The Genetic Characterization of Clinical and Agri-Food Multi-Drug Resistant *Salmonella enterica* serovar Heidelberg

by

Ashley Andrysiak

A Thesis submitted to the Faculty of Graduate Studies at
The University of Manitoba

In partial fulfilment of the requirements of the degree of
MASTER OF SCIENCE

Department of Medical Microbiology

University of Manitoba

Winnipeg

Copyright © 2007 by Ashley Andrysiak

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

The Genetic Characterization of Clinical and Agri-Food Multi-Drug Resistant *Salmonella enterica* serovar Heidelberg

BY

Ashley Andrysiak

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of

Manitoba in partial fulfillment of the requirement of the degree

MASTER OF SCIENCE

Ashley Andrysiak © 2007

Permission has been granted to the Library of the University of Manitoba to lend or sell copies of this thesis/practicum, to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film, and to University Microfilms Inc. to publish an abstract of this thesis/practicum.

This reproduction or copy of this thesis has been made available by authority of the copyright owner solely for the purpose of private study and research, and may only be reproduced and copied as permitted by copyright laws or with express written authorization from the copyright owner.

Abstract

Salmonella enterica serovar Heidelberg ranks amongst the most prevalent causes of human salmonellosis in Canada and an increase in multi drug resistant (MDR) isolates, including resistance to extended spectrum cephalosporins (ESC), has been observed. This study examined the genetic relationship between *S.* Heidelberg isolates from retail, abattoir and clinical samples to determine whether there is a link between the emergence of MDR *S.* Heidelberg from chicken agri-food sources and the increase in the amount of human salmonellosis attributable to *S.* Heidelberg. Chromosomal clonality was observed by Pulsed-field gel electrophoresis, sequence based typing and microarray based comparative genomic hybridization. Plasmid content was responsible for differences in antimicrobial susceptibility and some strain to strain diversity. Replicon typing and restriction fragment length polymorphism analyses identified two divergent plasmid types responsible for ESC resistance. Due to the limited genetic diversity among the isolates, chickens could not be identified as the sole source of human salmonellosis.

Acknowledgements

I would like to thank my supervisor and mentor Dr. Matt Gilmour for the opportunity to obtain a master's degree in an area of medical microbiology which I truly enjoy. Thank you for your invaluable assistance, suggestions and patience. Thank you for teaching me how to use a computer, improve my writing and presentation skills as well as so much more. I would not have achieved so much without your guidance. I would like to thank the present and past members of the Emerging Bacterial Pathogens Group at the National Microbiology Laboratory who have become friends as well as co-workers: Adam Olson, Dobryan Tracz, Keri Trout, Paul Backhouse, Connie Barton and Joanne McCrea. Thank you for making the lab an enjoyable place to work and for sharing your knowledge. Thank you to the PulseNet section at the National Microbiology Laboratory for performing PFGE. Thank you to the Office of the Chief Scientist, Dr. Lai King Ng and CIHR for study funding and stipend support. Thank you to the members of my committee: Dr. John Wylie, Dr. Rick Holley and Dr. Lai King Ng for valuable input and suggestions. Thanks to both the University of Manitoba department of graduate studies and department of medical microbiology for travel funding. Thank you to Phuong Nam Restaurant for providing food. Thank you to my mom, dad, sister, grandfather and the poochon for your never ending support. Thank you for putting up with journal articles spread out all over the kitchen and bad moods during exam periods and deadlines. Thank you to all of the great friends I have made during grad school, Melissa McCracken, Rhiannon Harris, Miles Majcher, and Jamie Borlang. Grad school would not have been the same without you. To close friends Sarah Cahill, Sara Reeves, Andrea Ormiston, Vanessa Parks, Bomediene Soufi and Antonella Sciangula thank you for always being there. To my best friend and my love Lev Kearney, thank you for your love and support throughout everything. I love you.

Foreward

This thesis is divided into four chapters and one appendix:

Chapter one is an introductory chapter which gives an overview of the current literature regarding *Salmonella* and includes a discussion about antimicrobial resistance in *Salmonella* as well as a discussion about the current methods which are used for typing and subtyping this organism. The chapter concludes with a brief description of the current study.

Chapter two details the materials and methodology used throughout the study.

Chapter three details the results of the study.

Chpater four is a discussion and interpretation of experimental results. The chapter finishes with a paragraph that summarizes the conclusions that can be drawn from this study and discusses their contribution to the field of study.

The appendix is composed of two published articles which include:

Gilmour, M.W., Tracz, D.M., Andrysiak, A.K., Clark, C.G., Tyson S., Severini, A., Ng, LK. (2006) Use of the *espZ* gene encoded in the locus of enterocyte effacement for molecular typing of shiga toxin-producing *Escherichia coli*. Journal of Clinical Microbiology **44**: 449-58.

Gilmour, M.W., Olson, A.B., Andrysiak, A., Ng, LK., Chui, L.. (2007) Sequence-based typing of genetic targets encoded outside of the O-antigen gene cluster is indicative of Shiga toxin-producing Escherichia coli serogroup lineages. Journal of Medical Microbiology. **56:** 1-9.

These articles detail work performed on an additional project undertaken during the course the Msc. degree. These projects focused on developing a molecular typing method as well as a molecular serogrouping method for shiga toxin-producing *Eshcerichia coli.*

Table of Contents

Chapter 1: Introduction 1
1.1 What are emerging pathogens? 2
1.2 Non-typhoid *Salmonella* 7
1.3 *Salmonella* Heidelberg 8
1.4 Canadian Integrated Program for Antimicrobial Resistance Surveillance 11
1.5 Antimicrobial Resistance 17
15.1 Plasmids 17
1.5.5.1 *Salmonella* Plasmids 18
1.5.2 Integrons 20
1.5.3 Salmonella Genomic Island I 23
1.5.4 Quinolone Resistance 26
1.5.5 β-lactamase Resistance 27
1.5.5.1 Extended Spectrum β-lactamase Resistance 28
1.5.5.2 AmpC β-lactamases 29
1.5.5.2.1 $bla_{cmy\text{-}2}$ Plasmids 30
1.6 The Dissemination and Origin of MDR Resistant *Salmonella* 32
1.7 The Human Health Consequences of Antimicrobial Resistance 35
1.8 Current Techniques for subtyping Salmonella 36
1.8.1 Phage Types 37
1.8.2 Molecular Methods 37
1.8.2.1 Pulsed-Field Gel Electrophoresis 38
1.8.8.2 Multi-locus Sequence Typing 40
1.8.8.3 Comparative Genomic Hybridizations 42
1.9 The Present Investigation 43

Chapter 2: Materials and Methods 44
2.1 Bacterial Strains and Determination of Resistance Phenotypes 45
2.2 DNA Extractions 50
2.3 Pulsed-Field Gel Electrophoresis 51
2.4 PCR 52
2.5 Sequencing 55
2.6 Comparative Genomic Hybridization 55
2.7 MLST 57
2.8 Plasmid Profiles 58
2.9 Electroporation 58
2.10 $bla_{cmy\text{-}2}$ Plasmid Isolation from Transformants 59
2.11 Restriction Fragment Length Polymorphism 59
2.12 Southern Blot 60
2.13 Conjugation 61
2.14 SGI1 Conjugation 61

Chapter 3: Results 63
3.1 Pulsed-Field Gel Electrophoresis 64
3.2 Resistance Gene PCR 72
3.3 Integron Characterization 76
3.4 MLST 79
3.5 Comparative Genomic Hybridization 79
3.6 Plasmid Analysis 82
3.7 RFLP 89
3.8 Replicon Typing and Resistance PCR 94
3.9 Southern Blot 94
3.10 Virulence Gene Detection 99
3.11 Conjugation 99
3.12 SGI1 Conjugation 99

Chapter 4: Discussion 104
4.1 Genetic Variation due to Chromosomal Determinants 105
4.2 Resistance Determinants 107
4.3 Genetic Variation Attributable to the Carriage of Plasmids 109
4.4 Conclusion 112

References 114

Appendix 127

List of Figures and Illustrations

Figure 1: *Salmonella* serovars responsible for human salmonellosis in Canada during 2004 9
Figure 2: Class I integron structure 20
Figure 3: Routes of *Salmonella* dissemination between animals and humans 33
Figure 4: Dendrogram of *S.* Heidelberg DNA macrorestriction patterns generated with *Xba*I 70
Figure 5: Genomic profiles as determined by CGH 80
Figure 6: CGH PCR confirmation 83
Figure 7: Complete plasmid profiles isolated from *S.* Heidelberg 87
Figure 8: A comparison of *S.* Heidelberg RFLP 90
Figure 9: bla_{cmy-2} plasmid *bgl*II RFLP 92
Figure 10: A) *bgl*II plasmid fingerprints 97
B) Southern blot probing for the bla_{cmy-2} gene 97
Figure 11: Sequence of the 18 bp imperfect repeat in the *thd*F gene from *S.* Heidelberg and *S.* Typhimurium 102

List of Tables

Table 1: Estimated illnesses, hospitalizations, and deaths caused by the four most prevalent foodborne pathogens in the United States 5
Table 2: The frequency of *S.* Heidelberg isolated from human and animal sources in 2003 as compared to 2004 13
Table 3: The prevalence of antibiotic resistance in *S.* Heidelberg isolates from human and animal sources in 2003 as compared to 2004 15
Table 4: Bacterial strains included in the study 46
Table 5: *Salmonella* and *E. coli* resistance breakpoints 48
Table 6: Oligonucleotides used and their respective annealing temperatures 53
Table 7: The total number of CIPARS *S.* Heidelberg isolates submitted to PulseNet Canada between 2001 and 2004 divided by source of isolation .65
Table 8: Bacterial strains included in the study 67
Table 9: The resistance phenotype and genotype of *S.* Heidelberg and *E. coli* isolates 73
Table 10: Characterization of Class I integrons from *S.* Heidelberg and *E. coli* 77
Table 11: The log_2 test/reference ratios for genes in the region STM691 - to STM704 85
Table 12: PCR-based replicon typing and resistance gene characterization of S. Heidelberg and *E. coli* bla_{cmy-2} plasmids 95
Table 13: The ability of bla_{cmy-2} plasmids from *S.* Heidelberg and *E. coli* to be transferred by conjugation to recipient *E. coli* RG 192 100

List of Abbreviations

A2C-Amp	Amoxicillin-clavulanic acid, ceftiofur, cefoxitin, and ampicillin
A3C-Amp	Amoxicillin-clavulanic acid, ceftiofur, cefoxitin, cephalothin and ampicillin
AMC	Amoxicillin
AMP	Ampicillin
CDC	Centers for Disease Control and Prevention, Atlanta
CEP	Cephalothin
CGH	Comparative genomic hybridization
CHL	Chloramphenicol
CIHR	Canadian Institutes of Health Research
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CRO	Ceftriaxone
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended spectrum cephalosporin resistance
ESC	Extended cephalosporin resistance
FOX	Cefoxitin
GEN	Gentamicin
HMW	High molecular weight
HCl	Hydrochloric Acid
Inc	Incompatibility group
KAN	Kanamycin

LB	Luria bertani
LFZ	Laboratory for Foodborne Zoonoses, Public Health Agency of Canada
LMW	Low molecular weight
MDR	Multi-drug resistance
MLST	Multi-locus sequence typing
NAL	Nalidixic acid
NaOH	Sodium hydroxide
NARMS	National Antibiotic Resistance Monitoring System
NESP	National Enterics Surveillance Program
NML	National Microbiology Laboratory, Public Health Agency of Canada
ORF	Open reading frame
PCR	Polymerase chain reaction
PHAC	Public Health Agency of Canada
PFGE	Pulsed-field gel electrophoresis
PT	Phage type
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulfate
SGI1	*Salmonella* Genomic Island 1
SMX	Sulfamethoxazole
SSC	Sodium chloride sodium citrate buffer
STR	Streptomycin
SXT	Sulfamethoxazole-trimethoprim
TE	Tris-EDTA

TET	Tetracycline
TIO	Ceftiofur
Vol	Volume

Chapter 1: Introduction

1.1 What are emerging pathogens?

Infectious diseases have been and continue to remain an important cause of morbidity and mortality for human populations. Dramatic examples include the Black Death and the Spanish flu. *Yersina Pestis* biovar *Mediaevalis* was the causative agent of the Black Death in Europe from the 13th to 15th centuries and is estimated to have been responsible for 17 to 28 million deaths (Perry *et al.*, 1997). The influenza A virus was responsible for the Spanish flu pandemic which occurred between 1918 and 1919. This worldwide pandemic is estimated to have caused 50 million deaths (Jawetz and Levinson, 2002). Following advent of improved public health measures, sanitation, antibiotics, vaccines, insecticides and surveillance programs there was a worldwide decrease in the burden of infectious diseases and the ability to control the spread of infectious diseases seemed to be within the grasp of the human population.

Infectious disease caused by viruses, parasites and bacteria continue to cause significant morbidity and mortality the human population each year. The Human Immunodeficiency Virus (HIV) / Aquired Immunodeficiency Syndrome (AIDS) is currently sweeping the globe especially in the developing world. As of 2005, an estimated 33.4 to 46 million people were infected with HIV causing an estimated 2.4 to 3.3 million deaths per year (UNAIDS, 2006). Tuberculosis is re-emerging as a significant human pathogen in both the developed and devolping world and approximately nine million new cases and two million deaths result from this disease each year (WHO, 2006). Typhoid fever caused by *Salmonella enterica* serovars Typhi and Paratyphi A has been almost eliminated as a serious health concern in many parts of the developed world due to improved sanitation and treatment options however it still remains a significant health threat in developing nations such as Southeast Asia, the Indian subcontinent, South America and increasingly Africa (Bhan *et al.*, 2005). An estimated 21.6 million cases of typhoid fever resulting in 220, 000 deaths occur each year (Crump *et al.*, 2004).

While the burden of infectious diseases including HIV, TB, and Typhoid fever is significantly lower in the developed world as compared to the developing world, food borne illnesses remain a significant cause of morbidity in both (Flint *et al.*, 2005). It is difficult to determine the burden of food borne disease in a population as very few cases can be linked to contaminated food products (Flint *et al.*, 2005). However, in the United States alone, food borne infections have been estimated to cause ~76 million illnesses, 325, 000 hospitalizations and 5000 deaths each year (Mead *et al.*, 1999). The most common foodborne bacterial pathogens in the United States and Canada include *Salmonella* serovars, *Shigella* species, *Campylobacter* species, *Escherichia coli* O157:H7, *Escherichia coli* non-O157:H7 STEC, enterotoxigenic *Escherichia coli*, and other diarrheogenic *Escherichia coli*. (Table 1) (Allos *et al.*, 2004; Mead *et al.*, 1999)

In 2001, a total 1415 species including viruses, bacteria, parasites, and fungi had been identified as human pathogens (WHO, 2004). However, this number is constantly increasing, with 35 new pathogens identified between 1972 and 1999 alone (Desselberger, 2000). These new pathogens are referred to as emerging pathogens and can arise in numerous different ways.

An emerging pathogen can represent a newly discovered species or a species linked for the first time to human disease. In 1983, *Helicobacter pylori* was identified as the cause of chronic gastric ulcers and gastric cancers, a condition previously attributed to noninfectious factors including stress and diet (Marshall *et al.*, 1984). Alternatively, the emergence of a significant new strain or trait from an already existing pathogen can result in a new emerging pathogen. The current global epidemic of *Salmonella enterica* serovar Typhimuirum definitive phage type (DT) 104 (hereafter referred to as DT104) is the result of the emergence of a multi drug resistant strain. The designation definitve phage type (DT) is used in the United Kingdom and is equivalent to the designation phage type (PT) used in Canada. DT104 was first described in the 1960's but in the

Table 1: Estimated illnesses, hospitalizations, and deaths caused by the four most prevalent foodborne pathogens (*Salmonella* spp, *Campylobacter* spp., *E. coli* and *Shigella* spp.) in the United States (Mead *et al.*, 1999).

Agent	Illnesses		Hospitalizations		Deaths	
	Total	Foodborne	Total	Foodborne	Total	Foodborne
Campylobacter spp.	2 453 926	1 963 141	13 174	10 539	124	99
E. coli O157:H7	73 480	62 458	2 168	1 843	61	52
E. coli non-O157 STEC	36 740	31 299	1 084	921	30	26
E. coli enterotoxigenic	79 420	55 594	21	15	0	0
E. coli other diarrheogenic	79 420	23 826	21	6	0	0
Salmonella, nontyphoidal	1 412 498	1 341 873	16 430	15 608	582	553
Shigella spp.	448 240	89 648	6 231	1 246	70	14

1980's a new multi drug resistant (MDR) variant harbouring the *Salmonella* genomic island 1 mediated resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline was identified (Threlfall, 2000). MDR-DT104 has since emerged worldwide as the predominant Typhimurium phage type implicated as the cause of human salmonellosis characterized by vomiting, nausea, diarrhea, myalgia and certain extreme cases acute febrile illness and death. Similarily, *Vibrio cholerae* emerged as a widespread human pathogen in 1815 – 1845 when it horizontally acquired a toxin gene along with other genetic factors which allowed it to colonize the human gut. The severe watery diarrhea characteristic of cholera facilitates the spread of the bacteria among human populations especially in regions without adequate sanitation such as refugee camps.

A dramatic increase in the incidence of a known pathogen can also represent an emerging pathogen. The apparent increasing incidence of a pathogen may be attributable to the advent of better diagnostic technique that allow for the accurate detection of pathogen which was previously under represented (Desselberger, 2000). In North America, gastrointestinal infections resulting from *E. coli* species are most often attributed to the O157:H7 serotype. There is a wealth of available of selective media and diagnostic techniques which facilitate the detection of *E. coli* O157:H7 as compared to non-O157 *E. coli*. This may bias detection toward the identification of *E. coli* O157:H7 although non-O157 serotypes including O26, O117, O145, O111 can cause a significant portion of *E. coli* infections (Blanco *et al.*, 1999; Werber *et al.*, 2002). Novel molecular diagnostic techniques may increase the ease of identifying *E. coli* non-O157:H7 serotypes leading to a more accurate account of the distribution of the *E. coli* serotypes responsible for human disease (Appendix 1).

The increase in incidence of a pathogen can result from the creation of a new niche for the pathogens or the development of an environment which allows for

increased human contact with the pathogen. The increase in domestic hot and cold water systems, leisure pools, and water cooled air conditioning systems creates conditions that suit growth of *Legionella* species and may put humans at a greater risk for contracting *Legionella* (WHO, 2004) (Desselberger, 2000). An increase in the incidence of a pathogen can also occur for unknown reasons. The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), a program of the Public Health Agency of Canada, has noted an increasing incidence of MDR *Salmonella enterica* subsp *enterica* serovar Heidelberg from 1995 to 2003 (CIPARS, 2004). Currently, the reason(s) for this increase are not yet fully elucidated.

1.2 Non-typhoid *Salmonella*

Salmonella species are gram negative rod-shaped zoonotic organisms that live in the intestines of mammals, birds and reptiles. Once shed into the environment via the feces of infected hosts, *Salmonella* is able to persist in water, soil and food products for extended periods of time (Angulo *et al.*, 2000). The Kauffman-White scheme delineates an estimated 2,500 serovars within the genus *Salmonella* based upon O-somatic and H-flageller antigens (Popoff and Le Minor, 2001; Popoff *et al.*, 2004). Some serovars are adapted to infect specific hosts, as in the case of *Salmonella enterica* subsp *enterica* serovar Gallinarium which infects only poultry while other serovars have a wider host range and can infect numerous different species (McDermott, 2006). *Salmonella* are responsible for a number of different disease syndromes in humans including asymptomatic colonization, gastroenteritis, severe extra intestinal illnesses (meningitis, septicemia and osteomyelitis) and auto-immune disorders such as Reiter's syndrome (Jawetz and Levinson, 2002). Human *Salmonella* infections usually result from the ingestion of contaminated food products such as milk, beef, pork, poultry, eggs and fresh

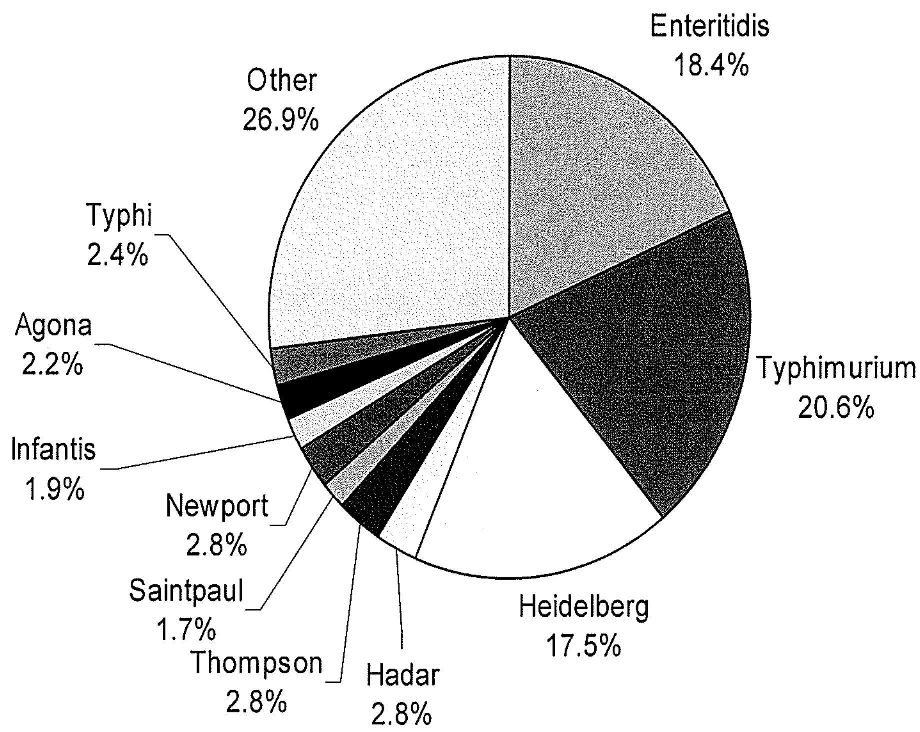
products. In some cases, direct contact with infected animals has also been implicated as a cause of infection (Fey *et al.*, 2000).

Salmonella serovars are one of the leading causes of bacterial food borne illnesses worldwide although the exact incidence remains unknown. In the United States alone, it is estimated that 1.4 million cases of salmonellosis occur each year resulting in at least 17,000 hospitalizations and 585 deaths (Mead, 1999). *Salmonella enterica* subsp *enterica* serovar Typhimurium and Enteriditis are responsible for the majority of human *Salmonella* infections, while other serovars, such as *S.* Heidelberg in Canada, that vary in regional prevalence are responsible for the rest (CIPARS, 2004) (Figure 1). Symptoms of *Salmonella* infections develop 12-48 hours following the consumption of contaminated food products and can consist of a combination of nausea, vomiting, diarrhea, fever, cramping, and myalgia (Jawetz and Levinson, 2002). These infections are normally self-limiting and require minimal treatment beyond fluid and electrolyte replacement therapy. However, if an individual is immunocomprimised at the onset of infection, elderly or a young child they may be at risk of developing a more severe systemic *Salmonella* infection which requires antimicrobial treatment (Jawetz and Levinson, 2002). In the United States, septicemia reported to occur in approximately 6% of all culture confirmed *Salmonella* cases (Mead *et al.*, 1999; Stutman, 1994). However, certain serovars like *S.* Heidelberg have a higher chance of causing severe invasive infections such as meningitis, myocarditis, acute febrile illness and death (Currie *et al.*, 2005).

1.3 *Salmonella* Heidelberg

S. Heidelberg belongs to *Salmonella enterica* subsp *enterica* serogroup B. Relatively little is known about this serovar compared to other common *Salmonella* serovars such as *S.* Typhimurium, *S.* Enteriditis or *S.* Newport. While highly prevalent in

Figure 1: ***Salmonella*** **serovars responsible for human salmonellosis in Canada during 2004. Based on data obtained from the National Enterics Surveillance Program (NESP).** ***Salmonella enterica*** **serovar Typhimurium, Enteriditis and Heidelberg are the three most prevalent** ***Salmonella*** **serovars in Canada accounting for a combined total of 56.5 % of human salmonellosis.**


Enteritidis
18.4%
Typhimurium
20.6%
Heidelberg
17.5%
Hadar
2.8%
Thompson
2.8%
Saintpaul
1.7%
Newport
2.8%
Infantis
1.9%
Agona
2.2%
Typhi
2.4%
Other
26.9%

Canada as well as the United States, *S.* Heidelberg is very infrequently reported in European countries such as Denmark (WHO Global-Salm-Surv, 2006) (http://www.who.int/salmsurv/links/GSSProgressReport2005.pdf). *S.* Heidelberg is one of the most common *Salmonella* serovars isolated from broiler chickens and egg laying flocks and accordingly, the majority of *S.* Heidelberg infections result from the consumption of contaminated food products including chicken and eggs (Currie *et al.*, 2005). Chicken nuggets contaminated with *S.* Heidelberg have been implicated as the cause of a human salmonellosis outbreak in Canada (Currie *et al.*, 2005). Person to person transmission as well as transmission due to direct contact with animals has rarely been reported (Currie *et al.*, 2005). Additonal information regarding *S.* Heidelberg is limited due to the lack of published information regarding this *Salmonella enterica* serovar.

1.4 Canadian Integrated Program for Antimicrobial Resistance Surveillance

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) of the Public Health Agency of Canada gathers information through ongoing integrated surveillance of antimicrobial resistance trends among enteric pathogens isolated from human, animal and animal derived food sources to assess the risks of the use of antimicrobials in food animal production and their impact on resistance in human pathogens. The integrated surveillance conducted by CIPARS includes both active and passive components. Active surveillance includes a Canada wide abattoir component wherein generic *E. coli* and *Salmonella* isolates are collected and characterized from the intestinal contents of healthy animals at the time of slaughter. In addition, active surveillance includes a retail component in Ontario and Quebec where isolates of generic *E. coli*, *Salmonella*, *Campylobacter*, and *Enterococcus* are collected and characterized from retail meat sources. Passive surveillance includes antimicrobial

resistant *Salmonella* from human and diseased animals collected by laboratories across Canada (i.e clinical samples). Available human antimicrobial use data is also incorporated into CIPARS studies (CIPARS, 2004).

While the total number of *Salmonella* isolates associated with human infection decreased between 1995 and 2003, the annual prevalence of *Salmonella enterica* serovar Heidelberg has increased over this time period (National Enterics Surveillance Program [NESP}, 2004; Currie *et al.*, 2005). According to the CIPARS 2004 annual report, *S.* Heidelberg accounted for 17.5 % of nationally reported human salmonellosis ranking it as the second most prevalent *Salmonella* serovar isolated from human infections in Canada (NESP, 2004). While prevalent in human clinical sources, *S.* Heidelberg was predominately isolated from chicken agri-food sources. The very high proportion of chicken agri-food sources that are contaminated with this serovar is reflected by the fact that 72 % of all *Salmonella* isolates recovered from retail chicken meats were *S.* Heidelberg (Table 2). During 2003 to 2004, the prevalence of antimicrobial resistance among *S.* Heidelberg increased. The number of *S.* Heidelberg isolates that were resistant to 1 or more antimicrobials increased form 46% to 56% (Table 3). A high prevalence of extended spectrum cephalosporin (ESC) resistance was seen among multi-drug resistant (MDR) *S.* Heidelberg in Canada. Greater than 30 % of human clinical isolates and greater than 50 % of chicken retail isolates exhibited the A2C-Amp phenotype which confers resistance to amoxicillin-clavulanic acid, ceftiofur, cefoxitin and ampicillin (Table 3). ESC resistance is a cause of concern as these antimicrobials are important for the treatment of human salmonellosis especially in pediatric pediatric patients (Giles *et al.*, 2004).

Table 2: The frequency of *S.* Heidelberg isolated from human and animal sources in 2003 as compared to 2004. Based on data obtained from the CIPARS 2004 annual report.

Source	2003[a]	2004
Human - clinical	20 %	18 %
Chicken – abattoir	50 %	35 %
Chicken – retail	72 %	56 %
Chicken – clinical	nd	52 %
Swine – abattoir	3 %	3 %
Swine – clinical	nd	3 %
Bovine – clinical	nd	4 %
Turkey – clinical	nd	17 %

a – nd; no data.

Table 3: The prevalence of antibiotic resistance amongst *S.* Heidelberg isolates from human clinical and animal sources in 2003 as compared to 2004. Based on data obtained from the CIPARS 2004 annual report.

Source and R - type[a]	2003	2004
R to more than 1 antimicrobial	46 %	56 %
A2C-Amp chicken – abattoir	6 %	45 %
A2C-Amp human – clinical – ON	17 %	36 %
A2C-Amp human – clinical – QC	31%	34 %
A2C-Amp chicken – retail - ON	11 %	53 %
A2C-Amp chicken – retail – QC	65 %	57 %

a – A2C-Amp; Amoxicillin-clavulanic acid, ceftiofur, cefoxitin and ampicillin resistance

1.5 Antimicrobial Resistance

The treatment of systemic *Salmonella* infections requires antimicrobial therapy which traditionally included ampicillin, chloramphenicol, and sulfamethoxazole-trimethoprim. Increasing antimicrobial resistance has reduced the effectiveness of these older antimicrobials and increase reliance on new antimicrobials such as the extended spectrum cephalosporins and flurorquinolones (Angulo *et al.*, 2000). The emergence of antibiotic resistant *Salmonella* limits available treatment options for extra intestinal infections which can result in clinical failure reinforcing the fact that emerging antimicrobial resistance remains paramount in any discussion of *Salmonella*. Three main types of antibiotic resistance are of concern in *Salmonella*; extended spectrum β-lactams resistance, quinolone resistance and MDR (Batchelor *et al.*, 2005; Boyd *et al.*, 2000; Hopkins *et al.*, 2005). Resistance to antimicrobials can be mediated by both structural and regulatory mutations to chromosomally encoded genetic elements, or by the acquisition of exogenous mobile elements such as plasmids or integrons which carry resistance determinants.

1.5.1 Plasmids

Plasmids are autonomously replicating extra-chromosomal elements which range from < 2 to > 100kbp in size (Rychlik *et al.*, 2006). They are composed of genes from many sources that contribute to their “mosaic” composition. All plasmids encode “backbone” genes required for vertical transmission to progeny host cells, such as those involved in replication and partitioning. In addition, plasmids may encode systems which allow for horizontal transfer between bacterial cells such as conjugation or mobilization systems (Boyd *et al.*, 1996). Plasmids will also often encode a variety of accessory genes beneficial to the host bacterium such as genes involved in antibiotic resistance,

virulence, or those that provide a selective advantage in a particular niche such as a hospital or a farm (Eberhard, 1989). Antibiotic resistance determinants are often carried in plasmid-mediated integrons or transposons (Guerra *et al.*, 2002).

The host range and copy number of plasmid are dictated by various replication systems. Plasmid classification is also based on the different plasmid replication control systems referred to as incompatibility (Inc) groups (Novick, 1987). The Inc group of a plasmid can be identified by traditional culture-based incompatibility grouping method or newer PCR based approaches (Carattoli *et al*, 2005). Incompatibility grouping is accomplished by introducing an unknown plasmid into a strain carrying a known resident plasmid through conjugation or transformation. Plasmids from the same Inc group will be unable to coexist in the same cell thus if the resident plasmid is eliminated the unknown plasmid is assigned to the same Inc group (Datta and Hedges, 1971; Datta and Hughes, 1983). The method of Inc group classification may prove to be an important tool to trace the spread and emergence of antibiotic resistance.

1.5.1.1 *Salmonella* Plasmids

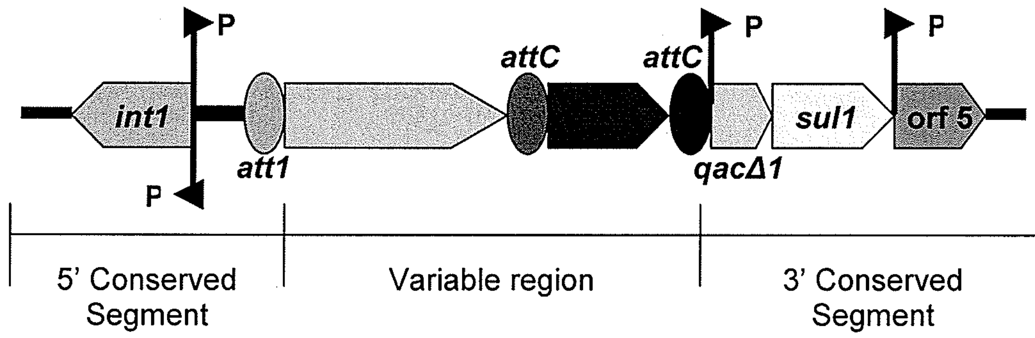
S. enterica serovars associated with human or agri-food animal infection are often shown to carry plasmids (Rychlik *et al.*, 2006). These plasmids include serovar-specific virulence plasmids, high molecular weight (HMW) as large as 200 kbp and plasmids encoding determinates beneficial to the host such as antibiotic resistance cassettes, and low molecular weight plasmids (LMW) of < 20 kbp (Carottoli *et al.*, 2005; Rychlik *et al.*, 2006). Eight *Salmonella* serovars including *Salmonella enterica* serovars Abortusovis, Abortusqui, Cholerasuis, Dublin, Enteriditis, Gallinarium-Pullorum and Sendai, Typhimurium have been shown to contain large virulence plasmids unique to each serovar (Akiba *et al* 1999; Barrow *et al.*, 1988; Beninger *et al.*, 1988; Chu *et al.*, 1999; Gulig *et al.*, 1988; Nakamura *et al.*, 1985). The majority of *Salmonella* virulence

plasmids do not encode genes required for mobilization or conjugation and thus rely on vertical transmission to progeny cells (Chu *et al.*, 1999). In addition, these plasmids encode accessory virulence determinants. The *spv* operon encoded on all known *Salmonella* virulence plasmids consists of five genes *spv*RABCD and is thought to be integral to the ability of *Salmonella* to survive in the low pH and nutrient limiting conditions found in the host macrophage (Fierer *et al.*, 1993; Valone *et al.*, 1993). The carriage of other virulence-associated factors is variable and can include genes such as *rck* (resistance to complement killing), *pef* (plasmid encoded fimbriae), *srgA* (putative disulphide bond oxidoreductase) or *mig-5* (macrophage inducible gene coding for putative carbonic anhydrase) (Hackett, *et al.*, 1987; Friedrich *et al.*, 1993; Rychlik *et al.*, 2006; Valdivia *et al.*, 1997). Virulence plasmids which contain antibiotic resistance gene cassettes in addition to virulence determinants have also been documented. A *S.* Typhimurium plasmid which carries the *spv* operon together with a novel trimethoprim resistance gene, *dhfrA23* has been described (Villa and Carattoli, 2005). *Salmonella* may also harbour high molecular weight plasmids up to 200 kbp in size. These plasmids can encode a variety of gene cassettes beneficial to the host including those that allow for the utilization of alternative carbon sources, resistance to heavy metal such as mercury, and resistance to antibiotics (Rychlik *et al.*, 2006; Timoney *et al.*, 1980; Ghosh *et al.*, 2000). Low molecular weight plasmids have also been identified in *Salmonella* isolates (Threlfall *et al.*, 1994; Ridley *et al.*, 1996). The biological function of these plasmids remains largely uncharacterized, although a LMW plasmid mediating resistance to sulphonamides has been identified in *S.* Cholerasuis (Haneda *et al.*, 2004). These plasmids are often too small to encode conjugation systems themselves, and have been shown to carry genes which allow for mobilization in the presence of another large conjugative plasmid (Gregorova *et al.*, 2004).

1.5.2 Integrons

Integrons are mobile elements which contain a site-specific recombination system capable of recognizing, capturing and integrating mobile gene cassettes. Integrons can be divided into 2 general categories 1) super integrons which are chromosomally located and carry gene cassettes with a wide variety of functions and 2) resistance integrons located on transposons, plasmids or the chromosome which carry antibiotic or disinfectant resistance genes (Fluit and Schmitz, 2004). Three classes of resistance integrons have been identified however only class I and class II have ever been documented in *Salmonella* species. Class I integrons are most frequently seen while class II are more rarely observed (Fluit and Schmitz, 2004; Orman et al., 2002). Class I integrons depicted in Figure 2 are composed of a 5' conserved segment (CS) which includes an integrase gene (*intI1*) and a recombination site (*attI1*). The 3' CS of a class I integron includes a *qacE*Δ*1* gene conferring low level resistance to some antiseptics, a *sul1* gene encoding sulphonamide resistance and an open reading frame of unknown function. Gene cassettes are inserted by *int1* mediated between *att1* and the 59 bp element of the cassette (Fluit and Schmitz, 1999). Inserted gene cassettes will normally lack a promoter and are transcribed from a promoter region present on the 5' conserved segment. A large variety of resistance gene cassettes have been reported in association with class I integrons, the most common of which is the *aadA* (streptomycin resistance) gene or a derivative of it (Fluit and Schmitz, 2004). Class I integrons are most often responsible for the dissemination of resistance determinants that mediate resistance to older classes of antimicrobials such as ampicillin, streptomycin and tetracycline (Daly and Fanning, 2000). Numerous different class I integrons have been identified among *Salmonella* serovars, including some unique to a single strain or serovar and some which are present across different serovars. Three class I integrons 0.65 kbp, 1.1 kbp and 2.0 kbp in length encoding *sat1* (aminoglycoside

Figure 2: Class I integron Structure. Class I integrons are typically composed of a 5' conserved segment (CS) which includes an integrase gene (*intI1*) and a recombination site (*attI1*). The 3' CS of a class I integron includes a *qacE*Δ*1* gene conferring low level resistance to some antiseptics, a *sul1* gene encoding sulphonamide resistance and an open reading frame (orf5) of unknown function. Adapted from Fluit and Schmitz, 2004.


P
attC
attC
P
P
int1
sul1
orf 5
att1
qacΔ1
P
5' Conserved
Segment
Variable region
3' Conserved
Segment

resistance), *aadA*, and *aadA* plus *dhfR* (trimthoprim resistance) respectively have been repeatedly identified among numerous *S*. Newport strains (Rankin *et al.*, 2002; Zhao *et al.*, 2003). Class I integrons are important contributors to the development of MDR in *Salmonella*.

1.5.3 *Salmonella* Genomic Island I

Whilst the emergence of multi-drug resistant (MDR) phenotypes is evident in many of the clinically significant *Salmonella* serovars, the most well characterized MDR *Salmonella* is *S*. Typhimuirum DT104. Initially described in the 1960's, DT104 was first identified as a causative agent of human illnesses in 1989 and has since emerged as the predominant Typhimurium phage type implicated in human infections creating a global health problem (Threlfall, 2000) .The global DT104 epidemic is actually the result of the spread of four closely related MDR strains DT104, DT104a, DT104b, and U302 (Lawson *et al.*, 2004; Mulvey *et al.*, 2006). The first MDR DT104 isolate was discovered in the United Kingdom in the 1980's and displayed what would become the classic DT104 associated MDR phenotype of resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (ACSSuT) (Threlfall, 2000). The origin of the ACSSuT phenotype remains uncertain, however DT104 is a zoonotic phage type often isolated from animals including cattle, pigs, sheep, and chickens. This may have provided an opportunity for the acquisition of the ACSSuT phenotype since the phenotype mediates resistance to 4 of the 5 most common antimicrobials (tetracyclines, β-lactams, aminoglycosides, and sulfonamides) used in veterinary medicine (Angulo *et al.*, 2000; Cloeckaert and Schwarz, 2001).

The chromosomally mediated 43kbp *Salmonella* Genomic Island 1 (SGI1) consists of 44 open reading frames (ORF) and contains a 13-kbp MDR region responsible for the characteristic ACSSuT penta-resistance phenotype (Boyd *et al.*,

2000; Briggs and Fratamico, 1999). The element has been shown to insert between *thdF* and *int2* (a cryptic retrophage) in *S.* Typhimurium strains or between *thdF* and upstream of *yidY* in non-Typhimurium serovars. The presence of these conserved integration sites indicates a site-specific insertion mechanism which is reinforced by the presence of imperfect 18bp direct repeats that flank SGI1 at the right and left junctions of the chromosome (Boyd *et al.*, 2000). The right junction direct repeat sequence is identical to the last 18bp of the *thdF* gene from *S. enterica* serovars which do not carry SGI1 while the left junction direct repeat is identical in all serovars (Mulvey *et al.*, 2006). The discovery of a chromosomal determinant which mediates MDR is a major cause of concern as the chromosomal integration of resistance determinants is likely to make them stable genetic structures able to persist even in the absence of antimicrobial selective pressure (Mulvey *et al.*, 2006).

SGI1 encodes genes with homology to known genes involved in DNA recombination, DNA replication, conjugal transfer, regulatory functions, drug resistance as well genes with no homology to any known genes (Boyd *et al.*, 2001). The 13 kbp MDR region of SGI1 is composed of the *floR* and *tetG* genes bracketed by two class I integrons: InC (1.0 kbp) carrying *aadA2* and a partial *sul1* and InD carrying bla_{Carb2} or bla_{pseI} and *sul1* (Sandvang *et al.*, 1998). The 3' of the InD integrase is replaced by a partial *groEL* gene (Briggs and Fratamico, 1999). The region flanking the MDR region contains the inverted repeats IRi and IRt which define the boundaries of the complex integron In104 (Levings *et al.*, 2005).

Molecular variants of SGI1 conferring resistance profiles other than ACSSuT have also been identified from DT104 as well as other *Salmonella* serovars. These SGI1 variants are classified as SGI1A-E. Variant SGI1s responsible for resistance phenotypes including ACSSuTTm (Tm = trimethoprim), SSuTM, ASu, SSu, ASSuT (Boyd *et al.*, 2002). Variants SGIs result from the integration of additional gene

cassettes as in the case of SGI1-A where the *dfrA1* gene conferring trimethoprim resistance and the putative transposase orf513 were acquired in an additional 4.1 kbp of DNA inserted downstream of the *sul1* gene (Boyd *et al.*, 2002). Variant SGI1s also arise due to the absence of gene cassettes as seen in SGI1-B which consists of only a single class I integron containing the *bla*$_{pse-1}$ cassette (Boyd *et al.*, 2002). Strains containing the variant SGI1-A with deletions of the 3' end and adjacent chromosomal DNA have also been described and are referred to as SGI1-AΔ1R, SGI1-AΔ2R, SGI1-AΔ3R (Boyd *et al.*, 2002).

SGI1 is emerging in *Salmonella enterica* serovars other than *S.* Typhimurium highlighting the horizontal transfer of the element. The complete *Salmonella* Genomic Island 1 or a variant has been detected in *Salmonella enterica* serovars Paratyphi B, Albany, Agona, Meleagridis, Newport, Derby, Cerro, Kiambu, Emek, Dusseldorf, and Infantis (Boyd *et al.*, 2001; Doublet *et al.*, 2003; Doublet *et al.*, 2004; Ebner *et al.*, 2004; Levings *et al.*, 2005; Meunier *et al.*, 2002). The exact method through which SGI1 is transferred between *Salmonella* strains is currently under investigation. SGI1 has been conjugally transferred from an *S. enterica* donor strain to both non-SGI1 *S.enterica* and *E. coli* but only in the presence of the conjugative IncC helper plasmid R55. The dependence on plasmid encoded conjugative functions indicates that the element is not self-transmissible but rather mobilizable (Doublet *et al.*, 2005). The conjugal transfer of SGI1 has been shown to occur in three steps; initially SGI1 excises from the donor chromosome using an SGI1 encoded integrase and through recombination between the direct repeats at the left and right ends of the element forms an extra-chromosomal circular form (Doublet *et al.*, 2005). Following the formation of the circular intermediate, the actual conjugative transfer of the element occurs. The final step of transfer, integration of SGI1 into the recipient chromosome, has been shown to occur by site specific recombination between an 18-bp portion of the *attP* gene in the circular

intermediate and a similar 18-bp sequence at the end of the *thdF* gene in *S. enterica* or the *attB* gene of *E. coli.* The conjugal transfer of SGI1 has been reported to occur at a frequency of 10^{-5} – 10^{-6} transconjugants per donor (Doublet *et al.*, 2005).

1.5.4 Quinolone Resistance

Quinolones are a class of broad-spectrum bactericidal drugs, including nalidixic acid and ciprofloxacin. Quinolones function by inhibiting DNA gyrase (encoded by *gyrA* and *gyrB*) and topoisomerse IV (encoded by *parC* and *parE*) thus blocking bacterial DNA synthesis (Hopkins *et al.*, 2005). Chromosomal mutations that modify bacterial DNA gyrase and topoisomerase are the primary mechanisms that mediate quinolone resistance, although modifications to bacterial outer membrane proteins resulting in reduced uptake of drug and over expression of drug efflux pumps can also be responsible (Hopkins *et al.*, 2005). Most recently plasmid mediated quinolone resistance due to the *qnr* gene was described in *Salmonella enterica* serovar Enteritidis (Cheung *et al.*, 2005). The *qnr* gene encodes a pentapeptide repeat protein Qnr which protects DNA gyrase from quinolone inhibition (Hopkins *et al.*, 2005). Chromosomal mutations which give rise to quinolone resistance cluster in a specific topoisomerase domain referred to as the quinolone resistance determining region (QRDR). Mutations in the QRDR result in a decrease of quinolone affinity for the enzyme-DNA complex allowing DNA replication to continue even in the presence of quinolone antimicrobials. Several amino acid changes can be responsible for conferring different levels of quinolone resistance. A single mutation in *gyrA* at Ser83 is most frequently seen in *Salmonella* and confers resistance to nalidixic acid along with decreased susceptibility to ciprofloxacin (McDermott, 2006). Double *gyrA* mutations alone or in combination with *parC*, *gyrB* and *parE* mutations are currently rare in *Salmonella* and correlate with higher levels of resistance to fluoroquinolones (McDermott, 2006). High levels of fluroquinolone

resistance are relatively uncommon in *Salmonella* species although an increased prevalence of low level resistance has been reported worldwide in certain serovars of *S. enterica* (Enteritidis, Hadar, and Virchow) (Hopkins *et al.*, 2005). Quinolone antibiotics, in particular fluoroquinolones are among the last line of defense, for the treatment of severe human salmonellosis. Thus, the emergence of quinolone resistance has extremely important implications for the treatment of human salmonellosis.

1.5.5 β-lactamase Resistance

β-lactam antibiotics (penicillins, cephalosporins, carbapenems and monobactams) are an important line of defense against human salmonellosis. β-lactam antibiotics disrupt bacterial cell wall synthesis by inhibiting transpeptidase enzymes essential for peptidodoglycan synthesis (Jawetz and Levinson, 2002). The disruption of peptidogylcan synthesis, an essential step of cell wall formation, results in the death of the bacterial cell. Resistance to β -lactam antibiotics is primarily mediated through chromosomal or plasmid mediated β -lactamase enzymes produced by the bacteria (Jawetz and Levinson, 2002). β -lactamases cleave the amide bond present in the β -lactam ring of the antibiotic rendering the drug inactive. Resistance to first and second generation β -lactam antibiotics has been seen for decades as a result of β -lactamase enzymes including TEM-1, TEM-2 and SHV which are capable of hydrolyzing penicillin and ampicillin, and to a lesser degree carbenicillin and cephalothin (Paterson and Bonomo, 2005). Third generation cephalosporins were introduced in the 1980s to combat emerging β-lactam resistance (Paterson and Bonomo, 2005). Almost immediately following the introduction of third generation cephalosporins into clinical practice, extended spectrum β-lactamses (ESBLs) and plasmid mediated AmpC β-lactamases were described. Both ESBLs and AmpC type enzymes have been identified in *Salmonella* (Alcaine *et al.*, 2005; Mulvey *et al.*, 2003).

1.5.5.1 Extended spectrum β lactamases

There is no consensus on the precise definition of an ESBL, however the most common definition is a β-lactamase enzyme which confers resistance to the penicillins, first-, second-, third-generation cephalosporins, monbactams and aztreonam, but not the cephamycins or carbapenems, and is inhibited by penicillin analogues / β-lactamase inhibitors such as clavulanic acid or sulbactam (Paterson and Bonomo, 2005).

ESBLs can be classified into nine families on the basis of amino acid sequence: TEM, SHV, CTX-M, PER, VEB, GES, TLA, BES, and OXA (Gniadkowski, 2001). The most common types of ESBLs are the TEM, SHV (*sul**h*ydryl *v*ariable) and CTX-M type enzymes all of which can either be located on the chromosome or plasmid mediated. All TEM and SHV ESBL enzymes are derived from the original TEM-1, TEM- 2, and SHV β -lactamases through point mutations which confer activity against extended spectrum cephalosporins (Paterson and Bonomo, 2005). CTX-M enzymes are named for their potent hydrolytic activity against cefotaxime and their clonal dissemination has been well documented worldwide (Paterson and Bonomo, 2005). CTX-M type β-lactamases share high sequence homology with the chromosomally encoded extended spectrum β-lactamases of *Kluyvera* species and are thought to have originated from the transfer of *Kluyvera* genes onto plasmids (Decousser *et al.*, 2001). ESBLs are most prevalent in nosocomial organisms including *E. coli*, *Pseudomonas* spp. and *Klebsiella* spp. ESBLs were not previously seen in *Salmonella* however they are being described with increasing frequency (Paterson and Bonomo, 2005).

1.5.5.2 AmpC β -lactamases

AmpC type β-lactamases are active serine site β-lactamases that exhibit hydrolytic activity against all β-lactam antimicrobials with the exception of carbapenems. These enzymes differ from ESBLs by the fact that they are not inhibited by β-lactamase inhibitors such as clavulanic acid (Philippon *et al.*, 2002). AmpC type β-lactamses were initially demonstrated to be chromosomal β-lactamase genes described in numerous gram negative bacteria including *Acinetobacter* spp., *Aeromonas* spp., *Chromobacterium violaceum*, *Citrobacter freundii*, *Enterobacter* spp., *E. coli*, *Morganella morganii*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica* (Philippon *et al.*, 2002). Wild type chromosomal *ampC* genes do not confer high levels of β-lactam resistance because they produce low levels of AmpC enzyme due to a weak promoter in conjunction with a transcriptional attenuator and are actually susceptible to ampicillin. High level β-lactam resistance mediated by a chromosomal *ampC* gene is only seen when mutations to the *ampC* promoter or attenuator allow for the over production of the AmpC enzyme (Hawley and McClure, 1983). These strains are subsequently highly resistant to β -lactam antibiotics. Plasmid-mediated *ampC* genes derived from the transfer of chromosomal *ampC* genes onto plasmids have emerged among the *Enterobacteriacea* (Papanicolaou *et al.*, 1990; Philippon *et al.*, 2002). Plasmid mediated *ampC* β-lactamases can be divided into 5-6 groups on the basis of their similarity to the chromosomal *ampC* genes of a particular species. The *C. fruendii* group includes *lat* and $bla_{cmy\text{-}2}$ types, the *Enterobacter* group includes *mir-1* and *act-1*, the *M. morganii* group includes *dha-1* and *dha-2*, the *Hafnia alvei* group includes *acc-1*, while the *Aeromonas* group includes *mox, fox*, and all of the other *cmy* enzymes (Philippon *et al.*, 2002). *Salmonella* do not naturally encode a chromosomal *ampC* gene thus all *ampC* mediated resistance in *Salmonella* is due to the acquisition of a plasmid mediated *ampC* β-lactamase (Medeiros, 1997). Although numerous AmpC enzymes have been

described in *Salmonella*, the $bla_{cmy\text{-}2}$ gene is most common and has been identified in numerous *Salmonella enterica* serovars (Allen and Poppe, 2002; Miriagou *et al.*, 2002; Navarro *et al.*, 2001; Rankin *et al.*, 2002).

1.5.5.2.1 $bla_{cmy\text{-}2}$ plasmids

In North America decreased susceptibility to extended-spectrum cephalosporins especially ceftriaxone is almost exclusively mediated by $bla_{cmy\text{-}2}$ (Dunne *et al.*, 2000; Gupta *et al.*, 2003; White *et al.*, 2001). This gene is exclusively plasmid mediated and is thought to be derived from the transfer of the *C. fruendii* chromosomal *ampC* gene onto plasmids (Bauernfeind *et al.*, 1996). At least three separate high molecular weight plasmids (> 100 kbp) which carry the $bla_{cmy\text{-}2}$ plasmid have been identified among *Salmonella* species. These three plasmids have been termed A, B, and C on the basis of *Pst*I RFLP patterns and $bla_{cmy\text{-}2}$ hybridization profiles. Initially, $bla_{cmy\text{-}2}$ plasmids were isolated only from *S.* Typhimurium, however beginning in 2000 were seen with a higher frequency in other serovars including *S.* Newport, Agona, and Reading (Carattoli *et al.*, 2002; Winokur *et al.*, 2001). A 10 kbp plasmid carrying the $bla_{cmy\text{-}2}$ gene has also been identified in *S.* Heidelberg and is referred to as the type D plasmid (Winokur *et al.*, 2001). Type A and C plasmids are considered highly related on the basis of their RFLP patterns, both encode resistance to multiple antibiotics (tetracycline, streptomycin, sulfonamides, and chloramphenicol) in addition to β-lactam resistance and both have low conjugal transfer efficiencies (Giles *et al.*, 2004) (Carattoli *et al.*, 2005; Carattoli *et al.*, 2006). Class I integrons carrying *aadA1* alone or combination with *cmlA* and *aadB* have been identified on some type A and C plasmids. The mechanism by which other resistance determinants were acquired remains unknown (Carattoli *et al.*, 2002). Type B plasmids only encode resistance to β-lactam antibiotics, have highly divergent RFLP patterns compared to type A and type C plasmids and are readily transferred by

conjugation (Giles *et al.*, 2004). The DNA sequence of the region surrounding the *bla*$_{cmy-2}$ gene was found to be highly conserved in all four *bla*$_{cmy-2}$ plasmid types although minor variations reinforcing the classification of *bla*$_{cmy-2}$ plasmids into four types were observed. The ISEcp1 element was identified upstream of the *bla*$_{cmy-2}$ gene (Giles *et al.*, 2004). Immediately downstream of *bla*$_{cmy-2}$ are two open reading frames with > 96% homology to the *blc* and *sugE* genes of *C. fruendii.* The *blc* gene is an outer membrane lipoprotein while *sugE*, is a small multidrug resistance family of multidrug efflux systems. Upstream of the *sugE* gene the region surrounding *bla*$_{cmy-2}$ gene begins to become divergent between plasmid types with type C and D plasmids carrying an additional 364 bp of DNA with 96.7% sequence homology to the *ecnR* gene of *C. fruendii.* In addition, an IS26 insertion sequence was identified in type C but not type D plasmids. The plasmid types are all divergent immediately adjacent to the left inverted repeat of ISEcp1 (Giles *et al.*, 2004). Independent acquisition of the *bla*$_{cmy-2}$ gene by different plasmid backbones is thought to be the means by which the four different plasmid types were generated (Carattoli *et al.*, 2002). Although, the mechanism through which the *bla*$_{cmy-2}$ gene was acquired by plasmids remains uncertain. Sequence analysis of the region surrounding *bla*$_{cmy-2}$ on the plasmids shows no evidence of the presence of an integron like structure eliminating this method of acquisition (Giles *et al.*, 2004). Recently *bla*$_{cmy-2}$ carrying transposon-like elements were detected on non-conjugative high molecular weight *bla*$_{cmy-2}$ plasmids alluding to a potential mechanism by which the plasmids could have acquired *bla*$_{cmy-2}$ (Su *et al.*, 2006). Similar and identical type A, B, and C *bla*$_{cmy-2}$ plasmids have been identified amongst a diverse group of temporally and geographically distinct *Salmonella* strains of varying serovars indicating that extended spectrum cephalosporin (ESC) resistance is emerging predominately through the horizontal transfer of *bla*$_{cmy-2}$ plasmids into separate genomic backgrounds rather than the transfer of the *bla*$_{cmy-2}$ gene itself onto multiple plasmid backbones (Carattoli *et al.*, 2002). Similar

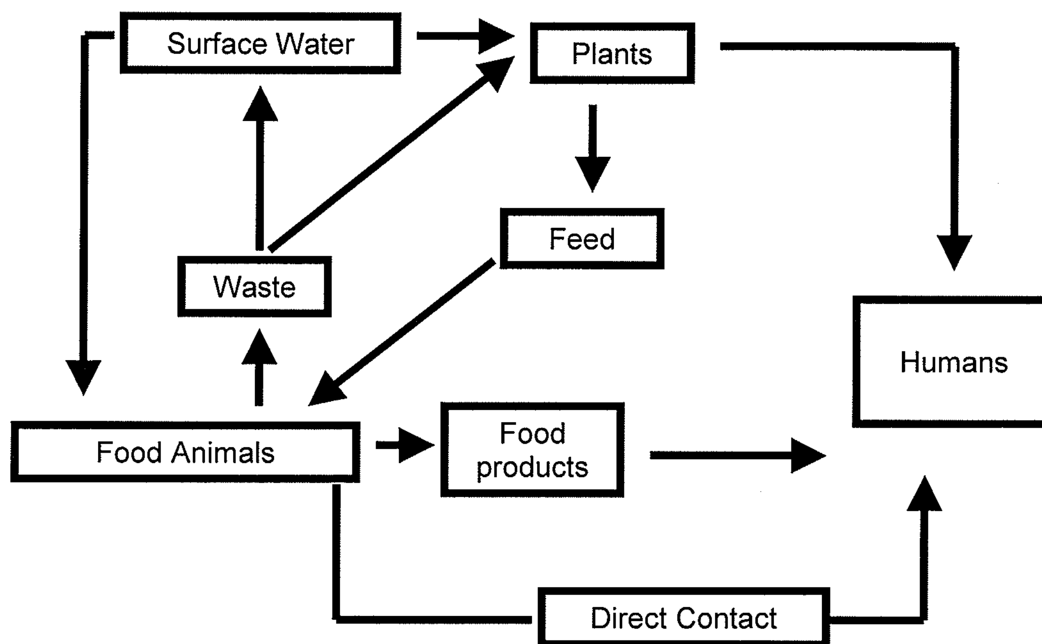
or identical *bla*$_{cmy-2}$ plasmids have been identified in both *Salmonella* and ESC resistant *E. coli* indicating that interspecies transfer of the *bla*$_{cmy-2}$ plasmids is occurring. In addition, distinct plasmids have been isolated from both species indicating that the independent acquisition of *bla*$_{cmy}$-2 by different plasmid backbones is also contributing to the emergence of ESC resistance in *E. coli* (Carattoli *et al.*, 2002; Rankin *et al.*, 2002; Winokur *et al.*, 2001).

In *Salmonella*, HMW plasmids have been shown mediate *bla*$_{cmy-2}$ (Giles *et al.*, 2004; Carattoli *et al*, 2002). PCR based Inc group identification has demonstrated the presence of *repAC* and *repI1* *bla*$_{cmy-2}$ plasmids in *S.* Typhimurium and *S.* Thompson isolates (Carattoli *et al.*, 2005). The *repAC* plasmids carried *bla*$_{cmy-2}$ in combination with a gentamicin resistance determinate while the *repI1* plasmids carried only *bla*$_{cmy-2}$ (Carattoli *et al.*, 2005).

1.6 The Dissemination and origin of MDR resistant *Salmonella*

Salmonella is mainly a foodborne or zoonotic infection. Human to human transmission of non-typhoidal *Salmonella* is rarely documented and ~ 95% of human salmonellosis is attributable to either direct or indirect contact with animals (Figure 3). Ingestion of contaminated food sources is quantitatively the most important mode of transmission of *Salmonella* from animal sources to humans (Stutman, 1994). The transfer of *Salmonella* from animals to humans through direct contact has also been documented to a lesser extent. The increasing prevalence of antimicrobial resistance among human *Salmonella* isolates has raised concerns about whether resistant *Salmonella* emerge in food producing animals and then are subsequently transferred to humans. This hypothesis is supported by the fact that only a small percentage of human salmonellosis is treated with antimicrobials. Thus the selective pressure at the human

Figure 3: Routes of *Salmonella* dissemination between animals and humans. Adapted from McDermott 2006 (McDermott, 2006).


Surface Water
Plants
Feed
Waste
Humans
Food Animals
Food products
Direct Contact

level is low and the emergence of resistance may be more readily attributable to antimicrobial use in food animals (Molbak, 2005).

Antimicrobial agents have been used in food animals since the 1950's to treat infections, for prophylactic purposes and to improve the growth and feed efficiency of animals. The use of antimicrobial agents in food animals may create selective pressure which favours the survival of antibiotic resistant pathogens and transmission up the food chain to humans (Anderson *et al.*, 2003). A steadily growing body of evidence based on epidemiologic investigations, outbreak studies, and molecular typing investigations supports the transfer of resistant bacteria from food farm animals to humans (Fey *et al.*, 2000). However, a definitive link between the use of antimicrobials in food animals and the subsequent emergence of resistance followed by transfer to humans cannot be made due to the lack of available information regarding the administration of antimicrobials to food animals in most countries.

1.7 The human health consequences of antimicrobial resistance

Regardless of origin, the human health consequences of antimicrobial resistance in *Salmonella* include limiting the available treatment options, decreasing the effectiveness of empirical treatments and increasing the cost of treatment. The use of antimicrobial agents in animals that are related to an agent used to treat human infections, or are themselves administered to humans, increases the chance that *Salmonella* from food animals will develop resistance to antimicrobial agents used in human medicine (Anderson *et al.*, 2003). Resistance to extended spectrum cephalosporins and fluoroquinolones is a cause of concern as these broad spectrum antimicrobials are commonly used for the treatment of human infections. Decreased susceptibility to ceftriaxone is particularly important, as this antimicrobial is used for the treatment of invasive infections in children whom cannot tolerate fluoroquinolone

antibiotics (Hohmann, 2001). Resistant *Salmonella* have been associated with higher rates of hospitalization and elevated mortality (Molbak, 2005). The use of antimicrobials can also select for resistant pathogens and thereby increase their transmission (Molbak, 2005).

1.8 Current techniques for subtyping *Salmonella*

Subtyping methods are invaluable to the understanding of infectious disease transmission to humans from animals (Fey *et al.*, 2000) (Sandt *et al.*, 2006). To track the emergence and spread of both MDR and non-MDR *Salmonella* accurate subtyping techniques are required. Serotyping is the traditional method used to differentiate *Salmonella enterica* subspecies 1. The Kauffman-white *Salmonella* serotyping scheme identifies more than 2500 serovars on the basis of antigenic variation present in the outer membrane lipopolysaccharide (O) phase, phase 1 (H1) and phase 2 (H2) flagella (Popoff and Le Minor, 2001). Serotyping remains a valuable method for the identification of *Salmonella* serovars however does not have the ability to robustly examine the genetic relationship between strains (Beltran *et al.*, 1988). In Canada, greater than 40% of all human salmonellosis is attributable to *S. enterica* serovars Typhimurium, Enteriditis and Heidelberg thus detecting isolates of these serovars from multiple sites is not evidence of an absolute link between isolates (CIPARS, 2004). Epidemiologic surveys, outbreak detection or examining chains of transmission requires that serotyping be used in combination with another more discriminatory method such as phage typing or molecular techniques including Pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and comparative genomic hybridization (Sukhnanand *et al.*, 2005).

1.8.1 Phage Typing

Phage typing is a phenotypic method based on the pattern of resistance or sensitivity of a bacterial isolate to a panel of distinct bacteriophage (Pelludat *et al*, 2005). Each unique pattern of resistance and sensitivity to phage is characteristic of a specific phage type (definitive phage type [DT]. Phage typing is often only carried out in certain reference laboratories since it requires access to an extensive phage library and the correct interpretation of results requires training and experience. In established reference facilities phage typing can be a rapid, convenient, low cost method of discriminating between isolates of the same serovar (Doran *et al.*, 2005). Phage typing has been an important method for differentiating between strains of the same serovar and has proved extremely valuable for tracking the emergence and spread of epidemic *S.* Typhimurium strains, in particular DT104 (Pelludat *et al.*, 2005; Threlfall *et al.*, 1994). One drawback of phage typing is that the method can be complicated by the conversion of phage types. The passage of a phage through a host harbouring a related prophage can allow for the production of recombinant phage which have plating properties that are different from the initial phage (Schmieger, 1999). Another drawback of an empirical method such as phage typing is the fact that there is only circumstantial evidence for the actual genomic content of the strain. Thus phage typing may need to be supplemented by molecular methods to get the level of discrimination required for outbreak investigation.

1.8.2 Molecular Methods

Numerous molecular typing methods have been used for *Salmonella* species including pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing

(MLST). Comparative genomic hybridizations have also emerged as a powerful new tool for investigating strain variation.

1.8.2.1 Pulsed-field gel electrophoresis

PFGE is currently the accepted gold standard for the molecular typing of *Salmonella* isolates and is used by PulseNet USA and Canada surveillance programs for the genetic characterization of *Salmonella* strains (Harbottle *et al.*, 2006; Murase *et al.*, 1995). PFGE involves the resolution of genomic DNA restriction fragments on an agarose gel to obtain a pattern representative of an isolates genome. *Salmonella* cells are re-suspended in buffer and added to liquid agarose to obtain a plug containing the agarose embedded organism. Cells are lysed in situ and following lysis, plugs are incubated with infrequently cleaving endonucleases such as *Bln*I, *Xba*I and *Spe*I to restrict genomic DNA. The restriction fragments are then resolved into a pattern of discrete bands by a contour clamped homogenous electric field (CHEF) apparatus which switches the direction of the current according to a pre-determined pattern (Tenover *et al.*, 1995). A molecular weight standard, in most cases a control strain with bands of known size, is also included to provide size orientations and to help normalize the gel during subsequent computer analysis. Isolate band patterns are compared by computer aided analysis using programs such as Applied Maths Bionumerics (Applied Maths, Austin, TX). To ensure accurate gel to gel comparisons between experiments, each gel is normalized according to the molecular weight standard/standard strain. Isolate band pattern is manually determined based on thresholds of stringency and optimization set by the experimenter (Tenover *et al.*, 1995). A dendrogram based on PFGE pattern can be created to depict genetic relatedness, but epidemiologic information remains essential to the ability to link strains with similar PFGE patterns. The major disadvantages of PFGE are the subjectivity of band analysis and the multi-step time

consuming process. A lack of standard criteria for band pattern analysis can result in varying interpretations of the same band pattern between investigators (Tenover *et al.*, 1995). Inter-lab and experimental variability can also complicate PFGE analysis. Band patterns of the same isolate may vary depending on the PFGE protocol used thus inter-lab reproducibility can be problematic. Experimental variation due to minor variability in the DNA concentration of plugs, gel composition, electrophoresis voltage, or buffer strength can result in different band patterns between experiments (Tenover *et al.*, 1995). The implementation of standard methods and band analysis criteria reduces experimental variation allowing PFGE to be used successfully as a molecular typing tool. In addition PFGE is a universal technique which requires limited prior knowledge of the genome for primer design, randomly probes the genome for strain to strain differences, and is inexpensive (Tenover *et al.*, 1995).

PulseNet USA at the Centers for Disease Control and and Prevention (CDC) has implemented standardized protocols resulting in the successful use of PFGE during outbreak investigations (Sandt *et al.*, 2006). Using the CDC PulseNet protocol, tomato-derived serovar *S. enterica* subsp *enterica* serovar Anatum isolates were successful linked to cases of human salmonellosis demonstrating the value of PFGE for short term epidemiologic investigations (Sandt *et al.*, 2006). While still considered the gold standard for the subtyping of *Salmonella*, PFGE has been shown to be unsuccessful at differentiating between strains of the same PT including *S.* Typhimurium DT104 and DT12 (Torpdahl *et al.*, 2006). The limitations of PFGE have prompted investigations into the utility of other potentially more powerful tools such as MLST and comparative genomic hybridizations which may prove useful for studying isolate relationships in epidemiologically unrelated populations.

1.8.2.2 Multi-locus sequence typing (MLST)

Emerging molecular typing methods such as MLST that have the ability to distinguish numerous genotypes but are based on genetic variation that accumulates relatively slowly may provide a new approach to monitoring infectious diseases (Enright and Spratt, 1999). MLST based schemes have been developed for numerous clinically important pathogens including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (Enright *et al.*, 1999). MLST characterizes a strain based on sequence data generated from selected bacterial genes (Harbottle *et al.*, 2006). MLST schemes must be composed of multiple unlinked loci to provide a meaningful level of resolution and will often include housekeeping, ribosomal, virulence genes (Enright and Spratt, 1999). Housekeeping genes encode determinants required of bacterial survival and as a result accumulate nucleotide changes in a slow most likely selectively neutral process (Enright and Spratt, 1999). The genes included in previously published *Salmonella* MLST schemes include combinations of the *fimA* (fimbrial gene A), *manB* (phosphomanomutase), *mdh* (malate dehydrogenase), *aroC* (chorismate synthase), *dnaN* (DNA polymerase III beta subunit), *hemD* (uroporphyrinogen III), *hisD* (histidinol dehydrogenase), *purE* (phosphoribosyl uminoimidazole carboxylase catalytic subunit), *sucA* (2-oxoglutarate dehydrogenase), and *thrA* (threonine synthetase) genes (Harbottle *et al.*, 2006; Sukhnanand *et al.*, 2005). Polymerase chain reaction (PCR) primers are designed to amplify internal fragments of the selected genes. Ideally short fragments are selected to allow for PCR amplification and sequencing based on a single pair of primers. During sequence analysis, DNA sequences that differ by even a single nucleotide are assigned different allele numbers. All sequenced loci can be concatenated to create an artificial allelic profile that defines the sequence type of an isolate, and phylogenetic trees can be created to display relatedness. As a sequenced based method, MLST offers numerous advantages. The analysis of nucleotide

sequences is relatively unambiguous compared to other methods such as PFGE (Enright and Spratt, 1999). The data generated can be readily compared between laboratories. MLST can be performed directly on clinical materials such as blood which would be advantageous for clinical diagnostics. MLST also defines all of the genetic variation present within an amplified gene fragment elucidating exact strain to strain differences (Enright and Spratt, 1999). Opinions on the utility of MLST as a typing method for *Salmonella* vary. Most often, MLST is shown to be capable of determining only the serovar of an isolate, and a seven gene MLST scheme based on *panB* (ketopantoate hydroxymethyltransferase), *fimA, icdA* (isocitrate dehydrogenase), *manB, mdh*, and *aceK* (isocitrate dehydrogenase kinase) was able to predict the serovar of *S.* Heidelberg and *S.* Schwarzengrund isolates (Sukhnanand *et al.*, 2005). MLST has minimal ability to distinguish between isolates of the same serovar especially when isolates are as closely related as those within the same PFGE cluster. Although some MLST schemes are able to provide limited discrimination between isolates of the same serovar but not to the same degree obtained with PFGE (Fakhr *et al.*, 2005). The seven gene MLST scheme was able to define only two to three subtypes within *S.* Agona, *S.* Typhimurium, *S.* Dublin, *S.* Javiana, and *S.* Newport (Sukhnanand *et al.*, 2005). The limited ability of MLST to distinguish between closely related isolates indicates that the higher discriminatory power of a method such as PFGE may be more useful for the examination of local outbreaks. Although, the capacity of MLST to identify a large diversity of *Salmonella* serovars indicates that it may prove to be useful tool for determination of *Salmonella* serovar or for the examination of evolutionary relationship on a large global scale.

1.8.2.3 Comparative genomic hybridization (CGH)

DNA microarrays are glass slides with DNA fragments (oligonucleotides or PCR products) immobilized on their surface. These DNA fragments each represent one open reading frame (ORF) and often an array will consist of all of the ORFs from a sequenced genome, although it can also consist of a combination of ORFs from different strains or genes of interest such as antibiotic resistance determinants and virulence genes. Comparative genomic hybridization (CGH) examines the similarities and differences in genetic content between different bacteria. Differentially labeled DNA from test and reference organisms are hybridized to the microarray and the presence or divergence of genes is determined based on fluorescence intensity at each individual spot (van Hoek *et al.*, 2005). The disadvantages of comparative genomic hybridizations include the cost and the high amount of optimization/validation required before the platform can be used. CGH based experiments are advantageous because they allow for examination of the whole genome of an organism. This dynamic technique can detect strain to strain differences not resolved by other techniques which could be useful for the subtying of *Salmonella*. Comparative genomic hybridization based on S. Typhimuirum specific probes have been use to subtype some serovar Typhimurium strains (Pelludat *et al.*, 2005). Other CGH studies have only been successful at identifying inter-serovar variations and documented very limited intra-serovar diversity amongst *S.* Enteriditis. (Porwollik *et al.*, 2005). Conflicting descriptions on the utility of comparative genomic hybridizations for examining strain to strain variation among closely related isolates indicate that this complex technique will need to be further evaluated before it can be compared to PFGE, MLST or phagetyping.

Each subtyping method described for *Salmonella* has both advantages and disadvantages. The ideal method would allow for the sensitive discrimination of subtypes while still being capable of tracking the emergence and spread subtypes

(Sukhnanand *et al.*, 2005). In the current absence of an ideal typing method, combining the results of different typing methods can provide more discriminatory power than each alone (Harbottle *et al.*, 2006).

1.9 The Present Investigation

The CIPARS group has noted that *S.* Heidelberg consistently ranks amongst the most prevalent causes of human salmonellosis in Canada (Figure 1). In addition, there has been an increase in the number of MDR isolates from human clinical and agri-food sources. This study aims to investigate whether the observed increase in the amount of human salmonellosis attributable to MDR *S.* Heidelberg is due to the increase in occurrence of MDR *S.* Heidelberg in chicken agri-food sources noted during abattoir, retail and passive surveillance by CIPARS. During the course of the study, the genetic relationship between *S.* Heidelberg isolates with different resistance profiles, provinces of isolation, years of isolation and sources (animal vs human vs food) were examined to determine whether molecular methods could provide a link between chicken agri-food sources and human isolates of MDR *S.* Heidelberg. The mechanisms by which MDR is disseminating among *S.* Heidelberg were also examined.

Chapter 2: Materials and Methods

2.1 Bacterial strains and determination of resistance phenotypes

The 44 bacterial strains included in this study were obtained from CIPARS with the exception of 1170, 539 and 564 which were obtained from The Laboratory for Foodborne Zoonoses (LFZ) and 00-5440 and 05-1147 obtained from the National Microbiology Laboratory (NML) Bacteriology and Enteric Disease culture collection (Table 4). The panel of strains contained 39 *S.* Heidelberg isolates which were selected to represent a diverse range of PFGE patterns, phage types, years of isolation (2001 – 2004), provinces of isolation (ON, QC, AB, BC, PEI), as well as sources of isolation including clinical (passive) isolates of human, bovine, porcine and chicken origin as well as retail and abattoir chicken isolates. Isolates were also selected to represent a diverse array of resistance phenotypes including sensitive, and ESC resistance alone or in combination with resistance to other classes of antimicrobials or resistanance to a combination of antimicrobials excluding ESC. The panel of bacterial isolates also included 5 *E. coli* strains that exhibited resistanance to ESCs alone or in combination with resistance to other classes of antimicrobials. The *E. coli* isolates included chicken and abattoir isolates as well as bovine retail isolates. Resistance to antimicrobials was determined either at the LFZ or the NML using a broth microdilution method performed using the Sensititre™ ARIS Automated Microbiology System (Trek Diagnostic System Ltd, Clevland Ohio) automated system (CIPARS, 2004). The breakpoints for susceptible, intermediate and resistant isolates were taken from the NCCLS/CLSI (NCCLS/CLSI M100-S15 Table 2A) except breakpoints for ceftiofur (NCCLS/CLSI M31-A2 Table 2) and streptomycin (NARMS, 2001) and are presented in Table 5.

Table 4: Bacterial strains included in the study. Isolates were selected to represent a diverse range of phage types, resistance phenotypes, sites of isolation (animal vs human vs retail), provinces of isolation, and years of isolation

Strain	Isolation Year	PT	Prov[a]	Specimen Source	Antibiogram[b]
01-7169	2001	29	ON	Human Stool	AmpChlStrSxt
02-0102	2001	11a	ON	Human Stool	ChlStrSxtTcy
02-2339*	2002	19	ON	Human Stool	Sensitive
02-4660*	2002	19	ON	Human Stool	AmpChlStrSxtTcy
02-5785*	2002	AT02-5785	ON	Human Stool	AmpChlStrSmxTcy
02-5980	2002	29	QC	Human Blood	AmpTcy
03-0262*	2003	41	QC	Human Blood	AmcAmpFoxTioCroCep
03-0845*	2003	29	ON	Human Stool	AmcAmpFoxTioCepChlGenKanStrSmxTcy
03-3012	2003	4	QC	Human Blood	AmcAmpFoxTioCroCep
03-4601	2003	AT03-4601	QC	Human Blood	AmcAmpFoxTioCroCepChlStrSmxTcy
03-4690	2003	32	ON	Human Stool	AmcAmpFoxTioCroCepGenStrTcy
03-7402*	2003	29	ON	Human Blood	AmcAmpFoxTioCroCep
04-0346*	2004	54	QC	Human Stool	AmcAmpFoxTioCroCepChlStrSmxTcy
04-1511	2004	41	ON	Human Stool	AmcAmpFoxTioCepStrSmxTcy
04-3194	2004	29	ON	Human Blood	AmcAmpFoxTioCro
04-3293	2004	29a	QC	Human Blood	AmcAmpFoxTioCro
04-4717	2004	29	QC	Human Blood	AmcAmpFoxTioCro
05-5435	2004	29	QC	Human Blood	AmcAmpFoxTioCroTcy
04-5511*	2004	41	ON	Human Stool	Sensitive
05-4260	2001	29	ON	Bovine passive	AmcAmpCepFoxTio
05-4262*	2001	untypable	AB	Chicken passive	AmcAmpCepFoxChlGenKanSmxStrTcyTio
05-4263	2001	untypable	ON	Chicken passive	AmcAmpCepFoxTcyTio
05-4264	2002	Atypical	ON	Bovine passive	AmcAmpCepFoxChlGenKanSmxStrSxtTcyTio
05-4269*	2003	29	ON	Turkey passive	AmcAmpCepFoxTio
05-4272	2003	29	QC	Chicken retail	AmcAmpCepFoxTio
05-4275	2003	32	QC	Chicken retail	AmcAmpFoxTioCepGenStrTcy
05-4277	2003	29	QC	Chicken retail	AmcAmpFoxTioCep
05-4287	2003	29	ON	Chicken abattoir	AmcAmpFoxCepTio
05-4294	2004	29	ON	Chicken retail	AmcAmpFoxTioCep
05-4299	2004	29	ON	Chicken abattoir	AmcAmpFoxTioCep
05-4316*	2004	29	PE	Bovine passive	AmcAmpFoxTioCep
05-4354	2004	41	QC	Porcine passive	AmcAmpFoxCepTio
05-4355	2004	29	QC	Porcine passive	AmcAmpFoxCepTio
00-5440	2000	29	AB	Human	Sensitive
05-1147	2005	29	QC	Human	Sensitive
539		nd	ON	Chicken rinse	Sensitive
564		nd	ON	Egg yolk mix	Sensitive
1170		nd	ON	Cocoa beans	Sensitive
S-467	1948	nd	BC	Human	Sensitive
E.coli 830	2004	nd	AB	Chicken abattoir	AmcAmpFoxTioCepGenStrSmxTet
E.coli 831	2004	nd	QC	Chicken abattoir	AmcAmpFoxTioChlKanStrSmxTet
E.coli 832	2004	nd	QC	Porcine abattoir	AmcAmpFoxTioCepChlStrSmxTet
E.coli 833	2004	nd	ON	Chicken abattoir	AmcAmpFoxTioCepGenStrSmx
E.coli 834	2004	nd	QC	Bovine retail	AmcAmpFoxTioCep

a - Prov, Province; AB, Alberta; BC, British Columbia; PE, Prince Edward Island; QC, Quebec; ON, Ontario
b - Amc, amoxicillin-clavulanic acid; Amp, ampicillin; Fox, cefoxitin; Tio, ceftiofur;Cro, ceftriaxone; Cep, cephalothin; Chl, chloramphenicol; Gen, gentamycin; Kan, kanamycin; Nal, naladixic acid; Str, streptomycin; Sul, sulfamethoxasole; Tet, tetracycline; Smx, sulfizoxazole/sulfamethoxazole; Sxt, trimethoprim-sulfamethoxazole.
* CGH was conducted on these isolates.

Table 5: *Salmonella* and *E. coli* resistance breakpoints. Antimicrobial susceptibility testing was done by broth microdilution method performed using the Sensititre™ ARIS Automated Microbiology System using resistance breakpoints as determined by CIPARS. CIPARS resistance breakpoints were based on NCCLS/CLSI guidelines (NCCLS/CLSI M100-S15 Table 2A) with the exception of breakpoints for ceftiofur (NCCLS/CLSI M31-A2 Table 2) and streptomycin (NARMS, 2001) .

Antimicrobial	Susceptible (µg/ml)	Intermediate (µg/ml)	Resistant (µg/ml)
amikacin	≤ 16	32	≥ 64
amoxicillin-clavulanic acid	≤ 8/4	16/8	≥ 32/16
ampicillin	≤ 8	16	≥ 32
cefoxitin	≤ 8	16	≥ 32
ceftiofur	≤ 2	4	≥ 8
ceftriaxone	≤ 8	16-32	≥ 64
cephalothin	≤ 8	16	≥ 32
chloramphenicol	≤ 8	16	≥ 32
ciprofloxacin	≤ 1	2	≥ 4
gentamicin	≤ 4	8	≥ 16
kanamycin	≤ 16	32	≥ 64
nalidixic acid	≤ 16	-	≥ 32
streptomycin	≤ 32	-	≥ 64
sulfizoxazloe/ sulfamethoxazole	≤ 256	-	≥ 512
tetracycline	≤ 4	8	≥ 16
trimethoprim-sulfamethoxazole	≤ 2/38	-	≥ 4/76

2.2 DNA extractions

A single colony from each wild type *S.* Heidelberg or *E. coli* strain was used to inoculate 4 ml of Luria Bertani (LB) broth (Invitrogen Carlsbad, CA). Alternatively, a single colony from each transformant (a DH5α *E. coli* containing the *S.* Heidelberg bla_{cmy-2} plasmid) strain was used to inoculate 6 ml LB broth containing 20 µg/µl cefoxitin (Sigma). Cultures were incubated overnight at 37°C at 200 rpm in a Controlled Environment Incubator Shaker (New Brunswick Scientific co., Edison, NJ) and pelleted by centrifugation for 5 min at 5000 rpm in an Eppendorf centrifuge 5804R (Eppendorf, Hamburg, Germany). The cell pellet was resuspended in 2 ml TE buffer (Sigma, St. Louis, MO) (10 mM Tris-HCL, 1 mM EDTA, pH 8.0). Lysozyme (Roche Diagnostics, Indianapolis, IN) (0.5 mg/ml), RNase (Roche Diagnostics) (1.5 µg/ml), and proteinase K (10 mM Tris-HCL pH 7.5, 20 mM $CaCl_2$, 50% glycerol) (Sigma) (0.12 mg/ml) were added to the cell resuspension and following incubation at 37°C for 1 h in sodium dodecyl sulphate (SDS) (Ambion, Austin, TX) was added to a concentration of 0.1% (wt/vol) and the mixture was incubated at 65°C for 5 min or until clearing occurred. The mixture was then transferred to Phase Lock Light tubes (Eppendorf) for phenol-chloroform DNA extraction. A volume of phenol-chloroform:isoamyl (25:24:1) alcohol (Invitrogen, Burlington, ON) equal to that of the cell resuspension mixture was added to the phase lock tubes. Phenol-chloroform:isoamyl alcohol extraction was repeated until the aqueous layer was clear. Following a final extraction with an equal volume of chloroform (Fisher Scientific, Ottawa, ON), the aqueous layer was transferred to a new tube. DNA was precipitated at -20°C for 20 min using 0.6 vol of isopropanol (Fisher Scientific) and 0.1 vol 3 M sodium acetate (pH 5.5) (Ambion). DNA was collected by centrifugation at 15 000 rpm for 15 min in an Eppendorf centrifuge 5417R and subsequently washed with 70% ethanol (Commercial Alcohols Inc, Brampton, ON). Following resuspension in 200

µl TE buffer, DNA was quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE) and diluted to 20 ng/µl for use as PCR template.

2.3 Pulsed-field Gel Electrophoresis (PFGE)

PFGE was performed by PulseNet Canada according to the PulseNet USA protocol (Swaminathan *et al.*, 2001). Briefly, *Salmonella* isolates were grown overnight on Nutrient Agar +1.5% NaCl (wt/wt). Cells were resuspended in cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8.0) to an optical density of 0.48 – 0.52 as determined by a Dade Microscan Turbidity meter (Microscan Systems, Renton WA). The cell suspensions were combined with 10 µl proteinase K and 200 µl of 1% SeaKem Gold (Mandel, Guelph, ON):1 % SDS agarose mixed and poured into disposable plug molds. Following solidification, cells were lysed using cell lysis buffer [50 mM Tris, 50 mM EDTA, 1% sacrosine (Sigma-Aldrich, Oakville, ON)], and 0.1mg proteinase K for 2 h at 54°C with constant rotation at 175 – 200 rpm. Plugs were washed, rinsed with 18 MΩ H_2O and then washed twice with water and four times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 50°C. A 2 – 2.5 mm wide slice of plug was incubated in 150 µl of the appropriate restriction buffer plus *Xba*I (80 units / sample) at 37°C for a minimum of 2 h. The plugs were then loaded onto a 1 % Seakem Gold agarose gel. PFGE was performed using the CHEF-Mapper DR-III (Bio-Rad Laboratories Mississauga, ON) at 200V (6V/cm) with an initial switch time of 2.2 sec, a final switch time of 63.8 sec, and a run time of 18 – 20 h. The *Salmonella* serovar Branderup standard strain H9812 was used as the reference strain on all gels. Following electrophoresis, gels were stained for 20 min in ethidium bromide (Fisher) 2 µg/ml and images were captured using the BioRad GelDoc XR (Bio-rad). Pattern analysis and dendrograms were performed using the BioNumerics version 4.0 software package (Applied Maths, Austin, TX)

2.4 PCR

PCR reactions for *tetA*, *tetB* and *tetG* were performed using 1 unit of Fast start *Taq* DNA polymerase (Roche Diagnostics, Laval, QC) in a reaction mixture containing 1X Fast start *Taq* DNA polymerase buffer with $MgCl_2$ (Roche Diagnostics), 1 mM $MgCl_2$ (Roche Diagnostics), 0.2 µM dNTP mixture (Invitrogen Carslbad, CA), 0.2 µM each primer, 20 ng template DNA and distilled DNAase, RNAase free H_2O (Invitrogen) to 25 µl. All other PCR was performed using 1.0 unit of Platinum Hifi *Taq* DNA Polymerase High Fidelity (Invitrogen) in a reaction mixture containing 2 X High Fidelity PCR Buffer (600mM Tris-SO_4 [pH 8.9], 180mM ammonium sulfate) (Invitrogen), 0.2 mM dNTP mixture (Invitrogen), 2 mM $MgSO_4$ (Invitrogen), 0.3 µM each primer, 20 ng template DNA and distilled DNAase, RNAase free H_2O (Invitrogen) to 25 µl total volume. PCR was carried out using primers described in Table 6 using a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, Foster City, PA). The following parameters were used for *intA*, *potE* and *miaB* PCR reactions conducted with Platinum *Taq*: initial denaturation at 94°C for 5 min followed by thirty cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at a primer specific temperature listed in Table 6, extension for 1 min (*intA*) or 90 sec (*potE, miaB*) at 68°C followed by one final extension at 68°C for 7 min. The following parameters were used for all other reactions conducted with Platinum *Taq*: initial denaturation at 94°C for 5 min followed by30 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at a primer specific temperature listed in Table 6, extension for 30 sec at 68°C followed by one final extension at 68°C for 7 min. The following parameters were used for tetA, tetB and tetG reactions conducted using Fast start Taq DNA polymerase: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at a primer specific temperature listed in table 5 for 30 sec, extension at 72°C for 1 min followed by one final extension at 72°C for 7 min. PCR amplicons were resolved by gel electrophoresis in an electrophoresis

Table 6: Oligonucleotides used and their respective annealing temperatures. Oligo refers to oligonucleotide. Product size indicates the approximate length of the amplicon generated. Sequencing primers are denoted by "sequencing" in the annealing temperature column.

Oligo[a]	Target	Sequence 5'- 3'	Product Size (bp)	Annealing Temp	Reference
aadA1F aadA1R	*aadA1*	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484	54°C	Randall *et al.*, 2004
aadA2F aadA2R	*aadA2*	TGTTGGTTACTGTGGCCGTA GATCTCGCCTTTCACAAAGC	712	61°C	Randall *et al.*, 2004
cmy-2-1 cmy-2-2 cmy-2-3 cmy-2-4 cmy-2-5	bla_{cmy-2}	ACACTGATTGCGTCTGACG AATATCCTGGGCCTCATCG AGTTCTGGCCAGTATTTCG TGCAACCATTAAAACTGGC TTCCTTTTAATTACGGAAC	1143	60°C Sequencing Sequencing Sequencing	Mulvey *et al.*, 2005
dhfR1F dhrfRR	*dhfr*	GTGAAACTATCACTAATGGTAGCT ACCCTTTTGCCAGATTTGGTACCT	470	50°C	Randall *et al.*, 2004
fimAF fimAR	*fimA*	TCAGGGAGAAACAGAAAACTAAT TCCCCGATAGCCTCTTCC	760	55°C	Sukhnanand 2005
fldA-R fldA-F	*fldA*	TTAGGCGTTGAGGATGTCG GCAATCACTGGCATCTTTTTC	530	55°C	This study
floRF floRR	*floR*	AACCCGCCCTCTGGATCAAGTCAA CAAATCACCGGCCACGCTGTATC	548	60°C	Randall *et al.*, 2004
furR-F furR-R	*furR*	AAAGAAGGCTGGCCTGAAAG TTATTTAGTCGCGTCATCGTG	452	50°C	This study
intAF intAR	3' CS 5' CS	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGG	variable	52°C	Randall *et al.*, 2004
manBF manBR	*manB*	CATAACCCGATGGACTACAACG ACCAGCAGCCACGGGATCAT	893	55°C	Sukhnanand 2005
mdhF mdhR	*mdh*	GATGAAAGTCGCAGTCCTCG TATCCAGCATAGCGTCCAGC	849	50°C	Sukhnanand 2005
miaB-R miaB-F	*miaB*	TAGAATCCTACGCCCAGCTC GGGCTGTCAGATGAACGAG	1424	55°C	This study
potE-R potE-F	*potE*	AATTCAAAACGCGGTGAGAC TGTCGTGCAGCTCACAATTC	1319	55°	This study
rep1IF rep1IR	*RNAI*	CGAAAGCCGGACGGCAGAA TCGTCGTTCCGCCAAGTTCGT	139	50°C	Carattoli *et al.*, 2005
repA/CF repA/CR	*repA*	GAGAACCAAAGACAAAGACCTGGA ACGACAAACCTGAATTGCCTCCTT	465	50°C	Carattoli *et al.*, 2005
strAF strAR	*strA*	AGCAGAGCGCGCCTTCGCTC CCAAAGCCCACTTCACCGAC	684	61°C	Randall *et al.*, 2004
sul1F sul1R	*sul1*	TCACCGAGGACTCCTTCTTC AATATCGGGATAGAGCGCAG	631	60°C	Randall *et al.*, 2004
tetAF tetAR	*tetA*	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	70°C	Mendez *et al.*, 1980
tetBF tetBR	*tetB*	TTGGTTAGGGGCAAGTTTTG TTGGTTAGGGGCAAGTTTTG	659	64°C	Mendez *et al.*, 1980
tetGF tetGR	*tetG*	CCGGTCTTATGGGTGCTCTA CCAGAAGAACGAAGCCAGTC	693	56°C	Randall *et al.*, 2004
thdFF thdFR	*thdF*	TTGATTTTCCGGATGAGGAG AGGCATTGACAAAGGTCAGG	1553	55°C	This study

a - F; forward primer, R; reverse primer.
b - 3' CS; 3' conserved segment of class I integrons, 5' CS; 5' conserved segment of class I integrons.

chamber (OWL scientific, Portsmouth NH) on 1.5% agarose gels in 0.5X TBE buffer (Sigma-Aldrich) at 120 V for 60 min. Gels were visualized by UV transillumination as previously described in section 2.3.

2.5 Sequencing

PCR was conducted as previously described in Section 2.4 except in 100 µl reaction volumes. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions and eluted in 30 µl EB buffer (10mM Tris-Cl, pH 8.5). DNA was quantified on a NanoDrop ND-1000 and diluted to 50 ng/µl for use as sequencing template. Sequencing was conducted by the DNA Core at the NML using an ABI3730 apparatus (PE Applied Biosystems) with the primers used to generate the template. Sequencing of the *cmy-2* PCR product which was in excess of ~1 kbp required the design of additional sequencing primers listed in Table 5. Sequence data was analyzed using SeqMan II™ (DNAStar Inc. Madison, WI).

2.6 Comparative Genomic Hybridization

DNA microarrays were constructed as previously published using 4492 commercially supplied 70-mer oligonucleotides (Qiagen) representing the coding sequences of *S.* Typhimurium LT2 genome as well as all putative open reading frames from *Salmonella* Genomic Island I (SGI1) (Golding, *et al*, 2007). Oligonucleotides were resuspended in 50 % Dimethylsulfoxide (DMSO), printed onto UltraGAP slides (Corning, Acton, MA), with each spot duplicated on the array in a top and bottom grid format. After spotting all slides were post-processed by UV cross linking at 600 mj. Genomic DNA was isolated by phenol-chloroform extraction as previously described in section 2.2 and 30 µg was sheared using nebulizers (Invitrogen) at 10 psi for 3 min

according to manufacturer's directions. The BioPrime Array CGH Genomic Labeling System (Invitrogen) was used according to manufacturer's directions to label 2 µg of nebulized DNA with either Cy3-dCTP or Cy5-dCTP (Amersham Biosciences) by random priming. Labeled probes were purified using the BioPrime CGH purification module (Invitrogen) as suggested by the manufacturer. A NanoDrop ND-1000 was used to quantify probes and determine the efficiency of label incorporation. The differentially labeled probe from the reference sample *S.* Typhimurium LT2 and the test sample *S.* Heidelberg strain were combined and 20 µg of salmon sperm DNA (Invitrogen) was added. Each test versus reference comparison was conducted in quadruplicate or minimally in triplicate, with at least one dye swap. Combined probes were dried in a Vacufuge^{TM} (Eppendorf), resuspended in 10 µl of sterile H_2O and then denatured by heating at 95°C for 5 min and holding on ice for an additional 5 min. Finally 50 µl of Digoxigenin (DIG) Easy Hyb (Roche Diagnostics) was added to the mixture. Slide pre-hybridization was conducted in DIG Easy Hyb buffer at 42°C for 45 min. Following pre-hybridization, slides were briefly washed twice in ddH_2O and isopropanol and dried by a 15 sec spin in a Galaxy mini centrifuge (VWR, Mississauga, ON). An M-Series LifterSlip^{TM} (Erie Scientific Co., Portsmouth, NH) was placed on top of the slide and the entire volume of probe mixture was pipetted underneath. Slide hybridization was conducted in a Genetix hybridization chamber (Genetix Inc, Boston, MA) containing DIG Easy Hyb Buffer (Roche) for 16-20 h at 42°C in a water bath. Following hybridization, slides were sequentially washed in buffer 1 (1 X SSC [3.0 M sodium chloride, 0.3 M sodium citrate] and 0.2 % SDS) for 6 min at 56°C, buffer 2 (0.1 X SSC and 0.2 % SDS) for 4 min at room temperature, and twice in buffer 3 (0.1 X SSC) for 2 min at room temperature. Slides were dried by briefly spinning in a Galaxy mini centrifuge (VWR).

Slides were scanned using an Agilent DNA microarray scanner (Agilent Technologies, Mississauga, ON). Data analysis was conducted a previously described

in Golding *et al.*, 2007. Briefly, slide images were analyzed using Array-Pro Analyzer V. 4.5.1.48 (Media Cybernetics Inc., Silver Spring, MD). Net intensity data was calculated by subtracting the raw intensity data from background fluorescence. All mircoarray data generated from experiments conducted for a single strain was exported into PartekPro™ statistical analysis software (Partek Inc., St. Louis, MO). Using Partek data was log_2 transformed to introduce normality and the mean of each Gaussian distribution was centered about zero, thus shifting all data generated for a particular strain to the same linear scale. An analysis of variance (ANOVA) based estimation of the experimental variation attributable to technical effects including dye biases, slide-to-slide variation and hybridization date was also performed in Partek to allow for the removal of batch effects. Finally data was $antilog_2$ transformed to convert it back to its original scale so that the log_2 ratio between test and reference strains could be measured. Log_2 ratios were averaged across all replicates per spot for each test versus reference comparison. GeneMaths XT software (Applied Maths, Austin, TX) was used for hierarchical clustering of the data to examine the overall genetic relatedness between strains, and to identify specific loci that were absent or divergent between different *S.* Heidelberg strains.

2.7 MLST

MLST was conducted using a previously published three gene scheme based on *fimA, manB*, and *mdh* (Sukhnanand *et al.*, 2005). PCR and sequencing were conducted as previously described in sections 2.4 and 2.5 using primers and annealing temperatures listed in Table 5. Sequences were concatenated to create an allelic profile.

2.8 Plasmid Profiles

A single colony from each strain was used to inoculate 8 ml of LB broth. Cultures were incubated overnight at 37°C with rotation at 200 rpm in a Controlled Environment Incubator Shaker. Complete plasmid profiles were isolated from 2 ml of overnight culture using a QIAGEN plasmid mini kit (Qiagen) according to manufacturer's directions. Plasmids were resuspended in 25 µl TE buffer (10 µM Tris-HCL pH 8.0, 1 µM EDTA) (Sigma). Plasmids were resolved by gel electrophoresis on 0.9 % agarose gels in 0.5 X TBE buffer (Sigma-Aldrich) at 90 V for 90 min. Following staining for 20 min in ethidium bromide (2 µg/ml) and destaining for 20 min in ddH_2O gels were visualized by UV transillumination using a BioRad GelDoc XR (Bio-rad).

2.9 Electroporation

Electroporation was carried out as described in Current Protocols in Molecular Biology (Sambrook and Russell, 2001). Briefly, 5 µl plasmid mini prep was added to 50 µl OneShot TOP10 Electrocompetant *E. coli* cells (Invitrogen) and the mixture was transferred to a 0.1 cm chilled cuvette. A BioRad Gene Pulser (Bio-rad) was used to apply a 1.25 kV pulse. Immediately following the pulse 1 ml of S.O.C medium (Invitrogen) was added to the cuvette and the contents transferred to a sterile culture tube. Following incubation of the transformation culture at 37°C for 60 min, 20 and 200 µl aliquots were plated onto LB agar (Invitrogen) plates containing 20 µg/ml cefoxitin (Sigma) and incubated overnight at 37°C. Potential transformant colonies were inoculated into 8 ml LB broth containing 20 µg/ml cefoxitin and incubated overnight at 37°C with agitation. DNA extractions were then conducted as previously described in section 2.2 and PCR using the bla_{cmy-2} primer set was performed as describecd in section 2.4 to verify transformants.

2.10 *bla*$_{cmy-2}$ Plasmid isolation from transformants

A single transformant colony from a selective plate was inoculated into 8 ml of LB broth with 20 µg/ml cefoxitin to create a starter culture. Starter culture was incubated overnight at 37°C with rotation at 200 rpm. Starter culture was diluted 1/500 into 150 ml LB broth with 20 µg/ml cefoxitin and grown overnight at 37°C with roatation at 200 rpm. Plasmids were isolated from 50 ml of overnight culture using a Qiagen plasmid midi kit according to the manufacturer\s directions with the following modification: plasmid DNA was precipitated using 2 ml 7.5M ammonium acetate (Sigma-Aldrich) in combination with 0.7 vol of isopropanol (Ficher Scientific). Plasmid DNA was resuspended in 100 µl TE buffer.

2.11 Restriction Fragment Length Polymorphism (RFLP)

Purified plasmid DNA (25 µl) was digested overnight at 37°C with 20 units of *Bgl*II (New England Biolabs, Pickering, ON). Resulting plasmid fragments were separated by gel electrophoresis on 0.7 % Tris-acetate-EDTA (TAE) agarose (Gibco BRL, Paisley, Scotland) agarose gels at 60 V for 6 h in TAE (400 mM Tris-acetate, 10 mM EDTA). A 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, CA) and Track it λ DNA/Hind III fragments (Invitrogen) were used as molecular size standards. Gels were stained with ethidium bromide 2 µg/ml, destained overnight in ddH_2O at 4°C, visualised with UV transillumination and photographed using a Bio-Rad Gel Doc XR. RFLP pattern analysis was conducted using Bionumerics version 4.0 software. A dendrogram based on RFLP patterns was generated in Bionumerics using the unweighted pair group method with a different bands fuzzy logic coefficient of correlation and positional tolerances as follows: 3 % optimization, 1 % tolerance, H >0.0 %, S >0.0 %.

2.12 Southern Blot

RFLP gels were depurinated in 250 mM HCL for 12 min, denatured in a 1.5 M NaCl, 0.5 M NaOH solution for 30 min and finally neutralized in a 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5 solution for 30 min. DNA was transferred by capillary blotting to a positively charged nylon Hybond-N+ (Amersham Biosciences, Little Chalfont, UK) membrane using the TurboBlotter system (Schleicher & Schuell, Keene, NH) according to manufacturer's directions with 10 X SSC (Ambion, Austin, TX) transfer buffer. Membranes were rinsed in 6 X SSC (Ambion) and DNA was fixed to the membrane by UV treatment. Nucleic acid labeling and detection was carried out using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Life Sciences, Little Chalfont, UK). Labeled probes were generated from PCR products. PCR amplicons were purified using the QIAquick PCR Purification Kit according to manufacturer's directions. Amplicons were quantified on a NanoDrop ND-1000 and diluted to 10 ng/µl. Amplicons (150ng) were denatured by boiling for 5 min and snap cooling on ice for an additional 5 min. Probes were labeled at 37°C for 10 min through the addition of equal volumes of DNA labeling reagent (Amersham) and gluteraldehyde (Amersham). Membranes were pre-hybridized at 42°C for 30 min in 25 ml pre-heated ECL gold hybridization buffer (Amersham) containing 0.5 M NaCl and 5 % (w/v) blocking agent (Amersham). Labeled probe was added to hybridization buffer and hybridization was allowed to proceed overnight at 42°C in a Fisher Isotemp hybridization oven (Fisher Scientific). Following hybridization, excess probe was removed by washing twice with primary wash buffer (0.5 X SSC, 0.4 % SDS) at 42°C for 20 min and twice with 2 X SSC for 5 min at room temperature. The presence of probe was detected on Hyperfilm ECL (Amersham) autoradiography film according to manufacturer's directions. Film was developed in a Feline™ file developer (Fisher).

2.13 Conjugation

Salmonella Heidelberg strains were used as bla_{cmy-2} plasmid donor strains. Recipient *E. coli* RG192 was serially passaged against rifampicin (Sigma-Aldrich) until resistance to 384 µg/ml was achieved. Cefoxitin (20 µg/ml) and rifampicin (384 µg/ml) (Sigma-Aldrich) were used as selective agents for the donor and recipient strains, respectively. Single colonies of donor and recipient strains from selective plates were inoculated into 8 ml of LB broth containing the appropriate selective antibiotic and grown overnight at 37°C at 200 rpm. Overnight cultures were then subcultured into LB broth without selective antibiotic and incubated at 37°C for 5 h at 200 rpm. Recipient and donor cells were combined in a 4:1 ratio in LB broth. Following overnight incubation at 37°C, transconjugants were selected by plating onto LB agar containing cefoxitin (20 µg/ml) and rifampicin (384 µg/ml). The transfer of bla_{CMY2} was confirmed by PCR to detect the presence of the bla_{cmy-2} gene followed by isolation of the bla_{cmy-2} plasmid from the recipient *E. coli* according to the methods described in sections 2.4 and 2.5

2.14 SGI1 conjugation

To determine whether *S.* Heidelberg could serve as a recipient for SGI1, the gene, *thdF*, previously shown to contain the SGI1 insertion point, was amplified by PCR and sequenced from five *S.* Heidelberg isolates using primers described in Table 5. The transfer of SGI1 was conducted using a plate conjugation method. Briefly, *E. coli* JSK 5K-12 containing the IncC R55 plasmid and SGI1 was used as the donor strain. Rifampicin resistant *S.* Heidelberg strains 05-4272R and 1170R obtained by serially passaging on increasing concentrations of rifampicin until resistance to 384 µg/ml was achieved were used as recipient strains. Tetracycline (15 µg/ml) (Sigma-Aldrich) and kanamycin (50 µg/ml) (Sigma-Aldrich) were used as selective agents for *E. coli* JSK 5K-

12. Rifampicin (128 µg/ml) was used a selective agent for *S*. Heidelberg. Single colonies of donor and recipient strains from selective plates were inoculated into 8 ml LB broth containing appropriate selective antibiotic and incubated overnight at 37°C at 200 rpm. Overnight cultures were then subcultured into 8 ml of LB containing appropriate selective antibiotic and incubated overnight at 37°C at 200 rpm. Overnight cultures were then subcultured into LB broth without selective antibiotic and incubated at 37°C for 5 h without rotation. Recipient and donor strains were streaked out onto MacConkey agar (BD Diagnositics, Sparks, MD) in a cross hatch pattern and incubated overnight at 37°C. Colonies at right angle intersections were resuspended in 1 ml of TE buffer and SGI1 transconjugants were selected by plating onto MacConkey agar containing 128 µg/ml rifampicin and 15 µg/ml tetracycline.

Chapter 3: Results

3.1 Pulsed field gel electrophoresis

The CIPARS group has noted that *S.* Heidelberg consistently ranks amongst the most prevalent causes of human salmonellosis in Canada (CIPARS, 2004). There has also been an increase in the number of MDR isolates from human and agri-food sources, particularily those resistant to one or more ESC antimicrobial. This study aimed to investigate whether the observed increase in the amount of human salmonellosis attributable to MDR *S.* Heidelberg is due to the increase in occurrence of MDR *S.* Heidelberg amongst chicken agri-food sources noted during abattoir, retail and passive surveillance by CIPARS. PFGE performed by PulseNet Canada was used to examine the genetic relationship among *S.* Heidelberg isolates. PFGE identified limited genetic diversity among the 114 CIPARS *S.* Heidelberg isolates submitted to PulseNet Canada between 2001 and 2004 (Table 7). These isolates represented temporally distinct isolates of different phage types from human (blood and stool), chicken (retail, abattoir and passive), bovine passive and porcine passive sources obtained from across Canada including Ontario (ON), Quebec (QC), Alberta (AB), British Columbia (BC) and Prince Edward Island (PEI). Despite the diverse origins of these strains, a single PFGE pattern SHEXAI.0001 predominated among the isolates. The majority of the other PFGE patterns observed were subtle variations on the SHEXAI.0001 pattern. Due to the limited genetic diversity identified by this method no obvious link between chicken and human isolates (implying chicken to human transmission) could be made.

A small panel of 44 strains (Table 8) was subjected to further genetic analysis to investigate whether the homogeneity observed among the isolates by PFGE represented chromosomal clonality among the isolates or whether any genetic traits characteristic of specific isolate types could be identified. The identification of any genetic traits that are exclusively or predominately shared between human and chicken

Table 7: The total number of CIPARS *S.* Heidelberg isolates submitted to PulseNet Canada between 2001 and 2004 divided by source of isolation.

Source	ON	QC	PE	BC	AB	Total
Bovine - passive	2		1			3
Chicken - abattoir	14	13		2		29
Chicken - passive	8	2			2	12
Chicken - retail	19	29				48
Porcine - passive		2				2
Turkey - passive	1					1
Human - blood	2	7				9
Human - stool	10					10
						114

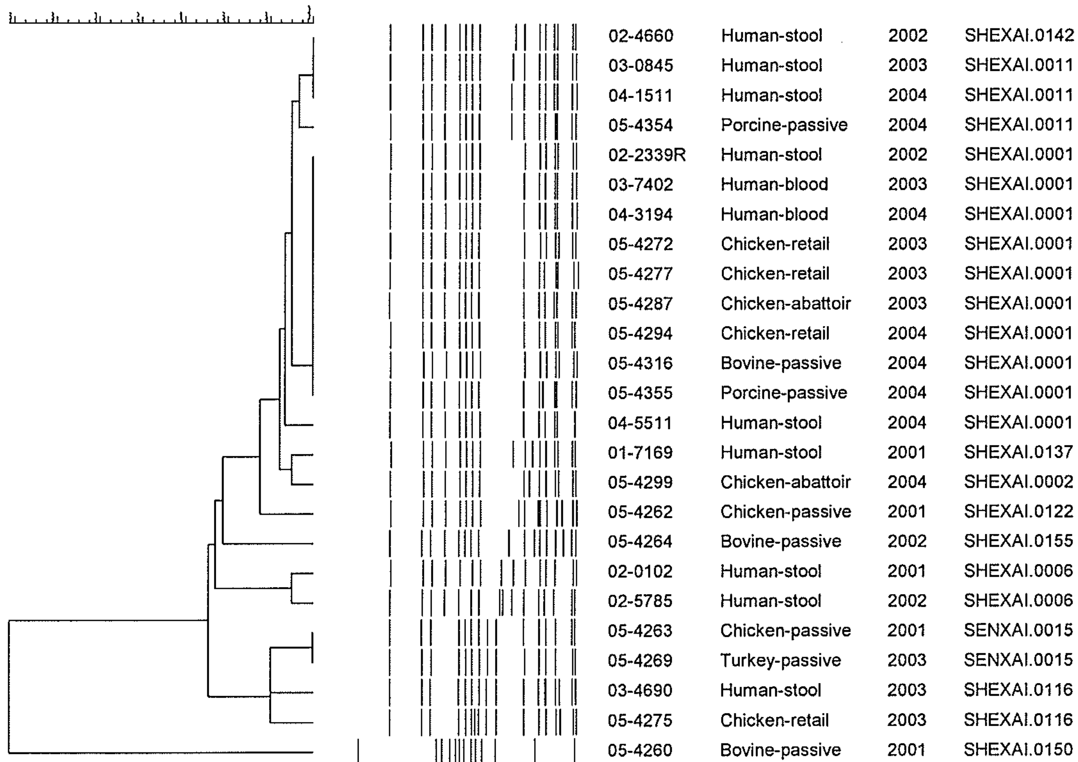
Table 8: Bacterial strains included in the study. Isolates were selected to represent a diverse range of phage types, resistance phenotypes, sites of isolation (animal vs human vs retail), provinces of isolation, and years of isolation.

Strain	Isolation Year	PT	Prov[a]	Specimen Source	Antibiogram[b]
01-7169	2001	29	ON	Human Stool	AmpChlStrSxt
02-0102	2001	11a	ON	Human Stool	ChlStrSxtTcy
02-2339*	2002	19	ON	Human Stool	Sensitive
02-4660*	2002	19	ON	Human Stool	AmpChlStrSxtTcy
02-5785*	2002	AT02-5785	ON	Human Stool	AmpChlStrSmxTcy
02-5980	2002	29	QC	Human Blood	AmpTcy
03-0262*	2003	41	QC	Human Blood	AmcAmpFoxTioCroCep
03-0845*	2003	29	ON	Human Stool	AmcAmpFoxTioCepChlGenKanStrSmxTcy
03-3012	2003	4	QC	Human Blood	AmcAmpFoxTioCroCep
03-4601	2003	AT03-4601	QC	Human Blood	AmcAmpFoxTioCroCepChlStrSmxTcy
03-4690	2003	32	ON	Human Stool	AmcAmpFoxTioCroCepGenStrTcy
03-7402*	2003	29	ON	Human Blood	AmcAmpFoxTioCroCep
04-0346*	2004	54	QC	Human Stool	AmcAmpFoxTioCroCepChlStrSmxTcy
04-1511	2004	41	ON	Human Stool	AmcAmpFoxTioCepStrSmxTcy
04-3194	2004	29	ON	Human Blood	AmcAmpFoxTioCro
04-3293	2004	29a	QC	Human Blood	AmcAmpFoxTioCro
04-4717	2004	29	QC	Human Blood	AmcAmpFoxTioCro
05-5435	2004	29	QC	Human Blood	AmcAmpFoxTioCroTcy
04-5511*	2004	41	ON	Human Stool	Sensitive
05-4260	2001	29	ON	Bovine passive	AmcAmpCepFoxTio
05-4262*	2001	untypable	AB	Chicken passive	AmcAmpCepFoxChlGenKanSmxStrTcyTio
05-4263	2001	untypable	ON	Chicken passive	AmcAmpCepFoxTcyTio
05-4264	2002	Atypical	ON	Bovine passive	AmcAmpCepFoxChlGenKanSmxStrSxtTcyTio
05-4269*	2003	29	ON	Turkey passive	AmcAmpCepFoxTio
05-4272	2003	29	QC	Chicken retail	AmcAmpCepFoxTio
05-4275	2003	32	QC	Chicken retail	AmcAmpFoxTioCepGenStrTcy
05-4277	2003	29	QC	Chicken retail	AmcAmpFoxTioCep
05-4287	2003	29	ON	Chicken abattoir	AmcAmpFoxCepTio
05-4294	2004	29	ON	Chicken retail	AmcAmpFoxTioCep
05-4299	2004	29	ON	Chicken abattoir	AmcAmpFoxTioCep
05-4316*	2004	29	PE	Bovine passive	AmcAmpFoxTioCep
05-4354	2004	41	QC	Porcine passive	AmcAmpFoxCepTio
05-4355	2004	29	QC	Porcine passive	AmcAmpFoxCepTio
00-5440	2000	29	AB	Human	Sensitive
05-1147	2005	29	QC	Human	Sensitive
539		nd	ON	Chicken rinse	Sensitive
564		nd	ON	Egg yolk mix	Sensitive
1170		nd	ON	Cocoa beans	Sensitive
S-467	1948	nd	BC	Human	Sensitive
E.coli 830	2004	nd	AB	Chicken abattoir	AmcAmpFoxTioCepGenStrSmxTet
E.coli 831	2004	nd	QC	Chicken abattoir	AmcAmpFoxTioChlKanStrSmxTet
E.coli 832	2004	nd	QC	Porcine abattoir	AmcAmpFoxTioCepChlStrSmxTet
E.coli 833	2004	nd	ON	Chicken abattoir	AmcAmpFoxTioCepGenStrSmx
E.coli 834	2004	nd	QC	Bovine retail	AmcAmpFoxTioCep

a - Prov, Province; AB, Alberta; BC, British Columbia; PE, Prince Edward Island; QC, Quebec; ON, Ontario
b - Amc, amoxicillin-clavulanic acid; Amp, ampicillin; Fox, cefoxitin; Tio, ceftiofur;Cro, ceftriaxone; Cep, cephalothin; Chl, chloramphenicol; Gen, gentamycin; Kan, kanamycin; Nal, naladixic acid; Str, streptomycin; Sul, sulfamethoxasole; Tet, tetracycline; Smx, sulfizoxazole/sulfamethoxazole; Sxt, trimethoprim-sulfamethoxazole.
* CGH was conducted on these isolates.

isolates as compared to isolates originating from other agri-food sources could be useful to establish a chain of transmission for *S.* Heidelberg from chicken agri-food sources to humans. The panel consisting of 44 strains was selected to represent a diverse array of isolate types available from across the agri-food spectrum and antimicrobial resistance profiles. The panel contained 39 *S.* Heidelberg strains obtained from CIPARS and NESP which were chosen to represent different PFGE patterns (Figure 4), phage types, years of isolation (2001 to 2004), provinces of isolation (ON, QC, AB, BC, PEI) as well as sources of isolation including clinical isolates from human, bovine, porcine and chicken sources as well as retail and abattoir chicken isolates. The panel also contained isolates which were sensitive, resistant to ESC alone or in combination with resistance to other classes of antimicrobials or were resistant to a combination of antimicrobials excluding ESC. A limited number of *S.* Heidelberg strains were isolated from non-chicken agri-food sources, thus all isolates from bovine (3), porcine (2) and turkey (1) sources were included in the study. The remaining isolates were human-blood, human-stool or chicken agri-food isolates. The PFGE pattern SHEXAI.0001 predominated amongst the original group of 114 isolates, and 10 isolates with this pattern were included in the study. These 10 isolates were selected to represent a variety of resistance phenotypes, sources and years of isolation. The 15 isolates of the original group of 114 isolates that had PFGE patterns other than SHEXI.0001 were included in the strain set to ensure all PFGE pattern types were represented. In addition to PFGE pattern, the human isolates included in the study were selected to represent different resistance phenotypes, years, provinces and sources of isolation. All of the eight available sensitive human isolates were included in the study. All five available non-ESC resistant isolates were also included in the study while the remaining human isolates were ESC resistant. When possible an equal number of human isolates was selected from each year, province and source of isolation (blood or stool) included in the

Figure 4: Dendrogram of *S*. Heidelberg DNA macrorestriction patterns generated with *Xba*I. Isolates were selected to represent a diverse range of PFGE fingerprints. Dendrogram was created using Applied Maths Bionumerics version 4.0 using unweighted pair group method (UPGMA) with a dice coefficient of similarity, 1 % band tolerance and 1.5 % optimization.



Different bands (Opt:3.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE-XbaI
PFGE-XbaI
02-4660 Human-stool 2002 SHEXAI.0142
03-0845 Human-stool 2003 SHEXAI.0011
04-1511 Human-stool 2004 SHEXAI.0011
05-4354 Porcine-passive 2004 SHEXAI.0011
02-2339R Human-stool 2002 SHEXAI.0001
03-7402 Human-blood 2003 SHEXAI.0001
04-3194 Human-blood 2004 SHEXAI.0001
05-4272 Chicken-retail 2003 SHEXAI.0001
05-4277 Chicken-retail 2003 SHEXAI.0001
05-4287 Chicken-abattoir 2003 SHEXAI.0001
05-4294 Chicken-retail 2004 SHEXAI.0001
05-4316 Bovine-passive 2004 SHEXAI.0001
05-4355 Porcine-passive 2004 SHEXAI.0001
04-5511 Human-stool 2004 SHEXAI.0001
01-7169 Human-stool 2001 SHEXAI.0137
05-4299 Chicken-abattoir 2004 SHEXAI.0002
05-4262 Chicken-passive 2001 SHEXAI.0122
05-4264 Bovine-passive 2002 SHEXAI.0155
02-0102 Human-stool 2001 SHEXAI.0006
02-5785 Human-stool 2002 SHEXAI.0006
05-4263 Chicken-passive 2001 SENXAI.0015
05-4269 Turkey-passive 2003 SENXAI.0015
03-4690 Human-stool 2003 SHEXAI.0116
05-4275 Chicken-retail 2003 SHEXAI.0116
05-4260 Bovine-passive 2001 SHEXAI.0150

study. In addition to PFGE pattern, the chicken agri-food isolates included in the study were selected to represent different resistance types, years, provinces and sources of isolation. All three of the chicken isolates which exhibited a MDR-ESC resistance phenotype were included in the study, while the remaining chicken isolates were ESC resistant. When possible, an equal number of chicken agri-food isolates were selected from each year, province and source (retail, abattoir, and passive-clinical) of isolation. The majority of *S.* Heidelberg isolates collected by CIPARS were ESC resistant thus the panel focused on isolates of this resistance phenotype. The subsequent genetic analyses focused on ESC resistance to determine whether the genetic elements mediating this common resistance phenotype could provide a link between human *S.* Heidelberg isolates and chicken agri-food isolates. To examine and control for other sources of ESC resistance in the agri-food continuum that could serve as sources of ESC resistance for *S.* Heidelberg the panel also contained 5 *E. coli* strains isolated from agri-food sources. The *E. coli* isolates included chicken and porcine abattoir isolates as well as bovine retail isolates representing either an A2C-Amp-MDR phenotype or A3C-Amp phenotype.

3.2 Resistance gene PCR

The panel of 39 *S.* Heidelberg and 5 *E. coli* strains was screened for the presence of the resistance gene cassettes *aadA1, aadA2,* $bla_{cmy\text{-}2}$, *dhfR, floR, tetA, tetB, tetG, strA, sul1* (Table 9). The genes responsible for the resistance phenotype of the strains were determined to examine whether any specific resistance determinants were exclusively present among human clinical and chicken agri-food isolates. No resistance gene

Table 9: The resistance phenotype and genotype of *S*. Heidelberg and *E. coli* isolates. The table is ordered according to both specimen source and year of isolation. Resistance phenotypes were determined using the sensititre™ ARIS Automated Microbiology System. The genes responsible for the resistance genotype of the isolate were determined by directed PCR screens targeting the *aadA1, aadA2, bla*$_{cmy-2}$*, dhfR, floR, strA, sul1, tetA, tetB,* and *tetG* genes.

Strain	Isolation Yr	Prov	Specimen Source	Antibiogram[a]	Resistance genes confirmed[b]
S-467	1948	BC	Human	Sensitive	-
00-5440	2000	AB	Human	Sensitive	-
01-7169	2001	ON	Human Stool	AmpChlStrSxt	*aadA2, strA,sul1*
02-0102	2001	ON	Human Stool	ChlStrSxtTcy	*aadA1, aadA2*
02-2339	2002	ON	Human Stool	Sensitive	-
02-4660	2002	ON	Human Stool	AmpChlStrSxtTcy	bla_{cmy-2}, *tetA, dhfR, strA, floR, sul1*
02-5785	2002	ON	Human Stool	AmpChlStrSmxTcy	bla_{cmy-2}, *tetA, strA, floR, sul1*
02-5980	2002	QC	Human Blood	AmpTcy	bla_{cmy-2}
03-0262	2003	QC	Human Blood	AmcAmpFoxTioCroCep	bla_{cmy-2}
03-0845	2003	ON	Human Stool	AmcAmpFoxTioCepChlGenKanStrSmxTcy	bla_{cmy-2}, *tetA, strA, sul1*
03-3012	2003	QC	Human Blood	AmcAmpFoxTioCroCep	bla_{cmy-2}
03-4601	2003	QC	Human Blood	AmcAmpFoxTioCroCepChlStrSmxTcy	bla_{cmy-2}, *tetA, strA, floR*
03-4690	2003	ON	Human Stool	AmcAmpFoxTioCroCepGenStrTcy	bla_{cmy-2}, *tetB, strA*
03-7402	2003	ON	Human Blood	AmcAmpFoxTioCroCep	*cmy-2*
04-0346	2004	QC	Human Stool	AmcAmpFoxTioCroCepChlStrSmxTcy	bla_{cmy-2}, *tetA, strA, floR*
04-1511	2004	ON	Human Stool	AmcAmpFoxTioCepStrSmxTcy	bla_{cmy-2}, *tetA, strA,*
04-3194	2004	ON	Human Blood	AmcAmpFoxTioCro	bla_{cmy-2}
04-3293	2004	QC	Human Blood	AmcAmpFoxTioCro	bla_{cmy-2}
04-4717	2004	QC	Human Blood	AmcAmpFoxTioCro	bla_{cmy-2}
05-5435	2004	QC	Human Blood	AmcAmpFoxTioCroTcy	bla_{cmy-2}
04-5511	2004	ON	Human Stool	Sensitive	-
05-1147	2005	QC	Human	Sensitive	-
05-4260	2001	ON	Bov passive	AmcAmpCepFoxTio	-
05-4262	2001	AB	Chi passive	AmcAmpCepFoxChlGenKanSmxStrTcyTio	bla_{cmy-2}, *tetA, dhfR, ,strA, floR, sul1*
05-4263	2001	ON	Chi passive	AmcAmpCepFoxTcyTio	bla_{cmy-2}, *tetB*
05-4264	2002	ON	Bov passive	AmcAmpCepFoxChlGenKanStrSxtTcyTio	bla_{cmy-2}, *tetA, dhfR, strA, aadA1, floR, sul1*
05-4269	2003	ON	Tur passive	AmcAmpCepFoxTio	bla_{cmy-2}, *tetB*
05-4272	2003	QC	Chi retail	AmcAmpCepFoxTio	bla_{cmy-2}
05-4275	2003	QC	Chi retail	AmcAmpFoxTioCepGenStrTcy	bla_{cmy-2}, *tetB, strA*
05-4277	2003	QC	Chi retail	AmcAmpFoxTioCep	bla_{cmy-2}
05-4287	2003	ON	Chi abattoir	AmcAmpFoxCepTio	bla_{cmy}
05-4294	2004	ON	Chi retail	AmcAmpFoxTioCep	bla_{cmy}
05-4299	2004	ON	Chi abattoir	AmcAmpFoxTioCep	bla_{cmy}
05-4316	2004	PE	Bov passive	AmcAmpFoxTioCep	bla_{cmy}
05-4354	2004	QC	Por passive	AmcAmpFoxCepTio	bla_{cmy}
05-4355	2004	QC	Por passive	AmcAmpFoxCepTio	bla_{cmy-2}
1170	1998	ON	Cocoa beans	Sensitive	-
539	1988	ON	Chi rinse	Sensitive	-
564	1988	ON	Egg yolk mix	Sensitive	-
E.coli 830	2004	AB	Chi abattoir	AmcAmpFoxTioCepGenStrSmxTet	bla_{cmy-2},*tetA, aadA1, sul1*
E.coli 831	2004	QC	Chi abattoir	AmcAmpFoxTioChlKanStrSmxTet	bla_{cmy-2},*,tet(A),aadA1,strA, floR, sul1*
E.coli 832	2004	QC	Por abattoir	AmcAmpFoxTioCepChlStrSmxTet	bla_{cmy-2}, *tetA, strA, floR*
E.coli 833	2004	ON	Chi abattoir	AmcAmpFoxTioCepGenStrSmx	bla_{cmy-2},*aadA1, floR, sull*
E.coli 834	2004	QC	Bov retail	AmcAmpFoxTioCep	bla_{cmy-2}, *aadA2*

a - Amc, amoxicillin-clavulanic acid; Amp, ampicillin; Fox, cefoxitin; Tio, ceftiofur; Cro, ceftriaxone; Cep, cephalothin; Chl, chloramphenicol; Gen, gentamycin; Kan, kanamycin; Nal, naladixic acid; Str, streptomycin; Sul, sulfamethoxasole; Tet, tetracycline; Smx, sulfizoxazole/sulfamethoxazole; Sxt, trimethoprim-sulfamethoxazole.

b - *aadA1*, streptomycin/spectinomycin adenyltransferase; *aadA2*, streptomycin/spectinomycin adenyltransferase; *dhfR*, dihydrofolate reductase; *floR*, efflux; *strA*, streptomycin phosphotransferase; *sull*, dihydropteroate synthase*;* *tetA*, efflus; *tetB*, efflux; *tetG*, efflux; bla_{cmy-2}, beta-lactamase.

cassettes could be amplified from the eight sensitive strains included in the study. In all cases where strains exhibited an ESC resistance phenotype (29/39), the *bla*$_{cmy\text{-}2}$ gene phenotypically observed streptomycin resistance in three of the *E. coli* strains. A streptomycin resistance determinant could not be amplified from the remaining strain was found to be responsible. The sequence of the *bla*$_{cmy\text{-}2}$ gene was identical for all isolates. Phenotypic testing indicated 13/39 strains were tetracycline resistant; the *tetA* gene was responsible for resistance in eight isolates while the *tetB* gene was responsible for resistance in four isolates. No tetracycline resistance determinant could be amplified from the remaining isolates. In all cases (12/39) where strains exhibited phenotypic resistance to streptomycin, the *strA* gene was found to be responsible. An additional streptomycin resistance determinant, either *aadA1*, *aadA2* or both was carried by five strains. Chloramphenicol resistance was seen in 9/39 isolates by phenotypic testing and the *floR* gene was found to mediate resistance in six of these isolates while the *cmlA* gene was found to mediate resistance in one. A resistance determinate conferring chloramphenicol resistance could not be amplified from two of the remaining resistant strains. Sulfamethoxazole – trimethoprim resistance was detected by phenotypic methods for three strains. The resistance genes responsible were *dhfR* and *sul1* in two cases, however in the remaining strain only *sul1* could be amplified and the resistance determinate conferring trimethoprim resistance was undetermined. A sulfamethoxazole resistance phenotype was detected in 7/39 strains and resistance was mediated by the *sul1* gene cassette in four cases whereas a resistance determinant could not be amplified in the three remaining cases. No resistance determinant was exclusively encoded amongst the human and chicken isolates.

The five *E. coli* isolates with an ESC resistance phenotype were also screened for the presence of resistance determinants (Table 8). The *bla*$_{cmy\text{-}2}$ gene was found to mediate ESC resistance in all cases. The sequence of the *bla*$_{cmy\text{-}2}$ gene was identical

among the *E. coli* isolates and in addition identical to the bla_{cmy-2} of the *S*. Heidelberg isolates. Tetracycline resistance was phenotypically observed for three of the isolates and in all cases was mediated by the *tetA* gene. The *strA* gene was responsible forwhich exhibited phenotypic streptomycin resistance. Phenotypic chloramphenicol resistance was observed in one strain and the gene responsible was determined to be *floR*. Phenotypic sulfamethoxazole resistance was detected in four of the *E. coli* strains, *sul1* was responsible for the phenotype in three of the strains while a resistance determinant conferring sulfamethoxazole resistance could not be amplified from the remaining strain.

3.3 Integron Characterization

To examine potential mechanisms for the acquisition of resistance determinants the strains were screened for the presence of Class I integrons. Class I integrons were amplified from a total of eight strains: four human-stool *S.* Heidelberg, one bovine-clinical (collected by passive surveillance) S. Heidelberg, and three *E.coli* chicken-abattoir strains (Table 10). Sequencing of the integron PCR amplicon identified resistance genes carried by the integron. The integron amplified from 03-0845 carried the chloramphenicol resistance determiant *cmlA* and aminoglycoside resistance determinant *aadB* and was located on a HMW plasmid later shown to be the bla_{cmy-2} plasmid. The integron amplified from 02-4660 was carried on a HMW plasmid (later shown to be the bla_{cmy-2} plasmid) and was 100% identical to the integron amplified from 05-4264 which carried the dihydrofolate reductase gene *dhfrA1*. The integron amplified from the genomic DNA of 01-7169 carried the streptomycin resistance determinant *aadA2*. The three *E. coli* isolates contained identical class I integrons encoding the streptomycin/spectinomycin adenyltransferase *aadA1*.

Table 10: Characterization of Class I integrons amplified from *S.* Heidelberg and *E. coli.* Integrons were amplified from the isolates using PCR primers which targeted the 3' conserved segment (CS) and 5' CS characteristic of class I integrons. The gene content of the integrons was determined by sequencing the PCR amplicon.

Isolate	Source	Location	Integron length (kbp)	Genes encoded[a]
01-7169	Human – stool	Unknown	1.2	*aadA2*
02-4660	Human – stool	$bla_{cmy\text{-}2}$ plasmid	1.2	*dhfRA1*
02-5785	Human – stool	Unknown	1.2	nd
03-0845	Human – stool	$bla_{cmy\text{-}2}$ plasmid	3.0	*aadB, cmlA*
05-4264	Bovine – passive	Unknown	1.2	*dhfRA1*
E. coli 830	Chicken – abattoir	Genomic	1.0	*aadA1*
E. coli 831	Chicken – abattoir	Genomic	1.0	*aadA1*
E. coli 833	Chicken – abattoir	Genomic	1.0	*aadA1*

a - *aadA1*, streptomycin/spectinomycin adenyltransferase; *aadA2*, streptomycin/spectinomycin adenyltransferase; *aadB*, aminoglycoside adenyltransferase; *cmlA*, chloramphenicol/florphenicol efflux; *dhfrA1*, dihydrofolate reductase; nd, not determined.

3.4 MLST

MLST was unable to identify any strain to strain variation among the isolates. A three gene MLST scheme based on *fimA, manB, mdh* identified no nucleotide polymorphisms among 2502 bp sequenced from each of ten *S.* Heidelberg isolates.

3.5 Comparative Genomic Hybridization

A diverse range of *S.* Heidelberg isolates was examined by CGH (Table 8). Isolates were selcted to represent all human and agri-food isolate types. Human strains were selcted to represent sensitve and resistance isolate types from blood and stool. Agri-food isolates were selected to represent non-chicken agri-food sources including bovine, porcine and turkey clinical (passive). A single chicken clinical (passive) isolate was analyzed. The panel of chicken isolates examined by CGH was not expanded because subsequent analysis of CGH data indicated that this technique was not a suitable method to examine the genetic content of *S.* Heidelberg strains. Comparative genomic analysis of *S.* Heidelberg strains indicated that there were regions which were consistently putatively divergent or absent amongst all of the *S.* Heidelberg strains with reference to *S.* Typhimurium LT2 (Figure 5). The majority of the putatively divergent regions consisted of prophage genes clustered in three regions of the *S.* Typhimurium genome. The first divergent region was STM0893 – STM0929 that encoded Fels-1 phage genes. The second divergent region, STM2584 – STM2636, encoded a portion of the Gifsy-1 prophage. The third divergent region was STM2694 – STM2739 which encoded a number of Fels-2 phage genes. CGH data was able to identify genetic differences between serovars *S.* Typhimurium and *S.*Heidelberg. However, genomic profiles of the strains showed limited strain to strain differences among the *S.* Heidelberg isolates. Human strains were not distinguished from bovine, turkey and chicken agri-

Figure 5: Genomic profiles as determined by CGH Dendrogram based on UPGMA created using GeneMaths XT software. White indicates genes present in both test and reference strains. Red indicates genes that are putatively duplicated in the test strain as compared to S. Typhimurium LT2. Green indicates genes which are putatively divergent in the test S. Heidelberg strains as compared to S. Typhimurium LT2. A represents STM0892-STM0929 which are Fels-1 prophage genes. B represents STM2584-STM2636 which are Gifsy-1 prophage genes. C represents STM2694- STM2739 which are Fels-2 prophage genes.

S-467-Human-sensitive
00-5440_Human_sensitive
05-1147_Human-sensitive
03-7402_2_Human_blood
03-7402_1&2_Human_blood
05-4262-Chicken_passive
04-845-Human_stool
02-4660_Human_stool
05-4269-Turket_passive
04-5511-Human-sensitive
05-4316_Bovine_passive
04-346-Human_stool
02-5785-Human_stool
03-0262-Human_blood
03-7402_1_Human_blood

A

B

C

food isolates. CGH results also did not differentiate between human strains with one exception. CGH data indicated that the region STM691 – STM704 was most likely to be putatively divergent among the *S.* Heidelberg isolates (Table 11). A set of four genes three (*fur, fldA, potE*) present in the putatively divergent region and one (*miaB*) found upstream of this region, were chosen to be amplified and sequenced from 5 *S.* Heidelberg strains (two in which the STM691 – STM704 region was divergent and three in which the STM691 – STM704 was not divergent) to confirm microarray results. This set of genes was sequenced from five *S.* Heidelberg isolates. Each gene was successfully amplified from all isolates and the sequence of each gene was identical between isolates (Figure 6). Notably, no nucleotide polymorphisms were observed in the region of each gene which corresponded to the 70-mer oligonucleotide spotted on the array. This indicated that microarray results were not fully representative of the actual genomic content of the isolates.

3.6 Plasmid Analysis

Complete plasmid profiles were obtained for all isolates to determine whether the plasmid content of the isolates exhibited strain to strain variation which could be used to supplement data obtained by PFGE, SBT, and CGH. Each *S.* Heidelberg strain contained one or more low molecular weight plasmids (LMW) ranging from 3 to 6 kbp (Figure 7). In addition, one HMW (~100 kbp) plasmid species was seen in all *S.* Heidelberg isolates. An additional HMW plasmid of > 100 kbp was seen in all cephalosporin resistant *S.* Heidelberg isolates. Plasmid profiles obtained for the 5 cephalosporin resistant *E. coli* also contained an additional HMW plasmid species of > 100 kbp. The HMW plasmids were further characterized by RFLP to determine whether the plasmids carried by different isolates were related.

Figure 6: CGH PCR confirmation. Sequence verification of the *miaB* and *potE* genes indicated to be putatively divergent in sensitive test *S.* Heidelberg isolates as compared to the reference *S.* Typhimurium LT2. The pink region corresponds to the CGH 70-mer oligonucleotide probe.

miaB – rRNA modification protein

```
miaB_05-4262 CCGGAACTGGTCAGTTTCCTGCATCTGCCGGTACAGAGCGGTTCCGATCGCGTGTTGAATCTGATGGGGCGCACCCACACTGCGCTGG AATATAAAGCAA
miaB_00-5440 CCGGAACTGGTCAGTTTCCTGCATCTGCCGGTACAGAGCGGTTCCGATCGCGTGTTGAATCTGATGGGGCGCACCCACACTGCGCTGG AATATAAAGCAA
miaB_02-4660 CCGGAACTGGTCAGTTTCCTGCATCTGCCGGTACAGAGCGGTTCCGATCGCGTGTTGAATCTGATGGGGCGCACCCACACTGCGCTGG AATATAAAGCAA
miaB_00-1147 CCGGAACTGGTCAGTTTCCTGCATCTGCCGGTACAGAGCGGTTCCGATCGCGTGTTGAATCTGATGGGGCGCACCCACACTGCGCTGG AATATAAAGCAA
miaB_03-7402 CCGGAACTGGTCAGTTTCCTGCATCTGCCGGTACAGAGCGGTTCCGATCGCGTGTTGAATCTGATGGGGCGCACCCACACTGCGCTGG AATATAAAGCAA
             701......710.......720.......730.......740.......750.......760.......770.......780.......790........
```

```
miaB_05-4262 TCATCCGTAAACTGCGCGCGGCGCGGCCGGACATTCAGATAAGCTCTGACTTTATCGT CGGATTCCCTGGCGAAACTACCGATGATTTCGAAAAAACCAT
miaB_00-5440 TCATCCGTAAACTGCGCGCGGCGCGGCCGGACATTCAGATAAGCTCTGACTTTATCGT CGGATTCCCTGGCGAAACTACCGATGATTTCGAAAAAACCAT
miaB_02-4660 TCATCCGTAAACTGCGCGCGGCGCGGCCGGACATTCAGATAAGCTCTGACTTTATCGT CGGATTCCCTGGCGAAACTACCGATGATTTCGAAAAAACCAT
miaB_00-1147 TCATCCGTAAACTGCGCGCGGCGCGGCCGGACATTCAGATAAGCTCTGACTTTATCGT CGGATTCCCTGGCGAAACTACCGATGATTTCGAAAAAACCAT
miaB_03-7402 TCATCCGTAAACTGCGCGCGGCGCGGCCGGACATTCAGATAAGCTCTGACTTTATCGT CGGATTCCCTGGCGAAACTACCGATGATTTCGAAAAAACCAT
             801......810.......820.......830.......840.......850.......860.......870.......880.......890........
```

potE – putrescine/ornithine antiporter

```
potE_05-1147 CACTATCGCTCAGGTCTTTAAATCCTCCGCTGACGAAGGCTATTTCCCGAAAGTCTTCTCTCGTGTAACGAAAGTCGACGCGCCGGTTCAGGGGATGTTG
potE_05-4262 CACTATCGCTCAGGTCTTTAAATCCTCCGCTGACGAAGGCTATTTCCCGAAAGTCTTCTCTCGTGTAACGAAAGTCGACGCGCCGGTTCAGGGGATGTTG
potE_00-5440 CACTATCGCTCAGGTCTTTAAATCCTCCGCTGACGAAGGCTATTTCCCGAAAGTCTTCTCTCGTGTAACGAAAGTCGACGCGCCGGTTCAGGGGATGTTG
potE_02-4660 CACTATCGCTCAGGTCTTTAAATCCTCCGCTGACGAAGGCTATTTCCCGAAAGTCTTCTCTCGTGTAACGAAAGTCGACGCGCCGGTTCAGGGGATGTTG
potE_03-7402 CACTATCGCTCAGGTCTTTAAATCCTCCGCTGACGAAGGCTATTTCCCGAAAGTCTTCTCTCGTGTAACGAAAGTCGACGCGCCGGTTCAGGGGATGTTG
             801......810.......820.......830.......840.......850.......860.......870.......880.......890........
```

```
potE_05-1147 ATTATTGTGATTATTCAGACTGGCCTGTCGCTGATGACCATTAGCCCGTCGCTGAATAGTCAGTTTAACGTCCTGGTTAACCTGGCGGTGGTGACGAACA
potE_05-4262 ATTATTGTGATTATTCAGACTGGCCTGTCGCTGATGACCATTAGCCCGTCGCTGAATAGTCAGTTTAACGTCCTGGTTAACCTGGCGGTGGTGACGAACA
potE_00-5440 ATTATTGTGATTATTCAGACTGGCCTGTCGCTGATGACCATTAGCCCGTCGCTGAATAGTCAGTTTAACGTCCTGGTTAACCTGGCGGTGGTGACGAACA
potE_02-4660 ATTATTGTGATTATTCAGACTGGCCTGTCGCTGATGACCATTAGCCCGTCGCTGAATAGTCAGTTTAACGTCCTGGTTAACCTGGCGGTGGTGACGAACA
potE_03-7402 ATTATTGTGATTATTCAGACTGGCCTGTCGCTGATGACCATTAGCCCGTCGCTGAATAGTCAGTTTAACGTCCTGGTTAACCTGGCGGTGGTGACGAACA
             901......910.......920.......930.......940.......950.......960.......970.......980.......990........
```

Table 11: The Log_2 test/reference ratios for genes in the region STM691 – STM704. Values greater than 0.5 indicated genes that were duplicated in the test *S.* Heidelberg strain as compared to the reference *S.* Typhimurium strain. Values less than 0.5 and greater than -0.5 indicated genes that were present in both the test *S.* Heidelberg strain and reference S. Typhimurium strain. Values less than -0.5 indicated genes that were putatively divergent in test *S.* Heidelberg strain as compared to the reference *S.* Typhimurium strain. The strains highlighted in pink (00-5440, 02-7402, 05-1147) have Log_2 test/reference ratios less than -0.5 for all genes in the region STM691 – STM704, indicating that this region is putatively divergent in these isolates compared to the other *S.* Heidelberg isolates examined by this method.

Gene	Human sensitive 00-5440	Human sensitive 04-5511	Human sensitive 05-1147	Human blood 03-0262	Human blood 05-7402	Human stool 02-4660	Human stool 02-5785	Human stool 04-0346	Human stool 04-0845	Chicken passive 05-4262	Turkey passive 05-4269	Bovine passive 05-4316
STM0691	-1.03397	0.138351	-1.634	-0.09358	-0.5857	0.065169	0.091086	-0.24553	0.194102	0.146521	0.151255	0.248833
STM0692	-1.27019	0.380729	-1.5162	0.216849	-0.7936	0.177245	0.219766	-0.12977	0.213424	0.051465	0.01437	0.290425
STM0693	-0.73272	0.151431	-1.37119	0.000895	-0.5233	0.099766	0.03223	-0.09949	0.310142	0.109882	0.109036	0.177003
STM0694	-0.42898	0.117639	-1.0722	-0.02709	-0.5778	0.150219	0.147879	-0.17299	0.186101	-0.11178	-0.04729	-0.00101
STM0695	-0.41036	0.184687	-0.91852	0.048924	-0.4828	0.08818	0.089201	-0.25223	0.223834	0.154802	0.187062	0.139808
STM0696	-0.83876	0.146665	-1.42494	-0.03946	-0.5947	-0.01548	0.050097	-0.4011	0.208108	0.08207	0.157395	0.028277
STM0697	-0.88454	0.073785	-1.39693	0.092882	-0.5702	0.147376	0.229963	-0.38447	0.278682	0.027002	0.132528	0.020676
STM0698	-0.45224	0.112756	-0.40377	-0.12006	-0.0981	0.063178	0.047683	-0.15616	0.224114	0.008528	0.032022	0.014839
STM0699	-0.57361	0.119712	-1.12201	-0.07302	-0.5403	0.145298	0.123346	-0.31974	0.187241	-0.02672	0.025945	-0.00255
STM0700	-1.0492	0.253563	-1.79586	-0.06376	-1.0207	0.191904	0.228371	-0.60707	0.102721	-0.0423	-0.00455	-0.02431
STM0701	-1.08125	0.157638	-1.72938	0.013219	-0.9251	0.187739	0.116205	-0.6691	0.22078	0.04778	0.112112	0.035506
STM0702	-0.92522	0.098872	-1.70677	0.027177	-0.8694	0.235984	0.14449	-0.29507	0.309622	0.139514	0.067476	0.144949
STM0703	-1.05328	-0.03117	-1.67189	0.186909	-0.5728	0.095913	0.105052	-0.12859	0.19403	0.355404	0.264106	0.343743
STM0704	-0.95439	-0.38821	-1.10136	0.135974	-0.5311	0.100506	0.072883	-0.08143	0.021583	0.541496	0.322374	0.226656

Figure 7: Complete plasmid profiles isolated from *S.* Heidelberg strains. Isolates were selected to represent sensitive human isolates, ESC-resistant chicken agri-food isolates and ESC-resistant human-stool or human-blood isolates. 'M' indicates supercoiled DNA molecular marker (Invitrogen). 'S' indicates sensitive isolates. R indicates resistant isolates. Lane 1 contains 1170, a sensitive isolate from chocolate. Lane 2 contains 539, a sensitive isolate from chicken rinse. Lane 3 contains 04-5511, a human stool sensitive isolate. Lane 4 contains 05-4272, a chicken retail resistant isolate. Lane 5 contains 02-4660, a human stool resistant isolate. Lane 6 contains 04-3194, a human blood resistant. The pink brackets highlight LMW plasmids ranging from ~ 8 kbp to ~ 2 kbp which are common to all isolates. The purple arrow highlights the ~ 100 kbp HMW plasmid species carried by both sensitive and ESC resistant isolates. The blue arrow highlights the > 100 kbp plasmid species carried by only ESC resistance isolates.

M S R M

1 2 3 4 5 6 7 8

Size (bp)

16, 210

14, 174

12, 138

10, 102

8, 066

7, 045

6, 030

5, 012

3, 990

2, 972

2, 067

HMW bla_{cmy-2} R plasmid

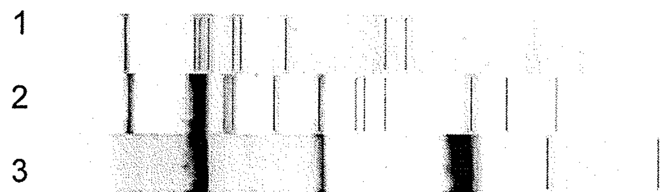
Common *S.* Heidelberg HMW plasmid

LMW plasmids

3.7 RFLP

RFLP was performed using *BglI* to determine whether the ~ 100 kbp plasmids common to all *S.* Heidelberg isolates were actually similar plasmids. RFLP was performed on the full plasmid profile of wild type sensitive S. Heidelberg (containing the ~ 100 kbp plasmid) isolates, wild type resistant *S.* Heidelberg (containing both the ~ 100 kbp and > 100 kbp plasmid) isolates as well as on solely the bla_{cmy-2} plasmid isolated from an *E. coli* transformant strain (Figure 8). The bands in the wild type plasmid profile consist of those contributed by both the > 100 kbp plasmid as well as the common ~ 100 kbp plasmid. These results indicate that the ~ 100 kbp plasmids seen in both the wild type resistant and wild type sensitive strains are similar between isolates on the basis of the RFLP pattern. Electroporation of the full plasmid profile from isolates with > 100 kbp plasmid into JA10 *E. coli* cells yielded cefoxitin resistant transformant colonies. Transformant colonies were not obtained for 02-5785 and 05-4264. The > 100 kbp HMW plasmid seen in wild type *S.* Heidelberg and *E. coli* isolates which exhibited ESC resistance was the sole plasmid isolated from the cefoxitin resistant transformants. The bla_{cmy-2} gene was amplified by PCR from all of the > 100 kbp plasmids (hereafter referred to as the bla_{cmy-2} plasmid) isolated from transformant strains. The bla_{cmy-2} plasmids were isolated from transformant strains and characterized by RFLP (Figure 9). The bla_{cmy-2} plasmids exhibited diverse RFLP patterns, however some isolates did share identical RFLP patterns. A few human and chicken agri-food bla_{cmy-2} plasmids shared identical RFLP patterns (Figure 9). The isolates 04-3293 (human-blood) and 05-4287 (chicken-abattoir) shared an indistinguishable RFLP patterns while 03-4690 (human-stool) and 05-4277 (chicken-retail) shared another indistinguishable RFLP pattern. Chicken agri-food isolates also shared indistinguishable plasmid RFLP patterns with isolates from different sources (porcine-passive) and different species (*E. coli*)

Figure 8: A comparison of *S*. Heidelberg plasmid RFLP profiles generated using BglI. Lane 1 contains the $bla_{cmy\text{-}2}$ plasmid isolated from an *E.coli* transformant strain. Lane 2 contains the full plasmid profile of a wild type MDR *S*. Heidelberg isolate (contains both the ~ 100 kbp and > 100 kbp $bla_{cmy\text{-}2}$ plasmid). Lane 3 contains the full plasmid profile of a wild type sensitive *S*. Heidelberg isolate (contains the ~ 100 kbp plasmid). The bands of the full wild type plasmid profile (lane 2) are contributed by both the $bla_{cmy\text{-}2}$ plasmid (lane 1) and the ~ 100 kbp plasmid (lane 3).

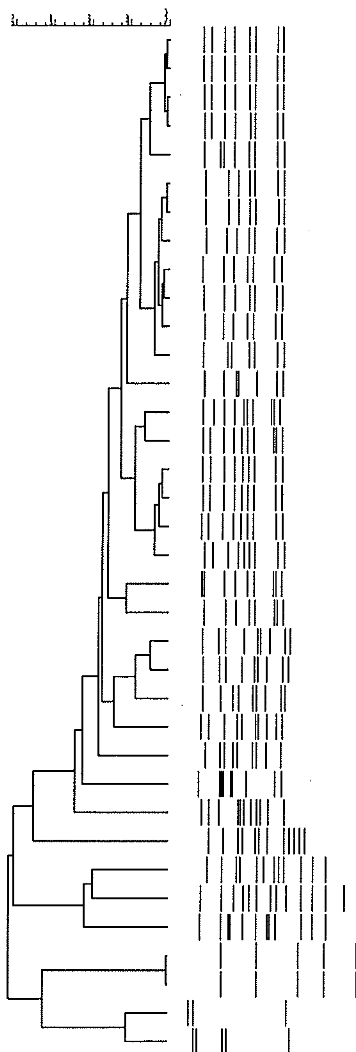


05-4272
05-4272_All
1170

Figure 9: *bla*$_{cmy-2}$ plasmid *bglI* RFLP. Dendrogram created with Bionumerics version 4.0 using UPGMA with a fuzzy bands coeffiecient of correlation, 2% optimization and 10% tolerance. All indicates RFLP performed on the full plasmid profile from the wild type strain. The *bla*$_{cmy-2}$ plasmid profiles from *S.* Heidelberg and *E. coli* isolates are highlighted in turquoise. The full plasmid profile of wildtype resistant *S.* Heidelberg strains is highlighted in blue. The full plasmid profile of wildtype sensitive *S.* Heidelberg strains is highlighted in purple.

Different bands(fuzzy) (Opt:2.00%) (Tol 10.0%-10.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
RFLP

RFLP



03-7402	Human-blood	ON	rep1I	cmy-2
05-4354	Porcine-passive	QC	unknown	cmy-2
05-4275	Chicken-retail	QC	rep1I	cmy-2
05-4299	Chicken-abattoir	ON	unknown	cmy-2
03-3012	Human-blood	QC	rep1I	cmy-2
04-3194	Human-blood	ON	rep1I	cmy-2
04-3293	Human-blood	QC	rep1I	cmy-2
05-4287	Chicken-abattoir	ON	rep1I	cmy-2
05-4269	Turkey-passive	ON	rep1I	cmy-2
05-4294	Chicken-retail	ON	rep1I	cmy-2
833 *E. coli*	Chicken-abattoir	ON	rep1I	cmy-2
04-1511	Human-stool	ON	rep1I	cmy-2
03-0845	Human-stool	ON	repAC	cmy-2, tetA, strA, sul1
03-0262	Human-blood	QC	rep1I	cmy-2
04-5435	Human-blood	QC	rep1I	cmy-2
03-4690	Human-stool	ON	rep1I	cmy-2
05-4277	Chicken-retail	QC	rep1I	cmy-2
05-4263	Chicken-passive	ON	rep1I	cmy-2
04-4711	Human-blood	QC	rep1I	cmy-2
05-4316	Bovine-passive	PE	rep1I	cmy-2
05-4355	Porcine-passive	QC	rep1I	cmy-2
03-4601	Human-blood	QC	repAC/rep1I	cmy-2, tetA, strA, floR
04-0346	Human-stool	QC	repAC	cmy-2, tetA, floR
831 *E. coli*	Chicken-abattoir	QC	repAC	cmy2, tetA, strA, floR
05-4262	Chicken-passive	AB	repAC/rep1I	cmy-2, tetA, strA, floR, sul1
832	Porcine-abattoir	QC	repAC	cmy-2, tetA, floR
05-4272	Chicken-retail	QC	rep1I	cmy-2
02-5980	Human-blood	QC	rep1I	cmy-2
02-4660	Human-stool	ON		cmy-2, tetA, dhfR, strA, floR, sul1
02-4660_All	Human-stool	ON		
04-3194_All	Human-blood	ON		
05-4272_All	Chicken-retail	QC		
1170	Chocolate-cocoa bea.	ON		Sensitive
539	Chicken-rinse	ON		Sensitive
830 *E. coli*	Chicken-abattoir	AB	unknown	cmy-2
834 *E. coli*	Bovine-retail	QC	rep1I	cmy-2, aadA2

3.8 Replicon Typing and resistance PCR

The *bla*$_{cmy-2}$ plasmids were further characterized using a PCR-based replicon typing scheme as well as using PCR and Southern blot to determine whether other resistance determinants were carried by the plasmid. Two different replicons, *repAC* and *repI1*, were identified among the *bla*$_{cmy-2}$ plasmids (Table 12). All seven *bla*$_{cmy-2}$ plasmids which carried a *repAC* replicon were found to carry additional resistance determinants including a combination of *aadA1, dhfR, floR, strA, sul1*, and *tetA* (Table 12). The plasmids which carried a *repAC* replicon included three human stool strains, one human blood strain, one passive chicken strains, and two abattoir *E. coli* isolates of chicken and porcine origin. The *repAC* human blood isolate was also shown to carry an additional *repI1* replicon. The majority of the *bla*$_{cmy-2}$ plasmids isolated from both *S.* Heidelberg and *E. coli* were shown to carry the *repI1* replicon in concert with only *bla*$_{cmy-2}$. The three remaining *bla*$_{cmy-2}$ plasmids carried only *bla*$_{cmy-2}$ however no replicon could be identified. The majority of resistance genes identified in each strain could be attributed to the carriage of the *bla*$_{cmy-2}$ ESC resistance or ESC-multi drug resistant plasmids.

3.9 Southern blot

The location of the *bla*$_{cmy-2}$ gene on the *bla*$_{cmy-2}$ plasmid was determined by Southern blot of RFLP patterns (Figure 10). A 1143 bp region of the *bla*$_{cmy-2}$ gene amplified by PCR and labeled using the ECl Direct Nucleic Acid Labelling and Detection System was used as the Southern blot probe. The *bla*$_{cmy-2}$ gene was identified on a ~ 20 kbp fragment in all but two isolates. In the remaining isolates, both Quebec human blood isolates, the *bla*$_{cmy-2}$ gene was carried on a slightly smaller fragment.

Table 12: PCR-based replicon typing and resistance gene characterization of *S.* Heidelberg and *E. coli* bla_{cmy-2} plasmids. The resistance gene content of bla_{cmy-2} plasmids was determined by PCR performed on bla_{cmy-2} transformants targeting the bla_{cmy-2}, *tetA, dhfR, strA, floR,* and *sul1* resistance determinants. The replicons carried by the bla_{cmy-2} plasmids were determined by PCR performed on the bla_{cmy-2} transformants targeting *repAC* and *rep1I.*

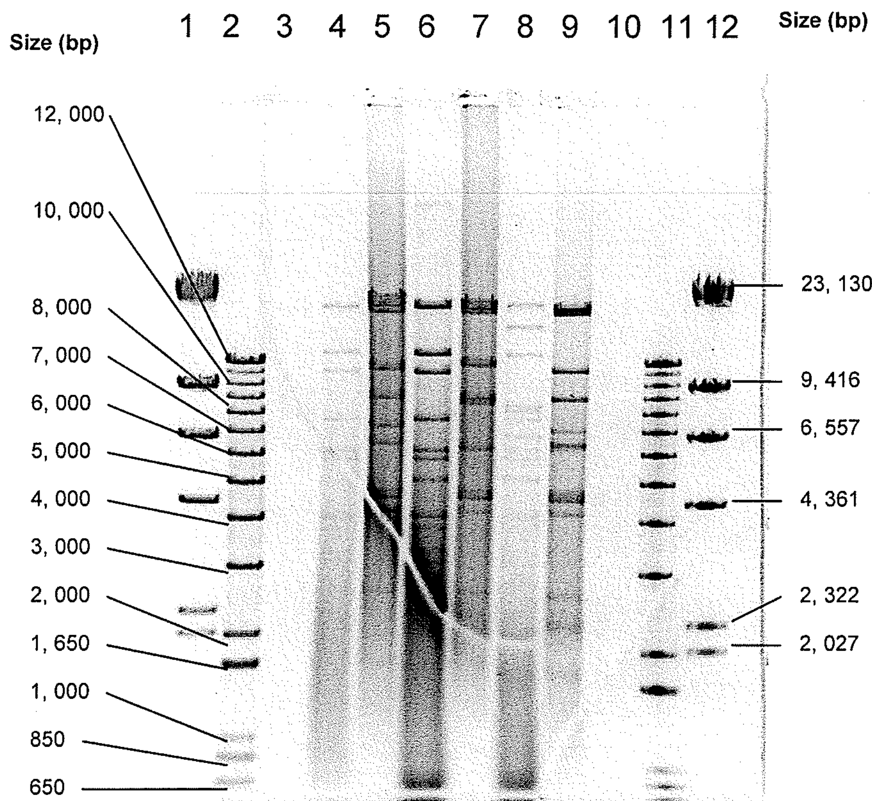
Strain	Isolation Yr	Prov	Source	Resistance genotype of strain[a]	*cmy2* plasmid mediated resistance determinants[a]	Rep[b]
02-4660	2002	ON	Human-stool	bla_{cmy-2}, *tetA,dhfR, strA, floR, sul1*	bla_{cmy-2}, *tet(A), dhfR, strA, floR, sul1*	AC
02-5980	2002	QC	Human-blood	bla_{cmy}	bla_{cmy}	1I
03-0262	2003	QC	Human-blood	bla_{cmy}	bla_{cmy}	1I
03-0845	2003	ON	Human-stool	bla_{cmy-2} *tetA, strA, sul1*	bla_{cmy-2}, *tet(A),strA, sul1*	AC
03-3012	2003	QC	Human-blood	bla_{cmy-2}	bla_{cmy-2}	1I
03-4601	2003	QC	Human-blood	bla_{cmy-2}, *tetA, strA, floR*	bla_{cmy-2}, *tet(A),strA floR*	AC/I
03-4690	2003	ON	Human-stool	bla_{cmy-2}, *tetB, strA*	bla_{cmy}	1I
03-7402	2003	ON	Human-blood	bla_{cmy-2}	bla_{cmy}	1I
04-0346	2004	QC	Human-stool	bla_{cmy-2}, *tetA, strA, floR*	bla_{cmy-2}*2, tet(A), strA, floR*	AC
04-1511	2004	ON	Human-stool	bla_{cmy-2}, *tetA, strA*	bla_{cmy}	1I
04-3194	2004	ON	Human- blood	bla_{cmy}	bla_{cmy}	1I
04-3293	2004	QC	Human- blood	bla_{cmy}	bla_{cmy}	1I
04-4717	2004	QC	Human- blood	bla_{cmy}	bla_{cmy}	1I
04-5435	2004	QC	Human- blood	bla_{cmy}	bla_{cmy-2}	1I
05-4262	2001	AB	Chicken-passive	bla_{cmy-2}, *tetA, dhfR, strA, floR, sul1*	bla_{cmy-2}, *tet(A), strA, floR, sul1*	AC
05-4263	2002	ON	Chicken-passive	bla_{cmy-2}, *tetB*	bla_{cmy}	1I
05-4269	2003	ON	Turkey-passive	bla_{cmy-2}, *tetB*	bla_{cmy}	1I
05-4272	2003	QC	Chicken-retail	bla_{cmy-2}	bla_{cmy}	1I
05-4275	2003	QC	Chicken-retail	bla_{cmy-2}, *tetB, strA*	bla_{cmy}	1I
05-4277	2003	QC	Chicken-retail	bla_{cmy}	bla_{cmy}	1I
05-4287	2003	ON	Chicken-abattoir	bla_{cmy}	bla_{cmy}	1I
05-4294	2004	ON	Chicken-retail	bla_{cmy}	bla_{cmy}	1I
05-4299	2004	ON	Chicken-abattoir	bla_{cmy}	bla_{cmy}	-
05-4316	2004	PE	Bovine-passive	bla_{cmy}	bla_{cmy}	1I
05-4354	2004	QC	Porcine-passive	bla_{cmy}	bla_{cmy}	-
05-4355	2004	QC	Porcine-passive	bla_{cmy}	bla_{cmy}	1I
E. coli-830	2004	AB	Chicken-abattoir	bla_{cmy-2}, *tetA, aadA1, sul1*	bla_{cmy}	-
E. coli-831	2004	QC	Chicken-abattoir	bla_{cmy-2}, *tetA ,aadA1, strA, floR, sul1*	bla_{cmy-2}, *tet(A), aadA1, strA, floR*	AC
E. coli-832	2004	QC	Porcine-abattoir	bla_{cmy-2}, *tetA, strA, floR*	bla_{cmy-2}, *tet(A), strA, floR*	AC
E. coli-833	2004	ON	Chicken-abattoir	bla_{cmy-2}, *aadA1, floR, sul1*	bla_{cmy}	1I
E. coli-834	2004	QC	Bovine-retail	bla_{cmy-2}, *aadA2*	bla_{cmy}	1I

a - *aadA1*, streptomycin/spectinomycin adenyltransferase; *dhfR*, dihydrofolate reductase; *floR*, efflux; *strA*, streptomycin phosphotransferase; *sulI*, dihydropteroate synthase; *tetA*, efflux; *tetB*, efflux; *cmy-2*, beta-lactamase.

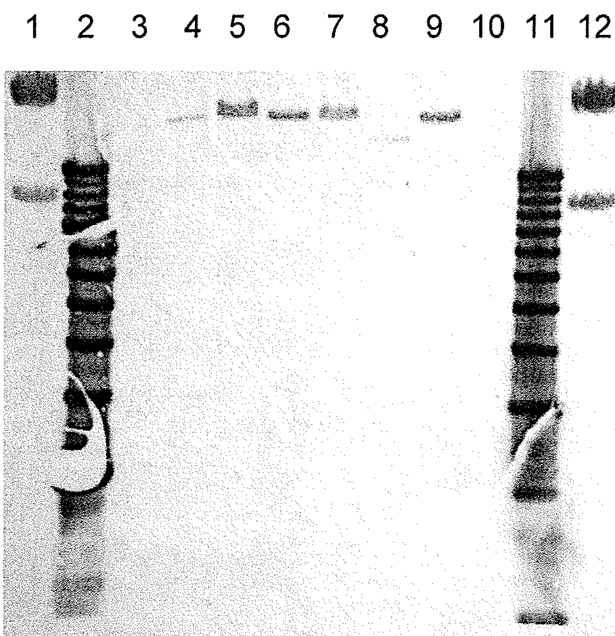
b – (-), unknown replicon

Figure 10: A) *bgl*II plasmid fingerprints and B) subsequent Southern blot probing for *bla*$_{cmy-2}$.gene. Lane 1 and 12 contain a λ *Hind*III digest DNA ladder (Invitrogen) for use as the molecular marker. Lane 2 and 11 contain the 1 kb+ extended DNA ladder (Invitrogen) for use as a molecular marker. Lane 3 contains 02-4660 (human stool). Lane 4 contains 03-0262 (human blood). Lane 5 contains 05-4262 (chicken passive). Lane 6 contains 05-4263 (chicken passive). Lane 7 conatins 05-4287 (chicken abattoir). Lane 8 contains 04-3194 (human blood). Lane 10 is blank. The *bla*$_{cmy-2}$ hybridizes to the highest molecular weight fragment (~ 20 kbp) in all cases with the exception of Lane 8 containing 03-3194 where it hybridizes to a slightly smaller fragement. Fragments were sized in Bionumerics version 4.0.

A)



B)



3.10 Virulence gene detection

The *bla*$_{cmy-2}$ plasmids were further characterized through PCR screening for the carriage of virulence genes previously identified on the HMW *Salmonella* virulence plasmids carried by *S.* Enteriditis, Choleraesuis, Typhimurium, Gallinarium and Dublin. None of the *spv* operon, *rck* (resistance to complement gene) and *pef* (virulence associated fimbriae) genes could be amplified from the *S.* Heidelberg *bla*$_{cmy-2}$ plasmids or genomic DNA.

3.11 Conjugation

Conjugation studies were attemped to determine whether the *S.* Heidelberg *bla*$_{cmy-2}$ plasmids could be transferred by this method. The majority of *bla*$_{cmy-2}$ plasmids could be transferred to the recipient *E.coli* RG192 by conjugation. All tested *bla*$_{cmy-2}$ plasmids encoding a *repI1* replicon (with the exception of the *bla*$_{cmy-2}$ plasmid isolated from 04-3293) could be transferred by conjugation (Table 13). All *repAC* *bla*$_{cmy-2}$ MDR plasmids with the exception of 05-4262 were not sucessfully transferred by conjugation. All tested *bla*$_{cmy-2}$ plasmids with an unknown replicon were able to transfer by conjugation.

3.12 SGI1 conjugation

SGI1 conjugation was attempted to determine if *S.* Heidelberg could serve as a recipient for SGI1. The sequence of the SGI1 insertion site in the *thdF* gene exhibited two nucleotide polymorphism as compared to *S.* Typhimurium indicating that the mobilization of SGI1 into *S.* Heidelberg should be possible (Figure 11). Attempts to transfer the SGI1 from the donor *E. coli* JSK 5K-12 to the recipient *S.* Heidelberg or to the control recipient *S.* Typhimurium were unsuccessful.

Table 13: The ability of *bla*$_{cmy-2}$ plasmids from *S*. Heidelberg and *E. coli* to be transferred by conjugation to recipient *E. coli* RG192. Y indicates that transformant colonies were successfully obtained by conjugation. N indicates that transformant colonies were not successfully obtained by conjugation.

Isolate	Species	Source	rep	Conjugative
02-5980	*S.* Heidelberg	QC-Human-blood	I	Y
03-7402	*S.* Heidelberg	ON-Human-blood	I	Y
04-3293	*S.* Heidelberg	QC-Human-blood	I	N
04-5435	*S.* Heidelberg	QC-Human-blood	I	Y
05-4272	*S.* Heidelberg	QC-Chicken-retail	I	Y
05-4287	*S.* Heidelberg	ON-Chicken-abattoir	I	Y
833	*E. coli*	ON-Chicken-abattoir	I	Y
03-4601	*S.* Heidelberg	QC-Human-blood	AC/I	N
05-4262	*S.* Heidelberg	AB-Chicken-passive	AC/I	Y
02-4660	*S.* Heidelberg	ON-Human-stool	AC	N
03-0262	*S.* Heidelberg	QC-Human-blood	AC	N
03-0845	*S.* Heidelberg	ON-Human-stool	AC	Y
04-0346	*S.* Heidelberg	QC-Human-stool	AC	N
831	*E. coli*	QC-Chicken-abattoir	AC	N
832	*E. coli*	QC-Porcine-abattoir	AC	N
05-4299	*S.* Heidelberg	ON-Chicken-abattoir	Unknown	Y
05-4354	*S.* Heidelberg	QC-Porcine-passive	Unknown	Y

Figure 11: Sequence of the 18 bp imperfect repeat in the *thdF* gene from *S.* Heidelberg and *S.* Typhimurium. SGI1 has been shown to insert by site specific recombination with this 18 bp imperfect repeat in the *thdF* gene of *S. enterica.* Two nucleotide polymorphisms are observed between *S.* Heidelberg and *S.* Typhimurium.

S. Typhimurium TTCTGTATCGGTAAGTAA
S. Heidelberg TTCTGTATCGGCAAATAA

Chapter 4: Discussion

Salmonella enterica serovar Heidelberg ranks amongst the most prevalent causes of human salmonellosis in Canada. An increase in multi drug resistant (MDR) *S.* Heidelberg, including resistance to extended spectrum cephalosporins (ESC), has been observed through integrated surveillance of clinical, retail and agricultural isolates by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS). The purpose of this study was to examine the genetic relationship between *S.* Heidelberg isolates from retail chicken, abattoir chicken ceca and clinical chicken, cattle, swine and human samples to determine whether there was a link between the emergence of MDR *S.* Heidelberg from chicken agri-food sources and the increase in the amount of human salmonellosis attributable to MDR *S.* Heidelberg.

4.1 Genetic Variation due to chromosomal determinants

PFGE is currently considered the gold standard for the subtyping of *Salmonella* (Harbottle *et al.*, 2006). PFGE was performed on all *S.* Heidelberg isolates entered into the PulseNet Canada database between 2001 and 2004. PFGE identified limited genetic diversity among this large panel of temporally and geographically distinct strains. The limited observable genetic diversity among these isolates may reflect chromosomal clonality and not the actual epidemiological relationship between the isolates therefore, no obvious link between animal and human isolates could be made. Thus a subset of 39 strains representing a diverse range of temporally and geographically distinct *S.* Heidelberg isolates with different PFGE types, sources, sites of isolation, and phage types and resistance phenotypes were selected for examination by additional subtyping methods.

PFGE analysis is a valuable tool for identifying isolates involved in outbreaks which by definition occur over a short period of time (Sandt *et al.*, 2006). The emergence of *S.* Heidelberg in Canada has occurred over a longer period of time, thus it

was hypothesized that another method such as MLST could provide a higher level of discrimination than PFGE for this study. MLST examines genetic variation which accumulates slowly and has been hypothesized to be a valuable method for examining genetic relationships over a long period of time (Harbottle *et al.*, 2006). MLST was conducted on *S.* Heidelberg strains using a previously published three gene scheme *fimA, manB*, and *mdh* (Sukhnanand *et al.*, 2005). No nucleotide polymorphisms were observed among 2502 bp sequenced from ten *S.* Heidelberg strains. The application of this method offered no additional discriminatory power as compared to PFGE.

CGH was used to determine whether the genetic homogeneity identified by PFGE and MLST was consistently maintained throughout the genome of *S.* Heidelberg CGH is capable of examining the entire genome of an isolate and is useful for detecting differences between isolates (van Hoek *et al.*, 2005). CGH was performed on *S.* Heidelberg isolates to identify regions which were potentially characteristic of particular isolate types. A diverse range of *S.* Heidelberg isolates was examined by CGH, including isolates from human, chicken, turkey and bovine sources (Table 8). Comparative genomic analysis of the *S.* Heidelberg strains indicated that there were regions that were consistently putatively divergent or absent among all of the *S.* Heidelberg isolates with reference to *S.* Typhimurium LT2 (Figure 5). The majority of these divergent regions encoded Fels-1, Fels-2 and Gifsy-1 prophage genes. Similar results have been obtained for *S.* Enteriditis PT13 isolates with reference to *S.* Typhimurium LT2 (A. Olson unpublished data). Thus this CGH platform was demonstrated to be capable of distinguishing between different *Salmonella* serovars and the observed divergence of prophage genes between serovars was expected. Previous CGH studies have identified that the carriage of prophage genes varies between members of *S. enterica* subsp *enterica* (Porwollik *et al.*, 2002; Porwollik *et al.*, 2005; Reen *et al.*, 2005)

Conversely, the genomic profiles of the *S.* Heidelberg isolates exhibited limited strain to strain variation between isolates with the exception of a single region. CGH data identified the region STM691 – STM709 as most likely to be putatively divergent in the *S.* Heidelberg isolates. Notably, no nucleotide polymorphisms were observed in the region of each gene which corresponded to the 70-mer oligonucleotide spotted on the array indicating that in all cases the gene should have been able to hybridize to the array (Figure 6). These results indicate that the minimal strain to strain differences identified by CGH may actually be due to experimental variation rather than actual strain to strain differences. The limited genomic diversity identified by this method may be representative of limited genetic diversity amongst the isolates. However, the lack of S. Heidelberg specific sequences on the array may have played a role in the inability of the platform to distinguish between isolates. However, in the absence of a sequenced *S.* Heidelberg genome CGH studies based on an *S.* Heidelberg specific microarray platform are not currently possible.

4.2 Resistance Determinants

The isolates were screened by PCR to determine which resistance genes were responsible for the observed resistance phenotype of the isolate and to determine whether there were resistance determinants exclusively shared between chicken agri-food and human isolates (Table 9). All S. Heidelberg isolates which exhibited an ESC resistance phenotype were PCR positive for the $bla_{\text{cmy-2}}$ gene, which is the most common plasmid mediated AmpC enzyme observed in *Salmonella (*Allen and Poppe, 2002; Miriagou et al., 2002; Navarro et al., 2001; Rankin et al., 2002). In addition, the $bla_{\text{cmy-2}}$ gene was also responsible for the ESC resistance phenotype of the 5 *E. coli* isolates included in the study. The sequence of the $bla_{\text{cmy-2}}$ gene was identical amongst

all of the isolates including *S.* Heidelberg and *E. coli* indicating that it may have been acquired from a common source.

Tetracycline resistance was mediated by a combination of *tetA* and *tetB* genes in *S.* Heidelberg and solely *tetA* in *E. coli.* The *tetA* and *tetB* genes were each also amplified from isolates determined to be tetracycline sensitive by phenotypic methods. The resistance cassette may not be functional which could explain the sensitive phenotype of the organism. Chloramphenicol resistance was mediated by *floR* and Streptomycin resistance was mediated by *strA* alone or in combination with either *aadA1* or *aadA2* or both. The carriage of two streptomycin resistance cassettes would seem to be redundant however; the *aadA* type genes confer resistance to spectinomycin in addition to streptomycin which may offer additional advantage to the isolate in certain niches (Randall *et al.*, 2004). Trimethoprim resistance was mediated by the *dhfR* gene and sulfizoxazole/sulfamethoxazole resistance was mediated by the *sul*1 gene. The presence of identical resistance determinants amongst the group of *S.* Heidelberg and *E. coli* isolates indicated that there may be similar mobile elements such as plasmids or integrons mediating resistance among all of the isolates.

Notably 9 of the S. Heidelberg isolates possessed the classic DT104 associated phenotype of resistance to ampicillin ($bla_{pse\text{-}1}$), chloramphenicol (*floR*), streptomycin (*aadA2*), sulfonamides (*sulI*), and tetracyclines (*tetG*) (Threlfal, 2000). The DT104 associated ACSSuT phenotype mediated by *Salmonella* genomic island I (SGI1) has been demonstrated in other *S. enterica* serovars thus it was hypothesized the ACSSuT phenotype of the *S.* Heidelberg isolates was due to the acquisition of SGI1(Boyd *et al.*, 2001; Doublet *et al.*, 2003; Ebner *et al.*, 2004; Levings *et al.*, 2005; Meunier *et al.*, 2002). However *tetG* did not mediate tetracycline resistance in the isolates which indicated that SGI1 was in fact not responsible for the phenotype of the isolates. The absence of SGI1 was also confirmed by CGH data (Figure 5).

SGI1 has previously been shown to be mobilizable and can insert into the *S. enterica* genome by site specific recombination with a specific imperfect 18bp repeat present at the end of the *thdF* gene (Boyd, 2000; Doublet *et al.*, 2005). The sequence of the 18 bp repeat from *S.* Heidelberg had two nucleotide polymorphism compared to *S.* Typhimurium. To determine whether the polymorphic sites would affect the ability *S.* Heidelberg to acquire SGI1 the transfer of the element was attempted. Attempts to transfer SGI1 to *S.* Heidelberg were unsuccessful, but the attempts to transfer SGI1 to the control S. Typhimurium strain previously shown to successfully acquire SGI1 were also unsuccessful. Thus it could not be determined if this was a productive insertion site.

The presence of the *sul1* gene in the isolates indicated the presence of Class I integrons. Class I integrons in fact were amplified from 8 isolates (5 *S.* Heidelberg and 3 *E. coli*) (Table 10). No class I integrons were amplified from chicken agri-food isolates indicating that these isolates have acquired resistance determinants by other mechanisms. The class I integrons were carried on HMW plasmids in 02-4660 and 03-0845. These HMW plasmids were later shown to also carry the $bla_{cmy\text{-}2}$gene. The class I integron from 02-4660 and 05-4264 carried the *dhfRA1* trimethoprim resistance determinant and were 100% identical indicating a common source. All three *E. coli* isolates carried an identical class I integron which encoded *aadA1* again indicating a common source.

4.3 Genetic Variation Attributable to the carriage of Plasmids

The genetic diversity between isolates examined by PFGE, MLST and CGH was limited at chromosomal determinants and thus could not be used to elucidate the relationship between isolates. Plasmid content exhibited variation amongst isolates and was seemingly responsible for differences in antimicrobial susceptibility most notably the

presence of absence of ESC resistance. The majority of differences in plasmids profile were due to the carriage of one or more low molecular weight (LMW) plasmids ranging from 3 to 6 kbp. In addition, one high molecular weight (HMW) ~ 100 kbp plasmid was seen in all *S.* Heidelberg isolates. These plasmids were demonstrated to have identical RFLP patterns (Figure 8). On the basis of the universal presence among the isolates and size this plasmid species was hypothesized to be the equivalent of the *Salmonella* virulence plasmid described in other *S. enterica* subsp *enterica* serovars (Chu and Chiu, 2006). The virulence determinants, including the *spv* operon, *pefA* and *rck* genes, demonstrated to be carried on other *Salmonella* virulence plasmids were not detected in the *S.* Heidelberg isolates by PCR thus the function of this common plasmid remain unknown and it appears to be a unique variant.

An additional HMW of > 100 kbp was seen in all cephalosporin resistant *S.* Heidelberg isolates (Figure 7). Plasmid profiles obtained for the 5 ESC resistant *E. coli* isolates also demonstrated the presence of a HMW plasmid species of > 100 kbp. The presence of this plasmid (referred to as the $bla_{cmy\text{-}2}$ plasmid) in isolates could thus be used a predictor of ESC resistance and accordingly, these all encoded $bla_{cmy\text{-}2}$.

The $bla_{cmy\text{-}2}$ HMW plasmids were isolated from transformant colonies and characterized by RFLP, southern blot, replicon typing and PCR targeting resistance determinants. The $bla_{cmy\text{-}2}$ gene was confirmed to be present by southern blot of the RFLP fragments. The $bla_{cmy\text{-}2}$ gene was localized to the largest RFLP (~20 kbp) fragment in all isolates with the exception of two human blood isolates from Quebec. Overall, the $bla_{cmy\text{-}2}$ plasmids exhibited diverse RFLP patterns, but a few isolates did share identical RFLP patterns (Figure 9). The presence of identical $bla_{cmy\text{-}2}$ plasmids in strains of different sources (ie. human and porcine, or human and chicken) indicates that ESC resistance is disseminating among *Salmonella* through the transfer of $bla_{cmy\text{-}2}$ plasmids to different genomic backgrounds. Identical RFLP profiles were also obtained

from *S.* Heidelberg 05-42494 and *E. coli* 833 indicating that *bla*$_{cmy-2}$ plasmids are successfully transferred between species (Figure 9). Distinctly different RFLP profiles were also noted among *bla*$_{cmy-2}$ plasmids isolated from *S.* Heidelberg. (Figure 9). This indicates that in addition to *bla*$_{cmy-2}$ plasmid transfer, the transfer of the *bla*$_{cmy-2}$ gene itself to different plasmid backbones is also playing a role in the emergence of ESC among *S.* Heidelberg. The apparent horizontal transfer of *bla*$_{cmy-2}$ plasmids between bacterial cells as well as the transfer of the *bla*$_{cmy-2}$ gene itself may complicate the use of the plasmids to examine the relationship between *S.* Heidelberg isolates. The *bla*$_{cmy-2}$ plasmids described in this study are different from those previously described by Giles *et al.* and may represent a new variant of *Salmonella bla*$_{cmy-2}$ plasmid.

A PCR-based replicon typing scheme was used to supplement RFLP data. Replicons represent a conserved portion of a plasmid which is vertically inherited by host progeny. Replicon typing schemes have been hypothesized to be a more accurate method of determining plasmid relationships than RFLP (Carattoli *et al.*, 2006). Two different replicons, *repAC* and *repI1*, were identified among the *bla*$_{cmy-2}$ plasmids indicating the presence of two divergent plasmids among the isolates (Table 12). The divergence of the plasmid types was reinforced when the *bla*$_{cmy-2}$ plasmids were screened for the carriage of resistance determinants in addition to *bla*$_{cmy-2}$. All seven *bla*$_{cmy-2}$ plasmids (5 *S.* Heidelberg and 2 *E. coli*) which carried a *repAC* replicon were found to carry additional resistance determinants including a combination of *aadA1, dhfR, floR, strA, sul1*, and *tetA* by targeted PCR screens and confirmation by Southern Blot (Table 12). The presence of *repAC bla*$_{cmy-2}$ plasmids which carry additional resistance cassettes has been demonstrated among S. Typhimurium isolates (Carattoli *et al.*, 2002). Two of the class I integrons were shown to be carried on the *bla*$_{cmy-2}$ plasmids from 02-4660 and 03-0262 demonstrating a potential mechanism for the generation of *bla*$_{cmy-2}$ – MDR plasmids. The *repAC* were unable to be transferred by

conjugation to the recipient *E. coli* strain RG192 which the exception of 03-7402 which was conjugative (Table 13). The *repAC* *bla*$_{cmy-2}$ plasmids may have been generated through the transfer of the *bla*$_{cmy-2}$ gene from different plasmid backbones rather that horizontal transfer. This would account for the great diversity among the RFLP patterns of *repAC* plasmids. The majority of the *bla*$_{cmy-2}$ plasmids isolated from both *S.* Heidelberg and *E. coli* were shown to carry a *repI1* replicon and only the *bla*$_{cmy-2}$ resistance gene by targeted PCR screen and confirmation by southern blot (Table 12). All *repI1* plasmids tested successfully transferred to a recipient *E. coli* strain with the exception of 04-3293 which did not transfer (Table 13). The conjugative ability of *repI1* plasmids can help explain their widespread presence amongst the *S.* Heidelberg isolates and conserved RFLP patterns. A single chicken-passive isolate was shown to carry both the *repAC* and *repI1* replicons along with the *bla*$_{cmy-2}$, *strA*, and *floR* resistance determinants. The three remaining *bla*$_{cmy-2}$ plasmids carried only the *bla*$_{cmy-2}$ resistance determinant however no replicon could be identified. The *repAC* MDR plasmids were isolated solely from clinical human and animal isolates thus did not provide a link between animal *S.* Heidelberg isolates and human illness. The *repI1* plasmids represented a diverse group of isolates and as such did not indicate a link between isolates of a single animal source and human *S.* Heidelberg isolates.

4. 4 Conclusion

There was limited genetic diversity at the chromosomal level amongst the *S.* Heidelberg isolates. Plasmid content exhibited strain to strain variation and was responsible for differences in antimicrobial susceptibility between isolates. No definitive molecular link between chicken-associated *S.* Heidelberg and human *S.* Heidelberg isolates could be made using differences in plasmid or genomic content. However, the

paucity of *S.* Heidelberg in other agri-food sources such as pigs or cows indicates that chicken agri-food products are the most likely source of human salmonellosis.

The resistance genotype of the *S.* Heidelberg isolates could be attributed to the carriage of HMW resistance plasmids. Although overall the HMW plasmids exhibit diverse RFLP patterns, there appears to be two plasmid species disseminating among S. Heidelberg in Canada. The *repAC* HMW plasmids encode ESC resistance in combination with other resistance determinants and are not transferred readily by conjugation. The *repI1* plasmids encode solely ESC resistance and are readily transferred by conjugation. The presence of HMW resistance plasmids in this serovar elucidates a mechanism by which MDR can disseminate between *S.* Heidelberg, other *S. enterica* serovars as well as other species such as *E. coli.*

The increasing incidence of ESC resistance demonstrated in numerous *Salmonella* serovars including *S.* Heidelberg is of particular concern as extended spectrum cephalosporin antibiotics represent the last line of defense for the treatment of invasive salmonellosis in children who cannot tolerate fluroquinolone antibiotics (Giles *et al.*, 2004). This study illuminates the elements responsible for MDR among *S.* Heidelberg and examines the mechanism by which ESC resistance is disseminating amongst *S.* Heidelberg. Elucidating the mechanisms responsible for the spread of ESC resistance contributes to a better understanding of the emergence of antibiotic resistance in *S.* Heidelberg and could be useful to mitigate the continued spread of resistance in this serovar.

References

Aarestrup, F.M., Hasman, H., Olsen, I., and Sorensen, G. (2004) International spread of bla(CMY-2)-mediated cephalosporin resistance in a multiresistant *Salmonella enterica* serovar Heidelberg isolate stemming from the importation of a boar by Denmark from Canada. *Antimicrob Agents Chemother* **48**: 1916-1917.

Akiba, M., Sameshima, T., Anzai, T., Wada R., Nakazawa, M. (1999) *Salmonella Abortusequi* strains of equine origin harbour a 95-kb plasmid responsible for virulence in mice. Vet Microbiol **68:** 265-272/

Alcaine, S.D., Sukhnanand, S.S., Warnick, L.D., Su, W.L., McGann, P., McDonough, P., and Wiedmann, M. (2005) Ceftiofur-resistant *Salmonella* strains isolated from dairy farms represent multiple widely distributed subtypes that evolved by independent horizontal gene transfer. *Antimicrob Agents Chemother* **49**: 4061-4067.

Allen, K.J., and Poppe, C. (2002) Occurrence and characterization of resistance to extended-spectrum cephalosporins mediated by beta-lactamase CMY-2 in *Salmonella* isolated from food-producing animals in Canada. *Can J Vet Res* **66**: 137-144.

Allos, B.M., Moore, M.R., Griffin, P.M., and Tauxe, R.V. (2004) Surveillance for sporadic foodborne disease in the 21st century: the FoodNet perspective. *Clin Infect Dis* **38 Suppl 3**: S115-120.

Anderson, A.D., Nelson, J.M., Rossiter, S., and Angulo, F.J. (2003) Public health consequences of use of antimicrobial agents in food animals in the United States. *Microb Drug Resist* **9**: 373-379.

Angulo F.J., Griffin P.M., Changes in antimicrobial resistance in *Salmonella enterica* serovar Typhimurium, Emerg. Infect. Dis. 6 (2000) 436-437.

Angulo, F.J., Johnson, K.R., Tauxe, R.V., and Cohen, M.L. (2000) Origins and consequences of antimicrobial-resistant nontyphoidal *Salmonella*: implications for the use of fluoroquinolones in food animals. *Microbial Drug Resistance* **6**: 77-63.

Barrow, P.A., Lovell M.A. (1988) The association between a large molecular weight plasmid and virulence in strain of *Salmonella pullorum*. *J Gen Microbiol.* **134:** 2307-2316.

Batchelor, M., Hopkins, K.L., Threlfall, E.J., Clifton-Hadley, F.A., Stallwood, A.D., Davies, R.H., and Liebana, E. (2005) Characterization of AmpC-mediated resistance in clinical *Salmonella* isolates recovered from humans during the period 1992 to 2003 in England and Wales. *J Clin Microbiol* **43**: 2261-2265.

Bauernfeild, A., Stemplinger, I., Jungwirth, R., Giamarellou, H. (1996) characterization of the plasmidic β-lactamse CMY-2, which is responsible for cephamycin resistance. Antimicrob Agents Chemother **40:** 221-224.

Beltran, P., Musser, J.M., Helmuth, R., Farmer, J.J., 3rd, Frerichs, W.M., Wachsmuth, I.K., Ferris, K., McWhorter, A.C., Wells, J.G., Cravioto, A., and et al. (1988) Toward a population genetic analysis of *Salmonella*: genetic diversity and relationships among strains of serotypes S. choleraesuis, *S*. derby, *S*. dublin, *S*. enteritidis, *S*. heidelberg, *S*. infantis, *S*. newport, and *S*. typhimurium. *Proc Natl Acad Sci U S A* **85**: 7753-7757.

Beninger, P.R., Chikami, G., Tanabe, K., Roudier, C., Fierer, J., Guiney, D.G. (1988) Physical and genetic mapping of the *Salmonella* dublin virulence plasmid pSDL2: relationship to plasmids from other Salmonella strains. J. Clin. Invest. **81:** 1341-1347.

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

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

Boyd, D., Peters, G.A., Cloeckaert, A., Boumedine, K.S., Chaslus-Dancla, E., Imberechts, H., and Mulvey, M.R. (2001) Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella* enterica serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *J Bacteriol* **183**: 5725-5732.

Boyd, D., Cloeckaert, A., Chaslus-Dancla, E., and Mulvey, M.R. (2002) Characterization of variant *Salmonella* genomic island 1 multidrug resistance regions from serovars Typhimurium DT104 and Agona. *Antimicrob Agents Chemother* **46**: 1714-1722.

Boyd, D.A., Peters, G.A., Ng, L., and Mulvey, M.R. (2000) Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhymurium DT104. *FEMS Microbiol Lett* **189**: 285-291.

Boyd, E.F., Hill, C.W., Rich, S.M., and Hartl, D.L. (1996) Mosaic structure of plasmids from natural populations of *Escherichia coli. Genetics* **143**: 1091-1100.

Brenner, F.W., Villar, R.G., Angulo, F.J., Tauxe, R.V., and Swaminathan, B. (2000) *Salmonella* nomenclature. *The Journal of Clinical Microbiology* **38**: 2465-2467.

Briggs, C.E., and Fratamico, P.M. (1999) Molecular characterization of an antibiotic resistance gene cluster of *Salmonella* Typhimurium DT104. *Antimicrob Agents Chemother* **43**: 846-849.

CIPARS (2004) Canadian Integrated Program for Antimicrobial Resistance Surveillance 2004 annual report.

Carattoli, A., Tosini, F., Giles, W.P., Rupp, M.E., Hinrichs, S.H., Angulo, F.J., Barrett, T.J., and Fey, P.D. (2002) Characterization of plasmids carrying CMY-2 from expanded-spectrum cephalosporin-resistant *Salmonella* strains isolated in the United States between 1996 and 1998. *Antimicrob Agents Chemother* **46**: 1269-1272.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., and Threlfall, E.J. (2005) Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* **63**: 219-228.

Carattoli, A., Miriagou, V., Bertini, A., Loli, A., Colinon, C., Villa, L., Whichard, J.M., and Rossolini, G.M. (2006) Replicon typing of plasmids encoding resistance to newer beta-lactams. *Emerg Infect Dis* **12**: 1145-1148.

Cheung, T.K., Chu, Y.W., Chu, M.Y., Ma, C.H., Yung, R.W., and Kam, K.M. (2005) Plasmid-mediated resistance to ciprofloxacin and cefotaxime in clinical isolates of *Salmonella enterica* serotype Enteritidis in Hong Kong. *J Antimicrob Chemother* **56**: 586-589.

Chu, C., Hong, S.F., Tsai, C., Lin, W.S., Liu, T.P., and Ou, J.T. (1999) Comparative physical and genetic maps of the virulence plasmids of *Salmonella enterica* serovars Typhimurium, Enteritidis, Choleraesuis, and Dublin. *Infect Immun* **67**: 2611-2614.

Cloeckaert, A., and Schwarz, S. (2001) Molecular characterization, spread and evolution of multidrug resistance in *Salmonella enterica* typhimurium DT104. *Vet Res* **32**: 301-310.

Crump, J.A., Luby, S.P., and Mintz, E.D. (2004) The global burden of typhoid fever. *Bull World Health Organ* **82**: 346-353.

Currie, A., MacDougall, L., Aramini, J., Gaulin, C., Ahmed, R., and Isaacs, S. (2005) Frozen chicken nuggets and strips and eggs are leading risk factors for *Salmonella* Heidelberg infections in Canada. *Epidemiol Infect* **133**: 809-816.

Daly, M., and Fanning, S. (2000) Characterization and chromosomal mapping of antimicrobial resistance genes in *Salmonella enterica* serotype typhimurium. *Appl Environ Microbiol* **66**: 4842-4848.

Datta, N., and Hedges, R.W. (1971) Compatibility groups among fi - R factors. *Nature* **234**: 222-223.

Datta, N., and Hughes, V.M. (1983) Plasmids of the same Inc groups in *Enterobacteria* before and after the medical use of antibiotics. *Nature* **306**: 616-617.

Decousser, J.W., Poirel, L., and Nordmann, P. (2001) Characterization of a chromosomally encoded extended-spectrum class A beta-lactamase from *Kluyvera cryocrescens. Antimicrob Agents Chemother* **45**: 3595-3598.

Demczuk, W., R. Ahmed, D. Woodward, C. Clark, and F. Rodgers. 2000. Laboratory surveillance for enteric pathogens in Canada, 2000 annual summary. National Laboratory for Enteric Pathogens, National Microbiology Laboratory, Health Canada. The Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba, Canada.

Desselberger, U. (2000) Emerging and re-emerging infectious diseases. *J Infect* **40**: 3-15.

Doran, G., Morris, D., O'Hare, C., DeLappe, N., Bradshaw, B., Corbett-Feeney, G., and Cormican, M. (2005) Cost-effective application of pulsed-field gel electrophoresis to typing of *Salmonella enterica* Serovar Typhimurium. *Appl Environ Microbiol* **71**: 8236-8240.

Doublet, B., Lailler, R., Meunier, D., Brisabois, A., Boyd, D., Mulvey, M.R., Chaslus-Dancla, E., and Cloeckaert, A. (2003) Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster in *Salmonella enterica* serovar Albany. *Emerg Infect Dis* **9**: 585-591.

Doublet, B., Weill, F.X., Fabre, L., Chaslus-Dancla, E., and Cloeckaert, A. (2004) Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster containing a novel 3'-N-aminoglycoside acetyltransferase gene cassette, aac(3)-Id, in *Salmonella enterica* serovar Newport. *Antimicrob Agents Chemother* **48**: 3806-3812.

Doublet, B., Boyd, D., Mulvey, M.R., and Cloeckaert, A. (2005) The *Salmonella* genomic island 1 is an integrative mobilizable element. *Mol Microbiol* **55**: 1911-1924.

Dunne, E.F., Fey, P.D., Kludt, P., Reporter, R., Mostashari, F., Shillam, P., Wicklund, J., Miller, C., Holland, B., Stamey, K., Barrett, T.J., Rasheed, J.K., Tenover, F.C., Ribot, E.M., and Angulo, F.J. (2000) Emergence of domestically acquired ceftriaxone-resistant *Salmonella* infections associated with AmpC beta-lactamase. *Jama* **284**: 3151-3156.

Eberhard, W.G. (1989) Why do bacterial plasmids carry some genes and not others? *Plasmid* **21**: 167-174.

Ebner, P., Garner, K., and Mathew, A. (2004) Class 1 integrons in various *Salmonella enterica* serovars isolated from animals and identification of genomic island SGI1 in *Salmonella enterica* var. Meleagridis. *J Antimicrob Chemother* **53**: 1004-1009.

Enright, M.C., and Spratt, B.G. (1999) Multilocus sequence typing. *Trends Microbiol* **7**: 482-487.

Fakhr, M.K., Nolan, L.K., and Logue, C.M. (2005) Multilocus sequence typing lacks the discriminatory ability of pulsed-field gel electrophoresis for typing *Salmonella enterica* serovar Typhimurium. *J Clin Microbiol* **43**: 2215-2219.

Fey, P.D., Safranek, T.J., Rupp, M.E., Dunne, E.F., Ribot, E., Iwen, P.C., Bradford, P.A., Angulo, F.J., and Hinrichs, S.H. (2000) Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. *N Engl J Med* **342**: 1242-1249.

Fierer, J., Eckmann, L., Fang, F., Pfeifer, C., Finlay, B.B., and Guiney, D. (1993) Expression of the *Salmonella* virulence plasmid gene *spvB* in cultured macrophages and nonphagocytic cells. *Infect Immun* **61**: 5231-5236.

Flint, J.A., Van Duynhoven, Y.T., Angulo, F.J., DeLong, S.M., Braun, P., Kirk, M., Scallan, E., Fitzgerald, M., Adak, G.K., Sockett, P., Ellis, A., Hall, G., Gargouri, N., Walke, H., and Braam, P. (2005) Estimating the burden of acute gastroenteritis, foodborne disease, and pathogens commonly transmitted by food: an international review. *Clin Infect Dis* **41**: 698-704.

Fluit, A.C., and Schmitz, F.J. (1999) Class 1 integrons, gene cassettes, mobility, and epidemiology. *Eur J Clin Microbiol Infect Dis* **18**: 761-770.

Fluit, A.C., and Schmitz, F.J. (2004) Resistance integrons and super-integrons. *Clin Microbiol Infect* **10**: 272-288.

Foley, S.L., White, D.G., McDermott, P.F., Walker, R.D., Rhodes, B., Fedorka-Cray, P.J., Simjee, S., and Zhao, S. (2006) Comparison of subtyping methods for differentiating *Salmonella enterica* serovar Typhimurium isolates obtained from food animal sources. *J Clin Microbiol* **44**: 3569-3577.

Friedrich M.J., Kinsey, N.E. Villa J., Kadner, R.J. (1993) Nucleotide sequence of a 13.9-kilobase segment of the 90-kilobase virulence plasmid of *Salmonella typhimurium*: the presence of fimbrial biosynthetic genes. Mol Microbiol **8:** 543-558.

Ghosh, A., Singh, A., Ramteke, P.W., Singh, V.P. (2000) Characterization of large plasmids encoding resistance to heavy metals in *Salmonella Abortusequi*. Biochem Biophys Res Commmun **50:** 6-11.

Giles, W.P., Benson, A.K., Olson, M.E., Hutkins, R.W., Whichard, J.M., Winokur, P.L., and Fey, P.D. (2004) DNA sequence analysis of regions surrounding blaCMY-2 from multiple *Salmonella* plasmid backbones. *Antimicrob Agents Chemother* **48**: 2845-2852.

Gniadkowski, M. (2001) Evolution and epidemiology of extended-spectrum beta-lactamases (ESBLs) and ESBL-producing microorganisms. *Clin Microbiol Infect* **7**: 597-608.

Gregorova, D., Matiasovicova, J., Sebkova, A., Faldynova, M., and Rychlik, I. (2004) *Salmonella enterica* subsp. *enterica* serovar Enteritidis harbours ColE1, ColE2, and rolling-circle-like replicating plasmids. *Can J Microbiol* **50**: 107-112.

Guerra, B., Soto, S., Helmuth, R., and Mendoza, M.C. (2002) Characterization of a self-transferable plasmid from *Salmonella enterica* serotype Typhimurium clinical isolates carrying two integron-borne gene cassettes together with virulence and drug resistance genes. *Antimicrob Agents Chemother* **46**: 2977-2981.

Gulig, P.A., Curtiss III, R. (1988) Cloning and transposon insertion mutagenesis of virulence genes in the 100-kilobase plasmid of *Salmonella* Typhimurium. *Infect Immum.* **56:** 3262-3271.

Gupta, A., Fontana, J., Crowe, C., Bolstorff, B., Stout, A., Van Duyne, S., Hoekstra, M.P., Whichard, J.M., Barrett, T.J., and Angulo, F.J. (2003) Emergence of multidrug-resistant *Salmonella enterica* serotype Newport infections resistant to expanded-spectrum cephalosporins in the United States. *J Infect Dis* **188**: 1707-1716.

Hackett, J., Wyk, P., Reeves, P., Mathan, v. (1987) Mediation of serum resistance in *Salmonella* Typhimurium by an 11 kDa polypeptide encoded by the cryptic plasmid. J Infect Dis **155:** 540-549.

Haneda, T., Okada, N., Miki, T., and Danbara, H. (2004) Sequence analysis and characterization of sulfonamide resistance plasmid pRF-1 from *Salmonella enterica* serovar Choleraesuis. *Plasmid* **52**: 218-224.

Harbottle, H., White, D.G., McDermott, P.F., Walker, R.D., and Zhao, S. (2006) Comparison of multilocus sequence typing, pulsed-field gel electrophoresis, and antimicrobial susceptibility typing for characterization of *Salmonella enterica* serotype Newport isolates. *J Clin Microbiol* **44**: 2449-2457.

Hawley, D.K., and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res* **11**: 2237-2255.

Hohmann, E.L. (2001) Nontyphoidal salmonellosis. *Clin Infect Dis* **32**: 263-269.

Hopkins, K.L., Davies, R.H., and Threlfall, E.J. (2005) Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. *Int J Antimicrob Agents* **25**: 358-373.

Jawetz, E., and Levinson, W. (2002) *Medical Microbiology and Immunology.* New York, NY: McGraw-Hill Companies.

Lawson, A.J., Desai, M., O'Brien, S.J., Davies, R.H., Ward, L.R., Threlfall, E.J. (2004) Molecular characterization of an outbreak strain of multiresistant *Salmonella enterica* serovar Typhimurium Dt104 in the UK. *Eur Soc Clin Microbiol Infect Dis* **10:** 143-147.

Levings, R.S., Lightfoot, D., Partridge, S.R., Hall, R.M., and Djordjevic, S.P. (2005) The genomic island SGI1, containing the multiple antibiotic resistance region of *Salmonella enterica* serovar Typhimurium DT104 or variants of it, is widely distributed in other S. enterica serovars. *J Bacteriol* **187**: 4401-4409.

McDermott, P.F. (2006) Antibiotic resistance in nontyphoidal *Salmonella*. In *Antibiotic resistant in bacteria of animal origin*. Aarestrup, F.M. (ed). Washington, DC: ASM Press, pp. 442.

Marshall, BJ., Royce H., Annear D.I., Goodwin C.S., Pearman, J.W., Warren, J.R., Armstrong J.A. (1984) Original isolation of *Campylobacter pylordis* from human gastric mucosa. Microbios Lett. **25:** 83-88.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. (1999) Food-related illness and death in the United States. *Emerg Infect Dis* **5**: 607-625.

Medeiros, A.A. (1997) Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. *Clin Infect Dis* **24 Suppl 1**: S19-45.

Mendez, B., Tachibana, C., and Levy, S.B. (1980) Heterogeneity of tetracycline resistance determinants. *Plasmid* **3**: 99-108.

Meunier, D., Boyd, D., Mulvey, M.R., Baucheron, S., Mammina, C., Nastasi, A., Chaslus-Dancla, E., and Cloeckaert, A. (2002) *Salmonella enterica* serotype Typhimurium DT 104 antibiotic resistance genomic island I in serotype paratyphi B. *Emerg Infect Dis* **8**: 430-433.

Miriagou, V., Filip, R., Coman, G., and Tzouvelekis, L.S. (2002) Expanded-spectrum cephalosporin-resistant *salmonella* strains in Romania. *J Clin Microbiol* **40**: 4334-4336.

Molbak, K. (2005) Human health consequences of antimicrobial drug-resistant *Salmonella* and other foodborne pathogens. *Clin Infect Dis* **41**: 1613-1620.

Mulvey, M.R., Soule, G., Boyd, D., Demczuk, W., and Ahmed, R. (2003) Characterization of the first extended-spectrum beta-lactamase-producing *Salmonella* isolate identified in Canada. *J Clin Microbiol* **41**: 460-462.

Mulvey, M.R., Boyd, D., Cloeckaert, A., Ahmed, R., and Ng, L.K. (2004) Emergence of multidrug-resistant *Salmonella* Paratyphi B dT+, Canada. *Emerg Infect Dis* **10**: 1307-1310.

Mulvey, M.R., Bryce, E., Boyd, D.A., Ofner-Agostini, M., Land, A.M., Simor, A.E., and Paton, S. (2005) Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. *Antimicrob Agents Chemother* **49**: 358-365.

Mulvey, M.R., Boyd, D.A., Olson, A.B., Doublet, B., and Cloeckaert, A. (2006) The genetics of *Salmonella* genomic island 1. *Microbes Infect* **8**: 1915-1922.

Murase, T., Okitsu, T., Suzuki, R., Morozumi, H., Matsushima, A., Nakamura, A., and Yamai, S. (1995) Evaluation of DNA fingerprinting by PFGE as an epidemiologic tool for *Salmonella* infections. *Microbiol Immunol* **39**: 673-676.

Nakamura, M., Sato S., Ohya, T., Suzuki, S., Ikeda, S. (1985) Possible relationship of a 36-megaDalton *Salmonella* Enteriditis plasmid to virulence in mice. Infect Immum **47:** 831-833.

Navarro, F., Perez-Trallero, E., Marimon, J.M., Aliaga, R., Gomariz, M., and Mirelis, B. (2001) CMY-2-producing *Salmonella enterica*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis* and *Escherichia coli* strains isolated in Spain (October 1999-December 2000). *J Antimicrob Chemother* **48**: 383-389.

NCCLS/CLSI. Preformance standars for antimicrobial susceptibility testing: fifteenth informational supplement. NCCLS/CLSI/ document M100-S15. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne Pennsylvania 19087-1898 USA, 2005.

NCCLS/CLSI. Preformance standars for antimicrobial susceptibility testing: twelfth informational supplement. NCCLS/CLSI/ document M100-S12. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne Pennsylvania 19087-1898 USA.

Novick R.P. (1987) Plasmid incompatibility. *Microbiol Rev.* **51**: 381-395.

Olsen, J.E., Skov, M.N., Threlfall, E.J., and Brown, D.J. (1994) Clonal lines of *Salmonella enterica* serotype Enteritidis documented by IS200-, ribo-, pulsed-field gel electrophoresis and RFLP typing. *J Med Microbiol* **40**: 15-22.

Orman, B.E., Pineiro, S.A., Arduino S. (2002) Evolution of multiresistance in nontyphoid *Salmonella* serovars from 1984 to 1998 in Argentina. Antimicrob Agents Chemother **46:** 3963-3970.

Osborn, A.M., and Boltner, D. (2002) When phage, plasmids, and transposons collide: genomic islands, and conjugative- and mobilizable-transposons as a mosaic continuum. *Plasmid* **48**: 202-212.

Papanicolaou, G.A., Medeiros, A.A., and Jacoby, G.A. (1990) Novel plasmid-mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **34**: 2200-2209.

Paterson, D.L., and Bonomo, R.A. (2005) Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev* **18**: 657-686.

Pelludat, C., Prager, R., Tschape, H., Rabsch, W., Schuchhardt, J., and Hardt, W.D. (2005) Pilot study to evaluate microarray hybridization as a tool for *Salmonella enterica* serovar Typhimurium strain differentiation. *J Clin Microbiol* **43**: 4092-4106.

Perry, R.D., Fetherston, J.D. (1997) *Yersinia Pestis*--etiologic agent of plague. Clin Microbiol Reviews **10:** 35-66.

Philippon, A., Arlet, G., and Jacoby, G.A. (2002) Plasmid-determined AmpC-type beta-lactamases. *Antimicrob Agents Chemother* **46**: 1-11.

Popoff, M.Y., Bockemuhl, J., Gheesling, L.L. (2004) Supplement 2002 (no.46) the Kauffamnn-White scheme. **155:** 568-570.

Popoff, M.Y., Le Minor, L. Antigenic formulas of the *Salmonella* serovars, eight revision, WHO collaborating centre of reference and research on *Salmonella*, Institut Pasteur, Paris, 2001.

Porwollik, S., Wong, R.M., and McClelland, M. (2002) Evolutionary genomics of *Salmonella*: gene acquisitions revealed by microarray analysis. *Proc Natl Acad Sci U S A* **99**: 8956-8961.

Porwollik, S., Santiviago, C.A., Cheng, P., Florea, L., and McClelland, M. (2005) Differences in gene content between *Salmonella enterica* serovar Enteritidis isolates and comparison to closely related serovars Gallinarum and Dublin. *J Bacteriol* **187**: 6545-6555.

Randall, L.P., Cooles, S.W., Osborn, M.K., Piddock, L.J., and Woodward, M.J. (2004) Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty-five serotypes of *Salmonella enterica* isolated from humans and animals in the UK. *J Antimicrob Chemother* **53**: 208-216.

Rankin, S.C., Aceto, H., Cassidy, J., Holt, J., Young, S., Love, B., Tewari, D., Munro, D.S., and Benson, C.E. (2002) Molecular characterization of cephalosporin-resistant *Salmonella enterica* serotype Newport isolates from animals in Pennsylvania. *J Clin Microbiol* **40**: 4679-4684.

Reen, F.J., Boyd, E.F., Porwollik, S., Murphy, B.P., Gilroy, D., Fanning, S., and McClelland, M. (2005) Genomic comparisons of *Salmonella enterica* serovar Dublin, Agona, and Typhimurium strains recently isolated from milk filters and bovine samples from Ireland, using a *Salmonella* microarray. *Appl Environ Microbiol* **71**: 1616-1625.

Rychlik, I., Gregorova, D., and Hradecka, H. (2006) Distribution and function of plasmids in *Salmonella enterica*. *Vet Microbiol* **112**: 1-10.

Sambrook, J., and Russell, D. (2001) *Molecular Cloning: A Laboratory Manual.* Cold Springs Harbour: Cold Springs Harbour Laboratory Press.

Sandt, C.H., Krouse, D.A., Cook, C.R., Hackman, A.L., Chmielecki, W.A., and Warren, N.G. (2006) The key role of pulsed-field gel electrophoresis in investigation of a large multiserotype and multistate food-borne outbreak of *Salmonella* infections centered in Pennsylvania. *J Clin Microbiol* **44**: 3208-3212.

Sandvang, D., Aarestrup, F.M., and Jensen, L.B. (1998) Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol Lett* **160**: 37-41.

Schmieger, H. (1999) Molecular survey of the Salmonella phage typing system of Anderson. *J Bacteriol* **181**: 1630-1635.

Stutman, H.R. (1994) *Salmonella, Shigella*, and *Campylobacter*: common bacterial causes of infectious diarrhea. *Pediatr Ann* **23**: 538-543.

Su, L.H., Chen, H.L., Chia, J.H., Liu, S.Y., Chu, C., Wu, T.L., and Chiu, C.H. (2006) Distribution of a transposon-like element carrying bla(CMY-2) among *Salmonella* and other *Enterobacteriaceae*. *J Antimicrob Chemother* **57**: 424-429.

Sukhnanand, S., Alcaine, S., Warnick, L.D., Su, W.L., Hof, J., Craver, M.P., McDonough, P., Boor, K.J., and Wiedmann, M. (2005) DNA sequence-based subtyping and evolutionary analysis of selected *Salmonella enterica* serotypes. *J Clin Microbiol* **43**: 3688-3698.

Swaminathan, B., Barrett, T.J., Hunter, S.B., and Tauxe, R.V. (2001) PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerg Infect Dis* **7**: 382-389.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., and Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**: 2233-2239.

Threlfall, E.J. (2000) Epidemic *Salmonella* Typhimurium DT 104--a truly international multiresistant clone. *J Antimicrob Chemother* **46**: 7-10.

Threlfall, E.J., Frost, J.A., Ward, L.R., Rowe, B. (1994) Epidemic in cattle and humans of *Salmonella* Typhimurium Dt 104 with chromosomally integrated multiple drug resistance. *Vet Rec* **134:** 577.

Timoney, J.F., Taylor, D.E., Shin, S., McDonough, P. (1980) pJT2: unusual HI plasmid in a highly virulent lactose-positive and chloramphenicol-resistant *Salmonella* Typhimuirum strain from calves. *Antimicrob Agents Chemother* **18:** 480-482.

Torpdahl, M., Sorensen, G., Ethelberg, S., Sando, G., Gammelgard, K., and Porsbo, L.J. (2006) A regional outbreak of *S.* Typhimurium in Denmark and identification of the source using MLVA typing. *Euro Surveill* **11**: 134-136.

United Nation Programme on HIV/AIDS. (2006) Report on the global AIDS epidemic 8.

Valdivia R.H., Falkow, S., (1997) Fluorescnece-based isolation of bacterial genes expressed within host cells. *Science* **277:** 2007-2111.

Valone, S.E., Chikami, G.K., Miller, V.L. (1993) Stress induction of the virulence proteins (SpvA, -B, and -C) from native plasmid pSDL2 of *Salmonella* Dublin. Infect Immun **61:** 705-713.

van Hoek, A.H., Scholtens, I.M., Cloeckaert, A., and Aarts, H.J. (2005) Detection of antibiotic resistance genes in different *Salmonella* serovars by oligonucleotide microarray analysis. *J Microbiol Methods* **62**: 13-23.

Villa, L., and Carattoli, A. (2005) Integrons and transposons on the *Salmonella enterica* serovar Typhimurium virulence plasmid. *Antimicrob Agents Chemother* **49**: 1194-1197.

Weill, F.X., Fabre, L., Grandry, B., Grimont, P.A., and Casin, I. (2005) Multiple-antibiotic resistance in *Salmonella enterica* serotype Paratyphi B isolates collected in France between 2000 and 2003 is due mainly to strains harboring *Salmonella* genomic islands 1, 1-B, and 1-C. *Antimicrob Agents Chemother* **49**: 2793-2801.

Werbe, D.A., Fruth, A., Liesegang, A., Littmann, M., Buchholz, U., Prager, R., Karch, H., Breuer, T., Tschape, H., Ammon, A. (2002) A multistate outbreak of Shiga toxin-producing *Escherichia coli* O26:H11 infections in Germany, detected by molecular subtyping surveillance. J. Infect. Dis **186:** 67-75.

White, D.G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., McDermott, P.F., McDermott, S., Wagner, D.D., and Meng, J. (2001) The isolation of antibiotic-resistant salmonella from retail ground meats. *N Engl J Med* **345**: 1147-1154.

WHO. (2006) WHO global salm-surv progress report 2000 ~ 2005. URL: http://www.who.int/salmsurv/links/GSSProgressReport2005.pdf

WHO. (2006) Tuberculosis fact sheet. acessed Jan. 24, 2007. URL: http://www.who.int/mediacentre/factsheets/fs104/en/index.html

Winokur, P.L., Vonstein, D.L., Hoffman, L.J., Uhlenhopp, E.K., and Doern, G.V. (2001) Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. *Antimicrob Agents Chemother* **45**: 2716-2722.

Zhao, S., Qaiyumi, S., Friedman, S., Singh, R., Foley, S.L., White, D.G., McDermott, P.F., Donkar, T., Bolin, C., Munro, S., Baron, E.J., and Walker, R.D. (2003) Characterization of *Salmonella enterica* serotype Newport isolated from humans and food animals. *J Clin Microbiol* **41**: 5366-5371.

Appendix 1

Gilmour, M.W., Tracz, D.M., Andrysiak, A.K., Clark, C.G., Tyson S., Severini, A., Ng, LK. (2006) Use of the *espZ* gene encoded in the locus of enterocyte effacement for molecular typing of shiga toxin-producing *Escherichia coli*. Journal of Clinical Microbiology **44**: 449-58.

This article details a molecular typing platform for Shiga toxin-producing *Escherichia coli*. A. Andrysiak contributed to the project by extracting template DNA, performing *esp*Z real time PCR, intimin and *stx* PCR as well as sequence data analysis.

Journal of Clinical Microbiology, Feb. 2006, p. 449–458 Vol. 44, No. 2
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.2.449–458.2006
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Use of the *espZ* Gene Encoded in the Locus of Enterocyte Effacement for Molecular Typing of Shiga Toxin-Producing *Escherichia coli*

Matthew W. Gilmour,* Dobryan M. Tracz, Ashleigh K. Andrysiak, Clifford G. Clark, Shari Tyson, Alberto Severini, and Lai-King Ng

National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada

Received 24 August 2005/Returned for modification 18 October 2005/Accepted 4 November 2005

Infections with Shiga toxin-producing *Escherichia coli* (STEC) result in frequent cases of sporadic and outbreak-associated enteric bacterial disease in humans. Classification of STEC is by *stx* genotype (encoding the Shiga toxins), O and H antigen serotype, and seropathotype (subgroupings based upon the clinical relevance and virulence-related genotypes of individual serotypes). The *espZ* gene is encoded in the locus of enterocyte effacement (LEE) pathogenicity island responsible for the attaching and effacing (A/E) lesions caused by various *E. coli* pathogens (but not limited to STEC), and this individual gene (~300 bp) has previously been identified as hypervariable among these A/E pathogens. Sequence analysis of the *espZ* locus encoded by additional STEC serotypes and strains (including O26:H11, O121:H19, O111:NM, O145:NM, O165:H25, O121:NM, O157:NM, O157:H7, and O5:NM) indicated that distinct sequence variants exist which correlate to subgroups among these serotypes. Allelic discrimination at the *espZ* locus was achieved using Light Upon eXtension real-time PCR and by liquid microsphere suspension arrays. The allele subtype of *espZ* did not correlate with STEC seropathotype classification; however, a correlation with the allele type of the LEE-encoded intimin (*eae*) gene was supported, and these sequence variations were conserved among individual serotypes. The study focused on the characterization of three clinically significant seropathotypes of LEE-positive STEC, and we have used the observed genetic variation at a pathogen-specific locus for detection and subtyping of STEC.

Gastrointestinal infection by Shiga toxin-producing *Escherichia coli* (STEC) is largely due to serotype O157:H7 in North America, but infections with other serotypes also result in human disease (25). In Canada, 48 different serotypes of STEC (as identified on the basis of O-somatic and H-flagellar antigens) have been isolated from humans, and strains of serotypes O26:H11, O121:H19, O103:H2, O145:NM, and O111:NM have represented a large proportion of non-O157 isolates (38). STEC serotypes are classified into five seropathotypes (A through E) based upon both virulence gene content and clinical relevance, among which seropathotype A is solely comprised of O157:H7 and O157:NM strains; infections with these strains can result in serious disease symptoms and lead to outbreaks (15). Infection with STEC can result in diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome, with possible disease outcomes including renal failure, neurological sequelae, and death (17, 33). Seropathotype B includes the non-O157 serotypes identified above, and while not known to cause large disease epidemics as frequently, infection with these pathogens can result in disease symptoms similar to those seen with seropathotype A strains (15). Notably, the differential capabilities for detection of O157 versus non-O157 serotypes in clinical laboratories may introduce reporting biases.

STEC disease manifestation correlates to the carriage of classical bacterial virulence determinants such as toxins and pathogenicity islands. The production of Shiga toxins, encoded by the stx_1 and stx_2 genes, is responsible for systemic disease symptoms, because necrotic and apoptotic cell death are induced after intracellular translocation (4). Stx1 is nearly identical to the cytotoxin produced by *Shigella dysenteriae* serotype 1 and is homogenous among *E. coli* carrying stx_1, whereas several variants of stx_2 have been identified, and the production of Stx2 is associated with hemolytic uremic syndrome (33). The carriage of *stx* genes is also variable among STEC serotypes, as the majority of seropathotype A strains encode both loci, whereas strains of the other seropathotypes typically encode a single toxin locus. A large subset of STEC strains (predominantly seropathotypes A and B) are also termed enterohemorrhagic *E. coli* (EHEC) strains and are partly characterized by attaching and effacing (A/E) lesions that they create on the intestinal epithelium, a histopathology resulting from the presence of the locus of enterocyte effacement (LEE) pathogenicity island (8, 19). While STEC strains are considered to be noninvasive, there is disruption of the brush border microvilli after actin rearrangements within epithelial cells. This process is induced by the LEE-encoded determinants that include 41 coding sequences (the majority of which are organized into five major operons) for type III secretion of effector and receptor proteins, including Tir, which allows the close association between the bacterial and the host epithelial cells via intimin (*eae*) present on the bacterial cell surface (6, 27).

Among the individual LEE-encoded genes present in different A/E pathotypes, including enteropathogenic *E. coli* (EPEC) and EHEC, a heterogeneous rate of genetic diversity has been observed (3, 27). It was our goal to select a LEE-encoded gene that would support the detection and subtyping of STEC, relying upon the ubiquity of the LEE among the most significant

* Corresponding author. Mailing address: National Microbiology Laboratory, 1015 Arlington Street, Winnipeg, Manitoba, Canada R3E 3R2. Phone: 204 784 5920. Fax: 204 789 5012. E-mail: Matthew_Gilmour@phac-aspc.gc.ca.

STEC serotypes and the large degree of genetic diversity observed at individual LEE genes. The loci that demonstrated the highest rates of diversity (measured as π) include those involved in interaction with the host cell (i.e., effectors and receptors), whereas the type III secretion apparatus had a lesser amount of diversity (3). Furthermore, the ratio of nonsynonymous mutation (dN) to synonymous mutation (dS) was markedly higher at those same loci involved in host interactions, and therefore these coding sequences may be undergoing positive selection (3), a process reflective of functional adaptation in different host environments and *E. coli* genetic backgrounds. The loci with the highest π estimates included *sepZ*, *tir*, *espA*, *espB*, *espF*, *espH*, and *eae*, and some of these have previously been used for molecular subtyping of A/E pathogens (1, 2, 21, 23, 29, 40). Corresponding variability of SepZ protein primary sequences was also observed among EPEC and EHEC strains (9, 14, 27). Notably, the *sepZ* coding sequence was renamed *espZ* after determining that the gene product is secreted and translocated to eukaryotic cells (14). The *espZ* locus was also estimated as having the highest rate (3), and the resultant variability between EspZ proteins likely arose due to functional adaptation to host proteins, which are currently unknown (14). Our study was of the *espZ* nucleotide sequences encoded in clinically significant STEC. Molecular techniques were developed for the detection and subtyping of STEC at this locus, and phylogenetic analyses were performed to estimate the evolutionary history of the relationship between STEC serotypes and the LEE pathogenicity island.

MATERIALS AND METHODS

Bacterial strains. The panel of STEC strains collected for this study (Table 1) included representative isolates from each of the major serotypes observed in Canada (classified as seropathotype A, B, or C), and among individual serotypes we included strains with different *stx* genotypes when available. All isolates were from the National Microbiology Laboratory (NML) Bacteriology and Enteric Diseases Program culture collection, which originated from human sources at various Canadian Provincial Health laboratories during 1985 to 2005, and serotype and toxin genotype were confirmed at the NML (Table 1). Existing *sepZ*/*espZ* sequence data deposited in GenBank were used to initiate our characterization of these Canadian strains as follows: for O26:H11 (strain 6549), accession number AF035656; for O26:H- (413/89-1), AJ277443; for O157:H7 (EDL933), AAC31516; for O157:H7 (Sakai), BAB37994; for O157:H7 (86-24), AF035655; for O15:H- (RDEC-1), AF035651; for O15:H- (EPEC 83/89), AF453441; for O111:NM (EPEC B171), AF035653; for O103:H2 (RW1374), AJ303141; for O127:H6 (EPEC E2348/69), X94450; and for O55:H7 (EPEC), AF035652.

PCR and sequencing. Template DNA was prepared by centrifuging 1 ml of log-phase cultures grown in brain heart infusion broth, resuspending the pellet in 1 ml of TE buffer (Sigma, St. Louis, MO) (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and boiling for 10 min. Boiled cells were pelleted, and the supernatant was removed and used as the template in real-time and standard PCRs. For quantitative determination of real-time PCR sensitivity, total genomic DNA was isolated from liquid cultures grown overnight in 8 ml brain heart infusion broth. After centrifugation, the bacterial pellet was resuspended in 2 ml of TE buffer with vortexing. Following the addition of lysozyme (Roche Diagnostics, Indianapolis, IN) (0.5 mg/ml), RNase (Roche Diagnostics) (1.5 μg/ml), and proteinase K (Sigma) (0.12 mg/ml), this mixture was incubated at 37°C for 1 h, and then sodium dodecyl sulfate (SDS) (Ambion, Austin, TX) was added to achieve a concentration of 0.1% (wt/vol) and the mixture was further incubated at 65°C until the suspension cleared. Organic extraction was performed using 15 ml Eppendorf Phase Lock tubes (Hamburg, Germany) with an equal volume of phenol:chloroform:isoamyl alcohol (Invitrogen, Burlington, ON) (25:24:1). Phenol-chloroform-isoamyl alcohol extraction was repeated until the aqueous layer was clear, and after a final extraction with 2 ml chloroform, the aqueous layer was transferred to a new 1.5 ml tube and 0.6 volumes of isopropanol and 0.1 volumes of 3 M sodium acetate (Ambion, pH 5.5) were added and DNA was precipitated at −20°C for 20 min. Following centrifugation, the DNA pellet was washed in 1 ml of 70% ethanol and resuspended in 200 μl of TE buffer. After quantification on a NanoDrop ND-1000 apparatus (NanoDrop Technologies, Rockland DE), the DNA was serially diluted from 33 ng/μl to 0.33 fg/μl.

Standard PCR was performed with Platinum *Taq* (Invitrogen), following the manufacturer's directions, and with the oligonucleotides described in Table 2. The thermocycling parameters for *espZ* required an initial denaturation at 94°C for 5 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 30 s, with a final extension at 68°C for 5 min. Conditions for PCR amplification of stx_2 were initial denaturation at 94°C for 5 min and 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 68°C for 30 s, with a final extension at 68°C for 7 min. PCR products for *espZ* and stx_2 were purified using a QIAquick PCR purification kit (QIAGEN, Mississauga, ON) and sequenced using the same primers used to generate this template. Sequencing was performed on an ABI3730 apparatus (Applied Biosystems, Foster City, CA). Subtyping of stx_2 variants was performed using oligonucleotides described by Wang et al. (36).

LUX (Light Upon eXtension) fluorogenic and unlabeled primer pairs (20) were designed using D-LUX designer software (Invitrogen) by targeting regions characteristic of each *espZ* allele (Fig. 1A) or for regions conserved in either stx_1 or stx_2 (see Results). LUX primers are self-quenching oligonucleotide primers that are labeled with a single fluorophore. Upon annealing to a specific DNA sequence, they become dequenched and the fluorescent signal increases. For LUX real-time PCR, Platinum quantitative PCR Supermix UDG (Invitrogen) was used for the amplification mixture with 200 nM of each LUX primer pair

TABLE 1. Bacterial strains used in this study

Serotype	Strain	Source[a]	Seropathotype[b]	stx_1[c]	stx_2	LEE[d]
O157:H7	87-1215	NML	A	+	+	+
O157:H7	01-8110	NML	A	+	+	+
O157:H7	05-0958	SK HPL	A	−	+	+
O157:H7	04-4319	SK HPL	A	+	−	+
O157:H7	03-2641	AB PLPH	A	+	+	+
O157:NM	01-6434	AB PLPH	A	+	−	+
O157:NM	03-5296	AB PLPH	A	+	+	+
O145:NM	03-4699	AB PLPH	B	+	−	+
O26:H11	00-3941	SK HPL	B	+	−	+
O26:H11	01-5870	MB CPL	B	+	−	+
O26:H11	01-6372	NS PHL	B	+	−	+
O26:H11	02-6738	BCCDC	B	+	−	+
O26:H11	03-2816	AB PLPH	B	+	−	+
O26:H11	03-4186	BCCDC	B	+	−	+
O26:H11	99-4610	BCCDC	B	+	−	+
O26:H11	02-6737	BCCDC	B	+	+	+
O121:H19	03-2636	AB PLPH	B	−	+	+
O121:H19	03-2642	AB PLPH	B	−	+	+
O121:H19	03-2832	AB PLPH	B	−	+	+
O121:H19	00-5288	BCCDC	B	−	+	+
O103:H2	99-2076	BCCDC	B	+	−	+
O103:H2	04-2446	MB CPL	B	+	−	+
O103:H2	01-6102	SK HPL	B	+	−	+
O111:NM	03-3991	AB PLPH	B	+	+	−
O111:NM	04-3794	MB CPL	B	+	+	+
O111:NM	98-8338	BCCDC	B	+	−	+
O111:NM	00-4748	SK HPL	B	+	+	+
O111:NM	00-4440	BCCDC	B	+	−	+
O111:NM	01-0252	BCCDC	B	+	+	+
O111:NM	01-1215	BCCDC	B	+	−	+
O121:NM	99-4389	NML	C	−	+	+
O165:H25	00-4540	BCCDC	C	−	+	+
O5:NM	03-2825	AB PLPH	C	+	−	+
O91:H21	85-489	NML	C	−	+	−
O113:H21	93-0016	NML	C	−	+	−

[a] AB PLPH, Alberta Provincal Laboratory for Public Health; BCCDC, British Columbia Centre for Disease Control; MB CPL, Manitoba Cadham Provincial Laboratory; NML, National Microbiology Laboratory standard strain; NS PHL, Nova Scotia Public Health Laboratory; SK HPL, Saskatchewan Health Provincial Laboratory.

[b] Seropathotype as defined in reference 15.

[c] Genotypes determined by standard PCR protocols (24).

[d] Carriage of the LEE was determined by PCR for *espZ*.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Target(s)	Sequence (5′ to 3′)[a]	Platform	Source or reference
GIL245	*espZ*	CAGCAAATTTAAGTCCTTCTGGC	PCR-sequencing	This study
GIL246	*espZ*	AGGCATATTTCATCGCTAATCCG	PCR-sequencing	This study
GIL246-L	*espZ*	Biotin-FEEOATATTTCATCGCTAATCCG	Microsphere array	This study
DOB56	*espZ*-γ1	cgctgGCTCTAGGTACAGGTATTGCAGcG (FAM)	RT-PCR[b]	This study
DOB57	*espZ*-γ1	GCCAGAAGTAATACCCAGGGCTAA		This study
DOB58	*espZ*-β1	ccgcGTCGTAATATCAGAATTGCAGCcG (FAM)	RT-PCR	This study
DOB59	*espZ*-β1	TTCTTGGGAGCTTGCATCTGTT		This study
DOB62	*espZ*-ε	cgatttAACCAGAAAATGGAACAAATcG (FAM)	RT-PCR	This study
DOB63	*espZ*-ε	CACCGATAGCGGCTAGAGCAC		This study
GIL294	*espZ*-γ2	cggtacACAGTGATGCGATACCAGTACcG (FAM)	RT-PCR	This study
GIL295	*espZ*-γ2	GCAACCAGAAGGTGAAACAAG		This study
DOB66	stx_1	cggctATTATTTCGTTCAACAATAAGCcG (Alexa 546)	RT-PCR	This study
DOB67	stx_1	CAGAGGGATAGATCCAGAGGAAGG		This study
GIL290	stx_2	cggacaCAGAGTGGTATAACTGCTGTCcG (FAM)	RT-PCR	This study
GIL291	stx_2	ATATCAGTGCCCGGTGTGACAA		This study
DOB70	*espZ*-γ1	TTAGCACTTACCACTACGGCT	Microsphere array	This study
DOB72	*espZ*-β1	GGTAAGTCGTAATATCAGAATTGC	Microsphere array	This study
DOB73	*espZ*-ε	GAACAAATCGTACCATTAGAATCC	Microsphere array	This study
DOB74	*espZ*-γ2	GCAACCAGAAGGTGAAACAAG	Microsphere array	This study
VT2v-5	stx_2 (13 bp upstream)	TGGTGCTGATTACTTCAGCC	PCR-sequencing	This study
VT2v-2	stx_2 (300 bp downstream)	GGGTGCCTCCCGGTGAGTTC	PCR-sequencing	35
ASH13	stx_2-internal	CAGAGATGCATCCAGAGCAG	PCR-sequencing	This study
ASH14	stx_2-internal	TGCTCAGTCTGACAGGCAAC	PCR-sequencing	This study
EA-B1-F	*eae*-β1	CGCCACTTAATGCCAGCG	PCR	2
EAE-B	*eae*-β1	CTTGATACACCTGATGACTGT	PCR	2
EAE-FB	eae-γ1; eae-γ2; ε-*eae*	AAAACCGCGGAGATGACTTC	PCR	2
EAE-C1	*eae*-γ1	AGAACGCTGCTCACTAGATGTC	PCR	2
EAE-C2	*eae*-γ2	CTGATATTTTATCAGCTTCA	PCR	2
LP5	*eae*-ε	AGCTCACTCGTAGATGACGGCAAGCG	PCR	2

[a] FAM, 6-carboxyfluorescein; O, C-phosphorothioate; F, A-phosphorothioate; E, G-phosphorothioate; lowercase bases at the 5′ end indicate those required for LUX primer hairpin formation and are not present in the target sequence; the penultimate 3′ base is tagged with the fluorescent molecule indicated in parentheses.
[b] RT-PCR, real-time PCR.

(Table 2) and 3 μl of template for a total reaction volume of 25 μl. Real-time PCRs were performed on the Cepheid SmartCycler 2.0 apparatus (Cepheid, Sunnyvale, CA), and samples were amplified after an initial denaturation at 95°C for 3 min and 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 15 s, and an extension step at 72°C for 15 s. Fluorescence was detected at the annealing step, and the threshold level was set at 30 fluorescence units. A real-time PCR result was considered positive when the log fluorescent signal exceeded this threshold after background subtraction.

Microsphere liquid-suspension arrays. Allelic discrimination of *espZ* was achieved after PCR amplification of biotin-labeled *espZ* target DNA from group A and B STEC seropathotypes (O157:H7, O26:H11, O121:H19, and O111:NM) by use of a GeneAmp 9700 thermocycler (Applied Biosystems) and the thermocycling parameters detailed above. The 100 μl amplification mixture consisted of the following: 10 μl of 10× HiFi buffer (Invitrogen), 2 μl of deoxynucleoside triphosphates (Invitrogen) (10 μM each), 4 μl of $MgSO_4$, 0.4 μl of Platinum Hi Fidelity *Taq* (Invitrogen), 61.6 μl of molecular biology grade water (Gibco, Grand Island, NY), and 10 μl each of primers GIL245 and GIL246-L (5′ biotinylated; Table 2), for a final primer concentration of 1 μM each. The template (2 μl), prepared from a boiled cell resuspension as described above, was added to the reaction mixture. Successful PCR amplification of *espZ* was confirmed by agarose gel electrophoresis. PCRs were purified with Qiaquick DNA purification kits (QIAGEN) and eluted with 50 μl of EB buffer (QIAGEN). Oligonucleotide GIL246-L contains four bases with phosphorothioate linkages, as well as a biotin molecule, all at the 5′ end. Of the two strands of the target DNA, the strand produced from GIL245 (*espZ* "sense" strand) is sensitive to T7 exonuclease digestion whereas the *espZ* antisense strand produced from GIL246-L is protected due to the phosphorothioate linkages (22). DNA digestion was performed by mixing 43 μl of purified PCR product with 5 μl of buffer 4 and 2 μl of T7 exonuclease (both from New England Biolabs, Ipswich, MA) (20 U total) and incubating at 37°C for 1 h. T7 exonuclease was inactivated by adding 2 μl of 0.5 M EDTA (Ambion). Selective degradation ensures elimination of the unlabeled target DNA strand, thereby preventing reannealing between the two target DNA strands during hybridization that, if left intact, would limit the intended hybridization between the biotin-labeled strand and the *espZ* allele-specific probes coupled to microspheres in subsequent steps.

Oligonucleotide probes for each *espZ* allele were designed matching the sense strand in highly variable regions characteristic of individual allele subtypes (Fig. 1A, Table 2). Oligonucleotides were screened using SBEprimer software (13) for potential secondary structures or cross-hybridization between probes. The oligonucleotide probes were synthesized with a 5′ C-12 amine and coupled to xMAP carboxylated fluorescently coded microspheres (Luminex Corporation, Austin, TX). Microsphere sets 103, 105, 108, and 110 were coupled to oligonucleotides DOB70, DOB72, DOB73, and DOB74, respectively (Table 2). Microspheres (5.0×10^6) were transferred to a 1.5 ml microcentrifuge tube, centrifuged at 8,000 × *g* for 2 min, resuspended in 50 μl of 0.1 M MES buffer [2-(*N*-morpholino)ethanesulfonic acid; Sigma] (pH 4.5), and vortexed, and 6 μl of capture oligonucleotide (100 μM) was added to the respective bead set. A fresh solution of EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimidehydrochloride; Pierce Biotechnology, Rockford, IL] (10 mg/ml) was prepared immediately before use, 2 μl was added to the bead-oligonucleotide mixture, and the mixture was vortexed and incubated at room temperature for 30 min in the dark. The EDC addition and incubation was repeated. Following incubation, beads were washed successively in 1 ml of 0.1% Tween and 1 ml of 0.1% SDS. Microspheres were resuspended in 100 μl of 0.1 M MES (pH 4.5) and enumerated on a hemocytometer.

For hybridization of biotin-labeled *espZ* target DNA strands to the capture probe-coupled microspheres, a reaction master mix was prepared in TE buffer at a concentration of 150 microspheres/μl for each of the four capture probe sets. Hybridizations were prepared in triplicate under low-light conditions as 50-μl reaction mixtures in Thermowell 96-well plates (Corning Incorporated, Corning, NY). Initially, 17 μl of the biotinylated PCR product was added to wells and denatured for 10 min at 95°C in a GeneAmp 9700 thermocycler, followed by addition of 33 μl of the reaction master mix and mixing by pipetting. Hybridizations were performed at 55°C for 20 min, after which 25 μl of the streptavidin R-phycoerythrin reporter dye (Molecular Probes, Eugene, OR) diluted to 10 μg/ml in 1.5× TMAC buffer (3 M tetramethylammonium chloride [Sigma], 0.1%

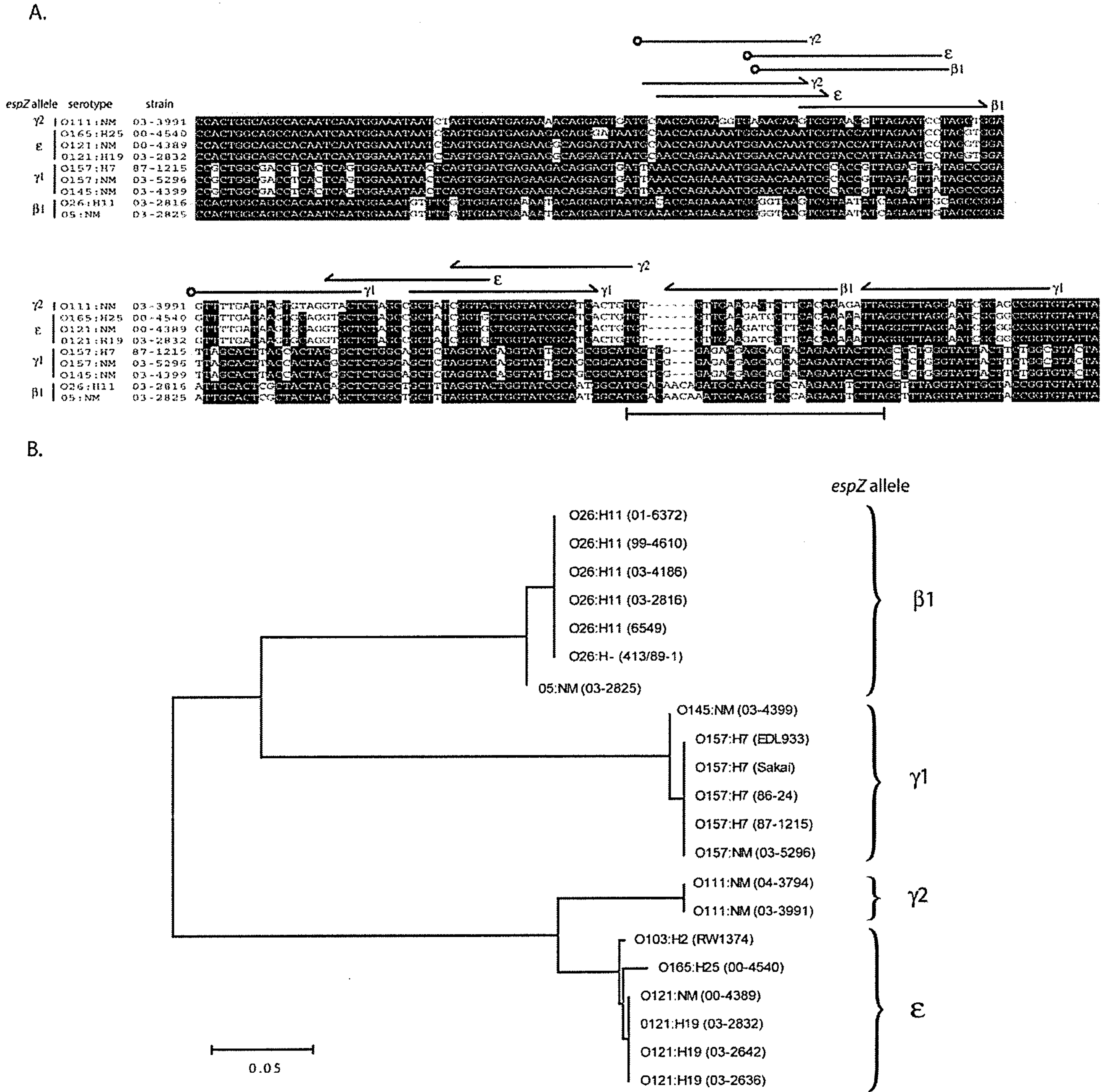


FIG. 1. Relatedness of *espZ* carried by STEC. (A) Multiple sequence alignment of the *espZ* central variable region, with LUX primers specific for individual *espZ* subtypes indicated by arrows (shown directly above the corresponding sequences; the arrowheads represent the 3′ end of the primers) and liquid microsphere suspension probes indicated by lines with spheres (the latter representing the 5′ end of the oligonucleotides where the microsphere is covalently attached). See Table 2 for the nucleotide sequence of these primers and probes. The double-bracketed line indicates the intervening loop-encoding region between the two surrounding predicted transmembrane domain-encoding regions (not indicated). Although sequences are broken into two sections asymmetrically, each of the *espZ* sequences is contiguous from the top to the bottom panel. The corresponding *espZ* allele subtype, serotype, and strain number for each sequence are indicated. (B) Phylogenetic distance between *espZ* encoded by different STEC serotypes represented by a neighbor-joining tree, and the scale bar indicates distance scores. Strain identification numbers are indicated in brackets. The *espZ* allele subtypes, corresponding to each cluster, are indicated by curly brackets. Nucleotide accession numbers are included in Materials and Methods and the text.

SDS, 50 mM Tris-HCl [pH 8.0], 4 mM EDTA, pH 8.0) was added and mixed by pipetting. Plates were incubated at 55°C for an additional 10 min. Flow cytometry with a QIAGEN LiquiChip workstation was used to quantify hybridization events, with the following settings: reading gates set at 8,300 to 16,500; minimum of 100 events read; and microplate handler heater block maintained at 55°C during measurements. Mean fluorescence intensity signals for each *espZ* target DNA sample and the negative control (TE blank) were averaged among the triplicate wells.

TABLE 3. Molecular characterization of Shiga toxin-producing *E. coli* by use of PCR and real-time PCR

Serotype	No. of isolates	*stx* genotype(s)	Intimin subtype	LUX real-time PCR[a]					
				espZ-γ1	*espZ*-β1	*espZ*-ε	*espZ*-γ2	stx_1	stx_2
O157:H7	3	stx_1, stx_2	γ1	+	−	−	−	+	+
	1	stx_1	γ1	+	−	−	−	+	−
	1	stx_2	γ1	+	−	−	−	−	+
O157:NM	1	stx_1, stx_2	γ1	+	−	−	−	+	+
	1	stx_2	γ1	+	−	−	−	−	+
O145:NM	1	stx_1	γ1	+	−	−	−	+	−
O26:H11	7	stx_1	β1	−	+	−	−	+	−
	1	stx_1, stx_2	β1	−	+	−	−	+	+
O121:H19	4	stx_2	ε	−	−	+	−	−	+
O103:H2	3	stx_1	ε	−	−	+	−	+	−
O111:NM	3	stx_1	γ2	−	−	−	+	+	−
	4	stx_1, stx_2	γ2	−	−	−	+	+	+
O121:NM	1	stx_2	ε	−	−	+	−	−	+
O165:H25	1	stx_2	ε	−	−	+	−	−	+
O5:NM	1	stx_1	β1	−	+	−	−	+	−
O91:H21	1	stx_2		−	−	−	−	−	+
O113:H21	1	stx_2		−	−	−	−	−	+

[a] +, exceeded fluorescent threshold of real-time PCR assay; −, below threshold.

Bioinformatics. Multiple sequence alignments were completed using ClustalW (www.ebi.ac.uk/clustalw/) and Boxshade (www.ch.embnet.org), neighbor-joining trees were constructed using MEGA3 (16), and genetic diversity statistics were calculated using DnaSP 4.10.3 (30). Pairwise global alignments were calculated using Align (www.ebi.ac.uk/emboss/align/#). Split decomposition analysis was performed using SplitsTree4 (12) and alignment inputs created by ClustalW, and calculations were performed using only parsimony-informative sites.

Nucleotide sequence accession numbers. The sequences determined in this study (see Results) have been deposited in GenBank under accession numbers DQ138070 to DQ138078 and DQ143180 to DQ143183.

RESULTS

Sequencing of the *espZ* hypervariable region. Molecular and phylogenetic characterization of the *espZ* locus was initiated by sequencing the central hypervariable region of this gene from seropathotype group A strains (O157:H7, O157:NM), group B strains (O26:H11, O121:H19, O111:NM, O145:NM) and group C strains which encode the LEE pathogenicity island (O5:NM, O121:NM, O165:NM). Oligonucleotides GIL245 and 246 (Table 2) were designed for the conserved C- and N-terminal encoding regions of *espZ*, observed in the sequence data from *espZ* (formerly *sepZ*) carried by various EPEC and EHEC strains (see Materials and Methods). These primers were able to produce an *espZ* product for each examined STEC serotype that was predicted to encode the LEE (Table 1), permitting sequencing of the intervening hypervariable region (Fig. 1A). The *espZ* PCR product was sequenced from four clinical strains of serotype O26:H11, three strains of serotype O121: H19, two strains of serotype O111:NM, and one strain for each of serotypes O5:NM, O145:NM, O157:NM, O157:NM, O121: NM, and O165:H25 (deposited in GenBank under accession numbers DQ138070 to DQ138078; strain numbers are indicated in Fig. 1B). Existing sequence data for serotypes O157:H7 and O26:H11 (see Materials and Methods) were identical to our data from Canadian strains; notably, the sequences of the amplified *espZ* product were identical for all strains of an individual serotype, but each examined serotype had a unique *espZ* allele (Fig. 1A), except for strains of serotype O121:H19 and O121:NM, in which the sequenced products were 100% identical.

Calculation of scores representing the distance between the *espZ* sequences for each STEC strain permitted construction of a phylogenetic tree (Fig. 1B), revealing four distinct clusters. Three clusters were constituted of multiple serotypes, and each of these clusters contained strains classified as representing different seropathotypes. The fourth STEC *espZ* cluster was constituted solely of O111:NM strains, and this group was most closely related to the O121-containing group and was 91% identical to O121:H19-encoded *espZ*. The overall pairwise sequence identity between *espZ* genes encoded by different STEC serotypes ranged from 67 to 100%. Sequence diversity existed in regions encoding both the predicted transmembrane domains as well as in the intervening loop region, in which the addition or deletion of up to two codons was observed (Fig. 1A). The genetic diversity at *espZ*, as calculated using data from 21 STEC strains identified in Fig. 1B, was $\pi = 0.23$, and this was similar to previous measurements calculated using data from six EPEC and EHEC strains (3). This diversity index was higher than that observed for any other LEE-encoded locus (3), and we calculated a synonymous mutation rate (dS) of 0.33 and a nonsynonymous rate (dN) of 0.18 using *espZ* sequence data from these 21 STEC strains. The resulting dN/dS ratio was <1, and therefore *espZ* would be classified as undergoing purifying selection, but the relatively high dN value is still suggestive of adaptive evolution at this locus.

Intimin typing. One of the prototypical virulence factors encoded in the LEE is intimin (*eae*), and significant sequence variation between STEC serotypes has been observed at this locus (40). PCR-based allelic discrimination was performed at the *eae* locus for each of the 35 STEC strains in our panel (Table 3), and these results correspond to the intimin subtypes previously observed in other strains of the same serotypes (2, 23, 32). Complete congruence between intimin and *espZ* subtypes was also previously observed (14), and this observation extends to our data set; therefore, we propose that the four

espZ lineages (Fig. 1A and 1B) should be named after the *eae* alleles they are coinherited with (β1, γ1, γ2, and ε).

Real-time PCR allelic discrimination of *espZ* subtypes. Sequencing of *espZ* indicated distinct sequence variation between serotypes, and the divergent regions characteristic of each proposed *espZ* lineage were targeted for development of real-time LUX PCR probes, a novel real-time system requiring only one self-quenching fluorescently labeled primer and one unlabeled primer (20). LUX primer pairs were designed for β1, γ1, γ2, and ε *espZ* alleles, and to provide additional strain characterization capabilities using the same platform, LUX primers for the Shiga toxin genes stx_1 and stx_2 were designed for conserved regions within each of these loci.

Real-time PCRs were performed with templates prepared from multiple clinical isolates for all LEE-positive serotypes classified in seropathotype group A, B, or C by use of each of the *espZ*-β1, -γ1, -γ2, and -ε LUX primer sets (Table 3). The specificity of each LUX primer set was 100% for each targeted serotype, based upon the known sequence data for other isolates of that same serotype. Notably, product was only detected with a single primer set for each examined template (Fig. 2A, showing data for O111:NM/*espZ*-γ2), and strains of the same serotype but of a different *stx* genotype had identical *espZ* alleles. STEC strains of serotype O91:H21 and O113:H21 that do not encode the LEE island were also tested with each *espZ* primer set (Table 3), and no products were detected, indicating that these LUX primers are only productive in the presence of *espZ*.

To determine the detection limits of the LUX real-time primer pairs, a dilution series of genomic DNA prepared from serotypes (O157:H7, O26:H11, O121:H19, and O111:NM) representing each of the four *espZ* allele subtypes was used as the template (Fig. 2B, showing data for O111:NM/*espZ*-γ2). The highest concentration of template examined was 100 ng/reaction, and a positive signal was produced for each sample. The lowest template concentrations that were productive (with fluorescence in excess of the threshold value) were 10 fg/reaction for O157:H7/*espZ*-γ1, 100 fg/reaction for O26:H11/*espZ*-β1, 100 pg/reaction for O121:H19/*espZ*-ε, and 10 pg/reaction for O111:NM/*espZ*-γ2; these lower detection limits correlated to $\sim2 \times 10^1$, 2×10^2, 2×10^4, and 2×10^3 genome copies per reaction, respectively. Therefore, the largest dynamic range was achieved with LUX primers specific for *espZ*-γ1, where 8 orders of magnitude in DNA concentration were detectable.

Detection of stx_1 and stx_2. To allow for the concurrent detection of *stx* genes in STEC strains by use of LUX real-time technology, primer sets were developed for each of stx_1 loci and the more variable stx_2 locus. Numerous molecular reagents have already been developed for real-time PCR detection of Shiga toxin-encoding genes and other STEC-associated virulence factors (11, 21, 29, 36), and our method provides additional platform-specific reagents. To assist in a comparison between different stx_2 alleles encoded by STEC required for primer design, the complete sequence of the stx_2 locus (encoding Stx2a and Stx2b subunits) was determined for the lone O26:H11 strain carrying stx_2 reported to the National Microbiology Laboratory (strain 02-6737), O111:NM strain 00-4748, and O121:H19 strains 03-2636 and 03-2642 (deposited in GenBank under accession numbers DQ143180 to DQ143183). These data identified a conserved region in the Stx2a subunit-encoding region that also matched LUX primer design parameters (data not shown), and all strains in our panel were examined for both stx_1 and stx_2 (Table 3). No discrepancies were observed between the LUX real-time results and the known *stx* genotypes and phenotypes of these strains. The sequence data for O26:H11, O121:H19, and O111:NM stx_2 loci were 99.3, 99.9, and 100% identical, respectively, to that of O157:H7 strain EDL933 carrying stx_2. Additionally, we subtyped the stx_2 loci encoded by the O91:H21, O113:H21, and O165:H25 isolates in our panel as stx_{2c} (data not shown), indicating that the stx_2-specific LUX primers are minimally capable of detecting the vh-a and c-type variants.

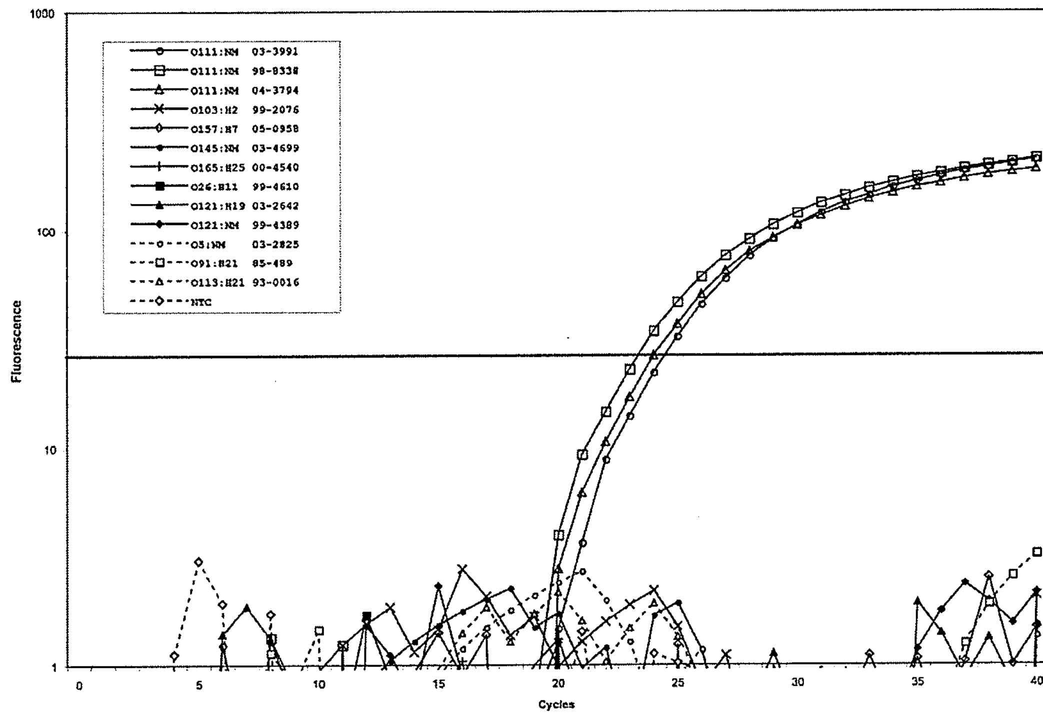
Liquid microsphere suspension array discrimination of *espZ* subtypes. Genotyping at bacterial loci can also be achieved using liquid microsphere suspension array technology, and the *espZ* gene meets many of the requirements for such a genotyping assay. This target is small (~300 bp) and contains a highly variable region (suitable for designing allele-specific probes) surrounded by highly conserved termini (suitable for designing universal primers for amplification of all alleles for that particular locus). Allelic discrimination of *espZ* was achieved for a representative strain of each of the four *espZ* lineages by addition of biotin-labeled, single-stranded *espZ* target DNA with four sets of differentially fluorescently coded microspheres covalently coupled with a probe specific for one of the four *espZ* alleles (Fig. 1A and Fig. 3). Single-stranded target DNA was selectively acquired after T7 exonuclease digestion of the unlabeled, exonuclease-sensitive strand in the GIL245/256-L *espZ* PCR product. After hybridizing individual targets with the full array of probe-coupled microspheres, the target was fluorescently labeled with streptavidin-R-phycoerythrin, and successful target-probe interactions were detected by Luminex flow cytometry. For each *espZ* target, only an interaction with the corresponding probe was detected.

DISCUSSION

To investigate genetic sequences that could potentially be used for molecular serotyping and characterization of STEC, we chose target loci common to the most frequently detected serotypes. The *espZ* gene is coinherited with other classical *E. coli* virulence determinants on the LEE, including sequences coding for intimin and effector proteins secreted by the type III secretion system. Our comparative DNA sequence analysis at the *espZ* locus revealed four distinct lineages among STEC strains, with heterogeneity observed between serotypes and conservation among strains of a single serotype. The observed sequence variation was developed into molecular tests that can accurately and rapidly identify toxin and *espZ* genotypes. These data also provide insight into the evolutionary history of the relationship between STEC serotypes and the LEE pathogenicity island.

Over 90% of STEC strains isolated in Canada are serotype O157:H7 or O157:NM (38), but it is estimated that between 20 and 50% of actual STEC infections result from non-O157 strains (17, 33). The bias towards O157 in clinical settings may be a result of the established methods for identification and the availability of selective media (39). To develop novel molecular methods specifically for non-O157 STEC, we had previously performed multilocus sequence typing on Canadian O26:H11

A.



B.

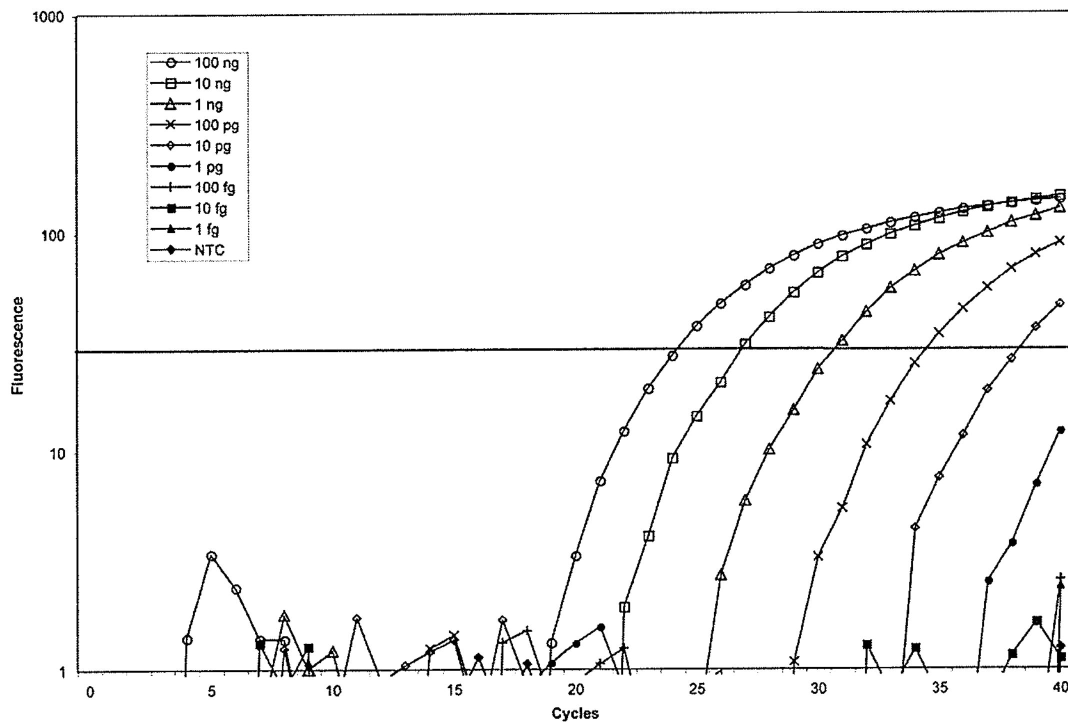


FIG. 2. Real-time LUX PCR using allele-specific *espZ* primers. (A) The *espZ*-$\gamma 2$ LUX primers were used on a panel of strains including three O111:NM isolates and a representative strain from other LEE-positive and -negative STEC (serotypes and respective strain numbers are indicated in the insets). (B) Detection limit testing on a dilution series of purified O111:NM genomic DNA. DNA amounts indicated in the legend represent the total amount of purified DNA present in the reaction tube. The horizontal line indicates the threshold value of 30 fluorescent units used to determine positive reactions. NTC, no template control.

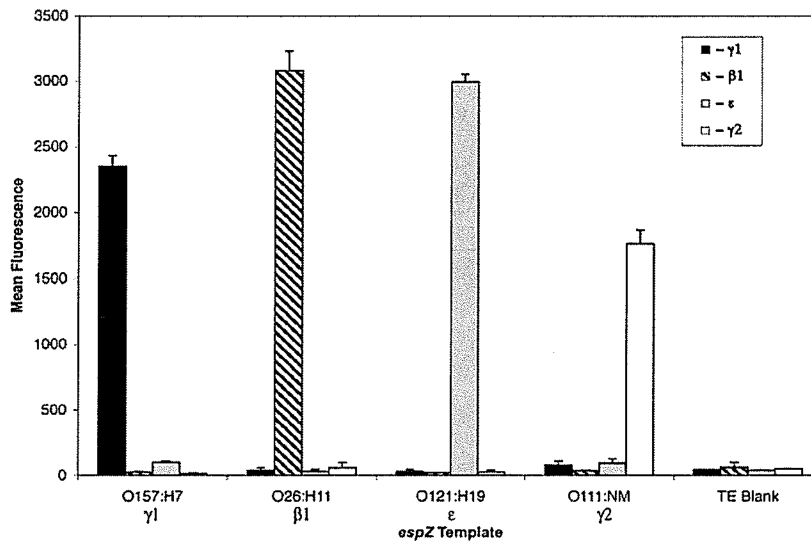


FIG. 3. Microsphere suspension array allelic discrimination of *espZ* encoded by different STEC serotypes. Biotin-labeled *espZ* template was amplified from strains of the indicated serotypes and incubated with a mixture of four fluorescently coded microspheres coupled with an oligonucleotide probe targeting the four *espZ* allele subtypes (see inset). The background fluorescence contributed by the microsphere-probe mixture was determined by using a no-template control (TE buffer), and standard errors are indicated by a vertical line on each bar.

STEC isolates but were unable to identify significant genetic diversity to subtype this collection of strains (10). Whereas the goal of multilocus sequence typing is to identify polymorphisms between related strains, other typing methodologies such as serotyping can identify more global characteristics that are indicative of corresponding genetic traits (i.e., serotype O157:H7 strains produce Shiga toxins). The real-time PCR primers and microsphere-coupled probes described here cumulatively allow for the detection and genotyping of toxin and pathogenicity islands by targeting stx_1, stx_2, and LEE subtype-specific alleles of *espZ*. These methods were not capable of molecular serotyping (e.g., *espZ*-β1 does not exclusively correlate to O26:H11 strains) and do not provide additional discrimination of STEC isolates compared to typing of the *eae* locus, but genotyping of toxin and pathogenicity islands can be used to infer different STEC lineages. Previously developed detection methods for O serotypes have been developed using targets from the O-antigen cluster genes *wzx* and *wzy* carried by EHEC O157, O103, O26, and O113 serotypes (5, 11, 26), and examination of additional loci, including the genes described here, or of genes unique to individual serotypes such as fimbria-encoding determinants (31, 34) may cumulatively provide a means for molecular serotyping.

To our knowledge, this study is the first example of allelic discrimination at a bacterial virulence locus determined using LUX primers. The design of traditional PCR primers which would amplify all alleles of *espZ* was facilitated by sequence conservation at regions adjacent to the start and stop codon. The first 20 codons of *espZ* are sufficient to direct EspZ translocation (14), and this is a possible explanation for why this 5′ segment is conserved among the different STEC serotypes. Alternatively, the sites within *espZ* selected for real-time LUX PCR and microsphere suspension array probe design occur principally in, or adjacent to, the segment encoding the first predicted transmembrane domain of EspZ (14). The intervening region between the two predicted transmembrane domains has been identified as the most divergent region of EspZ (14), and although significant nucleotide divergence exists in the region encoding the intervening loop between transmembrane domains, the probe design parameters for the microsphere suspension array and LUX technologies necessitated the design of primers and probes outside of this loop region (Fig. 1A) where subtle serotype-specific variations were available (e.g., O111:NM *espZ* versus O121:H19 *espZ*; Fig. 1A). The LUX real-time PCR technology offered quick resolution of *espZ* alleles (positive reactions within an hour after preparation of genomic template DNA); however, there are a greater number of *espZ* alleles than distinguishable fluorescent channels. Therefore, we did not multiplex this system to perform allele discrimination in a single reaction. Conversely, a single PCR using the *espZ* "universal" primer set was sufficient to initiate the microsphere-based allelic discrimination methodology, which was subsequently demultiplexed during flow cytometry of the probe-coupled microspheres. This latter method is therefore ideal for discrimination of loci that have a large number of alleles with definite sequence characteristics (for probe design), and additional targets could be incorporated into a single reaction mixture to provide additional typing capabilities.

The intimin-encoding gene *eae* was identified to exhibit a high degree of genetic diversity between A/E pathotypes of *E. coli* (3), and *eae* alleles are generally conserved between strains of the same STEC serotypes; therefore, this is also an appropriate target gene for molecular subtyping of *E. coli*. The diversity between *eae* alleles was calculated as $\pi = 0.14$ for six A/E pathogens (3) and $\pi = 0.14$ for the β1, γ1, γ2, and ε alleles of *eae* (data not shown). Molecular subtyping of *eae* utilizes the 3′ region encoding the Tir-binding domain (2, 40), and the diversity between the β1, γ1, γ2, and ε subtypes at this region was $\pi = 0.31$ (represented by 945 bp; data not shown). Al-

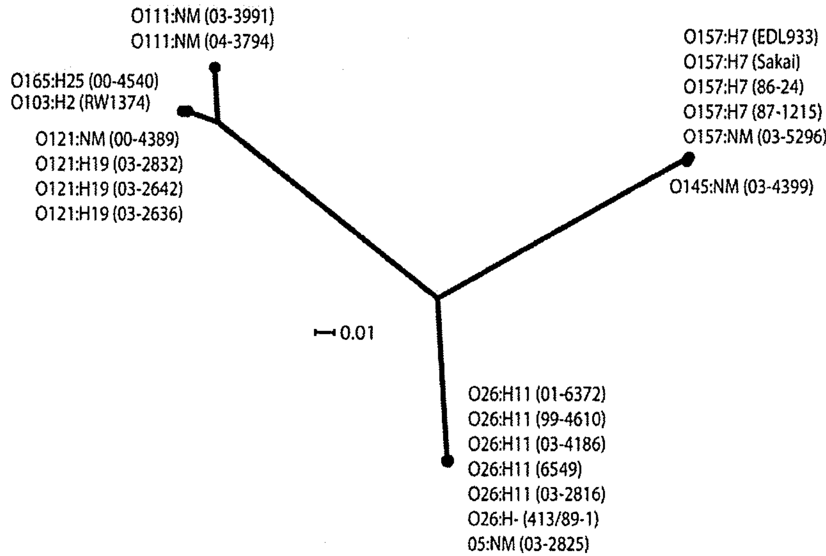


FIG. 4. Split decomposition analysis of *espZ*. Recombination between loci is indicated when the topology resembles a network rather than the single branching points seen in normal tree topologies. Strain identifications are indicated in brackets. Nucleotide accession numbers are included in Materials and Methods and the text.

though this region of *eae* is more diverse than *espZ* ($\pi = 0.23$), the *espZ* locus had ideal molecular characteristics for development of a liquid microsphere suspension assay, including conserved regions that surround the allele-specific sites (allowing universal amplification of all known *espZ* alleles and with a small fragment size suitable for this method). The allele-specific sites were also appropriate for LUX primer design. Furthermore, there was no evidence of recombination between *espZ* alleles (Fig. 4), whereas recombination was observed using split decomposition analysis for entire or partial coding sequences of *eae* subtypes (3, 40) or the 945-bp 3′ terminus of β1, γ1, γ2, and ε alleles (data not shown). The lack of observable recombination between *espZ* alleles is favorable for molecular typing, because the sites deemed characteristic for each allele are independently inherited in that lineage and are not recombined between unrelated lineages.

The LEE exhibits hallmark compositional traits that indicated that it was acquired through horizontal transfer (3), and our data suggest that it now appears to be stationary and is vertically transmitted by individual STEC lineages (i.e., serotypes). Congruence between STEC serotype and *espZ* allelic subtypes was observed, indicating that clonal dissemination of LEE variants (each categorized based upon *eae* and *espZ* allele carriage) occurred within individual serotypes. Between STEC strains of a single serotype, identical *espZ* alleles were observed, whereas between serotypes small to major variation occurred, ranging from 67 to 100% identity in pairwise sequence comparisons. The only observation of two STEC serotypes having identical *espZ* sequences was between O121:H19 and O121:NM strains. Furthermore, the congruence between serotype and *espZ* allelic variation does not support recent or frequent lateral transfer of the LEE pathogenicity island, and split decomposition analysis of STEC-encoded *espZ* did not indicate recombination between *espZ* alleles (Fig. 4). If the LEE is one of the founding genetic traits of STEC and if the *espZ* allele can be considered a marker for LEE evolution because it is highly polymorphic but not subject to recombination, then the lineage and serotype-specific variation observed at *espZ* could indicate the pattern of evolution for STEC serotypes, with a topology similar to that seen for *espZ* (Fig. 1B and 4). During the generation of three major lineages (β1, γ1, and ε/γ2) through point mutation in the central region of *espZ* and insertion or deletion of whole codons in the loop-encoding region, these precursor LEE variants may have segregated to the progenitors of the currently observed serotypes, wherein additional serotype-specific variation arose, albeit subtle in some instances (e.g., serotypes O157:H7, O157:NM, and O145:NM, each encoding *espZ*-γ1, are 99% identical to each other, and O121:H19 and O103:H2 strains are 98% identical at *espZ*-ε). Additionally, each STEC serotype also would have concurrently evolved with the LEE (7, 37) by the gain or loss of *stx* genes (18), plasmids, antigenic determinants, O islands (28), and other virulence determinants that contribute to the differential pathogenicity observed between serotypes.

ACKNOWLEDGMENTS

Our gratitude goes to L. Chui, A. Paccagnella, J. Wylie, K. Forward, and G. Horsman of the Provincial Health Laboratories in Alberta, British Columbia, Manitoba, Nova Scotia, and Saskatchewan, respectively, for providing strains. Members of the NML DNA Core Facility performed sequencing reactions and oligonucleotide synthesis, and members of the NML Serotyping, Identification, and Molecular Typing Programs conducted the initial strain characterization. We also thank V. Goleski for assistance with the Luminex assays, J. McCrea for assistance with LUX assays, and S. Tyler for assistance with stx_2 characterization.

This work was supported by Canadian Biotechnology Strategy Funds, administered by the Office of Biotechnology and Science, and Health Canada.

REFERENCES

1. **Bertin, Y., K. Boukhors, V. Livrelli, and C. Martin.** 2004. Localization of the insertion site and pathotype determination of the locus of enterocyte effacе-

ment of Shiga toxin-producing *Escherichia coli* strains. Appl. Environ. Microbiol. 70:61–68.

2. **Blanco, J. E., M. Blanco, M. P. Alonso, A. Mora, G. Dahbi, M. A. Coira, and J. Blanco.** 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from human patients: prevalence in Lugo, Spain, from 1992 through 1999. J. Clin. Microbiol. **42:**311–319.
3. **Castillo, A., L. E. Eguiarte, and V. Souza.** 2005. A genomic population genetics analysis of the pathogenic enterocyte effacement island in *Escherichia coli*: the search for the unit of selection. Proc. Natl. Acad. Sci. USA **102:**1542–1547.
4. **Cherla, R. P., S. Y. Lee, and V. L. Tesh.** 2003. Shiga toxins and apoptosis. FEMS Microbiol. Lett. **228:**159–166.
5. **DebRoy, C., E. Roberts, J. Kundrat, M. A. Davis, C. E. Briggs, and P. M. Fratamico.** 2004. Detection of *Escherichia coli* serogroups O26 and O113 by PCR amplification of the *wzx* and *wzy* genes. Appl. Environ. Microbiol. **70:**1830–1832.
6. **Donnenberg, M. S., and T. S. Whittam.** 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. J. Clin. Investig. **107:**539–548.
7. **Feng, P., K. A. Lampel, H. Karch, and T. S. Whittam.** 1998. Genotypic and phenotypic changes in the emergence of *Escherichia coli* O157:H7. J. Infect. Dis. **177:**1750–1753.
8. **Frankel, G., A. D. Phillips, I. Rosenshine, G. Dougan, J. B. Kaper, and S. Knutton.** 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. Mol. Microbiol. **30:**911–921.
9. **Gartner, J. F., and M. A. Schmidt.** 2004. Comparative analysis of locus of enterocyte effacement pathogenicity islands of atypical enteropathogenic *Escherichia coli*. Infect. Immun. **72:**6722–6728.
10. **Gilmour, M. W., T. D. Cote, J. Munro, L. Chui, J. Wylie, J. Isaac-Renton, G. Horsman, D. M. Tracz, A. Andrysiak, and L.-K. Ng.** 2005. Multilocus sequence typing of *Escherichia coli* O26:H11 isolates carrying *stx* in Canada does not identify genetic diversity. J. Clin. Microbiol. **43:**5319–5323.
11. **Hsu, C. F., T. Y. Tsai, and T. M. Pan.** 2005. Use of the duplex TaqMan PCR system for detection of Shiga-like toxin-producing *Escherichia coli* O157. J. Clin. Microbiol. **43:**2668–2673.
12. **Huson, D. H.** 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics **14:**68–73.
13. **Kaderali, L., A. Deshpande, J. P. Nolan, and P. S. White.** 2003. Primer-design for multiplexed genotyping. Nucleic Acids Res. **31:**1796–1802.
14. **Kanack, K. J., J. A. Crawford, I. Tatsuno, M. A. Karmali, and J. B. Kaper.** 2005. SepZ/EspZ is secreted and translocated into HeLa cells by the enteropathogenic *Escherichia coli* type III secretion system. Infect. Immun. **73:**4327–4337.
15. **Karmali, M. A., M. Mascarenhas, S. Shen, K. Ziebell, S. Johnson, R. Reid-Smith, J. Isaac-Renton, C. Clark, K. Rahn, and J. B. Kaper.** 2003. Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. J. Clin. Microbiol. **41:**4930–4940.
16. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5:**150–163.
17. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
18. **Mellmann, A., M. Bielaszewska, L. B. Zimmerhackl, R. Prager, D. Harmsen, H. Tschape, and H. Karch.** 2005. Enterohemorrhagic *Escherichia coli* in human infection: in vivo evolution of a bacterial pathogen. Clin. Infect. Dis. **41:**785–792.
19. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.
20. **Nazarenko, I., B. Lowe, M. Darfler, P. Ikonomi, D. Schuster, and A. Rashtchian.** 27 February 2002, revision date. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Res. **30:**e37. [Online.] http://nar.oxfordjournals.org/cgi/content/full/30/9/e37.
21. **Nielsen, E. M., and M. T. Andersen.** 2003. Detection and characterization of verocytotoxin-producing *Escherichia coli* by automated 5′ nuclease PCR assay. J. Clin. Microbiol. **41:**2884–2893.
22. **Nikiforov, T. T., R. B. Rendle, M. L. Kotewicz, and Y. H. Rogers.** 1994. The use of phosphorothioate primers and exonuclease hydrolysis for the preparation of single-stranded PCR products and their detection by solid-phase hybridization. PCR Methods Appl. **3:**285–291.
23. **Oswald, E., H. Schmidt, S. Morabito, H. Karch, O. Marches, and A. Caprioli.** 2000. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. Infect. Immun. **68:**64–71.
24. **Paton, A. W., and J. C. Paton.** 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx_1, stx_2, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfb_{O111}, and rfb_{O157}. J. Clin. Microbiol. **36:**598–602.
25. **Paton, J. C., and A. W. Paton.** 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. **11:**450–479.
26. **Perelle, S., F. Dilasser, J. Grout, and P. Fach.** 2005. Detection of *Escherichia coli* serogroup O103 by real-time polymerase chain reaction. J. Appl. Microbiol. **98:**1162–1168.
27. **Perna, N. T., G. F. Mayhew, G. Posfai, S. Elliott, M. S. Donnenberg, J. B. Kaper, and F. R. Blattner.** 1998. Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **66:**3810–3817.
28. **Perna, N. T., G. Plunkett III, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grotbeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamousis, J. Apodaca, T. S. Anantharaman, J. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner.** 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. Nature **409:**529–533.
29. **Reischl, U., M. T. Youssef, J. Kilwinski, N. Lehn, W. L. Zhang, H. Karch, and N. A. Strockbine.** 2002. Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. **40:**2555–2565.
30. **Rozas, J., J. C. Sanchez-DelBarrio, X. Messeguer, and R. Rozas.** 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics **19:**2496–2497.
31. **Shen, S., M. Mascarenhas, R. Morgan, K. Rahn, and M. A. Karmali.** 2005. Identification of four fimbria-encoding genomic islands that are highly specific for verocytotoxin-producing *Escherichia coli* serotype O157 strains. J. Clin. Microbiol. **43:**3840–3850.
32. **Tarr, C. L., T. M. Large, C. L. Moeller, D. W. Lacher, P. I. Tarr, D. W. Acheson, and T. S. Whittam.** 2002. Molecular characterization of a serotype O121:H19 clone, a distinct Shiga toxin-producing clone of pathogenic *Escherichia coli*. Infect. Immun. **70:**6853–6859.
33. **Thorpe, C. M.** 2004. Shiga toxin-producing *Escherichia coli* infection. Clin. Infect. Dis. **38:**1298–1303.
34. **Toma, C., E. Martinez Espinosa, T. Song, E. Miliwebsky, I. Chinen, S. Iyoda, M. Iwanaga, and M. Rivas.** 2004. Distribution of putative adhesins in different seropathotypes of Shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. **42:**4937–4946.
35. **Tyler, S. D., W. M. Johnson, H. Lior, G. Wang, and K. R. Rozee.** 1991. Identification of verotoxin type 2 variant B subunit genes in *Escherichia coli* by the polymerase chain reaction and restriction fragment length polymorphism analysis. J. Clin. Microbiol. **29:**1339–1343.
36. **Wang, G., C. G. Clark, and F. G. Rodgers.** 2002. Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 Shiga toxin family by multiplex PCR. J. Clin. Microbiol. **40:**3613–3619.
37. **Wick, L. M., W. Qi, D. W. Lacher, and T. S. Whittam.** 2005. Evolution of genomic content in the stepwise emergence of *Escherichia coli* O157:H7. J. Bacteriol. **187:**1783–1791.
38. **Woodward, D. L., C. G. Clark, R. A. Caldeira, R. Ahmed, and F. G. Rodgers.** 2002. Verotoxigenic *Escherichia coli* (VTEC): a major public health threat in Canada. Can. J. Infect. Dis. **13:**321–330.
39. **Zadik, P. M., P. A. Chapman, and C. A. Siddons.** 1993. Use of tellurite for the selection of verocytotoxigenic *Escherichia coli* O157. J. Med. Microbiol. **39:**155–158.
40. **Zhang, W. L., B. Kohler, E. Oswald, L. Beutin, H. Karch, S. Morabito, A. Caprioli, S. Suerbaum, and H. Schmidt.** 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. J. Clin. Microbiol. **40:**4486–4492.

Gilmour, M.W., Olson, A.B., Andrysiak, A., Ng, LK., Chui, L.. (2007) Sequence-based typing of genetic targets encoded outside of the O-antigen gene cluster is indicative of Shiga toxin-producing *Escherichia coli* serogroup lineages. Journal of Medical Microbiology. **56:** 1-9.

This article details a molecular serotyping method for Shiga-toxin producing *Escherichia coli*. A. Andrysiak contributed to the project by helping to extract DNA from samples, performing PCR on O117 *Escherichia coli* isolates, and analyzing O117 sequence data.

Journal of Medical Microbiology (2007), 56, 000–000 DOI 10.1099/jmm.0.47053-0

Sequence-based typing of genetic targets encoded outside of the O-antigen gene cluster is indicative of Shiga toxin-producing *Escherichia coli* serogroup lineages

Matthew W. Gilmour,[1,2] Adam B. Olson,[1] Ashleigh K. Andrysiak,[2] Lai-King Ng[1,2] and Linda Chui[3]

[1]National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington Street, Winnipeg, Manitoba R3E 3R2, Canada

[2]Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, Manitoba, Canada

[3]Alberta Provincial Laboratory for Public Health, Edmonton, Alberta, Canada

Correspondence
Matthew W. Gilmour
Matthew_Gilmour@phac-aspc.gc.ca

Serogroup classifications based upon the O-somatic antigen of Shiga toxin-producing *Escherichia coli* (STEC) provide significant epidemiological information on clinical isolates. Each O-antigen determinant is encoded by a unique cluster of genes present between the *gnd* and *galF* chromosomal genes. Alternatively, serogroup-specific polymorphisms might be encoded in loci that are encoded outside of the O-antigen gene cluster. Segments of the core bacterial loci *mdh*, *gnd*, *gcl*, *ppk*, *metA*, *ftsZ*, *relA* and *metG* for 30 O26 STEC strains have previously been sequenced, and comparative analyses to O157 distinguished these two serogroups. To screen these loci for serogroup-specific traits within a broader range of clinically significant serogroups, DNA sequences were obtained for 19 strains of 10 additional STEC serogroups. Unique alleles were observed at the *gnd* locus for each examined STEC serogroup, and this correlation persisted when comparative analyses were extended to 144 *gnd* sequences from 26 O-serogroups (comprised of 42 O : H-serotypes). These included O157, O121, O103, O26, O5 : non-motile (NM), O145 : NM, O113 : H21, O111 : NM and O117 : H7 STEC; and furthermore, non-toxin encoding O157, O26, O55, O6 and O117 strains encoded distinct *gnd* alleles compared to STEC strains of the same serogroup. DNA sequencing of a 643 bp region of *gnd* was, therefore, sufficient to minimally determine the O-antigen of STEC through molecular means, and the location of *gnd* next to the O-antigen gene cluster offered additional support for the co-inheritance of these determinants. The *gnd* DNA sequence-based serogrouping method could improve the typing capabilities for STEC in clinical laboratories, and was used successfully to characterize O121 : H19, O26 : H11 and O177 : NM clinical isolates prior to serological confirmation during outbreak investigations.

Received 7 November 2006
Accepted 28 January 2007

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are bacterial pathogens that result in both outbreak and sporadic occurrences of human mortality and disease. Symptoms can include bloody and non-bloody diarrhoea, and children are susceptible to renal failure due to haemolytic uraemic syndrome. STEC are transmitted to humans by consumption of contaminated food or water, person-to-person contact or animal-to-person contact, where natural reservoirs include cattle, pigs and sheep (Karch *et al.*, 2005). Serogroup classifications based upon the O-somatic or H-flagellar antigens of STEC provide significant epidemiological information on clinical isolates, and this measure can provide the first indication of relatedness between strains during outbreak investigations. The serogroup is also indicative of the overall genetic relatedness between *E. coli* strains, including virulence gene content, such as the locus for the enterocyte effacement (*LEE*) pathogenicity island, and the *stx1* and *stx2* loci encoding Shiga toxins (Prager *et al.*, 2005; Girardeau *et al.*, 2005; Karmali *et al.*, 2003).

Abbreviations: NM, non-motile; STEC, Shiga toxin-producing *Escherichia coli.*

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this paper are DQ472524–DQ472651.

47053 © 2007 Crown Copyright *Printed in Great Britain*

The predominant O-serogroup of STEC that is observed clinically in North America is O157 (Johnson *et al.*, 2006); however, biased sampling likely results from the availability of clinical media and detection reagents that target this serogroup. Directed studies for the isolation and characterization of both O157 and non-O157 STEC from clinical samples have indicated that the proportion of non-O157 in North America is likely higher than clinical records have indicated (Thompson *et al.*, 2005; Jelacic *et al.*, 2003; Fey *et al.*, 2000). In Canada, over 90 % of STEC strains detected are serotype O157 : H7 or O157 : non-motile (NM) (Woodward *et al.*, 2002). The global prevalence of non-O157 includes significant outbreaks of O26, O121, O103, O111 and O145, and in some countries it is recognized that these serogroups exceed the prevalence of O157 STEC (Karch *et al.*, 2005). Furthermore, non-O157 strains have been identified along with O157 strains in clinical samples (Paton *et al.*, 1996), so it is possible that a diagnostic bias towards O157 may prevent the detection of the aetiological STEC serogroup during human illness.

Molecular methods for the characterization and identification of O-antigen determinants have been devised using restriction profiling and allele-specific PCR. The entire O-antigen-encoding gene cluster could be amplified using primers that targeted conserved regions in the neighbouring *gnd* sequence (encoding 6-phosphogluconate dehydrogenase) and JUMPstart sequences, and enzymic digestion of this amplicon identified RFLPs correlating to O-antigen determinants (Coimbra *et al.*, 2000). This method was problematic due to the length of the amplicon (upwards of 20 kbp) and the absence of unique restriction profiles for all serotypes. Within the O-antigen gene cluster the *wzx* and *wzy* loci encode the O-antigen flippase and polymerase, respectively, and distinct alleles corresponding to each O-serogroup have been used for molecular serogrouping of O103, O157, O26, O113 and O111 strains (Perelle *et al.*, 2005; DebRoy *et al.*, 2004; Paton & Paton, 1999a; Fratamico *et al.*, 2005; D'Souza *et al.*, 2002). It has been suggested that these assays could replace traditional serological methods (DebRoy *et al.*, 2005); however, the individual tests currently detect only one to three O-serogroups. In the absence of a priori knowledge of a serogroup, a large number of reagents may be required to confirm serogroup identity with these methods. Robust platforms such as DNA microarrays containing *wzx* and *wzy* probes targeting up to four *E. coli* serogroups are currently being investigated (Liu & Fratamico, 2006), and broad subtyping of STEC has been achieved using allelic variants of a *LEE*-encoded determinant (Gilmour *et al.*, 2006).

Multilocus sequence typing has been attempted for each of the STEC serotypes O26 : H11, O121 : H19, O103 : H2 or O157 : H7, but this method was not appropriate for subtyping because very few polymorphisms were observed between strains of the same serotype (Gilmour *et al.*, 2005; Tarr *et al.*, 2002; Noller *et al.*, 2003; Beutin *et al.*, 2005). The genetic differentiation and subtyping of *E. coli* serotype O26 : H11 was attempted by sequencing 10 loci for 30 strains encoding *stx1*, or both *stx1* and *stx2* (Gilmour *et al.*, 2005). Amongst the O26 : H11 strains all loci were identical, with the exception of three alleles of *mdh* and two alleles of *ppk* that each differed by a single point mutation. Notably, comparative analyses of the *mdh*, *gnd*, *gcl*, *ppk*, *metA*, *ftsZ*, *relA* and *metG* alleles encoded by O26 : H11 STEC cumulatively distinguished this serotype from O157 : H7 (Gilmour *et al.*, 2005). The conservation of these loci between O26 : H11 strains, and the genetic distance from the other *E. coli* serotypes suggested that sequence-based typing of additional STEC might reveal serotype-specific alleles. In this study, additional DNA sequence data at these loci was obtained for a range of STEC and a single locus was observed to encode allelic variants correlating to individual STEC O-serogroups. We therefore present a simple molecular method for the identification of STEC serogroups, including both O157 and non-O157 strains.

METHODS

Bacterial strains. STEC strains (Table 1) were obtained from the reference stocks of the Enteric Diseases Programme at the National Microbiology Laboratory that originated from human sources at various Canadian provincial health laboratories during 1985–2005, or were recent clinical isolates obtained from the Alberta Provincial Laboratory for Public Health (nomenclature XX-YYYY, where XX generally refers to the year of isolation). During the course of these studies, five outbreak-associated STEC isolates were provided by Nova Scotia Public Health, Halifax, Nova Scotia, Canada. Confirmation of O : H serotype was completed with antisera prepared at the National Microbiology Laboratory (Ewing, 1986).

PCR and sequencing. Template DNA was prepared by centrifuging 1 ml exponential phase culture grown in brain heart infusion broth, resuspending the pellet in 1 ml TE buffer (Sigma; 10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and boiling the cells for 15 min. Boiled cells were pelleted, and the supernatant was removed and used as the DNA template in PCR.

Oligonucleotide primers used to amplify segments of *mdh*, *gnd*, *gcl*, *ppk*, *metA*, *ftsZ*, *relA* and *metG* are presented in Table 2. PCR was performed with high fidelity Platinum *Taq* (Invitrogen), following the manufacturer's directions. The thermocycling parameters for *ftsZ*, *relA* and *metG* included an initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C for 45 s and extension at 68 °C for 45 s, with a final extension at 68 °C for 5 min. The annealing temperature for *metA*, *mdh*, *gcl* and *ppk* was 58 °C, and 52 °C for *gnd*. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced using the same primers that generated these amplicons. Sequencing was performed on an ABI3730 (Applied Biosystems) and the data were deposited in GenBank with accession nos DQ472524–DQ472651. Existing genomic sequence data for *E. coli* O157 : H7 EDL933, O157 : H7 Sakai, O6 : H1 CFT073 and K-12 (GenBank accession nos NC_000913, BA000007, NC_002655, NC_004431) was included in our dataset for each of the above loci. From directed studies against the *gnd* locus (Tarr *et al.*, 2000; Paton & Paton, 1999b; Wang *et al.*, 1998), we included sequence data from O157 : H7 and O157 : NM (GenBank accession nos AF176359, AF176358, AF176357, AF176356, AF176360, AF176361 and AB008676), O113 : H2 (AF172324), O111 (AF078736) and non-toxin encoding O157 and O55 (AF176368, AF176367, AF176366, AF176363, AF176362, AF176369 and AF176373). Our

Table 1. Bacterial strains used in this study

Strains characterized during outbreak investigations are identified (O).

Seropathotype*	Serotype	Strain ID	Source†	Sequencing scheme‡	*stx1*	*stx2*	*LEE*§	Reference
A	O157:H7	87-1215	NML	8 loci	+	+	+	Gilmour *et al.* (2006)
	O157:H7	01-8110	NML	4 loci	+	+	+	Gilmour *et al.* (2006)
	O157:H7	05-0958	SK HPL	8 loci	−	+	+	Gilmour *et al.* (2006)
	O157:H7	04-4319	SK HPL	4 loci	+	−	+	Gilmour *et al.* (2006)
	O157:H7	03-2641	AB PLPH	4 loci	+	+	+	Gilmour *et al.* (2006)
	O157:NM	01-6434	AB PLPH	8 loci	+	−	+	Gilmour *et al.* (2006)
	O157:NM	03-3088	AB PLPH	4 loci	+	+	+	This study
	O157:NM	03-5296	AB PLPH	8 loci	+	+	+	Gilmour *et al.* (2006)
B	O26:H11	01-6372	NS PHL	8 loci	+	−	+	Gilmour *et al.* (2005)
	O26:H11	03-2816	AB PLPH	8 loci	+	−	+	Gilmour *et al.* (2005)
	O26:H11	05-6544	NS PHL (O)	*gnd*	+	−	+	This study
	O103:H2	99-2076	BCCDC	8 loci	+	−	+	Gilmour *et al.* (2006)
	O103:H2	04-2446	MB CPL	8 loci	+	−	+	Gilmour *et al.* (2006)
	O103:H2	01-6102	SK HPL	8 loci	+	−	+	Gilmour *et al.* (2006)
	O103:H2	03-3967	AB PLPH	4 loci	+	−	+	This study
	O103:H11	04-3973	MB CPL	*gnd*	+	−	+	Thompson *et al.* (2005)
	O103:H11	06-4464	MB CPL	*gnd*	+	−	+	This study
	O103:H25	03-1028	MB CPL	*gnd*	+	−	+	Thompson *et al.* (2005)
	O103:H25	03-1030	MB CPL	*gnd*	+	−	+	Thompson *et al.* (2005)
	O103:H25	04-3972	MB CPL	*gnd*	+	−	+	Thompson *et al.* (2005)
	O103:H25	03-2444	MB CPL	*gnd*	+	−	+	Thompson *et al.* (2005)
	O111:NM	03-3991	AB PLPH	4 loci	+	−	+	Gilmour *et al.* (2006)
	O111:NM	04-3794	MB CPL	8 loci	+	+	+	Gilmour *et al.* (2006)
	O111:NM	98-8338	BCCDC	4 loci	+	−	+	Gilmour *et al.* (2006)
	O111:NM	00-4748	SK HPL	8 loci	+	+	+	Gilmour *et al.* (2006)
	O111:NM	00-4440	BCCDC	4 loci	+	−	+	Gilmour *et al.* (2006)
	O111:NM	01-0252	BCCDC	8 loci	+	+	+	Gilmour *et al.* (2006)
	O111:NM	01-1215	BCCDC	8 loci	+	−	+	Gilmour *et al.* (2006)
	O121:H19	03-2636	AB PLPH	4 loci	−	+	+	Gilmour *et al.* (2006)
	O121:H19	03-2642	AB PLPH	*gnd*	−	+	+	Gilmour *et al.* (2006)
	O121:H19	03-2832	AB PLPH	8 loci	−	+	+	Gilmour *et al.* (2006)
	O121:H19	05-6541	NS PHL (O)	*gnd*	−	+	+	This study
	O121:H19	05-6542	NS PHL (O)	*gnd*	−	+	+	This study
	O121:H19	05-6543	NS PHL (O)	*gnd*	−	+	+	This study
	O121:H19	00-5288	BCCDC	8 loci	−	+	+	Gilmour *et al.* (2006)
	O145:NM	03-4699	AB PLPH	8 loci	+	−	+	Gilmour *et al.* (2006)
	O145:NM	04-7099	MB CPL	*gnd*	+	−	+	This study
	O145:NM	04-7194	MB CPL	*gnd*	+	−	+	This study
	O145:NM	04-1449	MB CPL	*gnd*	+	−	+	This study
	O145:NM	03-6430	MB CPL	*gnd*	+	−	+	Thompson *et al.* (2005)
	O145:NM	02-5149	BCCDC	*gnd*	+	−	+	This study
C	O5:NM	03-2825	AB PLPH	8 loci	+	−	+	Gilmour *et al.* (2006)
	O5:NM	03-2682	MB CPL	*gnd*	+	−	+	Thompson *et al.* (2005)
	O91:H21	85-489	NML	8 loci	−	+	−	Gilmour *et al.* (2006)
	O113:H21	93-0016	NML	8 loci	−	+	−	Gilmour *et al.* (2006)
	O113:H21	04-1450	MB CPL	*gnd*	−	+	−	Thompson *et al.* (2005)
	O121:NM	99-4389	NML	8 loci	−	+	+	Gilmour *et al.* (2006)
	O121:NM	03-4064	AB PLPH	4 loci	−	+	+	This study
	O165:H25	00-4540	BCCDC	8 loci	−	+	+	Gilmour *et al.* (2006)
D	O6:H34	03-5166	MB CPL	*gnd*	−	+	−	Thompson *et al.* (2005)
	O45:H2	05-6545	NS PHL	*gnd*	+	−	+	This study
	O45:H2	04-2445	MB CPL	*gnd*	+	−	+	Thompson *et al.* (2005)
	O55:H7	05-0376	NML	*gnd*	+	−	+	This study
	O85:H1	03-3638	AB PLPH	4 loci	−	+	−	This study

Table 1. cont.

Seropathotype*	Serotype	Strain ID	Source†	Sequencing scheme‡	*stx1*	*stx2*	*LEE*§	Reference
	O115:H18	03-3645	AB PLPH	4 loci	+	+	−	This study
	O117:H7	05-0379	NML	*gnd*	+	−	−	This study
	O117:H7	02-0035	BCCDC	*gnd*	+	−	−	This study
	O117:H7	02-4495	BCCDC	*gnd*	+	+	−	This study
	O146:H21	02-7808	BCCDC	*gnd*	+	−	−	This study
	O146:H21	02-1628	BCCDC	*gnd*	+	−	−	This study
	O177:NM	03-3974	AB PLPH	4 loci	−	+	+	This study
	O177:NM	06-5121	NS PHL (O)	*gnd*	−	+	+	This study
NA	O1:H7	03-3964	AB PLPH	4 loci	−	−	−	This study
	O2:H4	03-2815	AB PLPH	4 loci	−	−	−	This study
	O4:H5	03-3266	AB PLPH	4 loci	−	−	−	This study
	O6:H1	03-2638	AB PLPH	4 loci	−	−	−	This study
	O8:H19	03-2639	AB PLPH	4 loci	−	−	−	This study
	O25:H1	03-2637	AB PLPH	4 loci	−	−	−	This study
	O26:H6	01-5872	MB CPL	8 loci	−	−	−	Gilmour *et al.* (2005)
	O26:H32	99-4328	SK HPL	8 loci	−	−	−	Gilmour *et al.* (2005)
	O51:NM	04-2640	MB CPL	*gnd*	−	−	−	This study
	O91:H10	03-3269	AB PLPH	4 loci	−	−	−	This study
	O98:NM	02-7464	NB PHL	*gnd*	−	−	−	This study
	O117:H25	02-0714	NB PHL	*gnd*	−	−	−	This study

*NA, Not applicable. Strains that do encode *stx* are not classified in the seropathotype scheme (Karmali *et al.*, 2003).
†AB PLPH, Alberta Provincial Laboratory for Public Health; BCCDC, British Columbia Centre for Disease Control; MB CPL, Manitoba Cadham Provincial Laboratory; NML, National Microbiology Laboratory standard strain; NB PHL, New Brunswick Public Health Laboratory; NS PHL, Nova Scotia Public Health Laboratory; SK HPL, Saskatchewan Health Provincial Laboratory.
‡DNA sequencing was performed for 8 loci (*mdh, gnd, gcl, ppk, metA, ftsZ, relA* and *metG*), 4 loci (*gnd, gcl, ppk* and *relA*) or solely the *gnd* locus.
§As determined by PCR screening for the *espZ* gene (Gilmour *et al.*, 2006).

previously acquired sequence data from O26:H11, O26:H6 and O26:H32 strains were also included (GenBank accession nos AY973395–AY973421; Gilmour *et al.*, 2005).

Bioinformatics. Multiple sequence alignments were completed using ClustalW (www.ebi.ac.uk/clustalw/), neighbour-joining trees were constructed with Hasegawa–Kishino–Yano (HKY85) distance correction using SplitsTree4 (Huson, 1998), and genetic diversity statistics were calculated using DnaSP 4.10.3 (Rozas *et al.*, 2003). Pairwise global alignments were calculated using Align (www.ebi.ac.uk/emboss/align/#).

RESULTS AND DISCUSSION

Sequence typing correlates to O-antigen serogroups

The alleles of *mdh, gnd, gcl, ppk, metA, ftsZ, relA* and *metG* encoded by O26:H11 STEC cumulatively distinguished this serotype from O157:H7 (Gilmour *et al.*, 2005), and the corresponding segments of these loci were sequenced for STEC serotypes O111:NM, O113:H21, O157:NM, O145:NM, O91:H21, O121:H19, O121:NM, O103:H2, O165:H25 and O5:NM. This panel of STEC strains included isolates from each of the most predominant O-serogroups and O:H-serotypes observed in Canada (Gilmour *et al.*, 2005, 2006), and amongst individual serotypes, strains with different *stx* genotypes were included when available (Table 1). This sequence dataset was compared to previously published sequence data for STEC serotypes O157:H7 and O26:H11, as well as non-toxin producing O26:H32, O26:H6, K12 and O6:H1 (strain CFT073) strains using the 4464 nucleotide concatenate of the eight genetic determinants (Fig. 1). Each of the examined serogroups had distinct sequence types, including NM STEC strains of O121 and O157, were 99.8 and 99.9 % identical to O121:H19 and O157:H7 strains, respectively. The observed phylogenetic separation between serogroups, and homogeneity within strains of the same serogroup, indicated that these genetic traits have been acquired by and vertically inherited within individual STEC serogroup lineages.

Molecular-based serogrouping with four loci

Additional sequencing was performed at selected loci in an expanded panel of strains to determine if the phylogenetic separation observed between serogroups was maintained in a larger dataset (Table 1). The genetic determinants that contributed the majority of the observed genetic diversity (*gnd* and *gcl*; Table 3) or encoded putative serogroup-specific regions (*ppk* and *relA*; data not shown) were selected for further study. This panel included further

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Target	Sequence (5′ to 3′)	Product size (bp)	Reference
GIL213	*ftsZ*	GATCACTGAACTGTCCAAGCATG	450	Gilmour *et al.* (2005)
GIL214	*ftsZ*	TCAAGAGAAGTACCGATAACCAC		
gcl-F	*gcl*	GCGTTCTGGTCGTCCGGGTCC	758	Adiri *et al.* (2003)
gcl-R	*gcl*	GCCGCAGCGATTTGTGACAGACC		
gnd-F	*gnd*	GGCTTTAACTTCATCGGTAC	712	Noller *et al.* (2003)
gnd-R	*gnd*	TCGCCGTAGTTCAGATCCCA		
mdh-F	*mdh*	CAACTGCCTTCAGGTTCAGAA	580	Noller *et al.* (2003)
mdh-R	*mdh*	GCGTTCTGGATGCGTTTGGT		
metA-F	*metA*	CGCAACACGCCCGCAGAGC	601	Adiri *et al.* (2003)
metA-R	*metA*	GCCAGCTCGCTCGCGGTGTATT		
GIL219	*metG*	TGGCTGACCCGCAGTTGTAC	503	Gilmour *et al.* (2005)
GIL220	*metG*	GGTCAACTTTGGCGAAGTCGTC		
ppk-F	*ppk*	TGCCGCGCTTTGTGAATTTACCG	758	Adiri *et al.* (2003)
ppk-R	*ppk*	CCCCGGCGCAGAGAAGATAACGT		
GIL215	*relA*	TCTGTTTCCTCCGAACAGGTCG	470	Gilmour *et al.* (2005)
GIL216	*relA*	ACAATACGTACCGCACGCACATC		

strains from the serotypes represented in Fig. 1, as well as seropathotype D and non-toxin encoding *E. coli* strains recovered from paediatric stool samples (L. Chui, unpublished data). The overall genetic distinction between STEC serogroups (as determined in the eight locus scheme) was also represented amongst these four loci, and the additional strains and serogroups (Fig. 2).

Molecular-based serogrouping with the *gnd* locus

The *gnd* locus was the most genetically diverse of all examined loci (Table 3), and notably, this determinant is immediately adjacent to the O-antigen gene cluster. Additional sequencing of the 643 bp region of *gnd* was performed (Table 1), and *gnd* sequence data available in GenBank for O157, O113 and O111 STEC, as well as non-toxin encoding O157 and O55 strains, was also included in comparative analyses. In total, *gnd* DNA sequences were collected from 144 strains and 26 O-serogroups (comprised of 42 O:H-serotypes). The overall genetic distinction between serogroups (as determined in the eight and four loci schemes) was also represented in this single locus, as each examined STEC O-serogroup encoded a unique *gnd* allele (Fig. 3). For some of the most clinically significant STEC serogroups (O157, O26, O121, O145, O111 and O103) the *gnd* DNA sequences were compared between multiples strains (from 5 to 43 sequences), and for each serogroup all STEC strains encoded an identical *gnd* allele (Fig. 3). The only exception was O157:H7 strain 87-16 (GenBank accession no. AF176360), which encoded a

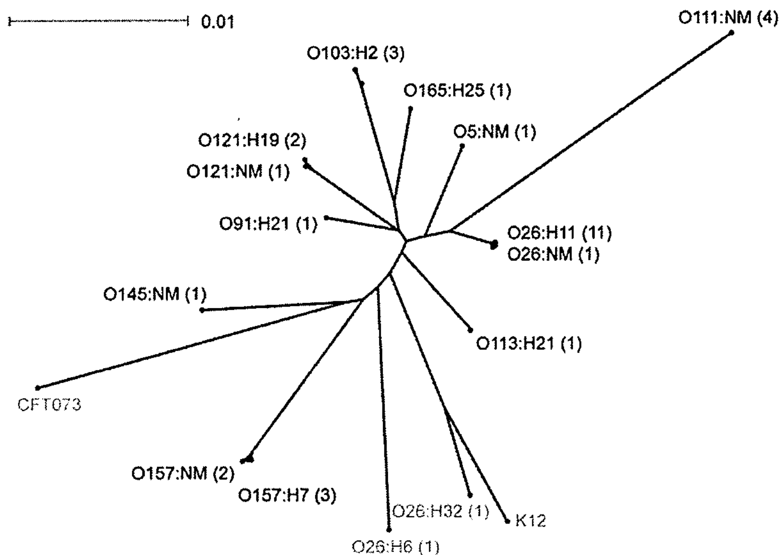


Fig. 1. Phylogeny of the concatenated segments of *mdh*, *gnd*, *gcl*, *ppk*, *metA*, *ftsZ*, *relA* and *metG* encoded by *E. coli*. This is based upon a neighbour-joining tree constructed with Hasegawa–Kishino–Yano (HKY85) distance correction. Sequences obtained from GenBank are identified in Methods. The serotype of strain K-12 was not designated, and the serotype of uropathogenic strain CFT073 was O6 : K2 : H1. Shiga toxin-producing serotypes are indicated in black type, and strains not encoding *stx* are indicated in grey. The number of sequences per serotype is indicated in parentheses. Bar, scale of the distance score.

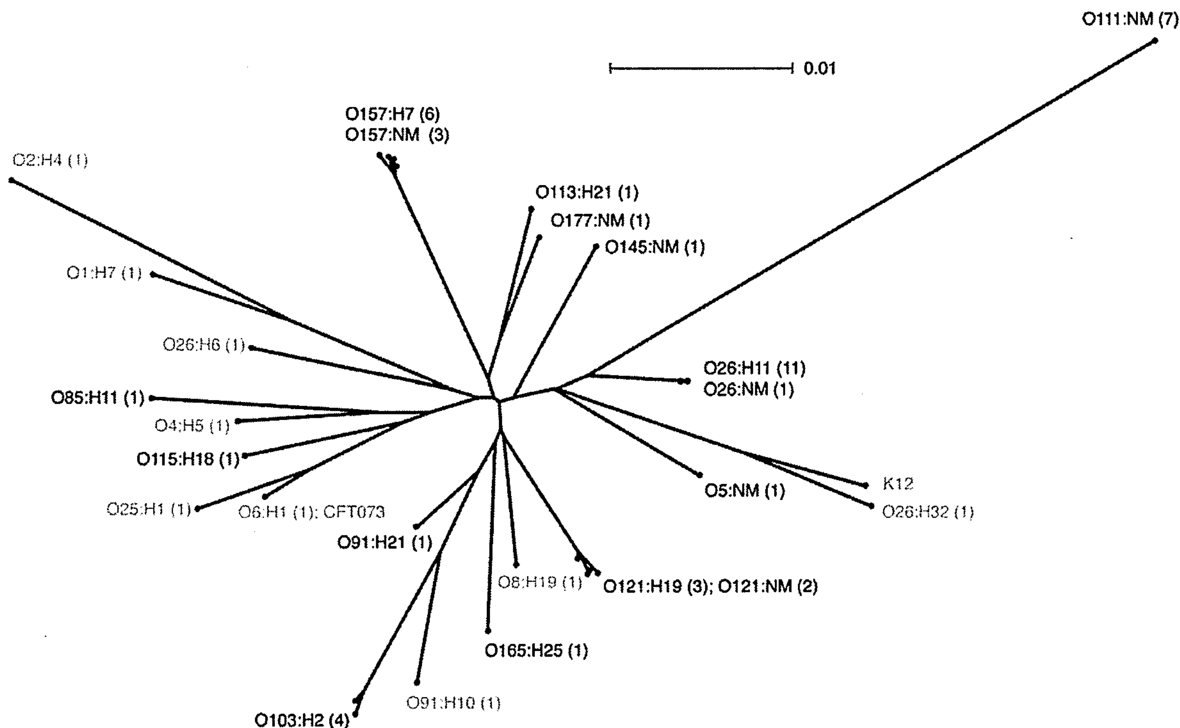


Fig. 2. Phylogeny of the concatenated segments of *gnd*, *gcl*, *ppk* and *relA* encoded by *E. coli*. This is based upon a neighbour-joining tree constructed with Hasegawa–Kishino–Yano (HKY85) distance correction. Sequences obtained from GenBank are identified in Methods. Shiga toxin-producing serotypes are indicated in black type, and strains not encoding *stx* are indicated in grey. The number of sequences per serotype is indicated in parentheses. Bar, scale of the distance score.

single nucleotide polymorphism compared to the other O157 strains, but otherwise the *gnd* alleles were conserved within STEC serogroup classifications. Furthermore, non-toxin encoding strains of O157, O26, O55, O6 and O117 encoded distinct *gnd* alleles compared to STEC strains of the same serogroup. Sequence typing of *gnd* was, therefore, a promising molecular method correlating minimally with the O-serogroup of clinical STEC strains. The O111 : NM STEC and non-toxin-producing O55 strains encoded *gnd* sequences outlying from the main cluster (Fig. 3) and these were homologous to *Citrobacter* spp. *gnd* alleles (Nelson & Selander, 1994). However, since pure bacterial isolates are preferred for preparation of DNA sequencing template, all isolates undergoing *gnd* DNA sequence-based serogrouping should previously be classified as STEC.

During the course of this study, outbreak-related isolates of non-O157 STEC were sent to the National Microbiology Laboratory for serotyping and genetic characterization. The *gnd* sequence data for each of isolates 05-6541 to 05-6543 clustered with known O121 strains (Fig. 3). A concurrent non-O157 sporadic isolate (05-6544) was also examined at *gnd* and this sequence clustered with known O26 : H11 strains (Fig. 3). Strain 06-5121 was isolated from a hospitalized patient with haemolytic uraemic syndrome and the *gnd* sequence of this strain was 99.8 % identical to a known O177 : NM isolate (Fig. 3). In correlation with these molecular data, subsequent serotyping using traditional methodologies characterized these isolates as O121 : H19, O26 : H11 and O177 : NM. The *gnd* DNA sequence-based serogrouping method therefore provided an advantageous alternative to O-specific immunoreagents during these crises. Over 55 serogroups of STEC have been reported to be associated with human disease (Johnson *et al.*, 2006), and an international panel of STEC strains from each serogroup, including the emerging sorbitol-fermenting O157, will be required to further validate this method.

The proportion of synonymous and nonsynonymous mutations were calculated for each locus from the accumulated DNA sequence data (Table 3). As expected for core loci, the majority of mutations were synonymous (dN/dS <1), but the *gnd* locus had the greatest number of nonsynonymous sites. This locus has already been identified as a polymorphic *E. coli* locus compared to other core loci (Bisercic *et al.*, 1991; Nelson & Selander, 1994; Dykhuizen & Green, 1991). A comparable ratio of synonymous versus nonsynonymous mutations was also reported by Bisercic *et al.* (1991). Genetic diversity at *gnd* arose in parallel to the extensive diversity and recombination that occurred at the neighbouring O-antigen gene cluster, and it is likely that these two genetic traits were

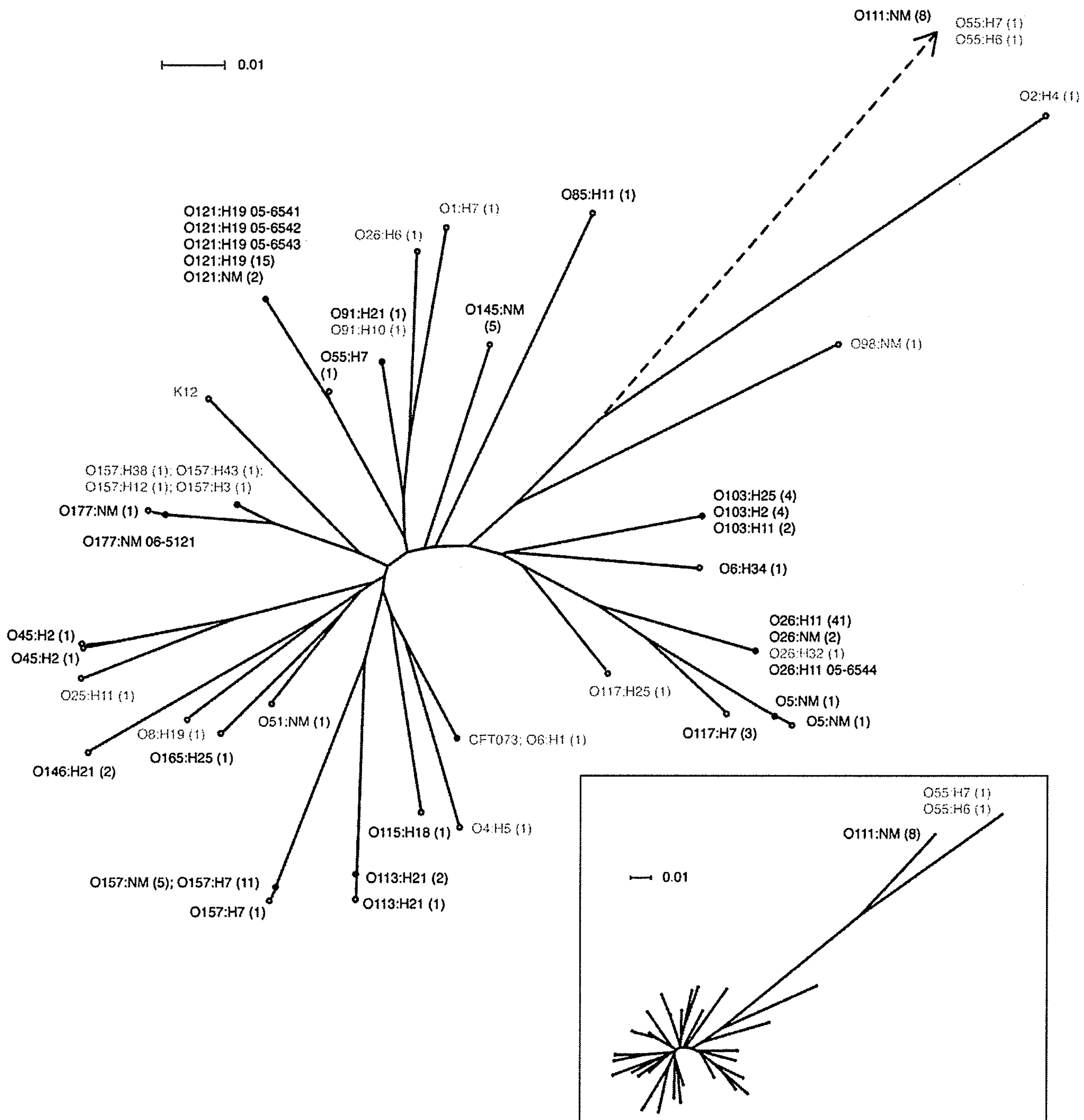


Fig. 3. Phylogeny of the *gnd* locus encoded by *E. coli*. This is based upon a neighbour-joining tree constructed with Hasegawa–Kishino–Yano (HKY85) distance correction. Sequences obtained from GenBank are identified in Methods. Shiga toxin-producing serotypes are indicated in black type, and strains not encoding *stx* are indicated in grey. The number of sequences per serotype is indicated in parentheses. Strain identification number is indicated for outbreak-associated clinical isolates. The dotted line indicates outlying *gnd* sequences, which are presented in relation to the entire dataset in the inset. Bar, scale of the distance score.

Table 3. Genetic diversity of the protein-encoding loci of *E. coli* sequenced in this study

For comparative purposes, multiple statistics for the *gnd* locus are presented as increasing numbers of serotypes and strains were analysed.

Target	No. of sequences*	No. of serotypes†	Size of target (bp)	No. of polymorphic sites (π)‡	No. of synonymous polymorphic sites	No. of nonsynonymous polymorphic sites	dN/dS§
gnd	47	42	643	210 (0.067)	189	21	0.035
	27	26	643	198 (0.061)	179	19	0.028
	17	16	643	173 (0.062)	154	19	0.030
gcl	26	26	654	68 (0.023)	61	7	0.023
relA	30	26	425	42 (0.019)	41	1	0.002
mdh	18	16	644	31 (0.010)	28	3	0.018
ftsZ	16	16	404	17 (0.010)	17	0	0.000
metA	16	16	559	36 (0.015)	29	7	0.115
metG	16	16	434	46 (0.024)	42	4	0.021
ppk	28	26	701	40 (0.013)	39	1	0.005

*Identical DNA sequences belonging to the same O : H serotype were not included.
†Minimally includes the serotypes indicated in Fig. 1 (when no. of serotypes=16), in Fig. 2. (when no. of serotypes=26) or in Fig. 3 (when no. of serotypes=42).
‡π; Measure of genetic diversity.
§Rate of nonsynonymous and synonymous mutations.

co-inherited between lineages (Tarr *et al.*, 2000; Nelson & Selander, 1994). To our knowledge, there is no indication that O-serogroups that encode similar *gnd* alleles (e.g. STEC O121 and O55) also encode similar O-antigen gene clusters, nor are the antigens themselves similar. The potential utility of a locus subject to recombination between genera might be seemingly limited for the purpose of molecular-based serogrouping; however, we currently observed conserved STEC serogroup-specific genetic polymorphisms at the *gnd* locus. Between strains of an individual STEC O-serogroup we observed conserved *gnd* alleles, and no serogroup encoded a *gnd* allele that was identical to another serogroup. This study provides a simple method for molecular-based serogrouping of *E. coli* strains encoding *stx*, which can be detected by a wealth of molecular reagents (Gilmour *et al.*, 2006; Hsu *et al.*, 2005; Nielsen & Andersen, 2003; Reischl *et al.*, 2002; Wang *et al.*, 2002). This method was used to characterize O121 : H19, O26 : H11 and O177 : NM clinical isolates prior to serological confirmation during an outbreak investigation, and could, therefore, improve the scope of STEC molecular diagnostics beyond the O157 serogroup.

ACKNOWLEDGEMENTS

We thank Tim Mailman and Nova Scotia Public Health for providing outbreak-associated strains, and John Wylie at the Manitoba Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada, Ana Paccagnella at the British Columbia Centre for Disease Control, Vancouver, British Columbia, Canada, and Yvonne Yaschuk at New Brunswick Public Health, Saint John, New Brunswick, Canada, for providing strains. We also thank Julie Walsh, Helen Tabor, Dobryan Tracz and Clifford Clark for helpful discussions. Oligonucleotide synthesis and DNA sequencing was performed by the DNA core facility, and serology was performed by the Serotyping and Identification Unit at the National Microbiology Laboratory. This work was supported by the Office of Biotechnology and Science.

REFERENCES

Adiri, R. S., Gophna, U. & Ron, E. Z. (2003). Multilocus sequence typing (MLST) of *Escherichia coli* O78 strains. *FEMS Microbiol Lett* 222, 199–203.

Beutin, L., Kaulfuss, S., Herold, S., Oswald, E. & Schmidt, H. (2005). Genetic analysis of enteropathogenic and enterohemorrhagic *Escherichia coli* serogroup O103 strains by molecular typing of virulence and housekeeping genes and pulsed-field gel electrophoresis. *J Clin Microbiol* **43**, 1552–1563.

Bisercic, M., Feutrier, J. Y. & Reeves, P. R. (1991). Nucleotide sequences of the *gnd* genes from nine natural isolates of *Escherichia coli*: evidence of intragenic recombination as a contributing factor in the evolution of the polymorphic *gnd* locus. *J Bacteriol* **173**, 3894–3900.

Coimbra, R. S., Grimont, F., Lenormand, P., Burguiere, P., Beutin, L. & Grimont, P. A. (2000). Identification of *Escherichia coli* O-serogroups by restriction of the amplified O-antigen gene cluster (*rfb*-RFLP). *Res Microbiol* **151**, 639–654.

DebRoy, C., Roberts, E., Kundrat, J., Davis, M. A., Briggs, C. E. & Fratamico, P. M. (2004). Detection of *Escherichia coli* serogroups O26 and O113 by PCR amplification of the *wzx* and *wzy* genes. *Appl Environ Microbiol* 70, 1830–1832.

D'Souza, J. M., Wang, L. & Reeves, P. (2002). Sequence of the *Escherichia coli* O26 O antigen gene cluster and identification of O26 specific genes. *Gene* 297, 123–127.

DebRoy, C., Fratamico, P. M., Roberts, E., Davis, M. A. & Liu, Y. (2005). Development of PCR assays targeting genes in O-antigen gene clusters for detection and identification of *Escherichia coli* O45 and O55 serogroups. *Appl Environ Microbiol* 71, 4919–4924.

Dykhuizen, D. E. & Green, L. (1991). Recombination in *Escherichia coli* and the definition of biological species. *J Bacteriol* **173**, 7257–7268.

Ewing, W. H. (1986). The genus *Escherichia*. In *Edwards & Ewing's Identification of Enterobacteriaceae*, 4th edn, pp. 93–134. Edited by P. R. Edwards and W. H. Ewing. New York: Elsevier.

Fey, P. D., Wickert, R. S., Rupp, M. E., Safranek, T. J. & Hinrichs, S. H. (2000). Prevalence of non-O157 : H7 shiga toxin-producing *Escherichia coli* in diarrheal stool samples from Nebraska. *Emerg Infect Dis* **6**, 530–533.

Fratamico, P. M., DebRoy, C., Strobaugh, T. P., Jr & Chen, C. Y. (2005). DNA sequence of the *Escherichia coli* O103 O antigen gene cluster and detection of enterohemorrhagic *E. coli* O103 by PCR amplification of the *wzx* and *wzy* genes. *Can J Microbiol* **51**, 515–522.

Gilmour, M. W., Cote, T., Munro, J., Chui, L., Wylie, J., Isaac-Renton, J., Horsman, G., Tracz, D. M., Andrysiak, A. & Ng, L. K. (2005). Multilocus sequence typing of *Escherichia coli* O26 : H11 isolates carrying *stx* in Canada does not identify genetic diversity. *J Clin Microbiol* **43**, 5319–5323.

Gilmour, M. W., Tracz, D. M., Andrysiak, A. K., Clark, C. G., Tyson, S., Severini, A. & Ng, L. K. (2006). Use of the *espZ* gene encoded in the locus of enterocyte effacement for molecular typing of Shiga toxin-producing *Escherichia coli*. *J Clin Microbiol* **44**, 449–458.

Girardeau, J. P., Dalmasso, A., Bertin, Y., Ducrot, C., Bord, S., Livrelli, V., Vernozy-Rozand, C. & Martin, C. (2005). Association of virulence genotype with phylogenetic background in comparison to different seropathotypes of Shiga toxin-producing *Escherichia coli* isolates. *J Clin Microbiol* **43**, 6098–6107.

Hsu, C. F., Tsai, T. Y. & Pan, T. M. (2005). Use of the duplex TaqMan PCR system for detection of Shiga-like toxin-producing *Escherichia coli* O157. *J Clin Microbiol* **43**, 2668–2673.

Huson, D. H. (1998). SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* **14**, 68–73.

Jelacic, J. K., Damrow, T., Chen, G. S., Jelacic, S., Bielaszewska, M., Ciol, M., Carvalho, H. M., Melton-Celsa, A. R., O'Brien, A. D. & Tarr, P. I. (2003). Shiga toxin-producing *Escherichia coli* in Montana: bacterial genotypes and clinical profiles. *J Infect Dis* **188**, 719–729.

Johnson, K. E., Thorpe, C. M. & Sears, C. L. (2006). The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. *Clin Infect Dis* **43**, 1587–1596.

Karch, H., Tarr, P. I. & Bielaszewska, M. (2005). Enterohaemorrhagic *Escherichia coli* in human medicine. *Int J Med Microbiol* **295**, 405–418.

Karmali, M. A., Mascarenhas, M., Shen, S., Ziebell, K., Johnson, S., Reid-Smith, R., Isaac-Renton, J., Clark, C., Rahn, K. & Kaper, J. B. (2003). Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J Clin Microbiol* **41**, 4930–4940.

Liu, Y. & Fratamico, P. (2006). *Escherichia coli* O antigen typing using DNA microarrays. *Mol Cell Probes* **20**, 239–244.

Nelson, K. & Selander, R. K. (1994). Intergeneric transfer and recombination of the 6-phosphogluconate dehydrogenase gene (*gnd*) in enteric bacteria. *Proc Natl Acad Sci U S A* **91**, 10227–10231.

Nielsen, E. M. & Andersen, M. T. (2003). Detection and characterization of verocytotoxin-producing *Escherichia coli* by automated 5′ nuclease PCR assay. *J Clin Microbiol* **41**, 2884–2893.

Noller, A. C., McEllistrem, M. C., Stine, O. C., Morris, J. G., Jr, Boxrud, D. J., Dixon, B. & Harrison, L. H. (2003). Multilocus sequence typing reveals a lack of diversity among *Escherichia coli* O157 : H7 isolates that are distinct by pulsed-field gel electrophoresis. *J Clin Microbiol* **41**, 675–679.

Paton, A. W. & Paton, J. C. (1999a). Direct detection of Shiga toxigenic *Escherichia coli* strains belonging to serogroups O111, O157, and O113 by multiplex PCR. *J Clin Microbiol* **37**, 3362–3365.

Paton, A. W. & Paton, J. C. (1999b). Molecular characterization of the locus encoding biosynthesis of the lipopolysaccharide O antigen of *Escherichia coli* serotype O113. *Infect Immun* **67**, 5930–5937.

Paton, A. W., Ratcliff, R. M., Doyle, R. M., Seymour-Murray, J., Davos, D., Lanser, J. A. & Paton, J. C. (1996). Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. *J Clin Microbiol* **34**, 1622–1627.

Perelle, S., Dilasser, F., Grout, J. & Fach, P. (2005). Detection of *Escherichia coli* serogroup O103 by real-time polymerase chain reaction. *J Appl Microbiol* **98**, 1162–1168.

Prager, R., Annemuller, S. & Tschape, H. (2005). Diversity of virulence patterns among Shiga toxin-producing *Escherichia coli* from human clinical cases – need for more detailed diagnostics. *Int J Med Microbiol* **295**, 29–38.

Reischl, U., Youssef, M. T., Kilwinski, J., Lehn, N., Zhang, W. L., Karch, H. & Strockbine, N. A. (2002). Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. *J Clin Microbiol* **40**, 2555–2565.

Rozas, J., Sanchez-DelBarrio, J. C., Messeguer, X. & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**, 2496–2497.

Tarr, P. I., Schoening, L. M., Yea, Y. L., Ward, T. R., Jelacic, S. & Whittam, T. S. (2000). Acquisition of the *rfb-gnd* cluster in evolution of *Escherichia coli* O55 and O157. *J Bacteriol* **182**, 6183–6191.

Tarr, C. L., Large, T. M., Moeller, C. L., Lacher, D. W., Tarr, P. I., Acheson, D. W. & Whittam, T. S. (2002). Molecular characterization of a serotype O121 : H19 clone, a distinct Shiga toxin-producing clone of pathogenic *Escherichia coli*. *Infect Immun* **70**, 6853–6859.

Thompson, L. H., Giercke, S., Beaudoin, C., Woodward, D. L. & Wylie, J. L. (2005). Enhanced surveillance of non-O157 verotoxin-producing *Escherichia coli* in human stool samples from Manitoba. *Can J Infect Dis Med Microbiol* **16**, 329–334.

Wang, L., Curd, H., Qu, W. & Reeves, P. R. (1998). Sequencing of *Escherichia coli* O111 O-antigen gene cluster and identification of O111-specific genes. *J Clin Microbiol* **36**, 3182–3187.

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

Woodward, D. L., Clark, C. G., Caldeira, R. A., Ahmed, R. & Rodgers, F. G. (2002). Verotoxigenic *Escherichia coli* (VTEC): a major public health threat in Canada. *Can J Infect Dis* **13**, 321–330.