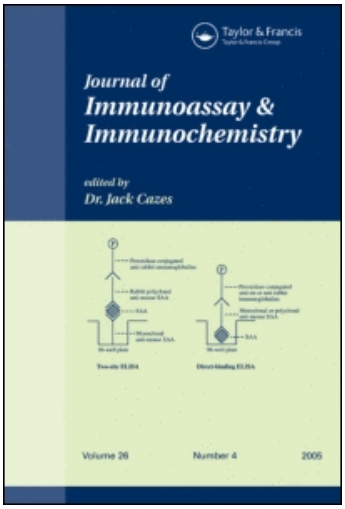

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Detection of *Eschericia coli* O157:H7 by Fluorescence Polarization Assay and Polymerase Chain Reaction

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Abstract: It is recognized that cattle and other domestic animals can be a reservoir of pathogenic *Escherichia coli*, including serotype O157:H7. To contain this potential health hazard, the first step is the identification of the carrier animals. For these purposes, a rapid serological screening test, a fluorescence polarization assay (FPA) was developed and results obtained from a randomly selected cattle population as well as cattle immunized with *E. coli* O157:H7 were compared to those obtained with an indirect enzyme immunoassay (IELISA). To identify pathogenic strains in carrier animals, polymerase chain reactions (PCR) for Shiga-like toxins I and II were implemented using agarose electrophoresis. The sensitivity of the fecal extracted *E. coli* for Shiga-like toxin I and II was approximately 200 CFU per reaction using multiplex hot-start nested PCR. The sensitivity of the fecal extracted *E. coli* varied from approximately 5×10^2 to 2.5×10^3 CFU per reaction depending on the commercial kits used. The combination of the serological screening FPA and hot-start nested PCR confirmatory assays provided rapid identification of the pathogen.

Keywords: E. coli, Fluorescence polarization, Polymerase chain reaction

INTRODUCTION

Escherichia coli is commonly found in the gut flora of most animals. Most *E. coli* are beneficial in that they prevent adhesion of pathogenic bacteria to the mucosal surface. However, some strains are pathogenic to man causing

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fever, urinary tract infections, alterations in blood cell populations, shock, gastroenteritis, the latter frequently severe and sometimes causing death, usually due to organ failure.[1–3] It appears that the reservoir of some of the pathogenic strains is domestic animals, most frequently cattle but also other species such as pigs and turkeys.[4–8] Pathogenic *E. coli* is usually transmitted to man through undercooked contaminated food and hence one strain was named 'hamburger disease' or through materials contaminated by bacteria excreted into the environment.[9–11] Disease is caused by groups of toxins produced by some strains of *E. coli*. The most notorious strain is *E. coli* O157:H7, a cause of gastroenteritis and renal failure, resulting in a substantial number of deaths of the young, old and immunologically incompetent individuals each year.[1–3]

While it is obviously important to detect pathogenic bacteria in food, elimination of the bacteria would create a safer food supply. Therefore, a combination of procedures to detect pathogens and eliminate them from the source is ideal. In the case of *E. coli*, a rapid inexpensive detection method, applicable to fecal samples with a minimum amount of handling, allows detection of carrier animals which may then be removed or treated to remove the organism.

A scan of the literature revealed that over 3000 scientific contributions involving *E. coli* 0157:H7 have been published since its first description in 1973.[2,12] Of these, about 400 deal with rapid detection. Detection methods vary considerably. The 'gold standard' method is demonstration of the causative organism by isolation, however, this is not a rapid or inexpensive technique. Faster techniques rely on either the demonstration of the presence of unique nucleic acid fragments[13,14] or using immunological tools to demonstrate antigens.[15,16] Nucleic acid fragments may readily be demonstrated, however, the technology is complicated, expensive and prone to false positive reactions.[17] It is, however, a useful procedure for confirming the presence of the bacterium. Immunlogically based tests generally make use of immobilized specific antibody used to capture antigens which are then detected using a second antibody with a signal system attached[18] or by allowing a secondary reaction such as agglutination to take place.[19] None of the immunologically based detection systems are sufficiently sensitive to detect the bacterium directly (i.e., small numbers) therefore requiring an increase in numbers of bacteria by preculturing the sample for a minimum of 6 hours but more often overnight.[18]

The 0157 somatic antigen molecule (lipopolysaccharide) found on the cell surface in large numbers would be useful for detection (although some heterogeneity in the O157 molecule has been described);[20] however, not all *E. coli* O157 strains cause disease.[21] Therefore, detection of 0157 antigen may be useful as a screening assay only. The same is true for the flagellar antigen H7.[22] Shiga-like toxins I and II would be more suitable as antigens for detection as either or both are present in the pathogenic strains, however, only a small number of copies are available in each cell for detection, compared to the number of lipopolysaccharide molecules per

cell.[23,24] The Shiga-like toxins are present in most enteropathogenic strains, including 0157:H7 and other *E. coli* serotypes as well as *Shigella sp*.[25,26]

Rapid detection of exposure to *E. coli* O157 may be accomplished by detection of serum antibody. This has been done in human cases[27] and in cattle[28] using enzyme immunoassays.

Most immunologically based assays are reasonably rapid to perform, however, because of their complex nature, they are best performed in a laboratory setting, therefore delaying results. Homogeneous assays are rapid assays requiring a single step to perform, eliminating the need for wash steps, dilutions etc. An example of a homogeneous assay is the fluorescence polarization assay (FPA). Molecular aspects of the FPA were recently reviewed by Nielsen et al.[29] The premise of the FPA is that a molecule in solution rotates at a rate inversely proportional to its size. The rate of rotation may be measured by conjugating the molecule with a fluorochrome and using polarized light to measure the rate of depolarization. If the molecular size is altered, so is the rate of rotation. Therefore, the rate of rotation of a small antigen molecule will be decreased if antibody is attached to the antigen. This type of assay has been used extensively for detection of drugs, however, only lately has the technology been applied to the diagnosis of infectious diseases. This is an attempt to apply fluorescence polarization technology to the rapid detection of *E. coli* O157 antibody in bovine sera as a rapid screening method for exposure and to a rapid PCR procedure for confirmation.

EXPERIMENTAL

E. coli Antigens

E. coli O157:H7 (American Type Culture Collection, Manassas, VA, ATCC #43894, contains Shiga-like toxins I and II) was propagated in nutrient broth for 24 hours at 37°C and transferred to nutrient agar plates, and incubated at 37°C for 24 hours. The bacterial cells were harvested into 0.15M NaCl. Part was autoclaved at 121°C for 15 min. and part was killed with 0.5% formalin. Other *E. coli* cultures were propagated in the same fashion.

Preparation of Antigens

Lipopolysaccharide (SLPS) was extracted by the hot water, hot phenol method.[30] Briefly, 5 gm of freeze dried bacteria suspended in 170 mL of distilled water were heated to 66°C in a waterbath. To this was added 190 mL phenol heated to the same temperature and the mixture was stirred for 15 min. After cooling, the mixture was centrifuged at 10,000 × g for

10 min at 4°C. The water and the phenol phases were collected, the SLPS precipitated with 500 mL cold methanol containing 1% v/v methanol saturated with sodium acetate. To each precipitate 80 mL of distilled water was added with stirring for 18 hours at 4°C. Soluble SLPS was recovered by centrifugation as above and the precipitates were treated with an additional 80 mL of distilled water, stirring for 1 hour. Soluble SLPS was recovered by centrifugation and added to the initial SLPS solution. The SLPS recovered from the water and the phenol phases were each treated with 8 gm of trichloroacetic acid stirring for 10 min. Precipitated material was removed by centrifugation and the soluble SLPS fractions dialyzed against distilled water. The crude SLPS preparations were freeze dried.

For the IELISA, the SLPS preparations were reconstituted in 0.01M phosphate buffer with 0.15M NaCl, pH 7.2 (PBS) to 1.0 mg/mL and stored frozen.

For the FPA, the SLPS was boiled in 2% v/v acetic acid for 1 hour. The O-polysaccharide (OPS) fraction was recovered by passing the neutralized digest through a polymyxin B column (BioRad Labs, Mississauga, ON) which binds lipid A. The OPS was then labelled with fluorescein isothiocyanate (isomer 1, from Sigma Fine Chemicals, St. Louis, MO) as previously described.[29]

Bovine Serum Samples

Sera from 7910 randomly selected animals were used to investigate the levels of antibody to *E. coli* O157 in the Canadian cattle population by IELISA. The sera were part of a national survey. Randomly selected sera from the same group (n = 913) were tested by FPA.

Five Hereford cross heifers, tested negative for *E. coli* O157 by fecal culture were immunized intramuscularly with 10^{10} heat killed *E. coli* O157 incorporated into Freund's complete adjuvant, followed by a second injection of the same number of bacteria without adjuvant 90 days later. The animals were bled weekly, the sera tested and then stored frozen.

Serological Tests

Indirect enzyme immunoassay: NUNC 269620 96 well plates were passively coated with 100 μL SLPS, 1.0 μg/mL in PBS, overnight at 18–20°C. After washing four times with PBS containing 0.15 M NaCl and 0.05% Tween 20 (PBST), 100 μL of serum, diluted 1:50 in PBST with 15 mM EDTA and EGTA, pH 6.3 was added and incubated at 18–20°C for 30 min. Following another wash cycle, 100 μL murine monoclonal antibody to bovine IgG1, conjugated with horseradish peroxidase and appropriately diluted in PBST was added to each well for 1 hour. After another wash cycle, 100 μL

1.0 mM hydrogen peroxide and 4.0 mM ABTS dissolved in 0.05M citrate buffer, pH 4.5 was added, shaken for 10 min and colour development was assessed using a spectrophotometer at 414 nm.

Each plate included a strongly positive control serum. The antibody level in each serum was calculated relative to the positive control (set at 100% positivity) using the following equation:

$$\%P = \text{optical density of test sample/optical density of positive control} \times 100$$

A weakly positive and a negative control serum as well as a buffer control (no serum added) were also included in each plate. The same control sera were used for the FPA.

Fluorescence polarization assay: The assay was performed as described[29] using serum diluted 1:100 in 0.01M tris buffer containing 0.15M NaCl, 10 mM EDTA and 0.05% Igepal CA630 in a 10 × 75 mm glass tube. After mixing, a background fluorescence reading was obtained with a Sentry FP Analyzer (Diachemix Corp, Whitefish Lake, WI). Ten μL of OPS labelled with fluorescein isothiocyanate isomer I (Sigma Fine Chemicals, St. Louis, MO) with total fluorescence intensity equivalent to 1.5 nM fluorescein was added and mixed, incubated for a minimum of 2 min and a final reading was obtained with the Analyzer (it automatically subtracts the initial reading) in millipolarization units (mP).

Fecal DNA Extraction

Fecal material was diluted by adding 200 mg to 1 mL of lysis PBS (0.73% of CTAB, 0.57 M NaCl in PBS) on ice before spiking with known amounts of *E. coli* O157:H7. Fecal samples were inoculated with different number of live *E.coli* O:H157 (ATCC#43894) and centrifuged one time at 1,300 × g for 1 min and 16,100 × g for 5 minutes. According to Stewart et al.,[31] the pellets were washed with 1 mL of lysis PBS two times at 16,100 × g for 5 minutes. The pellets were resuspended in 95 μL of resuspension buffer (25% sucrose in 10 mM Tris-HCl, pH 8.0, 5 μL of 10 mg/mL lysozyme and 200 mM EDTA) and incubated for 30 min at 37°C. Then, 3 μL of Proteinase K (20 mg/mL) were added and samples were incubated for 1 hour at 65°C. The samples were boiled in water for 20 min. Three volumes of water and 6 volumes of phenol: chloroform: isoamyl alcohol (25:24:1) were added and mixed by inversion. All samples were centrifuged for 10 min at 16,100 × g. The aqueous phases were mixed with 500 μL of chloroform and centrifuged for 10 min at 16,100 × g. DNA was precipitated with 60 μL 3 M sodium acetate (pH4.5) and 600 μL of isopropanol. The DNA was washed with 70% ethanol (cold) and dissolved in 50 μL of H_2O and stored at −20°C for the multiplex PCR. The flow chart of extraction of DNA summarizes the

protocol:

200 mg of fecal material in 1 mL lysis-buffer, spiked different numbers of bacteria

↓

Centrifuge at 1, 300 × g for 1 min, keep supernatant and centrifuge at 16, 100 × g for 5 min.

↓

Add 95 μl of 25% sucrose-Tris.Cl and 5 μl of lysosome-EDTA, incubate 30 min at 37°C

↓

Add 3 μL of 20 mg/mL proteinase K and incubate 1 h at 65°C

↓

Boil sample for 20 min. phenol:Chloroform:isoamyl alcohol (25:24:1) to extract DNA

↓

Isopropanol to precipitate DNA and store at −20°C for PCR

Polymerase Chain Reaction (PCR) was performed using Shiga-like toxin I forward primer (5′-CGCTGAATGTCATTCGCTC-3′), reverse primer (5′-CGTGGTATAGCTACTGTCACC-3′), internal forward primer (5′-AGGTA CGCCATTACAGACTA-3′) and internal reverse primer (5′-CAATGTAA CCGCTCTTGTAC-3′). The Shiga-like toxin II forward primer was (5′-CTTCGGTATCCTATTCCCG-3′), reverse primer (5′-CTGCTGTGA-CAGTGACAAAA-3′), internal forward primer (5′-ACGGACAGAGATAT CGACC-3′), and internal reverse primer (5′-CGCCAGATATGAT GAAACCA-3′). The first round PCR used 33 μl of distilled H_2O, 1 μL of 10 mM dNTP, 1.5 μL of 50 mM MgCl, 5 μL of 10 × PCR Buffer, 1 μL (25 μM) each of primers, 5 μL of DNA, and 0.5 μL (5 units/μL) of *Taq* polymerase added on ice. The PCR fragments were amplified using a program consisting of 1.5 min at 94°C, 40 cycles for 45 seconds at 94°C, 45 seconds at 53°C, and 1 min at 72°C, with a final extension at 72°C for 10 min. Twenty μL of PCR products were run on a 1% agarose gel. After the first PCR, 6 μL of PCR products, 32 μL of distilled H_2O, 1 μL of 10 mM dNTP, 1.5 μL of 50 mM MgCl, 5 μL of 10 × PCR Buffer, 1 μL of 25 μM of each internal primers and 0.5 μL (5 units/μL) were used to do multiplex hot-start nested PCR. The second round PCR products were amplified using a program consisting of 1.5 min at 94°C (held at 80°C to add *Taq*), 40 cycles for 45 seconds at 94°C, 45 seconds at 58°C, and 1 min at 72°C, with a final extension at 72°C for 10 min. Twenty μL of PCR products were loaded on a 1.0% agarose gel.

DNA was also extracted from bovine fecal samples using two commercial kits developed for extracting human fecal material. The procedures were performed according to the manufacture's recommendation. DNA from the fecal samples were used as templates. VT1-A

(5′-CGCTGAATGTCATTCGCTCTGC-3′) and VT1-B (5′-CGTGGTATAG CTACTGTCACC-3′) for Shiga-like toxin I and VT2-A (5′-CTTCGGTATCC-TATTCCCGG-3′) and VT2-B (5′-CTGCTGTGACAGTGACAAAACGC-3′) for Shiga-like toxin II.[32] Various volumes of distilled H_2O were used in the PCR mixture (0, 20, 34, 39 μL) and the template volumes (40, 20, 5, 1 μL) were adjusted correspondingly. All reaction contained 1 μL of 10 mM dNTP, 1.5 μL of 50 mM MgCl, 5 μL of 10 × PCR Buffer, 1 μL of 10 μM of each primer for Shiga-like I and II, and 1 μL (5 units/μl) of *Taq* polymerase. The PCR reagents were mixed on ice and the fragments were amplified using a program consisting of 2 min at 94°C, 40 cycles for 45 seconds at 94°C, 45 seconds at 56°C, and 1.5 min at 72°C, with a final extension at 72°C for 10 min. Twenty μL of PCR products were loaded on a 1.5% agarose gel.

Other *E. coli* species from ATCC and ADRI (Table 1) were tested with both PCR methods.

RESULTS

Lipopolysaccharide prepared from heat or formalin killed *E. coli* gave the same values in the IELISA. Based on the pre-treatment samples from the five immunized cows, values in the IELISA range from 22 to 64%P. Since no *E. coli* O157:H7 was isolated from these animals these values may be considered as normal background antibody levels to *E. coli*. Figure 1A shows an early rise in IELISA antibody levels, followed by a plateau and a decline. A

Table 1. Examination of other *E. coli* species

Bacteria	Number	Toxins
E. coli O157:H7	ATCC 35150	Cytotoxin
E. coli O157:H7	ATCC 43894	Shiga-like toxins I and II
E. coli O157:H7	ATCC 43889	Shiga-like toxin II
E. coli O157:H7	ATCC 43890	Shiga-like toxin I
E. coli O157:NM	ATCC 700376	Shiga-like toxin I
E. coli O157:NM	ATCC 700377	Shiga-like toxin II
E. coli O157: NM	ATCC 700378	Shiga-like toxin I and II
E. coli O111:H8	ATCC 700840	Shiga-like toxin
E. coli	ADRI[a] 43888	None
E. coli	ADRI # 7	None
E. coli	ADRI # 8	None
E. coli	ADRI # 9	None
E. coli	ADRI # 25922	None
E. coli	ADRI OH50CH	None

[a]ADRI: From the culture collection at the Animal Disease Research Institute, Nepean, Canadian Food Inspection Agency.

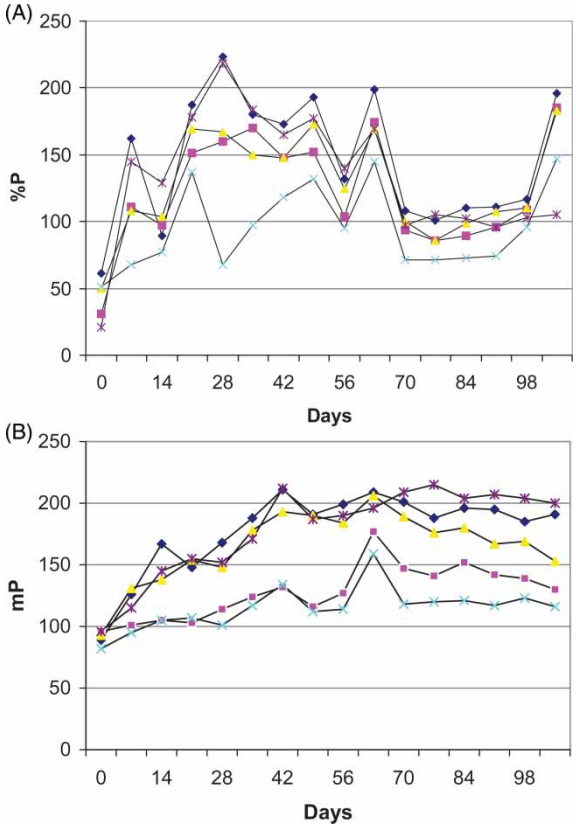

Figure 1. Antibody levels over time in cattle immunized with *E. coli* O157:H7 measured by an IELISA (A) and FPA (B).

secondary response after the 90 day re-immunization. The pattern for antibody reactivity in the FPA is different in that there is a gradual increase in antibody levels without a decline after immunization with no significant increase in antibody levels after re-immunization at 90 days. The pre treatment antibody levels were 82 to 96 mP. These data are presented in Figure 1B. From the data, it was assumed that the cut-off values between positive and negative animals would be approximately 65%P for the IELISA and 100mP for the FPA.

Using the IELISA to test 7,911 bovine sera from a random sample collected across Canada a skewed distribution (Figure 2A) was obtained with 95% of the sera giving values below 45%P, clearly negative while 32 sera (0.4%) gave a positive value (over 65% P). Using the FPA on 913 randomly selected samples from the original 7,911, a more normal distribution was obtained (Figure 2B); however, the results indicated higher levels of

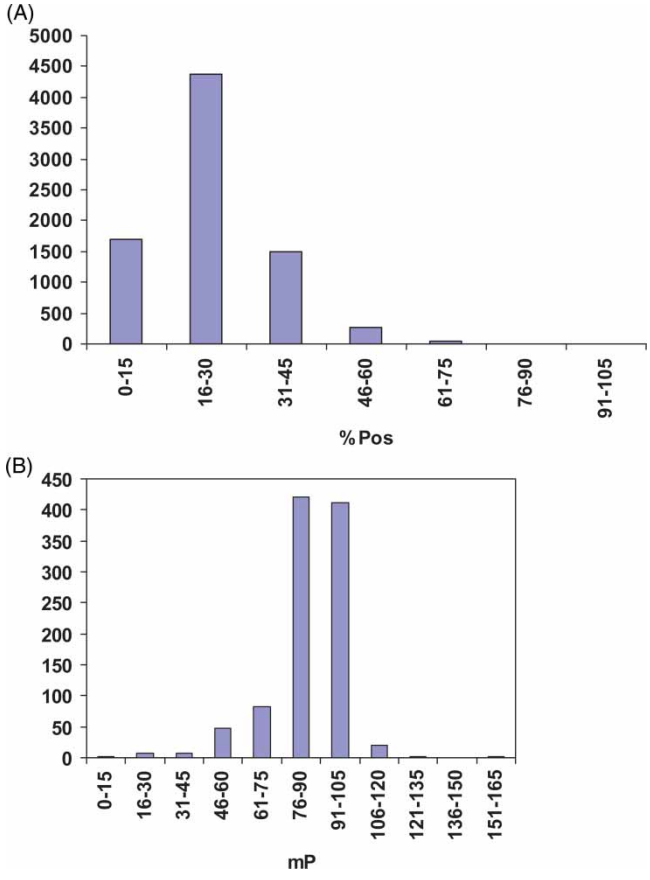

Figure 2. Distribution of antibody to *E. coli* O157:H7 in 7910 randomly selected Canadian cattle measured by an IELISA (A) and a subpopulation of 913 cattle measured by FPA (B).

antibody in a larger number of animals. Thus, this discrepancy is also evident in the low correlation coefficient between the FPA and the IELISA when testing the sera from immunized animals ($r = 0.40$).

In order to rapidly and inexpensively detect *E. coli* O157:H7 in animal feces, DNA was extracted from spiked samples using different methods, including some commercial kits, to compare sensitivity. DNA was used as a template for the PCR using *E. coli* O157 specific primers. As there are a number of inhibiting factors in fecal samples that may affect PCR amplification, a method to extract DNA from fecal material was developed. As described in the Experimental section, it was possible to detect approximately 200 live *E. coli* O157 bacteria per reaction (Figure 3) in feces by using 5 μL of DNA extracted from feces as a PCR template by multiplex hot-start nested

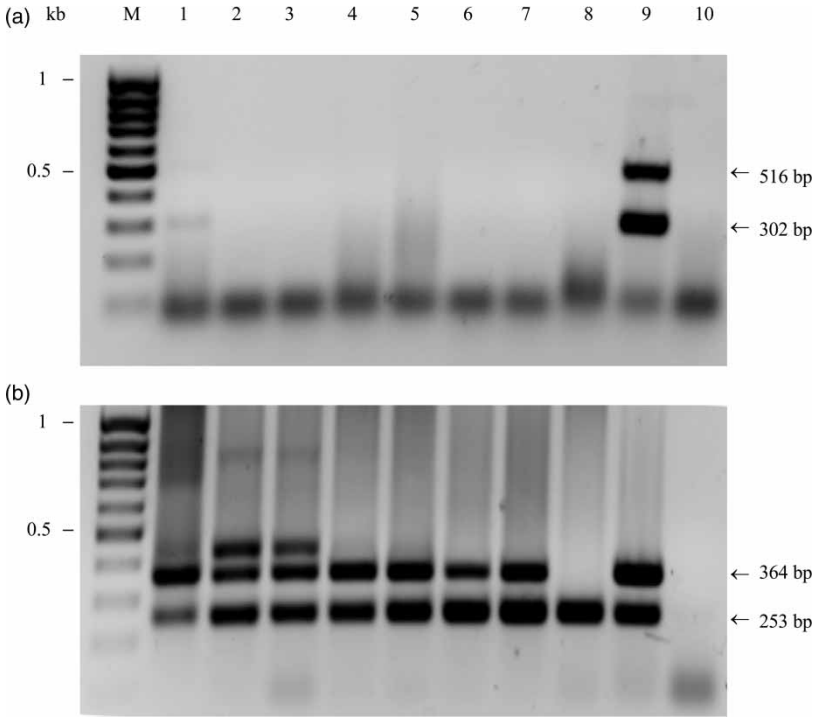

Figure 3. Sensitivity of PCR and hot-start nested PCR detecting *E. coli* O157 in feces. In both tests, PCR products for Shiga toxin I (302 bp) and II (516 bp) were amplified by first multiplex PCR (a) followed by hot-start nested PCR (b) for Shiga toxin I (253 bp) and Shiga toxin II (364 bp) as described in Experimental. M: 100 bp DNA marker. Lanes 1 to 7: the *E. coli* number from 2×10^6 to 2×10^0. Lane 8: fecal negative control. Lane 9: PCR positive control. Lane 10: PCR negative control.

PCR using specific primers for Shiga-like toxins I and II. The sensitivity for Shiga-like toxin II is lower compared with that of Shiga-like toxin I. Similar results were obtained using 1 or 5 μL of template DNA extracted using a commercial kit. In animal feces, the minimum number of *E. coli* O157 that could be detected was approximately 500 using the Shiga-like toxin I probe by PRC with 1 μL of DNA template with a second commercial kit. The second kit allowed detection of approximately 2.5×10^3 *E. coli* O157 in feces which could be detected by the PCR for Shiga-like toxin II. The results remained negative (or very weak bands in agarose electrophoresis) with different quantities of DNA extracted from the feces for the PCR.

Other strains of *E. coli.* O157 (Table 1) were tested with two methods of PCR by using specific primers for Shiga-like toxin I and Shiga-like toxin II. The PCR results were positive for the strains which contain either or both Shiga-like toxins and negative for the other strains.

DISCUSSION

Presence of *E. coli* in food or water is usually a result of contamination with fecal material. In the case of *E. coli* O157, a frequent source is feces from animals; in particular, cattle appear to be the main reservoir. One relatively easy solution to the problem is to manage the food or water supply in such a way that the contaminants are killed, for instance, by proper heating. However, this does not remove the problem which would always be a danger should food or water be under heated. A better solution would be to decrease or eliminate the bacterial reservoir in cattle and other species by identification of carriers for treatment. The first step in identification is to apply a rapid, inexpensive screening test which would then be followed by a more specific confirmatory test.

In this communication, a rapid, simple, and inexpensive serological screening test, the FPA, is described and is used to test a number of the general Canadian cattle population, as well as sera from cattle immunized with *E. coli* O157:H7. The results are compared to those obtained with an IELISA. It appears that there is little agreement between the two tests (correlation coefficient $r = 0.40$ for sera from the immunized group), perhaps as a result of low affinity antibody being removed by the washing procedures of the IELISA. The discrepancy is particularly noticeable with samples from days 65 to 90, during which time the IELISA dropped to a relatively low level of antibody in all five animals. Antibody affinity is of less concern in the FPA as no washing procedures are involved in a homogeneous assay. Assuming the FPA to provide the correct antibody levels, approximately 13% of Canadian cattle have been exposed to *E. coli* O157.

Polymerase chain reaction (PCR) was developed to be used to confirm carrier animals as PCR has become a rapid and reliable method for the diagnosis of some infectious diseases.[33] However, a typical diagnostic PCR for *E. coli* O 157:H7 in feces is limited because there are many inhibitors in fecal material, such as bile salts, bilirubins, polysaccharides, heparin, and hemoglobin degradation products[34] and plant tissue such as polyphenolic substances.[35] The substances could decrease the specificity and sensitivity of PCR for diagnosis O157:H7 in fecal material. A variety of methods have been developed to decrease or remove fecal inhibitors, including the use of different extraction and DNA purification methods,[31,36] or to enhance the efficiency of PCR, including real-time PCR.[37]

The current study focused on development of a rapid and inexpensive method to diagnose *E. coli* O 157:H7 carrier animal. Therefore, different methods to decrease inhibitors in DNA samples and increase PCR efficiency and sensitivity were tested; a combined PCR and FPA protocol was also tested. The sensitivity of PCR and PCR-FPA was approximately 1.5×10^3 CFU. However, insufficient PCR products were obtained to

perform the PCR-FPA test using DNA extracted from fecal samples. indicating that inhibitors in the extracted DNA inhibited DNA polymerase, bound magnesium, and/or denatured DNA.[14] As a simple solution, boiled fecal samples were extracted and to ascertain if inhibitors could be eliminated; the extracted DNA was tested in a multiplex PCR and hot-start nested PCR. The hot-start nested PCR was demonstrated to be more sensitive and efficient because the nested PCR results in exponential amplification of an already exponentially amplified PCR product.[38] The hot-start nested PCR could detect approximately 200 CFU per reaction in spiked fecal samples in total 50 μL volume by using 2.5 U of Taq polymerase for Shiga-like toxin I and II. The results indicated that the hot-start nested PCR performed well when used with the described scheme of common chemicals to extract DNA from stool samples (flow chart in Experimental) may be a good procedure for confirmation of the presence of pathogenic *E. coli* O157 in carrier animals. Results obtained, using commercial kits to extract DNA from fecal materials, indicated a considerable loss in sensitivity. From these results, it may be possible to utilize hot-start nested PCR to test for other toxins or to identify other pathogenic strains of O157:H7 in feces. It is interesting to note that, during the PCR protocol, negative fecal controls performed during nested hot-start PCR became positive for Shiga-like toxin I, although PCR for the first amplification of extracted DNA in the feces was negative. According to Trochimchuk et al.[36] *E. coli* O157:H7 could have been present in the feces; however, the background CFUs were likely between 1 and 10^2 CFU/100 mg feces and possibly non-viable. The increase in sensitivity might be the reason negative controls from the first PCR products became positive in hot-start nested PCR.

In summary, fluorescence polarization technology is a rapid test for *E. coli* O157 antibody in bovine sera that may be useful as an initial screen to detect exposed animals, offering an accurate and simple method to detect carrier animals. The hot-start nested PCR offers a rapid and inexpensive procedure for confirmation *E. coli* O157:H7 in feces from carrier animals, reducing the time taken to confirm pathogenic *E. coli* O157:H7 in carrier animals to 2 days. This scheme offers a clear cost advantage in large-scale screening to reduce the reservoir of pathogenic *E. coli* O157:H7, hopefully providing an initial step in the control and elimination of the pathogen.

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