Effects of sodium chloride treatment on the recovery of *Escherichia coli* O157:H7 from bovine feces for identification using a triplex PCR

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Andrew Patrick Jacobson

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Graduate College
Iowa State University

This is to certify that the Master's thesis of

Andrew Patrick Jacobson

has met the thesis requirements of Iowa State University

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ABSTRACT

Since 1982, *Escherichia coli* O157:H7 has emerged as a major threat to public health (Riley *et al.*, 1983; Doyle, 1991). Most illnesses in humans result from ingesting contaminated food products of bovine origin (Garber *et al.*, 1995). Isolation from cattle has implicated this species as the principle reservoir (Armstrong *et al.*, 1996) and entry of O157:H7 into the food supply is believed to occur during slaughter of infected animals (Voelker, 1994). To reduce the occurrence of O157:H7 at slaughter facilities, the development of timely and sensitive methods to detect O157:H7 in cattle has been proposed (Voelker, 1994).

Isolation of O157:H7 from feces has been used for diagnosing infections, however, this procedure is laborious and time consuming (Mermelstein, 1993). Polymerase chain reaction (PCR) offers a potential alternative, however, its sensitivity is limited by components in feces which inhibit the reaction (Widjojoatmodjo *et al.*, 1992). Enriching O157:H7 from feces in broth media increases sensitivity, but lengthens the time required to perform the procedure(Paton *et al.*, 1993; Padhye and Doyle, 1991). Minimum incubation times required to detect O157:H7 in PCR procedures may be influenced by methods used to prepare the feces for enrichment.

In this study, the effects of sodium chloride concentration of solutions used to treat bovine feces were evaluated. Wash solutions containing 0.0, 0.5, 0.9, and 1.5 percent sodium chloride were applied to feces inoculated with O157:H7 in a filtration procedure. The recovery of O157:H7 in filtrates were determined with each treatment and results were statistically compared. Filtrate samples were then enriched in broth media for eight, nine, and 10 hours prior to a triplex PCR to evaluate treatment effects on the development of positive PCR signals after these incubation times. Sequences targeted for amplification were from genes encoding verotoxin production (VT-1 and VT-2) and the *uid* A gene. Significantly higher recoveries from feces were achieved with 0.5 percent sodium chloride washes than with 1.5 percent washes (P=.05). The magnitude of positive results with

PCR where significantly higher after nine hours of incubation with 0.5 percent sodium chloride washes than with 1.5 percent sodium chloride (P=.05). No additional statistical differences were observed between any other treatment pairs.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) are a distinct group of enteric pathogens that produce toxins similar in structure and function to those of *Shigella dysenteriae* (Hull *et al.*, 1993; Mead and Griffin, 1998). Of this group, serovar O157:H7 is most frequently involved in human illness (Chart, 1998). The course of O157:H7 illness includes the development of diarrhea soon after infection. This may progress to hemorrhagic colitis (HC), a condition characterized by severe abdominal cramping and the production of grossly bloody stools. Colitis is thought to result from the cytotoxic effects of verotoxins (Feng, 1995). Approximately 10 percent of patients that have HC develop the hemolytic uremic syndrome (HUS). HUS is a systemic disease that affects renal function. This disease can be life threatening, particularly for young and elderly patients who are less likely to recover from renal failure which can potentially develop (Mead and Griffin, 1998; Chart, 1998). An initial description of O157:H7 as a food-borne pathogen was made following investigations on two isolated outbreaks of HC in 1982 in the United States. Ground beef patties distributed by fast food restaurants in Oregon and Michigan were identified as sources of infection (Dorn, 1993; Hao and Bruno, 1996). Approximately 500 confirmed cases and four deaths resulted from the Oregon outbreak. This included residents of adjacent states (Centers for Disease Control, 1993). Although the epidemiology of O157:H7 and its relationship to food-borne illness is poorly understood, ruminant species, particularly cattle, are considered by some to be the primary reservoir and directly involved in transmission to the food supply through fecal contamination of carcasses at slaughter (Reilly, 1998). Strategies to prevent O157:H7 related illnesses include early detection of positive herds and modification of practices during production and slaughter to reduce the prevalence of O157:H7 in the bovine population and transmission to the food supply (Reilly, 1998).

Isolation of O157:H7 on selective media followed by serovar specific biochemical and serological assays of suspect colonies is currently the most accurate method to diagnose carrier

animals (Tortorello *et al.*, 1996). The low sensitivity levels achieved (Armstrong *et al.*, 1996) and the time required to produce colonies limit the use of this procedure for routine surveillence of cattle or carcasses during production (Mermelstein, 1993). Other characteristics of O157:H7 carriage in cattle further minimize the efficiency of screening bovine populations through isolation on selective media. The low prevalence of O157:H7 in positive herds and the lack of clinical signs associated with infection in adult cattle make selection of suspect individuals difficult (Armstrong *et al.*, 1996; Vold *et al.*, 1998). It has also been demonstrated that age, diet, and seasonal patterns affect the level and duration of fecal shedding and consequently opportunities to isolate the pathogen from positive animals (Cray and Moon, 1995; Brown *et al.*, 1997). These observations prompt suspicion that the prevalence of O157:H7 among cattle may be higher than isolation rates suggest (Vold *et al.*, 1998). The development of sensitive detection techniques including polymerase chain reaction has become an increasingly important consideration for detection of O157:H7 in food animals and the food supply.

Polymerase chain reaction (PCR) is useful for the identification of pathogens in food and environmental samples through the production of amplicon products of unique genomic sequences. The advantages of PCR over immunoassays and culturing for identification include greater specificity and sensitivity, respectively, with potentially shorter lengths of time required between submission of samples and reportable results (Fratamico and Strobaugh, 1998). Amplification can be completed within three hours (Kato, 1993) and an additional one to two hours is usually required to interpret results. The use of PCR to detect low numbers of O157:H7 in fecal samples depends upon initial enrichment to increase populations of the organism to levels that are detectable. The time required to enrich samples may vary with levels of O157:H7, background competitor bacteria, and soluble inhibitors of PCR such as bile acids. Incubation for enrichment can lengthen the time required to screen samples by as much as 16 hours (Paton *et al.*, 1993; Padhye and Doyle, 1991).

To achieve optimal sensitivity with the shortest possible time for enrichment it is important to identify methods which most efficiently remove O157:H7 from bovine feces in initial separation steps and which produce intermediates that are low in concentrations of inhibitors relative to that of target organisms. In this study the effect of sodium chloride on the removal of O157:H7 from bovine feces during a centrifugal filtration procedure was evaluated. The selective removal and recovery of the pathogen by four wash solutions differing in saline concentration were compared. Filtrates produced from this procedure were then enriched in broth media prior to a triplex PCR procedure. Growth of cultures from each treatment were evaluated by measuring absorbency values (610 nm) of cultures at selected times into incubation. Cultures were then harvested at these times and subjected to a triplex PCR to determine the minimum length of time required for enrichment with each treatment to produce positive results. Results from this study were used to determine if differences existed among treatments in the length of time required for enrichment with this PCR procedure.

LITERATURE REVIEW

Description and Classification of Enterohemorrhagic *E. coli* (EHEC)

Escherichia coli are generally non-pathogenic commensal bacteria which inhabit the lower intestine of humans (Turi *et al.*, 1997). Many strains are opportunistic pathogens outside of this environment contributing to nosocomial infections involving the urinary tract and mammary glands (Aswapokee *et al.*, 1990). Of particular concern for public health and food safety are pathogenic serovars such as *E. coli* O157:H7 which exist in healthy cattle and other ruminants but cause enteric disease in humans (Pirro *et al.*, 1995; Brown *et al.*, 1997). Cattle are a recognized reservoir of O157:H7 and considered important in human infections through consumption of contaminated food products (Armstrong *et al.*, 1996). Bovine derived products are vehicles commonly involved in human illnesses, however infections resulting from consumption of other foods have been reported (Armstrong *et al.*, 1996; Feng, 1995). O157:H7 is shed into the environment from feces of infected cattle (Brown *et al*, 1997) and is capable of surviving in soil and water for extended lengths of time (Centers for Epidemiology and Animal Health, 1994; Doyle, 1991). These characteristics may enable the pathogen to chronically infect some bovine populations, thereby providing a constant source of O157:H7 which can potentially serve to contaminate the food supply.

Pathogenic *E. coli* have been recognized in enteric illness since the 1940s. Specific phenotypes which contribute to virulence have only recently been described for some pathogenic strains (Bokete *et al.*, 1997). Enterohemorrhagic *E. coli* (EHEC) are a group of Shiga toxin producing strains that have similar epidemiological, clinical, and pathogenic features to O157:H7, the prototype serovar (Blanco *et al.*, 1995). The two major diseases caused by EHEC are hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Schmidt *et al.*, 1996). Outbreaks of HC are characterized by a sudden onset of severe abdominal cramping followed by passage of grossly bloody diarrhea (Feng, 1995). HUS develops in approximately 2-7 percent of EHEC infections and usually follows HC

(Spencer, 1993). This disease results in impaired kidney function and can lead to death through renal failure (Bockemuhl *et al.*, 1992).

Emergence of O157:H7 as a Food-borne Pathogen

E. coli serovar O157:H7 was first described as a food-borne pathogen in 1982 after its isolation from patients during two independent outbreaks of HC in the United States (Riley *et al.*, 1983). Contaminated beef patties were identified as sources of infection in both outbreaks (Padhye and Doyle, 1992). The isolated strain was similar to those described previously in individual cases of HC, however, its association with outbreaks of such large scale and severity was unique. This led to further classification of pathogenic *E. coli* into a distinct group referred to as enterohemorrhagic E. coli (EHEC) (Padhye and Doyle, 1992). Further analyses showed the strain to express the type O somatic antigen 157 and the H flagellar antigen type 7. Analyses of toxins showed them to be similar to those produced by *Shigella dysenteriae* and distinct from the heat-labile and heat-stable enterotoxins associated with other pathogenic *E. coli* serovars (Armstrong *et al.*, 1996). Investigations spurred by numerous reports of HUS were being conducted simultaneously in Canada leading to the identification of O157:H7 and other strains of *E. coli* that produced the toxins as the etiologic agents. It has since been estimated that 85-95 percent of all HUS cases in North America are caused by O157:H7 and that it is responsible for an average of 20,000 illnesses and 250 deaths per year in the U.S. (Armstrong *et al.*, 1996).

The sudden emergence of O157:H7 has raised questions on possible sources of the pathogen and factors that may have promoted its association with food-borne illness in humans (Armstrong *et al.*, 1996). The bovine reservoir was suspected shortly after the initial description of O157:H7 in humans because of the association with ground beef consumption (Madico *et al.*, 1995). O157:H7 has been isolated from the intestinal tract of cattle in several studies conducted since 1982 supporting further a possible link between cattle and human illness. Specific details, which complete the

description of O157:H7's sudden appearance in the food chain, are lacking. In general cattle, are considered the most important source in human infections (Grif *et al.*, 1998), however, O157:H7 has been isolated from the intestinal tracts of other ruminant species and even asymptomatic humans (Kudva *et al.*, 1996; Berry *et al.*, 1982). It is difficult to determine when a relationship between O157:H7 and cattle developed because little is known about its existence prior to 1982. These dilemmas have generated theories on the emergence of O157:H7 which range from spontaneous acquisition of virulence traits through recombination and plasmid transfer between related species of bacteria to changes in herd management practices that created an ecological niche for O157:H7 in the bovine intestine (Armstrong *et al.*, 1996; Whipp *et al.*, 1994).

Epidemiology of *E. coli* O157:H7

Sources of infection

The epidemiology of O157:H7 is unclear, however, investigations of outbreaks have contributed to elucidating the organism's ecology and its increased involvement in human infections (Garber *et al.*, 1995). Although sources of infection vary, food products of bovine origin have been most commonly involved in outbreaks of HC and HUS (Garber *et al.*, 1995). In addition, strains isolated from patients with HC and HUS have been identical to those isolated from feces of healthy cattle (Centers for Epidemiology and Animal Health, 1994). As a result, cattle have been implicated as the principal reservoir of O157:H7 (Centers for Epidemiology and Animal Health, 1994). In recent outbreaks there has been increased involvement of foods from non-bovine origin, however, cross contamination from bovine sources are considered likely (Feng, 1995; Kudva *et al.*, 1996).

Entry of O157:H7 into the food supply is thought to occur through fecal contamination of carcasses at slaughter (Easton, 1997). The organism is believed to persist in the lower intestine of cattle from where it is shed intermittently in feces (Centers for Epidemiology and Animal Health, 1994; Garber *et al.*, 1995). Prevalence values generated by isolation of O157:H7 from feces vary

considerably because of irregular shedding patterns. Reported prevalence values in cattle based on fecal swab tests range from zero to four percent (Kudva *et al.*, 1996). Seasonal patterns have been shown to drastically affect fecal shedding and correlate positively with the incidence of human illnesses (Kudva *et al.*, 1996). Taken collectively, prevalence values are very low among individuals, but significantly higher among herds (Centers for Epidemiology and Animal Health, 1994). With the incidence of human infections, herd prevalence may be a more accurate indicator of O157:H7 occurrence in the bovine population.

Over the ten-year period following 1982, there were approximately 30 outbreaks of HC and HUS associated with O157:H7 (Feng, 1995). The largest outbreak in the United States occurred in 1993 resulting in 700 illnesses and four deaths (Armstrong *et al.*, 1996). Since this time reports of O157:H7 illnesses have risen, probably due to better surveillance and increased awareness by consumers, physicians, and clinicians (Feng, 1995). Although bovine products continue to be the major sources of infection, other products have been identified as vehicles in transmission. Many of these include acidic foods such as fresh pressed apple cider and mayonnaise. The ability of O157:H7 to persist in acidic foods became known during investigation of the 1993 outbreak in which mayonnaise was identified as a source of infection. Although it did not multiply, it was able to persist for up to 55 days prior to causing illness (Feng, 1995). Another outbreak involving the consumption of fresh pressed apple cider also demonstrated the pathogen's tolerance for acidic conditions. Follow up studies showed that O157:H7 could persist in apple cider for 10-31 days when stored at cold temperatures (Feng, 1995). O157:H7 can also remain viable in ground beef during freezer storage (Pearce *et al.*, 1994).

The occurrence of O157:H7 in a variety of foods has increased (Tauxe, 1997). This may correlate with increased cross contamination among products resulting from changes in the way foods are stored and transported. However, the isolation of O157:H7 from non-bovine meat products at grocery stores has aroused speculation on the existence of alternate reservoirs. In one survey of

grocery store meats, O157:H7 was isolated from two percent of all meats evaluated including beef, poultry, pork, and lamb (Padhye and Doyle, 1992). The natural occurrence of O157:H7 has been documented in sheep and the pattern of fecal shedding is similar to that observed in bovines (Kudva *et al.*, 1996). O157:H7 will colonize the ceca of one-day old chicks after experimental infection and can be isolated from the shells of eggs for up to 11 months (Schoeni and Doyle, 1994). Although it has been shown that O157:H7 can exist in a variety of animals, the bovine intestine is considered to be the primary source of infections in humans (Colombo *et al.*, 1998). Other foods involved in outbreaks include cantaloupe and items from salad bars which are believed to become contaminated through contact with meat or meat juices infected with O157:H7 (Feng, 1995). The role of these foods may be of considerable importance in the transmission of O157:H7 to humans. In one study it was demonstrated that the pathogen could survive and grow for up to 14 days on vegetables stored at 12°C and 21°C (Abdul-Raouf *et al.*, 1993).

Compared to beef, raw milk is relatively unimportant in O157:H7 infections, accounting for only two of 32 outbreaks between 1982 and 1994 (Centers for Epidemiology and Animal Health, 1994). This is probably due to high public awareness of illnesses which can result from consuming raw milk including those associated with *Yersinia, Salmonella*, and *Campylobacter* infections (Centers for Epidemiology and Animal Health, 1994). Raw milk is a good medium for several pathogens including O157:H7 because of the nutrient content and neutral pH. Mechanisms through which raw milk becomes contaminated is unknown, however, it is presumed that fecal contamination is involved. Sources that may serve to bring feces in contact with the udder include hands of dairy workers and equipment used in milking (Centers for Epidemiology and Animal Health, 1994). One outbreak associated with raw milk involved a kindergarten field trip to an Ontario dairy farm in 1986. Forty-eight of 60 students developed HC after consuming raw milk at the farm. O157:H7 was confirmed in 43 of the cases and the same strain was isolated from one of the 67 healthy cattle that were tested (Centers for Epidemiology and Animal Health, 1994). Both drinking and recreational

water can also serve as vehicles in the transmission of O157:H7 to humans (Feng, 1995). Infection in recreational water probably occurs through ingestion of water that becomes contaminated by feces of carriers sharing the area. The infectious dose of O157:H7 is believed to be low in cases where recreational water is involved. When introduced into large volumes of water, the pathogen is diluted out considerably and the amount of water ingested during recreation is usually small. Outbreaks of this kind also suggest that O157:H7 can remain viable in water for long periods of time. In an outbreak involving the use of lake water in Portland, Oregon, some individuals became infected after swimming in the area three weeks after the first reports of illness (Feng, 1995).

The identification of unknown sources of O157:H7 is important for a full description of the pathogen's epidemiology. Although there is high correlation between O157:H7 and cattle, it is important to realize that this relationship is not absolute (Chart, 1998). It is believed that the ability of O157:H7 to persist in the environment may contribute to the incidence of positive animals in herds (Kudva *et al.*, 1996). Strains of O157:H7 seem to tolerate environmental stresses well and can be distributed into the environment through water courses and several animal species (Chart, 1998). O157:H7 can be washed into streams and rivers from land previously grazed by infected animals or fertilized with manure slurries containing the organism (Chart, 1998). The pathogen has also been isolated from seabirds which may serve to efficiently distribute O157:H7 to cattle in other areas (Chart, 1998). O157:H7 has also been isolated from feces of deer providing further evidence for the existence of alternate reservoir hosts and complex pathways through which the organism can enter the food chain (SCWDS Briefs, 1997).

Ruminant reservoir

Cattle have been considered the principal reservoir of O157:H7 since it was first described in human illness in 1982 (Chart, 1998). Dairy cattle are considered a major source of human infections because they represent a large proportion of the cattle used in the production of ground beef which is

implicated as a vehicle in 58 percent of illnesses (Armstrong *et al.*, 1996). O157:H7 is among several serotypes of verotoxin producing E. coli (VTEC) present in bovine feces. All VTEC by definition produce toxins which are cytopathic to vero cells, a line of African green monkey kidney cells (Whipp *et al.*, 1994). These toxins are similar to those produced by *Shigella dysenteriae* and for this reason have been collectively referred to as Shiga-like-toxins or SLTs. Therefore VTEC are also sometimes referred to as Shiga-like-toxin producing *E. coli* or SLTEC. Differences exist among specific strains in the diseases produced and the animal species affected. Through the action of SLTs, SLTEC inhibit protein synthesis in host cells. VTEC that cause enteric disease in humans are commonly found in the gastrointestinal tract of healthy cattle. Differences among species in the distribution of specific glycolipid receptors for SLTs on surfaces of cells determines species variation and organ specificity (Whipp *et al.*, 1994).

There are more than 200 serotypes of SLTEC isolated from cattle and more than 160 serovars have been identified in human sources (Gyles *et al.*, 1998). Strategies for the prevention of HUS and HC in humans include the elimination of EHEC in cattle (Voelker, 1994). Serovar O157:H7 accounts for 70 to 80 percent of all human illnesses associated with SLTEC infections (Gyles *et al.*, 1998) and is the predominant serovar associated with HUS and HC (Armstrong *et al.*, 1996). The association of O157:H7 with cattle has prompted several on farm surveys to determine prevalence levels in the reservoir population (Cray *et al.*, 1996). Prevalence data vary considerably among studies due to variations in shedding patterns and methods used to identify infected cattle (Vold *et al.*, 1998). It has been demonstrated experimentally that O157:H7 is shed in higher amounts and for longer lengths of time in feces of younger animals (Cray *et al.*, 1996). Factors which affect fecal shedding include diet and seasonal variation (Vold *et al.*, 1998). The onset and duration of shedding may coincide with the incidence of human infections. A study conducted in Washington State concluded that O157:H7 related illness in humans peaked during the summer months. Sixty percent of the cases occurred between June and September paralleling heightened periods of shedding observed in cattle (Cohen

and Gianella, 1991; Hancock *et al.*, 1997). These trends may, however, reflect increased activities associated with food-borne illnesses during these months such as picnics and barbecues.

To conduct valid epidemiological investigations on SLTEC in cattle there are some critical factors that should be considered in designing studies. Methods used for detection should be specific for serovars that cause disease in humans. Strains of VTEC that are less virulent to humans than O157:H7 and other EHEC are common in the feces of cattle (Gyles *et al.*, 1998) and toxin production alone does not provide proof of pathogenicity (Orden *et al.*, 1998). The identification of VTEC in bovine feces alone is therefore not sufficient to demonstrate a risk for food-borne illness (Wilson *et al.*, 1992). The use of probes to exclusively detect genes conferring toxin production, for example, could result in a substantial number of false positives in surveys. Several methods have been developed to screen specifically for the presence of O157:H7 and other EHEC that are virulent to humans in bovine and food sources. Included in these are assays that detect combinations of virulence factors associated with HC and HUS such as SLT production and intimin, a surface protein important for adherence of the pathogen to enterocytes and the formation of attaching and effacing lesions (Gyles *et al.*, 1998). A flow chart scheme using combinations of tests is also useful in screening populations for specific serovars. Wilson *et al.* (1992) used this to measure the distribution of specific serovars in dairy cattle in southern Ontario.

Experimental design is also important for the generation of valid data. Many studies on VTEC prevalence in cattle fail to use formal random selection of animals from well-defined populations (Gyles *et al.*, 1998). In one investigation, cattle randomly chosen at an abattoir were screened for the presence of VTEC (Clarke *et al.*, 1988). It was concluded that 19.5 percent of dairy cows were positive for VTEC. This is not a valid estimate of the true prevalence of VTEC in the dairy cattle population of the area because only animals at abattoirs were screened. The infection rate among these animals may be different from that in the rest of the population. Dairy cows at abattoirs come from many different sites and cross infection through close association prior to slaughter (holding

pens, etc.) is likely (Gyles *et al.*, 1998). This experimental design also failed to use animals that represent a well-defined population. The majority of dairy cows at abattoirs are older animals which have been culled (Gyles *et al.*, 1998). Prevalence studies on O157:H7 use either the herd or the individual animal as the experimental unit. Individual prevalence values are useful because they provide a way to assess the risk from contact with any individual animal. Values of herd or farm-level prevalence, however, may be as important from a public health perspective because they measure the risk associated with exposure to any farm that was randomly selected from a population of farms (Wilson *et al.*, 1992). Detection of O157:H7 in herds may become increasingly useful in measuring the occurrence of O157:H7 in populations because of the variation associated with detection of the pathogen in individual animals.

Identification of a dairy herd associated with O157:H7 related illness in humans gave Mechie *et al.* (Mechie *et al.*, 1997) the opportunity to evaluate fecal shedding of O157:H7 in a naturally infected population. The study was initiated in May 1993 following the outbreak and conducted over a 15-month period. Factors considered in fecal shedding were age, seasonal variation, and management practices. The detection methods used were specific for O157:H7 and included immuno-magnetic separation followed by plating for isolation and characterization of suspect colonies using probes for verotoxin genes. Of 3538 rectal swabs obtained, O157:H7 was isolated from 147 or 4.2 percent. Comparisons were made among lactating and non-lactating cows, calves, and heifers. The farm was visited 28 times with lapses between visits varying from one to four weeks. On individual visits prevalence ranged from zero in all groups to 14 percent in lactating cows, 40 percent in non-lactating cows, 68percent in heifers, and 56 percent in calves. Peak shedding occurred between May and July of 1993. Between November of 1993 and May 1994, there was no fecal shedding in any group. During May of 1994, at the close of this study, shedding of O157:H7 resumed in all groups suggesting that the organism was maintained in the herd or re-infection occurred through the farm environment.

This study supports previous observations of age as a factor in fecal excretion of O157:H7 (Blanco *et al.*, 1996; Wells *et al.*, 1991) and could reflect the effects of rumen development on survival of O157:H7 in the bovine reservoir (Mechie *et al.*, 1997; Rasmussen *et al.*, 1993). Shedding varied for all groups during this study, but seasonal patterns were similar. Variations in the incidence of O157:H7 in herds due to seasonal patterns were consistent with the incidence of human illness in the area. Effects due to change in diet were considered when increased shedding among cows after calving was observed. Among lactating cows, fecal shedding occurred most frequently at 1-month post parturition. In this operation non-lactating cows were maintained on a high roughage diet consisting mostly of grass and silage. After calving, cows were switched to diets consisting mostly of compound dairy concentrate. Shedding decreased during the five to 6-month period following the initial peak at one month, but peaked again at month seven when lactation decreased and cows were fed diets containing less concentrate (Mechie *et al.*, 1997). These observations suggest that change in diet is an important factor in fecal shedding of O157:H7.

To reduce the occurrence of human illnesses, it is important to understand the biological relationship between O157:H7 and the ruminant reservoir to allow identification of production practices that may enable the pathogen to persist in bovine populations and the environment. It is believed that rumen development is related to the age variation associated with fecal shedding of O157:H7 (Cray and Moon, 1995). In comparison to underdeveloped rumens of young animals, the rumens of adult cattle have higher concentrations of volatile fatty acids (VFA) and lower pH values. This serves as a barrier to O157:H7 infection in adults (Rasmussen *et al.*, 1993).

High VFA concentration and low pH of rumen and hindgut environments inhibit the growth of enteric pathogens such as Salmonella (Rasmussen *et al.*, 1993). Rasmussen *et al.* (1993) compared the growth of O157:H7 in rumen fluids from fasted and well-fed cattle and observed that O157:H7 grew better in the former. To isolate and evaluate these effects, they conducted in vitro experiments using media having similar volatile fatty acid concentrations and pH values as rumen fluids from

well-fed animals and found the results to be consistent with what was previously observed. From this study the authors concluded that growth of *E. coli* is affected by the levels of these two variables which fluctuate with changes in diet. The growth of *E. coli* is poor in the rumens of well-fed animals when pH values are less than 6.5 and volatile fatty acid concentrations exceed 100 mM. During fasting these inhibiting effects are minimized as volatile fatty acid concentrations decrease to less than 50 mM and pH values exceed 7.0 (Rasmussen *et al.*, 1993).

Changes in microbial flora that occur with change in diet resemble the successional events leading to rumen development in pre-ruminant calves, with the adult rumen after a period of fasting or intermittent feeding being analogous to the pre-rumen (Rasmussen *et al.,* 1993). The low VFA concentration in these conditions enable enterobacteria to exist until normal microbial species colonize (Rasmussen *et al.,* 1993). Disturbances in the normal flora of the rumen could predispose cattle to infection with *E. coli*. Hindgut fermentation directly affects the fecal shedding of *E. coli*, and as occurs in the rumen, microbial populations are influenced by changes in diet (Rasmussen *et al.*, 1993). If the normal flora is disturbed, enterobacteria will initially dominate and increased shedding of *E. coli* occurs (Rasmussen *et al.*, 1993).

Acid tolerance seems to be an important factor in the transmission of *E. coli* from cattle to humans (Diez-Gonzalez *et al.,* 1998). When grown in mildly acidic conditions in vitro, *E. coli* develop very high tolerance for low pH and can survive in environments with pH values as low as 2.0 (Diez-Gonzalez *et al.*, 1998). The development of acid tolerance by *E. coli* was used by Diez-Gonzalez *et al.* (1998) to describe the results of a study that evaluated the effects of VFA concentration and pH on *E. coli* populations in the bovine colon. Their results conflict with those of Rasmussen *et al.* (1993) by concluding that low pH and high VFA concentrations in the rumen do not inhibit growth of *E. coli*, but instead promote the development of acid tolerance which increases the survival of *E. coli* in the colon.

Like Rasmussen *et al.* (1993) and Diez-Gonzalez *et al.* (1998) acknowledged that changes in pH and volatile fatty acid concentration occur with changes in diet. Diez-Gonzalez *et al.* (1998) measured the pH and VFA concentration of the rumen and colon before and after increasing the amount of grain fed to cattle. They also determined changes in the total rectal *E. coli* population and acid tolerance of the *E. coli* under these conditions. By increasing the percentage of grain in diets, the VFA concentration of the colon increased and the pH decreased while there were no significant changes in the rumen (Diez-Gonzalez *et al.*, 1998). In comparison to hay, grain has a higher starch concentration which, when fermented in the colon, results in the accumulation of volatile fatty acids which reduce the pH (Diez-Gonzalez *et al.*, 1998). The colonic pH of cattle that were fed hay only was >7.0 and the total *E. coli* count was approximately 20,000 cfu per gram of digesta. Nearly all of these were killed by acid shock treatment. After increasing the amount of grain to 60 percent of the diet, the total *E. coli* population increased more than 100 fold (6.3×10^6 cfu/gram digesta) and the acid resistance of that population was higher (Diez-Gonzalez *et al.*, 1998).

Transmission of O157:H7

E. coli O157:H7 is transmitted through the fecal oral route (Garber *et al.*, 1995). Several animal species including humans, the environment, and foods can be sources of O157:H7 infection (Whipp, 1994; Paros, 1993). Efforts to decrease the incidence of O157:H7 illness in humans include elimination of the pathogen in the cattle population (Voelker, 1994). The determination of on-farm prevalence and the identification of sources of infection are important for minimizing the transmission of O157:H7 among cattle. Isolation from feces is currently the most definitive method to identify infected individuals (Qadri and Kayali, 1998). With a pre-enrichment step using broth media, this procedure can detect less than one cfu per gram in ovine feces (Kudva *et al.*, 1995). However, intermittent shedding can minimize the accuracy of results through the occurrence of false negatives (Pirro *et al.*, 1995).

Transmission of O157:H7 among cattle is difficult to study because there are several factors that influence fecal shedding (Garber *et al.*, 1995). Weaning has been shown to be positively associated with fecal excretion of O157:H7 in infected calves. Calves may be important in the transmission of O157:H7 in herds because they shed the pathogen for longer periods of time and are colonized with *E. coli* shortly after birth (Garber *et al.*, 1995). In experimental infection, colonization of the gastrointestinal tract can occur as early as eight weeks following birth and the extent of colonization may be greater than that observed in adults (Spencer, 1993). This suggests that there are predisposing factors among calves such as rumen development and behaviors such as suckling and licking that result in increased colonization and higher rates of infection, respectively. The number of calves positive for O157:H7 increase when post-weaned animals are housed together. It is not clear if this increase reflects increased transmission among groups or elevated stress levels associated with competition within these groups. A positive relationship between fecal shedding and stress in calves has been demonstrated (Garber *et al.*, 1995).

The presence of O157:H7 in bovine feces has importance beyond the direct threat to human health. Cattle can become infected through sources contaminated with feces of infected herdmates. Hancock defines two possibilities that explain transmission of O157:H7 in herds (cited in Spencer, 1993). In the first explanation, O157:H7 is continuously spread by infected individuals to others in the herd. Any member of a positive herd is a potential source of O157:H7 at any time. The second possibility is transmission by chain infection. In chain infection there are always a few positive individuals in the herd that are infected for shorter periods of time. During infection, the pathogen is spread to susceptible individuals. The proportion of infected animals at any time is relatively constant as animals recover and become infected at consistent rates (Spencer, 1993).

Infection among cattle is difficult to control (Wang, 1996). The presence of O157:H7 in manure could contribute to this by increasing the likelihood of exposure to individuals in the population (Wang *et al.*, 1996). Concentrations of O157:H7 in the feces of infected calves range from

$<10^2$ to 10^5 cfu per gram (Zhao *et al.*, 1995). It is suspected by some that the use of cow manure slurry for irrigation since the early 1980s could have enabled O157:H7 to persist in the farm environment (Wang *et al.*, 1996). Studies on the survival of O157:H7 in bovine feces after excretion from cattle have revealed that the pathogen can survive for up to eight weeks at 37°C and nine weeks at 22°C (Wang *et al.*, 1996).

Transmission to humans can occur through several routes. Although food products are commonly sources of O157:H7 infections in man, several other potential sources exist (Jackson *et al.*, 1998). Contact with infected animals, contaminated water and soil have all resulted in illness (Renwick *et al.*, 1993; Chart, 1998; Cieslak *et al.*, 1993). The low infectious dose of O157:H7 and its tolerance for environmental stresses allow efficient transmission of the pathogen to humans (Karch *et al.*, 1996; Chart, 1998). Secondary human transmissions have been reported in institutional environments such as day care centers and nursing homes (Karch *et al.*, 1996; Dorn, 1993). Human to human transmission is also common in families when one individual becomes infected and serves as a source of infection for others (Dorn, 1993). Efficient transmission is likely in families, particularly when good hygiene is lacking. Rural families may be at greater risk for transmission of O157:H7 because of more frequent contact with livestock (Mead and Griffin, 1998). Fecal excretion of O157:H7 in humans can occur for several weeks following recovery (Mead and Griffin, 1998), and like cattle, younger children shed O157:H7 for longer periods of time. In one study on patterns of fecal shedding in children less than five years of age, O157:H7 was isolated from fecal samples for a median duration of 17 days following the onset of illness (Belongia *et al.*, 1993).

Virulence

The identification of specific phenotypes associated with disease is useful for administering accurate epidemiological surveys of infectious diseases and understanding the disease process. The production of disease by O157:H7 and other EHEC is believed to be mediated by a combination of

virulence factors. The presence or absence of specific factors in isolates associated with specific syndromes can be useful in diagnosing and preventing disease.

Shiga-like toxins

All strains of O157:H7 isolated from human patients produce at least one of two toxins that are cytotoxic to vero cells (Weertna and Doyle, 1991). To distinguish them from heat labile and heat stable enterotoxins produced by other pathogenic *E. coli*, these are referred to as verotoxins 1 and 2 or VT1 and VT2 (Pollard *et al.*, 1990; Dorn and Angrick, 1991). The toxins are alternatively referred to as Shiga-like-toxins (SLT) 1 and 2 because they are similar in structure and function to Shiga toxin produced by *Shigella dysenteriae* (Whipp *et al.*, 1994).

SLT-1 is almost identical to Shiga toxin in amino acid and nucleotide sequences (Gannon *et al.*, 1990). Antibodies to Shiga toxin in sera neutralize SLT-1 and biological activities of the toxins are similar (Dorn and Angrick, 1991; Cohen and Gianella, 1991). Both toxins are composed of two subunits, A and B (Noda *et al.*, 1987; O'Brien and Leveck, 1983). Subunit A prevents protein synthesis in host cells by binding to the 60s ribosomal subunit. Subunit B interacts with host cell receptors to initiate entry of subunit A (Ogasawara *et al.*, 1987, 1988). SLT-2 shares less sequence homology with Shiga toxin and is not neutralized by Shiga toxin antisera (Dorn and Angrick, 1991). A variant of SLT-2 is common in strains of SLTEC that produce disease in pigs. This variant, SLT-2e, differs from toxins associated with human illness in its lack of cytotoxicity to HeLa cells and heat sensitivity (Whipp *et al.*, 1994). SLT-2e is neutralized by antisera raised against SLT-2 and shares approximately 90 percent of the amino acid sequence (Gannon *et al.*, 1990; Cohen and Gianella, 1991). Nucleotide sequences between the two variants are less homologous than the amino acid sequences (55-60 percent) (Gannon *et al.*, 1990).

All O157:H7 strains isolated from humans with HUS express SLT-2 (Louise and Obrig, 1995). This suggests that there is a higher risk for the development of HUS in SLTEC infections if the strain

produces SLT-2 (Whipp *et al.*, 1994) and that SLT-2 has a role in the development of HUS. In studies comparing the cytotoxic activities of SLT-1 and SLT-2 on human renal microvascular endothelial cells (HRMEC), it was demonstrated that SLT-2 is 1000 times more cytotoxic than SLT-1 (Louise and Obrig, 1995). The presence of antibody to SLT in sera during infections suggest that toxins enter the circulation and can have systemic effects (Whipp *et al.*, 1994). It is also known that damage to endothelial cells is necessary for the development of HUS. SLTs bind to globotriaosyl ceramide (Gb3) receptors on the surfaces of endothelial cells promoting entry of subunit A into the cell. This results in inhibition of protein synthesis and cell death (Kaplan *et al.*, 1990).

Adherence and attaching and effacing activity

Persistence of bacteria in the gastrointestinal tract requires close association with intestinal mucosa or epithelial surfaces (Schultz *et al.*, 1997). Hydrophobic and electrostatic properties on bacterial surfaces can serve to facilitate this association (Turi *et al.*, 1997). More specific association is provided through interactions of bacterial adhesins with host cell receptors (Turi *et al.*, 1997). Attachment functions to resist peristaltic clearance of bacteria from the intestinal tract and allows toxins to have close contact with host cells (Cohen and Gianella, 1991). Patterns of colonization among pathogenic *E. coli* include localized adherence (LA), diffuse adherence (DA), and aggregative adherence (AggA) (Cohen and Gianella, 1991). EHEC colonize host cell surfaces in an LA pattern which is characterized by the formation of microcolonies on host cell surfaces. In diffuse adherence, bacteria are evenly distributed upon the cell surface and in aggregative adherence bacteria adhere to one another in a pattern that resembles a stacked brick arrangement (Zepeda-Lopez and Gonzalez-Lugo, 1995).

Attachment of some EHEC requires the expression of intimin, a 97-KDa surface protein transcribed from the eae gene (Sandhu *et al.*, 1996). EPEC also produce intimin. This protein is 97 percent homologous to intimin produced by EHEC at the N-terminus and central regions. There is

less homology between the C-terminal regions which confer specificity of the protein for receptors on host cells (Sandhu *et al.*, 1996). This is apparent in the different sites colonized by the two groups of pathogenic *E. coli* along the intestinal tract. The role of intimin is attachment of the bacteria to the intestinal epithelium (Jerse *et al.*, 1990). Intimin is also involved in the production of attaching and effacing lesions on intestinal surfaces, however products of other genes are required to initiate adherence and signal transduction (Wieler *et al.*, 1998).

Hemolysin production

A common characteristic of EHEC is the expression of enterohemolysins (Schmidt *et al.*, 1994). In *E. coli* O157:H7, the genes for hemolysin are encoded on the large 90Kb plasmid and their production is strongly associated with that of SLT (Schmidt *et al.*, 1994). The enterohemolysins belong to the repeating toxin (RTX) or pore forming family and have been shown to be related to alpha hemolysins through hybridization studies (Schmidt *et al.*, 1994). The role of EHEC hemolysins (enterohemolysins) is ill defined, however, it is thought that their role in disease is significant (Schmidt *et al.*, 1994).

Clinical Diseases caused by *E. coli* O157:H7

Hemorrhagic colitis (HC)

The clinical manifestations of infection with O157:H7 range from asymptomatic carriage to life threatening diseases including thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) (Mead and Griffin, 1998). The most common clinical manifestation that develops from O157:H7 infection in humans is hemorrhagic colitis (HC). It is estimated that 20,000 cases of HC occur annually in the United States as a result of O157:H7 infection (Nauschuetz, 1998). This condition is characterized by grossly bloody diarrhea resulting from the destruction of intestinal epithelium by EHEC colonizing the bowel (Jacewicz *et al.*, 1999). There is an average length of

three days between the ingestion of O157:H7 and the onset of symptoms. However, lengths of incubation have ranged from as little as one to as many as eight days (Mead and Griffin, 1998). Most patients recover within seven days (Mead and Griffin, 1998).

The diagnosis of EHEC infection early in the development of HC is important for the administration of proper therapy and the identification of sources of infection. Diagnosis of EHEC infections should not be made exclusively on the basis of clinical symptoms. The wide spectrum of clinical signs associated with EHEC infections can be misleading to clinicians (Griffin *et al.*, 1988). The acute abdominal pain, low-grade fever, low fecal leukocyte counts, and hemorrhagic diarrhea can resemble several non-infectious illnesses (Griffin *et al.*, 1988). Intussuseption and inflammatory bowel disease can be suspected in children and younger adults, respectively. EHEC infection can also be mistaken for ischemic colitis in elderly patients because the bloody diarrhea and abdominal cramping are common to both diseases (Griffin *et al.*, 1999; Alapati and Mihas, 1999). If other illnesses are initially suspected, invasive and potentially harmful techniques required for treatment or definitive diagnosis may be administered unnecessarily (Griffin *et al.*, 1988).

Grossly bloody stools do not occur in all individuals that are infected with EHEC. Asymptomatic carriage has been reported in outbreaks and some patients develop no more than watery diarrhea (Griffin *et al.*, 1988). In some outbreaks, reports of bloody stools are as low as 31 percent among the infected population (Qadri and Kayali, 1998). The severity and duration of the early signs of infection may correlate with the potential for progression of the illness to HUS. HUS is less likely to develop when patients have relatively mild symptoms such as watery diarrhea (Griffin *et al.*, 1988). The duration of diarrhea and bloody stools is also longer among patients that develop HUS (Oshima, 1997). Leukocytosis with counts exceeding 15,000 per microliter could also indicate a greater potential for development of HUS (Oshima, 1997). Patients that have fever and vomiting during HC are also more likely to develop HUS (Oshima, 1997). Early intervention with gamma

globulin, urinastatin, aspirin, and dipyridamole have all been effective in preventing HUS in patients considered to be high risk by the criteria described above (Oshima, 1997).

Hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP)

Hemolytic uremic syndrome or HUS was first described by Gasser in 1966 (cited in Aria and Matsuzaki, 1996). HUS can result from pregnancy, chemotherapy, cancer and transplant surgeries. Its association with VTEC infection was apparent following the first reported O157:H7 food-borne outbreak in 1983 (Karmali *et al.*, 1985). In sporadic cases of EHEC infection, HUS occurs in three to seven percent of patients. In outbreaks this figure can be as high as 20 percent (Mead and Griffin, 1998). Currently, the majority of HUS cases are attributed to VTEC infection (Aria and Matsuzaki, 1996). Young and elderly patients are most prone to the development of HUS and thrombotic thrombocytopenic purpura (TTP), respectively (Griffin *et al.*, 1988). HUS consists of a triad of disorders including micorangiopathic hemolytic anemia, severe thrombocytopenia, and renal failure (Gordon and Kwaan, 1999).

Experimental evidence is not conclusive, but strongly supports the involvement of SLTs in the development of HUS (Williams, 1999). Experiments using mice have shown that the renal tubules of the kidney are the primary sites affected by SLTs. Intravenous injection of SLT-2 results in necrosis within renal tubules with no change in the structure of glomeruli (Uchida *et al.*, 1997). There is usually little or no fever suggesting that the pathogen itself is non-invasive (Padhye and Doyle, 1992). SLTs enter the circulation from sites of infection in the intestine and bind to Gb3 receptors on endothelial cells from where they initiate cytotoxic effects (Mead and Griffin, 1998). Renal endothelial cells have high concentrations of Gb3 receptors, rendering them particularly susceptible to destruction by circulating toxins. Endothelial cell damage also triggers the clotting mechanism causing fibrin and platelet deposition. This results in the occlusion of the renal microvasculature leading to renal failure (Mead and Griffin, 1998). Patients often appear jaundiced and hypertensive

and there is an accumulation of waste products in the blood accompanying renal failure (Padhye and Doyle, 1992). Laboratory results are inconclusive, however leukocyte levels in the blood are usually high (Mead and Griffin, 1998). Circulating LPS may contribute by facilitating aggregation of platelets in the kidney microvasculature leading to the development of thrombocytopenia (Mead and Griffin, 1998).

HUS due to EHEC infection is the leading cause of renal failure in children (Noel and Boedeker, 1997). The disease most frequently affects children ten years of age or younger (Qadri and Kayali, 1998). The triad of diseases occur acutely in the development of HUS and patients may experience other disorders involving the cardiovascular and nervous systems (Padhye and Doyle, 1992). Death occurs in three to five percent of patients that develop HUS (Mead and Griffin, 1998). Approximately 50 percent of patients require dialysis and 75 percent have erythrocyte transfusions (Mead and Griffin, 1998). Those who recover often experience chronic proteinuria years later (Mead and Griffin, 1998). There are other sequelae that may develop including cholelithiasis, colonic stricture, pancreatitis, glucose intolerance, and cognitive impairment (Mead and Griffin, 1998). Thrombotic thrombocytopenic purpura (TTP) usually affects adults and is essentially the same disease as HUS when the causative agent is O157:H7 or other EHEC (Padhye and Doyle, 1992). In contrast to HUS, the central nervous system is the major organ system involved (Mead and Griffin, 1998).

Detection Methods

Several methods have been developed for the rapid identification of O157:H7 and other EHEC in clinical and environmental samples. These include enzyme linked immunosorbent assays, amplification of unique genomic sequences by polymerase chain reaction (PCR), toxicity on vero cells in culture, and nucleic acid probes specific for verotoxin production on suspect colonies (Ramotar *et al.*, 1995). The most definitive method of diagnosis is isolation of the pathogen on

differential media (Reilly, 1998). Differences in the rates of sorbitol fermentation exist between most strains of O157:H7 and other *E. coli* (Padhye and Doyle, 1992). This has been exploited for use in differential culture by supplementing MacConkey agar with sorbitol instead of lactose (Padhye and Doyle, 1992). The phenotypic difference is readily observed after 24 hours of incubation at 37°C on Sorbitol MacConkey Agar (SMAC) (March and Ratnam, 1986). Most strains of O157:H7 produce colorless colonies while most other isolates produce pink colonies (Mead and Griffin, 1998). Some non-O157:H7 bacteria do not ferment sorbitol and as a result produce colonies similar in appearance to O157:H7 colonies. These can be readily distinguished serologically with antisera specific for the O157 antigen (Mead and Griffin, 1998).

The use of SMAC media to isolate O157:H7 from environmental and clinical samples is limited by its low sensitivity of detection. Many samples of interest, including feces, have high populations of background bacteria which outgrow lower numbers of O157:H7 in culture (Sanderson *et al.*, 1995). To increase sensitivity levels, selective enrichment of samples prior to isolation on SMAC has been used (Sanderson *et al.*, 1995). Supplementing enrichment and SMAC media with compounds such as cefixime and tellurite increase the sensitivity of detection by inhibiting the growth of other competing bacteria during culture (Zadik *et al.*, 1993). Initial isolation of O157:H7 from clinical and food samples using O157 specific antibody coated beads also increase the sensitivity of detection with isolation procedures (Sanderson *et al.*, 1995).

To decrease the incidence of O157:H7 in the food supply, the development of methods to rapidly detect the pathogen in cattle and foods have been proposed (Mermelstein, 1993). There are several available, including PCR, which have the potential to contribute to these objectives. Advantages of PCR over other available methods include specificity for O157:H7 and other EHEC which are of considerable importance to human health. Although sensitive, the use of probe, H. 1992. Fort Collins, CO: Autho been shown to cross react with surface proteins of other speciesr including *Yersinia enterocolitica* and *Citrobacter freundii* (Feng, 1995). The probe is also present on other

non-H7 serotypes (Aleksic *et al.*, 1992). The use of multiplex PCR allows for selection of target sequences which in combination are highly specific for O157:H7 and other EHEC associated with severe illness in humans (Gannon *et al.*, 1997, Meng *et al.*, 1998). An additional advantage of PCR over other methods such as the detection of verotoxin in stools or antigen neutralization assays is in the analysis of bacterial epidemiology (Wilde *et al.*, 1990). This has importance in identifying sources of infection and chains of infection in human outbreaks and bovine populations (Weber, 1997). This is not possible with these alternative methods since they are not as specific and may not distinguish between previous and current infections (Weber, 1997, Pirro *et al.*, 1995).

Although potentially sensitive, amplification of target sequences in fecal samples is inhibited by components such as bile salts and bilirubin (Widjojoatmodjo *et al.*, 1992). Methods to circumvent these effects include dilution of fecal samples to minimize the effects of inhibitory substances (Ramotar *et al.*, 1995). Dilution of samples can, however, reduce the sensitivity of PCR by diluting O157:H7 to levels that are not detectable (Panaccio *et al.*, 1994). There is a tradeoff between the purification methods used prior to PCR and the sensitivity of detection. Isolation methods which require the fewest steps should be favored for use in PCR procedures to minimize the potential for contamination of samples and loss of sensitivity (Panaccio *et al.*, 1994).

Bacteria can be separated from most inhibiting components in fecal samples by applying aqueous solutions in a filtration procedure and recovering disassociated bacteria in filtrates. It is possible that the time necessary to enrich O157:H7 from fecal samples for detection with PCR is dependent upon the salt concentration of the solutions applied to feces in this procedure. It seems reasonable that with adequate recovery, target organisms are more readily detected in filtrates than in feces with PCR since levels of inhibitors are lower in filtrates. Additionally, methods to increase PCR sensitivity, such as enrichment, are more effective on target organisms recovered in filtrates than in those present in the untreated feces. Factors likely to affect this potential would include the effects of solutions on the removal of the target organism, competitor bacteria, and other components from

the feces that impede enrichment or interfere with the PCR. In this study, aqueous solutions varying in sodium chloride concentration were applied to bovine feces containing O157:H7 to evaluate the effects of this salt on the removal of the pathogen from feces during filtration. Treatment effects on the time required to incubate filtrate preparations for enrichment to detect O157:H7 using a triplex PCR procedure were then evaluated.

MATERIALS AND METHODS

Experimental Design

This experiment was designed to evaluate the effects of sodium chloride (NaCl) on the removal of *E. coli* O157:H7 from bovine feces in preparation for polymerase chain reaction (PCR) for the diagnosis of carrier animals. Bovine fecal samples spiked with *E. coli* O157:H7 were washed with solutions of 0, 0.5, 0.9, and 1.5 percent NaCl during a centrifugal filtration procedure. The recovery of O157:H7 in filtrates was determined by plate counts on differential media. Samples of filtrates from each treatment were inoculated into Luria broth media and cultures were incubated for eight, nine, and 10 hours at 37°C. Absorbency measurements were obtained following incubation to compare relative cell density values among cultures from each treatment at these times. Cultures were stored at –22°C to terminate enrichment. The cultures were later thawed and prepared for the triplex PCR specific for O157:H7 to determine the minimum time required for the incubation of extracts from each treatment to produce positive results. A total of six replications were performed in subsets of two replicate runs.

Bovine Feces

All feces used in this experiment were obtained from one steer at the College of Veterinary Medicine at Iowa State University. The sample was collected from the floor of the stall housing the steer and transported to the food safety laboratory located in the College of Veterinary Medicine. The sample was divided into 20-gram portions and stored at –20°C.

Preparation of O157:H7 Inoculum

E. coli serovar O157:H7 strain ATCC 35150 provided by Dr. James Dickson (Iowa State University) was used in this study. Streaking bacteria onto brain heart infusion agar media and incubating cultures at 37°C overnight isolated colonies. One colony was transferred to nine ml. of

Luria Broth and this was incubated overnight at 37°C to produce a pure culture of O157:H7. The culture was harvested by centrifugation at 650 x g for 15 minutes followed by aspiration of the media from the pellet. The pellet was re-suspended in 0.5 ml of PBS and vortexed. A spectrophotometer adjusted to measure absorbency at 610 nm was set to read an optical density of zero with a cuvette containing PBS. Drops of the bacterial suspension were added to the PBS until transmittance was approximately 50 percent. Ten fold serial dilutions were prepared out to 10^{-6}. One hundred µl. of the 10^{-6} dilution was plated onto Sorbitol MacConkey agar (March, 1986) and incubated at 37°C overnight to obtain colonies. Colony counts were used to determine viable cell density of the O157:H7 suspension that was used to inoculate the feces and the final concentration of O157:H7 in fecal samples.

Inoculation of Fecal Samples

For each replicate of the experiment 60 grams of fecal sample were removed from the freezer and allowed to thaw in a warm water bath. After thawing, samples were removed from the stomacher bags and placed on absorbent bench paper. The feces was allowed to sit for two hours at room temperature to decrease the water content. Two individual 20-gram samples were obtained from the sample and placed into stomacher bags. Into each bag, 25 µl of the 10^{-3} dilution prepared in step two was pipetted. Stomacher bags were heat sealed and stomached for five minutes to provide a sample assumed to have 10^{2}-10^{3} cfu of O157:H7 per gram of feces.

Extraction of O157:H7 from Feces

Inoculated samples were aseptically removed from stomacher bags after stomaching and individual four gram samples were placed into one seven µm retention filter seated in the mouth of a centrifuge bottle. Four samples were produced from each 20-gram aliquot to produce a set that represented one replicate of this experiment. One sample from each of the two sets was designated for each wash treatment. Wash solutions were applied to samples and centrifuged at 200-400 x g to

drive solution with the disassociated bacteria through the filter. This was repeated intermittently until 25 ml of filtrate was collected in each bottle.

Preparation of Filtrates for Enrichment

Filtrates were centrifuged for 10 minutes at 1700 x g. 15 ml was aspirated and discarded from each to reduce the volume of filtrate. The remaining fractions were transferred to individual 15-ml centrifuge tubes. Samples were centrifuged for 12 minutes at 650 x g. Supernatants were aspirated down to one-ml volumes with pellets. The pellet was re-suspended and 0.4 ml. was plated onto Sorbitol MacConkey agar to obtain viable cell density values of the filtrate preparations for comparison of removal of O157:H7 from feces among treatments. Samples were reconstituted with sterile dH20 to bring volumes to three ml.

Inoculation of Broth Media and Enrichment of O157:H7

A preparation of 1 ml. was inoculated into nine ml of Luria broth media. This was repeated two times for each preparation to yield three cultures for each treatment. Cultures were vortexed and incubated at 37°C with shaking (200 rpm). One culture from each treatment was removed after eight hours and absorbencies were measured at 610 nm. Cultures were stored at –22°C until harvested for PCR. This was repeated at hours nine and 10 with the remaining cultures.

Preparation of Template DNA from Cultures

Cultures were removed from –22°C and thawed in a warm water bath. Cultures were centrifuged at 900 x g for 12 minutes and supernatants were aspirated from the bacterial pellets. Pellets were re-suspended in one ml of PBS (pH 7.4) and transferred to 1.5-ml microcentrifuge tubes. Suspensions were centrifuged for 10 minutes at 16,000 x g and the supernatants were decanted from the pellets. Each pellet was re-suspended in 490 μl of distilled water and 10 μl of 1N sodium hydroxide. Mixtures were boiled for 10 minutes and then placed on ice for an additional 10 minutes

to extract DNA. Samples were centrifuged at 16,000 x g for two minutes and 10 μl of the supernatant was added to the PCR reaction mixture.

Polymerase Chain Reaction

The triplex PCR was designed to amplify three target sequences specific for O157:H7 (Matise, 1997). Oligonucleotide primers were synthesized by the Iowa State University Nucleic Acid Facility. Target sequences included a 252 base pair sequence unique to O157:H7 in the *uid* A gene. Primers used for this sequence were (5'-3') TGATGCTCCATAACTTCCTG and GCGAAAACTGTGGAATTGGG. Sequences in both Shiga toxin genes were also targeted. In the SLT-1 gene a 614 base pair region flanked by sequences specific for the following primers was targeted for amplification: (5'-3') ACACTGGATGATCTCAGTGG and CTGAATCCCCCTCCATTATG. Primers used for an SLT-2 gene sequence of 779 base pairs were (5'-3') ACACTGGATGATCTCAGTGG and CCACGAATCAGGTTATGCCTC. A 50 μl reaction volume was prepared for each sample. The composition of the reagents for the reaction were as follows: 10mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM of each base (dATP, dGTP, dCTP, dTTP), 0.1 mM of each primer, 2.5 units of Taq DNA polymerase and 10 μl of template DNA. PCR was run for 40 cycles. Each cycle began with a four-minute separation of duplex DNA at 94°C. This was followed by one minute at 58°C and 1.5 minutes at 72°C for primer binding. A final extension of seven minutes at 72°C was used to complete one cycle of the reaction. PCR products were electrophoresed for 40 minutes at 70 volts in 0.8 percent agarose gels containing 0.01 percent ethidium bromide. Products were visualized with a transilluminator and photographed.

Statistical Analysis

Data were collected from the randomized complete block design of this experiment. Four treatments were evaluated for recovery of O157:H7 from bovine feces. An Analysis of Variance (ANOVA) was used to identify differences in the times required to enrich preparations from

treatments in broth culture for detection of target sequences in a triplex PCR procedure. For each treatment, mean values of viable cell density in filtrates were calculated from six replications and compared using Statistical Analysis Software (SAS) (SAS Institute Inc., Cary, NC). For each treatment, mean values of PCR scores were calculated and expressed as the fraction value of the amplicon products observed out of the amplicons targeted. Scores of 1 and 0 indicate full positive and full negative scores, respectively.

RESULTS AND DISCUSSION

These experiments evaluated the effects of sodium chloride concentration in wash solutions on the removal of O157:H7 from bovine feces in a filtration procedure. As shown in Table 1, statistically there was a difference among the treatments used (LSD= 48.36, α= .05). This difference existed between the 1.5% and the 0.5% NaCl wash treatments. The high NaCl concentration was not as effective as 0.5% NaCl in removing viable O157:H7 from feces that had been artificially inoculated (P=05). No statistical differences were observed among treatments with solutions containing 0.9%, 0.5%, and 0% NaCl and rates of recovery were generally low for all treatments (< 20%) suggesting that the potential recovery of O157:H7 from bovine feces is low with the use of this separation procedure (Table 1). The lower relative recovery of O157:H7 in filtrates with the 1.5% NaCl treatment could be due to lower survival of this particular strain of O157:H7 at this concentration of sodium chloride. Although no statistical differences exist among the 0, 0.5, and 0.9% treatments, some affects due to NaCl concentration within the range of 0-0.9% may have been observed in this experiment. Figure 1 shows that the recovery of O157:H7 tended to be 50 percent higher with the 0.5% NaCl wash than with either the 0% or 0.9% washes, although this difference

Table 1. Average recovery of O157:H7 for each treatment with the wash filtration procedure*

	Percent NaCl			
E. coli O157:H7	0%	.5%	.9%	1.5%
Average total cfu in feces washed (4 grams)	1013 ± 59	1013 ± 59	1013 ± 59	1013 ± 59
Average total cfu recovered	105.8 ± 49.3	187.9 ± 40.6	112.9 ± 34.5	37.5 ± 18.4
Average percentage recovered	11.1 ± 5.5	14.0 ± 3.6	10.9 ± 3.2	3.5 ± 1.6

*N=6; mean values recorded with standard error of the mean

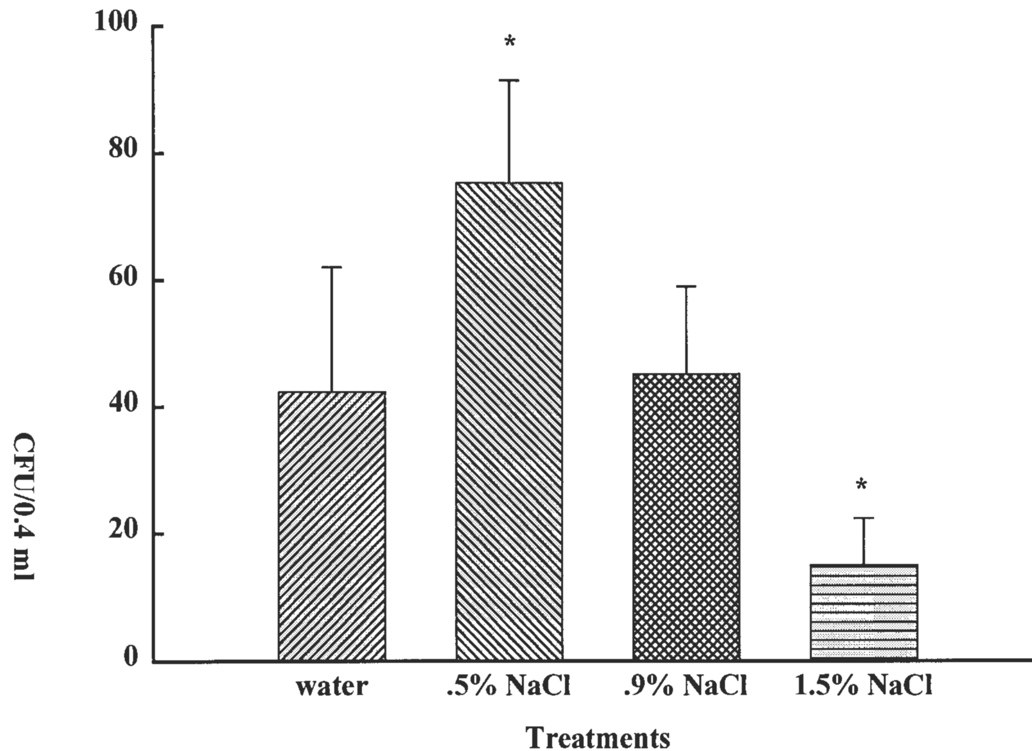


Figure 1. Average number of colonies (means) isolated on SMAC media from 0.4 ml of filtrates prepared for enrichment. Vertical lines represent standard errors of the mean (SEM). Statistical differences occur between the 0.5% NaCl and 1.5% NaCl treatments denoted by asterisks (*). There are no statistical differences between any other pair of treatments.

was not great enough to attain statistical significance in the study reported here. Assuming this trend continues with additional replicates of this experiment, it would be necessary to perform three additional blocks to detect a true difference in recovery (with significant differences being greater than or equal to 50 bacteria) among these treatments. It could then be stated that the use of solutions containing 0.5% sodium chloride increases the sensitivity of detecting O157:H7 from bovine feces by more than 50 percent over other treatments evaluated with this procedure (P=.05).

The low recoveries observed among all treatments suggest that removal of O157:H7 from bovine feces is inefficient with this procedure. However, there are advantages that may warrant use of this procedure for routine screening of bovine populations. Initial wash procedures may be more practical for use in screening large volumes of fecal material than direct plating or enrichment of fecal samples because the amount of feces that can be evaluated is not restricted to volumes of culture flasks or surface areas of plates. This has considerable importance for the isolation of O157:H7 from bovine feces because it is shed in varying amounts during infection (Pirro *et al.*, 1995). Secondly, the application and penetration of aqueous solutions into feces and the subsequent retrieval of solutions through filtration facilitate isolation of fecal bacteria. Bacteria in liquid media can be readily concentrated through techniques as simple as centrifugation or as sophisticated and specific as immunomagnetic capture (Panaccio *et al.*, 1994).

Several possibilities could explain the low recoveries observed in this experiment. Low survival of O157:H7 in sodium choride is doubtful since most strains of *E. coli* are tolerant of the levels used in this experiment (Garren *et al.*, 1998) and no statistical difference existed with the 0% NaCl wash. It is likely that most bacteria were not removed from the feces. There are two possible explanations for this. Either O157:H7 associates strongly with feces and is difficult to remove with the treatments used here, or the solutions did not permeate the fecal samples evenly and significant portions of samples were not adequately treated. It is difficult to accurately identify a causative factor or reasons for the low recoveries observed here, since several are possible.

Preparation of fecal samples in this experiment may have contributed to low recoveries of O157:H7 in filtrates. To distribute O157:H7 in samples evenly, feces were stomached for five minutes following inoculation. Stomached feces had a fine consistency that could have been more difficult to treat than if the feces had not been stomached. The feces used here compacted easily during centrifugation and became less permeable to the solutions as a consequence. This could have restricted washes to areas of lowest resistance within the sample leaving a large part of the sample

untreated. Stomaching would increase the surface area of samples, resulting in the production of smaller spaces within the matrix of the feces that could harbor significant numbers of O157:H7. This could have decreased the accessibility of solutions to these areas resulting in the lower recoveries observed. It is also possible that viable bacteria were lost during the series of centrifugations and transfers of filtrate intermediates between containers that occurred following the wash filtration procedure.

The absorbency of cultures from each of the treatments evaluated were measured during the times selected for harvest to compare relative cell density values during enrichment. These results are illustrated in Figure 2. There were no differences in absorbency values at 610 nm at any hour for the 0, 0.5, and 0.9% treatments (α= .05, LSD= .092). However, cultures from the 1.5% NaCl treatments had significantly lower absorbency values at all times (α= .05, LSD= .092). This is probably due to

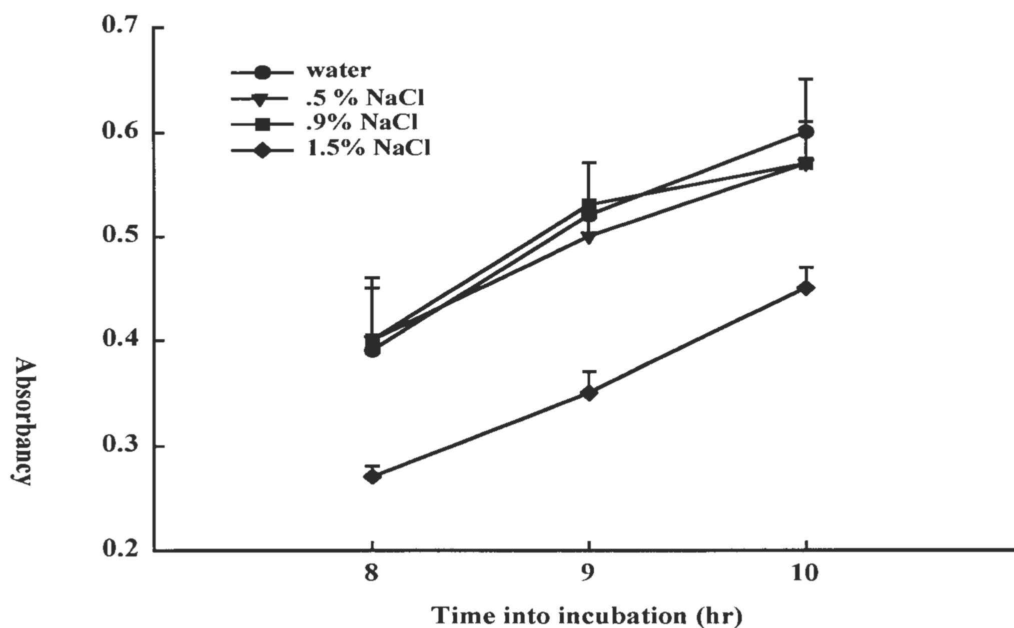


Figure 2. A comparison of mean values of absorbency at times evaluated during enrichment for each of the treatments (N=6). Vertical lines indicate the standard error of the means (SEM).

the lower recovery of O157:H7 achieved with this treatment and consequently the lower number of viable cells inoculated into the enrichment media at the start of incubation.

To compare the development of PCR signals for cultures harvested from each treatment at hours 8, 9, and 10, scores were calculated as the proportion of signals observed among the three sequences targeted. Scores for each treatment at each hour are the arithmetic mean of the six replicate scores expressed in decimal form. Comparisons of these composite PCR scores for treatments at each hour are illustrated in Figure 3. All treatments, except the 1.5% NaCl, produced signal after eight hours of enrichment, however only a small proportion of the targeted amplicons were detected as indicated in the graph for that time. There are no statistical differences in the PCR scores among treatments after eight hours of enrichment (LSD=.589, α=.05).

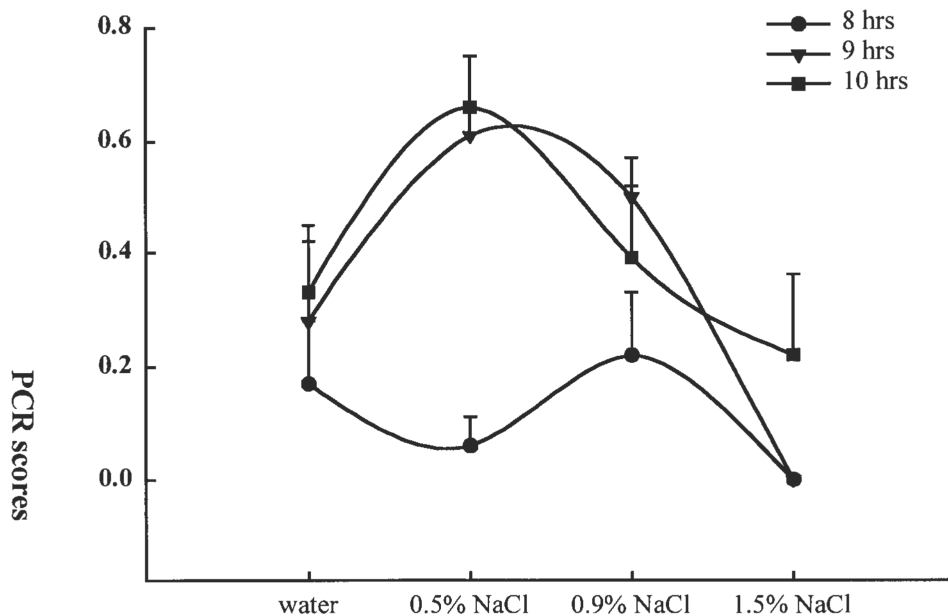
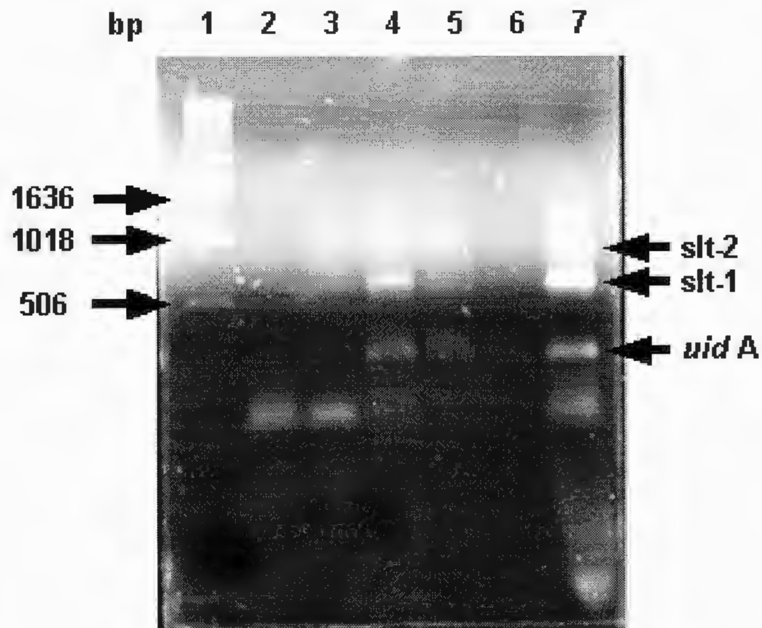


Figure 3. The development of a PCR signal during the hours of enrichment evaluated in this study. PCR scores for each treatment are the mean values calculated from the six replications. Values for standard error of the mean (SEM) are indicated with the vertical lines.

At hour nine of enrichment, there was no detectable signal with harvests of the 1.5% treatment, however, there was a substantial increase in scores from hour eight for harvests from all other treatments. This increase is greatest for the 0.5% NaCl treatment followed by that for the 0.9% treatment. It is probable that the absence of signal for cultures of the 1.5% NaCl treatment is a reflection of the comparatively lower cell densities in these cultures at this time (refer to Figure 2). Differences in the development of signal among treatments may be due to differences in treatment effects on the level of background bacteria or the amount of PCR inhibitors eluted from the feces into filtrates. There are, however, no statistical differences among the PCR scores for cultures from treatments of 0, 0.5, and 0.9% NaCl at nine hours (LSD=.589, α=.05). A statistical difference exists between scores for the 1.5 and 0.5% treatments at this time (LSD=.589, α=.05, P=.0002). At 10 hours, there are no statistical differences among any pair of treatments. PCR scores for the 0.5% treatments increase from the previous hour and 1.5% NaCl harvests increase from an average of 0 at the two previous hours to 0.22 at this time. The PCR score for the 1.5% treatment is consistent for this range of absorbency when comparing absorbency values of other treatments at previous hours and the correspondent scores. This suggests that any differences in effects due to background flora or the concentration of inhibitors with this treatment are negligible, insofar as this experiment was conducted.

PCR scoring was done immediately following electrophoresis of PCR products and does not account for differences in the intensity of bands produced among treatments. These differences could also result from differences in concentrations of inhibitors due to treatment effects. Figure 4 shows the signal produced for harvests after nine hours of enrichment. The signal is more easily observed in Lanes four and five which represent the harvests of 0.5 and 0.9% NaCl treatments, respectively. Although differences in the intensity of bands were not considered in this experiment, this is an important consideration. The scoring in this study was done by one individual and could have varied among individuals due to this subjectivity. The use of densitometric evaluation of gels could be



Lane 1: 1Kb DNA ladder (Life Technologies Inc.)
Lane 2: Negative control non-stx *E. coli* strain
Lane 3: Culture harvest from water wash
Lane 4: Culture harvest from 0.5% NaCl wash
Lane 5: Culture harvest from 0.9% NaCl wash
Lane 6: Culture harvest from 1.5% NaCl wash
Lane 7: Positive control *E. coli* ATCC strain ID 35150

Figure 4. The development of a PCR signal after nine hours of incubation for enrichment

valuable by reducing the ambiguous interpretation of PCR results inherent in the scoring system used here. Quantification of these results with densitometric evaluation would enable detection of existing differences in the concentration of amplicon products and a more valid evaluation of PCR sensitivity due to treatment effects.

CONCLUSIONS

Isolation of O157:H7 from Bovine Feces Prior to Enrichment for PCR

The purpose of this study was to determine if differences in sodium chloride concentration of aqueous solutions applied to bovine feces affects the recovery of *E. coli* O157:H7 from feces and the time required to enrich extracts in broth cultures for the detection of O157:H7 specific gene sequences using PCR. This was a two-part experiment which was designed to evaluate treatment effects on recovery of O157:H7 from feces and the time required to obtain positive diagnosis with triplex PCR. In the first part, O157:H7 recovery from bovine feces in a filtration procedure was determined by plating equal volumes of filtrates on differential media. Upon comparison of four concentration levels evaluated, significant differences ($P=.05$) in recovery were found to exist between two treatments. Washing feces with 0.5% sodium chloride solution resulted in significantly higher recovery of O157:H7 from bovine feces than washing with 1.5 percent solution. The time required to enrich extracts from each treatment prior to performing PCR were evaluated at three time points. Significant differences ($P=.05$) in the production of positive PCR results existed between extracts from the 0.5 percent and 1.5 percent treatments after nine hours of incubation. At hours eight and 10 there were no differences among any treatments.

Future Studies

The identification of factors which facilitate the removal of target pathogens from the feces of known reservoir species may benefit food safety and public health by providing the advantages of increased sensitivity of detection and efficiency of screening large populations of the reservoir species. PCR is a specific and potentially sensitive procedure that can detect pathogens in short lengths of time. This potential may depend upon the selection of treatments and procedures used to prepare or treat samples prior to subjecting them to PCR analyses.

If a sodium chloride concentration which most selectively recovers and isolates O157:H7 from clinical samples could be found, it would increase the sensitivity of detection with isolation and potentially decrease the time required to enrich samples prior to PCR. Sodium chloride concentration is one factor among possibly several which could affect the ability to detect O157:H7 in environmental samples using PCR. This study evaluated only four levels of sodium chloride concentration among an infinitesimal number of concentration levels. Other factors or combinations of factors may exist which are closer to meeting the objectives of short assay time and high sensitivity of detection with PCR. These may include variations in pH, temperature, or possibly the concentrations of other salts in wash solutions. The identification of biological competitive advantages of O157:H7 over other bacteria in nature and the ecological niche(s) of O157:H7 may facilitate the determination of possible factors. Factors which may have enhanced the persistence of O157:H7 in the environment, such as tolerance of low pH, may also be useful in selective isolation of this pathogen from samples prior to PCR.

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