Distribution, diversity and antimicrobial resistance of

Salmonella enterica isolated from urban and rural streams

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

This study presents the spatial and temporal variability of *Salmonella enterica* in urban and rural streams in a model watershed (Grand River watershed, Ontario, Canada), and examines the antimicrobial resistance (AMR) and genetic diversity of various serotypes. Using a swab collection method and various media types, *Salmonella* were detected in 78.4% of samples between November 2003 and July 2005. A diverse range of *Salmonella* serotypes (n=38) were isolated from water. Predominant serotypes and phagetypes (PT), including *S*. Typhimurium PT 104 and *S*. Heidelberg PT 19, and the proportion of isolates demonstrating AMR (33%), was similar to those for humans and farm animals locally and across Canada, a trend not commonly reported. There was a greater diversity of serotypes and AMR profiles in isolates from the urban stream compared to the rural/agricultural streams. Plasmid-borne resistance was observed in 28.6% of AMR isolates, with two different plasmids responsible for resistance; the TEM-1 plasmid (8.1Kb plasmids carrying bla_{TEM-1}, responsible for ampicillin resistance) and CMY-2 plasmid (95.5Kb plasmids carrying bla_{CMY-2}, responsible for 3rd generation cephalosporin resistance). CMY-2 plasmids were only found in the urban stream and did not create a biological burden under non-selective conditions, indicating the long-term stability of these plasmids. Seasonal differences in the overall diversity of serotypes and predominance of serotypes of human health significance (*S*. Typhimurium and *S*. Heidelberg) were observed. The lower occurrence of *S*. Typhimurium and *S*. Heidelberg in February and March was not the result of lower survival of these serotypes at low temperatures. Peaks in occurrence of *S*. Typhimurium and *S*. Heidelberg in the summer and spring, respectively, were pronounced in the rural/agricultural streams, as opposed to the urban stream. Pulsed-field gel electrophoresis and plasmid-typing revealed diversity within multiple drug resistant *S*. Typhimurium PT 104 isolates, indicating genetic differences among tributaries. The ubiquitous nature of *Salmonella* in water and the predominance of serotypes/phagetypes of human or veterinary health significance suggest that environmental exposure through consumption or contact with contaminated water is plausible. These streams may act as a vehicle for the dissemination of these organisms and their resistance genes between different hosts or environments.

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Table of Contents

List of Figures

List of Tables

Background & study objectives

1.1 Background

Waterborne disease and protecting water sources

Water has long served as a vehicle for the transmission of disease (WHO, 2004). Pathogens represent the most common etiological agent associated with waterborne illness (Dziuban *et al*, 2006; Craun *et al*., 2006; WHO, 2008). The consumption of contaminated water, either directly through drinking or indirectly through recreating in natural waters, represents a common exposure pathway for waterborne disease. The most common adverse health outcome associated with the consumption of waterborne pathogens is enteric illness (gastroenteritis), although more severe infections can result (Dziuban *et al.*, 2006).

The implementation of drinking water treatment more than a century ago significantly reduced the burden of illness, however, challenges still exist in underdeveloped countries where treatment is limited or non- existent. Despite major advances in drinking water treatment, waterborne outbreaks still occur in North America (Hrudey *et al*., 2002; Schuster *et al*., 2005; Craun *et al*., 2006). Thousands of illnesses are reported annually following the consumption of contaminated drinking water in the USA (Dziuban *et al*, 2006). These reported cases are assumed to be a significant

underestimation of the actual number of illnesses, with estimates reaching into the hundreds of thousands per year (Morris and Levin, 1995). Considerably fewer cases are reported in association with exposure to natural recreational water in the USA (Yoder *et al*., 2008). However, outbreaks and illness associated with recreational waters are difficult to quantify, therefore the extent of underestimation is unknown (USEPA, 2009a). In Canada, data on illnesses attributed to drinking water and recreation are not reported at the national level (Schuster *et al*., 2005).

Reducing the microbial concentration in water can reduce the risk to the population that may consume water directly or indirectly. With regard to drinking water, the multiple-barrier approach is considered the best strategy for providing safe drinking water (WHO, 2008). The first step in this approach involves protecting sources of drinking water from contamination, followed by adequate treatment and distribution prior to consumption. To provide enhanced protection from microbial contamination, several jurisdictions around the world have begun implementing source water protection upstream of drinking water intakes (e.g., USEPA, 1999; EU, 2000). Currently, in Ontario, regulations are being created to assist in the development and implementation of source protection plans across the province.

The Clean Water Act (CWA; OMOE, 2006) was created in Ontario following the recommendations of the Walkerton Inquiry, which was published following an outbreak of waterborne disease in Walkerton, Ontario, in 2000 (O'Connor, 2002). Rules and regulations (e.g., O. Reg 287/07, OMOE, 2007; OMOE, 2009) accompanying the CWA have been established to help define source protection zones and determine the level of risk that land uses and activities pose within a watershed. Additional rules and regulations are currently being completed to manage risk at the land use level. Although these rules are incomplete, the management of land use activities will likely include measures used in other jurisdictions such as: best management practices (BMPs) associated with farming, including secure manure storage and maximum land-application rates; advanced treatment of wastewater prior to discharge; and, in some instances, exclusion of certain activities deemed to be a significant risk.

In most jurisdictions, including Ontario, source water protection relates specifically to drinking water sources. The indirect benefit of this initiative on recreational waters

would largely depend on the proximity to drinking water intakes, as well as the level of land use management strategies implemented upstream. Some sources of pathogens, including bathers, sediment resuspension, and wildlife, which impact bathing beaches and other recreational waters, may not be captured under these initiatives. To prevent microbial contamination, sanitary surveys and management actions specific to bathing beaches would likely be required to protect recreational areas in source protection regions (WHO, 2003; Health Canada, 2009).

Awareness of water quality impairment is an important step in developing strategies to reduce contaminant levels. Water monitoring can reveal spatial and temporal trends of specific contaminants and can also help to determine predominant contamination sources upstream. Waters designated as bathing beaches are commonly monitored for microbiological contamination, mostly in the form of fecal indicator bacteria (i.e., *E. coli*), as per standards and guidelines produced by federal, state and provincial governments (e.g., USEPA, 1986; Health and Welfare Canada, 1992; USEPA, 2003; Health Canada, 2009). However, water monitoring rarely occurs outside of designated areas, although recreation still occurs. Similarly, monitoring water quality upstream of drinking water intakes is also rarely performed. In Ontario, regulations require monitoring of fecal indicator bacteria in the raw water at an intake (O. Reg. 170/03, OMOE, 2003). Water monitoring is not required upstream of an intake, therefore understanding of the microbiological quality of water within many watersheds is limited.

Monitoring for fecal indicator organisms, such as *E. coli*, can be used for a general assessment of water quality issues, however, the use of these organisms for determining the direct risk to the population is limited. The use of quantitative microbial risk assessments, which are increasingly being used to assist in the analysis of the human health risks associated with using specific water sources (WHO, 2003), require more specific knowledge of the occurrence of pathogens in water.

Transmission of waterborne pathogens

The transmission of waterborne pathogens is inherently complex (Figure 1.1). Several steps are needed for waterborne transmission to occur: 1) there must be a source of pathogens present on the landscape; 2) there must be movement of pathogens to a

watercourse; 3) the pathogens must survive within the aquatic environment long enough to reach a new host; and, 4) the consumption of pathogens by a host must be at a dose that can cause an infection.
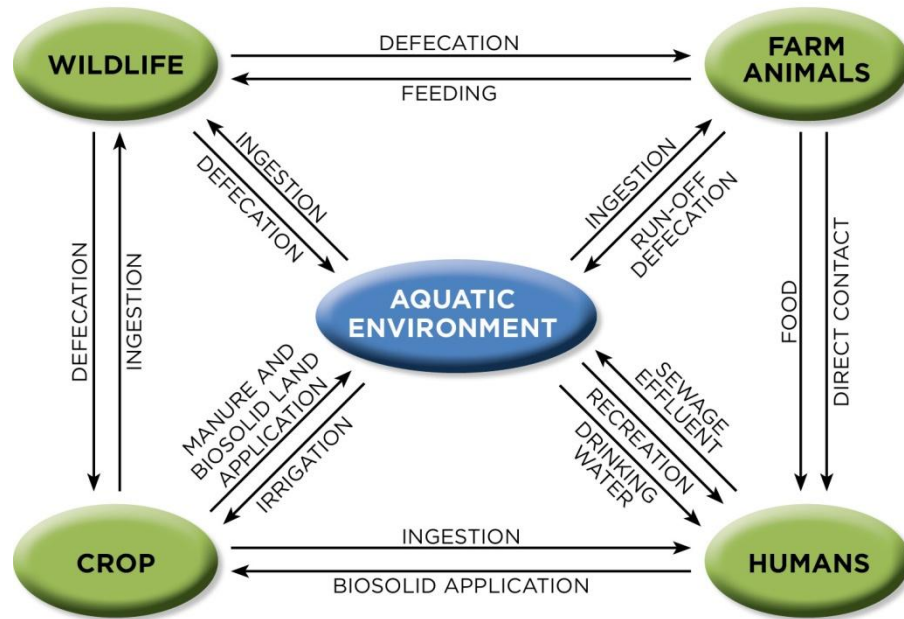
Figure 1.1. Complexity of the movement of fecally-derived pathogens in the environment and potential routes of exposure to subsequent hosts.

Most waterborne disease outbreaks have been associated with pathogens originating from feces. Fecal waste from humans and animals, both wild and domestic, can contain a range of enteric pathogens, including protozoa, bacteria and viruses (WHO, 2004; WHO, 2008). Within a watershed, sources of pathogens on the landscape include: agricultural operations where manure is generated, stored and land-applied; human waste management systems such as sewage treatment facilities, sewer lines and septic systems; and wildlife (Dorner *et al*., 2004a). Defining the source of specific pathogens within a watershed is difficult as enteric pathogens are transient in most host animals. Variability can also occur in the shedding intensity of pathogens, which can differ with the age and type of animal, seasonally, or in response to stress (Olson *et al*., 1997; Dorner e*t al*., 2004a; Fitzgerald *et al*., 2003).

In most instances, fecally-derived pathogens enter the watercourse after the mobilization of waste material. Hydrological events, such as heavy rainfall and snow melt, can create run-off from agricultural and urban areas where fecal contamination can reach a water course through overland transport or storm sewers (Atherholt *et al*., 1998; Curriero *et al*. 2001; Kistemann *et al*., 2002; Dorner *et al*., 2006). Hydrological events can also contribute to pathogen loading in aquatic environments following sewage treatment by-passes and combined sewer overflows (Jamieson *et al*., 2005; Dorner *et al*., 2007). Mobilization of pathogens that have accumulated in sediment can also occur when water flow increases (Dorner *et al*., 2007).

Pathogens can also enter a watercourse under base flow conditions. Direct fecal contamination from wildlife and livestock that are allowed access to water, as well as effluent discharge from sewage treatment facilities, can contribute to microbial loading during base flow. Pathogens can also reach surface water through tiles draining agricultural fields and groundwater discharge carrying contaminants from leaking on-site septic systems or sewage pipes (Scandura and Sobsey, 1997; Joy *et al*., 1998; Dorner *et al*., 2007).

The ability of enteric pathogens to survive in water is critical for transmission. Many factors can impact the survivability of waterborne pathogens including exposure to sunlight, predation, water temperature, and water chemistry (Johnson *et al*., 1997; Medema *et al*., 1997; Varnam and Evans, 2000; WHO, 2004). Enteric pathogens vary greatly in their ability to survive in the aquatic environment, with protozoan cysts/oocysts and bacterial spores generally surviving longer (Johnson *et al*., 1997; Medema *et al*., 1997). There is also evidence that some bacteria of enteric origin may be able to multiply in the aquatic environment which would contribute to enhanced environmental persistence (Whitman and Nevers, 2003; Byappanahalli *et al*., 2006; Kon *et al*., 2007; Vanden Heuvel *et al*., 2010).

To constitute a risk to the population consuming water, either directly through drinking or indirectly through recreation, waterborne pathogens must be at a concentration that can cause an infection in a host. The infectious dose varies greatly between pathogens and can also vary based on the susceptibility of the person ingesting the organism (WHO, 2004).

Non-typhoidal *Salmonella* and waterborne occurrence

Non-typhoidal *Salmonella,* which causes salmonellosis in humans, is rarely included in pathogen monitoring surveys in streams (Lamarchand and Lebaron, 2003; Patchanee *et al*., 2010), particularly in relation to drinking water. The exclusion of non-typhoidal *Salmonella* from many water monitoring studies is likely a reflection of the few drinking water outbreaks attributed to *Salmonella*, although some have been reported in nonchlorinated systems (e.g., Angulo *et al*., 1997; Craun *et al*., 2006), and the fact that foodborne transmission is reported to be the predominant exposure route for this pathogen (Mead *et al*. 1999; CDC, 2009a). In recognition of the gaps that exist in the study of waterborne *Salmonella,* the USEPA included this bacterium on the Contaminant Candidate List 3 (CCL 3), which was finalized in 2009 (USEPA, 2009b).

Salmonellosis is a major public health burden in many countries, including developed countries such as Canada (Thomas *et al*., 2006a). A typical infection caused by non-typhoidal *Salmonella* in humans is characterized by acute onset of abdominal pain, diarrhea, nausea, fever and sometimes vomiting (WHO, 2008). The incubation period is 6 to 72 h and symptoms generally last 3 to 5 days (WHO, 2008). Severe infections can occur resulting in extreme dehydration and septicemia, which can be life threatening (WHO, 2008).

It is estimated that only a small portion of infections are clinically recognized and as a result the rates of salmonellosis are greatly underestimated (Thomas *et al*., 2006a). Within Canada and the USA, it has been estimated that 13 to 38.6 illnesses occur for every confirmed case of salmonellosis (Thomas *et al*. 2006a; Voetsch *et al*. 2004). These estimates indicate that over 200,000 cases of salmonellosis in Canada and over one million cases in the USA may occur annually (Thomas *et al*. 2006a; Voetsch *et al*. 2004). The highest percentage of reported cases of salmonellosis occurs in children under the age of 5; an age at which hospitalization and treatment with antimicrobial drugs is most common (CDC, 2007a; Health Canada, 2003).

The majority of salmonellosis cases are considered to be sporadic or endemic, meaning they are independent of outbreaks and travel-related illness (PHAC, 2007a). The sources of endemic illness are considered of domestic origin and may include local food or water (PHAC, 2007a; 2007b). While foodborne exposure is considered the

predominant source of *Salmonella*, questions remain as to the role that water plays in sporadic or endemic cases. Recently, Denno *et al*. (2009) conducted a matched case-controlled study on sporadic salmonellosis in children and reported that non-foodborne exposure might be as important as foodborne exposure. In their study, exposure to specific water sources, including drinking untreated water from private wells and recreation in surface waters, were risk factors for sporadic salmonellosis in children.

The occurrence levels of non-typhoidal *Salmonella* in river water range greatly, with frequencies reported between 3% and 79.2% (McBride *et al*., 2002; Johnson *et al*., 2003; Gannon *et al*., 2004; Simental and Martinez-Urtaza, 2008; Haley *et al*., 2009; PHAC, 2007b; Edge *et al*., 2009). However, most studies report frequencies to be ≤ 20%, including all studies carried out in Canada (Johnson *et al*., 2003; Gannon *et al*., 2004; PHAC, 2007a; PHAC, 2007b; Edge *et al*., 2009; Wilkes *et al*., 2009). Many studies have reported variable survival rates for *Salmonella* in the aquatic environment, depending on the conditions of the study conducted (Johnson *et al*., 1997), though it is generally assumed that *Salmonella* can survive for considerable periods of time in water, or at least as long or longer than fecal indicator bacteria (Wright, 1989; Catalao Dionisio *et al*., 2000; USEPA, 2009a). The reported occurrence of waterborne *Salmonella*, as well as its survival capability in water, implies that the aquatic environment may play a role in the transmission of *Salmonella* between host animals. However few studies have reported similarities between the predominant *Salmonella* serotypes/phagetypes obtained from the aquatic environment and those of clinical relevance in humans (e.g., Haley *et al*., 2009; Patchanee *et al*., 2010). This lack of association leaves many unanswered questions regarding epidemiological connection between water and human health.

Antimicrobial drug treatment and resistance in *Salmonella*

Salmonellosis is generally considered a self-limiting infection that does not require treatment (Buyaye e*t al*., 2006; WHO, 2008). However, if the infection is serious or invasive, antimicrobial drugs are commonly administered (Foley and Lynne, 2008). Quinolones, including ciprofloxacin, are used to treat *Salmonella* infections in adults, however, due to the toxicity of ciprofloxacin, the 3rd generation cephalosporin, ceftriaxone, is commonly used to treat infections in children (Shea*, 2004; Buyaye e*t al*., 2006; CDC, 2007b). Earlier drugs used to treat salmonellosis, including

chloramphenicol, ampicillin, amoxicillin, and trimethoprim-sulfamethoxazole, are occasionally used as alternative treatments, however, their effectiveness can be limited as a result of the high levels of antimicrobial resistance (AMR) observed in *Salmonella* (WHO, 2005).

Zoonotic pathogens, including *Salmonella*, which demonstrate resistance to drugs of human health importance are of growing concern (Health Canada, 2002; Zhao *et al*., 2005; WHO, 2007). The overuse of antimicrobial drugs to prevent or treat infections in human and veterinary medicine contributes to the increased frequency of AMR (WHO, 2007). While still controversial, the use of drugs in livestock production, particularly for growth promotion, is thought to contribute to the maintenance and spread of AMR in *Salmonella* (Chopra and Roberts, 2001; Smith *et al*., 2007; WHO, 2007; Dutil *et al*., 2010).

Similar to other bacteria, *Salmonella* can acquire resistance through mutations in their genetic material or through the uptake of resistance genes on mobile genetic elements through a process referred to as horizontal gene transfer. Horizontal gene transfer occurs by three primary mechanisms: 1) the direct uptake of DNA from the environment (transformation); 2) phage-mediated transfer (transduction); and, 3) direct cell to cell contact (conjugation) (Davidson, 1999).

Horizontal transfer of extra-chromosomal elements, including plasmids, is often associated with a rapid rise in resistance (Kruse and Sorun, 1994; Davison, 1999; Foley and Lynne, 2008). Genes responsible for resistance to clinically significant drugs, such as 3rd generation cephalosporins, are commonly found to be plasmid-mediated in *Salmonella* (Winokur *et al*., 2001; Carattoli *et al*., 2002; Giles *et al*., 2004; Daniels *et al*., 2007; Li *et al*., 2007; Call *et al*., 2010). Conjugative plasmids often serve as the vehicles for inter- and intra-species transmission of AMR genes (Kruse and Sorun, 1994; Poppe *et al*., 2005; Smith *et al*., 2007). In the gut of animals, such as cattle, conjugation of AMR plasmids has been observed between pathogens and commensal bacteria, such as *Salmonella* and commensal *E. coli* (Poppe *et al*., 2005; Daniels *et al*., 2009). This scenario can aid in the rapid movement and permanence of AMR within the bacterial population.

The creation of international and national surveillance programs, such as the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) in Canada and the National Antimicrobial Resistance Monitoring System (NARMS) in the USA, have been instrumental in understanding the prevalence, movement, and emergence of drug resistant strains of *Salmonella*. Most surveillance programs focus on isolates obtained from infected humans, farm-animals and related food-products (Government of Canada, 2006; CDC, 2007b), but do not include an assessment of environmentally-derived isolates. These programs also primarily focus on the phenotypic assessment of drug resistance and exclude detailed analysis of the genes responsible for resistance and the way in which resistance is mediated.

Studies examining AMR in aquatic bacteria tend to focus on coliform and commensal bacteria, such as *E. coli*. Little attention has been given to AMR in waterborne pathogens, therefore the role that water plays in the movement and spread of these strains is largely unknown (Patchanee *et al*., 2010). Runoff from livestock production and sewage effluent are thought to be major contributors to AMR bacteria in a watercourse, as antimicrobial use predominates in humans and farm animals (Patchanee *et al*., 2010). Drug resistance in isolates originating from wildlife, which are not influenced by selective pressures of antimicrobial drugs, is thought to be low (e.g., Cole *et al*., 2005; Edge and Hill, 2005; Edge and Hill, 2007). To understand the extent to which the aquatic environment is associated with the spread of AMR *Salmonella*, and to determine if any epidemiological linkages exist between aquatic sources and human and animal health, the occurrence, AMR profiles, and genetic characterization of waterborne isolates are needed.

Pathogen monitoring - Grand River watershed

As the population around the world increases and more pressure is placed on drinking water sources, many jurisdictions are embarking on source water protection (SWP) initiatives to help maintain and improve water quality. To make informed decisions, policy makers continue to need concrete scientific information on the risk to the population using these waters, water quality impairment issues, sources of pathogen contamination in watersheds and measures needed to reduce microbial loading. In many instances, the study of model watersheds can help to focus these efforts.

In Canada, several watersheds have been included in intense waterborne monitoring surveys, including the Grand River watershed in southwestern Ontario, the South Nation watershed in eastern Ontario, and the Oldman River basin in Alberta. These watersheds have many attributes that make them ideal model watersheds for pathogen monitoring, including large portions of their basins used for farming, direct discharge from sewage treatment plants and large urban populations using these waters as drinking water sources. Also, these watersheds are in close proximity to research laboratories that are capable of collecting and analyzing water samples without long delays.

The Grand River watershed, which drains to Lake Erie, is the largest watershed in southwestern Ontario at approximately 6,800 km^2 (GRAR, 2010). The Grand River Source Water Protection Area was created to protect the five surface water intakes and over 200 municipal wells that supply water to 86% of the population living in the watershed, which is just under 900,000 (GRCA, 2010; LERSPC, 2010). In addition to its use for drinking water, the Grand River and its tributaries are used for numerous recreational activities including fishing, canoeing and swimming (www.grandriver.ca), as well as for agricultural activities including livestock watering and irrigation. Sources of fecal contamination in the Grand River watershed that will be captured as a "drinking water threat" related to pathogens under the Clean Water Act (OMOE, 2006) and associated rules and regulations will include; urban storm drains, sewage treatment effluent and bypasses, septic systems, livestock pasturing, and manure storage and spreading. Wildlife, which can be contributors to pathogen loading, is not considered a drinking water threat under the current rules and regulations.

Several large urban areas are found within this watershed, including the cities of Waterloo, Kitchener, Cambridge, Guelph and Brantford; however, the largest portion (70%) of the landscape is designated as agricultural/rural (LERSPC, 2010). Active farming occurs throughout the watershed, including activities such as livestock production and growing of crops. Cattle (beef/dairy), pigs and chickens make up the majority of livestock in the watershed (Dorner *et al.*, 2004a). The upper portion of the Grand River watershed has one of the highest manure production rates in Canada (Hofman and Beaulieu, 2006), with several tributaries showing the highest density of manure production in the watershed, including Canagagigue Creek and Conestogo River (Dorner *et al.*, 2004a; 2004b).

The Grand River receives waste effluent from approximately 80% of the population through 29 wastewater treatment facilities, all with varying levels of treatment (GRCA, 2010). By-passes, both untreated and partially treated, are common throughout the watershed (Grand River Municipal Water Managers Working Group, 2009). Septic systems or private treatment systems are used by the remainder of the population (GRCA, 2010).

The Grand River watershed has been previously included in a pathogen monitoring study between 2002 and 2003 (Dorner *et al.*, 2004b), which involved monitoring for several waterborne pathogens, including protozoa (*Cryptosporidium* and *Giardia*) and several bacterial pathogens (*Campylobacter* and *E. coli* O157:H7), as well as the creation of a watershed-scale transport model. This study helped to create an understanding of the shedding and loading rates of several pathogens in portions of the watershed.

Also within the Grand River watershed, in 2005, the Public Health Agency of Canada and Agriculture and Agri-food Canada, through the C-EnterNet Program, began conducting a large-scale epidemiological study in the Region of Waterloo. At this sentinel location in Canada, this surveillance program monitors enteric disease in the population and occurrence levels of enteric pathogens in various sources including farm animals, food and water. Salmonellosis is reported as one of the most common gastrointestinal diseases in the Region, with a rate of infection determined to be 22.8 to 31.3/100,000 person-years in 2005-2006 (PHAC, 2007a; 2007b). These rates are higher than reported in the province and country (PHAC, 2007b). Most cases of salmonellosis

11

in the Region of Waterloo are identified as endemic, meaning they are of domestic origin and not related to outbreaks or travel (PHAC, 2007a; 2007b). These endemic cases are primarily associated with infections caused by *S*. Typhimurium, *S*. Heidelberg and *S*. Newport (PHAC, 2007b).

1.2   Thesis objectives and organisation

Many questions remain as to the role that water plays in the transport and dissemination of *Salmonella*, which is primarily considered a foodborne pathogen, between host animals and humans. To fully understand the transmission of *Salmonella*, in particular serotypes of human and veterinary health significance, the environmental occurrence of strains needs to be further defined. Knowledge of the waterborne occurrence of *Salmonella* serotypes and phagetypes, both geographically and temporally, as well as the genetic variability of strains, is a first step and principal factor in understanding the risk to the population that uses these waters for recreation and as a source of drinking water, and is critical information needed prior to the implementation of prevention and control strategies for the future.

The overall objectives of this thesis were: 1) To assess the spatial and temporal distributions of *Salmonella* serotypes in three tributaries, two dominated by agricultural/rural activities and one urban; 2) to determine the frequency of drug resistance in waterborne isolates and compare these levels to those reported in isolates obtained from humans and agricultural animals in Canada; 3) to examine diversity and genes responsible for plasmid-mediated resistance in waterborne *Salmonella* and *E. coli*; and, 4) to examine the genetic relatedness of *Salmonella* serotypes of human health importance that are circulating within these tributaries.

Several factors make the Grand River watershed a suitable model watershed for this study:

- It is in close proximity to the university laboratory which allows for easier sample collection and more timely analysis;
- It is used as a source of drinking water and has recently been designated as a source protection area, and it is used for recreation;

- Significant portions of the landscape are impacted by anthropogenic activities that may contribute pathogen loading to water, including agricultural land and effluent from wastewater treatment facilities;
- Hydrometric, precipitation, and water chemistry data are available for many regions of the watershed; and,
- It has been previously included in a large-scale pathogen monitoring study (although *Salmonella* was not included in the previous survey; Dorner *et al.*, 2004b) and it is currently the study area for a large epidemiological investigation examining potential sources of enteric pathogens in the population.

The findings from this watershed can be representative of many temperate watersheds with similar land use impacts.

The thesis contains four data chapters (Chapters 2-5), and each chapter deals with a specific aspect of my overall research objectives.

Chapter 2 presents data on the occurrence of *Salmonella* and diversity of serotypes following a year and a half of monitoring in two agriculture/rural-influenced streams (Conestogo River and Canagagigue Creek) and a small urban stream (Laurel Creek). Factors that might influence the temporal variability of *Salmonella* were assessed, along with seasonal differences, to provide context to the dynamic nature of this waterborne pathogen. Data are presented on the survival differences of several environmentally derived *Salmonella* isolates, including serotypes of human health importance, at lower water temperatures. Chapter 2 also provides an assessment of various media combinations for the most efficient isolation of waterborne *Salmonella*.

Chapter 3 presents data on the phenotypic assessment of antimicrobial resistance (AMR) in waterborne isolates of *Salmonella*. Comparisons are made to the frequency and profiles of drug resistance in *Salmonella* isolates reported in both human and agricultural animals in Canada. A comparison of AMR levels between isolates from agricultural and urban streams is presented to determine if variability exists due to the predominant sources of contamination in these waters. In addition, data are presented on the drug resistance in waterborne *E. coli* isolates to look for similarities with

resistance levels in waterborne *Salmonella*, and determine if AMR *E. coli* could be a surrogate for monitoring AMR *Salmonella* in these waters.

In Chapter 4, genetic assessments of drug resistance in waterborne *Salmonella* and *E. coli* are presented. This chapter examines the proportion of isolates carrying drug resistance on plasmids and determines the genes responsible for resistance. Plasmid diversity is examined to determine the relatedness of plasmids carrying resistance to drugs of significance to human and animal health. Experiments examining the fitness of isolates carrying naturally occurring AMR plasmids, as well as the stability of plasmids under non-selective conditions, are presented to understand the potential permanence of AMR within the bacterial population.

Chapter 5 examines the genetic relatedness of serotypes of human and animal health importance: *S*. Typhimurium and *S*. Heidelberg. Data on the genetic diversity of isolates within and among tributaries are presented to determine if isolates are unique to specific tributaries or if similarities exist between isolates from various tributaries.

Chapter 6 presents the significance of this overall work, thesis conclusions and recommendations.

2

Temporal & spatial variability of *Salmonella* serotypes in streams impacted by various land-use activities

2.1 Introduction

Salmonellosis is predominantly considered a foodborne disease (Mead *et al*., 1999). Links between food and human illness have led to the development of government surveillance programs. In Canada these programs includes the National Enteric Surveillance Program (NESP) and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), which monitor and report on the prevalence of enteric pathogens, including *Salmonella*, in humans and farm animals. In the country, similarities between the predominant serotypes that infect humans and those isolated from a variety of farm animals are commonly reported, including serotypes such as *S*. Typhimurium and *S*. Heidelberg (Government of Canada, 2005; PHAC 2007c).

An understanding of the sources of infection, however, is slowly changing as more information comes forward on endemic disease within the population and potential environmental linkages. A recent case-controlled study on sporadic salmonellosis in children has shown that non-food sources might be as important as foodborne exposure (Denno *et al.*, 2009). This study by Denno *et al.* (2009) showed that exposure to specific water sources, including drinking untreated water from private wells and recreation in surface waters, were risk factors for developing salmonellosis.

To fully understand the role water plays in endemic/sporadic cases of salmonellosis, information such as occurrence and prevalence of specific serotypes of *Salmonella* in natural aquatic systems is needed. Identification of the different *Salmonella* strains at the serotype level is critical to understanding if clinically important isolates are present in water. Information of this type can also be helpful in understanding potential sources of fecal contamination and aid in developing strategies to protect sources of drinking water and recreational water. Despite its importance, serotyping data are often limited in aquatic studies as many laboratories do not have the capability to perform this analysis (Baudart *et al*., 2000).

Few researchers in Canada have included *Salmonella* as a pathogen in water monitoring studies. When *Salmonella* is included, the occurrence is low and few connections have been made between the predominant serotypes in water and those observed in infected humans and animals. One such study by Johnson *et al*. (2003) reported *Salmonella* in Oldman River, a highly agricultural watershed in southern Alberta, in 3% and 7.7% of water samples taken in 1999 and 2000, respectively. No serotyping data were given. In subsequent years, Gannon *et al*. (2004) detected *Salmonella* at similar values in the same watershed (Little Bow River and irrigation canals), in 5.5% of water samples in 2000 and 14.9% in 2001. Predominant serotypes in water did not correspond to those observed in farm animals or in the human population in the area (Gannon *et al*., 2004). Similar occurrence levels (9.6%) were observed by Wilkes *et al*. (2009) in the South Nation watershed in eastern Ontario between 2004 and 2006. No serotyping data were given in this study. Several reference sites that were located away from anthropogenic activities in this watershed, in particular agricultural operations, resulted in a lower proportion of water samples (3%) positive for *Salmonella* (Edge *et al*., 2009).

As part of a larger epidemiological study being conducted in the Region of Waterloo, the Public Health Agency of Canada (PHAC) and Agriculture and Agri-food Canada, through the C-EnterNet Program, began monitoring for *Salmonella* in the Grand River watershed in 2005 (Government of Canada, 2011). Between 2005 and 2007, *Salmonella* occurrence ranged from 10% to 20% (PHAC, 2007a and 2007b), using culture-based analysis. Using molecular detection, 17% to 36% of the locations were positive over the same time frame (PHAC, 2007a and 2007b). Between 2005 and

2007, no serotypes predominated; however, some serotypes of human health importance were observed in these waters.

In addition to understanding occurrence and predominant serotypes in water, making the connection between water and health also involves understanding environmental parameters that affect transport and survival of *Salmonella* in water (Haley *et al*., 2009). Many waterborne disease outbreaks have occurred following intense periods of precipitation in many countries, including Canada (Curriero *et al*., 2001; Hrudey *et al*., 2002; Schuster *et al*., 2005; Thomas *et al*., 2006b). This suggests a link between watershed hydrology and pathogen transmission (Dorner *et al*., 2007). However, few studies examining the occurrence of pathogens in rivers include hydrological data in their analysis (Wilkes *et al*., 2009).

Several studies from around the world have observed positive correlations between precipitation and occurrence of *Salmonella* in the aquatic environment (Baudart *et al*., 2000; Martinez-Urtaza *et al.*, 2004; Simental and Martinez-Urtaza, 2008; Haley *et al*., 2009; Setti *et al*., 2009; Wilkes *et al*., 2009; Walters *et al*., 2011), particularly when rainfall had occurred 1-3 days before sample collection (Haley *et al*., 2009; Wilkes *et al*., 2009). In Canada, Wilkes *et al*. (2009) observed considerable variability between hydrological conditions and occurrence of microorganisms in water, however, they did note that rainfall and discharge were positively associated with *Salmonella* detection.

Knowledge of the environmental fitness and survival capability of *Salmonella* is important for understanding the extent to which the aquatic environment might act as a vector for the movement of this bacterium. Many studies have shown that *Salmonella* remain viable for long periods of time in freshwaters (Wright, 1989; Catalao Dionisio *et al*., 2000; Santo Domingo *et al*., 2000; Moore *et al*., 2003), however, some researchers have suggested that differential survival rates exist between *Salmonella* serotypes (McFeters *et al*., 1974; Baudart *et al*., 2000; Haley *et al*., 2009; Setti *et al*., 2009). Several researchers have suggested that the lack of occurrence of *Salmonella* serotypes of human and animal health significance in water indicates different survival capabilities (Baudart *et al*., 2000; Haley *et al*., 2009).

Isolation and recovery of waterborne bacterial pathogens, including *Salmonella*, remain a challenge. In recent years, many researchers have moved to molecular detection of

waterborne pathogens, whereas others use traditional culture-based isolation techniques and others use a combination of both (Baudart *et al*., 2000). Most surveillance programs (e.g., CIPARS) that collect human and veterinary clinical samples still favour culture-based methodologies for *Salmonella* detection as isolates can be used for further analysis, including serotyping and antimicrobial resistance testing.

Although no single method exists for the recovery of *Salmonella* from water, Standard Methods for the Examination of Water and Wastewater (Standard Methods; APHA, 2005) suggests several isolation steps and medium types for culture-based isolation. In addition, many researchers use other medium types adapted from the food industry for isolation of waterborne *Salmonella* (Vassiliadis *et al*., 1981; BD, 2009). Traditionally, multiple media types are used to isolate *Salmonella* due to the variety of nutritional requirements and sensitivities of different serotypes. The variety of isolation techniques and varying water volumes used by researchers to isolate waterborne *Salmonella*, as well as the differences in medium isolation efficiencies, makes comparison between studies difficult. Maximizing the recovery of *Salmonella* from water is critical for understanding the role that water plays in the epidemiology of salmonellosis.

2.2   Research needs and objectives

Water has a great transmission potential, consequently, understanding the occurrence, distribution and survival characteristics of waterborne pathogens can aid in a better understanding of the risk associated with these bacterial pathogens in this environment. To establish the role of water in salmonellosis, information such as occurrence and prevalence of *Salmonella* serotypes in natural aquatic systems is needed. With all waterborne pathogens, a better understanding is needed between pathogen occurrence and the hydrology of aquatic systems to better predict times of increased contamination levels and also contribute to strategies to manage and prevent contamination. Understanding the long-term survival in pathogens, including various serotypes of *Salmonella*, in water allows for greater insight into the ecology of these organisms.

The specific objectives of the research presented in this chapter are to:

1. Determine the occurrence of *Salmonella* in three tributaries of the Grand River watershed and compare to the levels reported in other Canadian rivers;
2. Compare various media combinations to obtain optimal recovery of *Salmonella* from stream water;
3. Understand the predominant serotypes found in water and compare to those reported in humans and animals locally and in Canada;
4. Examine the spatial variability of *Salmonella* occurrence and the serotype diversity present in agricultural/rural and urban impacted waters;
5. Examine the temporal variability of *Salmonella* occurrence and serotype diversity, including seasonal changes; and,
6. Determine if the survival of *Salmonella* serotypes, in particular serotypes of human and animal health significance, differs at lower water temperatures.

2.3 Materials and Methods

Watershed characteristics and sample site locations

Water sampling was carried out in the Grand River watershed in southwestern Ontario, Canada. Three tributaries that flow into the Grand River were chosen for study: Canagagigue Creek (CAN); Conestogo River (CON); and Laurel Creek (LC).

Table 2.1. General land use and stream information for each tributary investigated in the study.

Tributary	Total area (m^2)	Percentage of land cover*				Average stream-discharge (m^3/s)**	Strahler stream order***
		Urban	Agri-culture	Forest/ Range	Wetland		
Laurel Creek (LC)	7.6 x 10^7	38%	44%	16%	1%	0.1	4
Canagagigue Creek (CAN)	1.1 x 10^8	4%	87%	12%	< 1%	1	5
Conestogo River (CON)	8.2 x 10^8	< 1%	82%	17%	1%	3	6

* percent land cover comes from a GIS layer created by the Ontario Ministry of Natural Resources and includes Ontario Land Cover data derived from LANDSAT Thematic Mapper data.
** Dorner *et al*. (2007)
*** determined at last sampling location on each stream

All three tributaries are in close geographical proximity (Figure 2.1). CON and CAN are located in some of the most intensive farming areas of the Grand River watershed (GRCA, 2010). Both of these tributaries have little land designated as urban (Table 2.1). Both are found within the clay till (low permeability) portion of the watershed and are highly (60%) tile drained (Dorner *et al*., 2004b and 2007). Both CAN and CON

have sewage treatment facilities. However, both sampling locations on CAN are upstream of the treatment facility.

Two sample locations are in Canagagigue Creek; CAN-2 is located just downstream of the Woolwich dam and CAN-2 is located to the east side of Elmira in a park. In Conestogo River, three sample location are monitored in this study; CON-3 is located upstream of St. Jacobs and CON-2 is located downstream of St. Jacobs approximately 4000 m downstream of the sewage treatment facility. CON-1 is located prior to where the Conestogo River enters the Grand River. The location of these sites can be seen in Figure 2.1. Universal Transverse Mercator (UTM) coordinates for each site are given in the appendix.

Compared to the other tributaries, LC has the highest portion of land designated as urban, although the percentage of urban land-cover is thought to be an underestimate based on the age of the GIS layer used for Table 2.1 and recent suburban developments. All sampling locations in LC were within the lower portion of the tributary, which is highly urbanized. The higher reaches of the tributary are designated as agricultural. Three sample locations are included in this study, with LC-1 being the most downstream location prior to discharge into the Grand River.
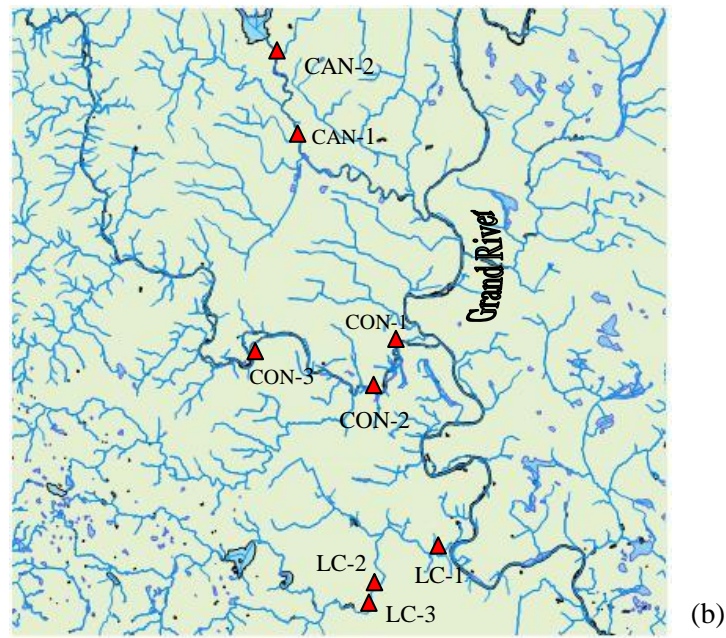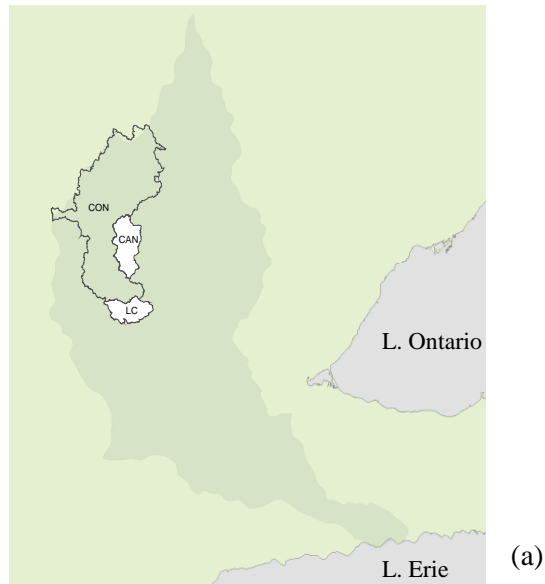
Figure 2.1.(a) The Grand River watershed and each of the three tributaries under study; Conestogo River (CON), Canagagigue Creek (CAN) and Laurel Creek (LC). (b) The locations of each sample site.

Salmonella isolation

Between November 2003 and 2004, samples were taken from each of the three tributaries. The aim of this sampling was to collect several samples per season throughout the year. Samples were usually taken from each tributary two times per month. Over the study period, samples were not taken in January, April or September of 2004. Although attempts were made to collect samples at each sampling location per sample event, there were several incidences when the swabs used for collection were lost or unsuitable for analysis due to changing river flow conditions. In 2005, several additional samples were taken between May and June in LC only. Appendix A contains the sampling record.

A swab collection technique was chosen for qualitative detection of *Salmonella*. A sterilized swab constructed from cheesecloth as specified by Standard Methods (APHA, 2005) was suspended under the water surface on a pig-tailed spike. After 3 to 5 days, the swab and approximately 100 mL of water were collected in sterile whirl-packs and transported on ice to the laboratory. Processing of the swabs began on the same day as they were collected.

In the laboratory, each swab was placed in a sterile bottle along with 90 mL of collected river water. Ten mL of 10x buffered peptone water was added as a pre-enrichment medium and the bottles were shaken at 100 rpm at 37°C overnight. Following pre-enrichment, the bottles were shaken for approximately 1 min. and 1 mL from each bottle was inoculated into 9 mL of each enrichment medium, tetrathionate broth (Tet) and Rappaport Vassiliadis broth (RV). Following incubation for 24 h at 42°C, each enrichment broth was vortexed and streaked onto Brilliant Green Sulpha Agar (BGSA) and 200 μL was also placed on the centre of Modified Semi-solid Rappaport Vassiliadis (MSRV) media and incubated for 24 h at 37°C and 42°C, respectively. One colony showing a positive presumptive result for *Salmonella* on BGSA and a loopful of migration on MRSV (taken approximately 15 mm from the inoculation point) were further transferred to MacConkey's agar for incubation at 37°C for 24 h. To help reduce the number of duplicate isolates, only one isolate was taken from each plate. Following incubation, lactose negative colonies were subjected to three biochemical tests: Triple Sugar Iron Agar (TSI), Lysine Iron Agar (LIA) and urea broth. All media were manufactured by BD Difco. Isolates showing positive

biochemical reactions for *Salmonella* were confirmed through slide agglutination using *Salmonella* O antiserum Poly A-I & Vi (Difco, Maryland) as recommended by the manufacturer. For comparison, a positive control strain of *Salmonella* (ATCC 11331) was used for each sample analysis.

Several fecal samples were collected in LC. On February 11, 2004, fecal samples (n=6) from mallard ducks (*Anas platyrhynchos*) were collected. These samples were freshly obtained from ice or snow at sample point LC-3. Fecal samples (n=10) of unknown origin were also collected on May 7, 2007, on the banks of LC at sample point LC-3. In all instances, fecal material was collected with a sterile cotton-tipped swab and placed in a sterile bag. Fecal sample was placed directly into pre-enrichment tubes and isolation continued as described above.

All confirmed *Salmonella* isolates were stored in 15% glycerol at -80°C until further use (Sambrook *et al.*, 2001).

Salmonella serotyping

Each *Salmonella* isolate was sent to the Public Health Agency of Canada's Laboratory for Foodborne Zoonoses (Office International des Epizooties (OIÉ) Reference Laboratory for Salmonellosis, Guelph, Ontario) for serotyping. Prior to overnight shipping, individual isolates were grown on LB slants overnight (37°C) and parafilmed. Slants were maintained at room temperature until tested.

Serotyping involves determining the O and H antigens on *Salmonella* isolates. The O or somatic antigens are determined through a slide agglutination technique (Ewing, 1986). The H or flagellar antigens are determined using a microtechnique (Shipp and Rowe, 1980) in microtitre plates. The antigenic formulae of Le Minor and Popoff (2001) were used to identify and name each serotype (Kauffmann-White scheme).

A serotype name was not designated without the detection of all the antigens specified in the Kauffman-White scheme for that serotype. In these instances, isolates missing one or more antigens are designated by a formula. Monophasic isolates that lack an H antigen are indicated by a minus sign ("-"). A designation of '-:-' means the isolate was non-motile. Variants were designated as 'rough' when the isolate did not express an O

antigen (CDC, 2007a). Results obtained from the OIÉ Laboratory for Salmonellosis described several isolates as *S*. Typhimurium var. Copenhagen. In this thesis the name of this variant was changed to *S*. Typhimurium variant 5 – (Var. 5-) to conform with current *Salmonella* serotype designations (CDC, 2007a).

This work assumes that all *Salmonella* isolates reported here are *Salmonella enterica*. For the following descriptions of *Salmonella* serotypes, and throughout this thesis, the species name '*enterica*' is inferred.

Stream conditions and water quality observations

To sort the samples collected during base flow or high flow conditions (event-flow), data were examined from flow gauge stations in each of the three tributaries. Data from November 2003 to June 2005 were extracted from the Water Survey of Canada's (Environment Canada) collection of discharge or level data from Hydat stations (CDRom, Hydat Version 2005 – 2.04, June 2007) and imported into the Web-based Hydrograph Analysis Tool (WHAT, Purdue University) to determine the base flow separation. Results were imported into Excel where they were visually examined after plotting hydrographs (water discharge or depth against time). Average daily discharge data were used for CAN and CON. Both of these tributaries have dams upstream of the sites and demonstrate a sluggish response to events that could be easily observed in the hydrograph. Determining if an event occurred in LC was more difficult, as there were rapid responses to smaller precipitation events. Therefore data for precipitation and field observations were used in conjunction with the level data to determine if event flow was occurring in the stream. The locations of the flow gauge stations are in Appendix A.

A sample was designated as being taken during an event if the flow or level data were above base flow or level during all or part of the time that the swab was in the water. Swabs that were placed in the water during the falling limb of the hydrograph were considered a base flow sample.

Rainfall data for Waterloo International Airport were extracted from Environment Canada's web site (www.climate.weatheroffice.gc.ca). Due to the distance between the precipitation collection point and the tributaries under study, the precipitation data

collected may not accurately represent specific events in the tributaries. Therefore, the precipitation data were averaged by month.

In addition to water temperature and pH, which were taken at each sampling point, additional water quality parameters were obtained from the Provincial Water Quality Monitoring Network (PWQMN) at the Ontario Ministry of the Environment. Data were obtained from two active water quality monitoring stations for Canagagigue Creek (Station No. 16018405102, Reid Woods Rd) and on Conestogo River (Station No. 16018402902, Northfield Dr) during the time of this investigation. No water quality data were obtained for Laurel Creek during the time of the study as there was no active PWQMN station during these years. Some data were for 2007-2008. These data and the locations of the PWQMN stations are in Appendix A.

Seasonal variability of *Salmonella* occurrence was determined based on three sample collections in winter (two in December of 2003 and one in February of 2004), six sample collections in spring (two in March of 2004 and four in May of 2004), six collections in summer (two in July of 2004 and 4 in August of 2004), and six collections in fall (two in November of 2003, two in October of 2004 and two in November of 2004).

Differential survival studies

Microcosm experiments were conducted on several *Salmonella* serotypes at 4°C. Each isolate used to inoculate microcosms was taken from frozen (-80°C) cultures and grown in LB broth at 37°C for 16 h. One mL of the *Salmonella* culture was centrifuged (10,000 rpms, 10 min.) and the cells were resuspended and washed twice with sterile saline (0.85%). Washed cells were then added to the microcosms containing 100 mL of sterile 0.85% saline (pH 8.04) in a 500 mL sterile flask.

Isolate information and length of time of each experiment is shown in Table 2.2. Each environmental isolate was tested in replicate. Microcosms were placed in a cold room (4°C) and continuously shaken at 30 rpm on an orbital shaker.

Table 2.2. *Salmonella* **isolate information and conditions for survival studies.**

Survival Expt No.	Isolate No.	Serotype	Isolate originated from	Original isolation date	Length of study (Days)
1	3A2	Typhimurium	LC	Dec/03	94
	3C4	Montevideo	CON	Dec/03	94
	Lab 1	Typhimurium ATCC 11331	-	N/A	94
2	21C1	Agona*	LC	Nov/04	121
	19D1	Heidelberg*	LC	Oct/04	121
	Lab 2	Typhimurium 971028*	-	N/A	121

*strain resistant to ampicillin

Direct counts on LB agar (experiment 1) or on LB containing ampicillin (64 µg/mL, experiment 2) were taken to determine the concentration of culturable cells in each flask at time zero. The starting cell concentrations for each microcosm were approximately 10^6 - 10^7 per mL. Aliquots were taken several additional times within the first 24 h of the study. Subsequently, samples were taken more frequently over the first two weeks and less frequently up to the end of the study (weekly to monthly). At each sampling time, aliquots were taken out, diluted in 0.85% saline and plated in duplicate.

Media comparison

Two evaluations were made on the media combinations used to isolate *Salmonella* from surface water. The first evaluation was a comparison of the media combinations that were used to isolate *Salmonella* from water throughout the main study period. The second evaluation was a targeted study to investigate differences between the media combination that were used throughout the investigation and additional media combinations that other researchers have used for *Salmonella* isolation. This latter study involved the collection of 29 additional swabs within Laurel Creek between October 2004 and May 2005. Swabs collected in 2004 were part of a study by Bartram (2007). Addition details are provided below.

Media evaluation one

The first evaluation involved a comparison of the media combinations used to isolate *Salmonella* throughout the study period. A comparison was first made between the two enrichment broths, Tet and RV, followed by an evaluation of the overall media combinations including enrichment broths and selective agars. The combinations included: Tet/MSRV, Tet/BGSA, RV/MSRV and RV/BGSA. MSRV was not supplemented with the antimicrobial novobiocin in this evaluation.

To make these comparisons, swabs that were positive for *Salmonella* from sampling dates between December 2003 to June 2005 were used. A swab was considered to be positive for *Salmonella* when one or more media combination(s) resulted in a confirmed *Salmonella* isolation. Of the 116 swabs analyzed, 84 were found to be positive for *Salmonella*.

To compare the media combinations, it was assumed that for each of the 84 swabs found to be positive for *Salmonella*, all media combinations originating from these swabs should, in theory, be positive for *Salmonella* also. For each of the 84 positive swabs, every medium or media combination that resulted in a positive isolation of *Salmonella* was given a score of 1. A score of zero was given if the media combination did not result in a positive isolation. The isolation efficiency was calculated by dividing the number of times that a specific medium or media combination was positive for *Salmonella* by 84 and expressed as a percentage.

Media evaluation two

For this study, 29 additional swabs were placed in the water as described previously. Each swab was subjected to three enrichment broths, Tet, RV, selenite cystine (SC), and six different selective media, including BGSA, MSRV with novobiocin (20 mg/L), MSRV without novobiocin, xylose lysine deoxycholate agar (XLD), bismuth sulfite agar (BS) and brilliant green agar (BGA). A positive control (*S*. Typhimurium, ATCC 11331) was run alongside each media combination. In all instances, each media combination was found to be positive for *S*. Typhimurium 11331.

Each step in the *Salmonella* isolation was carried out as described previously. Incubation times and temperatures using additional media were: SC, 48 h at 37°C ; XLD, 24 h at 37°C; BS, 24 h at 37°C; BGA, 24 h at 37°C; and, MSRV with novobiocin, 24 h at 42°C.

Similar to above, if one or more media combinations resulted in a positive isolation of *Salmonella*, then that swab was assumed to be positive for *Salmonella*. In this evaluation, 28 of the 29 swabs were found to be positive for *Salmonella*. Each media combination was then scored as described above and the isolation efficiency could be determined.

Data analysis

Percent occurrence data for *Salmonella* were calculated by determining the number of times that the site was positive for *Salmonella* divided by the total number of times that samples were taken at the site. Seasonal patterns in *Salmonella* were determined by examining the number of positive swabs and negative swabs during the designated months of the season.

The diversity of serotypes within each stream and between each season was calculated using the Shannon Index. Species richness was determined by the overall number of serotypes observed at a particular location or time over the study period.

Chi-square tests were used to determine if differences existed between the occurrence of *Salmonella* at each site, seasonally, between flow conditions and between different media. Fisher's exact test was performed if the expected value was < 5 or total value was < 50. The level of significance was set at a $P < 0.05$. Spearman rank correlations of monthly *Salmonella* occurrence data versus monthly precipitation, air temperature and water temperature were also conducted. Differences in survival among various serotypes were determined by examining differences between slopes of log concentrations (cells/mL) versus time using ANCOVA.

2.4   Results

Occurrence and spatial variability of *Salmonella*

A total of 116 swabs were analyzed for the presence of *Salmonella* between November 2003 and June 2005, and 91 (78.4%) of these swabs were found to be positive for one or more *Salmonella* isolates.

Two of the three tributaries had *Salmonella* occurrences of greater than 80%: Laurel Creek (LC) and Canagagigue Creek (CAN) had 89% (56/63) and 82% (23/28) positive, respectively. Samples taken from Conestogo River (CON) resulted in the lowest recovery of *Salmonella* at 48% (12/25). There was a significant difference in occurrence among these tributaries ($P < 0.001$). Regardless of the overall number of samples taken, a similar percent occurrence was observed at each site within a given tributary (i.e., between CON-2 and CON-3). No significant differences ($P = 0.52$ to 0.94) in the occurrence of *Salmonella* were observed between sites on the same tributary (Figure 2.2).
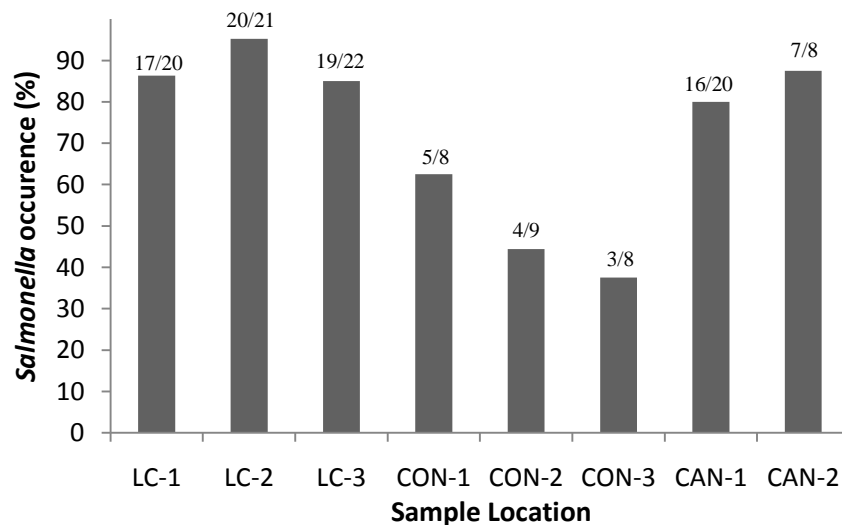
Figure 2.2. Percent occurrence of *Salmonella* at each sampling location. The number of positive swabs per site and the total number of swabs assessed are shown for each location.

Observed *Salmonella* serotypes and diversity within tributaries

Of the 235 waterborne *Salmonella* isolates obtained throughout the study, a total of 38 different serotypes were observed (Table 2.3), with the five most frequently observed serotypes being *S.* Typhimurium, *S.* Heidelberg, *S.* Thompson, *S.* Infantis and *S.* Kentucky. These serotypes comprised 43% of the total isolates observed (101/235). Half (17/34) of the *S.* Typhimurium isolates were designated as Var. 5-.

Of the five serotypes most often observed, three (*S.* Typhimurium, *S.* Heidelberg and *S.* Kentucky) were observed in all 3 tributaries. Despite its higher overall frequency, *S.* Thompson was only observed in LC but at all three sample locations in that creek.

A significant difference ($P < 0.001$) was observed in the number of serotypes obtained from each stream. The greatest diversity was in Laurel Creek (2.96 Shannon Index), where 27 different serotypes were observed. Of these serotypes, 14 were never isolated from CAN or CON. *S.* Thompson was the most frequently isolated serotype in LC. In CAN, 20 different serotypes were obtained with *S.* Typhimurium being the most frequently isolated. In CON, only 9 different serotypes were observed, with *S.* Heidelberg being the most common.

At two times during the study, fecal samples from wildlife were collected on the banks adjacent to LC-3. Fecal samples from mallard ducks collected on February 11, 2004, resulted in the isolation of *Salmonella*. Six samples were all positive and each isolate was found to be a monophasic serotype I:4,5,12:b:-. This serotype was only ever isolated from LC, where it was observed at the location that is impacted heavily by ducks (LC-3) in March 2004 and on two sampling times in May 2004 (LC-1). Supplemental sampling of fecal material from animals of unknown species in month May (2007) failed to isolate *Salmonella*.

Table 2.3. Occurrence of *Salmonella* serotypes overall and within each tributary. Frequency (%) and number of occurrences are given.

Serotype	Percent occurrence (Number of isolates)							
	Overall		Laurel Creek		Canagagigue Creek		Conestogo River	
Typhimurium*	14.5%	(34)	12.6%	(16)	**18.7%**	**(14)**	12.1%	(4)
Heidelberg	8.1%	(19)	5.5%	(7)	4.0%	(3)	**27.3%**	**(9)**
Thompson	7.7%	(18)	**14.2%**	**(18)**				
Infantis	6.4%	(15)	4.7%	(6)	12.0%	(9)		
Kentucky	6.4%	(15)	4.7%	(6)	6.7%	(5)	12.1%	(4)
Agona	5.1%	(12)	7.1%	(9)	4.0%	(3)		
Oranienberg	4.7%	(11)	4.7%	(6)	5.3%	(4)	3.0%	(1)
Kiambu	3.8%	(9)	7.1%	(9)				
Senftenberg	3.8%	(9)	2.4%	(3)			18.2%	(6)
Montevideo	3.4%	(8)	3.1%	(4)			12.1%	(4)
Tennessee	3.4%	(8)	1.6%	(2)	8.0%	(6)		
Mbandaka	3.0%	(7)	3.9%	(5)			6.1%	(2)
Berta	2.6%	(6)	4.7%	(6)				
Putten	2.6%	(6)			8.0%	(6)		
Uganda	2.6%	(6)			8.0%	(6)		
I:4,5,12:b:-	2.1%	(5)	3.9%	(5)				
Newport	2.1%	(5)	1.6%	(2)	4.0%	(3)		
Anatum	1.7%	(4)	0.8%	(1)	4.0%	(3)		
Hadar	1.7%	(4)	3.1%	(4)				
Saintpaul	1.7%	(4)	3.1%	(4)				
Derby	1.3%	(3)	2.4%	(3)				
I 4,5, 12:i:-	1.3%	(3)			1.3%	(1)	6.1%	(2)
I 28:y:-	1.3%	(3)			4.0%	(3)		
Indiana	1.3%	(3)			4.0%	(3)		
Orion	1.3%	(3)	2.4%	(3)				
Ago	0.9%	(2)	1.6%	(2)				
Enteritidis	0.9%	(2)	0.8%	(1)	1.3%	(1)		
Give	0.4%	(1)					3.0%	(1)
Hartford	0.4%	(1)	0.8%	(1)				
I 19:-:-	0.4%	(1)	0.8%	(1)				
I 4, 12:-:-	0.4%	(1)			1.3%	(1)		
I 23:d:-	0.4%	(1)			1.3%	(1)		
I Rough-O:fgt:-	0.4%	(1)	0.8%	(1)				
I Rough-O:d:l,w	0.4%	(1)			1.3%	(1)		
Litchfield	0.4%	(1)	0.8%	(1)				
Muenchen	0.4%	(1)	0.8%	(1)				
Pomona	0.4%	(1)			1.3%	(1)		
Worthington	0.4%	(1)			1.3%	(1)		
Species richness	38		27		20		9	
Diversity	3.279		2.96		2.686		1.983	

* includes *S*. Typhimurium Var. 5- and non-variant strains. Var 5- represents 5/16 in LC, 10/14 in and 2/4 in CON; Var. 5- refers to the O:5-negative variant of *S*. Typhimurium

Temporal variability of *Salmonella* within tributaries

On all sample collection dates there was always, at minimum, one sample location that was positive for *Salmonella*. This was true even for winter when the water was partially covered in ice and the water temperature was close to 0°C.

Influence of water flow/level on the occurrence of Salmonella

Between November 2003 and June of 2005, 26 high-flow events were captured. The remaining 34 sampling dates were classified as non-event or base flow. Of the 116 swabs collected, 43 were classified as event samples and 73 were classified as base flow samples (Table 2.4). Although there was a greater proportion of swabs positive for *Salmonella* during event flow vs. base flow, the difference was not significant ($P = 0.41$).

Table 2.4. Occurrence of *Salmonella* during event flow and base flow conditions throughout the study for each tributary.

Tributary	Event flow			Base flow		
	No. positive	No. negative	Percent positive	No. positive	No. negative	Percent positive
LC	24	4	85.7	32	3	91.4
CON	5	1	83.3	7	12	36.8
CAN	7	2	77.8	16	3	84.2
Overall	36	7	83.7	55	18	75.3

Significant differences within streams during event flow conditions were not observed ($P = 0.9$), however differences were observed during base flow ($P < 0.001$), as lower percentage occurrence was observed in CON compared to LC and CAN (Table 2.4). Overall, serotype diversity was similar between flow conditions with 28 different serotypes obtained during base flow and 25 during event flow. *S.* Typhimurium predominated during both flow conditions.

33

Precipitation, water and air temperature and Salmonella occurrence

Average precipitation, air temperature and water temperature for each month were compared to the occurrence of *Salmonella* in water (Figure 2.3). Spearman rank correlations between *Salmonella* occurrence and average monthly precipitation, air temperature, and water temperature were $r_s = 0.56$, $r_s = 0.39$ and $r_s = 0.53$, respectively. None of these Spearman rank correlations are significant at the 5% level (critical $r_s = 0.648$). The lowest occurrence of *Salmonella* did coincide with the month with the lowest observed precipitation, air temperature and water temperature, which was February 2004 when only 25% of swabs were found to be positive for *Salmonella* (Figure 2.3).
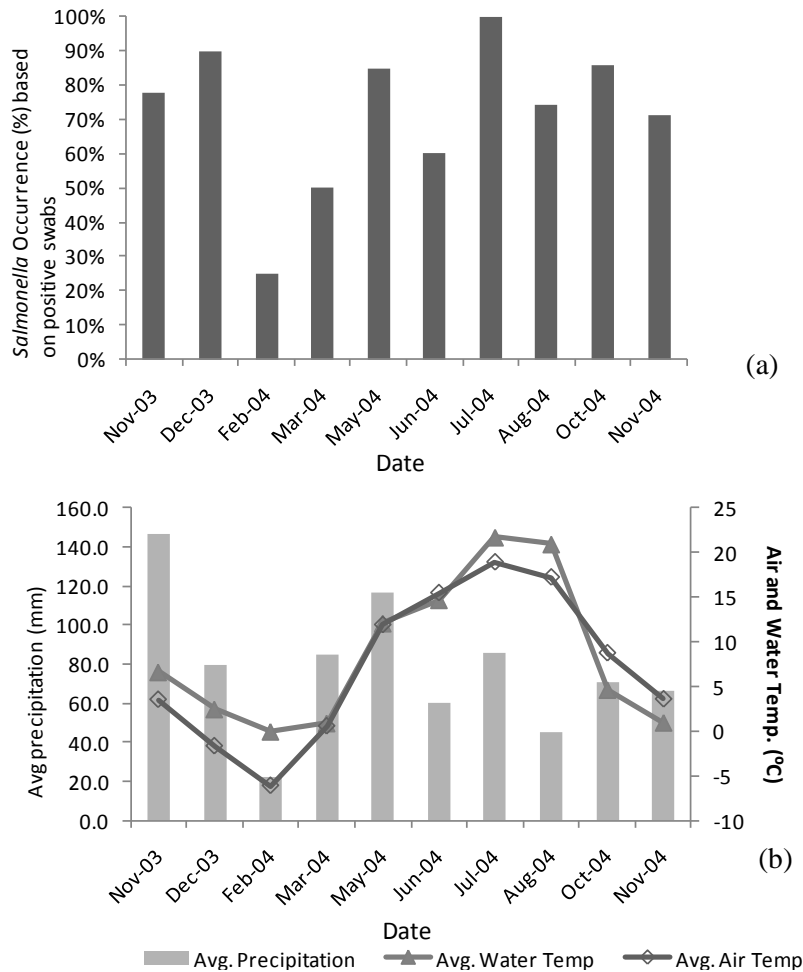
Figure 2.3. Occurrence of *Salmonella* per month based on the months in which samples were taken (a) and the corresponding average monthly precipitation, and air and water temperature (b).

34

Seasonal variability of Salmonella in water

Overall, significant differences were not observed among seasons ($P > 0.8$) as swabs were positive for *Salmonella* in the fall, winter, spring and summer at 78.4% (29/37), 71.4% (10/14), 72.7% (16/22), and 80% (28/35), respectively.

Although the overall occurrence of *Salmonella* was similar throughout the seasons, significant differences ($P = 0.002$) were observed in the serotype diversity between seasons. Overall, the winter and spring months had the lowest diversity. Similar diversity was observed in the summer and fall (Table 2.5).

Table 2.5. *Salmonella* serotype diversity by season (2003-2004).

Season	Months	No. of different serotypes	Shannon Diversity Index
Spring	Mar-May	10	2.00
Summer	Jun-Aug	22*	2.75
Fall	Sept-Nov	23	2.85
Winter	Dec-Feb	11	2.22

* value includes both variant (Var. 5-) and non-variant in
analysis, as this was the only season where both were observed

Only two serotypes were observed in all seasons; *S*. Typhimurium and *S*. Kentucky (Table 2.6). In the summer and fall, *S*. Typhimurium was the most commonly isolated serotype. The predominant serotypes over the winter months included serotypes that were less commonly observed overall and included *S*. Montevideo, *S*. Hadar, *S*. Senftenberg and *S*. Kiambu.

Table 2.6. Frequency of *Salmonella* serotypes by season (2003-2004).

	Spring (N=34)		Summer (N=75)		Fall (N=82)		Winter (N=24)	
Serotype	% (n)	Rank	% (n)	Rank	% (n)	Rank	% (n)	Rank
Typhimurium	3%(1)	6	28%(21)*	1	11%(9)	1	8%(2)**	4
Heidelberg	24%(8)	2	9%(7)	2	5%(4)	5		
Thompson			3%(2)	7				
Infantis			7%(5)	4	11%(9)	1	4%(1)	5
Kentucky	3%(1)	6	8%(6)	3	9%(7)	3	4%(1)	5
Agona			1%(1)	8	10%(8)	2	4%(1)	5
Oranienberg	26%(9)	1			1%(1)	8	4%(1)	5
Kiambu			5%(4)	5	1%(1)	8	13%(3)	3
Senftenberg	6%(2)	5			5%(4)	5	13%(3)	3
Montevideo					4%(3)	6	21%(5)	1
Tennessee	6%(2)	5			7%(6)	4		
Mbandaka	6%(2)	5	4%(3)	6	2%(2)	7		
Berta					7%(6)	4		
Putten					7%(6)	4		
Uganda			8%(6)	3				
I:4,5,12:b:-	15%(5)	3						
Newport					4%(3)	6	8%(2)	4
Anatum					5%(4)	5		
Hadar							17%(4)	2
Saintpaul			4%(3)	6	1%(1)	8		
Derby			4%(3)	6				
I 4,5, 12:i:-					2%(2)	7		
I 28:y:-	9%(3)	4						
Indiana			4%(3)	6				
Orion			3%(2)	7	1%(1)	8		
Ago			3%(2)	7				
Enteritidis			1%(1)	8			4%(1)	5
Give			1%(1)	8				
Hartford			1%(1)	8				
I 19:-:-			1%(1)	8				
I 4, 12:-:-			1%(1)	8	1%(1)	8		
I 23:d:-					1%(1)	8		
I Rough-O:fgt:-					1%(1)	8		
I Rough-O:d:l,w					1%(1)	8		
Litchfield			1%(1)	8				
Muenchen			1%(1)	8				
Pomona	3%(1)	6						
Worthington					1%(1)	8		

* 15 of 21 Var. 5-, ** all Var. 5-.

Overall, the occurrence of *S*. Typhimurium and *S*. Heidelberg appeared to vary by season (Table 2.6 and Figure 2.4). *S*. Typhimurium was found in all seasons but had a higher incidence in summer, where it represented 28% (21/75) of all isolates obtained, and a lower incidence in the spring at 3% (1/34) (Table 2.6). *S*. Typhimurium was the most frequently isolated serotype in both the summer and fall. Overall, S. Heidelberg was the 2nd most common serotype isolated in the spring and summer months where it represented 24% (8/34) and 9% (7/75) of the isolates obtained, respectively. In the fall months, this serotype was the 5th most common serotype observed at 5% (4/82). It was not observed in the winter months.
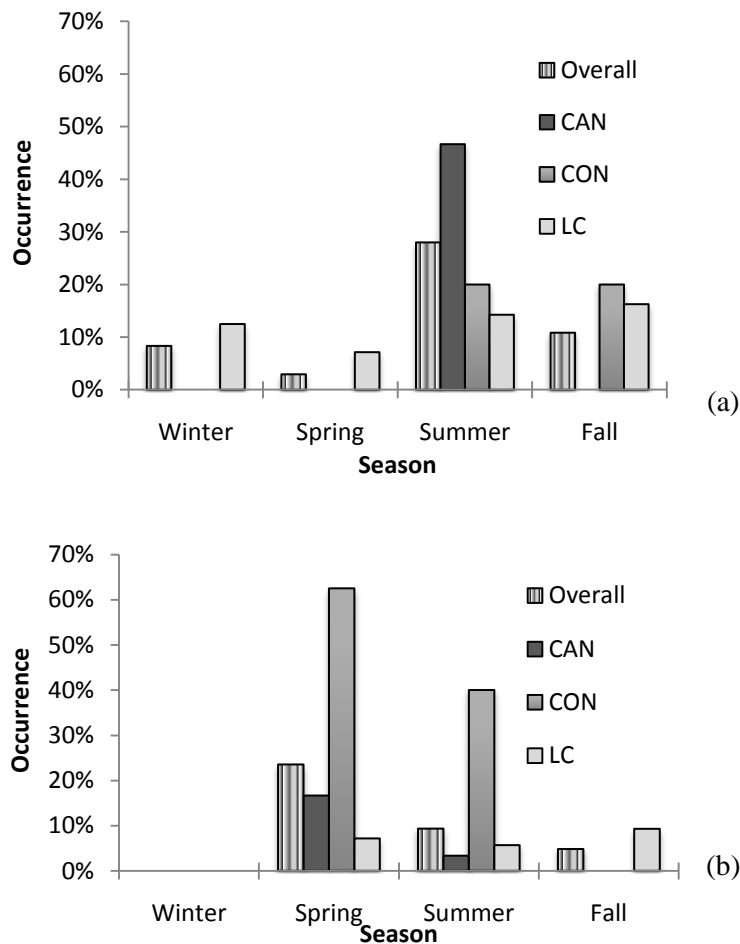
Figure 2.4. Occurrence of *S*. Typhimurium (a) and *S*. Heidelberg (b) by season in each tributary (2003-2004).

Both *S*. Typhimurium and *S*. Heidelberg demonstrated different seasonal trends when examined at the tributary level (Figure 2.4 a and b). *S*. Typhimurium was observed in all seasons in LC, where it represented the 3rd and 5th most commonly isolated serotype in winter and spring at 13% (2/16) and 7% (1/14), respectively, and it was the most commonly observed serotype in summer and fall at 14% (5/35) and 16% (7/43), respectively. In contrast to LC, this serotype was not observed in all seasons in both CAN and CON. In CAN, *S*. Typhimurium was observed only in the summer, where it was the predominant serotype representing 47% (14/30) of the isolates. Similarly, in CON this serotype was not observed in the winter and spring, but emerged in the summer and fall where it represented the 3rd and 2nd most common serotype, respectively.

During the summer months, the majority of the *S*. Typhimurium isolates obtained were Var. 5- (100% were Var.5- in CAN, 100% in CON and 60% in LC). No isolates in the spring and fall months were characterized as Var. 5- and only two isolates were characterized as variant strains in winter, both of which were found in LC.

Similar to what was observed with *S*. Typhimurium, *S*. Heidelberg showed a marked seasonal trend in CAN and CON compared to LC. Unlike *S*. Typhimurium, which peaked in the summer months in CAN/CON, this serotype showed the greatest occurrence in the spring (Figure 2.4 b). This serotype ranked 1st at 63% (5/8) in CON and 3rd in CAN at 17% (2/12) during this season. In the summer months *S*. Heidelberg continued to predominate in CON at 40% (4/10) of the isolates and dropped to the 4th most common serotype in CAN at 3% (1/30). Within these two tributaries, this serotype was not observed in the fall or the winter months. Similar to the observation seen with *S*. Typhimurium, S. Heidelberg showed more consistent levels of occurrence in LC, however, no *S*. Heidelberg was observed in this tributary in the winter months. In LC, this serotype ranked 5th overall in the spring (7%, 1/14) and 4th in the summer (6%, 2/35) and in the fall months (9%, 4/43).

Assessment of media for the recovery of *Salmonella* from water

Over the study period, 84 swabs where found to be positive for *Salmonella* through one or more of the media combinations used. If one assumes that all enrichment broths (84 Tet broths and all 84 RV broths) should be positive for *Salmonella*, then one can estimate the efficiency of these media by comparing these theoretical values (N = 84) to the actual number of enrichment broths positive for *Salmonella*. Using this method, Tet broths had a 95.2% isolation efficiency (80/84), while RV broth had 79.8% efficiency (67/84). All positive controls were positive for *Salmonella* occurrence regardless of the media used.

Overall, the combination of Tet/MSRV was more efficient than other media combinations (Table 2.7). Combinations with BGSA as the selective agar were the least efficient for the isolation of *Salmonella,* although combinations with RV seemed to have a lower level of isolation. Significant differences (*P* < 0.001) were observed among all media combinations.

Table 2.7. Recovery of *Salmonella* by different combinations of enrichment broths and selective media; Media evaluation one.

Media combination	Swab positive (N=84)	Isolation efficiency
Tet/MSRV*	75	89.3
RV/MSRV*	62	73.8
Tet/BGSA	45	53.6
RV/BGSA	22	26.2

* these trials did not include novobiocin supplement in MSRV media

For further comparison, supplemental studies were conducted with additional commonly used media for *Salmonella* isolation. Overall, the media combinations where Tet was the enrichment broth resulted in the highest percent isolation at 49% (83/168), followed by RV at 36% (61/168). SC had the lowest recovery of 18% (30/168). Significant differences ($P < 0.001$) were observed among these media types.

Similar to the first media comparison, Tet in combination with MSRV without novobiocin resulted in the greatest recovery of *Salmonella* from water (71.4% compared to 89.3% observed in the original media comparison; Table 2.8). Comparing the media combinations from the first trial, the supplemental study ranked the original media combinations in same order of efficiency; Tet/MSRV no novobiocin, followed by RV/MSRV no novobiocin, with combinations with BGSA showing lower recovery.

SC had the lowest overall recovery compared to the other enrichment broths. The only time that SC resulted in a greater than 50% recovery was when used in combination with MSRV without novobiocin.

Table 2.8. Recovery of *Salmonella* during supplemental sample collections by different combinations of enrichment broths and selective media; Media evaluation two.

Media combinations	Swab positive (N=28)	Isolation efficiency
Tet/MSRV no novo	20	71.4
Tet/BGA	18	64.3
Tet/MSRV w novo	17	60.7
RV/MSRV no novo	15	53.6
SC/MSRV no novo	15	53.6
Tet/XLD	14	50.0
RV/XLD	14	50.0
RV/BS	13	46.4
Tet/BS	12	42.9
RV/BGA	12	42.9
SC/MSRV w novo	9	32.1
RV/MSRV w novo	5	17.9
SC/XLD	4	14.3
Tet/BGSA	2	7.1
RV/BGSA	2	7.1
SC/BS	1	3.6
SC/BGSA	1	3.6
SC/BGA	0	0.0

When data were pooled for the selective media alone, without factoring in enrichment broth, several differences could be observed (see Appendix). MSRV without novobiocin resulted in the greatest recovery of *Salmonella* at 59.5% (50/84). Significant differences were observed for all comparisons including this selective media ($P = 0.005$ to < 0.001). MSRV without novobiocin resulted in a greater recovery compared to when this media included the antimicrobial (59.5% versus 36.9%, $P = 0.005$), however, pairwise comparisons revealed a significant difference ($P = 0.011$) only when these media followed enrichment in RV (see Appendix). Combinations with XLD, MSRV with novobiocin, BGA, and BS resulted in a 38.1% (32/84), 36.9% (31/84), 35.7% (30/84) and 30.9% (26/84), respectively. Media combinations with BGSA reported the lowest recovery of *Salmonella* at 5.9% (5/84), which was significantly different from all other selective media ($P < 0.001$). Significant differences ($P < 0.001$) were observed for pairwise comparisons between BGSA and BSA following isolation on all enrichment broths (see Appendix).

Differential *Salmonella* survival experiments

Comparisons of survival of various *Salmonella* serotypes at 4°C in 0.85% saline are shown in Figure 2.5. In both experiments, low rates of mortality were observed in the initial stages of each study. Culturability declined rapidly as each trial progressed. In all instances, culturable cells were detected in each microcosm at the end of each trial (94 to 121 d).
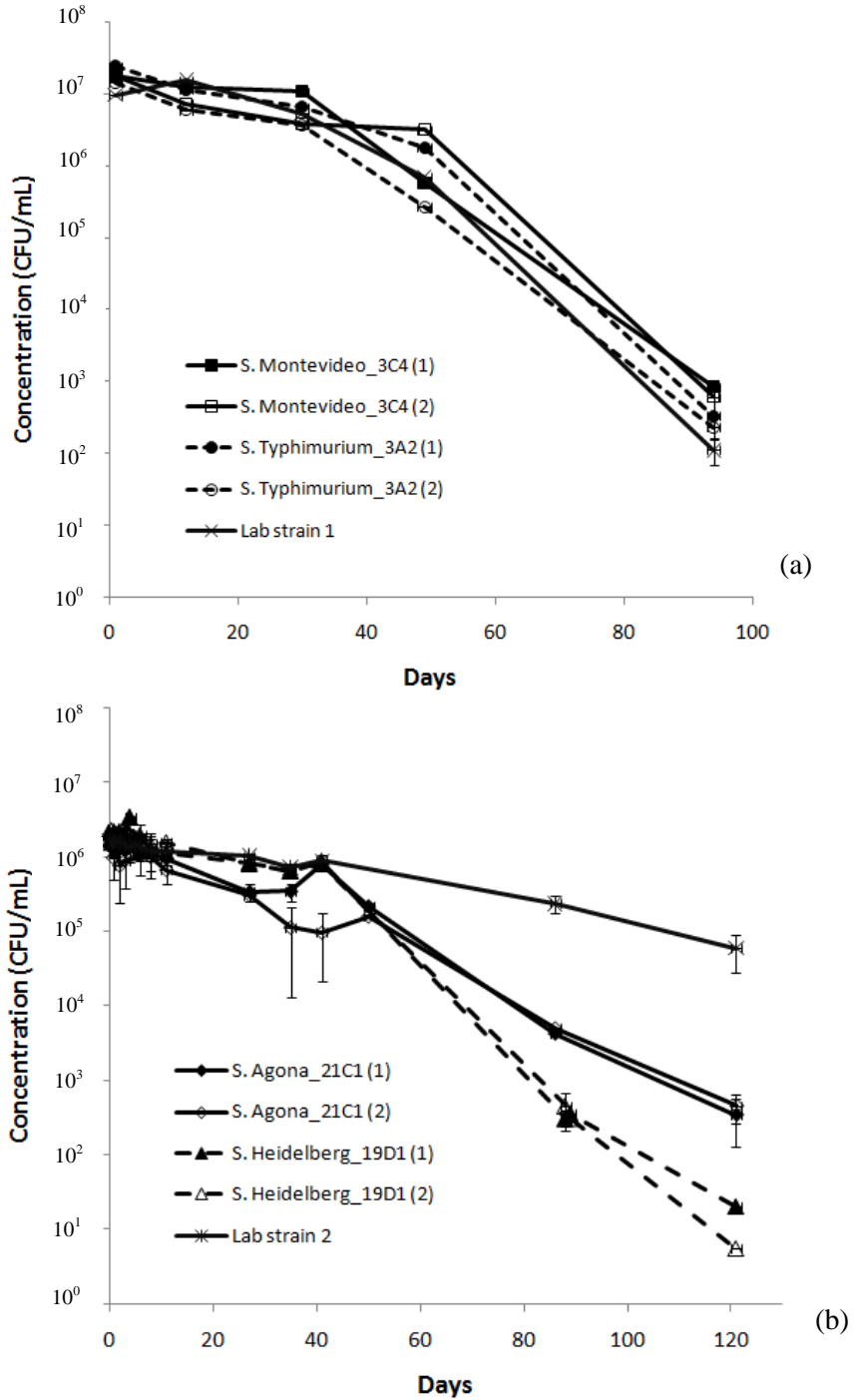
Figure 2.5. *Salmonella* **survival in saline water at 4⁰C. a) Expt. 1,** *S.* **Typhimurium and** *S.* **Montevideo, b) Expt. 2,** *S.* **Agona and** *S.* **Heidelberg. Replicate trials are shown for environmentally derived isolates. Standard deviations are shown for all. (Note: Connecting lines are shown for clarity)**

Serotype	Isolate No. (replicate)	Slope	P value
S. Agona	21C1 (1)	-0.02821	0.815
	21C1 (2)	-0.02764	
S. Heidelberg	19D1 (1)	-0.04192	0.501
	19D1 (2)	-0.04466	
S. Typhimurium	3A2 (1)	-0.05177	0.984
	3A2 (2)	-0.05202	
S. Montevideo	3C4 (1)	-0.04824	0.890
	3C4 (2)	-0.04635	
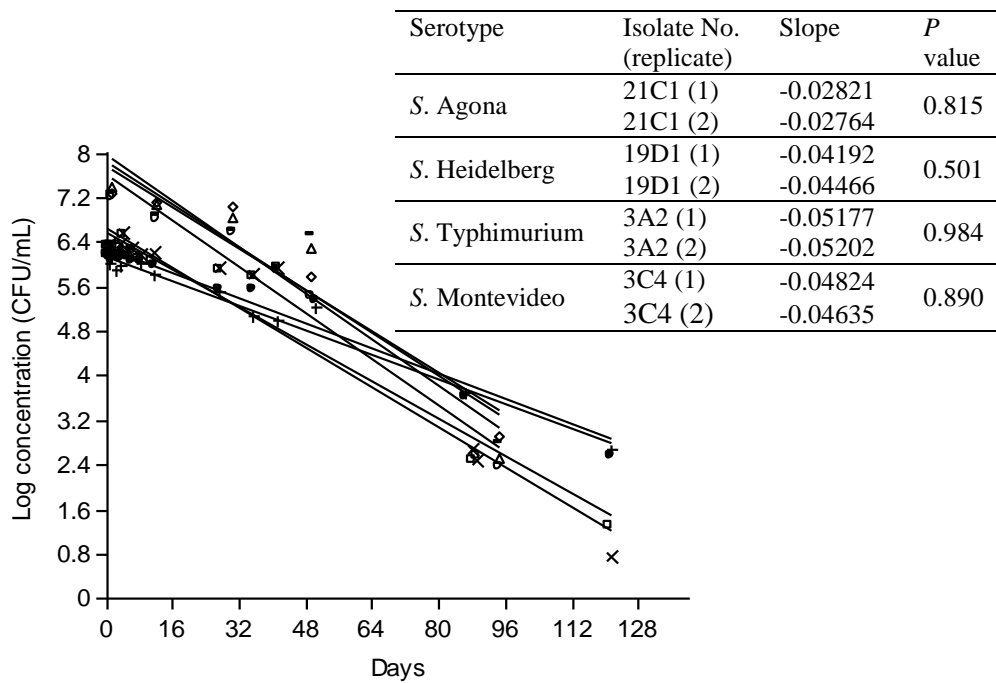
Figure 2.6. Comparison of mortality rates within serotypes using ANCOVA.

The slope of culturable cells vs. time showed no significant differences between replicate trials for each isolate (Figure 2.6), however, differences were observed among serotypes ($P < 0.001$), with *S.* Agona demonstrating differences when compared to the three other serotypes (Table 2.9). Differences were not observed among *S.* Agona, *S.* Typhimurium and *S.* Montevideo (Table 2.9).

Table 2.9. *P* values based on ANCOVA comparing mortality rates between serotypes at 4^{0}C.

Serotype	S. Agona	S. Typhimurium	S. Heidelberg	S. Montevideo
S. Agona	-	< 0.001	< 0.001	< 0.001
S. Typhimurium	-	-	0.351	0.969
S. Heidelberg	-	-	-	0.795
S. Montevideo	-	-	-	-

43

2.5 Discussion

Salmonella occurrence and isolation within the aquatic environment

This study revealed a substantially higher occurrence of waterborne *Salmonella* compared to other Canadian studies. *Salmonella* was obtained in 78.4% of the water samples (89% in LC, 82% in CAN, and 48% in CON), compared to 3-15% reported from studies on the Oldman River Basin in Alberta (Johnson *et al*., 2003; Gannon *et al*., 2004) and 3-10% in the South Nation watershed in Eastern Ontario (Wilkes *et al*., 2009; Edge *et al*., 2009). Following the completion of this study, the C-EnterNet Program observed *Salmonella* in 10-20% of water samples (PHAC, 2007a and 2007b) in the Grand River using culture based methods (4-21% in CAN and 7-21% in CON; PHAC, 2007b). While most studies world-wide describe similar occurrence levels to the Canadian studies described above, a recent study by Haley *et al*. (2009) reported *Salmonella* at comparable levels (79.2%) to the current study in highly agricultural rivers in Georgia, USA.

The difference between this study and other Canadian studies, particularly those observed in subsequent years in the Grand River (PHAC, 2007a and 2007b), is likely due to differences in collection techniques and/or the media combinations used. In 2008, C-EnterNet released a report on the methods used for the isolation of *Salmonella* in their Grand River study (PHAC, 2008). This information is useful in understanding what portion of the analysis likely accounted for the difference in *Salmonella* occurrence between these studies. Although the C-EnterNet study began sampling in the year that the field component of this study ended, it is unlikely that the difference in the occurrence levels is related to changes in land-use activities in the watershed between subsequent years.

Similar to other studies (Johnson *et al*., 2003; Gannon *et al*., 2004, Haley *et al*., 2009; Setti *et al*., 2009; Wilkes *et al*., 2009), the C-EnterNet study used a grab sample collection method to obtain water samples (500 mL). Grab samples involve collecting a specific volume of water, generally ranging from 100 to 1000 mL. The water is then filtered through a membrane which is placed in a pre-enrichment broth, or less commonly, the water samples are directly pre-enriched and used for further analysis. This research study and the C-EnterNet study used the same pre-enrichment and

enrichment media (Tet and RV), but differed in the type of selective agar used (C-EnterNet used BGA and XLD4, the current study used BGSA and MSRV). Media analysis conducted in this study showed that the media combinations used in the C-EnterNet study vary in their ability to select for *Salmonella*, although the combination of Tet followed by BGA and XLD was likely the most efficient for selection. Although differences in media combinations may have accounted for some difference in occurrence between these studies, it more likely that the collection technique used in the current study is the predominant reason for the enhanced recovery of *Salmonella*.

Studies that use identical media combinations, but a different water collection methods can be instructive to further evaluate the effect that the collection method has on the isolation of *Salmonella*. Two such studies are Johnson *et al*. (2003) and Gannon *et al*. (2004). Although these studies were conducted on different watersheds, both the Grand River and the Oldman River watersheds are known for their intense agricultural land-use activities and urban locations. Despite the use of almost identical media combinations, Johnson *et al*. (2003) and Gannon *et al*. (2004) reported the occurrence of *Salmonella* between 3% and 14.9%, compared to the current study at 78.4%. The collection technique would be the major factor in this observed variation as the authors used a small volume of water (90 mL) for direct enrichment. These authors likely would have observed greater occurrence levels if a larger volume of water was used for analysis and/or if a different collection technique was used.

Although the swab collection technique is one of the suggested methods for pathogen isolation in water (APHA, 2005), few studies employ this method for *Salmonella* isolation. One such study was Dondero *et al*. (1977), which reported *Salmonella* in 39% of water lakes and streams in New York State. In river samples downstream of agricultural feedlots, the authors found that 86% of the swabs were positive for *Salmonella*. This value is in line with the occurrence levels observed in this study.

Pathogens are known to vary greatly in space and time in aquatic systems (Johnson *et al*., 2003; Dorner *et al*., 2004a and 2004c). Due to the highly variable nature of fecally-derived pathogens in natural waters, it is not uncommon for small sample volumes that are collected by grab samples to result in limited isolation and therefore inconsistent outcomes. Small water volumes, such as 90 mL used by Johnson *et al*. (2003) and Gannon *et al*. (2004), may be too small to capture a particular pathogen and will

45

therefore give a false indication that the water is free of this pathogen. The resulting low levels of occurrence likely underestimate the ubiquitous nature of *Salmonella* and other bacterial pathogens in aquatic systems. A grab sample provides a snapshot of what is in the water at one moment in time, which can be highly variable over a short period. The swab collection technique may help to reduce this variability as isolates can be captured over a longer period of time.

As a result of the studies described above, a direct comparison of 'frequency' or 'percent occurrence' of waterborne pathogens between different studies is not easy, and in many instances, inaccurate. This is particularly true when no single standard exists for sample collection technique, volume of water used for analysis and media combinations. As a result, the occurrence levels in this study, as well as those reported by others, should be compared with caution.

Media combinations for optimal recovery of waterborne *Salmonella*

It is apparent that the sample collection method and volume of water used for analysis can affect the overall occurrence of *Salmonella*. However, as observed in the current study, the choice of media combinations can also play a critical role in the recovery of *Salmonella* from surface water.

Due to the long-standing knowledge of *Salmonella* as a food safety concern, enrichment broths and selective agars used to isolate *Salmonella* from water are largely adapted from those used for food products and stool samples (Vassiliadis *et al*., 1981; BD, 2009). Unlike these sources, enteric bacteria in aquatic environments are generally in lower concentrations and may be physiologically stressed, therefore some media used for isolation from food or fecal material may be less ideal for detection of waterborne isolates. Pre-enrichment, which involves incubating samples in buffered peptone water at 37°C, is a commonly utilized first step to help recover bacterial pathogens from natural water sources prior to isolation on selective media (Johnson *et al*., 2003; Gannon *et al*., 2004; APHA, 2005; Apel *et al*. 2009). Multiple selective media types are commonly employed to isolate *Salmonella* due to the variety of nutritional requirements and sensitivities of different serotypes (APHA, 2005).

In the current study, significant differences were observed between SC and the two other tested enrichment broths, Tet and RV. SC medium, which is a recommended media for the recovery of *Salmonella* in water (APHA, 2005) and commonly used by researchers (e.g., Arvanitidou *et al*., 2005; Morinigo *et al*., 1990a; Morinigo *et al*., 1993; Martinez-Urtaza *et al*., 2004; Setti *et al*., 2009), recovered substantially less *Salmonella* overall. Morinigo *et al*. (1990a) reported a poorer recovery of *Salmonella* from seawater when using selenite-based media. It has been suggested that selenite can have a toxic effect on waterborne *Salmonella* that are under physiological stress (Morinigo *et al*., 1990a; Morinigo *et al*., 1993). However, the pre-enrichment step is thought to help recover cells in this state (APHA, 2005; Apel *et al*. 2009). SC has shown to be more efficient if the concentration of cells in the pre-enrichment media is high (Corrente *et al*., 2004), which may not occur in natural water samples. It has also been suggested that selenite media do not adequately reduce the background levels of flora, which may be deleterious to *Salmonella* within the sample (Morinigo *et al*., 1990a; Morinigo *et al*., 1993). If this medium had been used as the sole enrichment broth in the current study, it is estimated that half as many samples would have been positive for *Salmonella*.

Both Tet and RV demonstrated high levels of *Salmonella* recovery from water compared to SC. Tet is currently a recommended media for *Salmonella* isolation in water and commonly used by researchers (APHA, 2005; Johnson *et al*., 2003; Gannon *et al*., 2004). Rappaport Vassiliadis (RV) broth, which was originally developed for *Salmonella* isolation from food and stool samples (BD, 2009) and not currently included in Standard Methods, has been used with increasing frequency for *Salmonella* isolation in water (e.g., Johnson *et al*., 2003; Gannon *et al*., 2004; Wilkes *et al*., 2009). In samples from seawater, Perales and Audicana (1989) and Morinigo *et al*. (1990a, 1993) reported that RV broth was an efficient media for *Salmonella* isolation compared to selenite-based media when paired with any combination of selective agar. RV broth reduced background flora better than selenite-based media (Morinigo *et al*., 1990a; 1993). Unlike the current study, Morinigo *et al*. (1993) reported that Tet broth was less selective than most selenite-based media.

The results of this research indicate, in addition to enrichment broths, subsequent recovery and isolation of *Salmonella* using various selective agars appeared to differ as well. Modified semi-solid Rappaport Vassiliadis (MSRV) media demonstrated the

greatest recovery when paired with any of the enrichment broths. Although MSRV is not included as a media in Standard Methods (APHA, 2005) it has been used with increasing frequency to detect waterborne *Salmonella* (Johnson *et al*., 2003; Gannon *et al*., 2004; Wilkes *et al*., 2009). Other studies have observed enhanced isolation of *Salmonella* when using MSRV compared to other media in various other matrices (e.g., human feces, Gomez *et al*., 1998). This media decreases background flora through the use of malachite green dye, magnesium chloride and a higher incubation temperature (Gomez *et al*., 1998; BD, 2009). In addition, it selects for *Salmonella* by using a lower pH semi-solid media that *Salmonella* can migrate through while many other *Enterobacteriaceae* cannot (Worcman-Barninka *et al*., 2001).

MSRV agar has several drawbacks related to its semi-solid state despite its higher recovery of *Salmonella* from water. Firstly, this media only allows for the selection of motile *Salmonella* isolates. In the current study, this was of minimal concern as less than 1% (2/235) of the waterborne *Salmonella* isolates obtained were non-motile. A small loss of certain isolates is outweighed by MSRV's ability to more efficiently isolate *Salmonella* from water. The use of additional selective agars in analysis, however, would help to recover any non-motile isolates excluded by MSRV. Secondly, unlike other selective media, a subsequent purification step is needed as the semi-solid state does not allow for colonies to form. Despite these limitations, the semi-solid state of MSRV does have an advantage over other selective media as it allows for the addition of greater volumes of enrichment broth to be plated. Greater volumes plated (200 µL) might contribute to the higher levels of recovery observed with this media type compared to other solid media where small volumes of enrichment broth are used for streaking plates.

The use of MSRV without the supplemental antimicrobial novobiocin resulted in significantly more positive samples compared to MSRV with this supplement (20 mg/L) overall. Novobiocin is included in this medium to further reduce background flora, including *Proteus* spp., in the sample while enhancing the recovery of resistant bacteria such as *Salmonella* (BD, 2009). Similar to other media used for *Salmonella* isolation in water, this medium is intended for use for food products and stool samples (BD, 2009). It has been suggested that the addition of this antimicrobial to media may inhibit environmental strains of *Salmonella* that continue to be under physiological stress (Morinigo *et al*., 1993). Previous researchers have noted that mutants of

48

Salmonella with altered outer membrane permeability have increased sensitively to this antimicrobial drug (Sukupolvi *et al*., 1984). Although Morinigo *et al*. (1990a and 1993) did not use MSRV, they did note that RV broth with and without novobiocin were both efficient for *Salmonella* isolation from water. However, they did note that when the concentration of novobiocin was high (40 mg/L), this media did not perform as well in seawater with low to moderate levels of fecal contamination. Further investigation would be needed to determine the cause of observed differences in the current study conducted in the Grand River watershed.

The combination of Tet followed by MSRV without novobiocin allowed for the recovery of more isolates than any other media combination tested in this study. If other enrichment broths or selective agars are used by other researchers, it is recommended that several media combinations be used as well. Unless followed by MSRV (without novobiocin), based on the results of this study, the use of selenite cystine is not recommended to isolate *Salmonella* from natural waters.

It appears that the media combinations used, as well as the sample collection technique, are factors in the recovery of waterborne *Salmonella*. The use of the swab collection technique was likely the most critical difference between this study and others previously carried out. The use of the swab technique has several pros and cons. Some of the disadvantages include the need to travel to a site to deploy the swab and then again to retrieve it several days later. Swabs can be lost with rapidly moving water or found to be suspended above the water if the level drops over the time of deployment. In addition, this technique cannot be turned into a quantitative method.

Although the swab technique of collection may have several shortcomings, one of its advantages is that it reduces the variability of pathogen detection in the aquatic environment. That is, the swab technique may better reflect the occurrence, if not the abundance, of *Salmonella* in water. A further advantage of obtaining a high occurrence is the ability to collect a greater number of isolates. A greater number of isolates can help to better understand and quantify the predominant serotypes that are within the aquatic environment, which may also assist in understanding linkages between this environment and the health of humans and other animals.

Predominant *Salmonella* serotypes observed in water

Identification of the different *Salmonella* strains at serotype level is critical to understanding whether clinically important isolates are present in river water. This study observed a close relationship between the predominant serotypes in water and those observed in animals and humans in the Region of Waterloo, as well as in Canada (PHAC, 2007a; 2007b). Overall, *S.* Typhimurium and *S.* Heidelberg were the most common serotypes observed in surface water samples.

The C-EnterNet study in the Region of Waterloo found the majority of *Salmonella* infections in humans to be associated with three serotypes, *S.* Typhimurium, *S.* Heidelberg and *S.* Enteriditis (PHAC, 2007a and 2007b). A comparison of travel versus endemic human cases of *Salmonella* indicated that all of the *S.* Typhimurium and *S.* Heidelberg were of domestic origin in the Region of Waterloo, whereas over half of the *S.* Enteriditis cases were travel related (PHAC, 2007b). Endemic cases of *S.* Typhimurium and *S.* Heidelberg might indicate that local environmental sources, including water, may play a role in exposure to the population. *S.* Enteriditis does not appear to be endemic in the farm animals in the region or in the country (PHAC, 2007b, Guerin *et al*., 2005a), which therefore likely explains the low occurrence of *S.* Enteriditis observed in the tributaries in this study (less than 1%).

Both *S.* Typhimurium and *S.* Heidelberg are predominant serotypes observed in farm animals, with *S.* Typhimurium being the most common serotype observed in swine and cattle in the Region of Waterloo (PHAC, 2007a and 2007b). *S.* Heidelberg predominates among poultry isolates (Government of Canada, 2005), however, it is also found among the top ten isolates in cattle and swine in Canada (Government of Canada, 2005). The predominance of these serotypes in farm animals is likely why these two serotypes were the most frequently isolated in the agricultural tributaries (CAN and CON).

Few studies have found an association between predominant serotypes in water and those observed in humans and animals (e.g., Yam *et al*., 1999; Gannon *et al*., 2004; Martinez-Urtaza *et al*., 2004; Haley *et al*., 2009; PHAC, 2007b; Patchanee *et al*., 2010). The reason for this disparity might be related to the limited number of *Salmonella* isolates obtained from water by other researchers. A greater number of isolates

obtained provides a better estimate of the overall prevalence of different serotypes. In humans and animals, hundreds to thousands of isolates are analyzed yearly through surveillance studies, such as CIPARS, to make estimates on serotype prevalence. In studies examining waterborne occurrence, usually much fewer than 100 isolates are used to make these assumptions.

Although there is no mention of the actual number of isolates obtained from the Gannon *et al*. (2004) study, of the 1629 water samples taken, 10.3% were positive for *Salmonella*. *S*. Rubislaw was the most commonly observed *Salmonella* serotype in two agriculture-impacted rivers under study in Alberta, where it represented over half of the isolates obtained (Gannon *et al*., 2004). However, as mentioned by the authors, this serotype is rarely isolated from humans or animals in Alberta or Canada (Gannon *et al*., 2004; Guerin *et al*., 2005b). Serotypes of human health significance, including *S*. Typhimurium and *S*. Heidelberg, were only a small portion of the isolates at 5% and 3%, respectively. It could be possible that obtaining more isolates may have changed these proportions.

The C-EnterNet study, which began monitoring for *Salmonella* in the Grand River following this current study in 2005, found few similarities between predominant serotypes in water and those found in animals and humans in the Region. Again, the few total isolates obtained may have limited the ability to make inferences about the predominant serotypes in these waters. For example, in 2005 and 2006 a total of 25 and 28 isolates were obtained, respectively. These few isolates represented 8-9 different serotypes, with no specific serotype distinctly predominating. While *S*. Typhimurium was observed, few *S*. Heidelberg were isolated from the water samples in that study (PHAC, 2007b).

The few studies that have isolated higher numbers of *Salmonella* isolates from water, in some instances, have observed a closer connection between serotypes obtained from water and those that are of clinical health significance in humans and animals. One example was Polo *et al*. (1999) who obtained over 800 isolates from Spanish waters (river, sea and reservoirs) and revealed that the predominant serotype to be *S*. Enteriditis, which is commonly isolated from infected humans. Similarly, Baudart *et al*. (2000) obtained over 500 isolates from French waters and found the predominant serotype to be *S*. Typhimurium. This serotype was reported to be the predominant

51

serotype in farm animals in the region. In contrast, some studies that have isolated higher numbers of *Salmonella* in water have not reported these similarities (e.g. Haley *et al*., 2009, n= 197; Patchanee *et al*., 2010, n = 104); the differences observed might be a reflection of the types of sources impacting these waters.

The detection of waterborne pathogens, including *Salmonella*, remains a challenging task. However, to represent the predominant serotypes circulating within the aquatic system, it is critical to maximize the number of isolates obtained. Better estimates of predominant serotypes in water can aid in linking sources of contamination within a watershed. In addition, observing similarities between predominant serotypes in water and those reported in animals and humans may be a first step in understanding if an environmental exposure source exists for *Salmonella*.

Spatial variability and serotype diversity of *Salmonella* between tributaries

A diverse group of *Salmonella* serotypes were observed in the aquatic environment, which suggests that many hosts are contributing to the loading of this pathogen to water. Of the 38 serotypes obtained, several serotypes rarely reported in humans and farm animals were observed, although at a lower frequency (e.g., *S*. Pomona, *S*. Kiambu and *S*. Uganda). The occurrence of these strains may be attributed to their occurrence in wildlife. In addition, several 'rough' strains and incomplete serotypes were observed in water. A similar finding was reported by Baudart *et al*. (2000) in river waters in France. These authors suggested that the occurrence of these strains of *Salmonella* in the natural environment may be related to resulting genetic modifications caused by exposure to environmental stresses. Future consideration should be given to the phenomenon of naturalization, as has been suggested in recent years with *E. coli* (Byappanahalli *et al*., 2003; Whitman and Nevers, 2003; Kon *et al*., 2007).

Pathogen monitoring studies are commonly conducted in streams impacted by agricultural operations due to the higher prevalence of pathogens, such as *Salmonella*, in farm animals. Similarly, streams impacted by sewage treatment effluent are frequently monitored for these reasons. Urban streams, on the other hand, are generally overlooked as significant sources of pathogens, particularly if there are no point sources of contamination (i.e., sewage treatment facilities). Although urban areas

would have a low rate of manure contamination by farm animals, these streams may still carry fecal waste through storm water runoff and from pets and wildlife (Dorner *et al*., 2004c). In Alberta, Johnson *et al*. (2003) reported that *Salmonella* was found in a high proportion (26.3%) of samples from municipal storm drains; one site even showed a prevalence of 80.0%.

Interestingly, Laurel Creek demonstrated the highest level of *Salmonella* occurrence and the greatest diversity of serotypes compared to the other tributaries. Of the 28 serotypes observed, 14 were only found in this stream. The great diversity of serotypes observed likely reflects the large number and variety of host species, in particular wildlife, that act as reservoirs of this bacterium in this tributary. *Salmonella* has a broad host range and is shed by a variety of wild animals, including birds (Hall and Saito, 2008; Daoust *et al*., 2000; Gorski *et al*., 2011), reptiles (Chambers and Hulse, 2006; Hahn *et al*., 2007), deer (Branham *et al*., 2005; Gorski *et al*., 2011) and small mammals (Gorski *et al*., 2011). A similar finding was recently reported by Patchanee *et al*. (2010), who observed a higher diversity of serotypes in a North Carolina watershed characterized as residential/industrial, compared to those designated as agricultural (crop or swine production) and forested. These authors also speculated that the greater diversity of serotypes observed is related to the larger variety of hosts in urban watersheds. In California, Walters *et al*. (2011) reported *Salmonella* occurrence and concentration to be positively correlated with urban land-cover, however, information on sewage treatment effluent in these urban watersheds was not mentioned; as well, serotyping data were not included in their study.

The significance of avian sources as carriers of *Salmonella* has been recognized with increasing frequency, although much remains unknown regarding the link between these sources and public health risk (Hall and Saito, 2008). Although birds can carry strains that are less commonly associated with human health, many birds are carriers of *S*. Typhimurium (Tizard, 2004; Gorski *et al*., 2011). Laurel Creek is impacted by many types of birds, including non-migratory geese and ducks that roost throughout the year. In addition, Laurel Creek waters are very turbid and allow limited light penetration which might enhance the survival of these bacteria once they have entered the water.

S. Thompson was the most common serotype in Laurel Creek. This serotype was not observed in the other two tributaries, which implies that the source of this serotype is

53

absent in agricultural/rural tributaries. This serotype is less common in humans and domestic animals compared to *S*. Typhimurium and *S*. Heidelberg, however it is often reported among the top ten serotypes in the Canadian population (Health Canada, 2003; Government of Canada, 2006). The prevalence of *S*. Thompson in wildlife is unknown, however, this serotype is more commonly reported in poultry (Guerin *et al*., 2005a; Government of Canada, 2006), therefore it might be associated with birds or waterfowl in the urban tributary.

Several other serotypes obtained in this tributary suggest avian sources, including *S*. Agona, *S*. Montevideo, *S*. Senftenberg and *S*. Litchfield (Kirk *et al*., 2002; Nesse *et al*., 2005; Hall and Saito, 2008). In addition, monophasic serotype I:4,5,12:b:-, which was only observed in Laurel Creek waters, was isolated from fecal samples taken from ducks in this tributary. Determining the genetic relatedness of isolates from water and birds may help to further make a connection between these sources (see Chapter 5 for this comparison). Birds, such as ring-billed gulls, mallard ducks and Canada geese, adapt well to urban environments. These sources could explain why *Salmonella* was commonly observed year round in LC, especially in the cold winter months, compared to Conestogo River and Canagagigue Creek. Regardless of the sources of *Salmonella* obtained in Laurel Creek, all of the serotypes obtained can pose a health risk to the human population.

Interestingly, the two streams designated as predominantly agricultural/rural, CAN and CON, showed marked differences in occurrence levels of *Salmonella* throughout the study. Overall, 48% of the samples taken from Conestogo River were positive for *Salmonella*, whereas 82% were positive from Canagagigue Creek sites. Both of these tributaries have similar land-uses, including livestock operations, crop land and small urban areas. Dorner *et al.* (2004c) characterized the counties in which both of these tributaries drain to be among the greatest for the production of manure within the Grand River watershed (> 20 kg/ha/day). These watersheds are also affected by the same climatic conditions as they are in close geographical proximity, are both located within clay till which produces run-off, and are also highly tile drained (Dorner *et al*., 2004c). Due to the similarities between these tributaries, it would be expected that both tributaries should produce a similar percent occurrence of *Salmonella*. It could also be expected that Conestogo River might actually demonstrate higher levels of occurrence as the tributary drains a larger portion of land than Canagagigue Creek and there was

one site that was located downstream of a sewage treatment facility (CON-2). In fact, the opposite was true.

There are several differences between Conestogo River and Canagagigue Creek which might account for the difference in *Salmonella* isolation and/or occurrence. These differences are largely related to the hydrology of the system and the size of the contributing area upstream of the sampling locations. Compared to Canagagigue Creek, Conestogo River drains water from a larger geographical area and has a greater discharge rate. Also, Conestogo River has a greater stream order than Canagagigue Creek. Interestingly, a larger stream order has been linked to lower pathogen occurrence and diversity (Edge *et al*., 2009). Although larger order streams generally have more streams feeding into them, and therefore more land-uses impacting the water, they are generally wider, have greater discharge rates and often are slower moving. These characteristics can result in greater dilution of bacterial contamination that results in lower frequencies of detection. Being wider and slower moving can also result in increased settling out of suspended particles and reduced turbidity which can allow for the greater penetration of UV light which contributes to the inactivation of bacteria in water.

As previously mentioned, *S*. Typhimurium was the predominant serotype in CAN and S. Heidelberg was the most common in CON, although both serotypes were observed in all three tributaries. The predominance of these two serotypes in these tributaries is likely a result of the high level of agriculture carried out in these watersheds and the elevated levels of manure production in these watersheds (Dorner *et al*., 2004c).

Over half of the *S*. Typhimurium isolates in this study were defined as *S*. Typhimurium Var. 5- isolates. Although the epidemiology of *S*. Typhimurium Var. 5- is poorly understood, studies have shown that Var. 5- is common in farm animals, particularly in cattle and swine (Frech *et al*., 2003; Zhao *et al*., 2005; Government of Canada, 2006; Farzan *et al*., 2008). Its higher occurrence in agricultural animals might explain why these variant strains were more predominantly obtained from the agricultural tributaries (n=12), over the urban tributary (n=5) in this study. Understanding the genetic relatedness of these isolates between tributaries may help to further define the ecology of these variant strains. This evaluation is carried out in Chapter 5.

Temporal variability and serotype diversity of *Salmonella* between tributaries

Climatic conditions and occurrence of Salmonella

The current study observed a large range of stream discharge/levels. Since all three tributaries have very different responses to precipitation events, it was important that data from discharge/level gauges in each tributary and in close proximity to sampling stations were used for analysis. These discharge/level data can help to determine when run-off has actually begun to impact the water. Daily precipitation data were not used in conjunction with discharge/level data in this study as no data could be obtained from rain gauges in close proximity to the sample locations.

While several studies have observed positive correlations between precipitation and occurrence of pathogens in the aquatic environment (Polo *et al*., 1999; Baudart *et al*., 2000; Simental and Martinez-Urtaza, 2008; Setti *et al*., 2009; Walters *et al*., 2011), overall, no significant relationship was observed between the occurrence of *Salmonella* and changes in flow conditions in this research study. However, at the tributary level, a difference was observed during base flow with samples from CON demonstrating a lower frequency compared to LC and CAN. Overall, the diversity of serotypes did not vary between event and base flow conditions. This is in contrast to Baudart *et al*. (2000) who observed a greater diversity of serotypes following river flooding events in France.

While studies have shown positive associations between precipitation/flow and *Salmonella* occurrence, many authors have acknowledged that the relationship is complex and not always strong (Martinez-Urtaza *et al*., 2004; Wilkes *et al*., 2009; Gorski *et al*., 2011). For examine, while Martinez-Urtaza *et al*. (2004) observed a positive correlation with *Salmonella* occurrence and precipitation, certain serotypes, including *S*. Typhimurium, did not follow this overall trend. Similar to the current study, a recent study by Gorski *et al*. (2011) reported few similarities between peaks in *Salmonella* occurrence and monthly cumulative precipitation in watersheds in California.

In the present study, few correlations observed between changes in hydrology and occurrence might be explained in several ways. Firstly, while pathogens might be entering the watercourse following increased precipitation and melt events, *Salmonella* and other pathogens might also be entering the stream from a variety of non-point sources after the hydrological event has passed. This might include the discharge from tile drains that do not flow during rain events, but discharge as the fields begin to drain off excess water over time. As mentioned previously, large portions of agricultural lands in both Canagagigue Creek and Conestogo River tributaries are artificially drained through tile drainage. Dorner *et al*. (2006), using hydrological modeling in Canagagigue Creek, observed that most pathogens were entering this stream through subsurface flow through tile drainage systems rather than overland transport. Secondly, continuous discharge from groundwater into streams during base flow may also contribute to the pathogen occurrence in these waters, although these loadings may be low. Thirdly, non-point sources including wildlife that continually load fecal wastes on the banks or directly into the stream, might be a factor in the continuous observance of *Salmonella* in these waters, particularly in Laurel Creek. Similarly, cattle wading into the streams may also contribute to *Salmonella* loading, a sight that was not uncommon in both Canagagigue Creek and Conestogo River during the time of this study. Lower occurrence in CON during base flow is likely explained by the hydrological differences described previously that contribute to increased pathogen settling and UV penetration in this stream compared to LC and CAN.

Lastly, the method of sample collection (swab technique) in this study might have reduced the ability to observe a direct correlation between hydrological events and occurrence. The high frequency of *Salmonella* occurrence, as a result of the collection technique, also limits the ability to make distinctions between event and base flow conditions. Similarly, Gorski *et al*. (2011) used a swab method of collection for some portion of water samples, which may have contributed to the lack of reported correlation between precipitation and *Salmonella* occurrence. Since the swab method is typically qualitative, it is unknown whether the concentrations in the waters were different between flow events. While monitoring streams in Georgia (USA), Haley *et al*. (2009) reported similar frequencies of *Salmonella* detection (79.2%) compared to the current study, however, their study included quantification data which allowed the

authors to observe a positive correlation between rainfall and concentration of *Salmonella*.

Regardless of the explanation, the similarity between the occurrence levels and serotype diversity among various flow conditions speaks to the ubiquitous nature of *Salmonella* in all three of these tributaries. The occurrence of *Salmonella* in these waters under various flow conditions also indicates that protection of source waters through the implementation of management practices should not be restricted to anthropogenic activities that only generate run-off following precipitation events.

Seasonal variability of Salmonella serotypes

Salmonella occurrence levels were comparable between seasons. This is in contrast to other studies in Canada that have demonstrated higher levels of occurrence in the summer (Johnson *et al*., 2003 and Gannon *et al*., 2004) or in the fall (Wilkes *et al*., 2009). Again, difference between this study and others might be related to the use of the swab collection technique and the resulting higher levels of *Salmonella* occurrence.

It is common for researchers to collect water samples during ice-free seasons in temperate climates, therefore, data are often absent for *Salmonella* occurrence over the winter months (Johnson *et al*., 2003; Gannon *et al*., 2004). This research study included many sample collections in winter to try to better represent the overall occurrence levels and the predominant serotypes in this season. During this study similar percent occurrences were observed over all seasons, including winter. This is in contrast to other studies that have reported low levels or no occurrence of waterborne *Salmonella* in the winter or colder months (e.g., Arvanitidou *et al*., 2005; Wilkes *et al*., 2009). When occurrence was examined on a monthly basis, however, February and March showed the lowest occurrence of *Salmonella* at 25% and 50%, respectively. During these months limited serotype diversity was observed as well. February demonstrated the lowest average precipitation and air and water temperature of the year. During winter, the temperatures of these streams can be low for weeks to months and they were partially ice-covered, which may limit pathogen entry to these water sources and may also affect survival once in the stream.

When examining the seasonality of *Salmonella* in the environment, most studies tend to focus on changes in the overall abundance, whereas few studies examine seasonality at the serotype level. As mentioned previously, many laboratories do not have the capability to perform serotyping analysis and as a result these data are not included (Baudart *et al*., 2000).

While no seasonal trends in occurrence of *Salmonella* where observed in this research study, there was a marked temporal diversity of serotypes with season. The greatest diversity was seen in the summer and fall months when there were two times as many serotypes observed compared to the winter and spring months. Haley *et al*. (2009) also observed a greater diversity of serotypes in surface waters in the summer months and lowest in the winter months in Georgia, USA.

In addition to overall seasonal diversity, a distinct seasonality was also observed in serotypes of human health significance; *S*. Typhimurium, and *S*. Heidelberg. Throughout the study, both *S*. Typhimurium and *S*. Heidelberg showed noticeable seasonal trends in their occurrence in water with peaks observed in the summer and spring months, respectively. A similar seasonal trend was also noted by Martinez-Urtaza *et al*. (2004) in marine waters in Spain, where the incidence of *S*. Typhimurium in water was significantly higher in the summer. Although data are not available on the seasonality of these serotypes in the human population within the Region of Waterloo, these trends correspond to the peaks in overall *Salmonella* infections in the region (PHAC, 2007a). Serotyping data at the national level, however, reveals that both *S*. Heidelberg and *S*. Typhimurium show strong seasonal trends in infection in the Canadian population (Ford *et al*, 2003; Health Canada, 2003), with peaks in infection generally reported between June and September. Although it is difficult to ascertain a relationship between environmental exposure and resulting illness, these data indicate that water may play a role in the overall distribution of these serotypes between various hosts at specific times of the year.

When examined at the tributary level, the seasonal peaks in occurrence for both of these serotypes appeared to occur only within Canagagigue Creek and Conestogo River. These peaks were not observed in Laurel Creek, which showed a consistent occurrence of these serotypes throughout the year. Further research would be needed to verify the observed seasonality of these serotypes, however it is possible that several

factors might be contributing to this observation. For example, farm animals are less apt to be a source of waterborne *Salmonella* in the colder months as animal pasturing is limited and manure is generally stored. Also run-off in these months is lower and the movement of *Salmonella* to the watercourse is minimal. Although manure spreading is decreased in the colder months, this practice is still carried out on many farms throughout the winter months in these watersheds. Differential survival of these serotypes in colder waters might also contribute to the observed differences, however, the year round occurrence of these serotypes in LC limits this assumption. Survival differences are discussed further below.

One additional reason for the observed seasonality might be related to the seasonal shedding of these serotypes within farm animals in these tributaries. Several researchers have noted seasonal differences in the overall occurrence and excretion rates of *Salmonella* in various animals, although serotype specific data are generally absent (Edrington *et al*., 2004; Guerin *et al*., 2005a; Farzan *et al*., 2009). Some reports in Canada have shown that there can be multiple peaks in *Salmonella* infections throughout the year in farm animals (Health Canada, 2003; Guerin *et al*., 2005a), with some of the larger peaks observed in the summer to early fall months (Health Canada, 2003). Many studies have observed significantly lower occurrence levels in farm animals in winter months compared to other times of the year (Edrington *et al*., 2004; Guerin *et al*., 2005a; Farzan *et al*., 2009). The seasonality of *Salmonella* serotypes in animals in the region is unknown, however, a recent study by Farzan *et al*. (2009) reported lower levels of *Salmonella* occurrence in swine manure over the winter months in the Waterloo Region. Unfortunately, no serotype data were given.

Differential survival of serotypes at low water temperatures

 Knowledge of the survival capability of *Salmonella* in the aquatic environment is important for understanding the extent to which this environment might act as a vector for the movement of this bacterium. In this research study, similar to several studies (Haley *et al*., 2009; Baudart *et al*., 2000), serotypes of human health significance were either absent or found at a lower prevalence in water at various times of the year. During colder months, *S*. Typhimurium only represented 8% of the isolates obtained in winter and dropped to 3% in the spring. *S*. Heidelberg was absent from winter samples, however, it was observed in the spring as the second most common serotype.

As mentioned above, the low occurrence or absence of these serotypes in colder months could be related to the particular pattern of *Salmonella* contamination in this region, including seasonal shedding of these serotypes or inability of contamination to reach the watercourse during this time of year. However, as suggested by others (Baudart *et al*., 2000; Haley *et al*., 2009) it is possible that differential survival rates occur between serotypes in water, particularly those of human health significance.

However, the primary objective of these trials was to examine the effects of low temperature on the survival of several different serotypes isolated from the aquatic environment. Survival was determined through culturability to reflect the methods used in the field evaluation. *Salmonella* survival was conducted under controlled and stable conditions, therefore the observed survival rates do not represent the actual survival in the aquatic environment where many other environmental factors can influence survival, including predation, sunlight intensity and water chemistry (Winfield and Groisman, 2003; USEPA, 2009c).

Significant differences were not observed between the survival rates of environmental derived isolates of *S*. Typhimurium, *S*. Heidelberg and *S*. Montevideo at $4^{\mathbf{o}}$C. Comparable survival between serotypes of human health significance, and *S*. Montevideo, which was the most common serotype obtained in the winter months, indicates that low water temperature alone is not a factor in the lower observed frequency of *S*. Typhimurium and *S*. Heidelberg at colder times of the year. This supports the notion that less shedding of these specific serotypes in farm animals or the lack of movement to the watercourse is most likely the explanation for the lower observed frequency in the colder months. From a human health perspective, the lower frequency of serotypes of clinical health significance may indicate that the overall risk to the population might also be reduced at certain times of the year.

Significant differences were observed between the survival of *S*. Agona at $4^{\mathbf{o}}$C compared to the other serotypes tested. This suggests that differential survival may exist between certain *Salmonella* serotypes in the aquatic environment at lower temperatures. Further experiments with other isolates of *S*. Agona, as well as other serotypes obtained from water, would help to determine the extent of these differences.

2.6 Conclusions

Similar to most fecally-derived pathogens, the frequency of *Salmonella* in water is highly variable over time and space. To help reduce this variability and enhance recovery of these bacteria, this study used a swab collection technique and effective media combinations. Using these techniques, the ubiquitous nature of *Salmonella* in the aquatic environment was revealed, as isolates were observed throughout the year and in all tributaries. Although the swab collection technique may have increased the likelihood of detection given the exposure period, it helped to increase the overall number of isolates obtained and allowed for a more comprehensive evaluation of the serotypes present in water.

Obtaining more isolates in this study demonstrated that serotypes that predominate in humans and farm-animals, *S.* Typhimurium and *S.* Heidelberg, were also the most common in water. Similarities between predominant serotypes in water and in the human population have not been previously observed in Canada. Although this does not indicate a direct correlation between exposure and illness, these data indicate that water may play an important role in the transmission of these serotypes between various hosts.

Seasonal variability, as opposed to hydrological events, appeared to be a predominant factor in the overall diversity and predominance of serotypes of human health significance in water. Although the occurrence of *Salmonella* was similar among seasons, marked differences in the diversity of serotypes was observed with the greatest number in summer and fall. Seasonality was observed for both *S.* Typhimurium and *S.* Heidelberg in the agricultural/rural-dominated tributaries (CAN and CON), although not in the urban tributary (LC). These differences might reflect changes in farm practices in the cooler months or the seasonal shedding of certain serotypes in domestic farm animals, whereas the consistent occurrence in the urban stream, particularly in the winter months, may reflect the continuous low level shedding of these serotypes by wildlife and/or outputs from other non-point sources.

Agricultural/rural watersheds are thought to be major contributors of non-point sources of contamination. However, small urban streams are often overlooked as a potential source of waterborne pathogens. The year round occurrence and the greater diversity of

Salmonella serotypes in Laurel Creek indicate that protection of source water should include small urban tributaries as well.

The ubiquitous nature of *Salmonella* in water, the presence of serotypes of human and veterinary health significance, as well as the long-term survival of *Salmonella* suggests that environmental exposure through consumption or contact with contaminated water is plausible. As our understanding of the occurrence of waterborne *Salmonella* increases, as well as the implementation of risk based assessment advances, our knowledge of the overall contribution of waterborne pathogens to enteric disease will expand. Surveillance and monitoring of *Salmonella* in environmental sources, such as water, is a step toward improving the understanding of the epidemiology of salmonellosis, as well as developing control strategies for the future.

2.7 Recommendations and future research needs

- While selenite cystine media may be useful for *Salmonella* recovery from waste waters, this media may not be optimal for the isolation of *Salmonella* from freshwater.

- Further monitoring should be conducted to determine if a seasonal trend continues for *S.* Typhimurium and *S.* Heidelberg in the agricultural/rural tributaries. As well, information on seasonal patterns of occurrence and/or shedding rates of these serotypes in agricultural animals in Ontario is needed.

- Follow-up investigations into survival difference between environmental strains, including *S.* Agona, are needed to confirm survival differences observed at lower water temperatures. Survival is considered greatest at lower temperatures; therefore trials at higher temperatures should also be conducted. This would provide a greater understanding of the persistence of various *Salmonella* serotypes in different seasons.

- A quantitative assessment of *Salmonella* in these waters is needed. These data can be used to perform a quantitative microbial risk assessment, which allows for a greater understanding of the actual risk posed to the population using these water sources for recreation and drinking water.

- Enteric disease surveillance in Canada has been instrumental in understanding the occurrence and predominance of *Salmonella* in humans and domestic farm animals. Initiatives like C-EnterNet, which include water monitoring, in addition to human and animal surveillance, are critical to understanding linkages between water and health. Enhancement of other government surveillance programs to include environmental sources, such as stream water, is warranted as it may allow for a broader perspective on the serotypes that are circulating in a watershed and the permanence of these serotypes in a particular geographical area.

3

Phenotypic assessment of antimicrobial resistance in waterborne isolates of *Salmonella* and *E. coli*

3.1   Introduction

The development and spread of antimicrobial resistance (AMR) represents a serious public health problem as it threatens the ability to fight infections (Health Canada, 2002; WHO, 2007). Drug resistance in bacterial pathogens is recognized as a global health problem in both human and veterinary medicine (Boerlin *et al*., 2005). The rise of resistance is attributed to the use of antimicrobial drugs in human medicine, veterinary medicine and livestock production (Threlfall, 2000; van den Bogaard and Stobberingh, 2000; Threlfall, 2002; Dutil *et al*., 2010).

National and international surveillance programs have been created to monitor the frequency and development of drug resistance in many zoonotic pathogens. In North America, these national programs include the National Antimicrobial Resistance Monitoring System (NARMS) in the USA and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) in Canada. These programs focus on AMR in enteric pathogens, such as *Salmonella,* and fecal indicator bacteria, such as *E. coli*, which originate from farm animals and humans, as antimicrobial drugs are mostly used in these hosts.

Between 2003 and 2005, CIPARS reported resistance to one or more antimicrobial drugs in 30-37% of *Salmonella* isolates obtained from human samples in Canada (Government of Canada, 2005; 2006; 2007). Farm animals showed a greater variability in the frequency of resistance with 30-68% of *Salmonella* isolates from cattle showing AMR, 47-78% from swine and 25-41% from chickens (Government of Canada, 2005; 2006; 2007). AMR in *E. coli* was reported in 27-33% of isolates from cattle, 80-88% from swine and 77-84% from chicken (Government of Canada, 2005; 2006; 2007). CIPARS does not report on *E. coli* isolates from humans, however, in a pilot study by NARMS in 2004/05, resistance was detected in 36 to 37% of *E. coli* isolates from human fecal samples (CDC, 2008).

Salmonella serotypes that predominate in humans and livestock, including *S.* Typhimurium and *S.* Heidelberg, commonly demonstrate AMR. One example is *S.* Typhimurium phagetype 104 (PT 104) which frequently demonstrates multiple drug resistance (MDR) to five drugs including ampicillin, chloramphenicol, streptomycin, sulfa-drugs and tetracycline (profile ACSSuT). This strain is commonly observed in human, swine, and cattle isolates in Canada (Government of Canada, 2006; 2007), although decreases in this MDR phagetype have been reported in recent years (Government of Canada, 2009). Drug resistance in *S.* Heidelberg is of particular importance as this serotype is frequently associated with invasive infections in humans and therefore likely to be treated with antimicrobial drugs (Government of Canada, 2007; Mataseje *et al*., 2009b). Resistance to drugs of human health importance, including 3rd generation cephalosporins, has been observed with increasing frequency in *S.* Heidelberg isolates from both human and animal sources (Shea *et al*., 2004; Government of Canada, 2007).

Health Canada has categorized antimicrobial drugs (Category I to IV) based on their importance to human medicine (Health Canada, 2011). Category I drugs, including 3rd generation cephalosporins and quinolones, are considered of very high importance in human medicine and represent the last line of defense for many invasive infections (Shea *et al*., 2004; Government of Canada, 2007). These drugs are also classified as critically important antimicrobials by the World Health Organisation (WHO, 2009). While the frequency of resistance to ceftriaxone, a 3rd generation cephalosporin used to treat serious *Salmonella* infections in children, remains low in the Canadian population, resistance to ceftiofur, a 3rd generation cephalosporin used only in animals, has

increased significantly (Shea *et al*., 2004; Government of Canada, 2007). Resistance to ceftiofur is generally associated with reduced susceptibility (intermediate resistance) to ceftriaxone (Government of Canada, 2009) and increases in resistance have been linked to the use of this drug in the poultry industry in Canada (Dutil *et al*., 2010).

The surveillance of AMR in zoonotic pathogens isolated from humans and livestock is exceedingly important for understanding the emergence and spread of AMR. Less attention has been given to AMR in enteric pathogens outside of the host environment. The occurrence of AMR bacteria and AMR genes in the aquatic environment is increasingly seen as an ecological concern (Bjorkman *et al*., 2000; Pruden *et al*., 2006; Mariano *et al*., 2009; Xi *et al*., 2009). Recently, resistance genes detected in bodies of water, independent of the bacteria from which they originate, have been described as 'emerging environmental contaminants' (Pruden *et al*., 2006). However, many questions remain as to the role natural water plays in the development and spread of resistance (Schartz *et al*., 2003). With regard to AMR in enteric pathogens, the aquatic environment may be an important reservoir for resistance and may aid in the rapid transmission of resistant bacteria between hosts, including wildlife that are not exposed to antimicrobial drugs.

Few studies have been performed to determine the distribution of drug resistant pathogens, such as *Salmonella,* in surface waters. The only Canadian study to examine AMR in waterborne *Salmonella* was conducted by Bell *et al*. (1980) in the Red River, Manitoba. The authors found that 18% of the *Salmonella* isolates carried some level of resistance to the tested drugs. No data were given on the types of AMR or the profiles demonstrated in these isolates. In other areas of the world, several studies have characterized AMR frequencies in waterborne *Salmonella*. Much of this research has coincided with concerns of *Salmonella* contamination in shellfish in coastal waters impacted by sewage. Several of these studies have been conducted in Spanish waters over the last few decades where frequencies of AMR have been reported in 12.7% to 90% of *Salmonella* isolated from sewage contaminated rivers and seawater (Alcaide and Garay, 1984; Morinigo *et al*., 1990b; Martinez-Urtaza *et al*., 2004).

Compared to *Salmonella*, considerably more studies have been conducted on AMR in waterborne *E. coli* (e.g., Edge and Hill, 2005; Hamelin *et al*., 2007; Edge and Hill, 2007). In recent years in Canada and other countries, much of this work has been

carried out in the context of microbial source tracking (MST) using resistance profiling (Parveen *et al*., 1997; Harwood *et al*., 2000; Edge and Hill, 2005 and 2007). AMR profiles in commensal bacteria, such as *E. coli*, reflect the antimicrobial drugs used by the host (Harwood *et al*., 2000; Scott *et al*., 2002); therefore, these profiles can be used in MST studies to determine the likely sources of contamination in water. Many MST studies, including one by Edge and Hill (2005) in Hamilton Harbor (Ontario, Canada), have observed a higher frequency and diversity of AMR in *E. coli* originating from human sewage compared to wildlife sources.

Improved knowledge of AMR in waterborne pathogens, including *Salmonella*, and commensal bacteria such as *E. coli*, may help in understanding the ecology of drug resistance in the aquatic environment. In addition, this information may assist in understanding whether an epidemiological connection exists between host and non-host sources, including water. Protecting water sources may not only be important for reducing pathogen loading to a watercourse, but might also be important for limiting the environmental spread of drug resistance.

3.2 Research needs and objectives

Current research on zoonotic pathogens carrying antimicrobial resistance (AMR) focuses mainly on isolates obtained from humans and farm animals intended for human consumption. However, as environmental exposure pathways for pathogens have been documented, it is important that waterborne pathogens be further characterized for drug resistance. While the role of the aquatic environment in the emergence of AMR bacteria is largely unknown, knowledge of AMR levels and profiles in waterborne pathogens may help to further define the spread of resistance outside of host animals.

Few studies have characterized the frequency of AMR in waterborne *Salmonella*. As a result, knowledge of similarities between AMR in waterborne isolates and those reported in infected humans and farm animals is largely unknown. Observed similarity between AMR levels and profiles could help elucidate if epidemiological linkages exist between aquatic sources and human and animal health.

Commensal *E. coli* is often used as an indicator organism to assess the extent and type of resistance emerging in the gastrointestinal tract of animals (van den Bogaard and

Stobberingh, 2000; Chopra and Roberts, 2001; Winokur *et al*., 2001; Salyers *et al*., 2004; Gow *et al*., 2008; Sharma and Topp 2008). Isolating and characterizing AMR in waterborne *E. coli* might help in understanding the frequency and prevalence of AMR circulating in animals within a watershed. Similarly, *E. coli* and *Salmonella* share the same ecological niche in the gut, therefore, it is not uncommon to see these two related bacteria carry similar types of AMR (Winokur *et al*., 2001; Poppe *et al*., 2005). Similarities between AMR levels and profiles in waterborne isolates of *E. coli* and *Salmonella* may allow *E. coli* to act as a surrogate representative for AMR frequencies in *Salmonella* in water. If similarities are observed, this might limit the need to detect *Salmonella* directly, which is more difficult to recover from water and takes several days longer to isolate than *E. coli*.

The specific objectives of the research presented in this chapter are to:

1. Determine the frequency and profiles of antimicrobial resistance in waterborne *Salmonella* and compare to those reported in isolates of human and animal origin;
2. Examine the spatial or temporal variability of drug resistance in *Salmonella* isolates obtained from urban and agriculturally/rural-impacted streams;
3. Examine the incidence of AMR in various serotypes and establish whether a relationship exists between serotype and resistance to single and multiple drugs; and,
4. Screen *E. coli* isolates from the same water source for comparable resistance frequency and patterns to *Salmonella*.

3.3 Materials and methods

E. coli isolation from water

Sixty five swabs were collected for the detection of *E. coli* between July 2004 and November 2004. These swabs were collected in conjunction with the swabs intended for *Salmonella* isolation.

Similar to the procedure used for *Salmonella*, as described in Chapter 2, these swabs were placed in buffered peptone water and shaken overnight at 37°C for recovery of bacterial cells. After this pre-enrichment, the bottles were shaken and 1 mL of water was filtered through a 0.45μm filter membrane (Pall Life Sciences) using a reusable sterile filter apparatus (Nalgene). Following filtration, using flame sterilized forceps, the membrane was laid flat on m-FC agar. All plates were incubated for 24 h at 44.5°C (APHA, 2005). A minimum of three colonies showing a positive reaction on m-FC agar were transferred for *E. coli* confirmation testing on EC MUG media (24h at 37°C). Following incubation, plates were examined under UV light for fluorescence. Isolates showing a positive reaction on EC MUG were further streaked onto MacConkey Agar. All media were obtained from BD Difco.

Susceptibility testing of waterborne *Salmonella* and *E. coli*

All 235 *Salmonella* and 147 *E. coli* isolates were subjected to antimicrobial susceptibility testing. This was performed using the standardized disc diffusion (Kirby-Bauer) method according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2005).

An 18-24 h culture grown on LB agar was used for AMR testing. A sterile cotton swab was used to create a bacterial suspension mixture in 0.85% saline equivalent to 0.5 McFarland Standard (1.175% wt/vol $BaCl_2$; CLSI, 2005). This standard is considered equivalent to approximately 1.5×10^8 CFU/mL. Following votexing, a fresh sterile swab was immersed into the suspension. Excess liquid was allowed to drain off by touching the side of the test tube. The entire surface of the Mueller-Hinton (MH) agar plate was swabbed edge to edge. Each plate was re-streaked two additional times by

rotating the plate a quarter turn to ensure an equal distribution of cells to create a uniform bacterial lawn.

Eight antimicrobials were chosen for initial assessment including: ampicillin (A), streptomycin (S), tetracycline (T), ceftazidime (Caz), ciprofloxacin (Cip), trimethoprim-sulfamethoxazole (SxT), sulfisoxazole (Su) and chloramphenicol (C) (BD Diagnostic Systems, NJ; Table 3.1). Isolates that were found resistance to ceftazidime were further assessed for susceptibility to ceftriaxone (Cro). Using sterile forceps, room temperature antimicrobial discs were placed on the agar surface within 5 min. of the application of cells. The plates were incubated inverted for 16-18 h at 35°C (CLSI, 2005).

Following incubation, the lawn was examined to determine if growth was uniform over the plate. The diameter of the zones of clearing (including the disc) was measured using a ruler. A single colony or a faint haze was regarded as no growth within the zone of clearing. These results were compared to standardized charts for interpretation as recommended by the manufacturer and following the criteria of CLSI (2005). Results were recorded as susceptible or resistant. Results showing intermediate resistance were considered to be susceptible in this study, unless otherwise stated. Isolates were considered to be multiple drug resistant (MDR) if they demonstrated phenotypic resistance to two or more antimicrobial drugs.

Table 3.1. Antimicrobial drugs used for phenotypic susceptibility testing, range of concentrations for minimum inhibitory concentration (MIC) testing and interpretive standard breakpoint values for resistance.

Subclass of antimicrobial drug	Antimicrobial drug	Drug code	Disc content (µg)	Concentration range (µg/mL) for MIC testing	Resistance breakpoint (µg/mL)*
Aminopenicillins	Ampicillin	A	10	32 - 512	32
Cephalosporins (3rd generation)	Ceftazidime	Caz	30	32 – 512	64
	Ceftriaxone	Cro	30	32 – 512	64
Phenicols	Chloramphenicol	C	30	32 - 512	32
Quinolones	Ciprofloxacin	Cip	5	N/A	N/A
Aminoglycosides	Streptomycin	S	10	32 - 512	64
Sulfonamides	Sulfisoxazole	Su	250	256 - 512	350
Tetracyclines	Tetracycline	T	30	16 - 512	16
Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole	SxT	1.25/ 23.75	N/A	N/A

* The concentration at which an organism is considered resistant to a specific antimicrobial drug. With the exception of streptomycin, all breakpoints values are from the CLSI (2005) MIC interpretive standards for *Enterobacteriaceae*. The resistance breakpoint for streptomycin was obtained from CDC (2007b).

Minimum inhibitory concentration of waterborne *Salmonella*

All resistant *Salmonella* isolates were further assessed to determine their minimum inhibitory concentration (MIC). This was carried out for each antimicrobial agent where full resistance was observed, with the exception of trimethoprim-sulfamethoxazole (SxT) due to its low prevalence.

MIC testing was carried out through agar dilution as described in the CLSI standard (CLSI, 2005). Following sterilization, MH agar was cooled to 50°C in a water bath before the addition of each antimicrobial drug. Each antimicrobial was added to the agar at two-fold increments and ranged in concentrations based on the break points of antimicrobial tested (Table 3.1). All antimicrobial drugs were purchased from Sigma Aldrich, with the exception of tetracycline, purchased from Alfa Aesar.

A suspension equivalent to 0.5 McFarland units was prepared in 0.85% saline from an 18 - 24 h culture grown overnight on LB agar at 37⁰C. Ten μL of the suspension were spot plated into a designated quadrant on each Mueller-Hinton agar plate containing the antimicrobial agent of varying concentrations. Plates were incubated right-side up at 35°C to prevent the inoculum from spreading. Growth/no growth was recorded from each plate after 16-18 h incubation at 35⁰C.

Phagetyping of selected *Salmonella* serotypes

Selected *Salmonella* serotypes, including *S*. Typhimurium, *S*. Heidelberg, *S*. Enteriditis and several monophasic strains, were phagetyped at the Public Health Agency of Canada's Laboratory for Foodborne Zoonoses (LFZ), Office International des Epizooties (OIÉ) Reference Laboratory for Salmonellosis (Guelph, Ontario). Only phagetypes demonstrating drug resistance are reported in this chapter. Additional details are given in Chapter 5.

Phagetyping is a specialized method performed in few laboratories in Canada. At the OIÉ Reference Laboratory for Salmonellosis, isolates were maintained at room temperature until tested. Briefly, prior to testing, all isolates were plated on nutrient agar and incubated at 37⁰C for 18 h. A colony was inoculated into 4.5 mL of Difco Phage Broth and incubated for 1.5 to 2 h in a shaking water bath at 37⁰C to obtain a turbidity equivalent to 0.5 McFarland Units. Difco Phage Agar plates were flooded with 2 mL of culture and excess liquid was removed. Plates were allowed to dry for 15 minutes at room temperature and approximately 20 μl of each of the serotype specific typing phages were inoculated onto the bacterial lawn using a multiple inoculating syringe method (Farmer et al., 1975). The plates were incubated overnight at 37°C and lytic patterns were examined and interpreted as described by Anderson and Williams (1956).

Isolates that reacted with phages, but did not conform to any recognized phagetype, were designated as atypical. Strains that did not react with any of the phages were designated as untypable. In this chapter, each phagetype is given the designation of 'PT' followed by the numerical value assigned to each isolate.

Data analysis

Groupings of various drugs are used in this chapter to compare their health significance to humans. These groupings are based on criteria set forth by Health Canada (http://www.hc-sc.gc.ca/dhp-mps/vet/index_e.html) and more recently updated (Government of Canada, 2007). This categorization has been used to give context to the types of antimicrobials tested. Category I drugs are considered of 'very high importance' in human medicine with no alternative antimicrobial for resistant infections (e.g., ceftriaxone and ciprofloxacin). Category II are considered 'high importance' (e.g., ampicillin, streptomycin, trimethoprim-sulfamethoxazole) and are alternative drugs when resistance occurs to category III drugs. Category III drugs are considered of 'moderate' or 'medium importance' and are often used as first line drugs (e.g., chloramphenicol, sulphamethoxazole, tetracycline, and 1st generation cephalosporins). Category IV drugs are considered of 'low importance' and are of limited use in human medicine due to the high levels of resistance observed in various bacteria. No Category IV antimicrobials were tested in this study.

Chi-square test was performed to determine if significant differences were observed between observations. Fisher's exact test was performed if the expected value was < 5 or total value was <50. The level of significance was set at a P <0.05. If the value was < 0.001, then this value was stated, to represent a highly significant difference.

Cluster analysis was performed to examine similarities between *Salmonella* resistance profiles. A binary data table for each resistance type for each isolate was created. Dendograms were generated using an unweighted –pair group algorithm (UPGMA) method with similarity measured by Dice coefficient using BioNumerics software (Applied Maths, Belgium).

3.4 Results

Occurrence of antimicrobial resistance in waterborne *Salmonella*

Overall, 32.8% (77/235) of the *Salmonella* strains isolated from surface water exhibited full resistance to one or more antimicrobial drugs. When the prevalence of resistance to individual antimicrobial drugs was assessed, resistance to sulfisoxazole was observed most frequently (25.1%, 59/235). Resistance to streptomycin was observed in 19.1% of the isolates (45/235), tetracycline resistance was observed in 13.6% (32/235) and 9.8% (23/235) were resistant to chloramphenicol. Among the β-lactams, resistance was greatest to ampicillin at 18.7% (44/235), followed by resistance to ceftazidime and ceftriaxone at 4.3% (10/235). Only one isolate was resistant to trimethoprim-sulfamethoxazole (<1%) and no resistance or intermediate resistance was observed to ciprofloxacin.

Minimum inhibitory concentration observed in waterborne Salmonella

There was a wide range of MIC for many antimicrobial drugs (Table 3.2). MIC exceeding the maximum tested range was common for ampicillin, sulfisoxazole and ceftriaxone. Isolates generally showed a range of MIC for tetracycline, ceftazidime, chloramphenicol and streptomycin.

Table 3.2. Distribution of MIC levels for all *Salmonella* isolates demonstrating resistance.

Antimicrobial	Resistance breakpoint (µg/mL)	No. of resistant isolates inhibited at antimicrobial concentration (µg/mL)						
		16	32	64	128	256	512	> 512
Ampicillin	32	-	0	0	0	0	0	44
Ceftazidime	64	-	0	2	3	4	1	0
Ceftriaxone	64	-	0	0	0	1	1	8
Chloramphenicol	32	-	0	0	0	14	9	0
Streptomycin	64	-	1	0	5	14	14	12
Sulfisoxazole	350	-	-	-	-	0	3	56
Tetracycline	16	0	0	0	16	1	15	0

Antimicrobial resistance profiles in Salmonella

Overall, 14 different resistance profiles were observed in the waterborne *Salmonella* isolates. In total, 75.3% (58/77) of resistant isolates demonstrated multiple-drug resistance (MDR) to two or more antimicrobial agents.

The most commonly observed resistance profile was penta-drug resistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole and tetracycline (R type - ACSSuT). This ACSSuT profile represented over a quarter of the resistant isolates observed (Figure 3.1). The second most predominate resistance profile was to streptomycin/sulfisoxazole (SSu) followed by ampicillin/sulfisoxazole (ASu) and streptomycin/sulfisoxazole/tetracycline (SSuT). These four different profiles accounted for half of the drug resistant isolates.

Resistance to only one antimicrobial drug was observed in 24.7% of the isolates. Single drug resistance (SDR) was observed in response to sulfisoxazole, ampicillin, streptomycin and tetracycline. Resistance to chloramphenicol, trimethoprim-sulfamethoxazole or ceftazidime was never observed alone. All isolates that demonstrated resistance to ceftazidime were also resistant to ceftriaxone and ampicillin, at a minimum. In total, three different resistance profiles were observed with ceftazidime and ceftriaxone (Figure 3.1). Resistance to chloramphenicol was only observed in combination with the penta-drug resistance profile ACSSuT or ACSSuTCazCro.
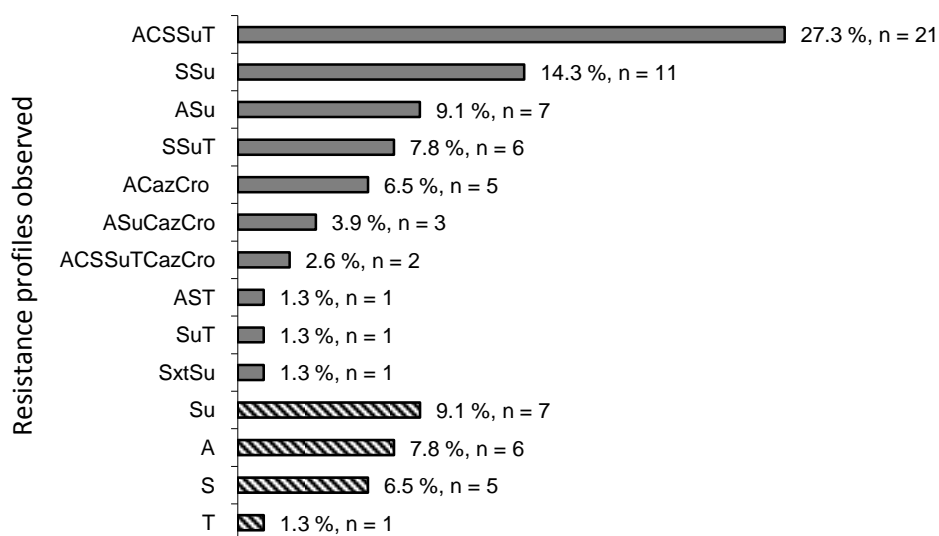
Figure 3.1. Frequency of antimicrobial resistance profiles in waterborne *Salmonella*. Multiple drug resistance is shown in the solid bars and single drug resistance in the hatched bars. Percentage and number (n) of isolates demonstrating the profile are also shown.

Drug resistance observed in each tributary

The greatest levels of AMR *Salmonella* were found in Canagagigue Creek at 60.9% (14/23 swabs yielded AMR isolates) and Laurel Creek at 58.9% (33/56). Apparently lower levels were observed in isolates obtained from Conestogo River at 33.3% (4/12) but comparisons yielded no significant differences among the tributaries ($P = 0.24$).

Drug resistance was observed at all sites with the exception of CON-2 (Figure 3.2). Each site in LC and in CAN demonstrated similar frequencies of AMR occurrence. This similarity between sites was not observed in CON as the frequency of AMR occurrence ranged from 0% to 60% at this site.
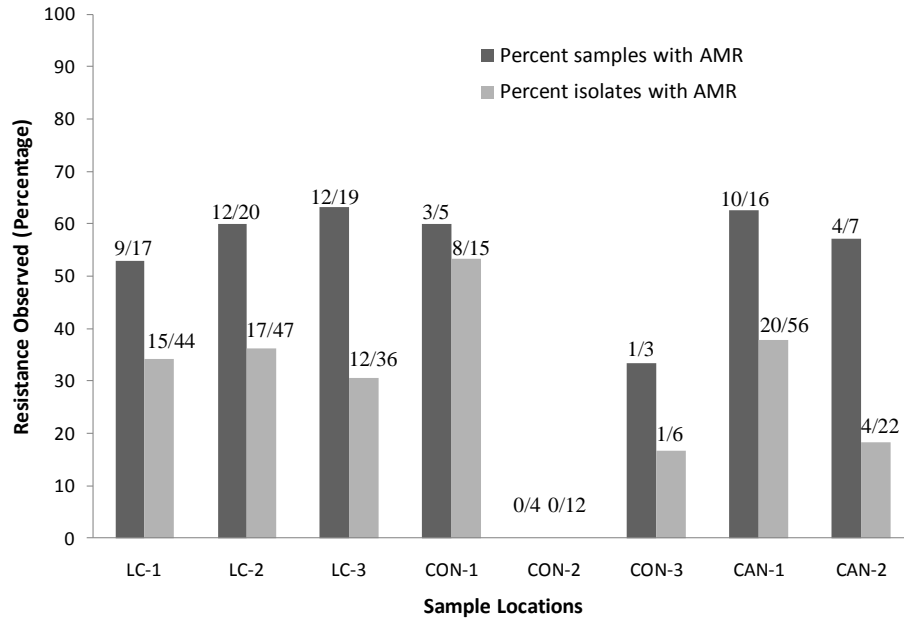
Figure 3.2. Samples positive for AMR *Salmonella* (total number of samples positive for AMR *Salmonella* / total number of samples positive for *Salmonella*) and the number of isolates demonstrating resistance at each sample location (number of isolates showing AMR / the total number of isolates obtained at the location) at the sampling locations.

In each tributary a higher proportion of isolates demonstrated MDR over SDR, with 83.3% (20/24) of isolates showing MDR in CAN, 72.7% (32/44) in LC and 66.7% (6/9) in CON.

Resistance to most Category II and III drugs appeared to be common (Table 3.3). Resistance to ceftazidime/ceftriaxone and trimethoprim-sulfamethoxazole were observed in LC, but never in CAN or CON.

Table 3.3. Antimicrobial resistance in *Salmonella* isolates by tributary throughout the study period.

Category of human health importance	Antimicrobial	LC N = 127		CAN N = 75		CON N = 33		Total N = 235	
		%*	n	%*	n	%*	n	%*	n
I	Ciprofloxacin	0.0	0	0.0	0	0.0	0	0.0	0
	Ceftazidime	7.9	10	0.0	0	0.0	0	4.3	10
	Ceftriaxone	7.9	10	0.0	0	0.0	0	4.3	10
II	Ampicillin	19.7	25	16.0	12	21.2	7	18.7	44
	Streptomycin	17.3	22	28.0	21	6.1	2	19.1	45
	Trimethoprim-sulfamethoxazole	0.8	1	0.0	0	0.0	0	0.4	1
III	Chloramphenicol	8.7	11	13.3	10	6.1	2	9.8	23
	Sulfisoxazole	24.4	31	28.0	21	21.2	7	25.1	59
	Tetracycline	14.2	18	14.7	11	9.1	3	13.6	32

N= total number of *Salmonella* isolates per tributary

n = number of isolates demonstrating resistance to selected antimicrobial agents

*percent of the number of AMR isolates (n) divided by the total number of isolates obtained in the tributary (N)

Of the 14 different AMR profiles observed in this study, the greatest diversity was in LC where 13 of these profiles were observed, compared to Canagagigue Creek (7 of 14) and Conestogo River (5 of 14).

Occurrence of drug resistance in relation to climatic conditions and seasons

When comparing samples that were AMR positive relative to flow conditions, 52% (17 out of 33) of *Salmonella* positive swabs were found to contain AMR *Salmonella* during an event flow, whereas 59% (34 out of 58) were positive during base flow conditions. There was no significant difference ($P = 0.66$) between the occurrence of AMR bacteria and different flow conditions.

Similarly, no difference ($P = 0.34$) was observed between the occurrence of AMR *Salmonella* positive samples in each season. In the fall, winter, spring and summer seasons, the samples collected contained AMR *Salmonella* in 58.3% (14/29), 60.0% (6/10), 75.0% (12/16) and 64.3% (18/28), respectively. A comparison between the percent of AMR positive samples in each tributary over each season was not conducted due to the low overall number of swabs.

When comparing the total number of isolates versus the number that were AMR, trends can be observed within each tributary throughout the different seasons. In LC, the winter and fall months showed the largest percentage of drug resistant isolates (Figure 3.3). In CAN, AMR isolates predominated in the spring and summer months. No AMR isolates were observed in this tributary in the winter months. Within CON, low numbers of AMR *Salmonella* were observed throughout all seasons.
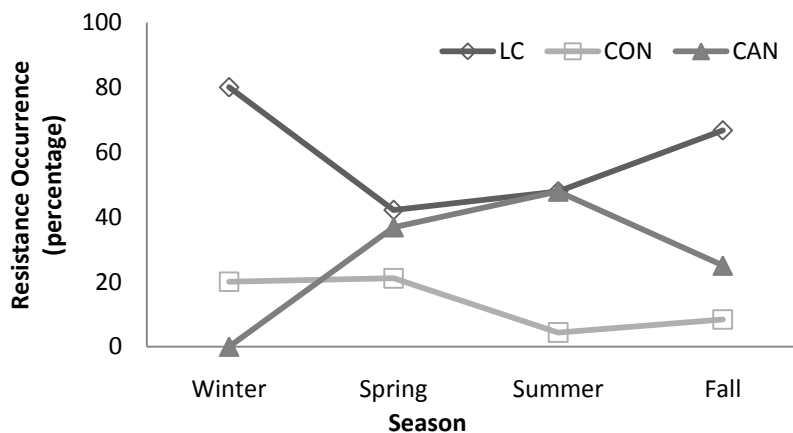
Figure 3.3. Percentage of *Salmonella* isolates demonstrating drug resistance throughout each season and in each tributary.

Resistance observed in various *Salmonella* serotypes obtained from water

Overall, 19 of the 38 serotypes obtained from water showed some level of drug resistance (Table 3.4). Susceptible and resistant isolates were observed within most serotypes (Table 3.4). Within the same serotype, many isolates demonstrated a broad range of the number of antimicrobial drugs included in AMR profiles. Only four serotypes showed resistance patterns to 5 or more drugs, including *S.* Typhimurium (including Var. 5-), *S.* Tennessee, *S.* Mbandaka and serotype I:4, 5, 12:i:-. Two isolates of *S.* Mbandaka demonstrated resistance to seven drugs.

Several serotypes demonstrated a higher portion of AMR over other serotypes (Table 3.4). Of the serotypes where over 5 isolates were obtained, over half of the isolates demonstrated antimicrobial resistance in serotypes: *S.* Typhimurium (including Var. 5-), *S.* Heidelberg, *S.* Tennessee and *S.* Mbandaka.

Several serotypes were more likely to carry resistance to multiple drugs than others, including *S.* Mbandaka, *S.* Typhimurium (and Var. 5-), *S.* Agona and *S.* Berta (Table 3.4). Serotypes that were susceptible to all antimicrobials tested were: *S.* Thompson, *S.* I:4,5,12:b:-, *S.* Senftenberg and *S.* Uganda. Serotype I:4,5,12:b:- obtained from duck fecal samples were also shown to be susceptible to all antimicrobials tested (data not shown in Table 3.4). Most other serotypes showed variable resistance with some isolates demonstrating MDR, SDR or no resistance.

Table 3.4. Proportion of waterborne *Salmonella* serotypes demonstrating drug resistance and number of antimicrobials included in resistance profile.

Serotype	N	Number of antimicrobials included in resistance profile								Isolates showing AMR (%)	AMR isolates showing MDR (%)
		0	1	2	3	4	5	6	7		
Typhimurium	17	8	1			1	7			52.9	88.9
Typhimurium Var. 5-	17	6					11			64.7	100.0
Heidelberg	19	8	3	6	2					57.9	72.7
Thompson	18	18								0.0	-
Infantis	15	12	2			1				20.0	33.3
Kentucky	15	13	1			1				13.3	50.0
Agona	12	5	2	3	2					58.3	71.4
Oranienberg	11	4	5	2						63.6	28.6
Kiambu	9	8		1						11.1	100.0
Senftenberg	9	9								0.0	-
Montevideo	8	6	1	1						25.0	50.0
Tennessee	8	3	2	2			1			62.5	60.0
Mbandaka	7	2		3					2	71.4	100.0
Berta	6	4			1	1				33.3	100.0
Putten	6	5		1						16.7	100.0
Uganda	6	6								0.0	-
I:4,5,12:b:-	5	5								0.0	-
Newport	5	4			1					20.0	100.0
Anatum	4	4								0.0	-
Hadar	4	0			4					100.0	100.0
Saintpaul	4	4								0.0	-
Derby	3	1		2						66.7	-
I:28:y:-	3	3								0.0	-
I:4, 5,12:i:-	3	2				1				33.3	100.0
Indiana	3	3								0.0	-
Orion	3	3								0.0	-
Ago	2	2								0.0	-
Enteritidis	2	2								0.0	-
Give	1	0	1							100.0	0.0
Hartford	1	1								0.0	-
I:19:-:-	1	1								0.0	-
I:23:d:-	1	1								0.0	-
I:4,12:-:-	1	0		1						100.0	100.0
I:Rough-O:d:l,w	1	1								0.0	-
I:Rough-O:fgt:-	1	1								0.0	-
Litchfield	1	0	1							100.0	0.0
Muenchen	1	1								0.0	-
Pomona	1	1								0.0	-
Worthington	1	1								0.0	-

N = total number of isolates per serotype
0 = isolate susceptible to all antimicrobials tested
1-7 = isolate resistant and shows profiles with resistance to 1 to 7 antimicrobial drugs

Similarity
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9

Serotype	Trib	Date (D/M/Y)	Season	Phage-type
Heidelberg	LC	31/05/2004	Spring	6
Heidelberg	CAN	31/05/2004	Spring	19
Heidelberg	CON	12/05/2004	Spring	Atypical
Heidelberg	CON	12/05/2004	Spring	Untypable
Heidelberg	CON	07/05/2004	Spring	19
Heidelberg	CON	07/05/2004	Spring	Atypical
Montevideo	LC	21/11/2003	Fall	-
Agona	LC	22/11/2004	Fall	-
Heidelberg	LC	21/10/2004	Fall	19
Heidelberg	Lc	18/10/2004	Fall	19
Heidelberg	CAN	31/05/2004	Spring	19
Montevideo	CON	10/12/2003	Winter	-
Oranienberg	LC	11/12/2003	Winter	-
Agona	LC	22/11/2004	Fall	-
Berta	LC	22/11/2004	Fall	-
Newport	LC	18/11/2004	Fall	-
Heidelberg	LC	10/08/2004	Summer	29a
Heidelberg	LC	21/07/2004	Summer	Untypable
Berta	LC	22/11/2004	Fall	-
Infantis	LC	18/10/2004	Fall	-
Kentucky	LC	07/05/2004	Spring	-
Agona	LC	11/12/2003	Winter	-
Litchfield	LC	16/08/2004	Summer	-
Infantis	LC	10/08/2004	Summer	-
Oranienberg	LC	07/05/2004	Spring	-
Oranienberg	LC	13/03/2004	Spring	-
Oranienberg	CAN	09/03/2004	Spring	-
Kentucky	CON	13/12/2003	Winter	-
Infantis	LC	13/12/2003	Winter	-
Agona	LC	26/08/2004	Summer	-
Oranienberg	LC	07/05/2004	Spring	-
Typhimurium	LC	07/05/2004	Spring	41
Tennessee	CAN	21/11/2003	Fall	-
Tennessee	CAN	21/11/2003	Fall	-
"1:4,5,12:-:-"	CAN	21/10/2004	Fall	-
Agona	CAN	21/10/2004	Fall	-
Agona	CAN	21/10/2004	Fall	-
Putten	CAN	18/10/2004	Fall	-
Agona	LC	21/07/2004	Summer	-
Mbandaka	LC	21/07/2004	Summer	-
Mbandaka	LC	21/07/2004	Summer	-
Mbandaka	CAN	12/05/2004	Spring	-
Tennessee	CAN	12/05/2004	Spring	-
Tennessee	CAN	09/03/2004	Spring	-
Oranienberg	CAN	09/03/2004	Spring	-
Oranienberg	LC	16/08/2004	Summer	-
Kiambu	CAN	26/08/2004	Summer	-
Derby	LC	04/06/2004	Summer	-
Derby	LC	04/06/2004	Summer	-
Hadar	LC	11/02/2004	Winter	-
Hadar	LC	11/02/2004	Winter	-
Hadar	LC	10/12/2003	Winter	-
Tennessee	LC	21/10/2004	Fall	-
Typhimurium	LC	06/06/2005	Summer	104
Typhimurium	LC	21/10/2004	Fall	104
Typhimurium	LC	21/10/2004	Fall	104
Typhimurium	LC	21/10/2004	Fall	104
Typhimurium	LC	18/10/2004	Fall	104
Typhimurium	LC	18/10/2004	Fall	104
"1:4,5,2:i:-"	CAN	26/08/2004	Summer	120
Typhim_Var!	CAN	23/08/2004	Summer	104
Typhim_Var!	CAN	16/08/2004	Summer	104
Typhim_Var!	CAN	10/08/2004	Summer	104
Typhim_Var!	CAN	10/08/2004	Summer	104
Typhim_Var!	CAN	10/08/2004	Summer	104
Typhim_Var!	CAN	30/07/2004	Summer	104
Typhim_Var!	CAN	30/07/2004	Summer	104
Typhim_Var!	CAN	21/07/2004	Summer	104
Typhim_Var!	CAN	21/07/2004	Summer	104a
Typhim_Var!	LC	13/12/2003	Winter	104
Typhim_Var!	LC	10/12/2003	Winter	104
Typhimurium	CON	19/11/2003	Fall	104
Typhimurium	CON	19/11/2003	Fall	104
Mbandaka	LC	07/05/2004	Spring	-
Mbandaka	LC	07/05/2004	Spring	-
Hadar	LC	10/12/2003	Fall	-
Give	CON	10/08/2004	Summer	-

Cluster labels (left of dendrogram): ASu, A, ACazCro, ASuCazCro, SuSxT, Su, S, SSu, SuT, SSuT, ACSSuT, ACSSuTCazCro, AST, T

Figure 3.4. Cluster analysis of drug resistance profiles associated with *Salmonella* serotypes.

The most common AMR profile, ACSSuT, was observed in 3 serotypes including 1 isolate of S. Tennessee, 1 isolate designated as I:4,5, 12:i:- and 19 isolates of S. Typhimurium (Figure 3.4).

Overall, 58.8% (20 of 34) of *S*. Typhimurium isolates demonstrated drug resistance, with the predominant resistance profile of ACSSuT (Figure 3.4). Only one isolate in this serotype demonstrated a profile other than ACSSuT, which was single drug resistance to streptomycin. All of these penta-drug resistant *S*. Typhimurium were designated as phagetype (PT) 104, with the exception of one that was found to be PT 104a. These isolates were obtained from all three tributaries, however, their frequency varied with season (Figure 3.4). S. Typhimurium PT 104 was more frequently isolated in the summer months (10 of 19 isolates), the majority of which were Var. 5- isolates and obtained from CAN (9 of 10). In the fall and winter months, PT 104 predominated in LC. No PT 104 isolates were detected in the spring in any of the tributaries.

S. Heidelberg isolates were also frequently observed to be drug resistant (57.9%, Table 3.4). Compared to *S*. Typhimurium, this serotype demonstrated diverse resistance profiles including SDR and MDR (Figure 3.4), however, resistance was always associated with one or more β-lactam drugs (ampicillin, ceftriaxone, ceftazidime). Several different phagetypes were associated with resistance, including PT 19, PT 6 and PT 29a. Isolates demonstrating atypical patterns or isolates that could not be phagetyped also demonstrated resistance. These AMR isolates were predominantly observed in the spring months (7 of 11) and observed in all tributaries (Figure 3.4). Lower frequencies of AMR *S*. Heidelberg was observed in summer (2 of 11) and fall (2 of 11), however, these isolates were only observed in LC. No AMR *S*. Heidelberg isolates were obtained in the winter months in any tributary.

Similar frequencies of AMR were observed in *S*. Typhimurium and *S*. Heidelberg isolates among streams. AMR was demonstrated in 56% (9/16), 64% (9/14) and 50% (2/4) of *S*. Typhimurium isolates from LC, CAN and CON, respectively. *S*. Heidelberg demonstrated AMR in 71% (5/7), 66% (2/3) and 44% (4/9) of isolates from LC, CAN and CON, respectively.

Several different *Salmonella* serotypes demonstrated identical resistance profiles (Figure 3.4). Single drug resistance profiles, including A, Su and S, were commonly

observed in different serotypes and found in isolates at various times of the year and in different tributaries.

Seven serotypes (n=10) displayed resistance to 3rd generation cephalosporins, ceftriaxone and ceftazidime, either in combination with ampicillin resistance or with ampicillin and sulfisoxazole (Figure 3.4). These serotypes included: *S*. Berta (n=2), *S*. Heidelberg (n=2), *S*. Mbandaka (n=2), *S*. Agona (n=1), *S*. Infantis (n=1), *S*. Kentucky (n=1), and *S*. Newport (n=1). As mentioned previously, these profiles were only observed in LC and never in CAN or CON. Resistance to these drugs occurred in different seasons in this tributary, with the exception of winter.

Comparison of drug resistance in waterborne *E. coli* and *Salmonella*

E. coli was isolated from additional swabs in each stream between July and November 2004 (Figure 3.5). *E. coli* was always present in these samples (64/64). Overall, AMR *E. coli* were observed in 26.6% (17/64) of the samples. Of the 147 *E. coli* isolates obtained, 11.5% (17/147) demonstrated resistance. Over the same period of time, AMR *Salmonella* was detected in 60% (30/50) of the samples found to be positive for *Salmonella*, and of these isolates, 31% (43/140) demonstrated drug resistance. The difference in AMR frequency between *E. coli* and *Salmonella* isolates was significant ($P < 0.001$).

The portion of samples that was positive for AMR *Salmonella* and *E. coli* were different ($P < 0.001$) in LC with 70.4% (19/27) of swabs positive for AMR *Salmonella*, compared to only 20.7% (6/29) for AMR *E. coli*. In CAN, AMR *Salmonella* was observed in 62.5% (10/16) of samples, compared to 55.6% (10/18) for AMR *E. coli* in CAN. CON showed the lowest levels of AMR *Salmonella* and *E. coli* at 14.3% (1/7) and 5.9% (1/17), respectively. The difference in AMR frequency between *E. coli* and *Salmonella* isolates was not significant in both CAN and CON ($P = 0.95$ and $P = 0.89$, respectively).

No samples were found to be positive for AMR *Salmonella* or *E. coli* at CON-2 (Figure 3.5a). When examined by sample date (Figure 3.5 b), the incidence of AMR *Salmonella* was more frequent than AMR in *E. coli*, with the exception of two dates. For swabs that were found to be positive for *Salmonella*, the occurrence of AMR

Salmonella ranged from 20% to 100%. Although *E. coli* was isolated from 100% of the swab samples, the occurrence of AMR ranged from 0% to 42.9% (Figure 3.5 b).

(a)

(b)
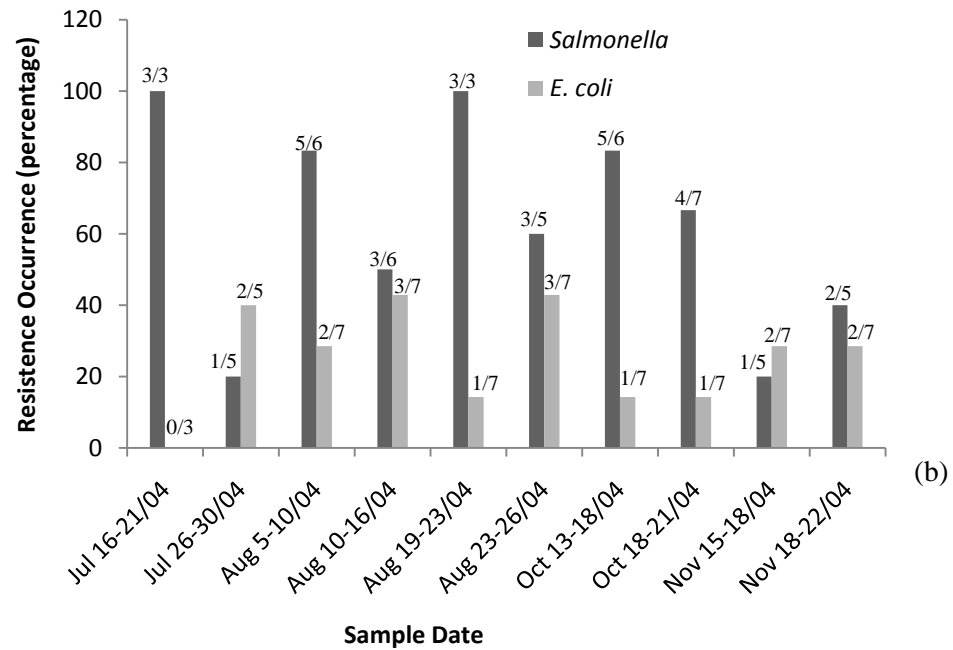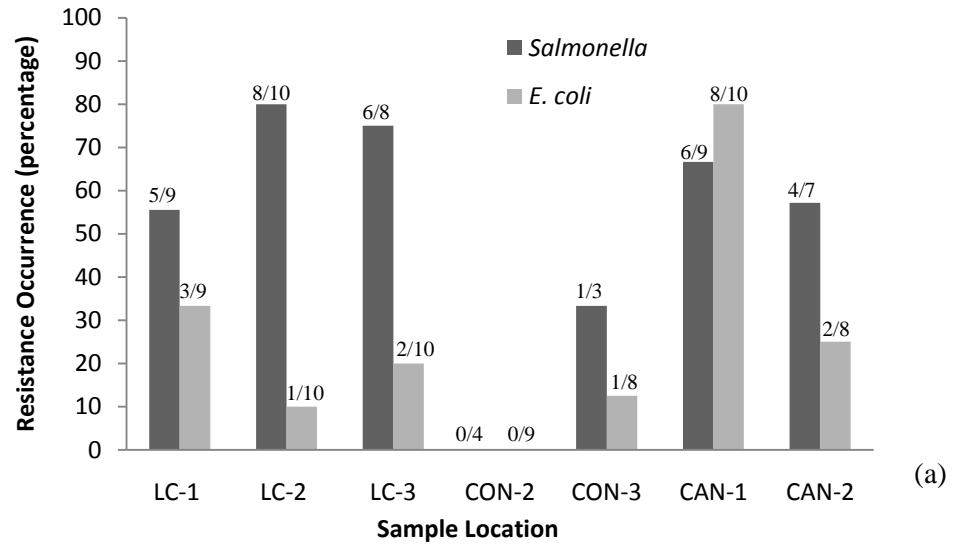
Figure 3.5. (a) Comparison of the incidence of samples demonstrating a positive result for AMR *Salmonella* and *E. coli* at each sampling location, and (b) comparison of the incidence of resistance in *Salmonella* and *E. coli* at each sample date between July and November 2004. Resistance percentage is the number of times a sample was positive for AMR isolates (n) divided by the number of samples positive for either *Salmonella* or *E. coli* (N).

Several similarities were observed between the proportions of isolates resistant to individual drugs (Figure 3.6). Both *Salmonella* and *E. coli* isolates showed similar frequencies of resistance to ampicillin and streptomycin. In *Salmonella*, higher proportions of resistance to sulfonamides were observed compared to *E. coli*. In *E. coli*, a greater proportion of resistance to tetracycline was observed compared to *Salmonella*. Ceftriaxone and ceftazidime resistance were only observed in *Salmonella* and only *E. coli* demonstrated resistance to trimethoprim-sulfamethoxazole during this sampling period.
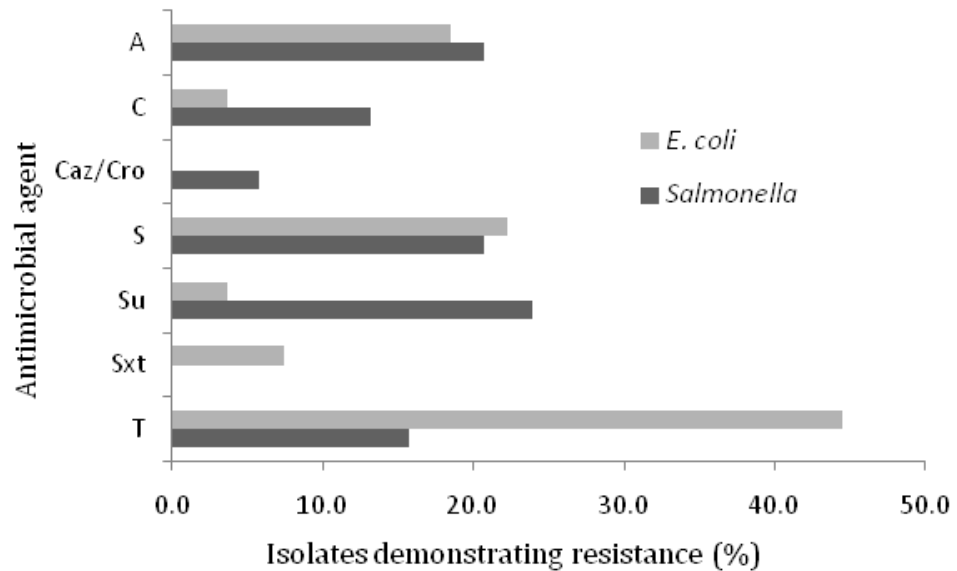
Figure 3.6. Proportion of *Salmonella* and *E. coli* isolates demonstrating resistance to each antimicrobial drug.

E. coli isolates were more likely than *Salmonella* to demonstrate resistance to a single drug, rather than resistance to several drugs (Table 3.5). Of the AMR isolates, 64.7% (11/17) demonstrated SDR, which was predominantly to ampicillin or tetracycline (Table 3.5). At this time, *Salmonella* isolates demonstrated SDR in only 15.8% (6/38) of isolates, compared to 84.2% (32/38) demonstrating MDR. Of the *E. coli* isolates demonstrating MDR (35.3%), the most predominant profile was a combined resistance streptomycin/ tetracycline (ST). Only two *E. coli* isolates demonstrated MDR profiles to more than two drugs (Table 3.5).

Between July and November, 11 different resistance profiles were seen in *Salmonella* isolates and 7 were observed in *E. coli*. *E. coli* isolates from Canagagigue Creek

87

showed the most diverse resistance profiles, with CAN-1 showing the highest level of resistant isolates and greatest diversity of profiles (Table 3.5). This was in contrast to *Salmonella* where the highest levels of AMR and the greatest diversity were observed at the LC sites.

Table 3.5. Resistance profiles for both *Salmonella* and *E. coli* between July and November 2004 at each sampling site location.

Sample Location	*Salmonella*		*E. coli*	
	Resistance profile	No. isolates with profile	Resistance profile	No. isolates with profile
LC-1	ACSSuT	2	SuT	1
	ACazCro	1	A	2
	SuT	1		
	A	1		
Percentage of total:		25 % (5/20)		13.6 % (3/22)
LC-2	ACazCro	3	ST	1
	ASuCazCro	1		
	ACSSuT	1		
	A	1		
	Su	1		
Percentage of total:		47 % (7/15)		4.2 % (1/24)
LC-3	ASuCazCro	1	A	1
	ACazCro	1	T	1
	ACSSuT	3		
	S	1		
	Su	1		
	SSu	3		
Percentage of total:		42 % (10/24)		9.5 % (2/21)
CAN-1	ACSSuT	7	S	1
	SSu	3	T	5
	SSuT	1	CSSxtT	1
			ASSxtT	1
Percentage of total:		35 % (11/31)		28.6 % (8/28)
CAN-2	ACSSuT	3	A	1
	SSu	1	ST	1
Percentage of total:		18 % (4/22)		12.5 % (2/16)
CON-3	T	1	ST	1
Percentage of total:		17 % (1/6)		7.1% (1/14)

The most commonly shared resistance profile in *Salmonella* and *E. coli* was single drug resistance to ampicillin, although this profile was not always observed in these genera at the same sample location (Table 3.5). Few identical MDR profiles were observed between *E. coli* and *Salmonella* in these water samples.

3.5 Discussion

Occurrence of AMR in waterborne *Salmonella*

Resistance to one or more of the antimicrobial drugs used in this study was observed in 33% of the waterborne *Salmonella* isolates obtained. The only other Canadian study to report AMR levels in waterborne *Salmonella* was carried out by Bell *et al*. (1980) in the Red River, Manitoba; which reported a lower frequency of resistance at 18%. Of the few studies available outside of Canada, varying levels of resistance in waterborne *Salmonella* are reported, with values as high as 90% when *Salmonella* was isolated from waters impacted by human waste effluent (Alcaide and Garay, 1984; Morinigo *et al*., 1990b). Several studies have also reported low frequencies of AMR or pan-susceptible isolates in water, including those from recreational waters and rivers (Whitman *et al*., 2001; Patchanee *et al*., 2010; Gorski *et al*., 2011).

Considerably more data are available on the occurrence of AMR in *Salmonella* from infected humans and farm animals. In Canada, CIPARS reports annually on these trends to create a better understanding of the epidemiological linkage between these sources. Between 2003 and 2005, rates of AMR in *Salmonella* obtained from infected people in Ontario and the Canadian population ranged between 30-37% (see Table 3.6; Government of Canada, 2005; 2006; 2007). Farm animals show a greater variability, with frequencies differing considerably between the type of animal and even between the types of sample taken (clinical samples vs abattoir). Between 2003 and 2005, resistance was reported in 30-68% of *Salmonella* isolates from bovine samples, 47-78% from swine samples and 25-41% from chickens (Table 3.6). The AMR frequencies reported by CIPARS, particularly in human samples, appear to be in line with those observed in the waterborne *Salmonella* isolates obtained in the current study.

In addition to similarities between AMR frequencies, waterborne isolates of *Salmonella* showed a higher proportion of MDR, which is commonly reported in isolates from humans and farm animals (Shea *et al*., 2004; Government of Canada, 2007). In the current study, three times more MDR was observed compared to SDR. Alcaide and Garay (1984) and Morinigo *et al*. (1990b) also observed this trend in *Salmonella* from Spanish waters, whereas Martinez-Urtaza *et al*. (2004) observed more SDR in waterborne isolates.

89

Table 3.6. Percentage of *Salmonella* isolates in human and animal isolates demonstrating resistance in Canada from 2003 to 2005 (Government of Canada, 2005; 2006; 2007).

Percentage of isolates resistant to one or more antimicrobial

Serotype	2003					2004					2005				
	Human Canada	Human Ont.	Bovine*	Swine*	Chicken*	Human Canada	Human Ont.	Bovine*	Swine*	Chicken*	Human Canada	Human Ont.	Bovine*	Swine*	Chicken*
Total	35.0	34.1	68.0/ND	73.0/49.0	41.0/41.0	37.0	36.3	57.0/ND	76.4/49.0	40.5/40.0	34.0	29.8	29.5/ND	78.3/47.0	25.0/40.0
Typhimurium	52.0	55.8	94.7/ND	70.0/83.0	50.0/NDG	47.0	51.5	66.7/ND	92.6/57.4	50.0/50.0	56.3	62.3	60.0/ND	92.7/94.1	50.0/0.0
Heidelberg	45.8	48.0	NDG/ND	NDG/50	31.6/40.0	56.0	54.8	75.0/ND	100.0/75.0	50.0/56.9	57.2	52.9	0.0/ND	87.5/100	11.1/37.9
Thompson**	< 13.7	2.08	0.0/ND	NDG/NDG	NDG/25.0	< 11.9	0.0	NDG/ND	NDG/NDG	0.0/33.0	NDG	2.8	0.0/ND	ND/NDG	0.0/12.5

NDG – no data given – usually due to the fact that this serotype was infrequently observed or not observed at all
ND - no data as samples are not collected for this animal type
*data given as percentages based on 'clinical/abattoir' isolates. Clinical isolates may not accurately represent the actual AMR in this commodity as these animals were sick at the time of sample collection, however, this is the only data given for cattle. This data was given for all farm animals as a comparison to cattle.
**Not a complete set of data given of S. Thompson in CIPARS as it is less frequently observed in humans and animals. If complete set of data not given, a 'less than' is used

The penta-drug resistant profile ACSSuT in *Salmonella*, which is among the most common observed in human, swine, and cattle isolates in Canada (Government of Canada, 2006; 2007), was also the most frequently observed in waterborne isolates where it represented the profile in 27.3% of AMR *Salmonella* isolates.

Similarities can be observed between the types of drug resistance in waterborne isolates and those reported in humans and farm animals. Resistance to Category II and III drugs was the most commonly observed in waterborne isolates. While resistance to the Category I drug ciprofloxacin was not observed in any waterborne isolates, resistance to 3rd generation cephalosporins (Cro and Caz) was detected in several isolates.

Resistance to Category III drugs is commonly reported in isolates obtained from humans and farm animals, which limits their use as a treatment option (Government of Canada, 2007; Zhao *et al*., 2007). The use of these drugs as growth promoters, including tetracyclines and sulfa-drugs, is thought to aid in maintaining and possibly promoting the development of resistance in *Salmonella* and other bacteria (Chopra and Roberts, 2001; GAO, 2004). Resistance to sulfisoxazole was the most commonly observed type of resistance among waterborne isolates, where it was detected in 25% of the AMR isolates. Resistance to tetracycline was also commonly observed in waterborne isolates at 14%. Despite the ban on use in food-producing animals and its limited use in humans (Gilmore, 1986; Government of Canada, 2007), resistance to chloramphenicol (Category III drug) was detected in 10% of the AMR waterborne isolates. Resistance to this drug, however, was only ever observed in combination with other types of resistance, including the ACSSuT and ACSSuTCazCro profiles. Similar findings have been reported in *Salmonella* isolates from other sources (Gow *et al*., 2008; Zhao *et al*., 2007). The genes responsible for chloramphenicol resistance are commonly integrated into the chromosome of *Salmonella* and linked to other resistance genes on integrons (Ng *et al*., 1999). This type of integration and co-selection with other resistance genes is likely contributing to the persistence of chloramphenicol resistance and makes the loss of this resistance more difficult, even when the selective pressure is reduced or eliminated (Travis *et al*., 2006; Gow *et al*., 2008).

Resistance to Category II drugs, which are considered of high importance to human health, was commonly observed in waterborne isolates, although lower frequencies of resistance to trimethoprim-sulfamethoxazole were observed. Resistance to both

ampicillin and streptomycin was observed in 19% of waterborne isolates. Isolates demonstrating resistance to these drugs also commonly showed MDR to other Category II or Category III drugs. The higher frequency of resistance to these two drugs is common in isolates obtained from humans and farm animals (Government of Canada, 2007; Zhao *et al.*, 2007).

Similar to isolates obtained from the Canadian population, resistance to Cro and Caz (Category I Drugs) in waterborne isolates was low (Shea *et al.*, 2004; Government of Canada, 2007), showing a frequency of 4.3%. Unlike reports in human isolates, waterborne isolates never demonstrated intermediate resistance to Caz or Cro. All isolates demonstrated high MIC values to these drugs, particularly to Cro. Resistance to these drugs has not been reported in waterborne *Salmonella* in Canada prior to this study; therefore there is a lack for data for comparison. However, the occurrence of resistance to these important drugs in waterborne isolates is of concern as these waters might aid in the transport of these isolates between hosts that are not generally exposed to these types of drugs, such as wildlife. Once in a variety of hosts, the permanence of this type of resistance is increased and may continue to reduce the usefulness of these important drugs in clinical settings.

Spatial and temporal difference in antimicrobial resistant *Salmonella* in the tributaries

It was originally assumed that differences in AMR frequency would be observed between the urban stream and the agricultural/rural streams. In a recent study by Patchanee *et al.* (2010), a stream impacted by swine operations in North Carolina showed higher levels of AMR in waterborne *Salmonella* compared those in the urban streams. Agricultural wastes, and to a lesser extent, human wastes, were expected to be the predominant source of *Salmonella* in CAN and CON. Since the use of antimicrobial drugs is common in humans and farm animals, higher levels of drug resistance were expected in the two agricultural/rural tributaries over the urban tributary. However, a significant difference was not observed between the frequency of AMR *Salmonella* in any of the three streams.

Human and agricultural sources were expected to be of limited impact on LC waters, therefore it was unexpected to observe high frequencies of drug resistance in this urban

watershed (58.9%, compared to 60% in CAN and 33% in CON). Isolates obtained from LC also showed a greater diversity of AMR profiles compared to those from CAN and CON. In addition, despite the use and reported increases in resistance to 3rd generation cephalosporins in human and animal isolates, LC was the only tributary where *Salmonella* isolates showed resistance to these drugs.

As mentioned in Chapter 2, wildlife were assumed to be major contributors to *Salmonella* in Laurel Creek waters as they produce a large amount of fecal waste in and around this stream. Compared to humans and farm animals, wildlife have limited exposure to antimicrobial drugs and are therefore expected to carry low levels of AMR bacteria (Whitman *et al*., 2001; Edge and Hill, 2005; Edge and Hill, 2007; Kozak *et al*., 2009). Limited data are available on the occurrence of AMR *Salmonella* in wildlife (e.g., Hudson *et al*., 2000), although studies examining AMR in *E. coli* isolates have demonstrated lower levels of drug resistance in wildlife compared to those isolated from human and farm animal waste (Cole *et al*., 2005; Edge and Hill, 2005; Edge and Hill, 2007; Kozak *et al*., 2009).

It is probable that the bird population is contributing to the movement of AMR *Salmonella* between watersheds. Ducks and geese, in particular, frequently travel between urban and agricultural sites. Their frequent feeding in farm fields where manure spreading occurs might result in the uptake of *Salmonella* strains carrying AMR. The transitory nature of these animals and frequent feeding at various locations might account for the greater diversity of AMR profiles obtained in these urban locations compared to the agricultural streams. Of the few duck samples that were found to be positive for *Salmonella* adjacent to Laurel Creek, no isolates showed AMR. However, this small number of fecal samples would not accurately reflect all *Salmonella* in isolates in the wildlife in this tributary.

Resistance to Category I drugs, including 3rd generation cephalosporins, is rarely reported in wildlife (Cole *et al*., 2005; Kozak *et al*., 2009), although it is possible that transitory birds may be responsible for the movement of strains carrying this resistance to the urban watershed after feeding downstream of sewage effluent or in farm land. However, if this were the case, then it would be expected that this type of AMR would be observed in the other tributaries as well.

The occurrence of resistance to Cro in waterborne isolates is suggestive of human sources of fecal contamination. Although Laurel Creek does not receive effluent from a sewage treatment facility, leaking sewage pipes and septic systems combined with a higher water table in the Region of Waterloo might contribute to the movement of these AMR *Salmonella* to the stream. Impacts from human waste may also contribute to the great diversity of AMR profiles observed in this creek. Due to the widespread use of a variety of antimicrobial agents in medicine, human isolates commonly demonstrate a wider variation in AMR profiles, including MDR, compared to animal isolates (Parveen *et al*., 1997; Edge and Hill, 2007). Further study in Laurel Creek would be needed to confirm if these sources are contributing to contamination in this stream.

As mentioned, a significant difference was not observed between the frequency of AMR positive samples in the three streams, however, there was considerable variability between sites on CON. This inconsistency was not observed between sites in LC or CAN. On CON, no samples were AMR positive at CON-2, 33% were positive at CON-3 and 60% were positive at CON-1. It is possible that the variability is related to the smaller number of samples taken in CON, compared to LC and CAN sites. Sites on CON were not always accessible; therefore samples taken may not represent the overall picture of AMR frequencies between sample sites on this river. This disproportionate AMR occurrence, if real, may speak to the differences of fecal inputs at these sites and the survival of *Salmonella* once in water (as discussed in Chapter 2). The sample point, CON-1, showed the highest frequency of AMR positive samples in this tributary. During sampling, grazing cattle were observed immediately upstream of CON-1 on several visits, which may have contributed to the higher percentages reported at this site.

Flow conditions were not correlated with occurrence of AMR strains, which was similar to Chapter 2. General trends, however, were observed in the seasonal occurrence of AMR *Salmonella* between streams. In LC a greater number of isolates showing AMR were obtained in the fall and winter months, whereas AMR isolates from Canagagigue Creek seemed to predominate in the spring and summer months. The seasonal patterns, as observed, may represent the increased use by waterfowl of the urban stream during the fall and winter months compared to the agricultural streams that tend to freeze over winter. Further study would be needed to verify any seasonal pattern. These observed *s*easonal differences may also be related to the predominance

of different serotypes, which are more commonly drug resistant, in these streams at certain times of the year. For example, in Canagagigue Creek over the summer months, there were numerous samples that contained *S*. Typhimurium Var. 5-, which demonstrated the resistance profile ACSSuT. This strain is frequently observed in farm animals, particularity cattle and swine (Government of Canada, 2006; Farzan *et al*., 2008) and its predominance at this time of year in water might relate to the greater number of pasturing cattle and/or the spreading of manure at this time of year.

Resistance observed in waterborne *Salmonella* serotypes

In the waterborne isolates obtained in this study, AMR was more clearly associated with some serotypes than others. A similar trend was also observed for SDR versus MDR. For example, over half of the *S*. Typhimurium (59%) and *S*. Heidelberg (58%) isolates were resistant to one or more drugs. Both were more likely to demonstrate MDR over SDR. These two serotypes are also among the most common to show drug resistance in humans and farm animals in Canada (Zhao *et al*., 2007; Government of Canada, 2009). The levels of resistance in these two serotypes in Ontario during 2003 and 2005 and in the Canadian population (46 to 62%) are similar to the levels observed in waterborne isolates (Table 3.6).

S. Typhimurium isolates from farm animals commonly demonstrate a high proportion of drug resistance (Zhao *et al*., 2007); higher than reported in humans (Table 3.6) and in waterborne isolates in this study. For example, CIPARS reported the frequency of AMR in *S*. Typhimurium to range between 60% and 94.7% in cattle (clinical isolates) and between 57.4% and 94.1% in swine (clinical and abattoir isolates). AMR in *S*. Typhimurium in poultry tended to be lower (Table 3.6). AMR levels in *S*. Heidelberg tend to vary in farm animals, with the levels in cattle ranging from 0% to 75% (clinical isolates), in swine from 50% to 100% (clinical and abattoir isolates) and in chickens ranging from 11.1% to 56.9% (clinical and abattoir isolates). Despite the frequency of AMR observed in isolates from farm animals, there was not more drug resistance in these serotypes in the agricultural/rural watersheds compared to the urban watershed.

S. Typhimurium and *S*. Heidelberg isolates obtained from water demonstrated MDR more often than SDR. In North America, MDR is a commonly reported phenomenon in these strains obtained from both human and farm animals (CDC, 2006; Poppe *et al*.,

2002; CDC, 2007a; Government of Canada, 2007). The occurrence of MDR was particularly prevalent in waterborne isolates of *S*. Typhimurium, where 19 of the 20 resistant isolates demonstrated the resistance profile of ACSSuT, a commonly reported resistance pattern in human and animal isolates (Government of Canada, 2006; 2007).

All waterborne isolates of S. Typhimurium, including Var. 5 – strains, demonstrating the ACSSuT profile were found to be phagetype PT 104 or PT 104a. PT 104 is among the most commonly reported phagetypes in swine and cattle in Canada, where it can represent over 80% of the *S*. Typhimurium isolates (Poppe *et al*., 2002). Similarly, in human isolates, this phagetype is commonly reported although the frequency is generally lower than in many types of farm animals (Government of Canada, 2006; 2007). The higher frequency of PT 104 in farm animals did not result in a higher occurrence of these isolates in the agricultural streams over the urban stream.

Outside of human and domestic farm animals, not much is known about the distribution of PT 104 in wildlife and domestic pets in Canada. However, studies from around the world have demonstrated the broad host range of this problematic phagetype (Poppe *et al*., 1998). Therefore, the occurrence of MDR S. Typhimurium PT 104 in LC could be originating from many sources in the watershed, including wildlife. MDR S. Typhimurium PT 104 appears to be ubiquitous in LC, as it was observed at all three sample locations and in all seasons. Knowledge of the genetic relatedness of these isolates, as described in Chapter 5, would aid in understanding if this MDR strain is a clone circulating within hosts in the watershed. Genetic variability of these isolates might indicate whether these strains are being introduced from areas outside of the watershed.

As mentioned previously, several studies in Spanish waters examining shellfish contamination have characterized AMR in waterborne *Salmonella*, although few report phagetype-specific data in their studies (Alcaide and Garay, 1984; Morinigo *et al*., 1990b). One study that did include this information was Martinez-Urtaza *et al*. (2004) who reported AMR in 30% (7/23) of *S*. Typhimurium isolated from shellfish. Less than half of these AMR S. Typhimurium were MDR, however, when MDR was observed it was more commonly associated with PT 104 (n=2). PT 41, which was the most common phagetype in their study, demonstrated SDR in half of the isolates (4/8). In the current study, one isolate of PT 41 was observed to carry drug resistance, showing SDR

to streptomycin. Similar to the current study, but in contrast to Martinez-Urtaza *et al.* (2004), Morinigo *et al.* (1990b) reported MDR to be common in *S.* Typhimurium isolated from Spanish waters, although there was no mention as to the AMR pattern or phagetypes observed.

Unlike S. Typhimurium, resistance in waterborne isolates of *S.* Heidelberg was not predominantly associated with one phagetype. *S.* Heidelberg demonstrated a diverse range of AMR profiles with resistance observed in three identified phagetypes (PT 19, PT 6 and PT 29a), as well as in several atypical phagetypes and two untypable isolates.

All AMR *S.* Heidelberg isolates showed full resistance to one or more β-lactam antimicrobial drugs, including ampicillin and/or 3rd generation cephalosporins. Resistance to 3rd generation cephalosporins was observed in 10.5% (2 of 19) of waterborne isolates of *S.* Heidelberg, which is higher than <1% reported in human isolates (Government of Canada, 2007), although reduced susceptibility (intermediate resistance) has been reported in human isolates at 26% (Government of Canada, 2006). *S.* Heidelberg, which is the predominant serotype in poultry, has shown increased resistance to ceftiofur, a drug used in this industry (Government of Canada, 2006; Dutil *et al.*, 2010). Cross resistance to this drug is thought to be related to the increased resistance to ceftriaxone in this serotype. Despite the increased occurrence in poultry, S. Heidelberg isolated from CAN and CON did not demonstrate resistance to 3rd generation cephalosporins. Isolates from these tributaries only demonstrated resistance to ampicillin and/or sulfisoxazole. *S.* Heidelberg demonstrating resistance to 3rd generation cephalosporins were only isolated in LC.

Resistance to 3rd generation cephalosporins, in addition to being observed in waterborne isolates of *S.* Heidelberg, was seen in several other serotypes, including; *S.* Agona, *S.* Berta, *S.* Infantis, *S.* Kentucky, *S.* Mbandaka and *S.* Newport. Similar to human isolates, resistance to ceftriaxone has been observed in a range of *Salmonella* serotypes with higher frequencies reported in both *S.* Anatum and *S.* Mbandaka and with lower levels reported in *S.* Newport, *S.* Heidelberg and *S.* Typhimurium (Government of Canada, 2006). No resistance was observed to the 3rd generation cephalosporins in any waterborne isolates of *S.* Typhimurium. Resistance to 3rd generation cephalosporins is commonly reported on plasmids (Winokur *et al.*, 2001; Alcaine *et al.*, 2005; Poppe *et al.*, 2005); which might explain why this type of resistance occurs in many different

serotypes isolated from water. Further investigation would be needed to determine if resistance in these isolates was plasmid-mediated and if these plasmids were transferable. The following chapter (Chapter 4) describes such a study.

Some serotypes obtained from water showed little to no drug resistance, one example being *S*. Thompson. This serotype was pan-susceptible in the current study. *S*. Thompson is commonly reported as one of the top ten serotypes in the Canadian population and is frequently obtained from poultry (Government of Canada, 2007). However, regardless of its prevalence and exposure to the selective pressures of antimicrobial drugs, resistance in the serotype has remained low in human and most animal isolates, although higher levels have been observed in chickens (Table 3.6, Government of Canada, 2006). It has been suggested by others (Poppe *et al*., 1998) that some serotypes are more likely than others to take up drug resistance genes, develop resistance or maintain resistance.

Comparison of drug resistance in waterborne *E. coli* and *Salmonella*

E. coli as an indicator of resistance in watersheds

Commensal bacteria, including *E. coli,* in the gut of farm animals and humans, are considered to be a reservoir for resistance and an indicator of the extent, type, and emergence of resistance within the GI system of animals (van den Bogaard and Stobberingh, 2000; Chopra and Roberts, 2001; Winokur *et al*., 2001; Salyers *et al*., 2004; Gow *et al*., 2008; Sharma and Topp 2008). Using waterborne *E. coli* as an indicator of resistance may aid in understanding the overall extent of resistance and the types of resistance circulating within host animals in a particular watershed. To understand whether *E. coli* would be a good indicator of AMR circulating in a watershed, particularly agricultural watersheds, comparisons between the AMR levels and profiles in agricultural animals, and those observed in waterborne isolates, can be informative.

AMR in waterborne *E. coli* was considerably lower (11.5%) than levels reported in *E. coli* from farm animals and humans (Government of Canada, 2005; 2006; 2007; Gow *et al*., 2008; CDC, 2008). In Canada, AMR *E. coli* is reported to range from 80-88% in isolates from swine, 77-84% in isolates from chicken and 27-65% in isolates from cattle

(Government of Canada, 2005; 2006; 2007; Gow *et al*., 2008). While fewer data exist on resistance in human isolates of *E. coli*, levels have been reported to range from 36 to 37% (CDC, 2008).

Although it is expected that fecal contamination in CON and CAN would originate primarily from agricultural operations, the frequency of AMR *E. coli* did not reflect AMR occurrence discussed above in farm animals. In these tributaries, AMR was observed in 22.7% (10/44) of *E. coli* isolates in CAN and in 2.8% (1/36) in CON. While not a source tracking study, these findings may indicate that a large portion of waterborne *E. coli* in these tributaries are originating from hosts that are not exposed or have limited exposure to antimicrobial drugs, including wildlife.

Despite differences in the frequency of AMR, some similarities were observed between the predominant types of AMR observed in *E. coli* from farm animals and those observed in water samples. Resistance to various Category II and III drugs, including tetracyclines, sulfonamides, ampicillin and streptomycin, which are commonly reported in *E. coli* isolates from farm animals in Ontario, was also observed in waterborne isolates (Boerlin *et al*., 2005; Kozak *et al*., 2009; Duriez and Topp, 2007). Tetracycline resistance, alone or in combination with other drugs, is among the most frequently observed resistance in isolates from farm animals including cattle and swine (Government of Canada, 2007; Gow *et al*., 2008; Kozak *et al*., 2009). Similarly, in waterborne isolates of *E. coli*, tetracycline resistance was the predominant type of resistance. In CAN, 80% of the AMR *E. coli* isolates demonstrated resistance to tetracycline.

The frequency of AMR in *E. coli* in water is much lower than that observed in farm animals in Canada. As a result, waterborne *E. coli* does not appear to be a good indicator for the AMR levels observed in these animals within a watershed. This lower frequency of AMR in these waters might reflect higher inputs from wildlife which carry more susceptible isolates (Scott *et al*., 2002; Edge and Hill, 2005 and 2007; Kozak *et al*., 2009). There may also be a possibility that some of these strains are not of enteric origin. Although AMR in *E. coli* do not correspond to the levels in farm animals, it is possible that monitoring for AMR levels in waterborne *E. coli* may in fact help to represent the overall incidence of AMR *E. coli* in a variety of hosts throughout a particular watershed.

E. coli as a surrogate for AMR in waterborne Salmonella

Enteric bacteria, such as *E. coli* and *Salmonella*, share the same ecological niche in the gut therefore it is not uncommon to see these two genetically related bacteria carry similar types of AMR (Winokur *et al.*, 2001; Poppe *et al.*, 2005). The ability of *E. coli* to act as a surrogate for AMR *Salmonella* in water might limit the need to detect this pathogen, which can be difficult to recover from water and takes several days to isolate.

In most farm animals, a greater frequency of resistance is generally reported in *E. coli* with almost two times the level of resistance compared to *Salmonella* (Government of Canada, 2007). This is in contrast to this study, where drug resistance in waterborne *E. coli* was less than half of that observed in *Salmonella* isolates. Over the same timeframe, 11.5% of *E. coli* isolates demonstrated AMR compared to 31% in *Salmonella* isolates. In addition, waterborne *Salmonella* isolates demonstrated considerably more multiple-drug resistance (84.2%) over *E. coli* isolates (35.5%) and also showed greater diversity in AMR profiles compared to those observed in waterborne *E. coli*. When examined at the tributary level, differences were observed between the frequency of AMR in *E. coli* and *Salmonella* isolates obtained from in the urban tributary (LC), however, similar levels were observed in isolates from the agricultural-influenced tributaries (CAN and CON). In most instances, few similarities were observed between the AMR profiles in waterborne *Salmonella* and *E. coli*. SDR to ampicillin was the most common shared resistance profile, however, genetic analysis would be needed to determine if these similarities were the result of the same genetic determinant in both genera (see Chapter 4 for this comparison).

Despite the increasing reports of *E. coli* resistance to 3rd generation cephalosporins (Boerlin *et al.*, 2005; Government of Canada, 2006; Dutil *et al.*, 2010), no waterborne isolates showed resistance or reduced susceptibility to these drugs. Unfortunately, if *E. coli* was used as a surrogate for *Salmonella* resistance in water, this important type of resistance would not have been observed. This would have underestimated the human health hazard associated with these samples.

E. coli does not appear to accurately reflect the frequency or types of resistance in *Salmonella* obtained from the same aquatic environment. However, it is possible that the difference between these organisms might be explained by the disproportionate

number of representative *E. coli* isolates obtained from these waters. *E. coli* is excreted from a large number and variety of warm-blooded animals and is generally found in higher concentrations in stream water compared to *Salmonella* (e.g., Haley *et al*., 2009). The small number of isolates obtained may not have adequately represented the *E. coli* population within the watershed. It might be expected that, waterborne *Salmonella* would only be originating from a subset of the hosts that excrete *E. coli* and would generally be found at a lower frequency in these waters; therefore the isolates obtained may be more representative of resistance levels in *Salmonella* in the watershed. Another possible explanation for the disproportionate AMR levels between *E. coli* and *Salmonella* in water might be that *Salmonella* isolates demonstrate higher frequencies of AMR regardless of the source from which they originate. While microbial source tracking studies have shown lower levels of AMR in *E. coli* from sources where antimicrobial use is low, such as wildlife (Edge and Hill, 2007), similar studies for *Salmonella* are limited. As observed in the current study and by others (Guerra *et al*., 2002; Zhao *et al*., 2007), AMR is often linked to serotype, therefore the serotype found in a host may be more important to AMR occurrence than a host's previous exposure to antimicrobial drugs.

Based on the results of this study, *E. coli* may not be reliably used to extrapolate *Salmonella* resistance profiles in all watersheds. *E. coli* is commonly used as an indicator of fecal contamination, however few studies support that it is a surrogate for pathogens (e.g., Ahmed *et al*., 2009; Wu *et al*., 2011). Although pathogens are more difficult to isolate from water than *E. coli*, to best represent the overall occurrence of AMR in waterborne pathogens, it is recommended that the actual pathogens be isolated.

3.6 Conclusions

Frequent isolation and year round occurrence indicates that the aquatic environment may be an important reservoir for antimicrobial resistant *Salmonella*. Once in the aquatic environment, water may play an important role in the movement and environmental spread of drug resistant *Salmonella* between a range of host animals, including wildlife not directly exposed to antimicrobial drugs.

Few reports on AMR in waterborne *Salmonella* in Canada limit the ability to compare studies, however, the frequencies observed in waterborne *Salmonella* in the current study were similar to those reported in humans and many farm animals in Canada. Comparable results were also observed between the predominant AMR profiles and resistance in specific serotypes and phagetypes. These similarities may indicate that an epidemiological connection exists between the aquatic environment and human and animal sources.

Surveillance programs, such as CIPARS, monitor for drug resistance in bacterial pathogens obtained from humans and animals intended for consumption, as the transmission between these sources plays a crucial role in the spread of AMR (Sharma *et al*., 2008). The integration of environmental samples into these monitoring programs may help to further define the spread of AMR outside of the host environment.

The high frequency of AMR in *Salmonella* in the urban tributary, Laurel Creek, was unexpected. Generally, run-off from livestock production and effluent from sewage treatment are thought to be major contributors to AMR pathogens in a watercourse, however, these sources do not impact this tributary. The greater diversity of resistance compared to the agricultural/rural tributaries (CAN/CON) suggest that many animals within this catchment, including wildlife, may carry AMR *Salmonella* despite the limited exposure to antimicrobial drugs. However, resistance to drugs of very high human health importance (3rd generation cephalosporins, Caz and Cro) in isolates obtained solely from the urban-impacted tributary may indicate that unknown human fecal sources are impacting these waters. Understanding the predominant sources of AMR *Salmonella* in this watershed may help to develop strategies to reduce this environmental pollution.

Protecting source waters, through the implementation of protective management strategies may not only be important for reducing fecally-borne pathogens from entering the water, but it might also help reduce public health risk by limiting the environmental spread of drug resistance by waterborne pathogens. Protecting urban waters may be as important as protecting waters impacted by agricultural activities.

In contrast to *Salmonella*, lower AMR frequencies were observed in waterborne *E. coli* compared to those reported in farm animals in Canada. The lower frequency of AMR in

waterborne isolates of *E. coli* might reflect higher inputs from wildlife which might carry fewer AMR bacteria. Few similarities were observed between the AMR frequency and profiles in waterborne isolates of *E. coli* and *Salmonella*. This may limit the use of *E. coli* as a surrogate for AMR occurrence in waterborne *Salmonella*. To understand the frequency and the types of drug resistance circulating within bacterial pathogens, it is the pathogen itself that should be isolated and tested.

3.7 Recommendations and future research needs

- A better understanding is needed regarding the significance of non-point sources to the contribution of AMR bacteria in the aquatic environment. This should include an assessment of AMR frequency in *Salmonella* in wildlife in these tributaries, in particular LC. Knowledge of environmental sources of resistance pathogens may help to further define the occurrence and spread of resistance outside of host animals.
- Future testing in LC is needed to determine if human fecal sources are impacting these waters. A source tracking study using a variety of tools might be needed to define the source of contamination.
- Moving beyond phenotypic AMR evaluation and understanding how drug resistance is mediated within these isolates (chromosomally or on mobile genetic elements) may further elucidate an epidemiological linkage to human and animal health.
- Further genetic analysis of the isolates obtained in the current study might provide insight into the genetic relatedness of AMR *Salmonella* isolates within the water. This information might help in understanding if drug resistance, particularly MDR, is related to clonal isolates circulating within hosts in each watershed. Genetic variability of these isolates might indicate that these strains are being introduced from areas outside of the watershed.

4

Characterization & diversity of plasmids carrying antimicrobial resistance in waterborne *Salmonella* & *E. coli*

4.1 Introduction

Conjugative transfer of genetic elements, such as plasmids, is an important and common way for many enteric pathogens, including *Salmonella,* to rapidly acquire antimicrobial resistance (AMR) (Kruse and Sorun, 1994; Davison, 1999; Foley and Lynne, 2008). Many plasmids can be exchanged between species or genera which can lead to their prevalence in a diverse group of microorganisms (Poppe *et al*., 2005; Smith *et al*., 2007). Commensal bacteria are thought to act as a reservoir of plasmid-borne antimicrobial resistance for pathogens, as plasmid transfer between *E. coli* and *Salmonella* has been shown to occur readily *in vitro* and *in vivo* (Poppe *et al*., 2005; Daniels *et al*., 2007; Boerlin and Reid-Smith, 2008; Daniels *et al*., 2009).

National surveillance programs, such as the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) in Canada and National Antimicrobial Resistance Monitoring System (NARMS) in the United States, report on the phenotypic expression of drug resistance in enteric pathogens and commensal bacteria. Despite its importance, genotypic characterization is rarely reported by these

programs (Boerlin *et al.*, 2005; Li *et al.*, 2007). Knowledge of the genes responsible for resistance, as well as the way in which bacteria carry resistance, either chromosomally or on plasmids, is imperative for understanding the development and spread of AMR.

Research examining resistance at the genomic level has helped in understanding the diversity, distribution and epidemiology of resistance genes in bacteria such as *Salmonella*. Analysis at this level has helped to recognize the clonal expansion of several drug resistant *Salmonella* strains, such as multiple-drug resistant (MDR) *S*. Typhimurium phagetype 104 (PT 104), which carries drug resistance chromosomally on a genomic island (Butaye *et al.*, 2006; Mulvey *et al.*, 2006), and *S*. Newport strains (MDR-AmpC) carrying plasmid-borne resistance to extended spectrum cephalosporins (Butaye *et al.*, 2006; Daniels *et al.*, 2007; Egorova *et al.*, 2008).

In addition to recognizing the clonal expansion of AMR in certain *Salmonella* strains, recent studies have also helped understand the occurrence and spread of common or epidemic plasmids in epidemiologically unrelated *Salmonella* (Carattoli *et al.*, 2005). Over the past decade, increasing attention has been focused on plasmids that harbour the resistance gene *bla* $_{CMY-2}$ (Li *et al.*, 2007). The *bla* $_{CMY-2}$ gene encodes an AmpC β-lactamase that hydrolyzes β-lactam drugs, including 3rd generation cephalosporins such as ceftriaxone, which is important for treating invasive *Salmonella* infections in children (Call *et al.*, 2010; Shea*,* 2004). In Canada, the first reported human case of *Salmonella* producing plasmid-borne AmpC β-lactamase occurred in 2002 during an outbreak of S. Newport (Pitout *et al.*, 2003). Since this time, the frequency of plasmid-borne *bla* $_{CMY-2}$ has increased in Canada and has been reported in several serotypes, with *S*. Heidelberg being one of the most common (Mataseje *et al.*, 2009b). Based on replicon-typing that classifies plasmids by incompatibility group, four plasmid types have been shown to carry the *bla* $_{CMY-2}$ in *Salmonella* (Carattoli *et al.*, 2002; Giles *et al.*, 2004; Carattoli *et al.*, 2005).

The genetic variability of many naturally occurring plasmids in *Salmonella*, as well as the biological cost associated with carriage of these plasmids, remains largely unknown (Bjorkman and Andersson, 2000; Arlet *et al.*, 2006; Enne *et al.*, 2005; Boerlin and Reid-Smith, 2008). Though AMR plasmids carry genes beneficial to their host, in the absence of antimicrobial drugs, biological or 'fitness' costs, expressed as decreased growth and invasion rates, can result (Lenski, 1998; Bjorkman *et al.*, 1999; Bjorkman

and Andersson, 2000; Dionisio *et al*., 2005). AMR plasmids may impose a metabolic load on a cell by creating energy expenditures associated with the production of extra proteins, particularly when enzymes such as AmpC β-lactamase are constitutively expressed (Bjorkman and Andersson, 2000; Zhang *et al*., 2006). In some instances, bacteria that experience fitness costs associated with plasmid carriage can segregationally eliminate plasmids during cell reproduction (Lenski and Bouma, 1987; Modi and Adams, 1991; Smith and Bidochka, 1998). However, some fitness costs associated with the expression of AMR can be restored by acquiring compensatory mutations (Bjorkman *et al*., 1999; Bjorkman and Andersson, 2000; Hossain *et al*., 2004; Maisnier-Patin and Andersson, 2004; Zhang *et al*., 2006). Several studies have reported fitness costs associated with plasmid carriage in *Salmonella* (e.g., Morosini *et al*., 2000), however, a more recent study by Hossain *et al*. (2004) reported that naturally occurring plasmids carrying the *bla* $_{CMY}$ gene do not confer fitness costs despite the high level of AmpC β-lactamase production.

Compared to isolates obtained from humans and livestock, information on the prevalence of plasmid-mediated resistance and the genes responsible for resistance in environmentally-obtained strains of *Salmonella* is limited. Bell *et al*. (1980), the only Canadian study to characterize drug resistance in waterborne *Salmonella*, reported that over half of the resistant isolates could transfer AMR to recipient cells, although no information was given on the type of resistance transferred. Despite limited characterization, these authors recognized that isolates carrying transferable resistance in the aquatic environment was concerning, however, no further studies have been conducted.

4.2 Research needs and objectives

Genotypic evaluation of drug resistance in environmentally-obtained strains of *Salmonella* and *E. coli* is limited. To understand the extent to which the aquatic environment is associated with the spread of AMR, the prevalence, diversity and distribution of resistance genes in waterborne isolates is needed. Ascertaining how resistance is mediated in waterborne isolates, whether it is on mobile genetic elements or chromosomally-encoded, is essential in understanding the complexity of resistance in environmental isolates and determining if similarities exist with AMR-carriage in

Salmonella isolates reported in various host animals. This type of information is a first step to determine if epidemiological connections exist between AMR in waterborne isolates and those associated with human and animal health. Establishing how widely distributed various plasmids are in waterborne *Salmonella* serotypes can help in understanding the ecology of plasmid-borne AMR in the aquatic environment. Few studies have examined fitness costs associated with naturally occurring AMR plasmids (Bjorkman and Andersson, 2000; Enne *et al*., 2005), particularly those in *Salmonella*. Understanding plasmid stability and fitness costs associated with the carriage of AMR plasmids, particularly those conferring resistance to drugs of human health significance, can help to determine the potential persistence of these plasmids in non-selecting environments.

The specific objectives of the research presented in this chapter are to:

1. Examine the prevalence and diversity of plasmid-mediated resistance in *Salmonella* and *E. coli* isolated from the aquatic environment, as well as the genes responsible for resistance;
2. Identify any association that may exist between plasmid carriage and specific serotypes of waterborne *Salmonella*;
3. Assess the distribution of AMR plasmids in *Salmonella* and *E. coli* spatially and temporally within the different tributaries;
4. Establish the genetic relatedness of plasmids carrying resistance to drugs of human health importance; and,
5. Examine the stability and potential fitness costs associated with carriage of naturally occurring AMR plasmids in *Salmonella* isolates.

4.3 Materials and methods

Plasmid extraction

All strains of *Salmonella* exhibiting resistance were subjected to a plasmid extraction assay. Two methods for plasmid extraction were used in this study. The predominant method was an alkaline lysis specifically used to extract large plasmids as discussed by Crosa *et al*. (1994) and Tolmasky *et al*. (2007), with slight modifications. The second method was conducted using a plasmid extraction kit (Qiagen, Mississauga, ON), using the manufacturer's recommendations for extraction of large plasmids.

The first method was as follows: isolates were streaked onto LB agar (or LB agar with selected antimicrobials) and incubated at 37°C for 24 h. Lysis buffer (4% SDS in TE, pH 12.4) was prepared on the same day prior to plasmid extraction. Lysis buffer (30 µL) was placed into sterile 1.5 mL microcentrifuge tubes. Using a sterile toothpick, colonies (size of a pinhead) were scraped off the plate, deposited on the side of the tube and gradually spread into the lysis buffer to reduce clumping. The tubes were gently inverted two times and then incubated at 37°C in a block heater (Fisher Scientific Dry Heater) for 20 min. The tubes were inverted gently two times and 50 µL of 2 M Tris-HCl (pH 7.0, 4°C) was added to the tubes to neutralize the solution. The tubes were inverted gently 25 times. Tubes were inverted again 25 times after the addition of 240 µL of 5 M NaCl (4°C) to precipitate chromosomal DNA. The tubes were placed on ice for 4 h followed by centrifugation at 13,600 rpm (Eppendorf Centrifuge 5415 D, Mississauga, ON) for 20 min. at room temperature. The supernatant was poured into new sterilized tubes and 550 µL of isopropanol (-10°C, Sigma, St Louis, Missouri) was added to each tube, followed by the addition of 100 µL of 7.5 M ammonium acetate (4°C, Sigma, St Louis, Missouri). Each tube was inverted 25 times after the addition of each solution and then placed for 30 min. in a freezer at -20°C. Tubes were then centrifuged again for 25 min. at 13,200 rpm at room temperature. Immediately after centrifugation, the supernatant was poured off and the tubes were inverted on paper towels to allow the pellet to air dry for 30 min. Each pellet was resuspended overnight at 4°C in 30 µL 1 x TES buffer and RNase A (1 mg/mL, Sigma, St Louis, Missouri) or other buffers depending on the subsequent experimental needs of the extract. Plasmid extractions were stored at -20°C until used for further analysis.

In several instances, additional purification of plasmid extracts was needed. To remove proteins and salts, ethanol precipitation was carried out by adding 3 µL of 3M sodium acetate (pH 5.2) to each 30 µL plasmid extraction. Into each tube, 2.5 times the volume of 95% ethanol (Sigma, St Louis, Missouri) was added and then inverted several times. Each tube was placed at -20°C for 30 min. and then centrifuged for 15 min. at 13,600 rpm (Eppendorf Centrifuge 5415 D, Mississauga, ON) at room temperature. The supernatant was removed and 1 mL of cold 70% ethanol (Sigma, St Louis, Missouri) was added without disrupting the pellet. Each tube was centrifuged again for 5 min., supernatant was poured off, and the tubes were inverted and allowed to air dry. The plasmid DNA was resuspended overnight in water at 4°C.

Examination of plasmid extracts

To examine and compare the size of plasmids between wildtype and transformants, plasmid extracts were subjected to gel electrophoresis. A 9 µL volume of the plasmid extract was mixed with 1 µL of gel loading buffer. Electrophoresis was carried out in 0.7% agarose (Sigma, St Louis, Missouri) gels in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 100V for 1.5 h. Gels were stained with ethidium bromide solution (1 mg/mL) for 30 min. and destained in water for 30 min. The gel was photographed under UV light (BioRad Gel Doc System, Mississauga, ON) using the imaging Quantity One software (BioRad, Mississauga, ON).

Plasmids of known molecular weight/size were used to estimate plasmid size. The plasmids used for comparison included: pDT285 (96-MDa); pDT369 (23-MDa); p971028 (60-MDa) from *S.* Typhimurium and 6 plasmids from *E. coli* V517 (1.4, 2.0, 2.6, 3.4, 3.7, and 36-MDa; Poppe *et al*., 2005). In some instances, a supercoiled DNA ladder (Sigma, St Louis, Missouri) was also used to approximate plasmid sizes.

Introduction of foreign plasmids into bacterial cells

The preparation of electrocompetent cells and electroporation was carried out as described in Sambrook *et al*. (2001), Shen *et al*. (1995) and by the manufacturer of the electroporation device (BioRad, Mississauga, ON), with slight modifications.

Preparation of electrocompetent E. coli DH5α cells

To prepare electrocompetent cells, a flask containing 500 mL of LB broth was inoculated with 25 mL of an overnight culture of *E. coli* DH5α and placed in an orbital shaking incubator (Lab-Line, Incubator Shaker) at 300 rpm at 37°C. After the desired cell density (OD_{600} of 0.5-0.6) was reached (approximately 4 h, Ultrospec 1100 pro UV Visible Spectrophotometer, Fisher Scientific) each flask was chilled on ice for 20 min. The total volume was portioned into multiple tubes and centrifuged (Beckman Coulter, Avanti J-301) at 4°C for 15 min. at 4000 x g. The supernatant was removed and the pellet was resuspended in the original volume of ice cold sterile water and centrifuged again. The pellet was then resuspended again in half the original volumes of ice cold 10% sterile glycerol. The cells were centrifuged again and resuspended in 10% glycerol at one tenth of the original volume. The cells were centrifuged for a final time and resuspended in 25% of the previous volume of 10% glycerol. A total of 40 µL of the concentrated cells were transferred into ice cold microcentrifuge tubes and frozen immediately in liquid nitrogen. These tubes were placed at -80°C until needed.

Electroporation procedure

Prior to electroporation the plasmid extract was subjected to ethanol precipitation and resuspended in water (as described above). Under sterile conditions, 1 µL of purified plasmid DNA extract was added to each tube containing electrocompetent cells and gently mixed using the pipette tip. The bacteria/plasmid mixture was left on ice for 20 min. to allow close association of DNA with whole cells. Under sterile conditions, bacteria/plasmid mixtures were then added to cuvettes with a 0.1 cm gap (VWR, Mississauga, ON) and electroporated (MicroPulser, BioRad, Mississauga, ON) using standard pre-programmed parameters (1.5 kV for approximately 5 ms).

Immediately following electroporation, 1 mL of pre-warmed (37°C) SOC media (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the cuvette. The contents were added to culture tubes and incubated at 37°C for 1 h while shaking at 225 rpm in an orbital waterbath to promote growth. The culture was then spread on LB agar plates containing appropriate concentration of antimicrobial drugs. The plates were incubated overnight at 37°C to allow for transformant growth. The positive control included electroporation of cells

with pGLO plasmid conferring ampicillin resistance (Bio-Rad Laboratory, Hercules, CA).

Conjugation procedure

Conjugation was performed as described by Provence and Curtiss (1994). Selected *Salmonella* isolates were taken from frozen (-80°C) stock and grown in LB broth overnight at 37°C in an orbital shaking waterbath. Naladixic acid (Nal) resistant recipients, *E. coli* C600N and *Salmonella* LB5000, were also grown overnight under the same conditions. Following overnight growth, each recipient and donor were streaked onto LB agar plates containing Nal (32 µg/mL) to ensure that the recipients maintained Nal resistance and that the donors were sensitive. A total of 9 mL of the overnight culture of each recipient culture was placed in a pre-warmed 50 mL flask at 45°C for 15 min. One mL of each donor was placed into one flask containing each recipient and swirled to mix cells. These flasks were incubated in a waterbath at 37°C without shaking. At 2 h and 24 h after the start of conjugation experiments, samples were taken from each flask, serially diluted and plated in duplicate on LB agar containing Nal and the appropriate antimicrobial drug (concentrations were as described in Chapter 3). Following 24 h incubation at 37°C, transconjugant cells that grew on LB + Nal + antimicrobial drug were examined and counted. Each conjugation experiment was replicated at least 2 times.

Phenotypic assessment of extended spectrum β-lactamase (ESBL) production in *Salmonella*

All *Salmonella* isolates showing reduced susceptibility to 3rd cephalosporins (Caz and Cro) were tested for the production of extended spectrum β-lactamase (ESBL) through phenotypic confirmatory testing (CLSI, 2005). First, discs of ceftazidime-clavulanic acid (30 µg/10 µg) and cefotaxime-clavulanic acid (30 µg/10 µg) were prepared. This was done by adding 10 µL of clavulanic acid (1000 µg/mL potassium clavulanate, Sigma, St Louis, Missouri) to ceftazidime (30 µg) and cefotaxime (30 µg) discs. The discs were allowed to dry in the dark for 30 min. prior to use. Standard disc diffusion protocols were used to streak Mueller-Hinton plates as described previously in Chapter 3. On each plate the four discs were added, allowed to dry for 10 min. at room temperature and incubated inverted overnight at 35°C.

Following incubation, the plates were examined and the zone size was determined. A positive result of ESBL production occurred when there was $\geq$ 5 mm difference in the zone measurement between discs with and without clavulanic acid (CLSI, 2005).

DNA amplification by PCR

Plasmid-mediated resistance genes

Each plasmid found to be responsible for drug resistance in *Salmonella* and *E. coli* was assessed for the genetic determinants causing this resistance. Polymerase Chain Reaction (PCR) was performed in an iCycler iQ thermocycler (BioRad, Mississauga, ON) using synthetic oligonucleotides synthesized by Sigma-Genosys (Oakville, ON). To amplify DNA fragments, plasmid extracts from transformed cells were used as templates. Each reaction mixture contained: 12.5 µL of 2 x master mix (ReadyMix Taq PCR Reaction mix without MgCl$_2$, Sigma, St Louis, Missouri), 2.5 µL forward and 2.5 µL reverse primers (1 µM), 1.5 µL of 25 mM MgCl$_2$, 1 µL template plasmids and up to 25 µL total volume with water (see details below).

Bacterial isolates demonstrating plasmid-mediated resistance to β-lactam antimicrobial drugs were tested for several β-lactamase-encoding genes by PCR using consensus primers (see Table 4.1).

Table 4.1. Primers used to determine the genes responsible for β-lactam resistance.

Primer designation	Sequence (5' - 3')	Gene	Size of product (bp)	Anneal-ing Temp (ºC)	Reference
OXA-F OXA-R	AATGGCACCAGATTCAACTT CTTGGCTTTTATGCTTGATG	bla_{OXA}	595	50	Chen *et al.*, 2004
SHV-F SHV-R	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	bla_{SHV}	867	50	Rasheed *et al.* 1997
CTX-M-F CTX-M-R	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	bla_{CTX}	948	60	Ahmed *et al.*, 2007
CMY-F CMY-R	GCCTCTTTCTCCACATTTG GGACAGGGTTAGGATAGC	bla_{CMY}	1066	55	This study*
TEM-F2 TEM -R2	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTATC	bla_{TEM}	1080	55	Ahmed *et al.*, 2007

*CMY primers were designed using Beacon Designer 5.1 (Premier Biosoft, Palo Alto, CA) using sequence Accession Number U77414 for the *bla* $_{CMY}$ sequence.

PCR cycles for the primers designed for the detection of β-lactamase-encoding genes included a 2 min. initial denaturation at 94°C followed by 35 cycles at 94°C for 30 s, annealing for 45 s (See Table 4.1 for specific temperature) and 72°C for 1.5 min. A 10 min. final extension step occurred at 72°C. Following PCR, 9 µL of each tube was mixed with 1 µL tracking dyes and subjected to gel electrophoresis in 1% agarose in 1x TAE buffer at 130 V for 35 min. The size of each PCR product was determined by comparing to an EZ Load 100 bp molecular ruler (BioRad, Mississauga, ON). Following electrophoresis the gel was stained and photographed as described previously. The expected size of each PCR product is shown in Table 4.1.

Isolates with plasmid-mediated resistance to tetracycline were subjected to multiplex PCR using a range of primers specific for various *tet* genes (See Table 4.2).

Primers for determining which *tet* gene was responsible for tetracycline resistance were grouped (Groups I to IV) and multiplexed for PCR, as described by Ng *et al*. (2001). Reaction mixtures for Group I contained primers for *tet*B (0.25 µM), *tet*C (0.25 µM) and *tet*D (2.0 µM) each (4.0 mM $MgCl_2$). Group II contained primers for *tet*A (1.0 µM), *tet*E (1.0 µM) and *tet*G (1.0 µM) each (3.0 mM $MgCl_2$). Group III contained primers for *tet*K (1.25 µM), *tet*L (1.0 µM), *tet*M (0.5 µM), *tet*O (1.25 µM) and *tet*S (0.5 µM) each (3.0 mM $MgCl_2$). Group IV contained primers for *tet*AP (1.25 µM), *tet*Q (1.25 µM) and *tet*X (1.25 µM) each (4.0 mM $MgCl_2$) (Ng *et al*., 2001).

PCR cycles for the primers designed for *tet* genes were described by Ng *et al*. (2001) and included a 2 min. initial denaturation at 94°C, followed by 35 cycles of 94°C for 1 min., annealing for 1 min. (See Table 4.2 for temperatures) and 72°C for 1.5 min. A 10 min. final extension occurred at 72°C. PCR products were analysed by gel electrophoresis and band size was determined as described previously.

Table 4.2. Multiplex PCR primers for genes responsible for tetracycline resistance.

Primer grouping	Primer designation	Sequence (5' - 3')	Gene	Size of product (bp)	Annealing temp (°C)
Group I	*tet*B-F	TTGGTTAGGGGCAAGTTTTG	*tet*B	659	
	*tet*B-R	GTAATGGGCCAATAACACCG			
	*tet*C-F	CTTGAGAGCCTTCAACCCAG	*tet*C	418	60
	*tet*C-R	ATGGTCGTCATCTACCTGCC			
	*tet*D- F	AAACCATTACGGCATTCTGC	*tet*D	787	
	*tet*D- R	GACCGGATACACCATCCATC			
Group II	*tet*A-F	GCTACATCCTGCTTGCCTTC	*tet*A	210	
	*tet*A-R	CATAGATCGCCGTGAAGAGG			
	*tet*E- F	AAACCACATCCTCCATACGC	*tet*E	278	60
	*tet*E- R	AAATAGGCCACAACCGTCAG			
	*tet*G- F	GCTCGGTGGTATCTCTGCTC	*tet*G	468	
	*tet*G- R	AGCAACAGAATCGGGAACAC			
Group III	*tet*K -F	TCGATAGGAACAGCAGTA	*tet*K	169	
	*tet*K-R	CAGCAGATCCTACTCCTT			
	*tet*L-F	TCGTTAGCGTGCTGTCATTC	*tet*L	267	
	*tet*L-R	GTATCCCACCAATGTAGCCG			
	*tet*M-F	GTGGACAAAGGTACAACGAG	*tet*M	406	55
	*tet*M-R	CGGTAAAGTTCGTCACACAC			
	*tet*O-F	AACTTAGGCATTCTGGCTCAC	*tet*O	515	
	*tet*O-R	TCCCACTGTTCCATATCGTCA			
	*tet*S-F	CATAGACAAGCCGTTGACC	*tet*S	667	
	*tet*S-R	ATGTTTTTGGAACGCCAGAG			
Group IV	*tet*AP-F	CTTGGATTGCGGAAGAAGAG	*tet*AP	676	
	*tet*AP-R	ATATGCCCATTTAACCACGC			
	*tet*Q-F	TTATACTTCCTCCGGCATCG	*tet*Q	904	55
	*tet*Q-R	ATCGGTTCGAGAATGTCCAC			
	*tet*X-F	CAATAATTGGTGGTGGACCC	*tet*X	468	
	*tet*X-R	TTCTTACCTTGGACATCCCG			

DNA sequence analysis

PCR products of the correct size for any of the β-lactamase-encoding genes were cut from the gel and purified with a gel extraction kit (Qiaquick Gel Extraction Kit, Qiagen, Mississauga, ON) prior to sequencing. This extraction involved excising the desired band, dissolving of the gel and removing any agarose, salts and unused primers. Following air drying, the pellet was resuspended overnight in the fridge at 4⁰C in water. The concentration of PCR products was determined by running a 1 µL portion of the extracted product along side of quantity ladder (EZ Load Precision Molecular Mass Standard, BioRad, Mississauga, ON). A total of 5 µL of PCR products (10-40 µg/µL) and 5 µL of primer (1 µM) were shipped overnight to Mobix Lab (McMaster University, Hamilton, ON) for sequencing.

All obtained sequences were uploaded into Sequence Scanner software (version 1, Applied Biosystems) to make minimal edits to each sequence. All edited sequences were entered into NCBI's basic local alignment search tool (BLAST) to find similarities between sequences in the database. The nucleotide BLAST program was used and each sequence was compared to the nucleotide collection database for matches. All sequences were submitted to Genbank and accession numbers were obtained.

Plasmid restriction fragment length polymorphism

Plasmid restriction fragment length polymorphism (pRFLP) was performed to determine the similarity between the larger-sized plasmids that demonstrated resistance to ACazCro. The plasmids were obtained from either a transconjugant or a transformant using the method previously described. Following extraction, these plasmids were resuspended overnight at 4⁰C in 5 µL 10x reaction buffer (Epicentre Biotechnologies, Madison, WI) with 43.5 µL sterile water.

To reduce the level of genomic DNA in the sample, each extract was treated with Plasmid-Safe ATP-Dependant DNase (Epicentre Biotechnologies, Madison, WI). Following significant losses of plasmids, troubleshooting revealed that half of the concentration of Plasmid Safe DNase and ATP (as specified by the manufacturer) was the most effective concentration to use. This reaction included the addition of 1 µL of

25 mM ATP and 0.5 µL Plasmid-Safe DNase (5 U) to each plasmid extract suspended in reaction buffer. After the addition of this enzyme, the sample was incubated at 37°C for 30 min. The enzymatic reaction was stopped by placing the samples in a heating block at 70°C for 30 min. To ensure concentrated plasmid extracts were obtained for additional analysis, three separate plasmid mini-prep extractions per plasmid-type were combined.

Phenol:chloroform extraction was carried out prior to additional enzymatic digestion (Tolmasky *et al*., 2007) by adding 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma, St Louis, Missouri) to each sample, inverting several times and removing the aqueous phase to a new microcentrifuge tube. The sample was then treated with 100 µL of chloroform: isoamyl alcohol (24:1) and the aqueous phase was removed again. An ethanol precipitation, as described previously, was carried out. After drying, the DNA was suspended in 25 µL of 1 x restriction enzyme buffer 3 (NE Buffer 3, New England BioLabs, Ipswich, MA) overnight (4°C) to ensure the pellet was fully dissolved.

The purified plasmid was digested for 4 h with *Pst*I (50 U) with 100 µg/mL BSA (New England BioLabs, Ipswich, MA) at 37°C. The reaction was stopped at 80°C for 20 min. The resulting products were separated by electrophoresis on 1.2% SeaKem LE agarose gel (Cambrex Bio Science, Rockland, ME) for 7 h at 65V. Two DNA ladders were included for size comparison (Direct Load Wide Range DNA Marker, Sigma, St Louis, Missouri; Fermentas Genomic Marker, Fermentas, Burlington, ON). The gel was stained, examined and photographed under UV light as previously described.

To critically assess banding patterns, images were uploaded into BioNumerics software (Applied Maths, Belgium) and resulting banding patterns were normalized to bands in the DNA ladder (Direct Load Wide Range DNA Marker, Sigma, St Louis, Missouri). Band matching was determined initially by the software and manual changes were made if bands were placed in the incorrect band class. The relatedness of each banding pattern was calculated using Dice coefficients with a 1.5% band position tolerance and optimization. Dendograms were generated using an unweighted –pair group algorithm (UPGMA) method.

Interpretation of the similarity between banding patterns was carried out based on criteria described by Tenover *et al*. (1995). Plasmids were considered indistinguishable if the similarity was $\geq$ 99%. Patterns were considered closely related if similarities were > 80%.

Fitness and plasmid stability studies

Plasmid curing

Attempts to cure *Salmonella* isolates of plasmids were performed as described by Poppe and Gyles (1988). Select isolates were taken from frozen (-80°C) stocks and grown in LB at 37°C overnight. After incubation, 0.1 mL were added to 100 mL LB in a 250 mL flask and shaken at 250 rpm at 44.5°C in an orbital waterbath shaker (New Brunswick Scientific). Every 48 h, 0.1 mL were again transferred to fresh LB broth. A portion of each culture was taken periodically, serially diluted, plated on LB agar plates and incubated overnight at 37°C. Using a sterile toothpick, up to 50 well isolated colonies were transferred to LB agar plates containing the appropriate antimicrobial agent (Amp or Amp and Caz). Any colony that grew on LB and not on LB containing the antimicrobial drug was presumed cured of their corresponding resistance plasmid. To further confirm, several isolates were assessed for plasmid loss through plasmid extraction (as described previously).

Several isolates of *Salmonella* containing resistance-carrying plasmids were chosen for curing, including isolates designated as 21C1 (*S*. Agona), 19C1 (*S*. Infantis), 22C1 (*S*. Berta), 22C2 (*S*. Berta) and 22D2 (*S*. Newport). Each of these strains carried plasmids responsible for the resistance profile ACazCro. A biological control was also run alongside, *S*. Typhimurium 970125, that contained a 95.5 Kb plasmid (p970125) carrying resistance to ampicillin. This curing experiment was run for up 100 days.

Fitness and plasmid stability

Several experiments were conducted to examine the reproductive fitness of isolates with and without plasmids. Differences in bacterial growth rates were monitored by measuring the turbidity of each culture and the colony-forming units (CFU). The optical

densities at 600 nm (Ultrospec 1100 pro UV Visible Spectrophotometer, Fisher Scientific) were recorded at 1 h intervals up to 12 h and in some instances again at 24 h. At various times throughout the experiment aliquots were taken and plated on LB in replicate to determine the concentration of the culture (CFU/mL). As described previously, replica plating onto LB agar containing the appropriate antimicrobial agent was conducted with a 50 to 100 well isolated colonies to determine the portion of cells that had lost resistance-carrying plasmids.

Each set of experiments began with the initial preparation of cultured cells. Cultures were started from frozen cells (-80°C) and grown overnight in LB or LB containing ampicillin (32 µg/mL) at 37°C. Following overnight growth, 1 mL of each culture was placed in a microcentrifuge tube and centrifuged for 5 min. at 10,000 rpms. These cells were resuspended in sterile saline and re-centrifuged. This washing took place three times in total. The cells were resuspended in saline and 0.1 mL of each culture was placed into 100 mL of LB (or 1/10 LB) in a sterile 250 mL flask. At time zero (T=0), a portion was removed to determine the initial starting concentration and also taken for a reading on the UV spectrophotometer.

In some instances, the number of generations (n) was calculated to normalize data between isolates and treatments. The number of generations was determined by the following (Madigan *et al*., 2003):

$$n = (\log_{10} N_t - \log_{10} N_o) / \log_{10} 2$$

Where n is the number of generations, N_t is the concentration of cells at stationary phase (approximately 12 h) and N_o is the initial cell concentration. The number of generations (n) for each strain was averaged from replicate growth curves. The average number of generations (n) was multiplied by the number of serial passages to determine the number of generations at which plasmid stability was investigated.

Fitness study No. 1 - Fitness in Salmonella isolates carrying plasmids and cured of plasmids

For this study, three strain variations were tested in duplicate, including: 1) the original wild-type *Salmonella* isolates containing plasmid(s); 2) *Salmonella* that lost resistance following curing; and 3) a *Salmonella* isolate that was exposed to curing conditions but maintained resistance. Five different *Salmonella* isolates were included in this reproductive fitness evaluation; 970125 (as a biological control, *S*. Typhimurium), 21C1 (*S*. Agona), 19C1 (*S*. Infantis), 22C1 (*S*. Berta), and 22D2 (*S*. Newport). During the reproductive fitness trial, all cultures were prepared as described above and grown at $37^{\circ}C$ throughout the experiment. The OD_{600} was measured hourly up to 12 h and then again at 24 h. Results were plotted as changes in OD_{600} over time to visually examine differences between isolates. Differences in growth rates between the three strain variations were also determined, as described below.

Fitness and plasmid stability study No. 2 – Wildtype Salmonella carrying plasmid-mediated resistance in LB and 1/10 LB

Several *Salmonella* isolates, including 21C1 (*S*. Agona), 19C1 (*S*. Infantis) and 22C2 (*S*. Berta), were subjected to growth at $44.5^{\circ}C$ in LB broth and in 1/10 LB broth. The cultures were prepared as described above and run in duplicate. For the first 12 h, the OD_{600} was measured and the concentration was determined. These data were used to determine the number of generations over time.

Each culture was propagated every 12 h by adding 0.1 mL into 100 mL of fresh LB, and continued to grow at $44.5^{\circ}C$. On occasion, a portion of culture was removed, serially diluted, and plated on LB agar to determine the concentration of cells. Up to 100 colonies were replica-plated onto LB agar containing ampicillin and LB agar containing ceftazidime to determine the proportion of cells maintaining resistance plasmids over time. These data were presented as the proportion of plasmids remaining in the culture over successive generations.

Fitness and plasmid stability study No. 3 - E. coli containing resistance plasmids originating from Salmonella

E. coli C600N cells carrying large-sized resistant-carrying plasmids conjugated from *Salmonella*, p21C1, p19C1 and p22C2, were used in this study. A non-transformed *E. coli* C600N culture was also grown alongside these cultures to compare the growth rate of isolates without the addition of these plasmids. The cultures were prepared in the same fashion as those described above. An experiment was conducted at 44.5⁰C in LB and 1/10 LB broth and at 37⁰C in LB broth for 12 h. Each experiment was performed in duplicate. Replica plating was carried out on agar plates containing both nalidixic acid and ampicillin.

Growth curves were used to determine the number of generations and also used to compare any differences in the growth between isolates carrying plasmids and those which were not. The proportion of cells that maintained resistance was also examined over successive generations. The OD_{600} over 12 h was plotted and growth rates among the original *E. coli* C600N strain and those with plasmids were compared, as described below.

Fitness and plasmid stability study No. 4: Competitive fitness evaluation between E. coli isolates with and without plasmids

Competitive fitness was carried out by culturing isolates with and without introduced plasmids in the same culture flask. *E. coli* C600N, and an isolate of *E. coli* C600N carrying the p21C1 originating from *S.* Agona (obtained through conjugation), were grown within the same flask at 37⁰C for up to 72 h. This trial was carried out in duplicate.

Every 24 h, 0.1 mL of the contents of each flask was transferred into a new flask containing 100 mL of LB. In addition, a portion of the culture was removed and plated onto LB agar plates in replicate. Up to 100 colonies were replica plated onto LB containing nalidixic acid and ampicillin to determine the proportion of isolates carrying the resistance plasmid and those that were plasmid free.

4.4 Data analysis

Chi-square tests were used to determine if differences existed between the occurrence of isolates carrying AMR plasmids in each tributary and at various times. Following reproductive fitness trials, growth rates of isolates with and without plasmids were determined as a measure of relative fitness. Growth rates were determined as slopes of the exponential portion of the log optical density (OD_{600}) versus time and compared using ANCOVA. The level of significance was set at a $P < 0.05$. If the value was < 0.001, then this value was stated, to represent a highly significant difference.

4.5 Results

Plasmid mediated resistance in waterborne *Salmonella* and *E. coli*

Number of isolates demonstrating plasmid-mediated resistance

A total of 77 *Salmonella* and 17 *E. coli* isolates demonstrating AMR were further characterized for the presence of plasmid-mediated resistance. Through conjugation and/or electroporation, 28.6% (22/77) waterborne *Salmonella* isolates demonstrated plasmid-mediated resistance. In total 41.2% (7/17) *E. coli* isolates demonstrating AMR carried resistance on plasmids. With the exception of one *Salmonella* isolate, all plasmids carrying resistance in *Salmonella* and *E. coli* were self transferable through conjugation (Table 4.3 and Table 4.4).

Table 4.3. Plasmid mediated resistance observed in waterborne *Salmonella*.

Tributary	Site locat-ion	Collection date (D/M/Y)	No.	Serotype	Phage-type	Wildtype AMR profile	AMR transferred to recipient		AMR plasmid size (Kb)	Gene for plasmid-mediated resistance	Sequence Accession No.
							Conjuga-tion	Electro-poration			
	LC-2	22/11/2004	22C1	Berta	-	ACazCro	ACazCro	ACazCro	95.5	*bla* CMY-2	GU393326
	LC-3	22/11/2004	22C2	Berta	-	AGCazCro	ACazCro	ACazCro	95.5	*bla* CMY-2	GU393330
	LC-3	22/11/2004	22D2	Newport	-	ACazCro	ACazCro	ACazCro	95.5	*bla* CMY-2	GU393327
	LC-2	18/11/2004	21C1	Agona	-	ACazCro	ACazCro	ACazCro	95.5	*bla* CMY-2	GU393329
	LC-1	21/10/2004	20D3	Heidelberg	19	A	A	A	8.1	*bla* TEM-1	GU727532
	LC-1	21/10/2004	20C3	Typhimurium	104	ACSˡSuT	A	A	8.1	*bla* TEM-1	GU727531
Laurel Creek	LC-2	18/10/2004	19C1	Infantis	-	AGCazCro	ACazCro	ACazCro	95.5	*bla* CMY-2	GU393328
	LC-3	18/10/2004	19D1	Heidelberg	19	A	A	A	8.1	*bla* TEM-1	GU727533
	LC-1	10/08/2004	15C3	Heidelberg	29a	ACazCro	ACazCro	ACazCro	95.5	*bla* CMY-2	GU393325
	LC-2	21/07/2004	12Ca	Heidelberg	Untypable	ACazCro	ACazCro	ACazCro	95.5	*bla* CMY-2	GU393324
	LC-1	31/05/2004	10Ca	Heidelberg	6	AG	A	A	8.1	*bla* TEM-1	GU727534
	LC-3	07/05/2004	8Db	Kentucky	-	AGCazCro	ACazCro	ACazCro	95.5	*bla* CMY-2	GU393323
	LC-1	10/12/2003	3D2	Hadar	-	AST	A	A	8.1	*bla* TEM-1	GU727535
	LC -2	11/12/2003	504b	Oranienberg	-	A	-	A	8.1	*bla* TEM-1	GU727536
	LC-1	21/11/2003	2Fa	Montevideo	-	AG	A	A	8.1	*bla* TEM-1	GU727537
Canagagigue Creek	CAN-1	31/05/2004	10Bc	Heidelberg	19	A	A	A	8.1	*bla* TEM-1	GU727538
	CAN-1	31/05/2004	10Cc	Heidelberg	19	AG	A	A	8.1	*bla* TEM-1	GU727539
	CON-1	12/05/2004	9Cb	Heidelberg	Atypical	AG	A	A	8.1	*bla* TEM-1	GU727540
Conestogo River	CON-1	12/05/2004	9Db	Heidelberg	Untypable	AG	A	A	8.1	*bla* TEM-1	GU727541
	CON-1	07/05/2004	8Cd	Heidelberg	19	AG	A	A	8.1	*bla* TEM-1	GU727542
	CON-1	07/05/2004	8Dd	Heidelberg	Atypical	AG	A	A	8.1	*bla* TEM-1	GU727543
	CON-1	10/12/2003	3C4	Montevideo	-	A	A	A	8.1	*bla* TEM-1	GU727544

Drug resistance observed on plasmids

Plasmid-mediated resistance was only observed to β-lactam drugs in *Salmonella* isolates. Of these 22 isolates, all showed resistance to ampicillin, and 8 also showed resistance to 3rd generation cephalosporins, ceftazidime (Caz) and ceftriaxone (Cro).

As described in Chapter 3, 44 *Salmonella* isolates showed AMR to ampicillin and half of these isolates (n=22) carried this resistance on plasmids. Of these 22 isolates, only 5 showed single drug resistance to ampicillin while the remainder carried multiple drug resistance. Some of these MDR isolates showed all resistance on plasmids, as in the case of the profile ACazCro, while others showed only a portion of resistance on plasmids. Plasmid-mediated resistance to Caz and Cro was observed in 80% (8/10) of the isolates that demonstrated resistance to these drugs. Plasmids carrying this type of resistance were always self transferable through conjugation.

As described in Chapter 3, 23 isolates demonstrated the penta-drug resistant profile, ACSSuT. This profile accounted for over half of the resistance observed to ampicillin. Of these ACSSuT profiles, however, only one isolate was observed to carry ampicillin resistance on a plasmid (Table 4.3).

In waterborne *E. coli*, the predominant drug resistance observed on plasmids was resistance to tetracycline (n=6). All of these plasmids could be self transferred through conjugation. In total, ampicillin resistance was observed in 2 isolates (Table 4.4), one of which also showed plasmid-mediated resistance to tetracycline.

Table 4.4. Plasmid-mediated resistance observed in waterborne isolates of *E. coli*.

Tributary	Collect Date (D/M/Y)	Site location	Isolate No	Wild-type AMR profile	AMR transferred to recipient		AMR plasmid size (Kb)	Gene for plasmid-mediated resistance*
					Conjuga-tion	Electro-poration		
Laurel Creek	22/11/2004	LC-3	22.B	A	A	A	55.4	*bla* TEM-1
	10/08/2004	LC-1	15.2	SuT	T	T	138.6	*tet*A
Canagagigue Creek	10/08/2004	CAN-1	15.1	T	T	T	138.6	*tet*B
	16/08/2004	CAN-1	16.2	T	T	T	138.6	*tet*A
	23/08/2004	CAN-1	17.2	T	T	T	138.6	*tet*B
	26/08/2004	CAN-1	18.2	CSSxtT	T	T	138.6	*tet*A
	18/11/2004	CAN-1	21.B	ASSxtT	AT	AT	138.6	*bla* TEM-1 /*tet*A

*Accession number for *bla* TEM-1 in isolate No. 22.B is GU727530 and for isolate 21.B is GU727529.

Size of resistance plasmids

Plasmid extraction comparisons showed that there were two different-sized plasmids responsible for β-lactam resistance in *Salmonella* isolates. Resistance to ampicillin alone was associated with a smaller-sized plasmid which was approximately 8.1Kb, whereas resistance to ampicillin and both 3rd generation cephalosporins, Caz and Cro, was observed on a larger-sized plasmid at approximately 95.5 Kb. Examples are shown in Figure 4.1.
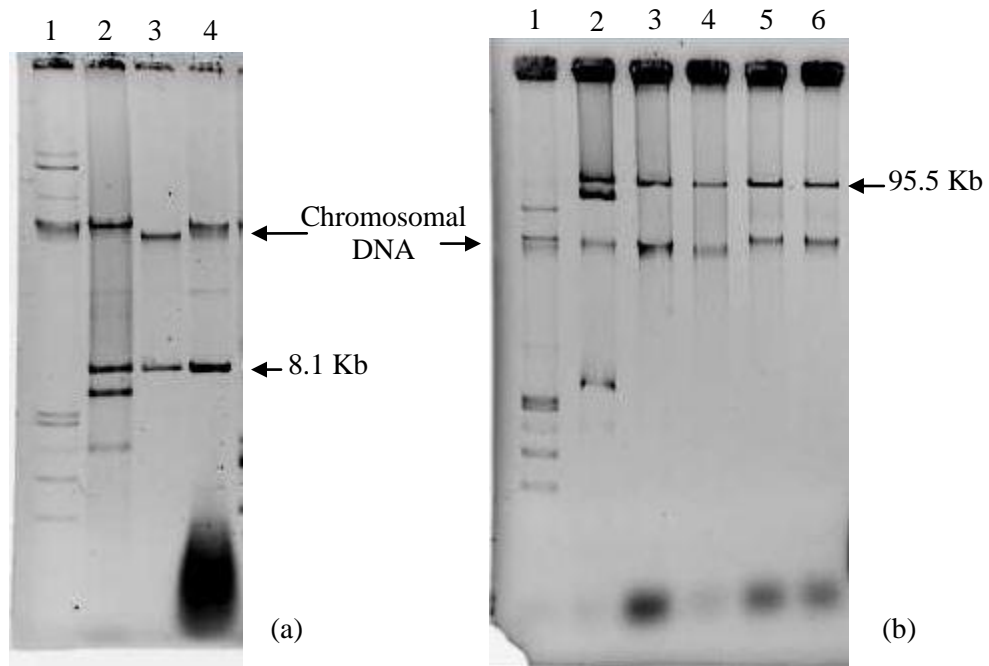
Figure 4.1. Visualization of two different-sized plasmids carrying drug resistance in waterborne *Salmonella*. (a) *Salmonella* **isolate 20C3, lane 1: plasmid ladder; lane 2: wildtype 20C3; lane 3: transformed strain grown on ampicillin (*E. coli* DH5α); and, lane 4: second transformed strain grown on ampicillin (*E. coli* DH5 α). (b)** *Salmonella* **isolate 22D2, lane 1: plasmid ladder; lane 2: wildtype 22D2; lane 3: transformed strain grown on ampicillin (*E. coli* DH5 α); lane 4: second transformed strain grown on ampicillin (*E. coli* DH5 α), lane 5: transformed strain grown on ceftazidime (*E. coli* DH5 α); and, lane 6: second transformed strain grown on ceftazidime (*E. coli* DH5 α).**

Two different sized plasmids were observed to carry resistance in waterborne *E. coli*: 55.4 Kb and 138.6 Kb (Table 4.4; image not shown). The 55.4 Kb-sized plasmid was observed in one isolate and carried single drug resistance to ampicillin. The larger-sized plasmid was observed in 6 isolates and carried resistance tetracycline. One of these plasmids also carried resistance to ampicillin.

Plasmid-mediated resistance in *Salmonella* serotypes

Ten different *Salmonella* serotypes (out of a total of 19 different serotypes showing AMR) were found to carry plasmid-mediated resistance. Table 4.5 shows all the serotypes that demonstrated AMR and the proportion of these serotypes that carried resistance to one or more antimicrobial drugs on a plasmid. Some resistant strains, including *S.* Heidelberg, *S.* Berta, *S.* Montevideo and *S.* Newport, showed a high proportion of AMR on plasmids. It should be noted that in some isolates AMR was found to be on plasmids as well as incorporated into the genomic DNA.

The smaller sized (8.1 Kb) plasmids carrying ampicillin resistance were found in five different serotypes of *Salmonella*. With the exception of one isolate, all 8.1 Kb plasmids were transferred through conjugation to recipients of *E. coli* and *Salmonella* (Table 4.3). After several attempts, one 8.1 Kb plasmid from an *S.* Oranienberg isolate (504b) could not be conjugated to a recipient of *E. coli* or *Salmonella*.

Table 4.5. Proportion of each AMR *Salmonella* serotype demonstrating plasmid mediated resistance to one or more antimicrobial drug.

Serotype	Total No. of isolates	Proportion of isolates showing AMR		Proportion of AMR isolates carrying AMR on plasmid		No. of isolates carrying plasmid:	
		%	No.	%	No.	TEM-1	CMY-2
Heidelberg	19	58%	11	100%	11	9	2
Berta	6	33%	2	100%	2	0	2
Montevideo	8	25%	2	100%	2	2	0
Newport	5	20%	1	100%	1	0	1
Kentucky	15	13%	2	50%	1	0	1
Infantis	15	20%	3	33%	1	0	1
Hadar	4	100%	4	25%	1	1	0
Agona	12	58%	7	14%	1	0	1
Oranienberg	11	64%	7	14%	1	1	0
Typhimurium	34	59%	20	5%	1	1	0
Kiambu	9	11%	1	0%	0	-	-
Tennessee	8	63%	5	0%	0	-	-
Mbandaka	7	71%	5	0%	0	-	-
Putten	6	17%	1	0%	0	-	-
I:4,5, 12:i:-	3	33%	1	0%	0	-	-
Derby	3	67%	2	0%	0	-	-
I:4, 12:-:-	1	100%	1	0%	0	-	-
Give	1	100%	1	0%	0	-	-
Litchfield	1	100%	1	0%	0	-	-

Gene responsible for resistance on Salmonella and E. coli plasmids

Prior to genetic analysis, preliminary phenotypic tests were carried out to determine if resistance to ACazCro was the result of extended spectrum β-lactamase (ESBL) production. In both trials, some isolates showed a ≥ 5 mm zone size difference between Caz with and without clavulanic acid, however, this result was not consistent between trials (Table 4.6). A ≥ 5 mm difference was never demonstrated with Cxt discs. These trials revealed that no isolate showed a clear phenotypic result for ESBL production; therefore genetic analysis would be needed.

Table 4.6. Zones of clearing during ESBL phenotypic testing in *Salmonella* isolates.

Isolate no.	Serotype	Trial no.	Zone of clearing (mm)					
			Caz	Caz+ ClavAcid	Diff*	Cxt	Cxt+ ClavAcid	Diff*
22C1	Berta	1	12	18	**6**	17	19	2
		2	13	18	**5**	16	17	1
22C2	Berta	1	14	18	4	18	20	2
		2	16	20	4	20	22	2
22D2	Newport	1	13	17	4	17	19	2
		2	12	17	**5**	16	17	1
21C1	Agona	1	9	13	4	14	15	1
		2	11	17	**6**	16	17	1
19C1	Infantis	1	10	14	4	15	16	1
		2	12	18	**6**	17	20	3
15C3	Heidelberg	1	11	14	3	15	15	0
		2	13	18	**5**	17	18	1
12Ca	Heidelberg	1	12	17	**5**	18	18	0
		2	13	17	4	17	18	1
8Dd	Kentucky	1	13	17	4	17	17	0
		2	14	17	3	19	19	0

* Difference - text is bolded when a ≥ 5 mm zone size difference between Caz with and without clavulanic acid was observed

Several different PCR reactions were carried out to determine the likely gene(s) responsible for plasmid-mediated resistance to ampicillin and multiple drug resistance to ACazCro in *Salmonella* isolates, as well as tetracycline and ampicillin resistance in *E. coli*.

The primers used to detect genes responsible for β-lactamase production were designed to capture most, if not all, of the length of these genes. The resulting PCR products were sequenced and matched within the BLAST database to determine the gene responsible for resistance. Quality control reports from Mobix Lab (McMaster University) show that all reads were long (> 600 bp) and were of good quality. In general, reads were usable for 850 to 900 bp.

PCR of the *Salmonella* isolates revealed that a *bla* $_{TEM}$-type gene was likely responsible for the ampicillin resistance on the smaller 8.1 Kb plasmid and a *bla* $_{CMY}$-type gene was responsible for resistance to ampicillin, ceftazidime and ceftriaxone on the 95.5 Kb plasmid. Nucleotide sequence analysis revealed that all the sequences of the screened genes had 99-100% similarity to their best-match known genes (i.e. *bla* $_{CMY-2}$ or *bla* $_{TEM-1}$) retrieved from the GenBank database. Sequencing results, when aligned with sequences in BLAST databases, showed that all ampicillin resistance on all smaller plasmids was the result of the gene *bla* $_{TEM-1}$ (TEM-1 plasmid). Alignment of sequences with the CMY PCR product from the larger-sized plasmid revealed matches with the *bla* $_{CMY-2}$ gene (CMY-2 plasmid). This sequence was observed in all isolates showing plasmid mediated resistance to ACazCro. GenBank accession numbers for the sequences obtained are shown in Table 4.3 and Table 4.4.

Despite resistance to ampicillin, ceftazidime and ceftriaxone, two S. Mbandaka isolates (8Cc and 8Dc) obtained from LC did not demonstrate plasmid-mediated resistance (entire profile ACSSuTCazCro). In addition, no PCR products were obtained for any of the tested genes using whole cell extracts of these isolates.

As shown in Table 4.4, tetracycline resistance was the most commonly observed resistance on *E. coli* plasmids. Of the 14 *tet* genes detected, *tet*A and *tet*B were the only types found to be responsible for tetracycline resistance. Resistance to ampicillin was observed on plasmids in two isolates, both of which were attributed to *bla* $_{TEM-1}$ genes following sequence alignment through BLAST.

128

Spatial and temporal distribution of AMR plasmids in *Salmonella* and *E. coli* in tributaries

Overall, a significant difference ($P \leq 0.006$) was observed between the number of *Salmonella* isolates bearing AMR plasmids in these tributaries, with the majority of isolates obtained from Laurel Creek (Table 4.3). Also, *Salmonella* carrying AMR plasmids were obtained from all three sampling locations in Laurel Creek. In contrast, plasmid-bearing *Salmonella* isolates were only found at one location in Conestogo River (CON-1) and Canagagigue Creek (CAN-1).

In *Salmonella*, the TEM-1 plasmid was observed in all three tributaries, with a greater number of isolates carrying this plasmid obtained from Laurel Creek (n=7), followed by Conestogo River (n=5) and Canagagigue Creek (n=2). A significant difference was observed between the frequency of isolates carrying the TEM-1 plasmid among seasons ($P = 0.02$). Fifty percent of *Salmonella* isolates carrying this plasmid were isolated in the late spring (May), whereas no isolates carried the TEM-1 plasmid in the summer months (June, July and August, Table 4.7).

When examining the tributaries individually, isolates from Laurel Creek showed the largest range of seasons over which TEM-1 plasmids were observed, with the greatest proportions observed in the fall months, followed by winter and spring. Both Canagagigue Creek and Conestogo River isolates showed a greater representation of this plasmid in the spring (Table 4.7).

Laurel Creek showed the greatest diversity of serotypes carrying the TEM-1 plasmids, with plasmids obtained from *S*. Heidelberg (n=3), *S*. Hadar (n=1), *S*. Oranienberg (n=1) and *S*. Montevideo (n=1). Considerably less diversity was observed in Canagagigue Creek and Conestogo River, as *S*. Heidelberg represented all of the isolates carrying the TEM-1 plasmid in Canagagigue Creek and 4 out of 5 isolates in Conestogo River. *S*. Heidelberg carrying the TEM-1 plasmid in both Canagagigue Creek and Conestogo River were only obtained in June.

Table 4.7. Proportion of isolates carrying ampicillin resistance on the TEM-1 plasmid by season in each tributary.

Season:	Winter		Spring		Summer		Fall	
Avg. water temp (range, ^{0}C):	1.3 (0 – 2.5)		6.5 (0.8-15.2)		19.1 (14-23.5)		4.1 (0 – 8.5)	
Tributary	%	(No.)	%	(No.)	%	(No.)	%	(No.)
LC	28.6	(2/7)	14.3	(1/7)	0	(0/7)	57.1	(4/7)
CAN	0	(0/2)	100	(2/2)	0	(0/2)	0	(0/2)
CON	20	(1/5)	80	(4/5)	0	(0/5)	0	(0/5)
Overall	21.4	(3/14)	50	(7/14)	0	(0/14)	28.6	(4/14)

Several different phagetypes of *S*. Heidelberg carried the TEM-1 plasmid, with the most common being PT 19. PT 19 isolates carrying this plasmid were obtained from all three tributaries. Only one isolate of *S*. Typhimurium carried the TEM-1 plasmid and it was isolated from Laurel Creek. It was found to be PT 104 (Table 4.3).

Isolates carrying the CMY-2 plasmid (n=8) were only observed from Laurel Creek and never from Canagagigue Creek and Conestogo River (also discussed in Chapter 4). *Salmonella* isolates bearing these plasmids were found at all three sampling locations in Laurel Creek and in every season, except winter (Table 4.3).

E. coli isolates carrying AMR plasmids (n = 7) were observed only in Laurel Creek and Canagagigue Creek. Within both of these tributaries, *E. coli* isolates carried plasmids containing both *tet* genes and *bla* $_{TEM-1}$ (Table 4.4). Similar to *Salmonella*, in Canagagigue Creek all *E. coli* isolates carrying AMR plasmids were obtained from one sample location (CAN-1). Although monitoring for *E. coli* only took place from July to November, most *E. coli* carrying plasmid-mediated resistance were observed in August where 5 of 7 isolates carrying AMR plasmids were obtained.

Genetic relatedness of CMY-2 plasmids in *Salmonella*

To determine the degree of genetic relatedness between the 95.5 Kb CMY-2 plasmids, a plasmid restriction fragment length polymorphism (pRFLP) analysis was carried out. Following restriction digest with *Pst*I, 5 separate banding patterns (a-e) were observed.

Differences between these patterns were examined through band matching and cluster analysis using BioNumerics software (Applied Maths, Belgium).
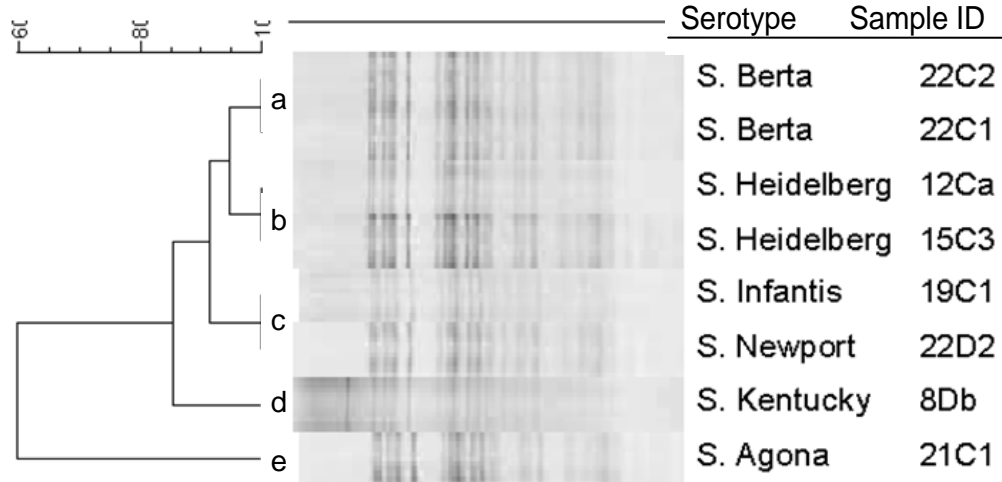
Figure 4.2. *Salmonella* **plasmid restriction fragment length polymorphism (pRFLP) of 95.5 Kb plasmids carrying** *bla* **CMY-2 gene (CMY-2 plasmid) with restriction enzyme** *Pst***I. Profiles a-e are shown.**

Based on software analysis, all plasmids showed a similar banding pattern and were found to have a ≥ 60% similarity to each other (Figure 4.2). Overall, 7 of the 8 plasmids were found to be > 80% similar to one another. Plasmids from several isolates demonstrated identical banding patterns and positions, including two *S*. Berta isolates (profile a), two *S*. Heidelberg isolates (profile b) and *S*. Infantis and *S*. Newport (profile c). *S*. Kentucky (profile d) and *S*. Agona (profile e) demonstrated unique patterns.

Fitness and plasmid stability in waterborne *Salmonella*

Plasmid curing in Salmonella isolates

Several isolates containing CMY-2 plasmids were subjected to curing conditions at 44.5°C. As shown in Figure 4.3, each isolate demonstrated differences in the loss of resistance plasmids. Within the first three days, all isolates demonstrated some losses. Two separate isolates of *S*. Berta (22C1 and 22C2) both demonstrated the greatest loss

131

of AMR throughout the experiment. Within 24 d at 44.5°C, one isolate (22C2) lost all ampicillin resistance and continued to demonstrate full susceptibility throughout the remainder of the study. After 34 d, the second *S.* Berta isolate (22C1) demonstrated a 97% loss in ampicillin resistance, however, at the next sampling time this isolate showed that only 33.3% of isolates had lost resistance, suggesting a > 60% recovery.

Ampicillin resistance remained fairly stable throughout the study (up to 82 d) in several isolates, including 21C1 (*S.* Agona) and 19C1 (S. Infantis). Isolate 22D2 (S. Newport) demonstrated losses at 34 d (to 52% isolates with ampicillin resistance), but appeared to increase again in the number of isolates demonstrating resistance until the end of the study. The biological control, *S.* Typhimurium 970128, which carried ampicillin on a large virulence plasmid, showed few losses in resistance throughout the study with only slight losses toward the end (81.5% of isolates carrying plasmids at 82 d and 88.9% at 100 d).
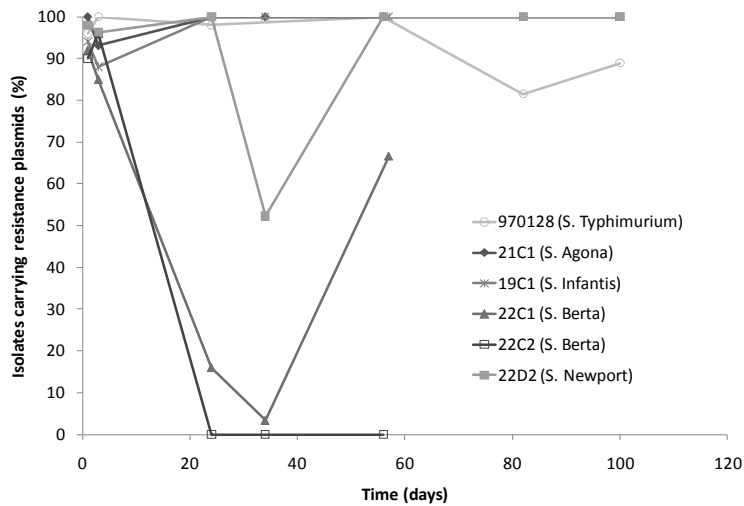
Figure 4.3. Proportion of *Salmonella* isolates carrying plasmids over time following exposure to curing conditions at 44.5⁰C. Strain 970128 is a biological control carrying a 95.5 Kb plasmid demonstrating resistance to ampicillin. All other strains are environmentally derived and carry the CMY-2 plasmid.

Fitness studies in isolates carrying plasmid-mediated drug resistance

In the first reproductive fitness trial (No. 1), the growth of 5 wildtype *Salmonella* isolates carrying resistance plasmids was compared to the growth of isolates that lost resistance following curing conditions (designated as 'plasmid loss') and to isolates that were subjected to curing conditions but maintained resistance (designated as 'plasmid maintained'). The results of these trials are shown in Figure 4.4 (a-e).
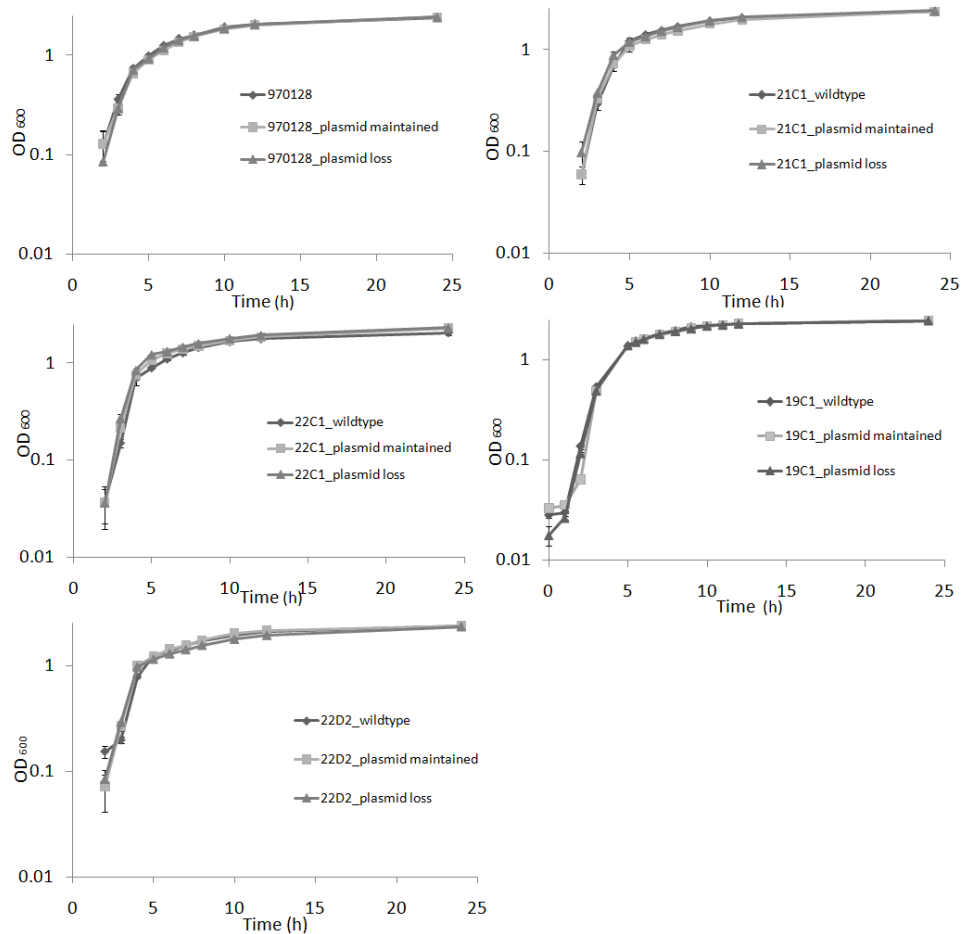
Figure 4.4. Growth curves examining reproductive fitness of five *Salmonella* isolates, *Salmonella* 970125, 21C1 (*S*. Agona), 19C1 (*S*. Infantis), 22C1 (*S*. Berta), 22D2 (*S*. Newport) in LB broth over 24 h at 37⁰C (Fitness Study No. 1). In each trial, wildtype isolates containing resistance plasmids, isolates that had been subjected to curing temperatures but maintained resistance (plasmid maintained) and isolates that lost resistance following curing (plasmid loss) were compared. Standard deviations are shown.

In no instances were significant differences observed between the growth rates of wildtype strains and strains that had maintained or lost AMR plasmids (Table 4.8).

Table 4.8. Differences in growth rate (h^{-1}) of *Salmonella* strains that maintained and lost resistance plasmids; reproductive fitness no. trial 1.

Strain No.	Serotype	Mean growth rate ($\pm$SD)*			P value
		Wildtype	Plasmid loss	Plasmid maintained	
970125	*S*. Typhimurium (Biological control)	0.30 (0.06)	0.35 (0.00)	0.29 (0.04)	0.79
21C1	*S*. Agona	0.43 (0.02)	0.37 (0.03)	0.41 (0.00)	0.96
19C1	*S*. Infantis	0.26 (0.01)	0.27 (0.00)	0.31 (0.01)	0.95
22C1	*S*. Berta	0.48 (0.01)	0.51 (0.06)	0.51 (0.06)	0.96
22D2	*S*. Newport	0.33 (0.02)	0.39 (0.01)	0.43 (0.06)	0.81

*determined as slopes of the exponential portion of the log optical density (OD_{600}) versus time

In fitness trial no. 2, several *Salmonella* isolates carrying the larger-sized CMY-2 plasmids were grown at elevated temperatures (44.5^{o}C) in varying concentrations of LB (full strength and 1/10 strength) to examine the percentage of plasmids lost over each generation. The 12 h growth curve for each isolate is shown in Figure 4.5 to determine the number of generations over time.
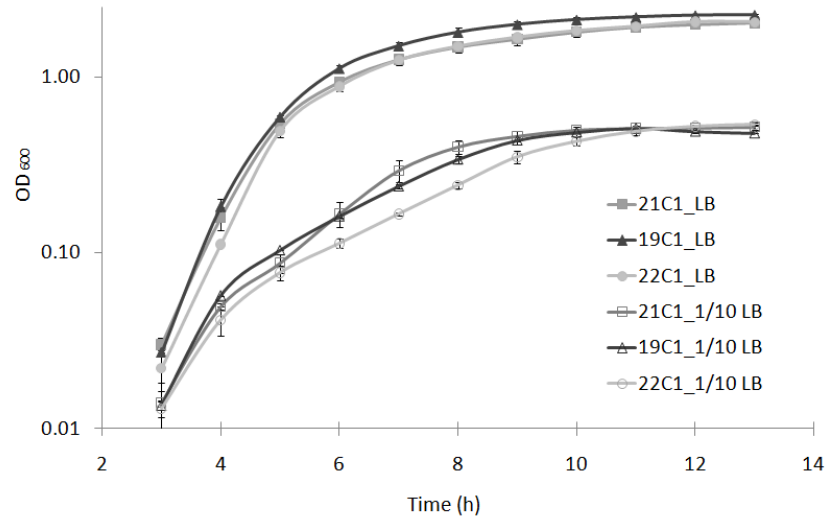
Figure 4.5. Growth curves of three *Salmonella* isolates, 21C1 (*S.* Agona), 19C1 (*S.* Infantis) and 22C1 (*S.* Berta) in LB broth and in 1/10 LB broth at 44.5⁰C. Standard deviations are shown.

As shown in Figure 4.6, in both LB and 1/10 LB, 21C1 (*S.* Agona) and 19C1 (*S.* Infantis) showed very few losses of resistance plasmids. 22C1 (*S.* Berta) showed the greatest losses over time in both media types. However, toward the end of the trial in 1/10 LB (154 generations) 95.5% of the isolates carried resistance plasmids.
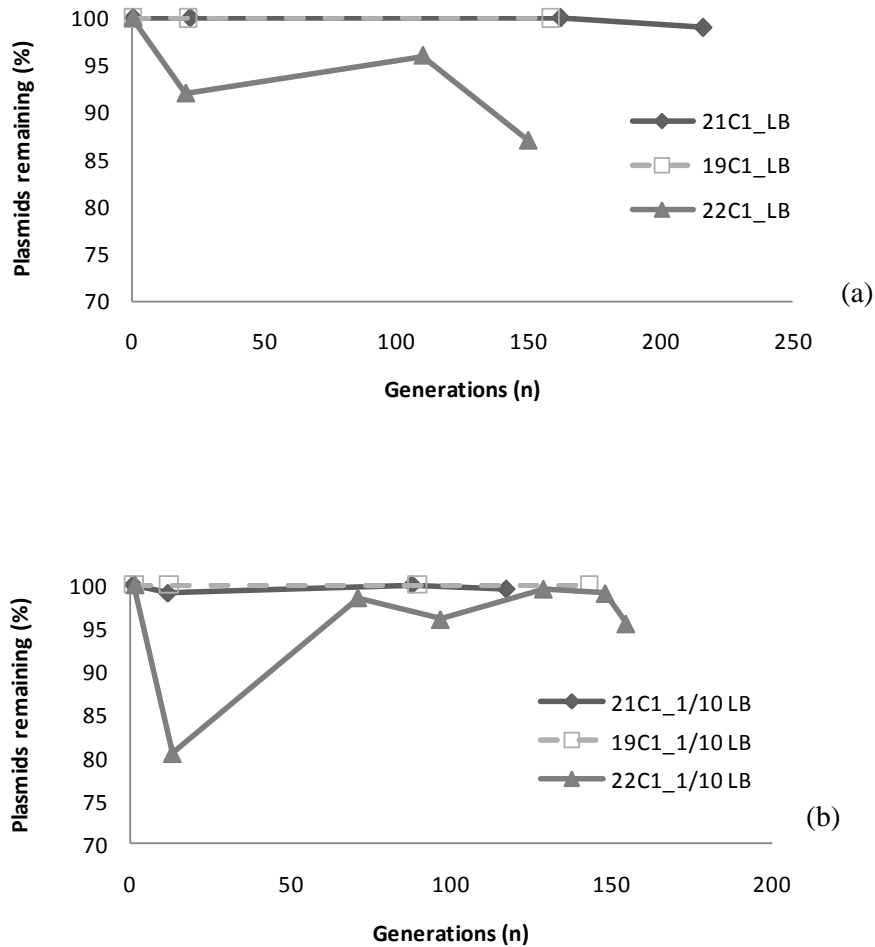
Figure 4.6. Proportion of *Salmonella* isolates carrying CMY-2 plasmids, 21C1 (*S.* Agona), 19C1 (*S.* Infantis) and 22C1 (*S.* Berta) in (a) LB broth and in (b) 1/10 LB broth at 44.5⁰C.

To further investigate if plasmid carriage affects reproductive fitness, several *E. coli* C600N isolates carrying CMY-2 plasmids, obtained through conjugation, were used for growth comparisons in fitness trial no. 3. The growth of these isolates was compared to growth of *E. coli* C600N that did not contain any resistance plasmids. These trials were carried out in both full strength LB and 1/10 strength LB at 44.5^{0}C and in LB at 37^{0}C. Growth curves for each isolate can be observed in Figure 4.7 (44.5^{0}C) and Figure 4.9 (37^{0}C). Plasmid losses in isolates grown at 44.5^{0}C are shown in Figure 4.8 (a-b). Results for plasmid losses are not shown for growth at 37^{0}C, as few isolates demonstrated a loss in resistance over time at this temperature.

136
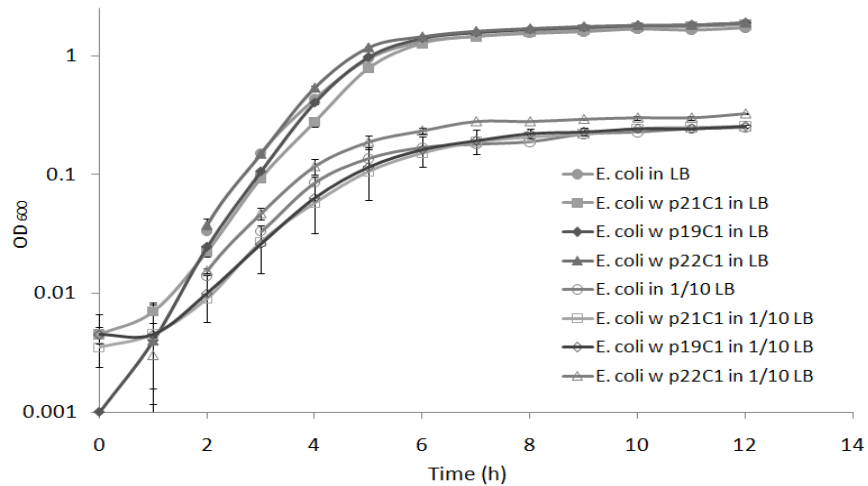
Figure 4.7. Growth curves of *E. coli* C600N containing three CMY-2 plasmids originating from *Salmonella* isolates, p21C1 (*S*. Agona), p19C1 (*S*. Infantis) and p22C1 (*S*. Berta) in LB broth and in 1/10 LB broth at 44.5⁰C. Original strains of *E. coli* C600N without plasmids were also grown in LB and in 1/10 LB broth. Standard deviations are shown.
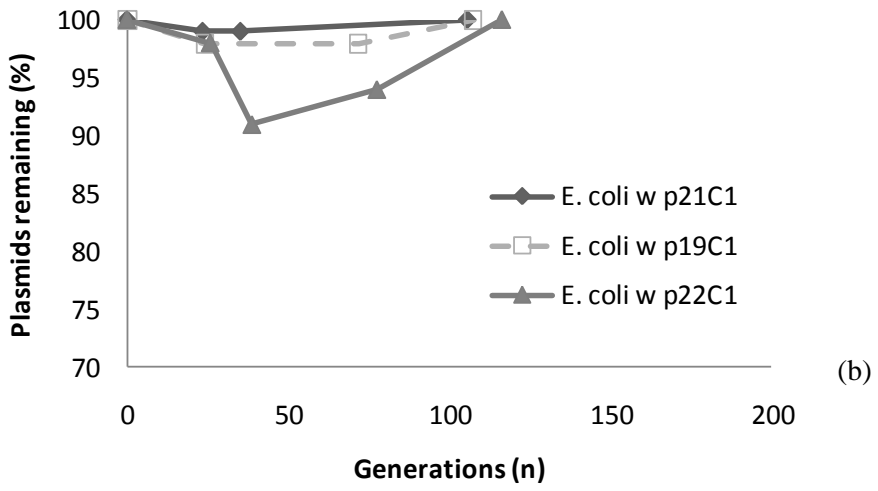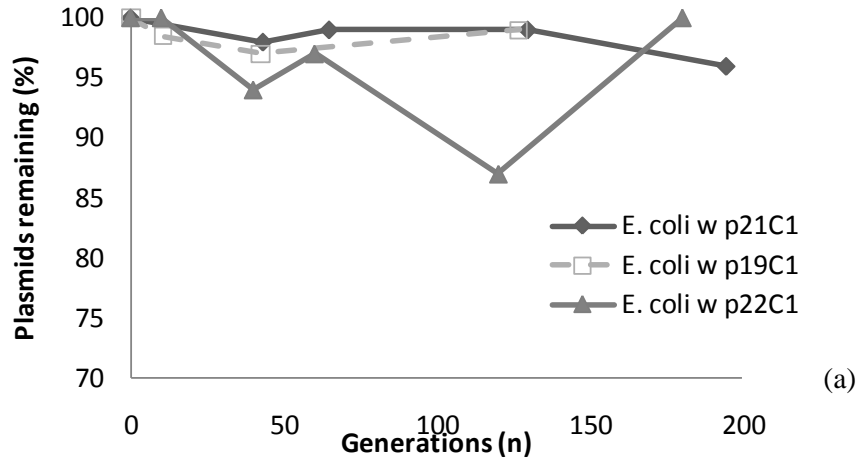
Figure 4.8. Proportion of *E. coli* C600N isolates containing introduced CMY-2 plasmids (p21C1, p19C1 and p22C1) grown in both (a) LB broth and in (b) 1/10 LB broth at 44.5⁰C.
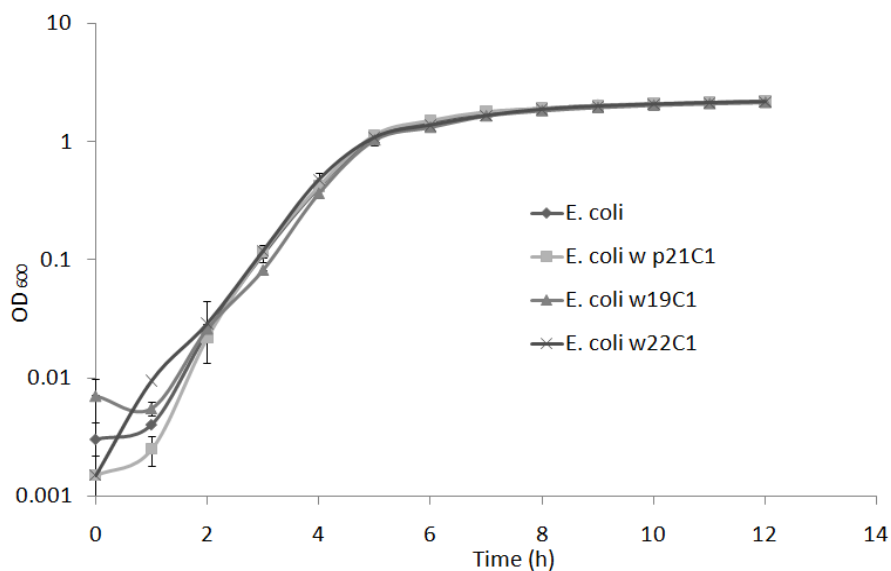
Figure 4.9. Growth curves of *E. coli* C600N containing three CMY-2 plasmids, p21C1, p19C1 and p22C1, originating from *Salmonella* isolates in LB broth at 37⁰C. *E. coli* C600N culture without plasmids was also grown in LB broth. Standard deviations are shown.

In no instances were significant differences observed between the growth rates of *E. coli* with and without introduced plasmids (Table 4.9).

Table 4.9. Differences among growth rates (h^{-1}) of *E. coli* strains without and with introduced plasmids (p21C1, p19C1 and p22C1) at select temperatures and nutrient conditions; reproductive fitness trial 3.

Conditions of trial		Mean growth rate (±SD)				
Media	Temp (⁰C)	*E. coli* C600N	*E. coli* C600N + p21C1	*E. coli* C600N + p19C1	*E. coli* C600N + p22C1	*P* value
LB	44.5	0.48(0.00)	0.51(0.00)	0.54(0.00)	0.50(0.02)	0.95
1/10 LB	44.5	0.34(0.00)	0.36(0.09)	0.36(0.01)	0.36(0.01)	0.37
LB	37.0	0.55(0.01)	0.57(0.00)	0.54(0.00)	0.54(0.07)	0.85

In a final attempt to determine if a fitness difference was measurable, a competitive fitness trial (no. 4) was carried out between *E. coli* isolates with an introduced resistance plasmid (p21C1) and isolates without plasmids. Within the first 24 h of the trial, similar proportions of plasmid containing cells were observed (Figure 4.10). However, after 48 h, 100% of isolates grew on agar containing ampicillin, meaning that all the isolates carried resistance plasmids by the end of the trial.
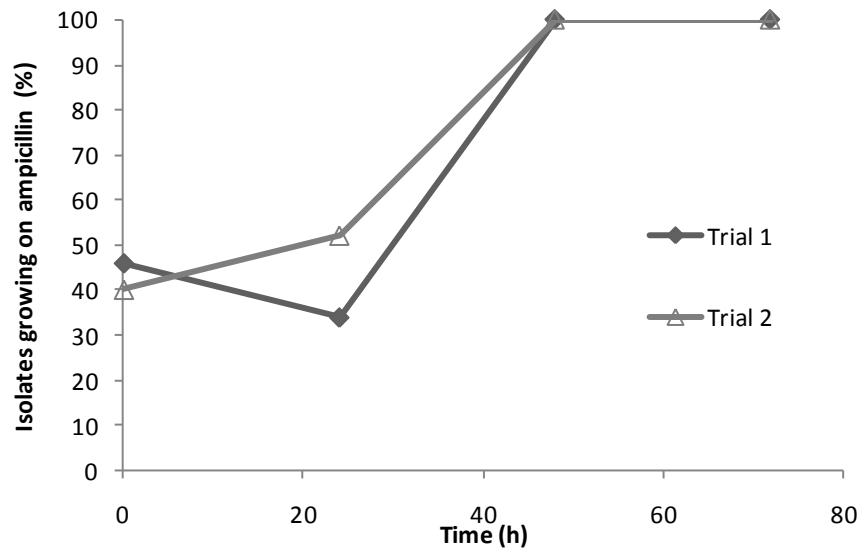
Figure 4.10. Competitive fitness trials between *E. coli* C600N and *E. coli* C600N carrying CMY-2 plasmid p21C1 with growth in LB at 37°C. Figure shows the proportion of *E. coli* C600N isolates carrying plasmid p21C1 over time in two trials.

4.6 Discussion

Prevalence and diversity of plasmid-mediated resistance in waterborne isolates of *Salmonella* and *E. coli*

Of the 77 waterborne *Salmonella* isolates that showed AMR, 28.6% demonstrated plasmid-mediated resistance. With one exception, all resistance plasmids were self-transferable through conjugation to recipient cells. As described in Chapter 3, *E. coli* showed a lower frequency of drug resistance compared to *Salmonella*, however, through genetic analysis, more AMR *E. coli* isolates (41.2%) demonstrated plasmid-mediated resistance compared to *Salmonella* (28.6%). Similar to *Salmonella*, plasmid-mediated resistance in *E. coli* was self-transferable through conjugation.

Few studies on AMR plasmid carriage in waterborne *Salmonella* are available for comparison. In Canada, the only study examining AMR in waterborne *Salmonella* was Bell *et al*. (1980) who observed that 57% of waterborne *Salmonella* transferred resistance to recipient cells. The type of AMR transferred and plasmid analysis was not discussed in the Bell *et al.* (1980) study. Studies examining AMR in waterborne *Salmonella* from other countries generally report solely on the phenotypic aspects of AMR (e.g., Alcaide and Garay, 1984; Morinigo *et al*., 1990b).

Human and animal research indicates that *Salmonella* and *E. coli* can carry resistance to numerous types of drugs on plasmids (Guerri *et al*., 2004; Foley and Lynne, 2008; Fricke *et al*., 2009). However, in waterborne *Salmonella* obtained in the current study, plasmid-mediated resistance was only observed to β-lactam drugs, including ampicillin and 3rd generation cephalosporins. *E. coli* isolates also demonstrated plasmid-mediated β-lactam resistance (only to ampicillin), but showed greater levels of plasmid-mediated resistance to tetracycline.

Plasmid-mediated β-lactam resistance is commonly reported in *Salmonella* isolates originating from humans and farm animals (Winokur *et al*., 2001; Carattoli *et al*., 2002; Giles *et al*., 2004; Daniels *et al*., 2007; Li *et al*., 2007; Foley and Lynne, 2008; Call *et al*., 2010). The current study demonstrated that two different sized plasmids were responsible for β-lactam resistance in waterborne *Salmonella*; a larger-sized plasmid (95.5 Kb) which carried resistance to ampicillin and the 3rd generation cephalosporins

(profile ACazCro), and a smaller-sized plasmid (8.1 Kb) which carried resistance to ampicillin alone.

The smaller-sized plasmid (8.1 Kb) was found to carry a *bla* $_{TEM-1}$ gene (TEM-1 plasmid), which is responsible for ampicillin resistance. Class A β-lactamases, resulting from the expression of the *bla* $_{TEM-1}$ gene, are among the most frequently expressed β-lactamase enzymes in gram-negative bacteria and commonly produced by a range of *Enterobacteriaceae,* including *Salmonella* (Chouchani *et al.,* 2006; Guerri *et al.* 2004; Wang *et al*., 2008). As a result of the widespread occurrence of the *bla* $_{TEM-1}$, it is not surprising that this gene was responsible for the most prevalent plasmid-mediated resistance observed in waterborne *Salmonella*. This gene was also detected in two isolates of *E. coli* that demonstrated plasmid-mediated resistance to ampicillin (one SDR to ampicillin and the other was MDR to ampicillin and tetracycline).

In *Salmonella,* resistance to extended spectrum cephalosporins, including 3rd generation cephalosporins, is commonly the result of the production of extended spectrum β-lactamase (ESBL) or AmpC β-lactamase (Miriagou *et al*., 2004; Poole, 2004). Recommended phenotypic tests utilizing clavulanic acid, which inhibits ESBLs, did not result in a clear and reproducible result in this study, therefore it was essential to examine resistance through molecular analysis. PCR and sequence analysis revealed that the *bla* $_{CMY-2}$ gene, which encodes CMY-2 AmpC β-lactamase, was solely responsible for the ACazCro resistance profile in waterborne *Salmonella* and was found exclusively on the 95.5 Kb-sized plasmid (CMY-2 plasmid).

CMY-2 AmpC β-lactamase provides reduced susceptibility to a range of β-lactam dugs, including ampicillin, amoxicillin-clavulanic acid, ceftiofur and ceftriaxone (Government of Canada, 2007; Mataseje *et al*., 2009b). In Canada, CMY-2 AmpC β-lactamase producing strains of *Salmonella* and *E. coli* have become more prevalent than those producing ESBLs in human clinical isolates (Government of Canada, 2005; Government of Canada, 2007; Baudry *et al*., 2008; Mataseje *et al*., 2009b). This is in agreement with waterborne isolates obtained in the current study, as no genes responsible for ESBLs were observed in *Salmonella*.

In contrast to most studies performed to date, isolates harbouring the CMY-2 plasmid found in this study demonstrated full resistance to ceftriaxone (Cro), as opposed to

reduced susceptibility (e.g., Zhao *et al*., 2007). It is possible that the plasmids carried several *bla* $_{CMY-2}$ genes (Call *et al*., 2010) or had a higher copy number. Further plasmid analysis would be needed to determine which attribute conferred this observed profile.

The literature shows that resistance to 3rd generation cephalosporins in *Salmonella* is primarily plasmid-mediated and disseminated through plasmid transfer (Winokur *et al*., 2001; Carattoli *et al*., 2002; Giles *et al*., 2004; Daniels *et al*., 2007; Li *et al*., 2007; Call *et al*., 2010). The current study demonstrated similar results with 80% of the waterborne *Salmonella* isolates demonstrating resistance to Caz and Cro carried resistance determinants to these drugs on plasmids. However, despite the high levels of resistance to Caz and Cro (MIC values of 128 and > 512 µg/mL, respectively; Chapter 3), two waterborne isolates of *S*. Mbandaka (entire profile ACSSuTCazCro) did not carry any of the tested genes for this type of resistance on a plasmid, nor were these genes found chromosomally. The lack of genes for β-lactamase resistance, in addition to the extensive resistance profile in these isolates, indicates that this resistance phenotype may be due to the loss or change of outer membrane porins, although this is rarely reported (Martinez-Martinez *et al*., 2000; Bellido *et al*., 1989; Miriagou 2004).

In Chapter 3, isolates were considered to be multiple drug resistant (MDR) if phenotypic resistance to two or more antimicrobial drugs was demonstrated. Under this definition, *Salmonella* isolates with the AMR profiles of ACazCro were considered to be MDR. Understanding that a single gene was responsible for resistance to these three β-lactam drugs reclassifies these isolates as SDR. While not a large difference, reclassifying these isolates changes the original frequency of MDR waterborne *Salmonella* from 75% to 70%.

Tetracycline resistance was the most commonly observed plasmid-mediated resistance in waterborne *E. coli*. Two genes were found to be responsible for this resistance, *tet*A and *tet*B, both of which are associated with an efflux pump mechanism (Roberts, 1996; Ng *et al*., 2001). In Canada, *tet*A and *tet*B genes are the most common types of *tet* genes responsible for tetracycline resistance in *E. coli* isolated from farm-animals; including chicken (Smith *et al*., 2007), swine (Boerlin *et al*., 2005) and cattle (Gow *et al*., 2008; Sharma *et al*., 2008). Despite the predominance of these *tet* genes, limited data are available on the incidence of these genes on plasmids. In the current study, plasmid-mediated *tet*A was observed in twice as many *E. coli* isolates as *tet*B. As

observed by others (e.g., Gow *et al*, 2008), the simultaneous presence of both of these genes in one isolate was not found. Interestingly, the plasmids carrying these different genes in waterborne *E. coli* appeared to be identical in size (138.6 Kb), with one plasmid even carrying a bla_{TEM-1} gene (in isolate No. 21.B). To further expose differences between *tet*-plasmids, additional genetic analysis would be needed.

Waterborne *Salmonella* and *E. coli* isolates showed a similar frequency of ampicillin resistance when examined over the same time period (July-November 2004; 20.5% in AMR *Salmonella* isolates and 18.5% in AMR *E. coli* isolates) following phenotypic analysis (Chapter 3). This similarity translated to the same level of resistance to ampicillin carriage on plasmids, with 40% of *E. coli* (2 of 5) and *Salmonella* (10 of 25) isolates demonstrating plasmid-mediated ampicillin resistance. Genotypic analysis revealed that the gene responsible for plasmid-mediated ampicillin-resistance in waterborne *E. coli* was solely bla_{TEM-1}. While some *Salmonella* isolates carried the bla_{TEM-1} gene, the majority of ampicillin resistance in *Salmonella* over this period of time was due to the bla_{CMY-2} gene.

Several studies have demonstrated significant homology between plasmids isolated from *E. coli* and *Salmonella* (Winokur *et al*., 2001; Poppe *et al*., 2005; Daniels *et al*., 2007; Call *et al*., 2010). Although there is some confirmation in this study of similar resistance genes in plasmids isolated from both *E. coli* and *Salmonella*, pronounced evidence of interspecies sharing of specific plasmids was not observed. Plasmid size comparisons and resistance profiles revealed no identical AMR plasmids in waterborne isolates of *Salmonella* and *E. coli*. This may indicate that plasmid-sharing between these genera does not occur readily enough within hosts in the watershed to be detected in water samples. In addition, the swab-based collection method may have limited the ability to detect similarities as the isolates entrapped are collected over a period of time and therefore reflect a wider potential source profile. This makes discrete comparisons between isolates more difficult than if isolates were collected over a finite timeframe (i.e., through a grab sample).

Approximately half of the waterborne *Salmonella* and *E. coli* isolates that carried resistance on plasmids also carried resistance chromosomally. Although further analysis would be needed, the ability of these bacteria to accumulate many types of

resistance determinants may indicate that few fitness costs are associated with carrying genes for drug resistance (further discussed below).

Waterborne *Salmonella* serotypes carrying plasmid-mediated resistance

Ten different *Salmonella* serotypes carried plasmid-mediated resistance to β-lactam drugs. TEM-plasmids were observed in 6 different serotypes and CMY-plasmids were observed in 5 different serotypes. All drug resistant isolates of *S*. Heidelberg, *S*. Berta, *S*. Montevideo and *S*. Newport carried resistance on plasmids, some of which also carried resistance chromosomally.

S. Heidelberg was the only *Salmonella* serotype to carry both TEM- and CMY-plasmids. *S*. Heidelberg represented 64% (9 of 14) of isolates carrying the TEM-1 plasmids and 25% (2 of 8) of isolates carrying the CMY-2 plasmids. In a Canadian survey of human clinical isolates, Mataseje *et al*. (2009b) reported that *S*. Heidelberg was the most common serotype to show plasmid-mediated resistance to 3rd generation cephalosporins, with the *bla* $_{CMY-2}$ gene accounting for the majority of this resistance. Resistance to these drugs is important as *S*. Heidelberg is among the most common invasive serotype in Canada (Government of Canada, 2007; Mataseje *et al*., 2009b).

Similar to isolates from the Canadian population (Mataseje *et al*., 2009b), plasmid-mediated resistance was observed in many different phagetypes of waterborne *S*. Heidelberg obtained in this study. The greatest prevalence was observed in PT 19 (5/11). This phagetype only harboured the TEM-1 plasmid and was observed in all three tributaries. In Laurel Creek, two S. Heidelberg isolates, PT 29a and one untypable isolate, carried the CMY-2 plasmid. In human isolates in Canada, plasmids carrying *bla* $_{CMY-2}$ were most commonly reported in PT 29 (Mataseje *et al*., 2009b).

The carriage of the *bla* $_{CMY-2}$ gene was observed in several waterborne *Salmonella* serotypes, in addition to *S*. Heidelberg. This is consistent with other research conducted on isolates from humans and farm-animals (Zhao *et al*., 2007; Mataseje *et al*., 2009b; Daniels *et al*., 2009). These additional serotypes included: *S*. Agona (n=1), *S*. Berta (n=2), *S*. Infantis (n=1), *S*. Kentucky (n=1) and *S*. Newport (n=1). Recent studies of human isolates and farm-animals in Canada and the USA have observe the *bla* $_{CMY-2}$

145

gene in all of these serotypes, with the exception of *S*. Berta (Zhao *et al*., 2007; Mataseje *et al*., 2009b). The occurrence of the CMY-2 plasmid in waterborne *Salmonella*, particularly *S*. Heidelberg, is concerning due to the human health importance of these drugs for treating invasive infections in children.

Waterborne *Salmonella* carrying the *bla* $_{CMY-2}$ gene have not been reported in Canada prior to this study. Similarities between waterborne isolates and those reported in humans and farm animals signify that the aquatic environment may be involved in the movement of these strains and their resistance genes between host animals. These results demonstrate that the aquatic environment is involved in the environmental spread of *bla* $_{CMY-2}$ genes in *Salmonella*, which can have important implications regarding the long-term control of these clinically-relevant genes.

In human clinical isolates in Canada, *S*. Typhimurium regularly demonstrates AMR on plasmids (Mataseje *et al*. 2009b). Although waterborne *S*. Typhimurium commonly demonstrated higher levels of phenotypic drug resistance (Chapter 3), only one isolate carried plasmid-mediated resistance (TEM-1 plasmid). The few isolates demonstrating plasmid-mediated resistance in this serotype is a reflection of the predominant phagetypes obtained in water, which was PT 104. PT 104 is well known for the accumulation of resistance genes within a region of the bacterial chromosome designated the *Salmonella* genomic island [SGI-1], which is responsible for the resistance profile ACSSuT (Mulvey *et al*., 2006). Nineteen of the 20 waterborne *S*. Typhimurium demonstrating AMR were designated as PT 104, all of which showed the profile ACSSuT, therefore plasmid-borne resistance in these waterborne isolates was expected to be low.

Spatial and temporal distribution of AMR plasmids in *Salmonella* and *E. coli*

The urban creek (LC) showed the greatest frequency of AMR and the greatest diversity of *Salmonella* serotypes carrying AMR-plasmids. As mentioned in previous chapters, this diversity is likely the result of the diversity of hosts within this watershed. *Salmonella* carrying the TEM-1 and CMY-2 plasmids were equally prevalent in this tributary, and found at all three sample sites. Due to the common occurrence of the *bla*$_{CMY-2}$ gene in human isolates of *Salmonella* in Canada (Mataseje *et al*., 2009b), the

incidence of CMY-2 plasmids in this tributary suggests human sources. These plasmids were not found in the isolates from the agricultural-rural tributaries.

In contrast to the CMY-2 plasmids, the TEM-1 plasmids appeared to be widespread within the aquatic environment as they were obtained from *Salmonella* isolates from all three tributaries and in numerous serotypes. Isolates carrying the TEM-1 plasmid were obtained over many months of the year, however, differences were observed between the frequency of isolates carrying the TEM-1 plasmid among seasons. A higher proportion of isolates carrying this plasmid were obtained in the spring, compared to no detection in the summer. The wide distribution of TEM-1 plasmids might indicate that it is found in many different hosts throughout the watershed. Although further genetic analysis would be needed, it is possible that the TEM-1 plasmids may be endemic in these watersheds.

In the agricultural tributaries (CAN and CON), the TEM-1 plasmid was the only AMR plasmid observed in *Salmonella*. Within these tributaries, TEM-1 plasmids were predominantly carried by *S*. Heidelberg where this serotype accounted for 100% (2/2) of the plasmid-mediated resistance in Canagagigue Creek and 80% (4/5) in Conestogo River. While low numbers of isolates containing TEM-1 plasmids limit extensive seasonal analysis between tributaries, TEM-1 plasmid was observed predominantly in the late spring months in isolates obtained from both CAN and CON, compared to a greater incidence of TEM-1 in the fall months in LC. The higher incidence of TEM-1, particularly in *S*. Heidelberg, in the spring in CAN and CON, might reflect agricultural inputs at this time of the year. The greater frequency may be the result of increased run-off following precipitation events following spring manure spreading. In any case, further study would be needed to confirm seasonal differences.

In contrast to *Salmonella*, twice the number of waterborne *E. coli* isolates carried plasmid-mediated resistance in Canagagigue Creek compared to Laurel Creek. However, as observed with *Salmonella*, *E. coli* isolates carrying plasmid-mediated resistance were only ever observed from one sample location (CAN-1) in Canagagigue Creek, whereas isolates from Laurel Creek were obtained from different sample locations. Although the CAN-1 sample location was solely responsible for plasmid-borne AMR in *Salmonella* and *E. coli* in CAN, these isolates were obtained at different times of the year, with AMR *Salmonella* predominating in May and *E. coli* in August.

147

The incidence of AMR-plasmid carrying *Salmonella* and *E. coli* at CAN-1 may indicate that a specific source of contamination is contributing to the input of these isolates in Canagagigue Creek. While this stream is found within an intensely farmed area, CAN-1 is located in a park in the town of Elmira. Although the sewage treatment discharge is located downstream of this sample location, it is possible that unknown human fecal inputs are impacting this location. Alternatively, it is possible that chemical contaminants in the environment, such as heavy metals and other pollutants, could be contributing to plasmid maintenance if genes encoding resistance to these contaminants exist. Further analysis of these plasmids would be needed to substantiate this assumption.

Diversity and genetic relatedness of plasmids carrying *bla* CMY-2 genes

A comparison of the genetic relatedness of the 95.5 kb-sized CMY-2 plasmids was conducted through plasmid restriction fragment length polymorphism (pRFLP) to better understand the dissemination of AMR plasmids in the aquatic environment.

In the literature, the *bla* CMY-2 gene is commonly reported on a variety of larger-sized plasmids (80 to 142 Kb) in *Salmonella*, although some have reported occurrences on smaller (10 - 33 Kb) plasmids (Pitout *et al*., 2003; Giles *et al*., 2004; Poppe *et al*., 2006; Mataseje *et al*., 2009a; Mataseje *et al*., 2009b; Mulvey *et al*., 2009; Call *et al*., 2010). In waterborne *Salmonella* isolates, the *bla* CMY-2 gene was only observed on 95.5 Kb-sized plasmids. While identical sizes and gene carriage suggest the CMY-2 plasmids may be indistinguishable, pRFLP and software analysis did not demonstrate absolute homology between these plasmids.

Despite comparable banding patterns, the software analysis showed five unique banding patterns and a 60% similarity among the 8 CMY-2 plasmids. However, the majority (7 of 8 plasmids) showed a greater than 80% similarity. These results suggest that, in most instances, the CMY-2 plasmids carried by different serotypes in Laurel Creek are closely related; however one endemic plasmid is not responsible for the occurrence and spread of the *bla* CMY-2 gene in this tributary. Although software analysis highlighted differences, additional genetic analysis should be conducted to confirm the degree of heterogeneity among CMY-2 plasmids.

In a few instances, indistinguishable pRFLP patterns were observed between CMY-2 plasmids in different isolates. These indistinguishable patterns were observed in several isolates of the same serotype (*S*. Berta and *S*. Heidelberg), obtained at different sample locations or on different sample dates. These similarities suggest that a consistent source may be contributing to the occurrence of these isolates. In one instance, identical pRFLP patterns were observed in two different serotypes, *S*. Infantis and *S*. Newport, both of which were isolated on different dates and at different locations in Laurel Creek. Identical CMY-2 plasmids in these different serotypes suggest that this plasmid may be endemic and circulating within hosts in this tributary. Further monitoring would be required to determine the prevalence of this plasmid in these waters.

Prior to this study, plasmids containing the *bla* $_{CMY-2}$ gene have not been characterized in waterborne *Salmonella* in Canada, however, a study on waterborne *E. coli* with similar plasmids was conducted by Mataseje *et al*. (2009a). Following pRFLP, these authors observed a great diversity among *bla* $_{CMY-2}$ bearing plasmids in *E. coli* obtained from beaches and drinking water sources in Canada. Despite the variety of restriction profiles, clustering tended to occur around replicon types (i.e., different incompatibility groups) and appeared to be independent of the source of the isolate (beach water versus drinking water) or location of origin (Alberta, Ontario or Quebec). Mataseje *et al*. (2009b) also characterized plasmids carrying *bla* $_{CMY-2}$ from human clinical isolates of *Salmonella* in Canada. Similar to their study on *E. coli*, the *bla* $_{CMY-2}$ plasmids in *Salmonella* demonstrated diverse restriction patterns, however, similarities were also observed among replicon types. In contrast to Mataseje *et al*. (2009b), waterborne isolates in the current study demonstrated limited diversity in pRFLP profiles of *bla* $_{CMY-2}$ bearing plasmids. While replicon typing was not conducted in the current study, the limited diversity in waterborne isolates suggests that all CMY-2 plasmids might be the same incompatibility group.

Previously, Carattoli *et al*. (2002) and Giles *et al*. (2004) reported that the *bla* $_{CMY-2}$ gene was encoded on 4 plasmid types in *Salmonella* with differing incompatibility groups, referred to as type A, B, C (all ~100 kb) and D (~10kb). Although plasmid incompatibility testing was not carried out in the current study, restriction analysis with the same enzyme revealed that the CMY-2 plasmids observed in waterborne isolates closely resemble that of the type B plasmid profile (also referred to as replicon type I1; Carattoli *et al*., 2006) . Giles *et al.* (2004) reported that type B plasmids were the most

likely to transfer through conjugation when compared with other plasmids. In addition, type B plasmids commonly demonstrate single drug resistance (i.e., only carry bla_{CMY-2} gene), a feature observed in the CMY-plasmids in these waterborne *Salmonella*. Plasmid types A/C encode multiple drug resistance and are less commonly observed in isolates in humans and farm-animals in Canada (Mataseje *et al*., 2009a; Mataseje *et al*., 2009 b; Call et al., 2010). They were not observed in the current study.

The results of this study demonstrate that the CMY-2 plasmid in waterborne isolates is not the result of the clonal expansion of one specific serotype, as has been reported in some *Salmonella* serotypes, including *S*. Newport, obtained from humans and farm animals (Butaye *et al*., 2006; Daniels *et al*., 2007; Egorova *et al*., 2008). Many different serotypes obtained from water carried similar or indistinguishable pRFLP profiles. This is in agreement with the results obtained by Mataseje *et al*. (2009b) in human clinical isolates in Canada. Although many CMY-2 plasmids in waterborne isolates of *Salmonella* were closely related, variation in these plasmids implies that one indistinguishable CMY-2 plasmid was not circulating within *Salmonella* in the LC watershed.

Stability and fitness costs of naturally occurring AMR plasmids in *Salmonella*

Few studies have examined biological/fitness costs associated with naturally occurring AMR plasmids (Enne *et al*., 2005), particularly in wildtype *Salmonella* carrying naturally occurring plasmids with bla_{CMY} genes. To date, no studies have examined fitness costs associated with plasmid carriage of bla_{CMY-2} genes in *Salmonella*.

Unlike chromosomal bla_{CMY} genes, no functional repressor gene is found on naturally occurring plasmids carrying these genes and, as a result, high-level production of AmpC β–lactamase occurs within the cell (Hossain *et al*., 2004). A previous study by Morosini *et al.* (2000) reported significant biological costs in *Salmonella* cells carrying a plasmid bearing an '*AmpC* gene', as indicated through decreased growth rates, cell size, and invasion rates. In contrast to Morosini *et al*. (2000), naturally derived CMY-2 plasmids in the current study did not pose a reproductive fitness cost (i.e., differences in growth rate) to *Salmonella* when wildtype isolates were tested versus isolates cured of these plasmids, or when these plasmids were introduced into a laboratory strain of *E*.

coli. Differences in growth rates among isolates were not observed when reproductive fitness was tested at different temperatures (44.5 or 37°C) or when grown in media with varying nutrient concentrations (full strength LB or 1/10 LB).

The lack of observed fitness costs in this study are in agreement with the results presented by Hossain *et al*. (2004), who reported no biological costs associated with carriage of a naturally occurring plasmid in *Salmonella* (originally isolated from an infected person) carrying a similar gene, *bla* $_{CMY-7}$. As discussed by Hossain *et al*. (2004), significant biological costs reported by Morosini *et al*. (2000) were likely related to introducing a cloned CMY gene into a plasmid. While Hossain *et al*. (2004) observed no functional repressor upstream of *bla* $_{CMY-7}$, they stated that some other plasmid-encoded factors must compensate for the biological cost of high-level AmpC production within *Salmonella*.

While differences in growth rates were not observed, exposure to plasmid curing conditions at 44.5°C (Poppe and Gyles, 1988), which was used to impose a metabolic burden on cells, generally resulted in some initial loss of CMY-2 plasmids, whether the plasmids were naturally occurring in *Salmonella* or the plasmids were introduced into *E. coli*. Bacteria that experience fitness costs associated with plasmid carriage can segregationally eliminate plasmids when the cells reproduce (Lenski and Bouma, 1987; Modi and Adams, 1991). Hossain *et al*. (2004) and Morosini *et al*. (2000) did not test plasmid stability in their trials so no direct comparisons can be made between these and the current study. However, Morosini *et al*. (2000) stated that under non-selective conditions some loss of resistance occurred, therefore, their fitness trials were conducted in culture media supplemented with ceftriaxone. While plasmid loss in the current study may indicate a biological burden on the cell, differences in growth rates were not observed. However, it is possible that the test used (i.e., comparison of growth rates following culture in LB media over 12 h) was not sensitive enough to detect differences. Alternatively, if biological costs did exist, these costs might not have impacted reproduction rates.

Unlike other *Salmonella* strains tested in this study, two isolates of *S*. Berta (22C1 and 22C2) lost a large portion of plasmid-containing cells in the initial curing trials. A similar observation occurred in subsequent fitness trials (at 44.5°C) with *S*. Berta (22C1) and when the p22C1 plasmid was introduced into *E. coli*. Following initial

151

losses, in most instances, cells containing plasmids dominated towards the end of the trial. This plasmid-loss/regain phenomenon, however, only occurred under the curing temperature of 44.5°C and not at 37°C.

Differences between *S*. Berta and other serotypes may indicate that the elevated temperature created a greater metabolic burden on this serotype compared to others thus causing greater segregation of the CMY-2 plasmid during reproduction. However, similar losses were observed when the plasmid (p22C1) originating from *S*. Berta was introduced into *E. coli* cells. This indicates that plasmid stability is not related to temperature sensitivity of this *Salmonella* serotype and that stability lies with the plasmid itself. As discussed previously, analysis through pRFLP demonstrated that, while similar, the CMY-2 plasmids in *S*. Berta (p22C1 and p22C2) were not identical to the other *bla* $_{CMY-2}$-carrying plasmids obtained from other *Salmonella* serotypes (Figure 4.2). It is possible that these differences might translate into a loss of genes that ensures the stable inheritance of these CMY-2 plasmids. Alternatively, these plasmids might have a lower copy number compared to other CMY-2 plasmids (Hossain *et al*., 2004), which might also have contributed to greater segregational loss of these plasmids. While these explanations might account for the initial losses of these plasmids, they do not account for the stable maintenance of the CMY-2 plasmids towards the end of the trial.

Cells can acquire compensatory mutations that rapidly abolish fitness costs associated with expression of drug resistance (Bjorkman *et al*., 1999; Bjorkman and Andersson, 2000; Hossain *et al*., 2004; Maisnier-Patin and Andersson, 2004; Zhang *et al*., 2006). Therefore, the CMY-2 plasmids originating from *S*. Berta, which might impose an undetected metabolic burden on its host, may be evolving over subsequent generations as mutational changes occur, thus allowing the cells to maintain these plasmids over time. Many studies have reported that plasmids, which initially conferred a fitness cost to a host cell, evolved over many generations to become less costly (Bouma and Lenski 1988; Modi and Adams 1991; Dionisio *et al*., 2005), with some evolved plasmids even creating a greater fitness level when re-introduced into ancestor cells (Dionisio *et al*., 2005).

How compensatory mutations restore fitness in AMR *Salmonella* is still not well understood (Zhang *et al*., 2006). Using *E. coli* as a model, to help compensate for the

costs of extrachromosomal elements, such as plasmids, studies have shown that co-evolution of a bacterium and plasmid can result primarily through mutations in the chromosome (reviewed in Maisnier-Patin and Andersson, 2004). Hossain *et al.* (2004) speculated that any fitness cost associated with high-level production of AmpC β-lactamases in *Salmonella* are compensated by some unknown plasmid-encoded functions and not related to repression of the *bla* $_{CMY}$ gene itself.

Accumulation of compensatory mutations in CMY-2 plasmids in waterborne *S*. Berta might be the reason that cells did not continue to lose plasmids as each trial progressed. The increased number of isolates carrying these plasmids at the end of each trial could be related to: 1) greater fitness in plasmid-bearing cells compared to plasmid-free cells, although no fitness differences were detected; or, 2) conjugation of CMY-2 plasmids between isolates as a result of the batch experiment, as observed in the competitive fitness trial (Figure 4.10). In any case, further analysis and experimentation would be needed on these isolates to determine the ultimate reason for initial plasmid loss and subsequent gain in plasmids extracted from waterborne *S*. Berta.

Despite losses at 44.5°C, stable maintenance at 37°C indicates that naturally occurring CMY-2 plasmids are likely stably maintained in many *Salmonella* isolates. This appears to be similar to studies that have shown naturally-occurring AMR plasmids to be stably maintained *in vitro* and *in vivo* in various bacteria, including *Salmonella* (e.g., Hossain *et al*., 2004). This differs from past studies where artificially-inserted plasmids were found to be less stably maintained over time (e.g., Lenski and Bouma, 1987; Lenski, 1998).

Similar fitness levels and plasmid maintenance in both *Salmonella* and *E. coli* containing the identical plasmid may indicate that once AMR plasmids spread to different species there might be a long-term persistence, which might continue to limit the use of antimicrobials, particularly 3rd generation cephalosporins, in treating disease. Similar results have been documented by others (e.g., Lenski, 1998; Dionisio *et al*., 2005) and have important implications both for the maintenance and spread of plasmids. As suggested by others, these results imply that even if general usage of a specific antimicrobial drug is stopped, conjugative plasmids can still persist in bacterial populations (Bjorkman and Anderson, 2000; Maisnier-Patin and Andersson, 2004; Dionisio *et al*., 2005; Zhang *et al*., 2006).

4.7 Conclusions

Phenotypic AMR analysis should be complimented with genotypic analysis to further understand the epidemiology of drug resistance in pathogens and commensal bacteria, particularly when resistance is expressed to drugs of high human health importance. Through genotypic analysis, this study provided further insight into the complexity of plasmid-mediated resistance in *Salmonella* outside of the host environment.

Plasmid-mediated resistance was widespread in waterborne *Salmonella* and was not the result of carriage by a single serotype, although *S*. Heidelberg commonly demonstrated AMR plasmid carriage. The type of resistance carried on plasmids, as well as plasmid size, was finite. Plasmid-mediated resistance in waterborne *Salmonella* was only observed to β-lactam drugs and found only on two different sized plasmids (95.5 Kb and 8.1 Kb). The 8.1 Kb plasmid carrying *bla* $_{TEM-1}$ (TEM-1 plasmid), demonstrating resistance to ampicillin, was found in many different serotypes and in all three tributaries. The widespread occurrence of the TEM-1 plasmid suggests that the *bla* $_{TEM-1}$ gene, if not the TEM-1plasmid, is endemic in the tributaries under study.

Plasmid-mediated resistance contributed significantly to the prevalence of resistance to 3rd generation cephalosporins in waterborne isolates of *Salmonella*. Similar to human and animal isolates in Canada, plasmid-mediated resistance to ceftriaxone was associated with the *bla* $_{CMY-2}$ gene. Several *Salmonella* serotypes carried this gene on a 95.5 Kb conjugative plasmid (CMY-2 plasmid). The occurrence of this CMY-2 plasmid in waterborne isolates is concerning due to the human health importance of these drugs and the potential transmission of strains carrying this plasmid in water. In addition, the occurrence of *Salmonella* strains carrying the CMY-2 plasmid in LC and not the other streams further suggests that human fecal waste is impacting this urban tributary.

Despite similar restriction patterns, software analysis revealed heterogeneity among most CMY-2 plasmids. Although further analysis would need to be conducted, this may indicate that no common plasmid is associated with *bla* $_{CMY-2}$ carriage in waterborne *Salmonella*. Many questions remain as to the future extent of the spread of resistance to 3rd generation cephalosporins. However, considering the ability to transfer through conjugation and the limited fitness costs associated with carrying plasmids

bearing bla_{CMY-2}, it is likely that the incidence of resistance to these drugs in environmentally derived strains will increase over time.

Naturally occurring plasmids carrying bla_{CMY-2} genes in *Salmonella* did not contribute detectable reproductive fitness costs, based on temperature and nutrient stress studies. As well, plasmid loss was minimal or was maintained over time after an initial measured reduction. These results indicate long-term stability of plasmids encoding bla_{CMY-2} genes under non-selective conditions. These results also suggest that even if general usage of 3rd generation cephalosporin drugs is stopped, conjugative plasmids may still persist in bacterial populations.

Though similarities were observed between resistance genes on plasmids acquired from *Salmonella* and *E. coli*, no common plasmids were observed between these genera. The difference in prevalence of AMR-carrying plasmids in these bacteria continues to bring into question the use of commensal bacteria as indicators of antimicrobial resistance in pathogens. Examining the molecular determinants of resistance in *E. coli* and *Salmonella* from specific animals may be more appropriate for understanding the significance of commensal bacteria in the development and transfer of drug resistance to pathogens, as opposed to a comparison between isolates obtained from the aquatic environment.

4.8 Recommendations and future research needs

- Further analysis should be carried out to determine the genetic relatedness of the TEM-1 plasmids obtained in this study to further understand the endemic nature of these plasmids in waterborne *Salmonella*.

- Additional restriction digests should be conducted on the CMY-2 plasmid extracts in order to confirm the degree of heterogeneity reported and to rule out that the differences observed were a result of the software analysis.

- Further study is needed on the CMY-2 plasmids originating from *S*. Berta strains to determine if these plasmids are more rapidly evolving. Trials should be conducted to determine if plasmid stability differs between isolates carrying wildtype plasmids compared to those carrying more evolved plasmids.

- Plasmid sequencing may also provide a more comprehensive understanding of the CMY-2 plasmids, as well as provide further insight into the stability of these naturally occurring plasmids and mutational changes that become evident over time.

- Further analysis is needed on the two isolates that demonstrated resistance to 3rd generation cephalosporins, however did not carry detectable genes responsible for β-lactamase production.

5

Genetic variability & diversity among waterborne *Salmonella* of human & animal health significance: *S.* Typhimurium and *S.* Heidelberg

5.1 Introduction

Serotyping is important in differentiating the over 2500 *Salmonella* serotypes, however, this technique has limited value for discriminating within more common serotypes, including *S.* Typhimurium and *S.* Heidelberg, both of which predominate in humans and animals (Government of Canada, 2005; 2006; 2007; Farzan *et al*. 2008). Different phenotypic and genotypic methods have proven useful for subtyping including phagetyping, antimicrobial resistance profiling, plasmid profiling, pulsed-field gel electrophoresis (PFGE) and rep-PCR (CDC, 2007a; Byappanahalli *et al*., 2009; Xia *et al*., 2009).

PFGE profiling, a molecular fingerprinting technique based on the restriction digestion of genomic DNA (Xia *et al*., 2009), is considered the 'gold standard' for *Salmonella* sub-typing. This technique is used in the PulseNet program, an international molecular subtyping network, to recognize the movement of clones of *Salmonella* strains associated with disease outbreaks and those responsible for the dissemination of drug resistance (Xia *et al*., 2009; CDC, 2010), such as the clonal expansion of multiple drug

resistant (MDR) *S.* Typhimurium phagetype 104 (PT 104) worldwide (Threlfall, 2000; Mulvey *et al.*, 2006).

PFGE is a well established molecular epidemiological tool used for outbreak investigations. In addition, PFGE is used to determine the diversity and genetic relatedness of *Salmonella* across large geographical areas and in isolates that are not epidemiologically linked (Martinez-Urtaza *et al.*, 2004; Galanis *et al.*, 2006; Zhao *et al.*, 2008; Xia *et al.*, 2009). Many studies of this nature have been carried out to investigate genetic relatedness in *Salmonella* isolates obtained from animals intended for human consumption, related food products and from infected humans (Nayak *et al.*, 2004; Patchanee *et al.* 2008; Zhao *et al.*, 2008). Several national studies in the USA examining the genetic relatedness of *Salmonella* isolates originating from farm-animals (Zhao *et al.*, 2007) and retail meat products (Zhao *et al.*, 2008) have reported the *Salmonella* population to be genetically diverse, however, there appears to be widespread dissemination of clones, particularly those demonstrating resistance to multiple drugs.

Although considerable research has been carried out to understand the molecular diversity of *Salmonella* serotypes in farm animals, food products and human isolates, knowledge of the genetic relatedness of environmentally-derived strains remains limited. To date, studies on *Salmonella* in the aquatic environment primarily focus on isolates at the phenotype/serotype level (Martinez-Urtaza *et al.*, 2004). Few studies characterize strains at the phagetype level or have used methods, such as PFGE, to examine genetic relatedness of environmentally-derived strains. Martinez-Urtaza *et al.* (2004) conducted one of the only studies to characterize the genetic relatedness of *Salmonella* obtained from the aquatic environment. Analysis of *S.* Typhimurium originating from shellfish off the coast of Spain demonstrated a high degree of homology within phagetypes following PFGE, with many isolates within the same phagetype demonstrating identical profiles. The identification of several indistinguishable isolates on different dates, but at the same location, allowed the authors to conclude that these isolates likely originated from one distinct source of contamination on the coast. A recent study conducted by Byappanahalli *et al.* (2009) observed considerable genetic similarity in *Salmonella* isolates obtained from beaches in Lake Michigan using rep-PCR. Clustering of isolates tended to occur around specific years and locations, however, it is unknown if this clustering was related to the

occurrence of similar or identical serotypes/phagetypes, as these attributes were not included in their testing.

5.2 Research needs and objectives

To further define the role that water plays in the movement of *Salmonella*, details on the genetic diversity of isolates are needed. Techniques such as phagetyping and PFGE can aid in determining the relatedness of *Salmonella* isolates circulating within and between tributaries and can help in understanding potential sources of contamination. Detailed analysis of serotypes of human and animal health significance, such as *S*. Typhimurium and *S*. Heidelberg, isolated from the aquatic environments may be a first step in understanding if epidemiological connections exist between isolates obtained from water and those from other sources within a watershed.

The specific objectives of the research presented in this chapter are to:

1. Determine the prevalence and diversity of phagetypes of *Salmonella* Typhimurium and Heidelberg found in urban and rural stream waters, and compare these findings to studies of humans and animal isolates in Canada;

2. Establish the genetic diversity among various serotypes of *Salmonella*, including *S*. Heidelberg, *S*. Typhimurium and monophasic serotypes obtained within and between three tributaries, and determine if clonality exists in isolates demonstrating antimicrobial resistance; and,

3. Ascertain the genetic relatedness of multiple drug resistant *S*. Typhimurium phagetype 104 circulating within urban and rural tributaries, using several typing techniques.

5.3 Materials and Methods

Pulsed-field gel electrophoresis

S. Typhimurium, *S.* Heidelberg and several monophasic isolates obtained from water samples were subjected to pulsed-field gel electrophoresis (PFGE) following the protocol described by Ribot *et al.* (2006) and PulseNet USA (CDC, 2009b), with minor differences.

Preparation of the agarose plugs

All isolates to be examined were taken from frozen (-80°C) stocks and streaked onto LB agar. These plates were incubated overnight (16 to 18 h) at 37°C. Using a sterile cotton swab, bacterial growth was transferred to a sterilized test tube containing 2 mL of cell suspension buffer (100 mM Tris and 100 mM EDTA at pH 8.0). Cells were added until OD$_{610}$ of 1.3 to 1.4 was obtained (Ultrospec 1100 pro UV Visible Spectrophotometer, Fisher Scientific). A total of 200 μL of each cell suspension was transferred to a microcentrifuge tube to which 10 μL of Proteinase K (0.5 mg/mL) and 200 μL of melted (55^{0}C) 1% SeaKem Gold:1% SDS agarose (prepared in TE buffer) was added. The SeaKem Gold agarose was purchased from Lonza Rockland Inc. (Rockland, ME) and SDS from Sigma (Mississauga, ON). The mixture was gently pipetted up and down several times and transferred into disposable plug molds (BioRad, Hercules, CA) and left at room temperature to solidify for 10 min. Two plugs per isolate were prepared.

Lysis of cells in plugs

After solidifying, the plugs were removed from the molds and placed into a 50 mL tube containing 5 mL of cell lysis buffer (50 mM Tris, 50 mM EDTA, 1% Sarcosyl at pH 8.0) and 0.1 mg/mL Proteinase K solution. Each tube was at 54°C in an orbital waterbath for 1.5 h at 150 rpm (Thermo Scientific Lab-line AquaBath). After incubation, the lysis buffer was removed and the plugs were washed 3 times with 15 mL sterile ultrapure water pre-heated to 50°C and then washed 4 times with 15 mL TE

buffer. Each washing was carried out for 15 min. at 50°C in a shaking waterbath at 150 rpm. Each plug was stored at 4°C in TE buffer until processed with restriction buffer.

Restriction digest of plugs

Each plug to be digested was cut in half using a razor blade and transferred to a microcentrifuge tube containing 200 μL of 1 x H buffer (New England BioLabs, Ipswich, MA). The plug was incubated at 37°C in a waterbath for 10 min. After incubation, the buffer was removed and 200 μL of the restriction enzyme solution was added, containing 1x buffer H and *Xba*I (New England BioLabs, Ipswich, MA) at 50 U per sample. Any *S*. Typhimurium phagetype 104 that produced similar patterns to other isolates following digestion with *Xba*I were further digested with *Bln*I (New England Biolabs, Ipswich, MA) at 30 U per plug sample. Digestion was carried out at 37°C in a waterbath for 4 to 5 h. Following incubation, the restriction buffer was removed and replaced with 200 μL of 0.5 x TBE for 5 min. at room temperature.

PFGE procedure

Using a spatula, each restricted plug slice was loaded into a well of a 1% SeaKem Gold agarose gel prepared in 0.5 x TBE buffer (45 mM Tris-borate, 1 mM EDTA at pH 8.0). Several plugs containing a lambda ladder (CHEF DNA size standard blocks, BioRad, Hercules, CA) were also included in the gel. Each well was sealed with melted 1% SeaKem Gold agarose.

The gel was placed into the CHEF-DR III electrophoresis system (BioRad, Hercules, CA) containing 0.5 x TBE cooled to 14°C. The conditions for pulsed field gel electrophoresis were: initial switch time of 2.2 s and a final switch time of 63.8 s, voltage of 6 V and an angle of 120°. Each gel was run for 19 h at 14°C with a buffer circulation rate of 1 L/minute. Following each run, the gel was stained with ethidium bromide (1 μg/mL) for 30 min. The gel was de-stained in water for 60 min. with a change of water every 20 min.

Interpretation of PFGE

Following PFGE, the DNA bands were visualized and photographed under UV light (Bio-Rad Gel Doc). Resulting banding patterns were analyzed using BioNumerics software (Applied Maths, Belgium) and normalized to the lambda ladder (CHEF DNA size standard blocks, BioRad, Hercules, CA). Band matching was determined initially by the software and manual changes were made if bands were placed in the incorrect band class by the software. The relatedness of each banding pattern was calculated using Dice's similarity coefficient with a 1.5% band position tolerance and optimization. Dendograms were generated by the unweighted pair group method with arithmetic means (UPGMA).

As described in Chapter 4, interpretation of the similarity between banding patterns was carried out based on criteria described by Tenover *et al*. (1995). Isolates were considered indistinguishable (clones) if the similarity was ≥ 99%. Patterns were considered closely related if similarities were ≥ 80%, which would show few differences in banding patterns. Isolates were considered unrelated if < 50% similarities were observed.

Distinct profiles were designated with the letter X (XbaI digests) or B (*Bln*I digests) in accordance with the restriction patterns observed for *S*. Typhimurium PT 104 isolates.

Plasmid typing in *S*. Typhimurium phagetype 104

All *S*. Typhimurium PT 104 (and PT 104a) isolates were characterized through plasmid typing. Plasmid DNA was extracted from each isolate with a plasmid extraction kit (Qiagen plasmid mini kit) and subjected to electrophoresis as described previously (Chapter 4). Following staining and imaging, plasmid sizes were determined by comparison to a supercoiled DNA ladder (Sigma, Mississauga, ON) and a mixture composed of plasmids of known size as described in Poppe *et al*. (2005): pDT285 (96-MDa); p971028 (62-MDa); pDT369 (23-MDa); and eight plasmids of *E. coli* V517 with molecular masses ranging from 1.4 to 35.8 MDa. To differentiate between the observed plasmid profiles, a plasmid typing number (P1 to P8) was assigned to different profiles for comparison.

Detection of virulence genes

S. Typhimurium DT 104 and DT 104a isolates were assessed for the presence of virulence plasmids. The primers used were from published sequences (Chiu and Ou, 1996) for the *spv*C gene and synthesized by Sigma-Genosys (Oakville, ON). Primer sequences were as follows: SPVC-1, 5'-ACTCCTTGCACAACCAAATGCGGA-3' and SPVC-2, 5'- TGTCTTCTGCATTTCGCCACCATCA -3'. *Salmonella* Typhimurium 971028 with a virulence-associated plasmid (p951028) was used as a positive control (Poppe *et al*., 2005).

The PCR mixture consisted of 12.5 µL Master mix (DyNAmo, Finnzymes, Finland), 2.5 µL of each primer pair (1 µM), 1 µL of purified plasmid DNA as the template and water that was added to make a total volume of 25 µL. The parameters for the amplification were; denaturation for 30s at 95°C, annealing for 30s at 50°C and primer extension for 1 min. at 72°C for a total of 35 cycles. A final extension at 72°C for 10 min was included at the end of the PCR reaction. To visualize the desired 524 bp product, PCR samples were subjected to electrophoresis and staining as described previously.

5.4 Results

Phagetypes of *S*. Heidelberg and *S*. Typhimurium observed in tributaries

Overall, eight different phagetypes of *S*. Typhimurium (Figure 5.1 a) were observed. PT 104 was the predominant phagetype and represented 53% of the *S*. Typhimurium isolates obtained from the river water. Over half of these isolates (11/18) were designated as variant 5 - (Var. 5-) strains, which are O:5 negative variants of *S*. Typhimurium. Six different phagetypes of *S*. Heidelberg were observed with the most common being PT 19 (Figure 5.1 b). Both of these predominant phagetypes, *S*. Typhimurium PT 104 and *S*. Heidelberg PT 19, were isolated in all three tributaries (Figure 5.1). Several phagetypes were only observed in a single tributary, however, these phagetypes were only represented by a few isolates. Several atypical or untypable phagetypes of *S*. Heidelberg were isolated, primarily from the Conestogo River.
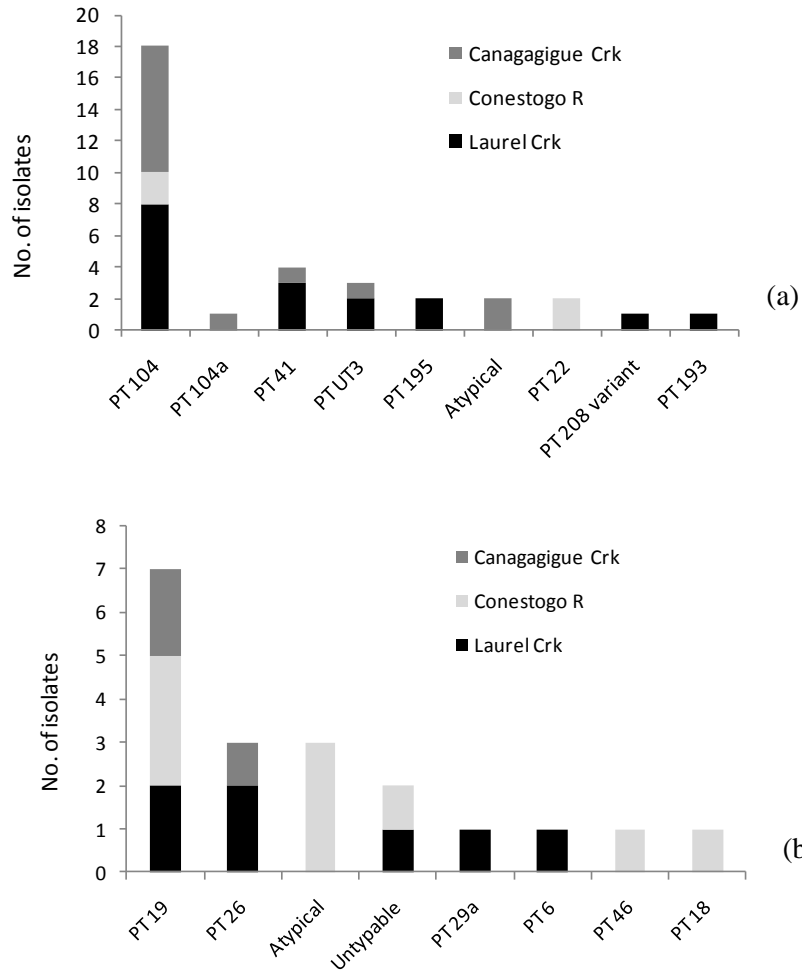
Figure 5.1. a) Phagetypes of *S.* **Typhimurium, and b)** *S.* **Heidelberg, observed in three tributaries of the Grand River.**

Phagetyping was also conducted on three isolates of *Salmonella* serotype I:4,5, 12:i:-, all of which demonstrated different phagetypes. One isolate obtained from CAN-2 was found to be PT 120 and two isolates from CON-3 on the same date were represented by PT 193 and PT 99 (Figure 5.3).

PFGE of waterborne *S*. Heidelberg isolates

Six unique PFGE banding patterns were observed in *S*. Heidelberg isolates following treatment with *Xba*I, all of which showed a $\geq 86.9\%$ similarity (Figure 5.2). Two main clusters, showing a $> 90\%$ similarity between isolates, were observed in the analysis, with PT 26 predominating in cluster A and PT 19 predominating in cluster B.

	Phage-type	Resist-ance profile	Sample location	Sample date (M/D/Y)	Isolate No.	Conjugative AMR plasmid size (Kb)
	26	-	LC-1	11/18/04	21D3	.-
	26	-	CAN-1	7/21/04	12Dc	.-
A	Atypical	-	CON-2	8/26/04	18C3	.-
	Atypical	ASu	CON-1	5/12/04	9Cb	.8.1 (A)
	26	-	LC-1	11/18/04	21C3	.-
	19	A	LC-1	10/21/04	20D3	.8.1 (A)
	19	A	LC-3	10/18/04	19D1	.8.1 (A)
	19	-	CON-2	8/26/04	18D3	.-
	19	A	CAN-1	5/31/04	10Bc	.8.1 (A)
	19	ASu	CAN-1	5/31/04	10Cc	.8.1 (A)
	19	-	CON-1	5/12/04	9Aa	.-
	46	-	CON-2	8/26/04	18A3	.-
	18	-	CON-2	8/26/04	18B2	.-
	29a	ACazCro	LC-1	8/10/04	15C3	.95.5 (A/Caz/Cro)
	Atypical	ASu	CON-1	5/7/04	8Dd	.8.1 (A)
	Untypable	ACazCro	LC-3	7/21/04	12Ca	.95.5 (A/Caz/Cro)
B	19	ASu	CON-1	5/7/04	8Cd	.8.1 (A)
	6	ASu	LC-1	5/31/04	10Ca	.8.1 (A)
	Untypable	ASu	CON-1	5/12/04	9Db	.-

Figure 5.2. Dendogram of *Xba*I-PFGE patterns among various phagetypes of *S*. Heidelberg obtained from three tributaries. Antimicrobial resistance carried on conjugative plasmids is indicated in parentheses (A, ampicillin; Caz, ceftazidime; Cro, ceftriaxone). Clusters denoted A and B are indicated.

Several isolates of the same phagetype that were obtained from different locations or sampling dates within the same tributary, as well as between different tributaries, demonstrated indistinguishable restriction patterns following digestion with *Xba*I. This was observed in isolates in cluster A and B. In addition, several isolates representing different phagetypes also demonstrated indistinguishable restriction patterns (Cluster B).

Several *S*. Heidelberg isolates shared indistinguishable *Xba*I PFGE patterns but were isolated from different tributaries and/or on separate dates, for example, isolates 21D3 and 12Dc which were both designated as PT 26, shared identical PFGE patterns but were found in different tributaries in November and July of the same year, respectively. This appeared to be common with PT 19 isolates as well.

As observed in the predominant cluster (Figure 5.2), indistinguishable *Xba*I patterns were observed between several PT 19 isolates and several other phagetypes, including PT 46, PT 18, PT 29a, an atypical phagetype and one untypable isolate. Isolates demonstrating this identical banding pattern were obtained from all three tributaries and at various sampling locations.

Conjugative plasmids responsible for antimicrobial resistance were observed in *S*. Heidelberg isolates in both clusters (Figure 5.2), however, these plasmids predominated in isolates from cluster B. Plasmid carriage was not related to a single PFGE profile, as many isolates with identical PFGE profiles either carried an AMR plasmid or did not.

The smaller of the two conjugative 8.1 Kb plasmids, which carried ampicillin resistance (TEM-1 plasmid, as described in Chapter 4), was observed in several phagetypes with similar or indistinguishable PFGE patterns, however this plasmid tended to predominate in PT 19 isolates. In many instances PT 19 isolates with identical PFGE patterns carrying this conjugative plasmid were observed in all three river systems and over several months of the year. The larger of the two conjugative 95.5 Kb plasmids, which carried resistance to ACazCro (CMY-2 plasmid, as described in Chapter 4), was observed in two isolates with indistinguishable PFGE patterns (PT 29a and an untypable phagetype) taken from Laurel Creek in July and again in August of the same year.

PFGE and plasmid-typing of *S*. Typhimurium and monophasic isolates

Following digestion with *Xba*I, 21 unique banding patterns were observed in the 33 isolates of *S*. Typhimurium and the 15 monophasic isolates. PFGE with *Xba*I grouped the majority of *S*. Typhimurium and monophasic serotypes into three major clusters (clusters A-C; Figure 5.3).

Serotype	Phage-type	Resistance profile	Sample location	Sample date (M/D/Y)	Isolate no.	Variant strain	Xba pattern (PT 104)
Typhimurium	208 var.	-	CAN-2	8/10/04	15C5	yes	
Typhimurium	UT3	-	CAN-2	8/10/04	15D5	no	
Typhimurium	Atypical	-	CAN-2	8/10/04	15A4	no	
Typhimurium	195	-	LC-2	8/16/04	16D2	yes	
Typhimurium	195	-	LC-3	8/16/04	16D1	yes	
Typhimurium	41	-	LC-2	8/23/04	17A2a	no	
Typhimurium	41	-	CAN-1	8/23/04	17C6	no	
Typhimurium	41	-	LC-2	7/30/04	14Db	yes	
Typhimurium	193	-	LC-2	7/23/04	17D2	no	
Typhimurium	22	-	CON-2	7/30/04	14Ae	yes	
Typhimurium	22	-	CON-2	7/30/04	14Ce	yes	
Typhimurium	UT3	-	LC-3	11/21/03	2A	no	
Typhimurium	UT3	-	LC-3	11/21/03	2Aa	no	
Typhimurium	41	S	LC-2	5/7/04	8Cb	no	
I:4,5 12:i:-	99	-	CON-3	10/21/04	20C5	-	
I:4,5 12:i:-	193	-	CON-3	10/21/04	20D5	-	
Typhimurium	104	ACSSuT	LC-1	5/6/05	24D1	no	X1
Typhimurium	104	ACSSuT	LC-1	10/21/04	20C3	no	X1
Typhimurium	104	ACSSuT	LC-2	10/21/04	20A2a	no	X1
Typhimurium	104	ACSSuT	CAN-2	8/23/04	17A7a	yes	X1
Typhimurium	104	ACSSuT	CAN-2	8/16/04	16A5a	yes	X1
Typhimurium	104	ACSSuT	CAN-1	8/10/04	15A3	yes	X1
Typhimurium	104	ACSSuT	CAN-1	8/10/04	15C4	yes	X1
Typhimurium	104	ACSSuT	CAN-1	8/10/04	15D4	yes	X1
Typhimurium	104	ACSSuT	CAN-1	7/30/04	14Ad	yes	X1
Typhimurium	104	ACSSuT	CAN-1	7/30/04	14Dd	yes	X1
Typhimurium	104	ACSSuT	LC-1	10/21/04	19C3	no	X3
Typhimurium	104	ACSSuT	CAN-1	7/21/04	12Ac	yes	X2
Typhimurium	104a	ACSSuT	CAN-1	7/21/04	12Cc	yes	X2
Typhimurium	104	ACSSuT	LC-1	12/13/03	4A2	yes	X2
Typhimurium	104	ACSSuT	LC-1	12/10/03	3A2	yes	X2
Typhimurium	104	ACSSuT	CON-1	11/19/03	S1	no	X2
Typhimurium	104	ACSSuT	CON-1	11/19/03	S1a	no	X2
Typhimurium	104	ACSSuT	LC-3	10/21/04	20C1	no	X4
I:4,5 12:i:-	120	ACSSuT	CAN-2	8/26/04	18A5	-	
I:4, 5, 12:b:-	-	-	LC-1	5/12/04	9Ca	-	
I:4, 5, 12:b:-	-	-	LC-1	5/12/04	9Da	-	
I:4, 5, 12:b:-	-	-	LC-1	5/31/04	10Ba	-	
I:4, 5, 12:b:-	-	-	LC-3	3/13/04	7B1	-	
I 4, 5, 12:b:-	-	-	LC duck feces	2/11/04	5A4a	-	
I 4, 5, 12:b:-	-	-	LC duck feces	2/11/04	5A4b	-	
I 4, 5, 12:b:-	-	-	LC duck feces	2/11/04	5D4a	-	
I 4, 5, 12:b:-	-	-	LC duck feces	2/11/04	5B4b	-	
I 4, 5, 12:b:-	-	-	LC duck feces	2/11/04	5C4	-	
I:4, 5, 12:b:-	-	-	LC-3	3/13/04	7D1	-	
I 4, 5, 12:b:-	-	-	LC duck feces	2/11/04	5D4	-	
I:4,5 12:-:-	-	-	CAN-1	10/21/04	20D6	-	
Typhimurium	104	ACSSuT	LC-2	10/18/04	19A2a	no	X5

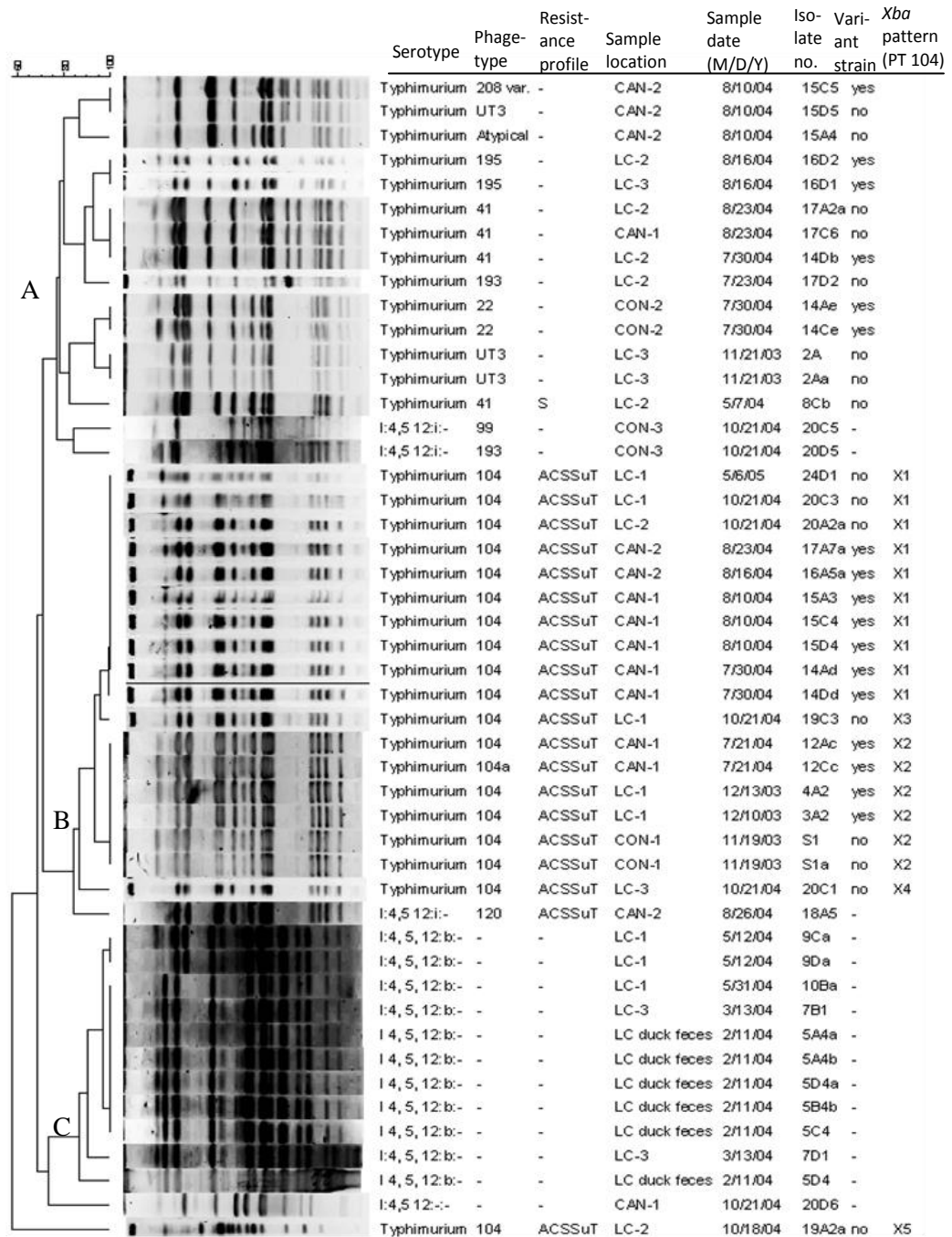**Figure 5.3. Dendogram of *Xba*I-PFGE patterns for *S*. Typhimurium and monophasic isolates obtained from three tributaries. Clusters denoted A, B and C are indicated.**

167

S. Typhimurium and monophasic serotypes did not demonstrate identical PFGE patterns, however similar patterns were observed in some instances. Monophasic serotype I:4, 5, 12:b:- isolates predominated in cluster C and demonstrated > 85% similarity. The majority of serotype I:4, 5, 12:b:- isolates (5 of 6 total) from fresh duck feces demonstrated indistinguishable restriction patterns. Identical or highly similar patterns (> 86% similarity) between isolates from feces and those obtained from Laurel Creek waters were also observed (Figure 5.3).

Cluster B, which demonstrated a > 80% similarity between PFGE profiles, was dominated by *S*. Typhimurium PT 104 strains demonstrating the ACSSuT resistance profile. In total, 18 of the 19 PT 104 isolates obtained from water were found in this cluster and showed a high degree of similarity between isolates (> 86.5%), with the majority of isolates showing indistinguishable restriction patterns following *Xba*I digestion. Many variant (Var. 5-) and non-variant isolates of PT 104 showed identical restriction patterns even when obtained from different tributaries, sample locations, and times of the year. Overall, more distinguishable *Xba*I patterns were observed in PT 104 isolates obtained from Laurel Creek than from the other tributaries with 8 isolates showing 5 different patterns (X1-X5), with pattern X1 being the most common (Figure 5.3). One PT 104 isolate (19A2a from Laurel Creek) showed the most unique profile (X5) when compared to all other isolates (Figure 5.3). This isolate demonstrated the lowest similarity (58%) to other waterborne *S*. Typhimurium or monophasic serotypes and was not observed in one of the three clusters (A-C).

Cluster A, where isolates demonstrated a ≥ 76.8% similarity, was composed of 7 distinct phagetypes of *S*. Typhimurium and two phagetypes of serotype I:4, 5, 12:i:-. Most isolates found to be of the same phagetype demonstrated indistinguishable restriction patterns (e.g., PT 41, PT 22 and PT 108). Many of these isolates that shared identical restriction patterns and phagetypes were found at different sample locations and/or on different dates. Cluster A also contained several variant strains of *S*. Typhimurium, many of which showed identical *Xba*I patterns to non-variant isolates designated with the same phagetype (e.g., PT 41).

PFGE and plasmid profiling PT 104 isolates

Following digestion with *Xba*I, all *S*. Typhimurium PT 104 isolates that demonstrated indistinguishable restriction patterns between two or more isolates (profiles X1 and X2) were further digested with *Bln*I (Figure 5.4). Following digestion, 4 patterns were observed (B1-B4) with most demonstrating a > 80% similarity. The profile designated as B1 was the most commonly observed.
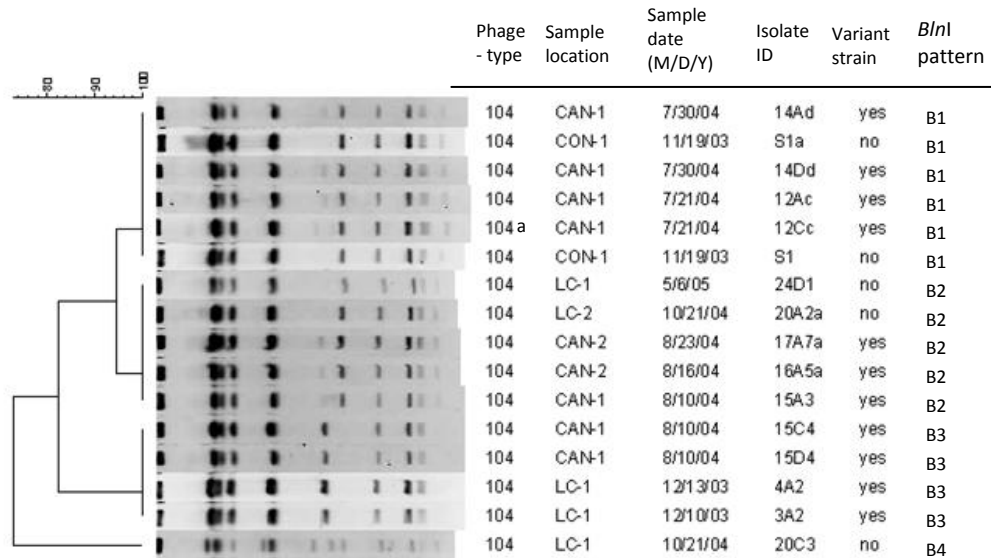

	Phage - type	Sample location	Sample date (M/D/Y)	Isolate ID	Variant strain	*Bln*I pattern
	104	CAN-1	7/30/04	14Ad	yes	B1
	104	CON-1	11/19/03	S1a	no	B1
	104	CAN-1	7/30/04	14Dd	yes	B1
	104	CAN-1	7/21/04	12Ac	yes	B1
	104 a	CAN-1	7/21/04	12Cc	yes	B1
	104	CON-1	11/19/03	S1	no	B1
	104	LC-1	5/6/05	24D1	no	B2
	104	LC-2	10/21/04	20A2a	no	B2
	104	CAN-2	8/23/04	17A7a	yes	B2
	104	CAN-2	8/16/04	16A5a	yes	B2
	104	CAN-1	8/10/04	15A3	yes	B2
	104	CAN-1	8/10/04	15C4	yes	B3
	104	CAN-1	8/10/04	15D4	yes	B3
	104	LC-1	12/13/03	4A2	yes	B3
	104	LC-1	12/10/03	3A2	yes	B3
	104	LC-1	10/21/04	20C3	no	B4

Figure 5.4. Dendogram of restriction digests with *Bln*I on *S*. Typhimurium PT 104 (and PT 104a) isolates that showed two or more isolates demonstrating indistinguishable restriction patterns after original digestion with *Xba*I.

Following plasmid analysis, 8 plasmid patterns (P1-P8) were observed in all PT 104 (and PT 104a) isolates. Plasmids ranged in size from 3.4 to 95.5 Kb and the number of plasmids ranged from 1 to 6 per isolate (Figure 5.5 and Table 5.1). An example of each plasmid profile is shown in Figure 5.5.
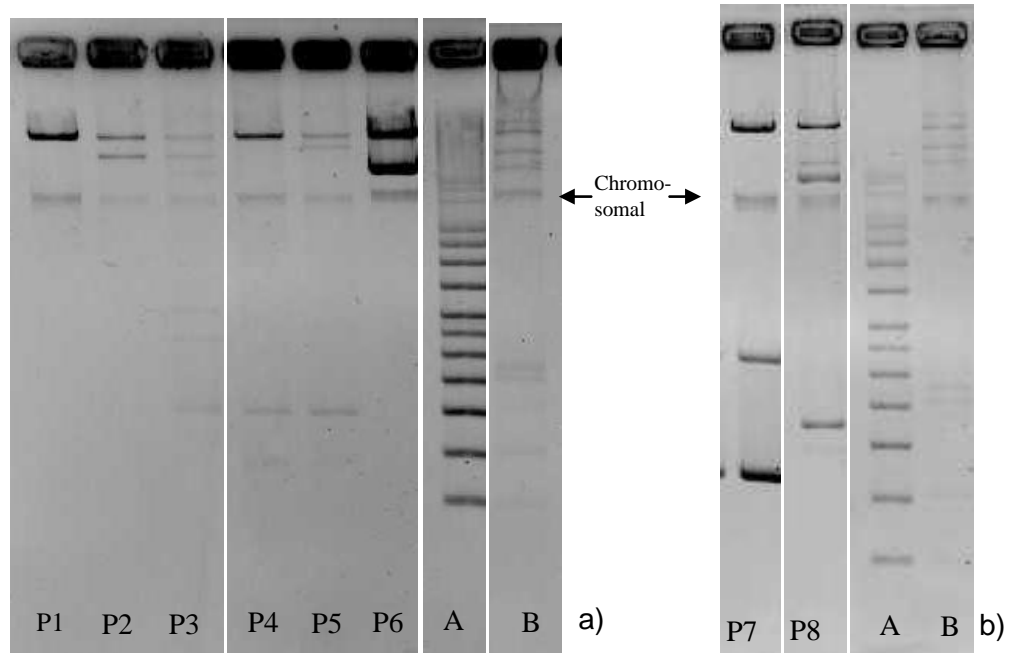
Figure 5.5. *S*. **Typhimurium PT 104 and PT 104a plasmid typing profiles. Example images of each plasmid profile (P1-P8) are shown. Plasmid profiles P1-P6 are shown in a) and P7-P8 are shown in b). Two ladders shown: A: Supercoiled DNA ladder and B: prepared plasmid ladder (see text for details).**

A positive amplification for the *spv*C gene fragment (524 bp) was generated for all PT 104 (and PT 104a) isolates (image not shown). All PT 104 (and PT 104a) isolates harboured a common plasmid (approximately 95.5 Kb) either alone or in combination with smaller plasmids (Table 5.1). Plasmid profile P1 harboured this common plasmid alone, which is the plasmid responsible for carrying the *spv*C gene. Plasmid profile P6 was the most common profile observed.

Table 5.1. Plasmid profiles observed in waterborne isolates of *S*. Typhimurium PT 104.

Plasmid profile	No. of plasmids in profile	Approx. plasmid size (Kb)	No. isolates with profile n = 19
P1	1	95.5	3
P2	2	55.1, 95.5	1
P3	6	4, 6.5, 8.1, 33.8, 55.1, 95.5	1
P4	2	4, 95.5	2
P5	3	4, 66, 95.5	1
P6	2	35.4, 95.5	6
P7	3	3.4, 6.6, 95.5	4
P8	4	4.4, 28.5, 35.4, 95.5	1

Five different banding patterns (X1-X5) were observed in all *S*. Typhimurium PT 104 isolates following PFGE with *Xba*I (Table 5.2). Isolates could be further separated into nine patterns following restriction with *Bln*I (combined genotypic profiles 1-9). Combined with the results of PFGE, the plasmid typing patterns could further separate 19 isolates of PT 104 (and PT 104a) into 13 distinct genotypic profiles (1a – 9a).

Several isolates shared identical *Xba*I and *Bln*I profiles, but different plasmid profiles, between tributaries. For example, isolates with the most common PFGE pattern X1/B2 carried plasmid patterns designated as P1, P4 and P6 (combined genotypic profile 2a-c) in Laurel Creek and Canagagigue Creek at different times throughout the study. Although less common, some isolates showed the same overall combined genotypic profile in the same river over different sampling dates. These included genomic patterns 2c (Isolate No. 15A3, 16A5a and 17A7a) and 6a (3A2 and 4A2). While identical *Xba*I and *Bln*I profiles were observed in isolates from different tributaries, no isolate demonstrated identical combined genotypic profile from different tributaries (Table 5.2).

Table 5.2. Combined genotypic profiles of *S.* Typhimurium PT 104 and PT 104a isolates obtained in three tributaries.

Isolate No.	Trib-utary	Collection Date (M/D/Y)	Var. 5-	Resist-ance Profile	*spv*C gene	PFGE banding pattern *		Plasmid typing pattern	Combined genotypic profile
						*Xba*I	*Bln*I		
14Dd	CAN	07/30/2004	Yes	ACSSuT	+	X1	B1	P6	1a
14Ad	CAN	07/30/2004	Yes	ACSSuT	+	X1	B1	P6	1a
24D1	LC	06/06/2005	No	ACSSuT	+	X1	B2	P1	2a
20A2a	LC	10/21/2004	No	ACSSuT	+	X1	B2	P4	2b
15A3	CAN	08/10/2004	Yes	ACSSuT	+	X1	B2	P6	2c
16A5a	CAN	08/16/2004	Yes	ACSSuT	+	X1	B2	P6	2c
17A7a	CAN	08/23/2004	Yes	ACSSuT	+	X1	B2	P6	2c
15D4	CAN	08/10/2004	Yes	ACSSuT	+	X1	B3	P7	3a
15C4	CAN	08/10/2004	Yes	ACSSuT	+	X1	B3	P7	3a
20C3**	LC	10/21/2004	No	ACS^{I}SuT	+	X1	B4	P3	4a
501a	CON	11/19/2003	No	ACSSuT	+	X2	B1	P1	5a
501	CON	11/19/2003	No	ACSSuT	+	X2	B1	P1	5a
12Ac	CAN	07/21/2004	Yes	ACSSuT	+	X2	B1	P6	5b
12Cc***	CAN	07/21/2004	Yes	ACSSuT	+	X2	B1	P8	5c
3A2	LC	12/10/2003	Yes	ACSSuT	+	X2	B3	P7	6a
4A2	LC	12/13/2003	Yes	ACSSuT	+	X2	B3	P7	6a
19C3	LC	10/18/2004	No	ACSSuT	+	X3	-	P5	7a
20C1	LC	10/21/2004	No	ACSSuT	+	X4	-	P2	8a
19A2a	LC	10/18/2004	No	ACSSuT	+	X5	-	P4	9a

* Distinct profiles were designated with the letter X (XbaI digests) or B (*Bln*I digests) in accordance with the restriction patterns
** Isolate designated as phagetype 104a.
*** Isolate demonstrated ampicillin resistance on a conjugative plasmid (8.1 Kb, TEM-1 plasmid).

Overall, PT 104 isolates from Laurel Creek showed the greatest variety of PFGE and plasmid patterns. For example, PFGE patterns X3, X4, X5 and B3, and plasmid profiles P2, P4 and P5 were unique to Laurel Creek isolates.

Similar to all *S*. Typhimurium PT 104 isolates, isolates designated as Var. 5- all contained, at a minimum, a large (95.5 kb) virulence plasmid. Although variant and non-variant strains commonly shared similar or identical PFGE patterns (e.g., X2/B1 and X1/B2), they never demonstrated the same combined genotypic pattern, regardless of the river system from which they were collected, as similarities between plasmid typing patterns were not observed.

5.5 Discussion

Phagetypes of *S*. Typhimurium and *S*. Heidelberg obtained from tributaries

Waterborne isolates of *S*. Typhimurium and *S*. Heidelberg demonstrated a diverse range of phagetypes. Overall, eight separate *S*. Typhimurium phagetypes were observed in water samples, with PT 104 being the most common. Six different phagetypes of *S*. Heidelberg were observed with the most common being PT 19.

Within Canada, PT 104 and PT 19 represent the most common *S*. Typhimurium and *S*. Heidelberg phagetypes obtained from human clinical samples, respectively (PHAC, 2007c; Government of Canada, 2003; 2005; 2006; 2007). Similarly, these phagetypes are also the most common reported in non-human isolates in Canada, where *S*. Typhimurium PT 104 is frequently obtained from cattle and swine and *S*. Heidelberg PT 19 is commonly found in chicken (Government of Canada, 2005; 2007). Both of these phagetypes have also been reported in a variety of other animals in Canada, although at a lower frequency, including PT 19 in other avian species, horses and dogs; and PT 104 in chicken and horses (PHAC, 2007c). Unlike the current research study, few studies have reported the predominance of these phagetypes in water. However, *S*. Typhimurium PT 104 has been reported in shellfish off the coast of Spain (Martinez-Urtaza *et al*., 2004) and more recently in water samples from the Grand River (PHAC, 2007a; 2007b), although in both instances this phagetype was not found to predominate overall.

Similar to the observations in waterborne isolates in this study, both *S*. Typhimurium PT 104 and *S*. Heidelberg PT 19 are often associated with drug resistance in human and animal isolates (Government of Canada, 2005; 2007). As previously mentioned

(Chapter 3), all waterborne *S*. Typhimurium PT 104 isolates demonstrated the classical resistance pattern ACSSuT (Figure 5.3) commonly reported in isolates obtained from humans and animals (Government of Canada, 2005; 2007). Five of the seven waterborne isolates of *S*. Heidelberg PT 19 demonstrated AMR, all of which carried resistance to ampicillin on conjugative plasmids (Figure 5.2). Both of these predominant phagetypes appeared to be ubiquitous, as they were observed in all three tributaries, at different sample locations and at various times of the year (Figure 5.1 and Figure 5.2).

Several other phagetypes of *S*. Typhimurium and *S*. Heidelberg that were observed in water are also reported in human and animal isolates in Canada. Many of the *S*. Typhimurium phagetypes that are less commonly reported in human and animal isolates in Canada, were also observed at a lower frequency in water. With the exception of UT3, all other phagetypes of *S*. Typhimurium isolated from water were reported in human or non-human sources in Canada during the time of sample collecting (PHAC, 2007c). With the exception of PT 46, all other phagetypes of waterborne *S*. Heidelberg were reported in human clinical isolates in Canada during the time of this study (PHAC, 2007c). PT 26, which was the second most common phagetype in waterborne isolates (obtained from LC and CAN), was reported to be among the top five phagetypes observed in human isolates in Canada (PHAC, 2007c). All *S*. Heidelberg phagetypes obtained from water samples have been reported in non-human isolates (PHAC, 2007c).

Similarities between predominant phagetypes in water and those reported in humans and farm animals signify that water may play a role in the movement of these strains between host animals. Knowledge of the genetic relatedness of these waterborne isolates, particularly those of clinical health importance, such as PT 104, would aid in understanding if clones are circulating within and/or between hosts in these tributaries or if genetic diversity exists among strains. This work is described below.

Genetic relatedness of waterborne *S*. Heidelberg

Limited genetic heterogeneity was observed between waterborne isolates of *S*. Heidelberg following digestion with *Xba*I ($\geq$ 86.9% similarity observed between all

isolates). Many isolates demonstrated indistinguishable restriction patterns within and between phagetypes, which demonstrates a close genetic relatedness among phagetypes. In addition, several isolates of the same phagetype, which varied spatially and/or temporally, demonstrated indistinguishable *Xba*I patterns, which might suggest that clones are circulating within and between tributaries. However, with the few isolates obtained and the use of a single enzyme for PFGE, limited assumptions regarding the absolute clonality of *S*. Heidelberg within these waters can be made.

Similar to the current study, following digestion with a single enzyme (*Xba*I), several studies have noted a high degree of genetic relatedness among *S*. Heidelberg isolates obtained from humans and farm-animals in the USA (Nayak *et al*., 2004; Patchanee *et al*. 2008), although information on specific phagetypes was not included. A national study conducted by Zhao *et al*. (2008) that examined *S*. Heidelberg in retail meat in the USA reported a higher degree of discrimination between isolates using a two enzyme analysis (*Xba*I and *Bln*I). While the authors observed many different restriction patterns between isolates, several clones (indistinguishable PGFE patterns) were observed circulating in various poultry meat products throughout the country.

PFGE groupings did not appear to be influenced by resistance genotypes in *S*. Heidelberg, as isolates showing resistance were observed in various clusters throughout the dendogram. Several PT 19 isolates that carried the TEM-1 plasmid shared identical PFGE patterns to other PT 19 isolates that did not carry this plasmid. This may indicate that some strains, while identified as clones through PFGE with *Xba*I, have developed drug resistance and expanded genetically through the acquisition of conjugative plasmids. Following analysis of retail meat products in the USA, Zhao *et al*. (2008) also reported differences in AMR carriage in many *S*. Heidelberg isolates that demonstrated identical PGFE patterns (*Xba*I and *Bln*I). Although plasmid analysis was not carried out, the authors speculated that subpopulations of *S*. Heidelberg clones may be developing through the acquisition of resistance plasmids. A similar conclusion can be drawn with isolates obtained in water, as strains are likely evolving in various host animals in these tributaries.

In this study, in addition to PT 19, several other phagetypes with differing PFGE patterns also carried the TEM-1 plasmid, supporting the notion that the TEM-1 plasmid

is not disseminated by one single *S*. Heidelberg clone and that this plasmid might be endemic within *Salmonella* strains.

Two *S*. Heidelberg isolates (No. 15C3 and 12Ca) representing PT 29a and an untypable phagetype with indistinguishable *Xba*I patterns and identical CMY-2 plasmid restriction patterns (Chapter 4), were obtained on different dates and locations in Laurel Creek. This finding suggests that a clone carrying this CMY-2 plasmid may be found within hosts throughout this watershed and/or could indicate a consistent host source. However, further investigations including the collection and analysis of additional *S*. Heidelberg carrying the CMY-2 plasmids in this tributary would be needed.

Genetic relatedness of waterborne *S*. Typhimurium and monophasic serotypes

PFGE with *Xba*I revealed that waterborne *S*. Typhimurium isolates were more genetically diverse compared to *S*. Heidelberg, with overall similarities in *Xba*I PFGE patterns > 60% (Figure 5.3). Compared to *S*. Heidelberg, *S*. Typhimurium PFGE with *Xba*I revealed a higher degree of genetic heterogeneity between phagetypes. Similar to *S*. Heidelberg, many isolates of the same phagetype shared indistinguishable PFGE patterns. High levels of genetic relatedness and clonality within specific *S*. Typhimurium phagetypes appears to be consistent with other studies that examined isolates from a range of samples, including human clinical samples (Baggesen *et al*., 2000; Guerri *et al*., 2004), fecal samples from farm-animals (Baggesen *et al*., 2000), and shellfish (Martinez-Urtaza *et al*., 2004).

Similar to this study, Martinez-Urtaza *et al*. (2004) demonstrated distinct clustering around phagetypes following PFGE of *S*. Typhimurium from shellfish taken from marine waters off the coast of Spain, with many isolates of the same phagetype exhibiting identical *Xba*I profiles. Martinez-Urtaza *et al*. (2004) suggested that a common source might be responsible for contamination in these waters as isolates with indistinguishable PFGE patterns were obtained at the same location over many different sampling dates. In the current study, strains with identical patterns were observed within the same tributary on different sampling dates, however, these identical patterns were also observed between strains obtained from different tributaries. This result likely indicates that the phagetypes represented by these strains

are highly clonal by nature and that no single source of contamination exists in these tributaries.

Clustering based on *Xba*I PFGE patterns placed most *S.* Typhimurium isolates into one of two distinguishable groups, with one cluster containing PT 104 (cluster B) and the other containing the non-PT 104 phagetypes (cluster A). This demonstrates how genetically distinct PT 104 is from other phagetypes of *S.* Typhimurium. Integrated prophage elements and the *Salmonella* genomic island 1 (SGI-1), which encodes drug resistance genes (Boyd *et al*., 2001; Mulvey *et al*., 2006), are thought to be the differences between MDR PT 104 and non-PT 104 isolates (Cooke *et al*., 2008; Guerri *et al*., 2004). In general, genomic islands in *Salmonella* are thought to contribute to the genetic diversity in this genus (Jacobsen *et al*., 2011).

Var. 5- strains of *S.* Typhimurium, which lack the O:5 antigen (Rabsch *et al*., 2002; Frech *et al*., 2003), did not cluster together following *Xba*I digestion, but were throughout the dendogram among other non-variant strains (Figure 5.3). Var. 5- and non-variant strains commonly showed identical *Xba*I patterns and tended to cluster around the phagetype classification. This is consistent with the findings of Zhao *et al*. (2007) who reported identical PFGE patterns among Var.5 – and non-variant isolates from a diverse group of farm-animals in the USA. In the waterborne isolates, it was common to see Var. 5- and non-variant isolates with identical *Xba*I patterns from different tributaries. This may indicate that limited genetic variability exists between Var. 5- and non-variant strains, regardless of the original source of the strain. Further discussion follows below with regard to PT 104 isolates.

Several monophasic isolates that were suspected to be related to *S.* Typhimurium based on their Kauffman-White scheme were assessed for genetic relatedness. Monophasic serotype I:4, 5, 12:i:-, which has been observed with an increased frequency in human cases of salmonellosis in North America in recent years (CDC, 2007a; PHAC, 2007c), clustered among *S.* Typhimurium serotypes and not other monophasic isolates following *Xba*I digestion (Figure 5.3). These results support the fact that monophasic serotype I:4, 5, 12:i:- is a variant of serotype Typhimurium (Guerri *et al*., 2004; Alcaine *et al*., 2006; CDC, 2007a)

The most common monophasic serotype obtained from water in this study, I:4, 5, 12:b:- (n=11), showed a high degree of genetic similarity following digestion with *Xba*I. This serotype, which is found in humans (CDC, 2007a; PHAC, 2007c), was ranked the 7th most common *Salmonella* serotype in human isolates in Ontario during the time of this study (PHAC, 2007c). The PFGE patterns generated by waterborne isolates demonstrated a ≥ 85% similarity between isolates, seven of which showed identical PFGE patterns. This monophasic serotype differed from other *S*. Typhimurium isolates and grouped within a distinct cluster (Cluster C, Figure 5.3). Similarity between cluster C and the other clusters was ~ 65%. These results suggest that I:4, 5, 12:b:- is genetically distinct from other *S*. Typhimurium isolates obtained. Interestingly, all serotype I:4, 5, 12:b:- isolates taken from fecal samples of ducks in February of 2004 showed indistinguishable *Xba*I patterns, which were also identical to waterborne isolates obtained in March and May of the same year within the same tributary (LC). This finding supports the suggestion that ducks are contributing to some level of *Salmonella* contamination in Laurel Creek.

Unlike S. Heidelberg, PFGE groupings were influenced by resistance genotypes in *S*. Typhimurium. All isolates in Cluster B demonstrated the resistance profile, ACSSuT. Zhoa *et al*. (2007) observed a similar trend in isolates obtained from various diseased animals in the USA, where clustering was also observed around isolates demonstrating this resistance profile. Unfortunately, no phagetyping information was reported in that study. The observed clustering of isolates demonstrating the ACSSuT profile is related to the predominant phagetype in this cluster, PT 104. Guerri *et al*. (2004) observed that PT 104 isolates that bear SGI-1, a large region of DNA which harbours the genes responsible for drug resistance in this phagetype (Boyd *et al*., 2001; Mulvey *et al*., 2006), clustered together following digestion. Isolates that did not contain SGI-1, and therefore did not display the classical resistance profile ACSSuT, did not cluster together. In recent years, the SGI-1 has also been observed in other phagetypes (Guerri *et al*. 2004), which might be the reason why serotype I:4, 5, 12:i:- PT 120 also demonstrated the ACSSuT profile and clustered with PT 104 isolates. Further analysis of this isolate would be needed to determine the presence of SGI-1.

Pulsed-field gel electrophoresis and plasmid typing in waterborne *S.* Typhimurium PT 104

Following treatment with *Xba*I, PFGE patterns revealed that 18 of the 19 PT 104 isolates obtained from water were found within the same cluster (Cluster B) and demonstrated a > 85% similarity. Similar to the current study, highly homogeneous PFGE patterns in PT 104 from epidemiologically unrelated isolates have been observed by other researchers (Baggesen *et al.*, 2000; Kim *et al.*, 2004; Guerri *et al.*, 2004; Martinez-Urtaza *et al.*, 2004; Cooke *et al.*, 2008).

Multiple drug resistant PT 104 is often described as a clonally spread pathogen, which generally results in non-discriminating PFGE patterns (Baggesen *et al.* 2000; Guerri *et al.*, 2004; Doran *et al.* 2005), particularly when a single-enzyme is used for PFGE. In this study, to help further understand the diversity among PT 104 isolates in different river systems, any PT 104 isolate that produced similar *Xba*I patterns were further digested with *Bln*I. As well, all isolates were subjected to plasmid typing analysis.

Five banding patterns were observed in *S.* Typhimurium PT 104 isolates following PFGE with *Xba*I and further separated into nine patterns with the use of *Bln*I (Table 5.1). Combined with the results of PFGE, the plasmid typing could further separate the 19 PT 104 isolates into 13 different patterns (Table 5.1), thus further showing the genetic diversity of PT 104 isolates in these tributaries.

PT 104 isolates appeared to have very diverse patterns of plasmid carriage. Overall, 8 different plasmid typing patterns (P1 to P8) were exhibited with the number of plasmids ranging from 1 to 6 per isolate. It was observed that many PT 104 isolates showed identical PFGE patterns but different plasmid patterns. Similar or identical PFGE patterns in isolates demonstrating different plasmid profiles suggests that a clone may be diverging through the addition or loss of plasmids over time.

In a few instances, the same overall genotypic profile (profile 2c and 6a) was observed in different isolates in the same tributary over several sampling dates, suggesting a common or consistent source within the river. Although there were isolates that showed identical PFGE patterns between river systems, there were never isolates that showed

the identical overall genotypic profile (PFGE and plasmid patterns) between river systems. This indicates that PT 104 isolates from one watershed, although demonstrating identical drug resistance patterns, are genetically distinct from those in nearby watersheds.

The PT 104 isolates obtained from the urban tributary demonstrated the greatest diversity in PFGE and plasmid patterns, which is likely a reflection of the large variety of host species that might act as a reservoir for this pathogen in this urban watershed (as discussed in Chapters 2 and 3). While commonly observed in human and domestic farm animals (Government of Canada, 2004; Farzan *et al*. 2008), not much is known about the distribution of PT 104 in wildlife and domestic pets in Canada. However, earlier studies have demonstrated the broad host range of this phagetype (Poppe *et al.* 1998).

Similar to observations in the waterborne isolates, previous studies have shown that *S.* Typhimurium PT 104 Var. 5- strains commonly exhibit similar resistance and PFGE patterns to other non-variant PT 104 isolates (Frech *et al*., 2003; Zhao et al., 2005). For example, Frech *et al*. (2003) observed identical *Xba*I patterns among variant isolates of PT 104 obtained from a variety of animals and could only observe minor variability following digestion with other enzymes, including *Bln*I. A similar observation was made in waterborne isolates in this study as digestion with *Xba*I and *Bln*I revealed few differences, however, plasmid typing revealed that variant and non-variant strains never carried the same plasmid-typing patterns. While similar PFGE patterns may indicate the genetic stability of these isolates, the accumulation of different plasmids might be a reflection of the evolution of variant and non-variant strains in different animal hosts.

5.6 Conclusions

The results of this research highlight the genetic relatedness of waterborne isolates of *Salmonella* Typhimurium and Heidelberg and offers insight into the complexity of these isolates circulating within and between tributaries.

Waterborne *S*. Typhimurium isolates demonstrated a greater genetic heterogeneity compared to *S*. Heidelberg following PFGE with *Xba*I. Among *S*. Typhimurium isolates, PT 104, which displayed the classical resistance pattern ACSSuT, appeared to be genetically distinct from other phagetypes of *S*. Typhimurium. As a result of the genetic relatedness of many isolates, the use of a two-enzyme PFGE analysis, particularly for *S*. Heidelberg isolates, is warranted to more accurately define the genetic variability in waterborne *Salmonella*.

Following PFGE with *Xba*I, monophasic isolates of serotype I:4, 5, 12:i:- shared significant homology with *S*. Typhimurium isolates indicating that these strains are most likely variants of the serotype *S*. Typhimurium, whereas the most commonly obtained waterborne monophasic serotype, I:4, 5, 12:b:-, appeared to vary genetically from other *S*. Typhimurium isolates. Serotype I:4, 5, 12:b:- strains taken from water samples were found to be genetically indistinguishable when compared to several isolates obtained from waterfowl samples in Laurel Creek, illustrating the direct link between wildlife impacts on water quality in these urban waters.

The commonly described clonal nature of PT 104 in the literature, as well as the identical phenotypic AMR profiles (ACSSuT) observed in waterborne isolates, was highly suggestive that clones were most likely circulating within and between these tributaries. Digestion with *Xba*I alone demonstrated limited diversity in PT 104, however, additional analysis with *Bln*I and plasmid-typing revealed a greater diversification in PT 104 in these watersheds.

Plasmid-typing allowed for the greatest differentiation between PT 104 isolates obtained in water, and proved to be particularly valuable in differentiating Var. 5-strains from non-variant strains. Many PT 104 isolates from different tributaries exhibited identical PFGE profiles, which suggest that clones may be circulating within

host animals in these tributaries. However, further analysis of these isolates revealed that plasmid profiles differed between tributaries, suggesting that PT 104 strains are distinctly different between these streams.

PFGE typing was not biased by the resistance genotype in *S*. Heidelberg, however, this association was observed in *S*. Typhimurium, in particular isolates demonstrating the resistance profile ACSSuT. This difference is a reflection of how these serotypes differ in their carriage of AMR genes, with waterborne *S*. Heidelberg isolates carrying AMR-genes predominantly on plasmids, which are not easily differentiated through PFGE, whereas certain *S*. Typhimurium phagetypes, including PT 104, carry AMR genes chromosomally on a genomic island which is reflected in the PFGE profiles.

Molecular typing methods, including PFGE, provided information on the genetic diversity of several serotypes of waterborne *Salmonella*, however, without examining the genetic relatedness of isolates originating from humans and animals in these watersheds, few epidemiological connections can be made presently.

5.7 Recommendations and future research needs

- Additional analysis of *S*. Heidelberg using several enzymes for PFGE and plasmid typing might aid in further differentiating among isolates in these watersheds.
- Comparison of PFGE results to those currently in the PulseNet Canada database should be conducted to understand if unique profiles exist in water, or if profiles correspond to those in humans and/or animals in Canada.
- Future analysis of locally derived isolates from humans and farm-animals may also help to determine if an epidemiological connection exists between these sources and water.
- Further detailed genetic analysis should be conducted on select isolates. Genome sequencing and comparison through bioinformatics approaches may help to understand the relatedness of isolates obtained from the environment and those from host animals.

6

Thesis conclusions & recommendations

The ubiquitous nature of *Salmonella* in the streams included in this research suggests that the aquatic environment is a reservoir for this bacterium, and therefore could be involved in the transport and dissemination of this pathogen between host animals. Predominant serotypes, phagetypes and antimicrobial resistance (AMR) profiles observed in *Salmonella* isolates from these streams were similar to those reported in human and farm animals living in the study area, as well as in Canada. The presence of clinically relevant *Salmonella* serotypes in natural waters has been previously documented, however, the predominance of these serotypes in the current study is rarely reported by others. These similarities indicate that water may play a role in the transmission of *Salmonella* to the population.

Efficient methods are needed for the analysis of waterborne pathogens, particularly when strains are present sporadically and in low concentrations. The method of sample collection and type of analysis used for the isolation of bacterial pathogens, such as *Salmonella*, from natural waters is critical and should be chosen to reflect the needs of the study. For example, the swab collection method used in this research can help in the collection of many isolates to determine serotype occurrence and predominance, however, this method is not appropriate if a quantitative assessment is needed. Similarly, detection using molecular (i.e., PCR) and or culture based analysis (i.e., solid and liquid culture media), should also be chosen based on the study needs. In this

research, the recovery of isolates was critical to characterizing the serotypes present in water and was the only way to determine if functional antimicrobial resistance was occurring in waterborne isolates. Based on the results of this study, the use of several media combinations for the isolation of *Salmonella* is recommended. Selenite cystine, while included as a medium in Standard Methods, may not be optimal for the isolation of *Salmonella* from water.

The common occurrence of drug resistant *Salmonella* strains in water may have important implications with regard to the spread and persistence of resistance in the environment. While national surveillance programs examining antimicrobial resistance in *Salmonella* tend to focus on farm animal and human sources, the results of the present study suggest that analysis of environmental sources, including stream water, might provide additional insight into the occurrence, emergence and spread of drug resistance. The natural aquatic environment provides an opportunity for the spread of AMR isolates to host animals that are generally not subjected to antimicrobials, in particular wildlife, but which might then in turn amplify the incidence of drug resistance in the environment.

The occurrence of waterborne *Salmonella* isolates carrying conjugative plasmids conferring the *bla* $_{CMY-2}$ gene is concerning as the spread of this gene can create problems for treating invasive infections in the human population. Many questions remain as to the future extent of the spread of resistance to 3rd generation cephalosporins. However, considering that the plasmids conferring the *bla* $_{CMY-2}$ gene were found in a variety of host serotypes, could transfer through conjugation, and exhibited limited fitness costs on host cells, it is likely that the incidence of resistance to these drugs in environmentally derived strains will increase over time. The long-term stability of plasmids encoding *bla* $_{CMY-2}$ genes under non-selective conditions suggests that even if general usage of 3rd generation cephalosporin drugs is stopped, conjugative plasmids may still persist in bacterial populations.

Urban tributaries, without point-sources of fecal contamination (e.g., sewage treatment effluent), are often overlooked as a source of waterborne pathogens. This is particularly true when regulations, such as the Clean Water Act in Ontario, target land-use activities without the examination of water quality. In Ontario, the protection of waters upstream of an intake will largely focus on watersheds designated as agricultural, as the land-use

activities associated with agricultural operations including the production, storage and land-application of manure are captured as drinking water threats under the Clean Water Act. As observed in this research, the urban tributary (Laurel Creek) demonstrated a similar frequency of *Salmonella* detection and occurrence of drug resistance compared to the agricultural-rural tributaries (Canagagigue Creek and Conestogo River). However, isolates obtained from the urban tributary revealed a greater diversity of serotypes and resistance profiles, including resistance to 3rd generation cephalosporins. Small urban watersheds can be an important source of pathogens, particularly pathogens such as *Salmonella* that have broad host ranges including wildlife. These results indicate that protecting urban waters may be as important as protecting waters impacted by agricultural activities.

Seasonal variability, as opposed to hydrological events, appeared to be a predominant factor in the overall diversity and predominance of serotypes of human health significance (*S*. Typhimurium and *S*. Heidelberg) in water. This research revealed that the lower occurrence of these serotypes in water in the colder months (February and March) was not the result of a lower survival rate in colder water temperatures compared to other serotypes. The seasonal trend in the occurrence of *S*. Typhimurium and *S*. Heidelberg was pronounced in the agricultural/rural tributaries, as opposed to the urban tributary. These differences might reflect changes in farm practices in the cooler months or the seasonal shedding of certain serotypes in domestic farm animals. These findings suggest that the implementation of seasonal management practices in agricultural watersheds could help to reduce the peak loading of these strains to the watercourse. These practices could include reducing the seasonal shedding of pathogens within animals themselves, as well as the pre-treatment of any manures prior to spreading at particular times of the year. In contrast to both the agricultural/rural streams, the consistent occurrence of these serotypes in the urban stream, particularly in the winter months, may reflect the continuous shedding/loading of these strains by wildlife and/or outputs from other non-point sources. The findings indicate that year round management practices would be needed in small urban streams, which could contribute to the protection of drinking water sources and recreational waters downstream.

A combination of genotypic and phenotypic markers was useful in studying variation among salmonellae populations in water and offered insight into the complexity of

strains circulating within and between tributaries. The commonly described clonal nature of *S*. Typhimurium PT 104 in the literature, as well as the identical phenotypic AMR profiles (ACSSuT) observed in waterborne isolates, was highly suggestive that clones were most likely circulating within and between these tributaries. However, diversity could be observed in waterborne isolates following pulsed field gel electrophoresis with two enzymes and plasmid-typing. The results showed that strains never shared the identical overall genotypic profile (PFGE and plasmid patterns) between river systems, indicating that PT 104 isolates from one watershed, although demonstrating identical drug resistance patterns, are genetically distinct from those in nearby watersheds. These results suggest that PT 104 isolates are not being introduced from nearby watersheds, for example through migratory birds and waterfowl, but may be evolving separately within hosts in each tributary. The greater genetic diversity observed in PT 104 isolates from the urban tributary, compared to the agricultural/rural tributaries, indicates that a variety of animals in this watershed may be hosts to this strain and contribute to the loading of this MDR pathogen to these waters.

As observed in this research, the ubiquitous nature of *Salmonella* in water, the presence of serotypes of human and veterinary health significance, as well as the long-term survival of *Salmonella*, suggests that environmental exposure through consumption or contact with contaminated water is plausible. Many factors remain unknown regarding the source of sporadic cases of salmonellosis and if water represents a transmission route related to a portion of these cases. To determine if an epidemiological connection exists, further examination of waterborne isolates compared to those found in food, humans, and animals is needed. This should include a genetic evaluation of isolates through a program such as PulseNet Canada. Examining isolates from the Grand River presents a unique opportunity as detailed assessments of isolates from other sources is currently being conducted through the C-EnterNet program.

Understanding the risk that waterborne pathogens pose to the population should be based on the detection and characterization of these pathogens in water, rather than on a lack of evidence of drinking water or recreational water outbreaks. This is particularly true for pathogens such as *Salmonella* that are considered primarily foodborne, but are found to be ubiquitous in water sources such as the tributaries included in this research and likely in many comparable temperate watersheds impacted by similar land uses. The use of risk assessment models, using quantitative data for *Salmonella* in water, can

186

help to make these associations. The next logical step in this research would include an evaluation of the concentrations of *Salmonella* in these streams followed by a quantitative microbial risk assessment. This type of analysis can add further context to the study of pathogen occurrence in water and how the levels of contamination might impact human health.

As our understanding of the occurrence of waterborne pathogens increases, as well as the implementation of risk based assessment advances, our knowledge of the overall contribution of waterborne pathogens, such as *Salmonella*, to enteric disease and its impacts on human health will expand.

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Appendix A

Dates are listed below in which samples were taken in each tributary throughout the study. Grey highlights indicate when a sample was taken and analyzed for *Salmonella* presence. The white boxes represent times when no sample was collected or if the swab was lost in the water prior to collection. In 2005 sample collection only occurred in LC.

**Table A.1. Dates (highlighted in grey) in which swabs were analyzed from each tributary**

Year	2003				2004							
Date	Nov 13-19	Nov 19-21	Dec 6-10	Dec 10-13	Feb 9-11	Mar 6-9	Mar 9-13	May 4-7	May 7-12	May 27-31	May 31-Jun 4	Jul 16-21
LC	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓
CON	▓		▓	▓	▓			▓	▓	▓	▓	
CAN	▓	▓	▓	▓		▓	▓	▓	▓	▓	▓	▓

Year	2004 (con't)									2005		
Date	Jul 26-30	Aug 5-10	Aug 10-16	Aug 19-23	Aug 23-26	Oct 13-18	Oct 18-21	Nov 15-18	Nov 18-22	May 26-30	June 3-6	Jun 17-20
LC	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓
CON	▓	▓	▓	▓	▓	▓	▓	▓	▓			
CAN	▓	▓	▓	▓	▓	▓	▓	▓	▓			

Table A.2. Water quality parameters for each tributary.

Stream	Date range for data	Temperature range during ice free times (°C) (Mar to Nov)	Turbidity min/max/ average (NTU)	Total Nitrogen KJELDAHL (mg/L) (mean ± stdev)	Total Phosphorous, unfiltered (mean ± stdev)	pH (mean ± stdev)
Laurel Creek	2007-08	2.0 – 22.7	ND	0.9 ± 0.43	0.12 ± 0.12	8.14 ± 0.23
Canagagigue Creek	2003-05	4.1 – 25.2	1.5/67/21	1.56 ± 0.52	0.14 ± 0.09	8.34 ± 0.12
Conestogo River	2003-05	4.3 – 26.5	1.6/83.5/15.3	0.82 ± 0.29	0.10 ± 0.11	8.48 ± 0.23

ND = no data
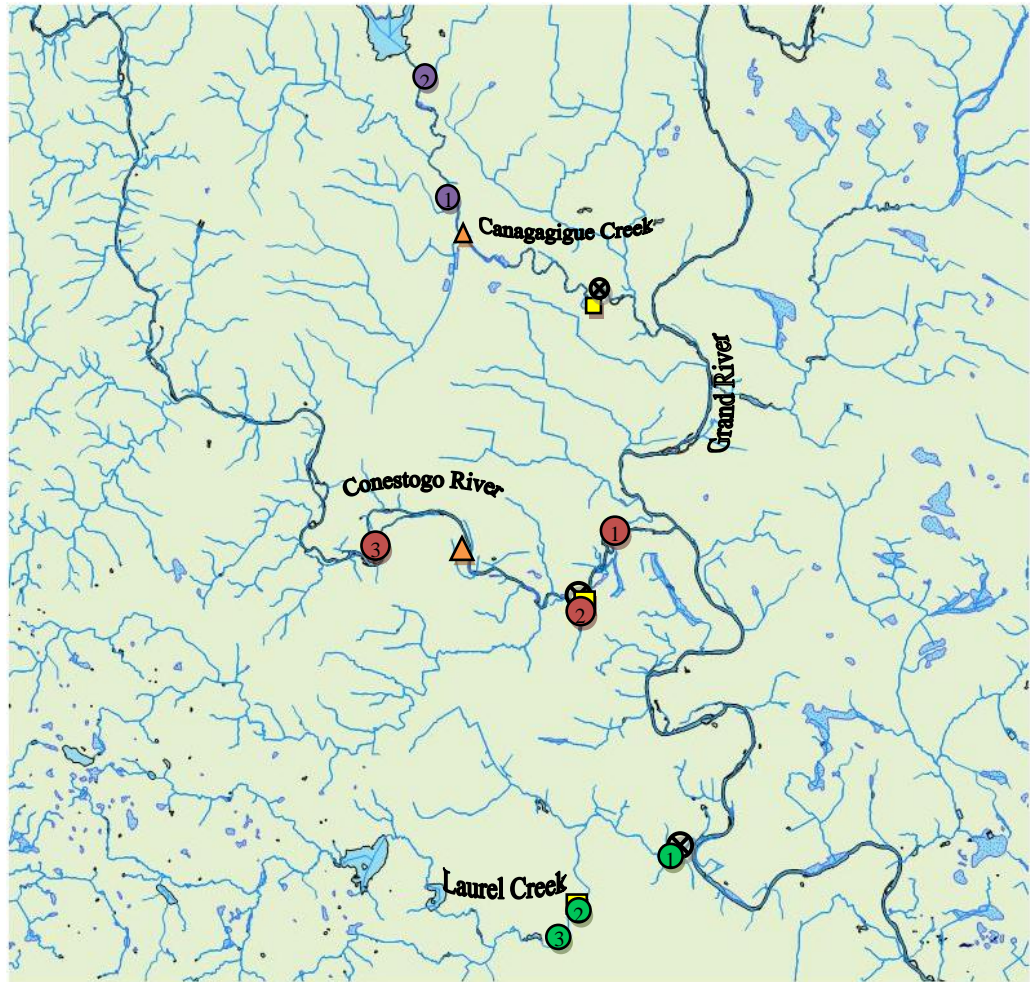
- ▲ Sewage treatment facility
- ▢ Level or flow gauge station
- ⊗ PWQMN station

Figure A.1. Detailed map of sample locations, sewage treatment facilities and points where other measurements were taken.

Table A.3. Georeferencing for each sample location.

| Stream | Station Name | Georeferencing (UTM coordinate position)* | |
		Easting	Northing
Canagagigue Creek	CAN -1	536082	4827572
	CAN - 2	535490	4829330
Conestogo River	CON - 1	539969	4820765
	CON - 2	539232	4819251
	CON - 3	534501	4820392
Laurel Creek	LC - 1	541909	4814601
	LC - 2	539300	4813355
	LC - 3	539223	4813294

*All in zone 17
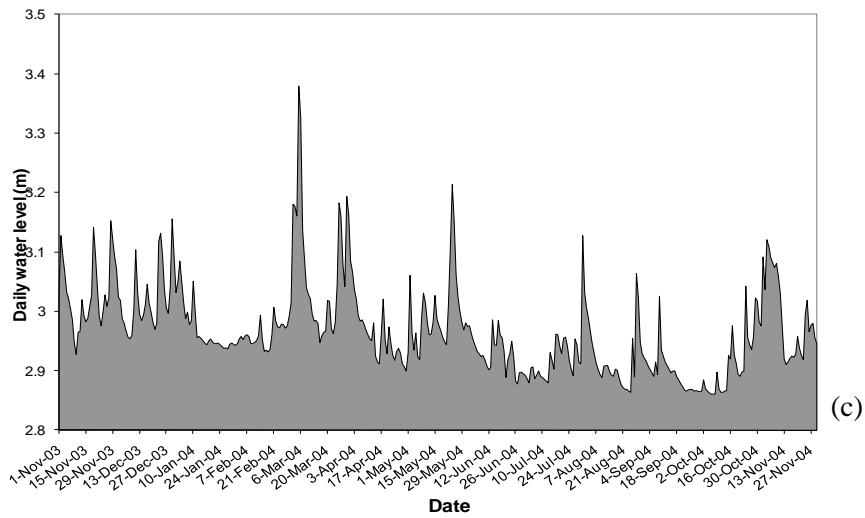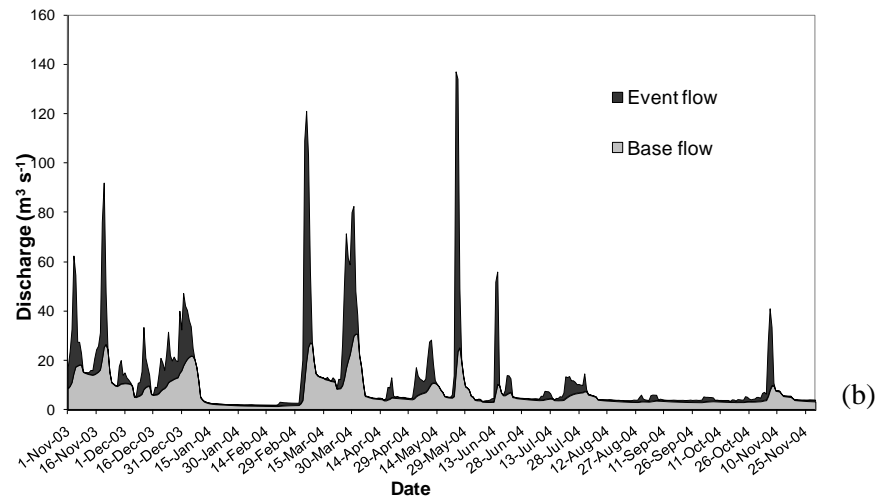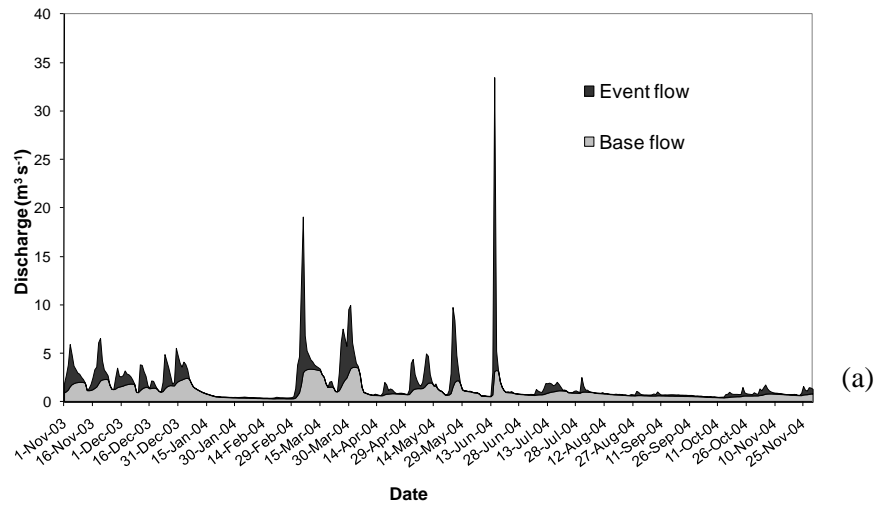
Figure A.2. Discharge from November 2003-2004 in Canagagigue Creek (a) and Conestogo River (b), with base flow separation shown. Level data are shown for Laurel Creek (c).

212

Table A.4. Differences observed between selective media expressed as *P* values following Chi-square Test. Significant values are in bold text.

Selective media	MSRV no novo	XLD	MSRV w novo	BGA	BS	BGSA
MSRV no novo	-	**0.009**	**0.005**	**0.003**	**< 0.001**	**< 0.001**
XLD		-	1	0.873	0.417	**< 0.001**
MSRV w novo			-	1	0.515	**< 0.001**
BGA				-	0.623	**< 0.001**
BS					-	**< 0.001**
BGSA						-

Table A.5. Differences between media combinations expressed as *P* values following Fisher`s Exact Test. Significant values are in bold text.

Enrichment broth	Selective media	Tet						RV						SC					
		BS	BGA	BGSA	XLD	MSRV w novo	MSRV no novo	BS	BGA	BGSA	XLD	MSRV w novo	MSRV no novo	BS	BGA	BGSA	XLD	MSRV w novo	MSRV no novo
Tet	BS	-	0.180	**0.004**	0.789	0.285	0.058	1	1	**0.004**	0.789	0.080	0.593	**< 0.001**	**< 0.001**	**< 0.001**	**0.037**	0.582	0.593
	BGA		-	**< 0.001**	0.418	1	0.775	0.282	0.180	**< 0.001**	0.418	**< 0.001**	0.588	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.031**	0.588
	BGSA			-	**< 0.001**	**< 0.001**	**< 0.001**	**0.002**	**0.004**	1	**< 0.001**	0.422	**< 0.001**	1	0.491	**< 0.001**	0.670	**0.040**	**< 0.001**
	XLD				-	0.591	0.171	1	0.789	**< 0.001**	1	**0.023**	1	**< 0.001**	**< 0.001**	**< 0.001**	**0.009**	0.277	1
	MSRV w novo					-	0.573	0.422	0.285	**< 0.001**	0.591	**0.002**	0.788	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.060	0.788
	MSRV no novo						-	0.102	0.058	**< 0.001**	0.171	**< 0.001**	0.270	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.007**	0.270
RV	BS							-	1	**0.002**	1	**0.04367**	0.790	**< 0.001**	**< 0.001**	**< 0.001**	**0.019**	0.412	0.790
	BGA								-	**0.004**	0.789	0.080	0.593	**< 0.001**	**< 0.001**	**< 0.001**	**0.037**	0.582	0.593
	BGSA									-	**0.001**	0.422	**< 0.001**	1	0.491	**< 0.001**	0.670	**0.040**	**< 0.001**
	XLD										-	**0.023**	1	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.277	1
	MSRV w novo											-	**0.011**	0.193	0.051	0.193	1	0.355	**0.011**
	MSRV no novo												-	**< 0.001**	**< 0.001**	**< 0.001**	**0.004**	0.177	1
SC	BS													-	1	1	0.352	**0.012**	**< 0.001**
	BGA														-	1	0.112	**0.002**	**< 0.001**
	BGSA															-	0.352	**0.012**	**< 0.001**
	XLD																-	0.205	**0.004**
	MSRV w novo																	-	0.177
	MSRV no novo																		-

213