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PREVALENCE OF SHIGA-LIKE-TOXIN PRODUCING *ESCHERICHIA coli* IN BEEF STEERS AND HEIFERS IN A SLAUGHTER HOUSE ON PRINCE EDWARD ISLAND

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

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Faculty of Veterinary Medicine

University of Prince Edward Island

R. Douglas Schurman

Charlottetown, P.E.I.

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ABSTRACT

Fecal swabs obtained from a random sample of 1000 beef slaughter steers and heifers from 123 different Prince Edward Island (P.E.I.) farms were examined for Shiga-like toxin producing *Escherichia coli* (SLTEC) using a Vero cell assay (VCA). Multiple isolates from each positive sample were tested similarly. Positive SLTEC colonies were confirmed as *E. coli* biochemically, tested for drug resistance, serotyped, and examined with monoclonal antibody toxin neutralization and polymerase chain reaction (PCR) techniques to determine the type of toxin. Cattle were classified as positive when a SLTEC isolate was positive on Vero cell assay and toxin production was confirmed by PCR. The prevalence of SLTEC in beef slaughter steers and heifers on P.E.I. was 4% (40/1000) with a confidence interval ranging from 2.3%-5.7% due to the clustering effect of farm. Most SLTEC isolates were sensitive to commonly used antimicrobials. Several SLTEC serotypes recovered in this study are associated with human illness. The most frequently isolated SLTEC serotype was *E. coli* O157 (5 isolates out of 40). Of these five *E. coli* O157 serotypes, four were *E. coli* O157:H7, a serious human pathogen. The types of shiga-like toxins produced by the SLTEC serotypes isolated during the study were identified by specific monoclonal antibodies. The monoclonal antibody toxin neutralization procedure was compared to the PCR procedure for toxin identification. The Kappa statistic was used to compare the results of the two procedures. Agreement between the two procedures was low.

A retail ground beef survey was conducted between June and September 1995 on 200 ground beef samples obtained from grocery stores and meat shops on P.E.I. All samples were tested by VCA for SLTEC and an ELISA test kit developed to identify *E. coli* O157 antigen in human stool. SLTEC were not detected by VCA. *E. coli* O157 antigen was detected in four of the samples.

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1.

General Introduction

1.1 SHIGA-LIKE-TOXIN PRODUCING *ESCHERICHIA COLI*

In the human population there are an increasing number of individuals who are susceptible to food borne illness. These individuals are susceptible to the effects of food borne illness due to their age and/or health status. Due to these demographic changes, the contamination of food sources with bacteria pathogenic to humans has created serious public health concerns. Our food supply comes from an environment in which microorganisms are widespread. Although many of these organisms are harmless to healthy animals and humans, others are less benign and can cause disease and even death.

Many different shiga-like-toxin producing strains of *E. coli* (SLTEC) serotypes associated with human illness have been isolated from foods of animal origin (1,2). SLTEC can cause several severe diseases in humans, including hemorrhagic colitis (HC), hemolytic uraemic syndrome (HUS) and thrombocytopenia purpura (TTP) (3). Over 50 serotypes of SLTEC have been associated with foodborne outbreaks in North America and elsewhere (4). Multiple sporadic cases of human illness associated with SLT-producing *E. coli* have also been reported (4). SLTEC is now the major cause of acute renal failure

in North American children and is usually among the top three bacterial causes of diarrhoea (4).

SLTEC organisms can produce several different toxin types. These toxins are either called verotoxins because of their lethal effect on African Green Monkey kidney cells (Vero cell culture) or Shiga-like toxins (SLT) due to their antigenic similarity to the toxin produced by *Shigella dysenteriae* serotype 1 (1). These toxins are known to be part of the virulence mechanism by which SLTEC cause serious illness in humans (1,4).

Cattle have been implicated as a source of *E. coli* that infect and cause disease in humans. SLTEC have been isolated from the feces of clinically normal and diarrhoeic cattle (5,6). This includes serotypes apparently unique to cattle and serotypes associated with disease in humans (5). Epidemiological investigations into outbreaks of human illness have incriminated undercooked ground beef and unpasteurized milk as sources of infection (5,6,7). There are many opportunities for animals to become contaminated with pathogenic microbes on the farm, during transport or at the slaughterhouse. Fecal contamination of meat surfaces during slaughter and dressing occurs randomly, usually on specific areas of a carcass. The flank, midline, and legs of a carcass are the areas most likely to be contaminated during these procedures (8). Basic microbial loads on routinely dressed carcasses will be low. The contamination these carcasses are exposed

to is often from cross-contamination due to the dressing procedures. The presence of a single pathogenic organism, however, is a significant food safety concern. An infectious dose for certain organisms can be low and improper cooling and refrigeration can allow multiplication (9).

The most common serotype identified in human illness is *E. coli* O157:H7 (4,10). The toxin produced by *E. coli* O157:H7 acts systemically action on vascular endothelial cells, thus causing HUS (11). Person-to-person and cattle-to-person transmission occur. Ground beef is the most common vehicle for *E. coli* O157:H7 transmission. Ground beef contaminated with *E. coli* O157:H7 can cause illness when meat is not properly cooked or when raw or undercooked meat cross-contaminates other food items (1,12,13,14).

1.2 THESIS OBJECTIVES

The objectives of this thesis can be grouped into three phases. Phase I was the determination of the prevalence of SLTEC in beef slaughter cattle at the time of hide removal and carcass evisceration. Fecal samples were obtained over a one year period (January-December, 1994) from 1000 beef animals and tested for the presence of SLTEC using the Vero cell assay (VCA). The VCA is the Gold Standard for the detecting SLT producing organisms in fecal and food samples. When SLTEC positive fecal samples were detected by the VCA, the Shiga-like-toxin (SLT) producing organisms were isolated from the sample, serotyped and tested for antibiotic sensitivity. Isolates determined biochemically to be lactose positive and sorbitol negative were tested by Enzyme Linked Immunosorbent Assays (ELISAs) from three different manufacturers to determine if the organisms contained O157 antigens.

Phase II was the determination of the type of toxin produced by each of the SLTEC organisms isolated from the positive fecal samples. Monoclonal antibody toxin neutralization assays and Polymerase Chain Reaction (PCR) procedures were used to identify the type of toxin produced by the SLTEC organisms. Since there are no Gold Standards to differentiate among toxin identification procedures, the two procedures were compared to each other to determine their agreement. Agreement between the two tests was also determined for isolates

categorized as low quantity SLT producers and high quantity SLT producers. These categories were based on the results of titration assays performed on all positive isolates.

Phase III was the examination of retail ground beef for SLTEC. Two hundred ground beef samples were collected from various retail outlets on Prince Edward Island between June and September of 1995. A VCA was performed on the samples to detect the presence of SLTEC. Additionally, all samples were tested for the presence of O157 antigen by a modified ELISA test. The test was originally intended to detect O157 antigen in human feces but the procedures were modified by staff at the Health of Animals Laboratory in Guelph, Ontario to detect O157 antigen in food. SLTEC organisms were isolated from ground beef samples that were ELISA positive and VCA positive. In particular, *E. coli* O157:H7 was to be identified by using immunomagnetic beads impregnated with antibody that binds O157 antigen and specialized agar biochemically formulated to enhance the growth of *E. coli* O157:H7.

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Chapter 2.

Survey for Shiga-like-toxin producing *Escherichia coli* in feces of beef cattle slaughtered on Prince Edward Island

2.1 INTRODUCTION

Presently, large scale changes are occurring in the food processing business and in particular the consuming public. Most food is provided by large operations that can manufacture massive quantities of product for global distribution. In the human population there are now many individuals who are susceptible to food borne illness due to their age (children, elderly) or health status (immunocompromised). Consequently, contamination of food sources with bacteria pathogenic to humans has created serious public health concerns. Shiga-like-toxin producing strains of *E. coli* (SLTEC), first recognized by Konowalachuk et al. (1), cause several severe diseases in humans, including hemorrhagic colitis (HC) and hemolytic uraemic syndrome (HUS). Among the virulence factors produced by SLTEC are a family of bacterial proteins toxic to Vero cells (African green monkey kidney cells) called verocytotoxins. These toxins are analogous to Shiga toxin, previously associated with *Shigella dysenteriae* serotype 1. Due to this association verocytotoxins are now usually referred to as Shiga-like-toxins (SLT) (2,3). SLTEC organisms produce two major classes of cytotoxins: SLT-I (immunologically similar to Shiga toxin) and SLT-II (immunologically distinct from Shiga toxin). Other so called SLT-II variant toxins have also been described.

These toxins belong to the SLT-II class but are called SLT-II variants (SLT-IIv). SLT-II and SLT-IIv toxins have been differentiated by tissue culture assay. Typical SLT-II toxin is cytotoxic to both Vero cells and HeLa cells whereas SLT-IIv is cytotoxic for Vero cells but not HeLa cells. SLT-IIv have been isolated from cattle and human beings and can cause disease. Another SLT-IIv causes edema disease in swine (3).

SLTs are bipartite toxins. They consist of five B-subunits and one A-subunit. The B-subunits bind to specific glycolipid receptors in the cell membrane which determine susceptibility of the cell to SLT. The A-subunit provides the biological activity by entering the cell and blocking protein synthesis (3).

There are at least 50 *E. coli* serotypes that produce these SLTs (4). The most common serotype identified in human illness is *E. coli* O157:H7 *(5)*. Apparently the toxin produced by *E. coli* O157:H7 acts systemically on vascular endothelial cells to cause HUS (6). Many different SLTEC serotypes associated with human illness have been isolated from foods of animal origin (2,7). An important reservoir for SLTEC appears to be cattle. Cattle have been implicated as a source of *E. coli* that infect and cause disease in human beings (3,8). Epidemiological investigations into outbreaks of human illness have incriminated undercooked ground beef and unpasteurized milk as sources of infection.

Considerable study has been done on determining the prevalence of SLTEC in mature dairy cows, heifers and calves, particularly with cattle on the farm. The prevalence of SLTEC in these classes of livestock is reported at 9-15% (3,7,9). Calves, between 3 & 6 months old, and heifers, shed more SLTEC than mature animals. Except for emergency removals, dairy heifers and calves between the ages of a few months and two years, are not routinely marketed prior to maturity. A significant number of calves are marketed as veal but veal is not used for ground beef. In the ground beef trade, large volumes of ground beef are prepared from cull dairy cows, cull beef cows, and portions from market age beef steers and heifers whose population numbers are several times that of the cow classes. The prevalence of SLTEC in beef steers and heifers, particularly at slaughter, has not been determined as frequently as it has for dairy animals.

SLTEC can be isolated from the feces of clinically normal and diarrhoeic cattle (3). This includes serotypes apparently unique to cattle, and serotypes associated with disease in humans (3). Studies of cattle as reservoirs of SLTEC pathogenic for humans include general surveys and investigations of dairy herds implicated as sources of human infection. In some studies, investigators have checked only for O157:H7, while others have looked for all SLTEC. There are non-O157:H7 SLTEC serotypes that cause O157:H7-like illnesses in humans. All studies of cattle as a source of human enteric pathogens should test for both O157:H7 and non-O157:H7 SLTEC (3).

In abattoirs, fecal contamination of carcasses with potential zoonotic organisms cannot be completely eliminated when livestock are slaughtered. The source of the contamination are primarily carcasses soiled with feces and mud (tag) or the escape of feces and ingesta from the digestive tract during the slaughter process. During slaughter, meat is most likely to become contaminated during hide removal and evisceration procedures. Thorough trimming and carcass washing to remove such contamination from the carcass prior to further processing is required. Control of contamination during these procedures will minimize the potential for carcass contamination and public health concern.

The standard method for identification of SLTEC is the cytotoxicity of culture supernatants for Vero cells (1). Production of cytotoxins active on Vero cells has been detected in strains of *E. coli* from cases of human and animal disease. These same toxins have been detected in strains of *E. coli* isolated from non-diseased animals (3,9,10,11). The objective of this study was to determine if beef steers and heifers harbor SLTEC as do dairy cattle. The prevalence of SLTEC in the feces of beef steers and heifers was determined at slaughter during carcass evisceration using the Gold Standard Vero cell assay for SLT detection. Individual isolates obtained from SLT positive fecal samples were serotyped and evaluated to determine the presence of those organisms known to cause illness in humans. The antibiotic sensitivity and resistance of SLTEC positive isolates to various veterinary antimicrobials was also determined.

2.2 MATERIALS AND METHODS

Sample collection

One thousand fecal swabs were collected from the distal rectum of randomly selected beef cattle (steers and heifers) slaughtered in a federally inspected abattoir on Prince Edward Island during 1994. The beef kill was scheduled in this abattoir on Monday, Tuesday, Wednesday, or Thursday. Seventy percent of the beef cattle were slaughtered on Monday, Tuesday, or Wednesday. Twenty-two samples per week were randomly collected from animals slaughtered on Monday, Tuesday or Wednesday. Thursday was not a test day; samples could not be consistently processed during the weekend for the one year period. To ensure that cattle were selected randomly on test day, each animal in the day's kill was assigned a sequential number starting at one. Twenty-two random numbers generated from the number of beef cattle to be slaughtered on the test days were calculated by the statistics program EpiInfo 5.0. One thousand animals from 123 different farms were sampled.

Preparation and storage of samples

A multi-stage technique was undertaken to test fecal samples and individual SLTEC isolates for the presence of Shiga-like toxin (SLT) (Figure 1). Fecal swabs

were placed in 9 ml of MacConkey broth (Appendix A) immediately after sampling and incubated overnight at 37°C. After incubation, 700 μL of each broth culture was added to 700 μL of glycerol and stored in individual vials at -70°C. Concurrently, 500 μL from each broth culture was added to 4.5 ml of brain heart infusion broth (BHIB, Difco) and incubated overnight at 37°C. The remaining broth culture was refrigerated at 4°C until it was determined if SLTEC were present in the sample.

Detection of SLTEC in fecal samples using Vero Cell Assay (VCA)

After incubation, the BHIB cultures were centrifuged (Eppendorf) at 12,000 g for 10 min. Fifty μL of supernatant from each sample was added to the top wells of a 96-well flat-bottomed micro titre plate (Becton Dickinson Labware) containing 200 μL of Eagles Minimal Essential Medium (EMEM, Gibco) in each well. Four serial dilutions of 1:5 were made vertically for each well to a final dilution of 1:625. One hundred μL of a Vero cell suspension (containing approximately 4.0 x 10^5 cells/ml) was added to each well. An SLT positive control well (Strain 321, Drs. Clarke and Read: Health Canada), an SLT negative control well, and a well with only EMEM and Vero cells were included on each plate. The positive and negative control wells were used as standards to assess the cytotoxic affect of SLT on the Vero cell monolayer. The third control well ensured that the medium was not verocytotoxigenic and that the Vero cells were healthy. The

micro titre plates were incubated at 37°C in a 5% CO_2 /95% atmospheric air incubator (Napco) for 48 h. The VCA titre endpoint (1:5, 1:25, 1:125 and 1:625) and degree of verocytotoxicity were and recorded at the end of incubation. Samples were determined to be either negative (-), weak(+/-) or positive (+). Samples were considered positive if 50% or more of the Vero cell monolayer was not intact after 48 h of incubation. This was a subjective observation that was made visually. Weak titres were samples that exhibited less than 50% verocytotoxicity. SLTEC colony isolation was attempted on samples exhibiting weak or positive verocytotoxicity.

SLTEC Isolation

The original MacConkey broths from positive samples were streaked onto MacConkey (MAC) and Sorbitol MacConkey agar plates (SMAC) and incubated overnight at 37°C (Appendix 1). With the assistance of a stereoscope, twelve colonies exhibiting different morphological (smooth, rough, mucoid, dry) and biochemical characteristics (lactose and non-lactose fermentation) were individually selected from each plate and inoculated into 1 ml of BHIB. MAC and SMAC agar assist in differentiating lactose fermenting and non-lactose fermenting bacteria colonies and sorbitol and non-sorbitol fermenting bacteria colonies from one another. After picking the colonies, a final sweep through the remaining colonies on the plate was made and inoculated into 1 ml of BHIB. The

picks and sweep were incubated overnight at 37°C and then tested for verocytotoxicity using the VCA. Cultures of all colonies positive on the VCA were stored at -70°C in 50% glycerol. If all individual picks were negative but the sweep was positive, BHIB from the sweep was streaked onto MAC and SMAC agar plates, incubated overnight at 37°C and another series of picks and sweeps were retested by VCA. If 25 subsequent individual picks were negative, the sample was considered to be negative for SLT-*E. coli*. If the sweep continued to be positive the samples were sent to the Agriculture and Agri-Food Health of Animals Laboratory (AAFCHAL) and were further examined to determine if SLTEC organisms were present in the sample.

Biochemical tests

Isolates that were VCA positive were verified as *E. coli* using the BIOLOG (Baxter Diagnostics Inc. Deerfield, IL, USA) and API (bioMérieux Vitek, Inc. Hazelwood, MO. USA) biotyping systems. Isolates not identified as *E. coli* by BIOLOG were tested by API. Isolates identified as *E. coli* either by BIOLOG or API were forwarded to the AAFCHAL for serotyping and toxin identification.

Antimicrobial tests

Antimicrobial sensitivities were determined for each isolate using the standard

disc diffusion Kirby Bauer Technique. Sensitest medium (Oxoid/Unipath) was inoculated with a suspension of each isolate adjusted to a density equivalent to a 0.5 MacFarland standard. Discs containing drugs at various concentrations were used (Table 3). Sensitivity and resistance were interpreted using the zone-size interpretation chart (12). For quality control, *E. coli* strain ATCC 25922 was used.

Latex Agglutination Kits

Positive VCA samples that were lactose positive and sorbitol negative were tested by a latex agglutination test to determine if the isolate was an *E. coli* O157. Three different kits [(*E. coli* O157-Latex Test Reagent Kit, Pro-Lab Diagnostics - Richmond Hill, Ontario), (Serobact *E. coli* O157-Latex Agglutination Test, Disposable Products Pty. Ltd. - South Australia) and (*E. coli* O157 Latex Test, Oxoid - Unipath Limited - England)] were used. For each kit, a suspension of pure culture was made equivalent to a MacFarland 4 standard. A drop of approximately 50μL of bacterial suspension was placed on a test card. Following the instructions included with the kits, two drops of O157 antibody-coated latex particles were placed on the card beside the bacterial suspension. The two reagents were mixed together and observed for agglutination. Positive and negative controls were included in two of the kits. In the Pro-Lab

Diagnostics kit, the positive control was an *Escherichia coli* O157:H7 antigen bound to latex particles. The negative control was a purified rabbit IgG which does not react with *Escherichia coli* O157:H7. In the Oxoid-Unipath kit, the positive control were latex beads coated with inactivated O157 cells in buffer and the negative control was a suspension of *Escherichia coli* O116 cells. Several negative samples were tested to check the kits for accuracy. No kit demonstrated agglutination reactions for non-O157 samples.

Analysis

Prevalence was calculated as defined in Veterinary Epidemiology: Principles and Methods (13). In this study prevalence is the number of cattle from which SLTEC were isolated divided by the total number of cattle sampled during the one year duration. Intra-herd correlation coefficient calculations were performed as demonstrated by Donald and Donner (14), and used to adjust the confidence interval of the prevalence estimates. Differences in the prevalence of SLTEC for sex, seasonality and day of slaughter were tested using chi-square (13).

2.3 RESULTS

SLTEC was recovered from forty of the one thousand beef steers and heifers randomly selected at slaughter on P.E.I. The prevalence of SLTEC on P.E.I. at slaughter in this class of animals was 4% (40/1000). The corresponding 95% confidence interval was 2.3% to 5.7%. The intra-herd correlation coefficient for infection with SLTEC in this class of livestock was 0.009.

There were 43 SLTEC isolates identified from the 40 positive animals. Three of the 40 positive animals yielded two SLTEC positive isolates each (Table 1). From the 43 positive isolates, 26 different serotypes of SLTEC were identified (Table 2). Ten of the 26 serotypes (38%) have been isolated in humans. Six of the 26 serotypes (23%) have been associated with illness in humans (2,8).

The 1000 animals in the study originated from 123 different Prince Edward Island farms. The 40 SLTEC positive animals were detected from 26% (n = 32) of the 123 farms. Animals sampled from 14.6% (18/123) of the farms yielded serotypes of SLTEC that have been isolated from humans. Animals with serotypes of SLTEC that have been associated with illness in humans were recovered from 10.6% (13/123) of the farms (Table 2). More than one SLTEC isolate was recovered from eight of the 32 positive farms. On three (5,95,122) of the eight farms, one animal on each farm yielded two different SLTEC

serotypes. On six of the eight farms, different serotypes of SLTEC were recovered from different animals on the same or different sampling days (Table 1).

Contingency table analysis determined that there was no difference in SLTEC infection between beef steers and heifers (χ^2, $p=0.41$). There was no evidence of seasonality detected in the study ($p=0.192$). There also was no evidence of a difference in SLTEC detection rate by day of slaughter ($p=0.36$).

All fecal SLTEC isolates were resistant to tilmicosin. Two isolates, (O?:NM, O8:H8) were resistant to oxytetracycline and streptomycin. A third isolate (O?:H7) was resistant to amoxycillin, cephalexin, and ampicillin. The other 24 isolates, including *E. coli* O157:H7, were susceptible to all antimicrobials tested (Table 3).

Each of the five O157 isolates identified during the study were detected by the latex kits. Four of the five isolates were serotyped and identified as *E. coli* O157:H7. The other isolate was *E. coli* O157:NM.

2.4 DISCUSSION

The prevalence of SLTEC in cattle has been demonstrated at 4-19% of animals in Canada, United States, Germany, and Britain (8,11,15,16,). The 4% prevalence of SLTEC detected herein is similar. SLTEC can be isolated from the stools of healthy beef animals at slaughter (3,8,10,15). In the present study SLTEC was isolated from the feces of healthy beef slaughter steers and heifers during carcass dressing procedures. The detection of SLTEC isolates in the stools of beef market cattle suggest that the emphasis placed on dairy cattle as a source for SLTEC that can cause human illness must also be shared by beef cattle (15,16).

Non-O157:H7 SLTEC was present in 3.7% of the beef cattle surveyed. This figure is lower than reported elsewhere. In the U.S., Wells and co-workers (8) reported finding non-O157:H7 SLTEC in 8.4% of adult dairy cows and 19% in heifers and calves. In a slaughter survey done in Canada by Clarke et. al. (15), non-O157:H7 SLTEC was detected in 10.5% of the beef cattle sampled. Another slaughter survey in Germany found non-O157:H7 SLTEC in 17% of dairy cows and 9.4% of young bulls (10).

Twenty-six different serotypes of O157:H7 and non-O157:H7 SLTEC were recovered. Ten serotypes (O113:H21, O103:NM, O?:NM, O153:H25,

O132:NM, O157:H7, O157:NM, O22:H8, O26:H11, O91:H21) encompassing about 38% of our isolates have been previously isolated from humans. Other results demonstrated that many SLTEC isolates recovered from cattle were of serotypes previously isolated from human stool (3,8,10).

Obtaining an accurate estimate of SLTEC prevalence in cattle is difficult. Comparisons between results of prevalence studies need to be made cautiously. Provided there is an awareness that differences between studies can occur due to such factors as the choice of the target population, differences in sampling strategies and variations in the microbial test methodologies such comparisons can be useful (9). The prevalence of SLTEC reported herein is somewhat lower than that reported in several other surveys. This difference may reflect how the animals were managed prior to slaughter and sampling. On certain days the cattle sampled were housed in the abattoir barn overnight without access to food. At slaughter some animals had empty rectums. This made it difficult to obtain an adequate amount of fecal material for testing. Since this study was undertaken to determine the prevalence of SLTEC in beef animals at slaughter and also obtain some indication of the potential for contamination of beef carcasses and the slaughter environment, animals randomly selected that had empty or near empty rectums were included in the survey. However, not having a copious amount of feces present in the rectum during sampling may have resulted in animals that were SLTEC positive not being detected because the

small volume of feces was not an adequate amount for isolation of SLTEC.

In this survey *E. coli* O157:H7, detected in 4 of 1000 beef cattle (0.4%), was one of the two most commonly isolated serotypes. *E. coli* O?:NM was also identified in four fecal samples. Presently, *E. coli* O?:NM has not been identified as a serotype of SLTEC pathogenic to humans although it has been isolated in human stools.

Although the overall SLTEC prevalence reported here is low, the prevalence of *E. coli* O157:H7 detected in this study is within the ranges presented elsewhere. Similar surveys indicated the prevalence of *E. coli* O157:H7 to be 0%-1.5%. In Canada, England, Germany and the U.S.A., the reported rates for *E. coli* O157:H7 were three of 200 beef cattle (1.5%), two of 207 beef cattle (1%), two of 212 young beef bulls (0.9%) and 0% for dairy heifers and calves respectively (15,11,10,8). In their study on beef cattle in feedlots and not at slaughter, Hancock and colleagues (16) reported *E. coli* O157:H7 in feces from 2 of 600 (0.33%) cattle.

The severe pathogenic consequences of O157:H7 in the human foodchain are well known. In this survey, *E. coli* O157:H7 isolates may have been missed from some of the animals fasted prior to sampling because a small quantity of feces is not adequate for detection of the organism. However, since these

animals did not have feces in their rectums, the threat of carcass contamination during dressing procedures is significantly reduced. The food safety concern, nonetheless, is whether or not these animals may have been shedding *E. coli* O157:H7 into the holding pens during the fasting period. Hide contamination creates a major potential for carcass contamination during the dressing process.

The impact of fasting on the incidence of fecal shedding is important to the epidemiology of SLTEC. Animals held off feed for 1-2 days prior to marketing undergo dietary stress that changes the internal environment of the rumen (3,5,17). Most surveys for SLTEC have been conducted using fecal swab specimens obtained from on-farm, well-fed animals in which nutritional stress is unlikely. The changes in rumen environment allow enteric bacteria that would not normally survive and grow within the rumen of well-fed animals to proliferate. In these feed restricted animals, fecal shedding of SLTEC may be notably different from those on full feed. Although carcass contamination by contaminated hides and/or rectal contents is less likely in fasted animals than unfasted animals, there is greater potential for contamination with *E. coli* O157:H7 when accidental spillage occurs (3). In this study, fasted and unfasted animals were not evaluated separately. Further study to clarify the risks involved in processing fasted animals is required.

On six farms from which beef cattle were sampled, SLTEC was isolated from

more than one animal. This indicates the occurrence of clustering in herds in which SLTEC positive individuals are detected. Similar results have been reported previously (8,16). In this study, the time frame in which the samples were taken suggests how SLTEC is maintained in a herd. There were occasions when positive isolates were detected from herdmates that were marketed on the same day. This suggests that in some herds SLTEC infection in multiple animals can occur simultaneously. Also, positive isolates obtained from animals originating on the same farm although marketed at different times suggests that SLTEC is able to maintain its presence within the herd. Due to this clustering effect, an intra-herd correlation coefficient must be calculated to establish an appropriate confidence interval (CI) for prevalence estimates. Basically, animals within a herd have a similar SLTEC status. If the clustering of SLTEC on farms was not considered, the estimated prevalence of SLTEC in beef animals would appear more precise than it is. This results in the wide CI in this study of 2.3% to 5.7%.

In this survey it was not determined if SLTEC were spread as a contagion from one animal to another, or if other unknown risk factors involving the animal, the environment, or the management of both were responsible for maintaining SLTEC infection in a herd over a period of time. To get this information, the incidence or herd SLTEC status must be measured.

One of the current efforts in SLTEC research is identifying SLTEC positive animals and their farm(s) of origin. After a farm has been identified with a herd of cattle from which SLTEC positive animal(s) originated, study of potential risk factors for SLTEC presence may be undertaken. Associations between certain risk factors and SLTEC infection at both the herd and individual animal level have been reported. However, these associations should be regarded as markers that suggest hypotheses for more targeted studies rather than clearly determined true associations (7,16).

In conclusion, SLTEC is present in at least 4% of beef cattle marketed in P.E.I., demonstrating that beef cattle are a significant reservoir for SLTEC. Further, SLTEC which cause human illness were detected in 1.5% of the beef animals marketed. *Escherichia coli* O157:H7 found in 0.4% of animals was one of the two most common serotypes detected. However, these rates most likely underestimate the true prevalence of SLTEC in the beef cattle population surveyed.

The contrast in this study between the low overall SLTEC rate and the mid-range pathogenic SLTEC rate suggests several food safety considerations. If the gastrointestinal tracts of fasted animals are an increased source of risk for contamination of meat and the environment, why is this not reflected in the results of this survey? Does this mean that although the fasted animal can

harbor pathogenic SLTEC, the potential for contamination is decreased because abattoir workers do not have to contend with the potential for spillage from full digestive tracts? Or, do abattoir workers have to be particularly careful when performing procedures on fasted animals since there may be an increased potential for carcass contamination with SLTEC organisms that cause illness in humans? Either scenario emphasizes the necessity to exert very effective control over slaughter activities to minimize the potential of carcass contamination.

As research into the natural history of SLTEC continues to expand our knowledge of this group of organisms, it is clear that there is no one area in the foodchain where stringent intervention will eliminate SLTEC as a concern. Highly controlled slaughter activities are critical; however to make these activities the only critical control point in the meat producing foodchain will result in continuation of devastating health problems.

Antimicrobial drug resistance was not a significant problem among the isolates in this study. Tilmicosin resistance was expected. Tilmicosin has no action on *Escherichia coli* or any other member of the Enterobacteriaceae family. It only inhibits gram positive bacteria (18). Tilmicosin was included in the survey to indicate if any of the isolates might be something other than a gram negative *Escherichia coli.* Antimicrobial resistance has been reported in SLTEC organisms isolated during other studies. Wilson et al. (9) reported that 41% of isolates

obtained from mature dairy cows and 73% of isolates from dairy calves three months of age or younger were resistant to at least one of the drugs they tested. In a study on retail ground beef, Read et al. (2) reported multiple antimicrobial resistance in two isolates of SLTEC serotype O117:H4 and resistance to tetracycline in SLTEC O113:H21. Read found 12% of isolates from ground beef were resistant to at least one drug (2).

Only 12% of the SLTEC isolates from beef animals tested in this study were resistant to antimicrobials other than tilmicosin. The difference in resistance patterns that apparently exists between beef animals in this study and dairy animals in Wilson's study may be partially explained by animal husbandry practices. Since dairy calves are frequently treated with antimicrobials, a detectable resistance pattern in SLTEC organisms isolated from calves is expected. Mature lactating dairy animals, however, should not be regularly treated with antimicrobials. Resistance to antimicrobials may be carried over from calfhood in mature dairy animals. This creates the concern that SLTEC antimicrobial resistant organisms could contaminate milk from these animals and create a serious health hazard.

TABLE 1.

Shiga-like-toxin producing *E. coli* serotypes recovered at slaughter from Prince Edward Island beef farms

Farm	Number of cattle	Serotype
1	2	O?:NM, O157:NM
3	1	O103:NM
5	1*	O153:H25, 0?:H2
9	1	OR:H25
13	1	O?:H38
14	2	O?:NM, O172:H21
15	1	O?:NM
17	1	O?:H2
19	2	O172:H21, O153:H25
24	3	O119:H25, O113:H21, O2:H27
26	1	O?:H2
27	2	O157:H7
29	1	O113:H21
30	1	O132:NM
31	1	O22:H8
39	1	O157:H7
42	1	O22:H8
43	1	O91:H21
50	1	O?:H25
59	1	O116:NM
61	1	O172:H21
69	1	O121:H7
77	1	O110:H2
78	1	O136:H12
84	1	O8:H8
89	1	O?:NM
94	1	O157:H7
95	1*	O136:H12, OR:H12
96	1	O113:H21
106	1	O43:H2
109	1	O26:H11
122	2*	O110:H2, O119:H16*, O121:H7*
Total 32	40	27

* 2 serotypes isolated from the same animal

TABLE 2.

Characteristics of Shiga-like-toxin producing *E. coli* serotypes recovered at slaughter from Prince Edward Island beef farms

E. coli serotype	Serotype isolated in humans	Serotype assoc with human illness	Number of isolates	Number of cattle	Number of farms	SLT*
O103:NM	✔		1	1	1	I
O110:H2			2	2	2	I
O113:H21	✔	✔	3	3	3	I,II
O116:NM			1	1	1	II
O119:H16[a]			1	1	1	
O119:H25			1	1	1	II
O121:H7 [a]			2	2	2	I
O132:NM	✔		1	1	1	I
O136:H12[b]			2	2	2	I,II
O153:H25[c]	✔	✔	2	2	2	I
O157:H7	✔	✔	4	3	3	I,II, I&II
O157:NM	✔	✔	1	1	1	II
O172:H21			3	3	3	I,II
O22:H8	✔	✔	2	2	2	I, I&II
O26:H11	✔	✔	1	1	1	I
O2:H27			1	1	1	
O43:H2			1	1	1	II
O8:H8			1	1	1	I
O91:H21	✔		1	1	1	II
O?:H2[c]			3	3	3	I,II
O?:H25			1	1	1	I
O?:H38			1	1	1	I&II
O?:H7			1	1	1	II
O?:NM	✔		4	4	4	I
OR:H25			1	1	1	I
OR:H12[b]			1	1	1	I
Total 26	10	6	43	40	32	

* Shiga-like-toxin

[a,b,c] 2 serotypes isolated from the same animal

TABLE 3.

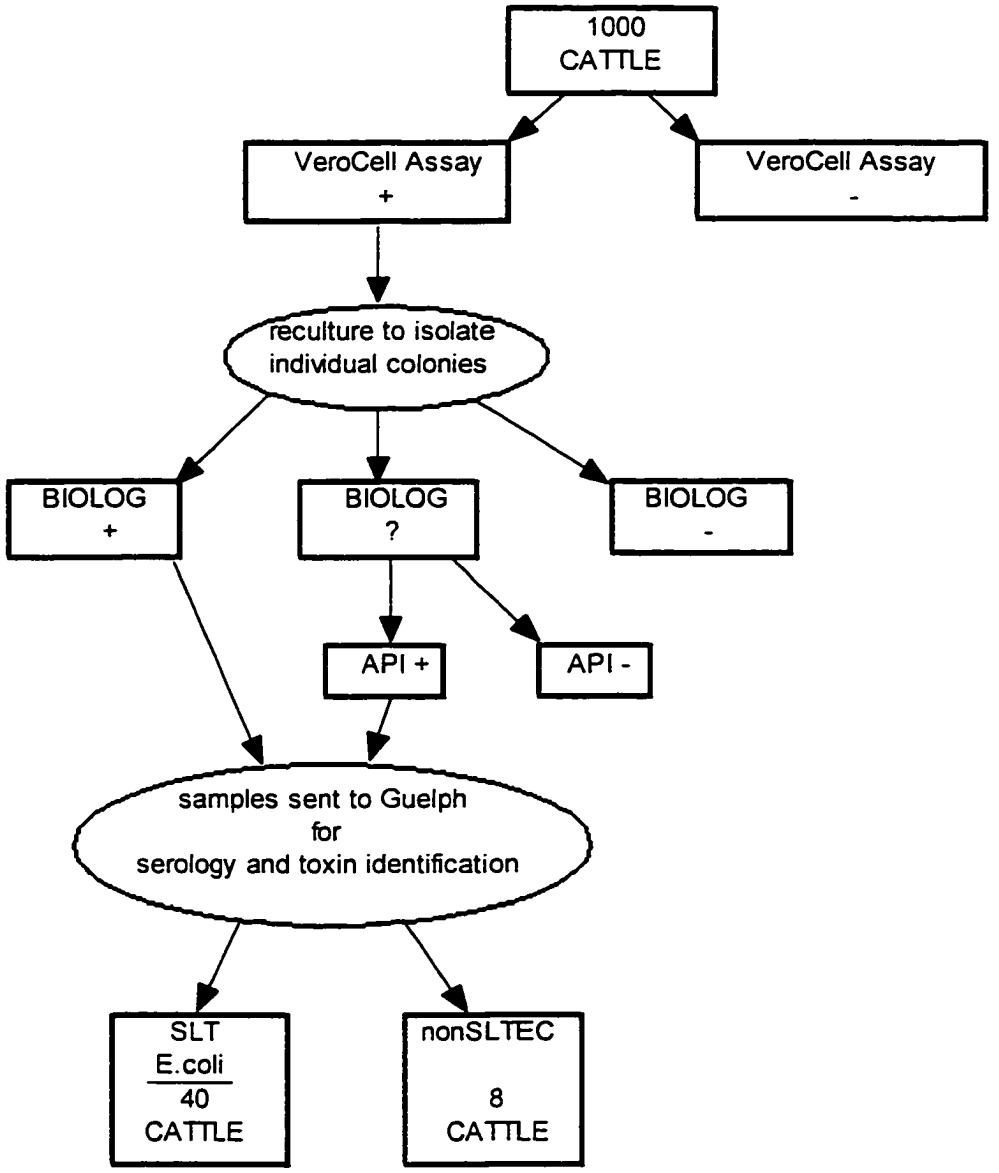
Resistance to antimicrobials* of Shiga-like-toxin producing *E. coli* serotypes recovered at slaughter

E. coli serotype	TIL	OXYT STREP	AMOX CEPH AMP	OTHER ANTIMICR
O103:NM	✔			
O110:H2	✔			
O113:H21	✔			
O116:NM	✔			
O119:H16	✔			
O119:H25	✔			
O121:H7	✔			
O132:NM	✔			
O136:H12	✔			
O153:H25	✔			
O157:H7	✔			
O157:NM	✔			
O172:H21	✔			
O22:H8	✔			
O26:H11	✔			
O2:H27	✔			
O43:H2	✔			
O8:H8	✔	✔		
O91:H21	✔			
O?:H2	✔			
O?:H25	✔			
O?:H38	✔			
O?:H7	✔		✔	
O?:NM	✔	✔		
OR:H25	✔			
OR:H12	✔			
TOTAL 26	26	2	1	0

✔ resistant

* TIL = Tilmicosin (15 µg)
OXYT, STREP = Oxytetracycline (30 µg) , Streptomycin (10 µg)
AMOX, CEPH, AMP = Amoxycillin (30 µg) , Cephalexin (30 µg), Ampicillin (10 µg)
OTHER ANTIMICR = Ceftiofur (30 µg), Gentamicin (10 µg), Neomycin (30 µg), Polymyxin B (300 units), Trimeth-sulpha (15 µg), Cefotaxime (30 µg), Cefixime (5 µg), Ciprofloxacin (5 µg), Apramycin (15 µg), Chloramphenicol (30 µg), Enrofloxacin (5 µg)

FIGURE 1. Flowchart showing multi-stage technique for identification of Shiga-like-toxin *E. coli*



BIOLOG and API provide a standardized micromethod of biochemical and assimilation tests for the identification of bacteria.

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Chapter 3.

Shiga-like toxin identification by monoclonal antibody neutralization and PCR: a comparison of procedures

3.1 INTRODUCTION

The identification of the toxin type produced by shiga-like-toxin producing *Escherichia coli* (SLTEC) is important. These toxins are part of the virulence mechanism by which SLTEC cause serious illness in humans. The illnesses include hemorrhagic colitis (HC), hemolytic-uraemic syndrome (HUS), thrombocytopenia purpura (TTP) and microangiopathic hemolytic anaemia (1,2). When the immunological status of an individual exposed to SLTEC is compromised due to immaturity, old age, or other debilitation, the consequences are likely to be severe illness or death.

SLTEC organisms can produce toxins called either verotoxins because of their lethal effect on African Green Monkey kidney cells (Vero cell culture) or Shiga-like toxins due to their antigenic similarity to the toxin produced by *Shigella dysenteriae* 1 (1,3). There are several types of Shiga-like-toxins (SLT): (SLT-I, SLT-II, and SLT-IIv). SLT-IIv describes several variant types of SLT-II. SLT-IIv is produced by strains of *E. coli* which cause edema disease in swine. It is cytotoxic for Vero cells, not HeLa cells. This characteristic differentiates SLT-IIv from SLT-I and SLT-II (3,4).

SLT-I is very similar to Shiga toxin. The two toxins share greater than 99% deduced amino acid sequence homology. SLT-I and SLT-II, however, share about a 60% deduced amino acid homology and are antigenically distinct (4). Neutralization of SLT-I can be accomplished with Shiga toxin antiserum raised from *Shigella dysenteriae* 1 or monoclonal antibodies raised against the B-subunit of SLT-I. SLT-II, however, can not be neutralized by either B subunit monoclonal antibodies or by Shiga toxin antisera. Antisera which neutralizes SLT-II can not neutralize SLT-I and can neutralize SLT-IIv (4).

Rapid, simple and specific tests are needed to detect SLT in food and fecal material. Numerous methods can accurately detect SLT in food. These procedures are usually based on one of two concepts. One way to identify the type of SLT produced by SLTEC organisms is by using antibodies that react with (neutralize) specific proteins unique to each type of SLT (5). A limitation that can be encountered, however, is the need for a large number of SLT producing organisms to be present in the sample to detect the toxin. The size of a sample, how it is handled and the presence of natural inhibitors can affect the number of viable SLT producing organisms present. If too few viable organisms are present to produce SLT, SLT will not be detected by methods utilizing the SLT antibody neutralization technique. To overcome this problem, many methods include an enrichment step to increase the number of SLT producing organisms in fecal or clinical samples. This step increases the likelihood of toxin detection once an

identification procedure is performed. This requirement adds significantly to the time needed to obtain results (5,6,7).

Another way to detect SLT in food and feces, without enrichment, is to detect organisms carrying SLT genes. DNA probes and polymerase chain reaction (PCR) are two procedures that can detect the presence of SLT genes in food and fecal samples. These procedures, however, cannot distinguish the DNA of live cells from that of dead cells (8,9). Although the DNA from a dead SLT producing cell may be present in a sample, the organism is not able to produce toxin and is no longer a potential threat (7).

Both of these methodologies, especially DNA detection, require significant technical expertise to ensure the procedures are meticulously completed. While these complicated characteristics can be accommodated in highly skilled laboratory environments, the same characteristics make many of these methods less applicable for use in diagnostic or epidemiological investigations where rapid processing of many samples is required.

In this study, the Gold Standard Vero cell assay (VCA) was used to screen fecal samples collected from beef steers and heifers at slaughter for SLT. Although the VCA is an excellent tool for identifying the presence of toxin in feces, the type of toxin present in the sample cannot be identified by VCA alone. To

identify the toxin(s) present in the VCA positive samples, a monoclonal antibody SLT neutralization procedure and a PCR procedure were compared to determine how well the procedures agreed. Monoclonal antibodies have been developed to bind to and neutralize specific subunits of SLT-I, SLT-II and SLT-IIv. The monoclonal antibody technique is not as involved as the PCR procedure but does require an enrichment step. PCR is a procedure that can be used to detect the presence of SLT toxin genes in SLTEC found in food or fecal samples. The PCR procedure is complex and requires specialized training and equipment.

3.2 MATERIALS AND METHODS

MONOCLONAL HYBRIDOMAS

Monoclonal antibodies against SLT-I and SLT-II were used to identify the type of toxin produced by each of the *E. coli* isolates. The monoclonal antibodies were produced by propagating hybridomas purchased from the American Type Culture Collection (ATCC). Three different mouse hybridoma cell lines were used:

i. ATCC CRL 1794 hybridoma cell line secretes an IgG_1 antibody that reacts with the beta subunit of SLT-1. It detects SLTEC strains that produce high levels of SLT-I only or both SLT-I and SLT-II. It will not neutralize SLT-IIv.

ii. ATCC CRL 1907 hybridoma cell line secretes an IgG_1 kappa antibody that reacts with the A subunit of SLT-II. It detects SLTEC strains that produce moderate or high levels of both SLT-I and SLT-II. It will partially neutralize SLT-IIv.

iii. ATCC CRL 1908 hybridoma cell line secretes an IgG_2a kappa antibody that reacts with the A subunit of SLT-II. It detects SLTEC strains that produce SLT-II. It can be used to differentiate SLT-II from SLT-I. It does not neutralize SLT-IIv.

Toxin neutralization using the three monoclonal antibody producing hybridomas was performed in 96 well microtitre plates (Becton Dickinson Labware). One hundred μL of EMEM (5% FBS, Gibco) was added to each well of the microtitre plates. One hundred μL of supernatant from each SLTEC isolate was added to the first well in each of two rows. Serial dilutions (1:2) of each supernatant were made horizontally for 6 wells. All SLTEC positive isolates were individually tested with the three different monoclonal antibodies. One hundred μL of the monoclonal antibody supernatant was added to the second row of each sample. The plates were incubated for 1 h at 37°C in a 5% CO_2 / 95% atmospheric air incubator to allow the monoclonal antibody to react with the SLT. One hundred μL of Vero cells (approximately 4.0×10^6 - counted with hemocytometer) was then added to all wells in the rows containing samples. A control well on each plate containing only EMEM, monoclonal antibody supernatant and Vero cells was included to ensure that the monoclonal antibody supernatants were not verocytotoxic. A SLTEC negative control (ATCC 25922) was included to ensure that the VCA was working correctly. Several positive control isolates obtained from the culture collections of R.C. Clarke and S.C. Read, Health Canada Laboratory, Guelph, Ontario (Strains 321, C600 and E325-11) were included to demonstrate how the monoclonal antibodies could effectively neutralize toxins. Plates were incubated at 37°C in a 5% CO_2 incubator for 48 h. The plates were read at 24 h and 48 h. The wells containing monoclonal antibody were compared to the wells without monoclonal antibody to determine if there had

been toxin neutralization.

To determine how well the monoclonal antibodies neutralized SLT, the viability of the Vero cell monolayer in microtitre wells containing monoclonal antibodies and SLTEC supernatant were compared to microtitre wells that contained SLTEC supernatant and no monoclonal antibody. Monoclonal antibody neutralization of SLT was determined to be effective if microtitre wells containing monoclonal antibody, had less than 50% of their Vero cell monolayer destroyed by SLT after 48 h of incubation.

TITRATIONS

To determine the level of Vero cell toxicity, each positive sample was titrated. Colonies from each sample were incubated overnight at 37°C in one ml of brain heart infusion broth. After incubation, the samples were centrifuged and 50 μL of supernatant from each was put into a microtitre well containing 200 μL of EMEM. Each sample was serially diluted (1:5) vertically in 12 wells. One hundred μL of Vero cells (approximately 4.0×10^6) was added to each microtitre well. The plates were then incubated at 37°C in a 5% CO_2 incubator for 48 h. Vero cell cytotoxicity was evaluated at 24 h and 48 h. A well was considered to contain SLT if 50% or more of the Vero cell monolayer was destroyed within the

48 h interval.

Polymerase Chain Reaction (PCR) Toxin Identification

All positive SLTEC isolates were sent to AAFCHAL in Guelph where the type of toxin produced by each was identified by PCR procedures (Appendix B).

Analysis

All data were entered into the computer data base program, Dbase III plus (Ashton-Tate). To calculate the agreement between the two tests, the Kappa Statistic was used (10).

3.3

RESULTS

Agreement between monoclonal antibody neutralization and PCR for identifying the type of toxin produced by SLTEC was poor. Of the 43 SLTEC isolates, 37 were screened for both monoclonal antibody neutralization and PCR. Four of the SLTEC isolates lost their verocytotoxicity during storage and were not tested.

PCR results were not obtained for two of the 43 SLTEC isolates. These two isolates were positive with generic toxin primers but negative with specific toxin typing primers. SLT-I DNA was detected by PCR in 24 of 41 SLTEC isolates. SLT-II DNA was detected in 14 of the 41 isolates; three of the isolates contained both SLT-I and SLT-II DNA (Table 1).

The Kappa Statistic was used to measure agreement between the monoclonal antibody neutralization procedure and PCR for toxin identification, beyond agreement expected by chance. The 37 SLTEC isolates with results for each toxin identification procedure were used to determine the Kappa values.

The Kappa value for all 37 isolates showed that the agreement between the two tests beyond that expected by chance was only 24% (Table 2). Moderate agreement between two tests is considered at Kappa values of 50% to 60%

(10). Kappa values for isolates considered high and low quantity toxin producers were also calculated. Isolates were considered to be high toxin producers if verocytotoxicity was observable at a toxin titre greater than or equal to 1/125. Isolates with toxin titres below this level were considered low level toxin producers. The agreement beyond chance between the two tests when the 24 high toxin producers were separated out was only 7% (Table 3). For the 11 low toxin producers the agreement beyond chance between the two tests was 38% (Table 4).

3.4 DISCUSSION

Epidemiological evidence suggests that the SLTs of *E. coli* cause disease in both humans and animals (1,3,11,12). Although no direct evidence proves the involvement of SLTs in pathogenesis, disease probably occurs after toxin absorbed from the gastro-intestinal tract impairs the endothelium of blood vessels that supply the colon, kidneys, and central nervous system (3,5). Because of this close association between SLTs and disease, an efficient, accurate and reproducible method for identifying the type of toxin produced by SLT *E. coli* strains in food would be useful both epidemiologically and industrially.

Although there are several methods available to determine the type of SLT produced by SLTEC, none have been clearly defined as the Gold Standard method against which other methods should be compared. The lack of a clearly defined Gold Standard means that these tests can only be compared against each other. In this study the monoclonal antibody SLT neutralization method was evaluated against the PCR SLT identification procedure. Since neither procedure is the Gold Standard, we evaluated the agreement of the two tests using the Kappa statistic.

The monoclonal antibody toxin neutralization procedure was not difficult to perform. However, tissue culture facilities are required which complicates

adaptation of the procedure into an industrial environment. The PCR system requires specialized equipment and highly trained personnel to perform the procedures. Considerable care is required for preparation of samples to avoid cross-contamination. This latter procedure, would also be difficult to perform in an industrial setting.

In this study, when the element of chance was removed via the Kappa statistic, the two SLT identification tests only agreed with each other in 24% of the comparisons. Since moderate agreement is considered when Kappa values are between 50% and 60%, the agreement between these two tests was low (10). When titrations were completed on the positive isolates, it was noted that 25/36 isolates had toxin titres that were still cytotoxic at dilutions between 1/125 and 1/3125. These isolates were considered to be high level toxin producers (13). However, there was very little agreement between the two SLT identification methods for high level toxin producers. Surprisingly, the Kappa results between the two methods were higher for low SLT producers than for moderate to high SLT producers. Kappa results for all levels of SLT production were low.

Speculating on the lack of agreement between the two tests is difficult. Difficulty in interpreting the results of the monoclonal antibody neutralization tests would impact on the agreement between the two tests. Another possible explanation is that some strains of SLTEC when tested at a later date, produce a

toxin different from that exhibited originally (14). It is hypothesized that a change from one type of SLT production to another is caused by the loss of a specific bacteriophage which is necessary to mediate the production of a particular SLT. It is also speculated that the amount of toxin produced by some strains of SLTEC may vary in culture media that is prepared at different times (14). In our study the monoclonal antibody neutralization assay and the PCR were performed at different times. Possibly a loss of bacteriophages as described above might explain some discrepancy between the two tests.

The monoclonal antibody SLT neutralization assay was difficult to interpret with variable results as reported elsewhere (8,13,14). Our findings using monoclonal antibodies and culture supernatants ranged from complete neutralization to partial or no neutralization. Contrary to other studies (4,13) we did not observe better neutralization results with moderate to high SLT producers compared to low SLT producers.

Cytotoxin neutralization tests performed on cell cultures can be difficult to interpret due to variable cell growth (14). Although not observed herein, reading of neutralization tests can also be faulty if toxin producing organisms only produce low quantities of toxin (4,14). The inability of the assay to detect low-level SLT producers may not be a disadvantage because SLTEC which produce low levels of SLT-I, unlike those SLTEC which produce moderate or high levels of

SLT-I, are not implicated in disease (4). This variability can cause inconsistent neutralization results for extracellular cytotoxicity with anti-Shiga toxin. Reliable readings are more consistently obtained when neutralization tests are performed on toxins produced by moderate to high-toxin-producing strains of SLTEC (4).

Inconsistent neutralization results occur in some cultures from lost SLT activity due to prolonged periods of freezing (15,16,17). In this study, the monoclonal antibody neutralization assays were performed on SLTEC that had been frozen for ten months. The loss of SLT production observed in several of the isolates might have resulted from freezing.

PCR is becoming the method of choice for the identification of SLT in food and clinical samples. PCR identified SLT genes in 41/43 SLTEC isolates in this survey. PCR detected SLT genes from four isolates which were no longer capable of producing SLT. The monoclonal antibody toxin neutralization procedure cannot be used to identify SLT if SLTEC no longer produce toxin. The capability of PCR, to detect SLT genes in isolates no longer producing toxin and provide results that are easier to interpret than results from the monoclonal antibody toxin neutralization procedure, indicate why PCR is becoming an accepted procedure. Although PCR can be applied directly to foods, the incorporation of an enrichment method to increase the number of SLTEC organisms in a food sample is recommended. Preparing food samples for PCR

presents several challenges due to differences in sample composition. Levels of total microflora may be high, while levels of the "target" organism may be quite low. The food matrix may also contain PCR-inhibiting substances (18).

Many SLTEC isolates are often present in low numbers in food and stool samples. For some SLTEC, even if the infective dose is very low there may still be illness. Low level toxin producers occur frequently. There is ongoing debate whether low level toxin producing SLTEC organisms cause illness. There also are epidemiological data, obtained from outbreak investigations indicating that SLT-II production is as important as SLT-I production as a potential virulence factor (14). Therefore, tests like PCR that can detect low levels of SLT-I and SLT-II are considered necessary and will facilitate testing for SLTEC. This will lead to a better understanding of the epidemiology of this group of *E. coli* isolates associated with human illness.

TABLE 1.

Toxin production Shiga-like-toxin *of* *Escherichia.coli* serotypes recovered at slaughter

E. coli serotype	PCR	MAb	Toxin Titre
O103:NM	I	?	Lost VT
O110:H2	I	I	High
O110:H2	I	?	Lost VT
O113:H21	I	II	Low
O113:H21	I	II	Low
O113:H21	II	?	Lost VT
O116:NM	II	II	High
O119:H16	generic only		?
O119:H25	II	I	High
O121:H7	II	II	Low
O121:H7	I	I	High
O132:NM	I	II	High
O136:H12	I	II	High
O136:H12	II	I	High
O153:H25	I	II	High
O153:H25	I	II	Low
O157:H7	II	I	High
O157:H7	I & II	II	High
O157:H7	I	I	High
O157:H7	I & II	I	High
O157:NM	II	I	High
O172:H21	I	II	Low
O172:H21	II	II	Low
O172:H21	II	II	High
O22:H8	I	II	High
O22:H8	I & II	I	High
O26:H11	I	I	High
O2:H27	generic only		?
O43:H2	II	II	Low
O8:H8	I	I	High
O91:H21	II	II	Low
O?:H2	I	II	High
O?:H2	II	II	Low
O?:H2	I	I	Low
O?:H25	I	I	High
O?:H38	I & II	I	High
O?:H7	II	?	Lost VT
O?:NM	I	I	Low
O?:NM	I	I	Low
O?:NM	I	I	High
O?:NM	I	I	High
OR:H25	I	II	High
OR:H12	I	I	Low
TOTAL 43	41	37	

TABLE 2. Agreement between PCR and MAb for Toxin Type*

		PCR Test		
		SLT 1	SLT 2	
Monoclonal Antibody	SLT 1	15	4	19
	SLT 2	10	8	18
		25	12	37

% Observed agreement	62.2%
% Expected agreement	50.5%
% Actual agreement beyond chance	11.7%
% Potential agreement beyond chance	49.5%
Kappa	0.24

*Shiga-like-toxin

TABLE 3. Agreement between PCR and MAb for High Toxin* Producers

		PCR Test: SLT 1	PCR Test: SLT 2	
Monoclonal Antibody	SLT 1	11	4	15
	SLT 2	6	3	9
		17	7	24

% Observed agreement 58.3%
% Expected agreement 55.2%
% Actual agreement beyond chance 3.1%
% Potential agreement beyond chance 44.8%
Kappa 0.07

*Shiga-like-toxin

TABLE 4. Agreement between PCR and MAb for Low Toxin* Producers

		PCR Test: SLT 1	PCR Test: SLT 2	
Monoclonal Antibody	SLT 1	4.5	0.5	5
	SLT 2	4.5	5.5	10
		9	6	15

% Observed agreement 66.7%
% Expected agreement 46.7%
% Actual agreement beyond chance 20.0%
% Potential agreement beyond chance 53.3%
Kappa 0.38

*Shiga-like-toxin

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Chapter 4.

Evaluation of a modified microwell ELISA kit for detection of O157 antigens in retail ground beef

4.1 INTRODUCTION

Our food supply comes from an environment in which microorganisms are widespread. Although many organisms are harmless to healthy animals and humans, many are less benign and can cause disease and even death. There are many opportunities for animals to become contaminated with pathogenic microbes on the farm, during transport or at the slaughterhouse. Fecal contamination of meat surfaces during slaughter and dressing occurs randomly, usually on specific areas of a carcass. The flank, midline, and legs of a carcass are the areas most likely to be contaminated during these procedures (1). Basic microbial loads will usually be low on such carcasses because most of the contamination is due to cross-contamination. The presence of a single pathogenic organism, however, is a significant food safety concern. Not only can an infectious dose be low for certain organisms, but improper cooling and refrigeration can cause multiplication (2).

Shiga-like toxin producing *E. coli* (SLTEC) are well-recognized causes of gastrointestinal illness, including both bloody and non-bloody diarrhoea, haemolytic uraemic syndrome (HUS), thrombocytopenia purpura, and death

(3,4). SLTEC is now the major cause of acute renal failure in North American children and is usually among the top three bacterial causes of diarrhoea. Over 50 serotypes of SLT-producing *E. coli* have been associated with foodborne outbreaks in North America and elsewhere. Multiple sporadic cases of human illness associated with SLT-producing *E. coli* are also reported in many areas globally (5).

Escherichia coli O157:H7, the most common SLTEC, was the first serotype recognized as a human pathogen following an outbreak of HC and HUS in 1982 (5,6). Outbreaks of severe illness due to *E. coli* O157:H7 have continued since this time in both Canada and the United States. Ground beef is the most common vehicle for *E. coli* O157:H7 transmission (7,8,9,10). Other vehicles of infection associated with human illness are: raw milk, apple cider, fermented sausage, cold sandwiches, mayonnaise, vegetables, and water (3). Person-to-person and cattle-to-person transmission occur (11,12). *Escherichia coli* O157:H7 can be recovered from the intestines of approximately 0.5%-2% of cattle. Because of processing practices, meat from many animals may comprise one hamburger (13). Ground beef contaminated with *Escherichia coli* O157:H7 can cause illness when meat is not thoroughly cooked (to an internal temperature of at least 68°C) or when raw or undercooked meat cross-contaminates other food items. Because the infectious dose is low, even limited deficiencies in food preparation or handling can result in exposure and infection.

Detecting pathogenic microorganisms in food is difficult. In such samples, the variable incidence of pathogen contamination and the presence of numerous competitive microbes make the development of accurate, rapid diagnostic methods challenging. Several different technologies have been developed to detect pathogenic microorganisms in food samples, based on biochemical, immunological, and genetic characteristics of the microorganism of interest (4,14).

The biochemical identification of microorganisms is primarily performed on culture-based procedures that rely on an organism's ability or inability to utilize or produce certain chemicals within specific temperature ranges. There are many organisms, however, that share biochemical properties. This leads to false-positive and false-negative results. This limitation in sensitivity or specificity can make biochemical identification of microorganisms difficult to interpret (7).

Immunological procedures, primarily ELISA methods, have been used for screening of pathogens in meat and poultry products. ELISA methods use an antibody specific to a particular pathogen and usually show a colorimetric reaction. Multiple samples can be analysed simultaneously, the procedure is semi-automated, and the assay can be completed in three hours. Some newer ELISAs take 15-30 min to perform and many samples can be run simultaneously

(2,7).

A variety of genetic based detection procedures have been developed to detect SLTEC in food and clinical samples. The identification of microorganisms with DNA probes and polymerase chain reaction (PCR) is common (2,7). DNA probes recognize and hybridize to a characteristic segment of the target organism's nucleic acid. Some probes can be specific for the detection of genus, species and sometimes strains. DNA probes require greater technical expertise and are more expensive than ELISAs. Some types of meat samples may contain DNA inhibitors that interfere with some newer molecular technologies. Some DNA probes are radioactively labelled, thus requiring appropriate containment and disposal controls in the laboratory (2,7).

Immunological and genetic-based detection procedures seem to address the problems associated with sensitivity and specificity that occur with culture-based procedures. A key factor, however, in testing for pathogens is the necessity to enrich samples to boost bacterial numbers to levels capable of detection. This is a limitation encountered with both ELISAs and DNA probes. The need to incorporate enrichment procedures into these methods may lengthen analysis time to 27-50 h (2).

The purpose of this study was to determine the prevalence of SLTEC in ground

beef available to the consumer from various retail outlets across Prince Edward Island. Since the prevalence of SLTEC in beef animals at slaughter on P.E.I. had been determined, it was necessary to perform this study on the opposite end of the production chain to determine what the potential exposure to SLTEC is for the consumer. The Vero cell assay (VCA) which had been used as the Gold Standard test for ascertaining the prevalence of SLTEC in beef steers and heifers was used to determine the prevalence of SLTEC in the retail ground beef sampled. Along with determining the prevalence of SLTEC in the ground beef samples an evaluation of an ELISA test kit was performed. The test kit designed to detect *E. coli* O157 antigen in fecal samples was modified for this study to detect the antigen in food samples. In ground beef samples in which the ELISA test detected O157 antigen, the usefulness of anti-*E. coli* O157 antibody coated immunocapture beads in conjunction with cefixime-tellurite sorbitol-MacConkey agar (TC-SMAC) for the isolation of *E. coli* O157:H7 was also evaluated.

4.2 MATERIALS AND METHODS

Sample collection

Two hundred ground beef samples were collected from 20 different grocery stores in five different communities on Prince Edward Island between June 01, and September 30, 1995. Samples were collected on Fridays and Saturdays and kept refrigerated until processed on Monday.

Preparation of samples

Bacteria numbers in the ground beef samples were increased by an enrichment step. Twenty-five g of ground beef from each sample was measured into a stomacher bag. Two hundred and twenty-five ml of modified-*E.coli* broth (Appendix 3), was added to each bag, and the sample was stomached for 2 minutes. All samples were incubated for 18 h at 37°C while shaking (120 rpm).

Detection of SLTEC in ground beef samples

After incubation, one ml aliquots of supernatant from each sample were centrifuged (Eppendorf) at 12,000 g for 10 minutes. A Vero cell assay was performed on each sample. Fifty μL of supernatant from each sample were

added to the top wells of a 96-well flat-bottomed micro titre plate (Becton Dickinson Labware) containing 200 μL of Eagles Minimal Essential Medium (EMEM, Gibco) in each well. Four serial dilutions of 1:5 were made vertically for each well to a final dilution of 1:625. One hundred μL of a Vero cell suspension (containing approximately 4.0×10^5 cells/ml - counted by hemocytometer) was added to each well. An SLT positive control well (Strain 321 - Health Canada), an SLT negative control well (ATCC 25922), and a well with only EMEM and Vero cells were included on each plate. The positive and negative control wells were used as standards to assess the cytotoxic affect of toxin produced by SLTEC present in the supernatant. The microtitre plates were incubated at 37°C in a 5% CO_2 / 95% atmospheric air incubator (Napco) for 48 h. The VCA titre endpoint was recorded at the end of incubation as negative (-), weak(+/-) or positive (+). Samples were considered positive if 50% or more of the Vero cell monolayer was not intact after 48 h of incubation. Weak titres were samples that exhibited less than 50% verocytotoxicity.

Detection of *E. coli* O157 antigen

The *E. coli* O157 antigen detection microwell ELISA kit (LMD Laboratories, Inc.) is an assay designed for qualitative determination of *E. coli* O157 antigen in feces. This assay is a double antibody (sandwich) ELISA using an anti-*E. coli* O157 antibody to capture the antigen from the supernatant. A second

antibody, conjugated to peroxidase (HRP), is then added which binds to the complex. This reaction is visualized by the addition of a chromogen (tetramethybenzidine) and a substrate. The resulting blue colour development indicates the presence of *E. coli* O157 antigens being bound by the anti-*E.coli* O157 antibodies. To assess the utility of this assay to detect *E. coli* O157 in ground beef, the following procedure was performed.

One hundred microlitres of positive (*E. coli* O157 cells in a formalin base) and negative (human *E. coli* O157 negative formalinized stool supernatant) control were added to individual microELISA wells. Along with the controls, 100 μL of supernatant from each ground beef sample was added to individual microELISA wells. All wells were incubated at 20°C for 20 min. After incubation, each well was washed 3 times with diluted wash buffer. Following washing, 100 μL of enzyme conjugate was added to each well and incubated for 10 min. Following incubation, the wells were washed 3 times with diluted wash buffer and a final rinse with deionized water completed this step. As per instructions, one drop of Substrate A (tetramethybenzidine) and one drop of Substrate B (peroxide solution in a citric acid base) were added to each well and incubated for 5 min. Two drops of stop solution (phosphoric acid) were then added to each well. The results were then read visually and via a plate reader at 450 nanometers. Optical density readings of 0.20 and above were considered positive.

Isolation of *E. coli* O157:H7

To isolate *E. coli* O157:H7 from ground beef samples identified as positive by the modified ELISA procedure, a one ml aliquot of each homogenate was mixed with 20 μL of magnetic polystyrene beads coated with O157 antibody (Dynabeads anti *E. coli* O157, Dynal Inc. Lake Success, NY) and incubated at 37°C for 60 min. After 60 min, a magnetic particle concentrator (Dynal MPC-M) was used to concentrate the immunobeads onto the side of the microfuge tube. The m-EC broth was decanted and replaced by one ml of wash buffer (PBS-Tween). The magnetic plate of the MPC-M was removed and the immunobeads were resuspended and the tubes were left to sit for 30 min. This wash step was repeated two more times. Washed beads were resuspended in one ml of m-EC broth and incubated for 6-hr. After incubation, 50 μL of the suspension was spread-plated on sorbitol-MacConkey agar (SMAC) and sorbitol-MacConkey agar supplemented with cefixime and tellurite (TC-SMAC) and incubated at 37°C overnight. The plates were read for colourless colonies and selected colonies were confirmed as *E. coli* O157:H7 by serological tests.

4.3 RESULTS

No SLT production was detected by VCA in any of the 200 retail ground beef samples surveyed. The LMD microELISA assay detected the presence of O157 antigen in 4 of the 200 ground beef samples. Since there were no SLT positive ground beef samples, no attempt was made to isolate *E. coli* O157:H7 organisms from any samples although O157 antigen was detected in four. The usefulness of the immunomagnetic bead separation procedure and the TC-SMAC agar in isolating *E. coli* O157:H7 was not evaluated because no SLT positive samples were identified during the survey.

4.4 DISCUSSION

The prevalence of SLTEC reported from other retail ground beef surveys was 36%-58% (5,8), not detecting any SLTEC during this study was completely unexpected. However, the expectation of finding *E. coli* O157:H7 in the 200 ground beef samples was not very high. Results reported from prior ground beef surveys indicated that the prevalence of *E. coli* O157:H7 in retail ground beef is 0.5%-3% (8,15).

Explaining why the results of this survey are so different from the results of others is difficult. Perhaps the ground beef sampled during the course of this survey was not contaminated with SLTEC. Another factor is that some of the grocery stores sampled during the survey used a mixture of fresh and frozen beef portions to prepare their ground beef. Researchers who have conducted similar surveys have indicated that verocytotoxicity in SLTEC can be lost during freezing or when subjected to prolonged periods of cold stress (5,17). The suitability of the enrichment broth used to increase bacteria numbers to detection levels in the ground beef is a factor that also must be considered. In this study, m-EC broth was used. Previous studies have reported that m-EC broth will reliably enrich food samples with low levels of SLTEC. In work completed by Acheson *et. al.* (5) in which they compared the success of detecting SLTEC toxin production in food samples enriched in MacConkey broth,

m-*E. coli* broth and BHI broth, the interpretation of the results was ambivalent. When they attempted to recover SLTEC from spiked ground beef samples, recovery was more successful in samples enriched in MacConkey broth. However, when they attempted to recover SLTEC from retail ground beef, there was very little difference in recovery between each of the three broths. Johnson et.al. compared how well they could recover *E. coli* O157:H7 from food samples enriched in m-EC broth and modified trypticase soy broth containing acriflavine (dm-TSB). Overall they found that both enrichment broths worked well but they were more likely to recover *E. coli* O157:H7 from food samples enriched in m-EC broth (6). Modified-EC broth is a proven enrichment broth and its use should not have had an impact on the results obtained during this study. However, since the results of this survey were so unexpected, it would be worthwhile in a repeat of this survey to compare m-EC broth with MacConkey broth because MacConkey broth was used to successfully select for SLTEC in feces.

Accurately assessing the detection of O157 antigen by the LMD microELISA assay in ground beef could not be done with certainty due to the low number of samples collected. However, based on the limited observations from the 200 ground beef samples assayed, the microELISA can detect O157 antigen in food. To determine if the O157 antigen detected the presence of *E. coli* O157:H7, further confirmatory procedures are required. One of the original objectives of

this study was to identify O157 antigen in ground beef via the microELISA test kit and then use the immunomagnetic bead separation (IMS) procedure in combination with highly selective TC-SMAC agar to attempt to isolate *E. coli* O157:H7 from the ground beef.

The evaluation of how well these two procedures, might perform in the isolation of *E. coli* O157:H7 was not attempted. No SLT was detected in any ground beef samples including the four samples in which an O157 antigen was detected by the microELISA test. The lack of SLT activity in the samples tested indicated that there was either no SLTEC or very few SLTEC present in the ground beef sampled. The other possibility was if SLTEC were present, they were not viable or were producing SLT at a level so low so as not to be toxic to Vero cells. Based on the 36%-58% prevalence reported in previous ground beef surveys we anticipated detecting at least 40-50 SLT positive samples. The prevalence of *E. coli* O157:H7 in ground beef is low; in 200 ground beef samples, there might not be any O157:H7 organisms isolated. Therefore in this survey in which no SLT activity was detected in any samples, the likelihood of isolating a SLTEC organism, let alone *E. coli* O157:H7, was so low that it was not practical to evaluate the IMS and TC-SMAC procedures. Due to limited study resources, it was not possible to evaluate the two procedures on laboratory spiked ground beef samples.

In the food manufacturing industry, there is significant need for tests that are rapid, sensitive and to some degree specific. The LMD microELISA O157 Antigen Detection Kit does possess some of these requirements. Within 16-20 hours of having obtained a food sample, results on whether or not detectable *E. coli* O157 antigen were present were available. Since the test kit is designed to detect O157 antigen it will detect both *E. coli* O157:H7 and non-*E. coli* O157:H7 organisms. In this survey, the four positive samples did not contain Shiga-like-toxin producing *E. coli* and therefore were not a food safety concern. The concern, however, is with the number of false-positives that indicate the possible presence of *E. coli* O157 organisms.

If large numbers of food samples containing *E. coli* O157 organisms are detected, the necessity to confirm the pathogenicity of the organisms would quickly overwhelm today's large scale food manufacturing businesses. Since only 2% of the food samples indicated the possible presence of such organisms, only 2% of the samples, instead of all samples, would need to be confirmed as SLT producing *E. coli* O157. The LMD microELISA screening test can be justified because many negative samples can be eliminated with minimal effort. Along with rapid return of results, this ELISA assay does not require extensive technical expertise or specialized facilities. Although the ELISA test is not a difficult procedure to perform, preparing samples for enrichment is time consuming. If enrichment broth is prepared on site and not purchased, its

preparation and storage are extra details that require attention.

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CHAPTER 5.

General Conclusions

5.1 CONCLUSIONS

The objectives of this thesis have been met. In Phase One using the Gold Standard VCA to detect SLT in feces, the prevalence of SLTEC in beef steers and heifers at slaughter was 4%. In Phase Two, a monoclonal antibody neutralization assay procedure and a PCR procedure were used to identify the type of toxin produced by the SLTEC organisms isolated from SLT positive fecal samples. The amount of agreement between the two procedures was determined to be low. In Phase Three the VCA was used to determine the prevalence of SLTEC in retail ground beef samples collected from various grocery stores across P.E.I. Surprisingly, SLT activity was not detected in any of the 200 samples. The effectiveness of the immunomagnetic bead separation procedure and the specialized TC-SMAC agar for isolation of *E. coli* O157:H7 were not evaluated because no SLTEC organisms were detected in the ground beef samples.

In Phase One, SLTEC was isolated from the feces of healthy beef slaughter steers and heifers during carcass dressing procedures. The overall 4% SLTEC prevalence reported herein is at the low end of reported prevalences from similar

studies (1,2,3,4). However, the 0.4% prevalence of *E. coli* O157:H7 detected in this study is within the ranges presented elsewhere (1,2,3,4,5). The lower SLTEC prevalence reported here may have been the result of how the animals were managed prior to slaughter and sampling. On certain days the cattle sampled were housed in the abattoir barn overnight without food. At slaughter some animals had empty rectums. This made obtaining a sample with an adequate amount of feces difficult. Animals with empty rectums were not excluded from this survey because we wanted to determine how the prevalence of SLTEC in feces might create potential for carcass contamination during evisceration procedures.

Non-O157:H7 SLTEC was present in 3.7% of the beef cattle surveyed. Twenty-six different serotypes of O157:H7 and non-O157:H7 SLTEC were recovered. Ten serotypes (O113:H21, O153:H25, O132:NM, O157:H7, O157:NM, O22:H8, O26:H11, O91:H21) encompassing about 38% of our isolates have been previously isolated from humans. In this survey *Escherichia coli* O157:H7 was detected in 4 of 1000 beef cattle. *Escherichia coli* O157:H7 was one of the two most commonly isolated serotypes. *Escherichia coli* O?:NM was also identified in four fecal samples. At this point O?:NM has not been identified as pathogenic to humans but has been isolated in human stools.

The severe pathogenic consequences of O157:H7 in the human foodchain are

well known (6). In this survey *Escherichia coli* O157:H7 isolates may have been missed because some animals were fasted prior to sampling and there may not have been sufficient feces in the sample to detect the organism.

On six farms, SLTEC was isolated from more than one beef animal. This indicates the occurrence of clustering in herds in which SLTEC positive individuals were detected (4,7). In this study, the time frame in which the samples were taken suggests how SLTEC is maintained in a herd. There were occasions when positive isolates were detected from herdmates that were marketed on the same day. This suggests that in some herds the presence of SLTEC infection in multiple animals can occur simultaneously. As well, positive isolates obtained from animals originating on the same farm but marketed at different times suggests that SLTEC is able to maintain its presence within the herd. We cannot make any further comments on SLTEC in herds because individuals were examined.

Further study at the herd level is needed to ascertain the prevalence of SLTEC within herds. Based on the results, SLTEC herd prevalence may be more important than individual animal prevalence. The prevalence of SLTEC in individuals is low. Thus it is easy to find negative animals and if one is not careful with these findings, it can be concluded that the overall prevalence of SLTEC in cattle is low. Since SLTEC appears to persists in a herd, prevalence

should likely be considered at the herd level (4). Testing an appropriate number of individuals within a positive herd could lead to a herd prevalence that is significantly higher than the individual animal prevalence we determined in this survey.

In Phase Two, an evaluation between a monoclonal antibody SLT neutralization assay and a PCR procedure to determine which method most accurately identifies the type of toxin produced by SLTEC was performed. Since neither procedure is considered the Gold Standard, we evaluated how well the two tests agreed with each other using the Kappa statistic. In this study, when the element of chance was removed via the Kappa statistic, the two SLT identification tests only agreed with each other in 24% of the comparisons. Since moderate agreement is considered when Kappa values are between 50% and 60%, the agreement between these two tests was low.

Although the monoclonal antibody SLT neutralization assay was not difficult to perform, it was difficult to interpret and gave variable results. Reasons for variability include variable cell growth, SLTEC toxin producing organisms producing low quantities of toxin, lost SLT activity due to prolonged storage, production of a toxin different from that exhibited originally, or a variation in the quantity of SLT produced in culture media prepared at different times (8,9,10,11,12).

PCR is becoming the method of choice for identification of SLT in food samples (13). The results obtained during this survey demonstrated that PCR provided more conclusive, easier interpreted data. Although PCR can be applied directly to foods, the incorporation of an enrichment method to increase the number of SLTEC organisms in a food sample is recommended (14). The PCR results used in this study were obtained from the Health of Animals Laboratory (H of A) in Guelph, Ontario which has an established reputation for SLTEC research and the use of PCR technology. Advice provided by the H of A technical staff familiar with PCR protocol indicated that technicians require 4-6 months to develop a PCR procedure that will consistently provide reliable results. Considering the effort that would have been required to perfect this procedure locally, forwarding the samples to the H of A laboratory was considered to be the best alternative.

In Phase Three, the prevalence of SLTEC in retail ground beef was determined to be zero. These results were completely unexpected. Very few, if any, *E. coli* O157:H7 organisms were expected to be isolated from the 200 samples tested (15,16). However, it was anticipated that at least 40-50 ground beef samples would be SLT positive (15,17). The microELISA test kit detected O157 antigen in four samples. Due to the low number of samples collected, accurately assessing how well the LMD microELISA assay detected the antigen could not

be performed with certainty. In the food processing industry, there is a requirement for tests that are rapid, sensitive and to some degree specific. Based on the limited observations from the 200 ground beef samples assayed, apparently the microELISA does have these capabilities. The intention was to test the microELISA positive samples for the presence of *E. coli* O157:H7 using the IMS procedure and TC-SMAC agar. The lack of SLT activity in all of the samples tested indicated that there were few if any SLTEC present to isolate and an evaluation of these two procedures would not have been meaningful. The final conclusion regarding food safety is that the consumer on P.E.I., during the Summer of 1995, was at low risk of SLTEC exposure from ground beef purchased on the weekends on which the samples were purchased for this study.

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APPENDIX A

MacConkey Broth and MacConkey Agar

MacConkey Broth

One litre

17 g peptone	Difco
3 g protease peptone	Difco
10 g lactose	Difco
1.5 g bile salts No. 3	Oxoid
5 g NaCl	Fisher
0.030 g neutral red	BDH
0.001 g crystal violet	BDH

Sorbitol - MacConkey Agar **One litre**

17 g peptone	Difco
3 g protease peptone	Difco
10 g sorbitol	BDH
1.5 g bile salts No.3	Oxoid
5 g NaCl	Fisher
0.030 g neutral red	BDH
0.001 g crystal violet	BDH
13.5 g agar	Oxoid

APPENDIX B

Generic SLT PCR and Toxin Typing Protocols

generic primers (4)

ES 149	cga aat ccc ctc tgt att tgc c
ES 151	gag cga aat aat tta tat gt

toxin typing primers

VT1c (3)	acc ctg taa cga agt ttg cg
VT1d (3)	atc tca tgc gac tac ttg ac
VT2a (1,2)	tta acc aca ccc acg gca gt
VT2b (2)	gct ctg gat gca tct ctg gt
VTEa (1)	cct taa cta aaa gga ata ta
VTEb (1)	ctg gtg gtg tat gat taa ta

1. SAMPLE PREPARATION

1.1 Inoculate a BHI broth tube with the isolate and incubate at 37°C overnight. Centrifuge the 1.0 mL BHI samples at 14,000 rpm for one minute.

1.2 Wash the pellet with 1.0 mL FA buffer and resuspend in 0.5 mL of sterile millique water.

1.3 Boil the samples for ten minutes and place on ice. Centrifuge for 1 m after cool. Place back on ice.

2. GENERIC PCR

2.1 Prepare Master Mix

Master Mix for generic PCR

Buffer, pH 8.3	Kcl	5 mM
	Tris-HCl	10 mM
dNTP's		200 µM
Primer ES 149		0.10 µM
Primer ES 151		0.10 µM

Taq polymerase	0.31 units/25 ul reaction
Magnesium Chloride	1.5 mM

Mix all ingredients ensuring everything is kept on ice.

QC Use Pipettors designated only for making up the master mix. These are considered clean and will not add contamination to the reaction.

2.2 Add 20μL of the master mix to each PCR tube. Add 5 μL of the DNA template.

2.3 Depending on the type of PCR thermal cycler used, a drop of sterile mineral oil may need to be added to each tube before capping.

QC A positive control containing the SLT genes and negative control not containing the SLT genes are used to ensure the reaction is being carried out properly. Also one of the PCR tubes contains only the Master Mix plus Millique water, to ensure that there was not any contamination during the reaction. If this sample appears positive that the reaction must be repeated from the beginning.

The thermal cycle for the reaction to occur contains the following steps.

Delay time at 72°C for 2 min

Denaturation at 94°C for 1 min
Annealing at 49°C for 1 min
Extension at 72°C for 1 min

These three steps make up one cycle. This cycle is repeated for a total of 35 times then:

Extension at 72°C for 5 min
Hold at 4°C

2.4 After the samples have finished being processed, remove them from the cycler.

2.5 Prepare a 2% agarose gel in a tray with a comb corresponding to the sample number.

2.6 Allow gel to set for 30 min

2.7 Remove comb, and place gel in electrophoretic gel box topped up with TBE buffer.

2.8 Add 2 μL of stop buffer to 5 μL of the sample. Add this combination to a well in the gel. In one of the wells, also include 5 μL of an 100 bp ladder molecular weight standard.

2.9 Run the gel at 100 V for 1.5 h.

2.10 Place the gel in a staining solution of 1 μL/mL EtBr solution for 30 min

2.11 Photograph the gel using a transilluminator and a polaroid camera.

2.12 SLTEC or NON SLTEC
An organism is considered to be SLTEC if, using the generic PCR there is a 323 bp fragment seen after electrophoresis.

3. TOXIN TYPING PCR

3.1 Prepare Master Mix

Master Mix for toxin typing	
Stoffel buffer, pH 8.3	Kcl 10 mM Tris-HCl 10 mM
dNTP's	200 μM
Primer VT1c	0.10 μM
Primer VT1d	
Primer VT2a	0.10 μM
Primer VT2b	
Primer VTea	0.10 μM
Primer VTeb	
Stoffel fragment	1.25 units/25 μL reaction
Magnesium chloride	3 mM

Mix all ingredients ensuring everything is kept on ice.

3.2 Add 20 μL of the master mix to each PCR tube. Add 5 μL of the DNA template.

3.3 Cap the tubes and place into a thermal cycler.

The thermal cycle for the reaction to occur is:

Denaturation at 94°C for 1 min
Annealing at 55°C for 2 min
Extension at 72°C for 1min

These 3 steps make up one cycle. This cycle is repeated for a total of 30

times, then:

Extension at 72°C for 5 min
Hold at 4°C

3.4 After the reaction is finished, run the samples on a 2% agarose gel as above. Except use a 1.0 Kbp ladder as a molecular weight marker.

QC A positive control for each toxin and a negative control are used to ensure the reaction is being carried properly. Also one of the PCR tubes contains just the Master mix plus Millique water, to ensure that there wasn't any contamination during the reaction.

3.5 Toxin Typing

SLT1 An organism has SLT1 if there is a 130 bp fragment present.
SLT2 An organism has SLT2 if there is a 346 bp fragment present.
SLT2e An organism has SLT2e if there is a 230 bp fragment present.

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APPENDIX C

Modified EC Broth

Difco EC medium (Cat. # 0314-17-2)
20 μg/ml Novobiocin (Sigma)

Deionized Water	1 L

Difco EC Medium - One litre

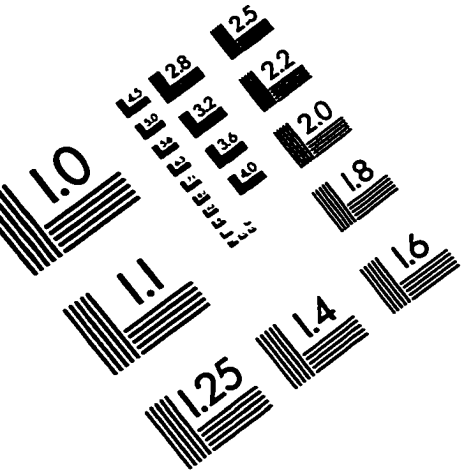
Bacto Tryptose	20 g
Bacto Lactose	5 g
Bacto Bile Salts #3	1.5 g
Dipotassium phosphate	4 g
Monopotassium phosphate	1.5 g
Sodium chloride	5 g

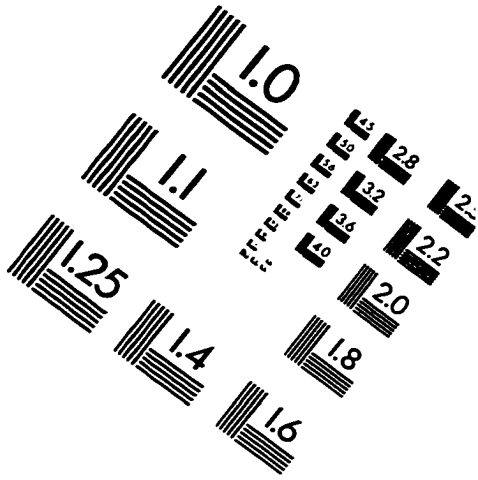
APPENDIX E

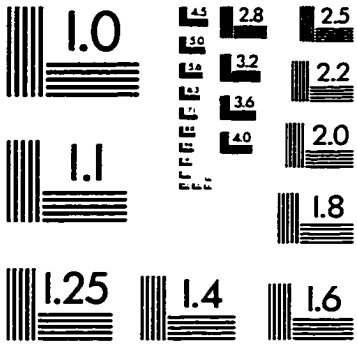
Commonly used Abbreviations

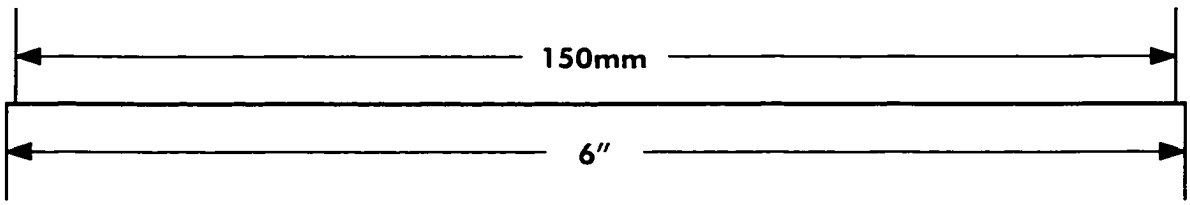
Term	Abbreviation
Standard units of measurements	
degree Celsius	C
gram	g
hour	h
international unit	IU
litre	L
minute	min
molar	M
revolutions per minute	rpm
year	yr
percent	%
microlitre	μL
millilitre	mL
probability	p
confidence interval	CI

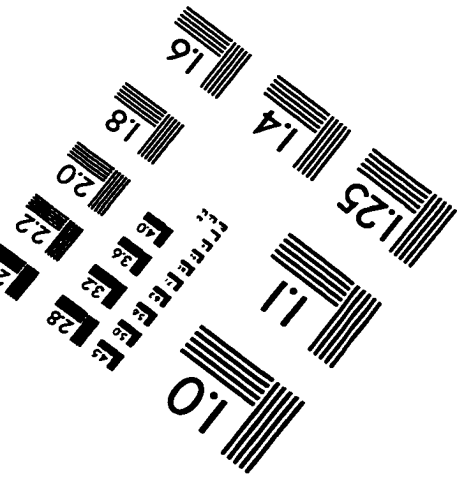
IMAGE EVALUATION
TEST TARGET (QA-3)

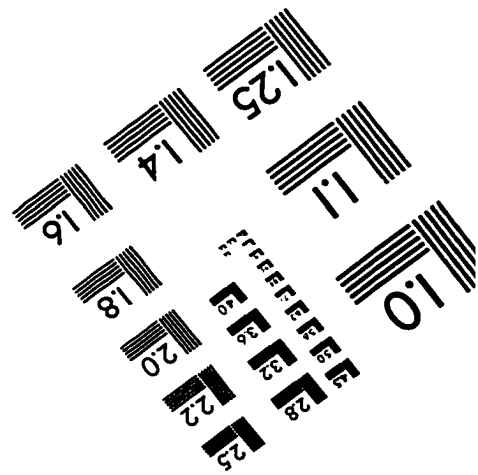












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

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