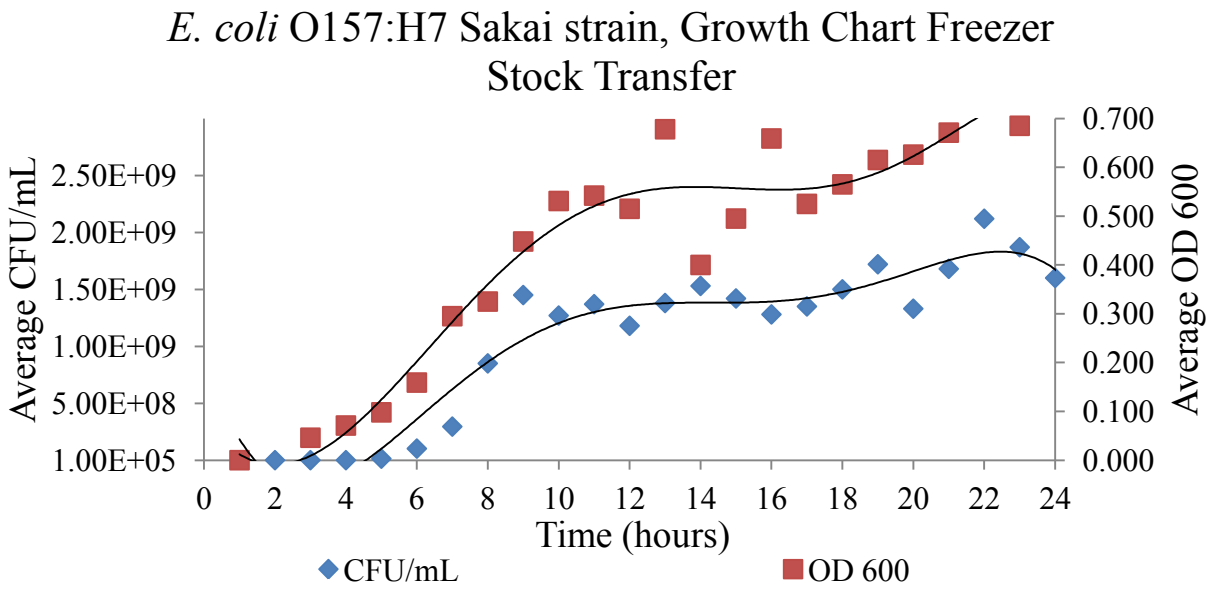

Figure 4.A.3: *E. coli* O157:H7 Sakai strain growth by hour of a loop of ice crystal inoculum into 10 mL TSB.

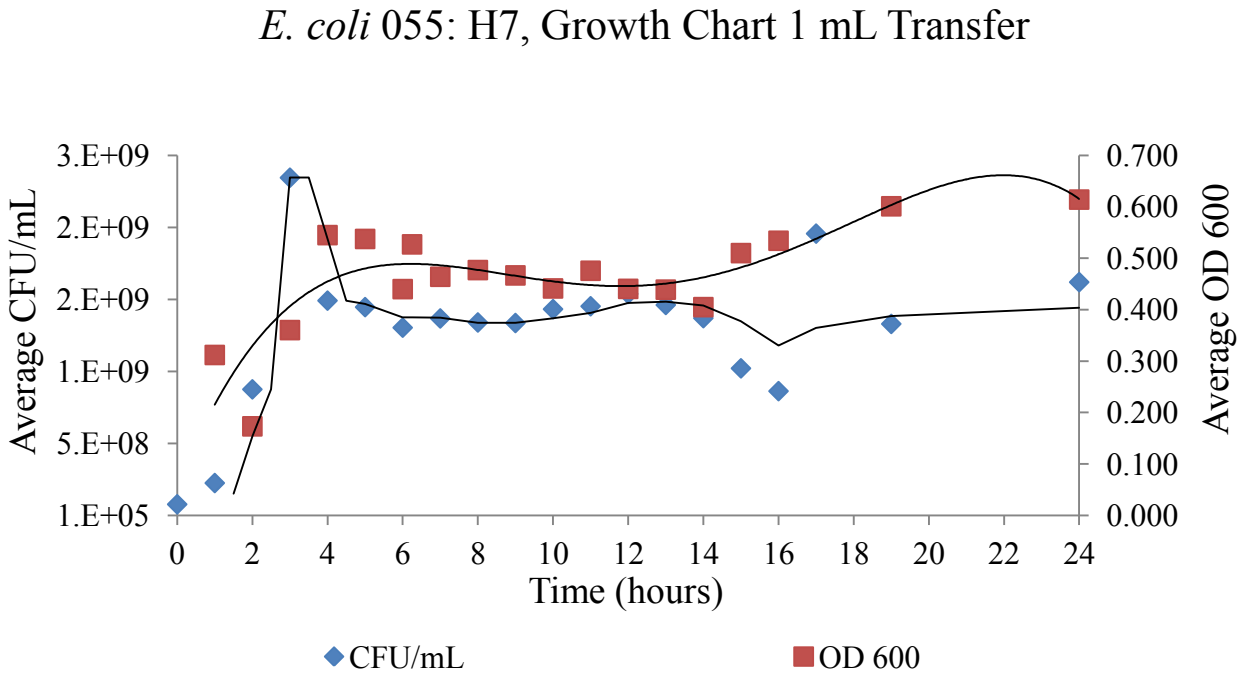

Figure 4.A.4: *E. coli* O55:H7 growth by hour of a 1 mL inoculum into 10 mL TSB.

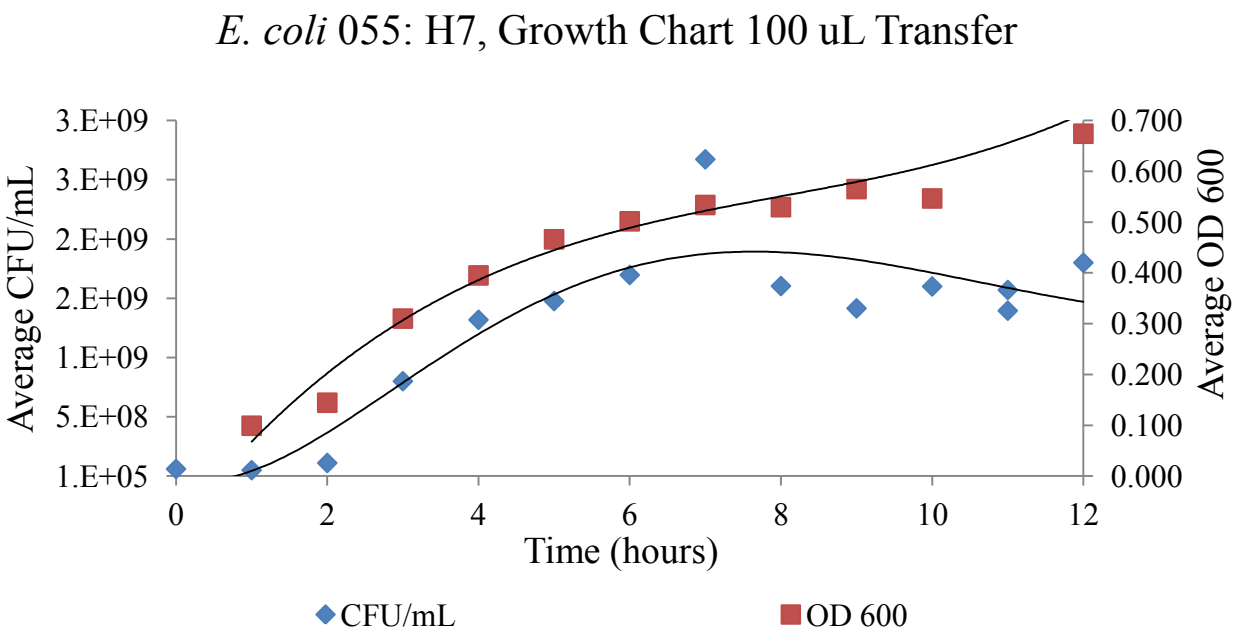

Figure 4.A.5: *E. coli* 055:H7 growth by hour of a 100 µL inoculum into 10 mL TSB.

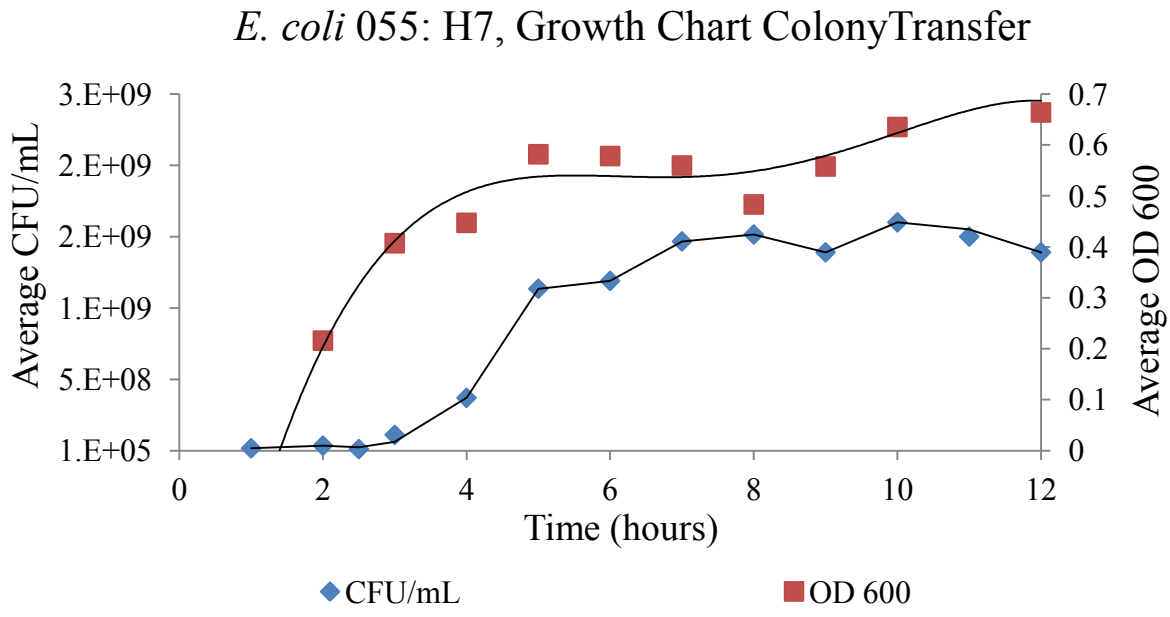

Figure 4.A.6: *E. coli* 055:H7 growth by hour of a single colony inoculum into 10 mL TSB.

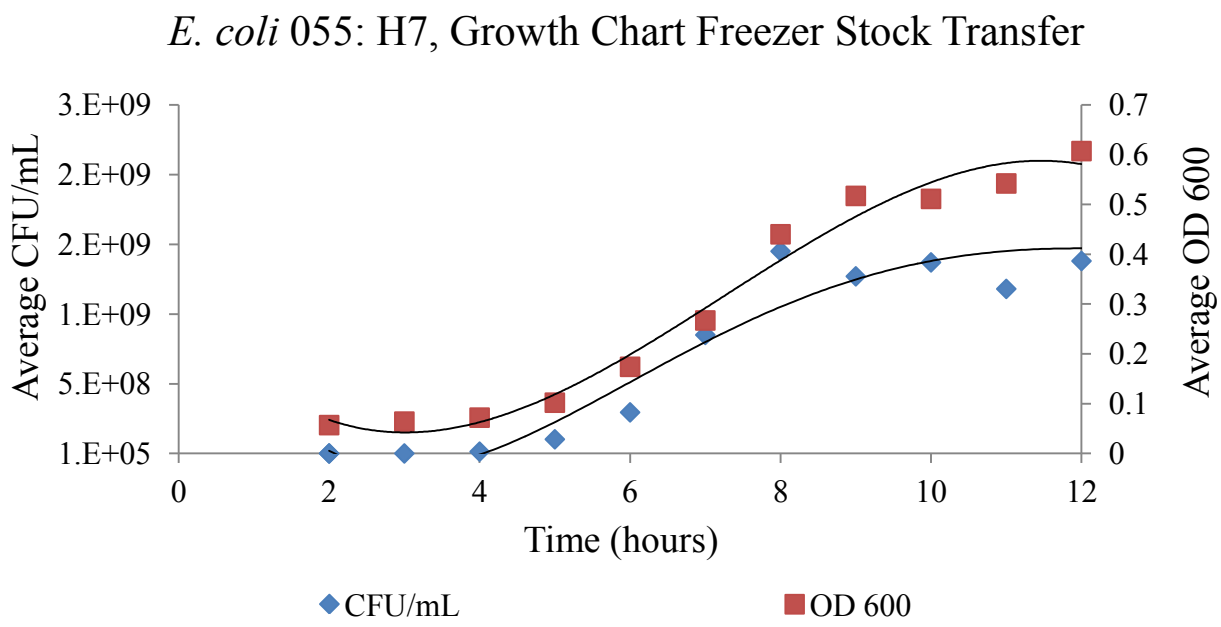

Figure 4.A.7: *E. coli* 055:H7 growth by hour of a loop of ice crystal inoculum into 10 mL TSB.

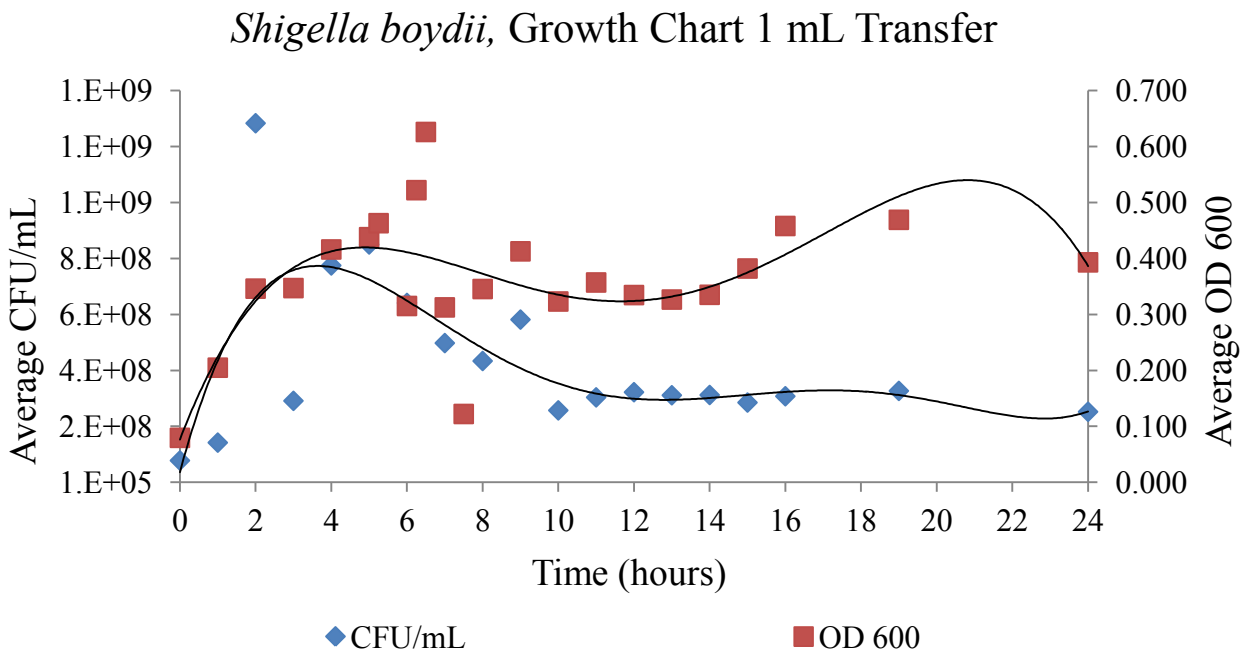

Figure 4.A.8: *Shigella boydii* growth by hour of a 1 mL inoculum into 10 mL TSB.

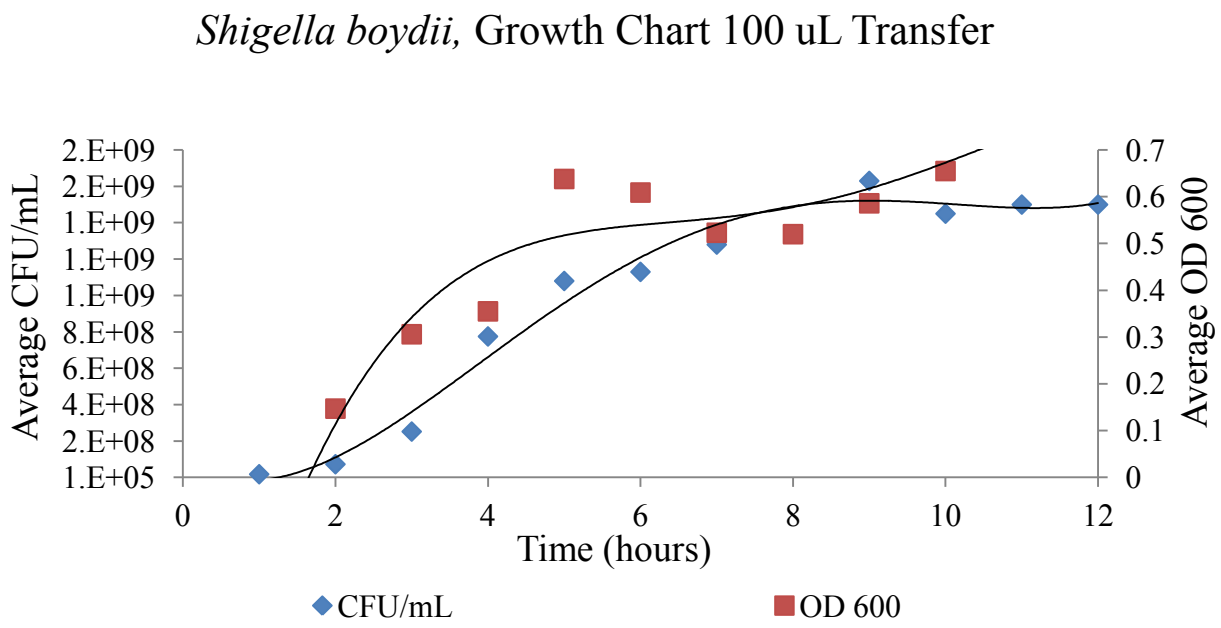

Figure 4.A.9: *Shigella boydii* growth by hour of a 100 µL inoculum into 10 mL TSB.

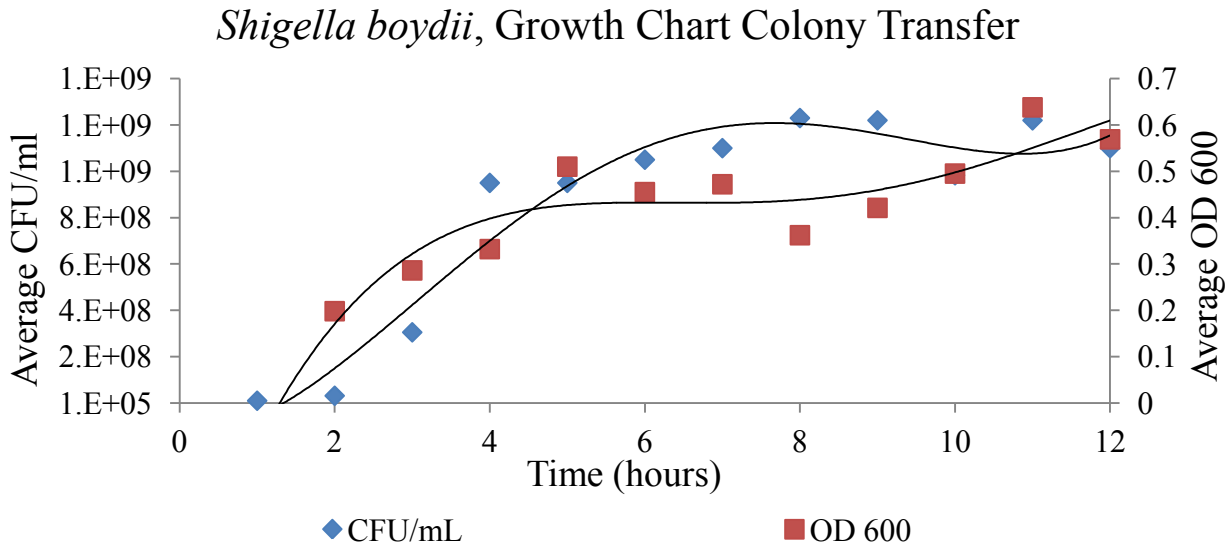

Figure 4.A.10: *Shigella boydii* growth by hour of a single colony inoculum of 10 mL TSB.

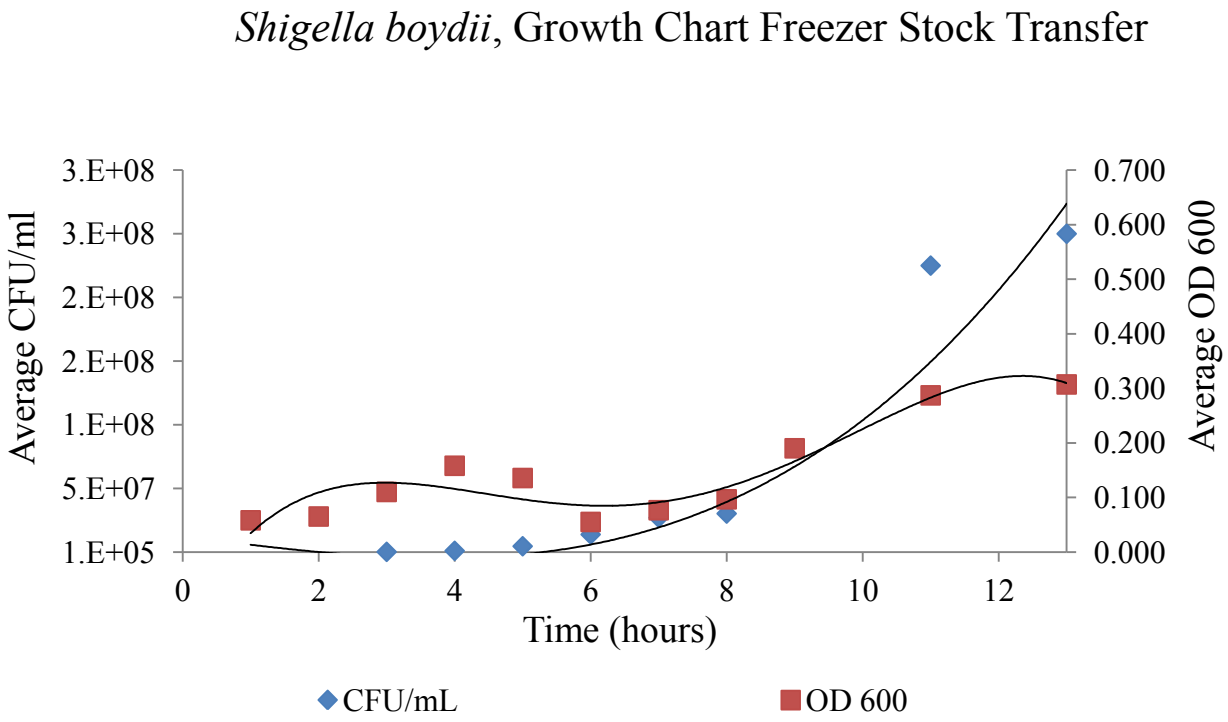

Figure 4.A.11: *Shigella boydii* growth by hour of a loop of ice crystal inoculum into 10 mL TSB.

REFERENCES

REFERENCES

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CHAPTER 5: Evaluation of the Limits of Detection for Electrically Active Magnetic Nanoparticles as Accurate and Efficient Microbial Extraction Tools

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Abstract

Food defense requires the means to efficiently screen large volumes of food for microbial pathogens. Even rapid detection methods often require lengthy enrichment steps, making them impractical for this application. There is a great need for rapid, sensitive, specific, and inexpensive methods for extracting and concentrating microbial pathogens from food. In this study, an immuno-magnetic separation (IMS) methodology was developed for *Escherichia coli* O157:H7, using electrically active magnetic nano-particles (EAMNPs). From broth cultures, the extraction protocol's lower limit of detection (LOD) was 1 CFU/mL and the limit of detection 50% (LOD_{50}) was 6-9 CFU/mL, using culture as the detection modality. The upper limit of detection was $1.0 * 10^9$ CFU/mL. The cost of producing one sample volume of EAMNPs conjugated with anti-*Escherichia coli* O157:H7 is ~ $0.43 and requires only 40 min from sample to result. The linear range of capture efficiency at 90% is from 10 CFU/mL to $1.0 * 10^7$ CFU/mL challenge concentrations. This was achieved with no attempt at holding the start concentrations of the bacterial culture age in the reported best range of 4-10 hours. Future studies employing techniques to estimate concentrations below the lower limit of detection reported here will be employed to further refine the true lower analytical limit of this extraction modality.

Introduction

Food-borne microbial pathogens comprise one of the single largest threats to maintaining a safe food supply. Food defense (securing food sources against malicious biological attack) and food safety (identifying and eradicating contamination from natural sources) (Spink, 2009) are growing increasingly relevant, as foods are processed and shipped further and faster than ever before (Zach, Doyle, Bier, Cxuprynski, 2012). Standard overnight culture methods for identifying microbial pathogens are no longer adequate, as the speed and breadth of food movement demands rapid, sensitive, specific, and economical means of extracting and detecting pathogens from food sources. Decreasing the cost of a first line evaluation of food, should allow a food company to test a greater percentage of their product, protecting their bottom line in preventing recalls and their brand reputation in the market. Moving the first line testing of food to the farm and field will allow both regulatory agencies and supply chain managers to find problems earlier before combination at the production or packing plant, benefiting both food safety and food defense.

The objective of this research was to develop an immuno-magnetic separation (IMS) methodology for food borne pathogens that is analytically sensitive and specific, highly inclusive and exclusive as well as inexpensive. Analytical sensitivity and specificity is the ability to isolate target cells with high efficiency, throughout the range of potential concentrations. One of the goals of effective biosensor evaluation is to evaluate the range and differentiation of the linear range results by cell concentrations (Theavnot, 1999). One of the most difficult, yet the most critical range of cell concentrations needing to be evaluated were the ranges of 0 to 100 CFU/mL (AOAC International, 2006). This range is below a concentration that provides

consistent results via microbiological culture, yet is within the range of infectious dose for many pathogens, such as *Escherichia coli* O157:H7 (FSIS, 2001).

IMS is a rapid method for extracting and concentrating a target analyte from its sample matrix. This is imperative due to the high level of interference the matrix of a food has on any diagnostic test (Ge & Meng, 2009). In IMS, micro- or nano-meter scale magnetic particles are immuno-functionalized with antibody, incubated with the sample to bind target cells, and separated from the sample matrix through application of a magnetic field. The magnetic particle-bound target can then be washed and concentrated removing the matrix interference. The possibility of concentrating target cells prior to detection can eliminate the need for time-consuming pre-enrichment steps with a greater real time analytical sensitivity. In comparison to centrifugation, filtration, or capture of target on an immuno-functionalized surface, the IMS is simpler, and generally results in higher capture efficiency due to the greater surface area available for target binding (Cheng et al., 2009; Jiang et al., 2011). This is especially true of nano sized particles. The surface chemistry of nano sized particles such as surface tension, magnetization and sheer volume of surface area improve the amount of functionalized space for reaction to occur and thus improve the capture ability and longevity of the resultant IMS particles (Cheng et al., 2009; Jiang et al., 2011).

Escherichia coli O157:H7, a type of entero-hemorrhagic *E. coli* (EHEC), was chosen as the target strain for this study because it is a common and highly infective food- and water-borne pathogen, with a median infectious dose of 23 colony forming units (CFU) (FSIS, 2001). The standard method of identifying *E. coli* O157:H7 from unknown samples is through enrichment in selective media, followed by growth on differential agar. These are identified phenotypically and serologically and toxigenically characterized by PCR, a process lasting

several days. The standard method is able to detect <1 CFU/g in foods (FDA, 2009). The IMS method presented here could be applied to extraction and concentration of *E. coli* O157:H7 from food samples, eliminating the standard method's overnight enrichment step, yet approaching the lower analytical detection limit. By pairing IMS with PCR or nearly any other rapid detection method, negative or presumptive positive results could be obtained in a few hours or less.

Experimental Design

To optimize the use of EAMNPs to extract and concentrate microbial targets the following hypothesis was tested: Mab-EAMNP extraction can be used to detect *E. coli* O157:H7 with a limit of detection of less than 10 CFU/mL and a capture efficiency of 90 – 100%, without pre-enrichment, as evaluated by culture.

In order to test the hypothesis stated above, this EAMNP IMS extraction protocol was evaluated in broth across the spectrum of culture concentrations ranging from zero to $1.0 * 10^9$ CFU/mL. A methodology called "dilute to extinction" was employed to ensure this range could be evaluated appropriately. All bacterial start solutions were diluted past the level where there were no cells to ensure a zero point was available. This methodology, combined with the limit of detection 50% (LOD_{50}) was obtained from the AOAC Task Force on Best Practices in Microbiological Methodology (2006).

Materials and Methods

E. coli O157:H7 strains, *E. coli* non H7 strains and non *E. coli* bacterial strains were obtained from the STEC Center collection at Michigan State University (MSU) (Shannon Manning, MPH, PhD), the Nano-Biosensors Laboratory at MSU (Evangelyn Alocilja, PhD), Neogen Inc. Research and Development, Lansing, Michigan (Jennifer Rice, DVM, PhD) and the

University of Georgia, Center for Food Safety (Dr. Michael Doyle, PhD). From frozen purified culture stocks (stored at -80° C), colonies were isolated by streak-plate method on trypticase soy agar (BD Biosciences, MD) plates. A single colony was used to inoculate a vial of tryptic soy broth (BD Biosciences, MD) and grown overnight at 37° C. A 1 mL aliquot of the liquid culture was transferred to a new vial of broth and stored at 37° C for up to 6 days. This culture was used to inoculate a new vial of broth with 1 mL of inoculum 10 to 24 h before each experiment to produce fresh bacterial cells which were serially diluted in 0.1% (w/v) peptone water (Fluka-Biochemika, Switzerland) prior to their use in the IMS procedure. Viable cells were enumerated by microbial plating on MacConkey agar with sorbitol (SMAC) (BD Biosciences, MD or Neogen Inc., MI), according to standard rules for plate counting (FDA BAM, 2009). Optical Density at 600 nanometers (OD 600) spectrophotometer readings (BIO-RAD Smartspec 3000, Hercules, CA) were taken from each culture before use as compared to blank Trypticase Soy Broth (TSB). Three readings were taken and averaged together.

EAMNP Production

Ferric chloride hexahydrate (EMD Chemicals, Bedford, MA), sodium acetate (CCI Chemicals, Vernon, CA), sodium acrylate, sodium chloride (NaCl), ethylene glycol, ethylenediamine, hydrochloric acid, aniline, iron (III) oxide nanopowder, ammonium persulfate, methanol, and diethyl ether were used as received from Sigma Aldrich (St. Louis, MO) in the synthesis of the EAMNPs. EAMNPs were synthesized by polymerization and acid doping of aniline monomer around gamma iron (III) oxide (γ-Fe_2O_3) nano-particles, using a slightly modified published procedure (Pal et al., 2008). Briefly, 0.650 g of iron (III) oxide nanopowder

were dispersed in 50 mL of 1 M HCl, 10 mL of deionized water and 0.4 mL of aniline monomer by sonication in an ice bath for 1 hour. A volume of 20 mL of 0.2 M ammonium persulfate (as oxidant) was added drop-wise to the above solution under continuous magnetic stirring. Color change from rust brown to dark green indicated formation of electrically-active (green) polyaniline over the smaller (brown) γ-Fe_2O_3 nano-particles. The solution was stirred for 2 hours in an ice bath and was filtered through a qualitative grade filter (2.5 μm pore size, Ahlstrom, grade 601). The supernatant thus obtained was successively filtered through a nitrocellulose membrane filter (1.2 μm pore size, Millipore) followed by washings with 10 mL each of 1M HCl, 10% (v/v) methanol, and diethyl ether. The particles were dried overnight at room temperature under vacuum. The particles ranged in size from 1.2 to 2.5 μm, and displayed a room temperature saturation magnetization of 30 emu/g.

EAMNP Antibody Conjugation

Nano-particles were immune-functionalized with monoclonal anti-*E. coli* O157:H7 antibodies obtained from Meridian Life Science, Inc. (Saco, ME). Polysorbate-20 (Tween-20), Triton X-100, phosphate buffered saline (PBS), Trizma base, casein, and sodium phosphate (dibasic and monobasic) were used in the IMS procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as follows: PBS buffer (10 mM PBS, pH 7.4), wash buffer (10 mM PBS, pH 7.4, with 0.05% Tween-20 or 0.05% Triton-X100), phosphate buffer (100 mM sodium phosphate, pH 7.4), blocking buffer (100 mM Tris–HCl buffer, pH 7.6, with 0.01% w/v casein). Magnetic separations were performed with a commercial magnetic separator (Promega Corporation,

Madison, WI). Hybridization of biological materials was carried out at room temperature with rotation on a tube rotisserie (Labquake, Thermo Scientific, MA). Scanning electron micrographs were acquired using field-emission scanning electron microscopy (JOEL 7500F, acceleration voltage of 5 kV). A superconducting quantum interference device magnetometer (Quantum design MPMS SQUID) was used for magnetic characterization of EAMNPs. Mab-conjugation of the EAMNPs was carried out by physical adsorption of antibodies onto the polyaniline surface. Electrostatic interactions between the negatively charged constant (Fc) portion of the antibodies and the positively charged polyaniline surface are thought to play a role in adsorption and orientation of the biomolecules onto the EAMNPs (Pal and Alocilja, 2009). Successful conjugation of antibodies onto EAMNPs was confirmed by measuring the quantity of antibody in the post-hybridization supernatant with a commercial fluorescence-based protein quantification kit. The measured protein concentration in the supernatant was significantly lower than the concentration of antibodies initially added to the MNPs (data not shown), indicating that antibodies were retained on the MNPs during hybridization.

EAMNPs were conjugated with monoclonal antibodies at an initial EAMNP concentration of 10 mg/mL (1% solid). A 100 µL aliquot of monoclonal, anti-*E. coli* O157:H7 antibody (suspended in 0.1 M phosphate buffer) was added to EAMNPs suspended in PBS, yielding a final antibody concentration of 1.0 mg/mL. The mixture was hybridized on a rotisserie-style rotator for 1 hour at room temperature, with 25 µL of 10X PBS being added after the first 5 min of hybridization, to increase the Sodium chloride content of the suspension to approximately 0.14 M. Following hybridization, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer (0.1M tris buffer with 0.01% casein) for 5 min. Again the conjugate was

magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer, this time for 1 hour with rotation. Finally, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 2.5 mL of 0.1 M phosphate buffered saline (PBS). The final concentration of EAMNPs in each solution was 1.0 mg/mL. Immuno-conjugated EAMNPs (Mab-EAMNPs) were stored at 4° C. Prior to experimental use, Mab-EAMNPs were further diluted in 0.1 M PBS, in order to obtain solutions of Mab-EAMNPs at 0.5 mg/mL EAMNPs.

Immuno-magnetic Separation (IMS) and Plating of Bacteria

Every experiment was applied to three different bacterial species individually: *E. coli* O157:H7 Sakai strain, *E. coli* O157:H7 2006 Spinach strain, pGFPuv (target species), *Shigella boydii* (non-target species). *S. boydii* bears less genotypic and phenotypic similarity to the target organism, but it is a commonly encountered food borne pathogen, and also produces shiga-toxin like *E. coli* O157:H7. The standard positive control used was *E. coli* O157:H7 2006 Spinach strain, pGFPuv. Its ability to fluoresce green in the presence of UV light provided confirmation that the positive samples were not cross contaminated from the positive control. The non-target organisms chosen for this study correspond with the recommendations made by the AOAC Task Force on Best Practices in Microbiological Methodology (AOAC, 2006). Serial dilutions of each bacterium were independently prepared in 0.1% (w/v) peptone water, along with subsequent negative, positive and blank controls. Three or four of the pure dilutions of each bacterium were plated (100 mL aliquots) on sorbitol MacConkey agar (SMAC) and incubated at 37° C overnight. For IMS, 50 mL of Mab-EAMNPs and 50 mL of the appropriate bacterial dilution were combined with 400 mL of 0.01 M PBS (pH 7.4), and hybridized with rotation at

room temperature for 30 minutes. After hybridization, the cell-Mab-EAMNP complexes were magnetically separated and the supernatant removed. Complexes were washed twice in wash buffer (0.01 M PBS containing 0.05% Triton-X100), and finally re-suspended in 0.5 mL of 0.01 M PBS. The IMS procedure required 40 min, and is depicted in figure 5.1.

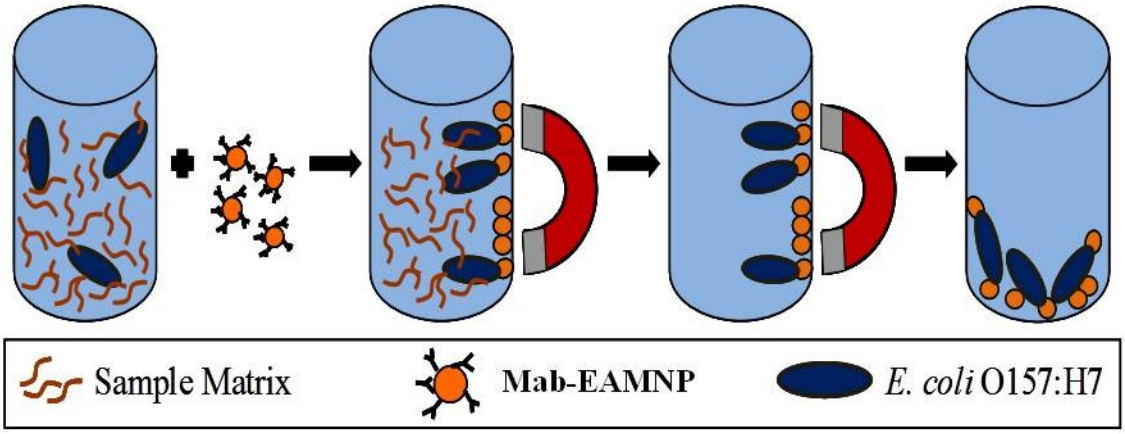

Figure 5.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes.

A 100 mL aliquot was placed on SMAC and incubated at 37° C overnight. The number of colony-forming units (CFU) in the 100-mL aliquot was determined by manually counting the colonies on each plate. For every experimental case (i.e., particular combination of Mab-EAMNP concentration, and bacteria), a minimum of two bacterial dilutions underwent IMS and were plated. In most cases a full spectrum of dilutions from 10^{-1} to 10^{-9} were run as independent units. For the lower dilutions from 10^{-1} to 10^{-5} the final IMS solution was diluted from 5 to 1 time respectively to obtain countable plates. For dilutions from the 10^{-8} and 10^{-9} series, all 500 µL present were plated.

Calculation of bacterial cell concentrations in both pure and IMS separated samples were carried out according to rules provided by the United States Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM, 2009). In addition, separate runs were performed where the final IMS solution was all plated to determine if any cells were collected at all, instead of the standard 100 µL. The CFU/mL calculations were adjusted accordingly to accommodate the change in plated dilution factor.

Capture efficiency as defined by the amount captured divided by the amount present in the original sample was calculated for each sample at each concentration for *E. coli* O157:H7, and each negative and positive control. Each similar start concentration was blocked together to facilitate evaluation. Groupings are 0-5.99 CFU/mL; 6-9.99 CFU/mL, 10-99 CFU/mL ($\log_{10}$ CFU/mL = 1) 100-999 ($\log_{10}$ CFU/mL = 2) and so on based on the start concentration. Capture efficiency was calculated by conversion of CFU/mL to $\log_{10}$ CFU/mL when above 10 CFU/mL. When calculating capture efficiency at the lower concentrations the log transformation is not performed since with a base 10 the result would be zero. Actual CFU/mL was used in this range to calculate capture efficiency. The limits of detection, high and low, were calculated at the lowest and highest levels that could be detected with culture from IMS extracted broth. The calculated concentrations of cells captured by IMS (in CFU/mL) were converted to their $\log_{10}$ values. The $\log_{10}$ conversion also normalizes the distribution (Mettler & Tholen, 2007).

Results

Immuno-magnetic capture of *E. coli* O157:H7 cells were quantified by plate counts, but capture was also visually confirmed by scanning electron microscopy (SEM). Figure 5.2 shows SEM images of (a) an individual EAMNP with diameter of approximately 1.3 μm, and (b) a Mab-EAMNP bound to an *E. coli* O157:H7 cell, after washing twice to remove non-specifically bound cells.

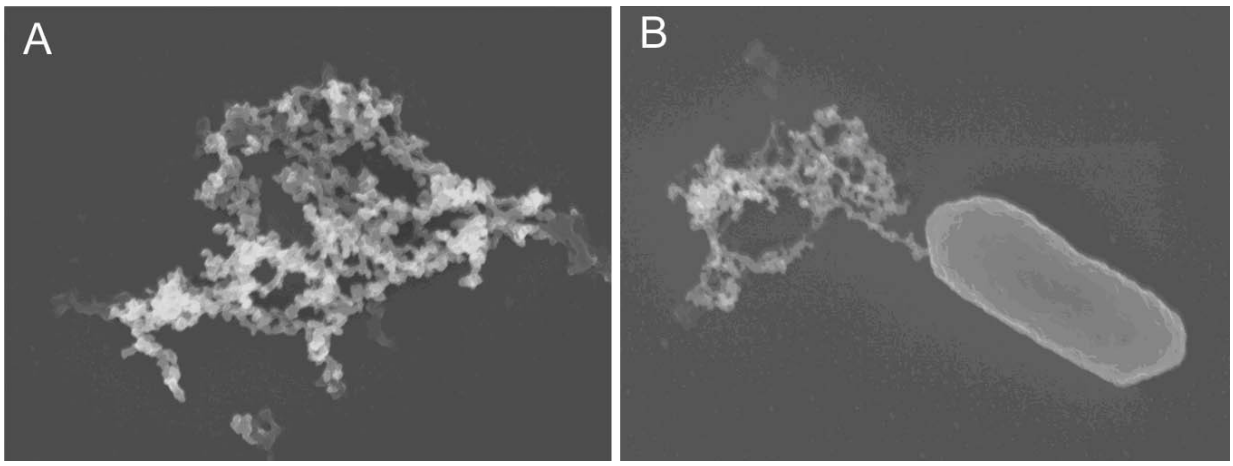

Figure 5.2: Scanning electron micrograph of (a) an individual EAMNP with diameter of approximately 1.3 μm, and (b) Mab-EAMNP bound to an *E. coli* O157:H7 cell.

Figures 5.7 through 5.10 show the capture efficiency of the EAMNPs with MLS MAb on the surface through the functional concentration range of the particles. Figure 5.7 and 5.10 are the entire range, figure 5.8 is the lower end of the range highlighted and figure 5.9 is the $\log_{10}$ CFU/mL actual capture compared to the available $\log_{10}$ CFU/mL in the start solution. Figure 5.9 demonstrates the linear range of functional capture efficiency at 90%.

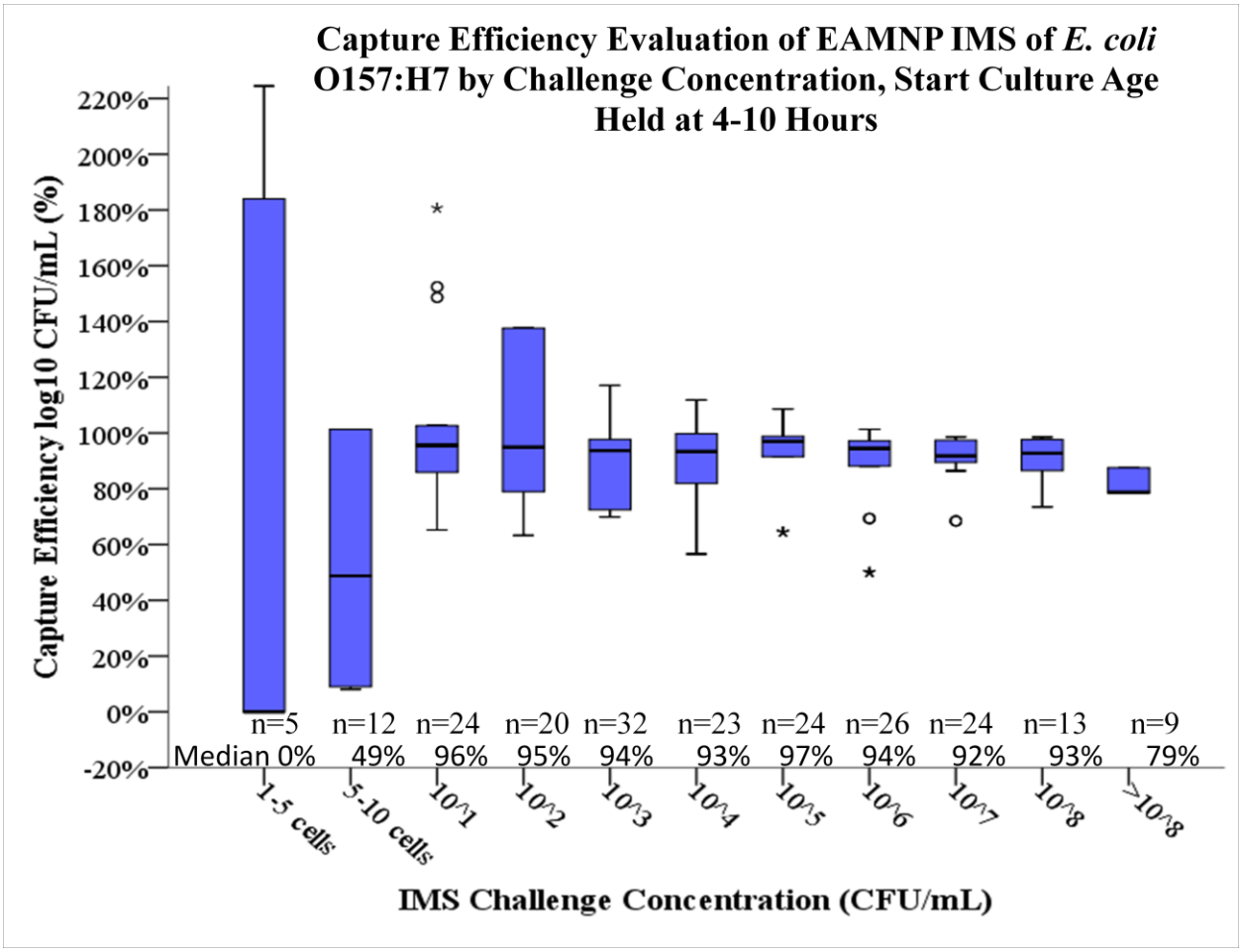

Figure 5.7: Capture Efficiency versus the $\log_{10}$ CFU/mLviable cells present in the start culture throughout the range of challenge concentrations. The culture age was held constant for these evaluations at 4-10 hours.

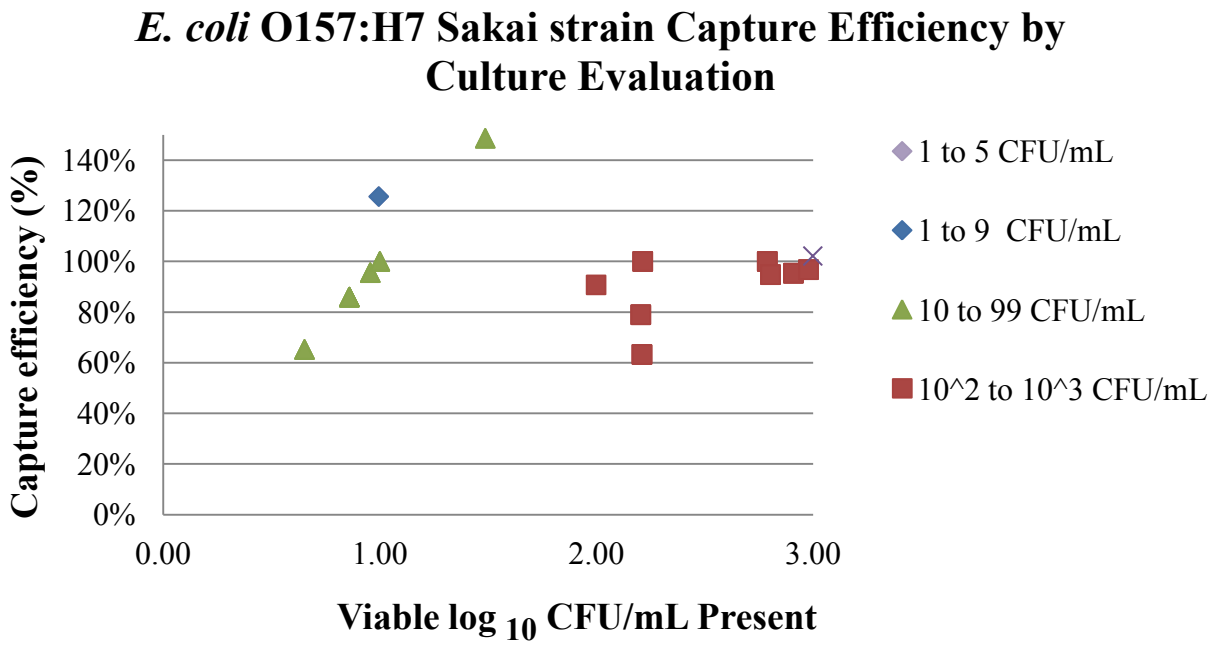

Figure 5.8: Capture Efficiency versus the $\log_{10}$ CFU/mL viable cells present in the start culture highlighting and expanding the lower range. Demonstrating the lower limists of detection of the EAMNPs extration modality of LOD = 1 CFU/mL and LOD_{50} = 6-9 CFU/mL. The culture age was not held constant for these evaluations.

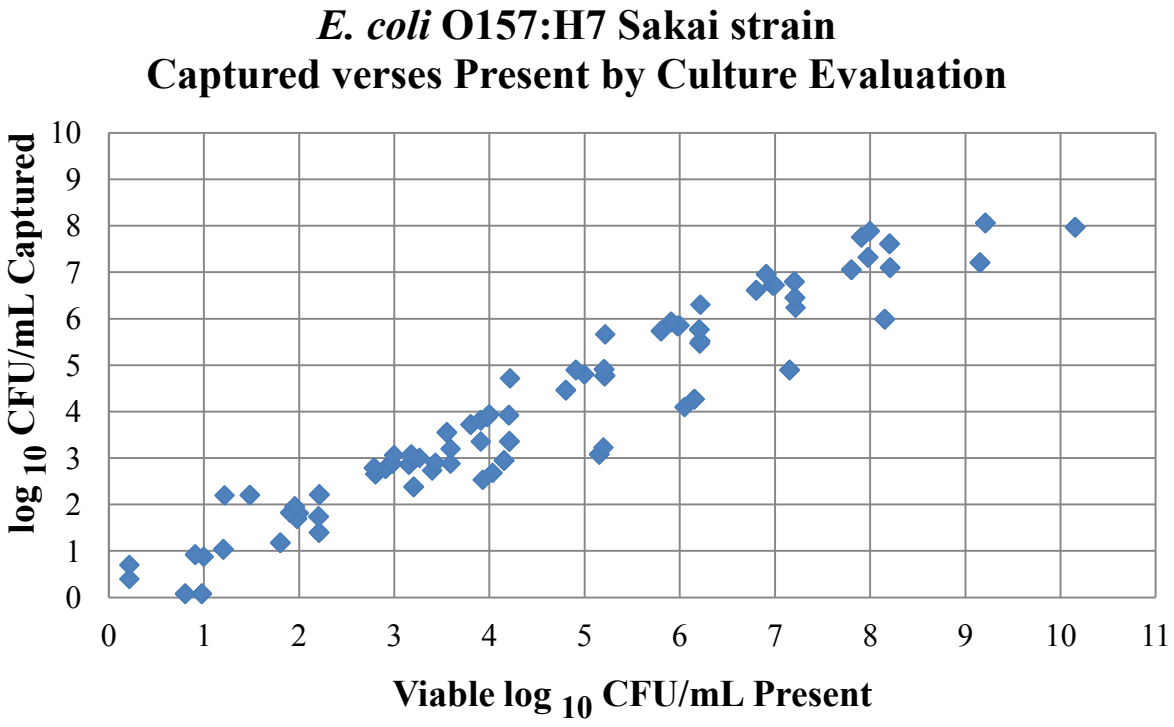

Figure 5.9: The $\log_{10}$ CFU/mL of captured cells versus the $\log_{10}$ CFU/mL viable cells present in the start culture by culture evaluation, demonstrating the upper limit of detection for the EAMNP extraction of $1.0 * 10^{9}$ CFU/mL. The culture age was not held constant for these evaluations.

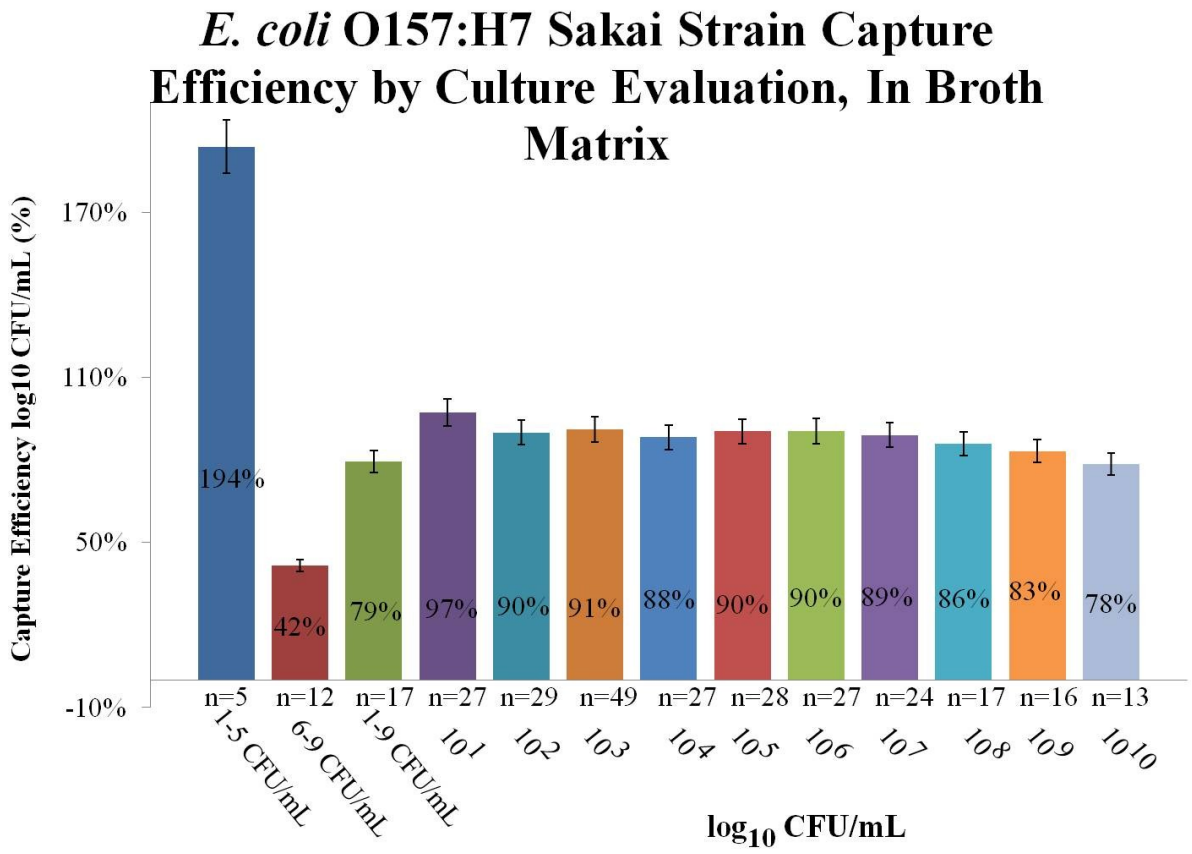

Figure 5.10: The average capture efficiency was consistently between 88% and 100% for the middle range of bacterial concentrations. This demostrates that the the functional range of consistent capture is between 6 CFU/mL and $1.0 * 10^9$ CFU/mL. Bacterial start composition concentrations were not held standard for this evaluation.

Discussion

As can be seen from the figures 5.7-5.10, even when more than $1.0 * 10^{10}$ CFU/mL are available, the IMS using EAMNP can only capture up to $1.0 * 10^9$ CFU/mL. Therefore, the upper limit of the extraction protocol is $1.0 * 10^9$ CFU/mL. The lower limit is 1-2 CFU/mL. While this lower limit may not hold true when the IMS solution is split into three fractions versus plating them all, this test is very sensitive in broth. When dealing with concentrations

below the countable range, often times the calculated CFU/mL ends up higher than physically possible due to plating error. In this case the added component of concentration of cells due to the IMS is also a factor. Accuracy at this level is difficult to evaluate. The same is true for limits of detection (LOD) reported in the literature of below 1 CFU/mL. Most of these are determined from most probable number tables and pre-enriched samples (AOAC, 2006). Further evaluation of this IMS technique will employ such methods to evaluate its capacity below 1 CFU/mL and clarify its true LOD. One method of comparison from test to test to deal with these discrepancies is the LOD_{50}. The LOD_{50} is similar to the Lethal Dose $_{50}$ (LD_{50}) used for pharmaceuticals (Casarett, Klaassen, Amdur, & Doull , 2001). LD_{50} is the concentration where 50% of the patients exposed will die. The LOD_{50} is the concentration where at least 50% of the samples are positive. It is a more consistent evaluation tool for comparing detection tools than the absolute limit of detection - which varies from run to run. As shown in figure 5.10, when using capture efficiency, the $LOD_{50,}$ in broth, is 6 -9 CFU/mL.

Care must be taken when interpreting capture efficiency as the sole evaluation tool. Above the limit of detection, there are not enough probes to capture all available bacteria, skewing culture results and thus capture efficiency. At the lower limit of detection, the non-homogeneous nature of bacteria in solution makes evaluating the capture efficiency difficult when using culture as the evaluation tool. Combined with that, when dealing with large numbers (i.e. $1.0 * 10^5$ or $2.0 * 10^5$), the coefficient (1.0 or 1.2) is already insignificant. Therefore, we calculate capture efficiency based on the $\log_{10}$ values discarding the coefficient (Mettler & Tholen, 2007). Capture efficiency as a ratio of what was captured versus what was present was

consistently between 78% and 100% with the overall average being 90% (n=195). As seen in figure 5.10, below 5 CFU/mL the capture efficiency exceeds 100% due to concentration of the bacteria during magnetic separation. Capture efficiency for this EAMNP IMS extraction has been maintained at 90-100%, on average, of the available bacteria when start cultures are used between 8-10 hours of a 1 mL transfer. Detection limits in broth are 1-2 cells as the lower limit and $1.0 * 10^9$ cells for the upper limit. This was achieved with no attempt at holding the start concentrations of the bacterial culture in the reported best range of 4-10 hours old. Future studies employing techniques to estimate concentrations below the lower culture limit of detection will be employed to further refine the true lower analytical limit of this extraction modality.

Conclusions and Limitations

This cumulative total of 450 repetitive broth challenges yielded statistically significant extraction and culture detection at all concentrations, with appropriate negative, positive and blank controls included. From broth cultures, the extraction protocol's lower limits of detection were LOD = 1 CFU/mL and LOD_{50} = 6-9 CFU/mL, using culture as the detection modality. The upper limit of detection was $1.0 * 10^9$ CFU/mL. The cost of producing one sample volume of EAMNPs conjugated with anti-*Escherichia coli* O157:H7 is ~ $0.43 and requires only 40 min from sample to result. The linear range of capture efficiency at 90% is from 10 CFU/mL to $1.0 * 10^7$ CFU/mL challenge concentrations, again by culture evaluation. Future studies employing techniques to estimate concentrations below the lower culture limit of detection will be employed to further refine the true lower analytical limit of this extraction modality. These criteria show that the EAMNP IMS methodology presented here is analytically

sensitive rapid, and inexpensive. It shows potential for extraction and concentration of microbial pathogens from food matrices, eliminating overnight enrichment steps, and could be paired with nearly any rapid detection method for practical applications in food defense, food and water safety, and clinical diagnostics.

Limitations of this extraction method include the fact that both viable and non-viable cells are extracted with this methodology. Further studies are designed and being implemented to evaluate the Mab-EAMNP to determine the reaction kinetics of non-viable verses viable cells on the antibody target region in broth cultures. Inclusivity and exclusivity of microbial families and biosensor platform experiments are necessary before validation trials of the whole biosensor can proceed. The ultimate goal of this extraction is to be able to multiplex many EAMNPs with different Mab targets to allow multiplexing. Future multiplexing with multiple EAMNP and multiple bacterial targets could have interactions between the EAMNPs or between the mixed antibodies. Certain matrices may remove the Mab from the surface of the EAMNPs and make their use in that matrix impossible. The largest drawback to this method is the need for refrigeration of the Mab-EAMNPs. When field based technologies are discussed, shelf stable reagents are an advantage.

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CHAPTER 6: Inclusivity and Exclusivity Evaluation of Electrically Active Magnetic Nanoparticles as Accurate and Efficient Microbial Extraction Tools

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Abstract

Food defense requires the means to efficiently screen large volumes of food for microbial pathogens. Even rapid detection methods often require lengthy enrichment steps, making them impractical for this application. There is a great need for rapid, sensitive, specific, and inexpensive methods for extracting and concentrating microbial pathogens from food, in the field. In this study, an immuno-magnetic separation (IMS) methodology was developed for *Escherichia coli* O157:H7, using electrically active magnetic nano-particles (EAMNPs). The entire IMS procedure requires only 40 minutes, and antibody-conjugated EAMNPs show no decline in performance up to 150 days after conjugation. A key aspect of a successful diagnostic different methodologies and practical implementation in practice is the ability to discern between non target bacteria and still include all strains of the target bacteria. These are the concepts of exclusivity and inclusivity, respectively. The microbiological inclusivity and exclusivity of the EAMNP IMS method was evaluated against 35 *E. coli* O157 strains and 29 other organisms many in the Enterobacteriacea family. The extraction protocol's inclusivity within strain is 94% and exclusivity outside the *E. coli* O157 family is 87%.

Introduction

Food-borne microbial pathogens comprise one of the single largest threats to maintaining a safe food supply. Food defense (securing food sources against malicious biological attack) and food safety (identifying and eradicating contamination from natural sources) (Spink, 2009) are growing increasingly relevant, as foods are processed and shipped further and faster than ever before (Zach, Doyle, Bier, Cxuprynski, 2012). Standard overnight culture methods for identifying microbial pathogens are no longer adequate, as the speed and breadth of food movement demands rapid, sensitive, specific, and economical means of extracting and detecting pathogens from food sources. Food and Drug Administration (FDA) inspections have dropped by 81% since 1972 and 47% between 2003 and 2006 (CSPI, 2007). Even with the new Food Safety Modernization Act (FSMA) the highest risk plants will only be inspected every three years by the FDA (Olsson, Weeda, Bode, 2010; Sjerven, 2012). FDA in the United States inspects less than 1% of the imported food supply before consumption and less than 0.2% of the imported food has laboratory analysis at all (CSPI, 2007). Ultimately, companies are responsible for their own products and must protect their own brands. They cannot depend completely on government inspectors or third-party auditors to ensure authenticity and safety of materials and products (Zach et al., 2012). Decreasing the cost of a first line evaluation of food, should allow a food company to test a greater percentage of their product, protecting their bottom line in preventing recalls and their brand reputation in the market. Moving the first line testing of food to the farm and field will allow both regulatory agencies and supply chain managers to find problems earlier before combination at the production or packing plant, benefiting both food safety and food defense. The objective of this research was to challenge an immuno-magnetic separation (IMS) methodology using electrically active magnetic nanoparticles (EAMNPs)

against a range of bacteria. Inclusivity and exclusivity are the ability to microbiologically discriminate against non-target cells yet include all versions of target cells. The AOAC Research Institute Performance Tested Methods Program (AOAC-PTM) recommends challenge against 30 in strain targets and 20 out of strain isolates to validate rapid microbiological methods for commercial use(AOAC, 2009).

Escherichia coli O157:H7, a type of entero-hemorrhagic E. coli (EHEC), was chosen as the target strain for this study because it is a common and highly infective food- and water-borne pathogen, with a median infectious dose of 23 colony forming units (CFU) (FSIS, 2001). Further rationale for selection of *E. coli* O157:H7 is that it is an important contributor to food borne outbreaks in the United States (Scallan, 2011) and many outbreak strains were available for test evaluation. The standard method of identifying *E. coli* O157:H7 from unknown samples is through enrichment in selective media, followed by growth on differential agar. These are identified phenotypically and serologically and toxigenically characterized by PCR, a process lasting several days. The standard method is able to detect <1 CFU/g in foods (FDA, 2009). The IMS method presented here could be applied to extraction and concentration of *E. coli* O157:H7 from food samples, eliminating the standard method's overnight enrichment step.

Experimental design

To optimize the use of EAMNPs to extract and concentrate microbial targets the following hypothesis was tested: EAMNP extraction is 100% inclusive and 98% exclusive when challenged by 30 in-strain *E. coli* O157:H7 serotypes and 20 non *E. coli* O157:H7 bacteria.

To test the above hypothesis, 74 different cultures were grown, pelleted, lysed and run on a western blot. The resultant gels were developed and designated as reactive or non reactive to the antibody used. Two commercial antibodies were evaluated. The best of the two antibodies

was conjugated to the EAMNPs and the same panel of bacteria repeated in an IMS extraction protocol using culture as the diagnostic tool.

Materials and Methods

Evaluation of inclusivity and exclusivity of the EAMNPs extraction protocol was accomplished in Trypticase Soy Broth (TSB). Using Western blot techniques, 74 strains of bacteria were challenged versus mouse anti *E .coli* O157:H7 Mab, purchased from Meridian Life Science, Inc. (Saco, ME) and against Goat anti *E. coli* O157:H7 Mab, purchased from KPL (Gaithersburg, MD). For the Western Blot, NuPAGE® Novex 4-12% Bis-Tris Gels, 1.0 mm thick, 10 well; PVDF membrane filter Sandwiches; Novex Sharp pre-stained ladder 2x250µL; NuPAGE® LDS sample buffer (4x); NuPAGE® sample reducing agent (10x); BCIP NBT sigma fast developer, NuPAGE®MES SDS running buffer; XCell *SureLock*™ Novex Mini-Cell vertical electrophoresis chamber and XCell II™ Blot Module (B) Western blot transfer kits were purchased from Invitrogen Inc. (Carlsbad, CA) and used as directed. Mouse anti-*E. coli* O157:H7 Mab; 1° MLS Mab; was purchased from Meridian Life Science, Inc. (Saco, ME). Goat anti-*E. coli* O157:H7 Mab; 1° KPL Mab; Goat anti-mouse Mab with Alkaline Phosphotase; 2° MLS Mab; and Rabbit anti-goat Mab with Alkaline Phosphotase; 2° KPL Mab; were purchased from KPL (Gaithersburg, MD). Remel® Wellcolex O157 agglutination kits (Lenexa, KS) and Neogen Inc, Reveal®O157:H7 kits (Lansing, MI) were purchased and used as directed. Polysorbate-20 (Tween-20), 20% Methanol, Sodium Chloride, Glycine, Tris buffered saline (TBS), trizma base, and non fat dried milk (Kroger Inc.; St. Johns, MI) were used in the Western

blot procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO).

All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as follows: TBS buffer (100 mM PBS, pH 7.4), wash buffer (TTBS) (100 mM TBS, pH 7.4, with 0.0005% Tween-20); Nonfat dried milk block (NFDM) (25 g/40 mL 1X TBS); 1° antibody was used at 1:1000 for both MLS and KPL Mabs and 2° antibody was used at 1:10,000; both in NFDM block. Loading buffer was prepared with 250 µL loading dye, 100 µL reducing agent and 650 µL DI water. Running buffer at 20 X was purchased commercially and diluted to 1X for use. 20X Transfer buffer (3.03g Trizma base, 14.4g glycine, 200 mL 20% methanol, 1000 mL DI water) was diluted to 1X for use.

EAMNP Production

Ferric chloride hexahydrate (EMD Chemicals, Bedford, MA), sodium acetate (CCI Chemicals, Vernon, CA), sodium acrylate, sodium chloride (NaCl), ethylene glycol, ethylenediamine, hydrochloric acid, aniline, iron (III) oxide nanopowder, ammonium persulfate, methanol, and diethyl ether were used as received from Sigma Aldrich (St. Louis, MO) in the synthesis of the EAMNPs. EAMNPs were synthesized by polymerization and acid doping of aniline monomer around gamma iron (III) oxide (γ-Fe_2O_3) nano-particles, using a slightly modified published procedure (Pal, Setterington & Alocilja, 2008). Briefly, 0.650 g of iron (III) oxide nanopowder were dispersed in 50 mL of 1 M HCl, 10 mL of deionized water and 0.4 mL of aniline monomer by sonication in an ice bath for 1 hour. A volume of 20 mL of 0.2 M ammonium persulfate (as oxidant) was added drop-wise to the above solution under continuous magnetic stirring. Color change from rust brown to dark green indicated formation of

electrically-active (green) polyaniline over the smaller (brown) γ-Fe_2O_3 nano-particles. The solution was stirred for 2 hours in an ice bath and was filtered through a qualitative grade filter (2.5 μm pore size, Ahlstrom, grade 601). The supernatant thus obtained was successively filtered through a nitrocellulose membrane filter (1.2 μm pore size, Millipore) followed by washings with 10 mL each of 1M HCl, 10% (v/v) methanol, and diethyl ether. The particles were dried overnight at room temperature under vacuum. The particles ranged in size from 1.2 to 2.5 μm, and displayed a room temperature saturation magnetization of 30 emu/g.

E. coli O157:H7 strains, *E. coli* non H7 strains and non *E. coli* bacterial strains were obtained from the STEC Center collection at Michigan State University (MSU) (Shannon Manning, MPH, PhD), the Nano-Biosensors Laboratory at MSU (Evangelyn Alocilja, PhD), Neogen Inc. Research and Development, Lansing, Michigan (Jennifer Rice, DVM, PhD) and the University of Georgia, Center for Food Safety (Dr. Michael Doyle, PhD). From frozen purified culture stocks (stored at -80° C), colonies were isolated by streak-plate method on trypticase soy agar (BD Biosciences, MD) plates. A single colony was used to inoculate a vial of tryptic soy broth (BD Biosciences, MD) and grown overnight at 37° C. A 1 mL aliquot of the liquid culture was transferred to a new vial of broth and stored at 37° C for up to 6 days. This culture was used to inoculate a new vial of broth with 1 mL of inoculum 10 to 24 h before each experiment to produce fresh bacterial cells which were serially diluted in 0.1% (w/v) peptone water (Fluka-Biochemika, Switzerland) prior to their use in the IMS procedure. Viable cells were enumerated by microbial plating on MacConkey agar with sorbitol (SMAC) (BD Biosciences, MD or Neogen Inc., MI), according to standard rules for plate counting (FDA BAM, 2009). Optical Density at 600 nanometers (OD 600) spectrophotometer readings (BIO-RAD Smartspec 3000,

Hercules, CA) were taken from each culture before use as compared to blank Trypticase Soy Broth (TSB). Three readings were taken and averaged together.

Nano-particles were immune-functionalized with monoclonal anti-*E. coli* O157:H7 antibodies obtained from Meridian Life Science, Inc. (Saco, ME). Polysorbate-20 (Tween-20), Triton X-100, phosphate buffered saline (PBS), Trizma base, casein, and sodium phosphate (dibasic and monobasic) were used in the IMS procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as follows: PBS buffer (10mM PBS, pH 7.4), wash buffer (10mM PBS, pH 7.4, with 0.05% Tween-20 or 0.05% Triton-X100), phosphate buffer (100mM sodium phosphate, pH 7.4), blocking buffer (100mM Tris–HCl buffer, pH 7.6, with 0.01% w/v casein). Magnetic separations were performed with a commercial magnetic separator (Promega Corporation, Madison, WI). Hybridization of biological materials was carried out at room temperature with rotation on a tube rotisserie (Labquake, Thermo Scientific, MA). Scanning electron micrographs were acquired using field-emission scanning electron microscopy (JOEL 7500F, acceleration voltage of 5 kV). A superconducting quantum interference device magnetometer (Quantum design MPMS SQUID) was used for magnetic characterization of EAMNPs.

EAMNP Antibody Conjugation

Mab-conjugation of the EAMNPs was carried out by physical adsorption of antibodies onto the polyaniline surface. Electrostatic interactions between the negatively charged constant (Fc) portion of the antibodies and the positively charged polyaniline surface are thought to play a role in adsorption and orientation of the biomolecules onto the EAMNPs (Pal and Alocilja, 2009). Successful conjugation of antibodies onto EAMNPs was confirmed by measuring the

quantity of antibody in the post-hybridization supernatant with a commercial fluorescence-based protein quantification kit. The measured protein concentration in the supernatant was significantly lower than the concentration of antibodies initially added to the MNPs (data not shown), indicating that antibodies were retained on the MNPs during hybridization. EAMNPs were conjugated with monoclonal antibodies at an initial EAMNP concentration of 10 mg/mL (1% solid). A 100 µL aliquot of monoclonal, anti-*E. coli* O157:H7 antibody (suspended in 0.1 M phosphate buffer) was added to EAMNPs suspended in PBS, yielding a final antibody concentration of 1.0 mg/mL. The mixture was hybridized on a rotisserie-style rotator for 1 hour at room temperature, with 25 µL of 10X PBS being added after the first 5 min of hybridization, to increase the sodium chloride content of the suspension to approximately 0.14 M. Following hybridization, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer (0.1M tris buffer with 0.01% casein) for 5 min. Again the conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer, this time for 1 hour with rotation. Finally, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 2.5 mL of 0.1 M phosphate buffered saline (PBS). The final concentration of EAMNPs in each solution was 1.0 mg/mL. Immuno-conjugated EAMNPs (Mab-EAMNPs) were stored at 4° C. Prior to experimental use, Mab-EAMNPs were further diluted in 0.1 M PBS, in order to obtain solutions 0.5 mg/mL EAMNPs.

Immuno-magnetic Separation (IMS) and Plating of Bacteria

Serial dilutions of each bacterium were independently prepared in 0.1% (w/v) peptone water. Three or four of the pure dilutions of each bacteria were plated (100 mL aliquots) on

sorbitol MacConkey agar (SMAC) and incubated at 37° C overnight. For IMS, 50 mL of Mab-EAMNPs and 50 mL of the appropriate bacterial dilution were combined with 400 mL of 0.01 M PBS (pH 7.4), and hybridized with rotation at room temperature for 30 minutes. After hybridization, the cell-Mab-EAMNP complexes were magnetically separated and the supernatant removed. Complexes were washed twice in wash buffer (0.01 M PBS containing 0.05% Triton-X100), and finally re-suspended in 0.5 mL of 0.01 M PBS. The IMS procedure required 40 min, and is depicted in Figure 6.1.

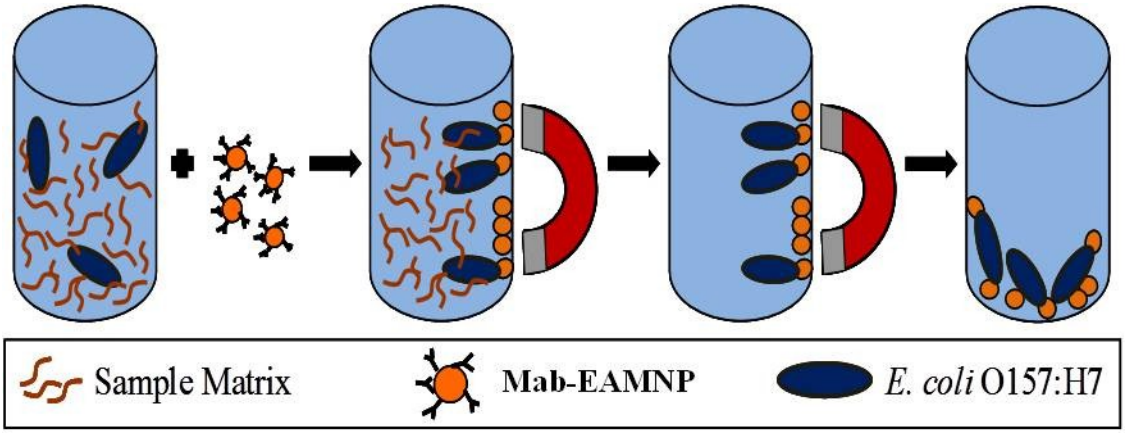

Figure 6.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes.

A 100-mL aliquot was placed on SMAC and incubated at 37° C overnight. The number of colony-forming units (CFU) in the 100-mL aliquot was determined by manually counting the colonies on each plate. For every experimental case (i.e., particular combination of Mab-EAMNP concentration, and bacteria), a minimum of two bacterial dilutions underwent IMS and were plated. In most cases a full spectrum of dilutions from 10^{-1} to 10^{-9} were run as independent units. For the lower dilutions from 10^{-1} to 10^{-5} the final IMS solution was diluted

from 5 to 1 time respectively to obtain countable plates. For dilutions from the 10^{-8} and 10^{-9} series, all 500 µL present were plated.

Calculation of bacterial cell concentrations in both pure and IMS separated samples were carried out according to rules provided by the United States Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM, 2009). In addition, separate runs were performed where the final IMS solution was all plated to determine if any cells were collected at all, instead of the standard 100 µL. The CFU/mL calculations were adjusted accordingly to accommodate the change in plated dilution factor.

Western Blot

For western blot analysis, cells were grown as described above and monitored until OD600 readings were 0.800, 1 mL of cells were pelleted in an Eppendorf 15 amp 5810R or 5415R centrifuge at 10,000 RPMs and stored at -80° C. If time did not permit waiting for the cultures to grow, OD600 readings were taken on an actively growing culture and divided by 0.800 to estimate the increase in volume over 1 mL of bacterial culture to centrifuge. The resultant cell pellet was re-suspended in 125 µL of loading buffer and freeze fractured with three, five minute rotations between a heating block at 95°C and the -80°C freezer. The resultant suspension was either loaded immediately or held at -80°C for later electrophoresis.

The XCell *SureLock*™ Novex Mini-Cell vertical electrophoresis chamber was filled with 1 X MES SDS Running buffer. 10 µg of fractured cells were loaded in each of 9 lanes per gel with 15µg of pre-stained ladder in lane 1. A Fischer Scientific FB200 power source was run on the chamber electrodes at 400 µAmps, 100 V for 20 minutes then 120 V for 1 hour or until

the dye front reached the bottom of the commercial12% Bis Tris gels. Sponges, filter paper and the electrophoresis gels were equilibrated for 10 min in 1X transfer buffer. The PVDF filters were primed for 30 seconds in 20% w/v methanol and soaked in 1X transfer buffer for 10 minutes prior to loading the sandwiches. Western blot sandwiches were made following manufacturer's directions, loaded into the XCell II™ Blot Module (B) Western blot transfer kits, placed into the XCell *SureLock*™ Novex Mini-Cell vertical electrophoresis chamber and covered with 1X transfer buffer. The blot was run for 1 hour at 30 V, 400 μAmps.

After transfer, the resultant filter was washed five minutes in TBS shaking on an Eppendorf orbital shaker and two hours shaking in NFDM block. Some gels were blocked overnight. After blocking, the appropriate 1:1000 1°Antibody was placed on the gels either for two hours or overnight as necessary. The 1°Antibody was kept at 4°C and used up to three times. After 1°Antibody incubation, two five minute washes with TTBS and a two hour 1:10,000 2°Antibody incubation the gels were washed, shaking, with two TTBS and one TBS five minute washes. The 2°Antibody was kept at 4°C and used up to three times. Development was accomplished according to manufacturer's directions with BCIP NBT sigma fast developer until color was visible. The final blot was rinsed in DI water, dried, and kept from light.

Statistical analysis

The gold standard used was microbial culture. Test positive was evaluated by either culture growth after IMS extraction on SMAC or Western immunoblot development of bacterial components. Inclusivity is calculated as the number of tests correctly identified as positive

divided by the total number of tests that were actually positive as identified by the gold standard. Exclusivity is calculated by the number of tests that were identified as negative divided by the total number of tests that were negative as determined by the gold standard.

Results

The gels are shown in Appendix 6.1 and the results by individual bacteria are shown in Appendix 6.2. In summary, 74 bacteria were challenged versus the MLS anti *E. coli* O157:H7 Mab and the KPL anti *E. coli* O157:H7 Mab. The MLS antibody correctly identified 31out of 35 *E. coli* O157:H7 strains, 8 of 10 *E. coli* O157 non H7 strains and excluded 25 of 29 non-*E. coli* O157 strains. The same 74 strains were challenged versus the KPL. The KPL Mab correctly identified 31 out of 35 *E. coli* O157:H7 strains, 7 of 10 *E. coli* O157 non H7 strains and excluded 24 of 29 non-*E. coli* O157 strains. The four *E. coli* O157:H7 strains that did not react were further evaluated by trace back of origins, Welcolux® O157:H7 and Neogen Reveal®. One was found to have been incorrectly identified as the Spinach 2006 outbreak strain and was actually an AEEC strain from Africa and should have been excluded as a non- *E. coli* O157:H7. The second did not agglutinate on the Welcolux® O157:H7 agglutination kit for the O157 antigen and was discarded due to lack of records on its origin. The last two strains #125 and #126 were traced back to their origin and verified as *E. coli* O157:H7. To evaluate if the missed cultures were *E. coli* O157:H7, an agglutination test using both Welcolux® O157:H7 agglutination kit and the Neogen Reveal 1 and Reveal 2 assay were performed. The results are shown in Tables 6.1 and 6.2. The potential for streak contamination was considered, to rule that out, two independent agglutination tests were performed. One test was from the original plates supplied by Neogen Inc. and were used directly from 4°C, while the second test utilized a freshly streaked plate (sub-

cultured from the original) and used fresh out of the 37°C incubator. To account for possible gel failure, the three fresh whole cell extracts were prepared for these three strains and were run again in a new gel. The results were the same and the two cultures were verified as *E. coli* O157:H7.

Table 6.1: O157 Agglutination reaction

Test	*E. coli* O157:H7 125	*E. coli* O157:H7 126	Culture type
Agglutination O157 #1	+	+	Original culture plate from 4°C
Agglutination O157 #2	+	+	Re-streaked from 37°C

Table 6.2: Results of Reveal 1.0 and Reveal 2.0 testing

Test	*E. coli* O157:H7 A110	*E. coli* O157:H7 GT 125	*E. coli* O157:H7 GT 126	*E. coli* O157:H43 GT 4136	*E. coli* O157:H19 GT 164	*E. agglomerans*	Culture type
Reveal 1.0 Grade Intensity	+ 2.5 571,000	+ 0.5 22,547	+ ghost 3.046	+ 2.5 533,000	+ 1.0 108,000	neg 0 1,585	New cultures stationary
Reveal 1.0 Grade Intensity	+ 3 816,000	+ ghost 5386	+ ghost 3382	+ 3 1.7 mil	+ 2.5 389,000	neg 0 689	New cultures log phase
Reveal 2.0 Intensity	+ 79,000	+ 970,000	+ 70,000	+ 843,000	+ 9700	neg 729	New cultures log phase
Reveal 2.0 #2 Intensity	+ 40,600	+ 831,000			+ 10,700		New cultures log phase

Table 6.3: MLS Mab; Inclusivity and exclusivity by culture or Western blot of IMS separated *E. coli* O157:H7 versus non-*E. coli* O157:H7

	E. coli O157:H7 positive	*E. coli* O157:H7 negative
IMS-Mab-EAMNP-culture or Western blot positive (MLS)	33	12
IMS-Mab-EAMNP – culture or Western blot negative (MLS)	2	27
	Inclusivity	94%
	Exclusivity	69%

Table 6.4: KPL Mab; Inclusivity and exclusivity by culture or Western blot of IMS separated *E. coli* O157:H7 versus non-*E. coli* O157:H7

	E. coli O157:H7 positive	*E. coli* O157:H7 negative
IMS-Mab-EAMNP-culture or Western blot positive (KPL)	33	14
IMS-Mab-EAMNP – culture or Western blot negative (KPL)	2	25
	Inclusivity	94%
	Exclusivity	64%

Table 6.5: MLS Mab; Inclusivity and exclusivity by culture or Western blot of IMS separated *E. coli* O157 versus non-*E. coli* O157

	E. coli O157 positive	*E. coli* O157 negative
IMS-Mab-EAMNP-culture or Western blot positive (MLS)	33	4
IMS-Mab-EAMNP – culture or Western blot negative (MLS)	2	27
	Inclusivity	94%
	Exclusivity	87%

Table 6.6: KPL Mab; Inclusivity and exclusivity by culture or Western blot of IMS separated *E. coli* O157 versus non-*E. coli* O157

	E. coli O157 positive	*E. coli* O157 negative
IMS-Mab-EAMNP-culture or Western blot positive (KPL)	33	5
IMS-Mab-EAMNP – culture or Western blot negative (KPL)	2	25
	Inclusivity	94%
	Exclusivity	83%

Discussion

The inclusivity of the EAMNP IMS method was evaluated against 35 *E. coli* O157 organisms and 29 other organisms, many in the Enterobacteriacea family. The antibody used an uncharacterized Mab to the O and Lipopolysaccharide (LPS) fraction of the *E. coli* O157:H7 bacteria cell wall and should detect O157 expressing organisms regardless of genus or H-type. The inclusivity results were largely as expected as the Mab was able to detect most of the O157

expressing serotypes, with the exception of two missed serotypes, GT 126 and GT 125. These two serotypes were expected to be positive but were negative on the Western blot and the EAMNP IMS extraction. There are three potential reasons for these two serotypes being missed by these two assays. The first is the potential the lack of expression of the O157 LPS in these two serotypes by their growth stage or by their response to the media, temperature and the microenvironment of the culture itself. Organisms change their surface proteins in all of these conditions. These two antibodies are not fully characterized as to which protein they target and the possibility exists that these two serotypes do not have the particular surface protein these antibodies are targeted at. If we were aware of the protein and its associated gene there could be a molecular evaluation available to solve this dilemma. The second reason is the potential for contamination or old cultures. Environmental sample such as these are isolates of outbreaks and purity is not necessarily guaranteed. This could be a mutated batch due to successive passes in culture or the older the culture is can change the microenvironment with metabolic byproducts, thus changing the surface protein expression. Again genetic analysis could resolve this. The third potential reason is that the Western blot process probes linear proteins only. As can be seen in Table 6.1 and 6.2, the two strains that were negative by Western blot came up positive on the agglutination test from two independent plates for each organism and in both Reveal assays. Therefore, the strains do express the O157 LPS. Repeating the analysis with fresh subcultures and new cultures eliminates the potential for culture age being responsible for the results. Since Western blots make the proteins linear perhaps the tertiary structure is necessary for recognition. The MLS Mab Western blots showed only a single, relatively high molecular weight band that corresponded to the fully assembled O157 LPS protein and not a typical LPS multiband ladder seen with the KPL LPS antibody. (Appendix 6.A) The KPL Mab included the ladder which

corresponds to the Mab recognizing the fragments of LPS, not just its whole fraction. (Appendix 6.A) The denaturing properties of the Western blot could still be a problem for single site Mab binding. The possibility that there is something different regarding the LPS structure of these strains also exists, therefore the Reveal assay was run on whole cells. This allowed for the screening of these and other strains against the antibodies used by Neogen in their Reveal 1.0 and Reveal 2.0 tests to determine whether an O157 polyclonal antibody test (Reveal 1.0) would react with these strains and whether these strains express the pathogenic form of the O157 LPS by testing with Reveal 2.0 which utilizes two separate antibodies. Reveal 1.0 uses a polyclonal antibody (Pab) to detect the O157 LPS antigen. Both organisms that were negative via Western blot were positive on Reveal 1.0. Reveal 2.0 uses a proprietary combination of a Pab and a Mab directed towards distinct epitopes of the O157 LPS. Again, both organisms that were negative via Western blot were positive on Reveal 2.0.

Of the 10 *E. coli* O157 strains that were non-motile or of another H- phenotype, all but one serotype of *E. coli* O157:H38 and one of *E. coli* O157:H19 reacted. There was cross reactivity with 4 of 29 organisms in the non *E. coli* O157 bacteria challenges, two *Citrobacter fruendii*, *Escherichia hermanni* and *Enterococcus fecalis.* The KPL Mab was selected as a potential correction for the missing strains. The KPL Mab correctly identified 33 out of 35 *E. coli* O157:H7 strains (miss classifying the same two strains as the MLS Mab test). Of the 10 *E. coli* O157 strains that were non motile or of another H- phenotype all but one strain of *E. coli* O157:H38 reacted with the KPL Mab. There was cross reactivity with 5 of 29 organisms in the non *E. coli* O157 strain challenge, the same 4 as the MLS Mab test plus another *Citrobacter* strain.

Conclusions and Limitations

The purpose of these tests was to screen the anti-O157 Mab for inclusivity and exclusivity. The AOAC Performance Test Method protocol for single laboratory validation studies recommends 30 in strain comparisons and 20 other bacterial strain comparisons (AOAC PTM, 2009). Using a combination of EAMNP extraction and Western blot characterization of the antibody, this study has accomplished this suggested criterion for testing. Challenging 35 strains of *E. coli* O157:H7; many of which were outbreak strains, and 39 other strains; many very closely related to the target organism, provided a comprehensive inclusivity/exclusivity screen for the Mab used on the EAMNPs. Using the Western Blot, this fast characterization and evaluation of the bacterial selection agent, the Mab, allows a large panel of inclusive and exclusive bacterial agents to be screened inexpensively without interference with the EAMNP itself or the biosensor detection. The MLS antibody correctly identified 33 out of 35 verified *E. coli* O157:H7 strains, 8 of 10 *E. coli* O157 non H7 strains and 25 of 29 other bacteria. The same 74 strains were challenged versus the KPL anti *E. coli* O157:H7 Mab. The KPL Mab correctly identified 33 out of 35 *E. coli* O157:H7 strains, 7 of 10 *E. coli* O157 non H7 strains and 24 of 29 other bacteria. KPL Mab will not correct the minor inclusivity problem the MLS Mab has exhibited, but will have poorer specificity. Other Mabs will be evaluated in the future, but for now missing two strains and incorrectly capturing four unrelated and 8 closely related organisms is acceptable for a series based screening tool such as this system is designed for.

The target for an ideal test would be 99 – 100% test sensitivity and 90% or greater test specificity (Hennekens & Buring, 1987). In pure cultures, challenged independently, exclusivity and inclusivity are used as the evaluation tool, but the same target percentages apply. The goal of a screening test run in series with a second diagnostic test is to cast a wide net and not miss

any *E. coli* O157:H7 pathogens. This IMS with EAMNPs is designed as a primary extraction for a primary screening tool. Higher inclusivity and lower exclusivity of the primary screen allows the secondary diagnostic test to have a higher prevalence of the target bacteria and thus a better positive predictive value. The primary screening tool missing a strain of *E. coli* O157:H7, however, is not tolerable. In order to overcome this problem, a second Mab or a new Mab will need to be added to the system, potentially in a 1:1 mixture with separate conjugation steps. This may decrease exclusivity even more. When evaluated against only non O157 organisms, the exclusivity for both antibodies increases to 87% and 83% from the high sixty percent range. In other words, the ability of both antibodies to exclude non O157 organisms is closer to the exclusivity goal of 90% for a screening tool. In the interest of time and to allow completion of the other objectives, changes to the type of antibody or the addition of a second antibody will be left for a later study.

Limitations of this extraction method include the fact that both viable and non-viable cells are extracted with this methodology. Further studies are designed and being implemented to evaluate the Mab-EAMNP to determine the reaction kinetics of non-viable verses viable cells on the antibody target region in broth cultures. Biosensor platform experiments are necessary before validation trials of the whole biosensor can proceed. The ultimate goal of this extraction is to be able to multiplex many EAMNPs with different Mab targets to allow multiplexing. Future multiplexing with multiple EAMNP and multiple bacterial targets could have interactions between the EAMNPs or between the mixed antibodies. Certain matrices may remove the Mab from the surface of the EAMNPs and make their use in that matrix impossible. Another drawback to this method is the need for refrigeration of the Mab-EAMNPs. When field based technologies are discussed, shelf stable reagents are an advantage.

Appendices Chapter 6

Appendix 6.A: Western Blot Gel Analysis of Selected Antibodies against the Inclusivity and Exclusivity Panel of Bacteria

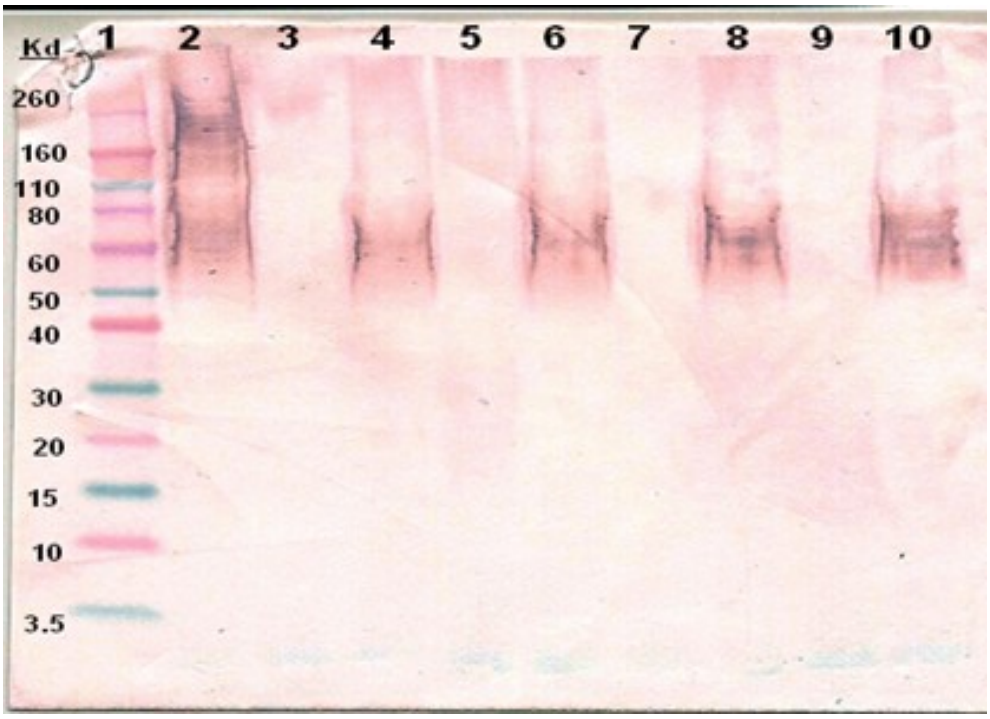

Figure 6.A.1: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #1. MLS 12-22-10 A.

Table 6.A.1: Lane identification key for Gel #1 in Figure 6.A.1. MLS 12-22-10 A

	Organism	**Result**
1	Ladder	
2	*Citrobacter freundii* CF3 GT 5742	pos
3	*Enterobacter agglomerans* GT 1611	neg
4	*E. coli* O157:H43 GT 4136	pos
5	*E. coli* O157:H19 164	neg
6	*E. coli* O157:H7 GT 632	pos
7	*E. coli* O157:H7 GT 127	neg
8	*E. coli* O157:H7 A110	pos
9	*E. coli* O157:H7 125	neg
10	*E. coli* O157:NM GT 4141	pos

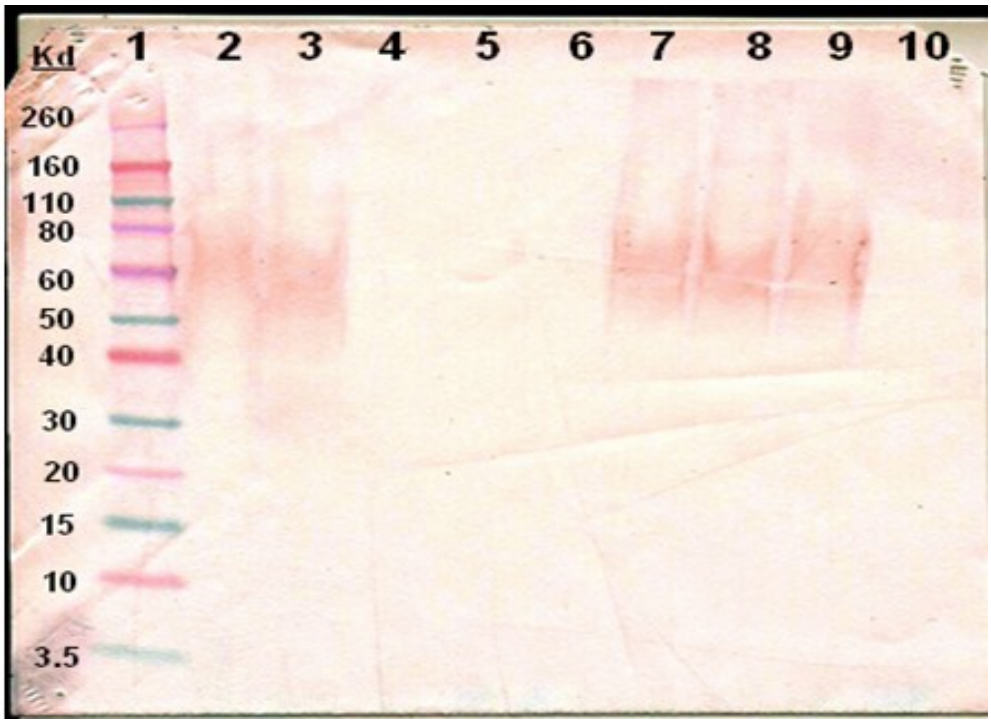

Figure 6.A.2: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #2. MLS 12-22-10 B

Table 6.A.2: Lane identification key for Gel #2 in Figure 6.A.2. MLS 12-22-10 B

	Organism	**Result**
1	Ladder	
2	*E. coli* O157:H16 GT4137	pos
3	*E. coli* O157:H38 GT 4138	pos
4	*E. aero genes GT 47*	neg
5	*E. cloacae GT 50*	neg
6	*C. freundii GT 9173*	neg
7	*E. coli* O157:H7 GT 4135	pos
8	*E. coli* O157:H7 GT 4132	pos
9	*E. coli* O157:H45	pos
10	*C. freundii GT 4885*	neg

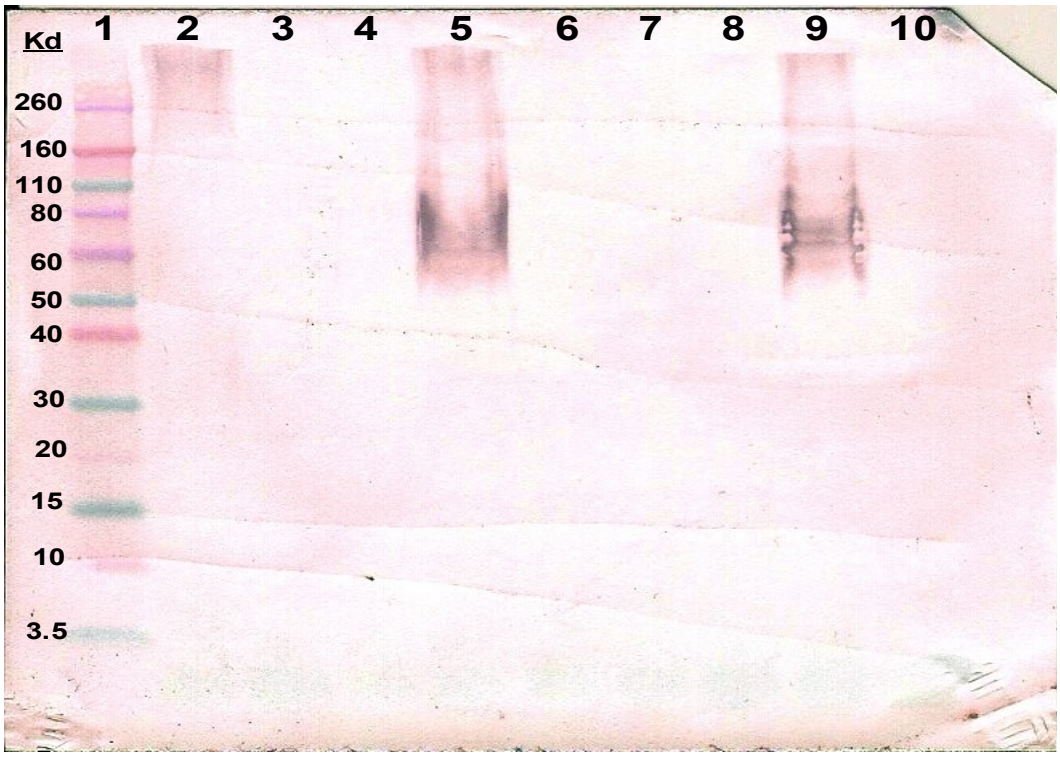

Figure 6.A.3: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #3. MLS 01-05-11 A

Table 6.A.3: Lane identification key for Gel #2 in Figure 6.A.3. MLS 01-05-11 A

	Organism	**Result**
1	Ladder	
2	*E. coli* O157:H19 GT 164 repeat	neg
3	*E. coli* O157:H7 GT 126 repeat	neg
4	*E. coli* O157:H7 GT 125 repeat	neg
5	*E. coli* O157:H7 Sakai strian	pos
6	*E. coli* O157:H7 AEEC strian	neg
7	*E. coli* O26:H11	neg
8	*E. coli* O55:H7	neg
9	*E. coli* O157:H7 Spinach pGFPuv	pos
10	*Shigella boydii*	neg

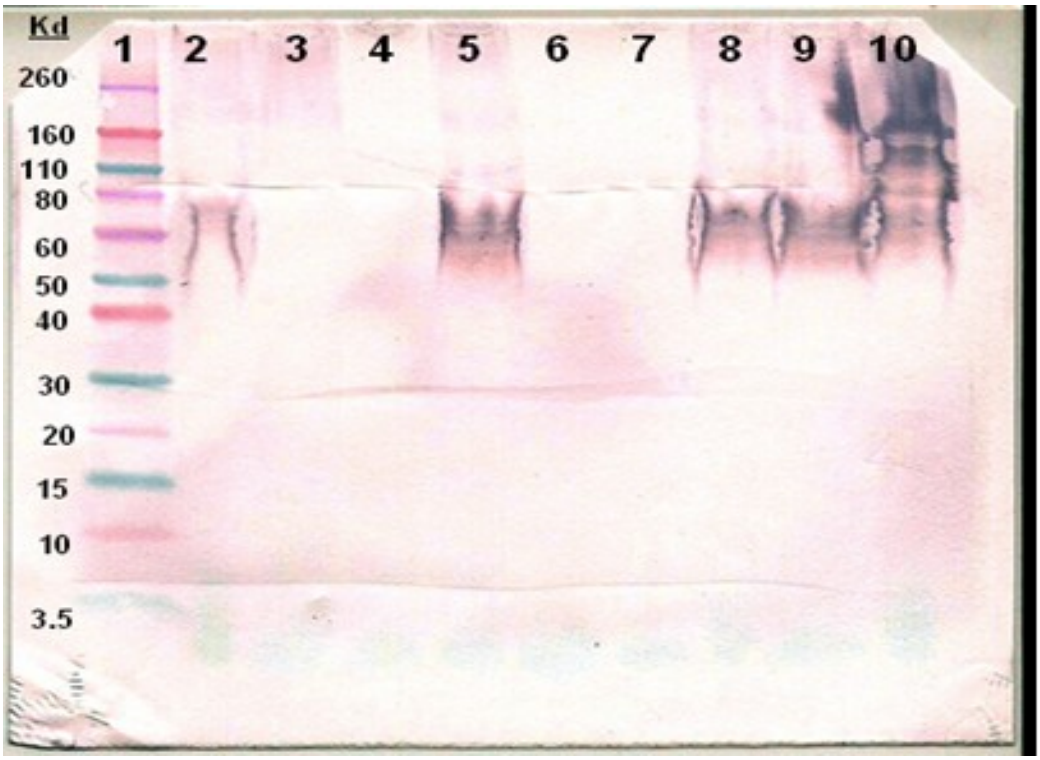

Figure 6.A.4: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #4. MLS 01-05-11 B

Table 6.A.4: Lane identification key for Gel #4 in Figure 6.A.4. MLS 01-05-11 B

	Organism	**Result**
1	Ladder	
2	*Escherichia coli* O157:H43 GT 4136	pos
3	*Escherichia coli* O157:H19 GT164	neg
4	*Enterobacter agglomerans* GT1611	neg
5	*Escherichia coli* O157:H7 GT 632	pos
6	*Escherichia coli* O157:H7 GT 126	neg
7	*Escherichia coli* O157:H7 GT 125	neg
8	*Escherichia coli* O157:NM GT 4141	pos
9	*Escherichia coli* O157:H7 A 110	pos
10	*Citrobacter freundii* (CF3) GT 5142	pos

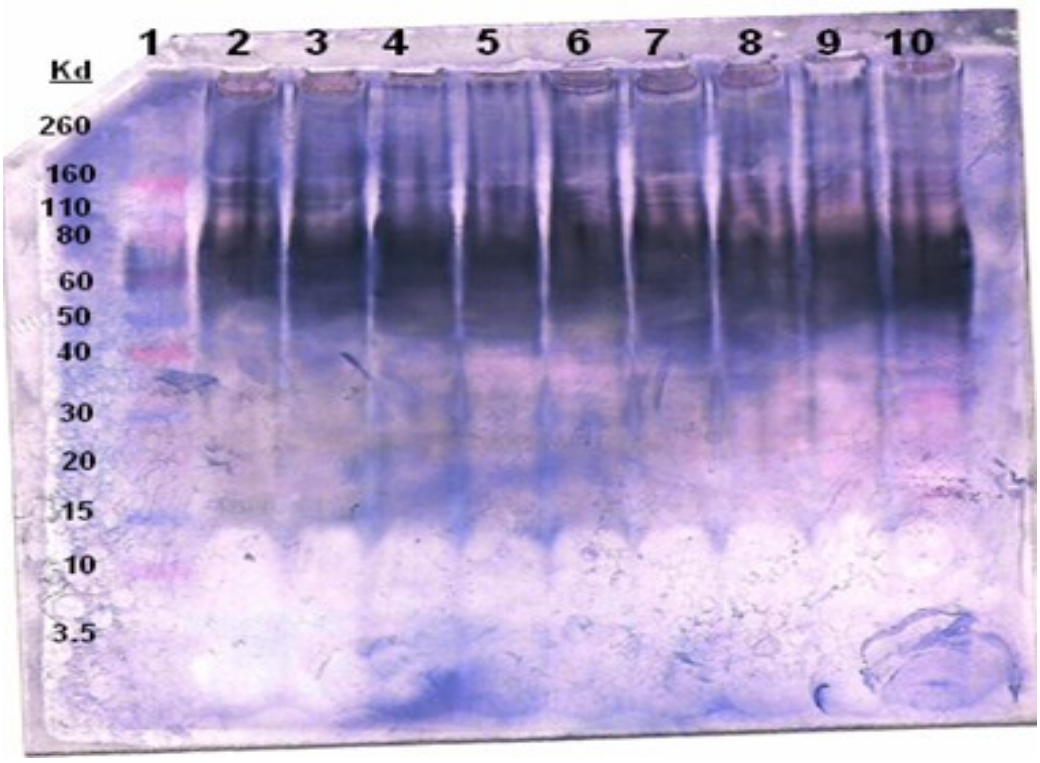

Figure 6.A.5: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #5. MLS 05-10-11 A

Table 6.A.5: Lane identification key for Gel #5 in Figure 6.A.5. MLS 05-10-11 A

	Organism	**Result**
1	Ladder	
2	SNP2 *E. coli* O157:H7	pos
3	SNP3 *E. coli* O157:H7	pos
4	SNP4 *E. coli* O157:H7	pos
5	SNP5 *E. coli* O157:H7	pos
6	SNP6 *E. coli* O157:H7	pos
7	SNP7 *E. coli* O157:H7	pos
8	SNP8 *E. coli* O157:H7	pos
9	SNP9 *E. coli* O157:H7	pos
10	SNP10 *E. coli* O157:H7	pos

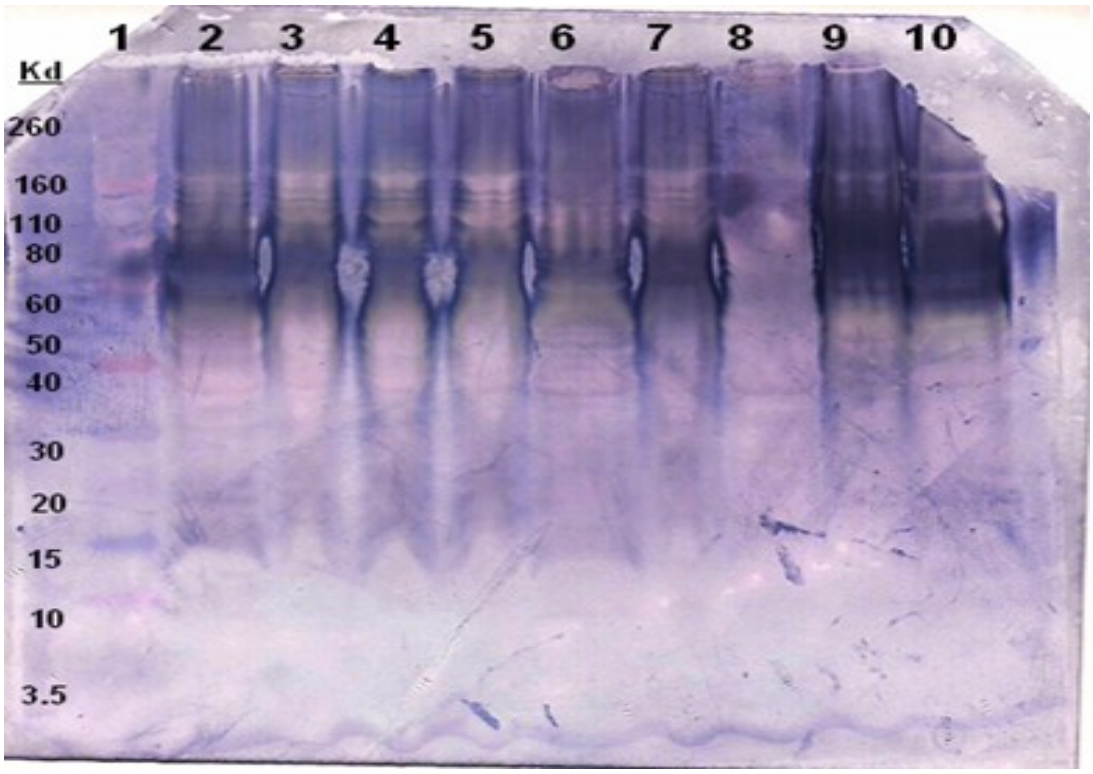

Figure 6.A.6: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #6. MLS 05-10-11 B.

Table 6.A.6: Lane identification key for Gel #6 in Figure 6.A.6. MLS 05-10-11 B.

	Organism	**Result**
1	Ladder	
2	SNP11 *E. coli* O157:H7	pos
3	SNP12 *E. coli* O157:H7	pos
4	SNP13 *E. coli* O157:H7	pos
5	SNP14 *E. coli* O157:H7	pos
6	SNP15 *E. coli* O157:H7	pos
7	SNP16 *E. coli* O157:H7	pos
8	4-22-10 (BSL #1)	neg
9	BSL #2 Bio Systems	pos
10	*Escherichia coli* ATCC 43895 (107)	pos

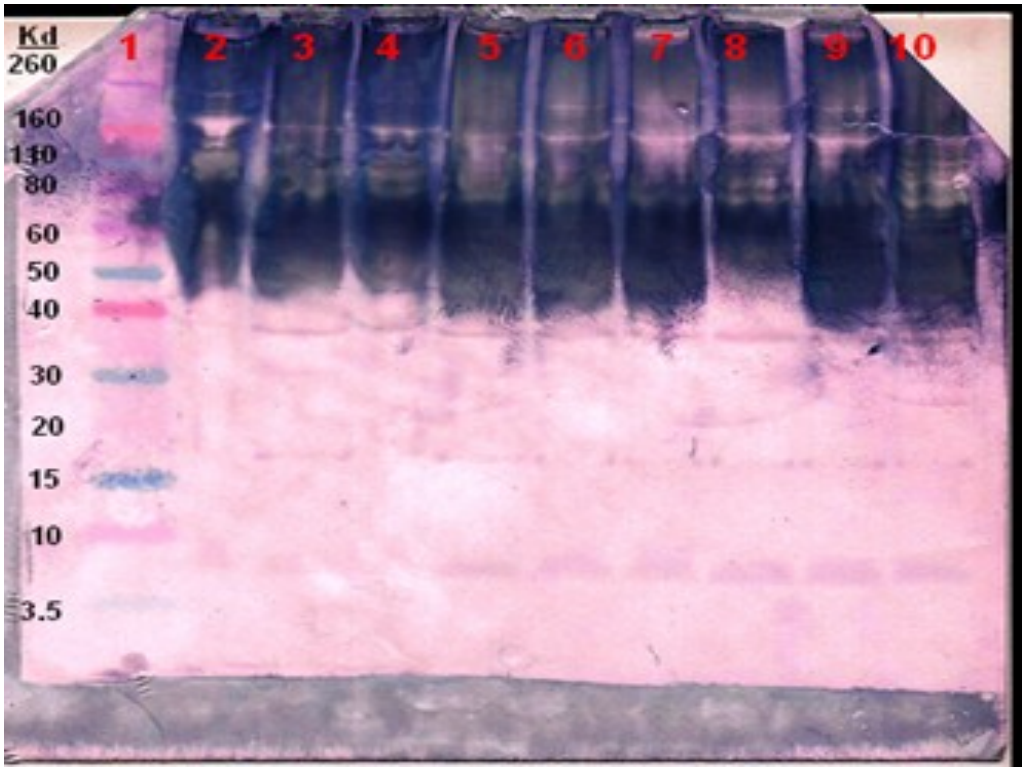

Figure 6.A.7: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #7. MLS 5-11-11 B.

Table 6.A.7: Lane identification key for Gel #7 in Figure 6.A.7. MLS 5-11-11 B.

	Organism	**Result**
1	Ladder	pos
2	SNP 17 *E. Coli* O157:H7	pos
3	SNP 18 *E. Coli* O157:H7	pos
4	SNP 19 *E. Coli* O157:H7	pos
5	SNP 20 *E. Coli* O157:H7	pos
6	EHEC1 #1 *E. Coli* O157:H7	pos
7	EHEC1 #2 *E. Coli* O157:H7	pos
8	EHEC1 #3 *E. Coli* O157:H7	pos
9	EHEC1 #4 *E. Coli* O157:H7	pos
10	EHEC1 #5 *E. Coli* O157:H7	pos

MLS 5-11-11 B

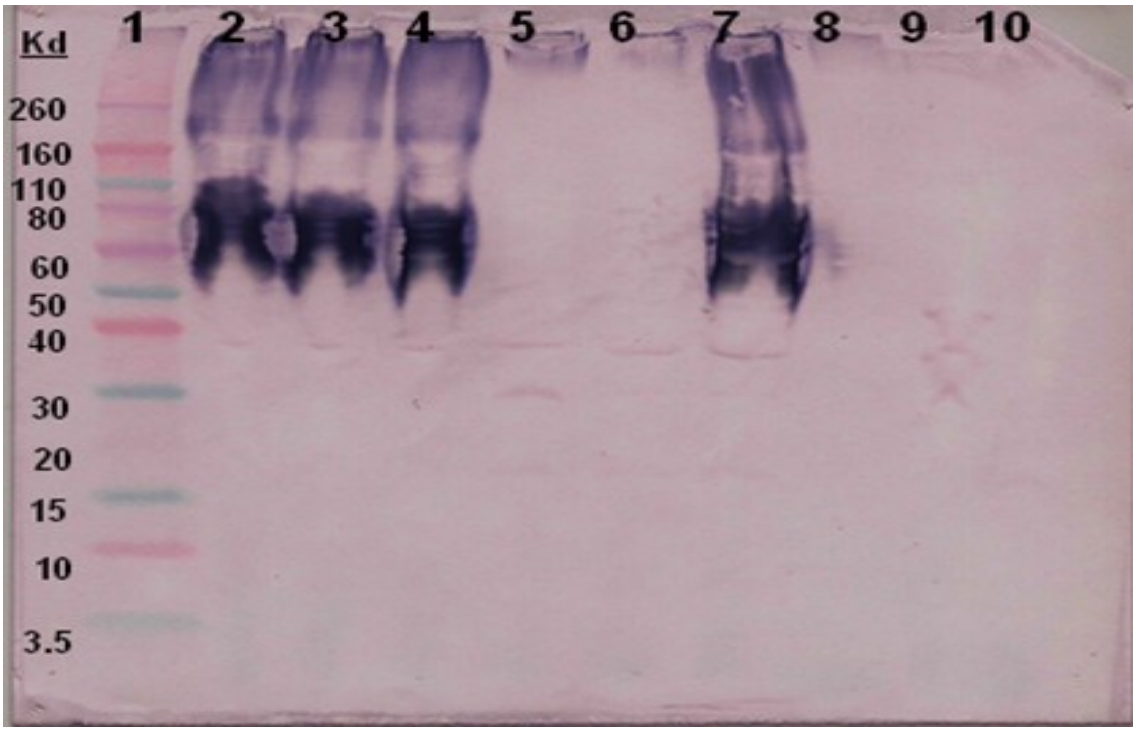

Figure 6.A.8: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #8. MLS 5-12-11 A.

Table 6.A.8: Lane identification key for Gel #8 in Figure 6.A.8. MLS 5-12-11 A.

	Organism	**Result**
1	Ladder	
2	EHEC1 #6 *E. coli* O157:NM	pos
3	EHEC1 #7 *E. coli* O157:NM	pos
4	*Eschericha coli* O157:H7 Ao317	pos
5	*Shigella flexnerii*	neg
6	*Eschericha coli* O26:H11 BSL 326	neg
7	*Citrobacter freundii* ATCC 8090	pos
8	*Salmonella enteritis* Typhimurium 0648 10/12	neg
9	*Klebsiella pneumonia* 6-21	neg
10	*Klebsiella pneumonia* ATCC 13883	neg

MLS 5-12-11 A

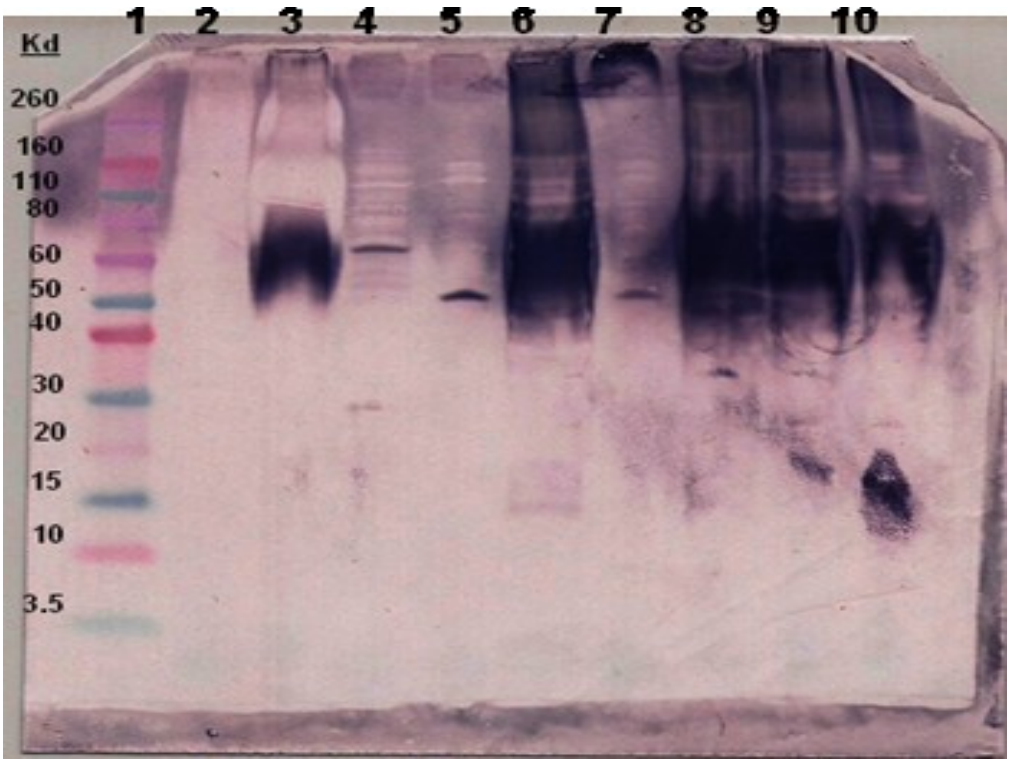

Figure 6.A.9: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #9. MLS 5-19-11 A.

Table 6.A.9: Lane identification key for Gel #9 in Figure 6.A.9. MLS 5-19-11 A.

	Organism	**Result**
1	Ladder	
2	*Pseudomonas aerugenosa*	neg
3	*Escherichia hermanni*	pos
4	*Staphalococcus aureus* 12600	neg
5	*Staphalococcus aureus* Ent AT #4	neg
6	*Enterococcus fecalis* ATCC 19433	pos
7	*Staphalococcus aureus* ATCC 25923	neg
8	*Citrobacter freundii* ATCC 8090	pos
9	EHEC1 #8 O157:H7 *E. coli*	pos
10	*Escherichia coli* O157:H7 Spinach TW 14359	pos

MLS 5-19-11 A

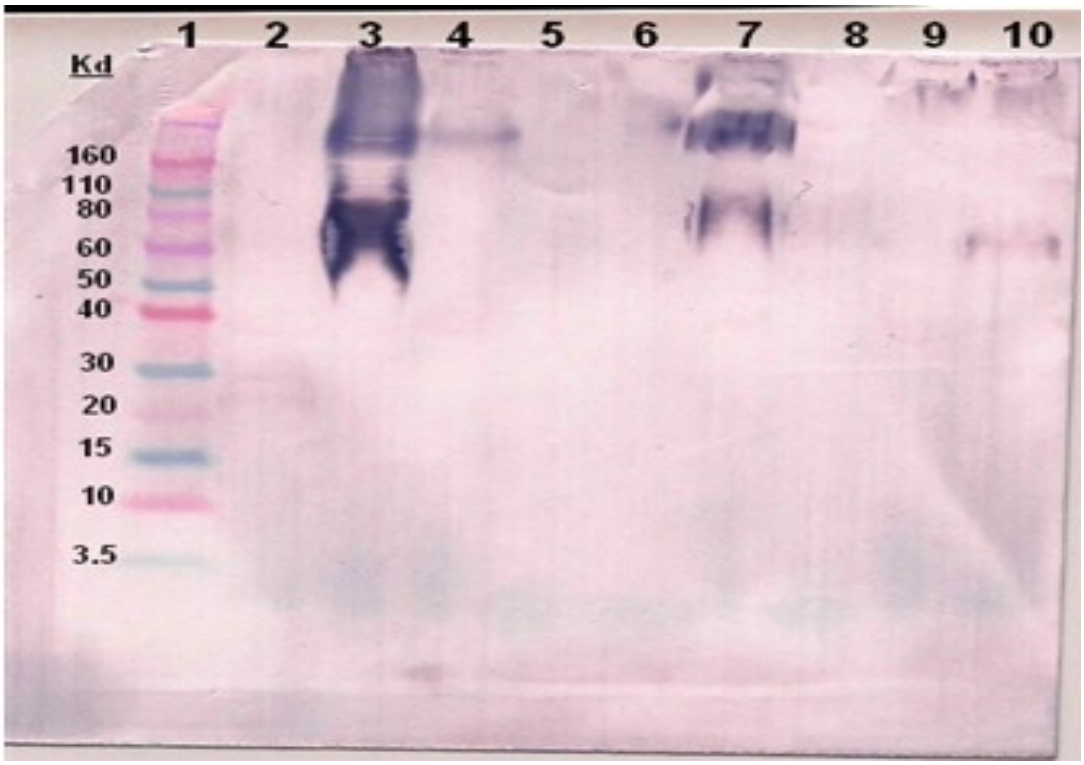

Figure 6.A.10: Western Blot of Meridian Life Sciences Anti *E. coli* O157:H7 Monoclonal Antibody Gel #10. MLS 6-9-11 A.

Table 6.A.10: Lane identification key for Gel #10 in Figure 6.A.10. MLS 6-9-11 A.

	Organism	**Result**
1	Ladder	
2	*Escherichia coli* O157:H38 Roe l A164	neg
3	*Escherichia coli* O157:H45 166	pos
4	*Escherichia coli* Mastitis 1368	neg
5	*Bacillus cerus*	neg
6	*Bacillus anthracis* Sterne Strain	neg
7	*Citrobacter freundii* GT 4885	pos
8	*Bacillus thuringersis*	neg
9	Bednark generic *E. coli* K12???	neg
10	*Enterobacter agglomerans* GT 1611	neg

MLS 6-9-11 A

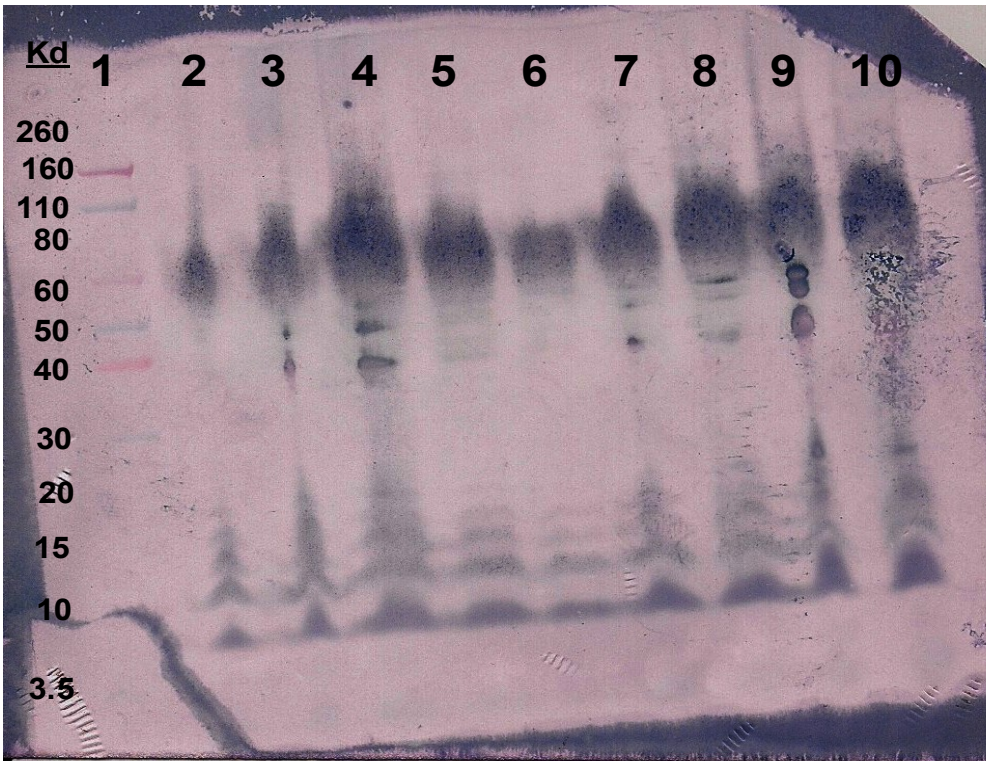

Figure 6.A.11: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #11. KPL 5-11-11 A.

Table 6.A.11: Lane identification key for Gel #11 in Figure 6.A.11.

	Organism	**Result**
1	Ladder	
2	SNP2 *E. coli* O157:H7 2ml	pos
3	SNP2 5ml	pos
4	SNP2 10ml	pos
5	SNP3 *E. coli* O157:H7 2ml	pos
6	SNP3 5ml	pos
7	SNP3 10ml	pos
8	SNP4 *E. coli* O157:H7	pos
9	SNP5 *E. coli* O157:H7	pos
10	SNP6 *E. coli* O157:H7	pos

KPL 5-11-11 A

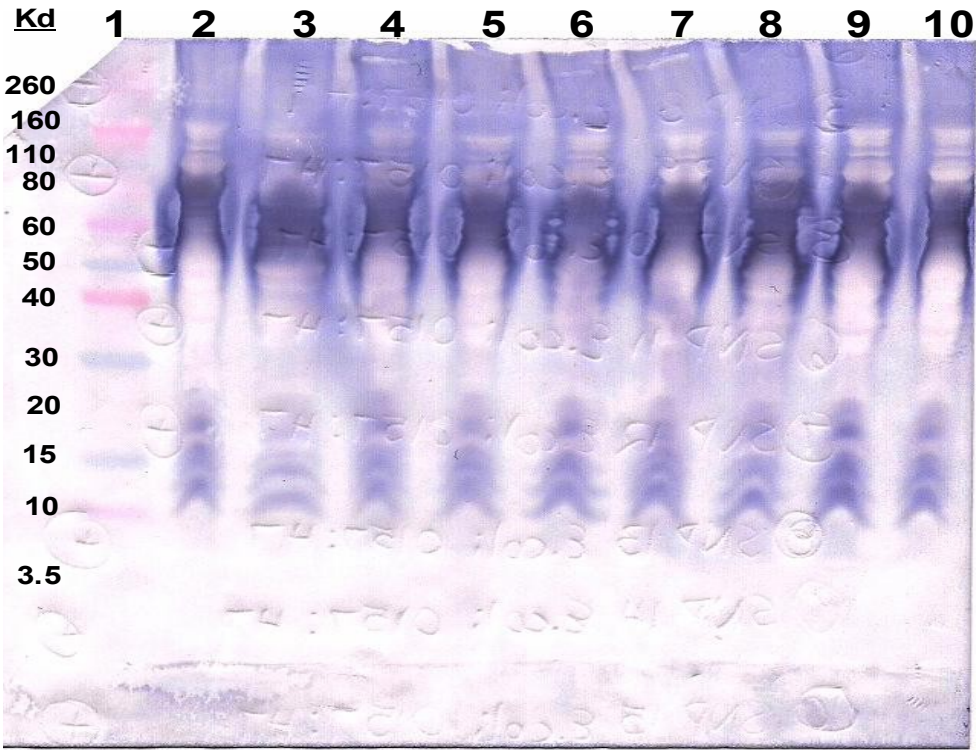

Figure 6.A.12: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #12. KPL 5-18-2011 A.

Table 6.A.12: Lane identification key for Gel #12 in Figure 6.A.12.

	Organism	**Result**
1	Ladder	
2	SNP 7 *E. coli* O157:H7	pos
3	SNP 8 *E. coli* O157:H7	pos
4	SNP 9 *E. coli* O157:H7	pos
5	SNP 10 *E. coli* O157:H7	pos
6	SNP 11 *E. coli* O157:H7	pos
7	SNP 12 *E. coli* O157:H7	pos
8	SNP 13 *E. coli* O157:H7	pos
9	SNP 14 *E. coli* O157:H7	pos
10	SNP 15 *E. coli* O157:H7	pos

KPL 5-18-2011 A

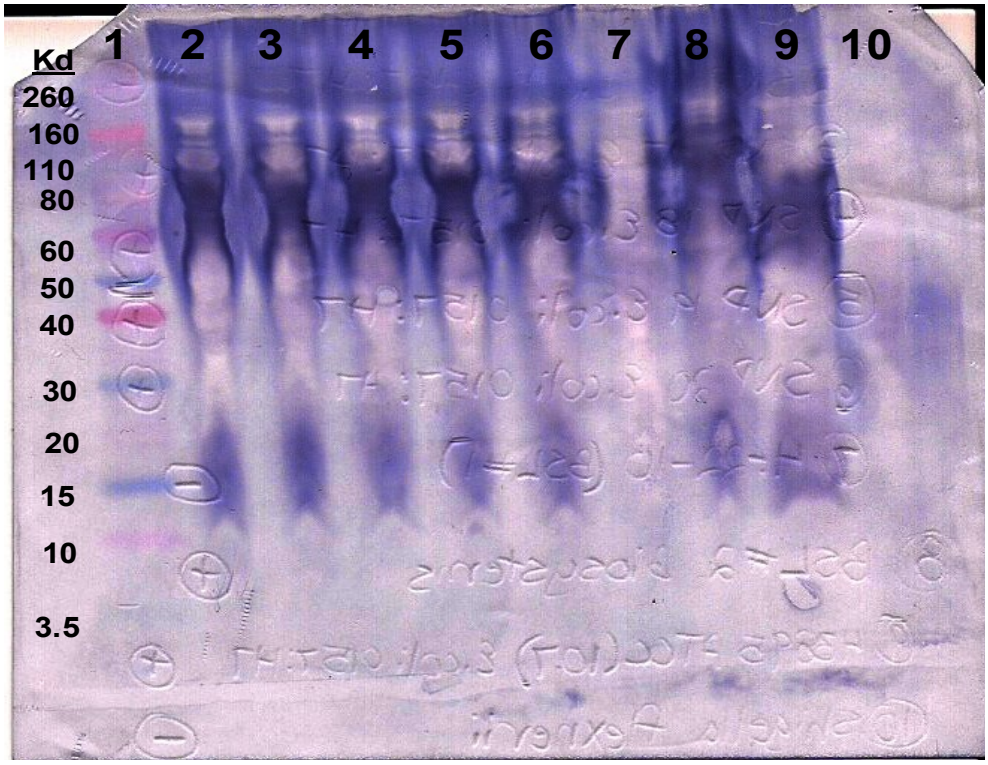

Figure 6.A.13: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #13. KPL 5-18-11 B

Table 6.A.13: Lane identification key for Gel #13 in Figure A.13.

	Organism	**Result**
1	Ladder	
2	SNP 16 *E. coli* O157:H7	pos
3	SNP 17 *E. coli* O157:H7	pos
4	SNP 18 *E. coli* O157:H7	pos
5	SNP 19 *E. coli* O157:H7	pos
6	SNP 20 *E. coli* O157:H7	pos
7	4-22-10 (BSL #1)	neg
8	BSL #2 Biosystems	pos
9	*E. coli* O157:H7 43895 ATCC (107)	pos
10	*Shigella flexnerii*	neg

KPL 5-18-11 B

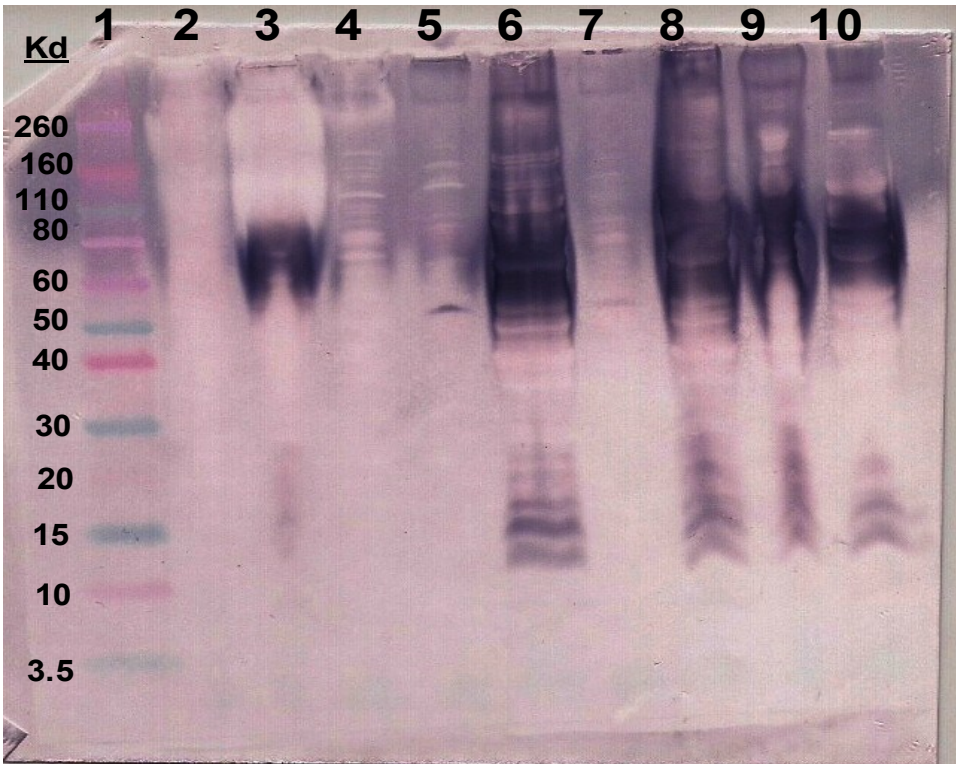

Figure 6.A.14: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #14. KPL 5-19-11.

Table 6.A.14: Lane identification key for Gel #14 in Figure 6.A.14.

	Organism	**Result**
1	Ladder	
2	*Pseudomonas aerugenosa*	neg
3	*Escherichia hermanni*	pos
4	*Staphalococcus aureus* 12600	neg
5	*Staphalococcus aureus* Ent A #4	neg
6	*Enterococcus fecalis* ATCC 19433	pos
7	*Staphalococcus aureus* ATCC 25923	neg
8	*Citrobacter freundii* ATCC 8090	pos
9	EHEC1 #8 O157:H7 *E. coli*	pos
10	*Escherichia coli* O157:H7 Spinach TW 14359	pos

KPL 5-19-11

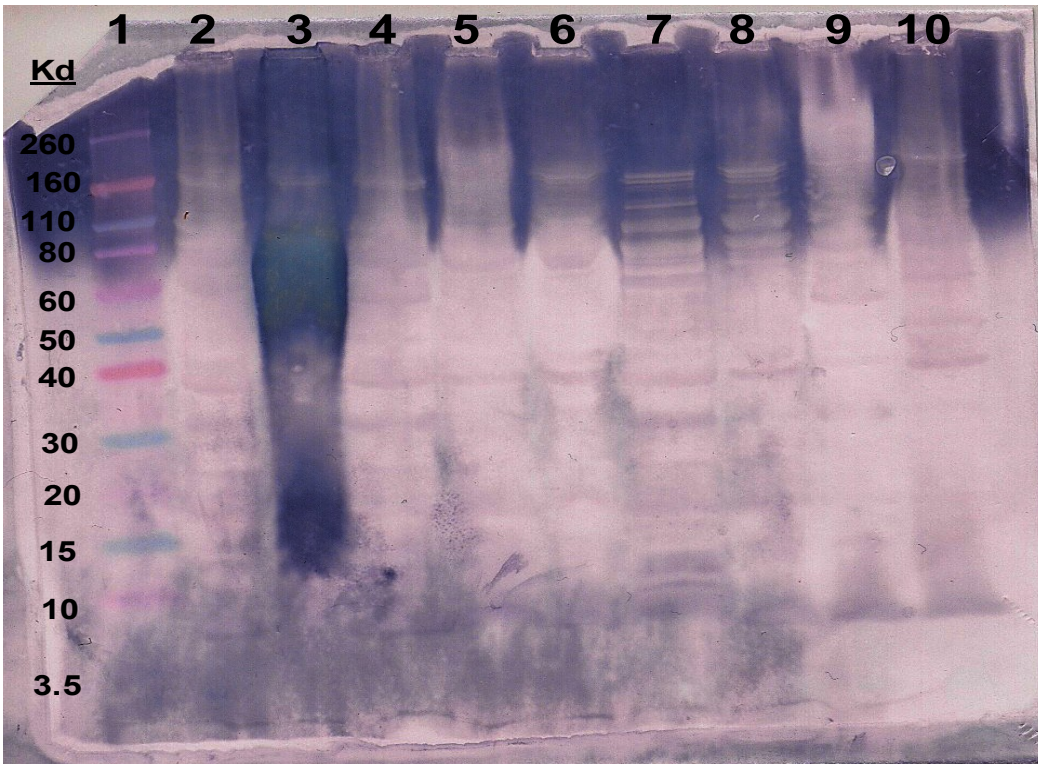

Figure 6.A.15: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #15. KPL 5-23-2011 A.

Table 6.A.15: Lane identification key for Gel #15 in Figure 6.A.15.

	Organism	**Result**
1	Ladder	
2	*Escherichia coli* O55:H7	neg
3	*Escherichia coli* O157:H7 GT 4135	pos
4	*Escherichia coli* AEEC (old spinach) TW 14549	neg
5	*Enterobacter cloacae*	neg
6	*Escherichia coli* O55:H6	neg
7	*Escherichia coli* O157:H7 126	neg
8	*Shigella boydii*	neg
9	*Citrobacter freundii* GT 4173	neg
10	*Escherichia coli* O157:H7 GT 4132	neg

KPL 5-23-2011 A

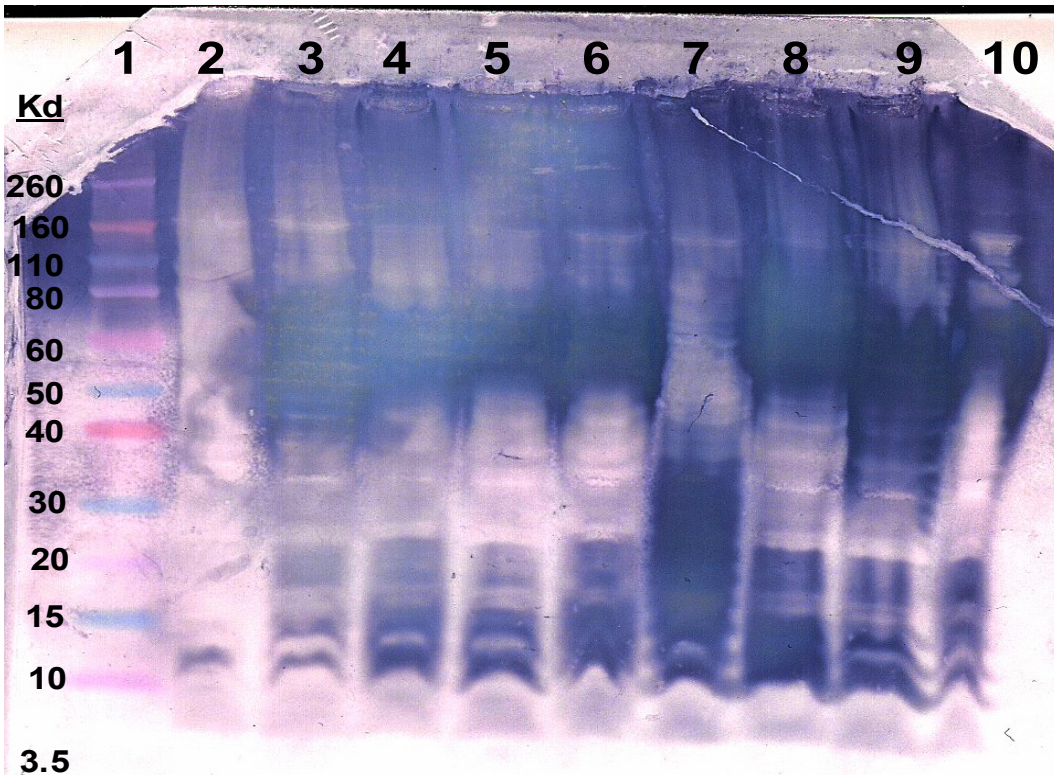

Figure 6.A.16: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #16. KPL 5-23-2011 B.

Table 6.A.16: Lane identification key for Gel #16 in Figure 6.A.16.

	Organism	**Result**
1	Ladder	
2	*Escherichia coli* O157:H7 125	neg
3	*Escherichia coli* O157:H38 GT 4138	pos
4	*Escherichia coli* O157:H43 GT 4136	pos
5	*Escherichia coli* O157:H7 Spinach pGFPuv	pos
6	*Escherichia coli* O157:H7 GT 632	pos
7	*Escherichia coli* O157:H19 #164	pos
8	*Escherichia coli* O157:H45 #174	pos
9	*Escherichia coli* O157:H16 GT 4137	pos
10	*Escherichia coli* O157:H7 Sakai	pos

KPL 5-23-2011 B

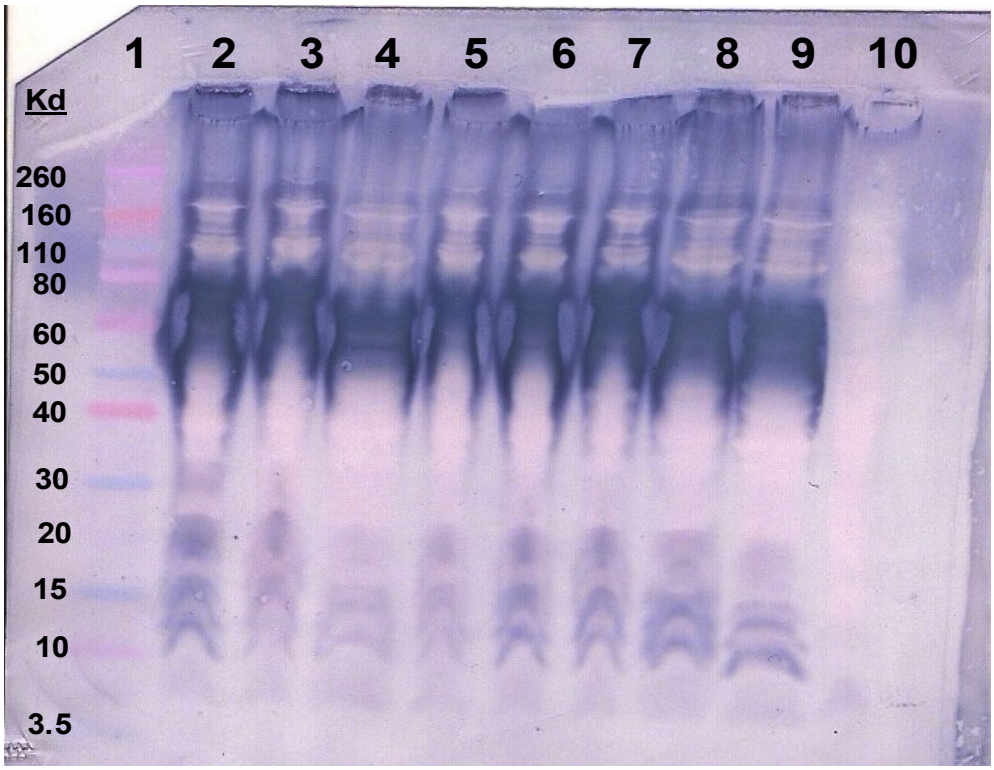

Figure 6.A.17: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #17. KPL 6-2-11 A.

Table 6.A.17: Lane identification key for Gel #17 in Figure 6.A.17.

	Organism	**Result**
1	Ladder	
2	*Escherichia coli* O157:H7 ATCC 35150 A110	pos
3	EHE1 #1	pos
4	EHEC1 #2	pos
5	EHEC1 #3	pos
6	EHEC1 #4	pos
7	EHEC1 #5	pos
8	EHEC1 #6	pos
9	EHEC1 #7	pos
10	*Escherichia coli* GT 47	neg

KPL 6-2-11 A

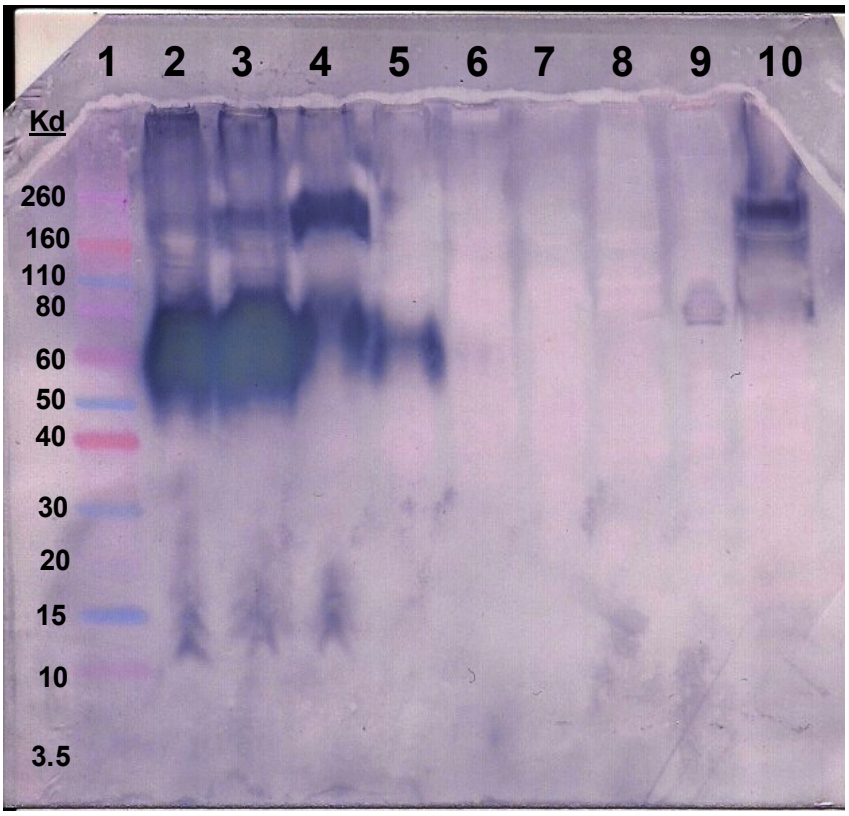

Figure 6.A.18: Western Blot of KPL Anti *E. coli* O157:H7 Monoclonal Antibody Gel #18. KPL 6-2-11 B.

Table 6.A.18: Lane identification key for Gel #18 in Figure 6.A.18.

	Organism	**Result**
1	Ladder	
2	*Escherichia coli* O157:H7 Ao317	pos
3	*Escherichia coli* O157:NM GT 4141	pos
4	*Citrobacter freundii* GT 4885	pos
5	*Enterobacter agglomerans* GT 1611	neg
6	*Escherichia coli* C3000	neg
7	*Klebsiella pneumonia* Cell 32 BSL K	neg
8	*Escherichia coli* O26:H11 BSL #26	neg
9	*Klebsiella pneumonia* # 13883	neg
10	*Citrobacter freundii* CF3 GT 5142	pos

KPL 6-2-11 B

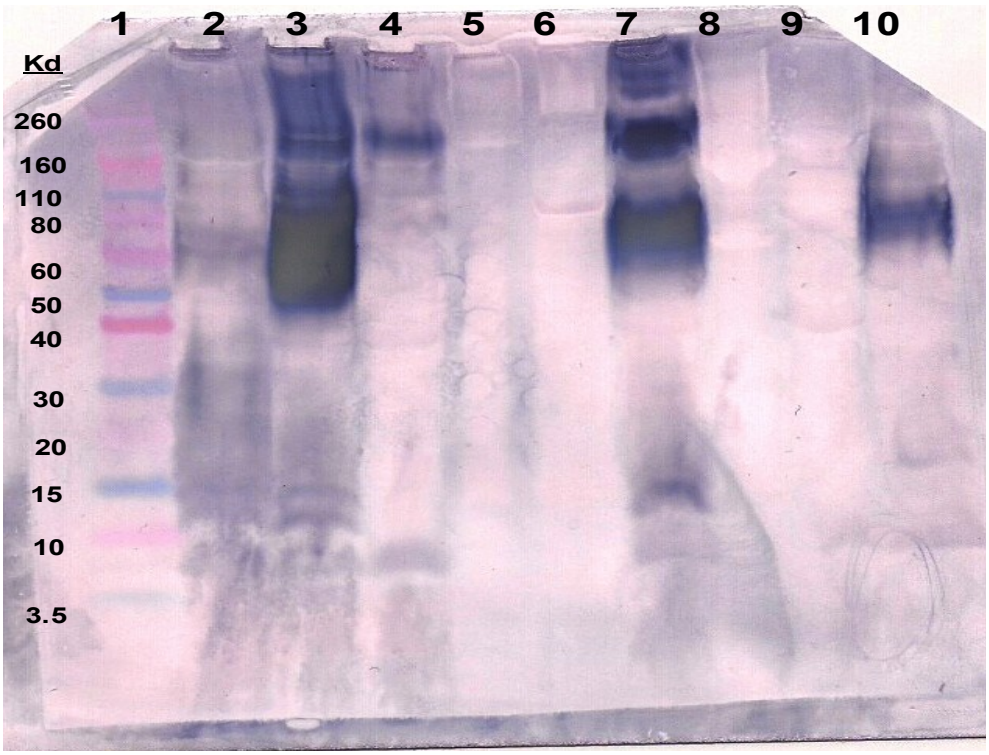

Figure 6.A.19: Western Blot of KPL Anti E. coli O157:H7 Monoclonal Antibody Gel #19 KPL 6-9-11 B.

Table 6.A.19: Lane identification key for Gel #19 in Figure 6.A.19.

	Organism	**Result**
1	Ladder	
2	*Escherichia coli* O157:H38 Roel A 164	neg
3	*Escherichia coli* O157:H45 166	pos
4	*Mastitis* 1368	neg
5	*Bacillus Cerus*	neg
6	*Bacillus Anthracis*	neg
7	*Citrobacter Freundii* GT 4885	pos
8	*Bacillus thuringersis*	neg
9	*Bednark generic* E. coli	neg
10	*Enterobacter agglomerans* GT 1611	pos

KPL 6-9-11 B

Appendix 6.B: Detailed information on bacteria in the Inclusivity and Exclusivity Panel

Master ID	Isolate	Co. # ID	ATCC	Genus Species Subgroup	MLS	KPL	EA-MNP	D-FSPCE
		BSL#1		*Escherichia coli* O157:H7	neg	neg	NA	5
		BSL #2		*Escherichia coli* O157:H7	pos	pos	pos	5
0043-0607		A110_# 124	35150	*Escherichia coli* O157:H7	pos	pos	pos	3
	GT A160	107 F2-87	43895	*Escherichia coli* O157:H7	pos	pos	pos	4
		Ao317		*Escherichia coli* O157:H7	pos	pos	pos	5
0043-0185	GT 632	1596		*Escherichia coli* O157:H7	pos	pos	pos	2
0043-0527	45956-52A; GT 4132	USDA 4100		*Escherichia coli* O157:H7	pos	pos	pos	4
0043-530	MF 1847; GT 4135	4103		*Escherichia coli* O157:H7	pos	pos	pos	3
0043-0608	A9218-C1	USDA #125		*Escherichia coli* O157:H7	neg	neg	neg	
0043-0609	A9167-1	USDA #126		*Escherichia coli* O157:H7	neg	neg	neg	
K 3995	Spinach pGFPuv			*Escherichia coli* O157:H7	pos	pos	pos	
TW14359	Spinach			*Escherichia coli* O157:H7	pos	pos	pos	5
TW08264	Sakai	SNP 1		*Escherichia coli* O157:H7	pos	pos	pos	25
TW 10022	H 2014	SNP 2		*Escherichia coli* O157:H7	pos	pos	pos	8
TW 04863	93-111	SNP 3		*Escherichia coli* O157:H7	pos	pos	pos	5
TW 08616	EK 8	SNP 4		*Escherichia coli* O157:H7	pos	pos	pos	3
TW 10012	F 6854	SNP 5		*Escherichia coli* O157:H7	pos	pos	pos	4

Appendix 6.B (cont'd)

Master ID	Isolate	Co. # ID	ATCC	Genus Species Subgroup	MLS	KPL	EA-MNP	D-FSPCE
TW 02302	EDL933	SNP 6		*Escherichia coli* O157:H7	pos	pos	pos	7
TW 07957	DA-31	SNP 7		*Escherichia coli* O157:H7	pos	pos	pos	5
TW 08613	EK 5	SNP 8		*Escherichia coli* O157:H7	pos	pos	pos	3
TW 11015	M102-88	SNP 9		*Escherichia coli* O157:H7	pos	pos	pos	3
TW 11052	M102-39	SNP 10		*Escherichia coli* O157:H7	pos	pos	pos	3
TW 01286	DEC 4A	SNP 11		*Escherichia coli* O157:H7	pos	pos	pos	6
TW 10246	M102-39	SNP 12		*Escherichia coli* O157:H7	pos	pos	pos	4
TW 07520	1541	SNP 13		*Escherichia coli* O157:H7	pos	pos	pos	6
TW 01663	87-1163	SNP 14		*Escherichia coli* O157:H7	pos	pos	pos	4
TW 07945	DA-19	SNP 16		*Escherichia coli* O157:H7	pos	pos	pos	3
TW 09098	M103-35	SNP 17		*Escherichia coli* O157:H7	pos	pos	pos	6
TW 11032	M102-55	SNP 18		*Escherichia coli* O157:H7	pos	pos	pos	3
TW 05356	G 5101	SNP 19		*Escherichia coli* O157:H7	pos	pos	pos	4
TW 06591	CB 1009	SNP 20		*Escherichia coli* O157:H7	pos	pos	pos	4
TW 07587	OK– 1	EHEC #3		*Escherichia coli* O157:H7	pos	pos	pos	6
TW 00116	86-24	EHEC #4		*Escherichia coli* O157:H7	pos	pos	pos	4
TW 00975	2886-75	EHEC #5		*Escherichia coli* O157:H7	pos	pos	pos	4
TW 05356	G 5101	EHEC #8		*Escherichia coli* O157:H7	pos	pos	pos	4

Appendix 6.B (cont'd)

Master ID	Isolate	Co. # ID	ATCC	Genus Species Subgroup	MLS	KPL	EA-MNP	D-FSPCE
TW 06555	493 / 89	EHEC #6		*Escherichia coli* O157:NM	pos	pos		20
TW 02883	E 32511	EHEC#7 _4109		*Escherichia coli* O157:NM	pos	pos		5
0043-0536	GT 4141	4104		*Escherichia coli* O157:NM	pos	pos		5
0043-0531	GT 4136	4105		*Escherichia coli* O157:H43	pos	pos		4
0043-0532	GT 4137	4106		*Escherichia coli* O157:H16	pos	pos		2
0043-0533	GT 4138			*Escherichia coli* O157:H38	pos	pos		4
	A164	Bio systems	roe 1	*Escherichia coli* O157:H38	neg	neg		
0043-0605		A174		*Escherichia coli* O157:H45	pos	pos		
		A166	3260-96	*Escherichia coli* O157:H45	pos	pos		4
0043-0600		164		*Escherichia coli* O157:H19	neg	pos		3
0029-0124	GT 5142			*Citrobacter freundii*	pos	pos		21
0029-0063	GT 4173	4141		*Citrobacter freundii*	neg	neg		
0029-0076	GT 4885	4690		*Citrobacter freundii*	neg	pos		
			8090	*Citrobacter freundii*	pos	pos		

Appendix 6.B (cont'd)

Master ID	Isolate	Co. # ID	ATCC	Genus Species Subgroup	MLS	KPL	EA-MNP	D-FSPCE
0037-0001	GT 47	1388	13048	*Enterobacter aerogenes*	neg	neg		8
0037-0066	GT 50	1395		*Enterobacter cloacae*	neg	neg	neg	4
0037-0030	GT 1611	801		*Enterobacter agglomerans*	neg	?	neg	6
TW 07545				*Shigella boydii*	neg	neg	neg	13
			12022	*Shigella flexneri*	neg	neg		8
TW 14549				*Escherichia coli* AEEC	neg	neg	neg	4
		3057-86		*Escherichia coli* 026:H11	neg	neg		8
TW 01188	DEC 10 C	EHEC2 #11		*Escherichia coli* 026:H11	neg		neg	
TW 00947	DEC 5D	EHEC1 #11		*Escherichia coli* 055:H7	neg	neg	neg	194
				Escherichia coli O55:H6	neg	neg	neg	
		2-7 6-21_1368		*Escherichia coli*	neg	neg		
			15597	*Escherichia coli* C3000	neg	neg	neg	6
		K12		*Escherichia coli* K12	neg	neg		
				Escherichia hermanni	pos	pos		3
		six - 21		*Klebsiella pneumonia*	neg	neg		
			13883	*Klebsiella pneumonia*	neg	neg		124
_	_	_	19433	*Enterococcus fecalis*	pos	pos		4
			10145	*Pseudomonas*	neg	neg	neg	8
			25923	*Staphalococcus aureus*	neg	neg		450

Appendix 6.B (cont'd)

Master ID	Isolate	Co. # ID	ATCC	Genus Species Subgroup	MLS	KPL	EA-MNP	D-FSPCE
		Ent A #4 9-03		*Staphalococcus aureus*	neg	neg		
			12600	*Staphalococcus aureus*	neg	neg		
		six - 21		*Staphalococcus aureus*	neg	neg		
		0648 10_12		*Salmonella enteritis* Typhimurium	neg	neg		
				Vibrio fischeri				16
				Bacillus anthracis Sterne	neg	neg		47

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CHAPTER 7: Development of a Universal, Shelf Stable, Electrochemical Detection Platform for use in the M^3 Biosensor

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Abstract

The largest hurdle to developing an efficient field based microbiological test is the ability to be run in resource limited settings with minimal refrigeration and climate control. In an effort to clear that largest hurdle, to monitor a greater percentage of the food supply at an affordable cost, a carbohydrate coated screen printed carbon electrode (D-FSPCE) was evaluated as a sensitive platform for multiplex evaluation of food samples extracted with electrically active magnetic nanoparticles (EAMNP). These nanoparticles provide the selectivity of the biosensor through their attached monoclonal antibody (Mab) while the carbohydrate coated chips provide the nonselective electrical detection platform. When combined, the D-FSPCE + Mab-EAMNP, using cyclic voltammetry for an electrical readout are named the M^3 Biosensor. Modified screens allow bacterial bead complexes to attach and empty bead complexes to be washed away, cleaning up the signal generation of the final electrochemical analysis. Statistically significant qualitative differentiation ($p = 0.0015$, $n = 188$) can be performed in broth with no pre-enrichment with *E. coli* O157:H7 as a target organism. Additionally, thirty nine organisms of 10 bacterial genera in both gram stain groupings all attached to the carbohydrate coated D-FSPCE, allowing for this platform to be used with many other organisms. This shelf stable multiplex detection platform can be used combined with any selective extraction modality and any electrical evaluation. The M^3 Biosensor can be used to detect bacterial contamination in broth without a pre-enrichment and is an inexpensive, field stable platform, with excellent multiplex capabilities in a wide variety of detection modalities.

Introduction

The search for an efficient and effective field based test to allow monitoring of a greater percentage of the food supply at an affordable cost by the military, government, or any food company is underway. The largest hurdles to developing such a test include food matrices, low contamination levels, low infective doses, competitive non-pathogenic organisms, and field portability (Fung, 2008; Ge & Meng, 2009). Before going into detail about food specific tests, it is helpful to discuss the requirements for a field based diagnostic test. The World Health Organization (WHO) Sexually Transmitted Diseases Diagnostics Initiative uses the term 'ASSURED tests' to describe the ideal characteristics of a diagnostic test for a resource limited settings (Mabey, Peeling, Ustianowski, & Perkins, 2004). The following criteria are listed:

1. Affordable by those at risk of infection.
2. Sensitive (few false-negatives).
3. Specific (few false-positives).
4. User-friendly (simple to perform and requiring minimal training).
5. Rapid (to enable treatment at first visit)
6. Robust (does not require refrigerated storage).
7. Equipment-free.
8. Delivered to those who need it.

Mabey et al. (2004) also states that developing a portable, field ready diagnostic test that matches all eight criteria is very difficult, but should not prevent the development of a useful test in the interim. There are multiple recent review articles that discuss the same criteria for food microbiological testing from the perspective of the microbiologist, the researcher and the food production manager, respectively (Fung, 2008; Ge & Meng, 2009; Jasson, Jacxsens, Luning, Rajkovic & Uyttendaele, 2010). Each of these supports, from different perspectives, the need for

the same criteria in food as ASSURED proposes for diagnostics. The largest hurdles to developing such a test include food matrices, low contamination levels, low infective doses, competitive non pathogenic organisms, and field portability. To take detection as far forward on the battlefield and in the farm field as possible, the chosen test must be able to be stored with limited refrigeration, work in a dirty, wet environment with a battery or small generator (Jasson et al., 2010). It should be small, portable, and light weight. Ideally for a military environment, but really any food company environment, the least amount of technical expertise and upkeep is necessary. Just as the ASSURED criteria show for diagnostic testing, there are no food based detection systems that fit this description on the market. Although sensitive, most of the currently available instrumentation does not work well in a field environment (Jasson et al., 2010). Another key component in all diagnostic arenas is the system should allow for multiplexing to further decrease cost and time to results.

The objective of this research is to produce a shelf stable universal detection chip for use with field based extraction modalities, especially the electrically active magnetic nano-particles (EAMNPs) developed in the Michigan State University (MSU) nano-biosensors laboratory. The published direct impedance method using EAMNPs measured the resistance of the captured cells on the electrical signal in the cyclic voltammogram, at a specific peak, that corresponded with the presence of polyaniline (Pal, Setterington & Alocilja, 2008; Pal & Alocilja, 2009). The theory was the larger peaks at the specific polyaniline response voltage would correspond with greater amounts of polyaniline. What was visualized in the initial studies was a lower peak height with higher concentrations of bacteria, not a larger one. It was hypothesized that the cellular presence "resisted" the conductance of the polyaniline coating on the EAMNPs in a DC voltage electrical system. This phenomenon was inconsistent and did not present a statistically

significant evaluation tool at most concentrations of bacteria when multiple trials were performed. Even as a qualitative; presence/absence evaluation, the system could not reliably give consistent results from run to run. Other research from the MSU biosensors laboratory was showing more consistent results by coating a screen printed carbon electrode (SPCE) with a polyclonal antibody (Pab) against *E. coli* O157:H7 (unpublished data; Jain, 2010) and against H1N1 (viral) organisms (Kamikawa et al., 2010). Both of these systems extracted the target using the using the same EAMNP extraction, combined with cyclic voltammetry. Pab binds to the target organism in a second site from the already bound Mab-EAMNP complex and holds it attached to the FSPCE. This allows rinsing of EAMNPs that are not bound to a target organism and a more direct correlation of the amount of polyaniline with the corresponding amount of bacteria. Unfortunately, Pab Functionalized SPCEs (Pab-FSPCEs) are not shelf stable, requiring controlled storage at 4°C. They also degrade rather quickly, the conjugation method used was reversible at room temperature and the Pab-FSPCEs are specific to one bacterial species, preventing multiplexing of the system. In order to address those issues, an alternative was identified as a stable way to attach the bacteria–Mab-EAMNP complex to the SPCE. Research has shown good results in using aminated D-mannose on magnetic particles to capture *Escherichia coli* (*E. coli*) without Mab (El-Boubbou, Gruden & Huang, 2007). D+ mannose is a sugar that is stable in the environment and *E. coli* O157:H7 has an affinity for *in vivo* entry into the cell. Many other organisms such as *V. cholera* and the entire *Enterobacteriacea* family do as well. (El-Boubbou, et al., 2007; Abraham, Sun, Dale & Beachey, 1988; Bouwman, Roep & Roos, 2006; Bhattacharjee & Srivastava, 1978; Caruso et al., 2010; Han, Ding, Jin, & Ju 2010; Sandoval-Bernal, Barbosa-Sabanero, Shibayama, Perez-Torres, Tsutsumi, & Sabanero, 2011; Van Staalduinen, Park ,Yeom, Adams-Cioaba, Oh & Jia 2010) A carbohydrate is more stable

than Pab and should allow multiple bacteria to bind; allowing the resultant aminated D+ mannose - Functionalized SPCE (D-FSPCE) to be used for more than one organism. Multiplexing allows easier and faster sample evaluation in the field.

Experimental design

The following studies developed the proof of concept using D-SPCEs, evaluated the inclusivity of the D-FSPCE, and the ability to detect from broth and whole liquid milk samples.

Hypothesis 1: Aminated D+ mannose can be bound irreversibly to the SPCE and remains biologically active when bound to the SPCE against bacteria that are bound to the Mab-EAMNP complex.

Hypothesis 2: Aminated D+ mannose can be used as a universal detection platform for multiple bacteria.

Hypothesis 3: Aminated D+ mannose can be used as a novel biosensor detection platform for multiple detection methods, but specifically cyclic voltammetry (CV) on a hand held potentiostat, in broth.

To test hypothesis 1, *E. coli* O157:H7 Sakai strain cultures were prepared as above and stained with acridine orange. Using a micropipette, 100 µL of the stained organisms were spread on SPCEs either plain, with blocked or unblocked glutaraldehyde, and with blocked aminated D+ mannose coated on the SPCE. These SPCEs were incubated for 15 minutes and rinsed two times with DI water. The resultant SPCEs were evaluated under a fluorescent light microscope (445 nm excitation and 520 nm emission) and then punched out and placed in a 96 well plate and evaluated with differing filter methods in the Victor spectrophotometer. Acridine orange was also applied to an SPCE without cells or blocking. *E. coli* O157:H7 Sakai strain captured by Mab-EAMNPs were also challenged in solution with aminated D+ mannose and their active

conjugation to the compound evaluated using D+ mannose's lectin, Con A, conjugated to FITC, in the Victor spectrophotometer. For the Victor spectrophotometer, relative fluorescence units (RFUs) were calculated using a blank D-FSPCE as the reference value of 1 RFU. To determine if the active site for D + mannose and the organism remained active when the Mab was attached to the organism, a second trial was performed using the lectin for D + Mannose, Con A. Commercially prepared Con A conjugated to FITC was obtained and mixed 1:1 with our aminated D + mannose in solution. That 1:1 solution was incubated 1:1 again with the same size sample of EAMNP captured *E. coli* O157:H7 Sakai strain as used during a potentiostat run. Con A FITC; D+ mannose Con A FITC and D+ mannose Con A FITC with EAMNP captured *E. coli* O157:H7 Sakai strain were evaluated in the Victor spectrophotometer. The EAMNP captured *E. coli* O157:H7 Sakai strain was magnetized out, washed and re-suspended in solution before placing in the Victor. The supernatant of that rinse was also evaluated to ascertain the decrease of RFU that would have moved with the magnetized Mab-EAMNP complex.

To test hypothesis 2, D-FSPCEs were incubated with various fluorescently stained bacterial cells from different families and visualized under a light microscope (445nm excitation and 520nm long pass emission) for the varying stains, the Victor spectrophotometer for relative fluorescence, the confocal microscope or the scanning electron microscope (SEM). A total of 29 different organisms were challenged on the D-FSPCE. Subsequently several unstained bacterial cultures were incubated on D-FSPCEs and evaluated in the field-emission scanning electron microscope (JOEL 7500F, acceleration voltage of 5 kV).

To test hypothesis 3, 100 µL of Mab-EAMNPs- *E. coli* O157:H7 Sakai strain from broth trial samples were placed on the D-FSPCE, incubated for 15 minutes and rinsed twice with DI water. They were allowed to dry, doped with 100 µL of 0.1 M HCl and evaluated, using cyclic

voltammetry, on PalmSens handheld potentiostat (Palm Instruments BV, Houten The Netherlands).

Materials and methods:

E. coli O157:H7 strains, *E. coli* non-H7 strains, and non-*E. coli* bacterial strains were obtained from the STEC Center collection at Michigan State University (MSU) (Shannon Manning, MPH, PhD), the Nano-Biosensors Laboratory at MSU (Evangelyn Alocilja, PhD), Neogen Inc. Research and Development, Lansing, Michigan (Jennifer Rice, DVM, PhD), and the University of Georgia, Center for Food Safety (Dr. Michael Doyle, PhD). From frozen purified culture stocks (stored at -80° C), colonies were isolated by streak-plate method on trypticase soy agar (BD Biosciences, MD) plates. A single colony was used to inoculate a vial of tryptic soy broth (BD Biosciences, MD) and grown overnight at 37° C. A 1 mL aliquot of the liquid culture was transferred to a new vial of broth and stored at 37° C for up to 6 days. This culture was used to inoculate a new vial of broth with 1 mL of inoculum 8 to 10 h before each experiment to produce fresh bacterial cells which were serially diluted in 0.1% (w/v) peptone water (Fluka-Biochemika, Switzerland) prior to their use in the IMS procedure. Viable cells were enumerated by microbial plating on MacConkey agar with sorbitol (SMAC) (BD Biosciences, MD or Neogen Inc., MI), according to standard rules for plate counting (FDA BAM, 2009).

EAMNP Production

The synthesis of the EAMNPs was performed using Ferric chloride hexahydrate (EMD Chemicals), sodium acetate (CCI Chemicals), sodium acrylate, sodium chloride (Sodium Chloride), ethylene glycol, ethylenediamine, hydrochloric acid, aniline, iron (III) oxide nanopowder, ammonium persulfate, methanol, and diethyl ether. Each was used as received.

EAMNPs were synthesized by polymerization and acid doping of aniline monomer around gamma iron (III) oxide (γ-Fe_2O_3) nano-particles, using a slightly modified published procedure (Pal et al., 2008). Briefly, 0.650 g of iron (III) oxide nanopowder were dispersed in 50 mL of 1 M HCl, 10 mL of deionized water and 0.4 mL of aniline monomer by sonication in an ice bath for 1 hour. A volume of 20 mL of 0.2 M ammonium persulfate (as oxidant) was added drop-wise to the above solution under continuous magnetic stirring. Color change from rust brown to dark green indicated formation of electrically-active (green) polyaniline over the smaller (brown) γ-Fe_2O_3 nano-particles. The solution was stirred for 2 hours in an ice bath and was filtered through a qualitative grade filter (2.5 μm pore size, Ahlstrom, grade 601). The supernatant thus obtained was successively filtered through a nitrocellulose membrane filter (1.2 μm pore size, Millipore) followed by washings with 10 mL each of 1 M HCl, 10% (v/v) methanol, and diethyl ether. The particles were dried overnight at room temperature under vacuum. The particles ranged in size from 1.2 to 2.5 μm, and displayed a room temperature saturation magnetization of 30 emu/g.

EAMNP Antibody Conjugation

EAMNPs were conjugated with Mab at an initial EAMNP concentration of 10 mg/mL (1% solid). Conjugation of antibodies onto EAMNPs was by direct physical adsorption and electrostatic interactions. A 100 μL aliquot of monoclonal anti-*E. coli* O157:H7 antibody (suspended in 0.1 M phosphate buffer) was added to EAMNPs suspended in PBS, yielding a final antibody concentration of 1.0 mg/mL. The mixture was hybridized on a rotisserie-style rotator for 1 hour at room temperature, with 25 μL of 10 X PBS being added after the first 5 min of hybridization, to increase the sodium chloride content of the suspension to approximately 0.14

M. Following hybridization, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer (0.1 M tris buffer with 0.01% casein) for 5 min. Again the conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer, this time for 1 hour with rotation. Finally, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 2.5 mL of 0.1 M phosphate buffered saline (PBS). The final concentration of EAMNPs in each solution was 1.0 mg/mL. Immuno-conjugated EAMNPs (Mab-EAMNPs) were stored at 4° C. Prior to experimental use, Mab-EAMNPs were diluted in 0.1 M PBS, in order to obtain solutions of Mab-EAMNPs at, 0.5 mg/mL EAMNPs (Setterington, Cloutier, Ochoa, Cloutier, Alocilja, 2011).

Immuno-magnetic Separation (IMS) and Plating of Bacteria

Every experiment was applied to three different bacterial species individually: *E. coli* O157:H7 Sakai strain, *E. coli* O157:H7 2006 Spinach strain, pGFPuv (target species), *Shigella boydii* (non-target species). *S. boydii* bears less genotypic and phenotypic similarity to the target organism, but it is a commonly encountered foodborne pathogen, and also produces shiga-toxin like *E. coli* O157:H7. The standard positive control used was *E. coli* O157:H7 spinach 2006 strain with a green ultra violet fluorescent plasmid inserted (*E. coli* O157:H7 2006 Spinach strain, pGFPuv). Serial dilutions of each bacterium were independently prepared in 0.1% (w/v) peptone water, along with subsequent negative, positive, and blank controls Three or four of the pure dilutions of each bacteria were plated (100 mL aliquots) on sorbitol MacConkey agar (SMAC) and incubated at 37° C overnight. For IMS, 50 mL of Mab-EAMNPs and 50 mL of the appropriate bacterial dilution were combined with 400 mL of 0.01 M PBS (pH 7.4), and

hybridized with rotation at room temperature for 30 minutes. After hybridization, the cell-Mab-EAMNP complexes were magnetically separated and the supernatant removed. Complexes were washed twice in wash buffer (0.01 M PBS containing 0.05% Triton-X100), and finally re-suspended in 0.5 mL of 0.01 M PBS. A 100 mL aliquot was placed on SMAC and incubated at 37° C overnight. The number of colony-forming units (CFU) in the 100 mL aliquot was determined by manually counting the colonies on each plate. Calculation of bacterial cell concentrations in both pure and IMS separated samples were carried out according to rules provided by the United States Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM, 2009).

Immuno-magnetic separation (IMS) was performed on samples with Mab-EAMNPs. A volume of 1 mL of cell culture was pelleted and re-suspended in acridine orange buffer for the spectrophotometer and microscopic evaluations. For the IMS separation, EAMNPs were immune-functionalized with monoclonal anti-*E. coli* O157:H7 antibodies obtained from Meridian Life Science, Inc. (Saco, ME). Polysorbate-20 (Tween-20), Triton X-100, phosphate buffered saline (PBS), Trizma base, casein, and sodium phosphate (dibasic and monobasic) were used in the IMS procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as follows: PBS buffer (10 mM PBS, pH 7.4), wash buffer (10 mM PBS, pH 7.4, with 0.05% Tween-20 or 0.05% Triton-X100), phosphate buffer (100 mM sodium phosphate, pH 7.4), blocking buffer (100 mM Tris–HCl buffer, pH 7.6, with 0.01% w/v casein). Magnetic separations were performed with a commercial magnetic separator (Promega Corporation, Madison, WI). Hybridization of biological materials was carried out at room temperature with rotation on a tube rotisserie

(Labquake, Thermo Scientific, MA). Scanning electron micrographs were acquired using field-emission scanning electron microscopy (JOEL 7500F, acceleration voltage of 5 kV).

Synthesis of aminated α D + Mannose

All chemicals used in the synthesis and evaluation of the aminated D+ mannose were purchased from Sigma Aldrich (St. Louis, MO) and used as purchased unless otherwise noted. Chemicals used to produce aminated D + mannose were: α D + mannose, α D + mannose pentacetate, pyridine, acetic anhydride, methanol (CH_3OH), methyl alcohol (anhydrous methanol), dichloromethanol (DCM), hexanes, ethyl acetate, nitrogen gas, tetrahedrofuran (THF), toluene, acetic acid, acetone, sodium azide, chloropropanol (1 chloro 3 hydroxypropane), sodium bicarbonate ($NaHCO_3$), sodium sulfate (Na_2SO_4), 5 M sodium methoxide (NaOMe), , BF_3•Et_2O, Triethylamine (Tea), chloroform-D, molecular sieves (4Å), 4-dimethylaminopyridine (DMAP), 10% hydrochloric acid (HCL), celite powder, amberlite IR120, paladium carbon (Pd/C) , and hydrogen gas.

Production and evaluation of the aminated D + mannose was accomplished in collaboration with Dr. Xuefei Huang's chemistry lab at MSU, using published protocols. Aminated D-mannose was synthesized starting either from the unprotected D-mannose or α-D-mannose pentaacetate, all commercially available. Due to the high price of α-D-mannose pentaacetate, starting with unprotected D-mannose was economically viable. At each step, a self poured silica gel column chromatography was used to purify, a rotovap (Büchi Rotovapor or Büchi Rotovapor R-114, Midwest AOAC Int., St. Louis, MO) was used to concentrate, and thin layer chromatography (TLC) or proton and carbon nuclear magnetic resonance imaging (NMR) (500 MHz, Varian Unity INOVA, Oxford Instruments, Concord, MA) and fourier transform

infrared spectroscopy (FTIR) (Perkin Elmer, Shelton, CT) were used to verify products. Procedures reported by (Teumelsan & Huang, 2007) were adopted for the synthesis of α-D-mannose pentaacetate. Briefly α-D-mannose was protected using acetic anhydride in pyridine with catalytic DMAP to achieve α-D-mannose pentaacetate. α-D-mannose pentaacetate was reacted with 3-chloropropanol and $BF_3.OEt_2$ in DCM overnight to give compound 1 shown in figure 7.1. Compound **2** was achieved by reacting compound **1** with sodium azide in DMF at 60 ^{O}C overnight (El-Boubbou, et al., 2007, Joosten, et al., 2004, Teumelsan & Huang, 2007). De-protection of the acetate groups was performed with freshly prepared sodium methoxide and produced compound **3.** Upon reduction of azide under hydrogenation condition, the final aminated α-D-mannose, compound **4,** was formed. The NMR spectra can be found in appendix 7.A. The reaction scheme is shown below in figure 7.1.

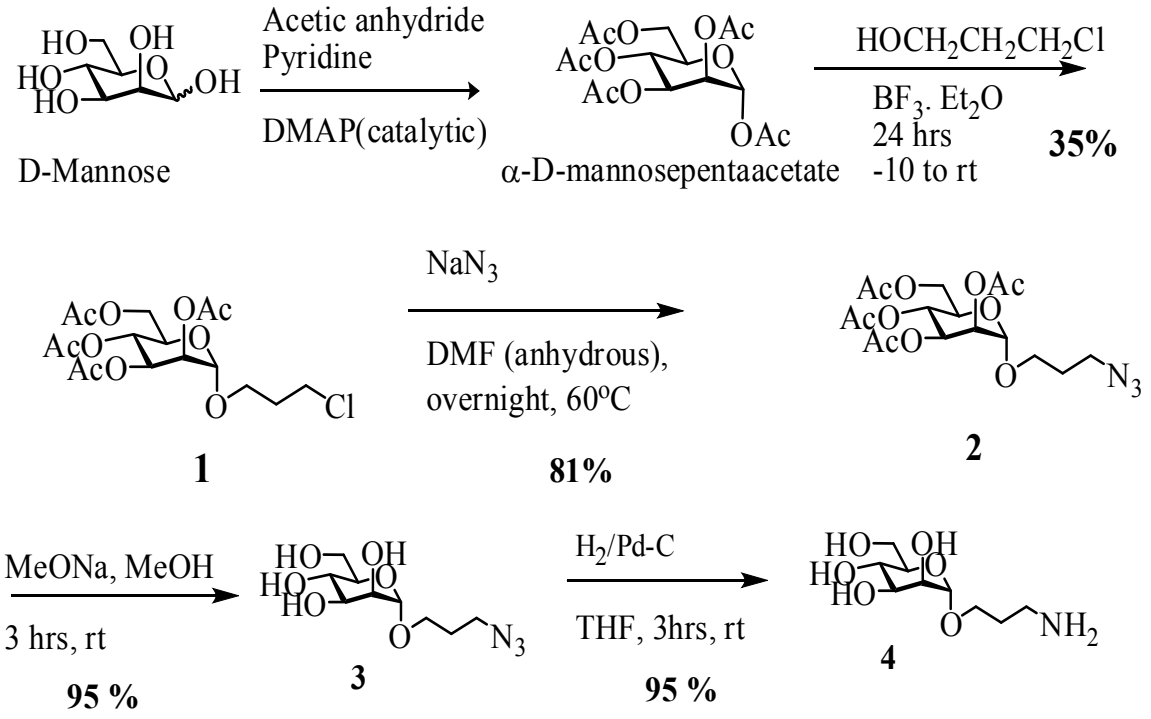

Figure 7.1: α-D-mannose was protected using acetic anhydride in pyridine with catalytic DMAP to achieve α-D-mannose pentaacetate. α-D-mannose pentaacetate was reacted with 3-chloropropanol and BF_3•OEt_2 in DCM overnight to give compound 1 shown in figure 7.1. Compound 2 was achieved by reacting compound 1 with sodium azide in DMF at 60°C overnight (El-Boubbou et al. 2007, Joosten, et al., 2004, Teumelsan & Huang, 2007). De-protection of the acetate groups was performed with freshly prepared sodium methoxide and produced compound 3. Upon reduction of azide under hydrogenation condition, the final aminated α-D-mannose, compound 4, was formed; IPAC chemical name: 3-α-Aminopropyl-D-mannopyranoside.

Functionalization of the SPCE

Chemicals used to functionalize the SPCE include: 2.5 mM glutaraldehyde solution, Citrate Gold Nanoparticles (AuNPs), stock ≈2.4 Au; ~15 nm in diameter**,** D+ mannose - amine @ 25 µg/mL**,** Deactivating buffer (0.2 M Tris in 0.01 M Phosphate Buffered Saline (PBS) plus 10 mM Cyanoborohydride)**.** Chemicals such as 0.1 M hydrochloric acid (HCl) solution, acridine orange (AO) stain (2 mg of AO into 1 mL of AO buffer = 100x); acridine orange buffer pH 3.8. (90 mL of 10mM (0.01 M) phosphate buffer + 585 mg of NaCl, qs to 100 mL), coupling buffer pH 7.4 (0.1 M phosphate buffer into 900 mL of dH_2O), Con A conjugated to FITC are used elsewhere in the protocol. SPCEs were purchased from Gwent Electronics Materials Ltd, United Kingdom and modified in house. Cyclic voltammetry was performed on the PalmSens handheld potentiostat (Palm Instruments BV, Houten, The Netherlands). Spectroscopic evaluations were performed on the Victor spectrophotometer.

To attach aminated D+ mannose to the SPCE, the rinsed, dried chips were incubated with 25 µL of 2.5 mM glutaraldehyde on the working center of the carbon electrode for two hours at 4°C. At completion, the excess glutaraldehyde was rinsed with DI water and dried. Citrate AuNPs (25 µL) were then incubated on the working center of the carbon electrode for two hours at 4° C. At completion, the excess citrate AuNPs were rinsed with DI water and dried. On the working center of the carbon electrode, 25 µL of aminated D+ mannose (25 µg/mL) was placed for 15 minutes at 21° C. At completion, the excess aminated D+ mannose was rinsed with DI water and dried and 50 µL of deactivating buffer was applied for 15 minutes at 21° C. (Hermanson, 2008) The resultant D-FSPCE were dried and stored at room temperature (protected from light). Both a blank SPCE and an aminated D+ mannose modified SPCE are

shown in figures 7.4 and 7.5 respectively. Scanning electron micrographs were acquired using field-emission scanning electron microscopy (JOEL 7500F, acceleration voltage of 5 kV).

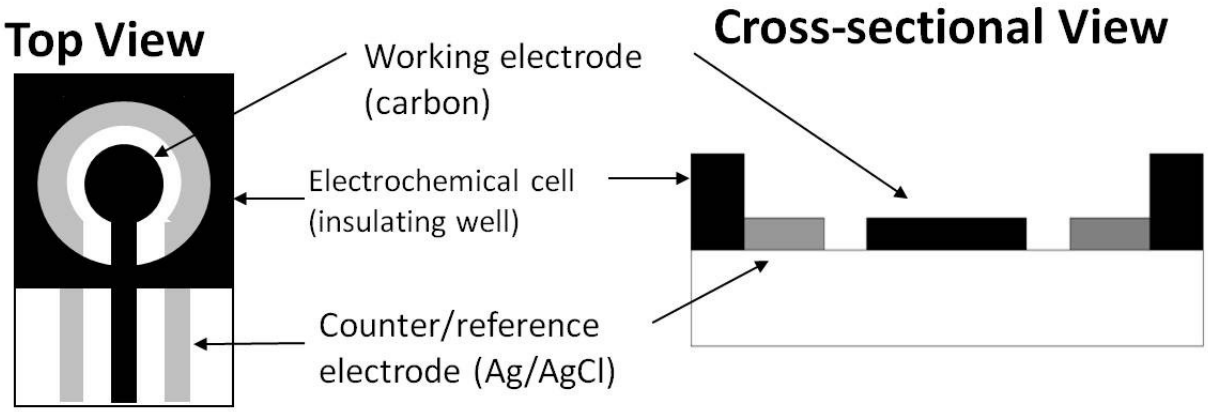

Figure 7.2: Schematic of the bare screen-printed carbon electrode (SPCE) sensor chip the detection is performed on, the circuit.

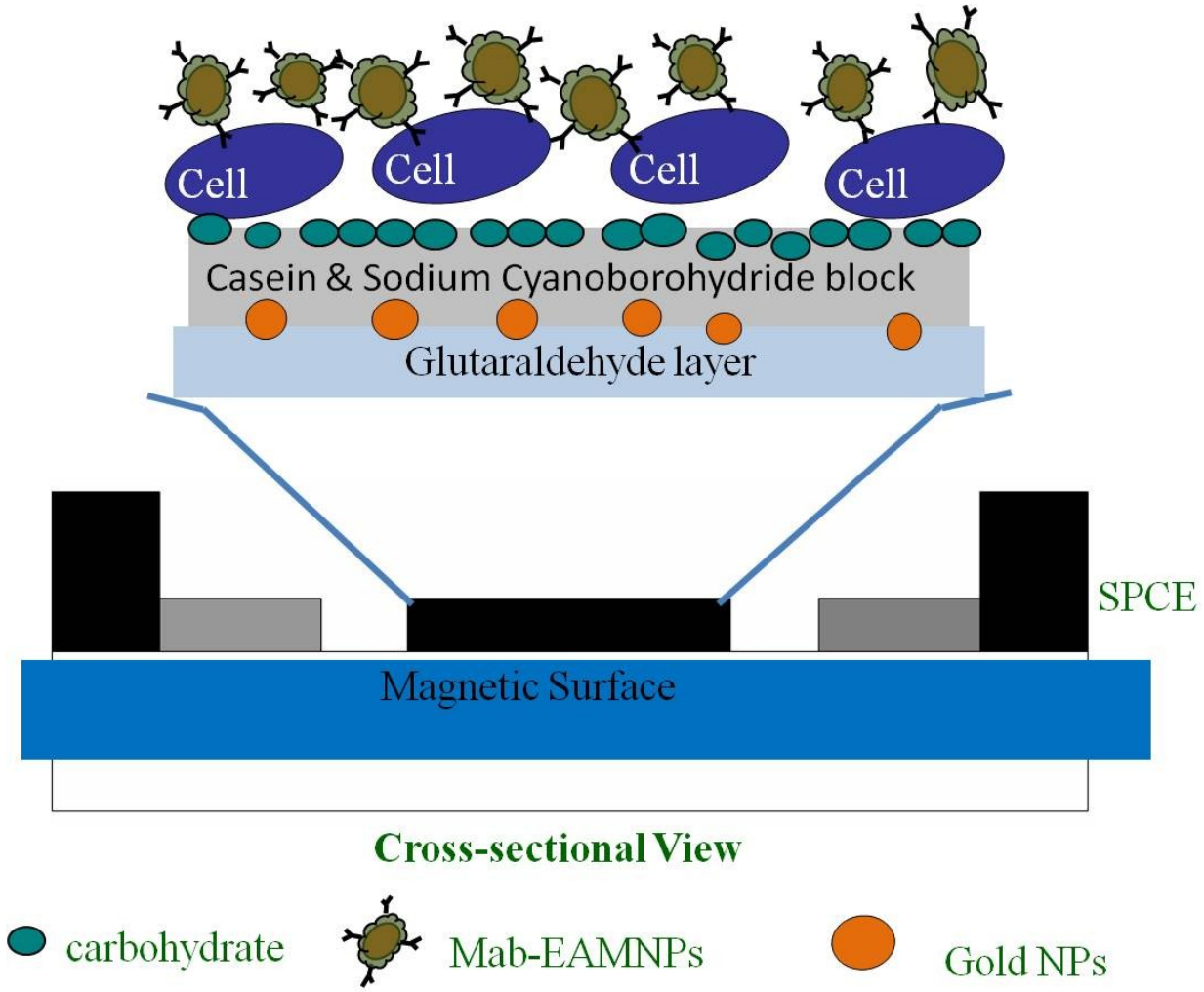

Figure 7.3: Layered schematic of the structure in the novel aminated D+ mannose functionalized screen printed carbon electrode (D-FSPCE) biosensor chip. The modifications are made to the center circle of the carbon electrode and HCL is used to connect the Ag/AgCl electrode during cyclic voltammetry.

Cyclic Voltammetry evaluation of D-FSPCEs:

Cyclic voltammetry is an electrochemical method where a potential is scanned from -1.4 V to 2.0 V and the resultant current is measured at each voltage. Varying parameters can be used on the resultant cyclic voltammogram, depending on the reporter used. Here polyaniline is electrically active and, if present, should provide a peak at a certain voltage in both the positive

and negative sweep of the current in the cycle. Larger levels of polyaniline should provide greater peak heights but other components of the system affect the polyaniline peak greatly as well. The current produced at a given potential is the combination of the entire electrochemical system and additional components can reduce the current as side reactions occur (Xu, Leng, Li, Lu, Wanga & Hu 2010; Li, Yang, Zhao & Du, 2011). Due to this, other parameters such as delta Q, peak shift, peak shape, resistance at each point in the cycle, and average resistance over the whole cycle were tabulated and evaluated in this study. Between the differing concentrations and the blank D-FSPCE, all the parameters collected were compared. The XY values of the highest, lowest, and expected polyaniline peaks were evaluated from the voltammogram. The shift positive or negative from the original position of the expected peak in a polyaniline coated D-SPCE without bacterial cells was evaluated and recorded for each run. The shapes of the characteristic peaks were also evaluated based on their width and tracing. From the collected data of voltage (V) and current (I) at each point on the voltammogram, the absolute resistance (R) was calculated from standard Equation 7.1, solved to Equation 7.2 and the result placed into Equation 7.3:

Equation 7.1: Ohm's Law used to calculate the electronic behavior in the circuit of each point taken during the cyclic voltammogram

$$V = IR$$

Equation 7.2: Solving of Ohm's Law to calculate the resistance at each point on the cyclic voltammogram.

$$R = \frac{V}{I}$$

Equation 7.3: Total average resistance across all points on three consecutive cyclic voltammograms.

$$\sum R = \frac{V}{I} \Big/ \#\, pts$$

Four scans of each D-FSPCE were conducted and the first scan was discarded as a system equilibration set. The last three provided at least 960 data points apiece across the cycle. For each of these 960 individual points the absolute resistance was calculated. The average resistance of the scans were averaged together to get the single average resistance across the system for each run at each concentration of bacteria.

Statistical Analysis

Independent two-tailed T-tests were performed on the calculated parameters of each concentration of Mab-EAMNP - *E. coli* O157:H7 Sakai strain on the D-FSPCE compared to the same parameters of a blank D-SPCE run through the same IMS extraction with the addition of sterile peptone water instead of bacterial culture in TSB. ($\alpha = 0.05$) In addition, the data from the broth trials were used to determine a cut point other than the blank parameters to call a fluid whole milk sample positive or negative.

Results

Hypothesis 1

Aminated D+ mannose remains biologically active when bound to the SPCE and against bacteria that are bound to the Mab-EAMNP complex. Using fluorescence, acridine orange stained *E. coli* O157:H7 Sakai strain were challenged and found to bind to the blocked D-SPCE chips more effectively than the nonspecific screen absorption that occurs with unblocked chips,

with or without glutaraldehyde. As shown in figure 7.3, the D-FSPCE captured more fluorescent organisms over plain unblocked SPCEs, and unblocked glutaraldehyde SPCEs and much more than blocked glutaraldehyde with no aminated D+ mannose, both visually and with the spectrophotometer. (data shown in Figure 7.4)

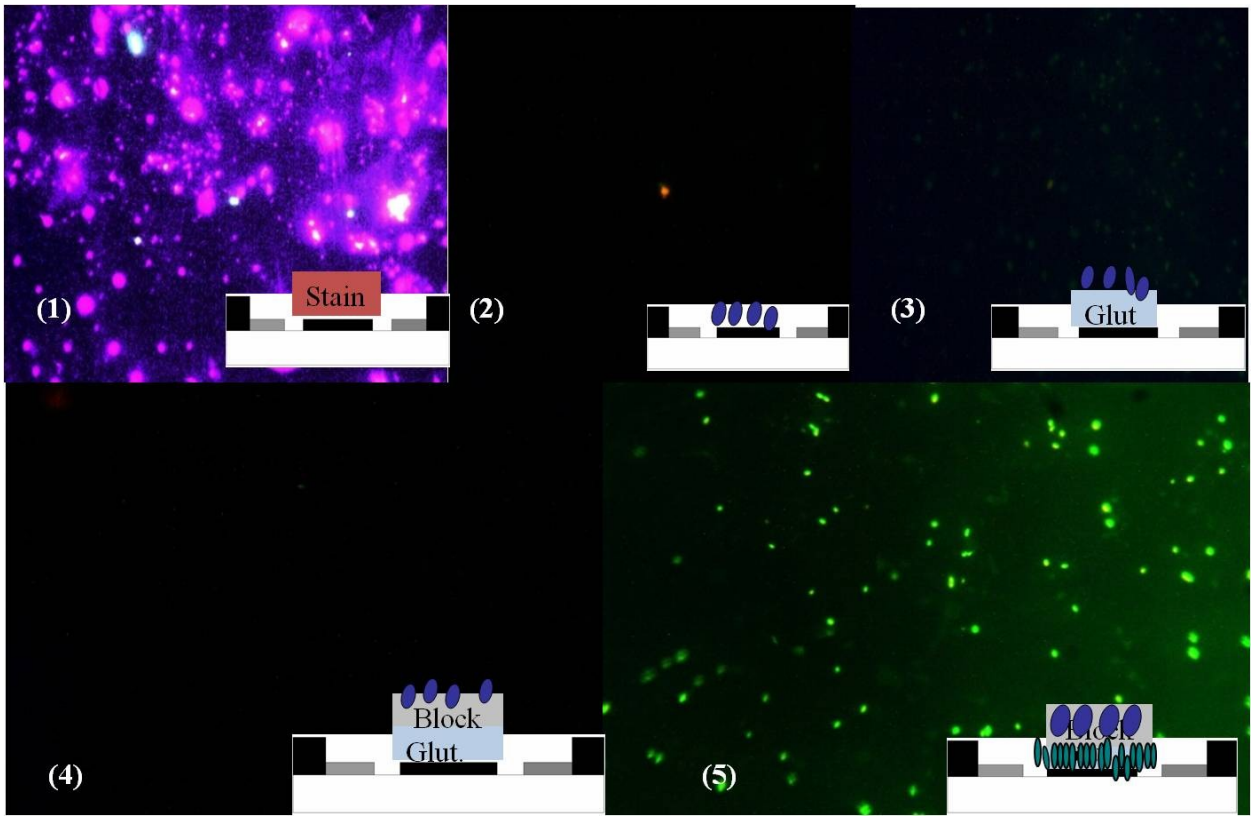

Figure 7.4: Successive electron microscope evaluations of acridine orange and acridine orange stained *E. coli* O157:H7 Sakai strain on varying SPCEs through a 445nm long pass filter 40X lens. 1: Acridine orange on SPCE (Non specific stain attachment) 2: Plain SPCE – no block (Non specific cell attachment) 3: glutaraldehyde coated SPCE (Non specific cell attachment) 4: Blocked glutaraldehyde coated SPCE - w/o carbohydrate and 5: Blocked D-FSPCE w/carbohydrate

As shown in Table 7.1, the resultant FITC fluorescence showed the appropriate reduction when diluted, but decreased too much when the EAMNPs were present. We have shown in the past that the EAMNPs absorb too much light to obtain good fluorescence data. To evaluate their

effect and determine how much actual Con A FITC-D+ mannose was captured by the bacteria, the cell mixture was heated to 95°C, spun briefly to remove the cells and the EAMNPs and retested in the Victor spectrophotometer. The results, as shown in columns 2-6, Table 4, show the 1:1 dilution gives roughly 150,000 RFUs or half that of the original 300,000 RFU. 108,000 RFU of that is left over with 9700 RFUs attached to the beads. When the cells attached to the beads are removed the original 17,000 units are returned to solution for a total of 135,383 RFUs. The aminated D + mannose active sites on the *E. coli* O157:H7 are still active when attached to the EAMNP-Mab.

Table 7.1: Con A conjugated FITC evaluation of the biological activity of aminated D+ mannose on Mab-EAMNP captured *E. coli* O157:H7 Sakai strain.

Combination	Fluorescent Units
Con A -FITC	324,275
Con A –FITC + D+ mannose 1:1 dilution	153,799 (≈ ½)
IMS → Wash, rinse Con A FITC+ D+ Mannose Residual in supernatant	108,588 (≈ ¾)
[Con A –FITC + D+ mannose] + [Mab -EAMNP – *E. coli* O157:H7]	9,756
Lyse cells → centrifuge Con A FITC **minus** [D+ mannose + Mab -EAMNP – *E. coli* O157:H7]	17,039

Hypothesis 2

Aminated D+ mannose can be used as a universal detection platform for multiple bacteria. The biological activity of aminated D+ mannose to *E. coli* O157:H7 on the SPCE and in solution to the *E. coli* O157:H7 attached to the Mab-EAMNPs was encouraging, but not enough.

To become a universal detection platform, a broad spectrum of biological activity must be demonstrated. To do this the same D-FSPCEs and acridine orange staining procedure was used to challenge multiple organisms without Mab-EAMNPs. The challenged organisms are multiple strains of *E. coli* O157:H- , *Bacillus anthracis* Sterne strain, *Staphalococcus aureus, Klebsiella pneumonia, E. coli* species, *Shigella* species*, Pseudomona aerugenosa*, *Vibrio fischeri* and *Enterobacter* species. Cell concentrations were not accounted for and the resultant RFUs are shown in Figure 4.4. All bacteria challenged attached to D-FSPCEs and had RFUs much larger than blank D-FSPCEs.

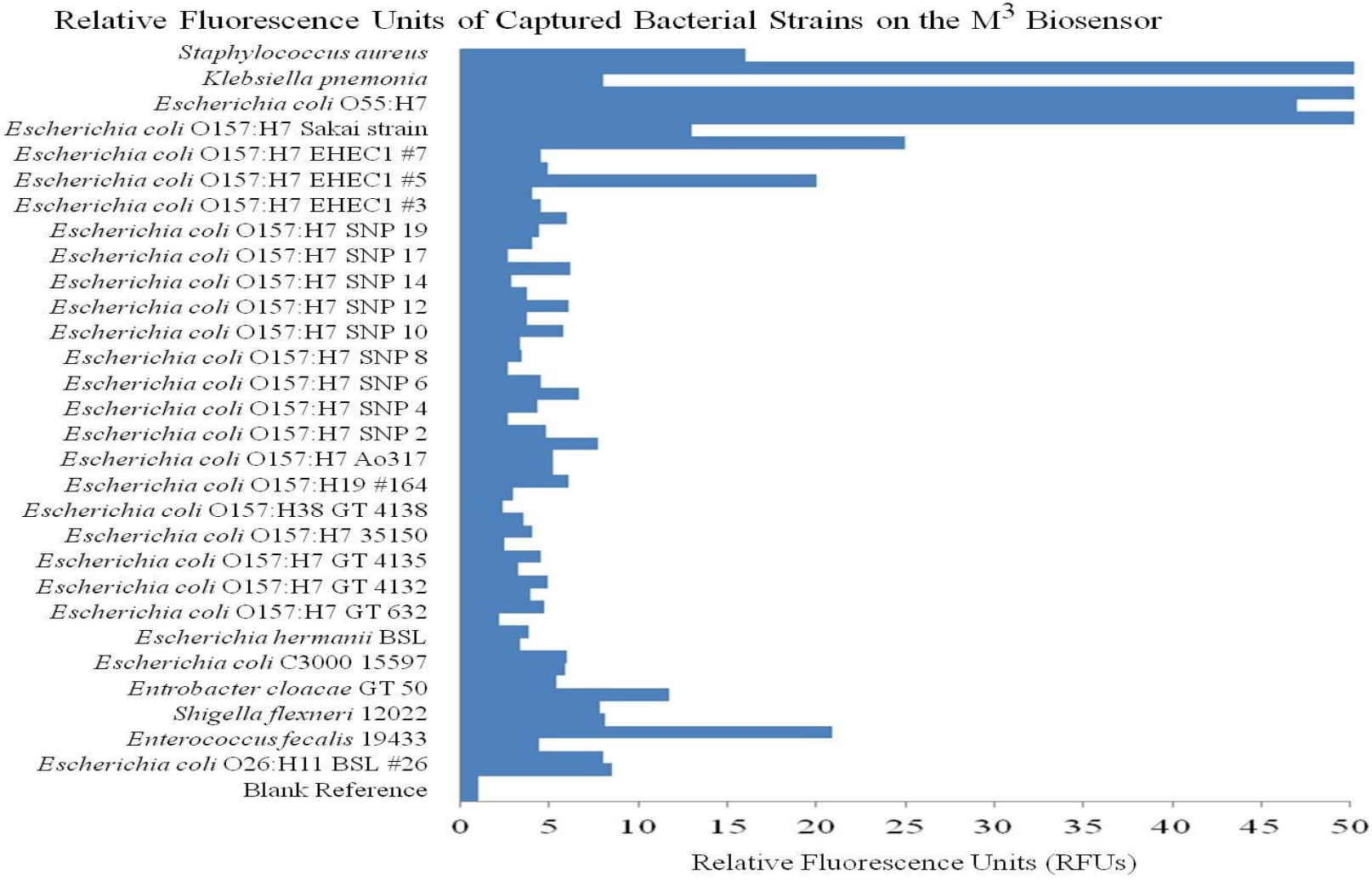

Figure 7.5: Blank D-FSPCE versus 29 bacterial organisms stained with acridine orange and captured on D-FSPCEs measured in Relative Fluorescence Units. (RFU)

Scanning electron micrographs of D-FSPCEs containing attached carbohydrate coating and various bacteria in single and mixed solutions are shown below in figures 7.5 and 7.6.

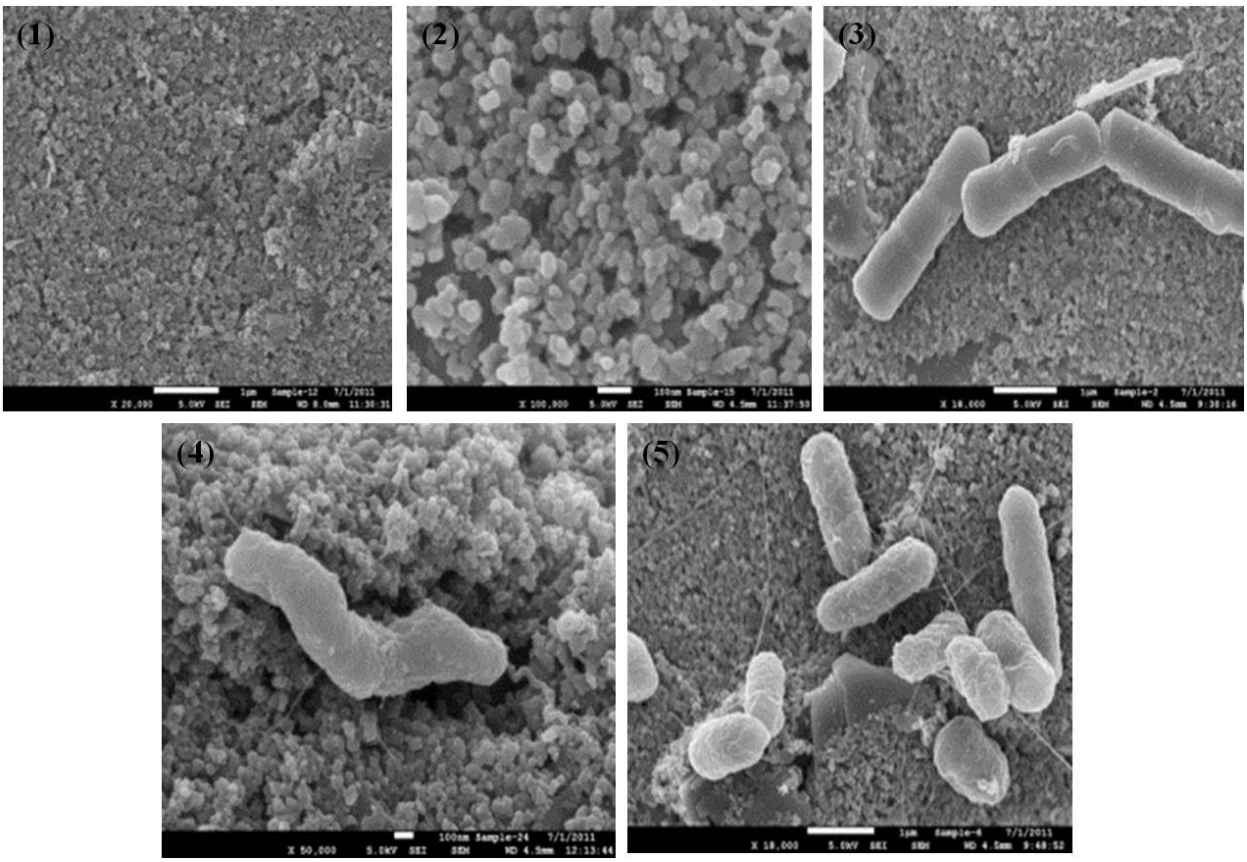

Figure 7.6: Scanning electron micrographs of the carbohydrate coating on the surface of the D-FSPCE and four individual bacterial solutions present after incubation and rinsing. 1 and 2: Carbohydrate coating on D-FSPCE 3: *Bacillus anthracis* Sterne strain on D-FSPCE 4: *Vibrio fischeri on D-FSPCE.* 5: *E. coli* O157:H7 on D-FSPCE.

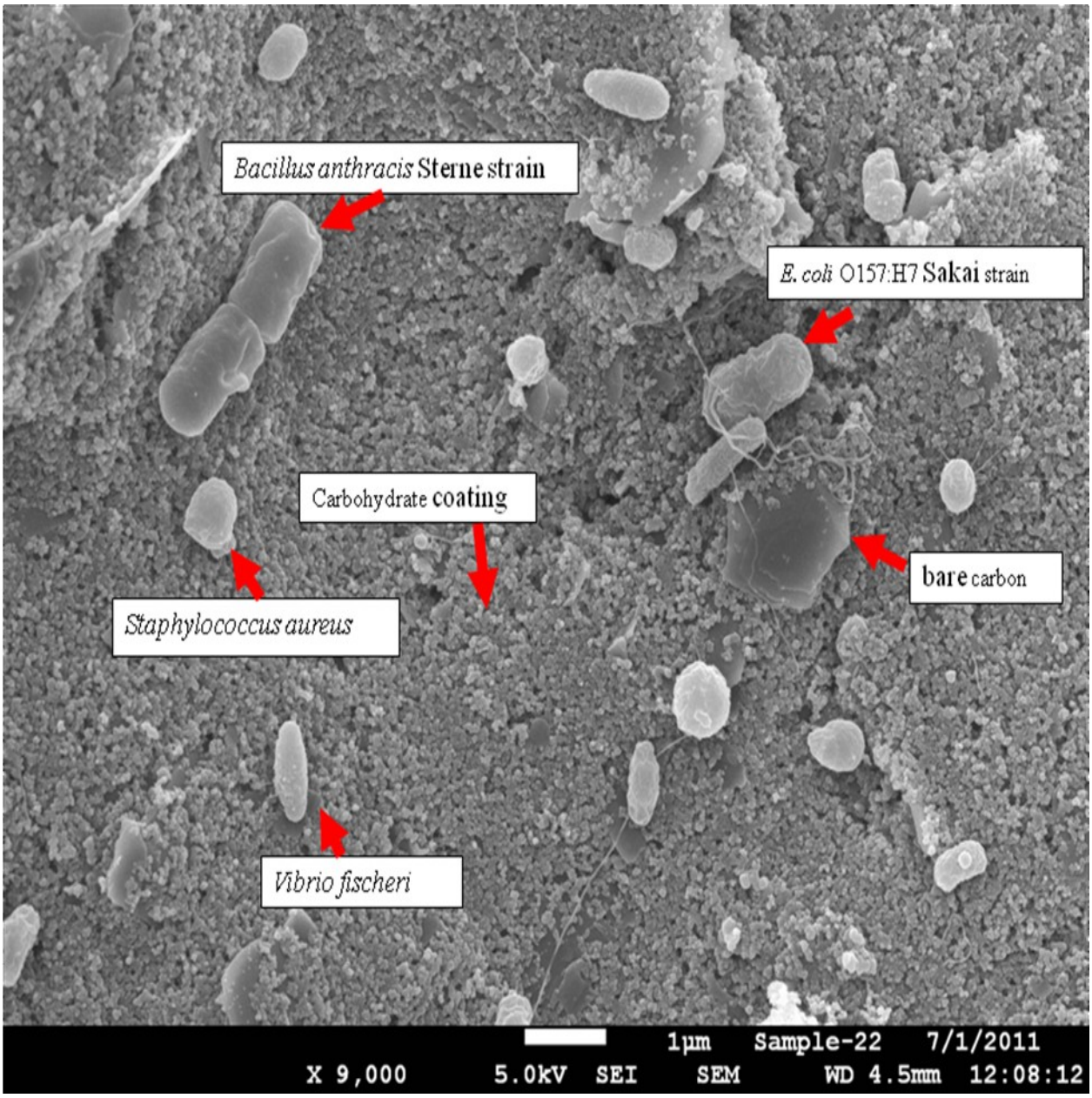

Figure 7.7: A scanning electron micrograph of the carbohydrate coating on the surface of a D-FSPCE and a mixed solution of 4 bacteria present after incubation and rinsing.

Hypothesis 3

Aminated D+ mannose coated SPCEs can be used as a novel biosensor detection platform for multiple detection methods. Here we use cyclic voltammetry (CV) on a hand held

potentiostat, from broth samples. Once biological activity was ensured and universality was probable, initial runs of several dilutions of Mab-EAMNP captured *E. coli* O157:H7 Sakai strains were placed on the potentiostat for cyclic voltammetry evaluation. Multiple calculations were evaluated to determine the best parameter for comparison of the concentrations and to determine if the biosensor is a quantitative or a qualitative test. One parameter in the initial runs showed promise to be discerning from the blank. That parameter was the calculation of average resistance across the whole voltammogram. The other parameters were inconsistent. Shown below is the qualitative evaluation of the broth based runs from the Mab-EAMNP IMS extraction run on the potentiostat using cyclic voltammetry and calculating the average resistance (figure 7.9) and the linear change as increasing concentrations of bacteria are run (figure 7.8).

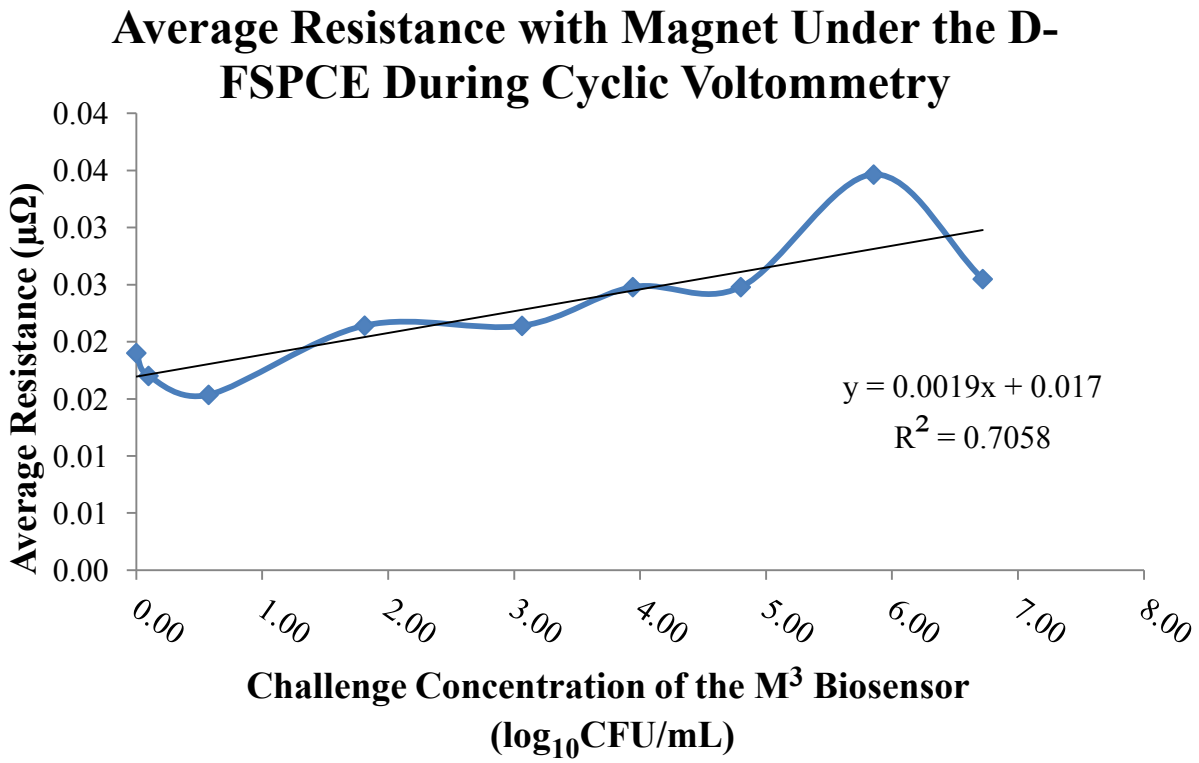

Figure 7.8: The average resistance change by $\log_{10}$ CFU/mL with a magnetic field under the D-FSPCE.

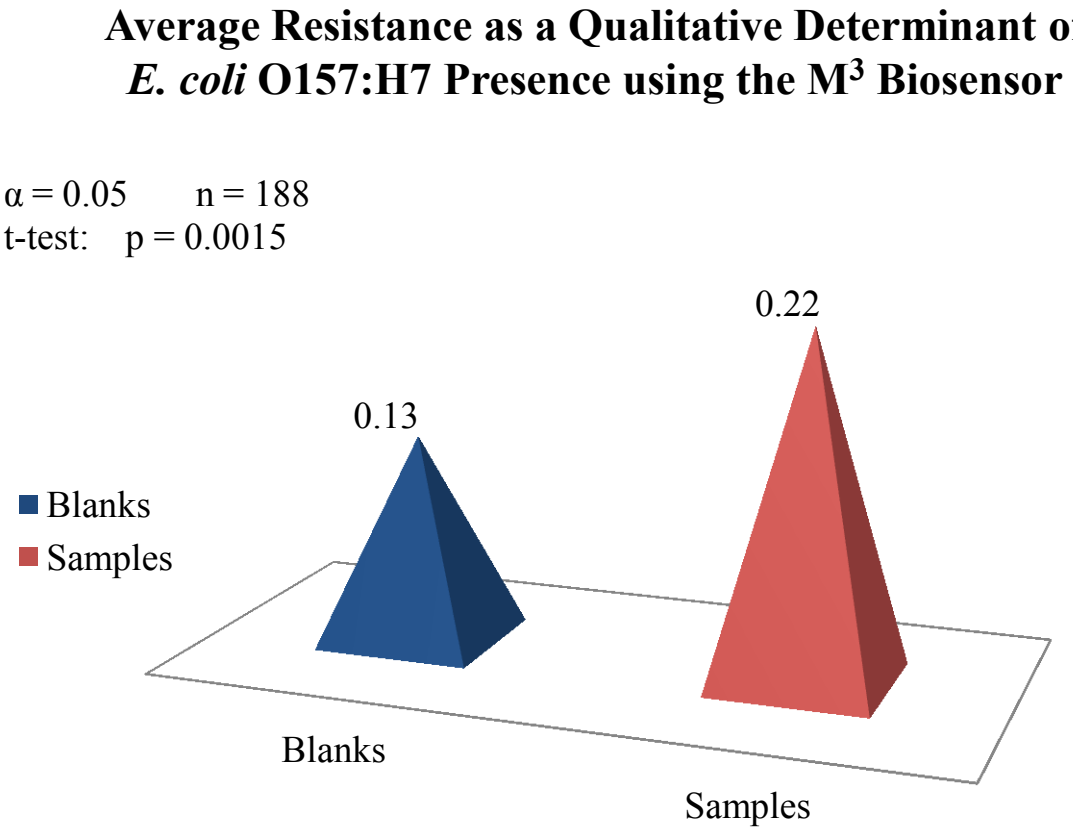

Figure 7.9**:** Qualitative evaluation of the M^3 biosensor for *E. coli* O157:H7.

Discussion

The aminated D+ mannose and glutaraldehyde chemically attach to each other and the SPCE via an amine bond. The addition of sodium cyanoborohydride (CH_3BNNa) converts those reversible amine bonds to amide bonds that are not reversible, giving the sensor its shelf stability (Hermanson, 2008; Dr. Xuefei Huang personal conversation.) The α D+ mannose with the amine group has successfully been attached to the SPCE and remains biologically active even

to Mab-EAMNP captured *E. coli* O157:H7. This indicates the active site of the Mab and the active site of the D+ mannose do not overlap for this bacteria and this antibody.

Multiple bacteria are reactive with α D + mannose even when functionalized to the surface of the SPCE**.** Cell wall glyco-carbohydrate binding site for bacterial cellular attachment in vivo has a high conservation among the entire *Enterobacteriacea* species. Of 39 organisms of 10 bacterial genera in both gram stain groupings, all attached to the carbohydrate coated SPCE. Attachment is selective for bacterial cells, but not differential for different bacteria showing that the D-FSPCE can be used as a multiplex sensor platform to detect multiple bacteria. Combining the D-FSPCE with the Mab-EAMNP or another selective extraction protocol creates a selective biosensor food testing procedure.

When combined, the D-FSPCE + Mab-EAMNP and using cyclic voltammetry have been named the M^3 Biosensor and have a patent pending. Modified screens allow bacterial bead complexes to attach and empty bead complexes can be washed away, cleaning up the signal generation of the final electrochemical analysis. Statistically significant qualitative differentiation can be performed in broth with no pre-enrichment. Even though the cells are attached to the D + mannose, adding a magnet to the underside of the D-FSPCE improves the conductance of the system by pulling the EAMNPs closer to the conducting layer in the SPCE. This allows clearer differences between concentrations of bacteria. The M^3 Biosensor can be used to detect bacterial contamination in broth without a pre-enrichment and is an inexpensive, field stable platform, with excellent multiplex capabilities in a wide variety of detection modalities.

Appendicies Chapter 7

Appendix 7.A: NMR spectra of Animated D + Mannose production

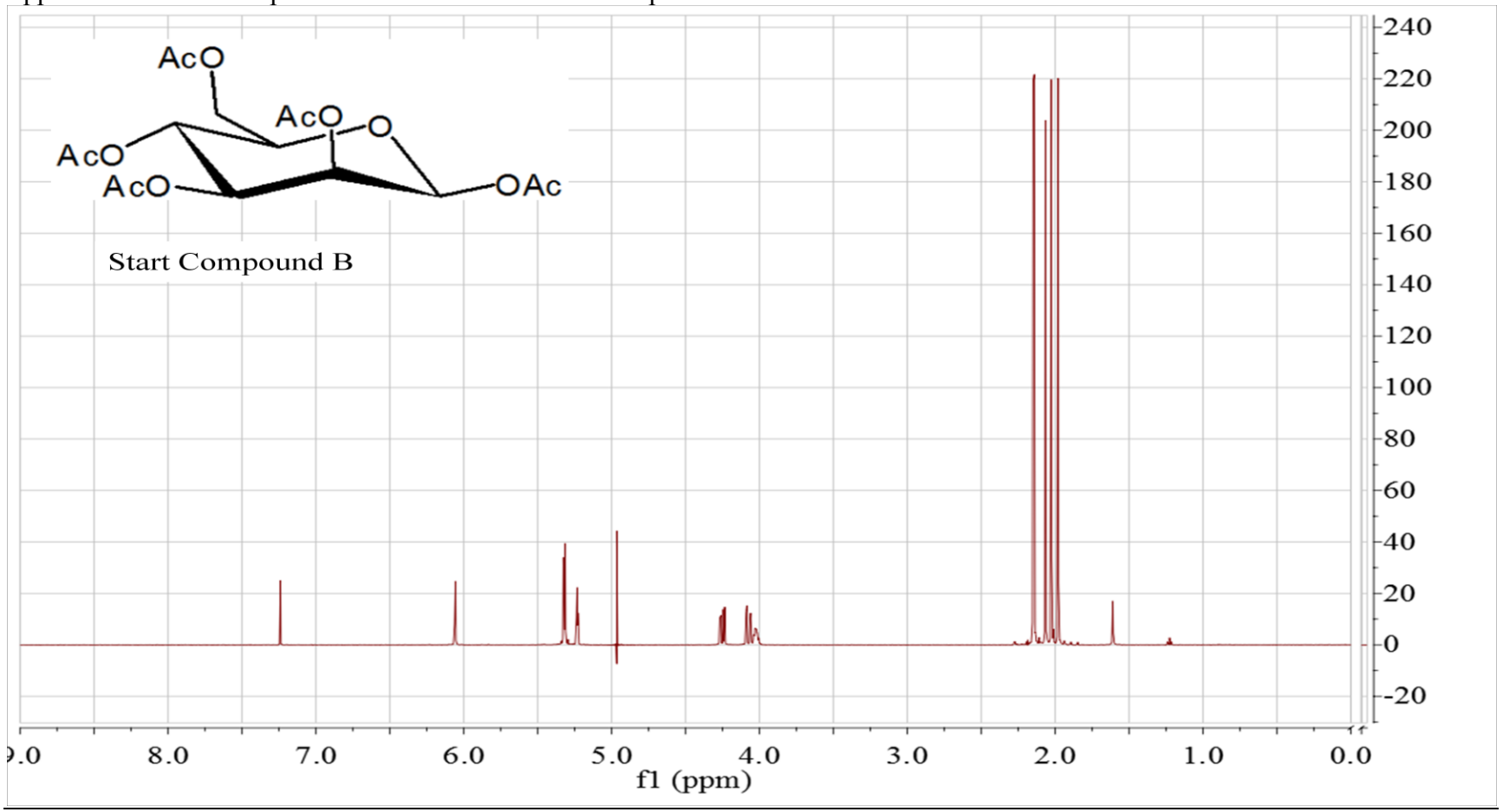

Figure 7.A.1: ^{1}H-NMR spectra of step one for the D+mannose pentacetate production. In this step the Ac groups are added to all the carbons of the 6 carbon ring. This is start compound B in figure 7.1 (500 MHz, $CDCl_3$).

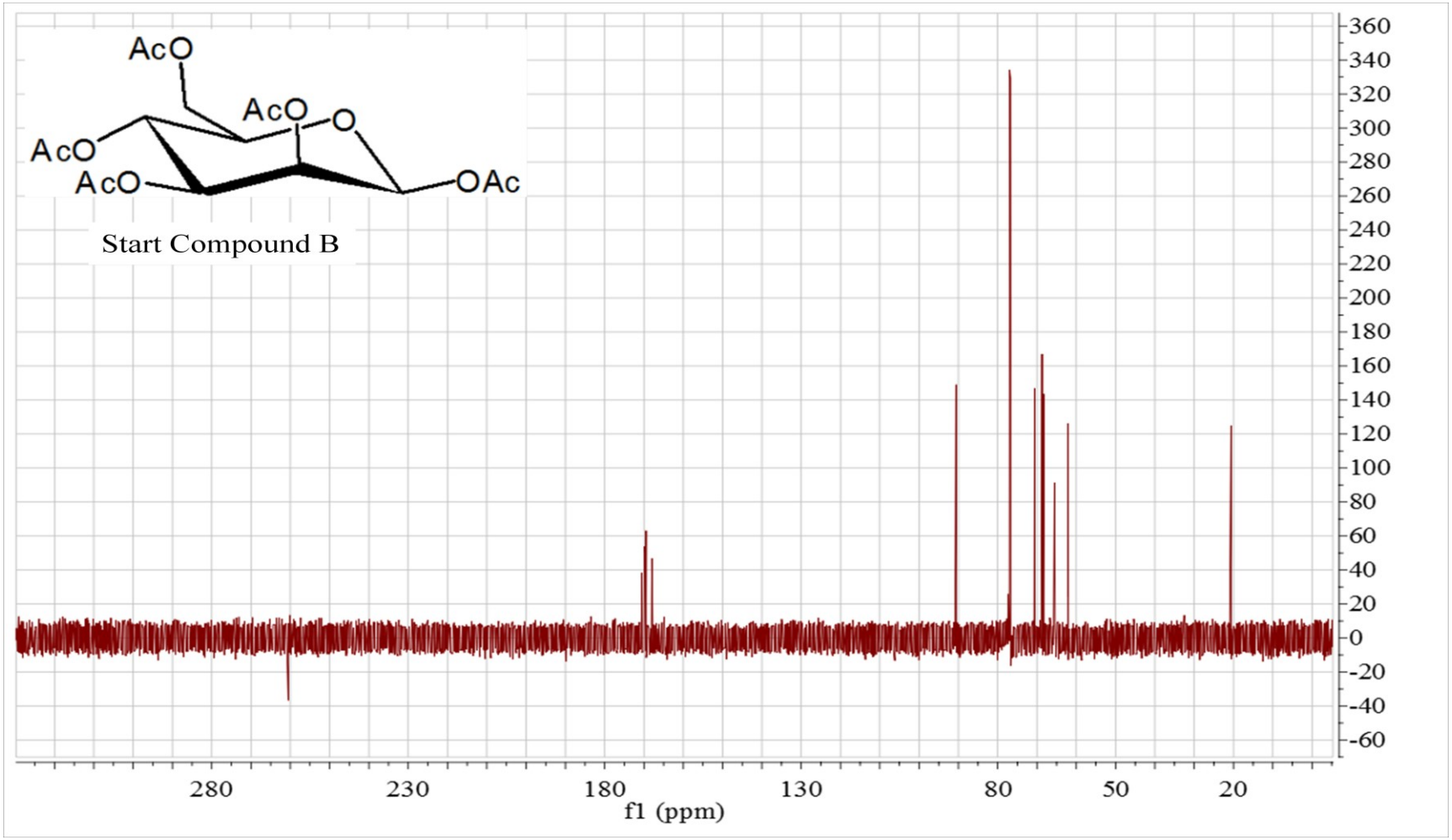

Figure 7.A.2: ^{13}C-NMR spectra of step one for the D+mannose pentacetate production. In this step the Ac groups are added to all the carbons of the 6 carbon ring. This is start compound B in figure 7.1 (500 MHz, $CDCl_3$).

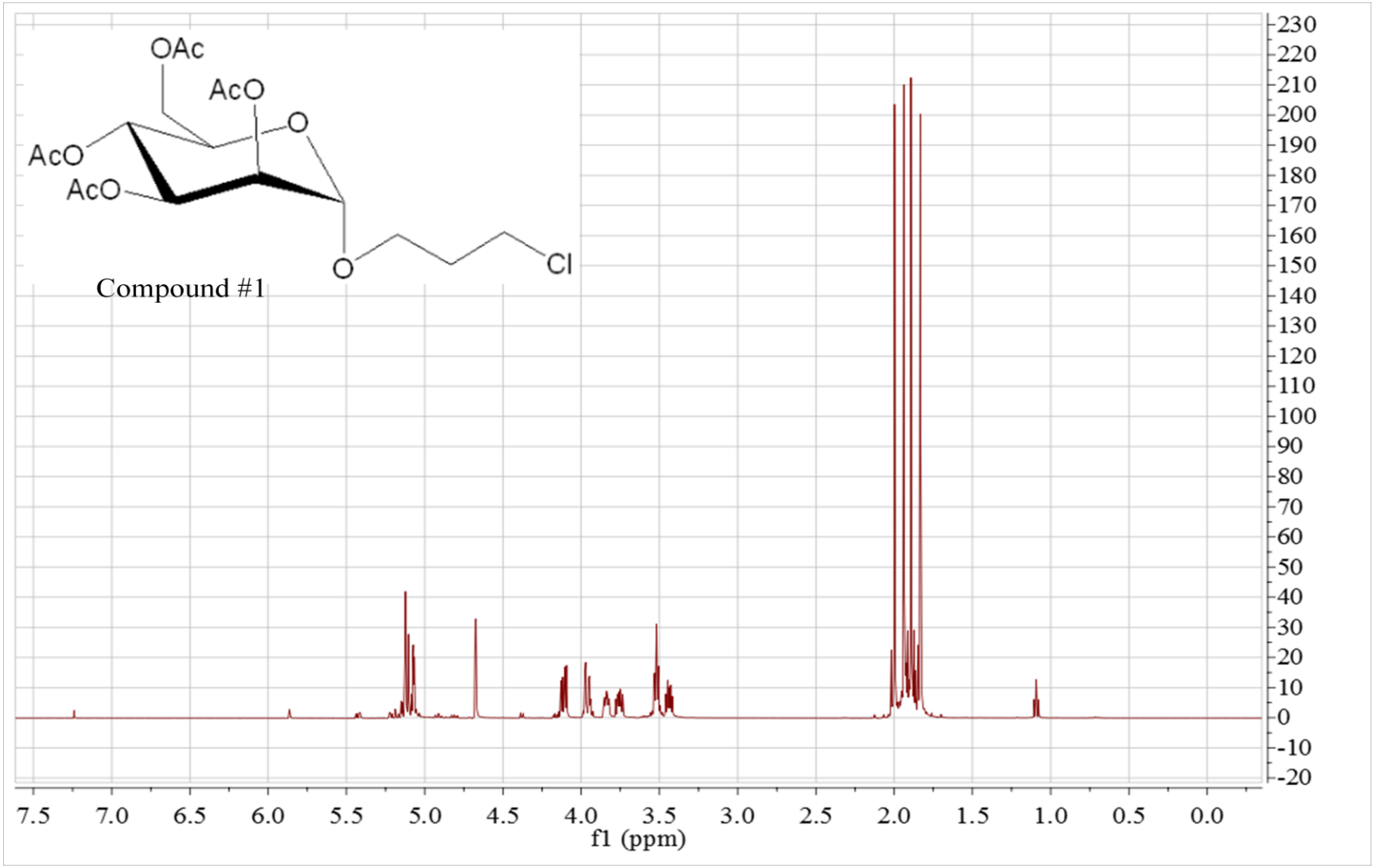

Figure 7.A.3: ^{1}H-NMR spectra of step one for the D+mannose pentacetate production, substitution on the anomeric carbon with a chloride linker. This is compound #1 in figure 7.1 (500 MHz, $CDCl_3$).

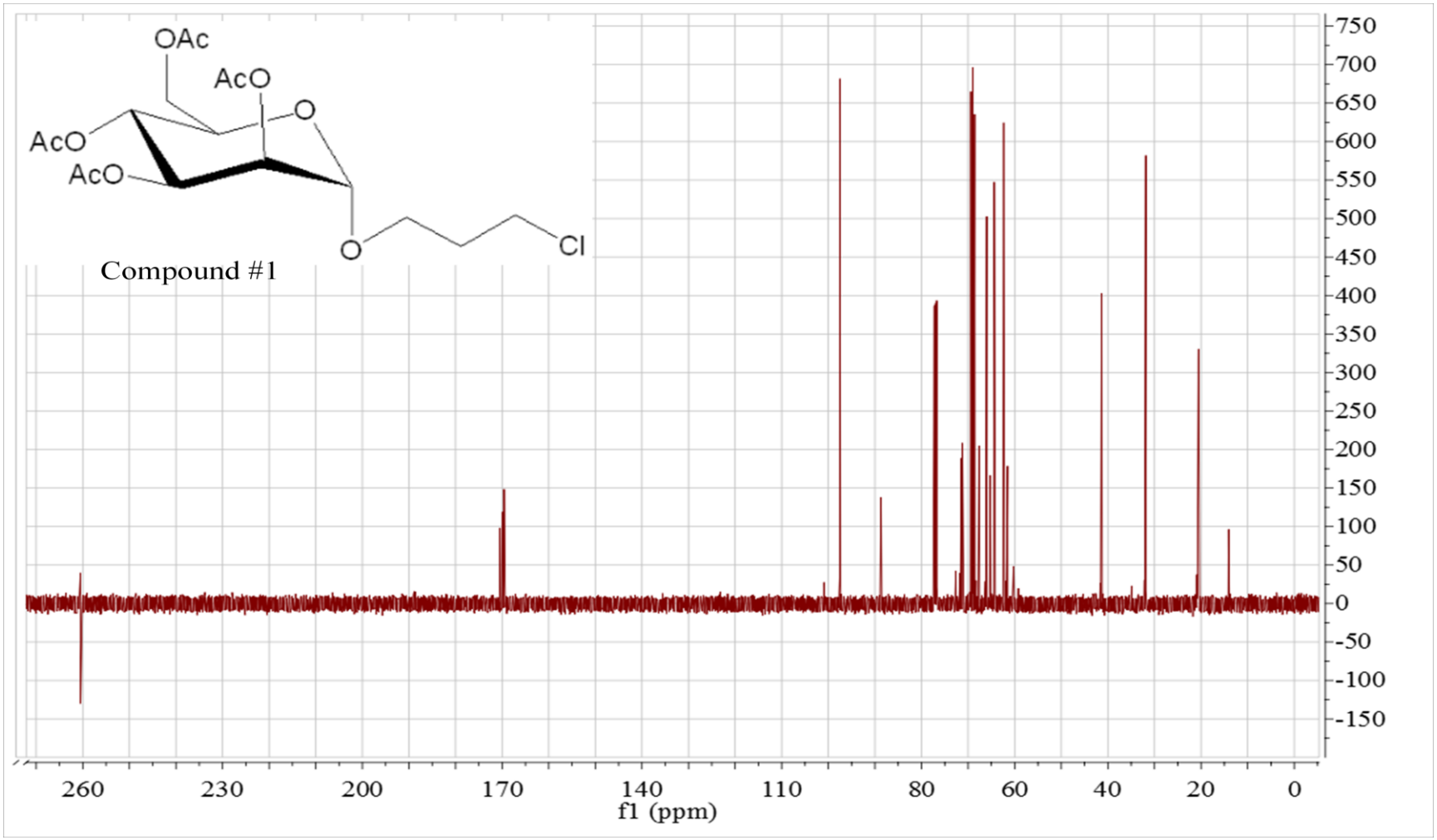

Figure 7.A.4: ^{13}C-NMR NMR spectra of step one for the D+mannose pentacetate production, substitution on the anomeric carbon with a chloride linker. This is compound #1 in figure 7.1 (500 MHz, $CDCl_3$).

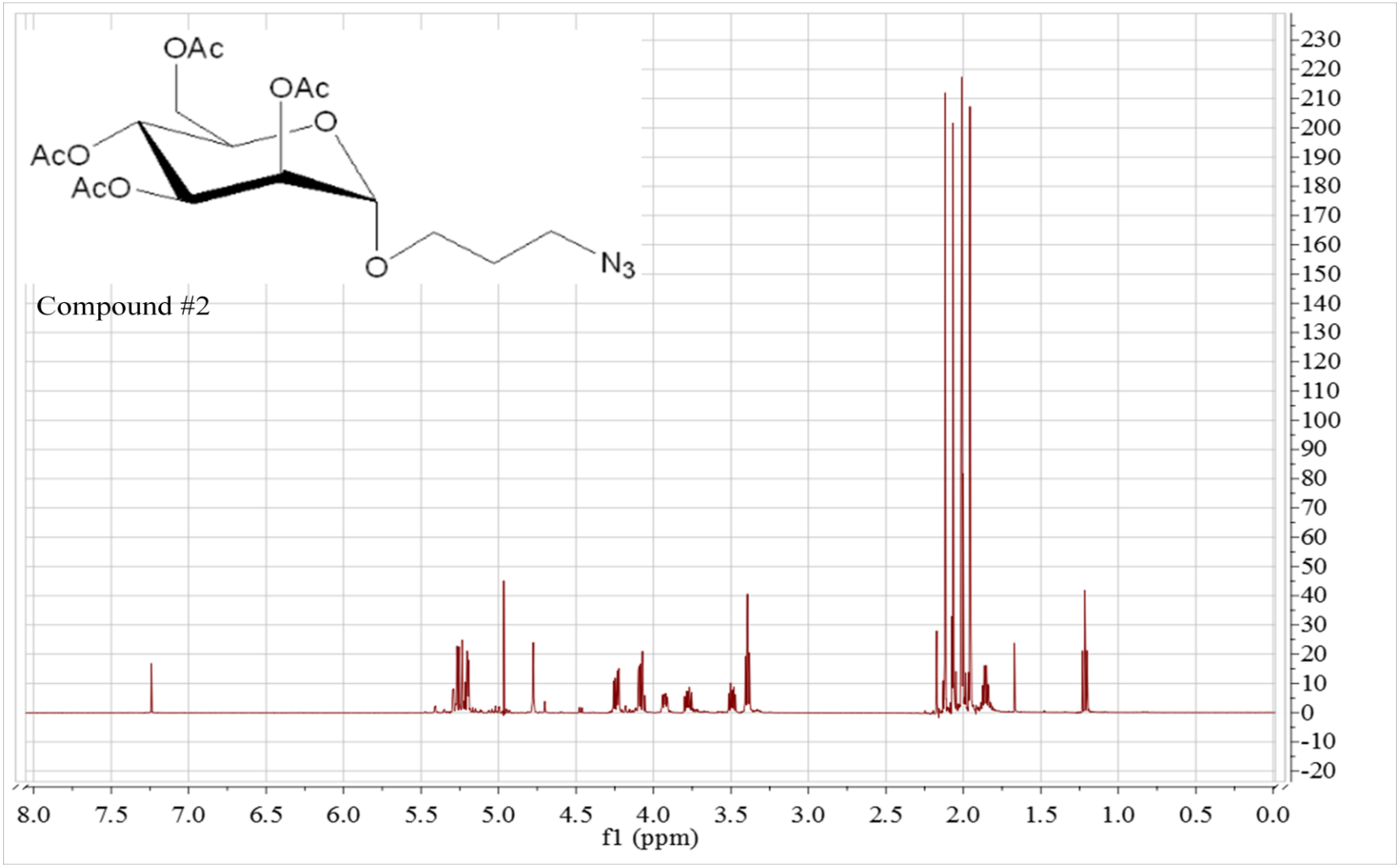

Figure 7.A.5: ^{1}H-NMR spectra of the third step for the D+mannose pentacetate production. In this step the chloride group is substituted with a N_3 group. This is compound #2 in figure 7.1 (500 MHz, $CDCl_3$).

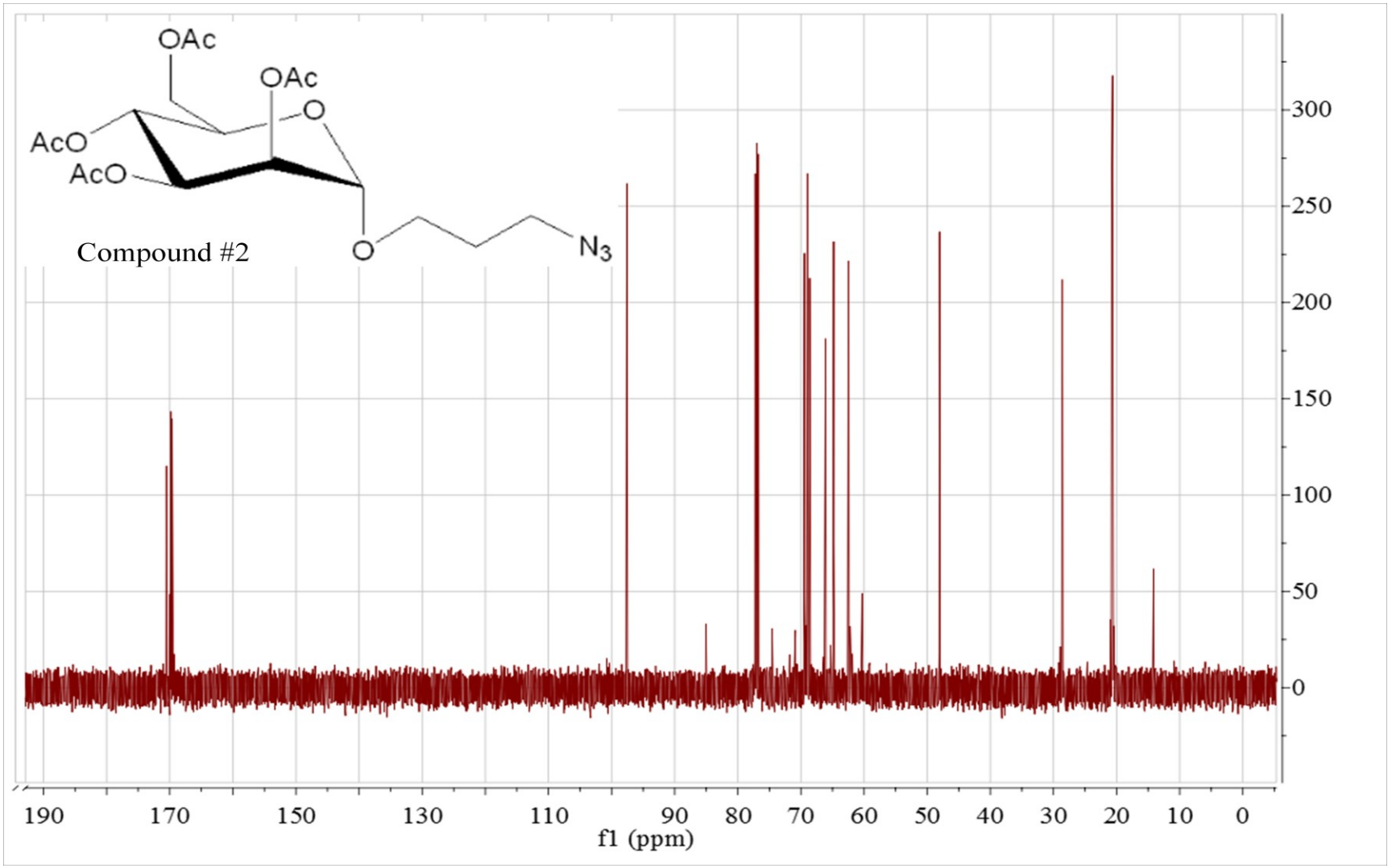

Figure 7.A.6: ^{13}C-NMR spectra of the third step for the D+mannose pentacetate production. In this step the chloride group is substituted with a N_3 group. This is compound #2 in figure 7.1 (500 MHz, $CDCl_3$).

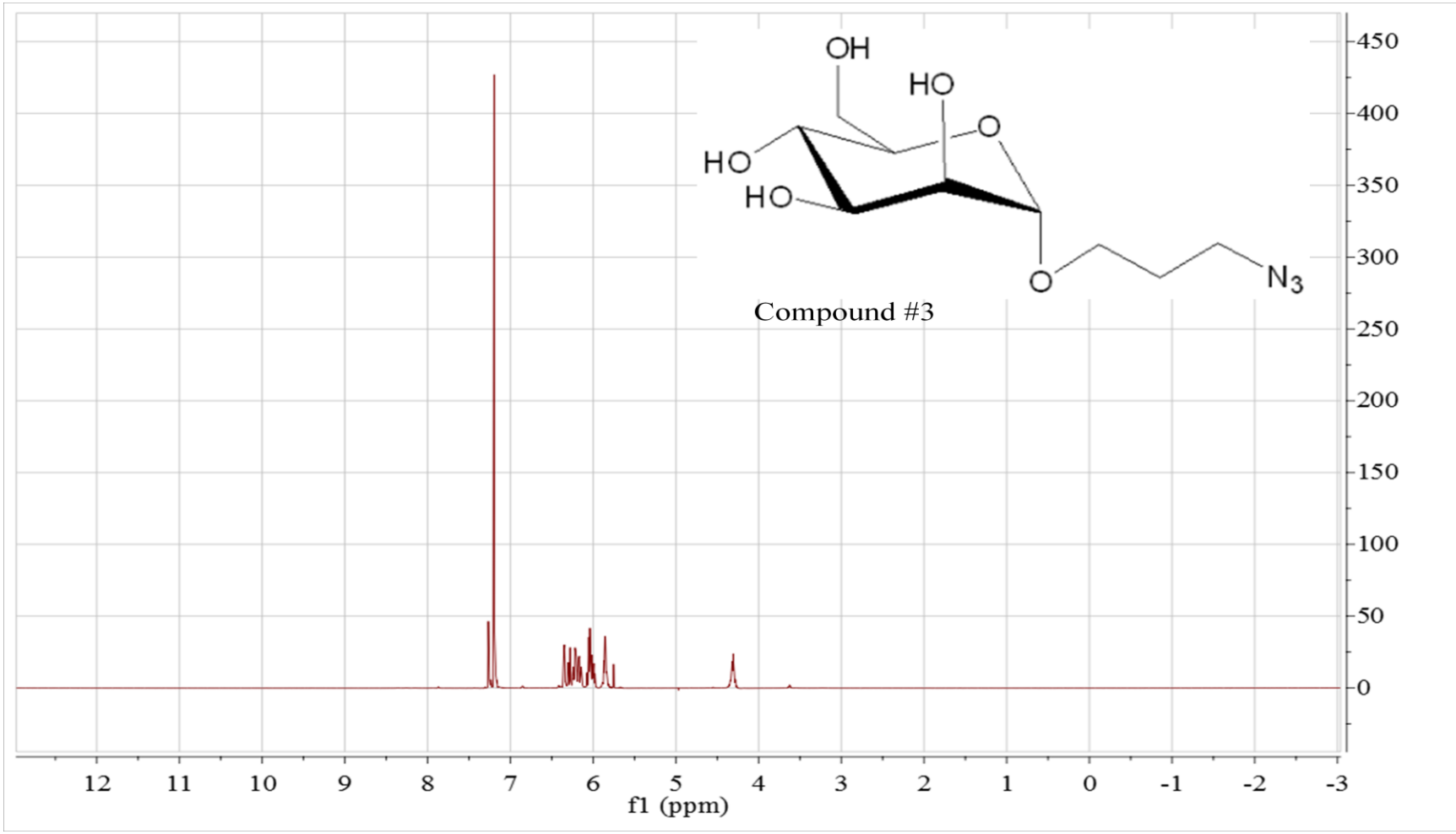

Figure 7.A.7: ^{1}H-NMR spectra of the third step for the D+mannose pentacetate production. In this step the Ac groups are removed. This is compound #3 in figure 7.1 (500 MHz, D_2O).

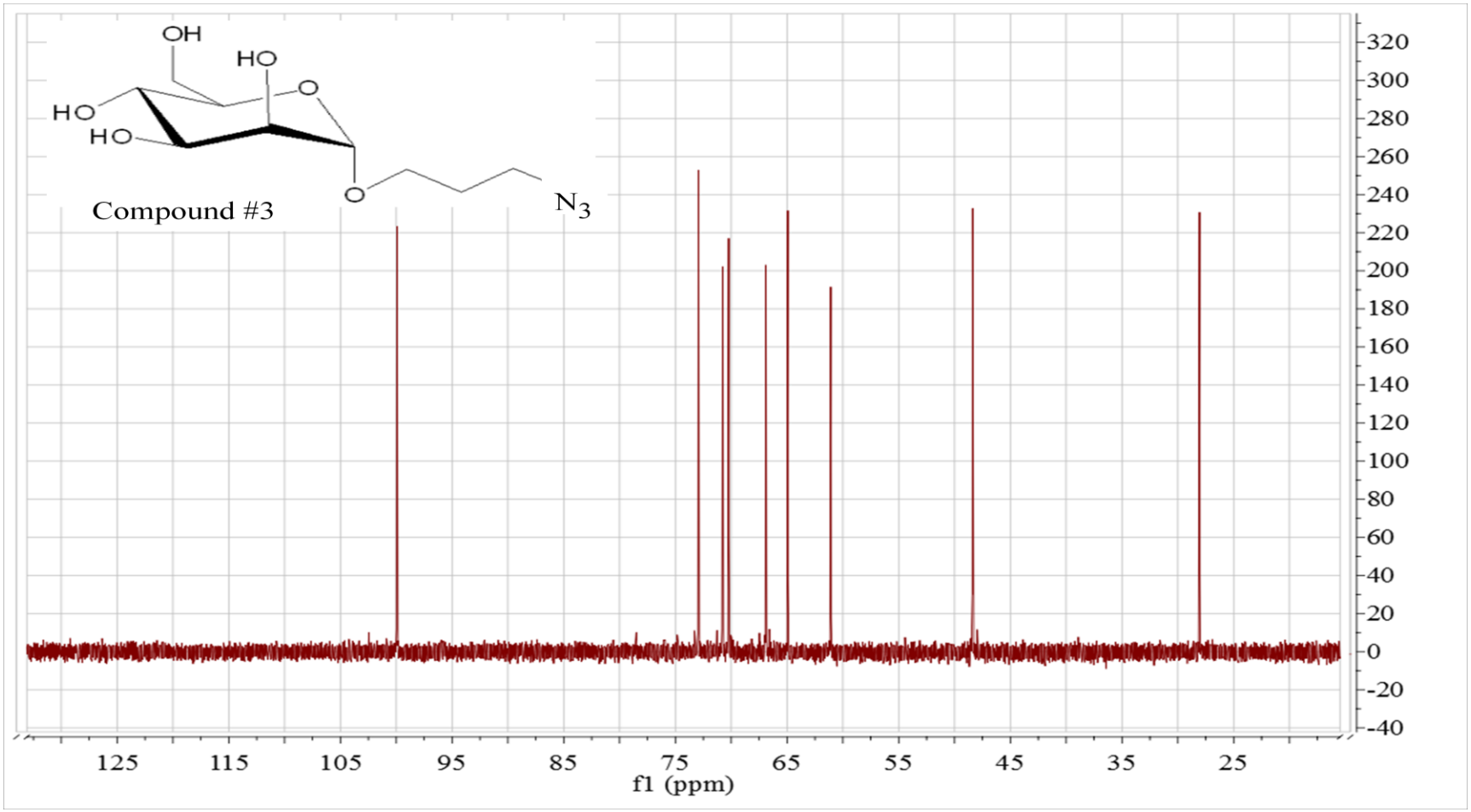

Figure 7.A.8: ^{13}C-NMR spectra of the third step for the D+mannose pentacetate production. In this step the Ac groups are removed. This is compound #3 in figure 7.1 (500 MHz, D_2O).

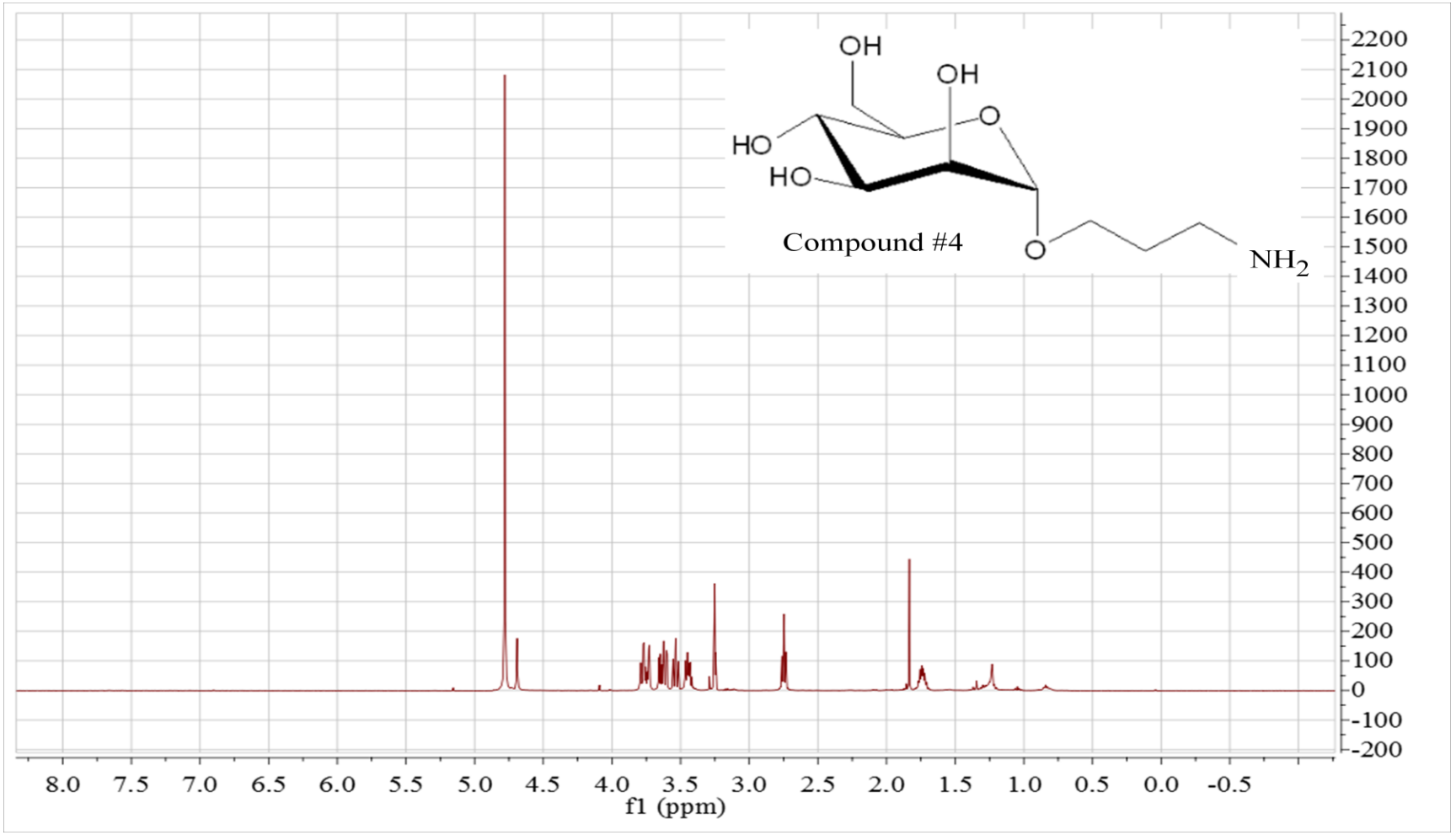

Figure 7.A.9: ^{1}H -NMR of the final step for the D+mannose pentacetate production. In this step the N_3 group is reduced to an NH_2. This is compound #4 in figure 7.1 (500 MHz, CD_3OD).

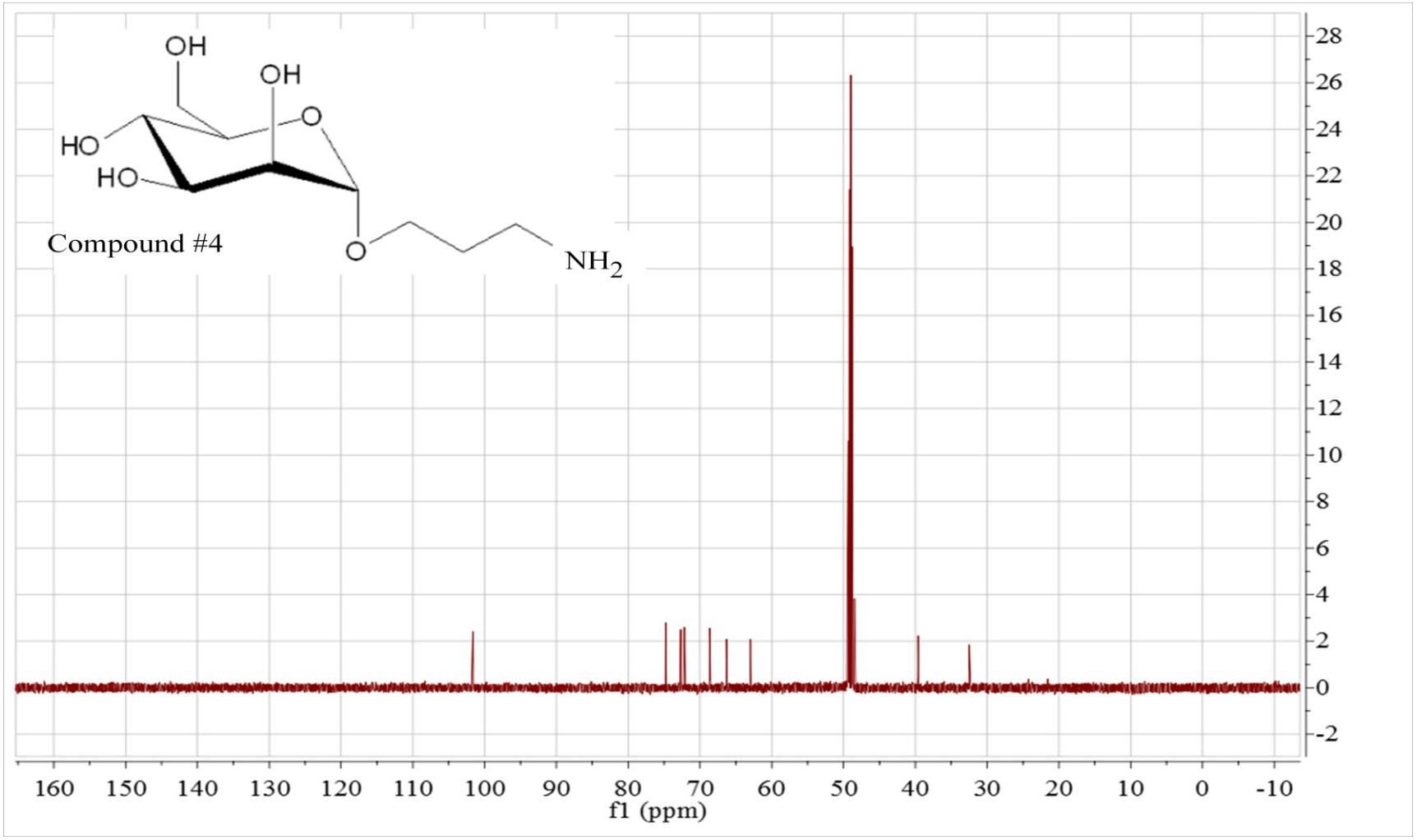

Figure 7.A.10: ^{13}C-NMR of the final step for the D+mannose pentacetate production. In this step the N_3 group is reduced to an NH_2. This is compound #4 in figure 7.1 (500 MHz, CD_3OD).

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CHAPTER 8: Validation of the M^3 Biosensor in Broth

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Abstract

Food defense requires the means to efficiently screen large volumes of food for microbial pathogens. Even rapid detection methods often require lengthy enrichment steps, making them impractical for this application. There is a great need for rapid, sensitive, specific, and inexpensive methods for extracting and concentrating microbial pathogens from food. In this work repetitive challenges to the M^3 Biosensor have shown reliable evidence for continued refinement of this sensor. The M^3 Biosensor has shown consistent ability to show presence/absence for bacteria consistently across a wide range on bacterial concentrations from 100 CFU/mL to $1.0 * 10^7$ CFU/mL. The M^3 Biosensor has a mean capture efficiency at 92% down to 5 CFU/mL; equivilant to a widely used commercial IMS methodolgy and a LOD_{50} of 6-9 CFU/mL. In previous work, the microbiological inclusivity and exclusivity of the EAMNP IMS method was evaluated against 35 *E. coli* O157 strains and 29 other organisms, many in the Enterobacteriacea family. The extraction protocol's inclusivity within strain is 94% and exclusivity outside the *E. coli* O157 family is 87%. Electrical chemical detection with statistically significant differences as low as 5 CFU/mL and a signal to noise ratio of 2:1. A linear range of 5 CFU/mL to $1.0 * 10^8$ CFU/mL for both IMS analysis and CV analysis is excellent performance through over 200 repeat analyses. The entire IMS procedure requires only 40 minutes, and antibody-conjugated MNPs show no decline in performance up to 149 days after conjugation. The cost of producing one sample volume of EAMNPs conjugated with anti-*Escherichia coli* O157:H7 is ~ $0.43. The M^3 Biosensor shows excellent progress toward a field ready bacterial food detection tool.

Introduction

The search for an efficient and effective field based test to allow monitoring of a greater percentage of the food supply at an affordable cost by the military, government or any food company is underway. The largest hurdles to developing such a test include food matrices, low contamination levels, low infective doses, competitive non pathogenic organisms, and field portability (Fung, 2008; Ge & Meng, 2009). Before going into detail about food specific tests, it is helpful to discuss the requirements for a field based diagnostic test at all. The World Health Organization (WHO) Sexually Transmitted Diseases Diagnostics Initiative uses the term 'ASSURED tests' to describe the ideal characteristics of a diagnostic test for a resource limited setting (Mabey, Peeling, Ustianowski, & Perkins, 2004). The following criteria are listed:

1. Affordable by those at risk of infection.
2. Sensitive (few false-negatives).
3. Specific (few false-positives).
4. User-friendly (simple to perform and requiring minimal training).
5. Rapid (to enable treatment at first visit)
6. Robust (does not require refrigerated storage).
7. Equipment-free.
8. Delivered to those who need it.

Mabey et al. (2004) also states that developing a portable, field ready diagnostic test that matches all eight criteria is very difficult, but should not prevent the development of a useful test in the interim. There are multiple recent review articles that discuss the same criteria for food microbiological testing from the perspective of the microbiologist, the research perspective and the from food production manager, respectively (Fung, 2008; Ge & Meng, 2009; Jasson,

Jacxsens, Luning, Rajkovic & Uyttendaele, 2010). Each of these supports from different perspectives the need for the same criteria in food as ASSURED proposes for diagnostics. The largest hurdles to developing such a test include food matrices, low contamination levels, low infective doses, competitive non pathogenic organisms, and field portability. To take detection as far forward on the battlefield and in the farm field as possible, the chosen test must be able to be stored with limited refrigeration, work in a dirty, wet environment with a battery or small generator (Jasson et al., 2010). It should be small, portable and light. Ideally for a military environment, but applicable to any food company environment, the least amount of technical expertise and upkeep is necessary. There are no food based detection systems that fit this description on the market. Although sensitive, most of the currently available instrumentation does not work in a field environment (Jasson et al., 2010). Another key component in all diagnostic arenas is the system should allow for multiplexing to further decrease cost and time to results (Laczka, Del Campo & Mu˜noz, 2007).

Food, with its unique challenges, is an excellent medium for biosensor development. Food diagnostics lag behind other diagnostics in the advancement of biosensor technology of any kind (Fung, 2008). Excellent food based biosensor overviews are available in Arora, et al., 2011; Ivnitski, Abdel-Hamid, Atanasov, Wilkins & Stricker, 1999; Leonard et al., 2003; Wang, 2006; and Wei, Bailey, Andrew & Ryhanen, 2006. Fung states that the minimum requirement for food microbiology pathogen detection is 1 viable CFU in 25 grams of food (2008). The most glaring problem in all reviews is the need for pre-enrichment to reach that level of analytical sensitivity. Pre-enrichment adds time, cost and amplification of non target bacteria, as discussed earlier.

Serial screening is a method of diagnostic testing that uses a highly sensitive pre-screen to collect a population of test subjects with a higher percentage of true positives followed by a

more specific secondary screen (Hennekens & Buring, 1987). Serial screening such as this are common in public health applications to allow for very sensitive and inexpensive first line tests with increased specificity and cost of second line tests on a smaller population with a higher percentage of true disease. Ideal screening tests should be reliable, valid, sensitive, and specific. They are most valuable for diseases where the disease can be detected in a preclinical or before symptoms stage and have a treatment available to stop the course of the disease before clinical signs. Food borne illness is one such disease. Finding organisms before they are eaten allows them to be removed from the food supply, never allowing exposure. Sensitivity and specificity are often a trade off. For many diagnostic tests, in to increase sensitivity, specificity is sacrificed and vice versa. The ideal balance between the two is a decision made from the relationship between the severity of the disease and the amount of disease in the population. For the organism chosen in this study, *Escherichia coli O157:H7,* the target for an ideal first line screening test would be ≥ 98% test sensitivity and ≥ 90% or greater test specificity with a false negative rate ≤ 2% and a false positive rate ≤ 10% (USDA MLG, 2002).

Using the AOAC Performance test methods (PTM) validation protocol (AOAC PTM, 2009) the goal for this research is to take the developed components of the M^3 biosensor and validate them, in broth, as a biosensor system. That protocol calls for tests of validity including inclusivity/exclusivity, sensitivity/specificity and false negative/false positive rates as compared to a gold standard, as well as repeatability and limits of detection. The PTM standards also require reliability testing, which is lot to lot stability and variation, and robustness of the established protocol. Since the inclusivity and exclusivity of the electrically active magnetic nanoparticle (EAMNP) extraction are already completed in Chapter 6 of this thesis and the second component the α D+ mannose functionalized screen printed carbon electrode (D-FSPCE)

is a universal detection platform capturing a multitude of bacteria, the inclusivity/exclusivity panel will not be repeated for the whole biosensor. The other PTM standards of validity and reliability will be evaluated.

An electrochemical biosensor is one that converts chemical events occurring in solution or at electrode surface into electrical signals (Palchetti and Mascini 2008). Impedance-based electrochemical biosensors use an analyte that directly impedes current flow in a DC circuit. Label based electrochemical biosensors have the analyte labeled with a compound (e.g., metal or semi-conductive polymer to catalyze redox reaction, which causes a measurable change in electronic signal. Electrochemical biosensors require simple, easily integrated (with electronic readout devices), and are less susceptible to the contamination and environmental challenges of a resource limited setting contaminants than other analytical techniques (Palchetti and Mascini 2008). The M^3 Biosensor uses a semi-conductive polymer coated on the outside of a magnetic iron core to capture and report a combination of electrical conductivity of the polymer coating and the resistive nature of the cellular components captured on the surface (Laczka, 2008; Maalouf, 2008; Varshney, 2007).

Experimental design

To validate the use of the M^3 biosensor to extract and detect microbial targets the following hypotheses were tested:

Hypothesis 1: The M^3 biosensor can selectively and repeatedly extract, concentrate and detect *E. coli* O157:H7 with a limit of detection of less than 10 CFU/mL and a capture efficiency of 90 – 100%, without pre-enrichment through the linear range of detection.

Hypothesis 2: The M^3 biosensor is equal to commercially available IMS separation products and holds the advantage of electrical activity that allows use in a variety of detection modalities.

To test hypothesis 1, 100 µL of broth extracted Mab-EAMNPs- *E. coli* O157:H7 Sakai strain were placed on the D-FSPCE, incubated for 15 minutes, rinsed twice with DI water and allowed to dry. Subsequently, the bacterial challenged D-FSPCE was doped with 100 µL of 0.1 M HCl and evaluated, using cyclic voltammetry, on PalmSens handheld potentiostat (Palm Instruments BV, Houten The Netherlands). Concurrently, a second fraction of 100 µL of broth extracted Mab-EAMNPs- *E. coli* O157:H7 Sakai strain samples were cultured on two plates essentially splitting the final 500 µL IMS extracted solution into three aliquots.

To test hypothesis 2, commercially produced and coated anti- *E. coli* O157:H7 Dynabeads® (Invitrogen, Grand Island, New York) were used to IMS extract *E. coli* O157:H7 Sakai strain. Capture efficiency was calculated and compared to the Mab-EAMNP's capture efficiency through the same range with the same conditions.

Materials and Methods

E. coli O157:H7 strains, *E. coli* non H7 strains and non *E. coli* bacterial strains were obtained from the STEC Center collection at Michigan State University (MSU) (Shannon Manning, MPH, PhD), the Nano-Biosensors Laboratory at MSU (Evangelyn Alocilja, PhD), Neogen Inc. Research and Development, Lansing, Michigan (Jennifer Rice, DVM, PhD) and the University of Georgia, Center for Food Safety (Dr. Michael Doyle, PhD). From frozen purified culture stocks (stored at -80° C), colonies were isolated by streak-plate method on trypticase soy agar (BD Biosciences, MD) plates. A single colony was used to inoculate a vial of tryptic soy

broth (BD Biosciences, MD) and grown overnight at 37° C. A 1 mL aliquot of the liquid culture was transferred to a new vial of broth and stored at 37° C for up to 6 days. This culture was used to inoculate a new vial of broth with 1 mL of inoculum 8 to 10 h before each experiment to produce fresh bacterial cells which were serially diluted in 0.1% (w/v) peptone water (Fluka-Biochemika, Switzerland) prior to their use in the IMS procedure. Viable cells were enumerated by microbial plating on MacConkey agar with sorbitol (SMAC) (BD Biosciences, MD or Neogen Inc., MI), according to standard rules for plate counting (FDA BAM, 2009).

EAMNP Production

Ferric chloride hexahydrate (EMD Chemicals), sodium acetate (CCI Chemicals), sodium acrylate, sodium chloride (Sodium Chloride), ethylene glycol, ethylenediamine, hydrochloric acid, aniline, iron (III) oxide nanopowder, ammonium persulfate, methanol, and diethyl ether were used as received in the synthesis of the EAMNPs. EAMNPs were synthesized by polymerization and acid doping of aniline monomer around gamma iron (III) oxide (γ-Fe_2O_3) nano-particles, using a slightly modified published procedure (Pal, Setterington & Alocilja, 2008). Briefly, 0.650 g of iron (III) oxide nanopowder were dispersed in 50 mL of 1 M HCl, 10 mL of deionized water and 0.4 mL of aniline monomer by sonication in an ice bath for 1 hour. A volume of 20 mL of 0.2 M ammonium persulfate (as oxidant) was added drop-wise to the above solution under continuous magnetic stirring. Color change from rust brown to dark green indicated formation of electrically-active (green) polyaniline over the smaller (brown) γ-Fe_2O_3 nano-particles. The solution was stirred for 2 hours in an ice bath and was filtered through a qualitative grade filter (2.5 μm pore size, Ahlstrom, grade 601). The supernatant thus obtained

was successively filtered through a nitrocellulose membrane filter (1.2 μm pore size, Millipore) followed by washings with 10 mL each of 1 M HCl, 10% (v/v) methanol, and diethyl ether. The particles were dried overnight at room temperature under vacuum. The particles ranged in size from 1.2 to 2.5 μm, and displayed a room temperature saturation magnetization of 30 emu/g.

EAMNP Antibody Conjugation

Nano-particles were immune-functionalized with monoclonal anti-*E. coli* O157:H7 antibodies obtained from Meridian Life Science, Inc. (Saco, ME). Polysorbate-20 (Tween-20), Triton X-100, phosphate buffered saline (PBS), Trizma base, casein, and sodium phosphate (dibasic and monobasic) were used in the IMS procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as follows: PBS buffer (10 mM PBS, pH 7.4), wash buffer (10 mM PBS, pH 7.4, with 0.05% Tween-20 or 0.05% Triton-X100), phosphate buffer (100 mM sodium phosphate, pH 7.4), blocking buffer (100 mM Tris–HCl buffer, pH 7.6, with 0.01% w/v casein). Magnetic separations were performed with a commercial magnetic separator (Promega Corporation, Madison, WI). Hybridization of biological materials was carried out at room temperature with rotation on a tube rotisserie (Labquake, Thermo Scientific, MA).

EAMNPs were conjugated with monoclonal antibodies at an initial EAMNP concentration of 10 mg/mL (1% solid). Conjugation of antibodies onto EAMNPs was by direct physical adsorption and electrostatic interactions. A 100 μL aliquot of monoclonal, anti-*E. coli* O157:H7 antibody (suspended in 0.1M phosphate buffer) was added to EAMNPs suspended in PBS, yielding a final antibody concentration of either 1.0 mg/mL. The mixture was hybridized on a rotisserie-style rotator for 1 hour at room temperature, with 25 μL of 10X PBS being added

after the first 5 min of hybridization, to increase the Sodium Chloride content of the suspension to approximately 0.14 M. Following hybridization, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 μL of blocking buffer (0.1M tris buffer with 0.01% casein) for 5 min. Again the conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 μL of blocking buffer, this time for 1 hour with rotation. Finally, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 2.5 mL of 0.1 M phosphate buffered saline (PBS). The final concentration of EAMNPs in each solution was 1.0 mg/mL. Immuno-conjugated EAMNPs (Mab-EAMNPs) were stored at 4° C. Prior to experimental use, Mab-EAMNPs were further diluted in 0.1M PBS, in order to obtain a solution of Mab-EAMNPs at 0.5 mg/mL.

Immuno-magnetic Separation (IMS) and Plating of Bacteria

Every experiment was applied to three different bacterial species individually: *E. coli* O157:H7 Sakai strain, *E. coli* O157:H7 2006 Spinach strain, pGFPuv (target species), *Shigella boydii* (non-target species). *S. boydii* bears less genotypic and phenotypic similarity to the target organism, but it is a commonly encountered foodborne pathogen, and also produces shiga-toxin like *E. coli* O157:H7. The standard positive control used was *E. coli* O157:H7 spinach 2006 strain with a green ultra violet fluorescent plasmid inserted (*E. coli* O157:H7 2006 Spinach strain, pGFPuv). Serial dilutions of each bacterium were independently prepared in 0.1% (w/v) peptone water, along with subsequent negative, positive, and blank controls. Three or four of the pure dilutions of each bacteria were plated (100-mL aliquots) on sorbitol MacConkey agar (SMAC) and incubated at 37° C overnight. For IMS, 50 mL of Mab-EAMNPs and 50 mL of the

appropriate bacterial dilution were combined with 400 mL of 0.01 M PBS (pH 7.4), and hybridized with rotation at room temperature for 30 minutes. After hybridization, the cell-Mab-EAMNP complexes were magnetically separated and the supernatant removed. Complexes were washed twice in wash buffer (0.01 M PBS containing 0.05% Triton-X100), and finally re-suspended in 0.5 mL of 0.01 M PBS. A 100 mL aliquot was placed on SMAC and incubated at 37° C overnight. The number of colony-forming units (CFU) in the 100 mL aliquot was determined by manually counting the colonies on each plate. Calculation of bacterial cell concentrations in both pure and IMS separated samples were carried out according to rules provided by the United States Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM, 2009).

Immuno-magnetic separation (IMS) was performed on samples with Mab-EAMNPs. A volume of 1 mL of cell culture was pelleted and re-suspended in acridine orange buffer for the spectrophotometer and microscopic evaluations. For the IMS separation, EAMNPs were immune-functionalized with monoclonal anti-*E. coli* O157:H7 antibodies obtained from Meridian Life Science, Inc. (Saco, ME). Polysorbate-20 (Tween-20), Triton X-100, phosphate buffered saline (PBS), Trizma base, casein, and sodium phosphate (dibasic and monobasic) were used in the IMS procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as follows: PBS buffer (10 mM PBS, pH 7.4), wash buffer (10 mM PBS, pH 7.4, with 0.05% Tween-20 or 0.05% Triton-X100), phosphate buffer (100 mM sodium phosphate, pH 7.4), blocking buffer (100 mM Tris–HCl buffer, pH 7.6, with 0.01% w/v casein). Magnetic separations were performed with a commercial magnetic separator (Promega Corporation, Madison, WI). Hybridization of

biological materials was carried out at room temperature with rotation on a tube rotisserie (Labquake, Thermo Scientific, MA). Scanning electron micrographs were acquired using field-emission scanning electron microscopy (JOEL 7500F, acceleration voltage of 5 kV).

Synthesis of aminated α D + Mannose

Production and evaluation of the aminated D + mannose was accomplished in collaboration with Dr. Xuefei Huang's chemistry lab at MSU, using published protocols. Aminated D-mannose was synthesized starting either from the unprotected D-mannose or α-D-mannose pentaacetate, all commercially available. Due to the high price of α-D-mannose pentaacetate, starting with unprotected D-mannose was economically viable. At each step, a self poured silica gel column chromatography was used to purify, a rotovap (Büchi Rotovapor or Büchi Rotovapor R-114, Midwest AOAC Int., St. Louis, MO) was used to concentrate, and thin layer chromatography (TLC) or proton and carbon nuclear magnetic resonance imaging (NMR) (500 MHz, Varian Unity INOVA, Oxford Instruments, Concord, MA) and fourier transform infrared spectroscopy (FTIR) (Perkin Elmer, Shelton, CT) were used to verify products. Procedures reported by (Teumelsan & Huang, 2007) were adopted for the synthesis of α-D-mannose pentaacetate. Briefly α-D-mannose was protected using acetic anhydride in pyridine with catalytic DMAP to achieve α-D-mannose pentaacetate. α-D-mannose pentaacetate was reacted with 3-chloropropanol and $BF_3.OEt_2$ in DCM overnight to give compound 1 shown in figure 7.1. Compound **2** was achieved by reacting compound **1** with sodium azide in DMF at 60 ^{O}C overnight (El-Boubbou, et al., 2007, Joosten, et al., 2004, Teumelsan & Huang, 2007). De-protection of the acetate groups was performed with freshly prepared sodium methoxide and

produced compound **3.** Upon reduction of azide under hydrogenation condition, the final aminated α-D-mannose, compound **4,** was formed.

Functionalization of the SPCE

Chemicals used to functionalize the SPCE include: 2.5 mM glutaraldehyde solution, Citrate Gold Nanoparticles (AuNPs), stock ≈2.4 Au; ~15 nm in diameter**,** α D+ mannose - amine @ 25 µg/mL**,** Deactivating buffer (0.2 M Tris in 0.01 M Phosphate Buffered Saline (PBS) plus 10 mM Cyanoborohydride)**.** Chemicals such as 0.1 M hydrochloric acid (HCl) solution, acridine orange (AO) stain (2 mg of AO into 1 mL of AO buffer = 100x); acridine orange buffer pH 3.8. (90 mL of 10mM (0.01 M) phosphate buffer + 585 mg of NaCl, qs to 100 mL), coupling buffer pH 7.4 (0.1 M phosphate buffer into 900 mL of dH_2O), Con A conjugated to FITC are used elsewhere in the protocol. SPCEs were purchased from Gwent Electronics Materials Ltd, United Kingdom and modified in house. Cyclic voltammetry was performed on the PalmSens handheld potentiostat (Palm Instruments BV, Houten, The Netherlands).

To attach aminated D+ mannose to the SPCE, the rinsed, dried chips were incubated with 25 µL of 2.5 mM glutaraldehyde on the working center of the carbon electrode for two hours at 4°C. At completion, the excess glutaraldehyde was rinsed with DI water and dried. Citrate AuNPs (25 µL) were then incubated on the working center of the carbon electrode for two hours at 4° C. At completion, the excess citrate AuNPs were rinsed with DI water and dried. On the working center of the carbon electrode, 25 µL of aminated D+ mannose (25 µg/mL) was placed for 15 minutes at 21° C. At completion, the excess aminated D+ mannose was rinsed with DI water and dried and 50 µL of deactivating buffer was applied for 15 minutes at 21° C. The

resultant D-FSPCE were dried and stored at room temperature (protected from light). Both a blank SPCE and a aminated D+ mannose modified SPCE are shown in figures 8.1 and 8.2 respectively.

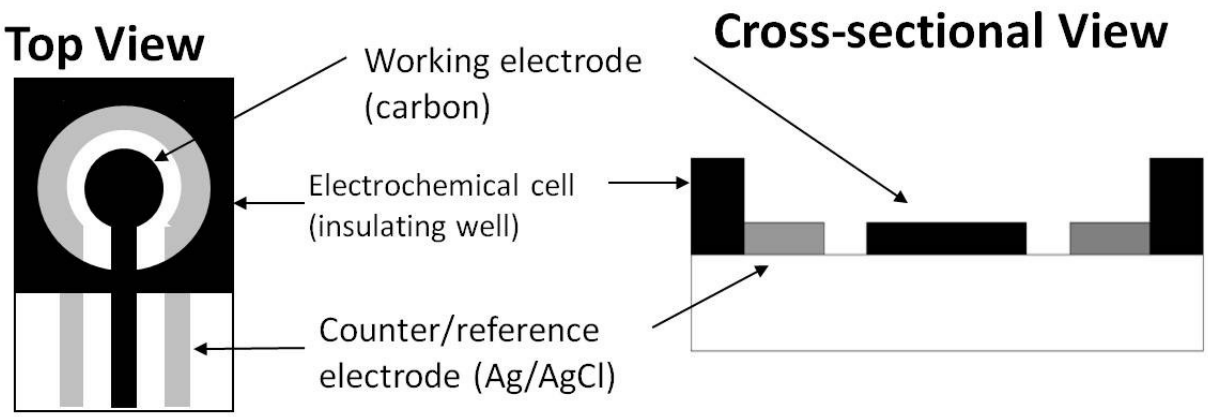

Figure 8.1: Schematic of the bare screen-printed carbon electrode (SPCE) sensor chip the detection is performed on, the circuit.

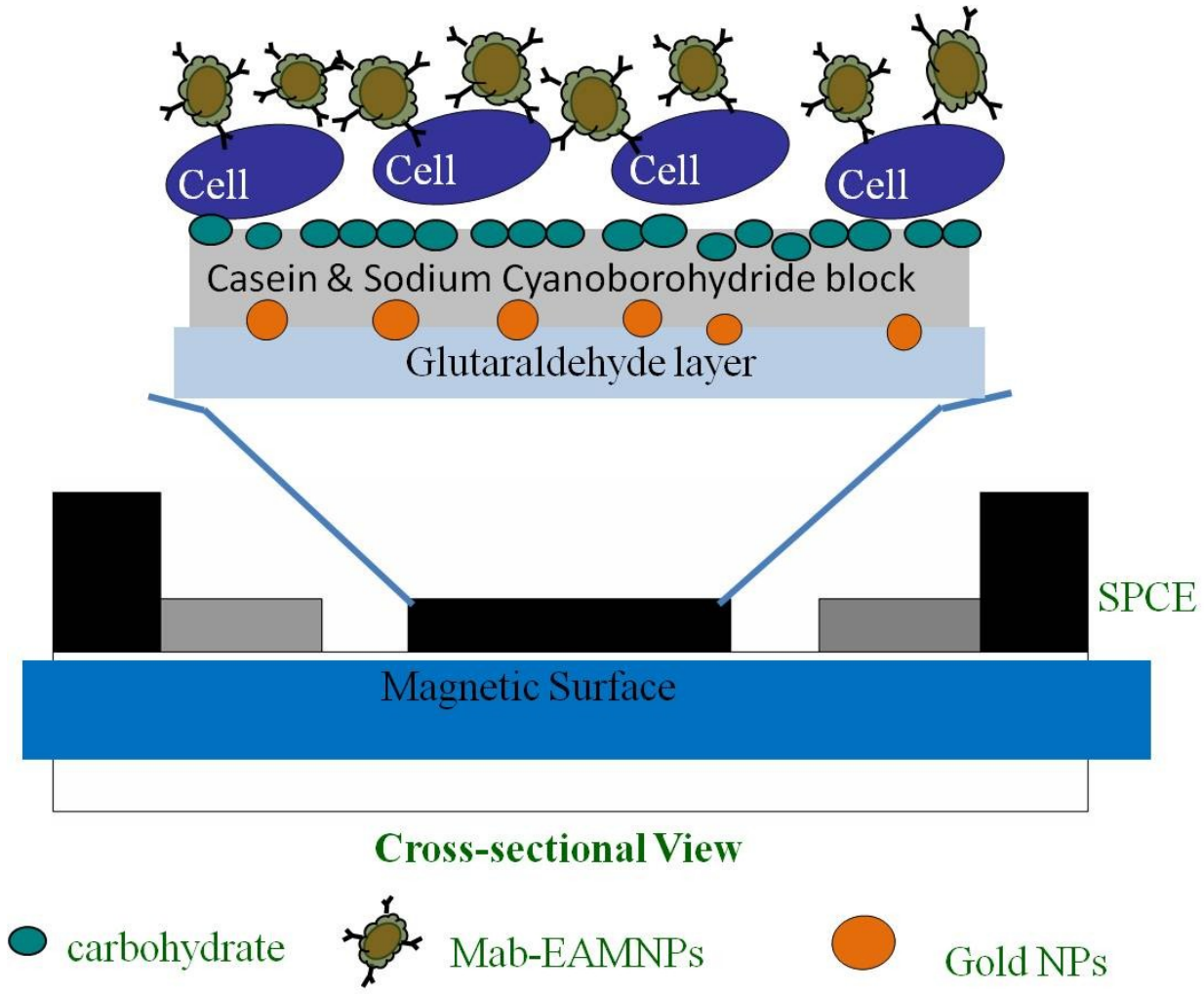

Figure 8.2: Layered schematic of the structure in the novel aminated D+ mannose functionalized screen-printed carbon electrode (D-FSPCE) biosensor chip.

Cyclic Voltammetry evaluation of D-F SPCEs:

Cyclic voltammetry (CV) is an electrochemical method where a potential is scanned from -1.4 V to 2.0 V and the resultant current is measured at each voltage. Varying parameters can be used on the resultant cyclic voltammogram, depending on the reporter used. Here polyaniline is electrically active and, if present, should provide a peak at a certain voltage in both the positive and negative sweep of the current in the cycle. Larger levels of polyaniline should provide

greater peak heights but other components of the system affect the polyaniline peak greatly as well. The current produced at a given potential is the combination of the entire electrochemical system and additional components can reduce the current as side reactions occur (Xu, Leng, Li, Lu, Wanga & Hu 2010; Li, Yang, Zhao & Du, 2011). Due to this, other parameters such as delta Q (ΔQ), peak shift, peak shape, resistance at each point in the cycle, and average resistance over the whole cycle were tabulated and evaluated in this study. Between the differing concentrations and the blank D-FSPCE, all the parameters collected were compared. The XY values of the highest, lowest, and expected polyaniline peaks were evaluated from the voltammogram. The shift positive or negative from the original position of the expected peak in a polyaniline coated D-FSPCE without bacterial cells was evaluated and recorded for each run. The shapes of the characteristic peaks were also evaluated based on their width and tracing. From the collected data of voltage (V) and current (I) at each point on the voltammogram, the absolute resistance (R) was calculated from standard Equation 8.1, solved to Equation 8.2 and the result placed into Equation 8.3. The ΔQ was calculated from the standard Equation 8.4 solved to Equation 8.5 and the result placed into Equation 8.6.

Equation 8.1: Ohm's Law used to calculate the electronic behavior in the circuit of each point taken during the cyclic voltammogram

$$V = IR$$

Equation 8.2: Solving of Ohm's Law to calculate the resistance at each point on the cyclic voltammogram.

$$R = \frac{V}{I}$$

Equation 8.3: Total average resistance across all points on three consecutive cyclic voltammograms.

$$\sum R = \frac{V}{I} \Big/ \#\, pts$$

Equation 8.4: The change in the rate of current transfer across the circuit at each point taken during the cyclic voltammogram.

$$I = \frac{\Delta Q}{\Delta t}$$

Equation 8.5: Solving the change in the rate of current transfer across the circuit for the unknown Δ QT.

$$\Delta Q = I\,\Delta t$$

Equation 8.6: Total average change of rate of current transfer (ΔQ) across three consecutive cyclic voltammograms.

$$Average\ \Delta Q\ =\ [\sum \Delta Q]/n\ scans$$

Four scans of each D-FSPCE were conducted and the first scan was discarded as a system equilibration set. The last three provided at least 960 data points apiece across the cycle. For each of these 2880 individual points the absolute resistance was calculated. The average resistance at all points in the three curves were averaged together to get the single average resistance across the system for each run at each concentration of bacteria. The same was repeated for the ΔQ values of each scan. The signal to noise ratio was calculated by averaging each category of cell concentration's final resistance value divided by the same treatment for the set of blank evaluations.

In addition to the above experimental design, to evaluate hypothesis 1, capture efficiency was calculated according to equation 8.7.

Equation 8.7: The calculation for capture efficiency of the EAMNPs in Milk. Equivalent to the spiked levels of available bacteria divided by the amount of bacteria captured after IMS.

$$Capture\ Efficiency\ (\%) = \left(log10\frac{CFU}{mL}start\right)/(log10\frac{CFU}{mL})\ IMS)$$

Statistical Analysis

Independent two-tailed student's T-tests were performed on the calculated parameters of each concentration of Mab-EAMNP - *E. coli* O157:H7 Sakai strain on the D-FSPCE compared to the same parameters of the blank D-SPCE run through the same IMS extraction with the addition of sterile peptone water instead of bacterial culture in TSB. ($\alpha = 0.05$) Percent positive calculations were performed and were used to determine potential cut points as a cut off values for determination of a food sample to be positive or negative in the next phase of development. Signal to noise ratios were calculated at each range of cell concentration by dividing the total signal of the spiked samples by the total signal of the blanks.

Results

Hypothesis 1:

The M^3 biosensor can be used to selectively and repeatedly extract, concentrate and detect *E. coli* O157:H7 1.0 to 1.0 * 10^9 CFU/mL of *E. coli* O157:H7 in broth samples, without a pre-enrichment, at a limit of detection of less than 10 CFU/mL and a capture efficiency of 90 – 100% through the linear range of detection.

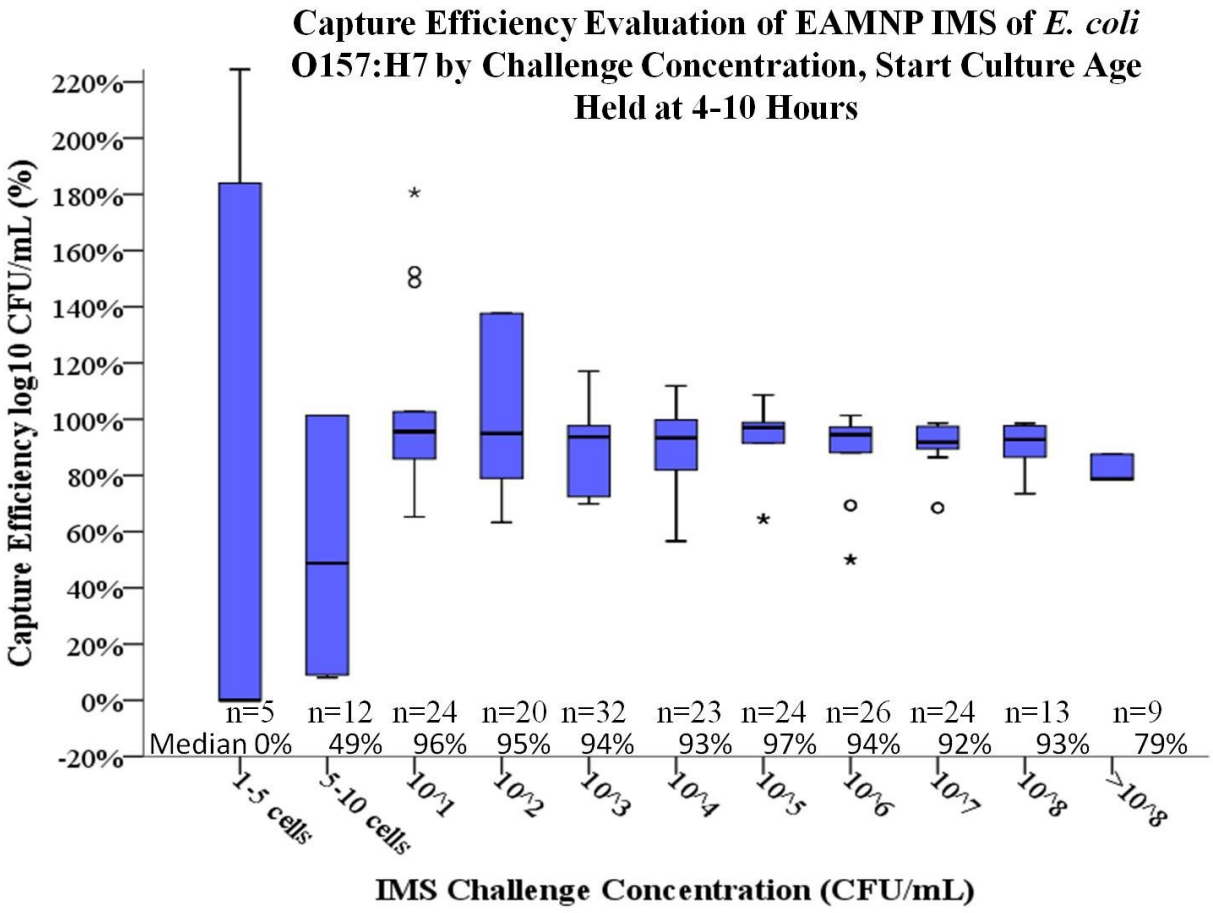

Figure 8.3: A quantitative dose based evaluation of capture efficiency vs IMS challanged concentrations of the M^3 Biosensor extracted and captured *E. coli* O157:H7 Sakai strain, in broth. Capture efficiency is steady from 10 cells to $1 * 10^8$ CFU/mL. with an overall mean value of 92% and an overall median value of 93%. All 213 sample cultures used were in the near optimum range of 3 to 10 hours after innoculation before serial dilution and testing.

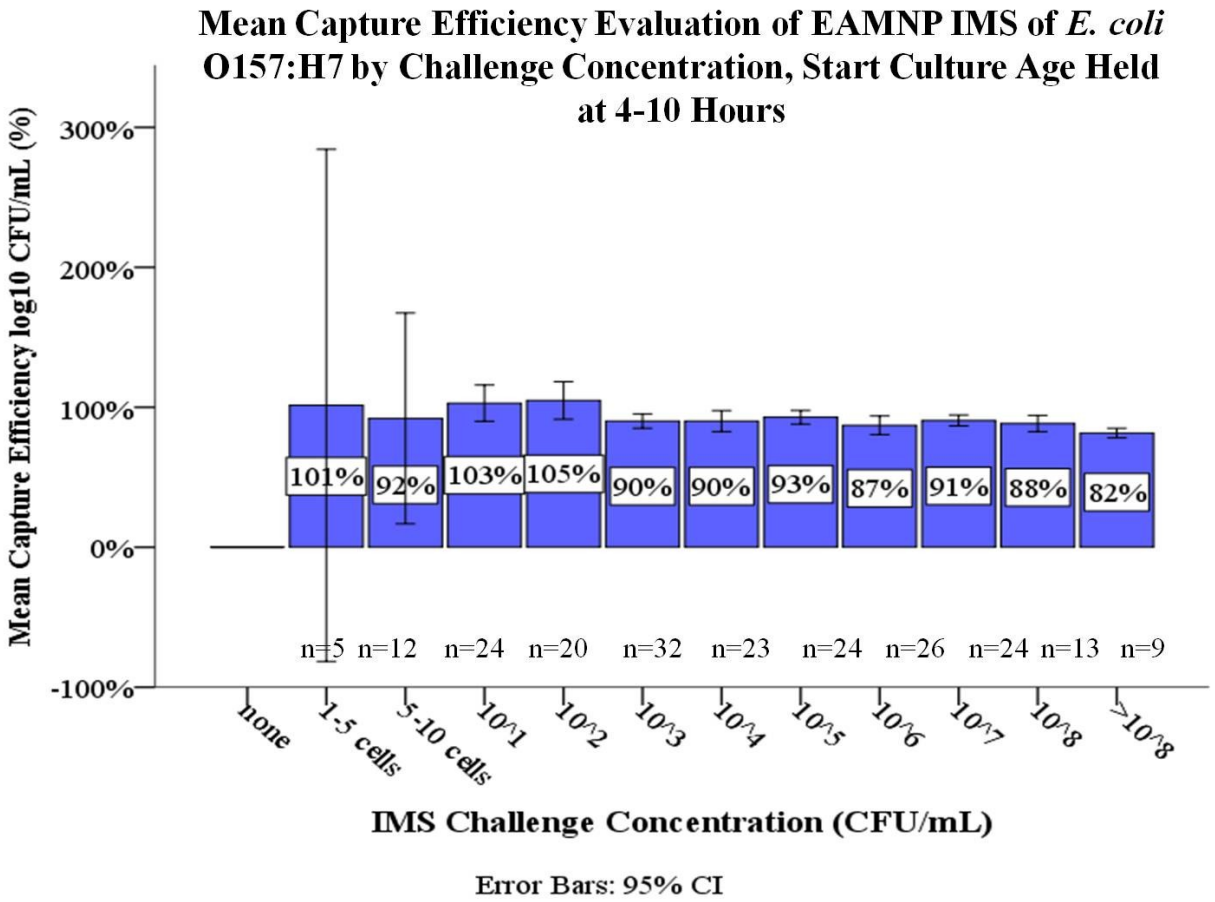

Figure 8.4: A quantitative dose based evaluation of mean capture efficiency vs IMS challanged concentrations of the M^3 Biosensor extracted and captured *E. coli* O157:H7 Sakai strain, in broth. Capture efficiency is steady from 10 cells to $1 * 10^8$ CFU/mL. with an overall mean value of 92% and an overall median value of 93%. All 213 sample cultures used were in the near optimum range of 3 to 10 hours after innoculation before serial dilution and testing.

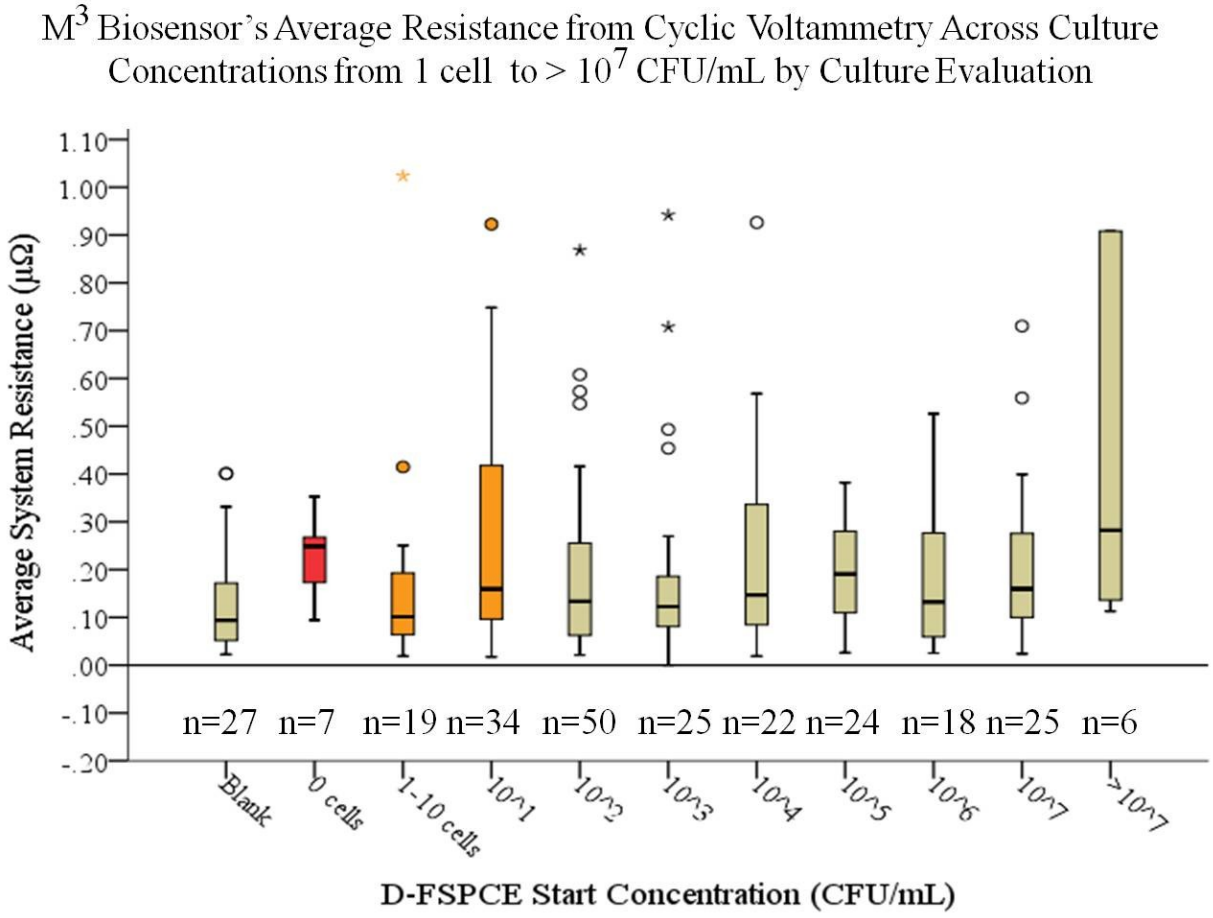

Figure 8.5: A quantitative dose based evaluation of average resistance vs $\log_{10}$ CFU/mL challenge concentrations on M^3 Biosensor captured bacteria, in broth. NOTE: "0 cells" (red) are concentrations that were at the limit of detection and although innoculated, did not grow cells. "1-100 cells" (orange) are below the consistent culture range reccommended by the FDA BAM (2009). All 257 sample cultures used were in the near optimum range of 3 to 10 hours after innoculation before serial dilution and testing.

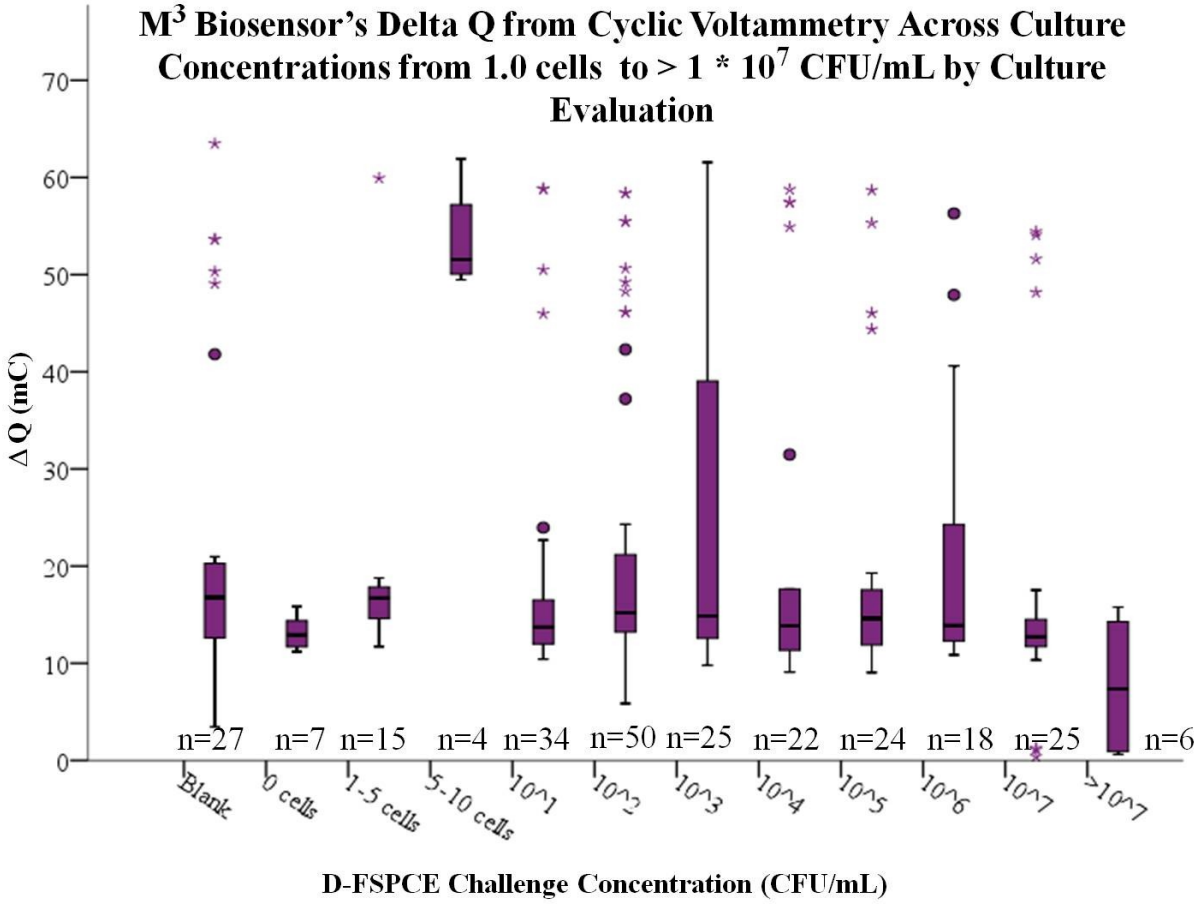

Figure 8.6: A quantitative dose based evaluation of Δ Q (mC) vs $\log_{10}$ CFU/mL challenge concentrations on M^3 Biosensor captured bacteria, in broth. All 257 sample cultures used were in the near optimum range of 3 to 10 hours after innoculation before serial dilution and testing.

Table 8.1: Average resistance using cyclic voltammetry on M^3 Biosensor on captured *E. coli* O157:H7 Sakai, in broth. Descriptive statistics and independent T tests for blank verses each category of bacterial concentration. Italic show statistically significant comparisons.

Average Resistance on the D-FSPCE by Challenge [ ] (log_{10} CFU/mL)	N	Mean	Median	Std. Deviation	Std. Error of Mean	T-test Blank vs. Cell [ ] $\alpha = 0.05$
Blank	27	.14	.09	.11	.02	N/A
0 cells	7	.23	.25	.08	.03	0.032
1-5 cells	15	.15	.10	.10	.03	0.433
5-10 cells	4	.28	.04	.49	.25	0.035
10^1	34	.24	.16	.22	.04	0.025
10^2	50	.23	.13	.31	.04	0.072
10^3	25	.23	.12	.31	.06	0.064
10^4	22	.34	.15	.59	.13	0.043
10^5	24	.19	.19	.11	.02	0.046
10^6	18	.18	.13	.14	.03	0.120
10^7	25	.21	.16	.17	.03	0.021
>10^7	6	1.48	.28	2.79	1.14	0.006
10^1 to 10^7	224					**0.047**
Total	257	.25	.15	.51	.03	

To evaluate the consistency of the results several parameters were used. Table 8.1 shows the descriptive statistics and the results of the student's T-tests for between group analyses. Each grouping of culture concentration was compared to the blank test category for the student's T

test. To determine an adequate cut point for subsequent analysis in food matrices, a "test positive cutoff" had to be calculated. Three values, the average resistance of the cyclic voltammograms at 0.1 μΩ, 0.14 μΩ, and 0.16 μΩ were evaluated as cut points. These correspond to the mean, median and a third arbitrarily higher resistance value of the blank category. As shown in figure 8.7, the percent positive at each range was changed dramatically with each adjustment in μΩ. Also determined and shown in figure 8.8 is the signal to noise ratio of the electrochemical analysis.

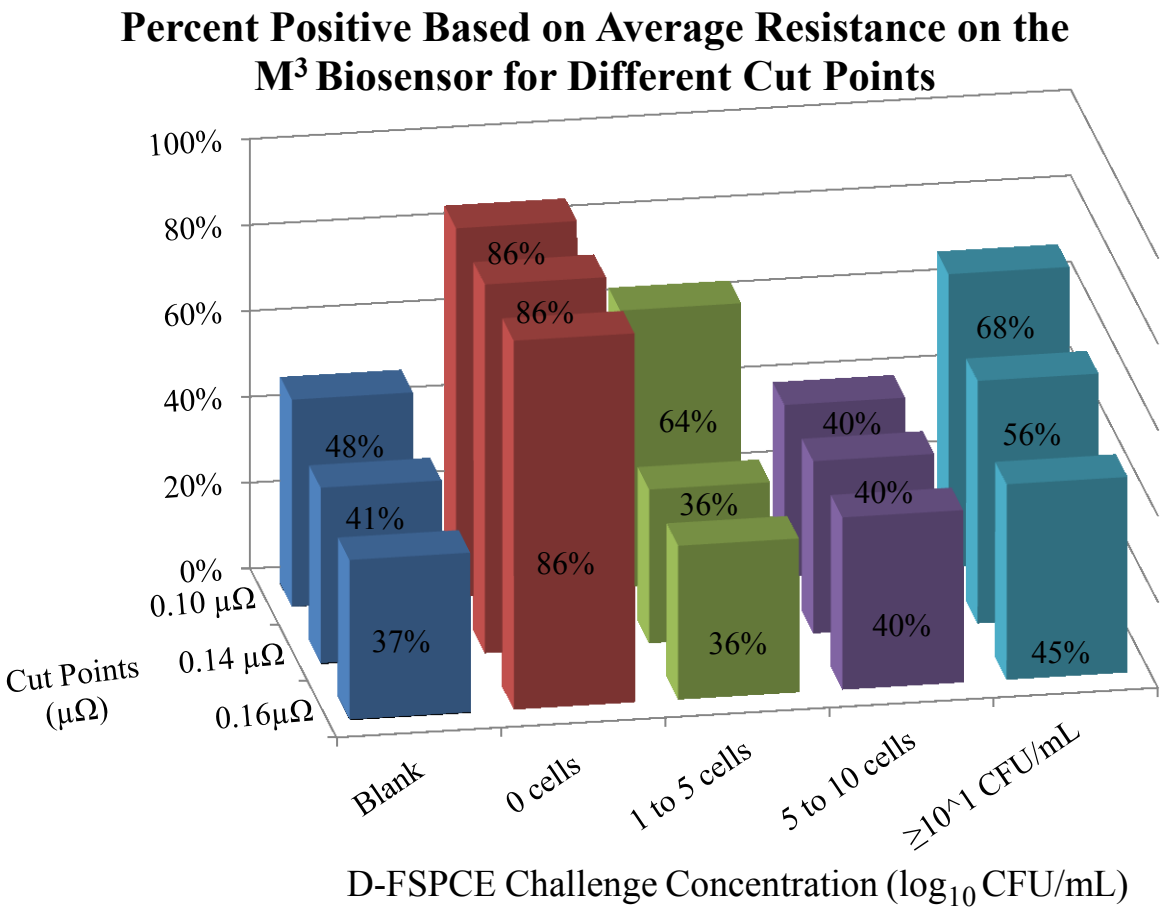

Figure 8.7: The percent of tests that would be considered positive when different cut points were selected in µΩ, by culture verified concentration challenged onto the M^3 Biosensor. The cut points selected correspond to the mean, median and an arbitrarily higher average resistance value of the blank category dataset. As the cut value decreases the percent considered positive at each concentration increases including the blank.

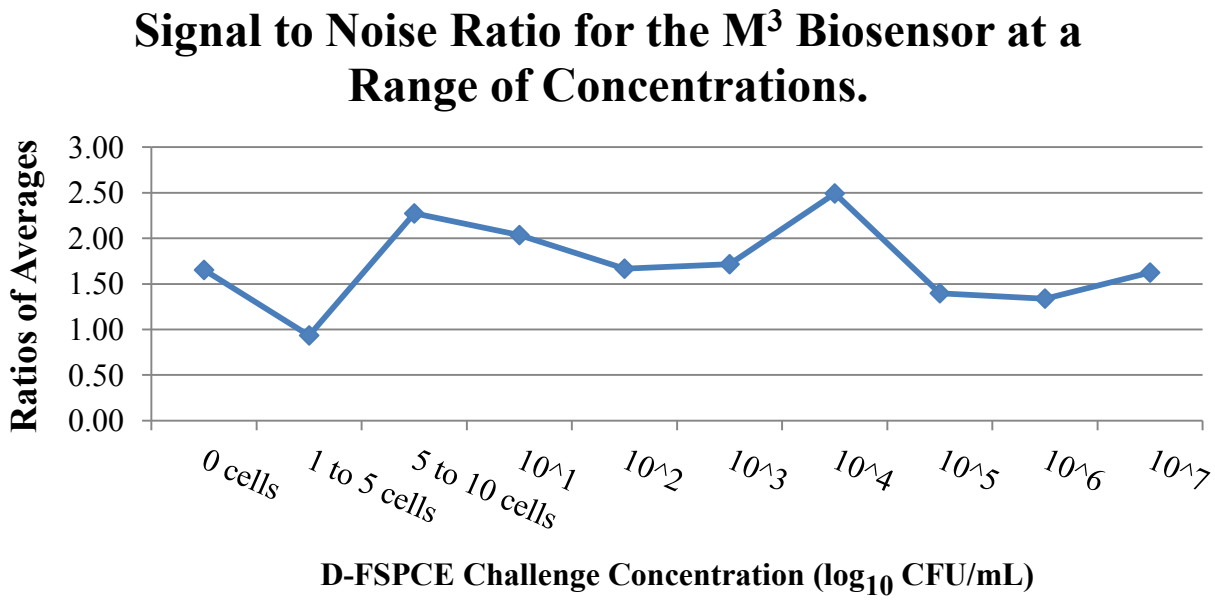

Figure 8.8: Electrochemical signal to noise ratio verses culture verified concentration challenged onto the M^3 Biosensor platform. Signal by culture concentration grouping was averaged and compared to the average of all the blank runs.

Hypothesis 2*:*

The M^3 biosensor is equal to commercially available IMS separation products and holds the advantage additional electrical activity for use in a variety of detection modalities. As shown in figure 8.9, capture efficiency of the commercial IMS technology had a median value of 86% compared to the median capture efficiency of the EAMNPs at 93% (shown in figure 8.3) using identical culture handling methodolgy.

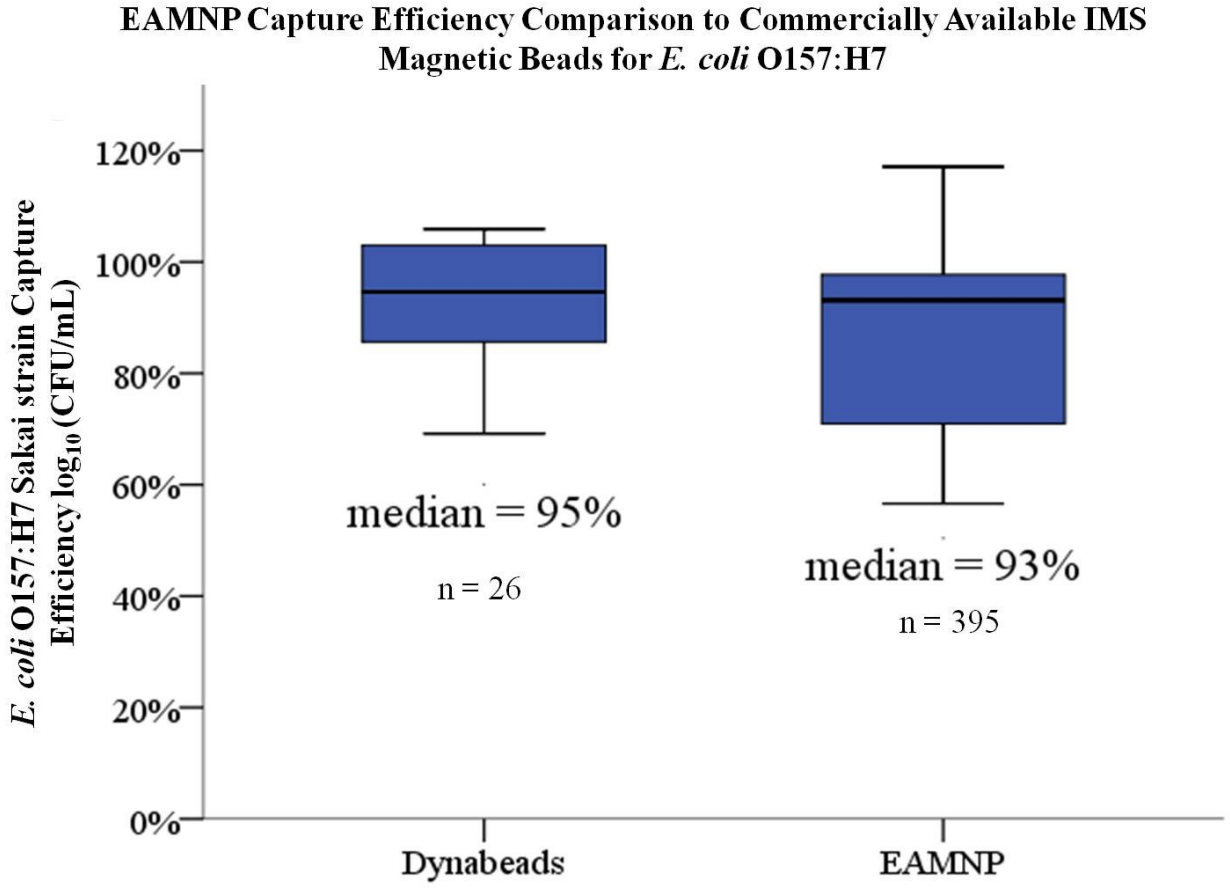

Figure 8.9: A quantitative evaluation of of the capture efficiency of IMS beads for bacterial concentration ranges of 10^2 and 10^7 CFU/mL. Dynabeads® extracted and captured *E. coli* O157:H7 Sakai strain, in broth and EAMNP extracted and captured *E. coli* O157:H7 Sakai strain, in broth are compared . Capture efficiency of the commercial IMS technology had a median value of 95% compared to 93% for EAMNPs. These were not significantly different.

Discussion

Throughout this entire analysis the M^3 Biosensor has been challanged through a range of bacterial challenge concentrations at both the IMS extraction level and at the electrochemical

level. No matter how robust the test, three distinct trends are seen. First, the M^3 Biosensor seems to be more sensitive than culture. Culture is inaccurate for enumeration below 100 CFU/mL due to sampling error (FDA BAM, 2009). Since culture was considered the gold standard, used as the verification tool and as the dividing factor into the categories for analysis, this introduces error into the system. The greatest evidence of this is figure 8.5 where the category of 0 cells grown are higher than the blank for their system resistance. These samples were at the dilute to extinction limit and could be truly negative for organisms. With the current analysis, that is impossible to know. The categories of 1 to 5 cells and 5-10 cells are also less reliable. EAMNPs do concentrate cells out of solution and therefore the actual concentration of these three categories is unknown at this time. One suggestion for evaluation of this low concentration is to pre-enrich the fraction of each sample that would have been plated day 1 and subsequently plate it after the enrichment. Capture efficiency calculations would not be possible, but a true evaluation of the presence or absence of bacteria in the solution that was placed on the biosensor would be.

The second trend is that the base electrical system, the D-FSPCE platform, still has too much variablitiy inherent in the current design to reach the kind of analytical level necessary for comercialization. Biological systems are by nature inexact because bacteria do not behave like elements or chemical mixtures. They are not homogeneous, they move, clump, attach and become viable but not cultureable without concentration gradients. Even so, in a system with a signal to noise ratio of only 1.5 to 2 over most concentration ranges needs to have a more precise base than the current sensor has. Optimization of the D-FSPCE was not performed. Exact concentrations of gold nanoparticles added to the SPCE surface in a stepwise fashion, are necessary to determine the optimum amount of gold conductance necessary to create consistently

low resistance D-FSPCE for comparison to the resisted cell challenged chips. Polyaniline is not a perfect redox reagent. The amount, character and electrical conductance of this semiconducting polymer are somewhat different for every run of this sensor depending on how many EAMNPs attach to each bacteria, how many bacteria there are and the consistancy and coating of each EAMNP particle. If elemental iron is showing, it will add to the conductivity of the system. Polyaniline changes it conductance not only in the microenvironment, but also by room temperature. Serial connections cause increases in noise in an electrochemical system. Using alligator clips to connect to the SPCE creates two connections before the potentiostat reciever and increases the noise measure from run to run. All of these proposed changes will only increase the abiltiy of an already effective biosensor system to reduce noise and subsequently the margin of error from sample to sample. Designing a box to perform the test analysis inside of, inline connections, optimizing the EAMNP coating of the IMS beads and optimizing the D-FSPCE to minimize variability are necessary next steps.

The third trend is that the M^3 Biosensor has shown consistent ability to show a presence/absence test for bacterial presence consistently across a wide range of bacterial concentrations. The M^3 Biosensor has shown a mean capture efficiency at 92% down to 5 CFU/mL, and equivilancy to a widely used commercial IMS methodolgy. The M^3 Biosensor has shown electrical chemical detection with statistically significant differences as low as 5 CFU/mL and a signal to noise ratio of 2:1. Its linear range of detection at 5 CFU/mL to $1.0 * 10^7$ CFU/mL for both IMS analysis and CV analysis is excellent performance through over 200 repeat analyses.

Conclusions and Limitations

Limitations of this extraction method include the fact that both viable and non-viable cells are extracted with this methodology. Further studies are designed and being implemented to evaluate the Mab-EAMNP to determine the reaction kinetics of non-viable verses viable cells on the antibody target regions of the chip and the IMS bead, in broth cultures. Optimization of the biosensor platform experiments and the polyaniline coating of the IMS beads are necessary before validation trials of the whole biosensor can proceed. The ultimate goal of this extraction is to be able to multiplex many EAMNPs with different Mab targets to allow multiplexing. Future multiplexing with multiple EAMNP and multiple bacterial targets could have interactions between the EAMNPs or between the mixed antibodies. Certain matrices may remove the Mab from the surface of the EAMNPs and make their use in that matrix impossible. The largest drawback to this method is the need for refrigeration of the Mab-EAMNPs. When field based technologies are discussed, shelf stable reagents are an advantage. This sensor is designed as a presence absence, screening test for the field. Cut values chosen are based on the goals of the detection. For *E. coli* O157:H7, with a zero tolerance federal limit, missing a case is the scenario to avoid. In that instance, choosing a cut value of 0.1 μΩ would ensure less would be missed at the price of more false positives.

Repeating these analysis with optimum culture ages and optimized EAMNPs and D-FSPCE designs with minimal connections between the chip and the potentiostat will produce a highly effective biosensor that can be moved into validation in food matrices. Its shelf stable, multiplex sensor platform and highly effective IMS extraction components can be used in a variety of clinical diagnostic and food diagnostic field based arenas. Other future work is to

validate in milk, ground beef and leafy greens as well as apply for Performance Tested Method (PTM) certification from the Association of Official Analytical Chemists (AOAC).

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CHAPTER 9: Proof of Concept for the M^3 Biosensor in Whole Fluid Milk

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Abstract

Food defense requires the means to efficiently screen large volumes of food for microbial pathogens. Even rapid detection methods often require lengthy enrichment steps, making them impractical for this application. There is a great need for rapid, sensitive, specific, and inexpensive methods for extracting and concentrating microbial pathogens from food. In this work repetitive challenges to the M^3 Biosensor have shown reliable evidence for continued refinement of this sensor. The M^3 Biosensor has shown consistent ability to show a presence/absence for bacterial presence consistently across a wide range on bacterial concentrations. M^3 Biosensor can capture and detect bacterial cells down to 1-4 MPN/mL from 200 mL of whole fluid milk in 1 hour. In previous work, the microbiological inclusivity and exclusivity of the EAMNP IMS method was evaluated against 35 *E. coli* O157 strains and 29 other organisms many in the Enterobacteriacea family. The extraction protocol's inclusivity within strain is 94% and exclusivity outside the *E. coli* O157 family is 87%. Additionally, thirty nine organisms of 10 bacterial genera in both gram stain groupings all attached to the carbohydrate coated D-FSPCE, allowing for this platform to be used with many other organisms. This shelf stable, multiplex detection platform can be used combined with any selective extraction modality and any electrical evaluation. The cost of producing one sample volume of EAMNPs conjugated with anti-*Escherichia coli* O157:H7 is ~ $0.43. The M^3 Biosensor shows excellent progress toward a field ready bacterial food detection tool with excellent multiplex potential.

Introduction

Food defense, food protection, and food safety are separate concepts that are all interrelated. Food defense (securing food sources against malicious biological attack) food protection (prevention of food fraud) and food safety (identifying and eradicating contamination from natural sources) (Spink, 2009b, CENS, 2008) are growing increasingly relevant as the global nature of the food supply has several inherent difficulties to its monitoring. The most daunting deterrence to use of CBRN terrorism in the food is the *technical knowledge* necessary. Knowledge of food and the microbes in food, the equipment to propagate the microbes, the volume of organisms necessary to pull off a widespread attack and access to the organism with the appropriate dissemination platform are all large deterrents. Having stated that, it is worthy to note that scientific knowledge across the world is increasing and access to it via the internet is tremendous (Garrett, 2001). This may lead to increased availability of the necessary knowledge to put together a food based CBRN attack. Most food based attacks listed in terrorism incident lists, or anywhere else, are on a very small scale such as individual attacks on coworkers, relatives or employer's interests (Dalziel, 2009). These facts demonstrate the need to find an adequate service level, farm, or field based method to prevention of intentional attacks while improving our ability to find unintentional contaminations.

Food itself is an impediment to large scale contamination and testing. The chemicals, natural and synthetic, compounds and bulk make extraction and detection of agents difficult but also make proliferation from a point source to multiple exposure points difficult as well. Dilution and agent death are significant protectors (Adams & Moss, 2008). The bulk of food and the non-uniform distributions of organisms in food make consistent attack exposures difficult to predict. Fat and other biological components of food have inhibitors for chemical and biological

growth and sustainment without the existence of temperature abuse or inadequate cooking on the consumer's end. This distribution hurdle makes the consistency and predictability of each attack's results and thus the terrorist's risk assessment for success more difficult, but it also makes designing a food based diagnostic test difficult.. Many food based evaluations of agent behavior in different food matrices are not known to the general scientific community and relate to the issues of diagnostic test development. All of these food based deterrents to terrorism are the same when designing diagnostic tests to detect bacterial contamination in food, no matter what the cause.

The immune-magnetic separation (IMS) extraction technique described here combined with the self stable, multiplex ready, detection platform seek to overcome the challenges listed above. Electrically active magnetic nanoparticles (EAMNPs) conjugated to a monoclonal antibody (Mab) extract out of a food matrix and their electrical activity allow them to be used directly on a D+ mannose coated screen printed carbon electrode (D-FSPCE) in a real time cyclic voltammetry, electrochemical evaluation. Removing the matrix interference assists in detection of the low levels of contamination necessary for organisms like *E. coli* O157:H7 with a median infectious dose of 23 CFU/mL (FSIS, 2001). This biosensor has been named the M^3 Biosensor. It was validated in broth and is challenged here in whole fluid milk. To ensure a true epidemiological sensitivity and specificity could be calculated the IMS extracted milk cultures were run in parallel with concurrent portions of the same spiked milk against the USFDA BAM method for the extraction of STEC *E. coli* O157:H7 (FDA BAM, 2009; Chapter 4a). Four items can be evaluated with this study design. They include: one the ability to concentrate and culture *E. coli* O157:H7 from a large volume of whole fluid milk samples at low levels of contamination without pre-enrichment; two the epidemiological sensitivity and specificity in whole fluid milk

with culture, M^3 Biosensor and PCR as the measurement tools: three the limits of detection in whole fluid milk; and four the ability of the resultant IMS extracted solution to be taken to PCR as the diagnostic tool. Most evaluation tools are unable to detect small amounts of bacterial cells in large volumes of fluid due to dilution, that's why the FDA BAM method uses pre-enrichment. The goal of this biosensor is to operate in the fractional recovery arena of 1 CFU in 200 mL of product without a pre-enrichment on a field stable, multiplex ready platform.

Experimental Design

Hypothesis 1: Mab-EAMNP extraction can be used as a precursor to cyclic voltammetry on a Screen Printed Carbon Electrode (SPCE), in milk, to detect *E. coli* O157:H7 with a limit of detection of less than 10 CFU/mL and a high capture efficiency of 90 – 100%, without pre-enrichment, as evaluated by culture. This hypothesis was divided into four sub-hypothesis for evaluation.

Hypothesis 1a: Mab-EAMNPs can extract and concentrate 1-10 CFU/mL of *E. coli* O157:H7 in 200 mL whole fluid milk samples without pre-enrichment.

Hypothesis 1b: Mab-EAMNP whole fluid milk extraction has a high sensitivity and high specificity when compared to the FDA BAM extraction protocol for *E. coli* O157:H7 in whole fluid milk samples, without pre-enrichment, as evaluated by culture.

Hypothesis 1c: Mab-EAMNP whole fluid milk extracted samples can be placed into the JBAIDS RT-PCR or other PCR methodology for verification of genetic identity.

Hypothesis 1d: Aminated D+ mannose can be used as a novel multiplex capable biosensor detection platform for detection of 1-10 CFU/mL of *E. coli* O157:H7 in 200 mL whole fluid milk samples without a pre-enrichment after a selective Mab-EAMNP extraction and concentration procedure.

To evaluate hypothesis 1a-c, a study was designed using a bulk tank of pasteurized liquid whole milk, off the shelf, to run 20 portions on the EAMNPs IMS extraction, 20 portions in the FDA gold standard method and concurrent 3 or 5 tube Most Probable Number (MPN) analysis. Scale up of the broth protocol from 500 µL to 200 mL included creating a larger magnet for IMS separation, creating a large volume rotator system for the incubation and increasing the time of incubation. The same volume and concentration of EAMNPs used in the 500 µL samples in broth (50µL of 0.5 mg/mL Mab-EAMNP) was used in 200 mL of whole fluid milk.

To test hypothesis 1d, 100 µL of Mab-EAMNPs- *E. coli* O157:H7 Sakai strain from milk trial samples were placed on the D-FSPCE, incubated for 15 minutes and rinsed twice with DI water. They were allowed to dry, doped with 100 µL of 0.1 M HCl and evaluated, using cyclic voltammetry, on PalmSens handheld potentiostat.

In addition, due to logistical issues beyond the researcher's control, the milk evaluations presented here are divided into three distinct entities. These entities, experiments 1-3, each have similar logistical problems within the group that prevent them from being evaluated together. The results and discussion sections are divided by experiment.

Materials and methods

Bacterial culture preparation

E. coli O157:H7 strains, *E. coli* non H7 strains and non *E. coli* bacterial strains were obtained from the STEC Center collection at Michigan State University (MSU) (Shannon Manning, MPH, PhD), the Nano-Biosensors Laboratory at MSU (Evangelyn Alocilja, PhD), Neogen Inc. Research and Development, Lansing, Michigan (Jennifer Rice, DVM, PhD) and the University of Georgia, Center for Food Safety (Dr. Michael Doyle, PhD). From frozen purified

culture stocks (stored at -80° C), colonies were isolated by streak-plate method on trypticase soy agar (BD Biosciences, MD) plates. A single colony was used to inoculate a vial of tryptic soy broth (BD Biosciences, MD) and grown overnight at 37° C. A 1 mL aliquot of the liquid culture was transferred to a new vial of broth and stored at 37° C for up to 6 days. This culture was used to inoculate a new vial of broth with 1 mL of inoculum 4 to 6 h before each experiment to produce fresh bacterial cells. Viable cells were enumerated by microbial plating specific to the methodology discussed below.

Every experiment was applied to three different bacterial species individually: *E. coli* O157:H7 (target species), *Shigella boydii* (both non-target species). *S. boydii* bears less genotypic and phenotypic similarity to the target organism, but it is a commonly encountered foodborne pathogen, and also produces shiga-toxin like *E. coli* O157:H7. The standard positive control used was *E. coli* O157:H7 spinach 2006 strain with a green ultra violet fluorescent plasmid inserted. Its ability to fluoresce green in the presence of UV light provided confirmation that the positive samples were not cross contaminated from the positive control. The non-target organisms chosen for this study correspond with the recommendations made by the AOAC Task Force on Best Practices in Microbiological Methodology (2006) and the FDA BAM (2009).

Milk Sample preparation

A bulk tank of whole fluid milk was spiked with a single concentration of *E. coli* O157:H7 and stored at 4°C for 48 hours to mimic environmental samples. According to the FDA BAM (2009), standard whole fluid milk sample sizes are 200 mL. Since the IMS method uses up the entire 200 mL sample during the concentration step, duplicate analysis of the same sample volume could not be performed. Due to this, AOAC Performance Test Method

Validation protocols require 5-15 out of the 20 portions to be positive for the sample to be positive (AOAC PTM, 2009). These sets of forty 200 mL inoculations with their controls are termed "Runs" for this analysis, 20 for the FDA method and 20 for the M^3 Biosensor method. They are numbered for reference from the first trial to the last trial and any n value discussed refers to the whole run unless designated as a portion number.

Four additional 200 mL samples, removed from the bulk tank prior to inoculation, per run, were inoculated with positive and negative control species with one used as a blank and one used as an unspiked control. For the positive control, *E. coli* O157:H7 Spinach 2006 pGFPuv and negative control *Shigella boydii,* 10 µL of stock culture solution were inoculated into the individual 200 mL sample bottles. This means the controls were spiked at high concentrations of 10^6 or 10^7 combined with the additional competitive inhibition of the normal flora in pasteurized milk. The third additional 200 mL sample per run was placed in a thin layer in the Biological Safety cabinet under UV light for one hour or X-rayed in Rainbow II Xray treatment machine (Rainbowfresh foods, Ann Arbor MI) at 70 KVP/150mA for 1 hour to further pasteurize the sample and designated it as the blank. The last 200 mL portion was designated as an unspiked milk control and processed through the M^3 Biosensor method. This was not repeated for the FDA BAM methodology. Remaining spiked milk from the bulk tank was used to inoculate the most probable number dilutions and store a backup portion from each run.

The total volume of spiked fluid whole milk was 9.6 L. This 9.6 L sample was inoculated with the appropriate volume of a 4-6 hour TSB broth, pure culture of *E. coli* O157:H7 Sakai strain to obtain differing target start concentrations of the target organism based off of equation 9.2 described in the most probable number methods section.

EAMNP production

Ferric chloride hexahydrate (EMD Chemicals, Bedford, MA), sodium acetate (CCI Chemicals, Vernon, CA), sodium acrylate, sodium chloride (NaCl), ethylene glycol, ethylenediamine, hydrochloric acid, aniline, iron (III) oxide nanopowder, ammonium persulfate, methanol, and diethyl ether were used as received from Sigma Aldrich (St. Louis, MO) in the synthesis of the EAMNPs. EAMNPs were synthesized by polymerization and acid doping of aniline monomer around gamma iron (III) oxide (γ-Fe_2O_3) nano-particles, using a slightly modified published procedure (Pal, Setterington & Alocilja, 2008). Briefly, 0.650 g of iron (III) oxide nanopowder were dispersed in 50 mL of 1 M HCl, 10 mL of deionized water and 0.4 mL of aniline monomer by sonication in an ice bath for 1 hour. A volume of 20 mL of 0.2 M ammonium persulfate (as oxidant) was added drop-wise to the above solution under continuous magnetic stirring. Color change from rust brown to dark green indicated formation of electrically-active (green) polyaniline over the smaller (brown) γ-Fe_2O_3 nano-particles. The solution was stirred for 2 hours in an ice bath and was filtered through a qualitative grade filter (2.5 μm pore size, Ahlstrom, grade 601). The supernatant thus obtained was successively filtered through a nitrocellulose membrane filter (1.2 μm pore size, Millipore) followed by washings with 10 mL each of 1M HCl, 10% (v/v) methanol, and diethyl ether. The particles were dried overnight at room temperature under vacuum. The particles ranged in size from 1.2 to 2.5 μm, and displayed a room temperature saturation magnetization of 30 emu/g.

EAMNP Antibody Conjugation

Nano-particles were immune-functionalized with monoclonal anti-*E. coli* O157:H7 antibodies obtained from Meridian Life Science, Inc. (Saco, ME). Polysorbate-20 (Tween-20),

Triton X-100, phosphate buffered saline (PBS), Trizma base, casein, and sodium phosphate (dibasic and monobasic) were used in the IMS procedure. All of the above reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). All solutions and buffers used in this study were prepared in de-ionized (DI) water (from Millipore Direct-Q system) as follows: PBS buffer (10 mM PBS, pH 7.4), wash buffer (10 mM PBS, pH 7.4, with 0.05% Tween-20 or 0.05% Triton-X100), phosphate buffer (100 mM sodium phosphate, pH 7.4), blocking buffer (100mM Tris–HCl buffer, pH 7.6, with 0.01% w/v casein). Magnetic separations were performed with a commercial magnetic separator (Promega Corporation, Madison, WI). Hybridization of biological materials was carried out at room temperature with rotation on a tube rotisserie (Labquake, Thermo Scientific, MA). Scanning electron micrographs were acquired using field-emission scanning electron microscopy (JOEL 7500F, acceleration voltage of 5 kV). A superconducting quantum interference device magnetometer (Quantum design MPMS SQUID) was used for magnetic characterization of EAMNPs. Mab-conjugation of the EAMNPs was carried out by physical adsorption of antibodies onto the polyaniline surface. Electrostatic interactions between the negatively charged constant (Fc) portion of the antibodies and the positively charged polyaniline surface are thought to play a role in adsorption and orientation of the biomolecules onto the EAMNPs (Pal and Alocilja, 2009). Successful conjugation of antibodies onto EAMNPs was confirmed by measuring the quantity of antibody in the post-hybridization supernatant with a commercial fluorescence-based protein quantification kit. The measured protein concentration in the supernatant was significantly lower than the concentration of antibodies initially added to the MNPs (data not shown), indicating that antibodies were retained on the MNPs during hybridization.

EAMNPs were conjugated with monoclonal antibodies at an initial EAMNP concentration of 10 mg/mL (1% solid). A 100 µL aliquot of monoclonal, anti-*E. coli* O157:H7 antibody (suspended in 0.1 M phosphate buffer) was added to EAMNPs suspended in PBS, yielding a final antibody concentration of 1.0 mg/mL. The mixture was hybridized on a rotisserie-style rotator for 1 hour at room temperature, with 25 µL of 10X PBS being added after the first 5 min of hybridization, to increase the Sodium chloride content of the suspension to approximately 0.14 M. Following hybridization, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer (0.1M tris buffer with 0.01% casein) for 5 min. Again the conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 250 µL of blocking buffer, this time for 1 hour with rotation. Finally, the EAMNP-antibody conjugate was magnetically separated, the supernatant removed, and the conjugate re-suspended in 2.5 mL of 0.1 M phosphate buffered saline (PBS). The final concentration of EAMNPs in each solution was 1.0 mg/mL. Immuno-conjugated EAMNPs (Mab-EAMNPs) were stored at 4°C. Prior to experimental use, Mab-EAMNPs were further diluted in 0.1 M PBS, in order to obtain solutions of Mab-EAMNPs at 0.5 mg/mL EAMNPs.

EAMNP Immuno-magnetic Separation (IMS) in Milk

The methodology for the EAMNPs (extract and concentrate) IMS method for food is the same as described for the broth trials of the author's earlier work. The scale up from 500µL to 200 mL required substantial coordination. To test hypothesis 1a - 1c, the same volume and the same concentration of Mab-EAMNPs was used as was used for the smaller 500 µL samples in broth (50µL of 0.5 mg/mL conjugated antibody). A new rotator system for the rotational incubation was designed using a 5 gallon bucket and a cement mixer, a cement mixer or a lab

designed rotator shown in figure 9.2. The rotator in figure 9.2 is modular. It snaps apart, folds and can be carried as seen in figure 9.3. The incubation period was extended by 10 minutes to account for the larger volume making it 50 minutes. A new larger size magnet was designed and used to magnetically extract the EAMNPs from the 200 mL portions. This magnet used 6 Rare Earth 4"x1"x1" neodydmium magnets from Applied Magnets (Plano, TX) placed on end in pairs separated by wood in a custom double sided wood stand capable of holding 6 - 200 mL bottles. A custom rack fitted on top held 6 - 50 mL tubes against the same magnets for the second step. The custom magnet is seen in figure 9.4. For the third and fourth step the magnetic separations were performed with a commercial magnetic separator (Promega Corporation, Madison, WI). Ten minutes was added to the rinse steps to allow more time for the magnet to pull the EAMNPs through the milk as opposed to broth. At each of the three washes with 0.1M PBS with 0.05% Triton X, the reconstitution volume was reduced as was the tube size. The 200 mL portion of the 9.6 L sample at concentration X was concentrated and re-suspended into smaller and smaller volumes during the wash steps, (50 mL then 2 mL and finally 600 µL) but the only real concentration step is the 600 µL level shown in equation 9.1.

Equation 9.1: Calculation of the amount of pure bacterial spike volume necessary to approximate a certain bacterial concentration in a bulk tank of whole fluid milk.

$$(200 * \text{X CFU}) / 0.6\ \text{mL} = 333 * \text{X CFU/mL}$$

Whatever the start concentration is in the bulk tank, the sample is concentrated 333 times.

Figure 9.1 shows a schematic of the IMS procedure.

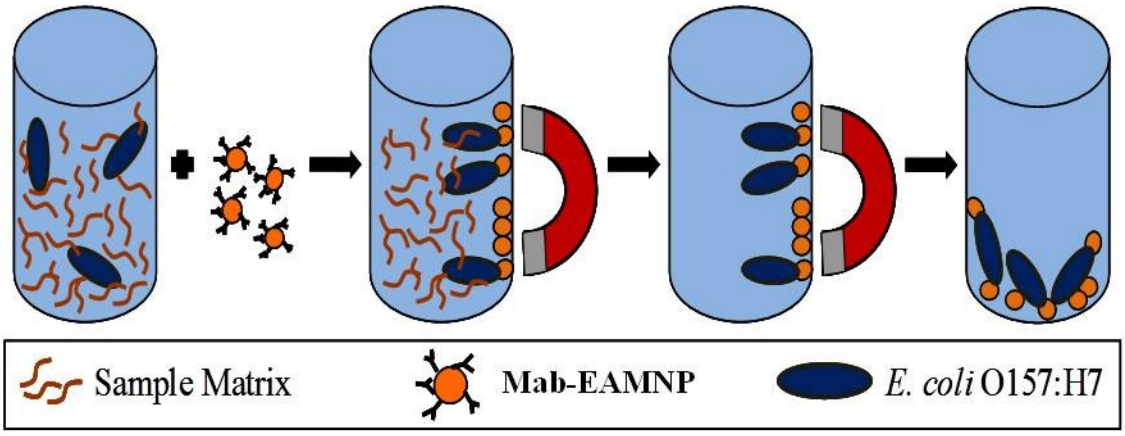


Figure 9.1: Immuno-magnetic separation procedure (IMS): sample plus Mab-EAMNPs → magnetic separation of target cells → removal of sample matrix → purified *E. coli* O157:H7-Mab-EAMNP complexes.

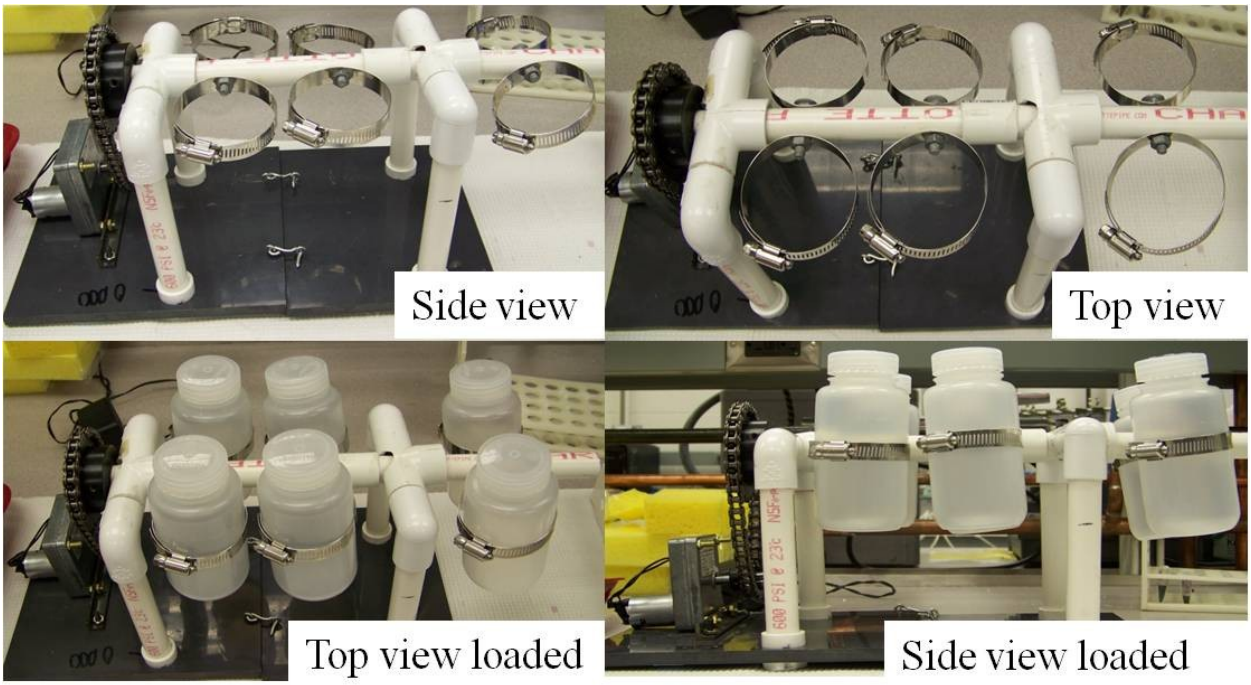


Figure 9.2: The lab designed, portable, 200 mL bottle rotator used for the 40 minute rotation as the EAMNPs capture the bacteria.

Figure 9.3: The lab designed, portable, 200 mL bottle rotator used for the 40 minute rotation as the EAMNPs capture the bacteria in its compacted transport formation.

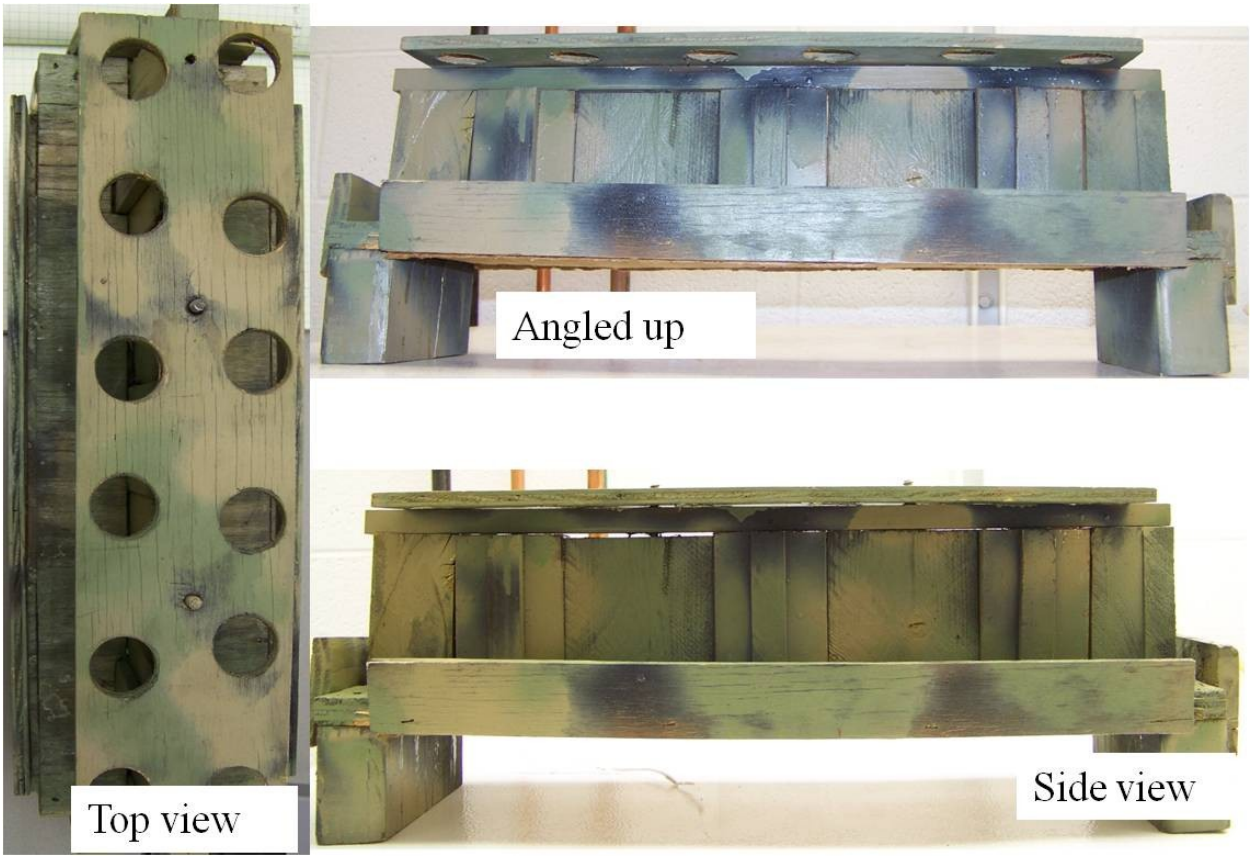


Figure 9.4: The lab designed, 200 mL to 50 mL magnetic separator used for the wash and concentration steps of the IMS with EAMNPs in whole fluid milk.

Enumeration and confirmation of extracted bacteria

The following reagents are used for the IMS protocol with EAMNPs milk portions and the FDA BAM methodology milk portions. Modified Buffered Peptone Water with pyruvate (mBPWp) (Neogen, East Lansing, MI), Acriflavin-Cefsulodin-Vancomycin (ACV) supplement, Tellurite/Cefixime (TC) supplement (Sigma-Aldrich St. Louis, MO), and Chromagar O157:H7 (DRG, Mountainside, NJ) were all used for the plating, enriching and MPN methods. Potassium Tellurite (0.64 mg/mL) stock solution , Cefixime (50mg/mL) stock solution and ACV stock solutions: Acriflavine HCL (2.25 mg/mL); Cefsulosin (2.25 mg/mL); Vancomycin (1.8 mg/mL), all purchased from Sigma-Aldrich (St. Louis, MO), were made ahead and kept at 4°C. Butterfield's phosphate buffer was made from stock chemicals in the laboratory using the FDA BAM protocol. TC SMAC was made according to manufacturer instructions with the addition of 3.9 mL of Tellurite stock and 1.0 mL of Cefixime stock per liter, during the cooling step. Chromagar O157:H7 was made according to the manufacturer's instructions with addition of 1.0 mL per L of agar of the Tellurite stock solution, during the cooling step.

Of the 600 µL concentrated sample, 100 µL went to a Chromagar O157:H7 plate, 50 µL went to a 1:10 dilution and then 100 µL to a Chromagar O157:H7 plate; 100 µL went to a TC SMAC plate, 50 µL went to a 1:10 dilution and then 100 µL to another TC SMAC plate; 100 µL went to the D-FSPCE biosensor and 200 µL were left for PCR confirmation in the JBAIDS or other PCR methodology (250 µL). The remaining volumes from the 1:10 dilutions were pooled back into the JBAIDS PCR reaction to approach the necessary 800 µL required in the protocol received with the instrument. These changes increased the assay time to 1 hour from each sample to results, plus 1.5 hours for JBAIDS confirmation. All agar plates were incubated overnight at 37°C. The number of colony-forming units (CFU) in the 100 mL aliquot was

determined by manually counting the colonies on each plate. Calculation of bacterial cell concentrations in both pure and IMS separated samples were carried out according to rules provided by the United States Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM, 2009).

For those concentrations which could be grown and counted, the capture efficiency as defined by the amount captured divided by the amount present in the original sample was calculated for each run for *E. coli* O157:H7. For some of those concentrations that were too low for adequate culture evaluation, the fractions normally plated were enriched (matching methodology to the MPN and FDA) and subsequently plated on Chromagar O157:H7 to determine a presence or absence of the target bacteria without enumeration. Representative samples of cultured bacteria were confirmed as *E. coli* O157:H7 by the Wellcolux® O157:H7 agglutination kit.

When it was possible to enumerate, capture efficiency was calculated by conversion of CFU/mL to $\log_{10}$ CFU/mL, above 10 CFU/mL. When calculating capture efficiency at the lower concentrations the log transformation is not performed since with a base 10 the result would be zero. Actual CFU/mL was used in this range to calculate capture efficiency. The calculated concentrations of cells captured by IMS (in CFU/mL) were converted to their $\log_{10}$ values. The $\log_{10}$ conversion also normalizes the distribution (Mettler & Tholen, 2007). Each similar start concentration was blocked together to facilitate evaluation. Groupings are 0-5.99 CFU/mL; 6-9.99 CFU/mL, 10-99 CFU/mL ($\log_{10}$ CFU/mL = 1) 100-999 ($\log_{10}$ CFU/mL = 2) and so on based on the start concentration.

Synthesis of aminated α D + Mannose

Production and evaluation of the aminated D + mannose was accomplished in collaboration with Dr. Xuefei Huang's chemistry lab at MSU, using published protocols. Aminated D-mannose was synthesized starting either from the unprotected D-mannose or α-D-mannose pentaacetate, all commercially available. Due to the high price of α-D-mannose pentaacetate, starting with unprotected D-mannose was economically viable. At each step, a self poured silica gel column chromatography was used to purify, a rotovap (Büchi Rotovapor or Büchi Rotovapor R-114, Midwest AOAC Int., St. Louis, MO) was used to concentrate, and thin layer chromatography (TLC) or proton and carbon nuclear magnetic resonance imaging (NMR) (500 MHz, Varian Unity INOVA, Oxford Instruments, Concord, MA) and fourier transform infrared spectroscopy (FTIR) (Perkin Elmer, Shelton, CT) were used to verify products. Procedures reported by (Teumelsan & Huang, 2007) were adopted for the synthesis of α-D-mannose pentaacetate. Briefly α-D-mannose was protected using acetic anhydride in pyridine with catalytic DMAP to achieve α-D-mannose pentaacetate. α D+mannose pentaacetate was reacted with 3-chloropropanol and $BF_3.OEt_2$ in DCM overnight. The resultant compound was reacted with sodium azide in DMF at $60^{O}C$ overnight (El-Boubbou, Gruden & Haung, 2007; Joosten, Loimaranta, Appeldoorn, Haataja, El Maate & Liskamp, 2004; Teumelsan & Huang, 2007). De-protection of the acetate groups was performed with freshly prepared sodium methoxide and finally reduction of azide under hydrogenation condition created the final aminated α-D-mannose.

Functionalization of the SPCE

Chemicals used to functionalize the SPCE include: 2.5 mM glutaraldehyde solution, Citrate Gold Nanoparticles (AuNPs), stock ≈2.4 Au; ~15 nm in diameter**,** α D+ mannose - amine @ 25 µg/mL**,** Deactivating buffer (0.2 M Tris in 0.01 M Phosphate Buffered Saline (PBS) plus 10 mM Cyanoborohydride)**.** Chemicals such as 0.1 M hydrochloric acid (HCl) solution, acridine orange (AO) stain (2 mg of AO into 1 mL of AO buffer = 100x); acridine orange buffer pH 3.8. (90 mL of 10mM (0.01 M) phosphate buffer + 585 mg of NaCl, qs to 100 mL), coupling buffer pH 7.4 (0.1 M phosphate buffer into 900 mL of dH_2O), Con A conjugated to FITC are used elsewhere in the protocol. SPCEs were purchased from Gwent Electronics Materials Ltd, United Kingdom and modified in house. Cyclic voltammetry was performed on the PalmSens handheld potentiostat (Palm Instruments BV, Houten, The Netherlands).

To attach aminated D+ mannose to the SPCE, the rinsed, dried chips were incubated with 25 µL of 2.5 mM glutaraldehyde on the working center of the carbon electrode for two hours at 4°C. At completion, the excess glutaraldehyde was rinsed with DI water and dried. Citrate AuNPs (25 µL) were then incubated on the working center of the carbon electrode for two hours at 4° C. At completion, the excess citrate AuNPs were rinsed with DI water and dried. On the working center of the carbon electrode, 25 µL of aminated D+ mannose (25 µg/mL) was placed for 15 minutes at 21° C. At completion, the excess aminated D+ mannose was rinsed with DI water and dried and 50 µL of deactivating buffer was applied for 15 minutes at 21° C. The resultant D-FSPCE were dried and stored at room temperature (protected from light). Both a blank SPCE and an aminated D+ mannose modified SPCE are shown in figures 9.4 and 9.5 respectively.

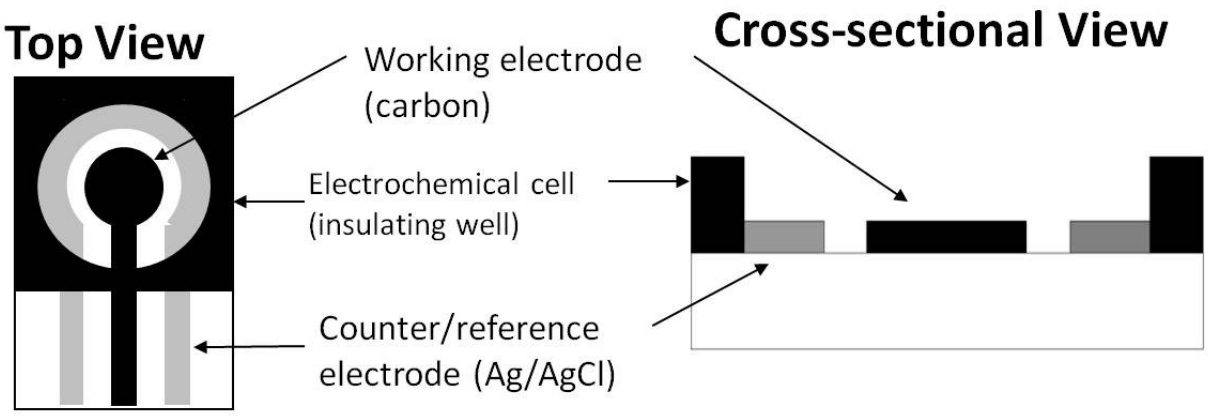


Figure 9.5: Schematic of the bare screen-printed carbon electrode (SPCE) sensor chip the electrochemical detection is performed on, the circuit.

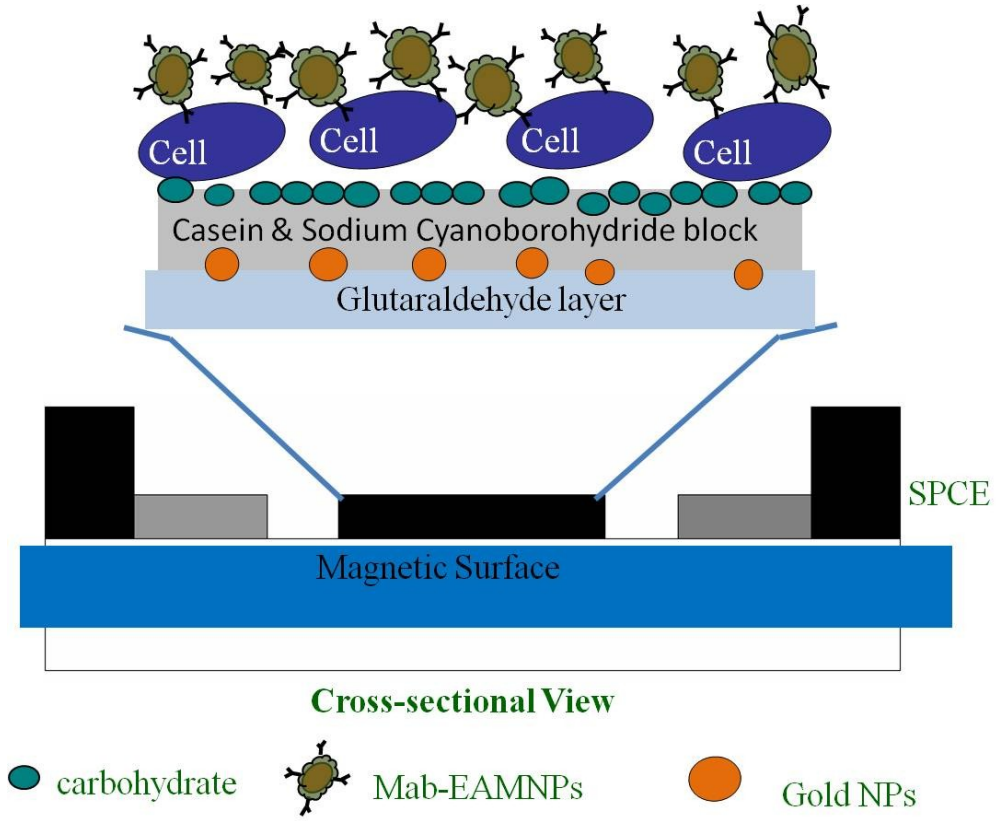


Figure 9.6: Layered schematic of the structure in the novel aminated D+ mannose functionalized screen-printed carbon electrode (D-FSPCE) biosensor chip.

Cyclic Voltammetry evaluation of D-F SPCEs:

Cyclic voltammetry (CV) is an electrochemical method where a potential is scanned from -1.4 V to 2.0 V and the resultant current is measured at each voltage. Varying parameters can be used on the resultant cyclic voltammogram, depending on the reporter used. Here polyaniline is electrically active and, if present, should provide a peak at a certain voltage in both the positive and negative sweep of the current in the cycle. Larger levels of polyaniline should provide greater peak heights, but other components of the system affect the polyaniline peak greatly as well. The current produced at a given potential is the combination of the entire electrochemical system and additional components can reduce the current as side reactions occur (Xu, Leng, Li, Lu, Wanga & Hu, 2010; Li, Yang, Zhao & Du, 2011). Average resistance over the whole cycle was tabulated with equation 9.4 and evaluated in this study. Between the differing concentrations and the blank D-FSPCE, average resistance of the three scans per sample was used as the system resistance and the measurement of presence/absence of bacteria.

From the collected data of voltage (V) and current (I) at each point on the voltammogram, the absolute resistance (R) was calculated from standard Equation 9.2, solved to Equation 9.3 and the result placed into Equation 9.4

Equation 9.2: Ohm's Law used to calculate the electronic behavior in the circuit of each point taken during the cyclic voltammogram

$$V = IR$$

Equation 9.3: Solving of Ohm's Law to calculate the resistance at each point on the cyclic voltammogram.

$$R = \frac{V}{I}$$

Equation 9.4: Total average resistance across all points on three consecutive cyclic voltammograms.

$$\sum R = \frac{V}{I} \Big/ \#\, pts$$

Four scans of each D-FSPCE were conducted and the first scan was discarded as a system equilibration set. The last three provided at least 960 data points apiece across the cycle. For each of these 2880 individual points the absolute resistance was calculated. The average resistance at all points in the three curves were averaged together to get the single average resistance across the system for each run at each concentration of bacteria. The signal to noise ratio was calculated by averaging each category of cell concentration's final resistance value divided by the same treatment for the set of blank evaluations.

FDA BAM Screening for serotype *E. coli* O157:H7 from foods – Gold Standard

All reagents listed for the culture and enumeration of the IMS protocol with EAMNP milk portions remain the same for the FDA BAM methodology milk portions. The procedure for the gold standard detection methods is published (FDA BAM, 2009). Briefly, the 200 mL samples are spun in a floor centrifuge at 15,000g. The supernatant was discarded and the resultant pellet was re-suspended in 225 mL mBPWp and incubated for 5 hours at 37°C. After five hours the ACV supplement was added at 1 mL of stock solution per supplement per portion and incubated for 18 more hours at 42°C. After incubation 50 µL: 450 µL serial dilutions were made in Butterfield's phosphate buffer from 10^{-1} to 10^{-4}. Two of the dilutions were plated depending on the original spike concentration, usually 10^{-2} and 10^{-4} and plated in duplicate one

on Chromagar O157:H7 and one on TC SMAC. All plates were incubated overnight at 37°C. Plates were counted according to FDA BAM published rules. Individual colonies that met published phenotypic characteristics on each agar were selected and challenged in a Wellcolux O157:H7 agglutination kit. Since the original sample was spiked, no further characterization was performed on the typical colonies.

Most Probable Number (MPN) quantization of spike rate

Multiple forms of quantification were used to estimate the actual CFU/mL or MPN/mL in the bulk tank. First, the spiked sample was estimated as to its cell concentration according to the OD600 and the growth curves already presented in the author's earlier work. Second, the target spiked concentration was identified and the spike volume of pure culture was calculated using a CFU/mL estimate by equation 9.5:

Equation 9.5: The calculation of the volume of bacterial pure culture to place in the bulk tank to obtain the targeted concentration per mL of contamination.

$$[Volume\ of\ milk\ (mL)\ *\ Target\ [\ ]\ (CFU/mL)]/\ Estimated\ Pure\ Culture\ [\ ] = Volume\ of\ spike(mL)$$

Care was taken on units as it was necessary to dilute the pure culture to obtain a measureable volume to spike. Third, the pure culture spike was diluted and plated to obtain the actual CFU/mL and the new estimated spiked milk concentration was back calculated. Fourth, a traditional 3 tube MPN was performed on day 1 of the EAMNP extraction method and the FDA method. Fifth, a traditional serial dilution of the milk sample is done on day one with Butterfield's phosphate buffer to obtain a direct plate count after the two day refrigeration hold time. The MPN was performed by making three serial dilutions of the spiked bulk tank milk

1:10, 1:100 and 1:1000. One mL of each of these dilutions was inoculated into a sterile glass centrifuge tube with 9 mL of mBPWp and an inverted small glass tube completely full of the peptone water. These MPN tubes were incubated for 5 hours at 37°C along with the FDA samples and subsequently 44 µL each of the ACV stock supplements were placed in each tube. The MPN tubes were returned to the incubator for 18 more hours at 42°C. After incubation, the resultant growth was evaluated, in each of the 9 tubes, noting turbidity and gas production in the inverted tube. How many of each dilution were positive (have growth and gas) were counted per dilution. For the MPN analysis, a 3 tube table of numbers then provides the estimate of the start concentration of the bulk milk tank on day one in MPN/mL.

Confirmation Wellcolux® Agglutination kits

Confirmation of any colonies on either TC-SMAC or Chromagar O157:H7 were confirmed for surface antigens by the Wellcolux® Agglutination kits for O157 for both the FDA method and the EAMNP method. Commercial kit instructions were followed for administration of the tests. Protocol for selecting a typical colony from the plate included colony morphology and color on the respective agar. If more than one type was present, a representative colony of each type was run on the test. Results were recorded and considered confirmed for E. coli O157:H7 typical colonies per agar type and agglutination positive on the test kit.

Confirmation JBAIDS

The Idaho Technology JBAIDS or the Joint Biological Agent Identification and Diagnostic System is a military specific, Real Time Polymerase Chain Reaction (RT-PCR) machine designed to detect pathogens of military concern. (Salt Lake City, UT) It is controlled

by International Trafficking in Arms Regulations (ITARs) and will not be discussed in detail. It is being used as a verification tool for the genetic presence of *E. coli* O157:H7 using reagents from a sister machine the Idaho Technology Ruggedized Advanced Pathogen Identification Device (RAPID LT). This machine uses PCR primers to the target DNA to amplify and a melt curve to identify *E. coli* O157:H7 and its H flagella proteins, respectively from a freeze dried field ready kit. These kits are standard for use with an 8-16 hour pre-enriched sample. For these experiments the 250-350 μL of remaining IMS separated sample was placed into the JBAIDS after a variety of DNA extraction protocols. The parameters, tests and extraction protocol varied according to experiment as did the actual sample volume.

For experiment #1 a Platinum Path ™ Extraction Kit (PPEK) (kit part # ASAY_ASY_0120, Idaho Technology, Salt Lake City, UT) was used to extract DNA after cell lysis with bead beater tubes or heat and the addition of a 4:1 concentration of protease; following the kit instructions. Heat lysis consisted of heating the sample to 95°C in a water bath prior to PPEK. Since the EAMNPs were still present, the heated sample and the bead beater tubes were placed on a magnetic separator and the supernatant was placed into the Platinum Path extraction kit. Lysed, extracted samples were prepared as directed in the JBAIDS *E. coli* O157:H7 detection kits (Kit # 3825, Idaho Technology, Salt Lake City, UT) and run on the JBAIDS. The volume of tests available limited the number of samples placed in the JBAIDS for this experiment. Samples were run in duplicate and kit positive and negative controls were run alongside samples, sample positive controls and sample negative controls. The blank and sample un-spiked were PCR confirmed as such in some experiments.

For experiment #2 the PPEK was used to extract DNA after cell lysis with bead beater tubes and the addition of a 4:1 concentration of protease for milk runs 5, 6, 9, & 16, following

the kit instructions. Since the EAMNPs were still present, the bead beater tubes were placed on a magnetic separator and the supernatant was placed into the PPEK. Lysed, extracted samples were prepared as follows and placed in the RAPID LT *E. coli* O157:H7 High Volume detection kits (product code # 32-C006, Idaho Technology, Salt Lake City, UT) and run on the JBAIDS machine using programming recommended by Idaho Technology. The directions on this portion of the samples in experiment 2 were misunderstood. Directions included placing 5 µL of enriched and PPEK extracted DNA into the sample capillary and an equivalent 5 µL of reconstituted reagent, reconstituted with 45 µL of reconstitution buffer, not in duplicate. For these 4 runs, we were already using un-enriched IMS samples from milk spiked below 20 MPN/mL. We also reconstituted the reagent into 90 µL and added 10 µL of reconstituted reagent and 10 µL PPEK extracted DNA into each capillary tube. This process was repeated in duplicate. Essentially, we diluted the DNA and the PCR reagents by 4 more times before attempting to run the PCR in the JBAIDS machine. The remaining PPEK extracted DNA for milk runs 5, 6, 9, & 16 were run again in the Q-PCR analysis discussed below, after shipping. Samples from milk runs 4, 7, 8, 10 -15 and 17 – 23 were not evaluated in the JBAIDS due to the limited number of kits left after the miscalculation. These samples were held at -4°C for three weeks, attempted to ship on ice, in the Texas summer heat three times, returning each time to be placed at -4°C in between attempts. On the fourth shipping attempt these same samples were held in Tennessee overnight under unknown conditions.

For experiment #3, heat lysis and ethanol precipitation was performed for all of the samples before analysis in the JBAIDS machine using the RAPID LT *E. coli* O157:H7 High Volume detection kits (product code # 32-C006, Idaho Technology, Salt Lake City, UT) with the

correct instructions. The ethanol precipitation protocol was the same as used in the QPCR confirmation section below.

Confirmation QPCR and 2% agarose gel MSU

QPCR analysis and the 2% agarose gel of some of the milk run portions were contracted out to another lab. Their 384 well format and large gel size expedited the testing of the 456 samples from experiment # 2 and the 170 cattle fecal samples from the MSU Nano-Biosensors lab summer project. The DNA was extracted by the below protocol and the extracted DNA was loaded into the 96 well plates as well as chose the primers and probes. A quality control test of the primers and probes chosen was run in the MSU Nano-Biosensors lab using the DNA Engine® Peltier Thermocycler (Biorad, Hercules, CA) and 2% agarose gel analysis to verify the protocol before providing the primers and probes in the samples to the MSU QPCR lab for analysis.

Extraction of the DNA for each of the milk samples and cattle fecal samples that were extracted by EAMNPs was performed using heat lysis and ethanol precipitation (Laird, 1991). Any and all IMS samples available from the corresponding milk runs in experiment #2 were mixed for analysis. Samples previously extracted for the JBAIDS with the PPEK, but remaining, were placed directly into the QPCR reaction without further manipulation. The resultant samples were spun at 13000 rpm for 10 min at 4°C and the supernatant removed. 500 µL of lysis buffer (Tris HCl 100 mM, EDTA 5mM, SDS 0.2%, NaCl 200 mM) was added to the pellet with 5 µL of proteinase K stock solution (Idaho Technology, Salt Lake City, Utah). The tubes were vortexed until the pellet was re-suspended. The samples were then placed in a water bath at 55°C for 75 min. At completion, 500 µL of cold 70% isopropanol (stored at -80° C) was added.

After mixing, each sample was placed into the -80° C freezer for 30 min or overnight. This procedure was completed two more times with 100% ethanol and then 70% ethanol. When completed, the pellet was dried inside the vacuum oven without heat to evaporate all the ethanol. 100 µL of milliQ reagent water (DNAse and RNAse free) (EMD Millipore, Bedford, MA) was added to re-suspend the pellet. Extracted samples were stored at -80° C until placed into the QPCR reaction. The FDA enriched sample (portion #1) verified by culture was used as the control per run. With milk protein present, those samples were doubly extracted using the PPEK.

After extraction, the DNA was loaded to final volume with 300 nano-molar of forward and reverse primers. Enzymes, buffers, dNPT's and Sybr green were loaded with 1 X Invitrogen fast cycle green **(**Life Technologies**,** Grand Island, NY). 75% of the sample was loaded to the QPCR (6 µL of 8 µL). STX 1 and STX 2 primer sets pretested in house, in QPCR by themselves and in tandem with positive culture dilutions. The STX 1 gene in O157:H7 is a cryptic phage that cannot lyse out but it is less specific to O157:H7 organisms (Bellin Pulz, Matussek, Hempen, & Gunzer, 2001). The STX 2 gene in O157:H7 is an intact phage and can come out of the genome (Bellin, et al., 2001). 80% (8 µL of 10 µL) of QPCR product was loaded on a large 2% w/v agarose gel with a 100 base pair ladder. Unfortunately, the 2% w/v agarose gel was discarded before removal and evaluation of the PCR product was possible.

Quality control testing

During experiment 2, difficulties in the consistency of the M^3 Biosensor results were encountered. The positive controls began to plate positive yet read negative on the M^3 Biosensor. Since three successful milk runs were accomplished in experiment 1, along with 257

broth runs, an examination of the components of the system, not a categorical failure of the sensor in milk warranted investigation. Several of the diagnostics used in the development of the EAMNPs and the D-FSPCEs were employed to evaluate their consistency, electrical activity, capture ability and visual appearance. Basically, the entire biosensor was pulled apart one component at a time until that component was ruled out as part of the problem. When broth runs were performed as previously published by this laboratory (Setterington, Cloutier, Ochoa, Cloutier & Alocilja, 2011) the problem could not be resolved. The original EAMNPs used were mixed in the room temperature, brown glass, shelf storage bottle after each production run. This fact did not allow an independent examination of lot to lot variation in their production nor were any quality control measures in place prior to this time. A quality control program was instituted and performed on older stocks of the EAMNPs and the D-FSPCE portions of the M^3 Biosensor and the PalmSens® potentiostat. The quality assurance procedures performed on the EAMNPs include electrical evaluation using blank SPCEs and a bench top potentiostat (Pal et.al, 2008). The CV analysis was run from 1 to -0.4 Voltage range for this evaluation. The new batches of EAMNPs, the batch used in experiment 2 and another batch from inside the laboratory were evaluated electrically with the same method. Those same batches were evaluated with a three point probe and the Transmission Electron Microscope (TEM) visualization using previously published protocols (Pal et.al, 2008). The new batches of EAMNPs were conjugated to Mab for *E. coli* O157:H7 (Meridian Life Sciences) and the capture efficiency calculated.

For the D-FSPCE, the gold nanoparticles, the aminated D mannose and the SPCE were examined with the same procedures as above for the EAMNPs. Storage conditions for each, their behavior on the CV with and without EAMNPs, and their visual appearance were considered as potential components of the failures.

Statistical analysis

In whole fluid milk, the sensitivity and specificity of culture results of IMS Mab-EAMNPs on Chromagar and TC SMAC, D-FSPCE biosensor evaluation with cyclic voltammetry and JBAIDS PCR were calculated versus the gold standard of FDA BAM results, separately. The same fractions were compared for the biosensor as a system evaluation after electrochemical and PCR challenge on those milk runs that could be evaluated. Sensitivity was calculated as the number of tests correctly identified as positive divided by the total number of tests that were actually positive as identified by the gold standard. Since the culture, biosensor and PCR are all from the same IMS portion they are replicates of each other. Their results were therefore compared in a statistical analysis as a group as well by portion and by milk run percent positives. Each similar start concentration was blocked together to facilitate evaluation. Groupings are 0-5 CFU/mL (or MPN/mL); 6-10 CFU/mL, 11-15 CFU/mL, 16-20 CFU and greater than 21 CFU/mL based on the spike concentration quantization. Capture efficiency was calculated as the captured concentration from the agar plates post IMS divided by the concentration in the spiked bulk tank, when the data was available. The limit of detection, low was calculated as the lowest start concentration that was detected by the one of the three different detection techniques and verified by the FDA BAM methodology and the MPN analysis.

Results

AOAC Performance test method validation requirements suggest that for a sample that cannot be tested with two different tests, the format for comparison to a gold standard is to run 20 portions for each test out of a bulk sample. These are considered parallel testing versus paired testing. We did that by spiking 8600- 9600 mL of milk and separating that sample into the 40 separate 200 mL portions with appropriate controls. 20 portions were tested under each of two

tests, one being the FDA BAM methodology and one being the M^3 Biosensor. The milk run testing results were considered positive if at least five of the portions were positive, by any of the three analytical tests on the EAMNP extracted portion. Due to that fact, results were separated into a per run result and a per portion results, by analytical test. System errors in each of the following three experiments prevent combination of the results easily. Therefore, the results will be discussed by experiment and then combined when possible.

Experiment 1

In the three milk runs done in experiment #1 138 portions were analyzed and are summarized in figure 9.7. The first was spiked at an estimated 10^4. A mathematical error took a target of 100 CFU/mL to spike concentration of 10^4. Since the experiment was set up for 100 CFU/mL the dilutions were too concentrated to count. These samples were used to determine if the heat or bead beater cell lysis would be more efficient for JBAIDs. Only four of the 20 portions were evaluated with PCR and all were verified correct as were the 20 Wellcolux agglutination kits. Of the 4 portions from the 10^4 run that could be evaluated, they were used to determine the best DNA extraction protocol for moving the EAMNP IMS separated sample into the JBAIDS via the PPEK. Heat lysis yielded DNA, but less consistently than using the bead beater tubes. For subsequent analysis, the bead beater tubes were employed. The second milk trial was contaminated by *Bacillus* species spores in the bulk tank causing the severe contamination of all milk samples, the MPN evaluation was diluted incorrectly the first time and two days later was repeated with the refrigerated sample. The average of the two MPNs was 29 MPN/mL.

The third milk trial was spiked low, based on calculated post spike plate counts at 10 MPN/mL. As shown in figure 9.9, culture alone cannot detect even concentrated samples at this level without pre-enrichment. At 10 MPN/mL the JBAIDS PCR verified that the culture results of the IMS EAMNPs fraction were incorrect showing that 13 of the 19 portions contained *E. coli* O157 DNA. This concentration was the only one where the majority of the portions could be evaluated in the PCR using the JBAIDS *E. coli* O157 kit without the melt curve for identification of the H7 portion. The IMS separated samples were split and cultured, scanned with the biosensor and evaluated via PCR. The M^3 Biosensor outperformed culture and PCR and correctly identified 20 of the 20 portions.

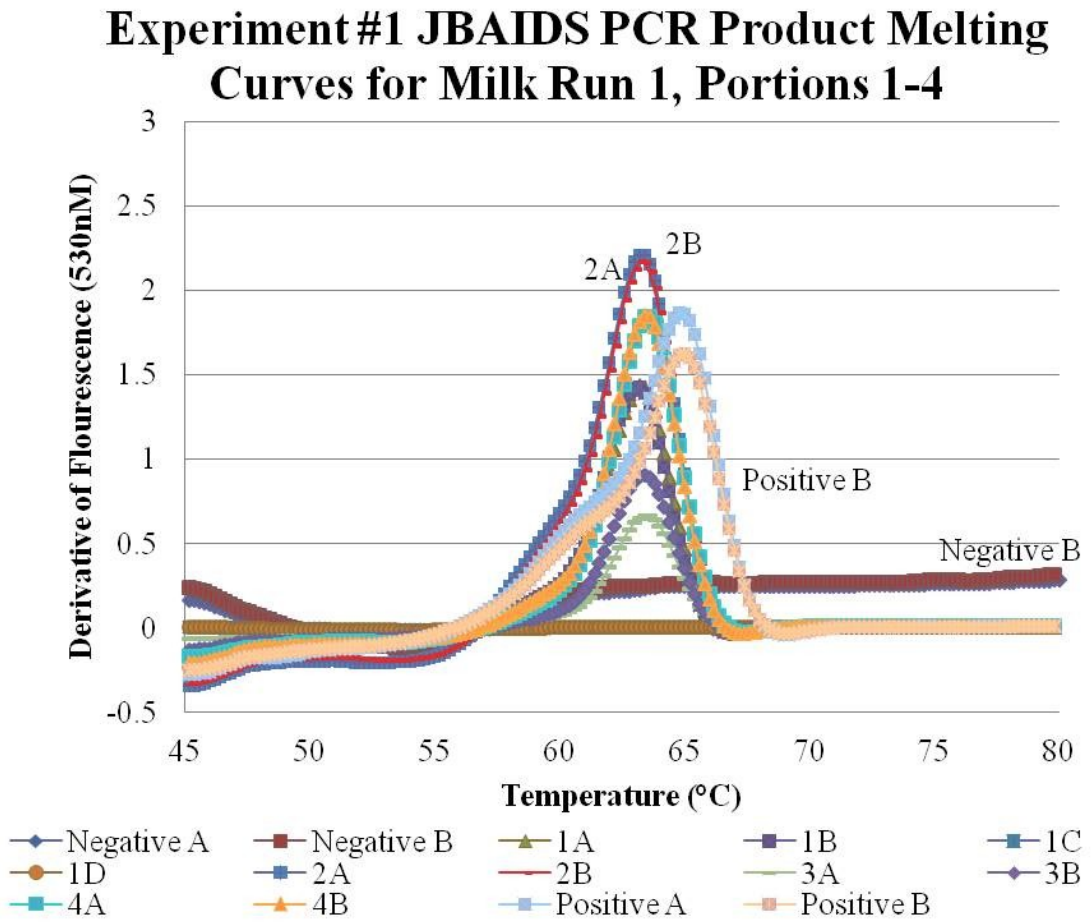


Figure 9.7: Milk Run # 1, portion 1-4 melt curve results on JBAIDS with the *E. coli* O157 test, 29 MPN/mL. This graph demonstrates the temperature difference at the melt curve for the kit positive control DNA (65°C) and the spiked milk positives from the *E. coli* O157:H7, Sakai strain (62°C).

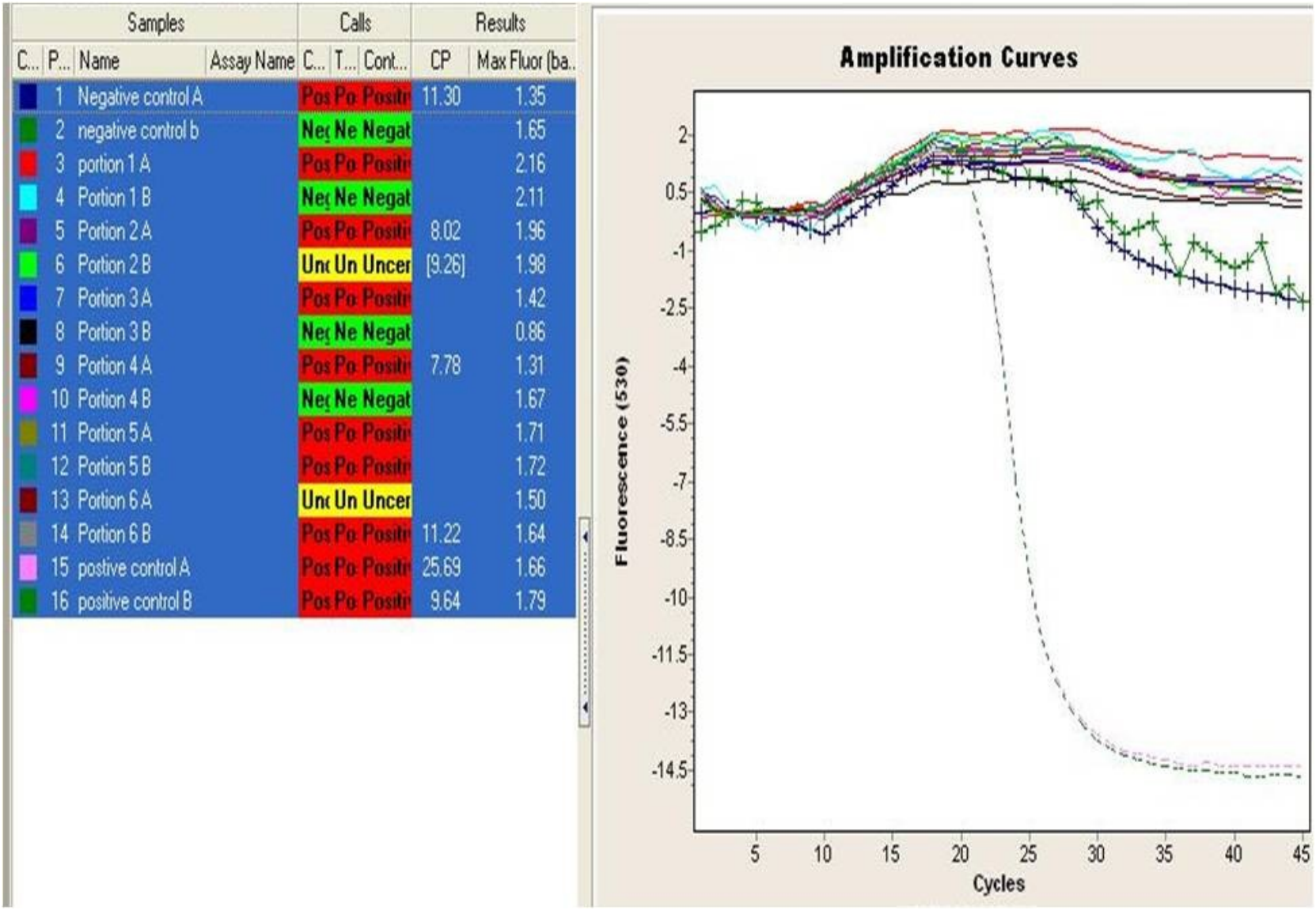


Figure 9.8: Milk Run # 3, portion 1-7 qualitative results on JBAIDS with the *E. coli* O157 test, 10 MPN/mL.

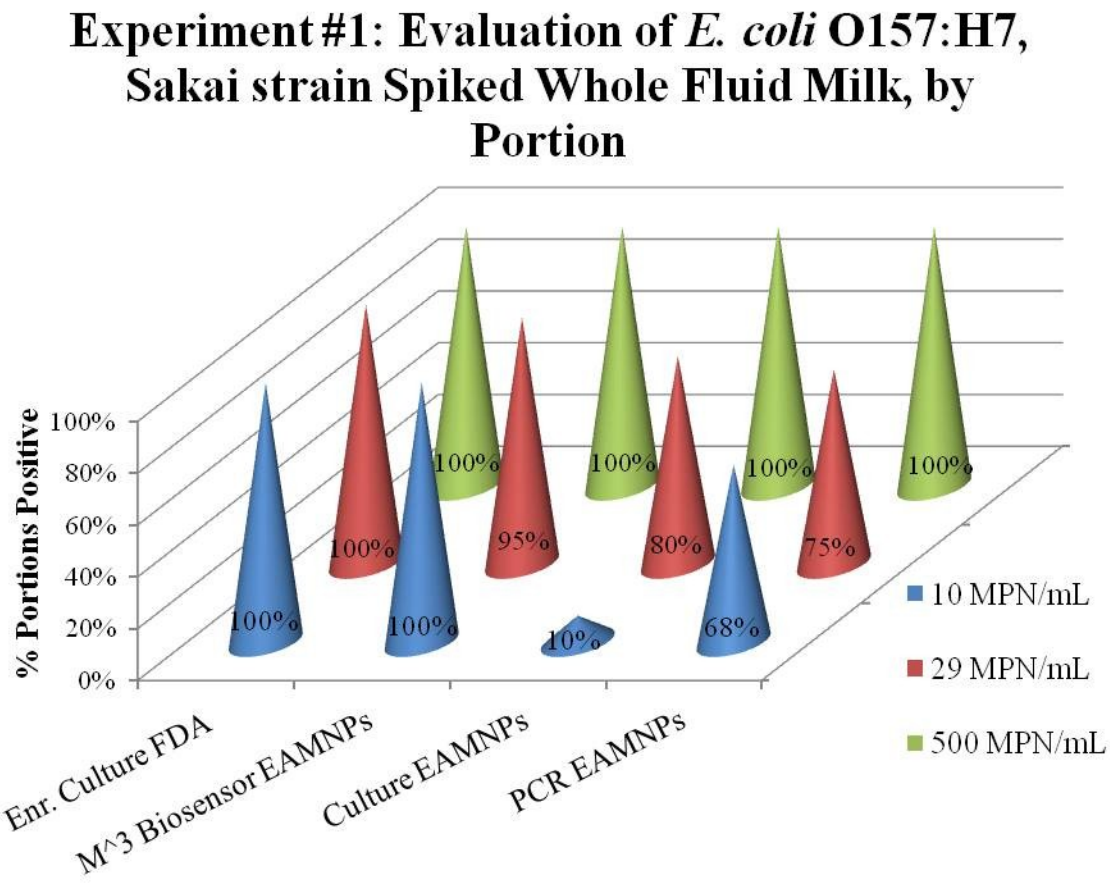


Figure 9.9**:** Experiment #1 by portion. Spiked bulk tank milk at three concentrations evaluated by 20 portions in the FDA BAM enrichment and culture verses 20 portions without enrichment and IMS separation with Mab-EAMNPs. The IMS separated portions were split and evaluated by M^3 Biosensor, culture and PCR.

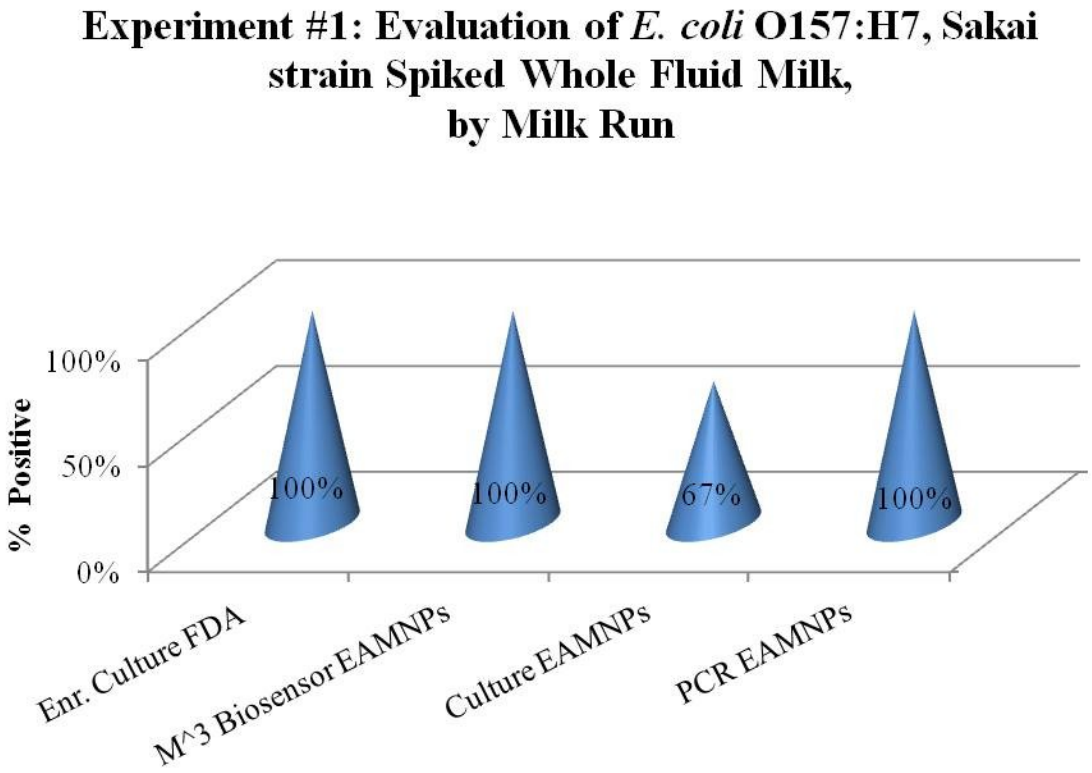


Figure 9.10: Experiment #1, by run. The percent positive samples by comparison methods, by run. The M^3 Biosensor matched the FDA gold standard 100% with all three runs showing at least 5 positive verified by PCR. The AOAC PTM standards only require 5 of the 20 for either method for the run to be called positive.

Experiment 2

For experiment #2, All 432 EAMNP extracted cellular portions were extracted for DNA and PCR results. All 432 matching FDA extractions were culture positive down to spike concentrations of 1 MPN/mL in whole fluid milk. Many evaluations were delayed due to the logistical, mechanical and component failures encountered during the move to the second laboratory. Biosensor results of the 23 runs evaluated in tandem with the FDA extraction protocol were not possible from this experiment. Attempts to resuscitate the extracted bacteria

and pre-enrich them failed, due to mishandling during shipment and the long delay from extraction to attempted pre-enrichment.

Quality control

In the quality control methods listed above, the electrical activity of the EAMNPs was tested by the potentiostat on blank SPCEs and after pellet production with a three point probe. Newly produced EAMNPs and old batches of EAMNPs matched the original published EAMNP data for both testing modalities (Pal et al, 2008). The EAMNPs taken to the second laboratory did not. The conductivity of the particles used in experiment 2 was less than half of the other three (figure 9.11).

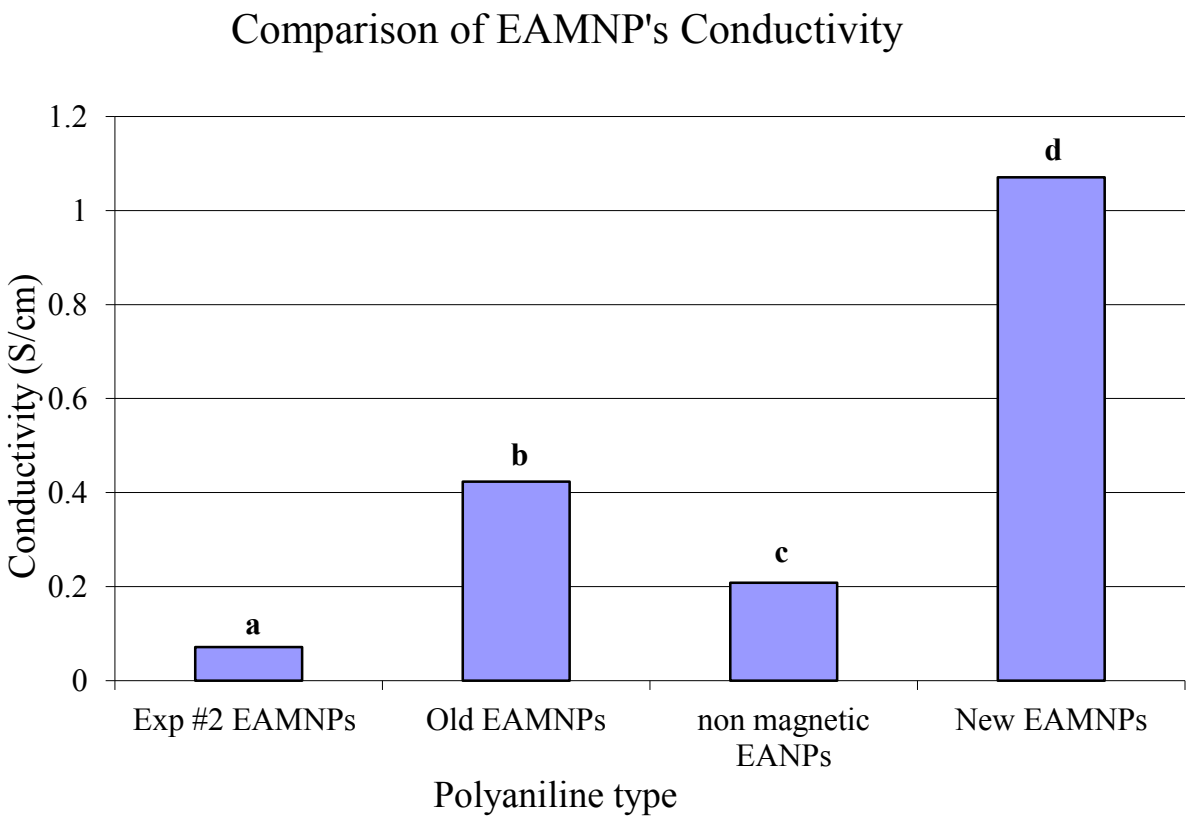


Figure 9.11 Conductivity of different EAMNPs. (a) Experiment #2 EAMNPs, (b) old EAMNPs, (c) non-magnetic Polyaniline (d) New EAMNPs.

Capture efficiency in previously unpublished studies with these EAMNPs using the published conjugation protocol (Setterington, et. al., 2011), was evaluated at multiple concentrations of bacteria in broth. Old EAMNPs in the laboratory captured at 85 – 100%, as did newly made EAMNPs. The EAMNPs used in experiment 2 captured 20% less than comparable EAMNPs that were not clumping up during the conjugation. The clumping experience exacerbated this drop in efficiency (data not shown).

Visually, the TEM images taken of archived original EAMNPs, old EAMNPs, experiment 2 EAMNPs, and the new EAMNPs are shown below in figure 9.12. As can be seen the magnetic particles used in experiment #2 (c) were clumped and nearly indiscernible from each other. The other three are very similar.

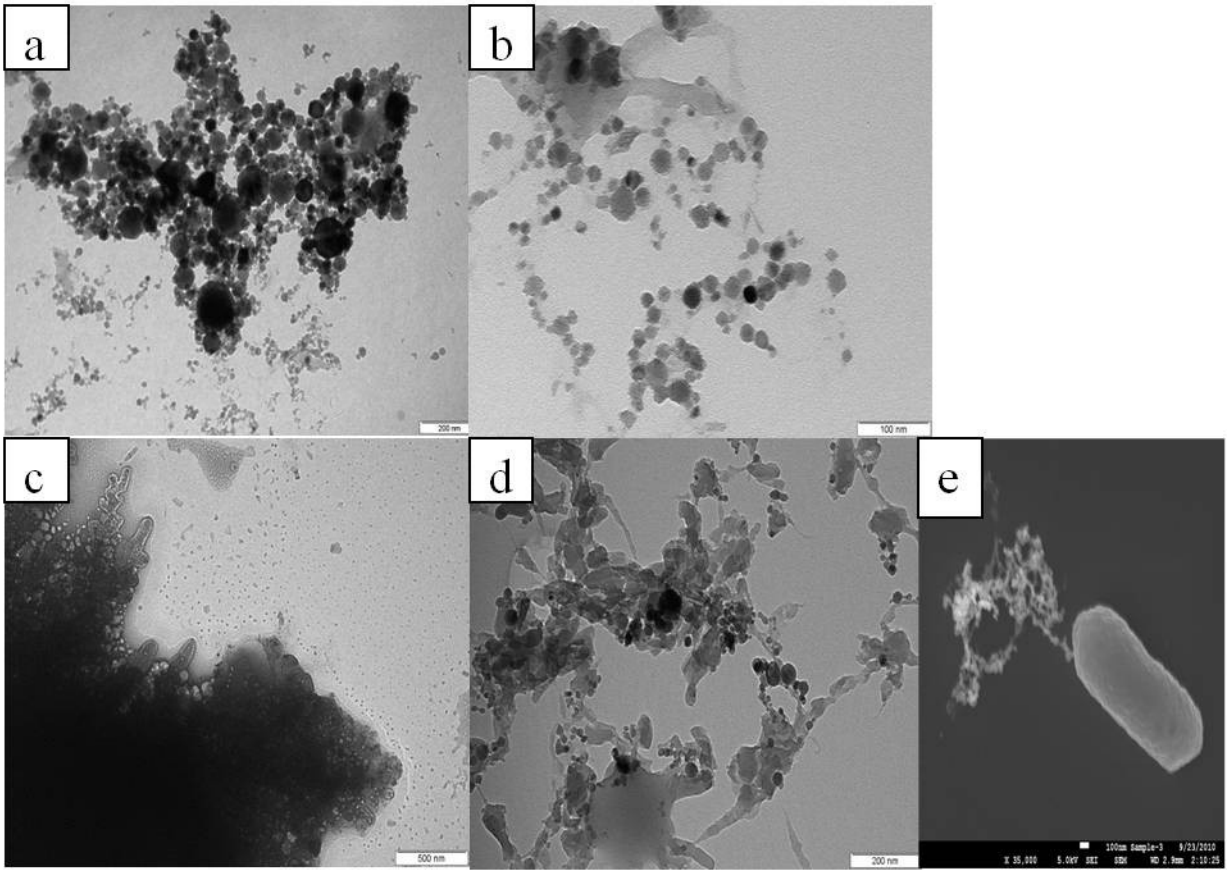


Figure 9.12: (a) TEM Original EAMNPs, (b) old EAMNPs, (c) Experiment #2 EAMNPs, (d) New EAMNPs, (e) an EAMNP captured *E. coli* O157:H7cell.

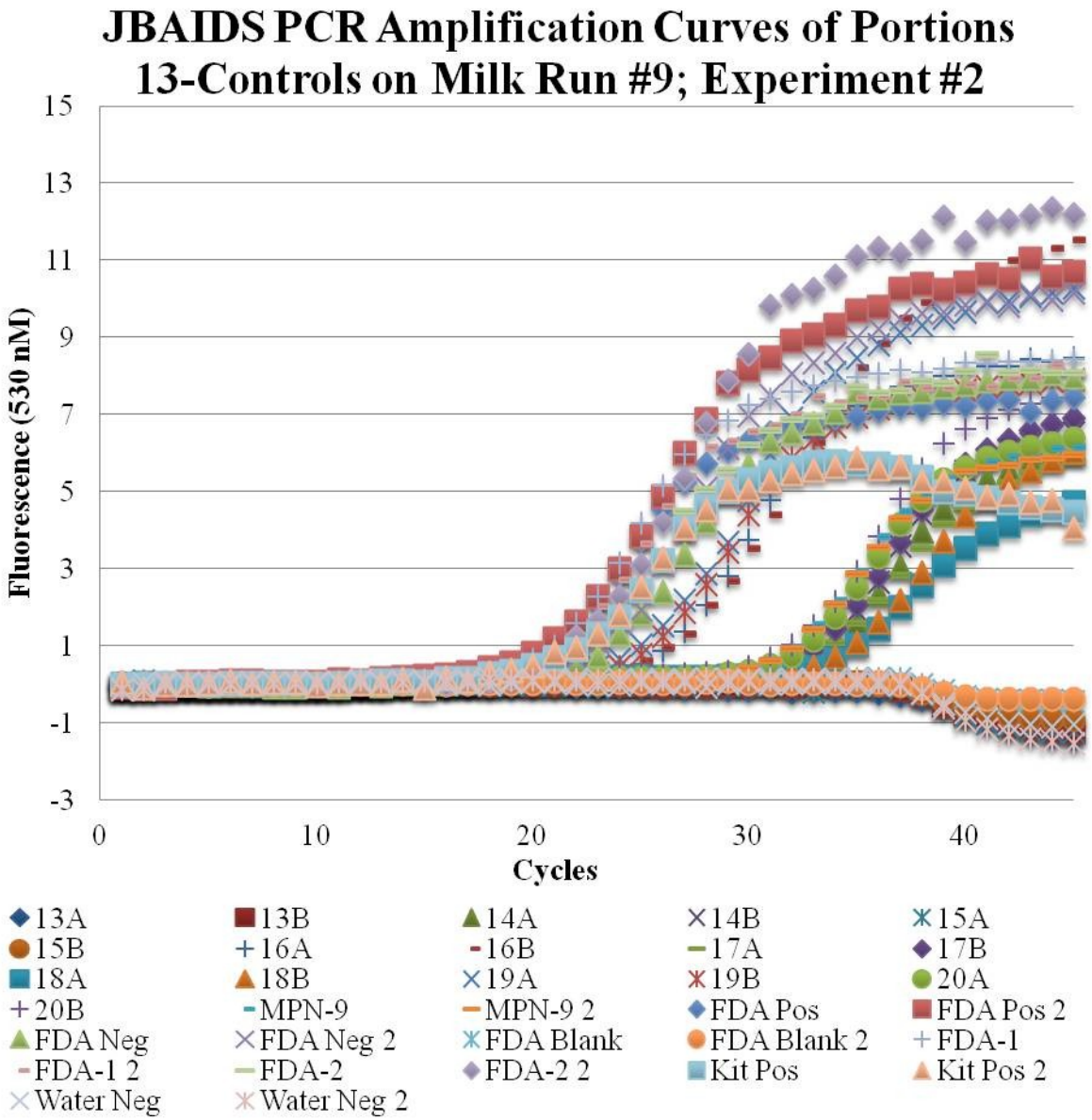


Figure 9.13: JBAIDS results for Milk run #9, portions 3-20 and the corresponding controls. The over dilution of the PCR primers decreased detection sensitivity, despite this at least five of the samples were confirmed positive by the JBAIDS at a milk spike concentration of 51 MPN/mL.

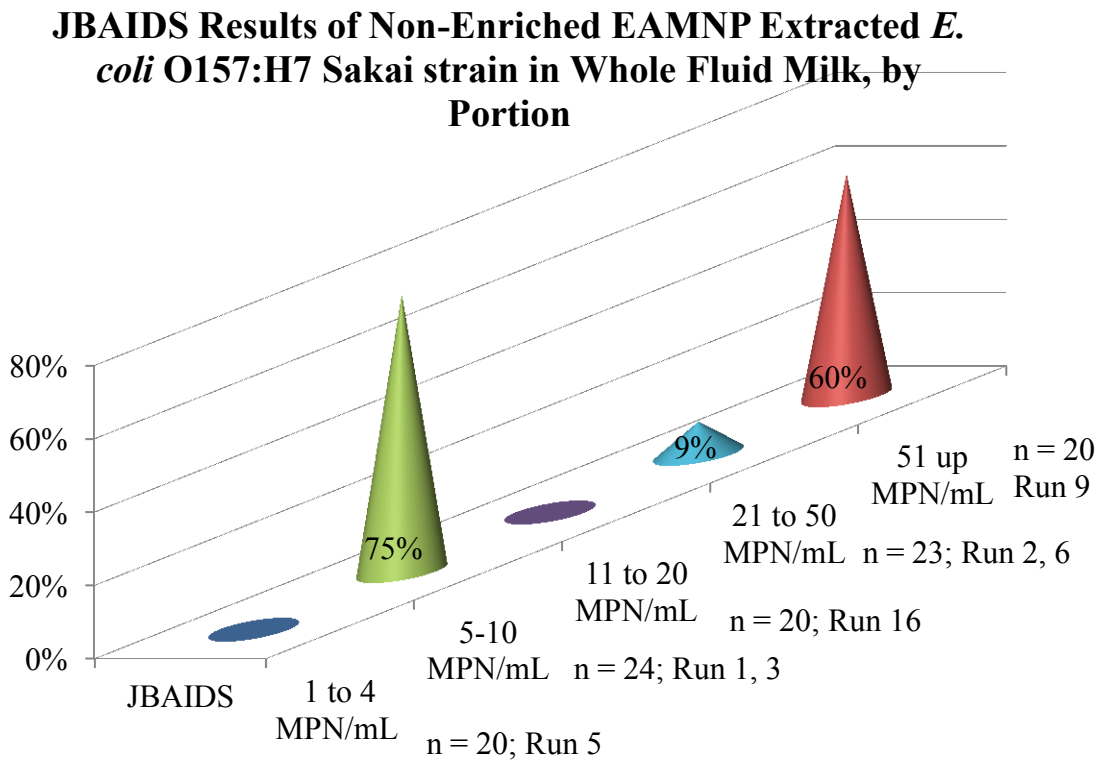


Figure 9.14: JBAIDS results in percent positive for each concentration. The over dilution of the PCR primers decreased detection sensitivity.

QPCR Results experiment # 2

As demonstrated in figure 9.15, the mixed set of STX 1 and STX 2 in lane 11, the two primer sets can be used on the *E. coli* O157:H7 Sakai strain together in the same reaction without primer-dimer interaction. The bands present at the appropriate sizes in the sample dilutions are discernible from each other. The in house gel was run slower and longer than the large QPCR gels were and that could be responsible for the clarity of the resultant gels that was not obtained in the larger gel. The STX 1 M is an in house designed primer we were testing. It was not used.

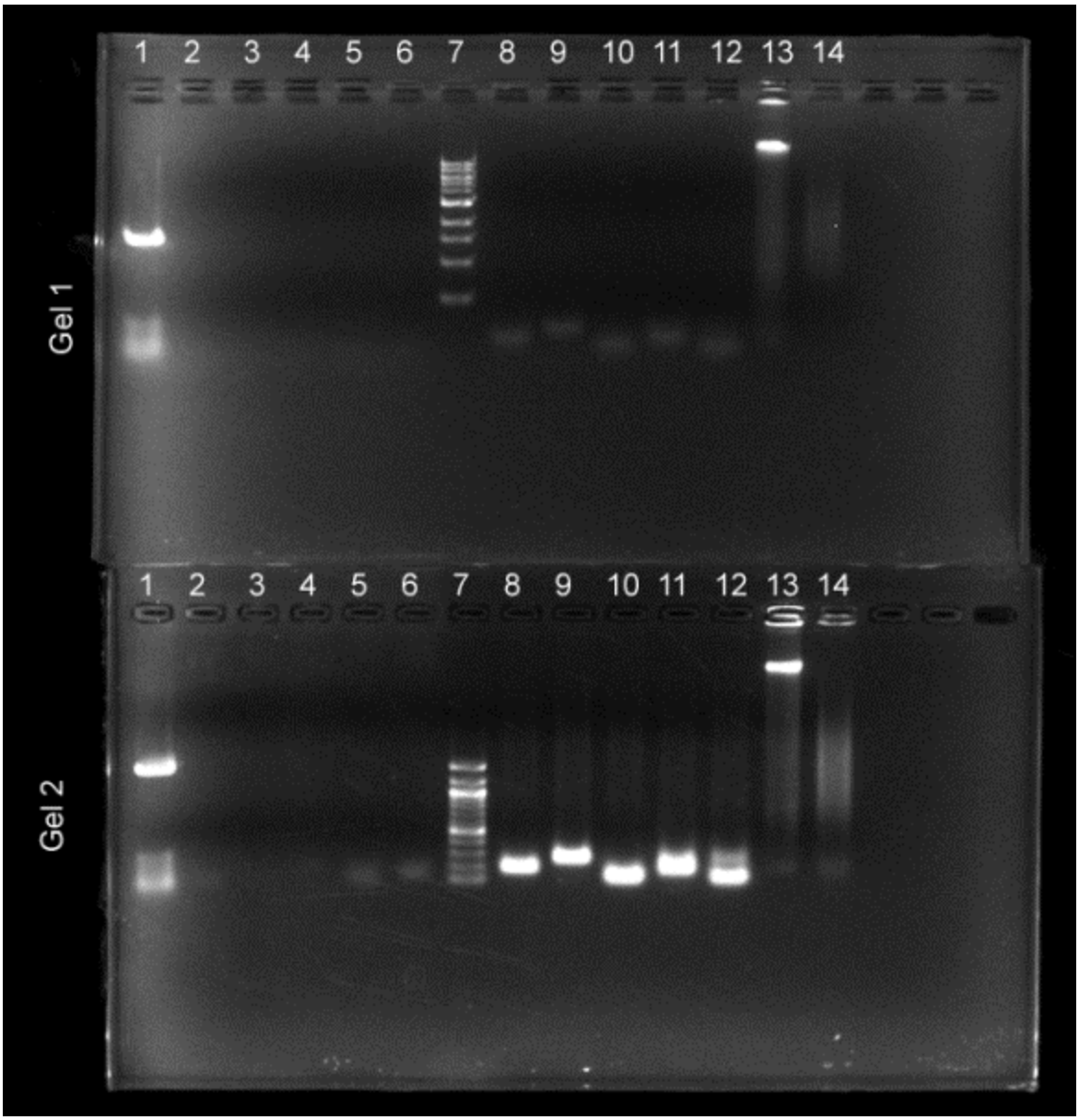


Figure 9.15: Practice 2% agarose gel on dilutions of a pure culture. Table 9.2 shows the lane designations

Table 9.1: Lane designations for the two agarose gels in figure 9.12.

1	Ladder	10bp
2	Primer Only	Stx1-c
3	Primer Only	Stx2-c
4	Primer Only	Stx1-M
5	Primer Only	Stx1-c & Stx2-c
6	Primer Only	Stx1-M & Stx2-c
7	Ladder	100bp
8	Template	Stx1-c
9	Template	Stx2-c
10	Template	Stx1-M
11	Template	Stx1-c & Stx2-c
12	Template	Stx1-M & Stx2-c
13	Template	Stock DNA - No Rxn
14	Template	No Primers

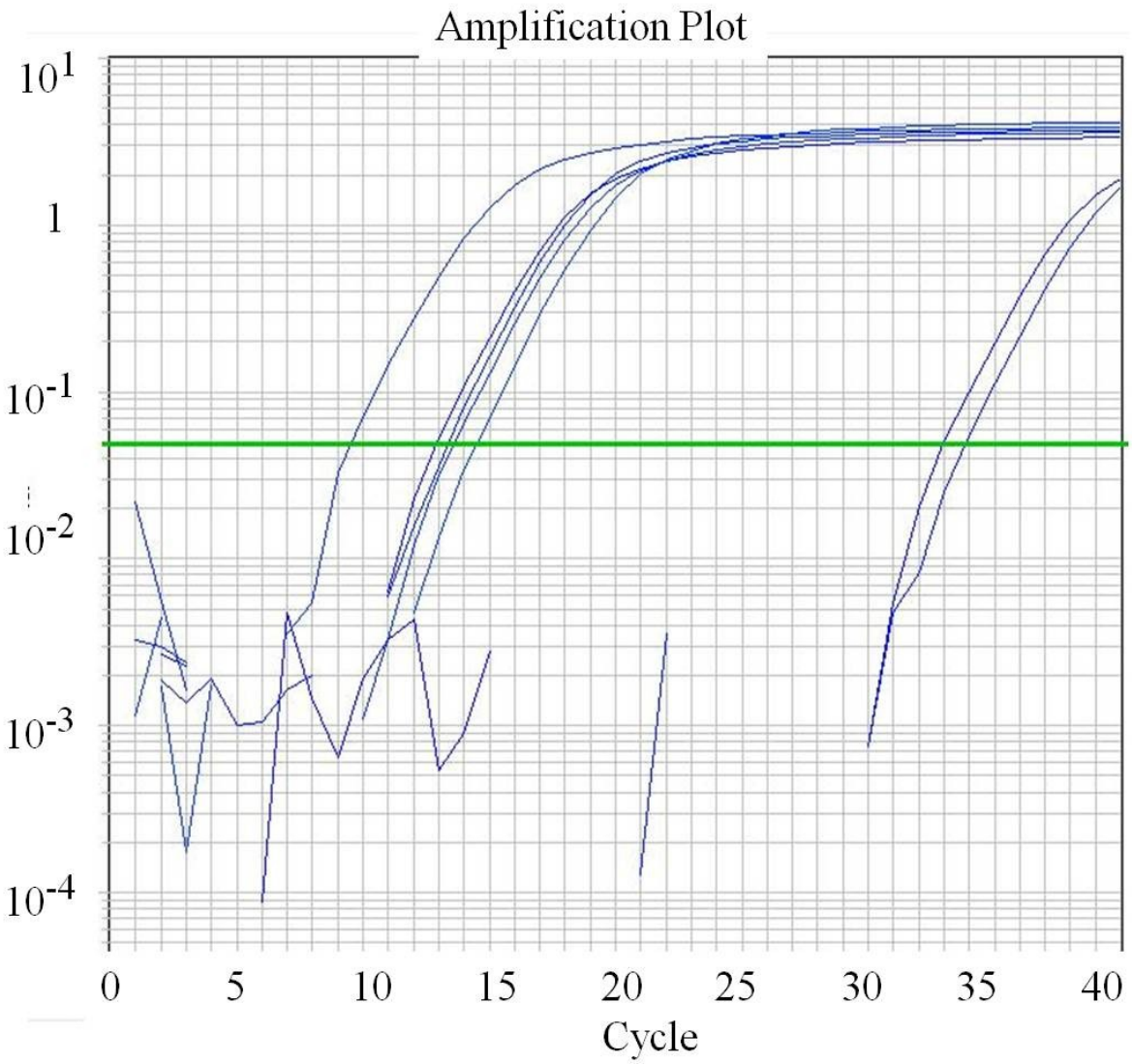


Figure 9.16: QPCR results from practice run with mixed primer sets.

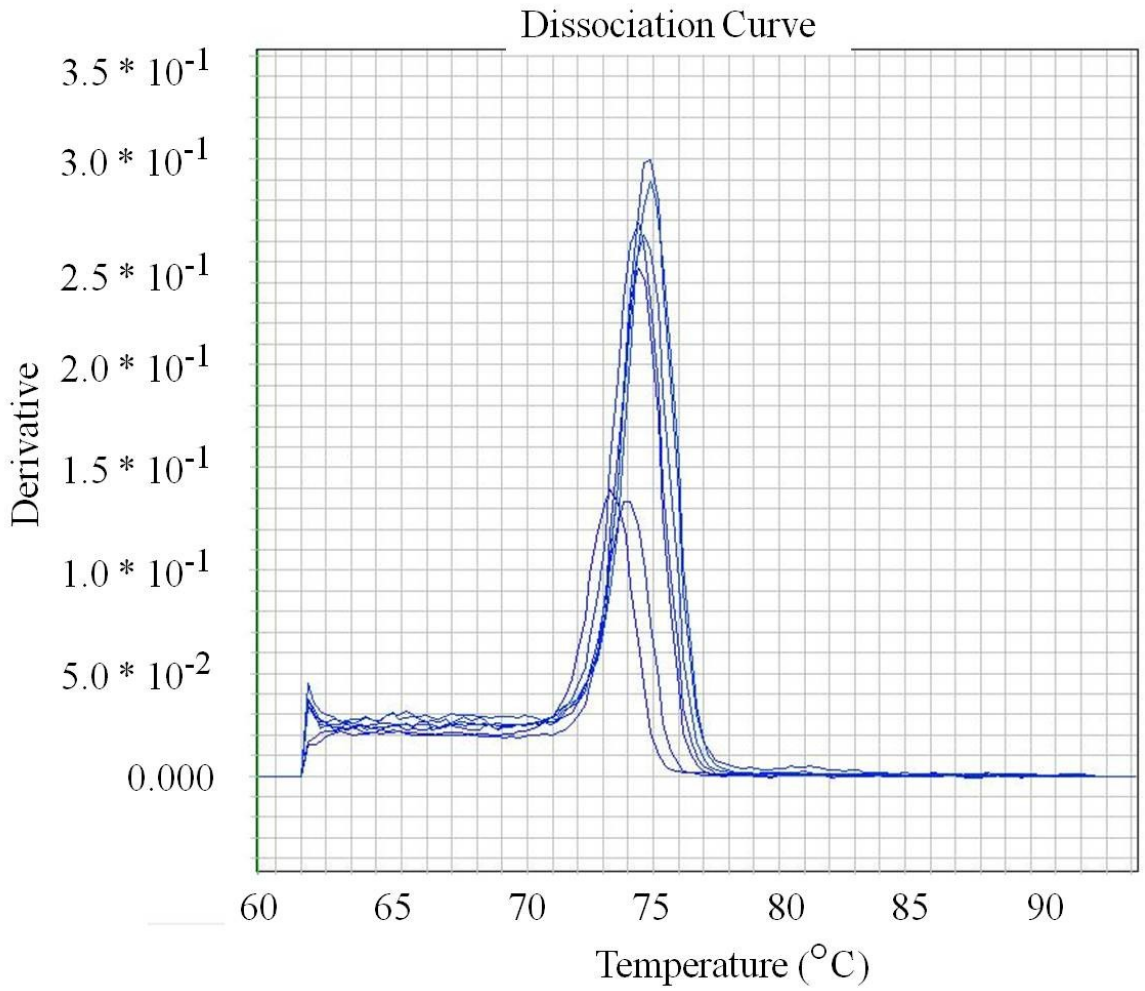
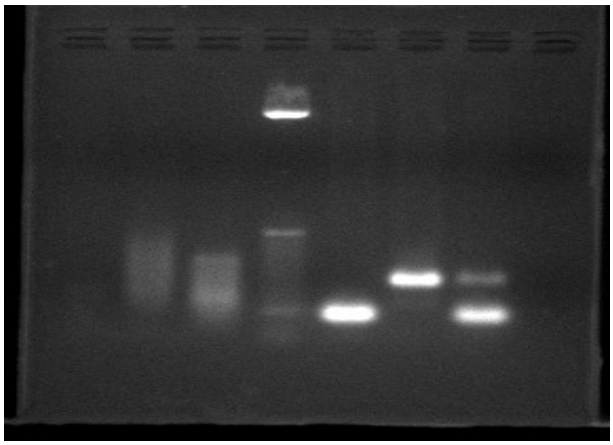


Figure 9.17: QPCR results from practice run with mixed primer sets.



1	**FDA + Run 9**	**STX 1**
2	**FDA + Run 9**	**STX 2**
3	**FDA + Run 9**	**STX 1 & 2**
4	**Broth 300ng**	**STX 1**
5	**Broth 300ng**	**STX 2**
6	**Broth 300ng**	**STX 1 & 2**

Figure 9.18: 2% agarose gel on QPCR product. FDA positive (+) samples were spiked with *E. coli* O157:H7 Spinach strain with a pGFPuv plasmid. Broth was a serial dilution of ethanol extracted DNA from *E. coli* O157:H7 Sakai strain broth cultures.

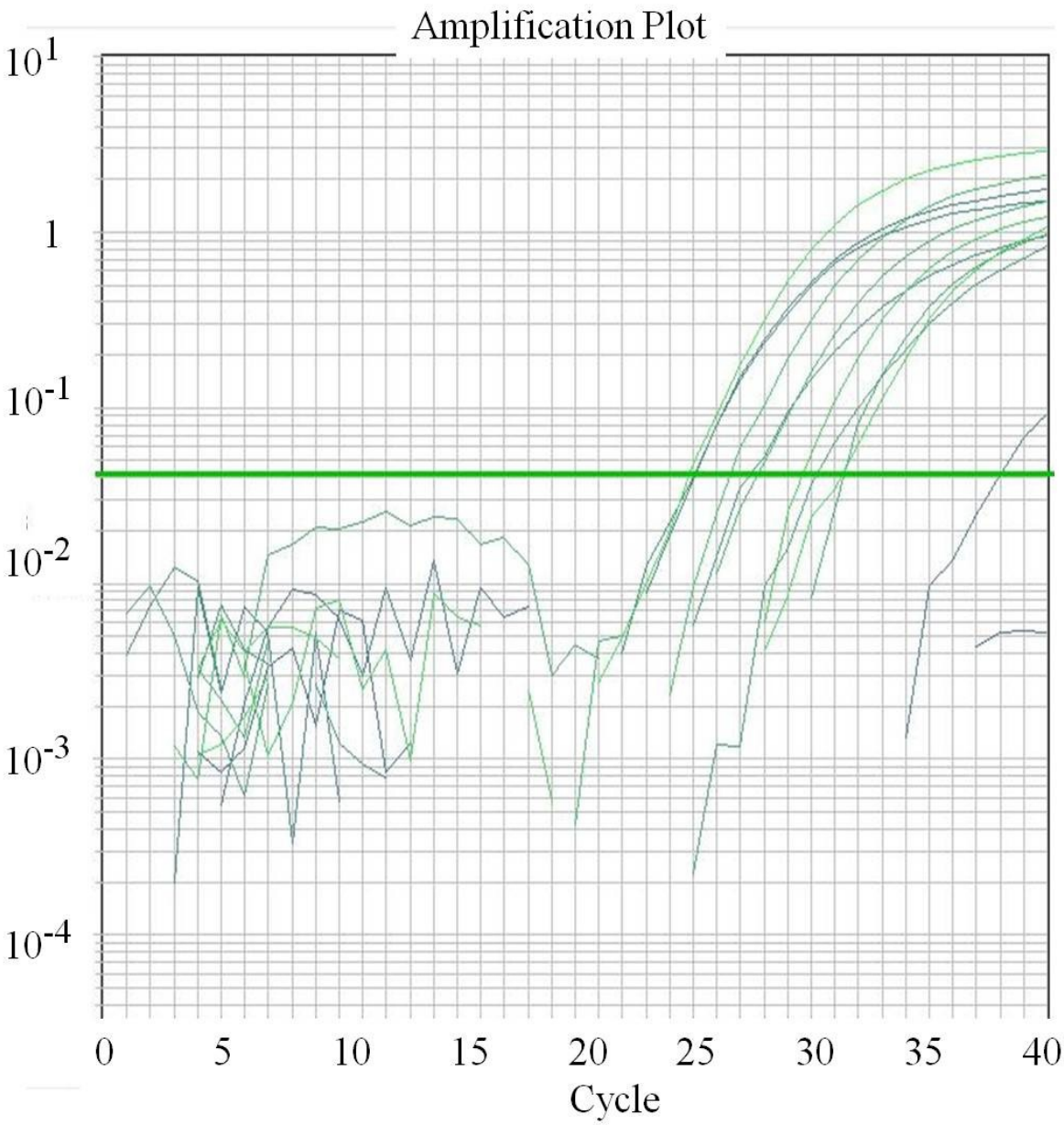


Figure 9.19: Run #17 Portion 1-12 QPCR Dissociation curves.

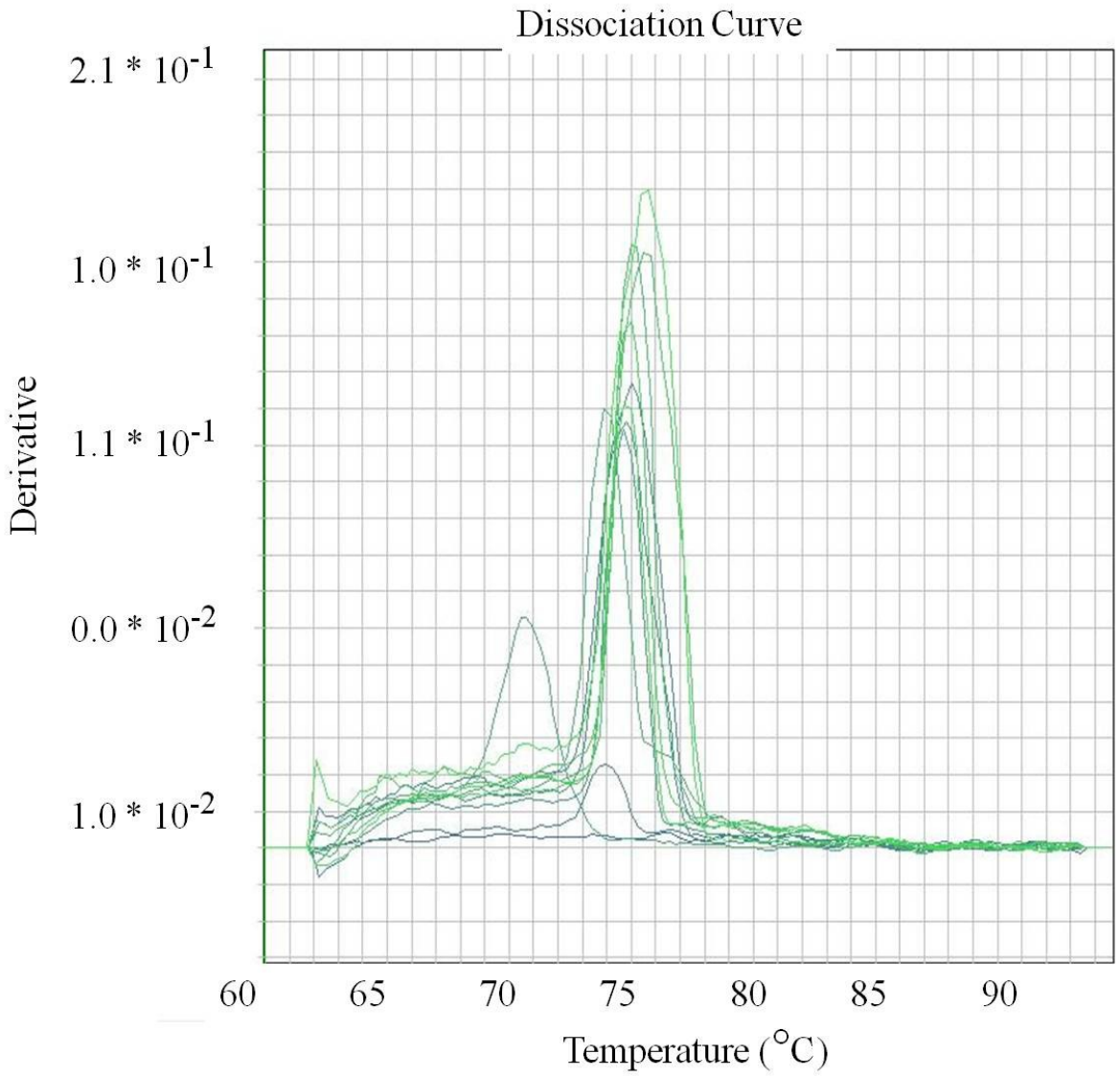


Figure 9.20: Run #17 Portion 1-12 QPCR Dissociation curves.

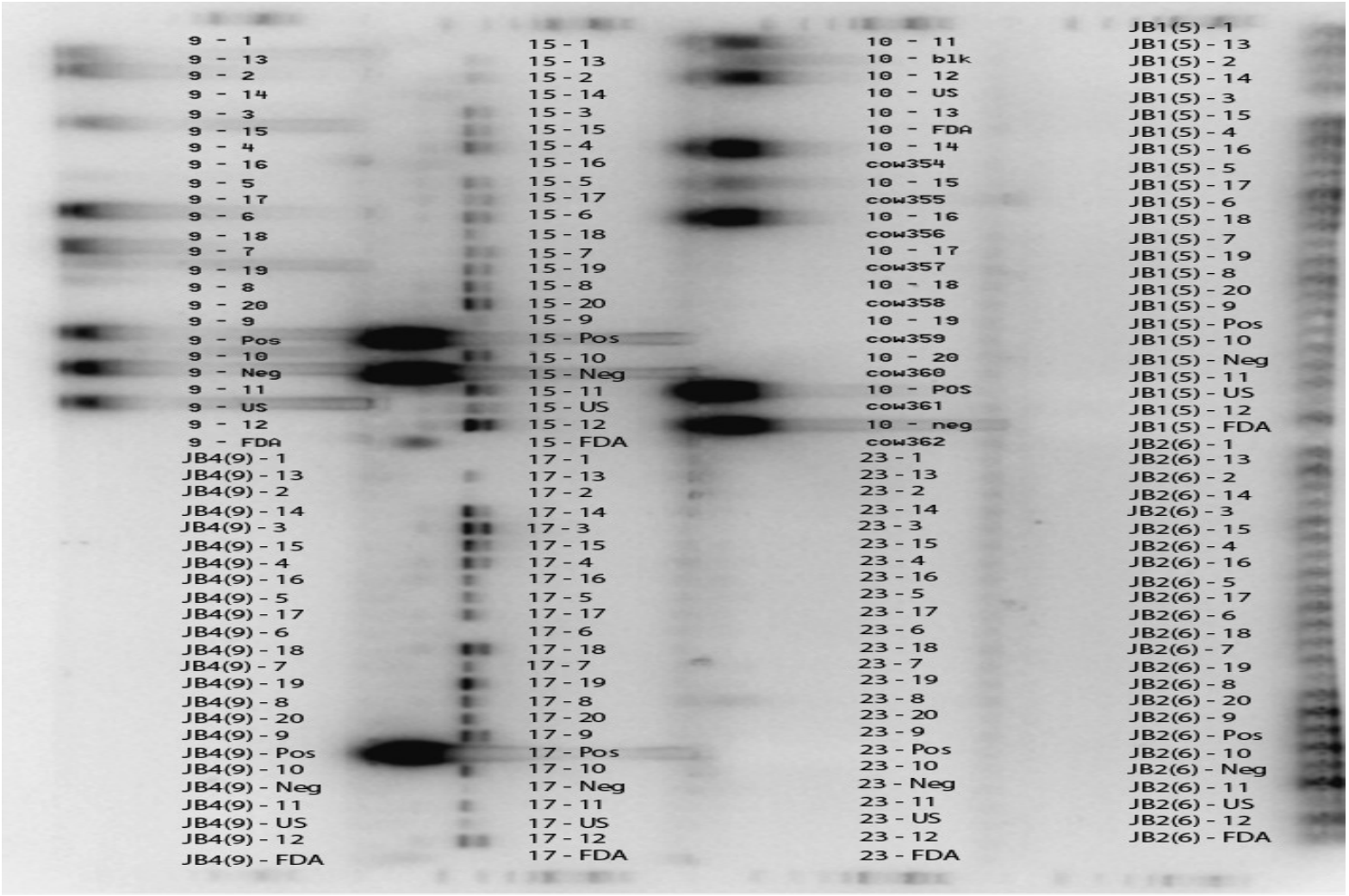


Figure 9.21: Large Gel # 2 of QPCR product. The 100 bp ladder is on either end of each of 4 rows. Positive bands are at 417 bp for STX 1 (narrow melt peak at 72^{o}C) and 263 bp (wide melt peak at 71.5^{o}C) (Bellin, et al, 2001). The remaining gel images are available in Appendix II.

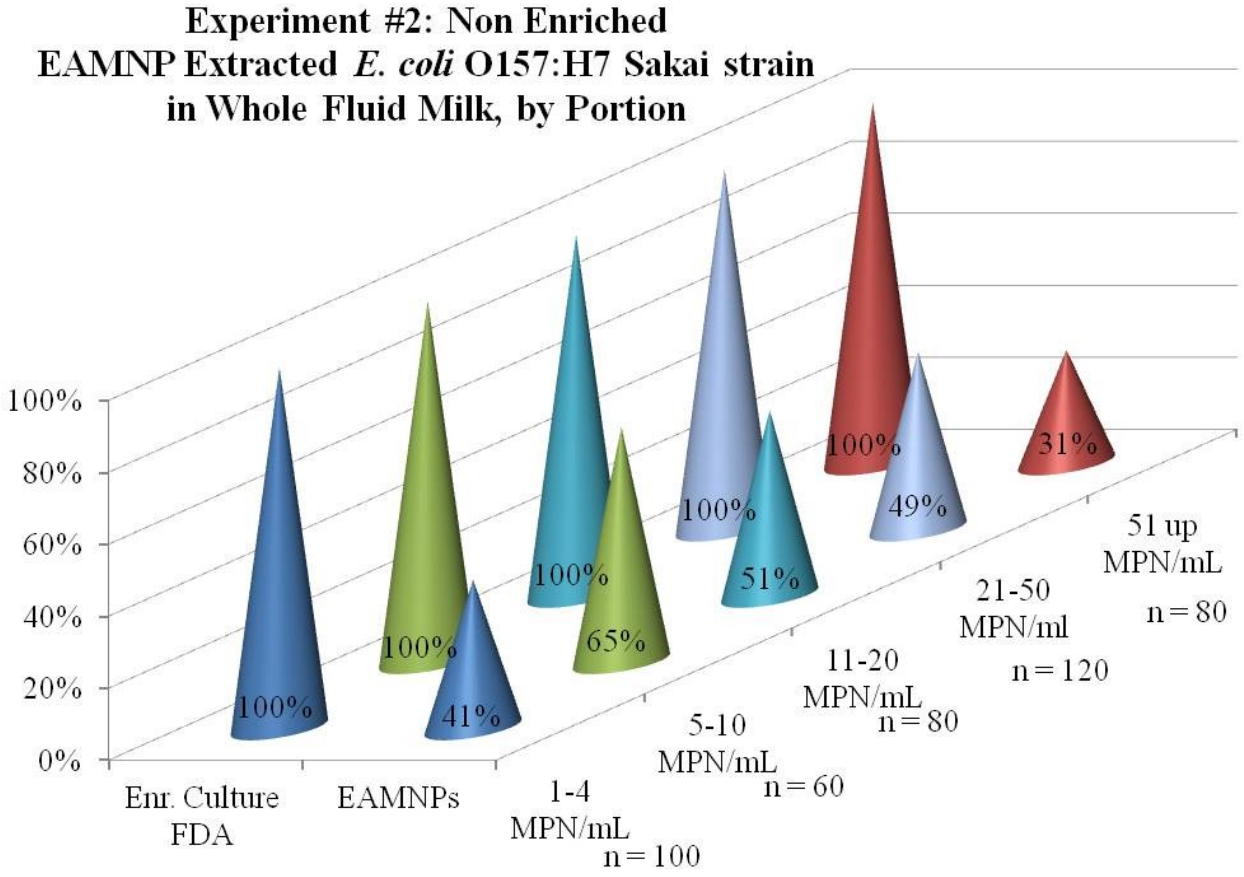


Figure 9.22: Experiment #2 EAMNP extraction results, by portion. Spiked bulk tank milk at varying concentrations evaluated by 20 portions in the FDA BAM enrichment and culture verses 20 portions without enrichment and IMS separation with Mab-EAMNPs. The IMS separated portions were split and evaluated by M^3 Biosensor, culture and PCR when possible.

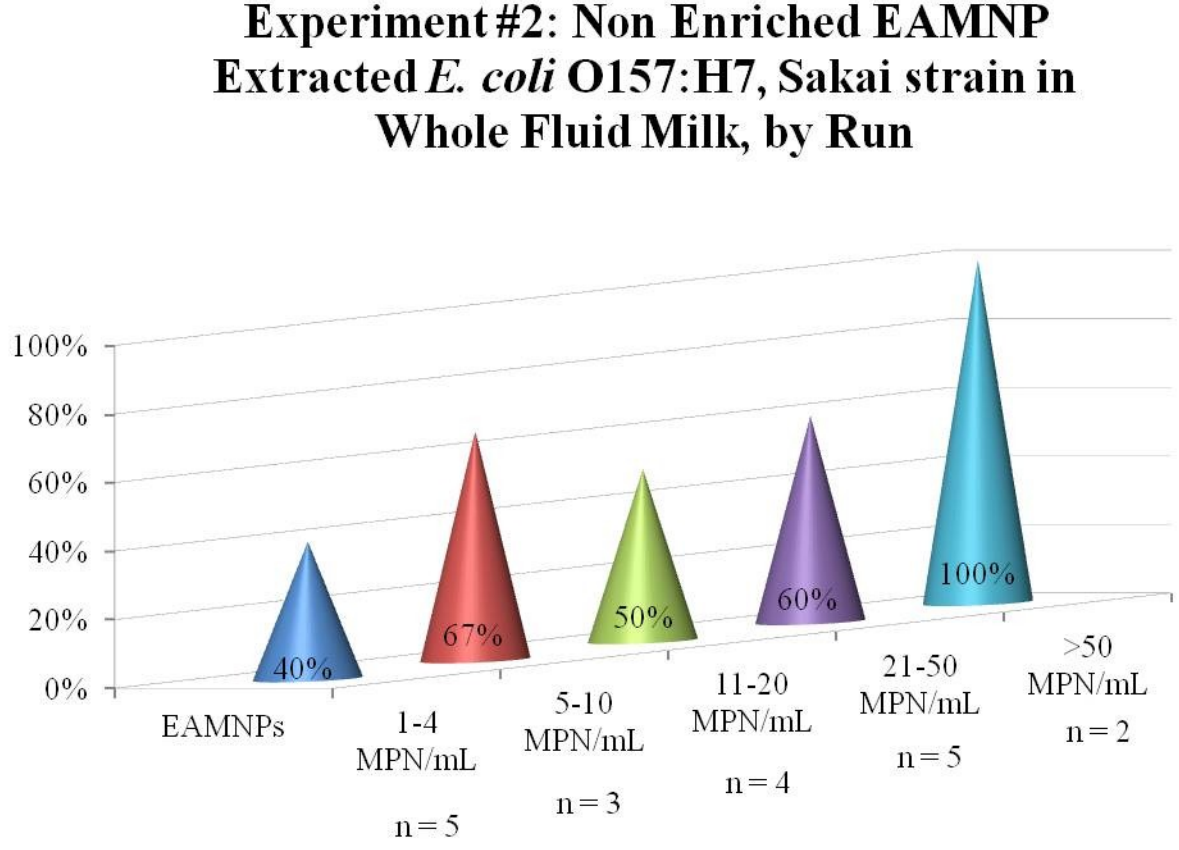


Figure 9.23: Experiment #2, by run. The percent positive samples by comparison methods for EAMNP extraction from whole fluid milk.

Experiment #3

Four more milk runs were attempted in experiment # 3 with newly produced EAMNPs and newly produced D-FSPCEs.

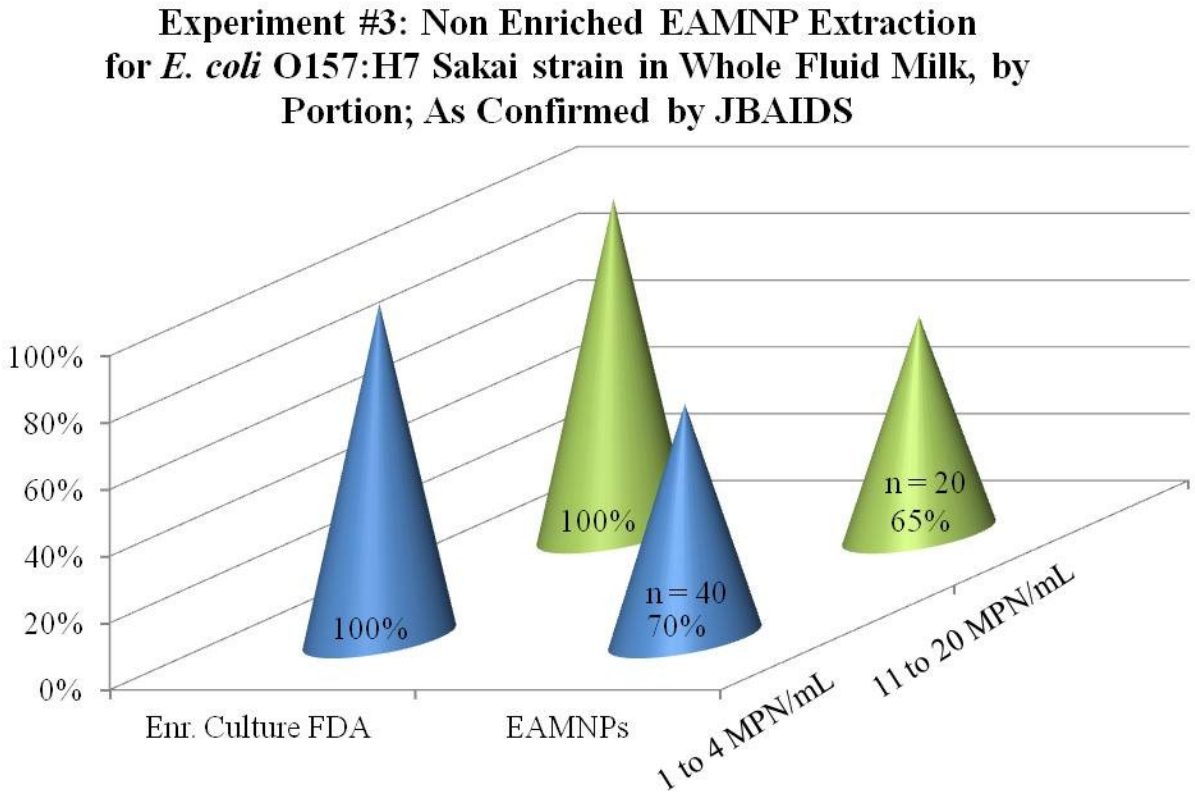


Figure 9.24: Experiment #3 EAMNP extraction results, by portion. Spiked bulk tank milk at varying concentrations evaluated by 20 portions in the FDA BAM enrichment and culture verses 20 portions without enrichment and IMS separation with Mab-EAMNPs. The IMS separated portions were split and evaluated by M^3 Biosensor, culture and PCR when possible

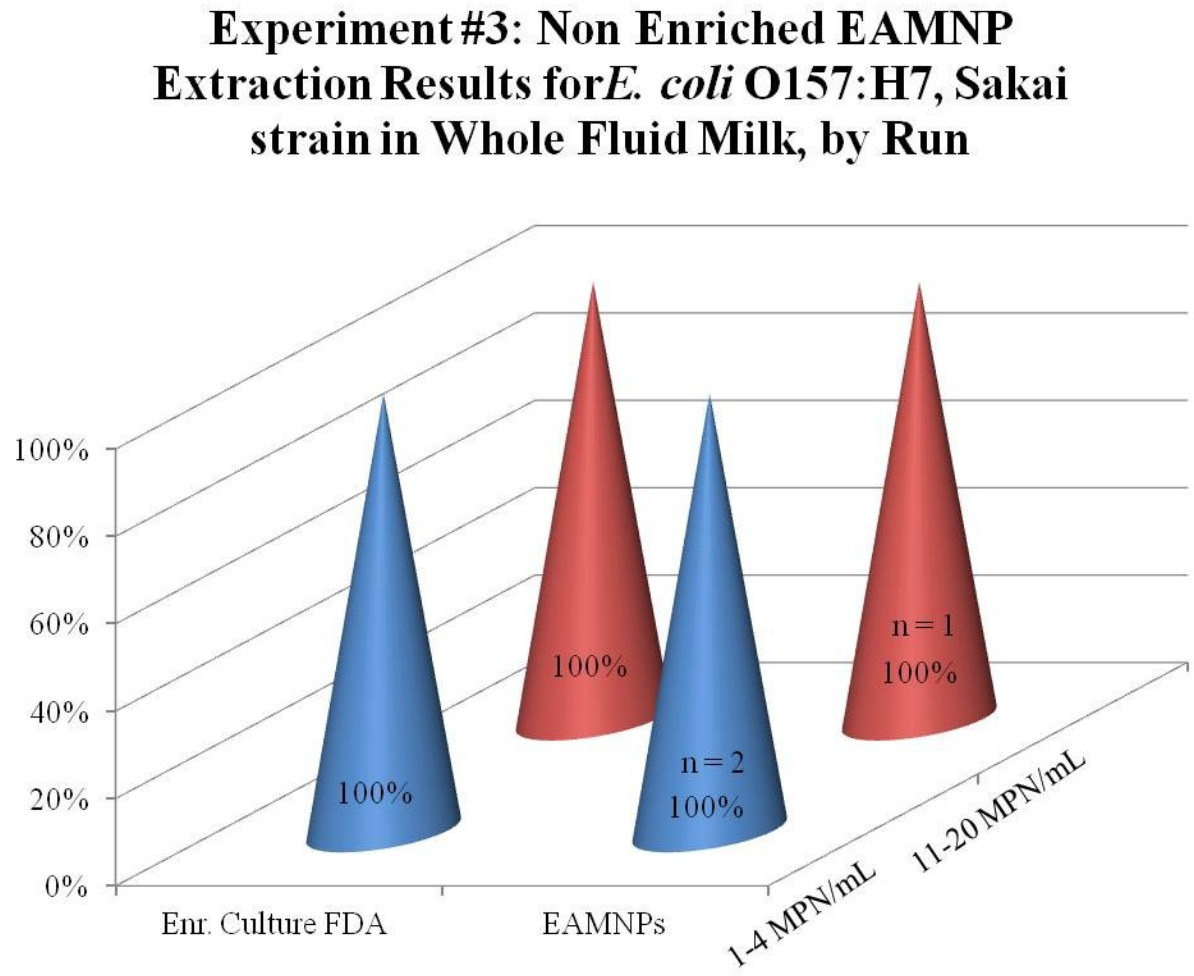


Figure 9.25: Experiment #3, by run. The percent positive samples by comparison methods for EAMNP extraction from whole fluid milk.

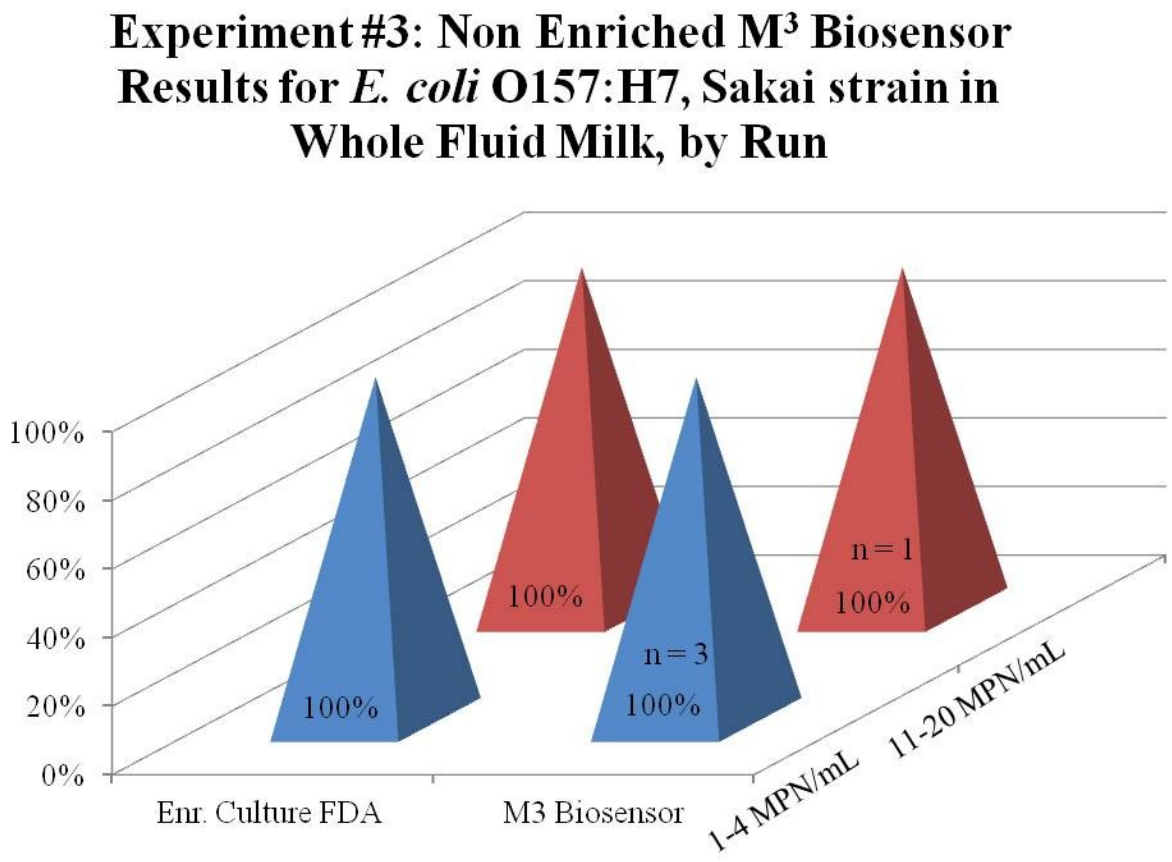


Figure 9.26: Experiment #3, by run. The percent positive samples by comparison methods for the M^3 Biosensor from whole fluid milk

Overall cumulative results of the M^3 Biosensor.

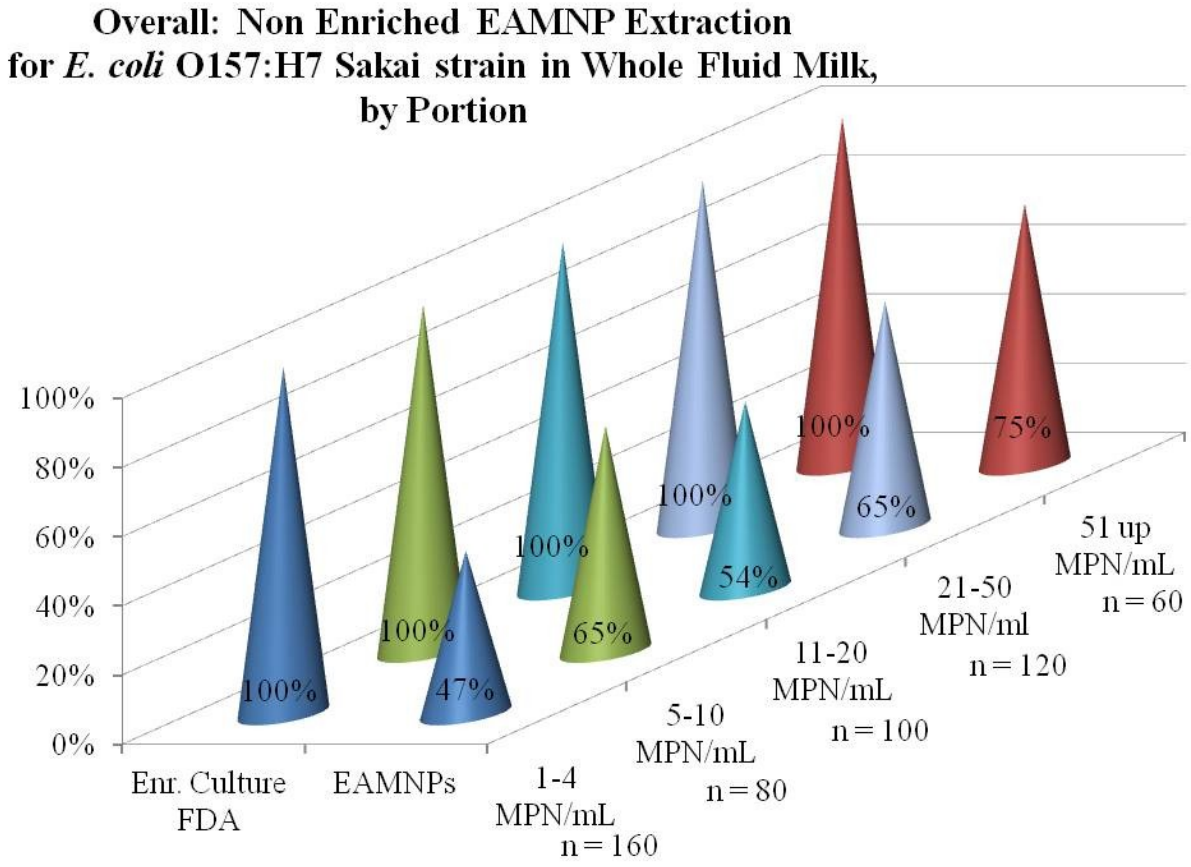


Figure 9.27: Complied results of all milk trials of the EAMNP extraction part of the M^3 Biosensor, by portion. Any one of the detection techniques positive made the portion positive.

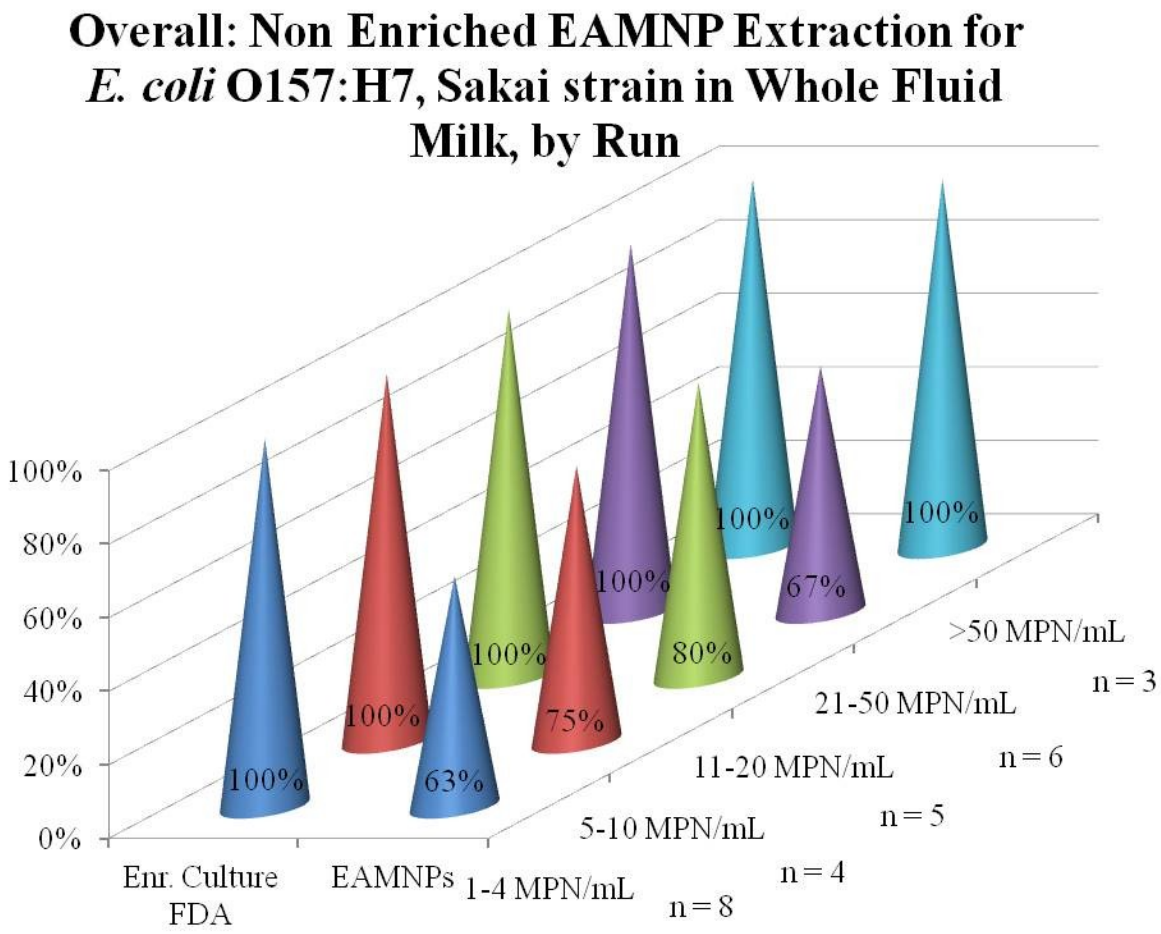


Figure 9.28: Compiled results of all whole fluid milk trials of the EAMNP extraction part of the M^3 Biosensor, by run. Detection by any one detection technique (JBAIDS, Culture, Q-PCR) was considered a verified positive sample by the M^3 system.

Discussion

Experiment # 1

Due to the non homogenous nature of food matrices and the low level of concentration, the AOAC PTM standards require 20 portion replicates per run. When treated as a unit, only 5-15 of the 20 need to be positive to consider the sample positive for the gold standard FDA

method or for the challenge method. The M^3 Biosensor had 100% agreement for all three of the runs with the FDA gold standard and by the JBAIDS PCR. This gives a 100% sensitivity and 100 % specificity for experiment #1. Capture, extraction and detection of *E. coli* O157:H7 is possible for samples at 10 CFU/mL using EAMNPs IMS and D-FSPCE cyclic voltammetry evaluations as the M^3 Biosensor with system resistance across the cycle as the parameter of interest. The resistance increasing from the blank in the presence of cells may mean that the conductance of the polyaniline is overridden by the resistance of the current by the cells present.

Only one run at the level of 10 MPN/mL has been accomplished, but this biosensor system was able to correctly identify 20 of the 20 portions at that level, where culture could only identify 2 of those portions. Figure 9.9 demonstrates the inability of culture to pick up even IMS separated samples at the 10 MPN/mL level. The splitting of the EAMNP IMS separated portions into three fractions may be responsible, but only 2 of 20 were positive on culture. The results were verified by portion in the JBAIDS PCR for 14 of 20 and concurrently verified in the FDA BAM gold standard method for 20 of 20 parallel samples. Further research is needed to determine the limits of detection and the LOD_{50} for this biosensor in whole fluid milk, but the preliminary results are promising. As for the lack of ability to culture the resultant captured and concentrated cells, a 1- 2 hour incubation of the 350 µL sample used for culture, could allow growth of the bacteria and subsequent culture visualization to confirm infectivity. Still, the science based decision to place the food on hold after a biosensor positive can be made in 2.5 hours with PCR confirmation. Culture on the IMS separated portion can be completed in 24 hours if infectivity is a concern instead of just contamination. Current regulatory response time is 36 hours.

This first experiment also shows that EAMNP separated bacteria can be placed into the JBAIDS PCR machine and find DNA. As shown in figure 9.9 at a concentration of 29 MPN/mL, portions 1-4 have a temperature difference on the melt curve from the kit positive control DNA (65°C) and the spiked milk positives from the *E. coli* O157:H7, Sakai strain (62°C). This is useful in future evaluation to demonstrate the strain differences in mixed strain PCR reactions. Using heat and bead beater tubes allowed adequate extraction and visualization of target DNA in the JBAIDS. Of the five portions tested, three were heat extracted, two were bead beater extracted. While all five were verified by one or the other of the duplicates as a positive, the two of the three heat extracted samples had inconclusive as one of the duplicate results. Bead beater tubes may be more advantageous when lower concentrations are evaluated.

Experiment #2

One of the problems identified in experiment #2 was the EAMNPs taken to the second laboratory were non functional. The TEM images in figure 9.12 show that all of the EAMNPs are visually similar except the batch used in experiment #2. They are not discrete particles and the area outside the large mass there can be seen multiple pieces of free iron oxide. Free iron oxide without the polyaniline coating cannot be conjugated to antibodies and therefore cannot capture bacteria. It is also not electrically active. This fact is verified with the bare SPCE results and the three point probe results shown in figure 9.11. Without electrical activity on the attached MNPs – the M^3 Biosensor cannot determine accurate resistance differences between cells versus no cells.

The second problem identified was that the D-FSPCEs were loaded with unverified gold nano-particles. Toward the end of 2011, the gold nano-particles that were being used on the D-

FSPCEs started to coagulate in the storage tube. Since this had never occurred before in more than two years, the origins of the particles in the tube were questioned. Instead of the stable commercial citrate gold nano-particles or the dextrin gold nano-particles made in the MSU Nano-biosensors laboratory, this batch that was now degrading was substituted on all of the D-FSPCEs used in experiment #2 and used on all the D-FSPCEs in the initial troubleshooting. These gold nanoparticles were made by a visiting technician with no training in gold nano-particle production nor any quality assurance measurements taken to determine their quality. As soon as the poor gold was eliminated, the M^3 Biosensor returned to closer to normal function. Therefore all the tests on the EAMNPs run in this experiment were unknowingly run on non-functional chips with non-functional beads.

The third problem with the M^3 Biosensor is the clumping of the EAMNPs during conjugation. Even when the same batch with the same buffers is used, it still occurs sporadically. This problem is not yet resolved but is shown to decrease the extraction capability of the EAMNPs by up to 20%. This may be a component to the decreased breadth of the detection method still present today, electrical activity in bulk may have returned to normal, but the continued sporadic clumping and the inconsistent detection in the subsequent broth trials indicate that when diluted, as in a food matrix, the electrical conductivity over all the individual EAMNPs is not the same as previous production was in previously published results. This will have to be resolved before a full validation can be performed.

The fourth problem involved the portable hand held potentiostat (PalmSens). That Palm Sens potentiostat is inconsistent as compared to other machines in the laboratory, but no diagnostic evaluations of the PalmSens have identified the cause of the inconsistency.

Poor quality EAMNPs, clumping of EAMNPs, poor quality gold nano-particles, physical equipment and supplies issues and damage to extracted samples by time and shipping all lead to a perfect storm of problems that caused the planned validation to be delayed. In order to determine if any data could be salvaged, the extracted samples were evaluated by multiple methods. The outstanding problems include the variable clumping of the EAMNPs and the Palm Sens machine damage. Surface charge and the addition of a surfactant into the production of the particles are being evaluated. We have hypothesized that the reason previous researchers have not seen this clumping phenomenon relates to concentration. In the original development, the EAMNPs were used at 10 mg/mL and now we use them at 0.5 mg/mL. At the higher concentration we lost specificity, but gained a cushion to the clumping affecting our capture. Hopefully, some of the testing being performed will solve the issue. If not increasing concentration or volume of EAMNPs added to each sample may have to be considered.

The use of the RAPD *E. coli* O157:H7 test kits on the JBAIDS had not been reported and there was no validated protocol at the time this experiment was started. This particular portion of our experiment was to show that the EAMNP extracted samples can be placed directly into the JBAIDS machine and can be detected with the RAPD *E. coli* O157:H7 test kits. Using the PPEK listed in the methods section, we were able to detect DNA from EAMNP extracted milk samples in as low as 51 MPN/mL in the JBAIDS as shown in figure 9.10. The sporadic results, at all concentrations, are consistent with the difficulty that occurred in the extraction process due to the clumping of the EAMNPs and the incorrect tube shape for optimum extraction. The damage to the shipped samples was not a factor as all JBAIDS evaluations were done within days of the extraction itself. For the runs that were completely negative 5, 16 and 6 were at concentrations 2, 19 and 23 MPN/mL respectively. The dilution of the PCR primers, probes and

reagents by four times by increasing the volume and splitting them into duplicates could very well have been responsible for this failure to visualize those three runs. Run 9, with positives at 51 MPN/mL, shows the extraction process followed by JBAIDS detection is possible even at low levels. With the benefit of the QPCR data having positives at all those concentrations under the same dismal extraction conditions, it is very possible that DNA was present in those three runs, but could not be detected by the JBAIDS. For the QPCR runs, we extracted the DNA with an ethanol precipitation, pelleting and thus concentrating the DNA if any was present. For the JBAIDS, we took the remaining 250 µL of EAMNP extracted sample and placed it directly into bead beater tubes with the appropriate solutions, placed the bead beater tube onto the magnetic separator and moved the subsequent supernatant to the PPEK. Without the concentration, and the poor extraction from the EAMNP step to start with, there could have been insufficient DNA concentrations for the JBAIDS to detect in the 2 µL that was placed into the reaction. The remaining Platinum Path extracted samples were tested after evaporation and rehydration in the QPCR reactions below, but even the positive samples ended up negative indicating a mismatch in the DNA sequences between the Platinum Path kits and the primers we used in the QPCR reaction. In conclusion, the EAMNPs extracted sample can be placed directly into the JBAIDS system. The limits of detection will need to be determined. This indicates that the use of the JBAIDS PCR system with the RAPD *E. coli* O157:H7 test kits as a verification tool for the M^3 Biosensor in the field a viable reality.

Positive QPCR samples included those with a melt temperature at around 75^{o}C and one or two bands at the corresponding 417 bp for STX 1 and 263 bp for STX 2 (Bellin, et al., 2001). Based on the QPCR gel, the samples were loaded with continuous electricity lending to the different spacing on the ladders from one side of the gel to the other and from the first row to the

other three rows. With all the damage these cells undertook, there were many with smears in the gel indicating DNA degradation and many with large blobs at the bottom of the gel that are unidentified. No questionable samples were determined positive. Samples that were extracted with the JBAIDS platinum path kit were all negative, including the controls and the JBAIDS positive samples from Run 9. That may be due to the DNA sequence on the platinum path kit extraction beads. The corresponding sequence that matches the primers we chose may have been excluded. As it is a commercial kit, we do not have access to this information. As well, a second amplicon at around a melt peak of 85^{o}C was seen in many samples. This is found also by another lab on campus in unpublished data. The smeared samples and the second peak samples could not be evaluated further because the gels and the PCR product were inadvertently discarded. An attempt to genomify the samples would have been nice to determine if there was another part of the genome that was amplifying also. Cross contamination is a potential with all the manipulation these samples underwent, but the sheer numbers of positive DNA samples implies that they are not all cross contamination.

EAMNPs can extract and concentrate *E. coli* O157:H7 in whole fluid milk samples, without pre-enrichment at the <10 CFU/mL level. With all the damage and poor quality of the EAMNPs used, a percent positive of 41% at concentrations of 1-4 CFU/mL and 67% at 11-20 CFU/mL, with no pre-enrichment is promising. Concentrations that low do not consistently culture, demonstrating the ability of the EAMNPs to concentrate bacteria from a large volume. At very low levels, culture is too unreliable as a gold standard. Of the culture negative fractions, most of them had *E. coli* O157:H7 DNA upon PCR analysis and should have been considered true positives if detected in the M^3 Biosensor not false positives.

Experiment #3

Experiment #3 contains four runs with corrected EAMNPs and corrected D-FSPCE. The EAMNPs were able to extract 70% at the 1-4 MPN/mL level over the previous 41% in experiment #2. The M^3 Biosensor was able to detect all four runs with at least 5 of the 20 samples positive, without pre-enrichment. Only 3 of the runs had JBAIDS kits available to evaluate them. Running exactly as the RAPD *E. coli* O157:H7 test directions stated, the JBAIDS verified positive DNA in all three runs in up to 18 of the portions down to 2 MPN/mL. Used correctly, the JBAIDS can detect the DNA off EAMNPs without a pre-enrichment. The caveat to this is that ethanol extraction was used, not PPEK. It is unknown how consistent this result would be with the more field stable DNA extraction method.

Experiment #3 shows promise that the M^3 Biosensor can be validated in whole fluid milk and can be used in the JBAIDS for confirmation in the field especially after the production issues with the EAMNPs are resolved.

Compiled Results of Experiments 1-3.

When all three experiments are combined, (figure 9.24) the M^3 Biosensor achieved a 63% capture rate at 1-4 MPN/mL out of whole fluid milk. Given the difficult conditions, this result is encouraging for further development of the system. Due to the diverse nature of the results and the high degree of missing data from run to run and portion to portion, no statistics were performed on this data. The individual data sets in any one analysis would have such low numbers that no valid conclusions could be drawn. This data and the information gained will be

used to launch a repeat study following the same protocols to adequately evaluate the whole M^3 Biosensor.

The EAMNPs can extract E. coli O157:H7 in 200 mL of milk at low levels of contamination under poor conditions. They can be used to go directly into the JBAIDS PCR with the RAPID LT *E. coli* O157:H7 detection kits using at least the Platinum Path DNA extraction kit and they can be used successfully on the D-FSPCE platform for electrochemical analysis. Overall, the M^3 Biosensor shows great promise to be a solution to the field based diagnostics needed by the industry.

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