POTENTIAL FOR PATHOGEN TRANSFER FROM HOG MANURE FERTILIZER TO GRAZED CATTLE AND GROUNDWATER

By

Joël Walkty

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Hog manure can contain pathogenic bacteria such as *Salmonella*, *Escherichia coli* and *Yersinia enterocolitica* that may increase the risk of human illness if manure is used as fertilizer. The objective of this work was to investigate whether it could be demonstrated that *Salmonella*, *E. coli* and *Y. enterocolitica* naturally present in hog manure, can be transferred to cattle grazing on fields previously fertilized with manure. Trials were conducted over two summer seasons. Hog manure was applied via splash plate at a calculated rate of 61.8 or 123.6 kg of available N/ha to each of 4 plots totalling 16.2 ha. An equal area, divided into two plots, served as a control without manure. Fecal samples were taken directly from 60-80 beef cattle each year, and cattle were divided into groups and introduced on pasture plots. Animals were removed or additional *Salmonella*, and *Y. enterocolitica* negative cattle were added to the fields in response to vegetation growth. There were 40-109 cattle on manured fields and 16-20 on control fields. The interval between manure application and cattle introduction ranged from 10-30 d. Fecal samples were taken monthly for 3 months. Soil and plant samples were taken before and 1-24 d after manure application. All hog manure, soil, forage and cattle fecal samples were negative for *Y. enterocolitica* in both years of the trial. Two thirds (2004) and 100% of hog manure samples (2005) were *Salmonella* positive each year. Twenty-nine *Salmonella* Krefeld and 17 *Salmonella* Derby cultures were isolated from the hog manure. Six *Salmonella* Typhimurium cultures were isolated from the vegetation up to 14 d after manure application. All isolates of each serovar were the identical serotype. *Salmonella* was not recovered from soil and was absent from the cattle fecal samples for the entire length of the study. Hog manure had *E. coli* levels of

4-5 log CFU/g and the organism was detected on vegetation 1-2 d after manure application. *E. coli* was not recovered from vegetation before cattle were grazed. Transfer of *E. coli* from hog manure to soil and cattle was examined by genetic fingerprinting (RAPD) of isolates. DNA fingerprints were compared one to the other using ClustanGraphics 7 software. No *E. coli* DNA fingerprints from hog manure were in common with genomic fingerprints of *E. coli* isolated from soil. One *E. coli* DNA fingerprint from hog manure was identical to one *E. coli* fingerprint from cattle and this was not considered to be a statistically significant observation (Fisher's Exact test, $p < 0.001$). During this study, *Salmonella* and *E. coli* present in hog manure did not appear to have been transferred to cattle grazing on the manure-treated pasture. *E. coli* was recovered in water from one background well and two wells on the experimental site before manure application in 2005 and from one well during the July (2005) sampling period. *Salmonella* serovars (Worthington and Manhattan) different from those in the hog manure were recovered from water in four wells 20 d after manure application in the fall of 2005.

Chapter 1

INTRODUCTION

Livestock production significantly contributes to the economy of Canada and the province of Manitoba. In 2004, Manitoba exported pork products worth a total of 426 million dollars to 25 different countries (Manitoba Pork Council, 2004). There were an estimated 2.9 million pigs in Manitoba at the end of the first quarter of 2006 (Statistics Canada, 2006). A consequence of raising large quantities of livestock is the production of enormous quantities of manure. In 2001, an estimated 177.5 billion kg of livestock manure was produced in Canada (Hofmann and Beaulieu, 2006). Manure production in 2001 by animal type was as follows: 86.2 % by cattle, 8.3 % by hogs, 2.7 % by poultry, 2.2 % by horses and less than 1% by sheep and goats (Hofmann and Beaulieu, 2006). Thus, it can be estimated that there was over 14.7 billion kg manure produced by hogs in 2001. Hog manure is usually stored in earthen lagoons or above-ground storage tanks. These storage facilities have limited capacities (maximum 380 d) and eventually the manure must be removed. Most livestock producers choose to spread manure on to agricultural fields as a fertilizer, replenishing the nutrients in the soil (Van Horn *et al.*, 1994). In Manitoba, approximately 237,000 ha of agricultural land receive a livestock manure treatment (Statistics Canada, 2001). Despite its rich nutrient content, livestock manure can contain bacterial pathogens such as *Escherichia coli*, *Salmonella* and *Yersinia enterocolitica*. These organisms are able to survive in diverse environmental conditions and can be transferred to humans and animals through contaminated food and water. Severe outbreaks of illness linked to the consumption of contaminated water have been reported involving thousands of people, some of whom died (Public Health Agency

of Canada, 2000). The potential for contaminating food crops is also a possibility with the application of manure to cropland. In 1992, an outbreak of illness of *E. coli* O157:H7 resulted from the consumption of contaminated vegetables from a manured garden (Cieslak *et al.*, 1993). More recently, during the fall of 2006, the consumption of fresh bagged spinach contaminated with *E. coli* O157:H7 from cattle feces caused illness in approximately 200 people across the U.S. (U.S. Food and Drug Administration, 2006).

Organisms from the environment may also be transferred to animals raised for food. These infected animals can infect other animals and the environment in a continuous cycle (Jensen *et al.* 2006, McGee *et al.*, 2004, Fedorka-Cray *et al.*, 1994). Once the animal is processed for human consumption, meat may become contaminated from infected animals and this increases the chance for foodborne disease.

A major objective of this research project was to determine whether cattle grazing on pastureland treated with hog manure could be infected with pathogens originating in the applied hog manure. Another objective was to follow the persistence of pathogens in the environment and their movement to the water table.

Chapter 2

LITERATURE REVIEW

2.1 *Escherichia coli*

E. coli is a Gram negative, facultative anaerobe that is naturally found in the intestines of warm-blooded animals including humans (Prescott *et al.*, 1995). The strains that normally inhabit the human intestinal tract are harmless but some strains are potentially harmful. One of the most well-known and important pathogenic *E. coli* strains is *E. coli* O157:H7. This organism is most commonly found in cattle but can also be found to a lesser extent in pigs, chickens, goats, sheep and deer, and can be shed in animal feces (Rasmussen *et al.*, 1993, Meng *et al.*, 2001).

2.1.1 Prevalence of shiga toxin-producing *E. coli* (STEC) O157 in pigs

Shiga toxin-producing *E. coli* O157 have been isolated from pigs in many parts of the world including Canada. However, the prevalence of STEC O157 in pigs around the world is relatively low with a few exceptions. Gyles *et al.* (2002) investigated the prevalence of STEC O157 in 44 pig herds in Ontario, Canada. Results from their study indicated that 9 % of herds and 3 % of pigs (35 % of pigs in positive herds) were positive for STEC O157. Fecal carriage rates of STEC O157 in Europe and the United States are estimated to be approximately 0.2 to 2 % (Caprioli, *et al.*, 2005). The prevalence in England is estimated to be 0.4 % (Synge and Paiba, 2000), 1.4 % in Japan (Nakazawa *et al.*, 1999) and 1.4 % in the Netherlands (Heuvelink *et al.*, 1999). Significantly higher prevalences were found in Germany (7.5 %) (Beutin *et al.*, 1993) and in Chile (8-10 %) (Caprioli *et al.*, 2005).

2.1.2 Clinical symptoms

The adverse effects of illness from STEC O157 are caused by the production of either one or two shiga toxins (Rusin *et al.*, 2000). Symptoms of infection include cramps, diarrhoea and, as the illness progresses, blood may appear in the feces. Most healthy adults recover from infection in about a week. However, for some people, especially young children, the infection can progress to Hemolytic-Uremic Syndrome (HUS) where there is a possibility of severe kidney damage. Further complications of HUS include fever and severe neurological symptoms, and this syndrome is called Thrombic Thrombocytopenic Purpura (TTP) (Rusin *et al.*, 2000).

E. coli O157:H7 is one of the most common STEC serotypes to cause human illness. However, over 100 different serotypes of *E. coli* produce shiga toxins (Bach *et al.*, 2002). Other shiga toxin producing, non- O157:H7 *E. coli* have been associated with the same severe illnesses caused by *E. coil* O157:H7 (Blanco *et al.*, 2004). Non-O157:H7 shiga toxin producing *E. coil* have been isolated from pigs and could also pose a significant health risk to humans (Kaufmann *et al.*, 2006). Other *E. coli* strains can cause milder types of illness and are also classified in specific categories. These categories include: Enterotoxigenic *E. coli* (ETEC) which are involved in traveller's diarrhoea via toxin production, Enteropathogenic *E. coli* (EPEC) which are involved in infantile diarrhoea, and Enteroinvasive *E. coli* (EIEC) which cause non-bloody diarrhoea by colonizing the intestinal wall (Meng *et al.*, 2001).

2.1.3 Outbreaks

In 2004, the Canadian average for *E. coli* O157:H7 infections was 3.36 cases / 100,000. The Manitoba average for this same period was 4.87 / 100,000 (Public Health Agency of Canada, 2004). Outbreaks of *E. coli* O157:H7 in Canada are usually due to contaminated beef products, unsafe drinking water and even by direct contact with infected animals.

Undercooked hamburger containing *E. coli* O157:H7 has been the cause of several outbreaks in Canada. In 2004, an outbreak of *E. coli* at a hockey camp in Sudbury, ON, resulted from the consumption of undercooked hamburger meat containing *E. coli* O157:H7 (Sutcliffe *et al.*, 2004). Contaminated drinking water has also been the vector for *E. coli* O157:H7 infections. In 2000, the town of Walkerton, ON, was victimized by this organism which was found in the town's drinking water. Approximately 2000 people became ill and 7 deaths occurred as a result of consuming the contaminated water. The source of *E. coli* O157:H7 was suspected to have originated from a nearby farm which served as a point source of contamination for the municipal water supply of the town of Walkerton (Public Health Agency of Canada, 2000). In 2003, an outbreak of *E. coli* O157:H7 occurred when children attended a petting zoo and became ill (David *et al.*, 2004). Investigations revealed that a goat at the petting zoo was positive for *E. coli* O157:H7. The pulsed field gel electrophoresis (PFGE) pattern was an exact match for the *E. coli* O157:H7 isolated from the feces of infected children and adults, indicating that it was probable that the children became ill through direct contact with the infected animal.

2.2 *Salmonella*

Salmonella is a Gram-negative, facultative anaerobic rod. This organism can be found in many cold and warm-blooded animals (Rusin *et al.*, 2000). There are over 2000 serovars of *Salmonella* and all of them are considered be pathogenic to humans to various degrees (Rusin *et al.*, 2000).

2.2.1 Prevalence of *Salmonella* in pigs

According to Olson (2004), prevalence of *Salmonella* in pigs ranges from 0-38%. Studies in Canada indicated that 5.2-14.3 % of pigs and 66.7-70.7 % of farms were *Salmonella*-positive (Rajic *et al.*, 2005, Letellier *et al.*, 1999a, Letellier *et al.*, 1999b). A survey in the U.S.A. reported the presence of *Salmonella* in 24.6 % of pigs and 83 % of farms (Davies *et al.*, 1997). In Denmark, 6.2 % of pigs and 22.2 % of farms were *Salmonella*-positive (Baggensen *et al.*, 1996) and 23 % of farms were *Salmonella*-positive in the Netherlands (van der Wolfe, 1999).

2.2.2 Salmonellosis

Typhoid fever and gastroenteritis are two different types of illness caused by salmonellae. Typhoid fever is caused by *Salmonella* Typhi and results from the consumption of water and food contaminated with feces. This disease is more prevalent in developing countries where there is a lack of adequate hygiene. The infective dose is thought to range between 1000 and 1 million cells, with an incubation period of 10 to 14 d (Bhan *et al.*, 2005). The most common symptoms of typhoid fever include mild fever evolving to a severe form after the first week of illness, plus headache, myalgia, anorexia and nausea (Bhan *et al.*, 2005). Illness can last for several weeks if left

untreated (Bahn *et al.*, 2005). A milder form of the disease called paratyphoid fever also exists and is caused by *Salmonella* Paratyphi.

Gastroenteritis is the most common form of illness caused by salmonellae in developed countries. This disease is caused mainly by serovars of *Salmonella enterica* (eg. Typhimurium). Clinical symptoms which usually manifest themselves between 12 and 36 h, last for 2-5 d and are characterized by abdominal cramps, diarrhoea and fever. Healthy adults usually recover from this illness without any complications. The severity of salmonellosis depends on the number and type of *Salmonella* ingested (Rusin *et al.*, 2000).

2.2.3 Outbreaks

Salmonellosis outbreaks in Canada are usually caused consumption of contaminated, undercooked foods. In 2004, the prevalence of Salmonellosis in Canada was 16.02 cases / 100,000 people while in Manitoba prevalence was 12.13 cases / 100,000 people (Public Health Agency of Canada, 2004).

In 2004, three outbreaks of *Salmonella* resulted from the consumption of contaminated Roma tomatoes in 18 U.S. states and one Canadian province causing 561 illnesses (Public Health Agency of Canada, 2005). In 2000, an outbreak linked to the consumption of baked goods made with egg products containing *Salmonella* made 11 people sick in British Colombia. Also in 2000, an outbreak involving the consumption of mung bean sprouts caused people eating at Vietnamese restaurants in Alberta and

Saskatchewan to get sick. The investigation discovered that the restaurants in Alberta and Saskatchewan had the same bean sprout supplier.

2.3 *Yersinia enterocolitica*

Yersinia enterocolitica is a Gram negative, facultative anaerobic rod. A characteristic that separates this organism from the two other organisms previously described is that it grows at refrigeration temperatures. This organism is found in the oral cavity and gastrointestinal tract of pigs (Schiemann *et al.*, 1981, Letellier *et al.*, 1999b) and has been isolated from other farm animals (cattle, sheep, goats and chickens) other mammals (rodents, foxes, hares, porcupines), pet animals (dogs and cats) and birds (Bottone, 1999). *Y. enterocolitica* has over 60 serogroups but only 11 of them are usually associated with disease in humans (Bottone, 1999). Pigs are considered an important reservoir for pathogenic *Y. enterocolitica* and the most common pathogenic serotypes isolated from pigs are 0:3, 0:8, 0:9 and 0:5,27 (Bottone, 1999).

2.3.1 Prevalence of *Yersinia enterocolitica* in pigs

The prevalence of *Y. enterocolitica* in pigs is variable. A study by Letellier *et al.* (1999b) found that the average prevalence of *Y. enterocolitica* in the cecal contents of finishing pigs at 6 slaughterhouses in three Canadian provinces (Québec, Ontario and Manitoba) was 20.9 ± 2.1 %. The most common serotypes isolated were 0:3 (85.5 %) followed by 0:5 (9.1 %). All isolates were screened for the presence of a virulence plasmid and 76.7 % of the isolates were found to be positive. Similar results were

observed by Bhaduri *et al.* (2005), who estimated that the prevalence of pathogenic *Y. enterocolitica* was approximately 13 % in finishing pigs on 77 farms across 15 eastern and mid-western U.S. states. They also found that approximately 53 % of farms had at least one animal positive for *Y. enterocolitica.*

Since *Y. enterocolitica* also colonizes the oral cavity of pigs, tonsils were examined to assess its prevalence. Hog tonsils were more frequently positive for *Y. enterocolitica* than fecal and gastrointestinal tract samples. The prevalence of *Y. enterocolitica* on the tonsils of pigs from 9 slaughterhouses in Finland was 37 % (Fredriksson-Ahomaa *et al.*, 2000). A similar result was reported in samples from an abattoir in Prince Edward Island where 42 % of hog tonsil samples were positive for *Y. enterocolitica* (Hariharan *et al.*, 1995). Sixty-seven percent of isolates were identified as serotype 0:3 and 20 % were serotype 0:5. Virulence characters were present in 95 % of serotype 0:3 isolates.

2.3.2 Yersiniosis

Pathogenesis by this organism is determined by chromosomal and plasmid virulence factors which include attachment and invasive proteins expressed by the *ail* and *inv* genes on the chromosome and the *yad* gene on a plasmid (Bottone, 1999). The infective dose for *Y. enterocolitica* is estimated to be approximately 6 log cells (Public Health Agency of Canada, 2006). The most common symptoms of yersiniosis include fever, inflammation of the intestinal tract, bloody, watery diarrhoea and abdominal pain (Public Health Agency of Canada, 2006). Symptoms can persist for 3 to 28 d in children

and for 1-2 weeks in adults (Bottone, 1997). This organism caused 11 reported cases of yersiniosis in Manitoba in 2004 and 15 cases in 2005 (Manitoba Health, 2006).

2.3.3 Outbreaks

Outbreaks of yersiniosis occur from time to time but are not as common as salmonellosis. Pork products such as chitterlings (intestines) have been the culprits of outbreaks on a few occasions (Jones, 2003, Lee *et al.*, 1990). Contaminated drinking water, tofu, and milk have also been involved in yersiniosis outbreaks (Walker and Grimes, 1985, Tacket *et al.*, 1985, Black *et al.*, 1978).

2.4 Pathogen survival in the environment

Human pathogens can be introduced into the environment by spreading manure on agricultural land and may pose a risk to human health should food or water be contaminated or by direct contact with contaminated animals (Pell, 1997). Applying manure on agricultural land is a common practice in North America, therefore the investigation of pathogen survival in different environments is crucial to estimating the risk this agricultural practice poses to human health.

2.4.1 Survival of pathogens in manure

Liquid hog manure is usually stored in earthen lagoons or above ground storage tanks until it can be disposed of. Survival of pathogens in manure environments is the first step to further distribution of pathogens throughout the environment.

Manure storage temperature is a major factor influencing the survival of pathogens in manure environments. Wang *et al.* (1996) followed the survival of *E. coli* O157:H7 in bovine feces at different temperatures. At 5 °C, *E. coli* O157:H7 inoculated in feces at 3 and 5 log survived 63 and 70 d, respectively. The survival time at 5 °C was longer than the survival at 22 °C (49 and 56 d) and 37 °C (42 and 49 d). These results agree with results from a study by Himathongkhan *et al.* (1999) in which the survival of *E. coli* O157:H7 was investigated in cattle manure and cattle manure slurry at different temperatures. Survival in cattle manure slurry was longer at 4 °C, followed by 20 °C and then 37 °C. Kudva *et al.* (1998) also reported that an increase in cattle manure temperature resulted in a decrease in the length of time *E. coli* survived.

The effect of temperature on the survival of *Salmonella* has also been documented. Himathongkhan *et al.* (1999) observed that *Salmonella* Typhimurium survived in cattle manure and cattle manure slurry for a longer period of time at 4 °C and numbers declined with increasing temperature where, at 37 °C, (maximum temperature of the experiment) *Salmonella* Typhimurium survival was the least. Jones (1976) also noticed an inverse relationship between cattle slurry temperature and survival times of *Salmonella* using a culture of *Salmonella* Dublin. Greatest survival was at temperatures ≤10 °C followed by 20 °C and then by 30 °C. No difference was observed between the survival times of *Salmonella* Dublin in manure at 5 °C and 10 °C.

Y. enterocolitica has not been as well studied as *Salmonella* or *E. coli*. However, the effect of temperature on the survival of *Y. enterocolitica* was similar to that for *E. coli* and *Salmonella*. Kearney *et al.* (1993) examined the survival of *Y. enterocolitica* in stored beef cattle slurry and observed that the organism experienced a log reduction in 20.8 d at 4 °C and 12.8 d at 17 °C.

Many of the experiments investigating the survival of pathogens in manure have only been done at a laboratory scale. While this can give an indication of the survival of pathogens as influenced by specific parameters such as temperature, moisture and pH, laboratory tests do not always accurately represent the overall survival capabilities of these pathogens exposed to changing environmental conditions. The temperature of stored manure changes in response to environmental conditions. A study by Arrus *et al.* (2006) examined the temperature fluctuations in an earthen manure storage lagoon and an above-ground storage tank at different depths over the course of the winter, spring,

summer and fall seasons in southern Manitoba. During the 4 seasons, temperatures in the above-ground storage tank ranged from –10.4 to 22.7 °C and from –8.2 to 26.5 °C in the earthen manure storage lagoon. To study the effect of different seasons on the survival of *Salmonella*, Plachà *et al.* (2001) exposed inoculated solid fractions of pig slurry to the environment during winter/spring and summer seasons. Results indicated that in winter/spring, *Salmonella* Typhimurium survived for 85 d with a decimal reduction time (T_{90}) of 12.9 d but only 26 d (T_{90}=2.3 d) in summer. Interestingly, these finding were consistent with laboratory acquired experimental data previously presented, indicating that *Salmonella* seemed to survive better at cooler temperatures.

2.4.1.1 Other factors influencing pathogen survival in manure

Along with higher temperatures, aeration can also help reduce the number pathogens like *Salmonella* in manure (Heinonen-Tanski, 1998). In a laboratory scale model, the aeration of manure slurries caused more pronounced decrease in the number of *Salmonella* compared to non-aerated manure when assessed using the Most Probable Number (MPN) method. The aeration of manure slurries caused *Salmonella* numbers to decrease from 10.1 MPN to undetectable (0.03 MPN) by day 14. In non-aerated manure slurry, Salmonella decreased in numbers from 10.1 MPN to 1 MPN over a period of 18 d (Heinonen-Tanski, 1998).

Manure contains a wide range of non-pathogenic organisms competing for the same nutrients as the pathogens. The effect of competitive organisms on the survival of *Salmonella* Dublin was reported by Jones *et al.* (1977). In this report, the survival of

Salmonella Dublin in sterilized cattle slurry was between 290 and 370 d, while survival in natural slurry ranged from 74 to 150 d.

2.4.2 Survival of pathogens in soil and on vegetation

Pathogens may be transferred to soil and vegetation when manure is applied. Survival of pathogens in soil and on vegetation will influence their ability to spread throughout the environment. *E. coli* O157:H7, salmonellae and *Y. enterocolitica* have been shown to survive very well in various types of soils and under a variety of conditions.

Hutchison *et al.* (2005) found that *E. coli* O157:H7 had a decimal reduction time ranging from 1.3 to 1.7 d when inoculated manure (6 log CFU/g) from various sources was spread on a grass pasture during the summer season. However, the organism could still be detected in the soil 16 to 63 d later. In another study, Franz *et al.* (2005) reported the survival time for *E. coli* O157:H7 in 4 different types of soil ranged from 2 to 56 days. A third study by Nicholson *et al.* (2005) also reported similar findings, where *E. coli* O157:H7 survived 32 d in soil with slurry and farmyard manure from sheep, dairy cattle, beef cattle or pigs spread on the soil's surface. Jiang *et al.* (2002) suggested that *E. coli* O157:H7 can survive from 56 d at 5 ^{o}C to 193 d at 21 ^{o}C. These survival times are much longer than in the studies previously mentioned. This increase in length of survival might have been due to the fact that Jiang *et al.* (2002) used enrichment procedures on samples in which *E. coli* O157:H7 were not initially detected by direct

plate count. This might indicate that *E. coli* O157:H7 is able to survive in an injured state in soil at very low numbers for a significant amount of time.

Salmonella is also able to survive in soils for an extended period of time. Studies have reported that survival of *Salmonella* in soil ranged from 180 to 299 d under controlled conditions (Baloda *et al.*, 2001, Holley *et al.*, 2006). Other studies indicate that *Salmonella* can survive from 7 d to 2 months in various types of soil. *Salmonella* has been reported to persist for 35 d in the soil of paddocks after the removal of *Salmonella*-positive pigs (Jensen *et al.*, 2005) and 54 d in soil-amended with liquid hog manure (Côté and Quessy, 2005). Franz *et al.* (2005) found that *Salmonella* Typhimurium survived from 28 to 56 d in 4 different types of soil, and Marsh *et al.* (1998) reported that *Salmonella* Typhimurium survived > 54 d in inoculated soil. In contrast, a few studies indicated that *Salmonella* did not survive very long in soil. *Salmonella* was not detected after 7 d in soil amended with manure inoculated with *Salmonella* (Gessel *et al.*, 2004). In this study by Gessel *et al.* (2004), *Salmonella* numbers in the hog manure applied to the field (at 6 to 14 °C) were low (3.36 log CFU/ml) and could have accounted for the shorter survival time observed. In another study, *Salmonella*, naturally present in the hog manure slurry was not detectable after 14 d when the slurry was spread over fields (Baloda *et al.*, 2001).

Y. enterocolitica at a concentration of 6 log CFU/g was undetectable after 7 d at 30 °C (Chao *et al.*, 1988). However, in a separate study at 4 °C, 5 (different) *Y. enterocolitica* strains were shown to survive from 11 to over 50 weeks and this was reduced to 3-18 weeks at 20 °C (Tashiro *et al.*, 1990) suggesting that *Y. enterocolitica*

can survive quite a long time at cool temperatures. This is not surprising since *Y. enterocolitica* is capable of growth at refrigeration temperatures.

Temperature is a major factor influencing the survival of organisms in the soil. In general, the cooler the temperature, the better the survival of the bacterial pathogens as seen from the data by Tashiro *et al.* (1990) previously mentioned. This trend was confirmed by Cools *et al.* (2001) who found *E. coli* to survive better at 5 °C than 25 °C in three different types of soil. Under actual environmental conditions, *E. coli* had a decimal reduction time of 3.3 d in summer and 13.4 d in autumn (Van Donsel *et al.*, 1967). In Manitoba, winter temperatures often reach – 20 °C, freezing the soil until the spring melt which can affect the survival of pathogens. A study by Holley *et al.* (2006) noticed a more rapid decline of *Salmonella* numbers over the first 30 days of their experiment when soil was incubated at – 18 °C than in soil incubated at 4 °C or 25 °C. The addition of manure to the surface or when mixed in the soil seemed to help slow the decline of *Salmonella* numbers compared to soil that did not receive a manure treatment. Organic matter and nutrients in the manure might help protect organisms from the effects of freezing.

Soil moisture is also a significant factor influencing the survival of pathogens in soil. Cools *et al.* (2005) evaluated the survival of *E. coli* in soil at moisture levels of 60, 80 and 100 % of field capacity (FC) at three temperatures (5, 15 and 25 °C). They found that over 80 d, there was better survival at a moisture of 100 % FC at 5 and 25 °C. At 15 °C, there was no difference in survival at all three moisture levels. There was also no difference in survival between 60 and 80 % FC for all 3 temperatures. In a similar study

by Holley *et al.* (2006), *Salmonella* survived longer in soils with a moisture level of 80 % FC compared to soils with moisture at 60 % FC.

Survival of pathogens on vegetation can become a source for animal infection during grazing on manured pastures or contribute to high pathogen numbers in runoff water. Under natural environmental conditions (15.6 °C, average daytime temperature), Taylor and Burrows (1971) found that *Salmonella* survived at least 11 d on vegetation at heights of 2.5 to 22.5 cm when added at 7 log/ml in manure, and *E. coli* survived for 11 d when initially present at 6 log/ml. Under simulated environmental conditions (16 h of light at 18 °C followed by 8 h of darkness at 15 °C and at a relative humidity of 80 %), *Salmonella* Typhimurium and *E. coli* (2.6 log CFU/g) were present for much longer periods compared to natural environmental conditions, both surviving for 56 d on grass (Johansson *et al.*, 2005).

2.5 Spread of pathogens in the environment

As described previously, pathogenic organisms present in manure are able to survive in the environment. Just as important as the ability of pathogens to survive in soil and manure is their ability to be transported throughout the environment. It would be ideal if pathogens introduced on fields as a result of manure application would stay confined to the area where the manure was spread. Unfortunately, through spring snow melting and periods of rain, organisms can be transported and contaminate surface and ground waters.

Soil type and composition are major factors influencing the speed with which pathogens can migrate through soil. The longer it takes a pathogen to move through soil, the less likely it is to survive and contaminate ground water (Mawdsley *et al.*, 1995). Soil acts like a sieve, filtering and retaining particles and organisms larger than the soil pore sizes. In general, the larger the pores are in the soil, the faster organisms can move through the soil (Mawdsley *et al.*, 1995). Moisture is also an important soil parameter because it provides a vehicle for bacteria to travel through the soil pores (Mawdsley *et al.*, 1995).

Another factor influencing the transport of microorganisms through soil is the tendency of organisms to attach to soil particles. If an organism is able to adsorb to soil particles, its movement through soil will be restricted. It is thought that adsorption is influenced by organic and inorganic material in soil as well as by the surface properties of the microorganism. Manure spread on fields is used as fertilizer for plants which changes the organic and inorganic composition of soil. This change in composition could have an effect on the adsorption of bacteria to soil. A study by Gruber *et al.* (2005) investigated the effect of bovine manure on the ability of *E. coli* to attach to soil particles. In three types of soil, the presence of bovine manure significantly reduced the attachment of *E. coli* to the soils. The mechanism of bovine manure action on the reduction of *E. coli* attachment to soils is uncertain. Competition between manure and bacteria for soil adsorption sites, bacterial adsorption on manure particles and modification of soil mineral surfaces are some explanations offered (Gruber *et al.*, 2005).

When soils are saturated with water, runoff from fields during spring snow melt or heavy rainfall can find its way into surface water. It has been shown that bacteria on the surface of the soil can be transported a significant distance after a heavy rainfall (Abu-Ashour and Lee, 1999). Oliver *et al.* (2005) demonstrated that at higher water flow rates on and through the soil, *E. coli* was more frequently transported to a water collection site. Another experiment by Dunigan and Dick (1980) showed that fecal coliforms in runoff from a sewage sludge-treated field remained high for the first 11-17 d after sludge application. Fecal coliforms were also detected in runoff from rainfall simulation experiments where bovine manure was applied to two types of both vegetation covered and bare soils. Higher numbers of fecal coliforms were detected in runoff from soils that did not have any vegetation compared to both types of soil that had vegetation (Roodsari *et al.*, 2005). When water flows over bare soil, large channels can be formed and it is easier for bacteria to be transported across the surface of soil (Roodsari *et al.* 2005). Vegetation slowed the flow of water across the surface of soil and limited transport of bacteria in the runoff (Young *et al.*, 1980, Roodsari *et al.*, 2005). However, as the flow of water was slowed by the presence of vegetation, more bacteria were able to penetrate into the soil.

2.6 Survival of pathogens in water

Surface and ground water sources provide drinking water for the human population. If the water being consumed is not treated, there is a risk that pathogens present in the water can cause illness in humans. The ability of some pathogenic organisms to survive for extended periods of time increases the risk that they may pose a threat to humans. In stream water McFeters and Silva (1991) observed a decrease in

numbers of *Y. enterocolitica* of approximately 3-4 log units in 21 d and a 4 log reduction in 21 d of *E. coli* numbers. Karapinar and Gönül (1991) showed that *E. coli* could survive 7 and 13 weeks in sterile spring water (4 °C) at an initial concentration of 2.9 and 4.9 log, respectively. *Y. enterocolitica* survived at least 64 weeks in sterile 4 °C spring water at an initial concentration of 5 log CFU/ml.

Survival of *Y. enterocolitica*, *E. coli* and *Salmonella* in sterile ground water at 10 °C was evaluated in a study by Filip *et al.* (1988). All three organisms survived at least 100 d and *Y. enterocolitica* survived the longest with < 1 log reduction. During this time *E. coli* was reduced by 3 log units and *Salmonella* Typhimurium showed a 4 log reduction. In non-sterile ground water, *Salmonella* Typhimurium and *E. coli* decay rates were about the same over 15 d with a reduction of about 2 logs (Bitton *et al.*, 1983).

Experiments investigating survival of bacteria in water have used water samples from different sources and, as a consequence, the physico-chemical properties and microbiological quality of the waters were different. This makes it difficult to generalize about the survival and decimal reduction times of various bacteria in water. It is valuable to look at the various water properties and environmental conditions that affect the survival of microorganisms in water.

Temperature is the most important factor affecting the survival of organisms in water. The survival of pathogens in water is affected by temperature in the same way it is affected in manure and soil. In general, the lower the water temperature, the longer the survival of microorganisms tends to be. Flint (1987) observed that *E. coli* K12 survived 2 d at 37°C, 4 d at 25°C, 8 d at 15°C and 15 d at 4°C in untreated river water. McFeters

and Stuart (1972) observed that the "half-life" of *E. coli* decreased as the temperature increased in creek water. Experiments by Wang and Doyle (1998) also showed similar trends with the best survival at 8 °C followed by 15 °C and then 25 °C.

Competitive microorganisms in water may play a role in reducing the length of survival of many pathogens. Numerous studies have compared the survival of *E. coli* or *Salmonella* in autoclaved water to their survival in untreated water. Studies by Wang and Doyle (1998), Scheuerman *et al.* (1988), Flint (1987) and Domingo *et al.* (2000) all indicate longer *E. coli* survival times in autoclaved water, suggesting that removing competitive organisms may allow longer survival of certain pathogens. Organisms such as protozoa can act as predators, reducing the numbers of bacteria in the water. In a relevant study Scheuerman *et al.* (1988) added eucaryotic cell inhibitors to water to inactivate protozoa and as a consequence, reduced their consumption of *E. coli*. In inhibitor-treated water, *E. coli* numbers were approximately 1.5 logs higher after 5 d than in non-treated water.

The role of nutrients on the survival of bacteria in water has also been investigated. When phosphate and/or amino acids were added to sterile lake water, *E. coli* increased 3 to 4 logs within 48 h and sustained this maximum concentration for at least 72 h. When *E. coli* was introduced into water without phosphate and/or amino acids there was no significant growth (Henis *et al.*, 1989). Similar results were obtained by Gordon and Toze (2002). In untreated water, *E. coli* numbers increased 1 log over 9 days when 0.1% peptone was added, and this was followed by a decrease in numbers until *E. coli* were no longer detected at 45 d. The addition of glucose alone not only

caused an increase in *E. coli* numbers, but also increased survival to 28 d compared to 5 d in glucose-free water.

2.7 Pathogen transfer from the environment to animals

After manure is spread on pastureland, livestock are often grazed on the treated fields. Grazing animals are at risk to acquire pathogens from the manure-treated vegetation or from direct contact with feces of other infected animals. Once infected, livestock can continue shedding these organisms and further contribute to the spread of pathogens throughout the environment.

Taylor and Burrows (1971) showed that cattle became infected with *Salmonella* Dublin and shed this organism as early as 1 d after grazing on a pasture treated with manure containing 6 log CFU/ml *Salmonella* Dublin. However, when manure containing 3 log CFU/ml *Salmonella* Dublin was applied to grazed fields, *Salmonella* Dublin was not detected in any of the cattle for the entire length of the trial (13 days). Similarly, Jack and Hepper (1969) reported that cattle became infected with *Salmonella* after they were exposed to a pasture that had received *Salmonella*-containing hog manure.

Hogs can also acquire pathogens from the environment. Jensen *et al.* (2006) observed that *Salmonella*-negative pigs became infected with *Salmonella* after introducing them into paddocks contaminated with feces from *Salmonella*-positive pigs. In another experiment, pigs became infected with *Salmonella* after they were exposed to *Salmonella*-positive feces (Fedorka-Cray, *et al.*, 1994). Fedorka-Cray *et al.* (1994) also

exposed a group of *Salmonella*–negative pigs to a group of pigs shedding *Salmonella* at a concentration of 2.69 log CFU/g. After 2 days, *Salmonella* was isolated from pooled fecal samples from pigs that were *Salmonella*-negative at the start of the trial.

The transmission of *E. coli* from the environment to cattle has also been investigated. In a study by Cobbold and Desmarchelier (2002), a dairy calf shedding a toxigenic *E. coli* O136:H16 strain was introduced into a pen containing seven *E. coli* O136:H16 negative calves and fecal samples were taken every day from each animal for 10 d. During the trial, *E. coli* O136:H16 was recovered from fecal samples of all animals at least four times during the 10 d study and as early as 1 d after the *E. coli* O136:H16 positive calf was introduced in the pen. Besser *et al.* (2001) found that E. coli O157:H7 negative calves became infected when they were housed in the same pen as a calf shedding *E. coli* O157:H7. In a similar experiment McGee *et al.* (2004) also found that animals became infected with *E. coli* O157:H7 after being exposed to a steer shedding *E. coli* O157:H7.

2.8 Molecular methods for the detection of pathogens from the environment

Isolating and identifying pathogens from environmental samples can involve enrichments, plating on selective agars and biochemical and serological tests. Culture methods and biochemical tests consume a significant amount of laboratory resources and are lengthy, taking many days or weeks to obtain conclusive results (Artz *et al.*, 2006). Some atypical strains might not present typical results on selective agars, resulting in false-negative or false-positive results during biochemical and serological testing

(de Boer and Heuvelink, 2000). A false-negative result could jeopardize human health if pathogens go undetected in contaminated food and water.

Advances in molecular biology methods such as the polymerase chain reaction (PCR) have made it possible to detect pathogens in environmental samples (Ibekwe and Grieve, 2003, Osek, 2002, Pulz *et al.*, 2003, Hu *et al.*, 1999). In concert with basic culture methods, PCR can help accurately identify organisms isolated from conventional media. However, the major advantage of using PCR for the detection of pathogens is the shorter time required to detect pathogens in environmental samples compared to conventional isolation methods (Campbell *et al.*, 2001). The faster results can be reported, the more quickly actions can be taken to reduce the risk to human health caused by contaminated food and water. Methods using PCR usually have three basic steps. The first step is DNA extraction and purification, followed by the PCR reaction and ending with the analysis of PCR products by electrophoresis. Polymerase chain reaction-based detection of pathogens is dependent on targeting one or many (multiplex PCR) genes specific for the organism in question. These genes often include virulence genes which can be responsible for toxin production. Pathogenic *E. coli* gene targets often include the shiga toxin genes (*stx1* and *stx2*), the intimin gene (*eaeA*) and the hemolysin gene (*hylA*) (Spano *et al.*, 2005). Additional genes can be used to specifically detect *E. coli* O157:H7 such as the flagellum H7 serotype gene ($flicC_{H7}$) and the somatic antigen O157 gene ($rfbE_{O157}$) (Wang *et al.*, 2002). Nucleic acid primers can be designed, based on target gene selection, to anneal to the target sequences and enable small fragments of DNA to be synthesized during the PCR reaction. If an organism does not have the target gene, the primers will not bind to the target gene and the PCR reaction

will not amplify any target DNA. A negative reaction will not produce fluorescent bands in an electrophoresis gel when exposed to ultraviolet light.

Many protocols have been used to identify the presence of pathogenic *E. coli* in environmental samples. An enrichment step is often used before the samples are processed by PCR to increase the sensivity of the method. Hu *et al.* (1999) used a multiplex PCR to detect *E. coli* O157:H7 in bovine feces with and without an enrichment step. This group had difficulty detecting *E. coli* O157:H7 directly from feces without an enrichment step, but when enrichment was used they were successful in detecting the organism. A study by Ibekwe and Grieve (2003) investigated the effect of enrichment time on the detection of *E. coli* O157:H7 in soil. They found that after 16 h of incubation, three target *E. coli* O157:H7 genes could be detected at initial cell concentrations of 10-100 cfu/g. When no enrichment was used, the lowest inoculation density of *E. coli* O157:H7 in soil necessary for detection was 3.5×10^8 CFU/ml. Campbell *et al.* (2001) used a double enrichment method for the detection of *E. coli* O157:H7 from drinking water, river water and soil using a multiplex PCR. They found that E. *coli* O157:H7 could be detected at levels ranging from 3-10 cfu/g in soil and 1-8 cfu/g in drinking and lake water.

Molecular methods are very specific for the organism in question and conclusive results can be obtained in a couple of days, however, there are some drawbacks to this technology. When samples are prepared, reaction tubes are opened and closed on repeated occasions and tubes may become cross-contaminated with DNA from other samples which may lead to false-positive results (Rys *et al.*, 1993, Vaneechoutte *et al.*,

1997). Detecting pathogens from environmental samples can be challenging due to the diverse chemical composition of the samples. There are many compounds that may inhibit the PCR reaction and result in a false-negative test. Phenolic compounds, humic acids and heavy metals are some of the compounds in environmental samples known to be involved in PCR inhibition (Wilson, 1997). Other inhibitors include non-target DNA, pollen, glove powder, cellulose, laboratory plasticware and bacterial cell components (Wilson, 1997). A solution to diminish the effect of PCR inhibitors is to dilute the inhibitors in the sample by using an enrichment method prior to DNA extraction (Wilson, 1997).

2.8.1 Real-time PCR

Real-time PCR offers a more rapid detection of microorganisms compared to conventional PCR. This type of PCR uses fluorescent dyes or probes in conjunction with primers that bind to target DNA sequences. When the target gene is successfully amplified fluorescent light is emitted and detected by the real-time PCR machine. With this method there is no need to pass the DNA through an electrophoresis gel because the fluorescence emitted from a positive test (amplification of target sequence) is directly detected by the real-time PCR machine.

2.8.1.1 DNA binding dyes and probes

The most common dyes and probes used for real-time PCR of environmental samples are SYBR Green I (dye), TaqMan (probe) and molecular beacon (probe) (Zhang and Fang, 2006). SYBR Green I has been used to detect *Salmonella* in water, animal feed soil, milk (Nam *et al.*, 2005) and feces (Fukushima *et al.*, 2003). *E. coli*, *Y.*

enterocolitica, *Campylobacter jejuni*, *Staphylococcus aureus*, various *Vibrio* species, and *Bacillus cereus* have also been detected in feces using SYBR Green I (Fukushima *et al.*, 2003). SYBR Green I is a DNA binding dye that only binds to double stranded DNA (dsDNA) (Zhang and Fang, 2006). When SYBR Green I is bound to dsDNA, its fluorescence increases 200 times. During the PCR reaction, target DNA sequences are copied increasing the number of dsDNA fragments after each cycle. SYBR Green I will bind to increased amounts of dsDNA and an increase in fluorescence will follow. The advantages of using SYBR Green I are that no probes need to be designed, it is less costly than probes and can be used with any primers (Zhang and Fang, 2006). The major disadvantage of this method is that SYBR Green I binds non-specifically to all dsDNA and may result in non-specific and unwanted DNA fragments being detected by the real-time PCR machine (Zhang and Fang, 2006).

The TaqMan probe is a linear oligonucleotide with a fluorophore (reporter) at one end and a quencher at the other extremity (Fig 2.1A) (Zhang and Fang, 2006). During the hybridization and annealing steps of the PCR reaction, the probe binds to a section of the target DNA sequence to be amplified. At this point, because of their close proximity to each other, the quencher absorbs the energy emitted from the reporter. During the extension step, the *Taq* DNA polymerase replicates the target DNA sequence and eventually gets to the location where the TaqMan probe is bound to the target DNA. The 5' endonuclease activity of the *Taq* DNA polymerase removes the probe from the target DNA and decouples the reporter and quencher dyes from the probe resulting in the quencher and reporter no longer being in close proximity to each other. The quencher is now unable to absorb the reporter's energy resulting in a release of

fluorescent light that is detected by the real-time PCR machine. After each PCR cycle, increasingly more target DNA is synthesized. As a consequence, more probe will hybridize to the target DNA sequences, and more probe will be cleaved by the *Taq* DNA polymerase yielding greater light emission. The more light that is emitted, the more target DNA has been amplified. The disadvantages of the TaqMan probe are that it is hard to design and usually only works well with small PCR products less than 150 bp (Zhang and Fang, 2006). Methods using the TaqMan probe have been successful in detecting *E. coli* O157:H7 in drinking water (Frahm and Obst, 2003), dairy and cattle wastewater (Spano *et al.*, 2005).

Another type of probe is the molecular beacon. Its structure is similar to the TaqMan probe but has the shape of a hairpin (Fig 2.1B). The hairpin structure of this probe enables the quencher to be in close proximity to the reporter and absorb most the energy emitted by the reporter. During the PCR reaction, the probe will hybridize with the target DNA sequence just like the TaqMan probe. To bind to the target DNA, the probe loses its hairpin structure and becomes linear. The quencher is now too far from the reporter to absorb the energy emitted from the reporter and a fluorescent signal is detected by the real-time PCR machine. During the extension step, the probe is dislodged from the target DNA and returns to its hairpin shape. The molecular beacon can be used for the next PCR cycle unlike the TaqMan probe which is cleaved into smaller fragments by the *Taq* DNA polymerase at each cycle. Methods using molecular beacon probes have been successful in detecting *E. coli* O157:H7 in milk and apple juice (Fortin *et al.*, 2001) and *Clostridium difficile* in feces (Bélanger *et al.*, 2003).

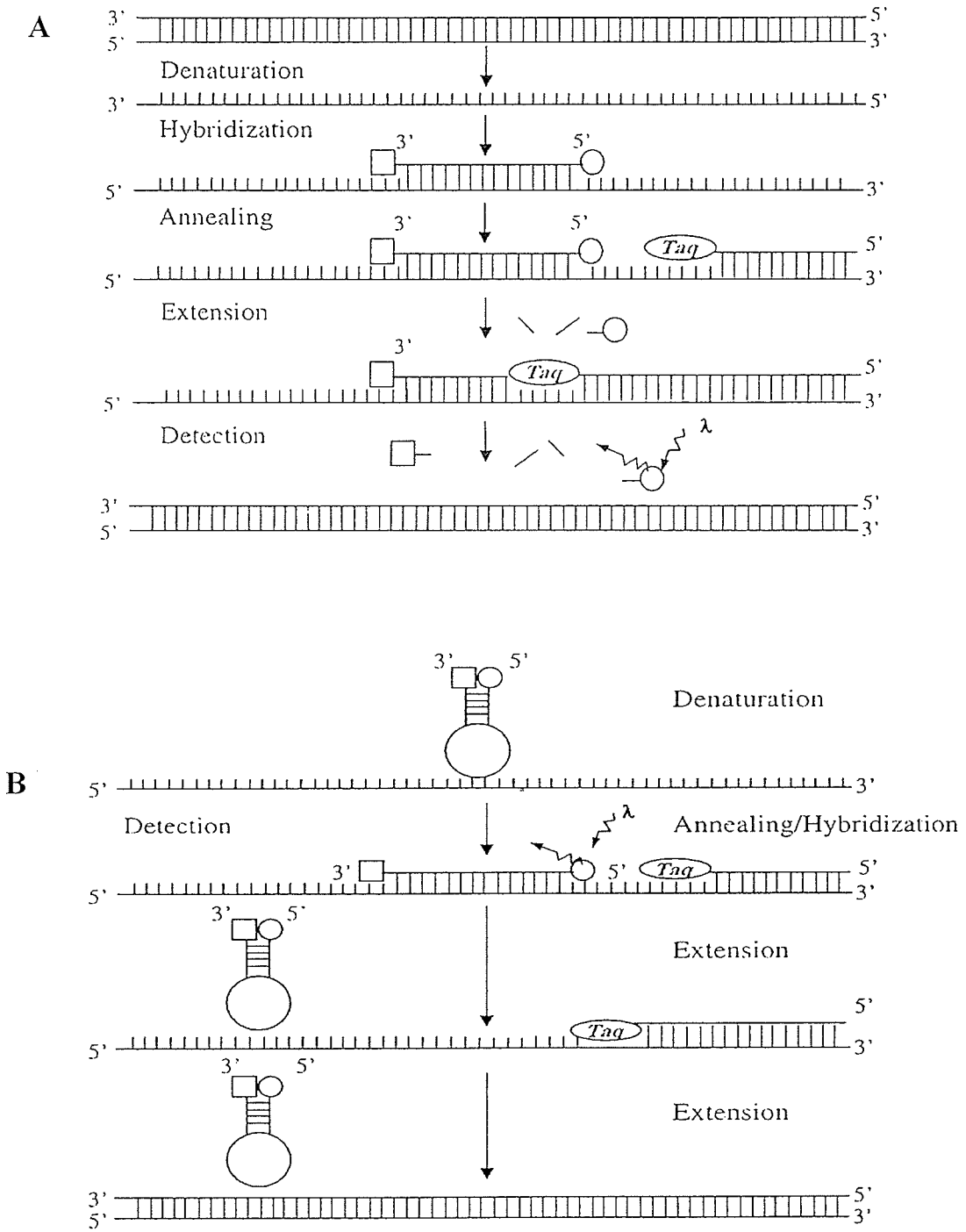


Figure 2.1 Schematic representation of TaqMan (A) and molecular beacon (B) probe mechanisms of action during real-time PCR (Zhang and Fang, 2006).

2.8.2 DNA fingerprinting

DNA fingerprinting is a popular PCR-based tool that can differentiate between strains of the same bacterial species in environmental samples. This method has been used to differentiate among strains of the same bacterial species originating from different species of animals (Somarelli *et al.*, 2006). The principal behind this technique is based on small differences in the genetic code between strains of the same species. Unlike other PCR methods, DNA fingerprinting methods do not target specific genes. This type of method uses a target sequence within the entire genome. Primers are usually designed to anneal to sequences that repeat themselves many times throughout the bacterial genome resulting in the formation of many fragments of various sizes being synthesized during the PCR reaction. Small differences in the genome of different bacterial strains will yield slightly different fragment sizes and form different banding patterns on an electrophoresis gel.

2.9 Summary

The presence of pathogens is frequent in livestock manure and most often the manure is not treated other than storage time to eliminate or substantially reduce their numbers. These observations are of concern because of the popularity of spreading manure on fields and the consequent risk to human and animal health posed by this activity. Pathogens are able to survive for extended periods of time in manure, soil and water. Their persistence in the environment allows these organisms to infect grazing animals or to contaminate plants or water consumed by humans. Temperature is an important variable influencing pathogen survival. In general, pathogens survive longer in cooler environments. It is clear that environmental conditions influence pathogen survival in manure. Since temperature, soil chemistry and moisture levels as well as soil porosity influence pathogen survival and movement in manure-treated soils, regional differences in pathogen survival have been reported. Further study is needed to assure that regionally prescribed minimum intervals (or delays) between manure application and use of the treated land for crops or grazing, realistically allow for pathogen elimination.

Chapter 3

MATERIALS AND METHODS

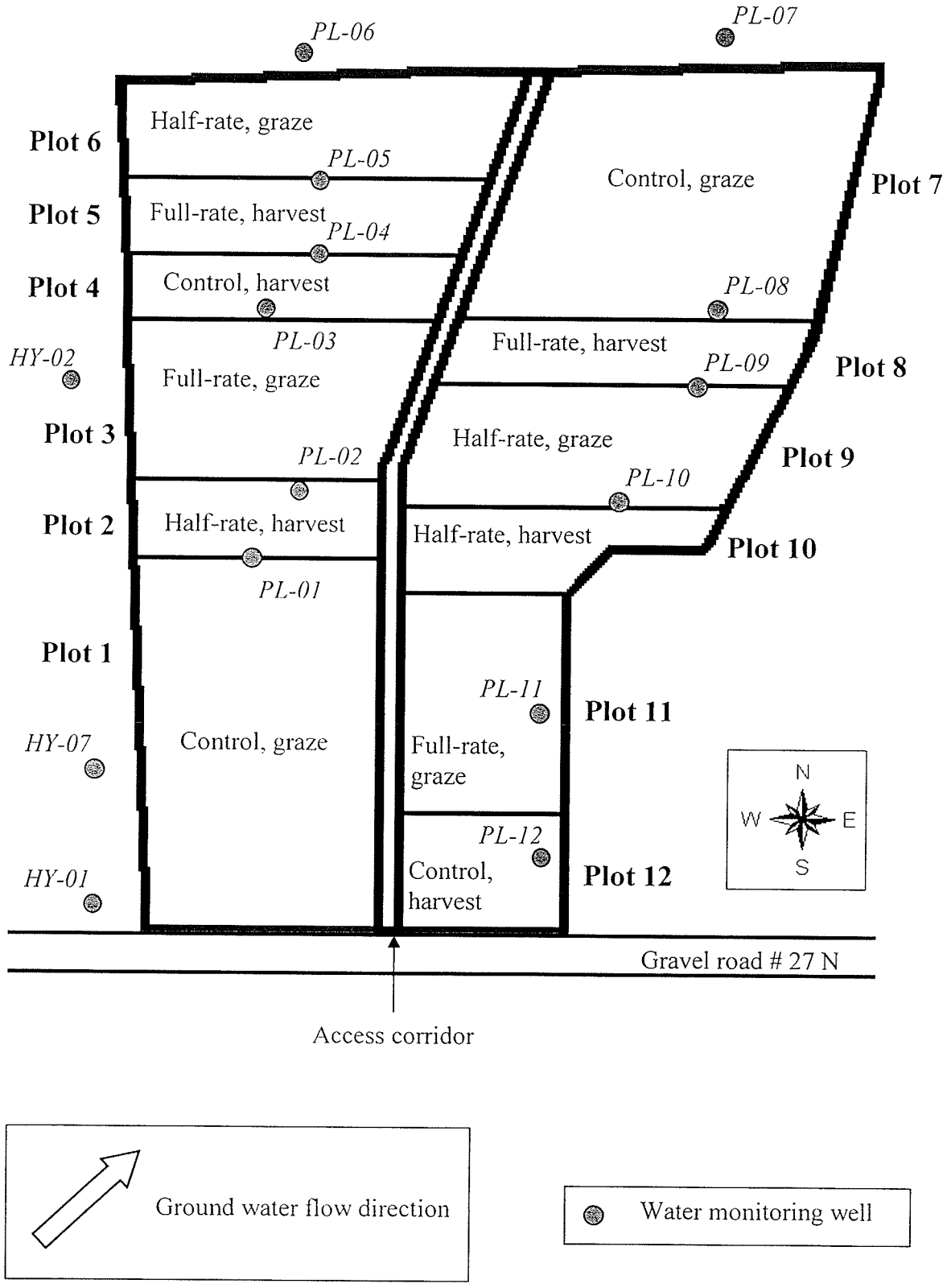
3.1 Experimental site description

The site where this project was carried out was located in the rural municipality of LaBroquerie, 13 km south of the town of LaBroquerie, Manitoba (SE20-5-8E) on 40.47 ha of fairly level pastureland. The pasture was divided by fences into 12 plots separated by an access corridor wide enough for passage of vehicles and farm machinery as illustrated in Fig 3.1.

The types of soil present in the area of the experimental site are Berlo loamy fine sand (70 %) and Kergwenan loamy sand to gravel (30 %). The Berlo soils have a Canadian Agricultural Capability Class of 3m. Soils with a 3m designation have rapid internal drainage which makes these kinds of soils prone to dryness and poor for annual crop production. Kergwenan soils agricultural capability class (4mw) indicates that its use for annual crop production is limited because of possible insufficient soil moisture or excess water, but this soil can still be used for forage production. Coarse textured surface materials are present at the eastern side of the experimental site from the ground surface to > 7.5 m below the surface and from ground level to 1.5-2.1 m below the surface on the western side. The western side also has the presence of a dense clay layer 3.0-4.5 m thick creating a perched water table (aquifer that occurs above the main water table) restricting water drainage. In consequence, excess water may pool on the surface during snowmelt and periods of heavy rain.

Three different hog manure application rates (no manure, half-rate and full-rate) and 2 different forage management practices (grazed cattle and mechanically harvested forage) were used, which provided a total of 6 treatments. Half-rate plots had half the amount of hog manure applied in the fall and the other half in the spring. Full rate plots had the desired amount of manure applied at one time in the spring (see section 3.2). Each treatment was repeated in duplicate where plots 1 to 6 represented replicate one and plots 7 to 12 represented replicate two. Cattle were grazed on plots 1 and 7 but plots were not exposed to hog manure (*control, graze*). Plots 2 and 10 were not grazed, instead forage was mechanically harvested and hog manure was applied to those plots twice at the half-rate *(half-rate, harvest*). Cattle were grazed on plots 3 and 11 and hog manure was applied once at the full-rate (*full-rate, graze*). Plots 4 and 12 did not have hog manure applied and were mechanically harvested (*control, harvest*). Plots 5 and 8 had manure applied at the full-rate and were also mechanically harvested (*full-rate, harvest*). Cattle were grazed on plots 6 and 9 and hog manure was applied twice at the half-rate. (*half-rate, graze*).

Figure 3.1 Well and plot treatment locations at the experimental site



3.1.2 Water monitoring wells

Fifteen wells were installed in and around the experimental site (Fig 3.1). The monitoring wells were constructed of schedule 40 threaded 5 cm diameter PVC pipe. Pipe sections were joined at the threaded ends without glue and joints were secured by a stainless steel screw. Part of the pipe had 0.025 cm slits (screen) to enable the water to flow through the well (Fig 3.2, Table 3.1). At the leading edge of the well a "push-point" was installed to facilitate soil penetration. The opening of the well was closed with a PVC screw cap. To protect the PVC pipe above ground, a metal pipe casing was installed around the plastic pipe and covered by placing a metal cap over the opening which was padlocked. Well depths (2.6-3.6 m) and screen lengths are listed in Table 3.1.

The locations of wells were decided after the direction of groundwater flow through the site was determined. Manitoba Water Stewardship determined that groundwater flowed from the southwest to northeast areas of the field (Fig 3.1). Three wells (HY-01, HY-02 and HY-07) were installed in the southwest corner of the site to monitor possible off-site contamination entering the experimental area. The 12 other wells were located in every plot, except for PL-06 and PL-07 which were placed just outside the north perimeter fence bordering these plots, respectively. All 12 wells were installed down gradient of the water flow direction which meant that the wells were installed near the fence line at the north end of each plot. Wells were positioned (and numbered) to monitor chemical and microbiological contamination which originated in each correspondingly numbered plot in response to the predominant ground water flow pattern. Microbiological analyses and results are subjects of the presently reported work.

Figure 3.2 Water monitoring well design

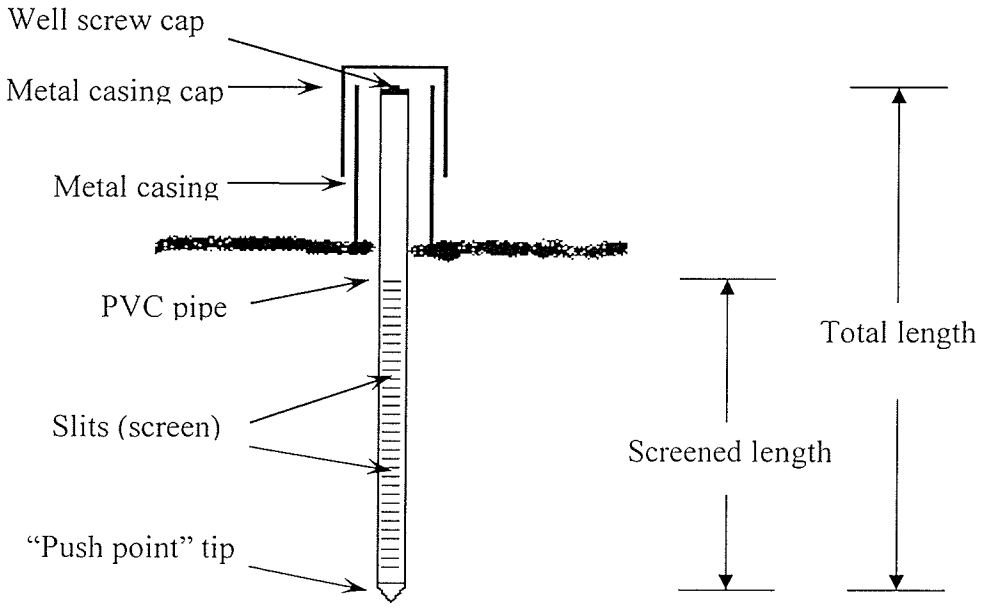


Table 3.1 Total length, depth to the top of the screen and screened length of wells on the experimental site

Well	Total length (m)	Top of well to top of screen (m)	Soil surface to top of screen (m)	Screened length (m)
PL-01	3.06	1.54	0.60	1.52
PL-02	2.60	1.53	0.62	1.07
PL-03	2.73	1.51	0.54	1.22
PL-04	2.77	1.55	0.63	1.22
PL-05	3.06	1.54	0.54	1.52
PL-06	3.07	1.55	0.59	1.52
PL-07	4.45	1.40	0.46	4.31
PL-08	4.57	1.52	0.60	3.05
PL-09	4.54	1.49	0.55	3.05
PL-10	4.38	1.33	0.35	3.05
PL-11	4.27	1.22	0.27	3.05
PL-12	4.44	1.39	0.47	3.05
HY-01	3.98	2.45	1.43	1.53
HY-02	3.35	1.82	0.89	1.53
HY-07	2.74	1.52	0.66	1.22

3.2 Hog manure application

Hog manure used on the field was taken from an earthen manure lagoon located 300 m from the experimental site next to 5 hog barns (Fig 3.3). The lagoon was on land separated from the test field by an above grade gravel road. Prior to application, a pump powered by a tractor agitated the manure for a minimum of 3 h. A tractor pulling a 19,000 L tanker equipped with a splash plate broadcast-spread the manure on the plots. The speed of the tractor was adjusted to accommodate each manure treatment and achieve the desired nitrogen addition to the field plots. Half-rate application was equivalent to 25 kg of available nitrogen per acre and 50 kg per acre was used for full-rate application. This nitrogen content represented a manure application of ~ 53,000 L/ha for the full-rate and ~ 26,500 L/ha for the half-rate in 2004. In 2005, ~ 39,600 L/ha was applied on full-rate plots and ~ 19,800 L/ha on half-rate plots.

The 2004 spring manure application started on May 10. On that day, plots 2, 5 and 6 received a manure treatment. The next application of manure was scheduled for the following day but a snowstorm delayed further application. The next application occurred on May 24 where plots 8, 9 and 10 were treated with hog manure. Finally, on May 25, applications to plots 3 and 11 were completed.

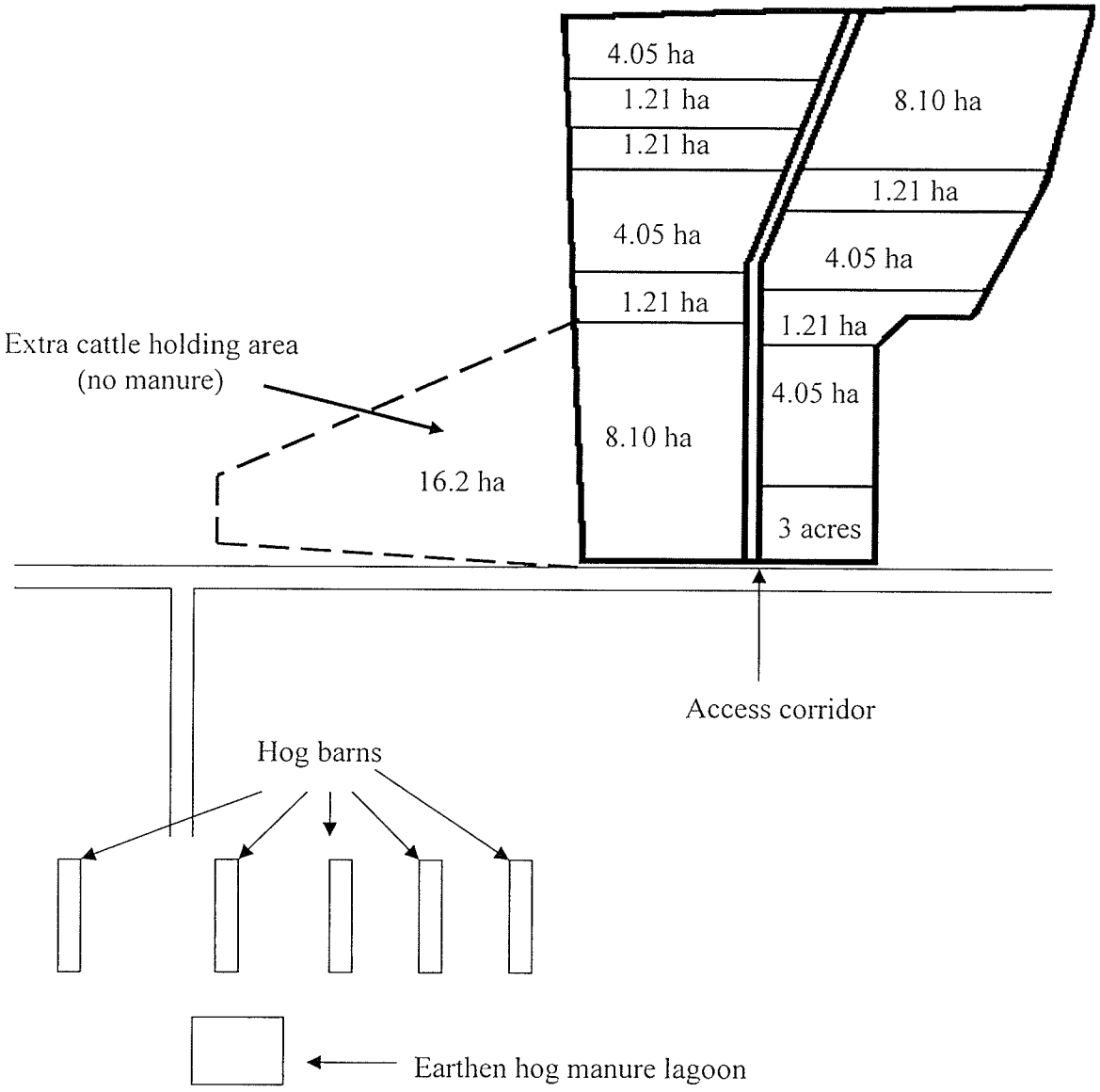
In 2005, manure application started on April 25 with plots 2, 3, 5, 6 receiving manure. On April 26, plots 8, 9, 10, and most of plot 11 had manure applied. Manure application to plot 11 was completed on April 27.

3.3 Cattle management and fecal sampling procedures

Each year, one year old steers of various British crossbreeds owned and managed by Hytek Ltd. were used for this study. Cattle were divided into groups and introduced on pasture plots. Animals were removed or cattle were added to the plots in response to vegetation growth. The extra cattle were held on a non-manured, 16.2 ha field next to the southwest corner of the experimental site (Fig 3.3). Throughout the 2 grazing seasons which were monitored, cattle on the control plots (no manure) ranged in number from 16-20 and from 40-109 animals were kept on manured plots.

Fecal samples were collected from cattle and examined to determine if they were shedding *Salmonella* or *Yersinia enterocolitica* before (period 0) and during each of two or three 28-day grazing periods in each year. Fecal samples were taken in 2004 during periods 1, 2 and 3 and in 2005 periods 1 and 2 (Table 3.2). Fecal samples taken at time 0 reflect the initial microflora of the animals before they came in contact with the hog manure-treated pasture. If cattle were added during a period, a fecal sample was taken from each animal before it was introduced onto a field. Fecal samples were obtained by gloved hand at the rectum of the steer and samples were kept in bags on ice in a cooler for < 10 h during transport and were stored at 1°C until analysed for *Salmonella, Yersinia enterocolitica and Escherichia coli.*

Figure 3.3 Plot size and hog manure lagoon location at the experimental site



3.4 Hog manure, forage, soil and water sampling procedures

3.4.1 Manure

All hog manure samples were taken in sterile plastic containers directly from the opening at the top of the tanker. One hog manure sample was taken at the start and again at the end of application each day for a total of 6 samples in 2004. In 2005, 1-2 hog manure samples were taken per plot during application. A total of 14 samples were collected.

3.4.2 Forage and soil

Forage and soil samples were collected before manure application and as close as possible to the date of manure application. Five samples areas, each being 2 m^2, were outlined in a "w" pattern, in each plot, with sample area number 1 located in the northwest corner of the plot. Both forage and soil samples were taken from the 2 m^2 sample area. Forage samples were removed by cutting the vegetation 4 cm above ground level. Once the forage sampling was completed, soil samples were taken to a depth of 5 cm with a tube soil sampler (JMC "backsaver" soil sampler, Clements Associates Inc., Newton, IA, USA). The cutting end of the soil sampler was sanitized by spraying it with a quaternary ammonium solution (ZEP, Atlanta, GA, USA) between each sample and again between sampling from each plot. After disinfection, the soil sampler was plunged into the soil next to the new sample area once or twice to remove the disinfectant. In 2004, 20 soil sub-samples were taken from each 2 m^2 sample area and pooled. These pooled samples (5/plot) were used for microbiological analysis. In 2005, material from each of the 5 composite samples per plot was again combined to make a single

composite sample for each plot. Material from this composite sample was used for microbiological analysis.

3.4.3 Water

Water samples were taken at 6 different times between April 25, 2005 and November 2, 2005 (Table 3.2). Teflon tubing (4.8 mm inside diameter, 6.4 mm outside diameter, Johnson Industrial Plastics, Winnipeg, MB, CAN) with # 24 Tygon tubing (6.4 mm inside diameter, Cole-Parmer Canada Inc., Anjou, PQ, CAN) connectors were used to collect water samples. Tubing was assembled by connecting one 4.0-4.5 m and one 0.5 m piece of Teflon tubing together using a 20-25 cm piece of Tygon tubing. The Tygon tubing was later inserted in the wheel of a peristaltic pump (Series 2 Geopump, Geotech Environmental equipment Inc., Denver CO, USA) and was used because of its flexibility. A 5-10 cm length of Tygon tubing was temporarily used to connect the extremities of the tubing to form a closed loop to minimise the possibility of contamination entering the tube. Complete tubing units were packaged in plastic bags, sealed and autoclaved to sterilize them. One set of tubing was used per well to avoid cross-contamination between wells. Once ready for sampling, a tubing unit was removed from its packaging and the 20-25 cm length of Tygon tubing was placed in the peristaltic pump. The smaller piece of Tygon tubing connecting both extremities was removed and the 4.0-4.5 m length of tubing was inserted the well. The 0.5 m Teflon section was used to deliver the water sample for collection. Tubing was placed in the well to a depth of 10 cm below the top of the screen (start of the slits) if the water level was above the screen or 10 cm below the water-line if the levels were within the screen. The peristaltic pump was activated and pumped water from the well into two sterile 1L plastic

containers. Samples were stored on ice in a cooler, transported to the University and analysed within 24h for *E. coli* and *Salmonella*.

Table 3.2 Chronological record of hog manure application, as well as animal and field sampling at the LaBroquerie, MB experimental site

Year	Date	Activity	Field location or period
2004	May10	Manure application	Plots 2, 5, 6
	May 24	Manure application	Plots 8, 9, 10
	May 25	Manure application	Plots 3, 11
	May 24	Forage and soil samples collected	Plots 2, 5, 6
	May 25	Forage and soil samples collected	Plots 8, 9, 10
	May 27	Forage and soil samples collected	Plots 3, 11
	June 3	Cattle fecal samples taken	Period 0 (Time 0)
	June 4	Cattle put on field	
	June 24	Cattle fecal samples collected	Period 1 Rep 2
	July 2	Cattle fecal samples collected	Period 2 Rep 1
	July 14	Cattle fecal samples collected	Period 2 Rep 2
	August 6	Cattle fecal samples collected	Period 3 Rep 1
	August 13	Cattle fecal samples collected	Period 3 Rep 2
2005	April 25	Manure application	Plots 2, 3, 5, 6
	April 26	Manure application	Plots 8, 9, 10, 11
	April 27	Manure application	Plot 11
	April 23	Forage and soil samples collected	All plots
	April 26	Forage and soil samples collected	Plots 2, 3, 5, 6
	April 27	Forage and soil samples collected	Plots 8, 9, 10,11
	May 20	Forage and soil samples collected	Plots 2, 3, 5, 6, 8, 9, 10, 11
	May 24	Cattle fecal samples taken	Period 0 (Time 0)
	May 25	Cattle put on field	
	June 6	Cattle fecal samples collected	Period 1 Rep 1
	June 14	Cattle fecal samples collected	Period 1 Rep 2
	July 6	Cattle fecal samples collected	Period 2 Rep 1
	July 12	Cattle fecal samples collected	Period 2 Rep 2
	April 25	Water samples collected	All Wells
	July 13 / 14	Water samples collected	All Wells
	September 27/ 28	Water samples collected	All Wells
	November 2	Water samples collected	All Wells

3.5 Microbiological isolation methods

3.5.1 *Salmonella*

3.5.1.1 Detection of *Salmonella* in hog manure, cattle feces, forage and soil

A double enrichment procedure in Tetrathionate broth (EMD Chemicals Inc., Gibbstown, NJ, USA) followed by Rappaport-Vassiliadis R10 Broth (RV) (Difco, Becton-Dickinson, Sparks, MD, USA) was used for the enrichment of *Salmonella* as stated in Guan and Holley (2003). Twenty-five grams of sample were introduced into a sterile stomacher bag containing 225 ml of Tetrathionate Broth, homogenized in a stomacher for 1 min (Stomacher Lab-Blender 400, Seward Laboratory, London, UK) and incubated at 35 °C for 24 h. After 24 h, 1 ml of Tetrathionate Broth was transferred to 99 ml of Rappaport-Vassiliadis R10 Broth (RV) and incubated at 35 °C for 24 h. Next, a loopful from RV was streaked on each of 2 plates of Hektoen Enteric Agar (HE) (Difco, Becton-Dickinson) and 2 plates of Xylose Lysine Desoxycholate Agar (XLD) (Difco, Becton-Dickinson) (Andrews *et al.*, 1981). These plates were also incubated at 35 °C for 24 h. Colonies with black centres were chosen on both media and restreaked again on the same medium to ensure culture purity. A *Salmonella* latex agglutination test (Oxoid, Hants, UK) was performed on colonies that fit the description of *Salmonella.* These were colonies on HE plates having a black centre with a clear border showing no change of colour in the medium, and on XLD agar were colonies having a black centre with a small amount of colour change in the medium (red to yellow) around the colony. If the agglutination test was inconclusive for a given isolate, an API 20E identification test (bioMérieux, Marcy l'Etoile, France) was performed on that same isolate to confirm its identity as *Salmonella.* Positive isolates were then submitted to the National

Microbiology Laboratory, Canadian Centre for Human and Animal Health in Winnipeg, Manitoba, for serotyping.

3.5.1.2 Detection of *Salmonella* in water

Water samples were analysed using a Hydrophobic Grid Membrane Filtration (HGMF) system with 0.45 μm filters (Iso-Grid, Neogen Corporation, Lansing, MI, USA). One liter of water was filtered through the membrane and with sterile tweezers the membrane was placed in a stomacher bag containing 225 ml of Tetrathionate Broth. In the event that the sediment in the water plugged the membrane, the latter was placed in the Tetrathionate Broth and a new filter was placed in the filter tower. The additional filter was added to the same bag as the plugged filter. The bag was then treated in the stomacher for 1 min and incubated at 35 °C for 24 h. The subsequent steps used for the isolation of *Salmonella* from water were identical to those used for *Salmonella* described in section 3.5.1.

3.5.2 *Yersinia enterocolitica*

3.5.2.1 Procedure (2004) used for the isolation of *Yersinia enterocolitica* from hog manure, cattle feces and forage

A double enrichment was used to evaluate samples for the presence *Y. enterocolitica*. A first enrichment in Alkaline Peptone Water (10g/L peptone, 10g/L NaCl, pH adjusted to 8.5 with 1M NaOH) was performed by incubation at 35 °C for 24 h (de Boer, 1992). The second enrichment was in Irgasan-Ticarcillin-Chlorate Broth (ITC) (Fluka, Sigma-Aldrich, USA) (de Boer, 1992). One ml of the Alkaline Peptone Water

enrichment was introduced into 99 ml ITC and incubated at 30 °C for 48 h. Two plates of Salmonella Shigella Agar (SS) (Difco, Becton-Dickinson) and 2 plates of *Yersinia* Selective Agar (CIN) (Difco, Becton- Dickinson) (Cox *et al.*, 1990) were streaked from the ITC enrichment. Colourless colonies on SS plates and colonies having the appearance of a "red bull's eye" on CIN plates were selected and re-streaked on the same medium. Confirmation tests were done according to the U.S. Food and Drug Administration (FDA) method from the Bacteriological Analytical Manual (Weagant *et al.*, 1998). Urea Agar slants (Difco, Becton-Dickinson), Bile Esculin Agar (Difco, Becton-Dickinson) plates and Lysine Arginine Iron Agar (LAIA) (Difco Lysine Iron Agar with 10g/L L-arginine added) slants were used to confirm the presence of *Y. enterocolitica* among colonies present on SS and CIN agars. For a sample to be considered presumptive positive for *Y. enterocolitica*, it would be positive for urease (pink colour), negative for esculin hydrolysis (no blackening of agar) and have an acid butt (yellow) and alkaline slant (purple) on LAIA agar slants. If the isolate fit the biochemical description of *Y. enterocolitica*, an API 20E test was performed to confirm its identity as *Y. enterocolitica.*

3.5.2.2 Procedure (2005) used for the isolation of *Yersinia enterocolitica* from hog manure, cattle feces, forage and soil

In 2005, the entire FDA method from the Bacteriological Analytical Manual was used for the detection of *Y. enterocolitica* (Weagant *et al.*, 1998). Twenty-five grams of sample were weighed in a sterile stomacher bag to which 225 ml of Peptone Sorbitol Bile Broth (8.23 g Na_2HPO_4, 1.2 g $NaH_2PO_4.H_2O$, 1.5 g Bile Salts No. 3, 5 g NaCl, 10 g Sorbitol, 5 g Peptone, 1L water) were added, treated in the stomacher for 1 min and

incubated at 10 °C for 10 days. Then, 0.1 ml of sample was transferred from the enrichment to a tube containing 1 ml of 0.5% KOH (Sigma-Aldrich, St.-Louis, MO, USA) in 0.5% NaCl (Sigma-Aldrich). An additional 0.1 ml of sample was transferred to a tube containing 1 ml of 0.5% NaCl. Both tubes were vortex-mixed for 5 sec and a loopful from each tube was streaked on 2 plates each of MacConkey (Difco, Becton-Dickinson) and CIN agars and incubated for 24 h at 30 °C. Presumptive positive isolates for *Y. enterocolitica* were deemed positive for urease, negative for esculin hydrolysis and had an acid butt and alkaline slant on LAIA slants. If all these criteria were satisfied, an API 20E test was performed to confirm the isolate's identity.

3.5.2.3 Survival of *Yersinia enterocolitica* in autoclaved hog manure

Six hundred grams of hog manure was introduced into each of six plastic 1 L bottles. The six bottles were autoclaved at 121 °C for 15 min. Once the manure was cool, 25 g was introduced into a stomacher bag to which 225 ml of 0.1 % peptone solution was added and treated in the stomacher for 1 min. A decimal dilution was done on the samples and 0.1 ml was spread onto the surface of CIN agar and incubated at 30 °C for 24 h. If colonies appeared on the CIN plates after incubation, the bottles containing the manure were autoclaved again. *Y. enterocolitica* previously isolated from hog manure (Rogasky, J., Department of Food Science, University of Manitoba, identified as *Y. enterocolitica* by the Ontario Ministry of Health, Etobicoke, ON.) was inoculated in 350 ml TSB and incubated at 30 °C for 24 h to reach levels of approximately 8 log CFU/ml. Fifty ml of culture was added to each of the six bottles containing the manure. Three bottles were incubated at 20 °C and three were incubated at 5 °C. Subsequent

steps for the isolation of *Y. enterocolitica* identical to the ones previously described in this section were used. One to two colonies from CIN plates from each sample were confirmed to be *Y. enterocolitica* using the API 20E test. If *Y. enterocolitica* was not detected on CIN agar, the FDA method from the Bacteriological Analytical Manual was used for the detection of *Y. enterocolitica* as described in section 3.5.2.2.

3.5.3 Escherichia coli

3.5.3.1 Detection of *Escherichia coli* in hog manure, forage and cattle feces

Twenty-five grams of sample was put into a sterile stomacher bag. Two hundred and twenty-five ml of 0.1% peptone solution was added and the mixture was treated in a stomacher for 1 min. Serial dilutions were performed on the samples and then 1 ml was plated onto EC Petrifilm™ (3M, Saint-Paul, MN, USA). Blue colonies with a gas bubble were counted as *E. coli* and pink colonies with a gas bubble were counted as coliforms. Blue colonies with a bubble were then individually picked from the Petrifilm™ and streaked on Levine Eosin Methylene Blue Agar (EMB) (Difco, Becton-Dickinson) plates to ensure that the isolate was a pure culture. Each isolate on EMB that had a green metallic sheen was subjected to IMViC biochemical tests which involve indole (I), methyl-red (MR), voges-proskauer (VP) reactions and citrate (C) utilization tests. The indole test was performed by inoculating 5 ml 1 % (w/v) Tryptone (Difco, Becton-Dickinson) in a tube with incubation at 35 °C for 24 h. After incubation, a drop of Indole Reagent (Becton-Dickinson) was introduced in each tube. A positive result (production of indole via degradation of tryptophan) was indicated by the formation of a red/pink ring at the top of the liquid. The MR reaction consisted of growing the isolate in MR-VP

medium (Difco, Becton-Dickinson) at 35 °C for 48 h, after which methyl-red reagent (0.1 g methyl red, 300 ml 95 % ethanol, 200 ml water) was added to the tube. A positive reaction (acid production by the degradation of glucose, pH < 4.4) occurred when the liquid in the tube changed colour to red. To test the VP reaction, the isolate was grown in MR-VP medium for 48 h at 35 °C after which 5 drops of an alpha-naphthol solution (5 g naphthol in 100 ml ethanol) was added and followed by 5 drops of 40 % NaOH solution. The appearance of a pink colour throughout the liquid within 15-30 min signified a positive reaction (production of acetyl methyl carbinol). The citrate test was done by streaking the isolate on a slant of Simmons Citrate Agar (BBL, Becton-Dickinson) and incubating at 35 °C for 24 h. A change in the slant colour from green to blue indicated a positive result (citrate utilization). Isolates considered to be *E. coli* were positive for indole (red ring at the surface of the tube) and methyl red (red colour) tests and negative for the VP (colourless) and citrate (green) tests. Positive *E. coli* isolates were streaked on Tryptic Soy Agar (Difco, Becton-Dickinson) slants and stored at 4 °C until they were used for genetic analysis.

3.5.3.2 Detection of *Escherichia coli* in soil

Twenty-five grams of soil were transferred to a stomacher bag containing 225 ml Tryptic Soy Broth (Difco, Becton, Dickinson) and incubated at 35 °C for 24 h. A serial dilution in 0.1 % peptone was done and 1 ml was plated on EC Petrifilm™ and incubated at 35 °C for 24 h. Blue colonies with a gas bubble were picked and streaked on EMB agar. Subsequent steps followed are described in section 3.5.3.1.

3.5.3.3 Isolation of *Escherichia coli* from water

Water samples were filtered using the HGMF system (described in section 3.5.1.2) and the filters were put into 225 ml of Buffered Peptone Water (Difco, Becton-Dickinson) and incubated at 35 °C. After 2 h of incubation, the bags of Buffered Peptone Water containing the filters were removed from the incubator. One millilitre of Buffered Peptone Water from each sample was plated on EC Petrifilm™ and incubated at 35 °C for 24 h to enumerate *E. coli* and coliforms after the 2 h resuscitation period. The bags were returned to the incubator for an additional 22 h at 35 °C. Decimal dilutions of the enrichment were done using a 0.1% peptone solution and 1 ml plated onto EC Petrifilm™ and incubated at 35 °C for 24 h. Suspect colonies were picked from Petrifilm™ and subsequent steps used for identification are described in section 3.5.3.1.

3.6 Genetic analysis of *E. coli*

3.6.1 DNA Extraction

E. coli isolates from hog manure, cattle fecal samples, soil and water stored on TSA slants were picked with a sterile toothpick and grown in a 1.5 ml microcentrifuge tube with a secure lid closure (Fisher, Fairlawn, NJ, USA) containing 1.5 ml of Luria-Bertani (LB) (Fisher) broth for 48 h. Tubes were centrifuged (Microlite, Thermo IEC, Waltham, MA, USA) for 10 min at 9300 xg, the supernatant was discarded and 1 ml of sterile Milli-Q (Milli-Q water purification system, Millipore, Billerica, MA USA) water was introduced into the tube and vortex-mixed to resuspend the pellet. The microcentrifuge tube was then heated in a heating block (Isotemp 145D, Fisher) at 95 °C for 10 min and then frozen at – 20 °C until they were analysed using polymerase chain reaction (PCR).

3.6.2 PCR sample preparation and conditions

A PCR master mix was made using 2 μL 10x PCR reaction buffer (200 mM Tris-HCl pH 8.5, 500 mM KCl, 50 mM MgCl, Invitrogen, Burlington, ON, CAN) 1 μL 25 mM $MgCl_2$ (Fisher), 0.4 μL 10 mM nucleotide mix (Fisher), 0.2 μL primer (BOC a1r: 5′-CTACGGCAAGGCGACGCTGACG-3′, made at the Faculty of Medicine, University of Calgary) (Dombek *et al.*, 2000), 0.2 μL Taq DNA polymerase 5000 IU/ml (New England Biolabs, Ipswich, MA, USA) and 16.2 μL water per sample. The PCR cocktail was mixed and 19.5 μL was put into 0.2 ml Thermowell PCR tubes with a domed cap (Corning Inc., Corning, NY, USA). When all the tubes were filled, 0.5 μL of DNA isolated in section 3.6.1 was introduced into the Thermowell tubes containing the master mix and put into the PCR machine (Tenius, Techne, Duxford, Cambridge, UK). In each set of PCR reactions, one sample of 0.5 μL water was used instead of DNA and served as a control. The PCR program had an initial denaturation step of 95 °C for 2 min followed by 30 cycle sequences of: 94 °C for 3 sec (denaturation), 92 °C for 30 sec (denaturation), 50 °C for 1 min (annealing) followed by 65 °C for 8 min (extension). After 30 cycles, an additional extension step was performed at 65°C for 8 min (Dombek *et al.*, 2000). Once the PCR program was finished, the samples were frozen at –20 °C until gel electrophoresis was performed.

3.6.3 Gel electrophoresis

Agarose gels were made by introducing 2.25 g of agarose (Promega, Madison, WI, USA) into 150 ml of 0.5x Tris-boric acid-EDTA (TBE) made from a 5x TBE solution (5x TBE: 54 g Tris, 27.5 g boric acid, 20 ml 0.5 M EDTA, water to 1000 ml total volume). The agarose-TBE solution was boiled in the microwave to dissolve all the

3.6.4 Statistical analysis of *Escherichia coli* genomic fingerprints

The presence or absence of 23 bands ranging from 425 to 1625 base pairs (bp) were evaluated for each *E. coli* DNA fingerprint. Data for each fingerprint was entered in a spreadsheet where the presence of a band was indicated by the numeral 1 and the absence of a band by the numeral 0, forming a matrix. ClustanGraphics 7 software (Clustan Ltd.) was then used to calculate the similarity between different DNA fingerprints using the Jaccard similarity coefficient (Dombec *et al.*, 2000). This same software was also used to cluster the data using the Average Linkage clustering method (Unweighted Pair-Groups Method Average) (Birch *et al.*, 1996) and then generated a dendrogram. An example of a generic matrix can be found in Appendix 1 and the resulting dendrogram can be found in Appendix 2. To determine the level (similarity) at which patterns could be grouped into distinct clusters (Appendix 2), a cut-off point was determined using Simpson's index of diversity (Appendix 3) (Hunter and Gaston, 1988). Pairwise group comparisons were made between different DNA fingerprint patterns of *E. coli* isolates from soil, cattle and hog manure (Table 3.3) to determine the number of common fingerprint patterns among the groups being compared. Fisher's Exact test (Schutze *et al.*, 1998) was used to determine if the number of patterns in common between two groups of fingerprints were statistically different.

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Table 3.3 Comparisons made between groups of DNA fingerprint patterns from *E. coli* isolated from hog manure, soil and cattle during 2004 and 2005

Comparison		
Group1		Group 2
Hog Manure 2004	vs	Cattle 2004-P0
	vs	Cattle 2004-P1
	vs	Cattle 2004-P2
	vs	Cattle 2004-P3
Hog Manure 2004	vs	Cattle 2005-P0
	vs	Cattle 2005-P1
	vs	Cattle 2005-P2
	vs	Cattle 2005-P3
Hog Manure 2004	vs	Soil 2004
	vs	Soil 2005-BM
	vs	Soil 2005-AM
Hog Manure 2005	vs	Cattle 2005-P0
	vs	Cattle 2005-P1
	vs	Cattle 2005-P2
	vs	Cattle 2005-P3
Hog Manure 2005	vs	Soil 2005-BM
	vs	Soil 2005-AM
Cattle 2004-P0	vs	Cattle 2004-P1
	vs	Cattle 2004-P2
	vs	Cattle 2004-P3
Cattle 2005-P0	vs	Cattle 2005-P1
	vs	Cattle 2005-P2

P0, P1, P2, P3: Sampling period 0, 1, 2, and 3
BM: Soil sampled before manure application
AM: Soil sampled after manure application

Chapter 4

RESULTS

4.1 Hog manure

4.1.1 Detection of *Salmonella*, *Yersinia enterocolitica* and *E. coli* in hog manure during spring application in 2004 and 2005

Most hog manure samples (4 of 6) in 2004 and all samples (14 of 14) in 2005 taken from the manure tanker at various times during hog manure application were positive for *Salmonella*. *Salmonella* Derby and Krefeld were isolated from hog manure during both the 2004 and 2005 seasons (Table 4.1). *Y. enterocolitica* was not detected in any hog manure samples taken during application in 2004 and 2005 (Table 4.2). Hog manure samples had *E. coli* numbers ranging from 4 to 5 log CFU/g in 2004 and 4 log CFU/g in 2005 (Table 4.3).

4.1.2 Survival of *Yersinia enterocolitica* in autoclaved hog manure

The survival of *Y. enterocolitica* in autoclaved hog manure stored at 5 °C and 20 °C was evaluated. After 14 d, *Y. enterocolitica* inoculated at 6.8 log CFU/g could not be recovered after enrichment from manure stored at 20 °C. In contrast, at 5 °C, *Y. enterocolitica* could be detected at a concentration of 3.7 log CFU/g after 64 d of storage (Fig. 4.1).

4.2 Soil and forage

4.2.1 Detection of *Salmonella* and *Yersinia enterocolitica* in soil and on forage in 2004 and 2005

Forage samples taken before cattle were grazed on pastureland were negative for *Salmonella* and *Y. enterocolitica* for both experimental years (2004 and 2005). Hog manure application in 2004 started on May 10 and plots 2, 5, and 6 received a manure treatment. The arrival of a snowstorm on May 11 delayed further application until May 24 for plots 8, 9, and 10 and May 25 for plots 3 and 11. *Salmonella* was detected on the vegetation in 3 of 5 samples on plot 6, 14 d after manure application and in 1 of 5 samples on plot 9, 1 d after manure application. The serotype identified from forage was *S.* Typhimurium (Table 4.1) and this serotype was not isolated from the hog manure samples taken during application. In 2005, *Salmonella* was detected in 6 of 8 composite forage samples. The different types of *Salmonella* isolated were Derby and Krefeld and these had serotypes identical to the *Salmonella* isolated from hog manure during hog manure application (Table 4.1). Forage samples taken 23 to 25 d after hog manure application (before cattle were grazed) were negative for *Salmonella*. *Y. enterocolitica* was not recovered from forage at any time after manure application in 2004 and 2005 (Table 4.2). *Salmonella and Y. enterocolitica* were not recovered from soil at any time during the 2005 season (Table 4.1 and 4.2).

4.2.2 Detection of *E. coli* in soil and on forage in 2004 and 2005

No *E. coli* were recovered from non-manured forage samples in 2004, however, following manure application that year, the organism was recovered from forage on 5 of 8 plots at 2 log CFU/g. Again, in 2005, *E. coli* was not detected on forage but was detected in soil (Plot 3, 6, 9 and 11) taken before manure application. However, after manure application, 7 of 8 forage plots were *E. coli* positive at 2 log CFU/g (Table 4.4).

In 2005, soil samples taken from control plots and 4 of 8 plots to be treated with manure were negative for *E. coli* before manure application. *E. coli* positive plots had low numbers of *E. coli* (5 CFU/g) before manure application. After manure application, all 8 plots were positive for *E. coli* at a level of 1 log CFU/g. About 25 d later, before cattle were grazed on these manure-treated plots, *E. coli* was only detected on 2 of the plots at a level of 1 log CFU/g (Table 4.4).

4.3 Cattle

4.3.1 Detection of *Salmonella* and *Yersinia enterocolitica* and *E. coli* in cattle fecal samples

Fecal samples taken before cattle were exposed to hog manure-treated pasture (Period 0) were negative for *Salmonella* and *Y. enterocolitica* for both years of the trial. *Salmonella* and *Y. enterocolitica* were not recovered from cattle fecal samples after these animals were allowed to graze on control and manure-treated fields during the summer of 2004 and 2005 (Period 1 to 3 in 2004 and Period 1 and 2 in 2005) (Tables 4.1 and 2). *E. coli* numbers in the cattle fecal samples ranged from 4 to 6 log CFU/g in 2004 and 2005 (Table 4.3).

Table 4.1 Occurrence of *Salmonella* in hog manure, forage and grazing cattle from manure-treated fields during two experimental years.

Year	Type of Sample			Detection of *Salmonella*	*Salmonella* serotype recovered (# of isolates)
2004	Hog Manure			+ (4 of 6)	(4) *S.* Derby 4,12:f,g:- (3) *S.* Krefeld 1,3,19:y:1,w
	Forage	Before manure		—	
		After manure		+ (4 of 40)	(6) *S.* Typhimurium 4,5,12:i:1,2 (Plot 6 and 9)
	Cattle	Period[a]	0	—	
			1	—	
			2	—	
			3	—	
2005	Hog Manure			+ (14 of 14)	(13) *S.* Derby 4,12:f,g:- (26) *S.* Krefeld 1,3,19:y:1,w
	Forage	Before manure		—	
		After manure		+ (6 of 8)[b]	(6) *S.* Derby 4,12:f,g:- (Plot 3, 5, 6, 9, 11) (4) *S.* Krefeld 1,3,19:y:1,w (Plot 2, 5, 6, 9)
		Before cattle		—	
	Soil	Before manure		—	
		After manure		—	
		Before cattle		—	
	Cattle	Period	0	—	
			1	—	
			2	—	

[a]Period 0: Cattle fecal samples taken before cattle grazed on fields
Period 1, 2, 3: Cattle fecal samples were taken monthly during a 3 month grazing period
[b]Composite samples
— Negative for *Salmonella*
\+ Positive for *Salmonella*

Table 4.2 Occurrence of *Yersinia enterocolitica* in hog manure, forage and grazing cattle from manure-treated fields during two experimental years.

Year	Type of Sample			Detection of *Y. enterocolitica*
2004	Hog Manure			—
	Forage	Before manure		—
		After manure		—
	Cattle	Period[a]	0	—
			1	—
			2	—
			3	—
2005	Hog Manure			—
	Plants	Before manure		—
		After manure		—
		Before cattle		—
	Soil	Before manure		—
		After manure		—
		Before cattle		—
	Cattle	Period	0	—
			1	—
			2	—

[a]Period 0: Cattle fecal samples taken before cattle grazed on fields
Period 1, 2, 3: Cattle fecal samples were taken monthly during a 3 month grazing period
— Negative for *Yersinia enterocolitica*

Table 4.3 Numbers of *E. coli* in hog manure and cattle fecal samples during the 2004 and 2005 experimental years.

Year	Type of Sample	*E. coli* (log CFU/g)
2004	Hog manure	4.6 – 5.3
	Cattle (fecal)	4.2 – 6.1
2005	Hog manure	4.1 – 4.4
	Cattle (fecal)	4.3 – 6.4

Table 4.4 *E. coli* numbers[a] on forage and in soil during different sampling periods in 2004 and 2005

Plot	Manure Treatment	Forage Treatment	2004	2005					
			Forage	Forage			Soil		
			After manure	Before manure	After Manure	Before Cattle	Before manure	After manure	Before Cattle
1	Control	Grazed	—[b]	—			—		
4	Control	Harvested	—	—			—		
7	Control	Grazed	—	—			—		
12	Control	Harvested	—	—			—		
2	Split	Harvested	—	—	100	—	—	10	—
6	Split	Grazed	—	—	100	—	5	10	10
9	Split	Grazed	100	—	100	—	5	10	—
10	Split	Harvested	100	—	100	—	—	10	—
3	Full	Grazed	100	—	100	—	5	10	—
5	Full	Harvested	—	—	100	—	—	10	—
8	Full	Harvested	100	—	—	—	—	10	—
11	Full	Grazed	100	—	100	—	5	10	10

[a] All counts are in CFU/g
[b] — negative for *E. coli*

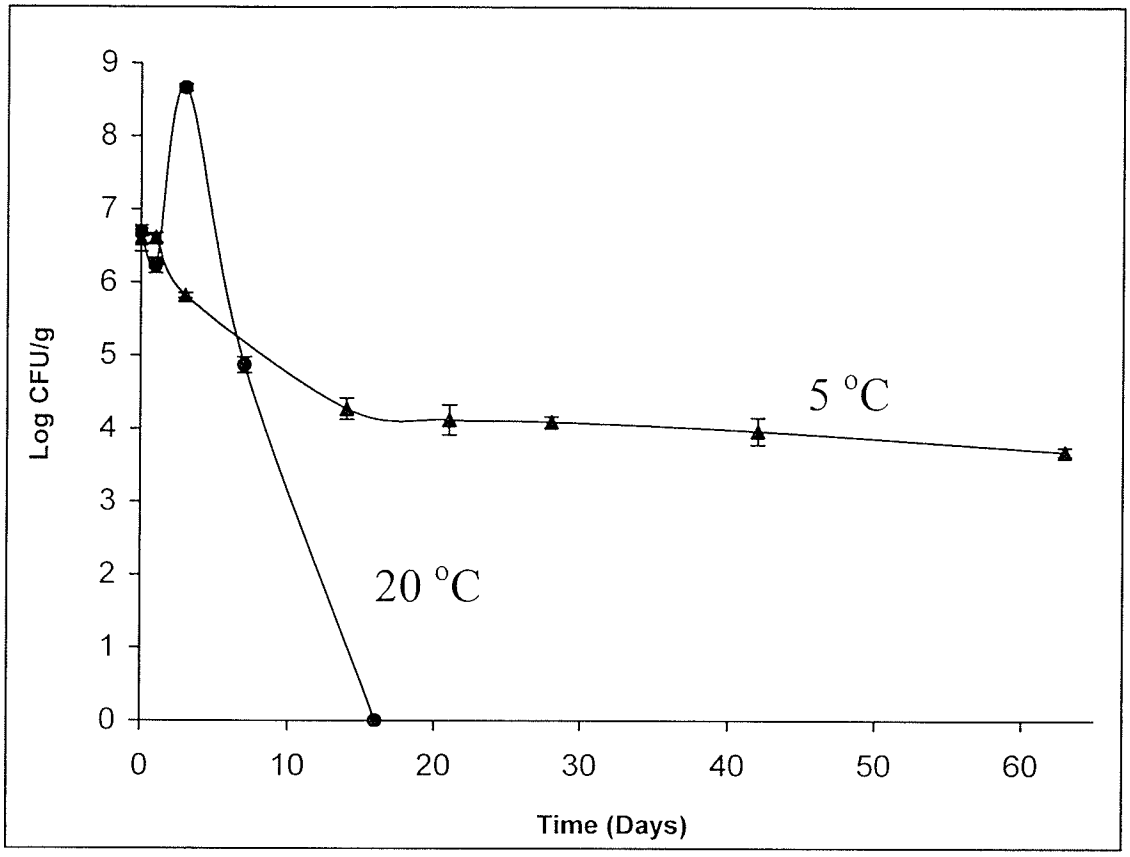


Figure 4.1 Survival of *Y. enterocolitica* in autoclaved hog manure stored at 5 °C and 20 °C. Data points are the average of three replicates plated in duplicate.

4.4 Detection of *Salmonella* and *E. coli* in groundwater

Water samples were taken at bi-monthly intervals between April 25th, 2005 and November 2nd, 2005. *E. coli* was detected in wells Hy-07, PL-01 and PL-06 on April 25th. *E. coli* was also detected in well PL-06 on July 13th (Table 4.5).

Salmonella was recovered from wells PL-02, PL-05, PL-10, PL-12 on November 2nd after the fall manure application in 2005 (Table 4.5). *Salmonella* Worthington and *S.* Manhattan were isolated from the water but these *Salmonella* serotypes were not recovered from any of the hog manure samples taken during hog manure application in 2004 or 2005.

Table 4.5 Occurrence of *E. coli* and *Salmonella* in groundwater from wells at the experimental site in 2005

Sample Dates	Method	Sample size	*E. coli* (well number)	*Salmonella* (well number and serotypes)
April 25th	Filtration	1 L	+ (PL-01) + (PL-06) + (HY-07)	—
July 13th / 14th	Filtration	1 L	+ (PL-06)	—
July 13th / 14th	[a]Quanti-Tray	100 ml	< 3 MPN/100 ml	N/A
September 27th / 28th	Filtration	1 L	—	—
September 27th / 28th	Quanti-Tray	100 ml	< 3 MPN/100 ml	N/A
November 2nd	Filtration	1L	—	+ (PL-02) + (PL-05) + (PL-10) + (PL-12) (*S.* Worthington[b] and *S.* Manhattan[c])

\+ Positive for *E. coli* or *Salmonella*
— Negative for *E. coli* or *Salmonella*
N/A Not applicable
[a]Quanti-Tray: Sample was processed using the Quanti-Tray method by EnviroTest, Winnipeg, Manitoba
[b]*S.* Worthington was isolated from wells PL-02 and PL-05
[c]*S.* Manhattan was isolated from wells PL-02, PL-10 and PL-12

4.5 *E. coli* DNA fingerprint profiles

E. coli DNA fingerprints from cattle, soil and manure samples taken during the 2004 and 2005 experimental years were compared for similarity using ClustanGraphics 7, which permitted the construction of similarity dedrograms (Fig. 4.2-4.4). A cut-off of 97 % calculated using the Simpson's index of diversity (Appendix 3) was used to cluster similar fingerprint patterns.

4.5.1 Transfer of *E. coli* from hog manure to cattle and soil

A total of 80 *E. coli* isolates (41 from 2004 and 39 from 2005) from hog manure was analysed by PCR and produced 20 different DNA fingerprint patterns from the 2004 isolates and 12 different patterns from the 2005 isolates. In soil, 18 patterns were identified from 26 *E. coli* isolates after manure application in 2004. In 2005, 20 patterns from 36 isolates taken before manure application were identified and 17 patterns from 35 isolates were identified in samples taken after manure application (before cattle were grazed). Cattle fecal samples yielded 54 DNA fingerprint patterns from 265 *E. coli* isolates in 2004 and 59 fingerprint patterns were detected from 353 *E. coli* isolates in 2005.

When *E. coli* DNA profiles from hog manure and cattle were compared, *E. coli* from hog manure did not appear to have been transferred to cattle. Only one fingerprint from cattle (Full-rate, P1) was in common with one fingerprint from hog manure (2004) (Fig. 4.3). The presence of one *E. coli* fingerprint in common between hog manure and cattle was considered not statistically significant using the Fisher's Exact test (Table 4.6). *E. coli* DNA fingerprints from hog manure were also compared to DNA

fingerprints of *E. coli* isolates from soil. No *E. coli* fingerprints from soil in 2004 and 2005 were in common with *E. coli* fingerprints from hog manure (Table 4.6 and Fig. 4.2).

4.5.2 *E. coli* diversity in cattle

E. coli DNA fingerprint patterns of organisms isolated from cattle sampled at period 0 (P0, before cattle were grazed) for each manure treatment (control, half-rate and full-rate) were compared to DNA fingerprints detected in *E. coli* from the same cattle at different sampling times (P1, P2, P3 in 2004 and P1 and P2 in 2005) to see if the *E. coli* population in the cattle changed over time (Table 4.7).

4.5.2.1 *E. coli* population of cattle on control plots

In 2004, 14 DNA fingerprint patterns were obtained from *E. coli* isolated from cattle before they were grazed (P0) on control plots. After cattle were allowed to graze, 5 of 8, 6 of 10 and 6 of 12 fingerprint patterns from *E. coli* isolated during P1, P2 and P3 sampling periods were in common with DNA fingerprints from cattle in P0. The Fisher's Exact test showed that the *E. coli* population in cattle grazing on control plots did not significantly change throughout all 3 periods ($p < 0.001$). The same result was obtained for both periods in 2005 where 8 of 18 and 6 of 10 fingerprint patterns from P1 and P2 were in common with 12 fingerprint patterns from cattle at P0 (Table 4.7).

4.5.2.2 *E. coli* population of cattle on half-rate (manured) plots

In 2004, cattle grazing on plots treated with manure applied at the half-rate at all three sampling periods seemed to have significantly different *E. coli* populations than when cattle were sampled before they were grazed. *E. coli* from cattle isolated during P1, P2 and P3 had 7 of 15, 9 of 21 and 9 of 20 patterns in common with *E. coli* isolated from cattle at P0 (18 patterns) (Table 4.7). In 2005, cattle sampled during P1 had 6 of 22 fingerprints in common with fingerprints identified during P0 (15 patterns). Statistical analysis of these results indicated that the *E. coli* population in the cattle at P1 was significantly different ($p < 0.001$) than that in cattle found at P0. This was not the case for P2 where 7 of 13 fingerprint patterns were in common with fingerprints at P0. Further analysis indicated that at both sampling times the populations were not statistically different from each other (Table 4.7). That is, the population of *E. coli* in the animals did not change during the course of the experiment.

4.5.2.3 *E. coli* population of cattle on full-rate (manured) plots

Twelve *E. coli* DNA fingerprint patterns were recovered from cattle before they were grazed in 2004. Grazing cattle sampled at P1, P2 and P3 had 4 of 9, 6 of 9 and 5 of 14 fingerprint patterns in common with patterns detected at P0. Only cattle grazing during P3 had had a significantly different *E. coli* population than before they were grazed (P0) (Table 4.7). In 2005, similar results were obtained from cattle on half-rate plots as for cattle on full-rate plots. The *E. coli* population in cattle at P1 was significantly different than in cattle sampled at P0 which had 7 of 24 patterns in common with P0 (17 different patterns). However, *E. coli* in cattle sampled at P2 had 9 of 17

patterns in common with *E. coli* in cattle at P0 and this indicated that the *E. coli* population in cattle was similar at both sampling times (Table 4.7).

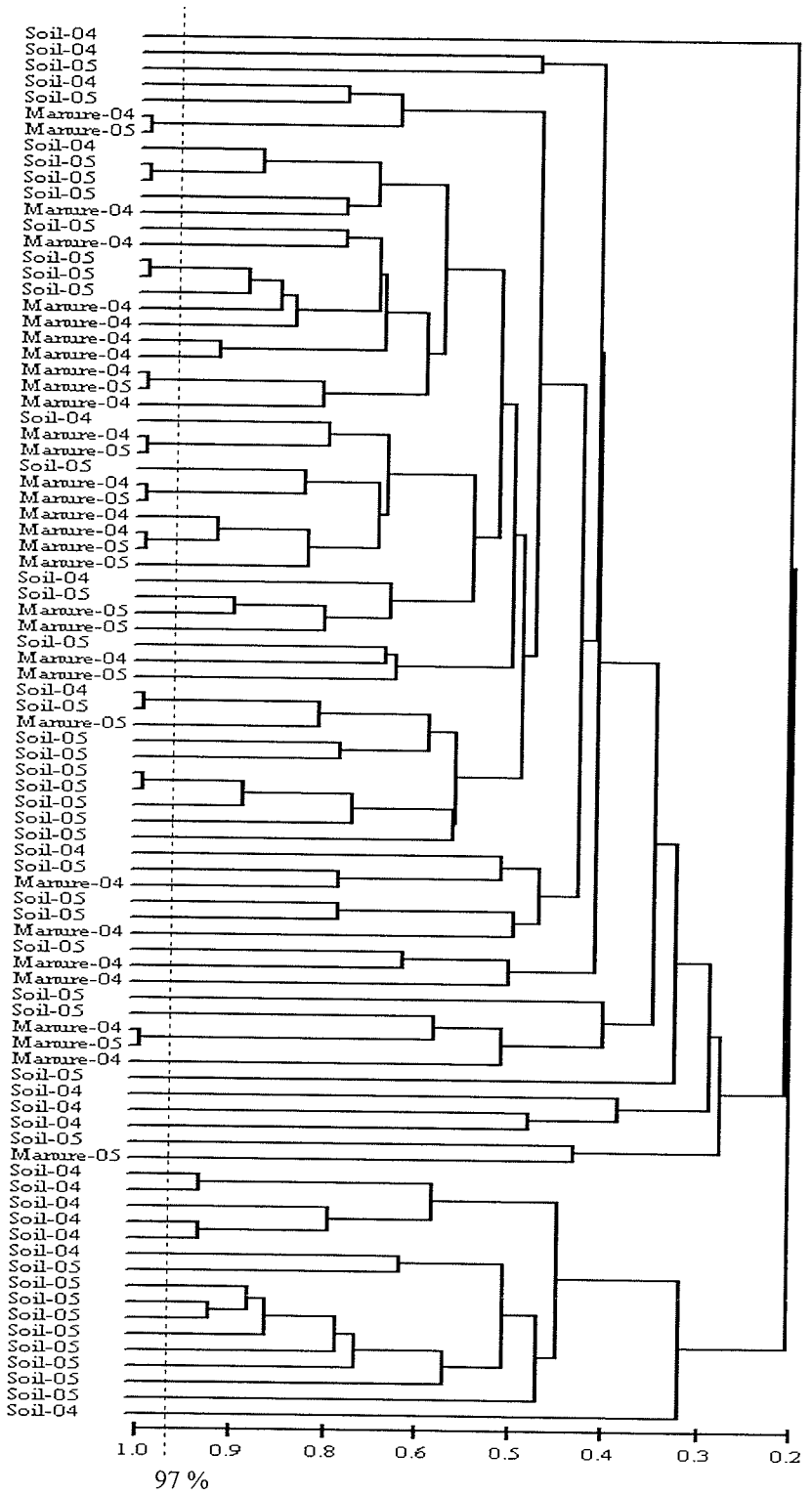


Figure 4.2 Dedrogram showing phylogenetic relationships based on the Jaccard coefficient and the average linkage clustering method (UPGMA) for the RAPD fingerprint patterns of *E. coli* isolated from soil and hog manure during the 2004 and 2005 experimental years. A cut-off of 97% (dashed line) was calculated using the Simpson's index of diversity.

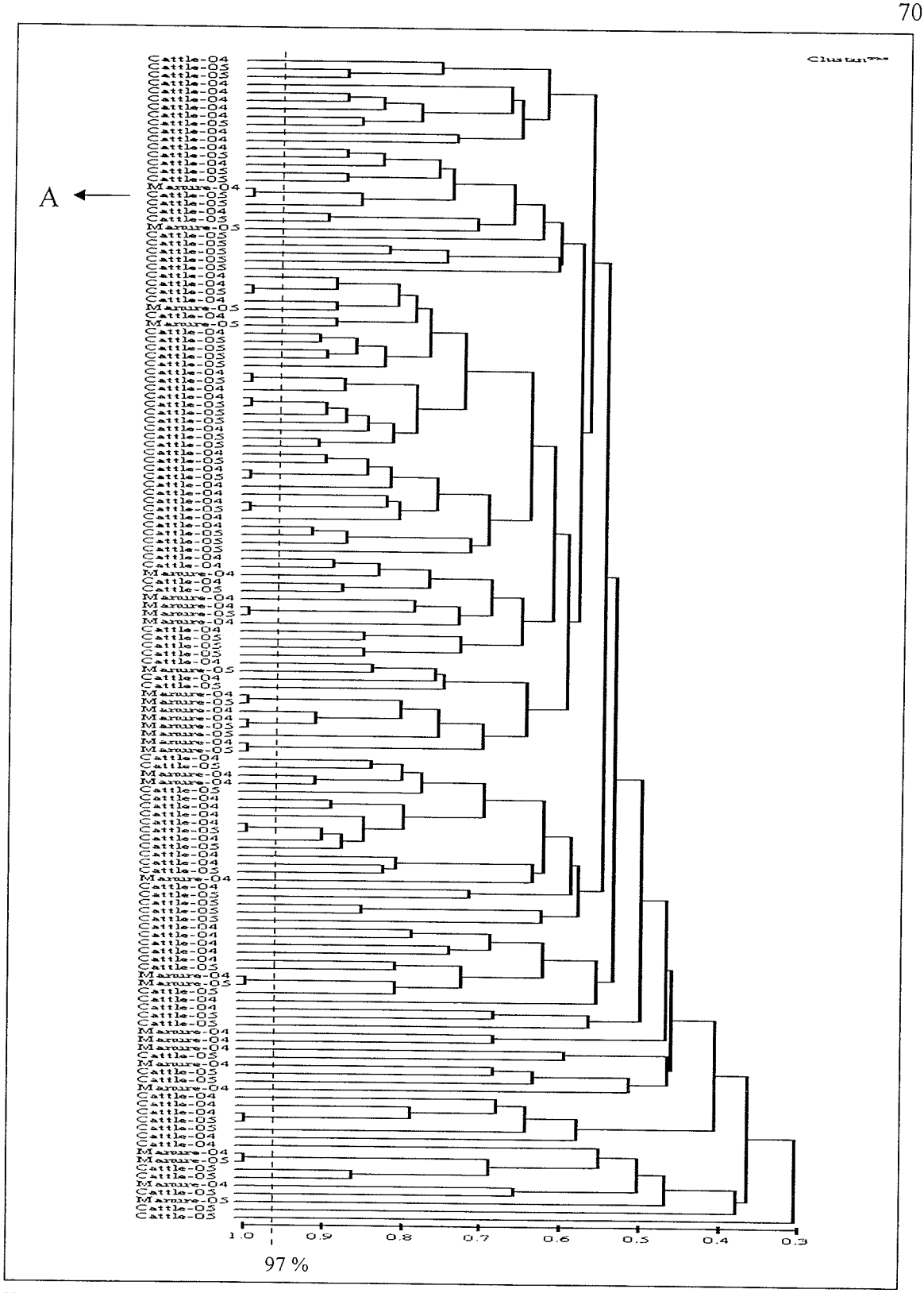


Figure 4.3 Dedrogram showing phylogenetic relationships based on the Jaccard coefficient and the average linkage clustering method (UPGMA) for the RAPD fingerprint patterns of *E. coli* isolated from cattle and from hog manure during the 2004 and 2005 experimental years. A cut-off of 97% (dashed line) was calculated using the Simpson's index of diversity.

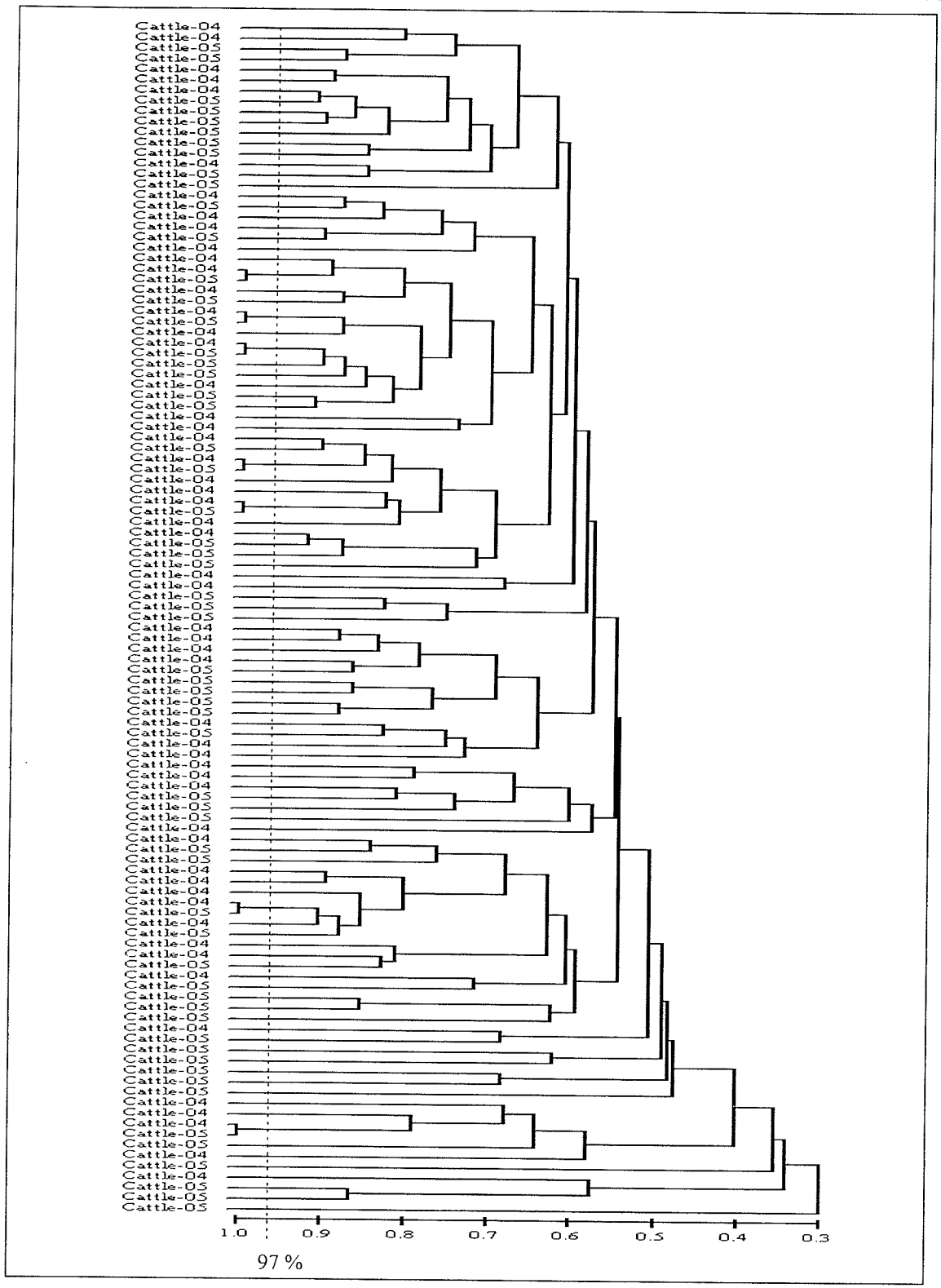


Figure 4.4 Dedrogram showing phylogenetic relationships based on the Jaccard coefficient and the average linkage clustering method (UPGMA) for the RAPD fingerprint patterns of *E. coli* isolated from cattle feces during the 2004 and 2005 experimental years. A cut-off of 97% (dashed line) was calculated using the Simpson's index of diversity.

Table 4.6 RAPD patterns in common between *E. coli* isolated from hog manure, cattle and soil during the 2004 and 2005 experimental years.

Comparison			Manure Treatment		
			Control	Half-rate	Full-rate
Hog Manure 2004	vs	Cattle 2004-P0	0	0	0
	vs	Cattle 2004-P1	0	0	0
	vs	Cattle 2004-P2	0	0	0
	vs	Cattle 2004-P3	0	0	0
Hog Manure 2004	vs	Cattle 2005-P0	0	0	0
	vs	Cattle 2005-P1	0	0	1
	vs	Cattle 2005-P2	0	0	0
	vs	Cattle 2005-P3	0	0	0
Hog Manure 2004	vs	Soil 2004	0	0	0
	vs	Soil 2005-BM	0	0	0
	vs	Soil 2005-AM	0	0	0
Hog Manure 2005	vs	Cattle 2005-P0	0	0	0
	vs	Cattle 2005-P1	0	0	0
	vs	Cattle 2005-P2	0	0	0
	vs	Cattle 2005-P3	0	0	0
Hog Manure 2005	vs	Soil 2005-BM	0	0	0
	vs	Soil 2005-AM	0	0	0

No result was statistically significant at $p < 0.001$

Table 4.7 RAPD patterns in common between *E. coli* isolated from cattle before grazing at different sampling times during the summer of 2004 and 2005.

Comparison			Manure Treatment		
			Control	Half-rate	Full-rate
Cattle 2004-P0	vs	Cattle 2004-P1	5	7*	4
	vs	Cattle 2004-P2	6	9*	6
	vs	Cattle 2004-P3	6	9*	5*
Cattle 2005-P0	vs	Cattle 2005-P1	8	6*	7*
		Cattle 2005-P2	6	7	9

*Statistically significant result ($p < 0.001$)

Chapter 5

DISCUSSION

5.1 Hog Manure

It is well known that pigs can harbour *Salmonella* in their gastrointestinal tract and that it can be shed in their feces. In fact, studies indicate that up to 24.6 % of pigs and 83 % of farms in Canada and the U.S.A. are *Salmonella*-positive (Rajic *et al.*, 2005, Letellier *et al.*, 1999, Letellier *et al.*, 1998, Davies *et al.*, 1997), therefore it was not surprising that *Salmonella* was recovered from hog manure samples taken during manure application in the spring of 2004 and 2005.

Y. enterocolitica was not recovered from hog manure samples taken during manure application in 2004 and 2005. *Y. enterocolitica* is usually found in the oral cavity and gastrointestinal tract of pigs (Schiemann *et al.*, 1981, Letellier *et al.*, 1999b), and in North America the detection of *Y. enterocolitica* in pigs can range from 13 to 42 % (Bhaduri *et al.*, 2005, Hariharan *et al.*, 1995). Therefore this organism may not be present consistently in hog manure. As in the present work, Watabe *et al.* (2003) failed to recover *Y. enterocolitica* from either unseparated pig slurry or separated solid and liquid fractions of the slurry. Failure to recover *Y. enterocolitica* from hog manure in the present study may have been because hogs supplying manure to the earthen lagoon which served as the manure source were not shedding this organism. A survey of the *Y. enterocolitica* status of the Hytek herd was not done. It is worth noting that in general, as pigs get older, the prevalence of *Y. enterocolitica* in these animals decreases (Bowman *et al.*, 2007, Nesbakken *et al.*, 2006). Nesbakken *et al.* (2006) suggested that

after the pigs reach a certain age (86-107 d), they develop immunity against *Y. enterocolitica* and will stop shedding the organism in the feces (>108 d). This might also help explain the absence of *Y. enterocolitica* in the hog manure samples used here since pigs in the barns supplying the manure lagoon were in the later part of their lives (77-196 d). If some of the animals were still shedding *Y. enterocolitica*, numbers of this organism might have been too low to detect once the organism was introduced into the lagoon and was diluted by rain, snowmelt and other fecal material.

Temperature is an important factor affecting the survival of pathogens in manure. During the present work, in autoclaved hog manure survival of *Y. enterocolitica* was greatest at 5 °C and *Y. enterocolitica* could still be detected after 64 d. In contrast, at 20 °C *Y. enterocolitica* could not be detected after 14 d. These results are similar to findings by Kearney *et al.* (1993), where *Y. enterocolitica* survived longer in pasteurized cattle manure slurry at 4 °C (Decimal reduction time (DRT) = 20.8 d) than at 17 °C (DRT = 12.8 d). Unlike organisms such as *E. coli* and *Salmonella, Y. enterocolitica* is capable of growing at refrigeration temperatures. Hog manure was spread during the spring of 2004 and 2005 and manure temperatures in the lagoon would have been <5 °C (Arrus *et al.*, 2006). This would have allowed the survival of *Y. enterocolitica* for at least 64 d and the organism should have been detected in the manure if it were present. However, the presence of competing microflora in non-sterile manure might be expected to shorten the survival of *Y. enterocolitica* in the lagoon.

5.2 Pathogen transfer from hog manure to cattle

Cattle fecal samples taken at various times during the summer of 2004 (P1, P2 and P3) and 2005 (P1, P2) were negative for *Salmonella*. Contrary to the presently reported results, Jack and Hepper (1969) found that cattle became infected with *Salmonella* after animals were exposed to a pasture that had received *Salmonella*-positive cattle slurry. A major difference between the two studies was the amount of manure applied to the pasture. The maximum amount of hog manure applied in the experiments reported here ranged from approximately 39,600 L/ha (2005) to 53,000 L/ha (2004) on full-rate plots. In Jack and Hepper's (1969) experiment, cattle slurry was applied to a pasture at a rate of approximately 230,000 L/ha. This large quantity of manure could have increased the probability that *Salmonella* present might infect grazing cattle. In another study, Taylor and Burrows (1971) also found that cattle became infected with *Salmonella* Dublin and shed this organism after grazing on a pasture treated with manure containing *Salmonella* Dublin at a concentration 6 log CFU/ml. Interestingly, when manure containing only 3 log CFU/ml *Salmonella* Dublin was applied to grazed fields, *Salmonella* Dublin was not detected in any of the cattle for the entire length of the trial (13 days), suggesting that *Salmonella* numbers in hog manure need to be quite high to infect grazing cattle (Taylor and Burrows, 1971). As in the tests by Jack and Hepper (1969), Taylor and Burrows applied manure on grass at a much higher rate (133,000 L/ha) than in the present work.

Comparisons made between *E. coli* DNA fingerprint patterns from hog manure and cattle showed with a high level of confidence that *E. coli* from hog manure was not transferred to cattle grazing on manure-treated fields. Only one fingerprint pattern from

hog manure was in common with another fingerprint from cattle and this result was not statistically significant. It is possible that by coincidence, cattle and hog manure shared the same type of *E. coli* without cattle having picked it up from hog manure. If *E. coli* were transferred from hog manure to cattle, more isolates in common between both types of animals would have been detected.

It is always difficult to conclude with absolute certainty that cattle did not acquire *E. coli* from the environment because only a proportion of the entire *E. coli* population was isolated and tested. In our study, 265 *E. coli* isolates from cattle were tested in 2004 and 353 isolates from cattle were tested in 2005. To achieve the most accurate representation of the *E. coli* population in cattle, thousands or even tens of thousands of *E. coli* isolates should be studied following recovery from fecal samples. However, *E. coli* strains are not evenly distributed and some types are more dominant than others in a given population. *E. coli* strains that are more common will have a greater probability of being isolated from a sample, therefore most of the *E. coli* isolated from cattle and hog manure would have been the most common *E. coli* species with a few exceptions.

If *E. coli* and *Salmonella* were being transferred from hog manure to cattle, the negative consequences and the hazard posed could be substantial to human, animal and environmental health. Recycling pathogens among animal species by repeated application of contaminated manure could select for better host-adapted and more virulent strains as well as increase their numbers present at any point in the cycle. *E. coli* and *Salmonella* strains from hog manure that were able to colonize the intestinal tract of

cattle could cause a significant shift in the microbial population of the new host's intestinal tract. Once established, one would expect the new strain(s) to be shed in the animal's feces and be detected in samples taken during the present study. The absence of evidence for interspecies transfer of pathogens may suggest that the management strategies used for handling manure and cattle in this study were adequate to interrupt pathogen recycling.

If a microorganism such as *E. coli* from pigs (or hog manure) were to establish a population in a different host such as cattle, it must be able to reproduce in the new host's intestinal tract. Several studies have suggested that it is difficult for introduced microorganims to successfully compete with an established microflora (Davis and Gordon, 2002, Freter *et al.*, 1983, Levy *et al.*, 1980, Williams Smith, 1975). New organisms must be superior to the native strains in competing for nutrients. The frequency of challenge and the number of new microorganisms to which the host is exposed are also factors influencing the establishment of a microorganism in a new host (Gordon and Cowling, 2003). In the present study, low numbers of *E. coli* and *Salmonella* in the environment were likely important factors influencing the lack of transfer of *E. coli*, and *Salmonella* to cattle (*Salmonella* were not quantified). In addition, competition among the introduced strain(s) and the host microflora might also have played a role.

5.3 Survival of *E. coli* and *Salmonella* on forage and soil

The delay between manure application and the introduction of cattle on the manured pasture is an important factor determining the risk of grazing cattle being infected by *E. coli* or *Salmonella* from the hog manure. The longer the period between manure application and cattle being allowed to graze, the more pathogens will be inactivated. Taylor and Burrows (1971) exposed cattle to manured pasture as early as 1 d after manure application and cattle became infected with *Salmonella*. In the present study, the time between hog manure application and the introduction of cattle on the manured pasture was 10 to 25 d in 2004 and 29 to 30 d in 2005. These delays were likely in part responsible for decreasing levels *E. coli* and *Salmonella* on the forage and in soil below numbers allowing for cattle infection.

5.3.1 *E. coli*

Up to 2 log CFU/g *E. coli* could be detected on forage samples shortly after manure application in 2004 and 2005. In 2004, *E. coli* was only detected on forage from plots that had manure applied after the snowstorm (Plots 3, 8, 9, 10, 11). The absence of *E. coli* in samples from plots which had manure applied before the snowstorm (Plot 2, 5, 6) was probably due to factors such as the 14 d delay before taking samples after hog manure application, freezing temperatures and snowmelt washing the organism from the vegetation. In 2005, forage samples were also taken before cattle were grazed and were negative for *E. coli*. The DRT of *E. coli* on vegetation has been reported to be between 1.2 to 1.7 d under natural environmental conditions (Hutchison *et al.* 2005, Taylor and Burrows, 1971). In the present tests, the delay between manure application and cattle

introduction to treated fields may have allowed enough time for *E. coli* to become undetectable on forage.

DRT's reported for *E. coli* in soil under natural environmental conditions (soil temperature between 0 °C and 24 °C) are variable and studies have reported DRT values ranging from 0.67 to 19.6 d (Huchison *et al.*, 2005, Van Donsel *et al.*, 1967, Nicholson, 2005, Côté and Quessy, 2005). The delay between manure application and cattle grazing in the present study would have been sufficient to reduce *E. coli* numbers in soil to undetectable levels since *E. coli* numbers only reached 10 CFU/g shortly after manure application.

5.3.2 *Salmonella*

In 2004, *Salmonella* was only detected in forage samples from plot 6 (manure applied before the snowstorm), 14 d after manure application and from plot 9 (manure applied after the snowstorm), 1 d after manure application. Most of the *Salmonella* from hog manure applied before the snowstorm probably did not survive freezing temperatures or were washed away by snowmelt and rain. The serotype isolated from plot 6 was *Salmonella* Typhimurium and this organism was not isolated from hog manure. On plot 6, 3 of 5 samples were positive for *Salmonella* and since sample sites were well distanced from one another, random contamination of the vegetation by wild animals was probably not the source. The same serotype of *S.* Typhimurium was also recovered from plot 9, 1 d after manure application (following the snowstorm). The source of *Salmonella* Typhimurium may have been hog manure if this organism was not

the dominant serovar of *Salmonella* present. Enrichment methods used would have obscured its detection in favour of more abundant serovars present.

In 2005, *Salmonella* was recovered in 6 of 8 composite forage samples and the *Salmonella* serotypes isolated were identical to the ones isolated from hog manure. It is puzzling why, in 2004, *Salmonella* was only detected in forage samples from plot 9, 1 d after manure application (after the snowstorm). *Salmonella* should have been detected more frequently from plots that received a manure treatment after the snowstorm and sampled 1-2 d after the manure application in 2004. The recovery of *Salmonella* on more plots in 2005 (plots 2, 3, 5, 6, 9, 11) shortly after manure application (1 d) could have been due to differences in physical properties of the hog manure used each year. The moisture content of the hog manure was 93.4 % in 2004 and 87.9 % in 2005. The difference in moisture caused the hog manure in 2005 to have a thicker consistency and seemed to increase its ability to adhere to forage. Also, the vegetation density at the start of the 2005 season was greater than the previous year due to good growth caused by manure treatment. Manure was not used prior to 2004 on test fields. This increased plant density could have prevented a significant amount of hog manure from reaching the soil, concentrating the manure on the vegetation and therefore increasing the number of *Salmonella* on the vegetation in 2005. That might also be the reason why *Salmonella* was not detected in soil shortly after manure application and before cattle were grazed in 2005. In 2004, the greater fluidity of the manure may have allowed much of the manure and any *Salmonella* present to drip from the vegetation to the soil. Unfortunately, no soil samples were analysed for *Salmonella* after manure application in 2004 to confirm its presence at that time. More moisture in the manure in 2004 could have diluted

Salmonella numbers and in consequence, made it less likely to be detectable on the forage.

5.4 Detection of *E. coli* and *Salmonella* in groundwater

Three wells were positive for *E. coli* on April 25, 2005 (HY-07, PL-01 and PL-06). Wells sampled on this date were sampled before manure application therefore it is unlikely that these wells were contaminated with *E. coli* from experimental manure treatments. In spring, snowmelt caused the water table to be high and probably facilitated the transport of *E. coli* among the fields. Since hog manure was spread on fields adjacent to the experimental site in spring, it is probable that *E. coli* responsible for contaminating the above wells came from neighbouring fields. One of the positive wells (HY-07) was considered a background well (for monitoring properties of water entering the experimental site), further suggesting that *E. coli* detected in wells before manure application did not come from the experimental site. *E. coli* was also detected in well PL-06 (plot 6) on July 13th. During this sampling period, the water table was approximately 30 cm above ground level at this well due to heavy precipitation. The plot next to this well was grazed by cattle and a large portion of the plot was also saturated with water which could have resulted in contamination of the surface water with cattle fecal material.

Salmonella was recovered from wells PL-02, PL-05, PL-10, PL-12 on November 2nd, after fall manure application in 2005. The types of *Salmonella* isolated were *S.* Worthington and *S.* Manhattan. These S*almonella* serovars were not recovered from any of the hog manure samples taken during hog manure application in 2004 or 2005.

Hog manure from the same earthen lagoon used in the present study was applied to other pastures in the area, and could have contributed to the contamination of the well water.

E. coli and *Salmonella* have been shown to be able to survive <100 d in autoclaved or natural water from different sources (Karapinar and Gönül, 1991, Henis *et al.*, 1989, Filip *et al.*, 1988, Bitton *et al.*, 1983). Temperature is a major factor influencing pathogen survival in water and studies indicate that the colder the temperature of the water, the longer these organisms will survive (Wang and Doyle 1998, Flint, 1987, McFeters and Stuart 1972). In the present study, most of the contamination in the wells was detected in spring or late fall when the water would have been cold and could have enabled *E. coli* and *Salmonella* to survive for extended periods of time, increasing the risk of human illness following consumption of contaminated water or crops. The presence of *E. coli* and *Salmonella* in test wells, although not necessarily as a result of hog manure application on the experimental site, illustrates the potential risk associated with application of untreated manure on fields as a fertilizer.

Chapter 6

CONCLUSIONS

- *Salmonella* and *E. coli* were present in hog manure applied on experimental plots during both years studied. *Yersinia enterocolitica* was not detected in hog manure at anytime during the study.

- In a laboratory study, *Yersinia enterocolitica* inoculated into autoclaved hog manure was able to survive at least 64 d when stored at 5 °C but only survived 14 d when stored at 20 °C. If present in a manure lagoon, *Yersinia enterocolitica* could possibly survive for long periods of time especially at low spring and fall temperatures when manure is being applied to fields.

- *Salmonella* and *E. coli* were detected on forage shortly after manure application but were not detected on forage before cattle were grazed. *Salmonella* was not detected in soil at any time before or after manure application during both years of the study.

- During this study, *Salmonella* and *E. coli* present in hog manure did not appear to have been transferred to cattle grazing on manure-treated fields. Low numbers of *E. coli* and *Salmonella* on vegetation and in soil at the time cattle started to graze may have been the main reason cattle did not acquire organisms from the applied hog manure.

- *E. coli* was detected in one background well as well as in two wells on the experimental site before manure application in 2005, suggesting that the source of contamination might be not be linked to experimental tests conducted. Contamination observed may have been from fields adjacent to the test site that were also treated with manure.

- *Salmonella* was detected in 4 wells 20 d after manure application in the fall of 2005. *Salmonella* serovars recovered from these wells could not be traced back to serovars isolated from hog manure.

Chapter 7

RECOMMENDATIONS FOR FUTURE RESEARCH

- Further study of technologies to reduce bacterial loads in manure such as composting, aeration, and anaerobic digestion should be continued along with work to make these technologies accessible and affordable to animal producers.

- Work should continue to investigate the survival of pathogens in the environment to eventually establish safe minimum intervals between manure application and the introduction of grazing cattle on these manure-treated fields to prevent pathogen transfer and provide a scientific basis for new regulations.

- Work is needed to optimize manure application to fields to achieve the greatest vegetation yield while minimizing the microbial load introduced on pastures to reduce widespread environmental contamination caused by excessive manure application.

- The effect of soil type on the survival of pathogens and their transport to groundwater needs to be further studied. The risks of manure application should be determined in different geographic areas having different soil types. This will enable manure management practices to be customized for each area.

- Research should be done to better characterize the ability of zoonotic pathogens in manure to establish themselves in the cattle gastrointestinal tract and be shed by these animals.

- Work should be undertaken to determine whether changes in the composition (nutrients, pH) of hog manure slurry during storage can accelerate declines in zoonotic pathogen survival.

References

Abu-Ashour, J. and Lee, H. 2000. Transport of bacteria on sloping soil surfaces by runoff. Environ. Toxicol. 15:149-153.

Andrews, W.H., Poelma, P.L. and Wilson, C.R. 1981. Comparative efficiency of brilliant green, bismuth sulfite, *Salmonella-Shigella*, hektoen enteric, and xylose lysine desoxycholate agars for the recovery of *Salmonella* from foods: collaborative study. J. Assoc. Off. Anal. Chem. 64:899-928.

Arrus, K.M., Holley, R.A., Ominski, K.H., Tenuta, M. and Blank, G. 2006. Influence of temperature on *Salmonella* survival in hog manure slurry and seasonal temperature profiles in farm manure storage reservoirs. Livest. Sci. 102:226-236.

Artz, R.R.E., Avery, L.M., Jones, D.L. and Killham, K. 2006. Potential pitfalls in the quantitative molecular detection of *Escherichia coli* O157:H7 in environmental matrices. Can. J. Microbiol. 52:482-488.

Bach, S.J., McAllister, T.A., Veira, D.M., Gannon, V.P.J. and Holley, R.A. 2002. Transmission and control of *Escherichia coli* O157:H7- A review. Can. J. Anim. Sci. 82: 475–490.

Baggensen, D.L., Wegener, H.C., Bager, F., Stege, H., Christiensen, J. 1996. Herd prevalence of *Salmonella enterica* infection in a Danish slaughter herds determined by microbiological testing. Prev. Vet. Med. 26:201-213.

Baloda, S.B., Christensen, L. and Trajcevska, S. 2001. Persistance of a *Salmonella enterica* Serovar Typhimurium DT12 clone in a piggery and in agricultural soil amended with *Salmonella*-contaminated slurry. Appl. Environ. Microbiol. 67:2859-2862.

Besser, T.E., Richards, B.L., Rice, D.H. and Hancock D.D. 2001. *Escherichia coli* O157:H7 infection of calves: infectious dose and direct contact transmission. Epidemiol. Infect. 127:555-560.

Bélanger, S.D., Boissinot, M., Clairoux, N., Picard, J.L. and Bergeron, M.G. 2003. Rapid detection of *Clostridium difficile* in feces by real-tim PCR. J. Clin. Microbiol. 41:730-734.

Beutin, L., Geier, D., Steinrück, H., Zimmerman, S. and Scheutz, F. 1993. Prevalence and some properties of verotoxin (shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. J. Clin. Microbiol. 31:2483-2488.

Bhaduri, S., Wesley, I.V. and Bush, E.J. 2005. Prevalence of pathogenic *Yersinia enterocolitica* strains in pigs in the United States. Appl. Environ. Microbiol. 71:7117-7121.

Bhan, M.K., Bahl, R. and Bhatnagar, S. 2005. Typhoid and paratyphoid fever. Lancet, 366: 749-762.

Bitton, G., Farrah, S.R., Ruskin, R.H., Butner, J. and Chou, Y.J. 1983. Survival of pathogenic and indicator organisms in ground water. Ground Water, 21:405-410.

Birch, M., Denning, D.W. and Law, D. 1996. Rapid genotyping of *Escherichia coli* O157 isolates by random amplification of polymorphic DNA. Eur. J. Clin. Microbiol. Infect. Dis. 15:297-302.

Blanco, J.E., Blanco, M., Alonso, M.P., Mora, A., Dahbi, G., Coira, M.A. and Blanco, J. 2004. Serotypes, virulence genes, and intimin types of shiga toxin (Verotoxin)-producing *Escherichia coli* isolates from human patients: prevalence in Lugo, Spain, from 1992 through 1999. J. Clin. Microbiol. 42:311-319.

Black, R.E., Jackson, R. J., Tsai, T., Medvesky, M., Shayegani, M., Feeley, J.C., MacLeod, K.I.E. and Wakelee, A.M. 1978. Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. N. Eng. J. Med. 298:76-79.

Bottone, E.J. 1997. *Yersinia enterocolitica*: The charisma continues. Clin. Microbiol. Rev. 10:257-276.

Bottone, E.J. 1999. *Yersinia enterocolitica*: overview and epidemiologic correlates. Microb. Infect. 1:323-333.

Bowman, A.S., Glenening, C., Wittum, T.E., LeJeune, J.T. Stich, R.W. and Funk, J.A. 2007. Prevalence of *Yersinia enterocolitica* in different phases of production on swine farms. J. Food Prot. 70:11-16.

Campbell, G.R., Prosser, J., Glover, A. and Killham, K. 2001. Detection of *Escherichia coli* O157:H7 in soil and water using multiplex PCR. J. Appl. Microbiol. 91:1004-1010.

Caprioli, A., Morabito, S. Brugère, H. and Oswald, E. 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Vet. Res. 36:289-311.

Chao, W.-L., Ding, R.-J. and Chen, R.-S. 1988. Survival of *Yersinia enterocolitica* in the environment. Can. J. Microbiol. 34:753–756.

Cieslak, P.R., Barrett, T.J., Griffin, P.M., Gensheimer, K.F., Beckett, G., Buffington, J., Smith, M.G. 1993. *Escherichia coli* O157:H7 infection from a manured garden. Lancet, 342:367.

Cobbold, R. and Desmarchelier, P. 2002. Horizontal transmission of shiga toxin-producing *Escherichia coli* within groups of dairy calves. Appl. Environ. Microbiol. 68:4148-4152.

Cools, D., Merckx, R., Vlassak, K. and Verhaegen, J. 2001. Survival of *E. coli* and *Enterococcus* spp. derived from pig slurry in soils of different texture. Appl. Soil Ecol. 17:53-62.

Côté, C. and Quessy, S. 2005. Persistance of *Escherichia coli* and *Salmonella* in surface soil following application of liquid hog manure for production of pickling cucumbers. J. Food Prot. 68:900-905.

Cox, N.A., Bailey, J.S., Del Corral, F., Shotts, E.B. 1990. Comparison of enrichment and plating media for isolation of *Yersinia*. Poult Sci. 69:686-93.

David, S.T., MacDougall, L., McIntyre, L., Paccagnella, A.M., Schleicher, S., Hamade, A. 2004. Petting zoo-associated *Escherichia coli* O157:H7 - Secondary transmission, asymptomatic infection, and prolonged shedding in the classroom. Public Health Agency of Canada. Canada Communicable Disease Report. November 12 Vol. 33-22. URL : http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/04vol30/dr3020ea.html. Access date: August 28, 2006.

Davies, P.R., Morrow, W.E.M., Jones, F.T., Deen, J., Fedorka-Cray, J., Harris, I.T. 1997. Prevalence of *Salmonella* in finishing swine raised in different production systems in NC, USA. Epidemiol. Infect. 119:237-244.

Davis, S.A. and Gordon, D.M. 2002. The influence of host dynamics on the clonal composition of *Escherichia coli* populations. Environ. Microbiol. 4:306-313.

Davis, S.A. and Cowling, A. 2003. The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effect. Microbiology, 149:3575-3586.

de Boer, E. 1992. Isolation of *Yersinia enterocolitica* from foods. Int. J. Food Microbiol. 17: 75-84.

de Boer, E., and Heuvelink, A. E. 2000. Methods for the detection and isolation of Shiga toxin-producing *Escherichia coli*. Symp. Ser. Soc. Appl. Microbiol. 29:133S-143S.

Dombek, P.E., Johnson, L.-A.K., Zimmerley, S.T. and Sadowsky, M.J. 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Appl. Environ. Microbiol. 66:2572-2577.

Dunigan, E.P. and Dick, R.P. 1980. Nutrient and coliform losses in runoff from fertilized and sewage sludge-treated soil. J. Environ. Qual. 9:243-250.

Fedorka-Cray, P.J., Whipp, S.C., Isaacon, R.E., Nord, N. and Lager, K. 1994. Transmission of *Salmonella* Typhimurium to swine. Vet. Microbiol. 41:333-344.

Filip, Z., Kaddu-Mulindwa, D. and Milde, G. 1988. Survival of some pathogenic and facultative pathogenic bacteria in groundwater. Wat. Sci. Tech. 20:227-231.

Flint, K.P. 1987. The long-term survival of *Escherichia coli* in river water. J. Appl. Bacteriol. 63:261-270.

Fortin, N.Y., Mulchandani and Chen, W. 2001. Use of real-time polymerase chain reaction and molecular beacons for the detection of *Escherichia coli* O157:H7. Anal. Biochem. 289:281-288.

Frahm, E. and Obst, U. 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J. Microbiol. Methods 52:123-131.

Franz, E., van Diepeningen, A.D., de Vos, O.J. amd van Bruggen, A.H.C. 2005. Effects of cattle feeding regimen and soil management type on the fate of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in manure, manure-amended soil and lettuce. Appl. Environ. Microbiol. 71:6165-6174.

Fredriksson-Ahomaa, M., Björkroth, J., Hielm, S. and Korkeala, H. 2000. Prevalence and characterization of pathogenic *Yersinia enterocolitica* in pig tonsils from different slaughterhouses. Food Microbiol. 17:93-101.

Freter, R., Brickner, H., Fekete, J., Vickerman, M.M. and Carey, K.E. 1983. Survival and implantation of *Escherichia coli* in the intestinal tract. Infec. Immun. 39:686-703.

Fukushima, H., Tsunomori, Y. and Ryotaro, S. 2003. Duplex real-time PCR assays for the detection of 17 species of food or waterborne pathogens in stools. J. Clin. Microbiol. 41:5134-5146.

Gessel, P.D., Hansen, N.C., Goyal, M.S., Johnston, L.J. and Webb, J. 2004. Persistance of zoonotic pathogens in surface soil treated with different rates of liquid pig manure. Appl. Soil Ecol. 25:237-243.

Gordon, C. and Toze, S. 2003. Influence of groundwater characteristics on the survival of enteric viruses. J. Appl. Microbiol. 95:536-544.

Gruber, A.K., Shelton, D.R. and Pachepsky, Y.A. 2005. Effect of manure on *Escherichia coli* attachment to soil. J. Environ. Qual. 34:2086-2090.

Guan, T.Y. and Holley, R.A. 2003 *Hog Manure Management, the Environment and Human Health.* Plenum Publishing Corporation, 179pp.

Gyles, C.L., Friendship, R., Ziebell, K., Johnson, S., Yong, I., Amezcua, R. 2002. *Escherichia coli* O157:H7 in pigs. Expert Committee on Meat and Poultry Products Annual Meeting Report. pp. 48-49 (Sept. 17, Guelph ON). Canada Committee on Food, Canadian Agri-Food Research Council, Ottawa ON, Nov. 19.

Hariharan, H., Giles, J.S., Heaney, S.B., Leclec, S.M. and Schurman, R.D. 1995. Isolation, serotypes, and virulence-associated properties of *Yersinia enterocolitica* from the tonsils of slaughter hogs. Can. J. Vet. Res. 59:161-166.

Heinonen-Tanski, H., Niskanen, E.M., Salmela, P. and Lanki, E. 1998. *Salmonella* in animal slurry can be destroyed by aeration at low temperatures. J. Appl. Microbiol. 85:277-281.

Henis, Y., Gurijala, K.R. and Alexander, M. 1989. Factors involved in multiplication and survival of *Escherichia coli* in lake water. Microbial Ecol. 17:171-180.

Heuvelink, A.E., Zwartkruis-Nahuis, J.T.M., van den Biggelaar, F.L.A.M., van Leeuwen, W.J. and de Boer, E. 1999. Isolation and characterization of verotoxin-producing *Escherichia coli* O157 from slaughter pigs and poultry. Int. J. Food Microbiol. 52:67-75.

Himathongkham, S., Bahari, S., Reimann, H. and Cliver, D. 1999. Survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in cow manure and cow manure slurry. FEMS Microbiol. Lett. 178:251-257.

Hofmann, N. and Beaulieu, M.S. 2006. A geographical profile of manure production in Canada, 2001. Statistics Canada, Agriculture Division. URL: http://www.statcan.ca/cgi-bin/downpub/listpub.cgi?catno=21-601-MIE2006077. Access date: August 28, 2006.

Holley, R.A., Arrus, K.M., Ominski, K.H., Tenuta, M. and Blank, G. 2006. *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. J. Environ. Qual. 35:1170-1180.

Hu, Y., Zhang, Q. and Meitzler, J.C. 1999. Rapid and sensitive detection of *Escherichia coli* O157:H7 in bovine feces by a multiplex PCR. J. Appl. Microbiol. 87:867-876.

Hutchison, M.L., Walters, L.D., Moore, T., Thomas, D.J.I. and Avery, S.M. 2005. Fate of pathogens present in livestock wastes spread onto fescue plots. Appl. Environ. Microbiol. 71:691-696.

Ibekwe, A.M. and Grieve C.M. 2003. Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR. J. Appl. Microbiol. 94:421-431.

Jack, E.J. and Hepper, P.T. 1969. An outbreak of *Salmonella* Typhimurium infection in cattle associated with the spreading of slurry. Vet. Rec. 84:196-199.

Jensen, A.N., Dalsgaard, A., Stockmarr, A., Neilsen, E.M. and Baggesen, D.L. 2006. Survival and transmission of *Salmonella enterica* Serovar Typhimurium in an outdoor organic pig environment. Appl. Environ. Microbiol. 72:1833-1842.

Jiang, J., Morgan, J. and Doyle, M.P. 2002. Fate of *Escherichia coli* O157:H7 in manure-amended soil. Appl. Environ. Microbiol. 68:2605-2609.

Johansson, M., Emmoth, E., Salomonsson, A.-C. and Albihn, A. 2005. Potential risks when spreading anaerobic digestion residues on grass silage crops – survival of bacteria, moulds and viruses. Grass Forage Sci. 60:175-185.

Jones, T.F. 2003. From pig to pacifier: chitterling-associated yersiniosis outbreak among black infants. Emerg. Infect. Dis. 9:1007-1009.

Jones, P.W., Smith, G.S. and Bew, J. 1977. The effect of the microflora in cattle slurry on the survival of *Salmonella* Dublin. Br. Vet. J. 133:1-8.

Jones, P.W. 1976. The effect temperature, solids content and pH on the survival of Salmonellas in cattle slurry. Br. Vet. J. 132:284-292.

Karapinar, M. and Gönül, S.A. 1991. Survival of *Yersinia enterocolitica* and *Escherichia coli* in spring water. Int. J. Food Microbiol. 13:315-320.

Kaufmann, M., Zweifel, C., Blanco, M., Blanco, J.E., Blanco, J., Beutin, L. and Stephan, R. 2006. *Escherichia coli* O157 and non-O157 Shiga toxin-producing *Escherichia coli* in fecal samples of finished pigs at slaughter in Switzerland. J. Food Protect. 69:260-266.

Kearney, T.E, Larkin, M.J. and Levett, P.N. 1993. The effect of slurry storage and anaerobic digestion on survival of pathogenic bacteria. J. Appl. Bacteriol. 74:86-93.

Kudva, I.T., Blanch, K. and Hovde, C.J. 1998. Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. App. Environ. Microbiol. 64:3166-3174.

Lee, L.A., Gerber, A.R., Lonsway, D.R., Smith, J.D., Carter, G.P., Pohr, N.D., Parrish, C.M., Sikes, R.K., Flinton, R.J. and Tauxe, R.W. 1990. *Yersinia enterocolitica* O:3 infection in infants and children associated with the household preparation of chitterlings. N. Eng. J. Med. 322:984-987.

Letellier, A., Messier, S., Paré, J., Ménard, J. and Quessy, S. 1999a. Distribution of *Salmonella* in swine herds in Québec. Vet. Microbiol. 67:299-306.

Letellier, A., Messier, S., and Quessy, S. 1999b. Prevalence of *Salmonella* spp. and *Yersinia enterocolitica* in finishing swine at Canadian abattoirs. J. Food Prot. 62:22-25.

Levy, S.B., Marshall, B. and Rowse-Eagle, D. 1980. Survival of *Escherichia coli* host-vector systems in the mammalian intestine. Science, 209:391-394.

Manitoba Health 2006. Monthly summary of communicable diseases. Communicable Disease Control Unit. URL: http://www.gov.mb.ca/health/publichealth/cdc/surveillance/index.html#summary. Access date: August 28, 2006.

Manitoba Pork Council 2004. 2004 Annual report and financial statements. URL: http://www.manitobapork.com/library.php. Access date: August 28, 2006.

Marsh, P., Morris, N.Z. and Wellington, M.H. 1998. Quantitative molecular detection of of *Salmonella* Typhimurium in soil and demonstration of persistence of an active but non-culturable population. FEMS Microbiol. Ecol. 27:351-363.

Mawdsley, J.L., Bardgett, R.D., Merry, R.J., Pain, B.F. and Theodorou, M.K. 1995. Pathogens in livestock waste, their potential for movement through soil and environmental pollution. Appl. Soil Ecol. 2:1-15.

McFeters, G.A. and Stuart, D.G. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. Appl. Microbiol. 24:805-811.

McFeters, G.A. and Terzieva, S.I. 1991. Survival of *Escherichia coli* and *Yersinia enterocolitica* in stream water: Comparison of field and laboratory exposure. Microbial Ecol. 22:65-74

McGee, P., Scott, L., Sheridan, J. J., Earley, B. and Leonard, N. 2004. Horizontal transmission of *Escherichia coli* O157:H7 during cattle housing. J. Food Prot. 67:2651-2656.

Meng, J., Doyle, M.P., Zhao, T. and Zhao, S. 2001. Enterohemorrhagic *Escherichia coli*. Pages 193-213 in: *Food Microbiology: Fundamentals and Frontiers*. Doyle, M.P., Beuchat, L.R. and Montville T.J., ASM Press, Washington, D.C.

Nakazawa, M., Akiba, M. and Sameshima, T. 1999. Swine as a potential reservoir of Shiga toxin-producing Escherichia coli O157:H7 in Japan. Emerg. Infect. Dis. 5:833-834.

Nam, H.-M., Srinivasan, V., Gillespie, B.E., Murinda, S.E. and Oliver, S.P. 2005. Application of SYBR green real-time PCR assay for specific detection of *Salmonella* spp. in dairy farm environmental samples. Int. J. Food Microbiol. 102:161-71.

Nesbakken, T., Iversen, T., Eckner, K. and Lium, B. 2006. Testing of pathogenic *Yersinia enterocolitica* in pig herds based on the natural dynamic of infection. Int. J. Food Microbiol. 111:99-104.

Nicholson, F.A., Groves, S.J. and Chambers, B.J. 2005. Pathogen survival during livestock manure storage and following land application. Biores. Technol. 96:135-143.

Oliver, D.M., Heathwaite, L., Haygarth, P.M. and Clegg, C.D. 2005. Transfer of *Escherichia coli* to water from drained and undrained grassland after grazing. J. Environ. Qual. 34:918-925.

Osek, J. 2002. Rapid and specific identification of Shiga toxin-producing *Escherichia coli* in faeces by multiplex PCR. Lett. Appl. Microbiol. 34:304-310.

Pell, A.N. 1997. Manure and microbes: public and animal health problem? J. Dairy Sci. 80:2673-2681.

Plachà, I., Venglovsky, J., Sasakova, N. and Svoboda, I.F. 2001. The effect of summer and winter seasons on the survival of *Salmonella typhimurium* and indicator microorganisms during the storage of solid fraction of pig slurry. J. Appl. Microbiol. 91:1036-1043.

Prescott, L.M., Harley, J.P. and Klein, D.A. 1995. Chapitre 21: Les bactéries Gram-négatives d'importance générale, médicale ou industrielle. Pages 426-449 in Microbiologie, 2e Édition. Translated from English by Bacq-Calberg, C.-M., Coyette, J., Hoet, P. and Nguyen-Distèche, M.. De Boeck-Wesmael S.A., Brussels, Belgium.

Public Health Agency of Canada 2000. Waterborne outbreak of gastroenteritis associated with contaminated municipal water supply, Walkerton, Ontario, May-June 2000. Canada Communicable Disease Report October 15, Vol. 26-20 URL: http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/00vol26/dr2620eb.html. Access date: August 28, 2006.

Public Health Agency of Canada 2004. Notifiable disease incidence by province/territory. URL: http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/c_prv_e.html Access date: August 28, 2006.

Public Health Agency of Canada 2005. Outbreaks of *Salmonella* infections associated with eating Roma tomatoes - United States and Canada, 2004. Canada Communicable Disease Report November 1, Vol. 31-21 URL: http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/05vol31/dr3121eb.html. Access date: August 28, 2006.

Public Health Agency of Canada 2006. Material Safety Data Sheet – Infectious Substances: *Yersinia enterocolitica, Yersinia pseudotuberculosis*. URL: http://www.phac-aspc.gc.ca/msds-ftss/msds168e.html. Access date: August 28, 2006.

Pulz, M., Matussek, A., Monazahian, M., Tittel, A., Nikolic, E., Hartmann, M., Bellin, T., Buer, J. and Gunzer, F. 2003. Comparison of a shiga toxin enzyme-linked immunosorbant assay and two types of PCR for detection of shiga toxin-producing *Escherichia coli* in human stool specimens. J. Clin. Microbiol. 41:4671-4675.

Rajić, A., Keenliside, J., McFall, M.E, Deckert, A.E., Muckle, A.C., O'Connor, B.P., Manninen, K., Dewey, C.E. and McEwen, S.A. 2005. Longitudinal study of *Salmonella* species in 90 Alberta swine finishing farms. Vet. Microbiol. 105:47–56.

Rasmussen, M.A., Cray, Jr., W.C., Casey, T.A. and Whipp, S.C. 1993. Rumen contents as a reservoir of enterohemorrhagic *Escherichia coli*. FEMS Microbiol Lett. 114:79-84.

Roodsari, R.M., Shelton, D.R., Shirmohammadi, A., Pachepsky, Y.A., Sadeghi, A.M. and Starr, J.L. 2005. Fecal coliform transport as affected by surface conditions. Am. Soc. Agr. Eng. 48:1055-1061.

Rusin, P., Enriquez, C.E., Johnson, D. and Gerba, C.P. 2000. Environmentally transmitted pathogens. Pages 447-490 in: Environmental Microbiology. Maier, R.M., Pepper, I.M. and Gerba, C.P. (editors), Academic Press, San Diego, CA., USA.

Rys, P.N. and Persing, D.H. 1993. Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. J. Clin. Microbiol. 31: 2356–2360.

Santo Domingo, J.W., Harmon, S. and Bennett, J. 2000. Survival of *Salmonella* species in river water. Cur. Microbiol. 40:409-417.

Scheuerman, P.R., Schmidt, J.P. and Alexander, M. 1988. Factors affecting the survival and growth of bacteria introduced into lake water. Arch. Microbiol. 150:320-325.

Schiemann, D.A. and Fleming, C.A. 1981. *Yersinia enterocolitica* isolated from throats of swine in eastern and western Canada. Can. J. Microbiol. 27:1326-1333.

Schutze, G.E., Kirby, R.S., Flick, E.L., Stefanova, R., Eisenach, K.D. and Cave, M.D. 1998. Epidemiology and molecular identification of *Salmonella* infections in children. Arch. Pediatr. Adolesc. Med. 152:659-664.

Somarelli, J.A., Makarewicz, J.C., Sia, R. and Simon R. 2006. Wildlife identified as a major source of Escherichia coli in agriculturally dominated watersheds by BOX-A1R-derived genetic fingerprints. J. Environ. Manage. In Press.

Spano, G., Beneduce, L., Terzi, V., Stanca, A.M. and Massa, S. 2005. Real-time PCR for the detection of *Escherichia coli* O157:H7 in dairy and cattle wastewater. Lett. Appl. Microbiol. 40:164-171.

Statistics Canada 2006. Hog Statistics. URL: http://www.statcan.ca/cgi-bin/downpub/listpub.cgi?catno=23-010-XIE2006003. Access date: August 28, 2006.

Statistics Canada 2001. Applications of manure to the land, by province (2001 Census of Agriculture). URL: http://www40.statcan.ca/l01/cst01/agrc05h.htm. Access date: August 28, 2006.

Sutcliffe, P., Picard, L., Fortin, B., Malaviarachchi, D., Hohenadel, J. and O'Donnell, B. 2004. *Escherichia coli* O157:H7 outbreak at a summer hockey camp, Sudbury, 2004. Public Health Agency of Canada. Canada Communicable Disease Report. November 12 Vol. 33-22. URL : http://www.phac-aspc.gc.ca/publicat/ccdr-mtc/04vol30/dr3022ea.html. Access date: August 28, 2006.

Synge, B. and Paiba, G. 2000. Verocytotoxin-producing *E. coli* O157. Vet. Rec. 147:27-28.

Tacket, C.O., Ballared, J., Harris, N., Allard, J., Nolan, C., Quan, T. and Cohen, M.L. 1985. An outbreak of *Yersinia enterocolitica* infections caused by contaminated tofu (soybean curd). Am. J. Epidemiol. 121:705-486.

Tashiro, K., Kubokura, Y., Kaneko, K. and Ogawa, M. 1991. Survival of *Yersinia enterocolitica* in soil and water. J. Vet. Med. Sci. 53:23-27.

Taylor, R.J. and Burrows, M.R. 1971. The survival of *Escherichia coli* and *Salmonella* Dublin in slurry, on pasture and the infectivity of *S.* Dublin for grazing calves. Br. Vet. J. 127:536-542.

U.S. Food and Drug Administration, 2006. Nation wide *E. coli* O157:H7 outbreak: questions and answers. URL: http://www.cfsan.fda.gov/~dms/spinacqa.html. Access date: December 28, 2006.

van der Wolf, P.J., Bongers, J.H., Elbers, A.R.W., Franssen, F.M.M.C., Hunneman, W.A., van Exsel, A.C.A., Tielen, M.J.M. 1999. *Salmonella* infection in finishing pigs in the Netherlands: bacteriological herd prevalence, serogroup and antibiotic resistance of isolates and risk factors for infection. Vet. Microbiol. 67:263-275.

Van Donsel, D.J., Geldreich, E.E. and Clarke, N.A. 1967. Seasonal variations in survival of indicator bacteria in soil and their contribution to storm-water pollution. Appl. Microbiol. 15:1362-1370.

Van Horn, H.H., Wilkie, A.C., Powers, W.J., Nordstedt, R.A. 1994. Components of dairy manure management systems. J. Dairy. Sci. 77:2008-30.

Vaneechoutte M. and Van Eldere, J. 1997. The possibilities and limitations of nucleic acid amplification technology in diagnostic microbiology. J. Med. Microbiol. 46:188-194.

Walker, P.J. and Grimes D.J. 1985. A note on *Yersinia enterocolitica* in a swine farm watershed. J. Appl. Bacteriol. 58:139-143.

Wang, G., Clark, C.G. and Rodgers, F.G. 2002. Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 Shiga toxin family by multiplex PCR. J. Clin. Microbiol. 40:3613-3619.

Wang, G. and Doyle, M.P. 1998. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in water. J. Food Prot. 61:662-667.

Wang, G., Zhao, T. and Doyle, M.P. 1996. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. Appl. Environ. Microbiol. 62:2567-2570.

Weagant, S.D., Feng, P. and Stanfield, J.T. 1982. Methods for the isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual Online. URL: http://www.cfsan.fda.gov/~ebam/bam-8.html. Access date: August 28, 2006.

Williams Smith, H. 1975. Survival of orally administered *E. coli* K12 in alimentary tract of man. Nature, 255:498-502.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741-3751.

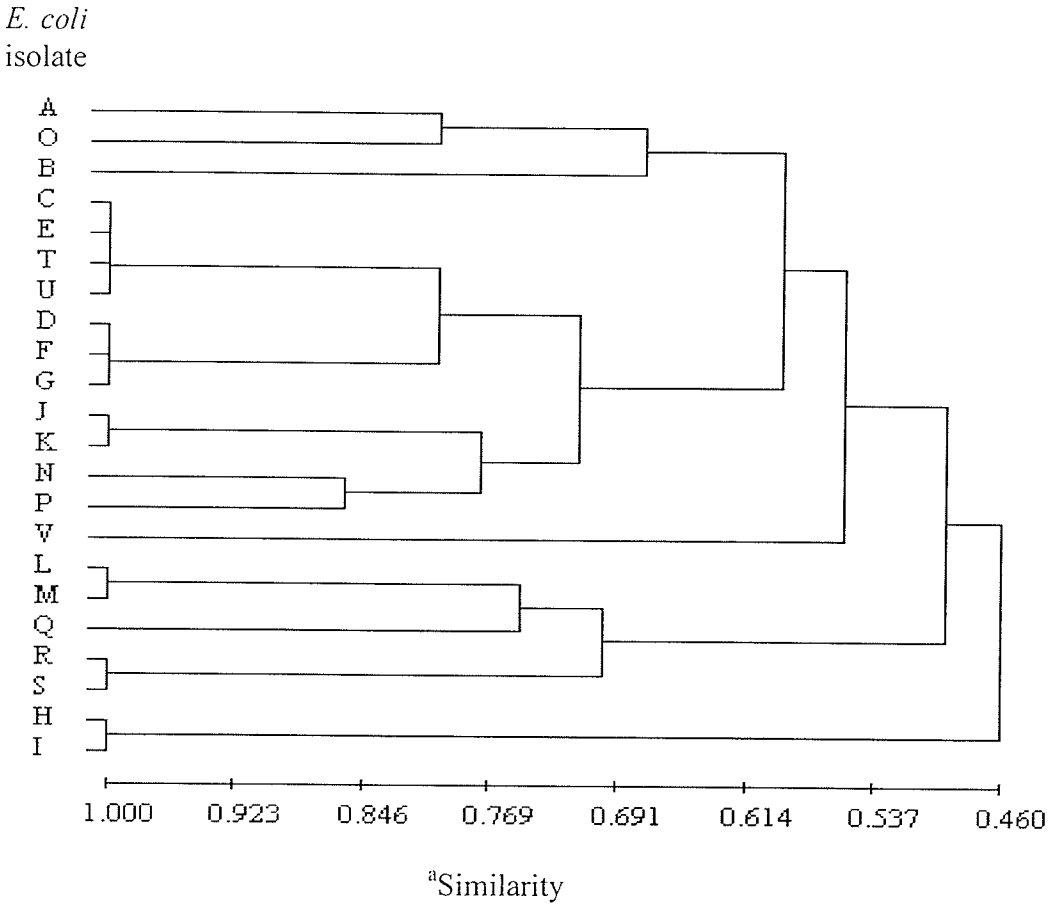
Young, R.A., Huntrods, T. and Anderson, W. 1980. Effectiveness of vegetated buffer strips in controlling pollution from feedlot runoff. J. Environ. Qual. 9:483-487.

Zang, T. and Fang, H.H.P. 2006. Application of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Appl. Microbiol. Biotechnol. 70:281-289.

Appendix 1: Generic matrix generated from the analysis of *E. coli* DNA fingerprint patterns using electrophoresis bands ranging from 900 to 1625 bp in length. The numeral 1 indicates the presence of a band and a 0 indicates the absence of a band.

E. coli Isolate	Electrophoretic band occurrence in bp length										
	1625	1425	1375	1325	1260	1250	1200	1125	1075	950	900
A	1	1	0	0	0	1	0	1	0	1	0
B	1	1	0	0	0	0	0	0	0	1	0
C	1	1	0	0	0	0	0	0	0	1	1
D	1	1	0	0	0	0	1	0	0	1	1
E	1	1	0	0	0	0	0	0	0	1	1
F	1	1	0	0	0	0	1	0	0	1	1
G	1	1	0	0	0	0	1	0	0	1	1
H	1	0	0	1	0	1	1	0	0	1	1
I	1	0	0	1	0	1	1	0	0	1	1
J	1	1	0	0	0	1	0	0	0	1	1
K	1	1	0	0	0	1	0	0	0	1	1
L	0	1	0	0	0	0	1	0	0	0	1
M	0	1	0	0	0	0	1	0	0	0	1
N	1	1	0	0	0	1	1	0	0	1	1
O	1	1	0	0	0	1	0	0	0	1	0
P	1	1	0	0	0	1	1	1	0	1	1
Q	1	1	0	0	0	0	1	0	0	0	1
R	0	1	0	0	0	0	1	0	0	1	1
S	0	1	0	0	0	0	1	0	0	1	1
T	1	1	0	0	0	0	0	0	0	1	1
U	1	1	0	0	0	0	0	0	0	1	1
V	1	1	1	0	0	0	0	0	1	1	1

Appendix 2: Dedrogram showing phylogenetic relationships based on the Jaccard similarity coefficient (Dombec *et al.*, 2000) and the average linkage clustering method (UPGMA) (Birch *et al.*, 1996) for the data of RAPD fingerprint patterns of *E. coli* presented in appendix 1. Calculation of the Jaccard similarity coefficients and the clustering of the data using the UPGMA method was done by using ClustanGraphics 7 software. [a] This axis shows the similarity between clusters of *E. coli* isolates. The scale ranges from 1 to 0 where the closer the value is to the numeral 1, the more similar the individuals in a cluster are to each other.



Appendix 3: Simpson's index of diversity (D) (Hunter and Gaston, 1988)

$$\mathbf{D} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

D: Simpson's index of diversity
N: Total number of isolates
S: Total number of different fingerprint patterns
$\mathbf{n_j}$: Number of isolates belonging to the *j*th fingerprint pattern