SIMPLIFIED HIGH-THROUGHPUT APPROACHES FOR MOLECULAR MARKERS RELEVANT TO HEALTH AND THE ENVIRONMENT

By

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ABSTRACT

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The use of nucleic acid-based approaches for detection and quantification of molecular targets in biology allows for rapid responses and potentially improved outcomes, whether it be successful treatment in human health situations, or protection of the environment. However, these approaches are often complex, requiring centralized laboratory facilities and skilled personnel. The use of simpler molecular approaches could allow these tests to be conducted in the field or at the point-of-care. Traditional nucleic acid-based approaches are also highly targeted, allowing the user to detect only a few targets per experiment. The use of higher-throughput technologies could allow full panel screening of many targets and samples in a single experiment. In this dissertation, the development and use of these simpler and high-throughput approaches has been shown for a number of biological applications relevant to human health and the environment. Direct nucleic acid amplification without sample processing has the potential to greatly reduce the time-to-results by allowing detection of molecular targets in the field or at the point-of-care. This was shown to be important for rapid detection of environmental DNA (eDNA) from aquatic invasive species, antimicrobial resistance genes (ARG) to rapidly determine treatment options, and human microRNAs directly from body fluids for diagnosis of diseases, cancers, or environmental exposure to toxicants. Similarly, higher-throughput molecular approaches were determined to be important for detection ARG panels in environmental and human samples to assess whether sources are anthropogenic or part of the natural resistome as well as for quantification of microRNA panels to determine differential

expression of microRNAs in response to environmental toxicants and members of the gut microbiome. Overall the development and use of these techniques can enhance a number of biological applications, resulting in improved environments and human health.

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KEY TO ABBREVIATIONS

3’ UTR: 3’ untranslated region

AMR: antimicrobial resistance

ANSORP: Asian Network for Surveillance of Resistant Pathogens

AR: antibiotic resistance

ARB: antibiotic resistance bacteria

ARDB: antibiotic resistance gene database

ARG: antibiotic resistance gene

BIP: backward inner primer

BPA: bisphenol A

BF: *Bacteroides fragilis*

CARD: Comprehensive Antibiotic Resistance Database

CARSS: Canadian Antimicrobial Resistance Surveillance System

CCD: charge coupled device

CDC: Center for Disease Control and Prevention

CFU: colony forming units

CLIA: Clinical Laboratory Improvement Amendments

CO1: cytochrome c oxidase subunit 1

CRE: carbapenem-resistant *Enterobacteriaceae*

CSF: cerebrospinal fluid

DEP: diethyl phthalate

DNA: deoxyribnucleic acid

dNTP: deoxyribose nucleoside triphosphate

EARS: European Antimicrobial Resistance Surveillance Network

eDNA: environmental DNA

EDTA: ethylenediaminetetraacetic acid

FIP: forward inner primer

GEAR: genome exponential amplification reaction

GF: germ-free

HDA: helicase dependent amplification

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome

IPA: integrated pathway analysis

KPC: *Klebsiella pneumoniae* carbapenemases

LAMP: loop-mediated isothermal amplification

LB: loop backwards

LF: loop forward

LOD: limit of detection

MGE: mobile genetic elements

mRNA: messenger ribonucleic acid

NARMS: National Antimicrobial Resistance Monitoring System

NASBA: nucleic acid sequence-based amplification

NDM-1: New Delhi metallo-β-lactamase-1

NTC: no template control

PAH: polycyclic aromatic hydrocarbons

PCR: polymerase chain reaction

POC: point-of-care

qPCR: quantitative polymerase chain reaction

RCA: rolling circle amplification

RDX: 1,3,5-trinitro-1,3,5,-triazine

RISC: RNA- induced silencing complex

RNA: ribonucleic acid

RPA: recombinase polymerase amplification

rRNA: ribosomal ribonucleic acid

RT-LAMP: reverse transcription loop-mediated isothermal amplification

SDA: stand displacement amplification

SFB: segmented filamentous bacteria

SMAP2: smart amplification process 2

SNR: signal-to-noise ratio

TAE: tris-acetate-EDTA

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

Th17: T helper 17

Tt: amplification time

TTP: time to positivity

UC: ulcerative colitis

CHAPTER I

Introduction and dissertation overview

1.1 Molecular approaches and technologies that allow simplified and high-throughput detection of nucleic acids

Since the invention of quantitative PCR, detection of nucleic acid markers in the laboratory has become greatly simplified and commonplace. Diseases, pathogens, and animals can all be detected using small amounts of clinical matrices such as blood, sputum, or urine, or environmental samples such as soil or water. Numerous methods have been derived from PCR, allowing for experimental customization of the target of interest. The usefulness of the method used is dependent on the specific application, for example, isothermal approaches allow for rapid, field-based detection of targets while the use of PCR or other approaches allow for highly parallel detection.

In general, methods used and evaluated in this dissertation can be divided into the following categories: i) amplification-based, and ii) label-free approaches. Both have advantages and disadvantages that allow them to be customized to the research questions and targets of interest. The lower-cost options of amplification-based approaches (such as quantitative PCR and isothermal amplification) allow for rapid detection of targets, provided the sequence of interest is known, allowing specific primers to be designed. The use of isothermal amplification techniques is advantageous for simplified or field-based applications because it does not require temperature cycling, making devices simpler, potentially more compact, and requiring lower energy costs. Commonly used methods for isothermal amplification include loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), nucleic acid sequence-based

amplification (NASBA), recombinase polymerase amplification (RPA), helicase-dependent amplification (HDA), genome exponential amplification reaction (GEAR), smart amplification process version 2 (SMAP2), and strand displacement amplification (SDA). Many of the methods described for isothermal amplification of DNA can also be modified to amplify RNA with the addition of reverse transcriptase (e.g., RT-LAMP). Each method is useful for different applications, as different isothermal temperatures are used, reaction times, and polymerases.

The use of traditional quantitative PCR which uses temperature cycling is useful for obtaining analytical sensitivities to single copy numbers as well as for allowing higher throughput detection. Though conventional thermal-cycling machines typically allow detection of 96 reactions per run, highly parallel options such as Wafergen SmartChip (which uses quantitative PCR) allow detection of 11,000 reactions simultaneously. This greatly increases the number of genes that can be evaluated in each run and/or the number of samples to be analyzed, making it particularly useful for detection of gene panels. Other approaches that allow high-throughput analysis of genetic markers including hybridization-based techniques such as nCounter by Nanostring. Using this approach, single copies of nucleic acids can be detected by direct counting.

1.2 Genetic markers for detection of aquatic invasive species

Aquatic invasive species are problematic to native species and local economies. For example, the cost of *Dreissena polymorpha* infestation to aquaculture is approximately $32.3 million per year in the Great Lakes (Pejchar and Mooney, 2009). The use of molecular approaches for detection of environmental DNA (eDNA) for aquatic invasive species (AIS) such as *Dreissena* sp. has the potential to increase the likelihood of early detection (Darling and

Mahon, 2011) thus enhancing the probability of successful eradication (Lodge et al., 2012). Compared to traditional survey methods, the detection of invasive species using eDNA may be 10- to 100- fold more economical (Hayes et al., 2005) and are presumed significantly more sensitive than traditional approaches. The most common genetic markers used for detection of aquatic invasive species are mitochondrial, due to increased specificity to the target of interest and greater variability between species (Hebert et al., 2003a, 2003b). A common gene analyzed is mitochondrial cytochrome c oxidase subunit 1 (CO1). The most commonly used method for detection of AIS eDNA is qPCR but this typically requires DNA extraction prior to amplification, thus requiring a centralized laboratory facility. Elimination of DNA extraction and purification by employing direct amplification simplifies the process and reduces time to results by eliminating the need for a centralized laboratory facility (Kanitkar et al., 2016; Kostic et al., 2015). However, direct amplification using PCR is difficult as PCR is often inhibited by components in the sample. For detection at very low abundance, sample concentration is often necessary, but this often leads to simultaneous concentration of substrates inhibitory to Taq polymerases used PCR (Harvey et al., 2009).

Isothermal amplification polymerases (such as *Bst* polymerase), are less impacted by PCR inhibitors (Koloren et al., 2011; Stedtfeld et al., 2015, 2014) and, compared to *Taq* polymerases, have been shown to work significantly better even when using crude lysates and whole cells (Kostic et al., 2015). The loop-mediated isothermal amplification (LAMP) technique is one such isothermal approach (63 °C) that utilizes *Bst* polymerase. LAMP could be well-suited for directly amplifying eDNA including cells, juveniles, eggs, or seeds, without extensive cell lysis as it has been shown to directly amplify unprocessed biological material in numerous other samples (Ebbinghaus et al., 2012; Gadkar and Rillig, 2008; Iwamoto et al., 2003; Misawa et al.,

2007; Qiao et al., 2007). Hence, direct isothermal amplification have the potential to complement eDNA-based surveillance programs for invasive species (Goldberg et al., 2015). In Chapter II, the use of direct isothermal amplification of eDNA from AIS is described.

1.3 Genetic markers for detection of antimicrobial resistance

Emergence of growing antimicrobial resistance (AMR) is a global crisis due to misuse of antibiotics accompanied by inaction or weak action (WHO, 2014). The Antibiotic Stewardship Program by the Centers for Disease Control and Prevention (CDC) is a U.S. initiative (CDC, 2014) to reduce bacterial selective pressure while improving patient outcomes. This requires accurate and rapid diagnostics of AMR though globally there are at least a dozen resistant organisms of concern (WHO, 2014) coupled with prudent use of antibiotics. Methods based on amplification of nucleic acids for detection of AMR are generally faster than traditional culture-based approaches but require extra time for transporting the sample to a centralized laboratory, processing of sample, and DNA purification and concentration. The use of POC devices are capable of rapidly diagnosing antibiotic-resistant infections which may help in making timely and correct treatment decisions. However, for most POC platforms, sample processing for nucleic acids extraction and purification is also generally required prior to amplification. Direct amplification has the potential to eliminate these steps without significantly impacting diagnostic performance. The potential impact of direct amplification on detection of AMR is reviewed in Chapter III.

However, currently there are thousands of known antimicrobial resistance genes (ARG) and mobile genetic elements that are important for dissemination of ARG via horizontal gene transfer. As such, the use of high-throughput technologies for detection of ARG is often needed,

particularly when characterizing ARG in the environment. The characterization of AMR in the environment is particularly challenging when it comes to separating the natural resistome from anthropogenic sources, though research in this area is on the rise. Chapter IV describes the characterization of AMR genes from Michigan surface waters, primary influent from three waste water treatment facilities, and ten clinical isolates from a regional hospital as evaluated via the highly parallel Wafergen system. The availability of a comprehensive database that differentiates the natural resistome from anthropogenic distribution of AMR in the environment would identify where changes are likely to be most effective for containment. Chapter IV also describes an application-based database that was developed to meet these challenges.

1.4 Detection of microRNAs as rapid biomarkers of disease and environmental exposure, and their role in gut health

MicroRNAs are short (~22 nucleotides), non-coding RNA molecules that account for >3% of all human genes (Bartel, 2004) and are important post-transcriptional regulators of gene expression. MicroRNAs regulate gene expression using a process of RNA-induced silencing complex (RISC) by which partial complementarity of the last 7-8 bases to the 3' untranslated region (3' UTR) of messenger RNAs (mRNAs), blocks translation and/or prevents mRNA degradation (Bartel, 2004). Though many mechanisms of gene expression regulation remain to be elucidated, it is known that a single microRNA can target many mRNAs and a single mRNA can have many microRNAs that target it (Taganov et al., 2007). It has also been suggested that microRNAs control noise during gene expression, by decreasing noise for lower expressed proteins while increasing noise for those highly expressed (Schmiedel et al., 2015). This allows microRNAs to have many regulatory roles in various cellular processes. As such, many

microRNAs are implicated in various diseases and cancers and forced overexpression of microRNAs has led to tumorigenesis in laboratory studies (He et al., 2005). In addition, microRNA levels in serum and plasma have been reported as up/down regulated between cancer patient samples and healthy controls, depending on the cancer type and microRNA studied. Recent evidence suggests that the inter-domain communication between the gut microbiome and host may in part occur via microRNAs which are often differentially expressed in the presence of bacteria and can even be released and taken up by bacteria. Chapter VI reviews this evidence and suggests that environmental exposure to toxicants impacts this communication.

Detection of microRNAs is often difficult, however, due to their short length and typically requires RNA extraction prior to detection, either via amplification or hybridization-based approaches. Amplification-based methods for measurement of microRNAs include stem-loop reverse transcription polymerase chain reaction (RT-PCR; Chen et al., 2005), rolling circle amplification (Harcourt and Kool, 2012; Liu et al., 2013; Zhou et al., 2010), loop-mediated isothermal amplification (LAMP; Li et al., 2011), exponential amplification reaction (EXPAR) (Wang et al., 2014; Zhang and Zhang, 2012). Isothermal approaches have the advantage of simplicity in terms of constant temperature and high amplicon yields (Mori et al., 2001), which allow for quantification with simpler devices at the point of care. However, sample concentration for microRNA is often challenging due to low abundances and because amplification-based techniques typically require RNA isolation in centralized laboratories. However, isothermal polymerases (e.g. *Bst*) are more robust and less impacted by inhibitory substrates compared to PCR polymerases (Kostic et al., 2015; Stedtfeld et al., 2014). Thus, an isothermal direct amplification approach has the potential to reduce analysis time and costs, without isolation and purification, and is therefore well suited for use at the point of care (Njiru, 2012). As such, in

Chapter V, a novel isothermal amplification methodology is described for the rapid, direct measurement of microRNAs in clinical matrices.

In cases where higher throughput analysis of microRNAs is desired, such as when evaluating the differential expression of microRNAs in response to gut microbes and environmental toxicants, the use of hybridization-based approaches such as Nanostring nCounter is more useful. A panel of 600 microRNAs can be detected and quantified, without amplification. Chapter VII describes the differential expression of microRNAs in response to environmental toxicants and members of the gut microbiome.

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CHAPTER II

Isothermal amplification of environmental DNA (eDNA) for direct field-based monitoring and laboratory confirmation of *Dreissena* sp.

The work in Chapter II has been published:

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Author contribution statement:

M. R. W., R. D. S., and S. H. are responsible for experimental design. M. R. W., C. E., P. S., and T. M. S. conducted experiments. M. R. W., U. F., and S. H. designed the application for training volunteers. J. L. arranged network of volunteers for sample collection. E. D., R. J. S., and S. H. are responsible for study design. M. R. W., R. D. S., E. D., R. J. S., J. L., and S. H. wrote and/or edited the manuscript.

Abstract

Loop-mediated isothermal amplification (LAMP) of aquatic invasive species environmental DNA (AIS eDNA) was used for rapid, sensitive, and specific detection of *Dreissena* sp. relevant to the Great Lakes (USA) basin. The method was validated for two uses including i) direct amplification of eDNA using a hand filtration system and ii) confirmation of the results after DNA extraction using a conventional thermal cycler run at isothermal temperatures. Direct amplification eliminated the need for DNA extraction and purification and allowed detection of target invasive species in grab or concentrated surface water samples, containing both free DNA as well as larger cells and particulates, such as veligers, eggs, or seeds. The direct amplification method validation was conducted using *Dreissena polymorpha* and *Dreissena bugensis* and uses up to 1 l grab water samples for high target abundance (e.g., greater than 10 veligers (larval mussels) per l for *Dreissena* sp.) or 20 l samples concentrated through 35 µm nylon screens for low target abundance, at less than 10 veligers per liter water. Surface water concentrate samples were collected over a period of three years, mostly from inland lakes in Michigan with the help of a network of volunteers. Field samples collected from 318 surface water locations included i) filtered concentrate for direct amplification validation and ii) 1 l grab water sample for eDNA extraction and confirmation. Though the extraction-based protocol was more sensitive (resulting in more positive detections than direct amplification), direct amplification could be used for rapid screening, allowing for quicker action times. For samples collected between May and August, results of eDNA direct amplification were consistent with known presence/absence of selected invasive species. A cross-platform smartphone application was also developed to disseminate the analyzed results to volunteers. Field tests of the direct amplification protocol using a portable device (Gene-Z) showed the method could be used in the field to obtain results

within one hr (from sample to result). Overall, the direct amplification has the potential to simplify the eDNA-based monitoring of multiple aquatic invasive species. Additional studies are warranted to establish quantitative correlation between eDNA copy number, veliger, biomass or organismal abundance in the field.

2.1 Introduction

The use of environmental DNA (eDNA) for aquatic invasive species (AIS) detection has the potential to increase the likelihood of early detection (Darling and Mahon, 2011) and enhance the probability of successful eradication (Lodge et al., 2012). Simplifying the analytical approach and decreasing the time-to-result is a key first step in developing rapid, field-deployable nucleic acid- based eDNA detection methods. Direct amplification, i.e., amplification without DNA extraction or purification, satisfies both these attributes. Elimination of DNA extraction and purification steps simplifies the process and may avoid the need for sample transport (Kanitkar et al., 2016; Kostic et al., 2015). For detection of invasive species at very low abundance, sample concentration is often useful and necessary. However, sample concentration may also lead to simultaneous concentration of substrates inhibitory to Taq polymerases used in polymerase chain reaction (PCR)-based eDNA assays (Harvey et al., 2009).

Isothermal amplification polymerases (such as *Bst* polymerase), have been found to be less impacted by the PCR inhibitors (Koloren et al., 2011; Stedtfeld et al., 2015, 2014). Compared to Taq polymerases, they have been shown to work significantly better even when crude lysates and whole cells are used as targets for amplification (Kostic et al., 2015). The loop-mediated isothermal amplification (LAMP) technique is one such isothermal approach (63 °C) that utilizes *Bst* polymerase. LAMP could be well-suited for directly amplifying eDNA including

cells, juveniles, eggs, or seeds, without extensive cell lysis and has been shown to directly amplify relatively unprocessed biological material such as cells, spores, and parasites (Ebbinghaus et al., 2012; Gadkar and Rillig, 2008; Iwamoto et al., 2003; Misawa et al., 2007; Qiao et al., 2007). Hence, direct isothermal amplification (i.e., amplification without carrying out DNA extraction and purification), combined with simpler field-deployable concentration approaches for samples containing much lower abundance of target species, have the potential to complement eDNA-based surveillance programs for invasive species (Goldberg et al., 2015).

To enhance the likelihood of detection, sample concentration (increasing the quantity of DNA or particles per unit volume) must be performed for low population abundances and is typically conducted in a laboratory either by filtration of 45 ml to 2 l water samples (Collins et al., 2012; Mahon et al., 2013; Takahara et al., 2013; Wilcox et al., 2015) through membranes of 0.45 to 10 µm pore size filters followed by eDNA extraction (Turner et al., 2014; Wilcox et al., 2015) or by eDNA precipitation (Ficetola et al., 2008). Filtration is time consuming and often leads to filter clogging. However, it is possible to filter large volumes which may be needed at very low abundances (Huq et al., 2012; Wilcox et al., 2013) by using larger pore size (e.g., 10 to 60 µm (Wilcox et al., 2015)) filters and simultaneously collect sloughed tissues, veligers, juveniles, and fecal matter. In fact, filtration of large volumes is routine using plankton net tows to collect and concentrate microscopic organisms (Horvath and Crane, 2010).

Overall, invasive species surveillance programs are currently hampered by the number of samples and the time required in getting them to the lab for processing. We hypothesize that by concentrating these cells using larger pore size filters in combination with direct amplification of eDNA in the field (both extracellular and present within these larger cells), we can increase likelihood of detection by providing a rapid methodology that could eliminate the need for

complex sample processing. Furthermore, providing a laboratory-based confirmation of results could increase sensitivity and enhance the likelihood of detection. In this study, a direct eDNA amplification approach based on loop-mediated amplification was developed for the rapid detection of *Dreissena* sp. in the field. This methodology is further confirmed by isothermal amplification in the laboratory using eDNA extracted from 1 l samples. To test the effectiveness of this method, a total of 318 surface water samples were collected and analyzed. The direct amplification protocol was also validated in a pilot experiment using a field-deployable, real time isothermal amplification device (Gene-Z) to evaluate amplification from sample-to-result under field conditions. To our knowledge, this study represents the first attempt of using a direct amplification approach for eDNA detection and has the potential for rapid (under 90 min), field-based detection of invasive *Dreissena* sp.

2.2 Methods and Materials

2.2.1 Loop-mediated isothermal amplification for *Dreissena* sp. detection

Loop-mediated isothermal amplification mixture consisted of 1X isothermal amplification buffer (New England BioLabs; Ipswich, MA), 1.4 mM each dNTP (Invitrogen; Carlsbad, CA), 0.8 M Betaine solution (Sigma-Aldrich; St. Louis, MO), 6 mM $MgSO_4$ (New England Biolabs; Ipswich, MA), 6.4 U *Bst* Polymerase 2.0 WarmStart (New England Biolabs, Ipswich, MA), 1 µl primer mixture (described in the next section), 20 µM SYTO82 Orange Fluorescent Nucleic Acid Stain (ThermoFisher Scientific; Waltham, MA), 2.8 µl DNA extract, and PCR-grade water to a 10 µl total reaction volume (Tomita et al., 2008). Incubation for amplification was performed using a Chromo4 real-time thermal cycler (BioRad; Hercules, CA) located in a separate room (to eliminate contamination) using an isothermal protocol of

incubation at 63 °C for 60 min with fluorescence measured at one-minute intervals. Filtered pipets, sterile pipet tips, autoclaved tubes, and PCR-grade sterile water were also used. Negative and positive controls (n=3 each) were run concurrently to ensure reagent quality and absence of contamination. Negative controls included PCR-grade water. Positive controls included DNA extracts containing *D. polymorpha* cytochrome c oxidase (CO1) target DNA. To prevent ambient contamination of amplicons after amplification, tubes were placed in zip lock bags and discarded in the separate room without ever opening the tubes. Benchtops were sterilized with 70% ethanol daily and 10% bleach weekly.

2.2.2 Primer design for *Dreissena* sp.

Species-specific isothermal amplification primers were designed for the CO1 gene for *D. polymorpha* (Accession #: AF120663) and *D. bugensis* (Accession #: DQ840132; Table 1) using sequences obtained from GenBank (Benson et al., 2013). One genus-specific sequence was also developed for *Dreissena* sp. using the 18S rRNA gene (Accession #: AF305702). Primer sets for each gene included six primers: loop forward (LF), loop backward (LB), forward (F3), backward (B3), forward inner primer (FIP), and backward inner primer (BIP). These were designed (Table 1) as per LAMP primer design requirements (Nagamine et al., 2002; Notomi et al., 2000; Tomita et al., 2008) using Primer Explorer V4 software and procured from Integrated DNA Technologies (Coralville, IA). The final primer mixture for the LAMP reaction contained 16 µM FIP and BIP, 8 µM LF and LB, and 2 µM F3 and B3.

To establish analytical sensitivity, standard curves were prepared using 10X serial dilutions of target DNA in the range of 10 to 100,000 copies per reaction (using synthesized sequences). Species-specific LAMP assays were numerically evaluated using Basic Local

Alignment Search Tool (BLAST (Altschul et al., 1990). Briefly, each primer sequence that was entered in BLAST was compared to sequences for mollusks and clams that are found in the same region. Individual primers were evaluated for specificity by analyzing the following four BLAST parameters: max score, % query coverage, E value, and % identity. Primers of non-target species that have matching values to the target species are most likely to be non-specific. As LAMP utilizes 6 primers that target 8 regions, increased specificity to the target species is often observed as compared to qPCR, which only utilizes 2 primers (Parida et al., 2008). Specificity was also determined experimentally by analyzing assays against related, non-target species.

Table 2.1: List of LAMP primers used in this study

Species/Gene	**Accession Number**	**Primer**	**Sequence (5' – 3')**
Dreissena sp./ 18S rRNA	AF305702	FIP	TGA AAG ATA CGT CGC CGG CGA ACT CGT GGT GAC TCT GGA C
		BIP	TGC CTA CCA TGG TGA TAA CGG GTG TCT CAT GCT CCC TCT CC
		LF	GTG CGA TCG GCA CAA AGT T
		LB	TAA CGG GGA ATC AGG GTT CG
		F3	GTT AGC CCA GAC CAA CGC
		B3	CTT CCT TGG ATG TGG TAG CC
Dreissena polymorpha/ cytochrome c oxidase (COI)	AF120663	FIP	AGA GAC AGG TAA AAC CCA AAA ACT AAT TGA TTG GTA CCA ATA ATA CTG AG
		BIP	ATT TTG TTC AGC TTT TAG GGA AGG AAA AAT CTA TCG CAG GGC C
		LF	CGA GGG AAA CCT ATA TCA GGA AGA
		LB	GGA TTC GGG GGT GGT TGA ACC
		F3	TAA TGG GGG GAT TCG GAA
		B3	GCT CCC CCA ATA TGA AGA G
Dreissena bugensis/ cytochrome c oxidase (COI)	DQ840132	FIP	AAG AAG CTC CAC CGA TAT GAA GAG CCA CCG TTA TCC AGG ATT
		BIP	AGA ACA TGA GGA AAT ATA CGT GCC CAC CAA TAG AAG TAC AAA ACA AAG
		LF	ATG GCT GGC CCT GAA TGC C
		LB	GGG TGT CAT CAG TTT TAT CGG GT
		F3	ATT TGG TGG GGG TTG AAC
		B3	GGC TAA AAC AGG TAT TGC TAA

2.2.3 Validation of direct loop-mediated isothermal amplification of *D. polymorpha and D. bugensis* tissues and whole veligers

Amplification mixture for direct amplification followed the LAMP protocol described above except that 2.8 µl of extracted DNA was replaced by the same volume of crudely lysed water sample. For validation of the direct amplification procedure, samples of tissue from *D. polymorpha* and *D. bugensi*s were obtained from organisms found at Muskegon Lake (Muskegon Co., MI). Crude lysate was obtained by removing shells, crushing the entire remaining organism using a pestle, and vortexing for 1 min. Four mg of tissue (wet mass) was diluted with 1 ml of deionized water and serially diluted (10X; ranging from 1.12 µg to 1.12 ng), then 1 µl was added directly to the amplification reaction, with three replicates per dilution. Standard curves were generated for *D. polymorpha* and *D. bugensis* tissue mass using CO1 primers. This experiment was repeated thrice to account for run-to-run variation and average standard curves were generated for each (9 total replicates). Assay sensitivity was calculated based on the amplification of 9 replicates. The probability of detection was calculated for each dilution as the number of successful calls divided by the total number of replicates (Hunter et al., 2015). Best-fit straight trend lines for each data set were fitted, and the corresponding equations were used to determine the mass of target tissue. Using these standard curves, the mass present in each reaction was estimated for environmental samples, by comparing to the time to positivity (TTP) obtained.

To further validate the performance of direct amplification at much lower concentrations of mostly veligers and tissues, field samples were collected from Klinger Lake (St. Joseph Co., MI) using a plankton tow net (Wildco; Yulee, FL). Approximately 500 l of lake water was concentrated to a final volume of 500 ml and immediately transported to the laboratory for

further analysis. The number of *D. polymorpha* veligers per ml of filtrate was counted under a microscope using a Sedgewick-Rafter counting cell (Wildco; Yulee, FL). Three serial dilutions of veligers were prepared in quadruplicate (0.09, 0.009, and 0.0009 veligers per µl) and subjected to: i) heat treatment at 95 °C for 3 min, ii) pestle crushing, iii) heat treatment at 95 °C for 3 min followed by pestle crushing, and iv) no treatment. Veligers were directly amplified without employing any DNA extraction procedure using *D. polymorpha* CO1 primers.

2.2.4 Collection, processing, and analysis of surface water samples

Surface water samples were collected (a total of 318 samples; Figure 2.1) from lakes and streams located in Michigan and northern Wisconsin with assistance from over 100 volunteers (see Acknowledgements). Sampling kits provided to volunteers included: i) a filter funnel made by attaching a 35 µm mesh filter to a modified 1 l bottle with 35 µm mesh netting (Wildco, Yulee, FL), ii) conical tubes (50 ml), iii) a 1 l bottle for collection of grab water samples, and iv) instructions. Two sample types (a field-concentrated sample and an unconcentrated sample) were collected and sent to the laboratory for analysis.

The field- concentrated samples (n = 318) were obtained using a filter funnel with 35 µm mesh netting to achieve a 1000-fold concentration (20 l to 20 ml). Volunteers dipped the filter-funnel in the surface water 20 times to achieve concentration of 20 l. Following filtration, the 35 µm mesh filter and particulates were added to a conical tube containing 20 ml of the same surface water. Samples were then frozen (-20 °C) immediately by volunteers for at least 12 h, then shipped to the laboratory via overnight shipping. Upon receipt samples were crudely lysed using a pestle, heated to 95 °C for 3 min, and then promptly stored at -20 °C until analysis to reduce chances for eDNA degradation (Strickler et al., 2015).

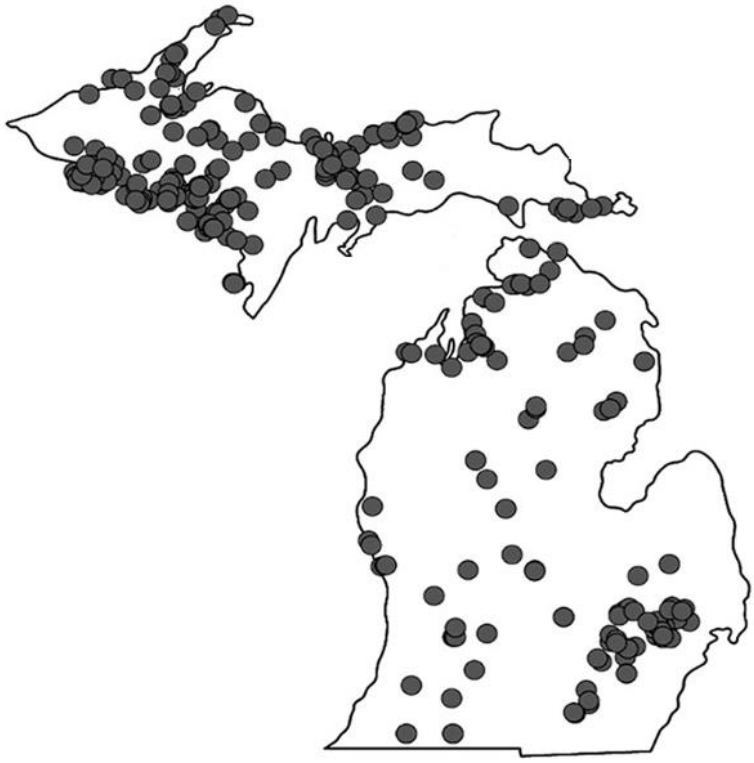

Figure 2.1: Location of 318 lake samples collected between November 2013 and August 2015.

To validate this sample concentration approach, samples were collected from two sites; one with a high population of *D. polymorpha* and another with a low population. At each location, two water samples were collected including an unfiltered water and concentrated water from the filter funnel (for 1000-fold concentration of AIS eDNA). To capture a high population abundance scenario (where there is a known infestation with peak reproduction seasons), samples were collected from Klinger Lake (St. Joseph Co., MI) in mid-June when high population densities have been previously observed.

To test how crucial the date/time of year of sample collection was for sensitivity, a selection of collected samples was obtained from the same location, but at multiple time points during the year. Concentrated samples (from 20 l to 20 ml) were collected from selected Michigan inland lakes including Klinger Lake (St. Joseph Co., MI), Au Train Lake (Alger Co., MI), Antoine Lake (Dickinson Co., MI), and Higgins Lake (Roscommon Co., MI).

A total of 174, 1 l grab un-concentrated surface water samples were also collected by volunteers to compare extracted DNA results with direct amplification. These were collected first by volunteers to ensure no contamination by the field- concentrated samples and also frozen immediately for at least 12 h before shipping overnight to the laboratory. Once received, surface water was filtered through 0.45 µm pore size filters (Millipore; Billerica, MA). DNA was then extracted using PowerWater DNA Isolation Kit (MoBio; Carlsbad, CA) following manufacturer's protocols. Total DNA was quantified using Qubit 2.0 fluorometer with the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific; Waltham, MA).

2.2.5 Volunteer training

A smartphone application, termed "eDNA" was developed to train volunteers in sample collection and disseminate results. A video detailing the sample collection protocol was included as part of the application. In the documentation provided with the sample collection kit, particular emphasis was placed on sample handling and prevention of sample cross-contamination. Though equipment was pre-sterilized, volunteers were instructed to avoid sample to sample contamination and wash equipment thoroughly with a 10% bleach solution if contamination is suspected. Furthermore, samples collected at the beginning of this study by volunteers we collected in parallel with scientists to ensure similar results. The protocols

provided emphasized that the sterilized sample collection bottles must only be opened once at the sampling location. To prevent DNA degradation within the collected sample, samples were frozen at -20°C within 4 h. Samples were then stored for at least 12 h until shipping to the laboratory for further processing and analysis. Samples were sent to the laboratory via overnight shipping and were typically still frozen upon arrival.

2.2.6 Pilot tests using Gene-Z for rapid, field-based *Dreissena* sp. detection

Field tests of a portable gene analyzer (Gene-Z) were conducted at two locations: Klinger Lake (St. Joseph Co., MI) in June 2014 and Muskegon Lake (Muskegon Co., MI) in August 2015. Briefly, Gene-Z is a battery-operated, handheld gene analyzer that utilizes isothermal amplification and microfluidic cards capable of analyzing 64 isothermal reactions simultaneously (Stedtfeld et al., 2012). The disposable cards are manufactured as previously described using a 40 W CO_2 laser (Stedtfeld et al., 2015) and prior to field use, primer sets were dispensed into the reaction wells of each chip, dried, and stored at -20 °C. At Klinger Lake, water samples were first collected using a hand filter and concentrated 1000-fold (from 20 l to 20 ml). At Muskegon Lake, water samples were collected without concentration step. Samples were then pipetted into a microfluidic chip which automatically distributes the samples into 64-wells using an airlock mechanism (Kostic et al., 2015), then sealed with an optically transparent tape and inserted into Gene-Z device. The device was operated at an isothermal temperature of 63 °C, with fluorescence measured every 15 seconds for each well. Fluorescence signals were tracked using an iPod touch, which also operated the device. Upon completion of the run, data was emailed from the iPod touch to a PC for further analysis in Excel.

2.2.7 Data and statistical analysis

In all experiments, the following statistical analysis process was used. First, using raw fluorescence data, signal to noise ratio (SNR) at time t was calculated as the raw fluorescence minus the median background divided by the standard deviation of the average background signal. The TTP (the time at which the reaction is first positive) was calculated as the time when SNR crossed a threshold of ten (Stedtfeld et al., 2014). All amplification reactions were performed in triplicate or higher. Based on positive amplification at the lowest copy numbers (1 target copy per well), a TTP of 50 min was selected as a cut-off for positive amplification. As stated earlier, the lower limit of detection for the assays was defined as the copy number at which at least 2 out of 3 replicates were positive (Stedtfeld et al., 2015). The limit of quantification required at 3 out of 3 replicates (or a 95% detection level as is recommended (Cai et al., 2008)) to establish a standard deviation. Environmental samples were considered positive for the target of interest if positive signals were observed in at least two of the replicates (Stedtfeld et al., 2015) but were not used for quantification. A student's t-test was used to determine significant differences between two means using n-1 degrees of freedom and cutoff p-values of 0.05.

2.3 Results and Discussion

2.3.1 Primer validation for analytical sensitivity and specificity with synthetic target gene DNA and extracted genomic DNA

From amplification reactions conducted with a dilution series of synthesized targets, the analytical sensitivity of the developed *D. polymorpha* and *D. bugensis* CO1 assays were calculated as 10,000 and 1,000 copies of target per reaction, respectively. For the 18S rRNA gene assay, the detection limit was 100 copies per reaction. In general, the primer set designed

for the 18S rRNA gene was more sensitive than those designed for mitochondrial genes. Based on the known ideal LAMP primer parameters, this increased sensitivity for the primer set can, in part, be attributed to higher GC content (Notomi et al., 2000) than the AT-rich CO1 genes. The mitochondrial CO1 genes have been reported to be more specific to the organism of interest, however, with more variability between species than other genes, making it ideally suited for eDNA detection (Hebert et al., 2003a, 2003b). These sensitivities were comparable with other studies (Hunter et al., 2015; Treguier et al., 2014). It has also been suggested that more copies of mitochondrial genes are present in cells than other genes (Robin and Wong, 1988), which may allow primers that target CO1 to overcome GC content limitations.

In specificity assays using extracted genomic DNA from the two closely related *Dreissena* sp. and other mussels expected in MI lake waters, the primers specific to *D. polymorpha* only amplified *D. polymorpha* extracted DNA (TTP = 13 ± 0 min.; Figure 2.2A). Similarly, the primers designed to be specific to *D. bugensis* CO1 gene only gave amplification product only from *D. bugensis* extracted DNA (TTP = 14.3 ± 2.3 min.; Figure 2.2B). Primers for *Dreissena* sp. 18S rRNA gene successfully amplified DNA from both *D. polymorpha* and *D. bugensis*. Species-specific *Dreissena* sp. CO1 primers were also determined to be specific when tested experimentally against *Sphaerium* sp., *Viviparus* sp., and *Corbicula fluminea*.

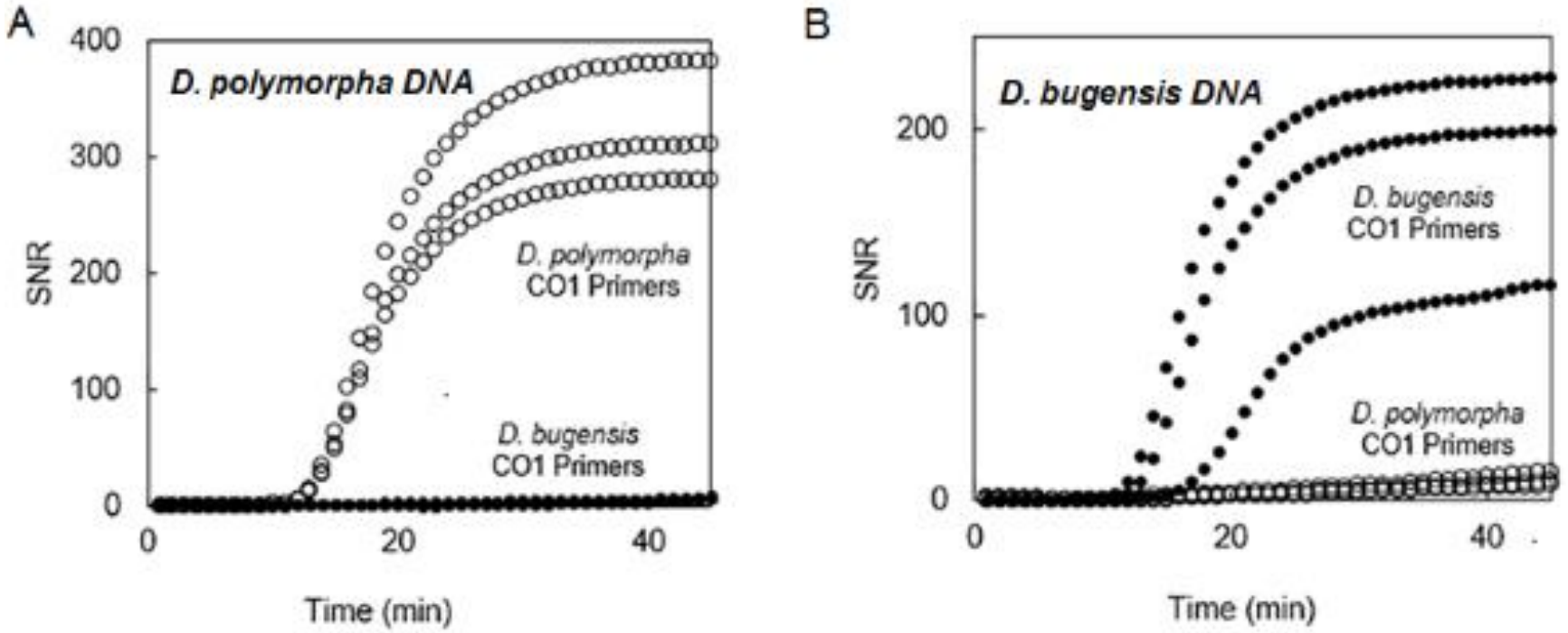

Figure 2.2: Specificity of assays validated with *D. polymorpha* and *D. bugensis.* A. *D. polymorpha* CO1 primers resulted in positive amplification only when *D. polymorpha* genomic DNA was present. B. Similarly *D. bugensis* CO1 primers gave amplification product only when *D. bugensis* genomic DNA was present.

2.3.2 Validation of primers for direct amplification from tissues and veligers

Using a dilution series prepared in the range of 0.1 ng/µl to 10 µg/µl of ground *D. polymorpha* and *D. bugensis* tissue samples, the sensitivity of the direct amplification of tissue was obtained. The detection limit was 0.01 µg tissue per reaction for the *D. polymorpha* CO1 gene assay, 0.001 µg tissue per reaction for the *D. bugensis* CO1 gene assay, and 0.0001 µg tissue per reaction for *Dreissena* sp. 18S rRNA gene assay (Table 2.2-a). For field applicability, the likelihood of detection at a given tissue concentration was also calculated based on the number of positive reactions per set of 9 replicates (3 replicates each across 3 separate runs). For *D. polymorpha* CO1 primer sets, the likelihood of detection at 0.112 µg per reaction was 0.89 with 8 of the nine replicates yielding positive amplification. At 0.0112 µg per reaction and below none of the replicates amplified indicating that the likelihood of detection was close to zero. For *D. bugensis* likelihood of detection at 0.112 µg per reaction was 0.56 with 5 out of 9 replicates yielding positive results, and at 0.0112 µg per reaction, it was 0.375 with 3 out of 9 replicates with positive amplification. As this is first work investigating direct amplification of biomass for eDNA detection, we were not able to directly compare biomass sensitivities (0.000112- 0.0112 µg tissue per reaction and 0.0009 veligers per reaction) to other studies, though other studies have linked eDNA results to organismal biomass (Doi et al., 2015). It is possible that a small amount of extracellular DNA may also be detected, though DNA size is much smaller than 35 µm and thus may not be concentrated by this approach.

Although, the whole genome information for *D. polymorpha* is still evolving, estimates are in the range of 1.7 pg per genome (Gregory, 2003). The total number of genes present in *D. polymorpha* (or other less studied mussels) is not yet fully assessed but studies related to *D. polymorpha* transcriptomics are emerging (Soroka et al., 2017). Based on the information

gathered about genomes size and an assumption of 10,000 genes per 1.7 pg of DNA and a DNA: tissue weight ratio of 0.1%, the lower limit of detection was approximately 10^4 gene copies per reaction for *D. polymorpha* at 0.112 µg tissue per reaction for CO1 gene. Further dilutions will of course lead to ~1 gene copy per reaction which will not always be present in each reaction well.

Direct amplification was also evaluated for *D. polymorpha* veligers in samples collected from Klinger Lake (St. Joseph Co., MI). Amplification was successful for as low as 0.09 veligers in the concentrated sample per reaction (TTP = 39.67 ± 1.53 min; Table 2-b), without any sample processing. For 0.009 veligers per reaction, only one of the three replicates was positive and at 0.0009 veligers per reaction, no amplification was observed. Heat treatment enhanced the limit of detection with three of six replicates amplifying (six replicates included three for the mixed samples and three for non-mixed samples) for 0.0009 veligers per reaction. Heat treatment also improved the likelihood of detection, particularly at 0.009 veligers per reaction. All three replicates were positive, as opposed to only one of three successfully amplifying for the non-heat-treated group. In general, differences between the heat-treated and control groups were statistically significant ($p = 0.0019$). The effect of cell crushing using a pestle was not statistically significant ($p = 0.065$).

Table 2.2: Results obtained for different sample types including: *Dreissena polymorpha* tissues, *Dreissena bugensis* tissues, *D. polymorpha* veligers, 1000X concentrated water, and un-concentrated water. Information for each sample includes the location, the month and year of sample collection, sample processing, primers used, estimated target per reaction, and measured TTP.

Sample Type	Location	Month, Year Collected	Sample Processing	Primers	Target/Reaction	Av. TTP ± SD
a. Direct amplification of Dreissena tissues						
Tissue (*Dreissena* sp.)	Muskegon Lake (Muskegon Co., MI)	July, 2015	Heat Treatment*	*Dreissena* sp. 18S rRNA	11.12 µg	19.67 ± 0.71
					1.12 µg	20.78 ± 0.44
					0.112 µg	20.11 ± 1.05
					0.0112 µg	22.56 ± 2.55
					0.00112 µg	28.33 ± 7.70
					0.000112 µg	35.83 ± 10.13
Tissue (*Dreissena polymorpha*)	Muskegon Lake (Muskegon Co., MI)	July, 2015	Heat Treatment*	*Dreissena polymorpha* CO1	1111.2 µg	22.00 ± 0.00
					111.12 µg	22.67 ± 2.08
					11.12 µg	24.22 ± 2.64
					1.12 µg	26.00 ± 2.55
					0.112 µg	31.00 ± 4.32
Tissue (*Dreissena bugensis*)	Muskegon Lake (Muskegon Co., MI)	July, 2015	Heat Treatment*	*Dreissena bugensis* CO1	11.12 µg	27.13 ± 5.41
					1.12 µg	31.13 ± 5.14
					0.112 µg	40.20 ± 9.78
					0.0112 µg	41.00 ± 3.46

Table 2.2 (cont'd)

b. Direct amplification of Dreissena polymorpha veligers

Veligers (*Dreissena polymorpha*)	Klinger Lake (St. Joseph Co., MI)	June, 2014	Heat Treatment*	*Dreissena polymorpha* CO1	0.09 veligers	33.67 ±	1.15
					0.009 veligers	32.33 ±	5.69
					0.0009 veligers	50.00[a] ±	N/A
			None		0.09 veligers	39.67 ±	1.53
					0.009 veligers	43.00 [a] ±	N/A
					0.0009 veligers	ND***	

c. Effect of sample collection date on results

Lake water concentrate (1000X)	Klinger Lake (St. Joseph Co., MI)	Oct., 2013	Heat Treatment*	*Dreissena polymorpha* CO1	N/A	ND**	
		May, 2014				ND**	
		June, 2014				28.67 ±	6.35
Lake water concentrate (1000X)	Au Train Lake (Alger Co., MI)	Nov., 2013	Heat Treatment*	*Dreissena polymorpha* CO1	N/A	ND**	
		July, 2014				29.67 ±	1.53
		Aug., 2014				ND**	
Lake water concentrate (1000X)	Antoine Lake (Dickinson Co., MI)	Nov., 2013	Heat Treatment*	*Dreissena polymorpha* CO1	N/A	ND**	
		Nov., 2014				22.67 ±	1.15
Lake water concentrate (1000X)	Higgins Lake (Roscommon Co., MI)	Oct., 2013	Heat Treatment*	*Dreissena polymorpha* CO1	N/A	ND**	
		July, 2014				32 ±	0.00

Table 2.2 (cont'd)

Lake water concentrate (1000X)	Gun Lake (Barry Co., MI)	Sept., 2013	Heat Treatment*	*Dreissena polymorpha* CO1	N/A	ND***	
		Oct., 2013				ND***	
		May, 2014				42.0 ±	5.20
		Aug., 2014				ND**	
		June, 2015				32.0 ±	0.00

[a]Only 2 of 3 replicates amplified.
*Heat Treatment = 95°C for 3 min.
**ND = Not Detected

2.3.3 Validation of filtration approach for sample concentration in the field

To validate the filtration approach for sample concentration, results from concentrated samples were compared with un-concentrated surface water. At high abundances (samples collected at Klinger Lake in St. Joseph Co., MI in June) positive results were obtained from both sample types, suggesting that with large population abundances no sample concentration is required (concentrated sample TTP = 22.3 ± 3.2 min and un-concentrated sample TTP = 23.3 ± 1.53 min; Figure 2.3). Similarities in TTPs obtained can be attributed to the plateau in decreasing TTP observed in the standard curves of organismal biomass (Table 2.2-a). For the lower population density case (where there is a known population but outside of reproduction peak season), samples were collected from Lake Lansing (Ingham Co., MI) in mid-November when veliger and tissue abundances are low. After concentrating the water sample by 1000-fold with the hand filter, positive amplification (concentrated sample TTP = 32.0 ± 3.0 min) was seen in all replicates. Without the concentration step, no amplification was observed in 60 min.

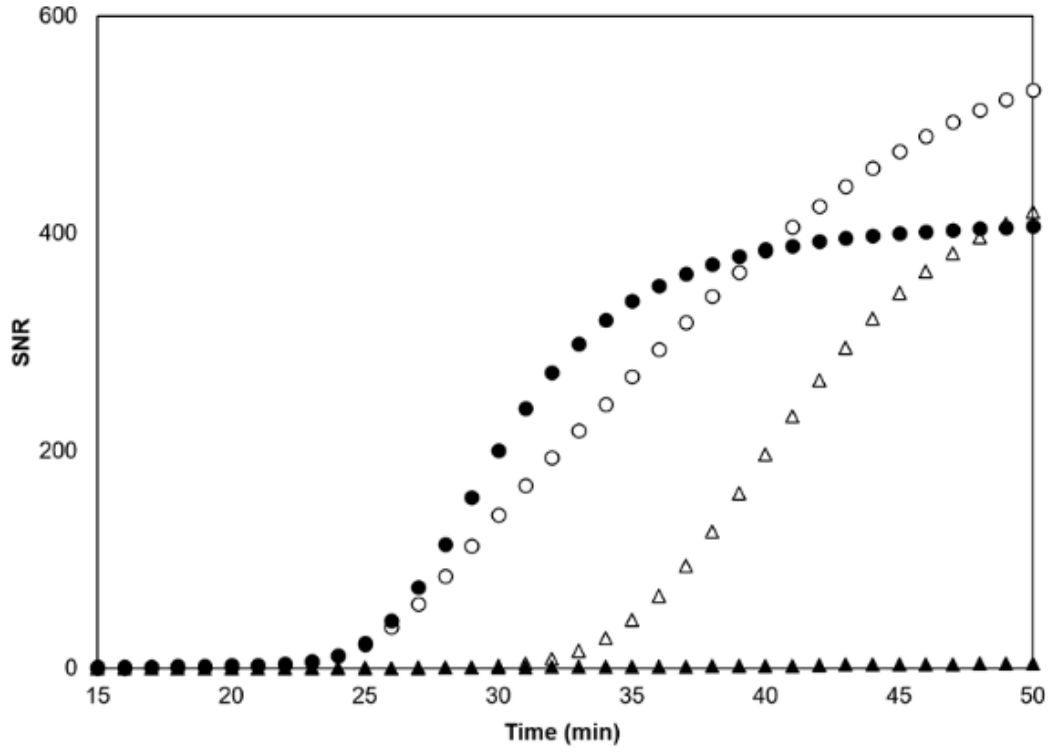

Figure 2.3: Direct amplification results for sample collection strategies including 1000X concentration (20 l hand-filtered to 20 ml) and un-concentrated water, at high initial population abundances (circles; open for concentrated and closed for un-concentrated) and low initial population abundances (triangles; open for concentrated and closed for un-concentrated). At high abundance, no change in TTP was observed between 1000X concentration and water-only. At low abundance, positive results were observed only after 1000X concentration.

2.3.4 Direct amplification of filtered surface water samples

In general, detection of *D. polymorpha* was significantly widespread, with 27 positive detections throughout the state (Figure 2.4). *D. bugensis* was only detected in 3 out of the 318 samples (Figure 2.4). A total of 168 out of 318 samples were also analyzed for *Dreissena* sp. and 59 samples were found positive. Increased observance of *Dreissena* sp. may be due to higher analytical sensitivity of the 18S rRNA gene primers compared to the species-specific primers.

Based on the results from the tissue mass presented in the above section, standard curves were generated for use in quantification of mass from field data using linear trendlines. While

this is the first use of these standard curves for estimation of quantification of AIS tissue mass from field data for direct amplification, it is commonplace for quantification of DNA from Ct values obtained with qPCR (Larionov et al., 2005; Whelan et al., 2003) and has also been presented with LAMP for quantification of cells (Samhan et al., 2017). For *D. bugensis* CO1 gene primers, the equation used was $y = - 2.02 \ln(x) + 32.571$, where x is the mass and y is the TTP obtained. Similarly, for *D. polymorpha* CO1 and *Dreissena* sp. 18S rRNA, equations were $y = -1.474 \ln(x) +27.238$ and $y = -1.315 \ln(x) +20.151$, respectively. Theoretical mass at each location was also calculated based on the earlier presented linear trendline equations for tissue mass for *D. polymorpha* CO1, *Dreissena* sp. 18S rRNA and *D. bugensis* CO1. A visual representation of the mass values at each sampling location are also shown in Figure 2.4.

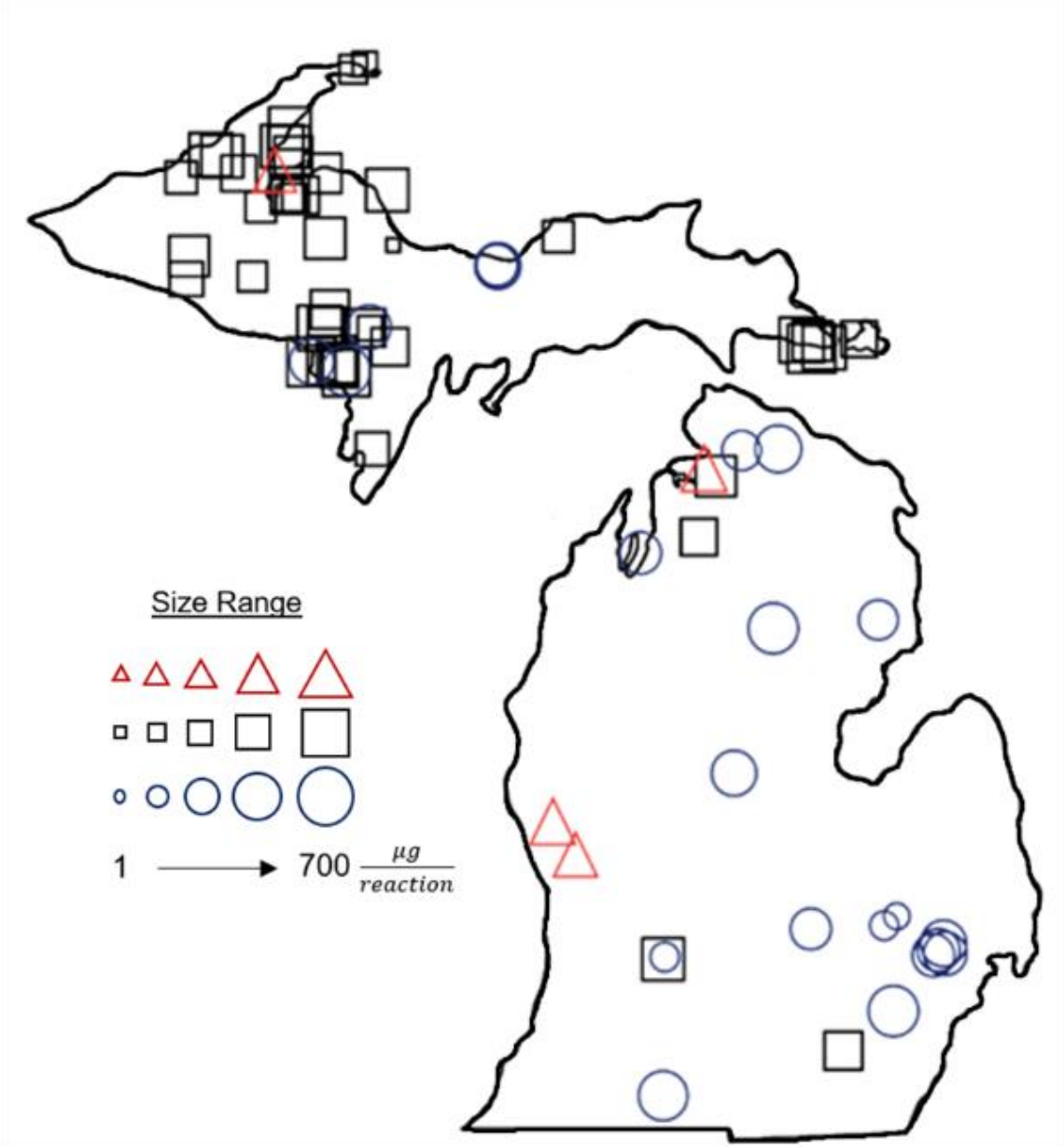

Figure 2.4: Results from direct amplification of environmental samples. Mass estimates for *D. polymorpha* CO1 (blue circles), *D. bugensis* CO1 (red triangles), and *Dreissena* sp. 18S rRNA (black squares). Larger shapes correspond to a high concentration tissue detected.

To obtain efficacy information about these results, known *D. polymorpha* infestation information was obtained from the United States Geological Survey (USGS) online database (USGS and USDOI, 2015). Of the total positive detections obtained from samples collected in

the sampling period (May 2014 to August 2014 and May 2015 to August 2015; 171 out of 318 samples), 65.4% of which corresponded with reported infestations. For 15.4% of the total samples, previous *D. polymorpha* infestations were reported but not detected by the eDNA protocol, suggesting future studies could focus on the improvement of the detection limit or variability due to sampling locations.

For most lakes, direct amplification of was positive from approximately May to August during a given year. Time to positivity values obtained from the same locations by date is shown in Table 1-c, using primers for *D. polymorpha* CO1. This may correspond with reproduction for *D. polymorpha,* which occurs when water temperatures exceed 12 °C and would suggest that the number of veligers in the water column is much higher (Fong et al., 1995). It also further confirms that the persistence of eDNA in the environment is important (Barnes et al., 2014; Dejean et al., 2011; Piaggio et al., 2014). In the summer months, there is a potential for mixing from recreational activities which is at its peak (Yousef et al., 1980). Summer months are also the recommended time for completing *D. polymorpha* veliger surveys as well as other eDNA analysis methods (Pilliod et al., 2014). This suggests that the implementation of the direct amplification method could complement these other approaches as they could be completed at similar times of the year. However, lakes (especially deep lakes) are typically stratified during warmer temperatures (Gorham and Boyce, 1989), which may complicate sample collection as there would not be complete mixing throughout the waterbody.

Of the 174 unconcentrated samples that were sent to the laboratory for DNA extraction and amplification analyzed and compared to their corresponding direct amplification sample, 11 were positive for *D. polymorpha* CO1 by both methods (Figure 2.5). A total of 5 positive results were obtained with direct amplification of filtrate samples and not with amplification of

extracted DNA. A total 12 positive results were obtained with amplification of extracted DNA and not with direct amplification of filtrate.

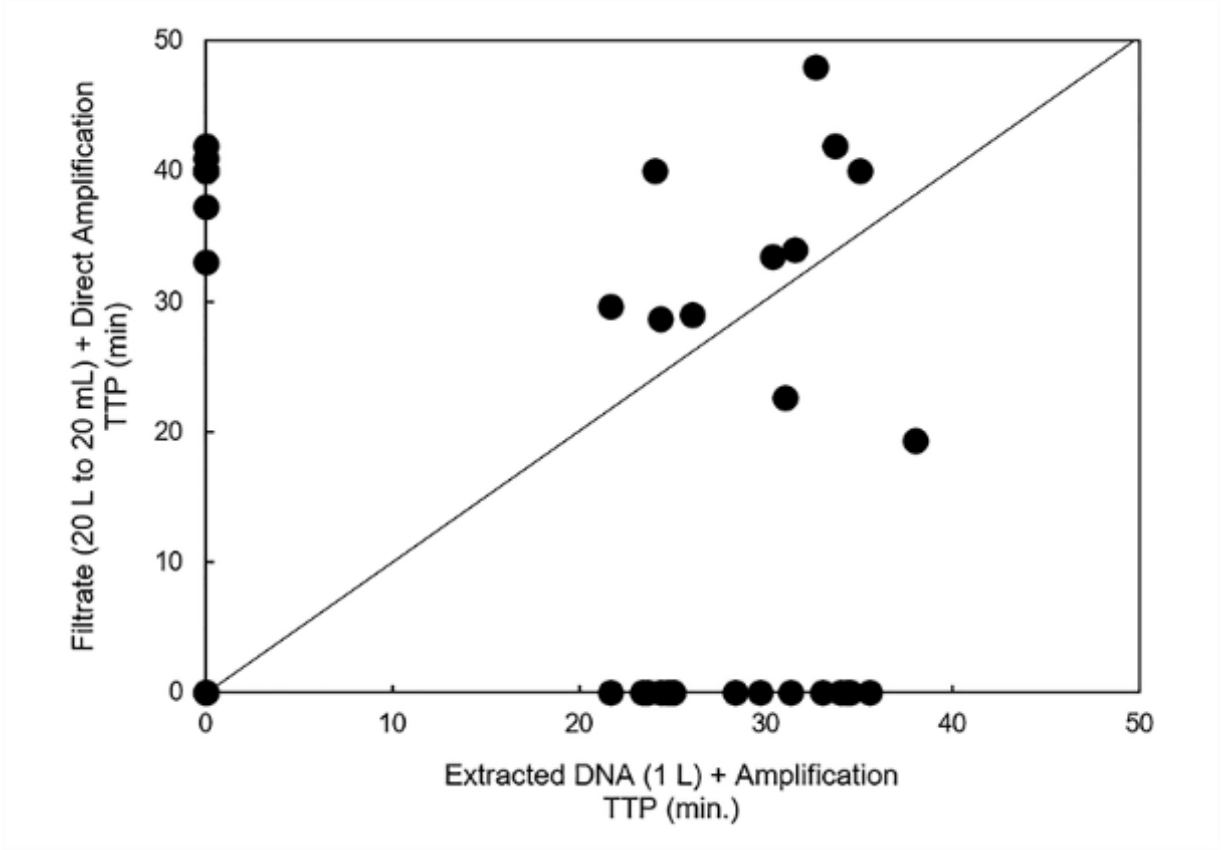

Figure 2.5: Comparison of results between the field-concentrated samples with direct amplification and the unconcentrated samples with DNA extraction and amplification. This 1:1 plot shows amplification results of the field-concentrated samples with direct amplification as compared to the results of 1 l unconcentrated samples following DNA extraction. Points along the y-axis only amplified with the field-concentrated samples and direct amplification while those along the x-axis only amplified with the unconcentrated method. Points in the center correspond to positive detections using both methods.

2.3.5 Results from pilot tests of Gene-Z for field-based detection of *Dreissena* sp

In a pilot scale test at Klinger Lake (St. Joseph, MI) of a field-deployable device (Gene-Z) concentrated lake water was collected using the field-concentration approach. Once the

filtrate was collected and crudely lysed as mentioned in the methods section, it was dispensed into the microfluidic cards and the card was sealed with optical film. Analyzing the concentrated lake water resulted in positive results for *D. polymorpha* using primers for the CO1 gene (TTP = 33.3 ± 3.8 min). When testing the un-concentrated water directly at Muskegon Lake (Muskegon, MI), positive detections were observed for *Dreissena* sp. (18S rRNA gene; TTP = 42.76 ± 8.8 min).

2.4 Conclusions

The results obtained in this study through the collection and analysis of 318 samples supports that direct amplification may be useful for field monitoring of aquatic invasive species. While this is not the first study to analyze large numbers of samples for eDNA from aquatic invasive species (Jerde et al., 2013) including *Dreissena* sp. (Peñarrubia et al., 2016), it is the first of its kind to analyze large numbers of samples for *Dreissena* sp. using LAMP. This highlights the advantages of a direct amplification-based eDNA approach, in that large numbers of samples are easily analyzed for dozens of species in a short time. Furthermore, through the laboratory-based confirmation of 1 l grab water samples, processed via filtration through a 0.45 µm filter following by DNA extraction, the likelihood of obtaining a positive result is significantly increased. The findings presented here show that extraction of DNA followed by LAMP may be slightly more sensitive than direct amplification and this is supported by the fold concentration of water that occurs with each (20 l – 20 ml for direct amplification; 1000-fold vs. 1 l – 50 µl for amplification following extraction; ~10,000 fold). The combination of both field-based direct amplification for rapid detection on- location combined with further laboratory

confirmation would give more power to results obtained by increasing likelihood of detection overall, but also allowing rapid responses should positive results be obtained in the field.

Experiments conducted as part of this study show that the developed concentration technique and direct isothermal amplification combined with a field-deployable device could be used as a rapid warning tool to detect invasive species, with a total time required (from filtration to results) of about 90 min. When sample concentration is not needed due to high abundances, less than 30 min may be sufficient. By increasing the efficiency of AIS screening, often spread over a larger geographic area, it allows for more samples to be analyzed and thus enhances the likelihood of detection if a species is present. Appropriate location for such samples must obviously be decided based on field data. Moreover, the inclusion of volunteers reduces travel requirements and helps to educate and involve the public. Taken together, the procedure and programs developed here provide a useful tool for AIS detection. Data presented here describe the performance of an approach and platform for basin-wide surveillance using primers for *Dreissena* sp. Future studies should optimize the particulate concentration protocol for detection of other species (invasive or native), such as plant seeds.

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CHAPTER III

Implications of direct amplification for measuring antimicrobial resistance using point-of-care devices

The work in Chapter III has been published:

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Author contribution statement:

M. R. W., R. D. S., H. W., T. M. S., and S. A. H. compiled literature. M. R. W. generated figures. M. R. W., R. D. S., H. W., T. M. S., W. K., B. E., M. H., J. M. T., and S. H. wrote and/or edited the manuscript.

Abstract

Antimicrobial resistance (AMR) is recognized as a global threat to human health. Rapid detection and characterization of AMR is a critical component of most antibiotic stewardship programs. Methods based on amplification of nucleic acids for detection of AMR are generally faster than culture-based approaches but they still require several hours to more than a day due to the need for transporting the sample to a centralized laboratory, processing of sample, and sometimes DNA purification and concentration. Nucleic acids-based point-of-care (POC) devices are capable of rapidly diagnosing antibiotic-resistant infections which may help in making timely and correct treatment decisions. However, for most POC platforms, sample processing for nucleic acids extraction and purification is also generally required prior to amplification. Direct amplification, an emerging possibility for a number of polymerases, has the potential to eliminate these steps without significantly impacting diagnostic performance. This review summarizes direct amplification methods and their implication for rapid measurement of AMR. Future research directions that may further strengthen the possibility of integrating direct amplification methods with POC devices are also summarized.

3.1 Introduction

Emergence of growing antimicrobial resistance (AMR) is now recognized as a global crisis with the accompanying dangers of inaction or weak action (WHO, 2014). Steps are finally being taken to address some of the key challenges to help sustain human health and quality of life (unimaginable before the advent of penicillin). The Antibiotic Stewardship Program by the Centers for Disease Control and Prevention (CDC) is a U.S. initiative (CDC, 2014) to reduce bacterial selective pressure while improving patient

outcomes. This requires accurate and rapid diagnostics of pathogens and AMR coupled with prudent use of antibiotics. Numerous national and international surveillance networks exist, focusing on various aspects contributing to such stewardships (Peirano et al., 2014). The "Core Elements of Hospital Antibiotic Stewardship Programs" recommends antibiotic "time outs" and tracking of "resistance patterns" to review and correct the empirical approach for prescribing the antibiotic in a timely manner. Policy changes that allow faster introduction of antibiotics are also being recommended (Bush and Pucci, 2011; Livermore, 2012; Woolhouse and Farrar, 2014). Among the challenges to stewardship are slow development of new antibiotics, weak control of prescription-based antibiotics, unnecessary use of these as growth promoting agents by the animal industry, costs of treatment, and the ultimate challenge to human suffering and loss of life in treating resistant organisms.

Globally there are at least a dozen resistant organisms of concern (WHO, 2014). Carbapenem- resistant enterobacteriaceae (CRE; *Klebsiella pneumoniae*, Enterobacter species, and extra-intestinal pathogenic *Escherichia coli*), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* are among the key organisms known to cause antibiotic resistant infections. CRE infections are on the rise in US hospitals and throughout the globe (Dortet et al., 2013; Huang et al., 2012; Kaiser et al., 2013; Kumarasamy et al., 2010; Li et al., 2014; Shoma et al., 2014; Székely et al., 2013; Tada et al., 2013). They are also associated with high mortality - often as high as 40-72% (CDC, 2012; Chetcuti Zammit et al., 2014; Daikos et al., 2014; Hussein et al., 2013). *Klebsiella pneumoniae* carbapenemases (KPC), first reported in the US in 1996, is now the most prevalent CRE in the US.(Nordmann et al., 2011) *E. coli* NDM-1 (New Delhi metallo-β-lactamase-1), first reported in New Delhi

in 2011 (Walsh et al., 2011), is now reported from more than 40 countries over all continents except Antarctica and South America (Bushnell et al., 2013; Johnson and Woodford, 2013). Increasing use of carbapenems is leading to the emergence of multi-drug resistant and pan-resistant CRE (McLaughlin et al., 2013). At present, tigecycline and colistin are among the few remaining antibiotics that continue to be effective against CRE (Denys et al., 2013; Kumarasamy et al., 2010; Stein and Babinchak, 2013). Even these may not persist in their efficacy without careful management (Stone et al., 2011). Introduction of new antibiotics has also slowed because of market forces. Timely and accurate information about CRE will help in the management of antibiotics with improved patient outcomes.

Overall, infections due to these and many other microorganisms often result in sepsis and affect more than 750,000 people in the US alone; of which 215,000 die annually (Angus et al., 2001). Survival rate for sepsis patients decreases by the hour and timely administration of correct antibiotics matters (Daniels, 2011; Kumar et al., 2006). If correct antibiotics are prescribed within the first hour, the survival rate could be as high as 80% but if treatment is delayed, it could be as low as 5% after 36 hours. For this reason, sepsis is treated as a medical emergency. Physicians based on other indicators and modify the course when the AMR test results become available through a combination of culture and nucleic acids-based analyses in a centralized laboratory which is generally after 1 to 4 days. At least one day is also required for sample transport.

For sepsis, the unmet need is to obtain the results in less than 1 hour, preferably within 15 min, be able to screen for multiple pathogens, and ideally at much lower concentrations (such as 1-10 colony forming units or cfu/ml). Point-of-care (POC) devices

capable of measuring nucleic acids have a critical role in providing such rapid diagnosis. However, sample processing, especially nucleic acids extraction and purification, must be integrated, automated, simplified, or eliminated for such POCs to be rapid and simple (Figure 3.1). Evidence suggests that elimination of the nucleic acids extraction step is possible for certain polymerases and especially those used for isothermal amplification (e.g., *Bst* polymerases). For polymerase chain reaction (PCR) polymerases, only some of the more recently developed polymerases may allow direct amplification when combined with cell lysis steps. This review evaluates the potential of direct amplification for measurement of antimicrobial resistant bacteria by POC devices. The focus of direct amplification is on isothermal amplification because most POC devices are isothermal. However, this is changing and many qPCR and PCR-based POC devices are also on the horizon and some of the literature related to direct PCR may also be useful for these qPCR and PCR-based POC devices.

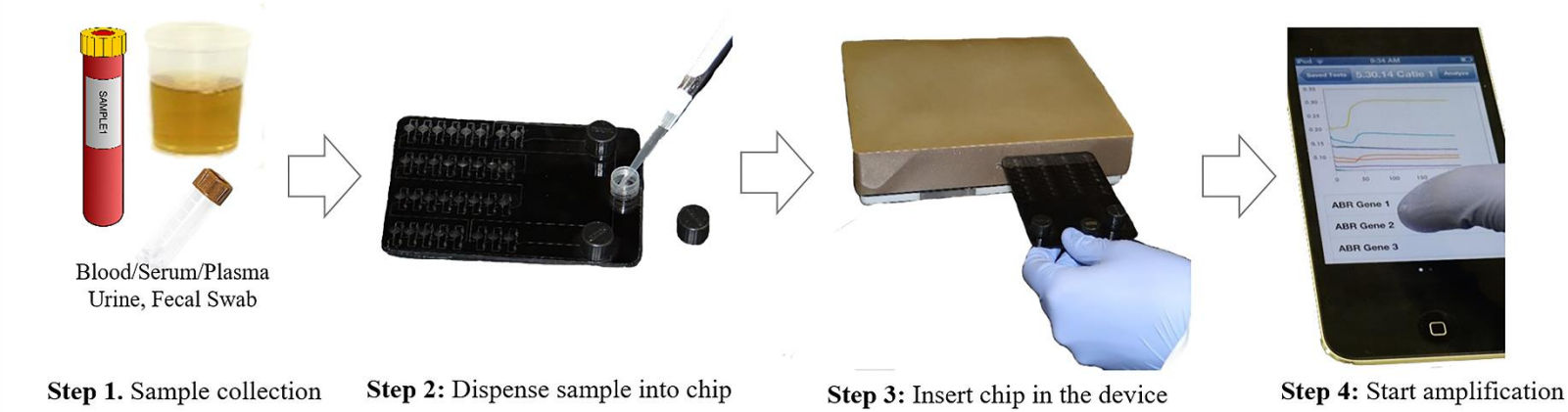

Figure 3.1: Schematic for point-of-care approaches, from sample collection to results

3.2 Rapid analysis at the best possible detection limit are key to measuring resistance in clinical settings

Because of the challenges faced at the lower detection limit, most methods do not start from blood, cerebrospinal fluid (CSF), pleural fluid, and urine – body fluids that are normally sterile. They employ blood culture or colony suspensions which take a minimum of 8 hours and more commonly 16-24 hours. This may already be too late for the majority of sepsis patients (Rivers and Ahrens, 2008). Most methods that claim to be rapid are actually referring to the time for "rapid confirmation of resistance - starting from positive blood culture or colonies" rather than "rapid detection of identity and resistance in normally sterile body fluids". Due to the need for large number of cells (as high as 10^5- 10^7 cells) to be present in a small volume of sample, most molecular methods require positive blood culture or colonies irrespective of the marker or principle used for antibiotic resistance confirmation. For example, detection and confirmation of CRE by quantitative polymerase chain reaction (qPCR) requires extracted and purified DNA starting from blood culture or colonies (Chen et al., 2011).

Direct qPCR of lysed cells from colonies (which must first be grown) helps eliminate the DNA extraction step but lysed cell volume must be kept low (2-5 µl) to avoid inhibition of qPCR. Thus direct qPCR may not be used for normally sterile samples. Other molecular methods are rapid but require blood culture or colonies. For example, matrix assisted laser desorption ionization – time of flight, a powerful rapid confirmation platform, can confirm a given species by comparative proteomics and the spectra can be obtained within a few minutes but the protocol requires processed mono-culture from positive blood culture (Inglis et al., 2012). Similarly, NP Carba, a rapid biochemical confirmation method

for carbapenemases less than 20 min, must use colonies (Dortet et al., 2012). These "rapid confirmation methods" are still quite useful compared to culture-based confirmation by antibiotic susceptibility profiles which takes an additional 1-3 days. Because of the need to use colonies or cultures, none of these methods can detect AMR within an hour starting with normally sterile samples. The challenge emanates from the very low concentration of organisms in normally sterile samples - as low as 1 cfu/ml. No culture-independent method known today can detect 1 cfu/ml and sample concentration steps are generally required. A number of assays are closer to this goal by one to two orders of magnitude (Figure 3.2). Idaho Technologies, for example, can detect 50-300 cfu/ml of several infectious agents including *Yersinia pestis* in whole blood (FDA, a). Similarly, SeptiFast from Roche can detect a set of 25 pathogens at 30 cfu/ml in whole blood (Lehmann et al., 2008) and 30-100 cfu/ml in synovial fluids, cardiac valves, and purulent exudates (Mencacci et al., 2011) but is currently available in Europe only. Roche cobas can detect 3-5 cfu/ml of *Neisseria gonorrhoea* in urine (FDA, b). To attain these superior detection limits, sample processing step involving cell lysis, DNA extraction and purification through the binding of magnetic glass particles, and PCR on the extracted DNA are generally integrated and often automated (Yoza et al., 2003).

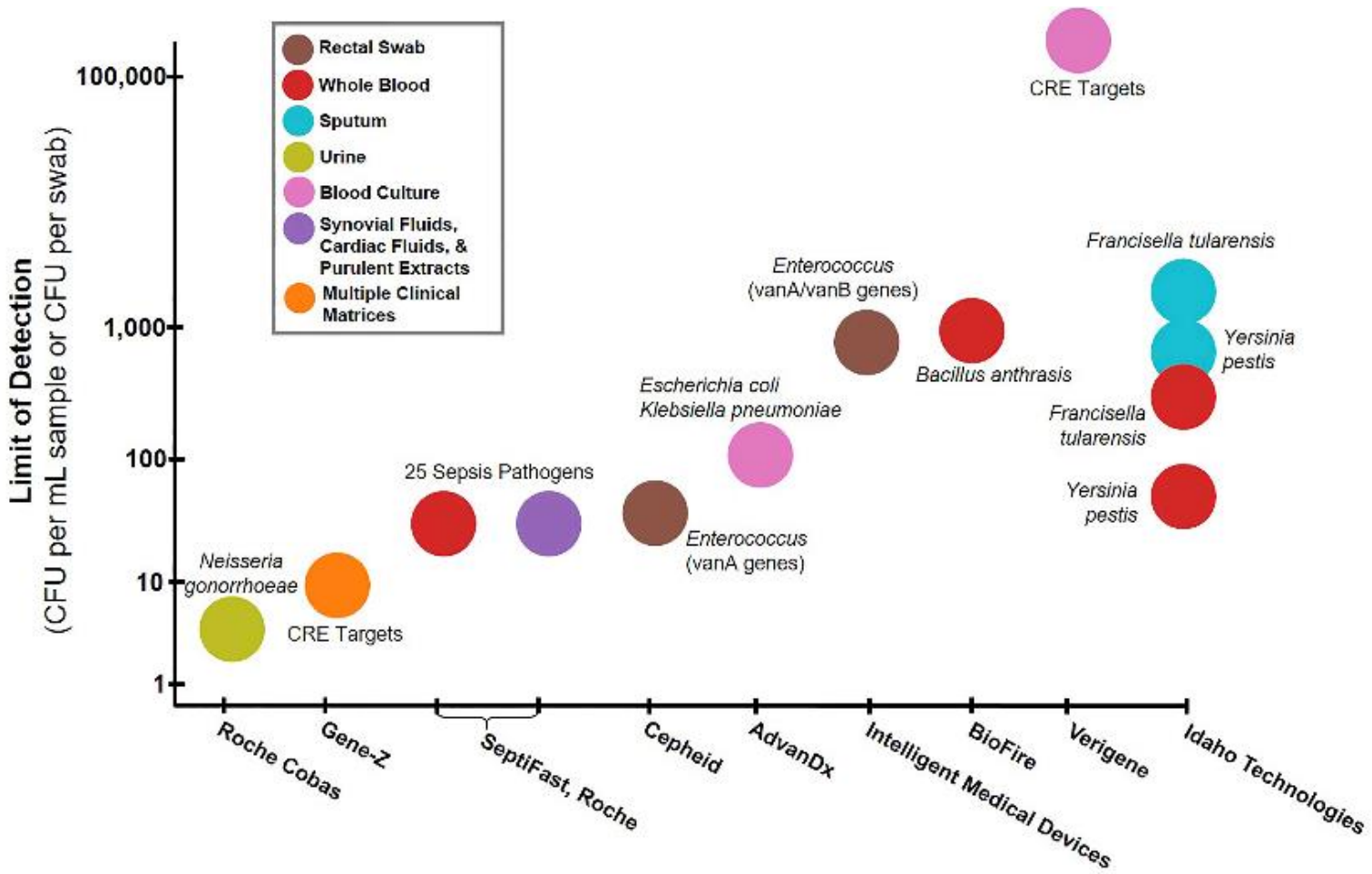

Figure 3.2: Direct amplification assays for sepsis-associated organisms that have been validated using nucleic acid amplification techniques. The Y-axis notes the detection limits from different clinical samples including rectal swabs, whole blood, sputum, urine, blood cultures, synovial fluids, cardiac extracts, purulent extracts, and other matrices. Data from US FDA 510(k) submissions (FDA, a, b, c, d, e) as well as publications for Gene-Z (Kostić et al., 2015; Stedtfeld et al., 2012) and SeptiFast (Lehmann et al., 2008; Mencacci et al., 2011).

The resulting instruments are expensive, require highly trained personnel, and cost per assay is also high. Many other FDA-cleared or commercially available assays for AMR bacteria have inferior sensitivities ranging from 100 cfu/ml (FDA, d, e) to 100,000 cfu/ml in samples that are normally known to contain much higher bacterial cell concentrations (blood culture, rectal swabs, colonies). One means of obtaining FDA clearance is that devices and assays can demonstrate substantial equivalence to another FDA-cleared device/assay. The assays provided in Figure 3.2 (with the exception of Gene-Z and SeptiFast) have been cleared through the demonstration of substantial equivalence, suggesting that their diagnostic performance is similar to other more traditional and commercially- available methods.

3.3 Direct amplification studies using isothermal or PCR polymerases

Loop mediated isothermal amplification (LAMP) and PCR have the maximum number of studies documenting direct amplification. Multiple direct amplification studies with LAMP support the notion that *Bst* polymerase (the amplification enzyme used in LAMP) in can tolerate higher levels of organic materials in the sample matrix that are inhibitory to PCR (Stedtfeld et al., 2014). Direct amplification studies with PCR or quantitative PCR (qPCR) have more commonly used pure culture or colonies s or sample matrices that do not contain significant amount of materials inhibitory to *Taq* polymerase. Even though qPCR is the current gold standard for nucleic acids amplification-based diagnostics, the availability of field-deployable PCR or qPCR POC devices lags behind the availability of isothermal POC devices. In the subsections below, selected studies that have

shown direct amplification with samples either using isothermal or PCR/qPCR are reviewed and their suitability for integration into POC devices is discussed.

3.4 Direct loop- mediated isothermal amplification (LAMP)

LAMP is an isothermal amplification technique that uses *Bst* polymerase with strand displacement activity eliminating the need for temperature cycling (Notomi et al., 2000). Its primer design approach uses three sets of primers. Since the first publication on LAMP in 2000, more than 4,500 studies have been reported using various targets. Because LAMP has the potential to be rapid (as low as 7 min for a very high target concentration with efficient primers, never taking more than an hour to detect a single copy per reaction), it has been used in many nucleic acids-based POC devices. Tolerance of LAMP to substances that are inhibitory to traditional PCR was recognized early on (Kaneko et al., 2007) and it was suggested that when using LAMP, DNA purification step may be skipped. It is perhaps ideally suited for direct amplification because of its simplicity and studies report at least 100-fold lower inhibition with numerous sample matrices as compared to PCR (Notomi et al., 2000). Amplification of methicillin-resistant *Staphylococcus aureus* was demonstrated directly with blood cultures by targeting the *MecA* gene encoding penicillin-binding protein-2 for conferring methicillin resistance (lower detection limit or LOD of 10^2 copies per reaction) and *Spa* gene encoding protein A (LOD of 10^3 copies per reaction; Misawa et al., 2007). In plasma, the direct detection of *Pseudomonas aeruginosa* was demonstrated with LOD of 2.8 ng total DNA per ml plasma (Yang et al., 2016). In faeces, amplification of DNA from pathogens has been demonstrated with a number of targets including *Clostridium difficile* cytotoxin B (Norén et al., 2011), *Campylobacter jejuni* and

Campylobacter coli (Yamazaki et al., 2008), *E. coli* (Hill et al., 2008), and others, without DNA isolation or other extensive sample processing. The use of LAMP for direct detection of pathogens in urine has also been demonstrated targeting the *malB* gene specific to *E. coli* (Hill et al., 2008). Though PCR is typically inhibitory at urea concentrations larger than 50 mM (Padmavathy et al., 2012). LAMP in this study was not inhibited. Direct amplification of targets from swab has been reported with enterovirus (Nie et al., 2012), with results in concordance of 86.8% and 100% sensitivity and specificity compared to DNA extraction and PCR. An LOD of 1 cfu per reaction was demonstrated in spiked human sputum samples for carbapenem-resistant *A. baumanii* using direct amplification of OXA-51 gene (*ISAba1*; Mu et al., 2016) Our group has extensively used direct amplification strategies including *Bst* polymerase and LAMP for a large number of matrices including bacterial cells spiked in blood, urine, sputum, and cerebral spinal fluid, protozoan cells in sludge, fish and veliger tissue suspended in water, crushed seeds and leaves, algae, bacterial cells in concentrated groundwater (Stedtfeld et al., 2014), and spores. It is now well established that direct isothermal amplification using LAMP may be easily achieved by a crude lysis of the cells. Lysing agents used in the past include heat (Nie et al., 2012), buffers (Nie et al., 2012), or portable mechanical-based lysis approaches such as simple bead-beating (Doebler et al., 2009) or portable sonication (Belgrader et al., 1999).

3.5 Direct amplification using qPCR

Clinical sample types for which direct qPCR has been demonstrated include whole blood (De Vries et al., 2001; Mccusker et al., 1992; Mercier et al., 1990; Nishimura et al., 2000), faeces (Kojima et al., 2002), urine (Lucchesi et al., 2004), buccal swab (Zimmerman et al., 2012), and cell culture (Pathmanathan et al., 2003). Even though the same approach

is relevant to PCR, due to the need for confirmation of amplification product using gel electrophoresis, direct amplification by PCR without measuring fluorescence is not relevant for the present discussion. Direct PCR amplification from blood-based samples is difficult due to inhibition by haemoglobin (Akane et al., 1994), lactoferrin (Al-Soud and Rådström, 2001), and immunoglobulin G (Al-Soud et al., 2000; Al-Soud and Rådström, 2001), anticoagulants (García et al., 2002; Satsangi et al., 1994). The common qPCR inhibitory substrates found in faeces include phytic acid, metabolic products, and complex polysaccharides (Monteiro et al., 1997; Thornton and Passen, 2004). For achieving direct amplification using PCR polymerases, reagents such as Ampdirect (Nishimura et al., 2000) or buffers with a higher pH are added to reduce the electrostatic interactions between proteins and genomic DNA (Bu et al., 2008; Kojima et al., 2002). Direct PCR detection of targets in urine is particularly useful for cytomegalovirus (Buffone et al., 1991) and *Leptospira* (Lucchesi et al., 2004). In urine, PCR is more notably inhibited by urea at concentrations greater than 50 mM (Padmavathy et al., 2012). A direct PCR method originally developed for plants has also been used with swabs of cheeks and skin, where clinical swabs are directly added into wells containing the buffer solution (Flores et al., 2012). Some direct PCR-based approaches are also available commercially as part of sample collection devices (e.g. buccal swabs; Wang et al., 2011) to collect and preserve DNA. Direct PCR on cell cultures may also be useful but the time to culturing (from 24-72 hr) may limit its usefulness for sepsis. Increasing the sensitivity of these tests may allow use in POC devices as it may eliminate the need for culturing.

3.6 Other isothermal approaches with limited evidence for direct amplification

Studies documenting direct amplification on other less commonly used isothermal amplification approaches (Chang et al., 2012; Craw and Balachandran, 2012; Zhao et al., 2015) are fewer, as expected. Because some of them use multiple enzymes or steps and lower temperatures, the complexity of these assays may also be a reason for the lack of studies reporting direct amplification.

Genome exponential amplification reaction (GEAR), for example, developed at CDC (Jothikumar Prithiviraj et al., 2012) is similar to LAMP in that it also uses 2-3 sets of primers and *Bst* polymerase with a reaction mixture incubated at 65 ^{o}C. It is expected that most of the direct amplification observed using the LAMP primer design approach, may also be possible using GEAR. This is supported by direct amplification of *E.coli* O157:H7 at 20 cfu per reaction obtained by concentrating the cells present in 100 l of water. Primers targeted the *rfbE* gene and the test was complete within 60 min (Jothikumar et al., 2014). Similar to LAMP, reverse transcriptase can also be integrated with GEAR.(Guan et al., 2016)

Recombinase polymerase amplification (RPA) exhibits rapid amplifications with the potential for relatively simplified instrumentation. Using this approach, direct detection of *Klebsiella pneumoniae* without DNA extraction was recently reported (Valiadi et al., 2016). After capturing and concentrating the pathogens present in urine samples, the cells were lysed by heating and extracts were used for the amplification of the *blaCTM-X-15* gene. The sample processing time was 10 min in addition to the 20 min amplification time normally required by RPA. An LOD of 10^3 cells per ml of urine was reported. Previous studies with *Francisella tularensis* using extracted DNA as a template has shown more

rapid results (10 min) with better LOD (10 to 100 copies per ml plasma prior to extraction; Euler et al., 2012). RPA has also been used for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) with an LOD of less than 10 copies of DNA per reaction (Hoff, 2006). Besides distinguishing the methicillin-resistant from a methicillin-sensitive *S. aureus* strain, RPA was also able to ascertain the presence of three different genotypes of MRSA.

Strand displacement amplification (SDA) is another isothermal amplification approach that uses either *Bst* polymerase or Klenow fragment DNA polymerase (Walker et al., 1992). It works at 37 ^{o}C and is somewhat slower, requiring a reaction time of ~120 min. Replication starts at nicks created by a strand-limited restriction endonuclease (HincII). The nicked site is ligated with each displacement step resulting in exponential amplification. Commercial platforms are also available e.g., BDProbeTec™ ET System with inbuilt heating and centrifugation system that separates the DNA from the samples. A number of pathogens have been validated using *Mycobacterium tuberculosis* (Alnimr and Alnemer, 2012), *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Akduman et al., 2002).

Helicase- dependent amplification (HDA) uses DNA helicase to generate single stranded templates which is then amplified using DNA polymerase (Vincent et al., 2004). Direct detection of MRSA from blood cultures using HDA is known (Goldmeyer et al., 2008). The samples required a dilution and heating step prior to the amplification of *nuc* and *mecA* genes and reaction time was around 60 min with an LOD of 50 cfu per reaction.

Smart amplification process (SMAP/SMAP2) is an isothermal amplification technique which can be used for direct detection of single nucleotide polymorphisms through its complete suppression of background signals (Mitani et al., 2007). SMAP has the potential to determine the presence or absence of metastasis to lymph nodes using a

cancer specific marker with a reaction time of only 30 min (Hoshi et al., 2007). Its updated version (SMAP2) has also been employed for the direct detection of many specific somatic and cancerous mutations including lung adenocarcinoma mutations (Araki et al., 2010). This technique has also been utilized for the detection of lamivudine resistance associated hepatitis B virus mutation with a sensitivity of 20 mutant copies per reaction (Yang et al., 2011).

For most PCR or isothermal approaches, primer design approach and reverse transcriptase enzyme may be integrated to accomplish mutation detection and RNA analysis. Other polymerases have some innate reverse transcription activity allowing their potential use in both amplification and reverse transcription (e.g., *Bst* polymerase and Klenow fragment DNA polymerase; Shi et al., 2015). Nucleic acid sequence- based amplification (NASBA), however, was developed specifically for amplification of RNA sequences. Features of NASBA include: i) utilization of three enzymes including T7 RNA polymerase, RNase H, and AMV reverse transcriptase (Deiman et al., 2002; Jean et al., 2004), ii) constant isothermal temperature of 37-41 °C, iii) reaction time of about 60-90 min, and iv) multiplex amplification capabilities (Jean et al., 2004). Due to its ability to quantify mRNA transcript of the target gene, it can be used as a useful tool for measuring gene expression and thus can be used as a strong predictor of resistant phenotype (Tuite et al., 2014). Differentiation of *blaKPC* variants from their pure culture is an example of such a phenotypic approach for combating with antibiotic resistance (Spanu et al., 2012). It has traditionally been used for rapid detection and quantification of different RNA viruses. Using an RNA-based NASBA, detection of Parvovirus B19 DNA with an LOD of 10 genome copies per reaction has also been reported (Bonvicini et al., 2015). The utility and

application of NASBA for direct detection and quantification of viable *Ralstonia solanacearum* cells has also been reported with an LOD of 10^4 copies per reaction (Bentsink et al., 2002).

3.7 Required analytical sensitivity for nucleic acids-based POC devices

It is rather common to aim for the best LOD possible for any new assay. However, poor LOD alone is not an indication that the method is not valuable (many routinely used antibody assays have poor LOD). In rare cases, a method that is highly sensitive may even be less desirable because some background level of the infectious agent, not indicative of disease, may always be present (e.g., nucleic acids based assay for *C. difficile*; Burnham and Carroll, 2013). On the other hand, there are many instances where assay LOD of 10^2-10^3 cfu/ml is sufficient to diagnose an infection and take action. This is especially true for limited resource settings where actionable diagnosis cannot be made based on non-nucleic acids-based test results which are seldom available anyway. A POC test providing viral load or genotyping data for HIV at a fraction of the cost may be extremely useful even if the treatment cut off of 50 viral particle per ml of blood is not achieved by the assay. This is because direction of response (from 50 million viral particles to say 50,000 and then to 500 viral particles convey the required information to the physician). The last bit of data i.e., the point at which treatment can be stopped (50 viral particles per ml) may then be obtained by tests conducted in a centralized lab. The same logic applies for genotyping by POC which may provide information about correctness of treatment. Similarly, the situations where POC genetic testing is envisioned, a patient may get significant benefit from a less powerful test with results available right away rather than a more powerful test

available with results available much later. This point was nicely illustrated in a report by BioVentures for Global Health (Mehta and Cook, 2010). The report states that a diagnostic test with lower clinical sensitivity e.g., a rapid antibody-based tests with 70% sensitivity done at the point of care may be able to treat more patients compared to a diagnostic test with higher clinical sensitivity e.g., genetic tests with 90% sensitivity done in a centralized lab. This is because the rate of return of patients in countries where travel distances to seek healthcare is long, is a significant determinant of how many patients get treated. In the case of POCs, the rate of return is 100% while for centralized testing it could be much lower (say 70%). When multiplied by the sensitivity, the total number of treated patients could be 70% for POCs with less sensitive assay and 63% with centralized lab tests with more sensitive tests. Consequently, if the more sensitive genetic assays could be conducted using POC devices, the number of patients treated should be much higher (90%).

If a nucleic acids-based POC test is being developed for emergency rooms in developed nations, then clearing them for use in diagnostics through the Clinical Laboratory Improvement Amendments (CLIA) waiver process or U.S. Federal Drug Administration's 510(k) clearance is required. In certain cases, lab-developed tests (LDTs) – tests developed and used within the healthcare facility may be acceptable but regulations are changing rapidly for these as well. The analytical sensitivity of these established assays for detection of AMR and other pathogens is crucial for its clinical utility, particularly when used directly with clinical samples. At present, genetic testing directly from clinical samples using POC devices is rare. It is not surprising that out of the hundreds of tests available at the more than 225,000 CLIA-certified laboratories in the U.S., only few are for bacterial and viral pathogens (e.g., for Hepatitis C, Strep A, Influenza A/B, HIV-1,

Helicobacter pylori, and *Borrelia burgdorferi*) and none are based on nucleic acids amplification. This is because CLIA-waived assays must be simple and rugged and nucleic acids-based assays are not yet simple enough because they require sample processing and DNA/RNA extraction. Cumbersome protocols and kits for nucleic acids extraction and purification have been an integral part of traditional genetic assays. This required the samples to be shipped to a centralized lab for processing by expert molecular biologists. The analytical sensitivity for nucleic acids-based POC devices, may therefore be guided by their beneficial use case scenario in a given geographical location rather than as a competing technology with centralized screening tools. In some cases, such as quickly determine the AMR which is not always dependent on LOD, somewhat inferior instrument detection limit may not be a hindrance in the usefulness of the test.

3.8 Nucleic acids-based POC devices

Multiple reviews exist on POC devices, highlighting potential for measuring antimicrobial resistance under field conditions (Rozand, 2014; St John and Price, 2014). Important features of POC devices for on-site detection include ease-of-use, cost, assay time, and ruggedness. Amplification chemistries and assays should also be sensitive and specific for clinical utility. Though POC devices are typically developed to detect a single assay, multiplexing for detection of multiple pathogens (e.g. sepsis) is crucial. This also allows for analysis of genes related to specific functions, as opposed to simple organism identification through 16S rRNA, for predicting virulence and potential resistance, enabling better treatment decisions. Self-digitizing microfluidics with multiple reaction wells is one such means of multiplexing. The simplicity of using plastic (Stedtfeld et al., 2012) or paper-

based (Rozand, 2014) materials for this purpose allows for more rugged (though still powerful) systems, while keeping equipment and consumable costs low, particularly when integrated miniaturization. Use of direct amplification with POC devices further reduces the time-to-results by potentially eliminating sample processing. Other POC devices (particularly qPCR-based devices) implement sample processing directly into these devices.

Some of the nucleic acids amplification- based POC devices exist that are commercially-available or near commercialization include Twista (TwistDx, n.d.), Illumigene, NucleSENSEasyQ, and Gene-Z. Twista (TwistDx, United Kingdom) uses RPA with capabilities for 8 samples per experiment while a number of devices use LAMP, detecting either fluorescence (Tomita et al., 2008; such as SYBR Green or SYTO82) or turbidity (e.g., Illumigene; MerdianBiosciences, n.d.) Meridian Biosciences, United States, capable of detecting 8 assays per run). NucleSENSEasyQ (Shumoski, n.d.; BioMérieux, France) uses NASBA and can detect 8-48 assays per run. For qPCR, the options are many since it has been the gold standard but fewer can be considered POC devices. Portable, low-cost qPCR devices that integrate sample processing into the workflow and may be used for direct amplification protocols include Hunter (InstantLabs, n.d.; Instant Labs, United States) which can detect 6 assays per run or GenePOC (GenePOC Diagnostics, United States) which can detect 94 assays per run. Open-source qPCR- POC devices are also on the horizon (e.g., OpenPCR; ChaiBiotechnologies, n.d.) Chai Biotechnologies, United States) which is currently available for under $3,200. It was manufactured through crowd-funding.

3.9 Direct amplification and antimicrobial resistance

It is obvious that if the sample processing step is eliminated or simplified, it will save time and equipment design and cost. Indirectly, it will also help save lives by providing timely analysis of resistance. Direct amplification is well established and approaches to enhance direct amplification are also becoming available. These include development of better polymerases, addition of nanomaterials that most likely puncture the cells, integration of sonication in microfluidic chips that may help break the cells, etc. Therefore, it should be possible to eliminate DNA extraction and purification steps for some of the sample matrices either entirely or partially. For a given sample matrix, it may be appropriate and beneficial to first explore if the DNA extraction and purification step is needed for the chosen amplification process and polymerase, before deciding to integrate a complex and cumbersome sample flow scheme. A simplified platform is more likely to be deployed globally for AMR surveillance – a critical component of antimicrobial resistance stewardship programs. However, some additional work summarized below needs to be done to demonstrate the value of direct amplification and integrate the overall scheme with existing and emerging POCs.

Most studies reporting direct amplification at present are carried out with limited number of samples. Validation studies with clinical specimens similar to those required for 510(k) approval for a given target may provide further confidence and also identify the limitations of direct amplification in a clinical setting. Such validation studies invariably are designed to address analytical precision and reproducibility, analytical sensitivity and specificity, clinical sensitivity and specificity, lower limit of detection, reportable range, and accuracy among other method development parameters.

3.10 Validation of existing assays for use with direct amplification

Many primer sets already exist for detection of sepsis- causing bacteria and those that are resistant to antibiotics. It follows that the implementation of direct amplification in this area may not be difficult, since assays have already been developed and validated (Figure 3.3). Primers for qPCR exist for thousands of organisms and genes and can be found in the literature and primer databases (Alm et al., 1996; Jaziri et al., 2014; Loy et al., 2003). Furthermore, some direct POC- based assays for pathogens including sepsis- causing organisms (e.g., those described in Figure 3.2**)** have shown detection limits as low as 5 cfu/ml sample, though some methods require culture prior to detection. The important work that remains includes evaluation of these assays using other direct amplification protocols in more clinical matrices, particularly ensuring that sensitivity is not significantly impacted when the inherent target concentration that occurs during nucleic isolation is removed.

3.11 Primer coverage for nucleic acids-based assays

Thousands of assays are developed for single organisms relevant to AMR targeting usually a single genetic marker and using extracted DNA (Bonomo, 2011; Hanaki et al., 2011; Metwally et al., 2014; Nawattanapaiboon et al., 2016; Qi et al., 2012; Su et al., 2014)**.** However, when multiple pathogens or markers must be targeted, the problem becomes more complex. Even for the presumably simple case of CRE, primer and coverage issues may pose a challenge because of the diversity in AMR gene and their allelic variability. The detection of CRE, for example, may require the use of multiple genetic markers including *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase-

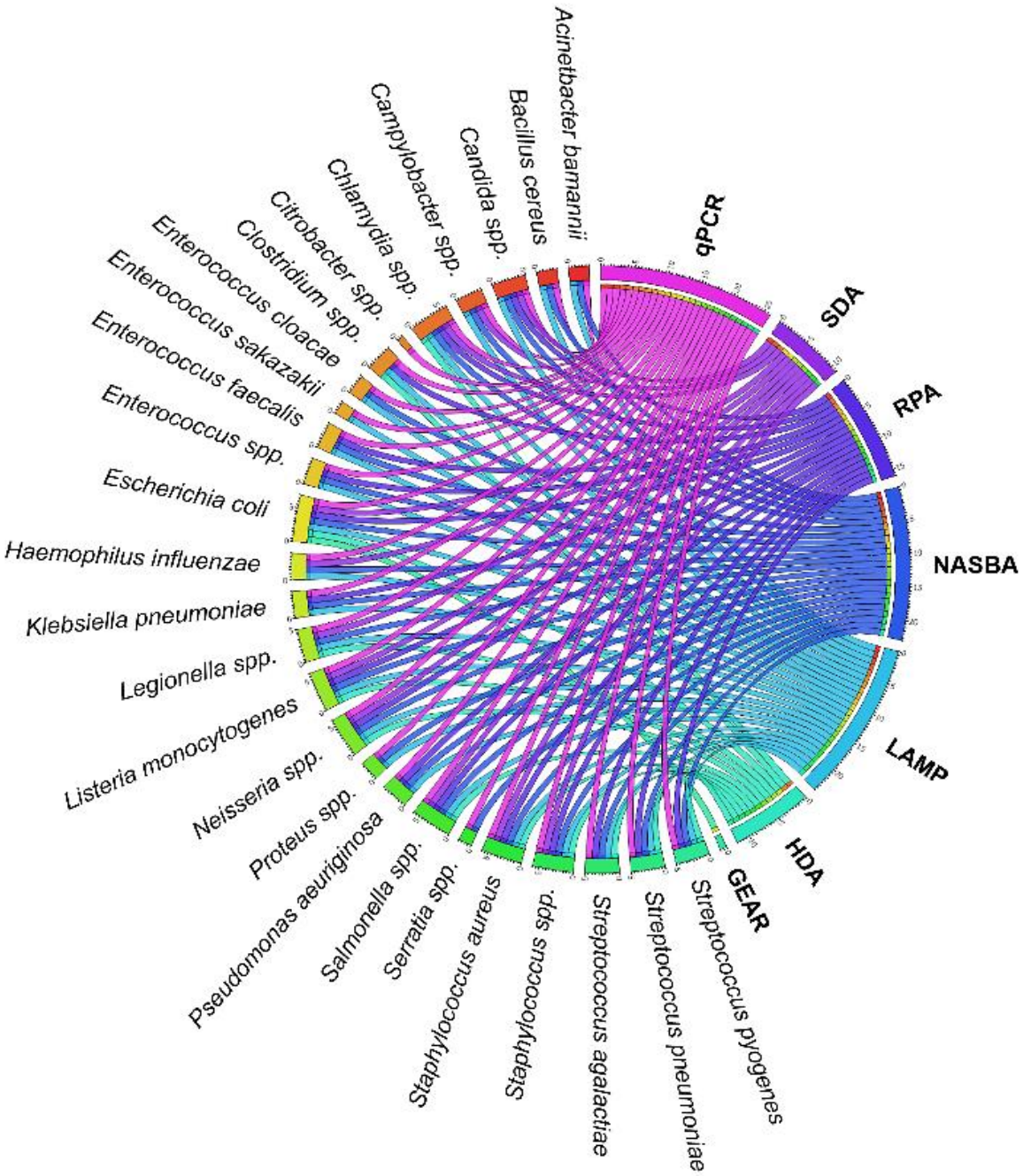

Figure 3.3: Analysis of studies reporting primers for select sepsis- causing organisms by nucleic acid amplification method (plotted using Circos Krzywinski et al., 2009). Results obtained from Web of Science keyword searches.

1 (NDM-1), oxacillinase (OXA), Verona imipenemase (VIM), imipenemase (IMP), cefotaximase (CTX-M), and extended spectrum β-lactamase (TEM, and SHV), all of which are markers of carbapenem resistance.

When designing primers, it will be important to address the allelic variability in each. Sometimes it may be easy because of similarity in sequence. For example, analysis of about 239 full-length NDM gene sequences shows that for the 810 base positions along the NDM gene, only 17 bases have some degeneracy. This indicates that universal primers are possible for NDM-1 (and indeed qPCR-based methods utilize this sequence conservation for primer design to provide thorough coverage of most NDM-1 sequences. The same is true for the KPC gene. However, other antibiotic resistance gene markers may have higher allelic variability. For example, the CTX-M gene must be separated into three groups: Group I (402 sequences) with 172 degenerate positions out of 930 base positions; Group II (228 sequences) with 125 degenerate positions out of 930 base positions. While Group III had 33 degenerate bases out of the 930 bases. What is evident is that nucleic acids-based molecular measurement of AMR will require multiple primer sets to theoretically achieve good coverage of most known sequences. For markers with high allelic variability (high diversity), the coverage may be unacceptably low highlighting the limitations of amplification-based tools compared to sequencing.

3.12 Sample concentration to enhance the lower limit of detection

When target concentration is low (e.g., at levels where sample volume used does not ensure at least one cell or organism per well), sample concentration must be performed using filtration, magnetic or dielectrophoretic separation. Cartridges and approaches that

can concentrate and then allow for lysis and/or direct amplification are being developed(Ahmad et al., 2017). We have also developed a technique in which filters were amplified directly within disposable Gene-Z chips (Stedtfeld et al., 2016, 2012). The method does not require a step to elute concentrated cells from filters and reduces lose that would occur due to DNA extraction. As such, a detection limit of 10^2 *Dehalobacter* cells per 100 mL of groundwater was observed. Others provide methods that can sort or trap the bacterial cells by other mechanisms (e.g., dielectrophoresis) in order to concentrate the cells into a smaller volume starting with say 1-10 ml of blood or other body fluid. It is envisioned that over time, sample concentration schemes that need smaller sample volumes (e.g., 10 ml of blood, or 50 ml of urine, 100 ml water etc.) will be integrated in the POC itself. This will be important to adapt direct amplification in POC devices. Sample concentration for matrices or applications where much larger sample volumes must be processed in order to obtain few cells may still use a separate concentration step (e.g., hollow fibre membrane used for concentration of viruses from water; Jothikumar et al., 2014).

3.13 Multiplexing and genotyping

The development of multiplexed approaches where multiple assays (specifically AMR) are detected simultaneously for a given sample has greater potential to reduce costs and decrease turnaround time-to-results. As of now, the most highly parallel AMR gene detection systems are based on PCR and require DNA extraction (Looft et al., 2012; Wang et al., 2014), but the development of POC technologies for AMR genes using isothermal direct amplification are being developed (Kostić et al., 2015). When using microfluidic cards with parallel reaction wells combined with LAMP, 20 AMR genes could be detected

simultaneously in a single experiment (Kostić et al., 2015). Results were obtained with heat-lysed *S. aureus* isolates, without DNA extraction or other concentration methods. When comparing the direct isothermal amplification method with results obtained through DNA extraction and qPCR, a 98.2% agreement was observed.

3.14 Integrating cell viability and antibiotic susceptibility

Nucleic-acid based methods at the DNA level will measure both DNA from live cells as well as extracellular, if any, from dead cells of the target organism. Hence, applications where live/dead differentiation must be made and viability established may not be well served by NA-based POC devices. In these cases, a number of chemical agents including propidium monoazide (Nocker et al., 2007) and ethidium monoazide (Rudi et al., 2005) have been used to block the extracellular DNA from amplifying. In some instances, this blockage is partial in that amplification is delayed. One of the steps in the treatment with blocking agents is exposure to light so that the blocking agent forms a covalent bond with DNA, preventing denaturation and amplification. This is an extra step at present and could be integrated into POC devices. Similarly, the ability to carry out rapid antibiotic susceptibility testing that may take a little longer (e.g., few hr), in parallel to molecular tests that are available much sooner (e.g., within 1 hr) will be ideal. For example, one study used a short cultivation step in the absence and presence of different antibiotics prior to padlock probe detection of the bacterial target DNA to allow a determination of susceptibility and genetic identification at a total assay time of 3.5 hr (Mezger et al., 2015).

3.15 Developing more robust polymerases that do not require DNA extraction or purification

The mechanism by which amplification enzymes, such as *Bst* polymerase, displays higher tolerance to inhibitors compared to PCR polymerases is mostly unknown. What is well established is that *Bst* polymerase can work in the presence of inhibitory substrates, at up to 100-fold higher concentrations, and is more successful in achieving direct amplification than *Taq* polymerases. Some insight can be gained by comparing the steps taken to minimize inhibition during PCR and isothermal amplification for various sample matrices. Amplification of nucleic acids by polymerases may be inhibited due to a number of factors including binding of chemical to active sites of the polymerases, alteration of DNA melting temperatures, reduced availability of Mg^{2+} required for polymerase activity, sequestering DNA from the reaction mixture, or interference with cell lysis (Schrader et al., 2012). Cellular debris, for example, is thought to interfere with PCR by causing sequestration of nucleic acids and primers due to interaction with proteins (Wilson, 1997). Increased primer concentration was proposed as a mechanism to alleviate some of this inhibition (Notomi et al., 2000). Urea, an inhibitor to and possible degrader of polymerases (Schrader et al., 2012), may be present up to 330 mM concentration in human urine (Rauter et al., 2005). Direct amplification is generally successful for up to 20% urine implying a tolerance of approximately 50 mM urea (Hill et al., 2008). *Bst* polymerase may have somewhat higher tolerance for urea compared to traditional PCR polymerases. Similarly, direct amplification in blood is inhibited by a number of components including hematin, heme, lactoferrin, and EDTA. Hematin may alter DNA melting temperature and enhance metal chelation (Opel et al., 2010). Binding of heme to DNA polymerase resulting in

feedback inhibition has been known for decades (Byrnes et al., 1975) and addition of bovine serum albumin is shown to reverse this inhibition.(Akane et al., 1994) Blood specimens often contain EDTA as preservative which chelates Mg^{2+} ions (Al-Soud and Rådström, 2001). For isothermal polymerases to work efficiently, the available Mg^{2+} concentration must be between 4 to 8 mM.(Notomi et al., 2000) Lactoferrin, another component that is present in body fluids releases Fe^{3+} ions which decreases primer specificity (Abbazadegan et al., 1993). Because isothermal amplification, and especially LAMP uses 4 to 6 primers, this decrease in specificity may still not significantly impact the outcome.

3.16 Summary

The amplification approaches for detection of AMR exist, using both isothermal platforms and qPCR-based polymerases. Isothermal approaches have shown better promise for samples with higher levels of substrates inhibitory to amplification. For direct amplification, qPCR may be useful if a buffer if added to prevent or reduce inhibition during direct amplification, but results typically take longer (with a reaction time of 1-3 hr**;** Table 3.1). An advantage to using qPCR for detection of ARG is the availability of published primers, as it is the gold standard for DNA amplification and thus most widely used. As there are only 2 primers required for qPCR, the issue of primer coverage is also much more easily faced. However, qPCR-based POC devices may be more costly and complex due to temperature cycling, whereas with isothermal amplification, this is not an issue. Other isothermal approaches requiring less primers, such as GEAR, RPA, SDA, SMAP/SMAP2, and NASBA, may mitigate this issue, while also allowing the use of isothermal POC devices. Though LAMP requires 6 primers, of the isothermal approaches,

LAMP has shown the most significant evidence for direct amplification, presumably due to its use of *Bst* polymerase, which is highly tolerant to clinical matrices. As it can also detect targets as low as a single copy in under an hr, it may be best suited for direct amplification. Furthermore, the development of POC devices that use LAMP are simple, due to the isothermal temperature requirement as well as its ability to detection either fluorescence or turbidity. This would allow rapid field deployability of the assays to rapidly diagnose AMR infections. The use of direct amplification in POC systems by isothermal or PCR polymerases has the potential to rapidly diagnose AMR infections.

Future research focusing on the validation of direct amplification approaches for detection and diagnosis of AMR infections, preferably using POC platforms may further provide confidence in this emerging approach. The reduction in time-to-result will help expedite decision-making by clinicians. It may also allow better limit of detections because recovery losses during sample processing may be avoided. Studies focusing on the mechanisms and reagent conditions that allow direct amplification, primer design to provide the needed coverage of AMR, and development of more robust polymerases may also need to be explored. Finally, while the ability to detect 1 cfu per ml or 1 cfu per reaction is a good target, it is best to link such objectives to the decision-making process of the physicians, especially for goals related to prevention of antimicrobial resistance through better diagnostic tools.

Table 3.1: Summary of some direct amplification approaches and the LOD achieved in some sample types

Name	Enzyme(s) Required	Reaction Temperature	Time to Results	LOD with Direct Amplification	Reference
PCR, qPCR	*Taq* Polymerase	Denaturation: ~94-96°C Annealing: 45-60°C Extension: 72°C	1-3 hour	~100 cfu per ml	(Vuong et al., 2016)
LAMP	*Bst* Polymerase	60-65°C	60 min	10^2 copies per reaction	(Misawa et al., 2007)
NASBA	Reverse Transcriptase; *T7* RNA Polymerase; RNase H	37-41°C	60-90 min	10^4 copies per reaction	(Bentsink et al., 2002)
HDA	Helicase; DNA Polymerase	37; 60-65°C	60-90 min	50 cfu per reaction	(Goldmeyer et al., 2008)
GEAR	*Bst* Polymerase	65°C	60 min	20 cfu per reaction	(Jothikumar et al., 2014; J Prithiviraj et al., 2012)
SDA	DNA Polymerase; Restriction Enzyme	37°C	120 min	99% sensitivity	(Akduman et al., 2002)
SMART-AMP/ SMAP2	*Aac* DNA Polymerase; Taq MutS	41°C	30-45 min	1% mutant DNA	(Araki et al., 2010)
RPA	*Bsu* Polymerase; Recombinase	37°C	10-20 min	1000 cfu per reaction	(Valiadi et al., 2016)

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CHAPTER IV

Antimicrobial resistance (AR) dashboard application for mapping environmental occurrence and resistant pathogens

The work in Chapter IV has been published:

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Author contribution statement:

M. R. W. collected and processed surface water samples. R. D. S. analyzed surface water samples. M. R. W. and R. D. S. analyzed results and developed results pertaining to surface water samples. M. R. W. and U. F. developed the dashboard application. T. A. J., T. S., and F. W. collected, processed, and analyzed results from wastewater treatment plants. W. K., M. H., B. E. E. collected, processed, and analyzed results from clinical isolates. R. D. S., M. R. W., J. M. T., and S. A. H. wrote the paper. All coauthors participated in editing.

Abstract

An antibiotic resistance (AR) Dashboard application is being developed regarding the occurrence of antibiotic resistance genes (ARG) and bacteria (ARB) in environmental and clinical settings. The application gathers and geospatially maps AR studies, reported occurrence and antibiograms, which can be downloaded for offline analysis. With the integration of multiple data sets, the database can be used on a regional or global scale to identify hot spots for ARGs and ARB; track and link spread and transmission, quantify environmental or human factors influencing presence and persistence of ARG harboring organisms; differentiate natural ARGs from those distributed via human or animal activity; cluster and compare ARGs connections in different environments and hosts; and identify genes that can be used as proxies to routinely monitor anthropogenic pollution. To initially populate and develop the AR Dashboard, a qPCR ARG array was tested with 30 surface waters, primary influent from three waste water treatment facilities, ten clinical isolates from a regional hospital and data from previously published studies including river, park soil and swine farm samples. Interested users are invited to download a beta version (available on iOS or Android), submit AR information using the application, and provide feedback on current and prospective functionalities.

4.1 Introduction

The emergence of antibiotic resistance (AR) in pathogenic bacteria (ARB) is a global problem (WHO, 2014). While AR is increasing at an alarming rate (e.g. methicillin-resistant *Staphylococcus aureus* increased from less than 5% in the 1980s to 60% of cases today, (Cardo et al., 2004), development of new antimicrobials is occurring at a much slower rate (Wright, 2015). Links between humans and the environment can promote emergence, spread and

transmission of AR infections. Development and implementation of surveillance tools are among the major goals identified in the National Action Plan for Combating Antibiotic-Resistant Bacteria (The White House, 2015).

In response, numerous national and international networks are currently focusing on aspects of stewardship and surveillance (Peirano et al., 2014) including: World Health Organization's Global Report on Surveillance (WHO, 2014), CDC's National Antimicrobial Resistance Monitoring System (NARMS; FDA, 2010), European Antimicrobial Resistance Surveillance Network (EARS-Net; ECDC, 2012), Canadian Antimicrobial Resistance Surveillance System (CARSS; PHAC, 2015) and Asian Network for Surveillance of Resistant Pathogens (ANSORP; www.ansorp.org). Most of these programs have databases to disseminate and coordinate AR related information primarily focused on health care settings. However, AR associated risk assessment must include tracking AR bacteria and genes (ARG) in the environment (Berendonk et al., 2015). A curated database that combines healthcare and environmental ARB and ARG occurrence could bridge this gap. In addition, the availability of a comprehensive database to help differentiate the natural resistome from anthropogenic distribution of AR in the environment would identify where changes are likely to be most effective for containment, whether through public policies or community actions.

We are developing an AR Dashboard Application for geospatial mapping of ARGs, mobile genetic elements (MGEs) and ARB occurrence in environmental and clinical samples. The term ‘Dashboard’ relates to mapping functionality. The Dashboard App is well-timed with the increasing use of technologies including next generation sequencing (Li et al., 2015), bioinformatics tools to mine metagenomes (Gupta et al., 2014; McArthur et al., 2013; Rowe et al., 2015; Wang et al., 2015), qPCR arrays to survey hundreds of ARGs in parallel (Looft et al.,

2012; Wang et al., 2014), the increasing availability of point of use tools to routinely track genetic markers (Kostić et al., 2015; Stedtfeld et al., 2012) and curated sequence database (e.g. Antibiotic Resistance Gene Database, ARDB; (Liu and Pop, 2009); and Comprehensive Antibiotic Resistance Database, CARD; (McArthur et al., 2013); Structured ARG Database, SARG; (Yang et al., 2016). The AR Dashboard is being developed to provide two key tools. The first will be in the form of an antibiogram dashboard, and the second for geographically linking information on ARG and ARB occurrence. Antibiograms, a matrix of the percent of infectious agents resistant to specific antimicrobials regionally encountered, enhance awareness of ARB present at a given location, and help clinicians formulate appropriate treatment strategies and inform policy formulation, implementation and evaluation. Our expectation is that by making the AR Dashboard freely available globally, regional antibiograms will be more accessible to all physicians, scientists and policy makers to observe clinical resistance trends at national and international scales. Similarly, mapping AR information geographically will allow for identification of regional hotspots and actions for containment. Together, the AR Dashboard may also provide baseline information necessary to link the environmental spread of AR to routes of transmission.

The AR dashboard is still under development and should be considered at beta stage. This manuscript is to demonstrate current and potential functionalities of the database and also invite interested physicians and researchers to i) download a beta version of the application (publically available by March 2016, search respective application stores for keywords ‘AR Dashboard’), ii) submit information using the application and iii) provide feedback regarding current and future functionalities. Information including antibiograms, studies and ARG/ARB occurrence can currently be added using submission pages available on the app (Figure 4.1). Our

goal is to have a publically available beta version by March 2016 and a more complete application and database by September 2016. The application is cross platform and can be downloaded for free using respective stores available on iOS, Android or Windows smartphones.

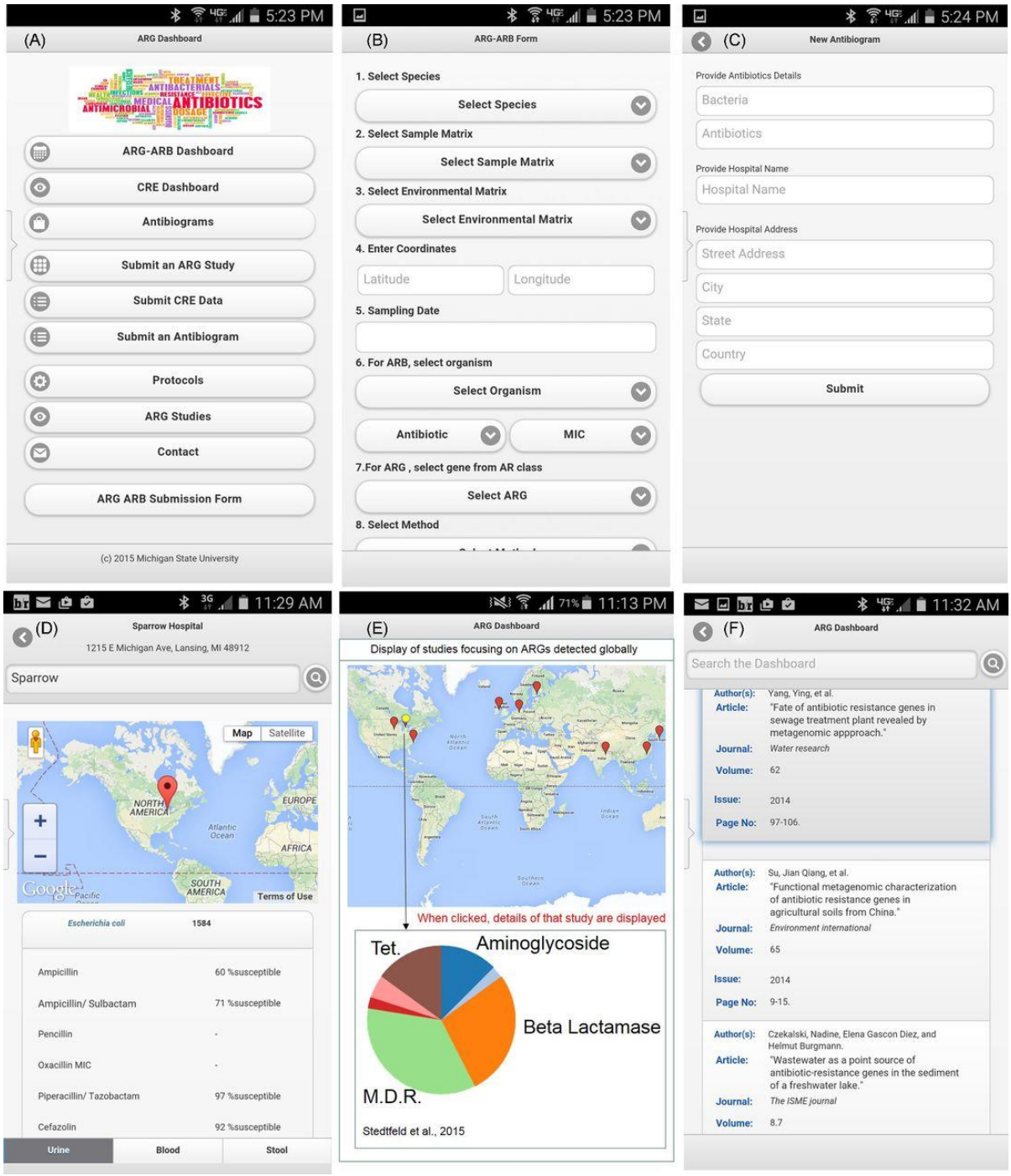

Figure 4.1: Screenshots of AR Dashboard functions including A**.** home screen, B**.** submitting ARG and ARB occurrence, C**.** submitting antibiograms, D. mapping results of antibiograms and providing data about antibiotic susceptibility, E. mapping results of submitted AR occurrence and F. curated AR studies.

To demonstrate utility, populate and develop the AR Dashboard Database, a qPCR array targeting ARGs and MGEs was tested with multiple samples including i) 30 environmental surface waters throughout Michigan, ii) primary influent from three waste water treatment facilities in mid-Michigan and iii) ten clinical isolates from a regional hospital (Sparrow Hospital, Lansing, MI). Results of these samples were analyzed to demonstrate utility of the downloadable database for identifying ARG and AR hotspots, studying factors that influence levels of ARG in the environment, categorizing natural versus anthropogenic distributed ARGs and track ARB infections. Additional data from swine farm, park soil and river samples (Ouyang et al., 2015; Wang et al., 2014; Zhu et al., 2013) were assembled to demonstrate connections and clusters between sample types.

4.2 Materials and Methods

4.2.1 AR dashboard application

The AR Dashboard App was developed using a cross platform application program interface (API) technology developed by Appery.io (www.appery.io). The platform allows the use of existing APIs with the ability to incorporate new programs using JavaScript. Additional features can be incorporated using Java, css and HTML5. Integration with Google Maps and potentially other powerful online databases such as Google Charts (for including, sorting and visualizing different levels of environmental factors with presence of AR) or CARD (for linking with sequence information) are an integral part of the Appery.io. Perhaps the greatest functionality of the Appery.io, is that developed applications are readily usable on Android, iOS and Windows operating systems. Thus it can be deployed on most smartphones and personal computers.

4.2.2 Collection and processing of environmental samples

Surface water samples (50 ml) were collected from 28 lakes and two different samples sites from a single river in the state of Michigan. Primary influent samples were also collected from three waste water treatment facilities (East Lansing, Lansing, Jackson, MI, USA). Samples were immediately frozen at −20°C until solid and shipped to the laboratory overnight in insulated containers. Received samples were stored at −20°C prior to processing. Sample processing included filtration, DNA extraction and whole-genome amplification. To concentrate biomass, 3 ml of each sample was passed through a 0.45 μm filter (Millipore, Darmstadt, Germany). DNA was extracted directly from filters using the MoBio PowerWater DNA extraction kit (Mo Bio Laboratories, Carlsbad, USA) following suggested protocols and a final elution volume of 50 μl. DNA extraction yields were measured using a Qubit fluorimeter and the Qubit dsDNA high sensitivity kit.

Community DNA extracted from surface water samples was enriched further using whole-genome amplification (GE Healthcare Life Sciences' Illustra TempliPhi Little Chalfont, UK). Following the suggested protocol, 1 ng of extracted DNA was added to 5 μl of sample buffer followed by denaturation at 95°C for 3 min and cooling to room temperature. A premix containing 5 μl of reaction buffer and 0.2 μl enzyme mix was added to the DNA buffer mixture. Reaction mix was incubated at 30°C overnight. After incubation, the enzyme was heat-inactivated at 65°C for 10 min. The amplification product was fragmented via sonication and purified using QIAGEN's QIAquick PCR Purification kit. Amplified DNA concentrations were measured using the Qubit fluorimeter and the Qubit dsDNA broad range kit (Life Technologies, Carlsbad, USA). Preliminary experiments were performed to evaluate bias of whole-genome

amplification prior to qPCR. A positive correlation of $R^2 = 0.74$ was observed between threshold cycle of samples with and without whole-genome amplification (Figure A4.1).

4.2.3 Collection and processing of clinical isolates

Bacterial isolates (Table A4.1) were collected at Sparrow Hospital's Microbiology, Immunology & Molecular Diagnostics Laboratory (Lansing, MI) using standard culture techniques in the Sparrow Clinical Microbiology Laboratory. Identification was performed using Siemens Microscan, BD Phoenix, and/or biochemical tests. Prior to revival, isolates were stored in 15% glycerol stocks at −80°C. Isolates were revived by growing on TSB media overnight at 37°C (no agitation). Genomic DNA was extracted from 1.5 ml of the revived stocks using the Qiagen Blood and Tissue kit (Qiagen, Hilden, Germany), following the protocol for lysing Gram-positive bacteria. DNA concentrations were measured using the Qubit fluorimeter and the Qubit dsDNA broad range kit (Life Technologies, Carlsbad, USA), and samples were run without previous whole-genome amplification.

4.2.4 qPCR array

The qPCR array contained 296 primer sets targeting AR mechanisms in all major classes of antibiotics. All primers sets were described in previous studies (Wang et al., 2014; Zhu et al., 2013). The *intI1* gene primer proposed by Gillings and coauthors (Gillings et al., 2015) was also included (run separately on the Chromo4 BioRad (Hercules, USA) using reagents and cycling parameters recommended with the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, USA). Quantitative PCR reactions with environmental samples were performed using a Wafergen SmartChip Real-time PCR system (Wafergen Biosystems, Inc, Fremont, USA) as

reported previously (Wang et al., 2014). Sample and primers were dispensed into the SmartChip using a Multisample Nanodispenser. PCR cycling conditions and initial data processing was performed as previously described (Wang et al., 2014), except that a threshold cycle of 28 was used as the detection limit, based on recommendations from Wafergen. Experiments with clinical isolates were performed on the OpenArray platform (Applied Biosystems, Carlsbad, USA) with reaction conditions and analysis as previously described (Looft et al., 2012; Stedtfeld et al., 2008). The OpenArray had the same primers as the 296 primer array, but contained 40 additional primer sets covering ARGs (these additional primers were not included in analysis). All qPCR reactions were run in triplicate reaction wells.

4.2.5 Data analysis

Estimated genomic copies and relative abundance were calculated as previously described (Looft et al., 2012; Wang et al., 2014). Relative abundance was calculated to normalize for inhibition and variations of bacterial DNA in each sample. The previously described (Looft et al., 2012) equation assumes an efficiency of one and estimates gene abundance. qPCR efficacy has been tested for our method of primer design and the small volume qPCR (Stedtfeld et al., 2008). To verify correct amplification of target genes, amplicons from ~30 primer sets have been sequenced. Multivariate analysis of ARG profiles between samples was performed in R using the Vegan package (Oksanen et al., 2014). Log_2 transformed values of the gene abundances relative to the 16S rRNA gene were used for Redundancy Analysis (RDA), which is a multivariate analysis tool for ecological studies (Oksanen et al., 2014). A heat-map was generated using TMEV (www.tm4.org/mev.html). Venn diagrams were prepared using R Studio v.3.1.3 (RStudio, Boston, MA, 2012). Figure showing population density with level of

ARGs in surface waters was made using Tableau Student Edition (Tableau Software, Seattle, WA). qPCR results from swine farms, park soils and river samples (Ouyang et al., 2015; Wang et al., 2014; Zhu et al., 2013) were included to compare sample type and connections in ARG occurrence. To demonstrate quantitative co-occurrence among various methods of measurement (e.g. qPCR and metagenomics), metagenomics data from two of the five primary influent waste water treatment plant (WWTP) samples (Li et al., 2015) were used for network analysis. Gene co-occurrence and RDA ordination were performed using the summed relative abundance for all primers targeting a given gene. Co-occurrence network analysis, based on Spearman correlation (Williams et al., 2014), was rendered using Cytoscape v. 3.3.0 (Institute for Systems Biology, Seattle, USA). Networks were organized by the prefuse force directed openCL layout based on correlation. Gene co-occurrence calls included detection in a majority of samples among a sample type (excluding lake samples in which the cutoff was 25%), false-discovery correction *q*-value < 0.05, $\rho > 0.75$ and *P*-value < 0.005. Node size was discretely mapped with number of connections between primers, and color of edges was continuously mapped with correlation (gray: $\rho \sim 0.75$; black: $\rho \sim 1.0$).

4.3 Results and Discussion

4.3.1 Wireframe and functionality of AR Dashboard

The AR Dashboard is being developed for participation and collaboration in tracking the occurrence, spread and emergence of ARB and ARGs (Figure 4.1). Currently, the App provides a central repository, mapping visualization and the ability to submit new information such as studies, antibiograms and occurrence of AR events. It was developed with the emphasis that information can be gathered and made available to researchers and care givers globally.

In detail, users of the AR Dashboard can submit information on AR type, the sample matrix in which the AR was observed, information regarding biological and technical replication, method for detection, latitudinal and longitudinal coordinates of sampling area and the date of sample collection. For submission of multiple results in parallel, users can download a submission form to be filled in a tabular format. Once completed, information is uploaded via the application, and automatically added to the database. Mapping capabilities will allow for an assortment of viewing capabilities based on type of ARG or ARB, location, sampling matrices, sampling date and method used for measurement. The database will be freely available (downloadable within the application), in which users can perform additional offline analysis.

The Dashboard app also provides a mechanism to collect and disseminate clinical antibiograms. Antibiograms are developed and maintained by many well-equipped hospitals, community health organizations and laboratories; as a tool to inform and aid physicians with decisions on antibiotic therapies. Currently, antibiograms are only available locally, however, the AR Dashboard will provide a page for users to access antibiograms globally and submit new antibiograms. Users can simply search for antibiograms based on hospital name, location or by finding with a map. The program will allow for users to sort and graphically visualize antibiogram information based on location of antibiogram, antibiotics and bacteria type. Currently, the application only has two antibiograms from local hospitals in Lansing, MI, but we are inviting researchers and clinicians to use the application to upload additional antibiograms. Antibiograms can be submitted via tabular or graphic formats.

Currently, the application also includes a CRE Dashboard specific to carbapenem resistance Enterobactericea, a global threat that is being tracked by many countries due to current impact on human health. Due to the clinical relevance of this group of organisms, it can be

handled independently of all other ARGs and ARB. A current version of the ARG array, not yet described in the literature also focuses on this group of organisms. In the future, this capability can be extended to other resistant pathogens of global concern. The Dashboard App also has a repository of key protocols for screening and tracking ARGs.

Prospective challenges of the AR Dashboard will include scaling-up with continual updates, participation and inconsistencies between measurement methods. Scaling challenges may require the use of additional database utilities. This was the reason for selection of the Appery.io, which can integrate other programs such as Goggle Maps and Charts. Oversight costs are also minimized using the Appery.io, which is a visual programming language that does not require developers to understand coding languages. As such, the Application can be maintained by relatively non-trained personnel.

For samples that require anonymity (e.g. detection of ARGs from a commercial farm or agricultural facilities), results do not need to be mapped with exact GPS coordinates. Submission of these occurrences can solely mention sample matrix and proximal location at a regional city or country level. In some cases, nomenclature of ARGs may not be consistent between reporting events (e.g. ARGs associated with aminoglycoside resistance); however, increased use of structured ARG databases should alleviate these issues (Yang et al., 2016).

Methods for detecting ARGs (e.g. metagenomics, qPCR, hybridization or susceptibility arrays) with varying levels of sensitivity, throughput and target coverage will influence quantitative comparisons between datasets. These variabilities may also cause discrepancies in reported occurrence, however, the database asks users to provide details on the tools used for testing, and different types of quantitative information. Relative abundance and concentrations of detected ARGs can be provided for all techniques to map results together. For example,

abundance ratios can be calculated as previously described (Li et al., 2015) for metagnomics results. In detail, length of ARG reads and reference sequences and the number of sequence reads can be used to calculate abundance ratio, which provides results similar to qPCR relative abundance. Only occurrence or presence of ARG/ARB can also be submitted if a given technique is not quantitative. The application is being designed so that users can choose to examine or request occurrence results from varies methods individually or together.

4.3.2 Mapping ARG hotspots and factors promoting distribution and persistence

One utility of the downloadable database is to help identify hotspots and factors contributing to AR occurrence. For samples tested within this study, ARG from nine major antimicrobial classes were detected. Initial analysis of these samples included a RDA of ARG relative abundance (Figure 4.2A). The RDA, which is a multivariate analysis (Oksanen et al., 2014), placed lake and primary influent samples into four groups. The three primary influent samples clustered into group 1, and the surface water samples with varying quantities of ARGs clustered into groups 2–4 (Figure 4.2B). A river sample collected one mile downstream of a waste water treatment facility (GR Walker, Figure 4.2A) contained the highest detected number of ARGs (n = 37) and clustered into group 2. The surface water sample with the lowest number of detected ARG (1) clustered into group 4. On average, 161 ARG targeted primers amplified in the primary influent samples (Figure A4.2B), which was 4–20 times higher than the average number of ARGs detected in the environmental waters (groups 2–4).

As expected, the river sample collected near the effluent line of the waste water treatment facility was a hotspot for ARGs, which has been previously observed (Xu et al., 2015). The level of ARGs detected in the group 4 samples may potentially provide a baseline for background

abundance levels in lakes (Czekalski et al., 2015). The elevated level of ARG observed in the other surface water samples (groups 2–3) may be due to a number of factors such as proximity, type and density of agricultural facilities (Hsu et al., 2014; Pruden et al., 2012), hospitals (Rodriguez-Mozaz et al., 2015), pharmaceutical and municipal landfills (Wu et al., 2015). Mapping the database of ARG and AR with these facilities may help identify factors potentially contributing to ARG abundance. For example, the environmental water samples were mapped with population density (Figure 4.2A). A number of samples with higher levels of ARG were from urban or more densely populated regions, which has previously been described (Ouyang et al., 2015). As mentioned above, the sample with the highest quantity of ARGs were collected from the river in Grand Rapids (MI), which was one mile downstream from the effluent line of the WWTP. However, one of the samples collected from an area with lower human population showed a high level of ARGs (Hubbard Lake, northeast Lower Peninsula) indicating additional influencing factors.

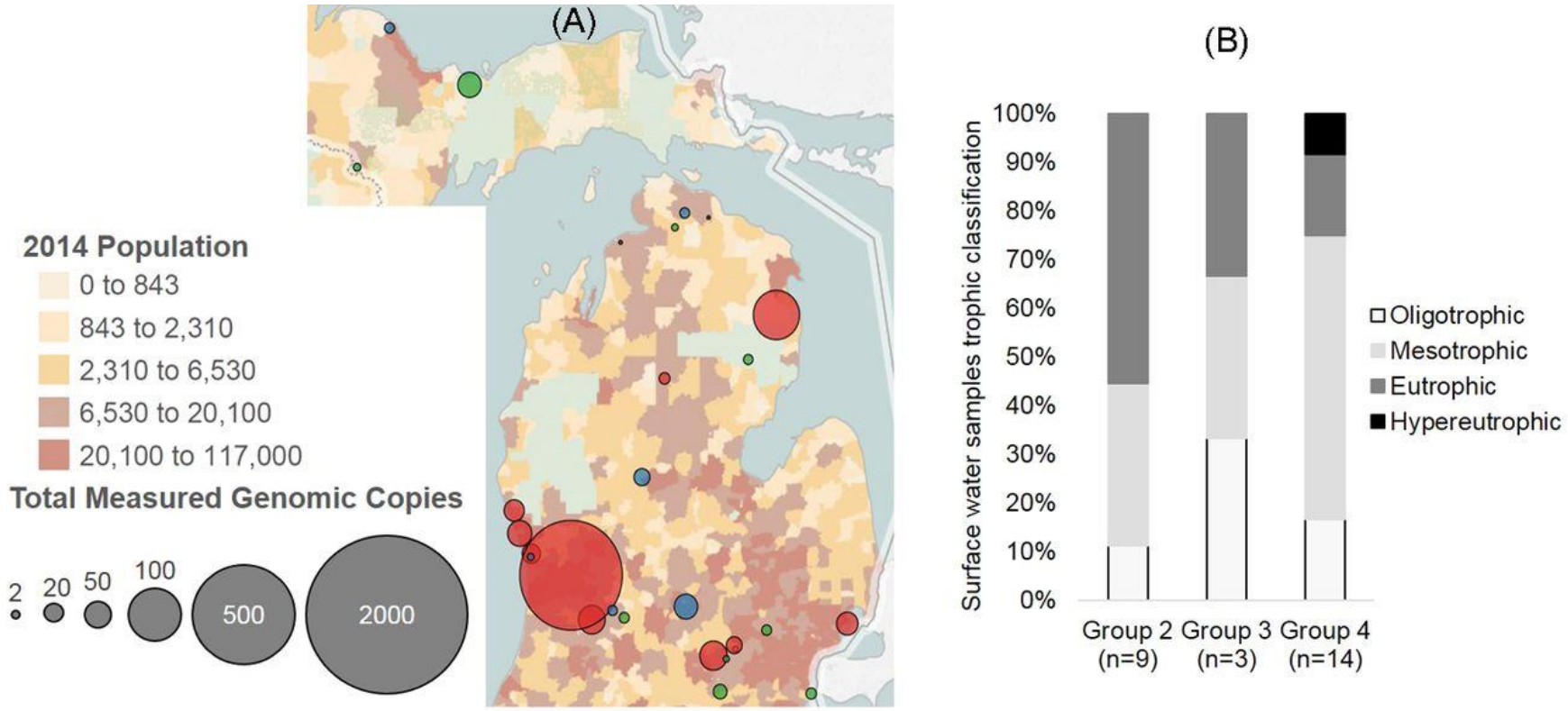

Figure 4.2: Mapping ARG database to identify hotspots and examine occurrence, co-occurrence and persistence of ARGs. A. Total ARG copies versus population density for all 30 surface water samples, size of dot relates to total number of detected ARG copies, red, blue and green dots indicate samples from group 2, 3 and 4, respectively. Green portions of the map indicate no population information was available. B. Trophic state of environmental surface waters, based on sample grouping via RDA (Figure A4.2).

Surface water ARG clusters were examined further using trophic conditions (Figure 4.2B) reported by the USGS (not available for four sampled water bodies). A higher percentage (55%) of surface water bodies clustered within group 2 (highest level of ARG) were eutrophic, and a lesser percentage of water bodies were eutrophic in group 3 (33%) and group 4 (27%).

4.3.3 AR Dashboard database to examine natural and anthropogenic distribution, clustering and connections among ARGs

Within the limited set of samples tested to develop the application, inferences can be made regarding detection of ARGs from the natural resistome, and genes that may serve to indicate human fecal contamination in surface water samples. Concerning Michigan surface water and primary influent samples, 190 ARG primers amplified in one or more of the tested samples. A total of 60 primers amplified in one or more of the surface water samples (Figure A4.3), and 178 primers amplified in one or more of the three primary influent samples (Figure 4.3A). In detail, 27 primers amplified in both primary influent samples and one or more group 2 surface water samples. Two primer sets (targeting the f*ox5* and *blaOXY* genes) appeared in all three primary influent waste water samples, seven or more of the surface water samples, and had a high Pearson correlation (>0.90) to total ARG copies. In addition, neither of these genes were detected in the group 4 surface water samples (with lowest level of ARGs). The *fox5* and *blaOXY* genes are both associated with beta-lactam resistance (Arakawa et al., 1989; Gonzalez et al., 1994) and are typically observed in gram negative enteric organisms (e.g. *E. coli* and *Klebsiella*). In addition, the primer targeting the integron-integrase (*intI1*) gene, had a Pearson correlation of 0.76 compared to total abundance of genomic copies within all

tested samples (Figure 4.3B). The correlation between total ARGs and the *intI1* gene in all water samples indicate potential for quantifying overall ARG abundance. Studies have examined and suggested using the *intI1* (Gillings et al., 2015) or other genes (e.g. *tetM*; Li et al., 2015) as a potential proxy for human pollution due to widespread occurrence, and co-occurrence with other ARGs in environmental, human and animal fecal samples. Confidence in selection of certain ARGs as proxies for human activity can be heightened using the populated AR Dashboard Database. Once selected, markers can be used to routinely monitor environmental samples via point-of-use tools (e.g. Kostić et al., 2015; Shu et al., 2015; Stedtfeld et al., 2012).

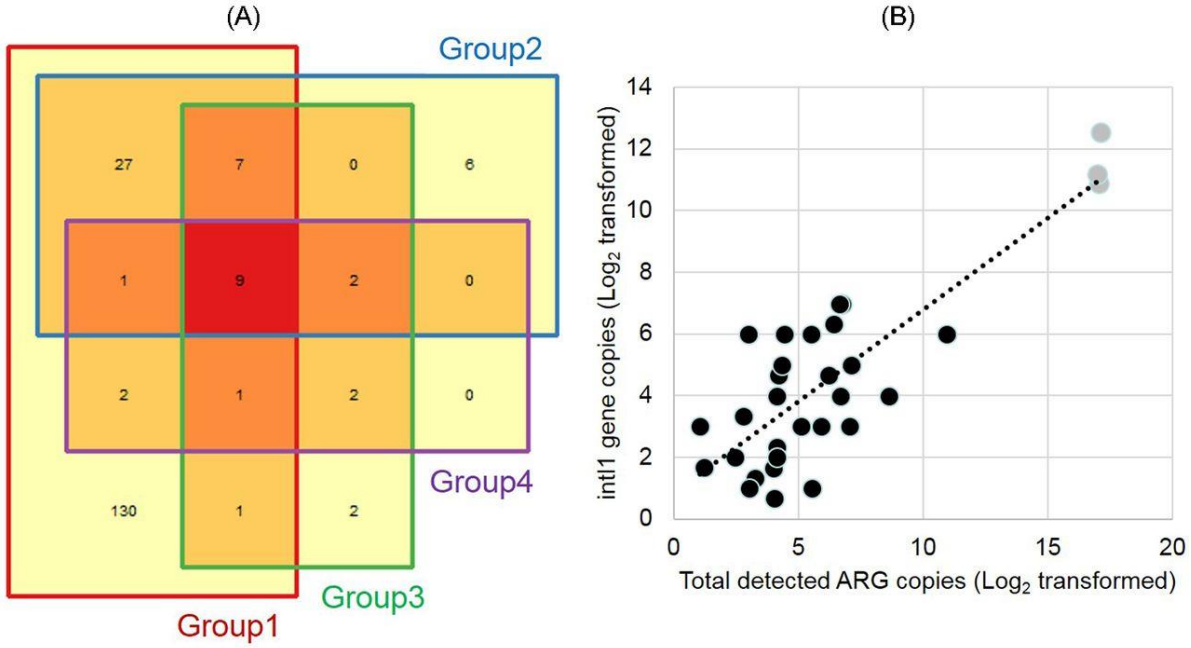

Figure 4.3: Utility of comprehensive database for differentiating ARGs from natural resistome, fecal contamination and identifying proxies for anthropogenic activity. A. Number of amplified ARG primers clustered by RDA, group 1 includes primary influent samples, and groups 2–4 are surface water samples with varying levels of ARGs. B. Correlation between total detected ARG copies (*x*-axis) and *intI1* gene copies (*y*-axis), gray circles are the three primary influent samples, and black circles are 30 surface water samples.

The database could also be used to differentiate naturally occurring ARGs that would only be expected in unsullied environments, and should not be included in screening or tracking studies. In our sample set, 12 primers (e.g. *ermC*, *tetK*) amplified in one or more surface water samples, but were not detected in any of the primary influent samples. These ARG may occur more often in natural environments. In fact, absence of these ARGs may indicate human activity, in which bacteria harboring them are deselected. Nine primer sets (e.g. *mexF* and *oprD* genes) amplified in all four clusters also indicating natural occurrence or genes that were less recently distributed. Studies of pristine samples from Antarctic soils also detected the *mexF* gene in multiple samples (Wang et al., 2016).

The inclusion of additional sample data sets and quantitative information will also help identify supplemental factors related to presence and persistence of AR including co-occurrence and co-selection of ARGs in different environments and hosts. To demonstrate, qPCR data from previously described studies were assembled including eight park soils (Wang et al., 2014), three river samples (Ouyang et al., 2015) and nine pig farm samples (Zhu et al., 2013). Ordination of relative abundance revealed significant ($P = 0.001$) clusters related to sample type (Figure 4.4A). The cluster spread associated with a given sample type varied, with river samples showing the largest cluster, which may be expected as some of the samples would be considered pristine, while other samples, such as the site downstream from the WWTP effluent had a higher number of ARGs. Pig farm samples also had a wide cluster, which is expected as these samples consisted of manure, compost and soil from three different farms (Zhu et al., 2013).

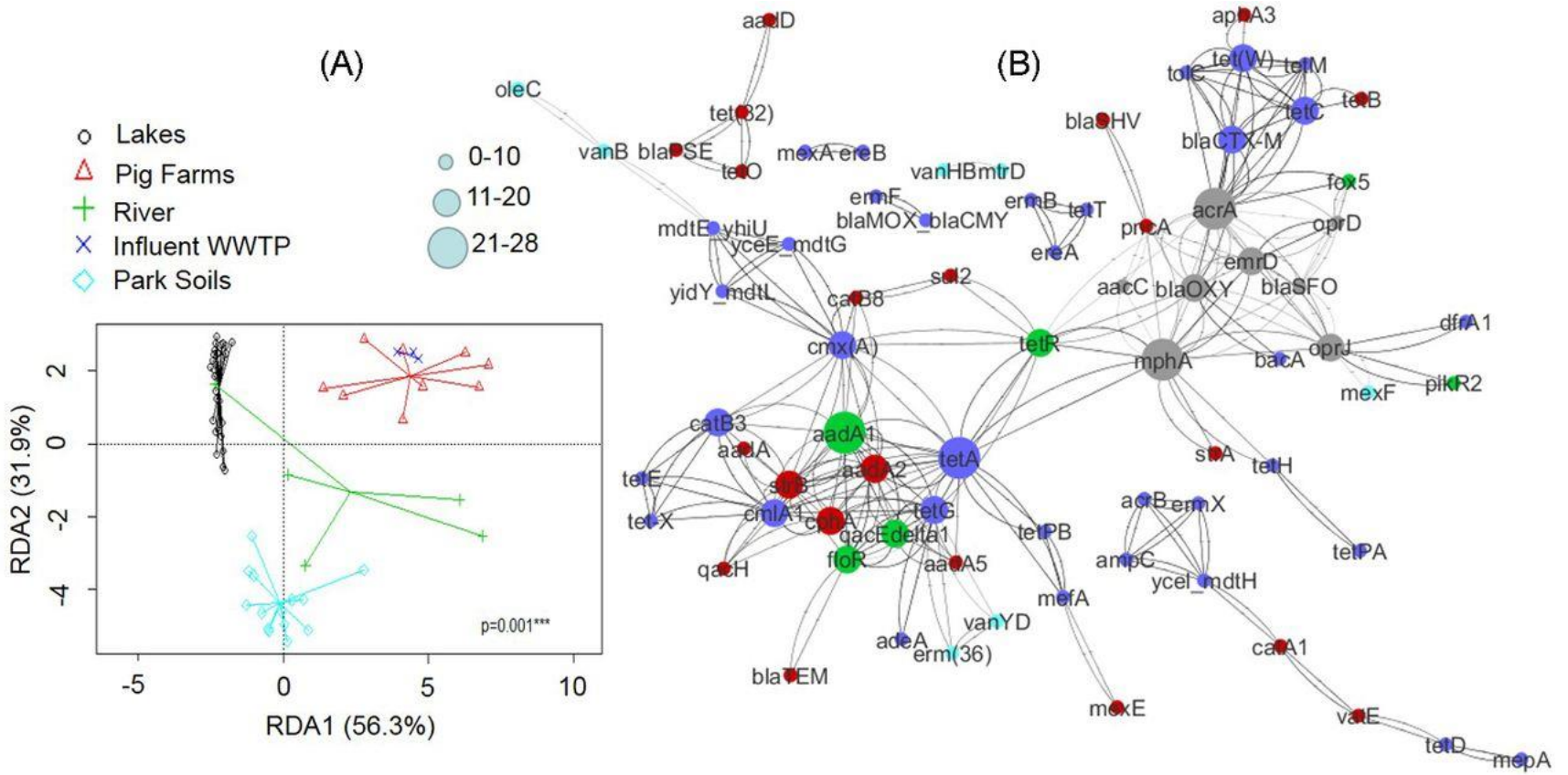

Figure 4.4: Clustering and co-occurrence of ARGs with multiple sample types including previously published data (Ouyang et al., 2015; Wang et al., 2014; Zhu et al., 2013). A. RDA of ARG Log_2 transformed relative abundance. B. Co-occurrence network with primer names as nodes constructed using Log_2 transformed relative abundance values. Nodes connected by an edge have a statistically significant Spearman correlation and are co-occurring (q value < 0.05, and $\rho > 0.75$). Node color indicates sample type in which a higher majority of connections occur (gray from lake, red from pig farm, green from river, dark blue from primary influent of waste water treatment facility, light blue from park soils). Node size indicates number of connections, and edge color maps level of correlation (darker indicate higher ρ).

Using assembled qPCR data and results observed via metagenomics from the primary influent of two waste water treatment facilities (Li et al., 2015), networks of connected ARGs that co-occur within tested sample types was also observed (Figure 4.4B). Co-occurrence was based on a significant Spearman correlation ($\rho > 0.75$, P-value < 0.005) of ARGs that amplified in 50% or more for a given sample type (excluding a cutoff of 25% for lake samples). The co-occurrence network contained 79 nodes, 302 edges and three modules containing more than four ARGs. Larger nodes within the two largest modules (*aadA1* and *tetA* in one module, and *mphA* and *acrA* in the second module) indicate that the occurrence and abundance of these genes may serve as indicators for ARG abundance (Li et al., 2015) within these sample types. Larger *acrA* and *mphA* nodes with the second module (with a majority of connections between lake samples) target sequence alleles from *E. coli*, which is routinely used as indicator of fecal pollution in environmental waters.

Mapping occurrence and abundance over space and time, the database can potentially be used to establish connections and risk considerations among environments, animals and humans. As reviewed previously (Allen et al., 2010; Huijbers et al., 2015), some environments will harbor ARGs (in some instances irrespective of anthropogenic influence due to spread via environment or wild animals), and more studies are necessary to quantity contributions of varying environments to exposure and transmission. It should be mentioned that some ARGs have functional roles that are independent of resistance, such as efflux and electron transport, and will not always be associated with human activity (Dietrich et al., 2008). With culture-based AR analysis accurately identifying sources of host fecal contamination (Park et al., 2015), molecular screening of ARGs may also provide a means of source tracking (Li et al., 2015). Thus, the AR

Dashboard Database could be used to aid in differentiation of genes for various environmental and clinical screening applications.

4.3.4 Linking regional occurrence of AR infection with ARG

A vital utility of the AR Dashboard Database includes potentially linking occurrence with spread and transmission of AR infections on a regional and global scale via the integration of hospital generated antibiograms, ARGs in the environment, and ARGs in clinical infections. For demonstration, the ARG array was tested with ten clinical isolates. The distribution of AR classes in ARGs detected from isolates and the different surface and waste water samples from Michigan were mapped (Figure 4.5). Genes detected with the ARG array were also compared with hospital tested culture based susceptibility (Table A4.1), in which the array successfully identified 40 of 52 AR events. The AR events that were not detected are thought to be due to incomplete coverage of ARGs and other resistance mechanisms (e.g. sequence mutations). Molecular based assays of course cannot determine whether that trait is functionally expressed, but larger data sets of ARGs should provide information on population selection which is a complementary route into understanding ARG and ARB ecology and aid improvements in surveillance and stewardship. With global participation, the AR Dashboard could perhaps bridge this gap and help identify links between emergence, spread and transmission of AR pathogen infections.

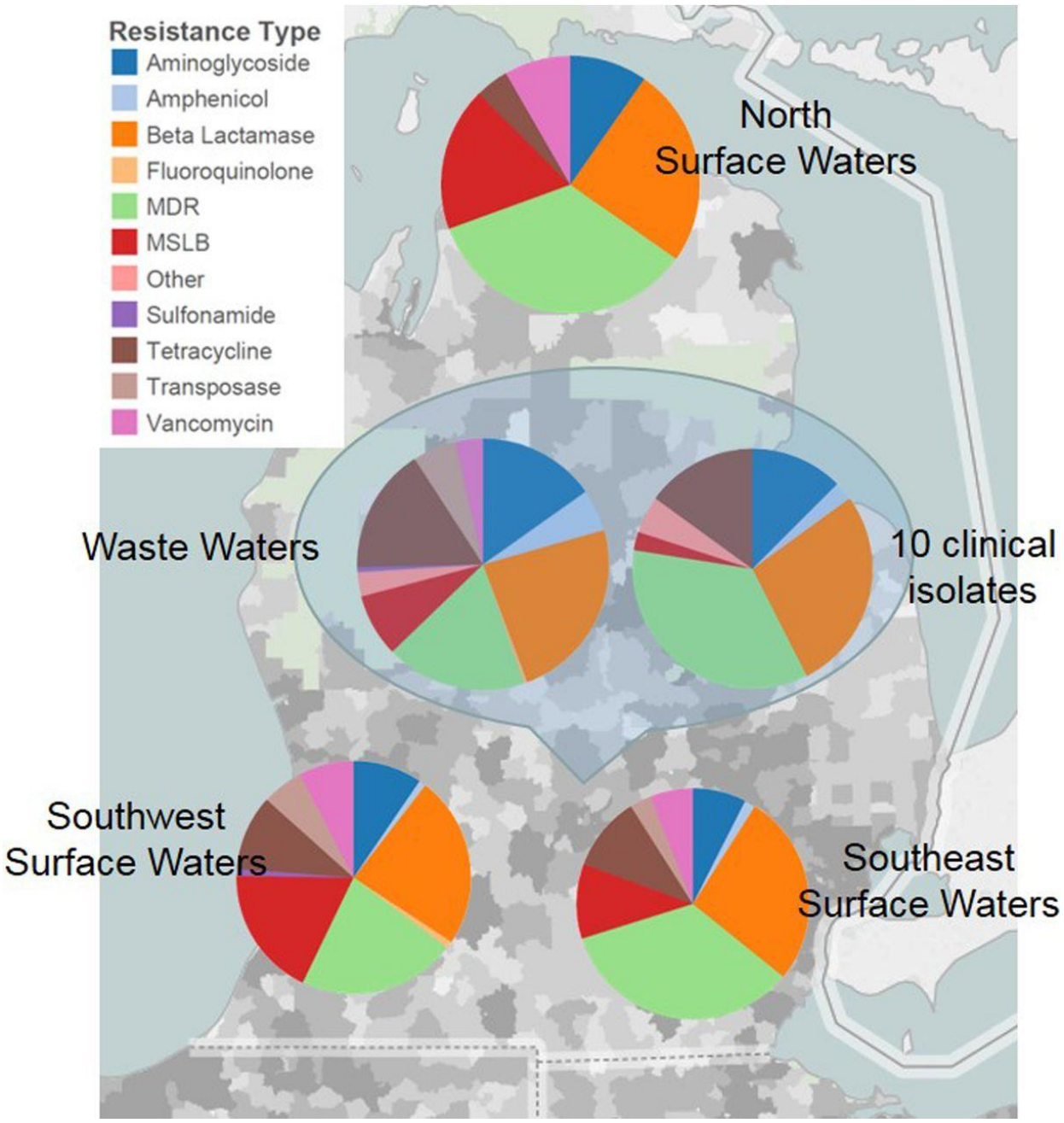

Figure 4.5: Demonstration using database to regionally or globally study AR infection with environmental distribution of ARGs. Map shows distribution of AR classes in ARGs detected from different surface water throughout MI, the primary influent waste water samples, and from 10 isolates collected from patients at a local hospital. MDR: multiple drug resistance, MSLB: macrolide, lincosamide and streptogramin B.

APPENDIX

APPENDIX

Additional figures and tables

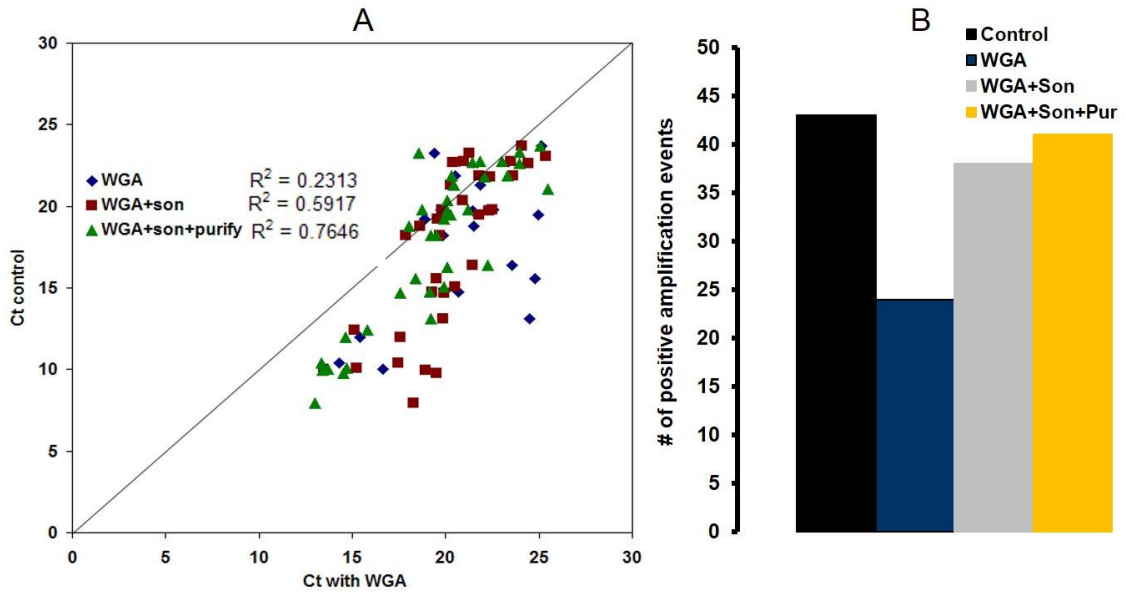

Figure A4.1: Testing whole genome amplification (WGA) and method of purification prior to qPCR. Experiment included running qPCR on samples directly after WGA (WGA), running qPCR after WGA and sonication (WGA+Son), running qPCR after WGA and sonication and purification (WGA+Son+Pur). Sonication was to reduce size of WGA amplicons and purification was to remove random primers. The same mass of DNA was used for each experiment.

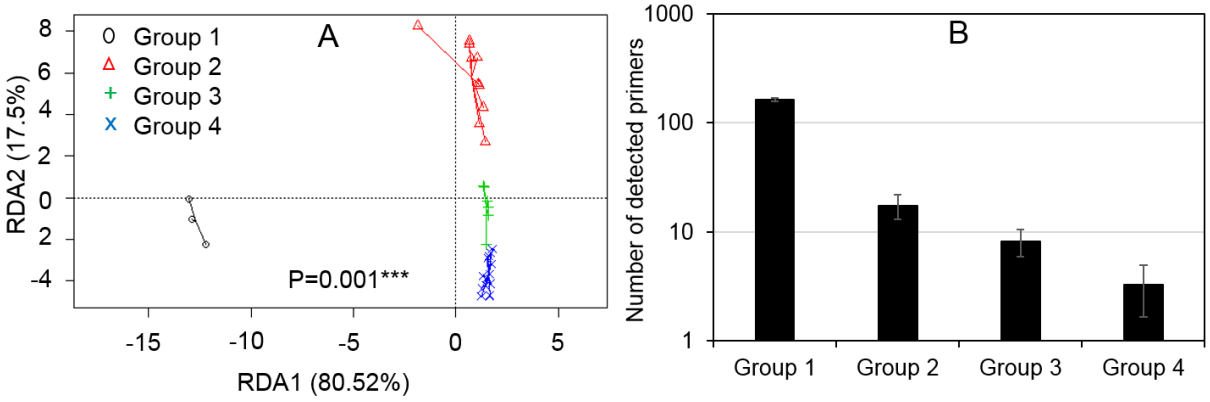

Figure A4.2: Surface water samples were grouped via A. redundancy analysis (RDA) of ARG relative abundance (Log_2 transformed). Waste water samples clustered into group 1, and the 30 surface water samples clustered into groups 2-4. B. Average number of primer sets that amplified in samples clustered among four groups. Error bars represent standard deviation.

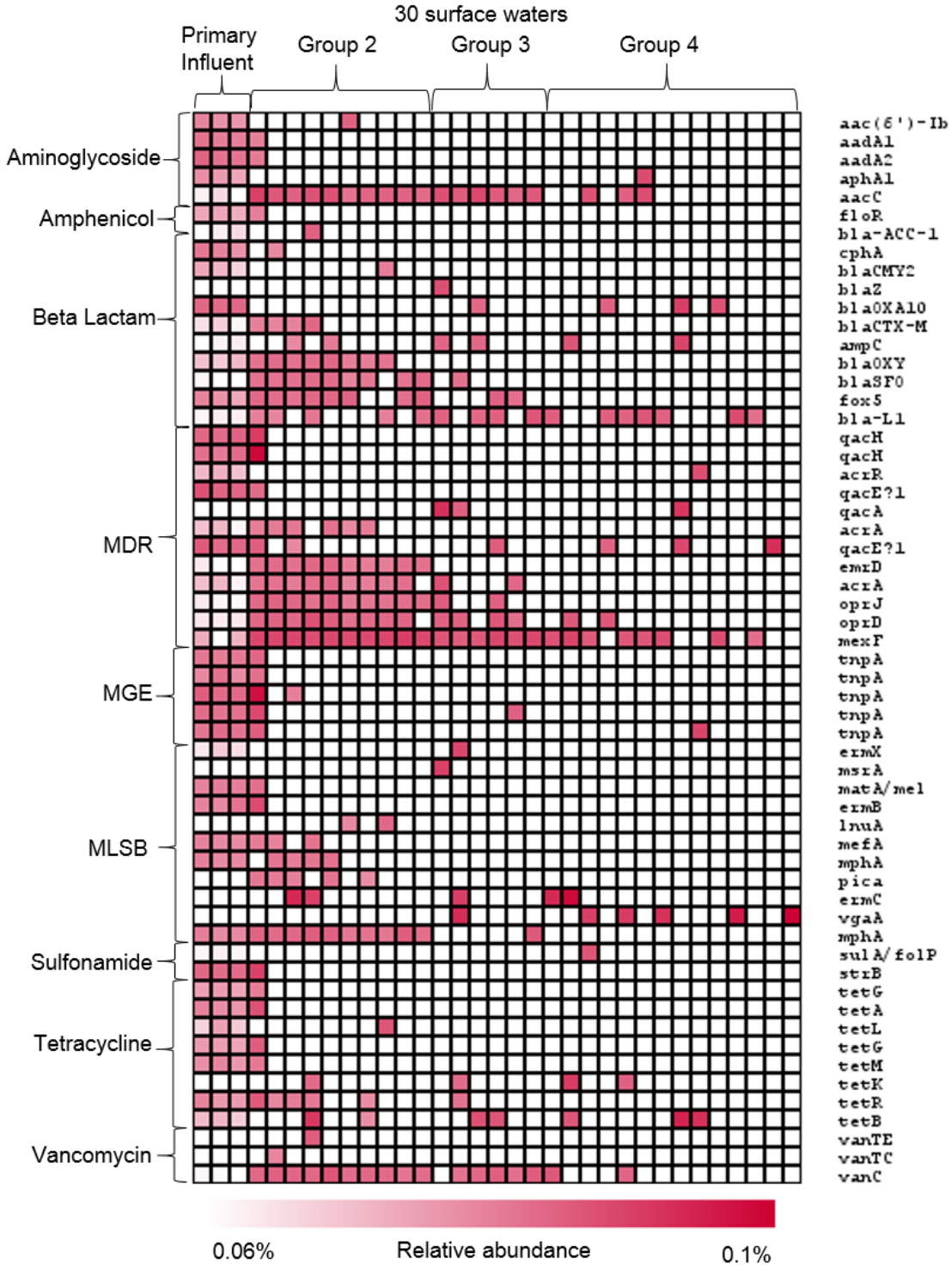

Figure A4.3: Heat map displaying primers detected in one or more surface water samples. The color scale indicates relative abundance to the 16S rRNA gene. White indicates genes not present or below the detection limit.

Table A4.1: Correspondence of culture based susceptibility and ARGs detected with qPCR array. No false positive events were observed (i.e. all ARGs that amplified were associated with a culture-based resistance event), and 40 of 52 culture based AR events corresponded in detection of an associated ARG. The twelve AR events not detected were suspected a result of ARGs not targeted on the array.

Clinical Isolates Run Separately on the ARG array	Culture based AR event	ARGs detected	Culture based AR events not detected by ARG array
Corynebacterium spp.	3	11	2
Staphylococcus aureus	5	16	1
Methicillin-resistant *Staphylococcus aureus*	14	10	1
Staphylococcus epidermidis	13	8	2
Streptococcus pyogenes	1	13	1
Streptococcus agalactiae	1	12	0
Proteus mirabilis	2	15	2
Eschericia coli	3	17	0
Klebsiella pneumoniae	1	19	0
Enterococcus faecalis	9	11	3
Total	52		12

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CHAPTER V

Quantification of microRNAs directly from body fluids using a base-stacking isothermal amplification method in a point-of-care device

The work in Chapter V has been published:

Williams M. R., Stedtfeld R. D., Stedtfeld T. M., Tiedje J. M., Hashsham S. A. 2017. Quantification of microRNAs directly from body fluids using a base-stacking isothermal amplification method in a point-of-care device. Biomedical Microdevices 19 (3): 45.

Author contribution statement:

M. R. W. and S. A. H. developed the microRNA amplification approach. M. R. W. and T. M. S. conducted experiments. M. R. W. and R. D. S. developed figures. All coauthors wrote/edited the manuscript.

Abstract

MicroRNAs have been proposed to be a class of biomarkers of disease as expression levels are significantly altered in various tissues and body fluids when compared to healthy controls. As such, the detection and quantification of microRNAs is imperative. While many methods have been established for quantification of microRNAs, they typically rely on time consuming handling such as RNA extraction, purification, or ligation. Here we describe a novel method for quantification of microRNAs using direct amplification in body fluids without upstream sample preparation. Tested with a point-of-care device (termed Gene-Z), the presence of microRNA promotes base-stacking hybridization, and subsequent amplification between two universal strands. The base-stacking approach, which was achieved in < 60 min, provided a sensitivity of 1.4 fmol per reaction. Tested in various percentages of whole blood, plasma, and faeces, precision (coefficient of variation = 2.6%) was maintained and comparable to amplification in pristine samples. Overall, the developed method represents a significant step towards rapid, one-step detection of microRNAs.

5.1 Introduction

Point-of-care (POC) nucleic acids-based methods and technologies have the potential to offer minimally invasive alternatives to biopsies and routine examinations allowing early detection and therapeutics. MicroRNAs (short, non-coding RNA molecules) are one such potential marker of disease and cancer with varied expression levels in tissues (Esquela-Kerscher and Slack, 2006; Gaur et al., 2007), faeces (Link et al., 2012, 2010), saliva (Michael et al., 2010; Shao et al., 2012), and blood (Schwarzenbach et al., 2014) between patient samples and healthy controls; depending on the disease (Ruepp et al., 2010). For example, increased expression of

miR-30b, miR-29b, miR-142-2p, miR-144, miR-203, and miR-223 (> eight fold in some instances) has been observed in oral squamous samples collected from cell carcinoma patients compared to healthy controls (Manikandan et al., 2016). In serum, miR-141 expression levels up to 46 fold between patients with prostate cancer and healthy controls (Mitchell et al., 2008). Thousands of microRNAs have been identified in humans (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008, 2006) and efforts to associate microRNAs to various types of cancer and disease is extensive and ongoing (Ruepp et al., 2010).

Methods to quantify microRNA require superior limit of detection, large dynamic range, and precision (Tricoli and Jacobson, 2007). Existing amplification-based methods for measurement of microRNAs include stem-loop reverse transcription polymerase chain reaction (RT-PCR; Chen et al., 2005) and reverse transcription-free PCR (Lu et al., 2011). Isothermal approaches such as rolling circle amplification (Harcourt and Kool, 2012; Liu et al., 2013; Zhou et al., 2010), loop-mediated isothermal amplification (LAMP; Li et al., 2011), exponential amplification reaction (EXPAR; Wang et al., 2014; Zhang and Zhang, 2012), and others have also been described for microRNA (Table 5.1). Isothermal approaches have the advantage of simplicity in terms of constant temperature and high amplicon yields (Mori et al., 2001), which allow for quantification with relatively simpler devices (e.g. turbidity meters, Illumigene, NucleSENSE easyQ, Gene-Z). However, upstream sample preparation for microRNA is challenging. This is because amplification-based techniques typically require RNA isolation by skilled personnel in a centralized laboratories. However, isothermal polymerases (e.g. *Bst*) are more robust and less impacted by inhibitory substrates compared to PCR polymerases (Kostic et al., 2015; Stedtfeld et al., 2014). Thus, an isothermal direct amplification approach has the

potential to reduce analysis time and costs, without isolation and purification, and is therefore well suited for use outside of laboratories with specialized infrastructures (Njiru, 2012).

The developed isothermal technique for direct microRNA detection is a variant of a qPCR-based approach reported by Lu and coauthors (Lu et al., 2011) and enhanced by Yu and coauthors (Yu et al., 2013). In detail, the presence of microRNA promotes a base-stacking, hybridization of two universal strands and amplification under isothermal conditions (Figure 5.1A). The developed approach for microRNAs was demonstrated in a conventional thermal cycler and a compact, low-cost real time isothermal amplification device (termed Gene-Z) that uses disposable microfluidic cards (Figure 5.1B; Stedtfeld et al., 2012). The direct amplification method was also tested in mouse body fluids (whole blood, plasma, and faeces) using real time fluorescence and confirmed via gel electrophoresis.

Table 5.1: Summary of selected isothermal amplification approaches for measurement of microRNAs

Validated microRNA(s)	Sample processing Required	Reaction name; Polymerase; Detection	Detection limit*	Reaction volume	Dynamic range*	Turn-around-Time (TAT)	Incubation temperature(s)	Reference
Isothermal approaches requiring RNA extraction prior to amplification of microRNAs								
miR-319a	RNA extraction, biotin-labeling	Poly (U) polymerase-mediated isothermal signal amplification; Poly(U); Electrochemical	$8.5x10^{-6}$ fmol (8.5 zmol)	5 µl	10-1000 fM	4 hr	37 °C	(Zhou et al., 2016)
Random sequence	Probes spiked into biological samples	Enzyme-assisted target amplification; Klenow fragment; Colorimetric	$50x10^{-6}$ fmol (50 zmol)	100 µl	0.5 fM–1 nM	2 hr	90 °C, 37 °C	(Yan et al., 2013)
miR-1, miR-122, miR-150, miR-143, and let-7a	RNA Extraction; reverse transcription, phosphorylation, ligation	Isothermal ramification amplification (RAM); *Bst*; SYBR Green	$25x10^{-6}$ fmol (25 zmol)	25 µl	1 fM-10 nM	8.5 hr	37 °C, 41-37 °C, 55 °C, 65 °C	(Yao et al., 2009)
let-7a	RNA extraction after ligation	Rolling circle amplification (RCA); Φ-29; probes	10 fmol	50 µl	200 pM-10 nM	30 hr	30 °C	(Harcourt and Kool, 2012)

Table 5.1 (cont'd)

miR-21, let-7d	RNA extraction, ligation	RCA; Φ-29; SYBR Green	20 $x10^{-6}$ fmol (20 zmol)	20 µl	1 fM-100 nM	8 hr	37 °C, 30 °C	(Zhou et al., 2010)
N/A	RNA extraction	Branched RCA; Φ-29; bioluminescence	0.1 fmol	10 µl	10 pM – 7.5 pM	2.5 hr	37 °C	(Mashimo et al., 2011)
miR-1	RNA extraction	Cascade RCA-NESA-DNAzyme amplification; Φ-29; Color change	$0.2x10^{-6}$ zmol (0.2 zmol)	100 µl	10 aM - 10 µM	6 hr	37 °C	(Wen et al., 2012)
miR-16	RNA extraction, ligation	RCA; Φ-29; Northern blot	$0.5x10^{-3}$ fmol (0.5 amol)	N/A	N/A	10 hr	37 °C, 30 °C	(Jonstrup et al., 2006)
let-7a	RNA extraction	Loop-mediated isothermal amplification (LAMP); *Bst*; SYBR Green	$1x10^{-3}$ (1 amol)	10 µl	0.1 pM-0.1 µM	~2 hr	55 °C	(Li et al., 2011)
let-7a	RNA extraction	Strand displacement amplification (SDA); Klenow fragment; SYBR Green	$16x10^{-6}$ fmol (16 zmol)	10 µl	16 fM-0.1 µM	90 min	37 °C	(Shi et al., 2014)

Table 5.1 (cont'd)

Isothermal approaches employing direct amplification of microRNAs								
miR-486-5p	Demonstrated directly and after RNA extraction	Hairpin probe RCA; Φ-29; SYBR Green 11	$0.5x10^{-6}$ (0.5 amol)	50 µl	0.2 fM-1 nM	4 hr	35 °C	(Li et al., 2013)
let-7a	Demonstrated directly and after RNA extraction	Cascade signal amplification; Klenow fragment; Molecular beacon	1 fmol direct; $0.1 x10^{-6}$ fmol after RNA extraction	10 µl	10 aM- 5 nM	80 min	37 °C	(Ma et al., 2014)
miR-141	Demonstrated directly and after RNA extraction	LAMP; *Bst*; SYTO82	1.4 fmol	10 µl	14 aM – 14 fM	60 min	65 °C	This study

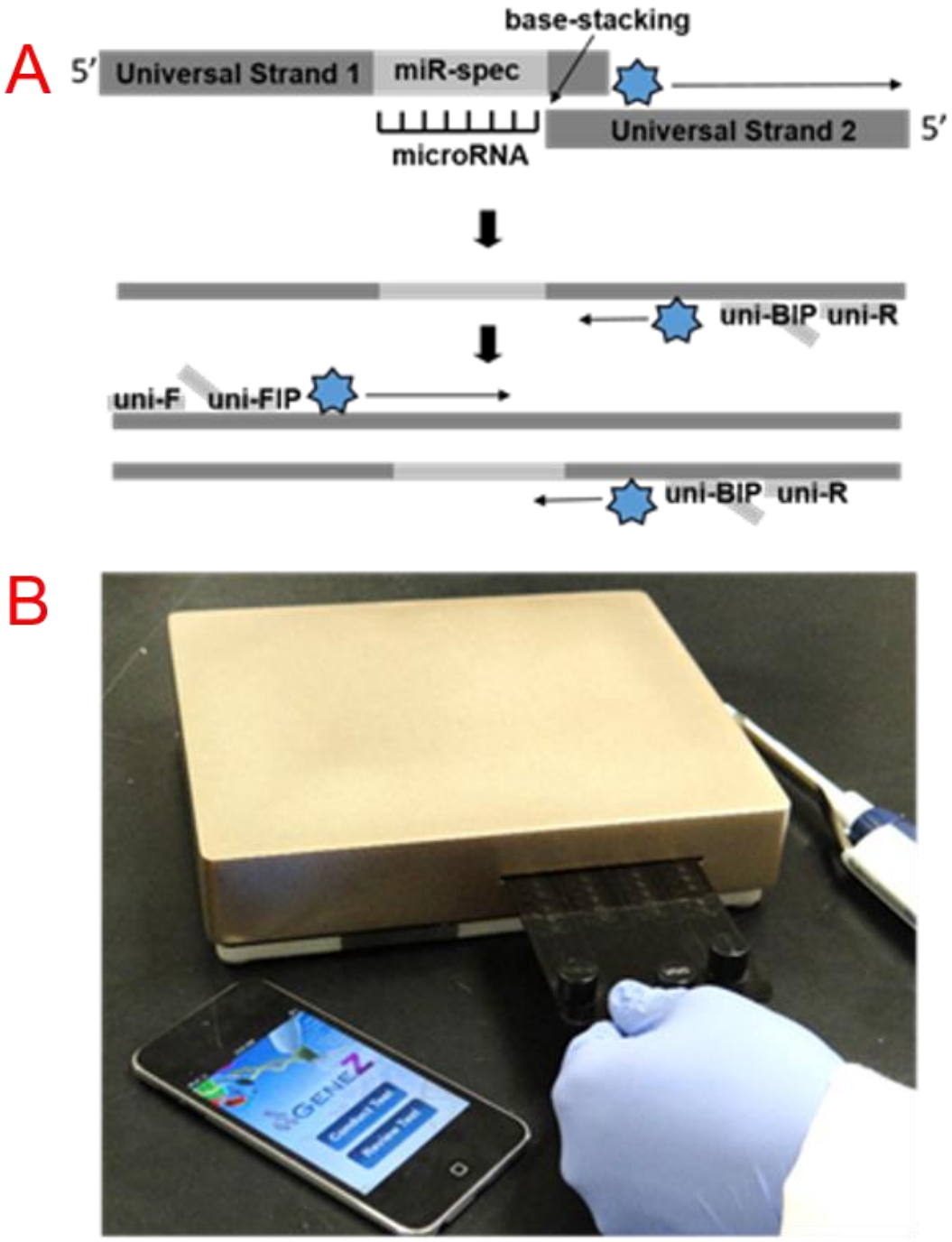

Figure 5.1: A. Schematic description of base-stacking microRNAs isothermal amplification. B. Real-time, Gene-Z and microfluidic chip for POC microRNA quantification.

5.2 Methods and Materials

Single-stranded universal template sequences were obtained from Integrated DNA Technologies (Coralville, IA) as HPLC-purified 4 nmole ultramers, rehydrated in nuclease-free water. Loop-mediated isothermal amplification primers specific to the universal strands, were designed using Primer Explorer Software V4 and obtained from Integrated DNA Technologies (Table 5.2). A 10X primer mix was created with 16 µM forward inner primer (FIP) and backward inner primer (BIP) and 2 µM forward (F) and reverse (R). The final 2X reaction included 1X primer mix, 2X Isothermal Buffer (New England Biolabs; Ipswich, MA), 0.28 mM dNTPs (Invitrogen; Carlsbad, CA), 1.6 mM Betaine solution (Sigma-Aldrich; St. Louis, MO), 12 mM $MgSO_4$ (New England Biolabs; Ipswich, MA) and sterile water (Thermo Fisher Scientific; Waltham, MA). A final 10 µl isothermal amplification reaction contained 1X reaction mix, 16 units *Bst* 2.0 Polymerase (New England Biolabs, Ipswich, MA), 20 µM SYTO82 orange fluorescent nucleic acid stain (Invitrogen), 3.75% formamide (Sigma-Aldrich; St. Louis, MO), 4 µg bovine serum albumin (BSA; New England Biolabs; Ipswich, MA), 0.4% Pluronic F-68 (Life Technologies; Carlsbad, CA), 1 µl clinical sample or water, 0.25 µM universal strands, and 1µl microRNA.

Table 5.2: Isothermal microRNA assay sequences, including universal strands, primers and oligos used in validation experiments.

Name	Sequence 5'-3'
miR-141 Specific Strand 1*	TGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAAC CACCACAGAAGTATTTAAATGGGATGGGGAAAAAAGGCT ATTCCCAG **CCATCTTTACCAGACAGTGTTA** *GGTCG*
miR-92 Specific Strand 1*	TGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAAC CACCACAGAAGTATTTAAATGGGATGGGGAAAAAAGGCT ATTCCCAG **ACAGGCCGGGACAAGTGCAATA** *GGTCG*
miR-141 Specific Strand 1* (Lower GC Content Overhang)	TGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAAC CACCACAGAAGTATTTAAATGGGATGGGGAAAAAAGGCT ATTCCCAG **CCATCTTTACCAGACAGTGTTA** *TGTCG*
miR-141 Specific Strand 1* (7 bp Overhang)	TGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAAC CACCACAGAAGTATTTAAATGGGATGGGGAAAAAAGGCT ATTCCCAG **CCATCTTTACCAGACAGTGTTA** *GGTCGCA*
miR-141 Specific Strand 1* (4 bp Overhang)	TGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAAC CACCACAGAAGTATTTAAATGGGATGGGGAAAAAAGGCT ATTCCCAG **CCATCTTTACCAGACAGTGTTA** *GGTC*
Universal Strand 2	CACTTCCTTAGACATGAGCTATACGACGAGCTAAATCTTG ATCGCCTAGGGTCATGTTCTT *CGACC*
Universal Strand 2 (Lower GC Content Overhang)	CACTTCCTTAGACATGAGCTATACGACGAGCTAAATCTTG ATCGCCTAGGGTCATGTTCTT *CGACA*
Universal Primer F3	TGCTTAATGCTTTGATCGG
Universal Primer B3	CACTTCCTTAGACATGAGCT
Universal Primer FIP	CTGGGAATAGCCTTTTTTCCCCACTTGAGCACCATAAGGC AA
Universal Primer BIP	AAGAACATGACCCTAGGCGAATACGACGAGCTAAATCTTG A
hsa-miR-141-3p	UAACACUGUCUGGUAAAGAUGG
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA
hsa-miR-92	UAUUGCACUUGUCCCGGCCUGU
miR-8 Family**	
hsa-miR-141-3p	UAACACUGUCUGGUAAAGAUGG
hsa-miR-200a-3p	UAACACUGUCUGGUAA**C**GAUG**U**
hsa-miR-200b-3p	UAA**U**ACUGCCUGGUAA**U**GAUG**A**
hsa-miR-200c-3p	UAA**U**ACUG**C**C**G**GGUAA**U**GAUGG**A**
hsa-miR-429	UAA**U**ACUGUCUGGUAAA**ACC**G**U**

*Underline represents microRNA-complementary regions. Italics represents overhangs
**Underline represents nucleotides that differ from miR-141-3p.

Reactions in the commercial real-time PCR machine were conducted in 96-well plates. Cycling included 58 seconds incubation at 65°C followed by a plate read, and repeated for 60 cycles. For reactions in the microfluidic chip, primers were dispensed into wells, dried at 70°C for 5 min, and sealed with optical adhesive film (Stedtfeld et al., 2015; Applied Biosystems; Foster City, CA). Pluronic and BSA were not used in experiments conducted in the microfluidic chip. Reaction mixture was injected into the chip and the chip was inserted into Gene-Z™. After reaching 65°C, fluorescence was measured every 16 seconds for 60 min (Stedtfeld et al., 2012). The sample is then added to the reaction mixture, mixed and then injected into the chip using a pipette. Once completed, raw data is emailed from the iPod to a PC for data processing and analysis.

Gene-Z performance has been demonstrated for many applications including bacterial pathogens important to water safety, human health, and assessing bioremediation performance in contaminated aquifers (Kostic et al., 2015; Stedtfeld et al., 2014). The work described here is its first demonstration for microRNA detection. Features of this real time device include: i) simple microfluidic chips consisting of up to 64 reaction wells each with 1 to 20 µl (Stedtfeld et al., 2015) ii) real time monitoring of amplification in less than 1 hr, iii) potential for wireless communication, automated data processing and reporting using a smartphone user interface, and iv) a hand-held format with internal rechargeable battery.

Experiments involving samples collected from mice were conducted in compliance with relevant laws and institutional guidelines, under Animal Use Form (AUF) Approval No. 02/14-030-00. Fecal pellets were collected, frozen, and stored at -80°C. Prior to direct amplification, fecal pellets were hydrated with nuclease free water, crudely lysed using a pestle, and vortexed for

1 min to homogenize. A portion of the whole blood was used for collection of plasma, via centrifugation at 2,000 x g for 10 min.

To verify true positive amplification in blood samples spiked with microRNA, reactions were also analyzed using 1% agarose gel electrophoresis in 1 x TAE buffer at 110 V at room temperature for 1 h. The gels were stained with SYBR safe, and images were captured using a smartphone.

All data was analyzed by calculating the signal to noise ratio (SNR). For the commercial real time PCR machine, the SNR was defined as a ratio between the differences in signal from the mean background to the difference in signal from the maximum signal. The amplification time was thus defined as the time the SNR crossed a threshold of 0.1. The amplification time (Tt) was defined as the time where the signal crossed a threshold of 5. The difference in amplification time between the target assay and the no template control (NTC) was defined as ΔTt.

5.3 Results

5.3.1 Base-stacking isothermal amplification method

The isothermal amplification method includes two universal strands, 100 and 80 nucleotides in length, one that has a complementary region to the microRNA of interest (Figure 5.1A). The other is a universal strand that can be used for detection of any microRNA. Directly adjacent to the microRNA-specific region at the 5’ end of the left strand is a five nucleotide long overhang sequence that is complementary to the first five nucleotides on the 3’ of the right strand. When the target microRNA is present in the reaction, it stabilizes the heterodimer (based on nearest neighbor base stacking interactions), thus allowing the binding of the overhangs to occur. When it is not present, annealing conditions are not favored – especially at the high

reaction temperature (65 °C), thus resulting in a significant delay in amplification time. Primers for the isothermal amplification were designed according to requirements for a one-hr loop-mediated isothermal amplification (LAMP; Mori et al., 2001; Notomi et al., 2000), which utilizes *Bst* polymerase, an enzyme minimally inhibited by complex sample components (Koloren et al., 2011; Stedtfeld et al., 2015, 2014). The forward primer (F) and forward-inner-primers (FIP) have the same sequences as universal strand 1, so are unable to bind and begin amplifying until the overhang connection has allowed the formation of its complementary strand. The same is true for the reverse primer (R) and the backward-inner primer (BIP) for universal strand 2 (Table 5.2).

5.3.2 Method optimization

Two aspects of the isothermal amplification reaction chemistry were systematically tested including overhang length and reaction chemistry. An overhang length of five nucleotides was determined to the optimal length to increase ΔTt (Figure 5.2A). This overhang length was similarly observed to be optimal for the reverse transcription-free qPCR method developed by Lu and coauthors (Lu et al., 2011). The addition of formamide in DNA amplification reactions lowers the melting temperature of DNA by ~ 2.4°C/mole of formamide and is destabilizing (Blake and Delcourt, 1996). In our system, it greatly increased the ΔTt at 3.75% (Figure 5.2B) but increased the amplification time at concentrations higher than 5%. Formamide concentrations above 7% resulted in no amplification. In addition, loop primers, which are typically used to decrease the amplification time (Nagamine et al., 2002), were not used as they also decreased the ΔTt.

5.3.3 Sensitivity and specificity

To validate the optimized method, a dilution series of miR-141 was prepared with four concentrations. Tested on a commercial real time cycler (used isothermally), the dynamic range was 1.4 fmol – 1.4 pmol per 10 µl reaction (Figure 5.3A). No template control (NTC) amplified after 65 min but amplification time compared to 1.4 fmol was significantly higher (p value < 0.05). Thus a detection limit of 1.4 fmol was observed using this technique with the miR-141 assay.

Specificity is crucial to the development of microRNA assays. Some amplification-based strategies that employ intercalating dyes may be unable to correctly differentiate one nucleotide mismatches, particularly when located near the 5' end (Shen et al., 2015). To assess specificity among closely related microRNAs, a universal strand specific to miR-141 was tested with members of the miR-8 family, which differ by 1-6 nucleotides, including miR-200a, miR-429, miR-200a, miR-200b, and miR-200c (Figure 5.3B). The concentration of miR used for the specificity experiments was 14 fmol. There was no difference between the no template control (NTC) and the assay targeting miR-429, while the ΔTt ranged from 1.3 to 3 min. A ΔTt of 10 min was observed for miR-141 compared to the NTC. Thus, the assay is indeed specific to miR-141.

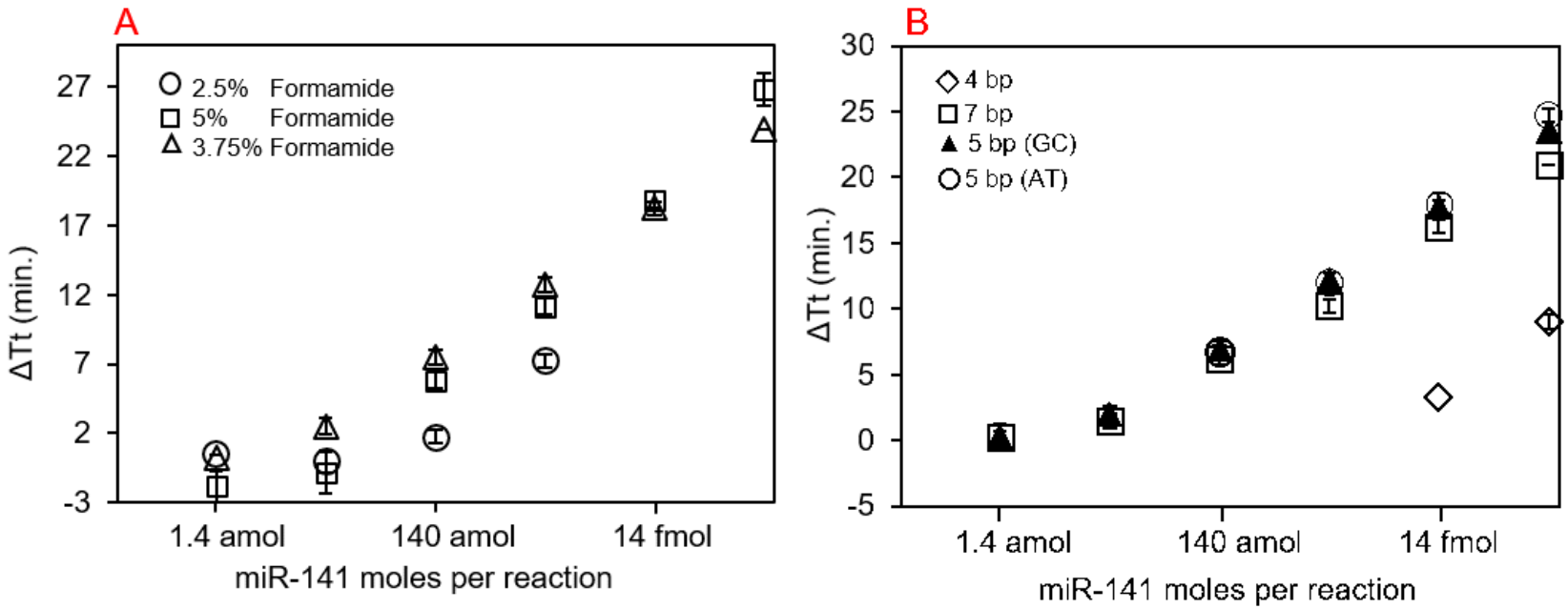

Figure 5.2: Optimization of the base-stacking microRNA isothermal amplification approach. **A.** Standard curves obtained with various overhang lengths (four, five, seven nucleotides), and five base overhang with AT content or GC content. **B.** Standard curves with formamide concentrations of 2.5%, 3.75%, and 5%. No amplification was observed at 7% and above. Points represent average and error bars are standard deviation of three technical replicates.

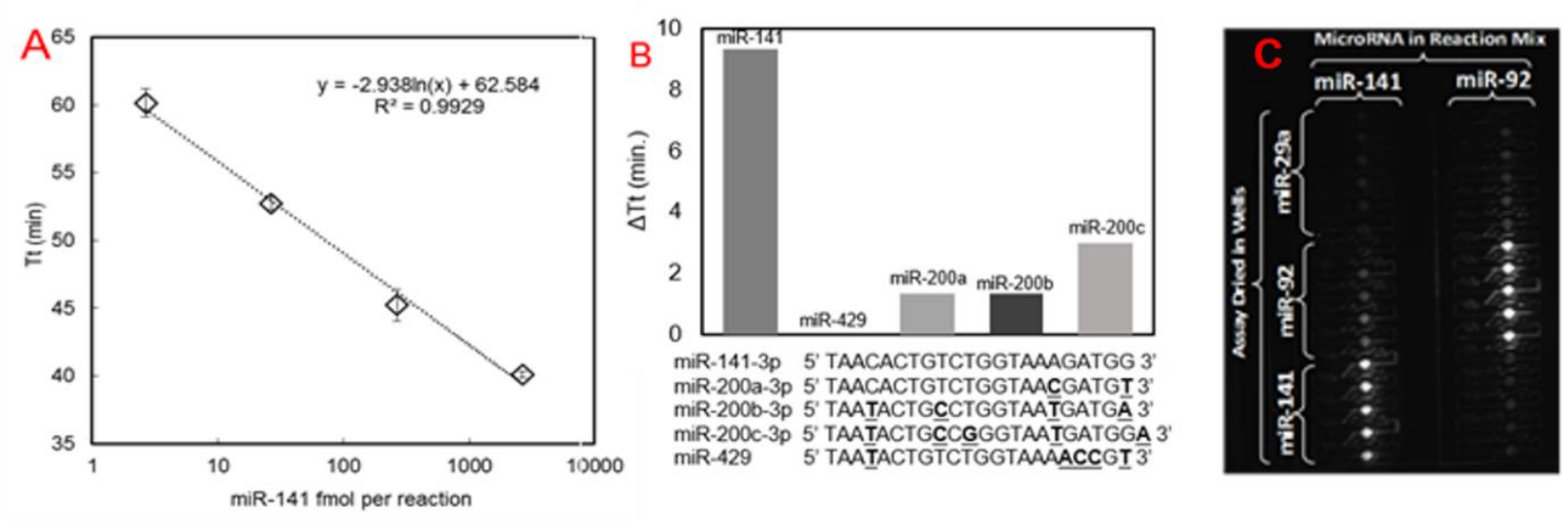

Figure 5.3: Sensitivity and specificity of base-stacking isothermal amplification method targeting miR-141. **A.** Dynamic range of isothermal amplification assay with miR-141 from 1.4-1400 fmol/reaction. **B.** Specificity of the miR-141 assay when tested with closely related members of the miR-8 family (14 fmol each). Points represent average and error bars are standard deviation of three technical replicates. C. Specificity as shown via imaging the Gene-Z microfluidic chip with a CCD.

Serial dilutions of microRNA were also tested on the Gene-Z device. The chip was divided into 8 groups (n = 4 wells each) and was preloaded with a ten-fold dilution series of the miR-141, and one group (n = 4 wells) was loaded without template to serve as the NTC. Results in Gene-Z were similar to the conventional thermal cycler with a significantly different ΔTt down to 1.4 fmol per reaction compared to the NTC. In a specificity experiment on the microfluidic chip used in Gene-Z, assays targeting miR-141 and miR-92 amplified in respective wells in which target microRNA was loaded. (Figure 5.3C). Thus specificity and sensitivity were maintained for assays tested using the Gene-Z device.

5.3.4 Direct amplification of microRNA from body fluids

To test the suitability of the developed methodology for use with clinical samples, miR-141 was spiked into hydrated faeces, whole blood, and plasma. The amount of body sample used per reaction was also tested for each matrix. Complete inhibition was only observed at higher concentrations of faeces (23 and 2.3 µg/µl; Figure 5.4A). Complete inhibition was not observed for any of the tested concentrations of whole blood and plasma though a slight delay in amplification time was observed in reactions with more blood and plasma. The NTC control was also delayed in higher concentrations of blood and plasma per reaction indicating that assay sensitivity was not influenced by direct amplification. For samples spiked with miR-141, gel electrophoresis of the reaction after 48 min of incubation further confirms correct amplification in body sample matrices (Figure 5.4B). Though still positive amplification product, the signal intensity of the amplicons on the gel was lower for faeces compared to the signal intensities for amplicons for whole blood and plasma.

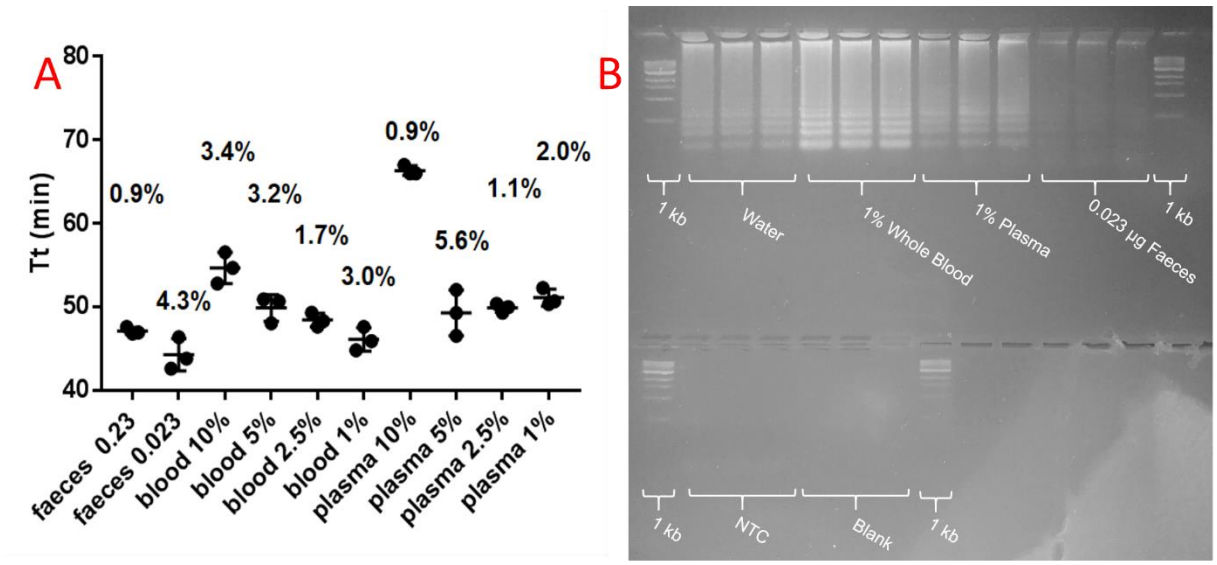

Figure 5.4: Precision and gel electrophoresis verifying utility of direct isothermal amplification in body samples spiked with miR-141. A. Amplification time (Tt) results after spiking 140 fmol miR-141 into different body sample matrices. Final concentration indicates amount of body matrix added to the amplification reaction (feaces indicate µg per µl). Error bars indicate standard deviation of three techinal replicates and and CV indicates coeffiecint of variation (%). B. Gel electrophoresis 48 min of incubation of base-stacking isothermal amplification for body samples spiked with or without miR-141.

5.4 Discussion

The described method appears to be well-suited for detection of microRNA via direct amplification from unprocessed biological samples, as *Bst* polymerase is less influenced by inhibition compared to qPCR (Koloren et al., 2011; Stedtfeld et al., 2015, 2014). To our knowledge, this is the most rapid method (< 60 min) described for quantitative detection of microRNAs (Table 5.1). The time to result was mainly achieved by eliminating sample processing and RNA extraction. A majority of previously described methods for microRNA require RNA extraction, ligation, and other steps that involve opening tubes and sample manipulation (Table 5.1). Two other studies described direct isothermal amplification; one of which required an incubation time of 4 hrs (Li et al., 2013). The other method appears to be

comparable in terms of hands-on-time, and was tested with cell extracts from lung; however, both precision and detection limits were questionable in that replication was not described, nor was a subsequent proof of amplification other than real-time fluorescence curves (Li et al., 2013).

A sensitivity of 1.4 fmol per reaction was observed using our base-stacking isothermal amplification method, which is comparable to other direct isothermal amplification studies (Table 5.1). Although sensitivity is important, it is not the most vital factor determining the clinical utility of a microRNA assay. For example, a single cell of human tissue may contain anywhere from 100,000 - 500,000 copies (0.5 - 1 amol) of total microRNAs or 0 – 40,000 copies (0 - 0.1 amol) of a single microRNA (Liang et al., 2007). This suggests that only ~10^3 cells would be required to measure microRNAs in higher abundance. Using a larger sample volume is always a possibility with human samples. For example, to measure 2.38x10^6 copies (4 amol) miR-224 per µl urine required to distinguish Type 1 diabetes mellitus patients from healthy controls (Bacon et al., 2015), approximately 350 µl of urine will be required.

Precision is a more important factor as the fold difference between diseased and cohort samples may be smaller for some microRNA/disease associations. Targeting mR-141 with our method, the slope of the standard curve was 6.74 min per 10 fold difference in template concentration. This slope is 2 times higher compared to conventional methods of qPCR (which is typically 3.3 cycles per 10 fold change in template concentration). This allows greater distinction between levels of microRNA. The coefficient of variation among all tested samples was also low (mean CV = 2.6%), which is near or below the CV observed in other studies using isothermal amplification following DNA extraction (Bosward et al., 2016; Brotons et al., 2016) or directly from groundwater samples (Stedtfeld et al., 2016).

Implications of having a direct isothermal approach for measuring microRNAs are far reaching. For example, oral cancer is among the leading causes of death in India due to use of chewing tobacco and betel nuts (Sen et al., 2002; Sinha et al., 2016). Thus, a simple microRNA-based test for early detection of oral cancer may influence timely treatment. Although the benefit of prostate specific antigen (PSA) assay are being questioned routinely, more than 30 million PSA tests are still conducted in the United States alone (Andriole et al., 2009). MicroRNA markers such as miR-141 reported by Mitchell and coauthors appears to be an extremely viable marker for prostate cancer (Mitchell et al., 2008). Using a rapid and easy to use direct amplification method, marker validation studies could potentially be extended to larger populations at a significantly lower cost.

Based on the review of literature presented in Table 5.1, an important observation was related to the usefulness of a NTC. It was noted that nearly all isothermal microRNA amplification approaches result in NTC amplification over an extended incubation period (Li et al., 2011; Ma et al., 2014). Thus, to establish the clinical utility of isothermal methods at the limit of detection, inclusion of NTC along with the standard deviations of NTC as well as the lowest concentration standard is critical. This information was not always available in many reported studies and must be included as part of any isothermal amplification method development for microRNAs.

5.5 Conclusions

The < 60 min time to results achieved with the base-stacking isothermal amplification method is among the most rapid methods currently known for quantification of microRNAs. Direct amplification of microRNAs from blood, plasma, sputum, or other body fluids could

reduce the necessity for hands-on training as well as need for sophisticated instruments. Combined with comparable sensitivities (Ma et al., 2014) and an inexpensive and quantitative device (Gene-Z), this method highlights the potential for microRNAs- based diagnostics under limited resource settings. Further validation is warranted using clinical specimens to establish clinical sensitivity, specificity, performance, and ruggedness under field conditions.

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CHAPTER VI

MicroRNAs-based inter-domain communication between the host and members of the gut microbiome

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Author contribution statement:

M. R. W. assembled literature and drew figure. M. R. W., R. D. S., J. M. T., and S. A. H. wrote/edited the manuscript.

Abstract

The gut microbiome is an important modulator of host gene expression, impacting important functions such as the innate immune response. Recent evidence suggests that the inter-domain communication between the gut microbiome and host may in part occur via microRNAs (small, non-coding RNA molecules) which are often differentially expressed in the presence of bacteria and can even be released and taken up by bacteria. The role of microRNAs in microbiome-host communication in intestinal diseases is not fully understood, particularly in diseases impacted by exposure to environmental toxicants. Here, we review the present knowledge in the areas of microbiome and microRNA expression-based communication, microbiome and intestinal disease relationships, and microRNA expression responses to intestinal diseases. We also examine potential links between host microRNA-microbiota communication and exposure to environmental toxicants by reviewing connections between i) toxicants and microRNA expression, ii) toxicants and gut diseases, and iii) toxicants and the gut microbiome. Future multidisciplinary research in this area is needed to uncover these interactions with the potential to impact how gut-microbiome associated diseases [e.g., inflammatory bowel disease (IBD) and many others] are managed.

6.1 Introduction

Inter-domain molecular communication plays a key role in host – gut microbiome interactions and symbiosis. MicroRNAs (small, non-coding RNAs that regulate gene expression post-transcriptionally) are emerging as a key mode for this communication (Zhou et al., 2017). It is hypothesized that pathogens modulate the expression of host microRNAs to enhance their

survival. Regulation of host microRNAs impacts various biological pathways (e.g., innate immune response) through the regulation of host gene expression (Bartel, 2004).

Dysbiosis of the commensal community has been linked with many gut-related diseases including irritable bowel syndrome (IBS), Crohn's disease, and gastric cancer (Round and Mazmanian, 2009), as well as many other disorders including those connected by the gut-brain axis such as autism, depression, and anxiety (Dinan et al., 2013) though many questions remain unanswered (Aziz et al., 2017; McCarville et al., 2016) and cause and effect has not been established (Degruttola et al., 2016). Three important interactions between the host and gut microbiome involving microRNAs are clear, however, including: i) microRNAs regulate host gene expression (Figure 6.1A), ii) gut microbiota influences host microRNA expression (Figure 6.1B), and iii) the host influences the gut microbiota through the release of microRNAs (Figure 6.1C). It has also been suggested that the host itself may regulate its microbiota but evidence for this is still in its initial stages (Liu et al., 2016).

At the time of this review, over 2,500 microRNAs are known in humans (miRBase Registry; www.mirbase.org; Griffiths-Jones et al., 2008, Kozomara et al., 2014). Primary microRNAs are initially transcribed in the cell nucleus then cleaved by the enzyme Drosha into pre-microRNAs. They are then exported into the cytoplasm where they are processed by the enzyme Dicer. To regulate gene expression, microRNAs are assembled with Argonaute and GW182 into the RNA-induced silencing complex (RISC). They bind by partial complementarity of the last 7-8 bases to the 3' untranslated region (3' UTR) of messenger RNAs (mRNAs), thus blocking translation and preventing mRNA degradation (Figure 6.1A; Bartel, 2004). It is well known that a single microRNA can target many mRNAs and a single mRNA can have many microRNAs that target it (Taganov et al., 2007). It has also been suggested that microRNAs

control protein expression variability by decreasing variability for lower expressed proteins and increasing variability for those highly expressed (Schmiedel et al., 2015). This allows microRNAs to have many regulatory roles in various cellular processes and many microRNAs are thus implicated in various diseases. In fact, forced overexpression of microRNAs has led to tumorigenesis in laboratory studies (He et al., 2005). In addition, over 100 microRNAs are shown to circulate in serum and their use as potential biomarkers of disease has been suggested (Chen et al., 2008). MicroRNA levels in serum and plasma have also been reported as up/down regulated between cancer patient samples and healthy controls, depending on the cancer type and microRNA studied (e.g. reviewed in Peng and Croce, 2016).

Regulation of host gene expression is one means of communication between the gut microbiota and host through the manipulation of host microRNA expression (Figure 6.1B). In fact, microRNA expression profiles are shown to be very different when comparing gut samples collected from traditional or colonized mice with germ free mice (Dalmasso et al., 2011; Singh et al., 2011; Xue et al., 2011). These differentially expressed microRNAs can in turn affect gene expression regulation of a number of gut related diseases. At present, the exact mechanism by which microbiota influence microRNA expression is unclear, though it has been suggested it may involve toll-like receptors and Myd88-dependent pathways (Larsson et al., 2012; Xue et al., 2011). However, the host may be influencing its gut microbiome through the release of microRNA in extracellular vesicles which are taken up by microbes and may affect microbial growth (Figure 6.1C; Liu et al., 2016).

Finally, the effects of outside influences on both the gut microbiome and microRNA expression are important. For example, environmental toxicants have been shown to be associated with differential microRNA expression and disease. MicroRNAs have been proposed

as biomarkers of environmental exposure as they are frequently differentially expressed following an exposure event. The gut microbiome is also often directly impacted by exposure to synthetic chemicals due to direct metabolism of chemicals, chemicals altering enzymatic activity, or the induction of dysbiosis (Claus et al., 2016). Dysbiosis could further impact disease, though there is no evidence to date except in the case of antibiotics (Becker et al., 2016).

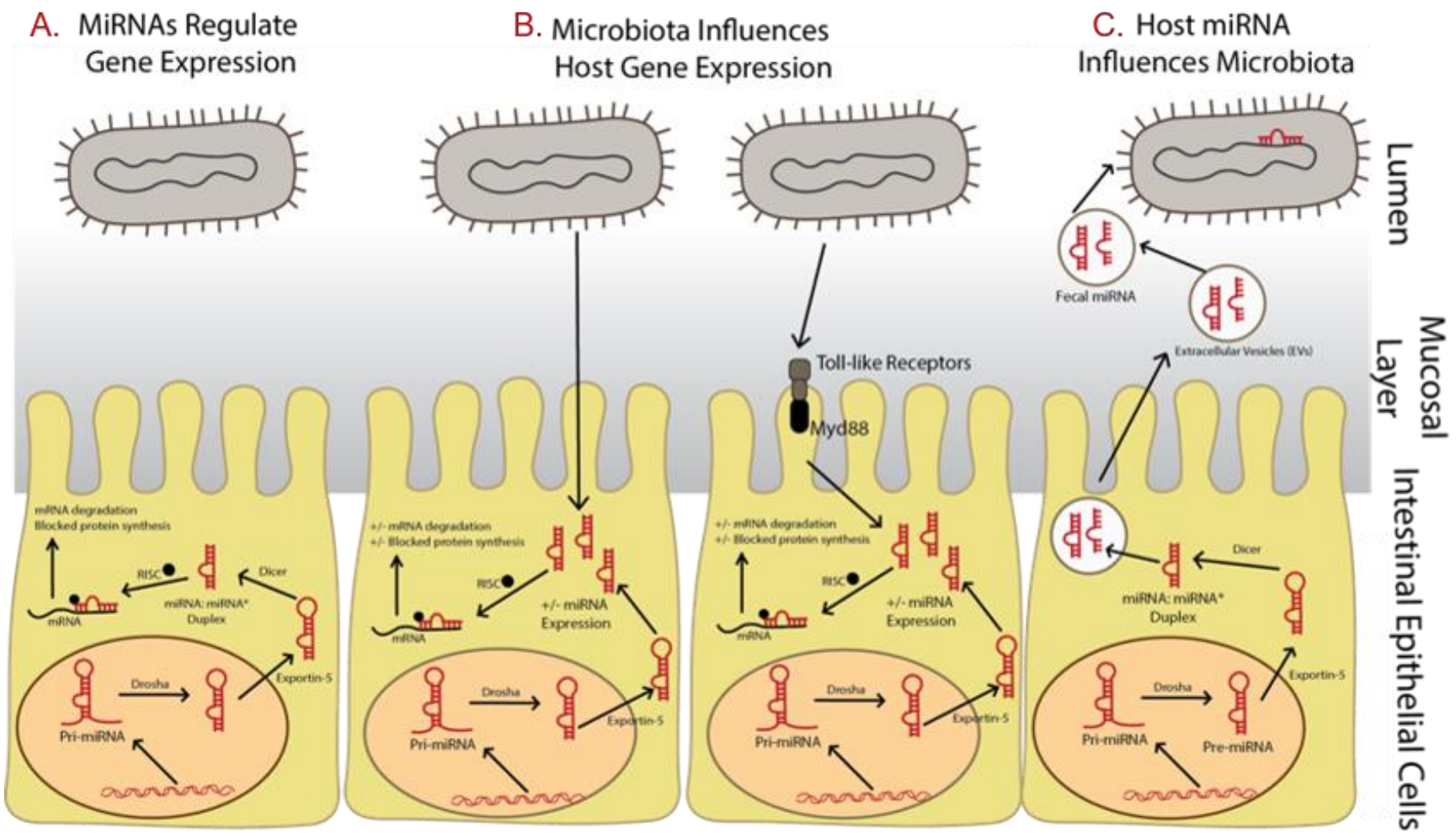

Figure 6.1: Summary of relationships between microRNAs and microbiota and the impact on gene expression regulation. A. MicroRNAs begin as precursor hairpin loops, generated in the cell nucleus, exported to the cytosol, and processed by Dicer into two structures, the mature microRNA strand and a rapidly degraded passenger strand (often labelled with *). B. Microbiota have been shown to regulated microRNA expression, possibly through toll-like receptor/Myd88 – dependent pathways. C. The host may be influencing its gut microbiome by releasing fecal microRNAs, which are taken up by bacteria.

6.2 Gut microbes influence host microRNA expression

The influence of pathogenic microorganisms (such as *Listeria monocytogenes*, *Salmonella enterica*, and *Helicobacter pylori*) on host microRNA expression has been extensively reviewed (e.g. Maudet et al., 2014). Far fewer studies exist reporting the influence of commensal bacteria on microRNA expression (Table 6.1; reviewed in Masotti, 2012; Runtsch et al., 2014) though specific gut commensals are not evaluated. Most studies focusing on the gut microbiome and host microRNAs have used mixed microbial communities as part of traditional mice and compared them to germ-free or colonized mice. These have focused on ileum, colon, and caecum because host microRNA expression is expected to be tissue-specific. Measurement of microRNA levels was carried out mostly by qPCR (Archambaud et al., 2013; Dai et al., 2015; Singh et al., 2011) but in certain studies microarray-based relative expression was obtained (Dalmasso et al., 2011; Xue et al., 2011). A total 4 studies measured larger mice microRNA panels for higher throughput screening (Archambaud et al., 2013; Dalmasso et al., 2011; Singh et al., 2011; Xue et al., 2011) while others measured only targeted microRNAs (Dai et al., 2015; Xue et al., 2011). One study that also included human ulcerative colitis (UC) patients, was interested in measuring a single microRNA (miR-193a-3p) because of its relevance to UC from earlier studies (Dai et al., 2015). As seen in Table 1, the number of differentially expressed microRNAs in response to commensal gut bacteria was between 5 and 16 in the three studies that measured the whole mouse panel of microRNAs. The changes in expression level are significant but seldom drastically different (e.g. in Singh et al., 2011 the maximum fold change for downregulated microRNAs was 0.2 and for upregulated microRNAs was 4.6).

Table 6.1: Studies relating mixed microbial communities from traditional or colonized mice to host microRNA expression

Comparison	Sex; Weight; Age of Individuals	Sample type	Differentially expressed microRNAs (↑ Upregulation, ↓ Downregulation)	Gene or functional targets of differentially expressed microRNAs (↑ Upregulation, ↓ Downregulation)	Reference
Germ-free (n= 6) vs. colonized Swiss Webster mice (n=6)	Female; N/A; 8 weeks	Ileum, Colon	Ileum: miR-298↑ Colon: miR-128↑, miR-200c*↑, miR-342-5p↑, miR-465c-5p↓, miR-466d-3p↓, miR-466d-5p↓, miR-665↓, miR-683↓	Abcc3↓ (directly targeted by miR-665 3' UTR and validated with in vitro studies)	(Dalmasso et al., 2011)
Germ-free (n= 5) vs. traditional Swiss Webster mice (n= 5)	Male; N/A; 5 weeks	Caecum	miR-21*↓, rno-miR-351↓, miR-351↓, miR-487b↓, miR-467a↓, miR-27b*↓, miR-148a↓, miR-145↑, miR-183↑, miR-133a↑, miR-150↑, miR-672↑, miR-181a*↑, rno-miR-664↑, miR-455↑, miR-138*↑, let-7g*↑	54 genes related to intestinal barrier function (potential targets determined computationally)	(Singh et al., 2011)
Not infected (n= 3) vs. infected conventional C57BL/6 mice with *Listeria monocytogenes* (n= 3) Not infected (n= 3) vs. infected germ-free C57BL/6 mice with *Listeria monocytogenes* (n= 3)	Female; N/A; 9-12 weeks	Ileum	Conventional: miR-143↓, miR-148a↓, miR-200b↓, miR-200c↓, miR-378↓ Germ-free: miR-194↓, miR-378↑	Protein encoding genes (potential targets determined computationally)	(Archambaud et al., 2013)

Table 6.1 (cont'd)

Germ-free (n= 3) vs. traditional C57BL/6 mice (n= 3)	Female; N/A; 8-10 weeks	Dendritic cells	miR-10a↓	Il-12/IL-23p40↑ (directly targeted by miR-10a 3' UTR and validated with in vitro studies)	(Xue et al., 2011)
Traditional (n=3) vs. germ-free Swiss Webster mice (n= 3)	Male; N/A; 10-12 weeks	Aorta	miR-204↓	Sirt1↑ (directly targeted by miR-204 3' UTR and validated with in vitro studies)	(Vikram et al., 2016)
Healthy (n=7) vs. colitis- induced C57BL/6 mice (n=7) Healthy (n=12) vs. active ulcerative colitis patients (n=11)	Mice: Female; 18-22 g; 8 weeks Humans: N/A	Colon	miR-193a-3p↓	PepT1↑ (validated with in vitro studies) Colonic inflammation↑	(Dai et al., 2015)

One of the earliest reports of the impact of the gut microbiome on microRNA expression used germ-free mice colonized with gut microbiota from specific pathogen-free (SPF) mice (Dalmasso et al., 2011). Briefly, Swiss Webster SPF mice (8 weeks; female) were colonized then introduced to germ-free mice cages. After 4 days, all mice were sacrificed and colons and ileum were collected. MicroRNA expression profiles were determined via microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR) by comparing tissues from germ-free (n=6) and colonized mice (n=6). Increased expression of miR-128, miR-200c*, and miR-342-5p and decreased expression of miR-465c-5p, miR-466d-5p, miR-665, and miR-683 was observed in colon tissues (Table 6.1). Increased expression of miR-298 was observed in ileum tissues. Using in vitro studies, it was confirmed that miR-665 targets the Abcc3 gene (ATP-binding cassette transporter) 3' UTR which was downregulated in colonized mice.

Caecal microRNA signatures have also been compared between germ-free (n=5) and conventional (n=5) Swiss Webster mice (male; 5 weeks; Singh et al., 2011). Overall, 334 microRNAs were detected in both groups with 16 differentially expressed between them (including miR-21*, rno-miR-351, miR-351 which were downregulated). Computational approaches and gene expression analysis revealed the potential targets of each microRNA which were involved in regulating intestinal barrier genes and immune system regulation, though these genes were not validated *in vitro* as targets of the microRNAs.

MicroRNA expression profiles from both traditional and germ-free C57BL/6 mice have also been shown to be influenced following oral *Listeria monocytogenes* infection (Archambaud et al., 2013). Briefly, 9-12 week old female mice were divided into four groups (n=3 per group) including i) germ-free, not infected, ii) germ-free, infected, iii) conventional, not infected, and iv) conventional, infected. After infection with *Listeria monocytogenes*, ileum samples were

collected and microRNA expression profiles were analyzed. Two microRNAs (miR-194 and miR-378) were differentially expressed between infected and non-infected germ-free mice while five microRNAs (miR-143, miR-194, miR-200b, miR-200c, and miR-378) were differentially expressed between infected and non-infected conventional mice. This suggests that the conventional mice were responding more to the *Listeria* infection than their germ-free counterparts. Finally, it was observed that the ten most highly expressed microRNAs in this study could be considered as "signature" microRNAs that are always present in high abundances.

Some studies have measured selected microRNAs focusing on one or two based on their role in specific functions. Xue and coauthors studied expression of miR-10a because of its role in regulating the innate immune response through targeting IL-12/IL-23p40 (Xue et al., 2011). It was reported that miR-10a expression in intestinal dendritic cells of conventional C57BL/6 mice was significantly lower compared to germ-free mice (female; 8-10 weeks old; n=3 per group). The microbiota was shown to regulate miR-10a using TLR-TLR ligand interactions and a MyD88-dependent pathway. Furthermore, the IL-12/ IL-23p40 was increased and the 3' UTR was determined to be a direct target of miR-10a (validated with *in vitro* studies). UC mice with high IL-12/IL-23p40 expression also had lower expression of miR-10a in intestinal tissues compared with healthy mice, suggesting the importance of miR-10a in disease, though this may not be due to direct affects.

The gut microbiome may also be regulating host microRNAs and function in regions beyond the gut. In a recent study, the decreased expression of miR-204 was observed in the aorta of germ-free (n=3) as compared to traditional Swiss Webster mice (n=3; Vikram et al., 2016). All mice in the experiment were males, 8-10 weeks in age. Following decreased expression of miR-204, its target, Sirt1 (sirtuin1 lysine deacetylase) was significantly increased. In fact, the

microbiome was shown to remotely govern miR-204. After administration of broad-spectrum antibiotics in mice to control the microbiome, Sirt1 expression was decreased which resulted in impaired endothelial function, specifically endothelium-dependent vasorelaxation.

Host genetics have been reported to shape the gut microbiome community structure which in turn influences host metabolism (Goodrich et al., 2014) but a recent report suggests that the host may also influence its gut microbiome through fecal extracellular microRNAs (Liu et al., 2016). It was observed that intestinal epithelial cells (IECs) are major sources of extracellular fecal microRNAs, due to their ability to secrete exosome-like vesicles. Fecal microRNAs are protected from degradation due to protection by i) entrapment in extracellular vesicles and ii) association to high-density lipoproteins or argonaute complexes (Creemers et al., 2012). Interestingly, overall abundance of fecal microRNAs was increased in germ-free mice versus SPF colonized mice. Furthermore, in intestinal epithelial cell-microRNA-deficient mice (mice without the enzyme Dicer), bacterial growth in the gut was uncontrolled but after undergoing fecal microRNA transplantation, homeostasis was observed, suggesting that control of bacterial growth may be due to the extracellular fecal microRNAs. However, microRNAs are involved in many important gene regulation processes (not just uptake into bacterial cells as the authors point out) which may impact dysbiosis via more indirect means. It is also noted that knocking out Dicer may not completely eliminate microRNAs processed in the cell, though they are significantly reduced.

These studies although limited in number indicate that microRNAs serve as an important communication channel between the gut microbiome and the host. Differential microRNA expression in turn regulates the host gene expression, potentially impacting pathways and host physiology and disease status. Unfortunately, a number of confounding factors exist as most of

the experiment designs are distinctly different. For example, the influence of the use of male or female mice can impact expression profiles as well as age, different tissue types and different number of replicates. Table 1 shows that all studies discussed in this section used different age animals and were split in their use of males or females. Influence of specific members of the gut microbiome on these expression profiles could also affect results. Future studies are needed to determine the context of these results in overall health.

6.3 MicroRNAs associated with select gut diseases

Differential expression of microRNAs as it pertains to diseases has been studied extensively for gut-associated diseases such as inflammatory bowel disease (IBD; Kalla et al., 2015), Crohn's disease (Kalla et al., 2015), UC (Kalla et al., 2015), and gastric cancer (Ishiguro et al., 2014). In fact, these microRNAs are both tissue-specific (Kalla et al., 2015) and circulating (Paraskevi et al., 2012) microRNAs are differentially expressed in patients with IBD compared to healthy controls. Circulating and other cell-free microRNAs could also serve as useful disease biomarkers (Zahm et al., 2011), as they only require a small blood or fecal sample employing relatively simple sampling procedures. It has also been suggested that circulating microRNAs may travel the bloodstream and regulate gene expression in distant cells (Creemers et al., 2012).

As such, extracellular microRNAs have been shown to be differentially expressed in various diseases as compared with healthy controls. For example, miR-29a collected from blood microvesicles, small bowel tissues, and colon tissues has been shown to be significantly increased in patients with IBS (Zhou et al., 2010). MiR-29a modulates an increase in intestinal barrier permeability due to its complementarity to the 3' UTR of the glutamine synthetase gene, thereby blocking production of glutamine synthetase, an enzyme that was reported in the study to

decrease intestinal barrier permeability (Zhou et al., 2010). Therefore, increased levels of miR-29a could lead to an increase in permeability which was observed during cell culture experiments. Specifically, IBS patients with increased intestinal barrier permeability showed increased expression of miR-29a compared with IBS with normal permeability. Similarly, expression levels of miR-150 and miR-342-3p in peripheral blood have also been shown to increase in patients with IBD, with a fold-change greater than 1.6 (Fourie et al., 2014). Though regulatory mechanisms were predicted using an Integrated Pathway Analysis (IPA) network, it was not experimentally investigated as part of the study. It was suggested that because miR-150 is interacts with protein kinase AKT2, it may thus affect inflammatory pathways associated with IBD. In addition, miR-342-3p may be important for pain signaling, motility in the colon, and smooth muscle function.

MicroRNAs have also been shown to be involved in the complications typically associated with Crohn's disease. For example, the miR-200 family are involved in fibrogenesis in the intestine (Chen et al., 2012). Using in vitro studies with intestinal epithelial cells, fibrogenesis was induced using transforming growth factor β1 (TGFβ1) which induces fibrosis in Crohn's disease. It was shown that TGFβ1 not only induced fibrosis but also inhibited the expression of miR-200b. Administration of miR-200b in vitro also protected intestinal epithelial cells, in part, from fibrosis and suggests this could be due to miR-200b inhibiting zinc finger E-box-binding homeobox 1 and 2 (ZEB1 and ZEB2). Furthermore, to determine if miR-200b could be used as a potential biomarker of fibrosis in Crohn's disease, serum of patients with Crohn's disease and fibrosis (n=10) were compared to serum from healthy controls (n=16) and Crohn's disease patients without fibrosis (n=10). MiR-200b was shown to be significantly upregulated in

all comparisons for patients with Crohn's disease and fibrosis, suggesting it could be used as a potential biomarker.

Select studies report that microRNAs could be useful biomarkers for the detection of cancers (e.g., Calin and Croce, 2006), due to significant differences in expression levels observed from a variety of samples between patients and healthy controls. Ohshima and coauthors have suggested that gastric cancer cells may use hsa-let-7a to promote oncogenesis (Ohshima et al., 2010). Results show that let-7a, which targets oncogenes (RAS, HMGA2) and suppresses the development of cancer, is released by gastric cancer cells (such as AZ-P7a cells) into their exosomes, thus maintaining oncogenesis. Indeed, it was found that let-7 family was abundant in both intracellular and extracellular environments, while the low metastatic cell line AZ-521 had much lower levels in both environments, which could result in increased expression levels in gastric cancer.

Characterizing the altered microRNA expression in certain gut diseases is important for understanding their role in disease as well as in the development of treatment options. Though the relationship between microRNA expression and gut diseases has been extensively studied, the full extent of this relationship and the importance of the gut microbiota on this is less clear.

6.4 Microbes associated with gut diseases

Dysbiosis of gut microbial communities that results in the loss of host-microbiota symbiosis often results in a shift from symbiont to pathobiont. This shift in microbial community structure is important in the development, incidence, recurrence, and treatment of major gut diseases such as IBD. Though many pathogenic organisms are also involved in these diseases (e.g. Helicobacter pylori infection increases the risk of gastric cancer; Wroblewski et al., 2010),

this section focuses on commensals or pathobionts that are not always pathogenic. The importance of commensals in maintaining overall gut homeostasis is clear, as evidenced by the success of fecal transplantation in the treatment of gut diseases such as UC (Borody et al., 2003).

One well-characterized change is the differences in the phylum Firmicutes in IBD. *Faecalibacterium prausnitzii* is an important gut commensal as it is a major producer of butyrate, which plays a key role in gut physiology and modulation of the immune system (Wrzosek et al., 2013). Specifically, reduced abundance of *F. prausnitzii* has been observed in Crohn's disease patients (n=22) as compared to healthy controls (n=27; Sokol et al., 2008). Other reports have suggested increased abundances of *F. prausnitzii* in pediatric Crohn's disease patients (n=13) as compared to healthy controls (n=12; Hansen et al., 2012). This increased abundance was also correlated with overall reduced bacterial diversity, something that has been observed in many other studies for Crohn's disease, IBD, and UC (Hansen et al., 2012; Lepage et al., 2011; Ott et al., 2004). Interestingly, the decrease in abundance of *F. prausnitzii* in UC and Crohn's disease has also associated with an increase in other gut commensals such as *Bifidobacterium* and *Lactobacillus* (Wang et al., 2014).

Another predominant commensal of the gut microbiome is *Bacteroides* including *Bacteroides fragilis*, which expresses polysaccharide A (PSA), a compound that modulates the host immune response by inducing Treg and cytokine expression (Troy and Kasper, 2010). PSA in itself can provide protection against colitis by repressing pro-inflammatory cytokines (Mazmanian et al., 2005). In an elegant review by Zhou and coauthors, based on an extensive analysis of the literature from 1990 to 2016, it was determined that abundances of *Bacteroides spp.* in Crohn's disease and UC patients were significantly lower than in healthy controls (Zhou and Zhi, 2016). Unfortunately, though typically commensal, some strains of *Bacteroides fragilis*

produce enterotoxins which can cause illness and diarrhea. Due to its ability to induce cytokine expression and widespread abundance, enterotoxigenic *Bacteroides fragilis* can result in persistent inflammation and induction of colitis and colonic tumors, as validated in multiple intestinal neoplasia mice (Wu et al., 2009). This occurs through a Stat3 (signal transducer and activator of transcription-3) and T helper type 17 (Th17)-dependent pathway, as Wu and coauthors noted.

Only one study has connected the differential expression of microRNAs in response to the microbial community observed in gut diseases (Dai et al., 2015). Specifically, in C57BL/6 mice (18-22 g; 8-week old; female; n=7) colitis was induced providing mice with filtered sterile water containing 3% dextran sodium sulfate for 8 days while control mice (n=7) were given untreated water. In colitis- induced mice, miR-193a-3p was downregulated while colonic PepT1 (di/tripeptide transporter) and overall colonic inflammation was upregulated (Table 1). In fact, PepT1 uptakes bacterial products suggesting a direct relationship between microRNAs and microbiota and increased expression of PepT1 is associated with IBD and indeed treatment with antibiotics resulted in reduced inflammation. Furthermore, after treatment with miR-193a-3p mimics, inflammation was also reduced. Similar expression profile results were also observed in human subjects when comparing healthy (n=12) and active UC patients (n=11).

Dysbiosis of the gut microbiome is also linked with gut diseases such as Crohn's disease and UC. Through evaluating specific community shifts (such as *F. prausnitzii* and *Bacteroides*), probiotic-based treatments targeting that dysbiosis could be used to revert to homeostasis, though much more research is required in this area as cause and effect has not been evaluated and conflicting reports exist (Aziz et al., 2017; Degruttola et al., 2016; McCarville et al., 2016). Though the relationship of microRNA expression to gut diseases as well as the relationship of

gut microbiome dysbiosis to gut diseases has been studied, their investigation together (namely the communication that may be occurring therein) has not. For example, *F. prausnitzii* is decreased in Crohn's disease (Hansen et al., 2012) but this may be affecting the microRNA-based communication in some way. As both microRNAs and gut microbes are important in the development of certain gut diseases, it is evident that role of microRNAs cannot be overlooked in studies focusing on gut microbiome associated diseases.

6.5 Links between exposure to environmental contaminants, microRNA expression, and the gut microbiome

Exposure to environmental contaminants can increase the risk for many diseases. A number of these contaminants alter genetics through DNA sequence mutation, DNA methylation, histone modifications, and differential microRNA expression in the host (Hou et al., 2012). In fact, host microRNAs have been shown to be biomarkers of environmental exposure to various chemical agents (Vrijens et al., 2015) as they are differentially expressed following exposure to contaminants (Hou et al., 2012). Examples of environmental contaminants that may alter host microRNA expression include cigarette smoke, aluminum, arsenic, bisphenol A, diethyl phthalate (DEP), formaldehyde, polycyclic aromatic hydrocarbons (PAH), hexahydro-1,3,5-trinitro-1,3,5,-triazine (RDX) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (as reviewed in Vrijens et al., 2015).

Some of these reports have associated environmental exposure with differential microRNA expression and gut diseases. For example, TCDD and other AhR activators are associated with colitis (Benson and Shepherd, 2011) and colorectal cancers (Xie and Raufman, 2015), BPA is implicated in colorectal cancer (Chen et al., 2015), and PAH may lead to digestive

tract cancers (Diggs et al., 2011). Though most of the research involving differential microRNA expression in response to contaminants involves other tissues such as liver (Szabo and Bala, 2013; Zhang and Pan, 2009), some effects related to the gastrointestinal tract (particularly those related to gastric and colorectal cancer) are also reported. For example, Mullany and coauthors observed an association between host differential microRNA expression, cigarette smoke, and rectal or colorectal cancer (Mullany et al., 2016). In this study, 306 host microRNAs were differentially expressed in smokers, with 200 directly associated and 41 inversely associated with tumor phenotypes for either colon or rectal cancer. These findings strongly suggest that the exposure to cigarette smoking may impact cancer development through differential microRNA expression. Smoking has been associated with other gut related diseases (Mahid et al., 2006) though conflicting reports exist (Rosenfeld and Bressler, 2012).

It has been suggested that the gut microbiome may be impacted by exposure to environmental contaminants by four possible mechanisms: i) direct metabolism, ii) metabolism following conjugation in the liver, iii) interfering with enzymatic activity, and iv) induction of dysbiosis (Claus et al., 2016; Figure 6.2). Certain chemicals are directly metabolized by gut microbiota including PAH, Nitrotoluene, DDT, PCB, and pesticides (Claus et al., 2016). Some of these, such as nitrated PAH, can form conjugates after metabolism by microbiota that are carcinogenic and more hazardous to the host than the initial chemical (Möller, 1994). Exposure to environmental contaminants has also been associated with dysbiosis of the gut microbiome. For example, smokers with active Crohn's disease were found to have significantly higher levels of *Bacteroides* than healthy controls (Benjamin et al., 2012). Specifically, healthy controls who also smoked had higher levels of *Bacteroides-Prevotella* than non-smokers, while Crohn's disease patients who smoked had higher *Bifidobacteria* and *Bacteroides-Prevotella* and lower *F.*

prausnitzii. Unfortunately, research related to connecting dysbiosis of the gut microbiome with gut diseases is still in its initial stages. Further efforts are needed to define the impact of these linkages on disease.

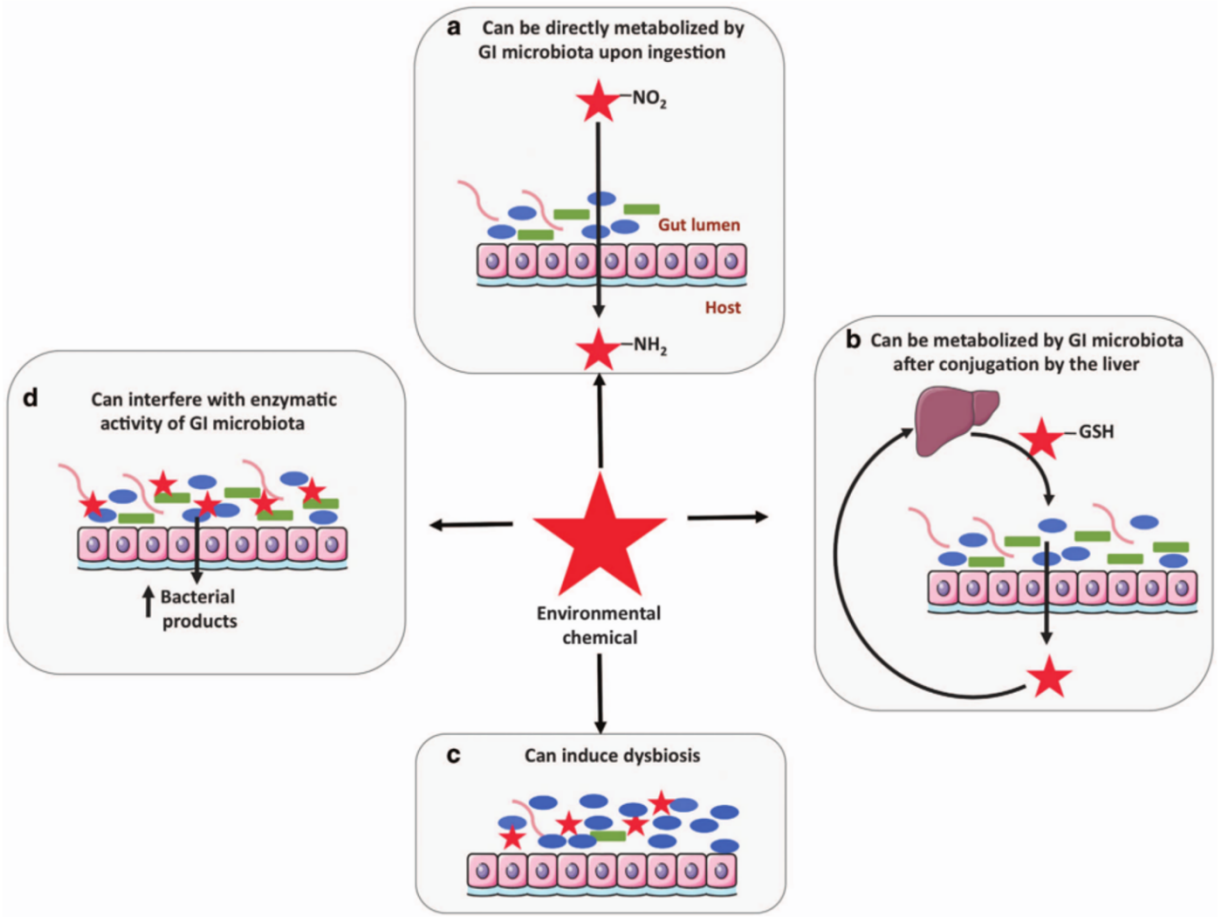

Figure 6.2: Four mechanisms the gut microbiome may be influenced by exposure to environmental contaminants include: a) direct metabolism, b) metabolism following conjugation in the liver, c) interfering with enzymatic activity, and d) induction of dysbiosis (Claus et al., 2016). Reproduced with permission under the Creative Commons License by Nature Publishing Group.

6.6 Challenges ahead and concluding remarks

Overall, the research reviewed here suggests that microRNAs may play a crucial role in communications between the gut microbiome and the host to maintain gut homeostasis and

prevent disease. In this review, we have discussed potential interactions between the gut microbiome and host microRNAs, microRNAs and gut diseases, and gut diseases and the gut microbiome. These complex relationships suggest that perhaps the symbiotic relationship we share with the gut microbiome is indeed co-evolved, down to the nucleic acid level. Furthermore, as recent research highlights the host regulation of the microbiome through fecal microRNAs (Liu et al., 2016), the microbiome may not be simply regulating host homeostasis, but the relationship between the host and the microbiome works together to maintain symbiosis. The question of "who is controlling whom?" is an interesting one, though the answer remains unclear and highlights that maybe the host and its bacteria are continually controlling each other to maintain ideal circumstances for both.

Despite these relationships, many aspects of the gut microbiome-host interactions remain unknown. First, the relationship and communication between the gut microbiome and microRNAs as they relate to gut diseases has not been fully evaluated. There is also the need to define the link between gut diseases and dysbiosis. Second, many outside factors (such as environmental exposure to toxicants) impact the gut microbiome, differential microRNA expression, and gut diseases. Unfortunately, studies that investigate the combined effect of all of these factors together do not yet exist. These may prove to be important in microRNA- based communication with the gut microbiome, particularly since they have all been shown to be connected separately. Until all aspects are researched together, cause and effect cannot be defined. Future studies investigating the impact of toxicants on human health would also benefit from evaluating the outside variables such as the gut microbiome and differential microRNA response. Interdisciplinary studies that include the fields of toxicology, microbiology, and human

health in particular would help bridge the gaps in current knowledge related to microRNA-based communication with the gut microbiome.

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CHAPTER VII

Influence of dioxin exposure on ileal microRNA expression is dependent on the presence of gut microbiota

Abstract

The gut microbiome impacts important host functions such as the innate immune response and is an important modulator of host gene expression. Though this occurs by many mechanisms, evidence suggests that it may occur, in part, via microRNAs (small, non-coding RNA molecules that regulate gene expression post-transcriptionally). Environmental toxicants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; an aryl hydrocarbon receptor), also impact the immune response and alter the gut microbiota community structure, though their combined relationship to both host microRNA expression and the gut microbiome is unknown. Toxicants, microRNAs and the microbiome are important players in gut diseases and intestinal homeostasis. Differentially expression of microRNAs has been previously observed in response to i) toxicant exposure and ii) members of the gut microbiome. However, the co-influence of these interactions has not been studied together. In this study, C57BL/6 gnotobiotic mice were colonized with specific gut microbes (segmented filamentous bacteria; SFB and *Bacteroides fragilis*) and administered 30 µg/kg TCDD every 4 d for 28 d. Using nCounter mouse microRNA panel, ileal microRNA expression profiles revealed significant differential expression for both SFB mono-colonization alone and SFB mono-colonization with TCDD treatment. Bacterial association with SFB and *B. fragilis* resulted in the higher number of differentially expressed microRNAs than TCDD treatment alone, indicating that members of the gut microbiome impact host microRNA expression. Analysis of transcriptomic and metabolic networks may be necessary to decipher the physiological effects this differential expression of microRNA may have. Overall, these results provide insight into host microRNA – microbiota interactions and perturbations via TCDD, with implications in immune modulation and gene regulation.

7.1 Introduction

Inter-domain molecular communication between the gut microbiome and its host is important for maintaining intestinal homeostasis. Intestinal microRNAs (small, non-coding RNAs that regulate gene expression post-transcriptionally) are emerging as one such communication molecule (Zhou et al., 2017). Control and regulation of host microRNA expression impacts many biological pathways (Bartel, 2004). It has also been suggested that pathogens modulate host microRNA expression to alter host responses to infection, thereby enhancing their survival. The host itself may regulate its gut microbiome growth through microRNAs (Liu et al., 2016) but this aspect of host associated control of microbiome is still emerging.

Significant information exists indicating that microRNA expression varies in response to changes in microbial communities. Such changes may be associated with under various disease conditions and the expression of microRNAs is generally expected to be tissue-specific. For example, differential microRNA expression was observed in germ-free (GF) mice as compared to specific pathogen-free (SPF) mice in different locations along the gut such as the ileum (Dalmasso et al., 2011), colon (Dalmasso et al., 2011), and cecum (N. Singh et al., 2012) and in specific cell types such as dendritic cells (Xue et al., 2011). Differential microRNA expression has also been observed in host diseases, such as inflammatory bowel disease (IBD; Dalal and Kwon, 2010)). It has been shown that miR-10a was downregulated in IBD and targets IL-12/IL-23p40, an important gene which may contribute to immune system homeostasis (Xue et al., 2011). There is far more information available, however, on host differential microRNA expression in response to specific pathogens such as *Listeria monocytogenes* (Izar et al., 2012), *Salmonella typhimurium* (Schulte et al., 2011), and *Helicobacter pylori* (Fassi Fehri et al., 2010).

Events of perturbation, such as environmental toxicant exposure (Williams et al., 2017) can disrupt this homeostasis, resulting in microRNA- based altered immune responses. Indeed this was observed with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an aryl hydrocarbon receptor (AhR) agonist), which has been shown to alter microRNA expression in zebrafish embryos (Jenny et al., 2012), liver cells (Yoshioka et al., 2011), thymus (N. P. Singh et al., 2012), and T cells (Chinen et al., 2015). Certain commensal organism such as *Candidatus Savagella* (also called segmented filamentous bacteria, SFB, known to induce Th17 development; Ivanov et al., 2009) and *Bacteroides fragilis* (which induces T_{reg} through production of polysaccharide A; Round and Mazmanian, 2010) can counteract the effects of TCDD on the immune response by reversing the imbalance of Treg/Th17. Though Liu et al., 2016 found that SFB was more abundant in dicer knockout mice as compared to wild-type mice, the impact of SFB on host microRNA expression has not be investigated. As SFB is an important immune modulator in mice (Ivanov et al., 2009) and influences host gene expression (Stedtfeld et al., 2017), it is also likely that it impacts host microRNA expression (as microRNAs controls gene expression). Furthermore, though microRNA expression in T cells after AhR activation by TCDD has been studied (Chinen et al., 2015), the microRNA expression profiles specific to the ileum following TCDD exposure and the influence of gut commensals on this relationship is unknown.

In this study, C57BL6 germ-free mice were used to evaluate microbiota- host communication by determining i) microRNA expression responses after colonization with key gut commensals (SFB and *B. fragilis*) and ii) the ileal microRNA response to an environmental toxicant, TCDD, following oral exposure. MicroRNA expression profiles were compared to gene expression data to determine possible modes of gene regulation by microRNAs. This work

highlights the importance of gut microbiome – host communication through microRNAs following TCDD exposure. To our knowledge, it is the first study to assess differential ileal microRNA expression in response to both bacterial groups and an environmental toxicant.

7.2 Methods and Materials

7.2.1 Bacterial association of animal models

Germ-free mice (female C57BL/6) were maintained and bred at the Germ-Free Mouse Facility at the University of Michigan's Unit for Laboratory Animal Medicine (Ann Arbor, MI). At 4 to 6 weeks after birth, three groups of mice were orally inoculated with bacteria in groups i) Segmented filamentous bacteria (SFB), *Candidatus* Savagella, ii) *B. fragilis*, iii) co-culture of SFB and *B. fragilis*, and iv) germ-free (n = 8 per group). *B. fragilis* (DSM 2151) was grown in Brucella broth (AS-105, Anaerobe Systems, Morgan Hill, CA). *Candidatus* Savagella SFB-mouse-Japan was isolated as described (Kuwahara et al., 2011). *B. fragilis* colonized groups were also inoculated with additional gut species including *Faecalibacterium prausnitzii* DSM 17677, *Ruminococcus bromii* Strain VPI 6883, *R. obeum DSM 25238, Butyrivibrio fibrisolvens* DSM 3071, and *Eubacterium rectale* DSM 17629, but were not detectable in fecal pellets after association. To confirm inoculation in mice, qPCR was used, followed by confirmation with Sanger sequencing. For qPCR, reaction mixture (20 µl) included 18 µl master mix, 1 µl of 10 mM primer solution, and 1 µl of 1 ng DNA extracted from fecal pellets. Cycling included 95 °C for 5 min, 40 cycles of 95 °C for 55 sec, 60 °C for 55 sec and 72 °C for 1.5 min (Bouskra et al., 2008). Humane treatment of the mice was provided in compliance with an animal use protocol approved by the University of Michigan Animal Welfare Assurance (A3114-01).

7.2.2 Treatment of animal models with TCDD

Beginning 4 wk after inoculation, mice were dosed with 30 μg/kg TCDD (Dow Chemical Company, Midland, MI) by oral gavage with sesame oil (0.1 ml; Sigma-Aldrich, St. Louis, MO) used as vehicle and control. Groups were as follows: i) untreated (sesame oil vehicle only), un-colonized germ-free (n = 4), ii) TCDD-treated (with sesame oil vehicle) un-colonized germ-free (n = 4), iii) untreated (sesame oil vehicle only) with SFB mono-colonization (n = 3), iv) TCDD-treated (with sesame oil vehicle) with SFB mono-colonization (n = 4), v) untreated (sesame oil vehicle only) with *B. fragilis* mono-colonization (n = 4), vi) TCDD-treated (with sesame oil vehicle) with *B. fragilis* mono-colonization (n = 4), vii) untreated (sesame oil vehicle only) with co-colonization of both SFB and *B. fragilis* (n = 4), and viii) TCDD-treated (with sesame oil vehicle) with co-association of both groups (n = 3). Prior to completion of the study, two mice died, one mouse from the untreated (sesame oil vehicle only), SFB mono-colonization group, and one mouse from the TCDD-treated (with sesame oil vehicle) with co-association group.

7.2.3 Tissue collection and total RNA isolation including small RNAs

After humane euthanasia, mice were weighed and intestinal tissues were collected. Ileal segments <0.25 cm in length were cut and placed immediately in RNA stabilizer (DNA/RNA Shield; Zymo Research; Irvine, CA). Samples were then stored at -80 °C until RNA isolation. Small RNAs from the collected ileal tissues were extracted using miRNeasy Mini Kit (Qiagen; Germantown, MD) which uses a QIAzol-chloroform extraction protocol. Total RNA including small RNAs were further digested to removed residual DNA using DNAase 1 (Thermo Fisher Scientific; Waltham, MA) and Turbo DNAse I kit (Thermo Fisher Scientific; Waltham, MA). Total RNA was then quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific;

Waltham, MA) and the Qubit RNA BR Assay Kit (Thermo Fisher Scientific; Waltham, MA). Purity of the total RNA was assessed using a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop; Wilmington, DE). Handling of tissue was carried out under Michigan State University Environmental Health and Safety under Animal Use Form number 02/14-030-00.

7.2.4 MicroRNA expression analysis using nCounter

Total RNA including small RNAs at concentration 33 ng/µl was submitted for nCounter analysis. The data obtained was then processed using nSolver software v3.0 (http://www.nanostring.com/products/nSolver/) as recommended by the manufacturer. Counts from the nCounter system was normalized as recommended by the manufacturer. Briefly, raw counts were normalized to the geometric mean of the top 100 most abundant microRNAs, with those removed where coefficient of variation was >60%, effectively normalizing to the total microRNA counts. The background signal obtained with the negative controls (provided with the nCounter panel) was also subtracted from the total counts. Normalized counts were exported for further expression analysis.

7.2.5 Statistical Analysis

Significance for each microRNA for all comparisons was determined using a one- way ANOVA test to increase power as compared to a traditional t test. Once significance for all groups was established with ANOVA, multiple comparison tests were used to determine which pairwise comparison was significant. For determining the effect of bacterial association (associated vs. germ-free) on microRNA expression, each group was compared to a single control group (germ-free; vehicle control) so Dunnett's multiple comparison test (for comparing

means to a control mean) was used. For determining the effect of TCDD-treatment on microRNA expression, each treated group was compared to its vehicle group (e.g. germ-free and vehicle was compared with germ-free and TCDD-treated) so Sidak's multiple comparison test (for pre-selected mean comparisons) was used. Each multiple comparison test provided an adjusted p value. Fold change for each comparison was calculated by dividing average treated counts by average control counts. MicroRNAs were deemed significant if fold change >|1.5| and adjusted p value < 0.05. Standard deviation of fold change values in the expression data were calculated by dividing each replicate of the treatment group by the average of the control group. Standard deviations of the control group (shown in error bars) were similarly obtained by dividing each replicate in the control group by the mean of the same group.

7.2.6 Prediction of mRNA targets and functional pathway clusters

To identify potential gene function impacts of microRNA expression, mRNA targets were predicted using available databases that evaluated the target's 3'-UTR for microRNA binding sites. MicroRNA functions were identified using mirBase (http://www.mirbase.org; Kozomara and Griffiths-Jones, 2011) and potential gene targets of the significant microRNAs were determined using TargetScan Mouse v.7.1 (http://www.targetscan.org/mmu_61; Agarwal et al., 2015). Unions of gene categories were generated using DIANA miRPath v.3 (Vlachos et al., 2015) to establish global function cluster predictions for the microRNAs that were differentially expressed. The analysis was conducted using Gene Ontology (GO) analysis. To establish microRNA regulation of mRNA results obtained in Stedtfeld et al., 2017, mRNA targets of differentially expressed microRNAs were also identified using TargetScan Mouse v.7.1 and results were screened for significantly expressed mRNAs. Predicted pathways were identified

using DAVID functional annotation tool (https://david.ncifcrf.gov/summary.jsp) under analysis of "Gene Ontonology (GO) BP Direct".

7.3 Results

7.3.1 Expression of most abundant microRNAs is independent of intestinal colonization or dioxin treatment

To establish a characteristic microRNA expression profile of murine ileal tissues, we investigated microRNAs which were most abundant and where differential expression was not observed in all comparisons (TCDD vs. vehicle or colonized vs. germ-free). We determined the average number of counts for each microRNA and established the most abundant microRNAs out of all 569 microRNAs analyzed. Regardless of treatment and bacterial association conditions, the 38 most abundant microRNAs were conserved across all samples (Table A7.1). These microRNAs accounted for 84.2% of the total microRNA counts and no significant differences were determined in any of the comparisons. MiR-145 was the most abundant, accounting for 12.6% of the total counts, followed by miR-22 (4.2%), let-7b (4.1%), let-7g (3.9%), and let-7c (3.9%). When ANOVA analysis was conducted with raw counts instead of the normalized counts, 92% of these 38 microRNAs still did not have any differential expression in any of the comparisons.

7.3.2 Differential microRNA expression is dependent on the gut microbial community

Host microRNA expression is known to be altered between germ-free and specific pathogen-free mice (Dalmasso et al., 2011), but differential microRNA expression due to specific commensals such as segmented filamentous bacteria (SFB) and *Bacteroides fragilis* remains unclear. To investigate the effect SFB and *B. fragilis* on host microRNA expression,

ileal microRNA counts from C57BL6 germ-free (GF) mice (administered sesame oil as controls) were compared to the ileal microRNA counts of the SFB mono-associated (SFB), *B. fragilis* mono-associated (BF), and SFB and *B. fragilis* co-associated groups (SFB + B). Of the 600 microRNAs analyzed in this study, 36 were differentially expressed ($log_2FC > |0.59|$, adjusted p value < 0.05) in at least one of the comparisons (either SFB v. GF, SFB + BF v. GF, or BF v. GF; Table A7.2). In general, the influence of association resulted in significantly more upregulation (FC = Association/GF; n=31) than downregulation (n=5). Of each group, the influence of SFB mono-association was most significant, accounting for 24 of the 36 significantly expressed microRNAs (Figure 7.1). A total of 3 microRNAs were differentially expressed when comparing *B. fragilis* colonized to germ-free mice and 9 microRNAs were significant when comparing the SFB + *B. fragilis* co-colonized group to GF. Though most differentially expressed microRNAs were specific to each group, one (miR-511) was differentially expressed when comparing both *B. fragilis* (B) and SFB co-colonized (SFB + B) groups to germ-free (GF) where it was downregulated (Table A7.2; $log_2FC = -1.39$ for BF and $log_2FC = -1.48$ for SFB +B). MicroRNAs with the largest change in expression included miR-1952 ($log_2FC = 2.29$, adjusted p = 0.0270), miR-2136 ($log_2FC = 3.01$, adjusted p = 0.0005), miR-466i ($log_2FC = 2.12$, adjusted p = 0.0210), miR-468 ($log_2FC = 2.20$, adjusted p = 0.0196), miR-471 ($log_2FC = 2.25$, adjusted p = 0.0027), miR-499 ($log_2FC = 2.07$, adjusted p = 0.0312), miR-329 ($log_2FC = 3.18$, adjusted p = 0.0165), and miR-3474 ($log_2FC = 2.03$, adjusted p = 0.0247).

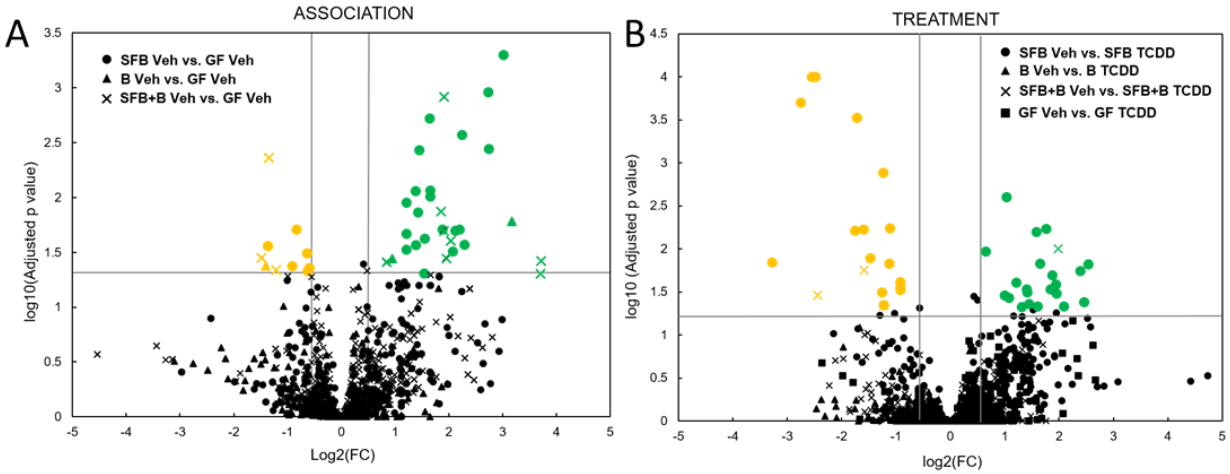

Figure 7.1: Differential expression of microRNAs in response to A. bacterial association of B, SFB, and SFB + B as compared to GF and B. TCDD treatment for each group (SFB, SFB+ B) as compared to vehicle controls

7.3.3 Differential expression of microRNAs in response to TCDD within bacterial groups

To determine the effect of TCDD on microRNA expression, TCDD-treated mice were compared to controls within each bacterial group (SFB, SFB + BF, BF, and GF; Table A7.3). We observed that microRNA expression modulation due to dioxin was highly dependent on gut community association, as no differentially expressed microRNAs were observed in TCDD-treated vs. vehicle controls in the GF group (Figures 7.1B, 7.1C). The most differentially expressed microRNAs were in the SFB mono-colonized group (n=27), the majority of which were downregulated (n=16; Figure 7.2A Right), contrary to the results obtained investigating the effect of SFB alone (n=4 downregulated; Figure 7.2A Left). The total differentially expressed microRNAs was significantly smaller in the SFB co-colonized group with only three microRNAs, all of which were downregulated. There was no significant difference between TCDD-treatment and vehicle controls in the *B. fragilis* mono-associated group. Those microRNAs with the largest change in expression included miR-188-3p (log_2FC = -2.75,

adjusted p = 0.0002), miR-2132 (log_2FC = -2.54, adjusted p = 0.0001), miR-2136 (log_2FC = -2.48, adjusted p = 0.0001), miR-668 (log_2FC = -2.44, adjusted p = 0.035), and miR-466c-5p (log_2FC = 2.39, adjusted p = 0.018) are shown in Figure 7.2B. On its own, TCDD did not elicit a differential microRNA expression response in germ-free mice.

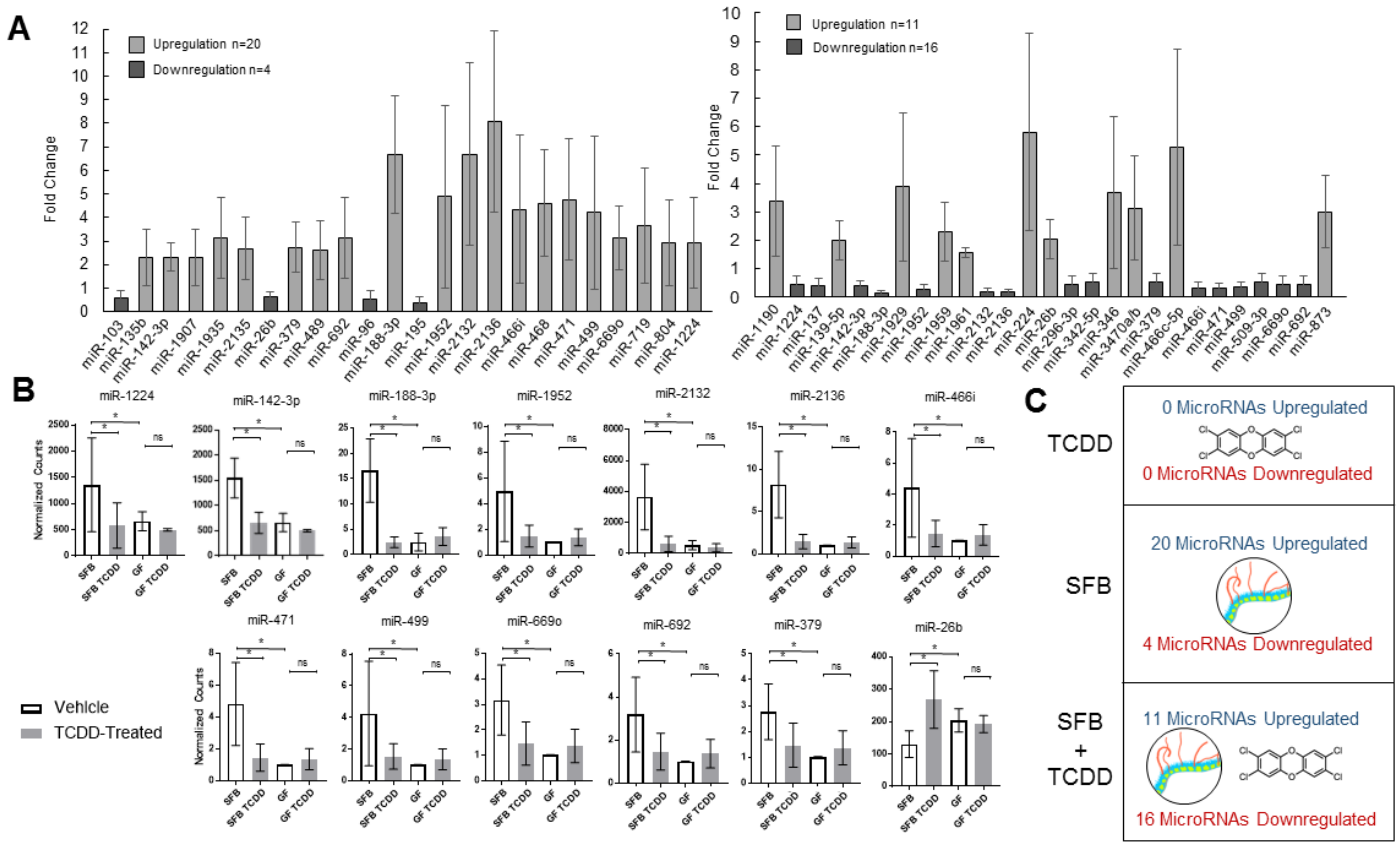

Figure 7.2: A. Fold change values for differentially expressed microRNAs in response to SFB mono-colonization alone (SFB v. GF; Left) and SFB mono-colonization with TCDD treatment (SFB: TCDD v. vehicle; Right). B. Normalized counts for microRNAs that exhibited significant differential expression for both SFB mono-colonization alone (SFB v. GF) and SFB mono-colonization with TCDD treatment (SFB: TCDD v. vehicle). Statistically significant comparisons are noted with an asterisk (* denotes adjusted p values <0.05; ** denotes adjusted p value < 0.01; *** denotes adjusted p value < 0.005). **C.** Visualization of the total differentially expressed microRNAs for TCDD treatment alone (GF: TCDD v. vehicle), SFB mono-colonization (SFB v. GF), and SFB mono-colonization with TCDD treatment (SFB: TCDD v. vehicle). As more microRNAs are upregulated for SFB mono-colonization alone while more are downregulated for SFB mono-colonization with TCDD-treatment, it would follow that TCDD treatment alone (GF: TCDD v. vehicle) would elicit in more downregulated microRNAs. This however is not the case as TCDD treatment alone has not effect on microRNA expression, suggesting the effects of SFB and SFB + TCDD treatment are not additive.

7.3.4 TCDD and bacterial modulation of host gene expression potentially regulated by microRNAs

To determine the regulatory role of microRNAs on host gene expression regulation, the list of differentially expressed microRNAs were compared to data from the same mouse experiment where nCounter was used to test a panel of 600 immunology genes (expression data published in Stedtfeld et al., 2017). Potential targets of microRNAs were identified and the differentially expressed microRNAs were compared to differentially expressed mRNAs in response to i) bacterial association alone and ii) bacterial association and TCDD exposure.

Potential gene targets of the bacterial association-induced differentially expressed microRNAs (n=32) were evaluated from 88 differentially expressed mRNAs reported in Stedtfeld et al., 2017. Most differentially expressed mRNAs were potential targets of at least one of the differentially expressed microRNAs, except for *Ccbp2, Cfi, Cxcr6, Gpr44, Gzma, H2-Aa, H2-Ea-ps, H2-K1, Il1b, Jak3, Klrb1, Klrd1, Lck, Nos2, Pla2g2a, Psmb9, Psmc2,* and *Tnfrsf17* which were targeted by none of the microRNAs. Three differentially expressed mRNAs were targeted by ten or more microRNAs, including *Fkbp5*, *Ikzf2*, and *Il7*. A total of 27 differentially expressed genes were targeted by between 5 and 9 differentially expressed microRNAs. Conversely, 6 differentially expressed microRNAs did not target any of the differentially expressed mRNAs (miR-103-3p, miR-489-3p, miR-96-5p, miR-499-5p, miR-1224-5p, and miR-379-5p). A total of 5 differentially expressed microRNAs had greater than 25 potential mRNA targets (miR-1190, miR-188-3p, miR-3474, miR-466i-3p, and miR-692).

Potential gene targets of the TCDD-induced differentially expressed microRNAs (n=28) were identified and compared to the 22 differentially expressed mRNAs reported in Stedtfeld et al., 2017. Most genes were potential targets of at least one microRNA, with the exception of

Gpr44, *H2-Ea-ps*, *Jak3*, and *Klra6*. Three genes (*Cd3d*, *Il10*, and *Il7*) were potential targets of 8 microRNAs, the highest number of the set. Conversely, 10 differentially expressed microRNAs did not target any of the mRNAs (miR-1224-5p, miR-137-3p, miR-296-3p, miR-379-5p, miR-346-5p, miR-668-3p, miR-873a-5p, miR-466c-5p, miR-499-5p, and miR-224-5p). A total of three microRNAs targeted higher than 8 mRNAs (miR-466i-3p, miR-692, and miR-188-3p).

To investigate potential regulation of microRNAs on the immune response specific to the AhR and dioxin exposure, genes related to Treg and Th17 were identified. Of those differentially expressed mRNAs (in response to TCDD for all groups), *Ciita*, *H2-Ea-ps*, and *Il1b* genes have been linked to Th17 differentiation and *Il10* has been linked to Treg. Differentially expressed microRNAs for which *Ciita* is a potential target include miR-509-3p (downregulation), miR-692 (downregulation), miR-1190 (upregulation), miR-188-3p (downregulation), miR-2136 (downregulation), miR-3470a (upregulation), miR-3470b (upregulation), and miR-142-3p (downregulation). All microRNAs targeting *Ciita* were differentially expressed only in the SFB mono-associated group. Only one differentially expressed microRNA targeted *Il1b* (mmu-miR-692; downregulated in the SFB mono-associated group). None of the differentially expressed microRNAs targeted *H2-Ea-ps*. A total of 8 differentially expressed microRNAs had *Il10* for a potential target including miR-105 (downregulation in the SFB co-associated with *B. fragilis* group), miR-1952 (downregulation in SFB mono-associated group), miR-1961 (upregulation in SFB mono-associated group), miR-669o-5p (downregulation in SFB mono-associated group), miR-692 (downregulation in SFB mono-associated group), miR-1190 (upregulation in SFB mono-associated group), miR-188-3p (downregulation in SFB mono-associated group), and miR-3470a (upregulation in SFB mono-associated group).

To obtain insight into potential biological functions impacted by differential microRNA expression, gene categories unions were established using DIANA miRPath to obtain functional gene categories (Figure 7.3). This model uses all potential mRNA targets of each microRNA and clusters them in functional groups. Though more microRNAs were differentially expressed upon bacterial association, the function categories affected by either SFB mono-association (Figure 7.3A) or TCDD-treatment following SFB mono-association (Figure 7.3B) were the same. Affected categories were crucial Gene Ontology (GO) biological processes (BP) including cell, intracellular, cellular components, biological processes, molecular functions, organelles, anatomical structure development, and cellular differentiation.

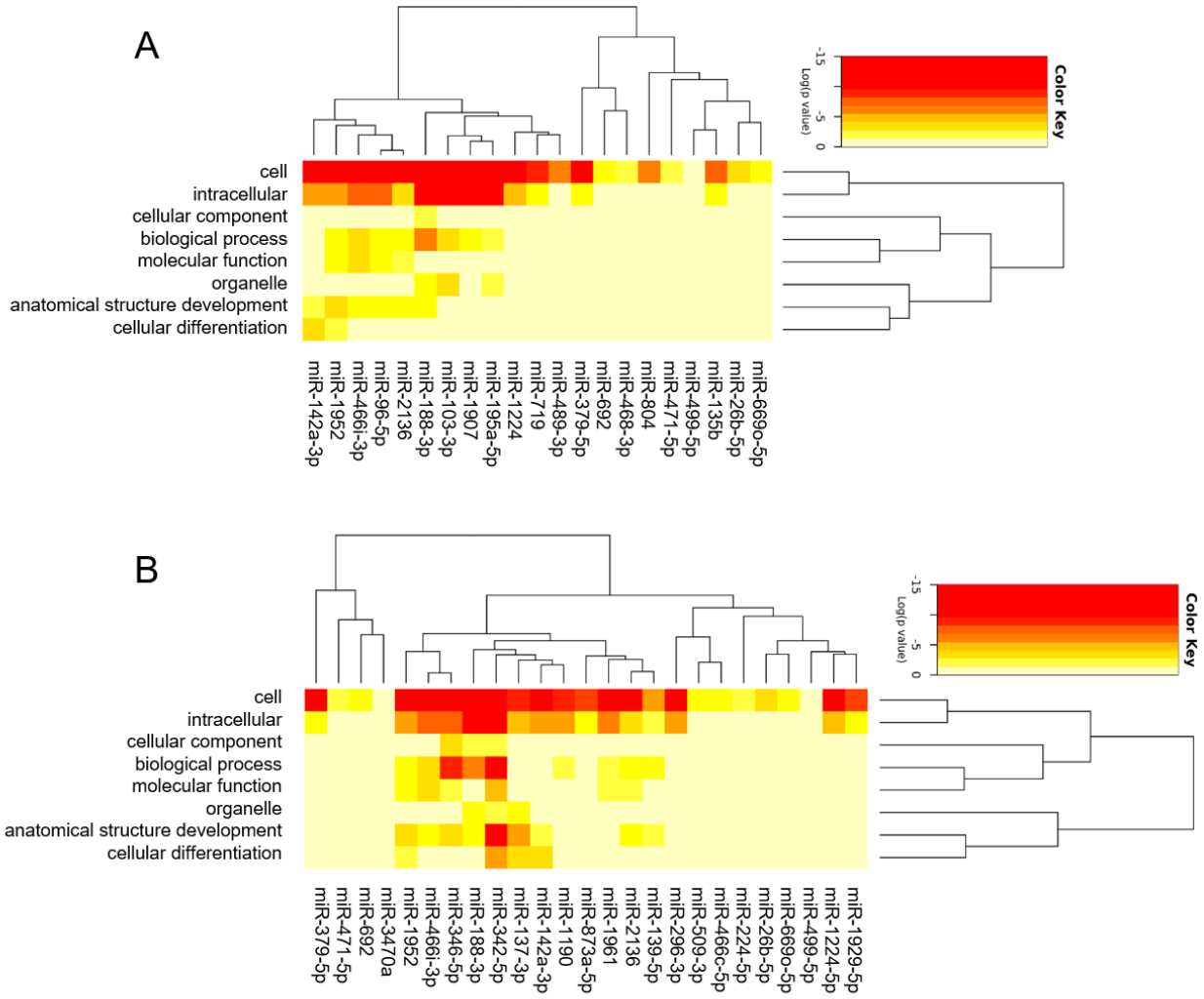

Figure 7.3: Theoretical GO BP (Gene Ontology Biological Process) clusters regulated by differentially expressed microRNAs results with union of gene categories impacted by A. SFB mono-associated and B. SFB mono-associated treated with TCDD. Clusters obtained using DIANA miRPath v3.

To evaluate functional pathways, microRNAs that target mRNAs differentially expressed in Stedtfeld et al., 2017 were evaluated. Differentially expressed mRNAs were grouped into two functional clusters including inflammatory response and positive regulation of transcription from RNA polymerase II promoter (Stedtfeld et al., 2017; Figure 7.4). Of all the differentially expressed microRNAs responding to TCDD (n=30), sixteen were identified as targeting genes in those clusters (Figure 7.2). While some mRNAs responded to TCDD in the germ-free group, no microRNAs were differentially expressed in this group, suggesting differential microRNA expression may be dependent on the presence of bacterial groups.

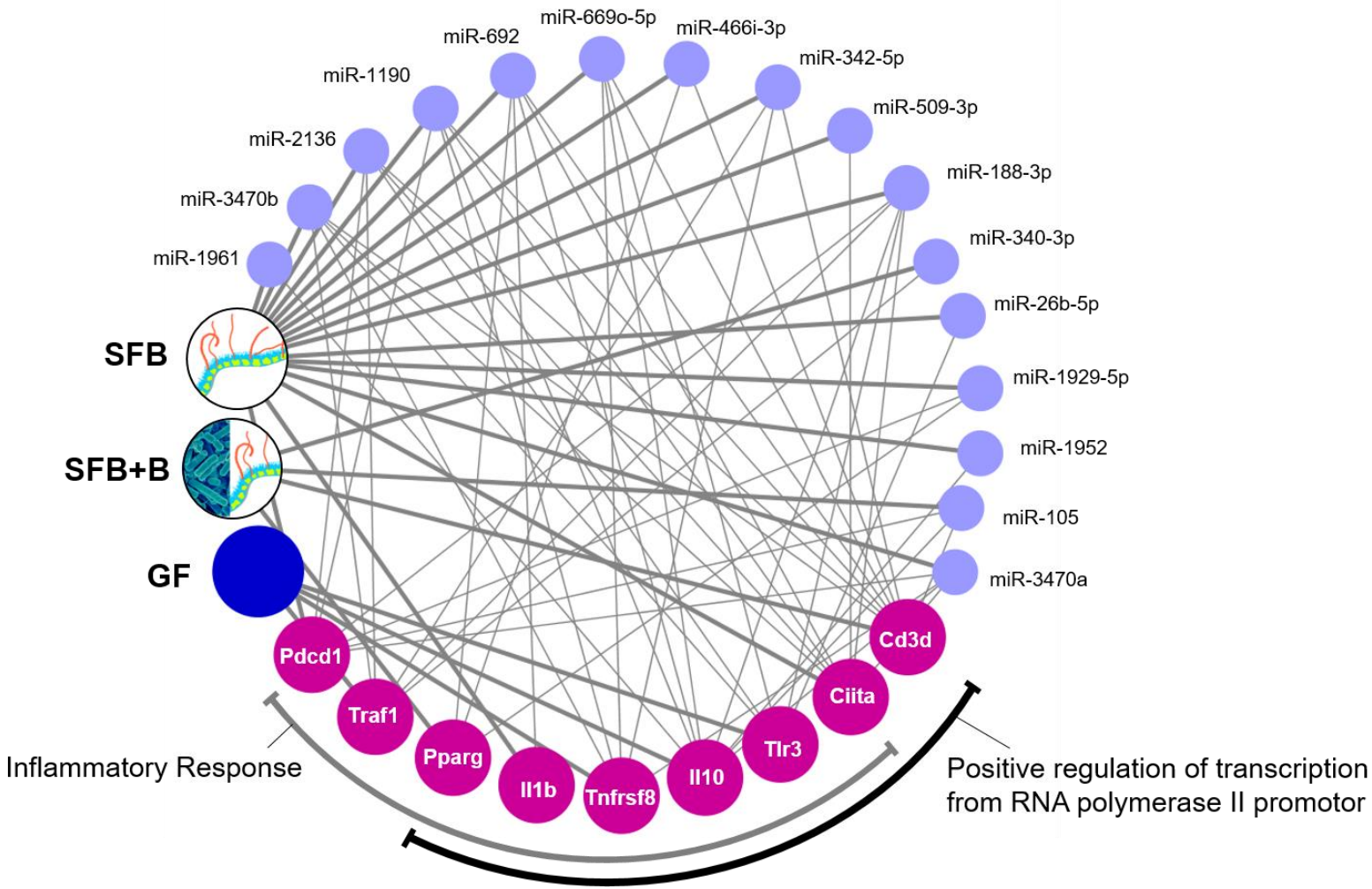

Figure 7.4: MicroRNA regulation possibilities for the gene expression pathways affected by TCDD treatment. The impact of this regulation by each association group is connected. Includes microRNAs that are differentially expressed between TCDD-treatment and vehicle controls in the SFB mono-association group, SFB co-colonized group, and germ-free group. No microRNAs were differentially expressed in the germ-free group but some mRNAs were. Neither microRNAs nor mRNAs were differentially expressed in the *B. fragilis* group.

7.4 Discussion

In this study, differential murine ileal microRNA expression was measured in response to i) bacterial association with SFB and *B. fragilis* and ii) treatment with TCDD.

7.4.1 Presence of a characteristic murine ileal signature could be used in data normalization

Overall, the top 38 most abundant microRNAs in all samples were not differentially expressed in any comparison. This suggests that the most abundant microRNAs could make up a characteristic murine ileal signature which could be used for assay normalization and controls. Indeed, the presence of a dominant "core" microRNA set has been observed in ileal samples of conventional mice as compared to germ-free mice colonized by *Listeria monocytogenes* (Archambaud et al., 2013). There, the top ten microRNAs were miR-215, miR-143, miR-192, miR-21, miR-378, miR-200c, miR-194, let-7b, miR-30a/d, and miR-200b. While miR-145 was not observed in that study in the top ten, it has been shown to be dominantly expressed in the murine ileum (Archambaud et al., 2013). Similarly, though miR-143 was not the most dominantly expressed microRNA in our study, we did observe that it was present in high abundances (in the top 12 of all microRNAs expressed).

7.4.2 Response of microRNA expression to bacterial association

MicroRNA expression measured in the ileum was impacted by both TCDD and bacterial association potentially modulating genes related to specific functions (including inflammatory and immune responses). Overall, bacterial association resulted in more differentially expressed microRNAs than TCDD treatment, suggesting the importance of members of the gut microbiome

on host microRNA expression. The influence of specific bacterial groups on gut microRNA expression has also been shown in many studies (e.g., *L. monocytogenes* (Izar et al., 2012), *S. typhimurium* (Schulte et al., 2011), and *H. pylori* (Fassi Fehri et al., 2010)) though all are pathogens.

7.4.3 Response of microRNA expression to TCDD-treatment is dependent on bacterial association

TCDD alone also did not elicit a differential microRNA expression response in germ-free mice. Indeed, little to no changes in microRNA expression as a result of TCDD exposure have been observed in other murine tissues, such as liver (Moffat et al., 2007), though conflicting results have been reported in other studies (Yoshioka et al., 2011). In this study, differential expression of microRNAs between TCDD-treated and vehicle controls were seen, however, when mice were associated with SFB or *B. fragilis*. One would expect that if TCDD had no effect of microRNA responses, no differential expression would be observed, regardless of whether or not bacteria are present. This suggests the changes in microRNA expression among the treated and associated groups are not additive and instead of SFB and dioxin affecting microRNA expression distinctly and separately, either i) dioxin is affecting how SFB interact with the host microRNAs or ii) SFBs are changing the host's microRNA response to dioxin in some way. Unfortunately, existing studies relating the gut microbiome, microRNA expression, and environmental exposure do not exist. The closest studies investigating these interactions are limited to two of the three (e.g., gut microbiome and microRNA expression or microRNA expression and environmental exposure). These relationships are particularly important as

dysbiosis of the gut microbiome in response to environmental exposure has been connected to gut diseases (Benjamin et al., 2012).

Claus and coauthors also characterizes potential interactions between the gut microbiome and environmental exposure to chemicals as one of four mechanisms: i) direct metabolism of chemicals, ii) direct metabolism of chemicals following conjugation in the liver, iii) interfering with enzymatic activity, or iv) induction of dysbiosis (Claus et al., 2016). As TCDD exposure can result in dysbiosis of the gut microbiome (Neamah et al., 2017), it is likely this may be the primary mechanism. This was indeed observed reported earlier by our group (Stedtfeld et al., 2017) where differences in SFB abundances following TCDD exposure were observed. At this time, it is unclear whether SFB metabolizes TCDD, either directly or after conjugation in the liver, or if TCDD is affecting the physiological activity of SFB in addition to causing dysbiosis.

7.4.4 TCDD and bacterial modulation of functional gene clusters potentially regulated by microRNAs.

Furthermore, this differential microRNA expression was connected to differential mRNA expression as reported earlier by our group (Stedtfeld et al., 2017) to gain insight into regulation of potential gene pathways by microRNAs. When grouped into functional clusters, TCDD treatment combined with SFB association impacted the inflammatory response and positive regulation of transcription from RNA polymerase II promoter. The RNA polymerase II promotion is an important regulator of gene expression. The impact of TCDD treatment on the inflammatory response cluster is not altogether unexpected as it is known that TCDD impacts the host immune response through the Treg/Th17 balance (Fantini et al., 2007; Peck and Mellins, 2010) through the aryl hydrocarbon receptor (Marshall and Kerkvliet, 2010). Individual genes

that are possibly targeted by microRNAs include *Pparg* (upregulation associated with the anti-inflammatory response; Youssef and Badr, 2004), *Il10* (which is shown to regulate immunity to infection; Couper et al., 2008), and *Ciita* (an important regulator of the innate immune response and has been suggested may be targeted by pathogens; (Accolla et al., 2001).

Though it is known that host - microbiome communication occurs, the mechanisms are largely unknown. Some researchers suggest microRNAs-based communication could occur through Myd88-dependent pathways, which can recognize bacterial-related metabolites and initiate microRNA responses (Larsson et al., 2012; Xue et al., 2011). Others suggest that the host could be regulating its gut microbiome by releasing microRNAs, which can be taken up by bacteria and alter their growth (Liu et al., 2016). The increased expression of most the differentially expressed microRNAs in response to SFB observed here suggests potential communication between the host and its microbiome. In fact, most differentially expressed microRNAs responded to SFB alone, supporting the expected role of SFB as an important modulator of the host gene expression. This is the first report studying the microRNA response to SFB in the presence of TCDD. Future studies should investigate microRNA expression in response to other members of the gut microbiome and evaluate their role in disease and homeostasis.

APPENDIX

APPENDIX

Table A7.1: Expression of the 38 most abundant microRNAs per associated group, presented as a percentage of the total counts per group

SFB mono-association			*Bacteriodes fragilis* mono-association			SFB + *B. fragilis* co-association			Germ-free		
MicroRNA	Average Counts	% of Total Counts	MicroRNA	Average Counts	% of Total Counts	MicroRNA	Average Counts	% of Total Counts	MicroRNA	Average Counts	% of Total Counts
mmu-miR-145	16747.4	11.18	mmu-miR-145	16965.0	11.32	mmu-miR-145	19586.9	13.07	mmu-miR-145	20801.1	13.88
mmu-miR-22	7065.2	4.71	mmu-miR-22	6859.1	4.58	mmu-let-7c	7124.5	4.75	mmu-let-7c	6442.2	4.30
mmu-let-7b	5929.5	3.96	mmu-let-7b	5204.8	3.47	mmu-let-7b	7083.7	4.73	mmu-let-7b	6201.9	4.14
mmu-let-7g	5838.8	3.90	mmu-let-7g	6283.0	4.19	mmu-let-7g	5603.8	3.74	mmu-miR-200b	5690.0	3.80
mmu-miR-200b	5507.5	3.68	mmu-let-7c	4721.9	3.15	mmu-miR-200b	5430.9	3.62	mmu-miR-194	5649.8	3.77
mmu-miR-16	5096.5	3.40	mmu-miR-200b	6150.7	4.10	mmu-miR-22	5084.6	3.39	mmu-miR-22	5577.9	3.72
mmu-miR-194	4824.5	3.22	mmu-miR-194	6943.9	4.63	mmu-miR-1944	4979.0	3.32	mmu-let-7g	5447.6	3.64
mmu-let-7c	4758.2	3.18	mmu-miR-16	5612.7	3.75	mmu-miR-429	4727.2	3.15	mmu-miR-429	4698.0	3.14
mmu-miR-429	4546.2	3.03	mmu-miR-429	4996.9	3.33	mmu-miR-143	4457.1	2.97	mmu-miR-16	4557.7	3.04

Table A7.1 (cont'd)

mmu-miR-126-3p	4402.8	2.94	mmu-miR-1944	3463.6	2.31	mmu-miR-126-3p	4370.4	2.92	mmu-miR-143	4344.9	2.90
mmu-miR-143	3918.6	2.61	mmu-miR-126-3p	4070.2	2.72	mmu-miR-194	4338.2	2.89	mmu-miR-1944	4124.0	2.75
mmu-miR-200c	7.23863.3	2.58	mmu-miR-143	3586.4	2.39	mmu-miR-16	4164.5	2.78	mmu-miR-192	3650.9	2.44
mmu-miR-1944	3852.1	2.57	mmu-miR-200c	3543.1	2.36	mmu-let-7a	3698.4	2.47	mmu-miR-200c	3551.3	2.37
mmu-miR-21	3535.1	2.36	mmu-let-7d	3610.6	2.41	mmu-let-7i	3593.6	2.40	mmu-miR-126-3p	3522.7	2.35
mmu-let-7d	3397.7	2.27	mmu-miR-192	3844.7	2.57	mmu-let-7d	3529.8	2.36	mmu-miR-21	3229.5	2.16
mmu-let-7i	3313.9	2.21	mmu-miR-21	3468.7	2.31	mmu-miR-200c	3337.1	2.23	mmu-let-7d	3219.8	2.15
mmu-miR-720	2810.8	1.88	mmu-let-7i	2976.1	1.99	mmu-miR-29a	2935.7	1.96	mmu-miR-200a	2932.2	1.96
mmu-miR-192	2769.1	1.85	mmu-let-7a	2578.2	1.72	mmu-miR-720	2786.9	1.86	mmu-miR-29a	2926.5	1.95
mmu-miR-29a	2649.5	1.77	mmu-miR-200a	3208.6	2.14	mmu-miR-21	2615.7	1.75	mmu-let-7a	2865.1	1.91
mmu-miR-200a	2576.5	1.72	mmu-miR-29a	2531.8	1.69	mmu-miR-192	2594.2	1.73	mmu-let-7i	2863.8	1.91
mmu-let-7a	2261.0	1.51	mmu-miR-720	1413.6	0.94	mmu-miR-200a	2549.9	1.70	mmu-miR-23a	1587.4	1.06

Table A7.1 (cont'd)

mmu-miR-20a+mmu-miR-20b	2202.5	1.47	mmu-miR-20a+20b	2223.9	1.48	mmu-let-7f	1985.2	1.32	mmu-let-7f	1562.8	1.04
mmu-miR-25	1960.2	1.31	mmu-miR-25	1641.0	1.10	mmu-miR-20a+mmu-miR-20b	1712.2	1.14	mmu-miR-20a+mmu-miR-20b	1501.6	1.00
mmu-miR-27a	1790.6	1.19	mmu-miR-23a	1680.2	1.12	mmu-miR-25	1610.4	1.07	mmu-miR-25	1475.6	0.98
mmu-miR-23a	1751.2	1.17	mmu-let-7f	1595.1	1.06	mmu-miR-23a	1522.1	1.02	mmu-miR-10a	1431.1	0.95
mmu-miR-15a	1522.8	1.02	mmu-miR-27a	1470.3	0.98	mmu-miR-27a	1463.9	0.98	mmu-miR-27a	1383.4	0.92
mmu-miR-181a	1293.7	0.86	mmu-miR-15a	1663.0	1.11	mmu-miR-2146	1267.0	0.85	mmu-miR-2146	1342.2	0.90
mmu-let-7f	1249.6	0.83	mmu-miR-10a	1411.9	0.94	mmu-miR-375	1262.4	0.84	mmu-miR-15a	1270.4	0.85
mmu-miR-106a+mmu-miR-17	1240.7	0.83	mmu-miR-106a+mmu-miR-17	1397.3	0.93	mmu-miR-181a	1163.0	0.78	mmu-miR-720	1207.6	0.81
mmu-miR-30c	1222.4	0.82	mmu-miR-181a	1136.1	0.76	mmu-miR-125b-5p	1137.6	0.76	mmu-miR-181a	1147.0	0.77
mmu-miR-148a	1215.4	0.81	mmu-miR-2146	742.3	0.50	mmu-miR-106a+ 17	1108.0	0.74	mmu-miR-125b-5p	1089.3	0.73

Table A7.1 (cont'd)

mmu-miR-125b-5p	1126.2	0.75	mmu-miR-125b-5p	924.3	0.62	mmu-miR-15a	1085.5	0.72	mmu-miR-106a+mmu-miR-17	1068.7	0.71
mmu-miR-199a-3p	1076.8	0.72	mmu-miR-30c	1069.7	0.71	mmu-miR-10a	1002.6	0.67	mmu-miR-29c	996.8	0.67
mmu-miR-378	1075.9	0.72	mmu-miR-375	810.8	0.54	mmu-miR-199a-3p	929.0	0.62	mmu-miR-378	980.9	0.65
mmu-miR-10a	1066.4	0.71	mmu-miR-199a-3p	1087.6	0.73	mmu-miR-378	833.3	0.56	mmu-miR-375	973.2	0.65
mmu-miR-2146	1062.4	0.71	mmu-miR-29c	949.8	0.63	mmu-miR-29c	814.4	0.54	mmu-miR-30c	973.1	0.65
mmu-miR-29c	985.7	0.66	mmu-miR-378	839.0	0.56	mmu-miR-148a	801.4	0.53	mmu-miR-199a-3p	871.1	0.58
mmu-miR-375	973.0	0.65	mmu-miR-148a	930.1	0.62	mmu-miR-30c	787.8	0.53	mmu-miR-148a	720.7	0.48

Table A7.2: Differentially expressed microRNAs in response to TCDD as compared with vehicle controls by bacterial association.

Comparison that is significant (TCDD v. Vehicle)	MicroRNA	Adjusted P Value*	Fold Change (FC)	Log_2FC
SFB mono-colonized	mmu-miR-1190	0.0059	3.385	1.76
	mmu-miR-1224	0.0451	0.429	-1.22
	mmu-miR-137	0.0323	0.420	-1.25
	mmu-miR-139-5p	0.0347	2.007	1.00
	mmu-miR-142-3p	0.0013	0.425	-1.24
	mmu-miR-188-3p	0.0002	0.149	-2.75
	mmu-miR-1929	0.0329	3.876	1.95
	mmu-miR-1952	0.0062	0.296	-1.76
	mmu-miR-1959	0.0246	2.313	1.21
	mmu-miR-1961	0.0108	1.571	0.65
	mmu-miR-2132	0.0001	0.171	-2.54
	mmu-miR-2136	0.0001	0.179	-2.48
	mmu-miR-224	0.0152	5.807	2.54
	mmu-miR-26b	0.0025	2.052	1.04
	mmu-miR-296-3p	0.0149	0.460	-1.12
	mmu-miR-342-5p	0.0282	0.531	-0.91
	mmu-miR-346	0.0203	3.683	1.88
	mmu-miR-3470a+ mmu-miR-3470b	0.0149	3.139	1.65
	mmu-miR-379	0.0300	0.531	-0.91
	mmu-miR-466c-5p	0.0182	5.258	2.39
	mmu-miR-466i	0.0060	0.332	-1.59
	mmu-miR-471	0.0003	0.304	-1.72
	mmu-miR-499	0.0129	0.362	-1.46
	mmu-miR-509-3p	0.0244	0.531	-0.91
	mmu-miR-669o	0.0058	0.462	-1.12
	mmu-miR-692	0.0148	0.460	-1.12
	mmu-miR-873	0.0064	3.007	1.59
SFB co-colonized (SFB + B)	mmu-miR-105	0.0459	0.427	-1.23
	mmu-miR-340-3p	0.0177	0.333	-1.59
	mmu-miR-668	0.0350	0.185	-2.44

*Generated with Sidak's multiple comparison test

Table A7.3: Differentially expressed microRNAs in response to bacterial association as compared with germ-free controls.

Comparison that is significant (Group vs. germ-free)	MicroRNA	Adjusted P Value*	Fold Change	Log_2FC
SFB mono-colonized	mmu-miR-103	0.0197	0.56	-0.83
	mmu-miR-135b	0.0214	2.32	1.21
	mmu-miR-142-3p	0.0112	2.32	1.22
	mmu-miR-1907	0.0214	2.32	1.21
	mmu-miR-1935	0.0098	3.14	1.65
	mmu-miR-2135	0.0137	2.68	1.42
	mmu-miR-26b	0.0325	0.64	-0.64
	mmu-miR-379	0.0037	2.73	1.45
	mmu-miR-489	0.0087	2.61	1.39
	mmu-miR-692	0.0086	3.14	1.65
	mmu-miR-96	0.0425	0.53	-0.91
	mmu-miR-188-3p	0.0011	6.66	2.73
	mmu-miR-195	0.0276	0.39	-1.36
	mmu-miR-1952	0.0270	4.89	2.29
	mmu-miR-2132	0.0036	6.69	2.74
	mmu-miR-2136	0.0005	8.08	3.01
	mmu-miR-466i	0.0201	4.36	2.12
	mmu-miR-468	0.0196	4.60	2.20
	mmu-miR-471	0.0027	4.76	2.25
	mmu-miR-499	0.0312	4.20	2.07
	mmu-miR-669o	0.0019	3.13	1.65
	mmu-miR-719	0.0195	3.67	1.88
	mmu-miR-804	0.0239	2.93	1.55
	mmu-miR-1224	0.0495	2.91	1.54
B. fragilis colonized	mmu-miR-329	0.0165	9.06	3.18
	mmu-miR-363	0.0362	1.94	0.96
SFB co-colonized (SFB + B)	mmu-miR-511	0.0352	0.36	-1.48
		0.0421	0.38	-1.40
	mmu-miR-105	0.0201	3.74	1.90
	mmu-miR-1190	0.0357	3.85	1.95
	mmu-miR-19b	0.0043	0.39	-1.35
	mmu-miR-340-3p	0.0134	3.60	1.85
	mmu-miR-3474	0.0247	4.09	2.03
	mmu-miR-668	0.0012	3.76	1.91
	mmu-miR-1937a+ mmu-miR-1937b	0.0375	13.09	3.71
	mmu-miR-676	0.0389	1.78	0.83

*Generated with Dunnett's multiple comparison test

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CHAPTER VIII

Conclusions and Future Perspective

The use of nucleic acid-based approaches to detect and quantify molecular targets is important for many applications relevant to the environment and human health. The goal of the work presented in this dissertation was to assess the use of simpler and higher-throughput molecular approaches to enhance these techniques, thus allowing the investigation of complex biological questions.

The use of direct isothermal amplification was evaluated for detection of environmental DNA from aquatic invasive species (validated with *Dreissena* sp.) to serve as an early warning system for new infestations. Though the use of amplification has been used for detection of AIS previously, it typically requires centralized laboratories and time-consuming sample processing. The employment of LAMP and direct amplification is novel and eliminates the need for the centralized laboratory facility, allowing detection in the field in under 1 hr. Future research in this area could focus on the development and validation of assays for other AIS as well as enhancing the limit of detection to create a more comprehensive screening program.

The use of direct amplification in point-of-care devices for screening antimicrobial resistance is also reviewed. As the development of AMR is becoming a global crisis, the development of techniques that help make screening simpler and more rapid at the point-of-care could be beneficial. Typically used methods require time for sample transport, processing, and DNA purification and concentration. The use of POC devices in combination with direct amplification could be more capable of rapidly diagnosing antibiotic-resistant infections to help in making timely and correct treatment decisions.

High-throughput qPCR was used to assess 384 ARG from surface water samples throughout Michigan, primary influent from three waste water treatment facilities, and ten clinical isolates from a regional hospital. In addition, a smartphone application was developed to compile environmental characterization results across many studies. The use of this comprehensive database that differentiates the natural resistome from anthropogenic distribution of AMR in the environment would identify where changes are likely to be most effective for containment. Future research should focus on populating the application with updated studies.

A novel microRNA amplification approach was developed that allows direct amplification of targets from clinical matrices. By utilizing base-stacking interactions, the microRNA of interest acts as a "key" to initiate the amplification reaction, allowing for one-step detection of microRNAs. Employing this approach could allow for rapid diagnostics based on differential microRNA expression to diagnose many diseases and cancers at the point-of-care. Future research that focuses on enhancing the limit of detection would be beneficial as some microRNAs are only present in concentrations as low as a few copies per µl of blood or serum.

The role of microRNAs in communication with the gut microbiome and their implications in gut health is reviewed, as is its potential for perturbation by environmental toxicants. While in Chapter VII, high-throughput detection of microRNAs was used to assess the differential expression of ileal microRNAs in response to an environmental toxicant (TCDD) and the gut microbiome. Differential expression of microRNAs was dependent on the presence of certain members of the gut microbiome (segmented filamentous bacteria and *Bacteroides fragilis*) as microRNAs from the germ-free group were not differentially expressed between the treated and non-treated samples. Overall, this represents the first study to assess the impact of TCDD on ileal microRNA expression in response to members of the gut microbiome. As gut

health is significantly impacted by many members of the gut microbiome and complex communities, future research should focus on performing similar investigations with traditional mice. Furthermore, as evidence for SFB is limited in humans, evaluating these results with humanized models is important.