This is to certify that the

thesis entitled

Isolation of Salmonella and Escherichia Coli in Feces of Cull (Market) Dairy Cows at Slaughter

presented by

Ozlem Akpinar

has been accepted towards fulfillment
of the requirements for

M.S. degree in LCS

Major professor

Date 12-12-01

ISOLATION OF *SALMONELLA* AND *ESCHERICHIA COLI* IN FECES OF CULL (MARKET) DAIRY COWS AT SLAUGHTER

By

Özlem Akpinar

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Large Animal Clinical Sciences

2001

ABSTRACT

ISOLATION OF *SALMONELLA* AND *ESCHERICHIA COLI* IN FECES OF CULL (MARKET) DAIRY COWS AT SLAUGHTER

By

Özlem Akpinar

Objectives of this study were to evaluate the prevalence and concentration of *Salmonella* and EHEC (Enterohemorrhagic *E. coli*) in feces of dairy cows at slaughter, and to evaluate the effects of season on the isolation of pathogens from dairy cows at slaughter. Samples were collected at slaughter from cattle that had either been shipped directly or indirectly to slaughter. Fecal samples from 1006 cows were collected in winter and summer of 1996. *Salmonella* and *E. coli* isolates were analyzed with respect to animal disposition including body condition score, animal health, source of the animal, and season of the year. *Salmonella* was isolated from 94 of the 1006 fecal samples. Twenty-two serotypes were identified with the predominant isolates comprising of *S. typhimurium* (22/94), *S. senftenberg* (17/94), and *S. kentucky* (8/94). *Salmonella* was isolated almost three times more often in the August sampling (70/505 for 13.86%) than the February sampling (24/501 for 4.79%). Coliform bacteria (*Escherichia coli* and *Klebsiella*) were isolated from 829 of 1006 fecal samples and there was no growth from 127 of 200 frozen samples. Sorbitol-negative *E. coli* was isolated twice as often in the summer 39% (199/505) than in the winter 19% (59/301). Of the 265 sorbitol-negative *E. coli,* 6 samples were identified as positive (0.023%) to EHEC and 2 were serotype O157:H7. A total of 47 samples contained only *Klebsiella* spp., 34 samples in winter and 13 samples in summer.

DEDICATION

It gives me great honor to dedicate this work to my family,

Mustafa G. and Berkcan Akpinar

and my parents Salih and Süheylâ Yildirim.

Thank you for believing in me, your unfailing love, support, and

encouragement throughout my masters program at Michigan State University.

ACKNOWLEDGMENTS

I would especially like to extend sincere thank you to my major advisor, Dr. Philip M. Sears, for always having an open door and mind, his help initiating this project, for his patient assistance, wise counsel, and instructional help throughout its duration. Another heartfelt thanks to my graduate committee members Dr. Paul C. Bartlett, Dr. Ronald J. Erskine and Dr. Robert E. Holland for their strong support and advice that enhanced completion of this study. I never would have gotten through this without you.

I would like to thank you Dr. Frederick Derksen chair of the Large Animal Clinical Science department for excepting me to the program, and all the employees of the department for giving me an opportunity to do my masters in such a wonderful university.

I would like to thank you Dr. Fred Troutt for his help and sharing his knowledge on this project.

I would like to thank you Dr. Barbara E. Straw for her help during collecting the data working in the laboratory and for the final touches on my thesis.

I would like to thank you my colleague Anantachai Chaiyotwittayakun, laboratory technician Chris Gage and all the other students for being a wonderful friend, their support and assistance in the laboratory culturing samples and traveling to slaughter with us to collect the data.

Finally I would like to thank you my dear husband Dr. Mustafa Akpinar even with his busy schedule who has put his knowledge in through out this project and help me out with the house work, my son Berkcan Akpinar (10) who is a great fan of the Spartans,

for being such a nice child and patient during mom was gone early in the morning to school, my sister Izlem Yildirim for being a great support while studying for her mechanical engineering undergraduate degree at Kettering University. My dearest parents Süheylâ and Salih Yildirim even while you were so far away from me but kept your heart and thoughts always beside me.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

INTRODUCTION

The term 'food-borne disease' is any illness that results from ingestion of food. The epidemiology of food-borne disease is changing. New pathogens have emerged, and some have spread worldwide. The potential microbiological hazards for food-borne illness from healthy and cull dairy cows include *Salmonella* (with special attention to DT104), *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Listeria monocytogenes*, *Clostridium perfringens* and *Staphylococuss aureus*. *Salmonella* spp., and *Escherichia coli* O157:H7 have been the focus of outbreak investigations (Table 1,4).[37,48,97,109,149] These pathogens cause millions of cases of sporadic illness and death over many states and nations.[37] These reports have increased consumer distrust in the safety of the food supply.[48,97] The major food vehicles associated with outbreaks were beef, turkey, chicken, ice cream, pork, dairy products, and eggs.[161] From 1986 to1995, *Salmonella* spp., *S enteritidis, and E. coli* O157:H7 accounted for 48% of food-borne diseases (Table 2,3).[5,36,149]

Food safety was identified as a priority area in Michigan agriculture for extension and research. Food safety can be defined in terms of risk of pathogens and risk of drug residues. Drug residues on farms are often related to the level of clinical disease and treatment procedures used to manage these cases. Risks of preharvest *Salmonella* and *E. coli* are related to disease incidence on the farm, methods of managing cases and decisions to cull animals. Culling decisions may be precipitated by disease, but while cull animals are not likely to be considered an important population on the farm, they can be an important contributor to antibiotic residues in meat, and a source of pathogens at

slaughter. [48,97] Cull dairy cows that are removed from the herd due to health problems including diarrhea, mastitis and pneumonia may pose a higher risk of contamination at slaughter. Shedding of pathogens such as *Salmonella* spp., and *Escherichia coli* increases when animals are stressed due to secondary health problems.[48,76,153] Although the reason for culling may present no immediate human health concern, the increase in fecal shedding of *Salmonella* spp., *E. coli* and other pathogens could result in a risk of meat contamination.[48,49,76,153] Not all bacteria isolated from cull cows are pathogenic, but handling practices which influence non-pathogen microorganism shedding in feces, could also increase pathogens if present in the herd.

As a part of a larger study the Allendale slaughter facility was chosen for sampling dairy cows. Animals presented for slaughter were evaluated as to the health of the animal, body condition and any physical abnormalities.[48] The effects of cold winter and hot summer conditions on shedding of the *Salmonella and E. coli*[48,153] were also tested, the transportation of these animals as a variable was determined in this study.

On July 25, 1996, sweeping reforms on food safety regulations, known as the final rule on pathogen reduction and Hazard Analysis Critical Control Points (HACCP), was published by the United States Department of Agriculture (USDA).[178] Although targeted for slaughter and processing plants that handle meat and poultry, the requirements could have an impact on dairy farmers.[178] All plants were also being required to adopt and implement their own HACCP plan, and slaughter plants and plants that produce raw ground products were required to ensure that the rate of contamination from *Salmonella* spp. was below the current national baseline incidence. Beginning January 27, 1997, the Food Safety and Inspection Service (FSIS) required all slaughter

plants to conduct microbial testing for generic *Escherichia coli* and to prepare and implement standard operating procedures for sanitation (SSOP).[178]

Table 1: Bacteria isolated during investigations of 82 outbreaks of food-borne disease, 1986-1995[149]

BACTERIAL AGENTS	FREQUENCY (%)
Salmonella* sp	21 (24)
Salmonella enteritidis	14 (16)
***Escherichia coli* O157:H7**	13 (15)
Shigella sonnei (n=6), ***S flexneri*** (2)	8 (9)
Bacillus cereus	5 (6)
Campylobacter botulinum	5 (6)
Clostridium botulinum	5 (6)
Staphylococcus aureus	5 (6)
Listeria monocytogenes	4 (5)
Clostridium perfringens	3 (3)
***Escherichia coli* O104:H21**	1 (1)
Enteroxigenic E coli	1 (1)
Yersinia enterocolitica	1 (1)
Group-A *Streptococcus*	1 (1)
*Includes ***S typhimurium*** (5), ***S tyhi*** (4), ***S heidelberg*** (2), ***S newport*** (2), ***S agona*** (1), ***S infantis*** (1), ***S montivedeo*** (1), ***S oranienburg*** (1), ***S poona*** (1), ***S reading*** (1), and ***S stanley*** (1).	

Table 2: Major source of selected food-borne and water-borne pathogens.[36]

PATHOGENS	SOURCE
Escherichia coli	Raw or undercooked beef, poultry, pork, and lamb; cheese; raw or inadequately pasteurized milk; apple cider; green salads
***Salmonella* spp.**	Raw or undercooked beef, poultry, lamb, eggs, fish, shellfish, and pork; ice cream; raw or inadequately pasteurized milk
Campylobacter jejuni	Raw or undercooked poultry, pork, lamb, and beef; raw or inadequately pasteurized milk; untreated water; fresh mushrooms
Listeria monocytogenes	Seafood; raw or undercooked beef, pork, poultry, lamb, and eggs; fermented sausages; produce and vegetables; ice cream
***Cryptosporidium* spp.**	Water

Table 3: Food vehicles implicated during investigations of 82 outbreaks of bacterial food-borne disease, 1986 to 1995.[149]

SOURCE	FREQUENCY (%)
Other*	24 (29)
Meat	20 (24)
Eggs	12 (15)
Poultry	7 (9)
Milk	5 (6)
Fish or shellfish	5 (6)
Meat products	4 (5)
Unknown†	4 (5)
Poultry products	1 (1)

* Includes reports in which food vehicles is not otherwise listed;
† Includes reports in which a meal or buffet, not a specific food, was implicated.

Table 4: Food-borne disease rates in the United States.[a,109]

Pathogens	Cases per 100,000 persons	Total no. of cases
Campylobacter	25.4	3,359
Salmonella	15.6	2,069
Shigella	9.6	1,272
***E. coli* O157:H7**	2.9	388
Yersinia	1.1	149
Listeria	0.5	64
Vibrio	0.2	21
Total	55.4	7,322

[a] Data are from the FoodNet 1996 final report and are for all sites covered by FoodNet.

CHAPTER 1

SALMONELLA

Salmonellosis in farm livestock and its association with human infection has attracted a great deal of attention, particularly in recent years. *Salmonella* reside in the intestinal tracts of humans and other animals, including birds. While there are over 2,450 recognized serotypes of *Salmonella*, only 5 or 6 serotypes are involved in the majority of infections in cattle. These serotypes include *S. typhimurium, S. dublin, S. newport, S. montevideo,* and *S. anatum.* Recently *Salmonella typhimurium* DT104 with resistance to 5 antibiotics has been reported as the cause of human infections in the United Kingdom and United States. The proportion of *Salmonella typhimurium* isolates that were R-type ACSSuT increased from 2% in 1980, to 30% in 1999 in the United States.[190]

As cattle operations have grown in size and animal density, salmonellosis has gained importance as a disease of calves.[16] The type and severity of the disease are influenced by the *Salmonella* serotype, the infective dose, and the age, immunity, and health of the calves.[16,73,206] Salmonellosis in farm livestock and its association with human infection has attracted a great deal of attention, particularly in recent years.[9] Salmonellosis is an infectious disease that continues to plague human populations in both developed and developing countries, and is usually transmitted to humans by eating contaminated foods.[182] *Salmonella* are of major concern to the dairy industry because a variety of serovars have been incriminated in outbreaks of human salmonellosis that were associated with the consumption of dairy products.[59] *Salmonella* infections cause significant morbidity, mortality, and economic loss and are particularly severe in infants,

elderly, or immunocompromised patients.[5] Although incidents of human salmonellosis are frequently limited to single cases the size of the international list of large food-borne outbreaks of salmonellosis is alarming.[182] The reported incidence of *Salmonella* infections in the U.S. has increased substantially since reporting to the Centers for Disease Control (CDC) began in 1943.[5] Each year in the United States, there are an estimated 800,000 to 4 million *Salmonella* infections, and approximately 500 are fatal.[98] From 1983 to1987, *Salmonella* accounted for 28% of food-borne disease outbreaks and 45% of food-borne disease cases of known etiology in the U.S.[5] The major food vehicles associated with these outbreaks were beef, turkey, chicken, ice cream, pork, dairy products, and eggs although vegetables may become contaminated.[5,161]

Cull dairy cattle are especially important potential reservoirs for human salmonellosis because they are the source of much of the hamburger consumed in the United States about 17%.[190] Unlike fed cattle, cull dairy cows may be in poorer body condition; therefore, meat from these dairy cows often is used for ground beef.[190] Undercooked ground beef has been implicated as an important source for *Salmonella* infections in humans.[74,190]

Between 1994-1995, the serotypes most frequently isolated from cull dairy cattle were: *S. typhimurium, S. dublin, S. kentucky, S. montevideo, S. muenster, S. newport, S. anatum, and S. cerro.*[68,162] Primary serotypes isolated from cattle were S. typhimurium and S. dublin.[68,162] In 1996 a nation wide study has a new list of 10 most isolated serotypes from cull dairy cattle: *S. montevideo, S. muenster, S. kentucky, S. anatum, S. cerro, S. lille, S. typhimurium, S. mbandaka, S. give,* and *S. meleagridis* (Table 5).

Table 5: The 10 most frequently isolated *Salmonella* serotypes (July 1995 to June 1996 and July 1996 to June 1997) from human sources at the Centers for Disease Control and Prevention (CDC), from cattle submissions to the National Veterinary Services Laboratory (NVSL), and culled dairy cows (Dairy) at 5 non-fed beef slaughter establishments during winter and summer periods in 1996.[74,190]

	CDC	NVSL		Dairy[a]
Rank	**1996**	**1995/1996**	**1996/1997**	**1996**
1	*S Enteridiidis* (var Copenhagen)	*S Typhimurium* (var Copenhagen)	*S Typhimurium*	***S Montevideo***
2	***S Typhimurium***[b]	***S Typhimurium***	*S Dublin*	*S Muenster*
3	*S Heidelberg*	***S Montevideo***	***S Typhimurium***	*S Kentucky*
4	*S Newport*	*S Cerro*	***S Montevideo***	*S Anatum*
5	***S Montevideo***	*S Anatum*	*S Kentucky*	*S Cerro*
6	*S Javiana*	*S Muenster*	*S Anatum*	*S Lille*
7	*S Orianenburg*	*S Dublin*	*S Meleagridis*	***S Typhimurium***
8	*S Hadar*	*S Kentucky*	*S Muenster*	*S Mbandaka*
9	*S Agona*	*S Give*	*S Cerro*	*S Give*
10	*S Muenchen*	*S Meleagridis*	*S Menhaden*	*S Meleagridis*

Salmonella serotypes that are underlined appear on a NVSL list as well as the Dairy list. *Salmonella* serotypes in bold type are common to all 3 lists.

[a] If *S Typhimurium* (var Copenhagen) is included in the count of *S Typhimurium*, as was done by the CDC, *S Typhimurium* would move up to rank 5. [b] Includes *S Typhimurium* var Copenhagen.

ISOLATION AND PREVALENCE OF *Salmonella* AT SLAUGHTER

Prevalence on farm

In England over a three-year period (1969-72) four large calf units were examined for *Salmonella* infection. The 4 units had different husbandry and farm management. The incidence of *Salmonella* in fecal samples ranged from a low of 0.7% to a high of 11.1%. The dominant serotype was *S. dublin.*[34]

In 1985 after detecting a rise in human infection with *S. newport* in California, Los Angelos County Department of Health Services conducted a survey on dairies in California. Of the 75 dairies randomly selected, the median number of cattle on the dairies were 580 adult cows (range 80 to 2900) and 50 calves less than 2 month old (range 0 to 300). *Salmonella* was isolated from at least 1 of 4 sample sites on 12 of 75 dairies (16%).

Calves were the single best source (8 of 12 *Salmonella* positive dairies) for isolating *Salmonella* at dairies where the organism was detected. Four *Salmonella* serotypes were isolated: *S. newport* (6), *S. montevideo* (3), *S. dublin* (2) and S. *typhimurium* (1).[143] Wray *et al.*[215] examined calves for *Salmonella* in England and Wales between September 1985 and April 1986. At least 28 days after their arrival on farms, 589 animals distributed in 25 groups on 11 farms were examined for excretion of *Salmonella*. *Salmonella* was found in 212 calves. *Salmonella* were not detected in 7 of the 25 groups. In the other 18 groups, between 3% and 90% of animals were identified as excretors. On arrival from various markets, total incidence of *Salmonella* fecal excretion was 0.7% and it reached it's peak around 2 to 3 weeks after arrival on the farms. *Salmonella typhimurium* and *S. dublin* were the predominant strains isolated.

There was no difference in excretion rates between calves housed singly compared to calves group-housed in pens.

Between 1991 and 1992 *Salmonella* prevalence was 2.1% from 6861 preweaned dairy heifer calf fecal samples from 1063 dairy farms at 28 states in the USA.[34,140] *Salmonella* serotypes found in this national survey showed that, *S. typhimurium* prevalence was 27.6%, *S. dublin* prevalence was 10.3% and *S. mbandaka* prevalence was 8.9%.[140] In European countries, S. *dublin* and *S. typhimurium* are found to be the most common serotypes in cattle.[140]

The number of *S. typhimurium* DT104 cases in humans and in animals for England and Wales rose between 1990 and 1996.[151] In the United States, the frequency of *S. typhimurium* isolation has also increased recently. In cattle DT104 was most commonly recognized in 2 to 4 week old sick calves suffering from diarrhea. Calves that were recently purchased and had traveled became sick soon after arrival and experienced a mortality of 40% to 50%.[60] Fecal shedding is persistent after outbreaks of this type of salmonellosis, and the organism has been recovered from feces for up to 18 months after infection. A case control study in Great Britain reported that most outbreaks lasted for less than a week and less than 4% of animals within herds were clinically affected. The incidence of disease was about 33% in calves, compared with only 4% of adults in affected herds. Sub-clinical carriage was common and persisted for up to 18 months.[151]

In 1991-1992, the U.S. Department of Agriculture did a project named the National Dairy Heifer Evaluation Project (NDHEP) which determined *Salmonella* prevalence rates across the nation. According to this study *Salmonella* prevalence was

highest in the late summer (July-September) with 36.1 of every 1000 samples testing positive (Figure 1).

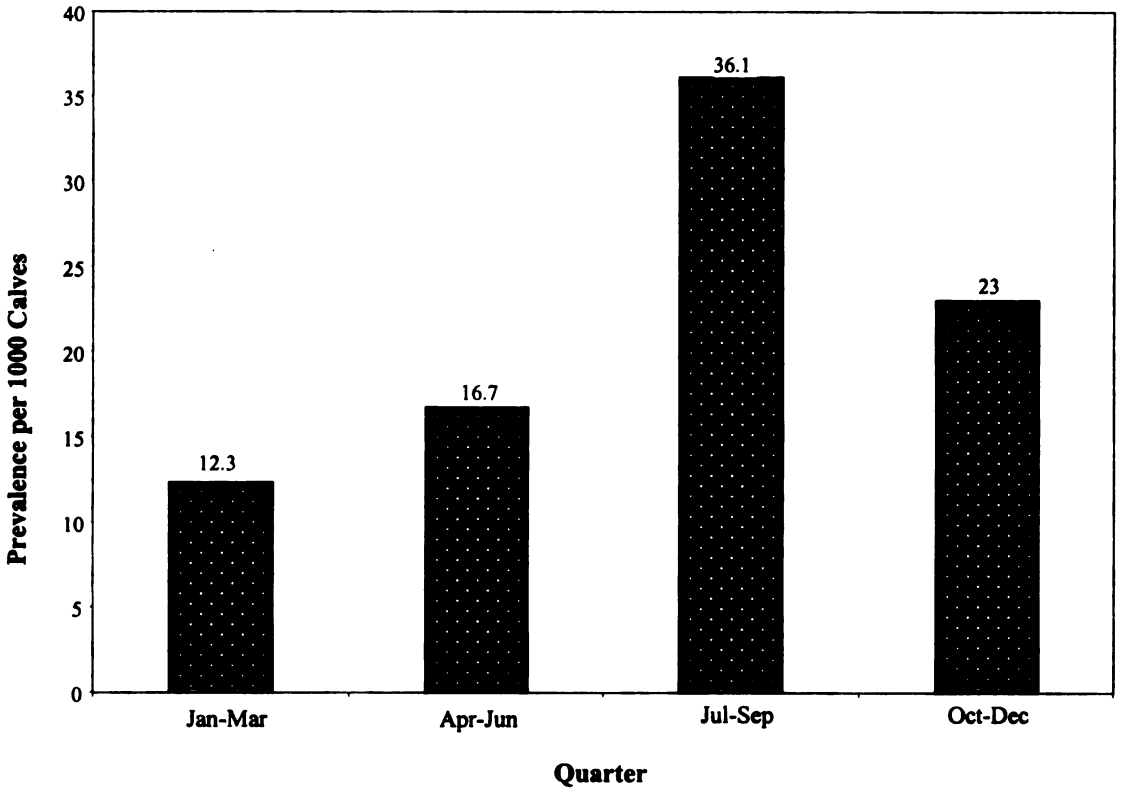


Figure1: Prevalence of *Salmonella* in dairy calves by season.[194]

Prevalence at slaughter

Rumen fluid is an important potential source of contamination of carcasses at slaughter and a reservoir of *Salmonella* for infection of the intestine.[84,86] Grau and Browlie,[84] found that 61 of 170 rumen samples (36%) and 39 (27%) of 146 fecal samples were positive for *Salmonella*. The prevalence of *Salmonella* infection in the rumen and feces of slaughtered cattle has been shown to increase with increased time between farm and slaughter and also to be higher in animals which have been fed once during the

holding period.[19] In an Australian slaughter project, the inspectors observed that animals that were slaughtered on the first two days of the week, Monday and Tuesday, had a higher prevalence of *Salmonella* in both rumen and mesenteric lymph nodes than animals slaughtered on other days of the week.[164,165,166] The difference was attributed to the cattle killed on Monday and Tuesday being held, and usually fed, over the weekend, after traveling considerable distances from their property of origin.[165,166]

Salmonella can persist in dairy cows and the surrounding environment for several years without showing evidence of clinical disease or production inefficiency.[79,82] There is a possibility that during such periods, cows which may have been *Salmonella* carriers are routinely culled for slaughter. If the time from farm to slaughter facilities is prolonged, the stress of transport and fasting prior to slaughter can increase the prevalence of *Salmonella* infection among animals.[126] Puyalto *et al.*[153] have shown that the prevalence of *Salmonella* was 8% (6/80) on leaving the farms and this number reached 25% (20/80) on arrival at the slaughter plant.

Studies[84,86,126] show that shedding of *Salmonella* is affected by rumen pH and volatile fatty acid level. Acidic pH and increased volatile fatty acid level prevent growth of enteric bacteria. Moderately elevated rumen pH and decreased concentrations of total acidity, as would occur in withholding of food, are conditions which foster growth of a variety of *Salmonella* serotypes in rumen fluid of cattle at slaughter.

In a study, Gay *et al.*[80] showed that the rate of fecal shedding of *Salmonella* in 1,289 cull dairy cows marketed in the state of Washington to be approximately 0.5%. In the same study mesenteric lymph nodes and rumen contents were cultured, a wide variety

of serotypes of *Salmonella* were isolated with a high prevalence (76%) from a population of 100 cull cows.[166]

The appearance of a chloramphenicol resistant strain of *Salmonella typhimurium* phage type 204 in calves in Great Britain highlighted potential public health risks and since then chloramphenicol resistant strains of the same organism, thought to have in some cases been derived from calves, have been isolated from sick humans.[187]

Though many of the more than 2,450 *Salmonella* serotypes can infect cattle, most infections are limited to a few serotypes. Recently, there has been an increase in the incidence of *Salmonella* outbreaks in dairy cattle in the Pacific Northwest of the USA.[11] Studies revealed that isolates from these outbreaks were *S. typhimurium* DT104, this was the first report of this definitive type of *Salmonella*.[11] *Salmonella enterica* serotype typhimurium characterized as definitive type 104 (DT104) is now the second most prevalent *Salmonella* in human beings and animals in the United Kingdom (England and Wales) and Europe.[101,198] In this study[11] investigators suggested that farmers can contract *S. typhimurium* DT104 by handling sick cows and calves but they didn't identify a single food stuff as responsible for the increasing number of human isolations of *S. typhimurium* DT104.[101,198,199]

The isolations referred to the Laboratory of Enteric Pathogens increased, 250 in 1990, 2873 in 1994, and 3837 in 1995.[185] The importance of this increase has been the epidemic spread of a *S. typhimurium* R-type ACSSuT strain multiresistant to five antimicrobial agents (A, ampicillin; C, chloramphenicol; S, streptomycin, Su, sulphonamides; T, tetracyclines).[185,199] A recent report[199] from England and Wales showed that infections caused by this multidrug-resistant typhimurium were associated

with greater morbidity and mortality than other salmonella infections. The DT104 strain is also resistant to drying and chemicals, which makes it a substantial potential zoonotic threat.[124] Molecular studies have demonstrated that in multiresistant DT104 all the resistance genes are located on the chromosome, which is rare phenomenon for *S. typhimurium*.[185]

Unlike *S. enteritidis* phage type PT 4, which is almost entirely associated with poultry and poultry products, the epidemiological evidence indicates that multiresistant DT104 is widely distributed in a variety of different food animals.[186] Although most commonly associated with cattle, the strain has also been isolated from sheep, pigs, goats, chickens, and turkey, and from a wide range of food products and processed foods in the United Kingdom.[2]

Another study[142] has demonstrated a high incidence of multiresistant DT104 in fresh raw sausages purchased from a range of retail outlets in the United Kingdom. The strain has also been isolated from farm workers and from domestic pets. It is now generally accepted that the incidence of *Salmonella* in farm livestock is related to husbandry methods and practices and there is a great deal of evidence to indicate that extensive systems in particular favor the spread of infection and a subsequent increase in the level of clinical disease.[9]

IDENTIFICATION AND IMPROVED METHODS OF ISOLATION IN THE INDUSTRY OF MONITORING

Salmonellae are part of a family of Gram-negative, rod-shaped bacteria known as Enterobacteriaceae, which occur in the intestinal tract of humans and in warm-blooded

and cold-blooded animals. To date, more than 2,300 serotypes of *Salmonella* are known to exist and new serotypes are being discovered each year. Of these recognized serotypes, only about 100 are routinely isolated from food, animals and man.[89] These facultative anaerobic Gram-negative bacteria produce gas from glucose and utilize citrate as their sole carbon source through their flagellated rods.[116]

The detection of food-borne pathogens is complicated because low numbers of the organism of interest are often present in a complex microbial flora and because of complex compositions of different foods.[152] In food microbiology, the presence of a single pathogenic organism is considered significant. Therefore, methods and media must be capable of enabling growth to occur from extremely low initial cell numbers. Five steps are common to most culture procedures for isolating and identifying *Salmonella* in foods. These include (1) pre-enrichment of a food sample in a nutritious, nonselective broth; (2) selective enrichment in a broth that allows salmonellae to grow but suppresses the growth of competing bacteria; (3) isolation of *Salmonella* by streaking onto selective plating agar; (4) biochemical characterization of isolates; and (5) serological confirmation of biochemically screened isolates.

Pre-enrichment

Pre-enrichment is the initial step in which the food sample is enriched in a nonselective medium to a stable physiological condition so that the bacteria can grow on the nutrients present in the medium.[40,161] Sublethal cell damage may have resulted from thermal processing of food, freezing, thawing, osmotic shock, or prolonged storage of low-moisture foods at elevated temperature.[40] Satisfactory resuscitation and pre-

enrichment generally require a nutritious nonselective medium. Generally, pre-enrichment media are nutritionally complex and may include trypticase soy broth, nutrient broth, reconstituted nonfat dry milk, or lactose broth. Pre-enrichment requires incubating cultures at 35-37°C for 16-24 hours.[8,131]

Enrichment

Selective enrichment is the step in which the sample is further enriched in a growth-promoting medium containing selective inhibitory reagents. This medium allows a continued increase of salmonellae while simultaneously restricting proliferation of most other bacteria.[4,40] Selectivity of the enrichment process is based on synergism between inhibitory agents in enrichment media and temperature of incubation.[40,153,206] Two media commonly used for selective enrichment are selenite cystine broth and tetrathionate broth.[179] The addition of cystine to selenite broth enhances *Salmonella* growth. All the variations of selenite broth are suitable for most serovars, including *S. typhi, S. dublin, and S. choleraesuis*. Selectivity of tetrathionate broth depends on the ability to suppress the growth of coliform organisms.[4,40,86,131] In addition to these two broths, Rappaport enrichment broth can be used that includes modifications for Rappaport-Vassiliadis (RV) enrichment broth, and Rappaport 25 (R_{25}).[40,46,131,153]

The temperature of incubation during selective enrichment significantly influences the successful recovery of *Salmonella* in food. Selective enrichment cultures are incubated for 16-24 hours at 35-37 or 43°C.[40,179,206]

Plating media and biochemical screening

Selective plating uses solid selective media that restrict growth of bacteria other than salmonellae.[4,179] Several agar media, including bismuth sulfite agar (BSA), brilliant green (BGA), xylose lysine desoxycholate (XLD), Hektoen enteric (Hek) agars, and xylose-lysine-tergitol 4 agar (XLT4) are widely used in standard methods for the isolation of *Salmonella* in foods.[4,40,46,86,131,179,206]

The BGA, XLD and Hektoen media are related in bacterial utilization of lactose and/or sucrose, low selectivity and incidence of numerous false-positive reactions, whereas the bismuth sulfite agar (BSA), a non-saccharide differential medium, shows good selectivity against non-salmonellae. Identification of salmonellae on this agar (BSA) is based on the development of black colonies resulting from the capture of metabolic H_2S gas as the insoluble FeS salt, and frequent appearance of a black halo around suspect colonies. Xylose-lysine-tergitol 4 (XLT4) is a new media in the 20th century, it was found to strongly inhibit *Proteus, Pseudomonas, Providencia* and many other non-salmonellae.[131,179] After 20 to 24h at 35 to 37°C, typical (H_2S-positive) *Salmonella* colonies on XLT4 media are smooth and creamy in texture and appear black or black-centered with a yellow (acid) periphery that changes to pink (alkaline) as the xylose is depleted.[46,131,179] It was concluded that the only genus capable of forming black colonies within 24h on XLT4 media was *Salmonella*, allowing easy differentiation from other organisms. None of the *Salmonella* plating media are fully selective, recovery of the widest possible range of *Salmonella* serovars requires two or more plating media.[86,152]

Salmonella-like colonies are selected and identified by biochemical tests. Two differential agars, that is, triple sugar iron agar (TSI) [salmonellae typically produce

alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar)] and lysine iron agar (LIA) [salmonellae typically produce an alkaline (purple) reaction in the butt, with or without production of H_2S], are commonly used in combination to provide initial biochemical data about the isolates.[103,191] The presence of glucose and an H_2S detection system in TSI facilitates screening of non-glucose-fermenting organisms such as *Pseudomonas* spp. and presumptive identification of *Salmonella*. The LIA medium is of equal diagnostic value because it screens for the presence of the lysine decarboxylase enzyme, which is commonly encountered in *Salmonella* spp.[40] Cultures typical of *Salmonella* in these media are then tested by biochemical tests to confirm the isolates. Biochemical tests typically used include urease (negative), lysine decarboxylase (positive), fermentation of dulcitol (positive), utilization of sodium malonate (negative), and production of indole (negative). Other tests occasionally used include fermentation of lactose and sucrose (both negative), Voges-Proskauer test (negative), and methyl red test (positive).[40]

Serotyping is the definitive step in providing a specific identification of the cultures.[4,86] Cultures are tested by agglutination assays with antisera specific for somatic (O), flagellar (H), and capsular (Vi) antigens. The heat-stable somatic antigens (O) of the bacteria are identified first, using the slide agglutination method. Unlike "O" antisera, "H" antisera are used in tube agglutination tests. If the slide technique is used, the "H" antisera either must be freed of "O" agglutinins by absorption or must be used in dilutions sufficiently high that "O" reactions do not occur.[62]

Rapid methods

Rapid detection methods for food samples have been a subject of research since the early 1980s, and these tests take 4 to 12h to complete.[104] Commercial diagnostic assays for *Salmonella* may be placed in five general categories: miniaturized biochemical tests; new media; instrumentation or automated systems; nucleic acid-based assays; and antibody-based assays.[40,66] Although many of these tests are referred to as "rapid methods", most of these *Salmonella* detection systems, regardless of the technology or assay format, still rely on cultural methods for selective amplification of *Salmonella* population in the broth culture. Therefore, pre-, selective-, or postenrichment procedures, or some combination of them, must be used in conjunction with these "rapid" methods for sensitivity and specificity.[41] Sensitivity of a test refers to the minimum amount of an organism or other substance that can be detected. Specificity is the ability of a test to distinguish exactly the component of interest with no other interactions. Most of the assay systems are screening assays and only provide for the presumptive identification of salmonellae. Negative results, therefore, are considered definitive, but presumptive positive results must be confirmed by conventional methods and serology.[66] Table 6 shows some of the commercial tests that are used for detecting *Salmonella* spp.

Table 6: Selected rapid methods/materials commercially available for the identification of *Salmonella* in foods.

Methods and materials	Assay format	Manufacturer	AOAC status
Miniaturized tests			
API 20E	Biochemical	Analytab	Final action
Enterotube II	Biochemical	Roche Diagnostics Systems	
Enterobacteriaceae Set II	Biochemical	BBL	Final action
MICRO-ID	Biochemical	Organon Teknika	Final action
Media			
HGMF/EF-18	Selective, differential	QA Life Sciences	First action
MSRV	Selective, differential	Various	First action
Oxoid SRT	Selective, differential	Oxoid Division of Unipath	None
Rambach agar	Selective, differential	Technogram (France)	None
Nucleic Acid-Based			
DNAH	DNA probe	GENE-TRAK	First action
Antibody-based			
Oxoid	Latex beads	Oxoid (UK)	None
MicroScreen	Latex beads	Mercia (UK)	None
Spectate	Latex beads	May and Baker (UK)	None
Bactigen	Latex beads	Wampole	None
Assurance	ELISA, polyclonal	BioControl	First action
TECRA	ELISA, polyclonal	Bioenterprises (Australia)	First action
Salmonella-Tek	ELISA, monoclonal	Organon Teknika	First action
Salmonella 1-2 Test	Immunodiffusion	BioControl	First action
UNIQUE	Dipstick	Bioenterprises (Australia)	None
PATH-STIK	Dipstick	LUMAC (The Netherlands)	None
Instrumentation			
GNI	Biochemical	BioMérieux Vitek	First action
Biolog	Carbonutilization	Biolog	None
VIDAS	ELFA	BioMérieux Vitek	None
Malthus	Conductance	Malthus Instruments (UK)	First action

Note: AOAC, Association of Official Analytical Chemists; ELISA, enzyme-linked immunosorbent assay; ELFA, enzyme-linked fluorescent immunoassay[39,41,42,104]

Nucleic acid-based assays

DNA Probe. A DNA probe is normally a short sequence of nucleotide bases that will bind to specific regions of a "target" sequence of nucleotides where the homology between the target and the DNA probe results in a stable hybridization. When a protein is the target of detection, as in an immunoassay, there is a risk that the nucleic acid sequences that coded for the amino acids that make up the protein might be changed or lost due to stress on microorganisms during food processing. Hybridization assays can detect the presence of bacterial cells, regardless of the physiological state of the organism or the status of proteins or lipids in or on the microorganism.[213]

Colorimetric DNA Hybridization Test. The first-generation DNA hybridization test used a radioactively labeled probe (^{32}P). The colorimetric assay employs *Salmonella* specific DNA probes and a colorimetric (instead of radioisotopic) detection system for the detection of *Salmonella* species in food samples following broth culture enrichment.

According to the studies, the GENE-TRAK *Salmonella* Assay appeared to be an effective screening procedure for rapid detection of salmonellae in meat and poultry products.[163,179] The major advantage of the colorimetric DNA probe assay is that large numbers of samples can be screened fairly rapidly for salmonellae.[163,179] However, all DNA probe-positive samples should be confirmed by culture.

Polymerase Chain Reaction (PCR). Genetically based, non-cultural, primer-mediated enzymatic amplification of target-DNA, called PCR, has been applied successfully for the detection of a large number of pathogens, including *Salmonella*.[179] The PCR method can specifically amplify a single copy to one million-fold of a gene or DNA segment unique to a target microbial pathogen. After amplification, the DNA segment can be

readily detected by DNA-DNA hybridization.[179] Using PCR-based probes and recombinant DNA hybridizations to detect pathogenic organisms has many advantages over classical culture techniques. Amplification of DNA sequences unique to an organism by PCR improves the speed and sensitivity at which organisms can be detected. PCR has been used to identify several bacterial species including *Salmonella* serovars from food and clinical samples.[33,38]

Antibody-based assays

The antibody-based assays are the largest group of commercial tests for detecting *Salmonella*. They can be classified into the following categories on the basis of their assay formats, including latex agglutination, enzyme-linked immunosorbent assay (ELISA), immunodiffusion, and dipstick.

Latex Agglutination Assay: These agglutination assays use latex particles coupled with polyvalent antisera to various *Salmonella* antigens. The latex particles with bound *Salmonella*-specific antibodies amplify agglutination reactions and allow visual identification of positive samples.[42]

The Oxoid *Salmonella* latex test, uses *Salmonella* antibodies that are specific both for somatic and flagellar antigens.[66] Positive results are confirmed by the reference culture method. In the MicroScreen® test, reagents of this latex agglutination kit respond to *Salmonella* flagellar antigens.[42] A study with isolated bacterial strains, including[124] *Salmonella* strains, showed high specificity as well as high sensitivity for this test (both 96%).[123]

Enzyme-Linked Immunisorbent Assays (ELISAs): Several ELISA's have been developed, using both polyclonal antibodies and monoclonal antibodies that will detect most *Salmonella* serotypes. These assays and others have been developed subsequently in kit form and are available commercially. Kit assays require enrichment steps to resuscitate injured cells and to selectively amplify salmonellae. All ELISA kits are designed in a "sandwich" or "capture format", that is, antibody-coated polystyrene wells are used to capture salmonellae antigen, and a second antibody to *Salmonella* conjugated with an enzyme is added to form an antibody-antigen-antibody (sandwich) complex. The sandwich complex is then determined by a colorimetric enzyme substrate, and the results are recorded either visually or with a spectrophotometer.

Most of the *Salmonella* ELISA kits use alkaline phosphatase or horseradish peroxidase enzyme conjugates with a colorimetric substrate system.[66] Polyclonal enzyme immunoassay (EIA) (Assurance) for *Salmonella* is configured in a microwell plate format. It is designed for the rapid detection of motile and non-motile *Salmonella*. Sensitivity is enhanced through the addition of another antibody that immunochemically links the bound *Salmonella* antigens with the enzyme conjugate. The colorimetric, monoclonal enyzme immunoassay (*Salmonella*-Tek) is a microtiter plate format, which was reported to be a promising test for the detection of *Salmonella* antigens with very low cross-reaction of the anti-*Salmonella* antibodies.[57,196] Detection of *Salmonella* antigens is based on EIA using specific monoclonal antibodies. This method is designed for detection of *Salmonella* in all foods. The test is not confirmatory because monoclonal antibodies used in this test may cross-react with a small percentage of non-*Salmonella*.[57]

Immunodiffusion: The *Salmonella* 1-2 Test is the only commercial assay for *Salmonella* in foods that uses the immunodiffusion format. It is a screening method for motile *Salmonella* in foods. One study reported a high false-negative rate for the 1-2 Test when the unit was inoculated from pre-enrichment broth and suggested that better productivity would likely be obtained with a modified enrichment protocol that included selective enrichment before inoculation of the unit.[39] Another study reported that use of tetrathionate brilliant green agar broth enrichment step following pre-enrichment enhanced the reliability of the 1-2 Test.[139]

Dipstick Assays: TECRA UNIQUE® *Salmonella* assay system uses an antibody-coated dipstick after pre-enrichment to selectively capture salmonellae. Because competing bacteria are not picked up by the dipstick, the UNIQUE system coupled with TECRA ELISA should produce in fewer false-positive reactions. Sensitivity is reported equivalent to that of standard culture methods. PATH-STICK is another assay that makes simultaneous use of *Salmonella* antibody, conjugated with enzyme, and a membrane-tipped dipstick bound with another antibody to *Salmonella*.[66]

Instrumentation and automated assays

Several automated and semi-automated systems using different technologies have been developed for *Salmonella* identification. The Vitek AutoMicrobic System with the Gram-Negative (GNI) Card uses a computer, optical reader, and test kits, with disposable plastic cards that have wells containing different biochemical substrates. Once a test kit has been inoculated with a suspension of the sample organism and has been loaded into the system, no additional biochemical reagents need to be added. A final report is printed

automatically for each test kit at the end of its cycle, which is 4h for *Salmonella*.[114] The GNI system correctly detected 96.7% of *Salmonella* species in food samples.[114] Other automated identification systems include the Biological Identification System, which measures the ability of the bacteria to oxidize 95 different carbon sources in order to generate identification and metabolic information, and VIDAS (Vitek Immuno Diagnostic Assay System), which uses the Enzyme-Linked Fluorescent ImmunoAssay (ELFA).[81,114]

METHODS OF CONTROL OF ORGANISM

Organism shedding and control in farm

Microbiological food safety is an important issue in beef products for human consumption. Cattle producers are implementers of management practices to reduce risk and are supportive of research for improvement. When cattle leave the farm or feed-lot for slaughter they will carry within their intestinal tracts and on their hooves and hides a large population of microorganisms. Under feed-lot conditions the hide may become heavily contaminated with feces. The percentage of animals carrying salmonellae in their intestinal tracts varies between different herds and at different times of the year (fall-winter-spring-summer).

Control measures for bovine salmonellosis have been well documented. In general, there are 3 main control points; (1) rodents and birds, which bring in *Salmonella* from outside sources or which act to maintain infection on premises as a vector into cattle feed, (2) contaminated feed sources, especially high moisture commodities in which *Salmonella* readily multiply after contamination by birds, rodents, or equipment, and (3)

infected cattle, either asymptomatic carrier cattle or ill and recovering animals, which magnify the number of *Salmonella* in the farm environment. [14,132,159,214]

Birds, rats and mice are frequently infected with *Salmonella*, particularly *Salmonella typhimurium*. Mice and rats may also be infected with *S. dublin* and should be eradicated as a part of the dairy control program. When feeds are contaminated by rodents and birds, multiplication of *Salmonella* in areas of high moisture occurs.[132] Fecal shedding of salmonellae by infected cattle is the main source of infection in calves, which are infected by the oral route.[214] Reduction of *Salmonella* in feeds is possible by use of organic acids.[13] Elimination of *Salmonella* from feeds may require high temperature pelleting or irradiation together with dehydration to reduce moisture content below 5% and proper handling to prevent wetting and recontamination.[13]

If farm waste or sewage sludge is applied to pasture, then it should have been stored for at least 4 weeks before application and there should be an interval of at least 4 weeks between application and grazing.[132] Animals should not graze pastures which have been flooded.

Whether the number of *Salmonella* in feces and the immediate surroundings is sufficient to cause clinical disease is not known, but it is also possible that close grazing during the late autumn may result in an increased infection rate. Clegg *et al.*[31] mentioned that disease couldn't be produced in calves allowed to graze on grass which had been sprayed with slurry containing *S. dublin*. During late summer, reliance on grass of deteriorating nutritional value may have precipitated the clinical infection.

In addition, septicemic infected calves shed salmonellae in nasal secretions and saliva, which can contaminate feeding equipment and farm personnel. Many calves are

infected by direct contact with their dams or from the calving environment during the first 24 hours of life and up to 2 month of age by *S. dublin*, similarly calves infected with *S. typhimurium* tend to be infected from one to 35 days old. During this time low numbers of organisms can establish an infection because the abomasum lacks protective acidity and there are no competing flora in the gut.

Carrier animals are important in transmission. One asymptomatic carrier cow can shed over 10 billion *Salmonella dublin* per day in feces and milk.[173] A combination of routine serology and bacterial culture of milk and feces from suspect animals can help identify persistently infected cattle, which can then be culled. Good husbandry and hygiene practices, such as housing calves individually and keeping the calving areas clean, will reduce the calves' exposure to salmonellae and other pathogens.

In 1996, the United States (particularly in western states) recognized a new and apparently more virulent phage type of *S. typhimurium*, DT104. Investigations in the United States have found associations between typhimurium DT104 infections in humans and the consumption of unpasteurized dairy products and direct contact with livestock. *Salmonella typhimurium* phage type (PT) or definitive type (DT) 104 is a virulent pathogen for humans and animals, particularly cattle. It has been isolated increasingly from humans and animals in the United Kingdom and several other European countries and, more recently, in the United States and Canada.

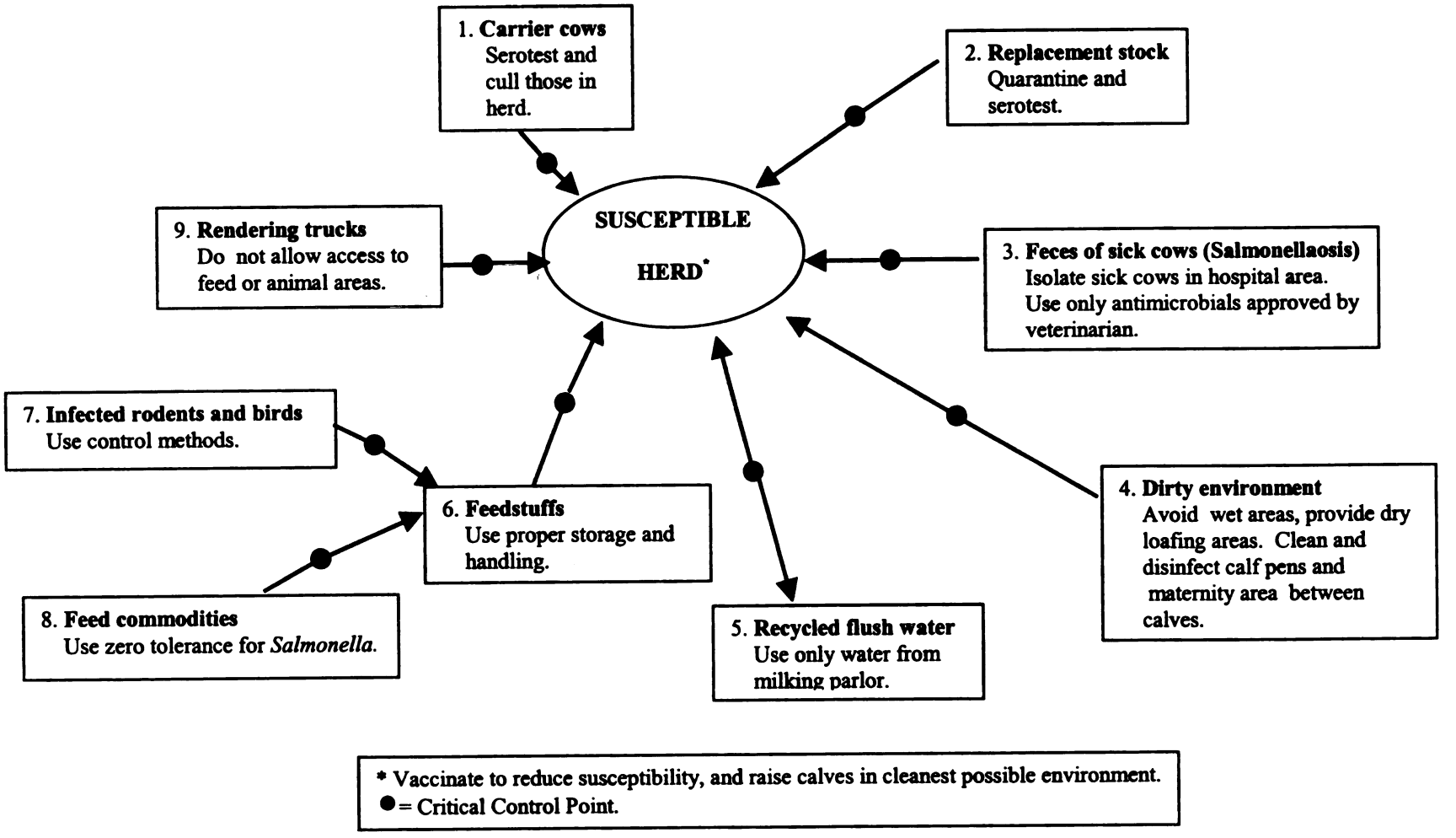
Farm families are particularly at risk of acquiring the infection by contact with infected animals or by drinking unpasteurized milk. *Salmonella typhimurium* DT104 infections in cattle may be prevented by purchasing replacement stock directly, rather than via livestock dealers, by maintaining a 4-week quarantine period for purchased

cattle, by housing sick animals in dedicated isolation areas, and by preventing wild birds from having access to feed for cattle.[60,61]

To prevent the contamination and spreading of *Salmonella* infection in the dairy, it is important to have good sanitary conditions and to minimize the contact of ill or carrier animals and their feces with the other healthy animals (Figure 2).[31] Some of the other methods can be summarized as:[14,120,129,132,214]

1. Because *Salmonella* infections are less likely to be found in calves individually penned as compared to calves housed in a group pen, individual housing systems are recommended for young calves.
2. Only strong, healthy calves should be purchased as replacement stock.
3. Purchased replacement stock should be serotested, cultured and quarantined.
4. Sick cows and calves should be isolated.
5. Avoid wet areas, provide dry areas such as free stalls for loafing, and clean and disinfect calf pens and maternity areas between calves.
6. Rendering trucks and other vehicles which may be contaminated or carry infectious material shouldn't be allowed on the farm near animals or feed. Front-end loaders used for dead animals or manure shouldn't be used for feed.
7. Do not use routine prophylactic antibiotics, as this promotes bacterial resistance and may harm cattle gut flora, predisposing to salmonella infection.
8. If there is clinical salmonellosis in the farm, vaccinate cows with a killed *Salmonella* bacteria specific for the serotype isolated. Killed vaccine also can cause side effects and side effects can increase with hot weather and administration with other vaccinations such as *Escherichia coli* bacterins and *Brucella abortus* live vaccine.

Figure 2: Critical control points in controlling *Salmonella* in cattle.[175]



CONTROL IN CONTAMINATION AND MONITORING AT SLAUGHTER PLANTS

Between the farm and the slaughter floor, the microbiological status of cattle can change. Food deprivation (high concentration of volatile fatty acids-VFA and low pH) and intermittent feeding that some herds undergo when they travel long distances to slaughter make such animals very sensitive to salmonellae and the prevalence of infection with *Salmonella* may increase markedly.[84,86,166]

Grau *et al.*[84], have shown that *Salmonella* may grow in the rumen of such animals and it was shown that large numbers of *Salmonella* may be present in the mesenteric lymph nodes as well as in the gut contents. Not only can the intestinal tract of these cattle become highly contaminated with salmonellae but these animals in turn contaminate the environment through which they pass; such as trucks, railway wagons, sale-yards and holding areas.[84,216] In these environments, hides and hooves are also contaminated. Even in clean areas, salmonellae shed in the feces of a few animals can get on the hooves, legs and, when cattle lie down, larger areas of hide.

Puyalto *et al.*[153] between April 1994 and May 1995, documented the increase in hair contamination by salmonellae in cattle between the farm and slaughter plant. Samples from animals and environment in which they were stationed were collected. Hair samples as well as the environmental samples were the most frequently contaminated (26% to 69%). Contamination of the hair had a frequency of 25%, during the time when the cattle were transported to slaughter.

At slaughter animal goes through a couple of steps, which gives an opportunity for food-borne pathogens to contaminate the products and the environment. These steps

are hide removal, evisceration, boning, chilling or freezing of boneless products, and temperature control in transport to the export market. While some of the same principles apply to both control of microbial contamination in a beef slaughter and to other meat species, there are a number of differences. There is considerably more vertical integration in the poultry and pig industries with a greater possibility of the one owner controlling handling from birth of the animal to final sale of the packaged meat. The skin is left on pig and poultry carcasses, and a heat treatment is applied to their surface tissues. The much larger size of a beef carcass, compared to poultry and sheep, increases the time required to chill it, and so influences the pattern of microbial growth. At the completion of slaughter and dressing, beef carcasses tend to carry a smaller load of microbial contaminants than is found on sheep, pig, and poultry carcasses.

There are multiple factors responsible for reductions in *Salmonella* prevalence within slaughter plants. Size, congestion, and maintenance of a slaughter plant, the number of animals slaughtered per day, the flow of carcasses through the plant, control of each step in the sanitary dressing procedure, sanitation of facilities and equipment, and personal hygiene are factors to be considered to reduce pathogens on carcasses. If all these measures are not possible, at least not shortly, consideration should be given to eliminating pathogens on the meat after slaughter procedures. The interventions available to plants to reduce pathogens on carcasses include; lowering water activity, reducing surface pH, using enzyme inhibitors, cooling (refrigeration or freezing), applying lactic fermentation, irradiation, and treating with organic acids, chlorine and hot water, sodium chloride, or sorbate.

Lowering water activity; surface drying of carcasses reduces the water activity and inhibits microbial growth.

Reduction of surface pH and treatment with organic acids; a low pH ranging 4.0-4.5 inhibits the growth of both spoilage and pathogenic micro-organisms.[22] This pH reduction has often been achieved by treating meat with organic acids such as acetic or lactic acids. These acids cause a transient drop in surface pH and affect the micro-organisms thereon. Spray treatment with a solution containing 2% lactic acid and 20% sodium chloride produced a shelf-life of 28h in wrapped and 36h in unwrapped carcasses stored in ambient temperature.[181]

Treatment with chlorine and hot water; although there is a real decline in numbers of bacteria after chlorination it does not have a significant effect on shelf-life. Hot water treatment appears to be more successful provided the surface reaches a high enough (60°C) temperature for a sufficient period.[176] There will be a slight discoloration of the meat, which may be regained during the holding period.

Sodium chloride treatment; sodium chloride lowers the water activity and inhibits microbial growth and also it is used to flavor and preserve a variety of meats.

Sorbate treatment; the primary inhibitory action of sorbate is against yeasts and molds. Sorbate inhibits many bacteria including *Salmonella*, *Escherichia*, *Staphylococcus*, and *Clostridium*.[177] Sorbate treatments are used for controlling microbial growth in beef carcasses held at a temperature of 15°C. Chemical dips containing potassium sorbate substantially reduce the counts of bacteria on unchilled beef and on beef stored at 30°C and 20°C and extend the shelf-life up to 32h at 30°C and 68h at 2°C.[115]

Enzyme inhibitors; administration of epinephrine controls the post-mortem autolysis of meat by inhibiting catheptic activity at ambient temperature.[117] It was suggested that this process could be useful for long-term storage and for more efficient meat distribution at ambient temperature. Anti-autolytic activity of urea was demonstrated in meat kept at ambient temperature.[156]

Cooling; refrigeration is the most commonly used method for carcasses immediately after slaughter, during transport and storage and for packed meat. At refrigeration temperature (4°C) the self-life of properly packaged retail meat is 72h, after which some discoloration can be expected to appear, while the shelf-life of ground meat is only one day.[22] Carcass chilling rooms are normally operated in the temperature range of -2°C to -4°C (28-25F) with relative humidity of 88-92%.[22] The faster the air movement, the more rapid is the cooling. Accelerated cooling is achieved by using extremely low temperatures (-15°C to -35°C) or by spraying with or immersion in cryogenic liquids. Liquid nitrogen is the ideal cryogenic agent.[22]

Freezing; is an effective method of storing cuts of large carcasses, whole small carcasses, and retail cuts in a fresh state for extended periods. Marketing of frozen meat is unsuccessful due to the appearance of the product. Frozen meat will not give the appearance of fresh meat due to the ice crystal formation on the meat surface. The recommended storage temperature for frozen meat is -18°C (0F). Freezing must be rapid. Rapid freezing produces smaller ice crystals on the surface of meat and damage to the meat tissues is very much less.[22] Thawing the meat is also important which is the reverse of freezing. While the meat is thawing the watery drip occurs which contains proteins,

vitamins, and minerals. The less damage that occurs to the tissues during freezing and frozen storage, the less drip loss during thawing.[22]

Lactic fermentation; meat preservation is attributed to the combined effect of several substances (lactic acid, volatile acids such as acetic acid, antibiotics and bacteriocins) produced by lactic acid bacteria (LAB) though lactic acid plays a vital role.[137] It is a simple, low-tech and inexpensive method that can be practiced at ambient temperatures.

Irradiation; has good potential in the elimination of pathogenic and spoilage microorganisms from carcasses, cuts and minced meat and in the preservation of meat. It has emerged as a cost-effective method and finds a place in developing countries. WHO clarified in 1980 the medical acceptability of irradiated foods and said "no health hazard results from consuming any food irradiated up to a dose of one megarad (1Mrad).[47] Irradiation reduces microbial levels and pathogenic microorganisms and eliminates parasites like *Trichinella spiralis.*[47] The USA permitted irradiation in pork and poultry.[22] The UK has permitted irradiation only in poultry. Several other countries have also permitted irradiation in meat, fish, and poultry.

Packaging; packaging protects the meat from moisture loss, contamination by microorganisms, changes in color and physical damage. Packaging fresh meat varies from simple wrapping to advanced systems like vacuum packaging (VP) and modified atmosphere packaging (MAP).[22] Carcasses and large size meat cuts are wrapped in simple polyethylene films to protect them from contamination during handling. Fresh retail meat cuts are packed in pouches (polyethylene or polyvinyl chloride). These pouches allow oxygen transmission which maintains the bright red color of meat and

reduces the moisture loss.[22,56] Shelf-life of these meat cuts varies between 3 and 5 days at 4C. Vacuum packaging provides at least three weeks shelf-life for the product under adequate refrigeration but the product looks dark. When the package is opened it regains its bright red color because of exposure to air (oxygen). In modified atmosphere packaging (MAP) three principal gases are used; 10% carbon dioxide (inhibits bacterial and mold growth), 85% nitrogen (inhibits the oxidation of fats and mold growth) and 5% oxygen (prevents anaerobic spoilage).[22,56] The expected shelf-life of fresh meat in MAP is ten days.[22]

FEDERAL AND STATE STANDARDS FOR PATHOGEN REDUCTION AND CONTROL

Ensuring the safety of food is an enormously complex task. Hazards can arise at every stage of the food production process: from the farm to the processing facility, in transportation and storage, in food service and retail establishments, and in the homes of consumers.[97] During each of these steps along the way, measures must be taken to prevent or minimize hazards. On July 25, 1996, the U.S. Department of Agriculture (USDA), Food Safety Inspection Service (FSIS), adopted Pathogen Reduction, Hazard Analysis and Critical Control Point Systems (HACCP) to improve food safety for meat and poultry.[193] HACCP system requires a detailed analysis of the whole process from the farm through to slaughter.

The hazards are scored according to the magnitude of risk to the consumer and a judgement is then made as to the necessary control points needed to eliminate or minimize the hazards. Once the critical control points (CCP) are in place, a monitoring

system to ensure that the CCP are working should be maintained. This kind of system requires the cooperation and motivation of everyone involved in the chain and independent auditing to ensure that problems are not overlooked.

Although the HACCP system is intended as a means of eliminating or minimizing microbial hazards, other hazards such as residues, contaminants and parasitic infestations are all open to the same approach. The purpose of the pathogen reduction and HACCP regulation is to improve food safety. However, the regulation will also improve industry's ability to compete in international markets. The HACCP regulation is consistent with the General Agreement on Tariffs and Trade (GATT), which requires countries to ensure that their sanitary or phytosanitary measures are based on science and risk assessment principles. The combination of performance standards and HACCP enables the United States to objectively demonstrate that the level of protection the U.S. system provides is science-based, addresses likely hazards, and is equivalent to foreign requirements.[12,97]

The new rules apply to both slaughter and processing plants that handle meat and poultry, but the requirements could have an impact on dairy farmers. The new rule includes the following four major topics:

HACCP- Every plant must adopt and carry out its own HACCP plan that systematically addresses all significant hazards associated with its products.[192]

Pathogen reduction performance standards for *Salmonella*- All slaughter plants and plants producing ground products must ensure that their *Salmonella* contamination rate is below the current national baseline prevalence. This regulatory performance standard for a

pathogen on raw meat and poultry will ensure progress in reducing pathogenic bacteria.[192]

Mandatory *Escherichia coli* testing in slaughter plants- Every slaughter plant must regularly test carcasses for *E. coli* to verify the effectiveness of the plant's procedures for preventing and reducing fecal contamination. *E. coli* is the best microbial indicator of fecal contamination currently avaliable.[192]

Sanitation standard operating procedures (SSOP)- As the foundation for HACCP, every plant must adopt and carry out a written plan for meeting its sanitation responsibilities. Effective sanitation in slaughter and processing plants is essential to prevent adulteration of meat and poultry products.[192]

HACCP is a system that identifies potential food safety risks, prevents or corrects them, records actions, and verifies that it worked. HACCP is a systematic approach to controlling potential hazards in post-harvest food production. HACCP tries to identify problems before they occur and then establishes control measures that are critical for maximizing food safety at each stage in food processing and production.[36] The principles of HACCP implementation for food production processes have been identified by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) of the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture.[183] These principles[31,36,178,183] are:

Conduct an analysis of potential hazards- Plants determine the food safety hazards reasonably likely to occur and identify the preventive measures the plant can apply to control these hazards. A food safety hazard is any biological, chemical, or physical property that may cause a food to be unsafe for human consumption.

Determine critical control points for the targeted hazard and hazards- A critical control point (CCP) is a point, step, or procedure in a food process at which control can be applied and, as a result, a food safety hazard can be prevented, eliminated, or reduced to an acceptable level.

Establish critical limits for each CCP- Each CCP will have preventive measures that must be properly controlled to assure prevention, reduction to a tolerable level, or elimination of hazards. Each preventive measure has critical limits associated with it that serve as boundaries of safety for each critical control point.

Establish critical control point monitoring requirements- Monitoring activities are necessary to ensure that the process is under control at each critical control point. FSIS is requiring that each monitoring procedure and its frequency be listed in the HACCP plan.

Establish corrective actions for each critical operation when the control data indicate that the operation is out of control- These actions are to be taken when monitoring indicates a deviation from an established critical limit. The final rule requires a plant's HACCP plan to identify the corrective actions to be taken if a critical limit is not met. Corrective actions are intended to ensure that no product injurious to health or otherwise adulterated as a result of the deviation enters commerce.

Establish record keeping procedures- The HACCP regulation requires that all plants maintain certain documents, including its hazard analysis and written HACCP plan, and records documenting the monitoring of critical control points critical limits, verification activities, and the handling of processing deviations.

Establish a system of verification to document that the HACCP program is being followed- Validation ensures that the critical control points and associated critical limits

are adequate and sufficient to control likely hazards. Plants will be required to validate their own HACCP plans. FSIS will not approve HACCP plans in advance but will review them for conformance with the final rule.[31] Verification ensures the HACCP plan is acceptably. Verification procedures may include such activities as review of HACCP plans, CCP records, critical limits, and microbial sampling and analysis. FSIS is requiring verification tasks to be performed by plant personnel and varied by FSIS inspectors. Both FSIS and industry will undertake microbial testing as one of several verification activities.

The Pathogen Reduction and HACCP systems regulation requires testing for *Salmonella* and *E. coli*. Fecal contamination from the gastrointestinal tract, hide, and feathers are primary means for contamination of livestock and poultry carcasses with enteric zoonotic pathogens.[13,97] Major sources of carcass contamination during slaughter include rupture of the intestine or crop during evisceration, contact of the hide or feathers with muscle of the same or adjacent carcasses, and airborne spread of materials during hide pulling or feather removal (Figure 3).[49,155,205]

The sample collection procedures required for *Salmonella* and *Escherichia coli* testing are the same. Cattle and swine carcasses must be sampled at the end of the slaughter process in the cooler. A sampling sponge is used to swab a 10cm by 10cm area at three sites on beef carcasses (flank, brisket, and rump) and three sites on pork carcasses (belly, jowl, and ham). Poultry carcasses must be sampled after the chill tank at the end of the drip line or the last readily accessible point prior to packaging or cut up. Carcass sampling for poultry carcasses is a nondestructive whole bird rinse.

Microbiological Contamination During Slaughter [43]

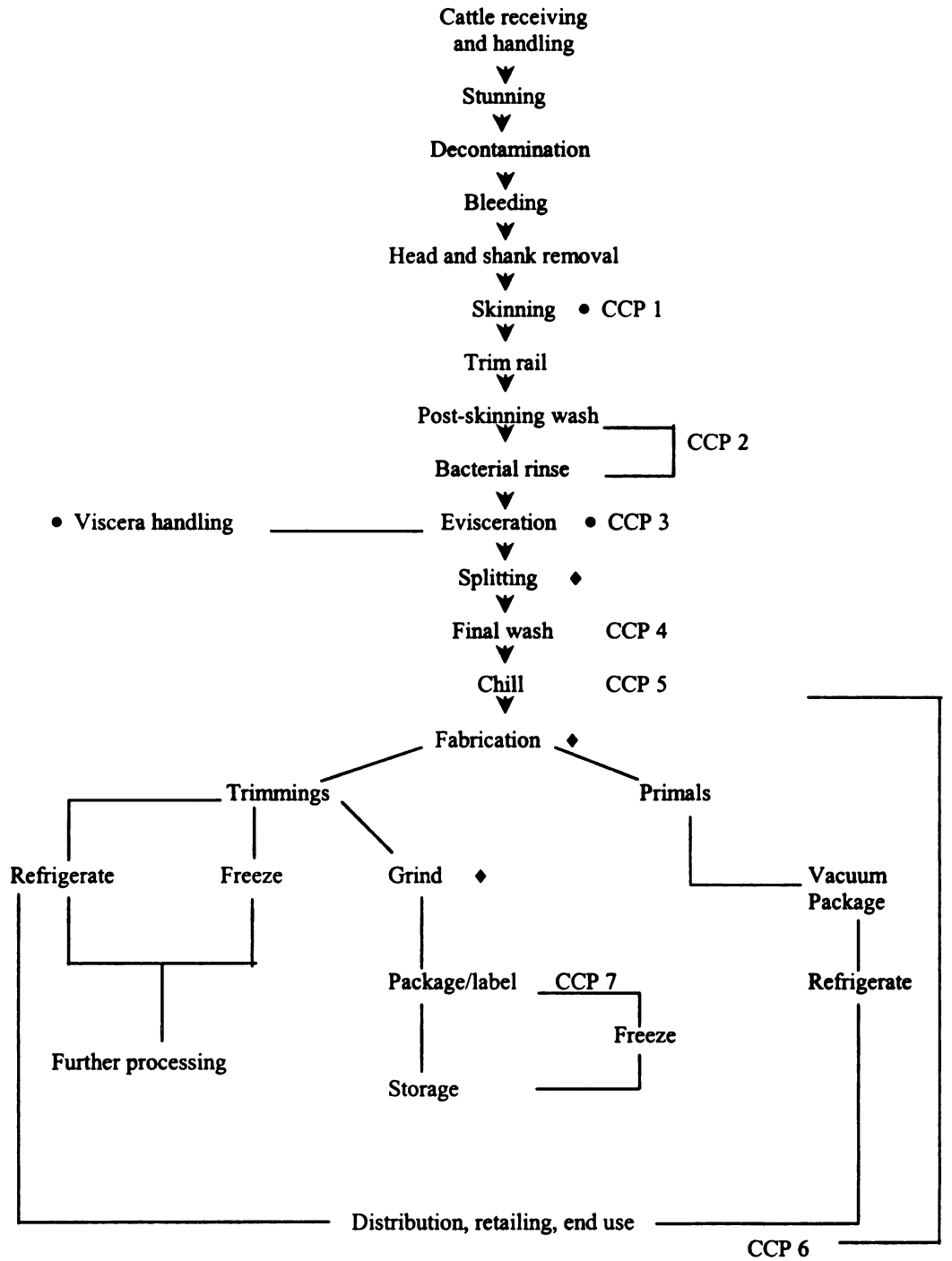


Figure 3: Generic HACCP for beef slaughter, fabrication and packaging. Potential site of minor contamination (♦); potential site of major contamination (•) (NAC, 1993).
•= Pens ; ♦= Holding

CHAPTER 2

ESCHERICHIA COLI

Escherichia coli O157:H7 as an important food-borne pathogen in human beings

Esherichia coli O157:H7 was first isolated in 1975 from a California woman with grossly bloody diarrhea.[160] The O157:H7 designates a serotype of the *E. coli* bacteria that was first identified as a cause of human illness in 1982 when 47 persons in Michigan and Oregon developed bloody diarrhea after eating hamburgers which were sold by a national fast-food chain.[37,87,160] Since 1982, more than 100 outbreaks of EHEC O157 have been documented, and of those outbreaks, 52% have been linked to food derived from cattle.[70]

Dairy cattle, especially young animals, have been implicated as a principal reservoir of *E. coli* O157:H7, with undercooked ground beef and raw milk being the major vehicles of food-borne outbreaks.[88,201,203] The public was generally unaware of *E. coli* O157's existence until a decade later, when more than 500 laboratory-confirmed infections occurred in four western States, also as a result of hamburger consumption.[1] Since then, several outbreaks of *E. coli* O157:H7 infection in the North America, Canada, United Kingdom, Japan have been reported. Because they cause bloody diarrhea and produce potent toxins, serotype O157:H7, O26:H11 and several others are classified as Enterohemorrhagic *E. coli* (EHEC). *Escherichia coli* O157:H7 is the EHEC serotype most often associated with human disease episodes and the EHEC most studied in food-producing animals.[160]

A single fast-food chain with restaurants in California, Idaho, Nevada, and Washington in late 1992 and early 1993 was associated with the largest outbreak involving ground beef. This western state outbreak resulted in more than 500 confirmed cases and four deaths.[45]

Between 1982 and 1995, *Esherichia coli* O157:H7 was implicated in 75 outbreaks involving 2,562 individuals.[45] The traceback studies support epidemiological data that link *Esherichia coli* O157:H7 with a bovine reservoir.[76,95,203]

ISOLATION AND PREVALENCE OF *E. coli* AT SLAUGHTER

Prevalence on farm

It is well known that Shiga Toxin-*E. coli* (Stx-*E. coli*) is commonly isolated from feces of clinically normal as well as diarrheatic cattle.[78,161] There is no direct evidence that *E. coli* O157:H7 is an animal pathogen.[217] There is great variation in *E. coli* O157:H7 prevalence in dairy cattle on farms. These variations are due to location of farms, herd management of the farms, age of the animals, seasonal effects, and isolation techniques. The prevalence of O157:H7 *E. coli* in cattle appears to be low, although the prevalence of other serotypes of Stx *E. coli* that are potential human pathogens are much greater. The prevalence of *E. coli* O157:H7 in the United States ranges from less than 1% to 61%.[58,125,217]

Verotoxin-producing *E. coli* in cattle was frequently detected on farm, but those isolates were comprised mostly of serotypes that have not been associated with human disease.[54,88,133] In Australian dairy herds, verotoxin producing *Escherichia coli* were

isolated from 16.7% of fecal samples from cattle.[211] Of those isolates, only 11.2% were serotype O157:H7 (prevalence of O157:H7 was 1.8%).[211]

In a survey of dairy herds in 14 different states from February to May 1993, *E. coli* 0157:H7 was isolated from 6 of 399 calves (1.5 %) that were between 24h old and weaning from 13 of 263 calves (4.9%) that were between weaning and 4 months.[203] In another survey of previously positive herds in the 11 states from June to August 1993, *E. coli* 0157:H7 was isolated from 5 of 171 calves (2.9%) that were between 24h old and weaning and from 7 of 132 calves (5.3%), that were between weaning and 4 months.[203,217]

Esherichia coli O157:H7 was isolated from 10 of 3,570 dairy cattle in the state of Washington (0.3%).[90] Another survey in 1991 and 1992, examined preweaned dairy calves in 28 states throughout the United States for *E. coli* O157:H7, and found that 0.4% (25 of 6,894) of the calves and 1.8% (19 of 1,068) of the herds tested positive.[96,217] In another study of nine farms that may have been sources of meat involved in an outbreak of hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura, five of the farms (55.5%) and 7 of 315 heifers (2.2%) tested positive for *E. coli* O157:H7.[202,203]

Although it is difficult to directly compare the results of previous studies because of different sampling and testing procedures employed, as well as missing information on the shedding of *E. coli* O157:H7, data from published reports suggests that the prevalence of *E. coli* O157:H7 in cattle ranges from 0.3 to 0.7% and the prevalence in cattle herds ranges from 1.8 to 16%.[90,96] In a study of 70 Wisconsin dairy farms, 5 of the farms (herd prevalence, 7.1 ± 4.5%) and 10 of 560 weaned calves (animal prevalence, 1.8%) tested

positive for *E. coli* O157:H7.[63] In this study, they sampled the weaned calves because in previous studies workers found that weaned calves are more likely to shed *E. coli* O157:H7 than cattle in other age groups.[76,90,212]

Hancock *et al.*[90] found a prevalence of less than 1% (0.28%) in over 3,500 fecal samples obtained from dairy cattle of various ages and a prevalence of 8.3% (5/60) of herds tested. *E. coli* O157:H7 was isolated from 2 of 1,273 lactating and 1 of 477 non-lactating dairy cows, and within the positive herds, *E. coli* O157:H7 was found in 1.7% (2/120) of lactating cows and 2.6% (1/39) of non-lactating cows.

In other studies the prevalence of *E. coli* O157:H7 in beef and dairy cattle on farms ranged from 0% to 68%, and the herd prevalence ranged from 1.8% to 100%.[48,168,210,212]

Prevalence at slaughter

Prevalence of *E. coli* in feces, hides and carcasses at and during processing may have been underestimated in the in the past, because of a lack of highly sensitive and specific methods for isolation of EHEC O157 from those elements.[70]

Studies[26,58] have been completed to determine the prevalence of EHEC O157 in cattle feces and on carcasses during slaughter processes. From cattle presented for slaughter in the United Kingdom, 0.83% of 6,495 bovine fecal samples in South Yorkshire found 4% of rectal swabs positive for EHEC O157. A study done at meat processing plants in Midwestern United States looked at the frequency of enterohemorrhagic *E. coli* O157:H7 in feces and on hides within groups of fed cattle from single lots that were going to slaughter, as well as investigating carcass

contamination.[49] From 29 lots that were sampled, 38% had positive hide samples, and 72% had at least one *E. coli* O157:H7 positive fecal sample. As a result the prevalence in feces and on hides was 28% (91 of 327) and 11% (38 of 355). There was a significant correlation between the prevalence of *E. coli* on hides and feces, and the frequency of carcass contamination indicating that control of *E. coli* O157 in live animal would have some potential for reducing rates of carcass contamination.[49] The carcasses were sampled at three points during slaughter: pre-evisceration, post-evisceration, and post-processing after the carcasses had been placed in the cooler.[29,58,158] Of 30 sampled lots, *E. coli* O157 was isolated from 87% pre-evisceration, from 57% post-evisceration (before antimicrobial intervention), and from 17% of post-processing samples.[58] The prevalence of *E. coli* O157:H7 at the three sampling points were as follows; 43% (148 of 341), 18% (59 of 332), and 2% (6 of 330). The decrease in carcass prevalence suggests that sanitary procedures of slaughter were effective in reducing bacterial load.[58]

More recent studies have isolated *E. coli* O157 from 3.6% and 13.4% of beef cattle, and 3.9% and 16.1% of dairy cattle at slaughter.[29,48,158]

Seasonal variation in *E. coli* O157:H7 excretion by cattle was demonstrated in several epidemiological studies. Shedding of *E. coli* O157:H7 at slaughter was significantly higher in summer than any of the other seasons. The prevalence of *E. coli* O157:H7 in fecal samples at slaughter was 19.7% in the summer and 0.7% in the winter (Figure 4).[48]

Prevalence of *E.coli* O157:H7 in cattle by season

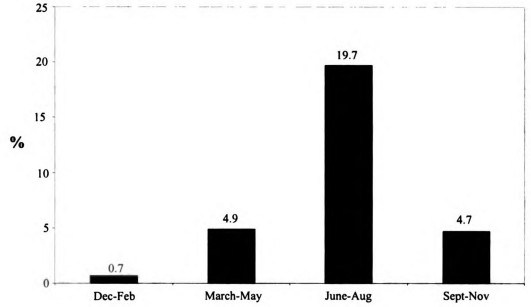


Figure 4: Prevalence of *Escherichia coli* O157:H7 in fecal samples from yearling cattle and cull cows at slaughter in Alberta by season.[48]

IDENTIFICATION AND IMPROVED METHODS OF ISOLATION IN THE INDUSTRY OF MONITORING

Esherichia coli, a member of the family Enterobacteriaceae is considered to be a part of the normal microflora of the intestinal tract of humans and most warm-blooded animals. They are Gram-negative, straight rods, (1.1 - 1.5μm x 2.0 - 6.0μm) that are oxidase negative.[5] Organisms of this species are generally lactose fermenters, but sometimes lactose fermentation is delayed.[131]

The enterohemorrhagic *E. coli* prototype, *E. coli* O157:H7, like all *E. coli*, is typical of the species (the O refers to the somatic antigen, and H to the flagellar antigen),

with the exception of sorbitol fermentation and β-glucuronidase activity.[52,109,201] About 93% of *E. coli* isolates of human origin ferment sorbitol within 24h; however, *E. coli* O157:H7 does not.[201] Additionally, 93% of *E. coli* strains posses the enzyme β-glucuronidase that is the basis for a rapid fluorogenic assay for *E. coli*.[67] This assay uses 4-methylumbelliferyl β-D-glucuronide (MUG) as an indicator which is hydrolyzed to a fluorogenic product by the enzyme β-glucuronidase. β-glucuronidase activity is not phenotypically expressed by these organisms.[67,109]

E. coli grow rapidly between 30-42°C, with generation times ranging from 0.49h at 37°C to 0.64h at 42°C.[52] The organism grows poorly at 44-45°C and does not grow within 48h at 10 or 45.5°C.[52,154] The organism can survive well in ground beef during frozen storage at -20°C. There is no major change in populations of *E. coli* O157:H7, in ground beef frozen at –80°C and held at –20°C for up to 9 months.[52] The organism is not unusually heat resistant (e.g. cooking ground beef to well-done to kill typical strains of *Salmonella* will also kill *E. coli* O157:H7).[209]

Escherichia coli usually remains confined within the intestinal lumen of mammals as a harmless saprophyte, but in the debilitated or immunosuppressed host or in the immunologically normal host with disruption of critical anatomical barriers, normal intestinal strains of *E. coli* are major causes of opportunistic infections. These strains cause diarrhea and other symptoms in humans and warm-blooded animals by producing different toxins. Researchers[112,119,160] have demonstrated that toxins produced by strains of *E. coli* O157:H7 are cytotoxic for vero (African green monkey kidney) cells. Infection with verotoxigenic strains of *E. coli* in people can cause diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, renal failure and death. Verotoxins are heat-labile proteins

that produce an irreversible cytopathic effect in vero cells.[112,119] Verotoxins have two subunits; an A (active) subunit and several B (binding) subunits. The B-subunit of the verotoxins binds to a receptor on the surface of a cell. The A-subunit is then internalized in the cell and cleaves to an active fragment, and then inhibits cellular protein synthesis. Because of their close homology to Shiga-toxin, VT1 and VT2 are often referred to as Stx-I and Stx-II. *Escherichia coli* strains that produce verotoxins or shiga-toxins have been referred to as verotoxigenic *E. coli* (VTEC), Stx-producing *E. coli*, and enterohemorrhagic *E. coli* (EHEC) (Table 7, Figure 5).

Table 7: Classifications and characteristics of *Escherichia coli* of the gastrointestinal tract in people.[37,109,148]

CLASSIFICATIONS	CHARACTERISTICS
Enteropathogenic *E. coli* **(EPEC)**	Appears to destroy microvilli without further invasion; bundle-forming pili mediating localized adherence; type III secretion system mediating attaching and effacing lesions. Only a minority of these organisms produce verotoxins. Human are the main reservoir.
Enteroinvasive *E. coli* **(EIEC)**	Invades and proliferates within epithelial cells and causes cell death. Human are the main reservoir.
Enterotoxigenic *E. coli* **(ETEC)**	Penetrates the mucous layer of the proximal small intestine. The organism adheres to mucosal cells and produces heat-stable or heat-labile enterotoxins. This type frequently causes traveler's diarrhea. Human are the main reservoir.
Enterohemorrhagic *E. coli* **(EHEC)**: *E. coli* O157:H7 and *E. coli* O126:H11	This is the most important group of *E. coli* in terms of food-borne disease. Shiga-toxins; type III secretion system mediating attaching and effacing lesions. Three principal syndromes have been linked to *E. coli* O157:H7; hemorrhagic colitis, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP). Its main animal reservoir is the rumens and intestines of cattle and sheep.
Enteroaggregative *E. coli* **(EAEC)**	The EaggEC strains have the ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. They produce a heat-labile-plasmid-encoded toxin (EnteroAggregative ST or EAST).
Diffusely adherent *E. coli* **(DAEC)**	Fimbrial and afimbrial adhesins; elongation of microvilli. Diarrhea in older children.
Uropathogenic *E. coli* **(UPEC)**	Virulence factors P and other fimbriae; hemolysin. Urinary tract infections.
E. coli that cause neonatal meningitis **(NMEC)**	Virulence factors capsules; S-fimbriae; cellular invasion. Sepsis and meningitis in neonates and infants. Transmission person-to-person.
Verotoxigenic *E. coli* **(VTEC)**	Strains of *E. coli* produce heat-labile toxins that are cytotoxic for vero cells in an invitro assay.

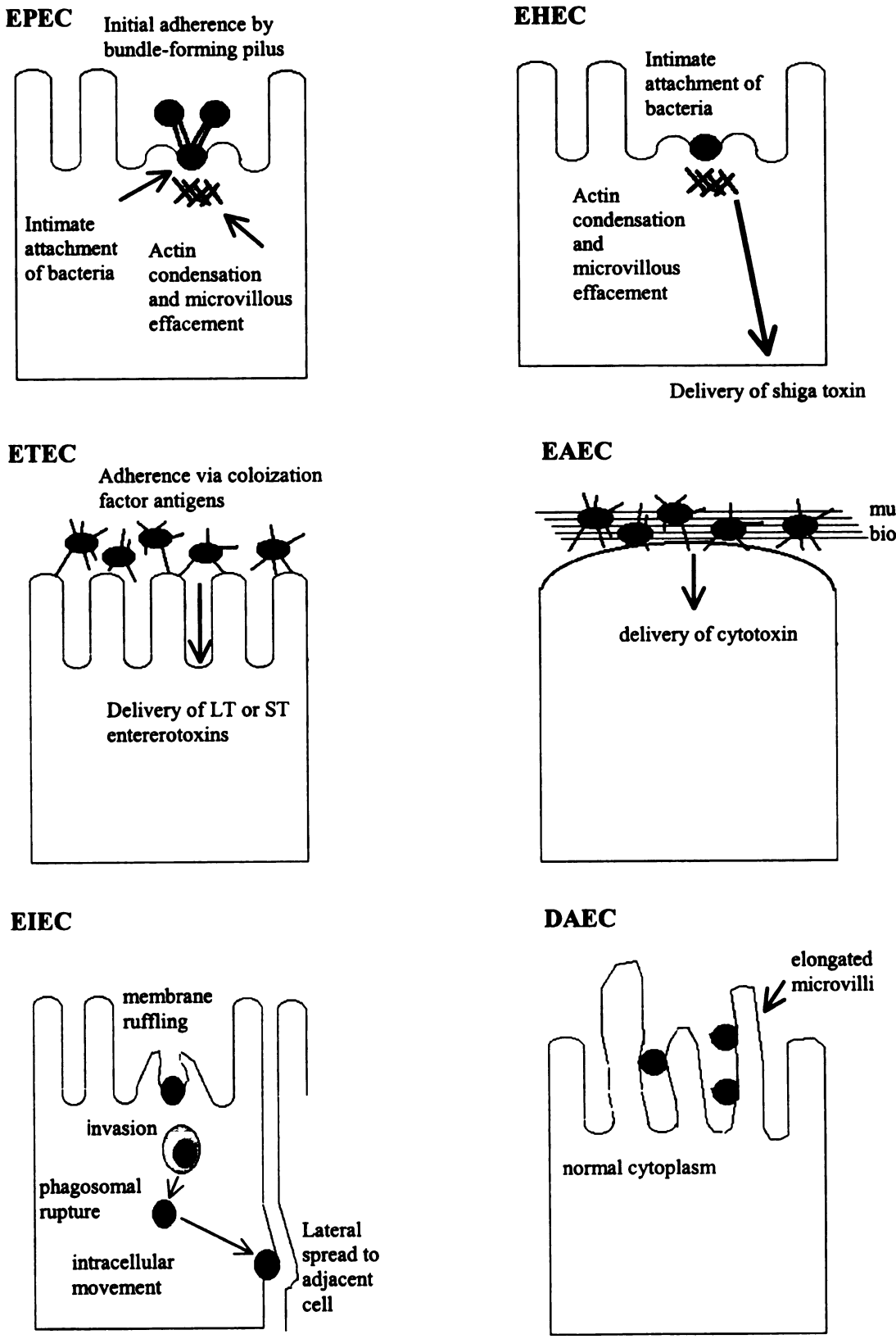


Figure 5: The molecular pathogenesis of *Escherichia coli* infections.[21]

For detection of *E. coli* O157:H7 , fecal and rumen content suspensions are placed on the primer media, MacConkey (MAC) and sorbitol MacConkey agar (SMAC). Lactose positive organisms (coliforms) are identified on MacConkey agar, as colonies that appear in 24h; are flat, pink to red and 2-4mm in size. Colonies have a characteristic pink halo of precipitated bile salts surrounding them. These colonies are transferred to sorbitol MacConkey agar and incubated at 37°C overnight. Many screening methods used for the isolation of EHEC from food products and stool specimens utilize sorbitol MacConkey agar (SMAC) as the primary isolation medium.[17,23,64] Sorbitol MacConkey medium is designed to detect only this serotype; *E. coli* O157:H7. Since *E. coli* O157:H7 does not ferment sorbitol within 24h and does not posses β-glucuronidase activity the pale white colonies will appear on the medium next day. Colonies that are sorbitol-negative are selected and confirmed as *E. coli* O157:H7 by morphology, biochemical, serological and vero cell cytotoxicity assays. Some of the biochemical tests used include; Triple Sugar Iron Agar (TSI), motility test, Indol test, Ornithine test, Simmons' citrate agar, Oxidase test, and Urease test to differentiate *E. coli* from *Salmonella, Klebsiella, Proteeae, Shigella*, and *Serratia* (Table 8,9). *Escherichia coli* can be serogrouped by slide, tube, or latex agglutination to detect the O157 antigen. Serotyping can be completed by looking for immobilization in H7 antisera-containing motility media; however, some strains may be nonmotile (H^-).

Table 8: Identification of coliforms and related organisms.[62]

Characteristic	Strong Lactose(+)			Weak Lactose (+)	Lactose (-)		
	E coli	*Klebsiella**	*Enterobacter**	*Citrobacter*	*Proteus**	*Serratia*	*Pseudomonos*
Oxidase	-	-	-	-	-	+	+
Motility	+/-	-	+	+	+	+	+
Indole	+	-/+	-	-/+	+	-	-
Methyl red	+	-	-	+	+	-	-
Voges-proskauer	-	+	+	-	-	+	-
Citrate	-	+	+	+	d	+	-
Urease	-	+	-	d	+		-
Lysine Decarboxylase	+/-	+	+	-	-	+	-
Ornithine Decarboxylase	d	-	+	d	-	+	-

+/- = most strains positive;
-/+ = most strains negative;
d = different biochemical types;
* = some species of the genus will show different reactions for some of the tests.

Table 9: Growth characteristics of *Shigella* on selective media.[55]

Organism	Medium	Colony Appearance
E. coli	MacConkey	Lactose fermentor; flat, pink colonies surrounded by darker pink region (indicates sorbitol fermentors, non-sorbitol fermentors form colorless colonies)
	Hektoen	Yellow
	XLD	Yellow
Salmonella	MacConkey	Colorless (lactose non-fermentors)
	Hektoen	Green
	XLD	Red with black center
Shigella	MacConkey	Colorless (lactose non-fermentor)
	Hektoen	Green
	XLD	Colorless

XLD: Xylose-lysine-desoxycholate agar

Although the inclusion of pre-enrichment incubations and immunomagnetic separation (IMS) and additional selective subculturing or secondary enrichment incubations have been reported to increase the detection rate of *E. coli* O157:H7 from foods and fecal specimens, these methods are dependent on isolating individual colonies from selective and/or indicator media and then characterizing them using immunological and biochemical/fermentation reactions.[7,28,30,72,110] In the use of immunomagnetic (IMS) method, O157-specific antibodies attached to superparamagnetic polystyrene beads (anti-*E. coli* O157 Dynabeads; Dynal, Inc., Lake Success, N.Y.) are added to an enriched sample and subsequently subjected to magnetic separation. *E. coli* O157 organisms, if present in the enrichment culture, are removed with the magnetic beads.[55,109] The sediment containing the bacterium-coated beads is streaked to selective plating medium such as SMAC or CT-SMAC (potassium tellurite and cefixime added to SMAC).[109] IMS increased the detection rate of O157 by 65% in a sampling of specimens associated with several outbreaks and was superior to molecular methods such as polymerase chain reaction (PCR), cytotoxicity assays, and direct plating.[109] IMS has the advantage of being simpler and easier to perform than similarly sensitive methods, such as PCR assays, and leads to the isolation of the targeted organism.[55,109] However, it will select only for *E. coli* O157 and not for other serogroups of STEC (Stx-producing *E. coli*), unless the beads are coated with antisera for the specific serogroup to be isolated.[109]

Antibody-based methods for the immunologic detection of STEC include colony immunoblot assays and antibody capture or toxin receptor-mediated EIAs. Immunological assays are used to determine if the O157 somatic and H7 flagellar antigens are present, while the biochemical/fermentation reactions determine in classical

taxonomic fashion the genus and species of the isolate. Combined with the initial replication steps in the isolation process, the current *E. coli* O157:H7 identification process takes 5 or more days to complete.[105,146,170] This adds considerably to the cost required to determine whether a sample contains *E. coli* O157:H7 and is a limiting factor in doing a large number of *E. coli* O157:H7 tests. A commercial EIA kit (Premier EHEC; Meridian Diagnostics, Inc., Cincinnati, Ohio) for detecting Stx in suspensions of stool or fecal enrichment cultures has recently become avaliable.[55,109,190] Another commercially available product is a latex agglutination kit (VTEC-RPLA; Denka Seiken Co., Tokyo, Japan), which detects both Stx-I and Stx-II from culture supernatants.[55,109] These commercial methods are easy to perform and do not require specialized equipment and laboratory skills, unlike cell culture and DNA-based methods.[50,55,64,109,113, 197]

Nucleic acid-based assays for detection of *E. coli* O157; colony hybridization assays with DNA probes for Stx genes, the 60-Mda plasmid present in *E. coli* O157, and the *eae* gene have been used to detect and characterize STEC.[105,109,146,170] Rapid methods for identifying *E. coli* O157:H7 in foods or fecal specimens have been directed at immunological or genetic targets. Antigenic targets have included the *E. coli* somatic (O157) or flagellar (H7) antigens, two low-molecular-weight antigens, and the virulence-associated Stx types I and II.[50,64,113,197] However, these assays are occasionally unable to distinguish certain other *E. coli* strains from *E. coli* O157:H7 strains and/or toxigenic from nontoxigenic *E. coli* O157:H7 strains.[105,146,170]

Most *E. coli* O157:H7 carry a 60-megadalton plasmid. This plasmid is required for expression of fimbrial adhesion and adherence to Henle 407 intestinal cells.[54] Toth *et al.*[189] developed a direct ELISA for detection of *E. coli* O157:H7 and other Stx-producing

E. coli.[189] The assay is based on the presence of two proteins of 82 and 92 kDa in *E. coli* which are associated with the 60-MDa plasmid commonly carried by VTEC. Hence, this ELISA is a specific test for detecting 60-MDa plasmid-associated proteins. The method works well in pure cultures.[128]

A monoclonal antibody (MAb) 4E8C12 specific for enterohemorrhagic *E. coli* of serotypes O157:H7 and O26:H11 was produced and characterized by Padhye and Doyle.[144] Using this MAb, a rapid and sensitive procedure was developed for the detection of *E. coli* O157:H7 from food in less than 20h.[144] The procedure involves enrichment of a food sample in a selective enrichment broth for 16-18h at 37°C with agitation. Enrichment culture is applied to a sandwich-enzyme-linked immunosorbent assay (ELISA) procedure that has a polyclonal antibody specific for *E. coli* O157 antigen as the capture antibody and the MAb 4E8C12 as the detection antibody.[144,189] In addition to being highly specific, sensitive, and rapid, this procedure is easy to perform and is amenable to use by laboratories performing routine microbiological testing. Also no cross-reactivity was observed with strains of *Salmonella* spp., *Yersinia enterocolitica, Shigella dysenteriae, Proteus* spp., *Escherichia hermanii, Klebsiella pneumoniae, Campylobacter jejuni, Serratia marcescens, Citrobacter spp., Enterobacter cloacae, Hafnia alvei, Aeromonas hydrophila,* and all except five strains of *E. coli* other than serotype O157:H7 (including strains of serotype O157 but not H7).[109,144]

A polymerase chain reaction (PCR) procedure for rapid and specific detection of verotoxin genes in *E. coli* was developed by Pollard *et al.*[150] PCR is an in vitro method for amplifying specific nucleic acid sequences by repeating cycles of DNA synthesis over a period of hours. Polymerase chain reaction-based detection procedures have been used

to identify *E. coli* O157:H7 and have targeted the Stx-I and Stx-II genes, the enterohemorrhagic *E. coli* (EHEC) *uidA* gene, and a portion of a 60-megadalton plasmid. With this procedure, DNA is amplified to increase the level of target DNA when VTEC are present in very low numbers. A variety of PCR amplification methods have been developed for detecting and characterizing STEC. Recently, Ganon *et al.*[75] reported the development of two multiplex PCR assays; one provides identification of *E. coli* O157:H7, other STEC strains, and potential enteropathogenic *E. coli* (EPEC) isolates; the other provides identification of only *E. coli* O157:H7 and other STEC strains. In the former assay, these organisms are detected by targeting Stx-I, Stx-II, a region of the *eae* gene that is conserved between STEC and EPEC, and H7 antigen-specific sequences in the flagellin gene, *fliC*.[50,64,75,109,113,197] The polymerase chain reaction technique is both sensitive and specific, hence it may be useful for rapidly screening clinical specimens for VTEC. But PCR can suffer from common problems such as contamination, the presence of inhibitors of the polymerase enzyme, and undesirable reaction conditions that influence laboratory assay detection limits, assay sensitivity, and assay specificity, which can lead to false-negative or false-positive test results.[188]

Although PCR can amplify DNA molecules thousands-fold, the specifically amplified product must be detected in order to prove its presence, and a variety of methods have been developed for this purpose. The most commonly used research technique, gel electrophoresis, does not show the specificity of the PCR and lacks sensitivity. Southern blots or dot blot hybridizations with probes demonstrate the specificity of the PCR, but they require multi-step processing and add considerable time

and expense to the detection process. Neither of these PCR detection processes is conducive to rapid, high-throughput, automated PCR detection schemes.[100]

METHODS OF CONTROL OF ORGANISM

Organism shedding and control on farm

Dairy cattle are the main reservoir of *E. coli* O157:H7. Also a wide variety of animals including sheep, and deer may carry the organism. In the farm environment, weaned calves have the highest rate of *E. coli* O157 shedding. Calves are exposed to *E. coli* during the first week of life and the infection rate after this exposure is generally steady, until weaning time around 8 weeks of age.[32,63,76,109,211] The pre-weaning period may play an important role in determining the farm prevalence of *E. coli* O157:H7. The prevalence of *E. coli* O157 in calves less than 8 weeks old was 1.4% and in calves 8 weeks or older was 4.8%.[76] Calves were three times more likely to shed *E. coli* O157 after weaning than before weaning.[76,212]

Farm management in the pre-weaning period is very important in controlling the overall rate of *E. coli* O157:H7 infection in herd. Farms in which calves are kept in groups from birth to weaning or grouped before weaning have higher rates of *E. coli* O157:H7 shedding.[32,76] Crowding may create stress in calves that are already susceptible to infections. Stress and nutritional deprivation increases the shedding of *E. coli* O157:H7.[76] Close contact with other calves and their by-products facilitates infection by the fecal-oral route. It is natural for calves to lick and suckle each other in the pre-weaning period.[76]

Another control point that influences *E. coli* O157:H7 shedding involves the feeding practices used on the farm. Sharing nipples and bottles between calves without rinsing or cleaning may contribute to spread of infection among calves. Also the use of open pails instead of nipple bottles for feeding calves is associated with increased rates of *E. coli* O157:H7 shedding.[211]

Composition of the feed can affect shedding of *E. coli* O157.[91] Some studies report conflicting results such as the inclusion of cottonseed which was reported by Garber *et al.*[76] to have a negative relationship with *E. coli* O157 shedding and yet Herriot *et al.*[99] could not find any association between cottonseed feeding and *E. coli* shedding by calves (even though cottonseed is toxic for baby calves) in their 1977 study. However, they did find positive relationships between feeding corn silage, grain screenings and ionophores, and *E. coli* O157:H7 shedding. Corn silage may provide a moist environment suitable for bacterial growth of *E. coli* O157. Also ionophores in the cow ration create an environment that favors the development of Gram-negative intestinal flora. Ionophores alter the ratio of proprionic acid and acetic acid in the rumen but their effects again are controversial.[76,99]

Hancock *et al.*[11,123] found that previous irrigation of grazing land with fecal slurry is a positive risk factor for carriage of *E. coli* O157 in a dairy herd. If fecal slurry is used on grazing land some months should elapse between spreading of slurry and grazing of animals.

In a survey[63] conducted to determine the prevalence and to identify the sources of *E. coli* O157:H7 isolates on Wisconsin dairy farms, only animal drinking water was identified as a non-fecal source of *E. coli* O157:H7 within the farm environment.

Because water can serve as a reservoir for *E. coli*, farm managers should take extra precaution to protect water sources from fecal contamination.

Vaccination is another control option on the farm against *E. coli* O157:H7 as in the case of other Gram-negative bacteria.[91] The purpose of vaccination would be to reduce the susceptibility of cattle to colonization with *E. coli* O157:H7 or to decrease the duration of such colonization.[91,109] This would presumably require targeting an adherence or other surface antigen with a mucosal immune response.[109] Currently, it is not clear if this is theoretically feasible, and a number of practical problems would need to be addressed even if effective immunization could be demonstrated under controlled conditions. Fimbrial vaccines are used especially by parenteral administration to pregnant cows to protect neonatal calves by increasing antibody in colostrum and milk.[91,109,134]

Another control point involves "niche engineering". Modifying the environment to make an ecosystem less susceptible to sustaining a particular agent has been called niche engineering.[109] For *E. coli* O157:H7 on cattle farms, the best candidates for niche engineering are related to feed and water trough management. Preventing multiplication of *E. coli* O157:H7 in moist cattle feeds would decrease exposure doses.[91,122] Frequent cleaning and appropriate sanitation of water troughs can reduce replication and maintenance of *E. coli* O157:H7 in sediments.[91]

SOURCE OF *Escherichia coli* O157:H7 INFECTIONS IN HUMAN BEINGS

The hemolytic uremic syndrome which is caused by O157:H7 was first described in 1955.[78] Zoonotic transmission of O157:H7 is thought to occur because epidemiologic

studies have found associations of HC and HUS with ingestion of ground beef and consumption of raw milk,[18,125,201,203] although outbreaks also have been associated with fresh-pressed apple cider,[10] unchlorinated drinking water,[180] and person-to-person transmission.[88,147] Among the 8 outbreaks with an identified food vehicle in the U.S., 6 were traced to ground beef and 2 to roast beef.[109,147,204]

Beef, beef products and untreated milk have been suggested as possible sources of VTEC (verotoxin-producing *E. coli*) infection for man.[58,63,125,160] In May-June 1992 there were 5 cases of VTEC O157 human infection in the Sheffield area of England, 3 of phage type 2 and 2 of phage type 8 that may have been associated with consumption of beef originating from a South Yorkshire slaughter plant. *E. coli* O157 was subsequently isolated from the rectal contents of 84 (4%) of 2103 cattle at the slaughter plant and it was suspected that they were the source of this organism. Seventy-eight (93%) of the 84 isolates were VT(+) and were of the same phage type, toxin and plasmid profile as strains implicated in human disease in the same area.[135] A comparison of human and bovine *E. coli* O157:H7 isolates by toxin genotype, plasmid profile, and bacteriophage λ-restriction fragment length polymorphism profile has linked 5 sporadic human cases to bovine origin.[184,203]

Other than fecal shedding, *E. coli* O157 has been isolated from beef carcasses (from excised meat and from the surface of carcasses) in slaughter plants and these isolated strains have been linked to human cases in the UK.[26]

An outbreak of VTEC O157:H7 infection among 60 children and 14 adults who had visited a dairy farm resulted in 48 cases of diarrhea and 3 of the affected people developed HUS. A significant association was found between infection and the

consumption of unpasteurized milk. VTEC O157:H7 was isolated from a fecal sample from one animal on the farm. All these findings provide direct evidence that cattle may be one of the main reservoirs of VTEC O157:H7 bacteria that are associated with human disease.[18]

EPIDEMIOLOGY OF *Escherichia coli* O157:H7

After HC and HUS outbreaks, in the course of investigation to identify the source of infection, *E. coli* O157:H7 was isolated from 5 (5.9%) fecal samples from 85 heifers and calves and none of 141 adult cows on two dairy farms in Wisconsin. Also in another HUS outbreak a related survey in dairy farms in Washington State revealed that 2.2% of 315 heifers and calves and none of 224 adult cows had *E. coli* O157:H7 in their feces.[202] Culture surveys not related to outbreaks were also conducted. The rate of *E. coli* O157:H7 isolation has been 15% or less in most surveys, with generally higher rates up to 5% in heifers and calves.

Escherichia coli O157:H7 has been isolated only rarely from animals with diarrhea, and it was not known whether the *E. coli* was the cause of the diarrhea or not.[87,160] Therefore, no animal illnesses have been conclusively attributed to *E. coli* O157:H7. Other EHEC serotypes, however, have been isolated from calves with bloody diarrhea.[51] The lack of observable illness in food-producing animals that are shedding *E. coli* O157:H7 in their feces, makes it more difficult to identify carrier animals so that they can be removed from the food chain.[40,172]

In herds, prevalence of fecal shedding of *E. coli* depends on; age of the cattle, pre- or post-weaning status of calves (calves older than 3 weeks do not develop attaching-and-

effacing A/E lesions with *E. coli* O157:H7, and a large inoculum is required to infect adult cattle), ecology of environment, effects of fasting-dietary, stress and seasons.[17,48,93,94,109] The highest prevalence of fecal shedding is seen at 8 weeks of age, although calves are less likely to be *E. coli* positive before weaning than after weaning, the pre-weaning period may play an important role in establishing and maintaining *E. coli* shedding on the farm. Herds in which calves are housed in groups from birth to weaning or in which previously individually housed calves are grouped before weaning are more likely to include calves that shed *E. coli*.[58,93,204]

One explanation can be related to stress from crowding and competition triggering shedding of organisms. A second possibility may be that multiple calf facilities can concentrate the bacteria that are shed. Sharing nipples and bottles among calves without rinsing or washing also may serve to spread infection from one calf to another.[58,76]

Certain feeding practices were negatively associated with *E. coli* shedding. None of the 6 herds in which clover hay was fed, were positive for *E. coli*. Additionally, 7 of 8 herds in which heifers were pastured on clover were negative for *E. coli*. Feeding whole cottonseed to heifers prior to first calving also was negatively associated with carriage of *E. coli*, with all of 7 herds were negative of *E. coli* in which this feeding practice was reported.[76] Shedding of *E. coli* was not associated with any of the signs of illness evaluated (poor condition, dehydration, diarrhea). These results were consistent with those of other studies[27,203] in which *E. coli* was found in healthy cattle.

Escherichia coli can not grow in the rumen under normal conditions because of rumen pH and volatile fatty acid (VFA) concentrations.[195] However, during times of

food deprivations or other nutritional stress, inhibitory conditions of the rumen are eliminated as pH rises and VFA concentration declines.[102,204] Such conditions allow enteric bacteria to survive and even multiply in the rumen (especially when feeding is briefly resumed after feed deprivation). Thus, rumen contents can become a reservoir of enteric pathogens. For that reason any stress factor, transportation from farms to slaughter plant, food deprivation, or illnesses, can cause an increase in *E. coli* shedding. Prevalence studies should be done in slaughter plants as well as on farms.[157,204]

CHAPTER 3

PREVALENCE OF *Escherichia coli* IN DAIRY CATTLE AT SLAUGHTER

ABSTRACT

The objectives of this study were to evaluate the prevalence and concentration of enterohemorrhagic *Escherichia coli* (EHEC) in feces of dairy cows at slaughter, and to evaluate the effects of winter and summer on the isolation of pathogens from dairy cows at slaughter. Samples were collected at slaughter from cattle that had either been shipped directly to slaughter or that had been sold through auction markets. Fecal samples from 1006 cows were collected in two seasons, winter and summer of 1996 to study *E. coli* shedding patterns. *Escherichia coli* isolated were analyzed with respect to animal disposition including body condition score, animal health, source of the animal, and season of the year. *Escherichia coli* was isolated from 829 of 1006 fecal samples. Sorbitol-negative *E. coli* was isolated twice as often (199/496) in summer 40%, compared to 22.6% (59/260) in winter. Of the 265 sorbitol-negative *E. coli,* six samples were positive (0.022%) by ELISA to EHEC and two were serotype O157:H7. Forty-five fecal samples were positive only for *Klebsiella* sp; 34 in winter and 13 in summer.

INTRODUCTION

This project was undertaken to evaluate the effects of: 1) season, 2) body condition score (BCS), 3) health (lameness, respiratory, non-ambulatory), and 4) route of shipping to slaughter, on shedding of *E. coli* in feces at slaughter. Earlier reports

investigated seasonal effect on fecal shedding of *E. coli*. Two studies reported the peak prevalence of fecal shedding of *E. coli* was most common in summer and early fall.[91,92] Other studies reported the highest occurrence of *E. coli* in the feces of cattle in the summer.[48,93,94,95]

Transmission of pathogens between animals during marketing, transport and waiting prior to slaughter is a possibility. Studies preformed by Brownie and Grau[19] in 1967, working with cattle and Grau *et al.*[85] in 1969 working with sheep, found evidence suggesting that transmission of *E. coli* between animals can occur at markets and during transport, especially if the time from farm to slaughter is prolonged. Another study[137] suggested that cattle that were fasted prior to slaughter were more susceptible to colonization with *E. coli* O157:H7. However, a Canadian study[48] did not show fasting as an important risk factor for the increased fecal shedding of *E. coli* O157:H7 in slaughter cattle under existing commercial transport.

The differences in prevalence estimates may be due to the factors listed above and may be affected by the time of year that the study was conducted and the geographical location of the study. In other studies[48,174] fewer *Escherichia coli* O157:H7 organisms were shed by feedlot cattle near the end of the feeding period than by newly arrived cattle. Moreover, there is less shedding of the organisms in cattle of slaughter age than in younger cattle. The prevalence of *E. coli* O157:H7 in feedlot cattle is similar to that in range cattle.

SAMPLE COLLECTION AND PROCEDURES

Slaughter animal collection

A total of 1006 fecal samples were collected. One group of 501 samples was collected during 6 days in February and a group of 505 samples was collected during 6 days in August. All samples were collected from cattle delivered to the same slaughter facility in Allendale, MI. Fecal samples were collected from the cecal-colon juncture of each study cow on the viscera table at slaughter. For each sample, a single-edged razor was used to make a linear incision and cecal-colon contents were collected and stored immediately on ice in sterile plastic bags. The specimen was approximately divided in half and each half placed in a separate sterile plastic bag which was labeled with the sample number, date, market tag number and location code. Samples were held on ice prior to being transported to the laboratory within 24h. From each cow one sample that was to be examined for *Salmonella* spp. was shipped directly on ice to USDA-Animal Health Laboratories (Ames, Iowa) by overnight Federal Express mail. The other samples which was held on ice examined for *E. coli* within 24h at the MSU laboratory.

Prior to slaughter each cow was examined for breed, body condition, ambulatory score (L= obvious lame, NR= moves easily), hide score (C= clean, D= dirty), udder condition (NR= normal, UP= problem), respiratory problems (NR= normal, RRP= problem), vulva problems (NR= normal P= problem), eyes (NR= normal, OD= defective), gastrointestinal problems (NR= normal, D= diarrhea), lumps (Y= yes, NR= normal). The same person performed the examination on each cow.

Sample preparation and handling for *Escherichia coli*

Upon arrival at the laboratory, 1.0g of feces was diluted in 9ml of peptone broth. This mixture was vortexed to assure a uniform dilution. Fecal debris was removed by straining the solution through sterile gauze. Fecal samples (1.0g) were serially (1:10) diluted in 9ml of peptone broth (0.85% PBS to 10^{-7} CFU/g.). Because of the heavy concentration of microorganisms 7 dilutions were made to facilitate counting. The dilutions were prepared by adding 0.2ml of strained fecal solution to a tube containing 1.8ml peptone broth and mixing thoroughly by vortex and serially transferring 0.2ml of the resulting mixture into1.8ml for seven dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}). The diluted samples were stored overnight at 4°C and plated on agar the next day. In the winter, 200/501 fecal samples were stored at -20°C for 5-7 days to accommodate the laboratory's culture capacity. Results between frozen and fresh samples were compared.

Media and organism isolation and detection

Culturing. All sorbitol-negative and EHEC positive *E. coli* samples were further screened with O157 antiserum. *E. coli* that were positive for the O157 serotype were further tested with H7 monoclonal antibody. A 0.1ml sample from each of the seven dilutions was plated on MacConkey medium to isolate lactose-fermenting microorganisms (coliforms) and on Sorbitol-MacConkey medium to identify sorbitol non-fermenting colonies. Plates were read after 18h of aerobic incubation at 32°C. The SMAC medium is designed to detect only the serotype O157:H7 by not fermenting the sorbitol within 24 hours.[37] Each sorbitol negative colony (colorless) and lactose fermenting colony (pink colonies) was subcultured to MacConkey agar and triple sugar

iron slants at 37°C for 18h. *Escherichia coli* isolates were confirmed biochemically using urease, Simons' citrate, oxidase, and indol.

Enzyme immunoassay for the detection of the toxins produced by Enterohemorrhagic E. coli in culture systems (EIA): All sorbitol-negative *E. coli* isolates were subcultured and tested for the presence of Stx using a commercial EHEC kit (Premier^{R}, Meridian Diagnostic).[50] The EHEC (Enterohemorrhagic *E. coli*) test utilizes monoclonal anti-Stx capture antibody absorbed to microwells. Diluted samples were added to the wells and incubated at room temperature, washed and enzyme conjugated anti-IgG polyclonal antibody applied. The presence of Stx-produces a reactive antibody-enzyme complex that can be interpreted visually.

Polymerase chain reaction (PCR.): A random selection of fecal samples from winter and summer collection periods were cultured on MacConkey's agar and 25 colonies selected to determine the prevalence of *E. coli* attaching and effacing gene type A (*eaeA*) and Stx gene strains. PCR was performed on all lactose fermenting isolates for amplification of both Stx I and Stx II. Isolates were serotyped against *E. coli* O157 and H7 monoclonal antibody.

Statistical analysis

Prevalence of sorbitol-negative *E. coli* was compared to season, slaughter using chi-square (X^2). Prevalence of *E. coli* and sorbitol-negative *E. coli* was compared to fresh versus frozen sampling for the winter samples. The *P*-value of significance was set at 0.05 with a degree of freedom at 1 to compare season. The degree of freedom was 2 when comparing fresh versus frozen samples.

RESULTS

Coliform bacteria were isolated from 829 of 1006 fecal samples cultured on MacConkeys agar (Table 10) during summer and winter periods. Two hundred samples were frozen in the winter and examined after being held at –20°C for 5-6 days. In the winter sampling, frozen samples yielded a lower recovery of *E. coli* than fresh samples with 260 from 301 (86.4%) for frozen samples as compared to 73 from 200 (36.5%). During the summer sampling period, all fecal samples were processed immediately and plated within 24 hours. Of the 829 coliform bacteria positive cultures, 265 were sorbitol negative of which 6 were positive on the EHEC Elisa test and 2 positive for serotype O157 (Table 10). The prevalence of coliform bacteria from fresh samples was compared between seasons with 260/301 (86.4%) isolated in the winter and 496/505 (98.2%) in the summer (Table 10). Of the 829 coliform bacteria positive cultures, 265 were sorbitol negative of which 6 were positive on the EHEC Elisa test and 2 positive for serotype O157 (Table 10). There were twice as many sorbitol-negative *E. coli* isolated in summer with 199/505 (39%) compared to 59/301 (19%) in winter, but EHEC positive were the same (2-3%) from these isolates (Table 11). A total of 47 samples were *Klebsiella* spp. positive (34 samples in winter and 13 samples in summer, Table 10).

Of the 501 samples, the *E. coli* prevalence for the fresh processed samples on sorbitol MacConkey-negative agar was 19.79% (59/301; Table 12) compared to frozen samples 3.5% (7/200; Table 12). Using fresh samples greatly increased the isolation of sorbitol-negative *E. coli* with a prevalence of 19% (59/301) and an OR=6.72, 95% confidence limits 2.88 –16.48 ($P<0.0001$) (Table 12). Both (2) *E. coli* O157 serotype isolates were identified from fresh samples. Also the growth of fresh samples on

MacConkey agar was significantly greater, with a prevalence of 86% (260/301) and an OR=11.03, 95% confident limits 6.97 –17.52 (P<0.0001) (Table 12).

DISCUSSION

In this study, two *E. coli* O157:H7 serotypes were isolated from 1006 fecal samples (0.2%). One of them was isolated in the summer and the other in winter. In other surveillance studies from North America and England the prevalence of *E. coli* O157:H7 was 0.7% and 19.7% for winter and summer respectively.

In the previous studies large variances were noticed in the *E. coli* O157:H7 prevalence due to methods of handling samples and different isolation and identification techniques. The first step in isolation of O157:H7 is based on non-fermentation of sorbitol and lack of β-glucronidase activity which is tested on sorbitol MacConkey agar (SMAC).

In our study, 265 of 829 *E. coli* cultures were SMAC and β-glucoronidase negative suggestive of the presence of O157. However, only 6 of these sorbitol negative cultures were found to be EHEC positive by using the EHEC Elisa that detects Stx I and II. This monoclonal anti Stx antibody test requires culturing of stool samples within two hours or if not possible placing the stool samples in transport media immediately and culturing within two to three days if they were stored at 2-8°C. In our study we were not able to place specimens into the transport media within two hours. This may have affected the detection of EHEC positive serotypes. Although all EHEC-positive serotypes were isolated from fresh samples, we can not make any assumption that fresh samples might yield better EHEC isolation.

Our study also detected a seasonal effect on sorbitol-negative *E. coli* shedding. Sorbitol-negative *E. coli* isolation was greater in the summer than in winter (199/505, 39% in summer compared to 59/301, 19% in winter). Enterobacteria infections in humans as well as animals may peak in summer months.[58,99,109,190] Several studies have shown seasonal differences in prevalence of bacterial shedding in cattle.[58] In a study done in Washington State, *E. coli* O157:H7 isolation in cattle fecal samples was highest during the period from June to September.[95] Donkersgoed *et al.*[48] found that the prevalence of *E. coli* O157:H7 was significantly higher in the summer than winter.

CONCLUSION

We investigated the prevalence of sorbitol-negative *E. coli* shedding in live cull (market) dairy cows presented for slaughter. According to our results the prevalence of sorbitol-negative *E. coli* was higher in summer than winter. The shedding of sorbitol-negative *E. coli* was not associated with the body condition scores (BCS) at slaughter. Similarily, the source of cattle by direct or indirect shipment did not influence the shedding of sorbitol-negative *E. coli* at slaughter. Overall we were only able to isolate two *E. coli* O157:H7 serotypes, one isolate in each season (winter and summer). These findings will be important considerations for researchers designing future studies to reduce shedding of pathogens at slaughter.

Table 10: Isolation of *Escherichia coli* and *Klebsiella* from feces of dairy cattle before slaughter.

				Escherichia coli						*Klebsiella*	
SEASON	**Samples**	**No growth Fresh**	**No growth Frozen**	**MacConkey Agar[a] Fresh**	**MacConkey Agar[a] Frozen**	**Sorbitol MAC Agar[a] Fresh**	**Sorbitol MAC Agar[a] Frozen**	**EHEC Positive[b] Fresh**	**Serotype O157[c] Fresh**	**MacConkey[a] Fresh**	**MacConkey[a] Frozen**
WINTER	501	41	127	260	73	59	7	2	1	25	9
SUMMER	505	9	0	496	0	199	0	4	1	13	0
TOTAL	1006	50	127	756	73	258	7	6	2	38	9

[a] One gram of feces in 9 ml of peptone and serially diluted for isolation on agar
[b] EHEC Elisa test (Premier®, Meridian Diagnostic) isolates from sorbitol MacConkeys agar
[c] O157 antigens identified by serotype from sorbitol negative isolate

Table 11: The prevalence of sorbitol-negative *E. coli* during winter and summer.

Sorbitol Negative *E. coli**	**WINTER**	**SUMMER**	**TOTAL**
Positive	59	199[a]	258
Negative	242	306	548
TOTAL	301	505	806

[a] OR = 0.37, 95% confidence limits 0.26 – 0.53; $P < 0.001$
* Only fresh samples

Table 12: Prevalence of *E. coli* sorbitol-negative and MacConkey positive growth of the fresh and frozen samples in winter.

MacConkey agar *E. coli - Klebsiella*	**FRESH**	**FROZEN**	**TOTAL**
Positive	260[a]	73	333
Negative	41	127	168
TOTAL	301	200	501
Sorbitol-Negative *E. coli*			
Positive	59[b]	7	66
Negative	242	193	435
TOTAL	301	200	501

[a] OR = 11.03, 95% confidence limits 6.97 – 17.52; $P < 0.001$
[b] OR = 6.72, 95% confidence limits 2.88 – 16.48; $P < 0.001$

CHAPTER 4

PREVALENCE OF *Salmonella* IN DAIRY CATTLE AT SLAUGHTER

ABSTRACT

The objectives of this study were to evaluate the prevalence and shedding of pathogens in feces of cull dairy cows at slaughter and also to evaluate the effects of winter and summer on the isolation of pathogens from cull dairy cows at slaughter. Samples were collected at slaughter from cattle sold direct or through auction markets. Fecal samples from 501 cows were collected in winter of 1996 and 505 samples in summer of 1996 to study *Salmonella* spp. shedding patterns. *Salmonella* spp isolates were analyzed against animal disposition including body condition score, animal health, source of the animal, and season of the year. *Salmonella* was isolated from 94 of the 1006 fecal samples. Twenty-two serotypes were identified. The predominant isolates comprising of *S. typhimurium* (22/94), *S. senftenberg* (17/94), and *S. kentucky* (8/94).

Season had a major effect on the prevalence of *Salmonella*. *Salmonella* prevalence was significantly higher ($P < 0.0001$) in the summer (13.86%) than in winter (4.79%). Both body condition scores and transportation (direct versus indirect) were significantly associated with prevalence in the summer. Thin animals having a BCS $\leq$ 2.0 in the summer ($P = 0.033$) had a significantly higher prevalence of *Salmonella*. Fecal samples from cull cow sent directly to slaughter were less likely (13.7%) to culture-positive for *Salmonella* from fecal samples than animals that were sent though auction markets (25.1%) in the summer ($P = 0.018$).

INTRODUCTION

This study was undertaken to evaluate the prevalence and shedding of pathogens in feces of cull dairy cows at slaughter and evaluate the effect of body condition and direct / indirect shipping to slaughter on prevalence of *Salmonella* at slaughter.

The distribution of *Salmonella* along the gastrointestinal tract and in associated with lymph nodes was studied in 100 sheep and 100 cattle at slaughter plant in a study conducted in United Kingdom. The carriage rate of *Salmonella* spp. was 77% at cattle and 43% at sheep.[164] In another study from Australia, livers from normal slaughter cattle were examined for surface contamination by *Salmonella* spp. immediately after evisceration and again after inspection. *Salmonella* spp. was isolated from 32% at evisceration and 82% after inspection.

Numbers of *Salmonella* present were low at evisceration, and rose after inspection. The sources of the *Salmonella* spp. were probably the contents of the gastrointestinal tract and the mesenteric lymph nodes, both of which may show high prevalence of infection in cattle which have been held before slaughter.[165] It was concluded that contamination of viscera during handling and inspection at the slaughter plant occurred frequently.

Anderson *et al.*[214], found that 0.5% of calves in the market were infected with *Salmonella*. An infection rate of 0.6% was found in calves if animals were kept in the premises few hours before slaughter. Infection rate was further increased if animals were slaughtered after staying 2-5 days in the premises.

Cattle are commonly exposed to *Salmonella* in feed and a variety of stresses can increase susceptibility to colonization.[91] Grau *et al.*[84] also found that the incidence of *Salmonella* in the bovine rumen was greater the longer the period between the farm and the abattoir. Also they found that persistence of *Salmonella* inoculated into the rumen of cattle were depend on the pre and post inoculation feeding of the cattle. Well-fed cattle before *Salmonella* inoculation eliminated *Salmonella* rapidly but starvation 2-3 days prior to inoculation increased intestinal prevalence of *Salmonella* and a further increase was seen when normal feeding was restarted.[86]

Frost *et al.*[65] examined aspects of *Salmonella* infection in cattle at slaughter. Three groups of cattle (15 in each group) taken from the sale yard, were transported to feedlots near slaughter. Animals were slaughtered in 2 days, 18 days and 80 days respectively and rumen contents, and mesenteric lymph nodes were examined for *Salmonella* infection. The first and second group (2 and 18 days) showed 7/15 and 15/15 *Salmonella* infection but there was no *Salmonella* isolation on third group. It was clear transportation, starvation and re-feeding was increasing the *Salmonella* shedding.

The hide is thought to be the immediate source of most bacterial contamination of carcasses.[58] During transportation, close contact between animals may cause hide contamination. Puyalto *et al.*[153] found that hair contamination increased from 8% at farm to 25% at slaughter after transportation.

However, these surveys may underestimate *E. coli* O157:H7 prevalence at time of slaughter because stress, health condition, feeding frequency, and fasting, can influence populations of *E. coli* in the gut. *Salmonella* spp. and *E. coli* are poorly adapted to rumen and hind gut fermentation due to their sensitivity to the low pH and

high volatile fatty acid [VFA] concentrations of these compartments.[157] In the rumen of well-fed animals, growth inhibition is greatest at pH<6.5 and [VFA]>100mM.[19,86] During periods of fasting, however, these factors of inhibition diminish as ruminal [VFA] decline (<50mM) and pH values exceed 7.0.[25,130] Under these conditions the rumen may be a potential reservoir of enteric pathogens instead of an obstacle to their growth.[73,166]

For that reason, the prevalence of *Salmonella* spp. and *E. coli* O157:H7 was evaluated in cull dairy cows at the slaughter plant.

To evaluate the effects of season on isolation of pathogens from cull dairy cows at slaughter, samples were taken in the winter and in the summer.

According to the CDC (Center for Disease Control) from 1973-1987 *Salmonella* outbreaks have mostly occurred in July and August warm months of the year.[40] In 1991-1992, the U.S. Department of Agriculture conducted a study called the National Dairy Heifer Evaluation Project (NDHEP) which has determined *Salmonella* prevalence rate according to season.[194] More *Salmonella* positive calves were found in late summer with prevalence of 36.1 of every 1,000 samples.[194] The prevalence was lowest 12.3 per 1,000 in the winter period.[194]

MATERIAL AND METHODS

Study design at slaughter

A USDA inspected slaughter plant located in Allendale, MI was used for surveillance because cull cows arrive for slaughter as the result of direct sale and through auction markets. Animals presented for slaughter were evaluated as to the health of the animal, the body condition, and any physical abnormalities. The study was conducted in

February, 1996 and August, 1996 to compare the shedding of bacteria in cull cows during winter and summer. The slaughter plant was visited for 3 to 5 days during each period until approximately 500 cull dairy cows were sampled. Since cull dairy cows were only a portion of all the cattle processed daily at the slaughter plant all dairy cows were sampled on each day until the desired number for each sampling period was obtained. The dairy cows in this study came from auction markets and directly from local farms. Each cow was identified with a back tag number, which enabled us to trace the origin of these cattle and viscera through the slaughter process.

Sampling and bacterial culture methods for feces

Fecal samples were collected from the cecal-colon juncture of each study cow on the viscera table at slaughtered. For each sample, a single-edged razor was used to make a linear incision and cecal-colon contents were collected and stored immediately on ice in sterile plastic bags. At the end of each day, all samples were shipped by overnight delivery to the Diagnostic Bacteriology Laboratory, National Veterinary Service Laboratory, USDA-APHIS (NVSL) in Ames, Iowa for isolation of *Salmonella*.

One gram of feces was removed and inoculated into a sterile culture tube with 10ml of tetrathionate broth and incubated at 37°C for 48hours[79,131,166] (Figure 6). After incubation, the tube was vortexed and a 0.1ml sample was pipetted into a tube with 10ml Rappaport-Vissiliadis R10 broth and also streaked onto Brilliant Green Agar plates with Novobiocin (BGN).[79] The Rappaport-Vissiliadis R10 tube and Brilliant Green Agar with Novobiocin were incubated at 37°C for 18-24h.[46,79] After incubation, the tube was vortexed and a streak sample was taken and plated to xylose-lysine-tergitol-4 (XLT-4)

agar and to another Brilliant Green Agar with Novobiocin plate.[46,131] The plates were streaked for colony isolation and incubated at 37°C for 18-28h. At least three suspected *Salmonella* colonies were picked from each plate and transfered to XLT-4 agar and BGN agar for identification (black in color on XLT-4 agar and red on BGN agar).[131] Each colony was transferred to separate tubes containing 5ml of triple sugar iron agar (TSI) and lysine iron agar (LIA) slants and incubated at 37°C for 18-24h for biochemical profiles.[80] The XLT-4 and BGN plates that were presumptive negative for *Salmonella* colonies were held at room temperature for an additional 18-24h and rechecked for suspected colonies. Suspect colonies (three colonies) were picked and differentiated by use of LIA and TSI agar slants.[46,62,131]

All isolates were serotyped for both *Salmonella* O-antigen and H-antigen determination for verification and complete serotyping.

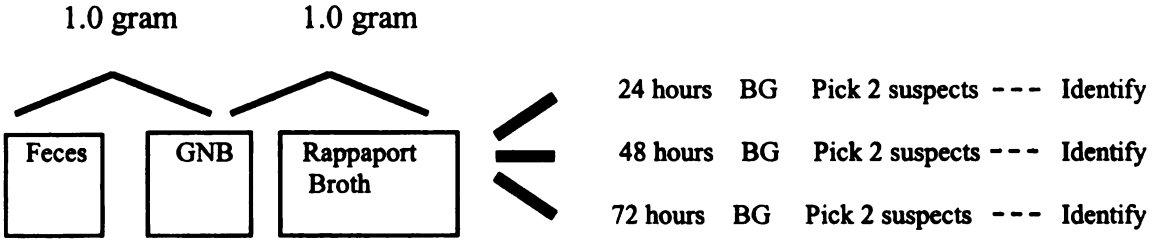


Figure 6: Diagram of *Salmonella* isolation procedure.

Statistical Analysis

Prevalence of *Salmonella* was compared to season, body condition and transportation route to slaughter using chi-square (X^2). The *P*-value of significance was set at 0.05 with a degree of freedom at 1 to compare season, transportation, and body

condition score. Because cattle came from Michigan, Ohio and Indiana the degree of freedom was 2 when comparing state of origin.

RESULTS

Of the 1006 samples, 94 (9.34%) were positive for *Salmonella* (Table 13). Of the summer samples, 70 of 505 (13.86%,) were positive for *Salmonella* with 16 different serotype isolated. *Salmonella typhimurium* (22), *S. seftenberg* (17), and *S. kentucky* (8) were the most frequently isolated serotypes (Table 13). Of the samples collected in the winter, 24 of the 501 (4.79%) were *Salmonella* spp. positive. There were 12 different serotypes of *Salmonella* isolated during winter, *Salmonella enteritidis* phage type (4), *S. kentucky* (4), *S. muenster* (4), and *S. lilie* (4) were the most frequently isolated serotypes (Table 13). *Salmonella typhimurium* was not isolated in any winter samples.

Salmonella isolation was significantly higher, (OR=0.31, 95% confidence limits 0.19 – 0.52, $P < 0.001$), in summer than winter (Table 14).

Of the 640 cull cows that could be tracked from place of origin, 243 cattle were sent to slaughter via auction markets. *Salmonella* was detected in 17 of the samples (17/243; 6.9%) during winter. Of the 102 cattle that were sold directly to slaughter from the farm, *Salmonella* was detected in 7/102 samples (6.8%). The difference was not significant. However, of the 179 cattle brought to slaughter from auction markets during the summer, *Salmonella* was detected from 45/179 samples (25%), as compared to 16/116 (13.8%) from the cattle sent directly to the slaughter. This difference was statistically significant with an OR=2.10, 95% confident limits 1.08 – 4.13, $P = 0.0187$, (Table 15).

Overall, cattle from out-of-state did not differ from cull cows shipped from Michigan. The only exception was cull cows shipped from Indiana in the winter (P = 0.0094; Table 16).

The body condition scores of the 974 cull cows were recorded for correlation with *Salmonella* prevalence. *Salmonella* isolation in the cull cows with a BCS ≤ 2.0 (thin cows) was compared to *Salmonella* isolation in the cull cows with a BCS > 2.0. *Salmonella* was isolated more often from thin cows in the summer with a prevalence of 18% (45/250) with an OR=1.94, 95% confidence limits 1.11 – 3.39, P = 0.012, (Table 17). Cows with BCS ≤ 2.0 were twice as likely to shed *Salmonella* than cows with BCS > 2.0 (Relative Risk=1.8).

DISCUSSION

In our survey, cull cows sent to slaughter in the summer were three times more likely to be shedding *Salmonella* than in the winter. *Salmonella typhimurium* was the most common isolate in the summer. But *S. typhimurium* was not isolated in winter. Other frequently isolated *Salmonella* serotypes in summer were *S. senftenberg* and *S. kentucky, S. kentucky, S. meunster*, and *S. lille* were most often isolated in the winter. Other surveys also determined *Salmonella typhimurium* as the most common serotype in the Northeast United States (USDA, 1994).

Cull cows coming through auction markets were three times more likely to shed *Salmonella* than those sent directly to slaughter. In winter, *Salmonella* prevalence was higher in the cows coming from auction markets than cows coming directly from the farm to slaughter.

Previous studies determined starvation and stress during transportation increases *Salmonella* shedding.[65,84,214] Thus introducing animals originating from different farms to auction markets causes cross contamination between the animals and increase shedding especially if there is a delay before slaughter.

According to this survey, the comparison between states may lack significance because of the low number of samples collected from states other than Michigan. Isolation was numerically higher for out-of-state cull cows and a large sample size might have concluded different results. However, this maybe related to the time in transit and indirectly shipping to slaughter.

Although our data set was limited, body condition of the cull cows were an affect on *Salmonella* shedding. Thin cows (BCS $\leq$ 2.0) were twice as likely to shed *Salmonella* in summer as heavier cows. The thin cull cow may be more susceptible to the effects of transportation, starvation, and stress than better conditioned cows. Summer heat may add to this stress as seen in this study.

Although animals were examined at slaughter for health conditions (lameness, hide score, udder condition, respiratory problems, vulva discharge, eyes, fecal consistency, and lumps and lesions) none of these health factors were shown to be a factor in the shedding of *Salmonella*.

The facts of high level of *Salmonella* isolation on cattle feces at slaughter require more attention for preslaughter management of animals. Further studies of control points on transportation conditions and managing holding facilities should be conducted in prospective, controlled manner. Duration of preslaughter period needs further study.

CONCLUSION

To make significant improvements in food safety, measures should be taken at all points in the farm-to-table chain including production, transportation, slaughter processing, storage, retail and food preparation. In our present study season, body condition scores and the source of cattle by direct or indirect shipment can affect the prevalence of salmonellae at slaughter. Because of these factors slaughter cattle will, at times, carry food-borne pathogens that can be transferred to carcass and meat. While care taken during the slaughter operations can minimize the transfer of food-borne pathogens from animal to meat, it cannot entirely prevent it. Furthermore, it can be predicted that the above factors will affect the shedding of *Salmonella* at slaughter. More controlled studies are needed to understand the precise roles of each of the factors in the shedding of *Salmonella* to be able to reduce the risks of food-borne diseases.

Table 13: Isolation of *Salmonella* from cull cows at slaughter.

Samples	*Salmonella*		
	Total	Winter	Summer
Total	1006	501	505
Salmonella Isolation	94 (9.34%)	24 (4.79%)	70 (13.86%)
Serotypes			
S. typhimurium	22	0	22
S. seftenberg	17	0	17
S. kentucky	12	4	8
S. muenster	8	4	4
S. mbandaka	6	0	6
S. lilie	5	4	1
S. enteritidis phage type 8	4	4	0
S. cerro	3	1	2
S. dublin	3	0	3
S. oranienburg	3	0	3
S. uganda	3	3	0
S. braenderup	2	2	0
S. havana	2	1	1
S. java	2	1	1
S. thompson	2	0	2
S. agona	1	0	1
S. montevideo	1	0	1
S. ohio	1	0	1
S. schwarzengrund	1	0	1
S. panama	1	1	0
*S. enteritidis**	1	1	0
S. bovismorbifican	1	1	0

* *phage untypable*

Table 14: Seasonal difference in *Salmonella* prevalence at slaughter.

	WINTER	SUMMER	TOTAL
***Salmonella* (+)**	24	70[a]	94
***Salmonella* (-)**	477	435	912
TOTAL	501	505	1006

[a] OR = 0.31, 95% confidence limits 0.19 – 0.52; $P < 0.001$

Table 15: Prevalence of *Salmonella* in cattle that were assembled prior to shipment (indirect) or directly shipped to slaughter.

WINTER	INDIRECT	DIRECT	TOTAL
***Salmonella* (+)**	17	7	24
***Salmonella* (-)**	226	95	321
TOTAL	243 **(6.9%)**	102 **(6.8%)**	345 **(6.9%)**
SUMMER			
***Salmonella* (+)**	45	16[a]	61
***Salmonella* (-)**	134	100	234
TOTAL	179 **(25.1%)**	116 **(13.7%)**	295 **(20.6%)**
SUM TOTAL	62/422	23/218	85/640

[a] OR = 2.10, 95% confidence limits 1.08 – 4.13; $P = 0.018$

Table 16: *Salmonella* prevalence in cull dairy cows originating from Michigan, Indiana and Ohio.

WINTER	MICHIGAN	INDIANA	OHIO	TOTAL
***Salmonella* (+)**	14	8[a]	2	24
***Salmonella* (-)**	354	55	54	463
TOTAL	368 **(3.8%)**	63 **(12.6%)**	56 **(3.5%)**	487 **(4.9%)**
SUMMER				
***Salmonella* (+)**	46	7	9	62
***Salmonella* (-)**	274	58	74	406
TOTAL	320 **(14.3%)**	65 **(10.7%)**	83 **(10.8%)**	468 **(13.2%)**
SUM TOTAL	60/688	15/128	11/139	86/955

[a] Chi-square = 9.33; $P = 0.0094$

Table 17: *Salmonella* prevalence related to body condition scores.

WINTER	BCS ≤ 2.0	BCS >2.0	TOTAL
***Salmonella* (+)**	9	15	24
***Salmonella* (-)**	137	317	454
Total	146 **(6.1%)**	332 **(4.5%)**	478 **(5%)**
SUMMER			
***Salmonella* (+)**	45[a]	25	70
***Salmonella* (-)**	205	201	426
TOTAL	250 **(18%)**	246 **(10%)**	496 **(14.1%)**
SUM TOTAL	54/396	40/578	94/974

[a] OR = 1.94, 95% confidence limits 1.11 – 3.39; $P = 0.012$

CHAPTER 5

CONCLUSION

Cattle-associated pathogens like enterohemorrhagic *E. coli* and *Salmonella* infections pose serious challenges for beef markets and constitute emerging threats to public health. Recent reports indicate that dairy cows account for about 8% of U.S. domestic beef production, 25% of U.S. non-fed beef available for consumption in the U.S., and about 18% of U.S. ground beef. Producers remove the majority of cull dairy cows for reproductive problems, udder or mastitis problems, poor production unrelated to disease, or lameness or injury. These reasons for culling are not usually related to ill health or systemic disease, which might preclude their wholesomeness as a human food source which makes cull dairy cows a likely source of food-borne microbiological hazards. For that reason we have recorded the health conditions along with body conditions for each cow that was slaughtered. As a result we found out that health conditions did not affect the shedding of either *E. coli* or *Salmonella* whereas body condition influenced the shedding of *Salmonella* at the slaughter plant during the summer. This finding may raise a question of "why body condition did not affect *E. coli* shedding"? One possibility may be the limited number of fecal samples being sampled at the time. We also examined fresh sampling versus frozen sampling. Using fresh samples significantly affected the prevalence of not only *E. coli* but probably also O157:H7. We only found 2 positive samples of O157:H7 both from fresh samples and from each season. In the case of *Salmonella* fecal samples were all processed fresh.

It has been estimated that approximately 17% of the nation's ground beef may come from cull dairy cows.[190] Nearly 77% of cows intended for beef slaughter are sent

to markets, auctions, and sale barns, while 22% are sent straight to slaughter facilities. This information indicates a relatively high amount of transportation involved in the movement of cull dairy cows to slaughter plants. Increased transportation poses risks of nutritional and environmental stresses, exposure to disease pathogens either from other cattle by contact or from feed deprivation that cattle go through during transportation. Transportation was another variable of our study. We observed that cows shipped indirectly to slaughter had a higher prevalence of *Salmonella* than cows shipped directly to the slaughter plant. This result also agreed with the previous studies. [48,58,65,84,214] We were unable to detect an influence on *E. coli* shedding due to transportation.

We also observed a seasonal effect on the shedding of both *E. coli* and *Salmonella* at the slaughter plant. Shedding of both was higher in the summer than winter as seen in the other studies.[48]

Practices that have been tentatively, but not consistently, associated with the fecal prevalence of *E. coli* O157:H7 and *Salmonella* include herd size, grouping, weaning, manure management, equipment sanitation (including water troughs), feed composition, feed additives (by products, ionophores), and parenteral antibiotics.[48,77,99]

In acknowledging that variable densities of microbial pathogens in gastrointestinal contents are likely to have a significant effect on subsequent contamination levels of beef carcasses, consideration of preharvest controls must be included in any farm-to-plate safety strategy. The management of food-borne pathogens will become part of an integrated program to enhance food safety, which includes the producer, the packer, the distributors, retailers and the consumer. Hazard Analysis and Critical Control Points (HACCP) type prevention programs, using scientifically based critical management

points, will help further reduce risk.[174] Caution must be exercised when making direct comparisons among prevalence estimates of bacteria from various studies. There are differences in culture techniques, including a large variability in the sensitivity of tests. Additionally, the number, frequency and timing of sampling (on-farm, and at slaughter); the handling, transport and storage of samples; the type and age of cattle; the type of sample (fecal pat, fecal swab, weight of fresh feces); the season of sampling; the unit of analysis (individual, herd, process lot); and the serotype of bacteria may affect prevalence estimates.[48]

At the farm level there should be an education/awareness program for farm workers, repeated and updated periodically as appropriate, to ensure they are aware:[148]

- of the existence, potential prevalence and nature of *E. coli* O157:H7 and *Salmonella*;
- of the potential for the spread of infection on farms, notably from fecal material, and of the consequent need for scrupulous personal hygiene;
- of the need for care in the use of untreated slurry or manure; and
- of the absolute requirement for the presentation of animals in an appropriate, clean condition for slaughter.

At the slaughter plant:[148]

- Good practices in slaughter procedures must be identified and promoted by Industry with the help and support of government departments, particularly in the areas of the presentation of clean cattle and of hide and intestine removal.
- Slaughter workers should be trained in good hygiene practice during slaughter and enforcement should concentrate on slaughter and subsequent handling of carcasses.

- The Hazard Analysis and Critical Control Point (HACCP) system should be enshrined in the legislation governing slaughter plants and the transportation of carcasses and meat. Meanwhile, enforcers and the trade should ensure that HACCP principles are observed.
- Further consideration should be given, involving the industry and consumer interests, to the potential use and benefits of end-process treatments such as steam pasteurization.

Finally it is clear that for many food-borne pathogens, the gastro-intestinal tract of clinically normal cattle is an important reservoir of infection for human beings. These organisms find ready access to the food chain at processing due to the inevitable transfer of bovine fecal flora onto carcasses. New opportunities are being sought to improve the microbial safety of beef products by applying interventions in both the 'preharvest' and 'postharvest' periods.[106] Preharvest control measures are those that can be implemented while cattle are on the farm, during marketing and transport, and while waiting at slaughter. Such measures have the appeal of not placing total reliance on the hygienic practices of processors, food handlers and consumers. They are also consistent with the belief that, where possible, control should be exercised at all possible points within the food chain.[106]

But still, as long as you cook the hamburgers really good keep eating those delicious 'Big Macs'.

REFERENCES

1. Anita Bleem. *Esherichia coli* O157:H7 in raw milk: A review. Animal Health Insight Spring/Summer:1994,1-9.

2. Anon. *Salmonella* in animal and poultry production 1992. Ministry of Agriculture, Fisheries and Food, Welsh Office, Agriculture Department, Scottish Office, and Agriculture and Fisheries Department, 1993.

3. Aspinall ST., Hindle MA. Hutchinson DN. Improved isolation of salmonellae from feces using a semisolid Rappaport-Vassiliadis medium. Eur J Clin Microbiol Infect Dis.1992;11:936-939.

4. Bailey JS., Cox NA., Blankenship LC. A comparison of an enzyme immunoassay. DNA hybridization, antibody immobilization, and conventional methods for recovery of naturally occurring salmonellae from processed broiler carcasses. J Food Prot.1991;54:354.

5. Bean NH., Griffin PM. Food-borne disease outbreaks in the United States. 1973-1987: pathogens, vehicles and trends. J Food Prot.1990;53:804.

6. Belongia EA., et al. An outbreak of *E. coli* O157:H7 colitis associated with consumption of precooked meat patties. J Infect Dis.1991;164:338-343.

7. Bennett AR., MacPhee S., Betts RP. The isolation and detection of *Escherichia coli* O157 by use of immunomagnetic separation and immunoassay procedures. Lett Appl Microbiol.1996;22:237-243.

8. Bergey's Manual of Systematic Bacteriology.1994;Vol 1:408-423.

9. Bernard M. Williams. Bovine Salmonellosis. Ministry of Agriculture, Fisheries and Food. England. November 1980. No. 15.

10. Besser RE, Lett SM, Weber T, et al. An outbreak of diarrhea and hemolytic uremic syndrome from *E. coli* O157:H7 in fresh-pressed apple cider. JAMA 1993;17:2217-2220.

11. Besser TE., Gay CC., et al. Salmonellosis associated with *S. typhimurium* DT104 in the USA.Vet Rec.1997;140-75.

12. Billy T: Presentation to the National Meat Association. San Diego, CA, February 12,1997.

13. Bisplinghof FD. Antimicrobial properties of commercial organic acid compounds in meat and bone meal. Proc. Symposium on the Diagnosis and Control of *Salmonella*, San Diego, 1991. USAHA, 32-42.

14. Bisplinghof FD. Progress in controlling *Salmonella* in animal by products. In Report of the Committee on *Salmonella*, USAHA Proceeding.1991;461.

15. Bitzon JT. Cytotoxic activity of *E. coli* O157:H7 culture filtrate on the mouse colon and kidney. Curr Microbiol.1984;11:335-342.

16. Blood DC. et al: Veterinary Medicine: A Text-book of the Disease of Cattle, Sheep, Pigs, and Horses. 6th Ed. Bailliere Tindall, London, England 1983; 576-581.

17. Bopp CA., Greene KD., Downes FP., Wells JG., et al. Unusual verotoxin producing *E. coli* associated with hemorrhagic colitis. J Clin Microbial.1987;25:1486-1489.

18. Borczyk AA., Karmali MA., et al. Bovine reservoir for verotoxin-producing *Escherichia coli* O157:H7. Lancet 1987;1:98.

19. Brownlie LE., Grau FH. Effect of food intake on growth and survival of salmonellas and *E. coli* in the bovine rumen. J Gen Microbiol.1967; 46:125-134.

20. Calderwood SB., Auclair F., Donohue-Rolfe A., et al. Nucleotide sequence of the Shiga-like toxin genes of *E. coli*. Prc Natl Sci USA 1987;84:4364-4368.

21. Cary JW., Linz JE., Bhatnagar D. Microbial food-borne diseases: Mechanisms of Pathogenesis and Toxin Synthesis. Technomic publication.2000;87-130.

22. Cassens RG. 1994 Mear Preservation, Food and Nutrition Press, Connecticut.

23. Centers for disease control and prevention 1991 surveillance of *E. coli* O157 isolation and confirmation US 1988 Morbid weekly report 40 SS-1: 1-6.

24. Centers for Disease Control and Prevention. Update: multistate outbreak of *Escherichia coli* O157:H7 infections from hamburgers-western United States, 1992-1993. MMWR 1993;42:258-263.

25. Chambers PG., Lysons RJ. The inhibitory effect of bovine rumen fluid on *Salmonella typhimurium*. Res Vet Sci.1979;26:273-276.

26. Chapman PA, Siddons DJ, et al. Cattle as a possible source of verotoxin-producing *E. coli* O157:H7 infection in man. Epidemiol Infect.1993;111:439-447.

27. Chapman PA., Wright DJ., Norman P. Verotoxin-producing *E. coli* infections in Sheffield: cattle as a possible source. Epidem Inf.1989;102:439-445.

28. Chapman PA., Cedan AT., et al. Use of commercial enzyme immunoassays and immunomagnetic separation systems for detecting *Escherichia coli* O157 in bovine fecal samples. Appl Environ Microbiol.1997;63:2549-2553.

29. Chapman PA., Siddons CA., et al. A 1-year study of *Escherichia coli* O157 in cattle, sheep, pigs, and poultry. Epidemiol Infect.1997;119:245-250.

30. Chapman PA., Wright DJ., Siddons CA. A comparison of strains of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from bovine faeces. J Med Microbiol.1994;40:424-427.

31. Clegg FG., Chiejina SN., Duncan AL., et al. Outbreaks of *Salmonella newport* infection in dairy herds and their relationship to management and contamination of the environment. Vet Rec.1983;112:580-584.

32. Cobbold R., Desmarchelier P. A longitudinal study of Shiga-toxigenic *Escherichia coli* (STEC) prevalence in three Australian dairy herds. Vet Microbiol.2000;71:125-137.

33. Cohen ND., Neibergs HL., et al. Genus-specific detection of salmonellae using the polymerase chain reaction (PCR). J Vet Diagn Invest.1993;5:368-371.

34. Corrier DE., Purdy CW., DeLoach JR. Effects of marketing stress on fecal excretion of Salmonella spp in feeder calves. Am J Vet Res.1990;51:866-869.

35. Cox VS. Nonsystemic causes of the downer cow syndrome.Vet Clin North Am Food Anim Pract.1988;4:413-433.

36. Cullor JS. Common pathogens that cause foodborne disease: Can they be controlled on the dairy? Food-Animal-Practice.1995;185-194.

37. Cullor JS. *Escherichia coli* O157:H7: The silent danger. Vet Med.1995;74-82.

38. Curiale MS., Klatt MJ., Bartlett CL. Colorimetric deoxyribonucleic acid hybridization assay for rapid screening of *Salmonella* in food: collaborative study. J Assoc Off Anal Chem.1990;73:248-256.

39. D'Aoust JY., Sewell AM., et al. Reliability of the immunodiffusion 1-2 Test system for detection of *Salmonella* in foods. J Food Prot.1988;51:853.

40. D'Aoust JY. *Salmonella* in Foodborne Bacterial Pathogens. Doyle MP. Ed. Marcel Dekker. New York. 1989;327.

41. D'Aoust JY., Sewell AM. Efficacy of an international method for detection of *Salmonella* in chocolate and cocoa products. J Food Prot.1984;47:111.

42. D'Aoust JY., Sewell AM., Greco P. Commercial latex agglutination kits for the detection of food-borne *Salmonella*. J Food Prot.1991;54:725.

43. Davis A., Broad R. The microbiology of meat and poultry. 1998;123.

44. Davies A., O'Neil P., Towers L., Cooke M. An outbreak of *Salmonella typhimurium* DT104 food poisoning associated with eating beef. Comm Dis Rep CDR Rev.1996;6(11):159-162.

45. Davis M., Osaki C., Gordon D., et al. Update: multistate outbreak of *Esherichia coli* O157:H7 infections from hamburgers-western United States, 1992-1993. Morbid Mortal Weekly Rep.1993;42:258-263.

46. De Smedt JM., Bolderdijk RF. Dynamics of *Salmonella* isolation with modified semi-solid Rappaport-Vassiliadis medium. J Food Protect.1987;50:658-661.

47. Demster JF. Radiation preservation of meat products: A review. Meat Sci. 1985;12:61-89.

48. Donkersgoed JV., Graham T., Gannon V. The prevalence of verotoxins, *Escherichia coli* O157:H7, and *Salmonella* in the feces and rumen of cattle at processing. Can Vet J.1999;40:332-338.

49. Donkersgoed VJ., Jericho FWK., et al. Preslaughter hide status of cattle and the microbiology of carcasses. J Food Protec.1997;60:1502-1508.

50. Donohue-Rolfe A., Acheson DWK., Kane AV., Keusch GT. Purification of shiga-toxin and shiga-toxin I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross-reactive monoclonal antibodies. Infect Immun.1989;57:3888-3893.

51. Dorn CR., Francis DH., Angrick EJ., et al. Characteristics of Vero cytotoxin-producing *Escherichia coli* associated with intestinal colonization and diarrhea in calves. Vet Microbiol.1993;36:149-159.

52. Doyle MP., Schoeni JL. Survival and growth characteristic of *E. coli* associated with hemorrhagic colitis. Appl Environ Microbial.1984; 48:855-856.

53. Doyle MP. Isolation of *E. coli* O157:H7 from retail fresh meats and poultry. Apply Environ Microbial.1987;53:2394-2396.

54. Doyle MP. *Esherichia coli* O157:H7 and its significance in foods. Int J Food Microbiol.1991;12:289-302.

55. Downes FP. *Shigella*. Compendium of Methods for the Microbiological Examination of Foods 4th edition. pp381-385.

56. Dushyanthan K. 1997. In Training Manual on Meat Processing and Packaging Technology, Department of Meat Science and Technology, Madras Veterinary College, Chennai, India. In: Davis A., Broad R. (Eds.), The microbiology of meat and poultry. 1998. Pp 259-260.

57. Eckner KF., Dustman WA., et al. Elevated temperature, colorimetric, monoclonal, enzyme-linked immunosorbent assay for rapid screening of *Salmonella* in foods: collaborative study, JAOAC Int.1994;77:374.

58. Elder OR., Keen EJ., Siragusa RG., et al. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. Appl Biol Scien.2000;97:2999-3003.

59. El-Gazzor FE., Martin EH. *Salmonellae*, salmonellosis, and dairy foods: a review. J Dairy Sci.1992;75:2327-2343.

60. Evans S., Davies R. Case control study of multiple resistant *Salmonella typhimurium* DT104 infection of cattle in Great Britain. Vet Rec.1996;139:557-558.

61. Evans SJ. A case control study of multiple resistant *Salmonella typhimurium* DT104 infection of cattle in Great Britain. Cattle Pract.1996;4:259-266.

62. Ewing WH. 1972. Differentiation of Enterobacteriaceae by biochemical reactions. Edwards and Ewing's Identification of Enterobacteriaceae. 3rded. Burgess Publ. Co. Inc. Minneapolis, Minnesota. Pp.21-48.

63. Faith NG., Shere JA., Brosch KW., et al. Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. App Environ Microbiol.1996;62:1519-1525.

64. Farmer JJ, et al. H7 antiserum-sorbitol fermentation medium: a single tube screening medium for detecting *E. coli* O157:H7 associated with HC. J Clin Microbial.1985;22:620-625.

65. Federal Register 1992. Use of sorbitol in cured pork products. Fed Regist 57:355.

66. Feng P. Commercial assay systems for detecting foodborne *Salmonella*: a review. J Food Prot.1992;56,197.

67. Feng PCS., Hartman PA. Fluorogenic assays for immediate confirmation of *Escherichia coli*. Appl Environ Microbiol.1982;41:1320-1329.

68. Ferris KE., Thomas LA. *Salmonella* serotypes from animals and related sources reported during July 1994-June 1995. In Proc. Annual Meeting of the US Animal Health Association, Reno, Nevada, 28 October-3 November. USAHA, Pat Campbell & Associates/Spectrum Press, Richmond, Virginia, 1995;510-524.

69. Fitts R., Diamond M., Hamilton C., Neri M. DNA-DNA hybridization assay for detection of *Salmonella* spp. in foods. Appl Environ Microbiol.1983;46:1146-1151.

70. Food Safety Unit, Programme of Food Safety and Food Aid (1997) Prevention and Control of Enterohemorrhagic *Escherichia coli* (EHEC) Infections (World Health Organization, Geneva).

71. Fratamico PM., et al. Virulence of an *E. coli* O157:H7 sorbitol-positive mutant. Appl Environ Microbial.1993;Vol.59;No.12:4245-4251.

72. Fratamico PM., Schultz FJ., Buchanan RL. Rapid isolation of *E. coli* O157:H7 from enrichment cultures of foods using an immunomagnetic separation method. Food Microbiol.1992;9:105-113.

73. Frost AJ., O'Boyle D., Samuel JL. The isolation of *Salmonella* spp. From feed lot cattle managed under different conditions before slaughter. Aust Vet J.1988; 65:224-225.

74. Galland JC., Troutt FH., Brewer RL., Osburn BI., et al. Diversity of *Salmonella* serotypes in cull (market) dairy cows at slaughter. JAVMA.2001;219:1216-1220.

75. Gannon VP., D'Souza S., Graham T., King RK., Rahn K., Read S. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. J Clin Microbiol. 1997;35:656-662.

76. Garber LP, Wells SJ, Hancock DD,et al. Risk factors for fecal shedding of *E. coli* O157:H7 in dairy calves. JAVMA.1995;Vol.207,No.1:46-49.

77. Garber L., Wells S., Schroeder-Tucker L., Ferris K. Factors associated with fecal shedding of Verotoxin-producing *Escherichia coli* O157 on dairy farms. J Food Prot.1999;62(4):307-312.

78. Gasser C., Gautier E., Steck A., et al. Hamolytisch-uramische syndrome: Bilaterale nierenrindenmek-rosen bei akuten erworbenen hamolytischen anamien. Schweiz med Wochenschr 1995;85:205-209.

79. Gay JM., Hunsaker ME. Isolation of multiple *Salmonella* serovars from a dairy two years after a clinical salmonellosis outbreak. J Am Vet Med Assoc. 1993;203(9):1314-1320.

80. Gay JM., Rice DH., Steiger JH. Prevalence of fecal *Salmonella* shedding by cull dairy cattle marketed in Washington State. J Food Protec.1994;57:195-197.

81. Gibson DM., Coombs P., Pimbley DW. Automated conductance method for the detection of *Salmonella* in foods. J AOAC Int.1992;75:293.

82. Giles N., Hopper SA. Persistence of *S. typhimurium* in a large dairy herd. Epidemiol Infect.1989;103:235-241.

83. Gransden WPR., Damm MAS., Anderson JD., et al. Further evidence associating hemolytic uremic snydrome with infection by verotoxin-producing *Escherichia coli* O157:H7. J Infect Dis.1986;154:522-524.

84. Grau FH., Brownlie LE., Roberts EA. Effects of some preslaughter treatments on the *Salmonella* population in the bovine rumen and feces. J Appl Bacteriol.1968; 31:157-163.

85. Grau FH., Brownlie LE., Smith MG. Effects of food intake on number of Salmonellae and *Escherichia coli* in rumen and faeces of sheep. J Appl Bacteriol 1969;32:112-117.

86. Grau FH., Brownlie LE. Occurrence of salmonellas in the bovine rumen. Vet J 1965;41:321-323.

87. Griffin PM., Ostroff SM., Tauxe RV., et al. Illnesses associated with *Escherichia coli* O157:H7 infections. Annals of Internal Medicine 1988;109:705-712.

88. Griffin PM., Tauxe RV. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemorrhagic uremic syndrome (HUS). Epidemiol Rev.1991;13:60-98.

89. Guthrie R. *Salmonella*. CRC Press, Inc., Boca Raton, Florida, 1991, 220pp.

90. Hancock DD., Besser TE., Kinsel ML., et al. The prevalence of *Escherichia coli* O157:H7 in dairy and beef cattle in Washington State. Epidemiol Infect 1994; 113:199-207.

91. Hancock DD., Besser TE., Lejeune J., Davis M., Rice DH. The control of VTEC in the animal reservoir. Int J Food Microbiol.2001;66:71-78.

92. Hancock DD., Besser TE., Rice DH. Ecology of *Escherichia coli* O157:H7 in cattle and impact of management practices. In: Kaper JB., O'Brien AD. (Eds.), *Escherichia coli* O157:H7 and Other Shiga Toxin-Producing *E. coli* Strains. ASM, Washington,1998;85-91.

93. Hancock DD., Rice DH., Herriot DE., Besser TE., Ebel ED., Carpenter LV. Effects of farm manure-handling practices on *Escherichia coli* O157:H7 prevalence in cattle. J Food Prot.1997a;60:363-366.

94. Hancock DD., Rice DH., Thomas LA., Dargatz DA., Besser TE. Epidemiology of *Escherichia coli* O157:H7 in feedlot cattle. J Food Prot.1997b;60:462-465.

95. Hancock DD, et al. A longitudinal study of *Escherichia coli* O157 in fourteen cattle herds. Epidemiol Infect.1997;118:193-195.

96. Hancock, DD., Wells SC., Thomas LA., Garber LP., Herd S. National prevalence study for *Escherichia coli* O157:H7 in United States dairy calves. J Am Vet Med. Assoc., in press.

97. Hathaway SC. Intensive (pasture) beef cattle operations: the perspective of New Zealand. Rev Sci Tech Off Inf Epiz.1997;16(2):382-390.

98. Helmick CG., Griffin PM., Addiss DG., Tauxe RV., Juranek DD. Infectious diarrhea, In: Everhart JE, ed. Digestive diseases in the United States: epidemiology and impact. Washington, D.C.; Government Printing Office,1994:85-123.

99. Herriott ED., Hancock DD., et al. Association of herd management factors with colonization of dairy cattle by Shiga toxin-positive *Escherichia coli* O157. J Food Proct.1998;61(7):802-807.

100. Holland PM., Abramson RD., et al. Detection of specific polymerase chain reaction product by utilizing the 5′ to 3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci. USA 1991;88:7276-7280.

101. Humphrey TJ., Mead GC., Rowe B. Poultry meat as a source of human salmonellosis in England and Wales. Epidemiol Infect.1988;100:175-184.

102. Hungate RE. The rumen and its microbes. New York: Academic Press Inc. 1966;533.

103. International Organization for Standardization, International Standard ISO 6579, Microbiology general guidance on methods for the detection of *Salmonella*, Geneva, Switzerland, 1981.

104. Jarvis B. A philosophical approach to rapid methods for industrial food control, in Rapid Methods and Automation in Microbiology and Immunology, Habermehl KO., Spriger-Verlag, New York, 1984,593.

105. Johnson RP., Durham RJ., et al. Detection of *Escherichia coli* O157:H7 in meat by an enzyme-linked immunosorbent assays, EHEC-Tek. Appl Environ Microbiol. 1995;61:386-388.

106. Jordan D., McEwen SA., Lammerding AM., McNab WB., Wilson JB. A simulation model for studying the role of preslaughter factors on the exposure of beef carcasses to human microbial hazards. Prev Vet Med.1999;41:37-54.

107. Kabagambe EK., Wells SJ., Garber LP., Salman MD., Wagner B., Fedorka-Cray PJ. Risk factors for fecal shedding of *Salmonella* in 91 US dairy herds in 1996. Prev Vet Med.2000;43:177-194.

108. Kain ML., Kochevar SL., Sofos JN., Smith GC. Detection and control of external pathogens through microbial mapping: The New York cull dairy cow project. Englewood, Colorado:National Cattlemen's Beef Association, 1997.

109. Kaper JB., O'Brien AD. *Escherichia coli* O157:H7 and Other Shiga Toxin-Producing *E. coli* Strains. ASM, Washington, 1998.

110. Karch H., Meyer T. Evaluation of oligonucleotide probes for identification of Shiga-like-toxin-producing *E. coli*. J Clin Microbiol.1989;27:1180-1186.

111. Karmali MA., Petric M., Lim C., Arbus PC., et al. The association between hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J Infect Dis.1985; 151:775-782.

112. Karmali MA. Infection by verocytotoxin-producing *Esherichia coli*. Clin Microbiol Rev.1989;2:15-38.

113. Kleanthous NK., Fry HR., Smith RJ., et al. The use of sorbitol-MacConkey agar in conjunction with a specific antiserum for the detection of Vero cytotoxin-producing strains of *E. coli* O157. Epidem Infect.1988;101:327-335.

114. Knight MT., Wood DW., Black JF., et al. Gram-negative identification of *Salmonella*, *Escherichia coli*, and other Enterobacteriaceae isolated from foods: collaborative study. J Assoc Off Chem.1990;73:729.

115. Kondiah N., Zeuthen P., Jul M. Chemical dips for extending the shelf-life of unchilled fresh beef at ambient temperature. J Food Sci Tech.1985;22:142-145.

116. Krieg, NR. *Salmonella*. In Bergey's Manual of Systematic Bacteriology. Vol. 1, Williams & Wilkins, Baltimore. 1984.

117. Lawrie RA. 1985 Meat Science, Pergamon Press, New York.

118. Lett PW., et al. Detection of pathogenic *E. coli* in ground beef using multiplex PCR. Food testing and Analysis December 1995-January 1996;34-38.

119. Levine MM. *E. coli* that cause diarrhea: Enterotoxigenic (ETEC), Enteroinvasive (EIEC), Enterohemorrhagic (EHEC), Enteroadherent (EPEC). J Infect Dis.1987; 155:377-389.

120. Linton AH., Howe K., Osborne AD. Epidemiology of *Salmonella* infection in calves (1): Its relation to their husbandry and management. Vet Rec.1974;94:581-585.

121. Lopez EL., Diaz M., Grinstein S., et al. Hemolytic uremic syndrome and diarrhea in Argentine children: the role of Shiga-like toxins. J Infect Dis.1989;160:469-475.

122. Lynn TV., Hancock DD., Besser TE., Harrison JH., Rice DH., Stewart NT., Rowan LL. The occurrence and replication of *Escherichia coli* in cattle feeds. J Dairy Sci. 1998;81(4):1102-1108.

123. Manafi M., Sommer R. Comparison of three rapid screening methods for *Salmonella* spp:MUCAP Test, MicroScreen® Latex and Rambach Agar, Lett Appl Microbiol.1992;14:163.

124. Marissa A., Miller, Sean FA. The President's national Food Safety Initiative. JAVMA.1998;Vol 213,No.12:1737-1739.

125. Martin ML., Shipman LD., Wells JG., et al. Isolation of *E. coli* O157:H7 from dairy cattle associated with two cases of haemolytic uraemic syndrome. (Letter). Lancet.1986;2:1043.

126. Mattila T., Frost AJ. The growth of *Salmonella* in rumen fluid from cattle at slaughter. Epidemiol Infect.1988;101:337-345.

127. McManus C., Lanier JM. *Salmonella*, *Campylobacter jejuni*, and *Yersinia enterocolitica* in raw milk. J Food Prot.1987;50:51-55.

128. Mechie SC., Chapman PA., Siddons CA. A fifteen month study of *Escherichia coli* O157:H7 in a dairy herd. Epidemiol Infect.1997;118:17-25.

129. Meyer H., Koch H., Methner U. Vaccines in salmonellosis control in animals. Int J Med Microbiol.1993;278:407-415.

130. Michalowski T. Effect of feeding frequency on the diurnal changes in microbialprotein, volatile fatty acids and ammonia contents of the rumen of sheep. J Agric Sci Camb.1979;93:67-70.

131. Miller RG., Tage CR., Malinson ET., Scherrer JA. Xylose-Lysine-Tergitol 4; an improved selective medium for isolation of *Salmonella*. Poultry Sci.1991;70:2429-2432.

132. Mitchell, GA., McChesney DG. A plan for *Salmonella* control in animal feeds. Proc. Symp on the Diagnosis and Control of *Salmonella*, San Diego, 1991;USAHA,28-31.

133. Montenegro MA., Bulle M., Trumpf T., Aleksic S., Reuter G., Bulling E., Helmuth R. Detection and characterization of fecal verotoxin-producing *Esherichia coli* from healthy cattle. J Clin Microbiol.1990;28:1417-1421.

134. Moon HW., Bunn TO. Vaccines for preventing enterotoxigenic *Escherichia coli* infections in farm animals. Vaccine.1993;11(2):200-213.

135. Morgan CM., Newman C., Palmer SR., et al. First recognised community outbreak of haemorrhagic colitis due to verotoxin-producing *Escherichia coli* O157:H7 in the UK. Epidemiol Infect.1988;101:83-91.

136. Myers LL., Firehammer BD., Border MM., Shoop DS. Prevalence of enteric pathogens in the feces of healthy beef calves. Am J Vet Res.1984;45:1544-1548.

137. Narasimha Rao D. 1995. Lactic fermentation in the preservation of animal products, in Microbes for Better Living, Bangalore Printing and Publishing Company, Bangalore-560 018.

138. Nataro JP, Baldini MM., Kaper JB., Black RE., Bravo N., Levine MM. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J Infect Dis.1985;152(3):560-565.

139. Nath EJ., Neidert E., Randall CJ. Evaluation of enrichment protocols for the 1-2 Test for *Salmonella* detection in naturally contaminated foods and feeds. J Food Prot.1989;52:498.

140. National Animal Health Monitoring System. *Escherichia coli* O157:H7 in U.S. Dairy Calves. Jan 1994.

141. Neil MA., Tarr PI., Clausen C., et al. *Escherichia coli* O157:H7 as the predominant pathogen associated with the hemolytic uremic syndrome: a prospective study in the Pacific Northwest. Pediatrics 1987;80:37-40.

142. Nichols GL., De Louvois J. The microbiological quality of raw sausages sold in the UK. PHLS Microbiol Digest.1995;12:236-42.

143. Pacer RE., Spika JS., Thurmond MC. Prevalence of *Salmonella* and multiple antimicrobial-resistant *Salmonella* in California dairies. J Am Vet Med Assoc.1989;195:59-63.

144. Padhye NV., Doyle MP. Production and characterization of a monoclonal antibody specific for enterohemorrhagic *E. coli* of serotype O157:H7 and O26:H11. J Clin Microbiol.1991;29:99-103.

145. Pai CH., Ahmed N., Lior H, et al. Epidemiology of sporadic diarrhea due to serocytotoxin-producing *E. coli*: a two year prospective study. J Infect Dis. 1988157:1054-1057.

146. Park CH., Vandel NM., Hixon DL. Rapid immunoassay for detection of *Escherichia coli* O157 directly from stool specimens. J Clin Microbiol. 1996;34:988-990.

147. Parry SM., Salmon RL., Willshaw GA., Cheasty T. Risk factors for and prevention of sporadic infections with vero cytotoxin (shiga toxin) producing *Escherichia coli* O157. Lancet 1998;351:1019-1022.

148. Pennington H. Factors involved in recent outbreaks of *Escherichia coli* O157:H7 in Scotland and recommendations for its control. J Food Saf.1998;18:383-391.

149. Petersen KE., James WO. Agents, vehicles, and casual inference in bacterial foodborne disease outbreaks: 82 reports (1986-1995). JAVMA.1998;212(12):1874-1881.

150. Pollard DR., Johnson WM., et al. Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. J Clin Microbiol. 1990;28:540-545.

151. Poppe C., Smart N., Khakhria R., et al. *Salmonella typhimurium* DT104: A virulent and drug-resistant pathogen. Can Vet J.1998;39:559-565.

152. Post DE. Food-borne Pathogens, monograph no.1, *Salmonella*, Oxoid/Unipath, UK.,1994.

153. Puyalto C., Colmin C., Laval A. *Salmonella typhimurium* contamination from farm to meat in adult cattle. Descriptive study. Vet Res.1997;28:449-460.

154. Raghubeer EV., Matches JR. Temperature range for growth of *Escherichia coli* serotype O157:H7 and selected coliforms in *E. coli* medium. J Clin Microbiol.1990;28:803-805.

155. Rahkio TM., Korkeala HJ. Airborne bacteria and carcass contamination in slaughterhouses. J Food Protec.1997;60:38-42.

156. Rao DN., Murthy VS. A preliminary study of the effect of urea in the preservation of meat. Meat Sci.1986;17:251-165.

157. Rasmussen MA. Rumen contents as a reservoir of EHEC. FEMS Microbiol Lett 1993;114:79-84.

158. Rice DH., Ebel ED., Hancock DD., et al. *Escherichia coli* O157 in cull dairy cows on farm and at slaughter. J Food Protect.1997;60:386-387.

159. Richardson AR. The transmission of *Salmonella dublin* to calves from adult carrier cows. Vet Rec.1973;92:112-115.

160. Riley LW., Remis RS., Helgerson SD., et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med.1983;308:681-685.

161. Roberts T. Salmonellosis control: estimated economic costs. Poult Sci.1988; 67:936.

162. Robinson RA., Bender JA. Descriptive epidemiology of *Salmonella* infection in Minnesota herds. Bovine Practit.1994;28:83-86.

163. Rose BE., et al. Evaluation of a colorimetric DNA hybridization test for detection of salmonellae in meat and poultry products. J Food Prot.1991;54,127.

164. Samuel JL., Eccles JA., Francis J. *Salmonella* in the intestinal tract and associated lymph nodes of sheep and cattle. J Hyg Lond.1981;87(2):225-232.

165. Samuel JL., O'Boyle DA., Mathers WJ., Frost AJ. The contamination with *Salmonella* of bovine livers in an abattoir. Aust Vet J.1980;56(11):526-528.

166. Samuel JL., et al. Isolation of *Salmonella* from mesenteric lymph nodes of healthy cattle at slaughter. Res Vet Sci.1980;28:238-241.

167. Sanderson MW., Gay JM., Hancock DD., Gay CC., Fox LK., Besser TE. Sensitivity of bacteriologic culture for detection of *Escherichia coli* O157:H7 in bovine feces. J Clin Microbiol.1995;33:2616-2619.

168. Sargeant JM., Gillespie JR., et al. The frequency of *E. coli* O157:H7 in beef cows and calves in Kansas: Preliminary Results. Proc 78th Annu Meet Conf Res Workers Anim Dis (Abstract). November 1997.

169. Scotland SM., Rowe B., Smith HR., et al. Vero cytotoxin-producing strains of *Escherichia coli* from children with haemolytic uraemic syndrome and their detection by specific DNA probes. J Med Microbiol.1988;25:237-243.

170. Sernowski LP., Ingham SC. Frequency of false presumptive positive results obtained using a commercial ELISA kit to screen retail ground beef for *Escherichia coli* O157:H7. J Food Prot.1992;55:846.

171. Shere JA., Bartlett KJ., Kaspar CW. Longitudinal study of *Escherichia coli* O157:H7 dissemination on four dairy farms in Wisconsin. Appl Environ Microbiol. 1998;64:1390-1399.

172. Shooter RA., Cooke EM., et al. Animal sources of common serotypes of *Escherichia coli* in the food of hospital patients. Possible significance in urinary tract infection. Lancet 1970;2:226-228.

173. Smith BP., Da Roden L., Thurmond MC., et al. Prevalence of Salmonellae in cattle and in the environment on California dairies. J Am Vet Med Assoc.1994;205:467-471.

174. Smith RA., Griffin DD., Dargatz DA. The risks and prevention of contamination of beef feedlot cattle. The Perspective of the United States of America. Rev Sci Tech. 1997;16(2):359-368.

175. Smith BP., House J. Prospects for *Salmonella* control in cattle. Am Assoc Bov Pract Conf.1992;1:67-73.

176. Smith MG., Graham A. Destruction of *Escherichia coli* and salmonellae on mutton carcasses by treatment with hot water. Meat Sci.1978;2:119.

177. Sofas JN., Busta FF. 1983. In Antimicrobials in Foods., Marcel Dekker, New York.

178. Stefan G. Food safety issues affecting the dairy beef industry. J Dairy Sci.1997;80(12):3458-3462.

179. Stone GG., Oberst RD., et al. Detection of *Salmonella* from Clinical Samples by Enrichment Broth Cultivation-PCR. J Clin Microbiol.1994;32;1742-1749.

180. Swedlow DL., Wooddruff BA., Brady RC., et al. A waterborne outbreak in Missouri of *Escherichia coli* O157:H7 associated with bloody diarrhea and death. Ann Intern Med.1992;117:812-819.

181. Syed ZK., Rao DN., et al. Extension of shelf-life of buffalo meat by pre-treatment-lactic acid and sodium chloride. Current Res.1995b;24:62-65.

182. Tauxe R. *Salmonella*: a postmodern pathogen. J Food Prot.1991;54:563.

183. The National Advisory Committee on Microbiological Criteria for Foods: Int J Food Microbiol.1992;16:1-23.

184. Thompson JS., Hodge DS., Borczyk AA. Rapid biochemical test to identify verotoxin-positive strains of *E. coli* serotype O157:H7. J Clin Microbiol. 1990;28:165-168.

185. Threlfall EJ., Frost JA., Ward LR., Rowe B. Epidemic in cattle of *S. typhimurium* DT104 with chromosomally-integrated multiple drug resistance. Vet Rec.1995; 134:577.

186. Threlfall EJ., Rowe B., Ferguson JL., Ward LR. Characterization of plasmids conferring resistance to gentamicin and apramycin in strains of *Salmonella typhimurium* phage type 204c isolated in Britain. J Hygiene.1986;97:419-426.

187. Threlfall EJ., Ward LR., Rowe B. Epidemic spread of a chloramphenicol-resistant strain *Salmonella typhimurium* phage type 204 in bovine animals in Britain. Vet Rec.1978;103(20):438-440.

188. Toit R., et al. Empirical evaluation of conditions influencing the polymerase chain reaction: Enterotoxigenic *Escherichia coli* as a test case. Eur J Clin Biochem. 1993;31:225-231.

189. Toth I., Barrett ML., et al. Enzyme-linked immunosorbent assay for products of the 60-megadalton plasmid of *Escherichia coli* serotype O157:H7. J Clin Microbiol. 1991;29:1016-1019.

190. Troutt FH., Galland JC., Osburn BI., Brewer RL., et al. Prevalence of *Salmonella* spp in cull (market) dairy cows at slaughter. JAVMA.2001;219:1212-1215.

191. US Food and Drug Administration, Bacteriological Analytical Manual, 6th ed. Association of Official Analytical Chemists, Washington, DC, 1992.

192. USDA, FSIS: Background on a science-based strategy for protecting public health. U.S. Department of Agriculture Printing, 1996.

193. USDA, FSIS: Pathogen reduction: Hazard Analysis and Critical Control Point (HACCP) Systems-Final Rule. Federal Register pp.38806-38989,1996.

194. USDA. *Salmonella* in Dairy Calves. National Animal Health Monitoring System. Jan 1994.

195. Uyttendaele M., Taverniers J., Debevere J. Effects of stress induced by suboptimal growth factors on survival of *Escherichia coli* O157:H7. Int J Food Microbiol. 2001;66:31-37.

196. Van Poucke LG. *Salmonella*-Tek, a rapid screening method for *Salmonella* species in food. Appl Environ Microbiol.1990;56:924.

197. Vanderzant C., Spittstoesser DF. Compendium of methods for microbiological examination of foods, 3rd ed. Edward Brothers, Ann Arbor. Mich.

198. Wall PG., Morgan K., Lamden M., Griffin EJ., Threlfall EJ., et al. Transmission of multi-resisrant strains of *Salmonella typhimurium* from cattle to man. Vet Rec.1995; 136:591-592.

199. Wall PG., Morgan D., Lamden K., et al. A case-control study of infection with an epidemic strain of multiresistant *Salmonella typhimurium* DT104 in England and Wales. Commun Dis Rep.1995;4:130-135.

200. Warnock JP., Caple IW., Halpin CG., et al. Metabolic changes associated with the 'downer' condition in dairy cows at abattoirs. Aust Vet J.1978;54:566-569.

201. Wells JG., Davis BR., Wachsmuth IK., et al. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. J Clin Microbiol.1983;18:512-520.

202. Wells JG., Shipman LD., Greene KD., et al. Isolation of *E. coli* serotype O157:H7 and other Shiga-like toxin producing *E. coli* from other cattle. J Clin Microbiol. 1990;29:985-989.

203. Wells JG., Shipman LD., Greene KD., Sowers EG., et al. Isolation of *Escherichia coli* serotype O157:H7 and other Shiga-like toxin-producing *E. coli* from dairy cattle. J Clin Microbiol.1991;29:985-989.

204. Whipp SC., et al. Animal as a source of *Escherichia coli* pathogenic for human beings. JAVMA.1994;204:1168-1175.

205. White PL., Baker AR., James WO. Strategies to control *Salmonella* and *Campylobacter* in raw poultry products. Rev Sci Tech Off Int Epiz.1997;16(2):525-541.

206. Williams BM. Bovine salmonellosis. Bovine Pract.1980;15:122.

207. Willshaw GA., Smith HR., Rowe B., et al. Cloning of genes determining the production of vero cytotoxin by *Escherichia coli*. J Gen Microbiol.1985;131:3047-3053.

208. Willshaw GA., Smith HR., Scotland SM., et al. Heterogeneity of *Escherichia coli* phages encoding vero cytotoxins: comparison of cloned sequences determining VT1 and VT2 and development of specific gene probes. J Gen Microbiol.1987; 133:1309-1317.

209. Willshaw GA., et al. Examination of raw beef products for the presence of verocytotoxin producing *Escherichia coli*, particularly those of serogroup O157. J Appl Bacteriol.1993;75(5):420-426.

210. Wilson JB., Clarke RC., et al. Verocytotoxigenic *Escherichia coli* infection in dairy farm families. J Infect Dis.1996;174:1021-1027.

211. Wilson JB., Renwick SA., Clarke RC., Rahn K., Alves D., et al. Risk factors for infection with verocytotoxigenic *Escherichia coli* in cattle on Ontario dairy farms. Prev Vet Med.1998;34:227-236.

212. Wilson JB., McEwen SA., Clarke RC., Leslie KE., et al. Distribution and characteristics of verocytotoxigenic *Esherichia coli* isolated from Ontario dairy cattle. Epidemiol Infect.1992;108:423-439.

213. Wolcott MJ. DNA-based rapid methods for the detection of foodborne pathogens. J Food Prot.1991;54:387.

214. Wray C., Sojka WJ. Reviews of the progress of dairy science: bovine salmonellosis. J Dairy Res.1977;44:383-425.

215. Wray C., Todd JN., Hinton M. Epidemiology of *Salmonella typhimurium* infection in calves: Excretion of *S typhimurium* in the faeces of calves in different management systems. Vet Rec.1987;121:293-296.

216. Wray C., Todd N., McLaren MI., Beedell YE. The epidemiology of *Salmonella* in calves: the role of markets and vehicles. Epidemiol Infect.1991;107:521-525.

217. Zhao T., Doyle MP., Shere J., Garber L. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. Appl Environ Microbiol. 1995;61:1290-1293.