Escherichia coli (*E. coli*) O157 and Non-O157 among Ruminants in Peninsular Malaysia: Pathogenicity, Antibiotic Resistance, and Survival under Sub-lethal Stress Conditions

Asanthi Perera

Bachelor of Science (Hons.)

University of Adelaide, South Australia (2010)

A thesis submitted for the degree of Doctor of Philosophy (PhD) at

School of Science

Monash University Malaysia

2015

Copyright notice

Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material, which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journals. The core theme of the thesis is characterisation of *Escherichia coli* O157 and non-O157. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Science under the supervision of Prof. Gary Dykes, Dr. Narelle Fegan, Dr. Charles Clarke and Dr. Lee Sui Mae.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 2, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	Characterization of Shiga toxigenic *Escherichia coli* (STEC) O157 and non-O157 isolates from ruminant feces in Malaysia	Accepted	Candidate devised the experimental design, conducted the experiments, analysed the results and drafted the manuscript

I (have) / have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: [redacted]

……

Date: **30 September 2015**

……

List of Research Outputs from the thesis

(1). Publication:

PERERA, A., CLARKE, C. M., DYKES, G. A. & FEGAN, N. 2015. Characterization of Shiga toxigenic *Escherichia coli* O157 and non-O157 isolates from ruminant feces in Malaysia. *BioMed Research International*, ID: 382403

(2). Poster Presentation at Conference:

PERERA, A., CLARKE, C. M., DYKES, G. A. & FEGAN, N. 2013. Shiga toxin-producing *Escherichia coli* O157 (STEC O157) and non-O157 (STEC non-O157) serougroups in ruminanats in Malaysia. 2013. Australian Society for Microbiology (ASM) Annual Scientific Meeting, Adelaide, South Australia.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my principal supervisors Prof. Gary Dykes and Dr. Narelle Fegan for the opportunity given to study my PhD under their supervision and for the continuous guidance and support provided throughout the years. Their patience, motivation and vast knowledge on the research field of microbiology has helped me immensely in completing my research work and writing of the thesis successfully. I could not have imagined having better advisors and mentors for my PhD study. I would also like to thank my co-supervisors Dr. Charles Clarke and Dr. Lee Sui Mae for their kind support and advice provided throughout the course of my PhD.

Besides my supervisors, I would also like to thank the rest of my thesis committee: Dr. Adeline Ting Su Yien, A/Prof. Song Keang Peng, and Prof. Sunil Lal for their insightful comments and encouragement during my PhD thesis review.

My sincere thanks also goes to Mr.Glen Mellor, Dr. Edward Fox, Mr. Shean Moor, Dr. Gan Han Ming, and Mr. Gary Gan Huan You for their advice and expert technical assistance provided during the research project. I am grateful to Ms. Lesley Duffy, Dr. Robert Barlow, Prof. Son Radu, Prof. Séamus Fanning, Mr. Juan Wang, Dr. Josée Harel and Mr. Garneau Philippe for providing bacterial strains as positive controls for my research work.

Many thanks to the School of Science, Monash University Malaysia and Jeffery Cheah Foundation for the opportunity and financial assistance provided to pursue my PhD. Special thanks to the administrative staff of School of Science for their efforts in dealing with the paperwork in relation to my PhD candidature.

I thank my fellow lab mates particularly from Prof. Gary Dykes' research team and Dr. Lee Sui Mae's research team for their kind support during the course of the research project. It was also a pleasure to work along with fellow research students of School of Science, Monash

University Malaysia during my study period. I also thank my friends who supported me countless times throughout the years of my research project.

Finally I would like to express my utmost gratitude to my husband, Lochana Maldeniya and my parents for their kind support, countless advice and encouragements and financial support given throughout the course of my PhD. I appreciate all their help very much in making this journey a success, which without would have been difficult to achieve.

Abstract

Pathogenic *Escherichia coli* (*E. coli*) are a common cause of diarrhoea and a broad range of extra-intestinal diseases in humans. Among these pathogenic strains, Shiga toxin producing *E. coli* (STEC), has emerged as one of the most virulent virotypes associated with cases of food borne disease in humans. Ruminants are considered an important reservoir of these pathogens. In Malaysia, data on the prevalence and characterisation of *E. coli* O157 and the non-O157 serogroups in ruminants is limited. Thus, *E. coli* including STEC O157 and serogroups of non-O157 STEC were isolated and characterised based on their virulence, antibiotic resistance and survival under sub-lethal heat, cold and acid stress conditions. A total of 136 ruminant feces samples were collected from six different farms in Peninsular Malaysia for the isolation and characterisation of *E. coli* O157 and the non-O157 serogroups O26, O103, O111, O121, O45 and O145. STEC O157:H7 was isolated from six (4.4 %) samples, from which 32 O157:H7 strains were obtained. All 32 STEC O157:H7 strains from this study were motile, carried s*tx*$_{2c}$, *eaeA-γ1* and *ehxA*, belonged to the less virulent lineage II, contained an occupied *sbcB* locus, and were negative for Stx production. Non-O157 STEC was isolated from 2 (1.5 %) samples, from which 2 non-O157 strains of unknown serotype were obtained. One of the STEC non-O157 strains carried s*tx*$_{1a}$, s*tx*$_{2a}$, s*tx*$_{2c}$, and *ehxA* and produced moderate amounts of Stx, while the other carried s*tx*$_{1a}$ alone and the production of Stx was below the level of detection. A total of six Shiga toxin producing *E. coli* strains including two STEC O157 and one non-O157 STEC strain obtained from Malaysia were used in the isolation of *stx* bacteriophage and subsequent infection of non-STEC. However, no infection was demonstrated from any of the induced *stx* bacteriophage from the STEC strains.

In addition to the STEC strains, a total of 161 non-STEC strains including ten strains of serogroup O157, ten strains of serogroup O103 and five strains of serogroup O26 were obtained

from the 136 ruminant feces collected. Of these *E. coli* strains, a total of 153 strains representing each feces sample and each serougroup obtained were used to determine antibiotic resistance. Analysis of antibiotic resistance among these *E. coli* indicated that complete resistance was most common for more traditional antibiotics such as tetracycline (11/153, 7.2 %), trimethoprim (6/153, 3.9 %), ampicillin (5/153, 3.3 %), and streptomycin (3/153, 2.0 %), while they were completely susceptible to more modern antibiotics such as cefuroxime and cetazidime. Multi-antibiotic resistance was observed in only 5.9 % (9/153) of the strains. Plasmid mediated resistance was apparent for tetracycline – *tet*(A), trimethoprim – *dhfr* I, V, VII, and XIII, ampicillin and cephalothin – bla_{TEM}, streptomycin – *aadA* and *strA–strB*, and chloramphenicol – *floR* and *cmlA*. Conjugation assays with selected strains indicated the transmissibility of antibiotic resistance determinants such as ampicillin, tetracycline, trimethoprim and streptomycin.

The effect of sub-lethal heat, cold and acid adaptation stress on the survival of two multi-antibiotic resistant *E. coli* strains, one positive (EC27) and the other negative for *stx* (EC135), in subsequent acidic conditions mimicking the acidic environment in the human stomach was also investigated. Acid adaptation and cold-stress adaptation of strain EC27 increased its susceptibility to subsequent acid stress, while strain EC135 was susceptible to acid stress regardless of pre-exposure to acid and cold stress. Heat shock treatment did not have any impact on the survival of both strains under post acid stress conditions.

The results of this study highlight that ruminants in Malaysia are a potential source of STEC and a significant reservoir of antibiotic resistance determinants with the propensity of antibiotic resistance dissemination. However, the presence of STEC O157 and non-O157 in a small percentage of ruminants in this study together with their virulence characteristics suggests that they may have limited impact on public health. Moreover, the lack of *E. coli* strains with resistance towards more modern antibiotics such as cefuroxime and ceftazidime and the

reduced survival observed from the multi-antibiotic resistant *E. coli* strains under severe stress conditions further indicates their low pathogenic potential towards humans.

Table of Contents

COPYRIGHT NOTICE ..... **i**
DECLARATION ..... **ii**
LIST OF RESEARCH OUTPUTS FROM THE THESIS ..... **iii**
ACKNOWLEDGEMENTS ..... **iv**
ABSTRACT ..... **vi**
LIST OF FIGURES ..... **xii**
LIST OF TABLES ..... **xiv**
LIST OF APPENDICES ..... **xv**
ABBREVIATIONS ..... **xvi**
CHAPTER 1. GENERAL INTRODUCTION **1**
1.1 *Escherichia coli* **2**
1.2 Pathogenic *E. coli* **4**
1.2.1 Antibiotic resistance 4
1.2.2 *E. coli* O157 8
1.2.3 *E. coli* non-O157 9
1.3 Reservoirs and Transmission **9**
1.4 Pathogenesis and important virulence factors of STEC **16**
1.5 Clinical features of STEC infections **17**
1.6 Global incidence of STEC infections **18**
1.6.1 Outbreaks of STEC O157 18
1.6.2 Outbreaks of non-O157 STEC 19
1.6.3 Annual incidence rates of STEC infections 21
1.7 Variation in disease severity of STEC infections **23**
1.8 STEC infections in Malaysia **25**
2.0 Research objectives **26**
DECLARATION FOR THESIS CHAPTER 2 ..... **27**
CHAPTER 2. CHARACTERISATION OF SHIGA TOXIGENIC *ESCHERICHIA COLI* (STEC) O157 AND NON-O157 STRAINS FROM RUMINANT FECES IN MALAYSIA **28**
2.1 Introduction **29**
2.2 Materials and methods **31**
2.2.1 Sample collection and preparation 31
2.2.2 Isolation and characterisation of *E. coli* O157 33
2.2.3 Detection, isolation and characterisation of non-O157 *E. coli* 34
2.2.4 Biochemical confirmation of *E. coli* strains 35
2.2.5 Bacterial strains 35

2.2.6 Pulsed-field gel electrophoresis (PFGE) 35
2.2.7 Subtyping of *stx* and intimin (*eaeA*) genes of *E. coli* O157 and non-O157 35
2.2.8 Detection of Shiga toxin expression 35
2.3 Results **37**
2.3.1 Presence of STEC O157 and virulence factors 37
2.3.2 Presence of non-O157 STEC and virulence factors 38
2.3.3 Characterisation of *E. coli* serogroups lacking *stx* 41
2.3.4 *stx* and *eaeA* subtyping 42
2.3.5 Shiga toxin production 42
2.4 Discussion **42**
2.5 Conclusion **45**
CHAPTER 3. PATTERNS OF ANTIBIOTIC RESISTANCE AND RESISTANCE GENE DETERMINANATS IN COMMENSAL *ESCHERICHIA COLI* (*E. COLI*) ISOLATED FROM RUMINANATS IN PENINSULAR MALAYSIA **46**
3.1 Introduction **47**
3.2 Materials and methods **49**
3.2.1 Isolation of *E. coli* 49
3.2.2 Resistance profiling of isolated *E. coli* 51
3.2.3 Detection of antibiotic resistance genes and class 1 and class 2 integrons 51
3.2.4 Screening for plasmid mediated resistance 59
3.2.5 Sequencing of integron gene cassettes and quinolone resistance genes 59
3.2.6 Conjugation assays 59
3.3 Results **64**
3.3.1 Antibiotic susceptibility of the *E. coli* strains 64
3.3.2 Occurrence of resistance determinants 65
3.3.3 Sequence analysis of the resistant determinants for nalidixic acid (quinolone) resistance 70
3.3.4 Integrons and gene cassettes 70
3.3.5 Conjugal transfer of antibiotic resistance 71
3.4 Discussion **71**
3.5 Conclusion **77**
CHAPTER 4. GENOMIC ANALYSIS OF SHIGA TOXIN ENCODING BACTERIOPHAGE INSERTION SITES (SBI) OF NON-O157 SHIGA TOXIGENIC *ESCHERICHIA COLI* (STEC) FROM AUSTRALIA AND MALAYSIA **78**
4. 1 Introduction **79**
4.2 Materials and methods **82**
4.2.1 Bacterial strains 82
4.2.2 Phage preparation 82
4.2.3 The standard dual layered agar dilution drop assay 83

4.2.4 Formation of *stx*-encoding DH5-α (*E. coli* K12) lysogen strains 87
4.3 Results **88**
4.4 Discussion **90**
4.5 Conclusion **92**
CHAPTER 5. IMPACT OF SUB-LETHAL HEAT, COLD AND ACID ADAPTATION STRESS ON SUBSEQUENT ACID TOLERANCE RESPONSE OF TWO MULTI-ANTIBIOTIC RESISTANT *ESCHERICHIA COLI* (*E. COLI*) STRAINS ISOLATED FROM MALAYSIA **93**
5.1 Introduction **94**
5.2 Materials and methods **96**
5.2.1 Bacterial strains and culture conditions 96
5.2.2 Inoculum preparation 96
5.2.3 Determination of heat shock treatment temperature 96
5.2.4 Heat shock treatment followed by acid tolerance assay 97
5.2.5 Acid adaptation followed by acid tolerance assay 97
5.2.6 Acid adaptation–heat shock treatment followed by acid tolerance assay 98
5.2.7 Cold stress adaptation–heat shock treatment followed by acid tolerance assay 98
5.2.8 Acid tolerance assay 98
5.2.9 Enumeration of viable *E. coli* cells 99
5.2.10 Statistical analysis 99
5.3 Results **99**
5.3.1 Effect of heat shock on subsequent acid tolerance 99
5.3.2 Effect of acid adaptation on subsequent acid tolerance 101
5.3.3 Effect of acid adaptation and heat shock treatment on subsequent acid tolerance 103
5.3.4 Effect of cold-stress adaptation and heat shock treatment on subsequent acid tolerance 105
5.4 Discussion **109**
5.5 Conclusion **112**
CHAPTER 6. GENERAL CONCLUSION **113**
REFERENCES **1288**
APPENDICES **1489**

List of Figures

Figure 2.1. Geographical distribution of farms A–F in Peninsular Malaysia, from which the ruminant feces samples were collected 32

Figure 4.1. Dual layered agar dilution drop assay. Phage suspensions were filtered with a 0.22 µm membrane filter and serially diluted in LB. Aliquots of 20 µl of respective dilutions were spotted onto the dual layered agar and incubated at 37°C. Adapted from Sim (2010) 84

Figure 5.1. Effect of heat shock at 48°C for 10 min on the survival of *stx* positive *E. coli* strain (EC27) in acidified BHI broth at pH 2.5. Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC27 strain from 0 to 10 min in acidified broth (pH 2.5)..... 100

Figure 5.2. Effect of heat shock at 48°C for 10 min on the survival of *stx* negative *E. coli* strain (EC135) in acidified BHI broth at pH 2.5. Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC135 strain from 0 to 10 min in acidified broth (pH 2.5)..... 101

Figure 5.3. Effect of acid adaptation for 1 h in acidified BHI broth at pH 5 (with conc. HCl) on the survival of *stx* positive *E. coli* strain (EC27) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 20 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC27 strain from 0 to 20 min in acidified broth (pH 2.5)..... 102

Figure 5.4. Effect of acid adaptation for 1 h in acidified BHI broth at pH 5 (with conc. HCl) on the survival of *stx* negative *E. coli* strain (EC135) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 20 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC135 strain from 0 to 20 min in acidified broth (pH 2.5)..... 103

Figure 5.5. Effect of initial acid adaptation for 1 h in acidified BHI broth at pH 5 using conc. HCl on the survival of heat shocked (at 48°C for 10 min) *stx* positive *E. coli* strain (EC27) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC27 strain from 0 to 10 min in acidified broth (pH 2.5) 104

Figure 5.6. Effect of initial acid adaptation for 1 h in acidified BHI broth at pH 5 using conc. HCl on the survival of heat shocked (at 48°C for 10 min) *stx* negative *E. coli* strain (EC135) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the

acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC135 strain from 0 to 10 min in acidified broth (pH 2.5) 105

Figure 5.7. Effect of cold-stress adaptation at 4°Cfor (a) 1 day and (b) 7 days on the survival of heat shocked (at 48°C for 10 min) *stx* positive *E. coli* strain (EC27) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC27 strain from 0 to 10 min in acidified broth (pH 2.5) 107

Figure 5.8. Effect of cold-stress adaptation at 4°Cfor (a) 1 day and (b) 7 days on the survival of heat shocked (at 48°C for 10 min) *stx* negative *E. coli* strain (EC135) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC135 strain from 0 to 10 min in acidified broth (pH 2.5)..... 108

List of Tables

Table 1.1. Prevalence of STEC among ruminants in different countries 12
Table 2.1. Distribution of ruminant feces samples collected from farms A–F 32
Table 2.2. Bacterial strains used in the study 366
Table 2.3. STEC O157 and non-O157 and their virulence profiles 388
Table 2.4. Occurrence of target virulence factors and 'big 6' non-O157 serogroups in the initial PCR screen of the enriched samples from each farm (A−F)..... 40
Table 2.5. Isolation and virulence profiles of *E. coli* O157 and 'big 6' *E. coli* non-O157 serogroups lacking stx 41
Table 3.1. Distribution of *E. coli* strains used in antibiotic susceptibility testing 50
Table 3.2. PCR primer characteristics..... 53
Table 3.3. Resistance profiles and resistance gene determinants of the transconjugants obtained during conjugation assays with the donor *E. coli* strains collected from the ruminant feces samples..... 61
Table 3.4. Occurrence of antibiotic resistance and the respective genetic determinants among the *E. coli* strains 67
Table 4.1. Bacterial strains used in the study 82
Table 4.2. Modifications of the standard dual layered agar dilution drop assay 85
Table 4.3. Volumes of *stx* phage lysate and DH5-α cells used in lysogen formation assay 1 87
Table 4.4. Phage induction, plaque formation and lysogen formation of the STEC strains used in the study 89

List of Appendices

Appendix 1. Dendogram indicating the PFGE band patterns of 46 *E. coli* O157 isolates 149

Appendix 2. Dendogram indicating the PFGE band patterns of 26 *E. coli* non-O157 isolates 150

Abbreviations

AK	Amikacin
Ala	Alanine
AMP	Ampicillin
ATCC	American type culture collection
ATR	Acid tolerance response
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
bp	Base pairs
BPW	Buffered peptone water
C	Chloramphenicol
cat	Chloramphenicol acetyl transferase
CAZ	Ceftazidime
CDC	Centers for Disease Control and Prevention
cfu	Colony forming units
CIP	Ciproflaxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamycin
CSIRO	Commonwealth Scientific and Industrial Research Organization
CSP	Cold shock protein
CT-SMAC	Sorbitol-MacConkey agar containing the cefixime, tellurite supplement
CTX	Cefotaxime
Cys	Cystine
CXM	Cefuroxime sodium
DAEC	Diffusely adherent *E. coli*
dhfr	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
dUTP	2'–Deoxyuridine 5'–Triphosphate
eaeA	Attaching and effacing
EAEC	Enteroaggregative *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
Gb_3	Globotriaosylceramide
Gln	Glutamine
HC	Hemorrhagic colitis
HCl	Hydrochloric acid
HSP	Heat shock protein
HUS	Hemolytic uremic syndrome
IMR	Institute of Medical Research

IMS	Immunomagnetic separation
IPM	Imipenem
K	Kanamycin
KF	Cephalothin
LB	Luria-Bertani
LEE	Locus for enterocyte effacement
LSPA-6	Lineage-specific polymorphisms–6
Lys	Leucine
NA	Nalidixic acid
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
OD600	Optical density at 600 nm
QRDR	Quinolone resistance-determining region
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RecA	Recombination Protein A
S	Streptomycin
SBI	Shiga toxin encoding bacteriophage insertion sites
Ser	Serine
SMAC	Sorbitol-MacConkey agar
SNP	Single Nucleotide Polymorphism
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga toxin
TE	Tetracycline
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TSBG	Trptone soya broth with glucose
TTP	Thrombotic thrombocytopenic purpura
UPM	University Putra Malaysia
UV	Ultraviolet
W	Trimethoprim
Wt/vol	Weight/volume composition of the reagents

Chapter 1

General Introduction

1.1 *Escherichia coli*

Escherichia coli (E. coli) are a genomically and phenotypically highly heterogeneous group of facultatively anaerobic, Gram-negative bacilli within the family *Enterobacteriaceae* (Nataro and Kaper, 1998). They were first described in 1885 by Theodor Escherich as Bacterium coli commune (the common colon bacterium) during his studies on neonatal and infant fecal microbiota (Neill et al., 1994). Most of its members are typically motile, non-pathogenic commensal inhabitants of the gastrointestinal tract of humans and animals. *E. coli* colonisation of the gastrointestinal tract occurs within hours or few days following birth. In humans, *E. coli* typically colonise the gastrointestinal tract of infants within 40 hours of birth and is the predominant facultative anaerobe found in the human colonic flora (Todar, 2005b). Once established in the gastrointestinal tract, strains of *E. coli* may persist for months or years.

Physiologically, *E. coli* are versatile bacteria well adapted to their characteristic habitats. They are capable of utilising a wide variety of substrates for growth. Glucose is the preferred carbon source of *E. coli* and growth can occur in media with glucose as the sole organic constituent (Martinez-Gomez et al., 2012). Wild-type *E. coli* have no growth factor requirements, and are capable of synthesising all essential growth factors such as purines, pyrimidines, amino acids and vitamins from the carbon sources being utilised (Todar, 2005a). As facultative anaerobes, *E. coli* utilise fermentation or anaerobic respiration for growth in the absence of oxygen. During fermentation *E. coli* employs the mixed acid fermentative pathway that produces alternative acidic end products such as lactate, acetate, formate and gases such as carbon dioxide and hydrogen in variable amounts (Sumbali and Mehrotra, 2009). In addition, *E. coli* growth is also supported by anaerobic respiration, which generates the majority of its cellular energy under anaerobic conditions (Sumbali and Mehrotra, 2009). The ability of *E. coli* to grow under aerobic and anaerobic conditions has enabled their adaptation to intestinal (anaerobic) and extra-intestinal (aerobic or anaerobic) environments in humans and animals.

E. coli cells respond to a multitude of environmental stimuli including temperature and pH. Optimal growth of *E. coli* occurs at 37^{o}C although it can still grow and multiply within the temperature range of 7–8^{o}C to 46^{o}C (International Commission on Microbiological Specifications for Foods, 1996). Some strains of *E. coli* have been shown to grow at temperatures of 48–50^{o}C and survive well in chilled (0–4^{o}C) and frozen food at −20^{o}C (Ministry for Primary Industries − New Zealand, 2001). The growth pH of *E. coli* ranges between 4.4–10.0, with an optimum of 6–7 (Desmarchelier and Fegan, 2003). Some *E. coli* strains have been shown to survive at pH 2.5–3.0 for over four hours or more (Molina et al., 2003). Prior exposure to acidic conditions increases survival of some *E. coli* strains under very low pH conditions such as that encountered in the human stomach (pH 1.5–3.5) for periods of more than three hours, the time generally required to clear an average meal (Ministry for Primary Industries − New Zealand, 2001). Shifts of temperature or pH within the normal growth range of *E. coli* generally result in quick, transient adjustments in the level of cell components and enzyme activity. However, deviations in temperature and pH above or below the normal growth range may lead to stress conditions in *E. coli* resulting in either reduced growth or survival potential. Under stress conditions, *E. coli* induces a general stress response providing cross protection against multiple stresses as well as a specific response towards the primary stress (Jones, 2012). The stress response serves as an immediate and long term strategy of adaption of *E. coli* cells to various stresses through changes in cellular physiology and morphology as well as gene expression.

Although *E. coli* are a highly diverse group of organisms, serotype analysis has facilitated their differentiation to a great extent. *E. coli* serotyping is based on their surface antigen profiles, namely somatic (O), flagellar (H), and capsular (K) (Nataro and Kaper, 1998), which was initially proposed by Kauffman (Kauffman, 1947). The O antigen of *E. coli* defines the serogroup while a specific combination of O and H antigens defines the serotype. Currently,

more than 700 serotypes of *E. coli* have been identified (Griffin and Tauxe, 1991). In *E. coli*, the serologic antigens themselves do not confer virulence but rather serve as readily identifiable chromosomal markers that correlate with specific virulent clones.

1.2 Pathogenic *E. coli*

Although the majority of *E. coli* strains are non-pathogenic commensals, some strains have evolved as pathogens by acquiring genes that enable them to cause a broad spectrum of human diseases, and which on rare occasions can lead to death. Pathogenic potential of particular *E. coli* strains depends on the repertoire of specific virulence factors they may possess including adhesins, toxins, secretion systems, resistance to antibiotics, and the ability to withstand host defences. Strains of pathogenic *E. coli* are categorised into two major groups: those that primarily cause gastrointestinal disease such as diarrhoea and those that primarily cause extra-intestinal disease such as urinary tract infection, meningitis, peritonitis and septicaemia (Nataro and Kaper, 1998, Sodha et al., 2011, von Baum and Marre, 2005). The *E. coli* that cause diarrheal disease in humans have been divided into several pathotypes based on their virulence factors and mechanisms, by which they cause disease (Nataro and Kaper, 1998). These pathotypes include, Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998). Among these pathotypes, STEC has emerged as one of the most important causes of food borne disease in humans world-wide. Evolution of STEC is characterised by the stepwise acquisition of multiple virulence factors through horizontal gene transfer mediated predominantly by bacteriophage transduction (Feng et al., 1998).

1.2.1 Antibiotic resistance

Acquisition of antibiotic resistance, among other virulence factors, has significantly increased the pathogenic potential of *E. coli* world-wide. The emergence and rapid increase of antibiotic

resistance has created an added burden to the treatment of infections caused by *E. coli*. Antibiotics are used to treat disease in human and veterinary medicine. In addition they are incorporated in to livestock and poultry feed at sub-therapeutic doses for growth promotion. The extensive use of antibiotics in humans as well as animals creates a selective pressure on the bacteria residing in humans and animals. *E. coli*, both pathogenic and commensal bacteria, are often subjected to this selective pressure leading to the emergence of antibiotic resistance. Resistance to antibiotics acquired by commensal bacteria can subsequently be spread to pathogenic bacteria through resistance determinants present on mobile genetic elements such as conjugative plasmids, transposons and integrons. Acquisition of antibiotic resistance by pathogenic *E. coli* strains through these mechanisms especially in food-producing animals creates a public health concern due to the potential dissemination of resistant bacteria to humans via the food chain. Moreover, *E. coli* are highly capable of exchanging genetic material with other bacterial species (Blake et al., 2003, Davison, 1999), and as a result antibiotic resistance determinants may be transferred to bacterial pathogens other than *E. coli* that can cause disease in humans.

A link between antibiotic use and development of antibiotic resistance in food-producing animals has been reported in several studies. In Alberta, Canada, a study was conducted to determine the effect of sub-therapeutic administration of antibiotics commonly used in the Canadian feedlot cattle industry including chlorotetracycline plus sulfamethazine, chlorotetracycline, monensin, and tylosin on the prevalence of antibiotic resistant *E. coli* in feedlot cattle (Alexander et al., 2008). The prevalence of cattle shedding tetracycline and ampicillin resistant *E. coli* were > 40 % and 30 % respectively prior to inclusion of antibiotics in the diet. Prevalence of cattle shedding tetracycline and ampicillin resistant *E. coli* increased with the inclusion of chlorotetracycline plus sulfamethazine in their diet. In addition to the *E. coli* which showed resistance to tetracycline (47.1 %) and ampicillin (13.9 %), 7.0 % of strains

were resistant to gentamycin, 1.8 % were resistant to ceftazidime and 2.0 % were resistant to cefpodoxime after antibiotic treatments. Similarly, use of antibiotics to treat diseases such as salmonellosis in cattle have been shown to increase resistance among commensal *E. coli* strains from cattle in comparison to the non-treated control groups in another study (DeFrancesco et al., 2004). Surveillance data indicate consistently high rate of antibiotic resistance in *E. coli* for antibiotics that have been in use for a long time in humans and animals (Food and Drug Administration – USA, 2010). However, major increase in antibiotic resistance in *E. coli* to more modern antibiotic compounds such as fluoroquinolones and certain cephalosporins has been observed in the past two decades (Levy and Marshall, 2004). A study in the USA tested antibiotic susceptibility of *E. coli* from human and animal sources, which included cattle, chicken and pigs collected between 1950 and 2002 to a total of 15 antibiotics (Tadesse et al., 2012). In this study *E. coli* strains of an animal origin showed an increasing trend in resistance to 11 antibiotic agents, which included ampicillin, sulphonamide, tetracycline, cephalothin, trimethoprim/sulfamethoxazole, streptomycin, chloramphenicol, cefoxitin, gentamicin, amoxicillin/clavulanic acid, and kanamycin. Resistance to ampicillin increased from 0 % to 69.4 % between 1950 and 2002, while the resistance to sulphonamide and tetracycline increase from 0 % to 73.7 % and 0 % to 85.5 % respectively. In addition, the study showed that the *E. coli* strains of animal origin were more resistant to the antibiotics tested compared to the *E. coli* strains of human origin. Of the 746 *E. coli* strains recovered from animal sources, resistance was most frequently observed for tetracycline (71.1 %), followed by streptomycin (59 %), sulphonamide (57.7 %), kanamycin (37.1 %), and ampicillin (34.1 %). In addition, multi-antibiotic resistance (≥ 3 antibiotic classes) was mostly observed among *E. coli* strains recovered from animals than humans.

Antibiotic resistant *E. coli* in hospital environments clearly pose a threat to the public health. Similarly, antibiotic resistant *E. coli* colonization in animals cannot be disregarded due to the

significant role they play in dissemination of antibiotic resistance determinants among animals as well as humans. A survey conducted in Japan in 2007 showed 9.2 % resistance to penicillin, 19.2 % resistance to streptomycin, 26.2 % resistance to tetracycline and 1.5 % resistance to fluoroquinolone among commensal *E. coli* isolated from cattle (Harada and Asai, 2010). Resistance to penicillin, streptomycin, and tetracycline antibiotics among commensal *E. coli* strains from cattle in the USA between 2002 and 2003 were 2.5 %, 10.2 %, and 23.1 % respectively (reviewed in Harada and Asai, 2010). A high prevalence of resistance to penicillin (48.5 %), tetracycline (82.4 %) and fluoroquinolone (25.5 %) antibiotics was observed among commensal *E. coli* isolated from cattle in the Netherlands in 2005 (Veterinary Antibiotic Usage and Resistance Surveillance Working Group, 2005). A very low prevalence of resistance was observed for tetracycline (3 %) antibiotics among commensal *E. coli* strains from cattle in Australia between 2003 and 2004 while none of the strains were resistant to penicillin, and fluoroquinolone antibiotics (Department of Agriculture, Fisheries and Forestry – Australia, 2007). In Korea during a study conducted between 2003 and 2004, the most frequently observed resistance among *E. coli* strains from cattle feces samples was to tetracycline (30.5 %), followed by resistance to streptomycin (20.4 %), ampicillin (12 %), and chloramphenicol (6.9 %) (Lim et al., 2007). In a study conducted in Germany, 25 % of *E. coli* isolated from cattle were resistant to antibiotics including sulfamethoxazole, tetracycline, streptomycin, ampicillin, kanamycin, chloramphenicol, trimethoprim and gentamycin (Guerra et al., 2003). The predominant antibiotic resistance gene(s) for each antibiotic were bla_{TEM1} (ampicillin), *catA* and *cmlA1* (chloramphenicol), *aac(3)-IV* (gentamycin), *aphA1* (kanamycin), *aadA1* and *strA/B* (streptomycin), *sul1*, *sul2* and *sul3* (sulfamethoxazole), *tet(A)* and *tet(B*) (tetracycline), and *dfrA1*, *dfrA12* and *dfrA12* (trimethoprim). In addition, 20 % of *E. coli* strains from cattle were positive for the class 1 integron, which carried genes conferring resistance to streptomycin and trimethoprim. Integrons (class 1 and class 2) carrying antibiotic resistance genes mostly

conferring resistance to streptomycin/spectinomycin were detected among *E. coli* strains from cattle in two studies conducted in Australia (Barlow et al., 2008, Barlow et al., 2009). Class 1 integrons carrying antibiotic resistance to aminoglycoside, trimethoprim, and β-lactams were identified among multi-antibiotic resistant *E. coli* strains recovered from Irish cattle (Karczmarczyk et al., 2011b). Although less frequent class 2 integrons were also identified in these strains carrying resistance to trimethoprim and streptomycin. In addition conjugative plasmids associated with resistance to ampicillin, neomycin, streptomycin, sulfonamaide, and tetracycline were identified among these *E. coli* strains.

In Malaysia, most studies have investigated antibiotic resistant *E. coli* in hospital settings (Akter et al., 2012, Ho et al., 2012, Kor et al., 2013, Lim et al., 2009) and there is very limited data on antibiotic resistance among commensal *E. coli* from food-producing ruminants. However, high resistance to antibiotics including ampicillin, chloramphenicol, cephradine, cefoperazone, ciprofloxacin, erythromycin, kanamycin, nalidixic acid, tetracycline, and trimethoprim has been observed among *E. coli* strains from poultry in one study (Myaing et al., 2005). In this study high prevalence of plasmid DNA was detected among the strains carrying resistance to a larger number of antibiotics. Another study reported high resistance of *E. coli* isolated from ducks in Penang, Malaysia to vancomycin, tetracycline, ampicillin, streptomycin and sulfamethoxazole-trimethoprim (Adzitey et al., 2013). Data available on antibiotic resistance and resistance determinants on commensal *E. coli* strains from different geographical regions around the world provide evidence that the use of antibiotics contributes to the resistance development in commensal *E. coli* and they constitute an essential reservoir of antibiotic resistance determinants.

1.2.2 *E. coli* O157

E. coli O157:H7, a predominant subtype of STEC, is one of the serotypes most frequently associated with cases of food borne disease in humans (Lim et al., 2010). Serotype O157:H7

is believed to be derived from an atypical EPEC serotype O55:H7 with the acquisition of the bacteriophage encoding Stx2, a virulence plasmid and an antigenic shift from O55 to O157 (Feng et al., 1998). Further genetic events have led to the acquisition of the bacteriophage encoding Stx1 and loss of ability to ferment sorbitol. The low infectious dose (can be < 50 to few hundred organisms) of *E. coli* O157:H7 (Bell et al., 1994), its ability to survive for long periods in the environment, its potential to cause severe disease with occasional life-threatening sequelae in humans, and the increased occurrence of food borne outbreaks over the past decades has raised public health concern. Due to the importance of *E. coli* O157 in human disease, STEC serotypes are commonly grouped into two major categories, *E. coli* O157 and non-O157.

1.2.3 *E. coli* non-O157

STEC strains belonging to more than 100 serogroups, which have been isolated from humans are collectively referred to as non-O157 STEC (Johnson et al., 1996). Less than ten of these serogroups are found to be responsible for the majority of cases of human disease (Gyles, 2007). The Centers for Disease Control and Prevention (CDC) has identified six non-O157 STEC serogroups, namely O26, O45, O103, O111, O121 and O145, as the most common non-O157 STEC associated with human disease in the United States (Brooks et al., 2005). Similar to the STEC O157 serogroup, the pathogenic potential and public health significance of non-O157 STEC is evident from their ability to cause severe disease such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans as well as their low infectious dose (Paton et al., 1996) and long term survival in the environment.

1.3 Reservoirs and Transmission

Pathogenic and non-pathogenic strains of *E. coli* are most commonly found in the intestinal tract of humans and animals. Even though STEC is not host specific, it has been found to be more prevalent in ruminants, especially cattle, than in other animals (Reimann and Cliver,

1998). Consequently, human infections caused by STEC are most frequently traced to cattle, which are considered as the major reservoir of STEC. It is not surprising therefore that most human STEC infections are attributed to consumption of animal derived food, particularly of bovine origin (meat and dairy products), which have been contaminated directly or indirectly with STEC-containing feces before or after processing. Since the initial STEC O157 outbreak in 1982 in North America, which was associated with the consumption of undercooked beef patties in hamburgers (Riley et al., 1983) the number of food vehicles implicated in O157 infections have increased. Recent outbreaks have been linked to fresh produce including spinach (Centers for Disease Control and Prevention, 2006), lettuce (Hilborn et al., 1999), unpasteurized apple juice (Besser et al., 1993) and fermented hard salami (Centers for Disease Control and Prevention, 1995) possibly contaminated by cattle feces. Other modes of STEC transmission include contaminated water (by drinking or swimming), person to person and contact with infected animals (reviewed in Caprioli et al., 2005).

The significance of cattle as a reservoir of STEC and a route of transmission to humans emphasise the importance of determining the prevalence and diversity of STEC strains among cattle. In addition, the presence of STEC in other ruminants such as buffalo, sheep and goat also indicates the importance of detecting STEC prevalence and their diversity among these ruminants. Many studies have been conducted especially in countries of Europe (Chapman et al., 1997, Fremaux et al., 2006, Monaghan et al., 2011), USA (Zhao et al., 1995, Elder et al., 2000), Canada (Van Donkersgoed et al., 2001), Australia (Fegan et al., 2004) and Japan (Fukushima and Seki, 2004, Sasaki et al., 2011) to determine STEC prevalence among cattle as well as other ruminants. A summary of STEC prevalence among ruminants in different countries is presented in Table 1.1. Variation in STEC prevalence among ruminants can be observed in different countries according to Table 1.1. Differences in the study design, sampling and isolation methods used in different studies would affect the prevalence of STEC

reported leading to the variation of prevalence. In addition, animal-associated factors such as intermittent shedding of STEC and differences in animal management practices as well as environmental factors between different geographical locations would also contribute to the variation of STEC prevalence observed.

In Europe, high prevalences as well as low prevalences of STEC have been reported in different studies. A study conducted in France reported a high prevalence of STEC (34.0 %) from cattle feces. A very low prevalence of STEC (0.2 %) in cattle feces was reported in a study conducted in Norway. In North America, one study conducted in USA reported a high prevalence of STEC (28.0 %), while another study showed a relatively low prevalence of STEC (8.2 %). Several studies from Argentina and Brazil in South America reported high prevalence of STEC in cattle. In Australia, prevalence of STEC ranged from 0.3–13.0 %. In Asia, few studies have been conducted in Japan on prevalence of STEC which ranged from 0.4–15.9 %. In contrast to countries such as UK, USA, and Australia, very few studies have examined the prevalence of STEC in most of the tropical countries. The study conducted in Bangladesh indicated a relatively high prevalence of STEC O157 (14.4 %) and non-O157 (23.6 %) among buffalo feces samples. In Vietnam, a high prevalence of STEC was observed in goat feces samples (38.5 %). Of the studies conducted in India, the prevalence of STEC ranged from 4.5–17.9 %. Unfortunately in Malaysia there are no reported studies so far on the prevalence of STEC O157 or non-O157 in ruminant feces.

Table 1.1. Prevalence of STEC among ruminants in different countries

Continent	Country	Year	Source	No. of + samples/total samples tested (%)	Serougroup(s)	Reference
Europe	Belgium	2007–2009	Cattle feces	24/399 (6.0)	O26, O103, O111, and O145	(Joris et al., 2011)
	France	1997–1998	Cattle feces	162/471 (34.0)	O157 and non-O157	(Pradel et al., 2000)
	France	2003–2004	Cattle feces	80/415 (19.3)	O157, O26, and O55	(Fremaux et al., 2006)
	Ireland	2007–2008	Cattle feces	23/1200 (1.9)	Non-O157	(Monaghan et al., 2011)
	Netherland	1996	Cattle feces	75/1152 (6.5)	O157	(Heuvelink et al., 1998)
	Norway	1998–1999	Cattle feces	3/1541 (0.2)	O157	(Johnsen et al., 2001)
	Spain	2000–2001	Lamb feces	7/697 (1.0)	O157	(Rey et al., 2003)
			Lamb feces	246/697 (35.0)	Non-O157	
	UK	1995–1996	Cattle feces	752/4800 (15.7)	O157	(Chapman et al., 1997)
			Sheep feces	22/1000 (2.2)	O157	

	UK	2003	Cattle feces	121/2553 (4.7)	O157	(Milnes et al., 2009)
			Sheep feces	21/2825 (0.7)	O157	
North America	Canada	1998–1999	Cattle feces	2/240 (0.8)	O157	(Van Donkersgoed et al., 2001)
	Central Mexico	2001	Cattle feces	3/240 (1.3)	O157	(Callaway et al., 2004)
	USA	1993	Cattle feces	7/85 (8.2)	O157	(Zhao et al., 1995)
	USA	1999	Cattle feces	91/327 (28.0)	O157	(Elder et al., 2000)
South America	Argentina	Not reported	Cattle feces	540/1440 (37.5)	O157 and non-O157	(Fernandez et al., 2010)
	Argentina	2000	Rectal swabs from cattle	37/59 (62.7)	O157 and non-O157	(Padola et al., 2004)
	Brazil	2001	Cattle feces	1/153 (0.6)	O157	(Irino et al., 2005)
				38/153 (24.8)	Non-O157	
	Brazil	Not reported	Cattle feces	20/344 (5.8)	Non-O157	(Leomil et al., 2003)

Oceania	Australia	2000	Cattle feces	39/310 (13.0)	O157	(Fegan et al., 2004)
	Australia	2008–2009	Cattle feces	1/300 (0.3)	Non-O157	(Barlow and Mellor, 2010)
	New Zealand	2008	Recto–anal swabs from cattle	10/309 (3.2)	O157	(Irshad et al., 2012)
Asia	Bangladesh	2006	Buffalo feces	25/174 (14.4)	O157	(Islam et al., 2008)
			Cattle feces	10/139 (7.2)		
			Goat feces	10/110 (9.1)		
			Buffalo feces	41/174 (23.6)	Non-O157	
			Cattle feces	18/139 (12.9)		
			Goat feces	1/110 (0.9)		
	Japan	2000–2001	Cattle feces	97/605 (15.9)	O157 and non-O157	(Fukushima and Seki, 2004)
	Japan	2007–2008	Cattle feces	218/2436 (8.9)	O157	(Sasaki et al., 2011)
				10/2436 (0.4)	O26	
	India	2003	Cattle feces	8/177 (4.5)	O157	(Manna et al., 2006)

	India	1999	Cattle feces	37/206 (17.9)	Non-O157	(Khan et al., 2002)
	Vietnam	2004–2005	Buffalo feces	64/237 (27.0)	O157 and non-O157	(Vu-Khac and Cornick, 2008)
			Cattle feces	29/126 (23.0)		
			Goat feces	79/205 (38.5)		
	Thailand	Not reported	Cattle feces	1/55 (1.8)	O157	(Vuddhakul et al., 2000)

1.4 Pathogenesis and important virulence factors of STEC

The pathogenicity of *E. coli* strains belonging to different pathotypes show considerable variation. Pathogenesis of STEC results from a number of virulence factors. Among them production of Shiga toxin (Stx) is considered as the most important virulence factor (Gyles, 2007), which contributes to the major manifestations of HUS (Tarr et al., 2005). Stx in STEC occurs in two antigenic forms, namely Stx1 and Stx2. Stx1 and stx2 genes are carried by distinct lambda-like temperate bacteriophage, which can be integrated into the host chromosome during lysogeny following phage infection (Allison, 2007). Both Stx1 and Stx2 comprise of different subtypes although Stx1 is a more homologous group with only few variants described so far (Scheutz et al., 2012). Stx2 is a more heterogeneous group compared to Stx1with currently identified subtypes of Stx2a (Stx2), Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g (Scheutz et al., 2012). Incidents of HC and HUS are found to be strongly associated with the possession and expression of Stx2a and its variant Stx2c (Persson et al., 2007, Ethelberg et al., 2004). All members of the Stx family consist of a conserved structure containing two subunits, A and B. The B subunit binds the toxin to a specific glycolipid receptor called globotriaosylceramide (Gb_3) (Mead and Griffin, 1998), which is present on eukaryotic cells such as renal glomerular endothelial, mesangial, and tubular epithelial cells (reviewed in Tarr et al., 2005). Stx is produced in the colon where STEC is attached to the gastrointestinal wall and is transported through the blood stream to the kidney which contains the Gb_3 receptors for toxin attachment (reviewed in Kaper et al., 2004). Once bound, the holotoxin is endocytosed and transported to the endoplasmic reticulum via the Gologi apparatus of the cell (reviewed in Sandvig and Van Deurs, 1994). The A subunit is then translocated to the cytoplasm of the cell where it acts on the 60S ribosomal subunit leading to the inhibition of protein synthesis. Disruption of protein synthesis by the toxin ultimately results in renal endothelial cell damage or damage of any other cells carrying receptors for the toxin (reviewed in Kaper et al., 2004).

Stx production by STEC is not in itself sufficient to cause disease in humans. Several other virulence factors have also been identified to enable the pathogenesis of STEC belonging to the major serogroups including O157. These accessory virulence factors include a 60 MDa plasmid and a chromosomal island (34 kb) called the 'locus for enterocyte effacement' (LEE) (Mead and Griffin, 1998). The plasmid carries the gene *ehxA* that encodes an enterohemolysin, a protein implicated in hemolysis of the red blood cells, which together with specialised transport systems may allow the bacteria cells to use the blood released in to the intestine as a source of iron (Law and Kelly, 1995). The LEE is identified to carry the attaching and effacing (*eaeA*) gene among other virulence genes, which facilitates the attachment of the bacterial cells to the gastrointestinal wall of the host (Nataro and Kaper, 1998).

1.5 Clinical features of STEC infections

The effects of infection caused by *E. coli* of different pathotypes vary significantly. The effects of STEC infections range from non-bloody diarrhoea to more severe cases of HC and HUS occasionally leading to death (Mead and Griffin, 1998). Initial illness is typically associated with abdominal cramps and non-bloody diarrhoea followed by HC over the next 1–2 days in many cases. HC is characterised by the presence of blood in stools (Karmali, 1989). In most outbreaks over 70 % of patients have been reported to develop bloody diarrhoea (Slutsker et al., 1997, MacDonald et al., 1996). The incidence of HC is mostly observed in people above 65 years of age with a 15.0–23.0 % mortality (Griffin and Tauxe, 1991). HUS predominantly affects children under five years of age and develops in 5–10 % of individuals infected with STEC (Griffin and Tauxe, 1991). It is defined by acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia, preceded typically by a bloody diarrheal illness similar to HC (Nataro and Kaper, 1998). In North America and Europe, HUS is the commonest cause of acute renal failure in children (Advisory Committee on the Microbiological Safety of Food, 1995). During the acute phase of the disease, approximately 5 % of HUS patients die. Although

kidneys are frequent targets of HUS infections, some other organs including the central nervous system, lungs and heart may also be affected (Gyles, 2007).

1.6 Global incidence of STEC infections

1.6.1 Outbreaks of STEC O157

The clinical and public health significance of STEC was not appreciated until the year 1982 when STEC O157:H7 was first recognised as a distinct class of human pathogens and a causative agent of food borne disease due to two outbreaks of distinctive gastrointestinal disease, which occurred in Oregon and Michigan, USA (Riley et al., 1983, Wells et al., 1983). The outbreaks affected at least 47 people who consumed hamburger sandwiches at the same fast food chain outlets. This disease was characterised by severe abdominal pain, watery diarrhoea followed by bloody diarrhoea with little or no fever, and was designated as HC. Increased surveillance following the two outbreaks of HC revealed STEC O157 as the etiologic agent of HUS (Karmali et al., 1983). It is believed that STEC O157:H7 is responsible for over 90 % of all HUS cases in industrial countries (Siegler, 1995).

Since the initial reports of STEC O157:H7 infections in 1982, an increasing number of sporadic cases and outbreaks have been reported, especially in the North America and Europe. During 1992–1993 North America experienced its largest STEC O157 outbreak due to consumption of hamburgers from a single fast-food restaurant chain (Karch et al., 1999). It affected 732 individuals with 55 people developing HUS (mostly children) leading to the death of four children. Another large outbreak associated with the consumption of spinach occurred in 2006, affecting multiple states in North America and resulting in high rates of hospitalisation, HUS and death (Centers for Disease Control and Prevention, 2006). In only six years, between 1990 and 1996, 25 outbreaks of *E. coli* O157 have occurred in Scotland affecting more than 700 people (Cowden, 1997). This included the largest European STEC O157 outbreak in central Scotland during 1996 (Karch et al., 1999)**,** which resulted in 272 laboratory-confirmed cases

and 20 deaths (Ahmed, 1997). Although most cases of infection appear to be sporadic, between 2 % and 28 % of infections have resulted from outbreaks (Locking et al., 2003, Michel et al., 1998, Willshaw et al., 2001). The severity of disease outcome from outbreaks has ranged from 3 % hospitalisations with zero deaths (Rodrigue et al., 1995) to 26 % hospitalisations (Pennington, 1998) and 17 deaths (Cowden et al., 2001). High rates of STEC O157 infections have also been reported in South America, especially in Argentina. In Argentina, HUS is endemic (Rivas et al., 2006) with a 5–10 times higher rate of incidence compared to North America (Lopez et al., 1989). STEC O157 infection has also been documented in countries such as Canada, Australia, and South Africa (Nataro and Kaper, 1998).

Outbreaks due to STEC O157 have not been reported in Asian countries except for Japan and China. A massive outbreak of STEC O157 occurred in Sakai city, Japan with 2764 microbiologically confirmed cases and 106 with HUS (mostly children), resulting from the consumption of white radish sprouts (Pennington, 2010). In China a large outbreak of STEC O157 occurred in 1999 with 195 hospitalised patients diagnosed with HUS and 177 deaths (Wang et al., 2004, Xu et al., 2002). Although outbreaks due to STEC O157 was not documented, isolation of STEC O157 from clinical sources was briefly reported in India (Gupta et al., 1992), Hong Kong (Yam et al., 1998) and Korea (Kim et al., 1998).

1.6.2 Outbreaks of non-O157 STEC

To date, STEC O157 has been the primary focus of most STEC prevalence studies due to its initial predominance in human clinical infection (Monaghan et al., 2011). Consequently, the significance of non-O157 serogroups as pathogens in humans is less well understood and underestimated (Huppertz et al., 1996, Johnson et al., 1996). This is partly due to the differences in reporting and health surveillance systems world-wide, the selective development and optimisation of culture and molecular methods for the detection of STEC O157, with little attention to the non-O157 serogroups, and also the lack of a convenient common culture media

to reliably screen for the non-O157 STEC compared to STEC O157. A distinctive feature of STEC O157 is their inability to ferment the sugar D-sorbitol rapidly (within 24 hours) in contrast to about 75–94 % of other STEC strains (Farmer and Davis, 1985, March and Ratnam, 1986). Thus STEC O157 can be reliably detected using sorbitol MacConkey agar, on which they grow as colourless colonies compared to the sorbitol fermenting *E. coli* strains, which grow as pink colonies.

However, with the increase of suitable diagnostic methods for non-O157 serogroups, the STEC strains of non-O157 serogroups are increasingly identified in cases of food borne disease in humans both in outbreaks and sporadic cases (Caprioli et al., 1997, Johnson et al., 1996, Paton et al., 1996, Caprioli et al., 1994). The non-O157 serogroups most commonly associated with human disease include O26, O103, O111, O113, O45 and O145 (Eblen, 2006). In some countries such as Australia (Vally et al., 2012), and Chile (Ojeda et al., 1995) non-O157 serogroups of STEC are found to be responsible for the majority of human STEC infections.

Compared to STEC O157, a relatively few outbreaks of non-O157 STEC are reported world-wide. In 1984 the earliest outbreak of non-O157 STEC occurred in Japan caused by the serotype O145:H^- (Johnson et al., 1996). However, the source of the infection was unable to be determined. Since then a number of outbreaks due to non-O157 STEC have been reported from Japan. The causative agents of most of these outbreaks belonged to the serogroups O26 and O111. Person to person contact was identified as the cause of most of these outbreaks while the vehicle of infection was not identified in the rest (Kasper et al., 2010). The pathogenic potential of non-O157 STEC strains was clearly evidenced following a large community outbreak reported from Australia in 1995 caused by the O111 serogroup due to consumption of uncooked, semidry fermented sausages (Paton et al., 1996). More than 200 individuals were affected with the majority of cases presenting with watery or bloody diarrhoea. HUS developed in 22 children, while adults were diagnosed with thrombotic thrombocytopenic purpura.

Among the STEC characterised so far in Australia, the most common serogroups reported are of non-O157 including the serogroups O111 and O26 (Vally et al., 2012).

In 2008, an outbreak in Oklahoma caused by the serotype O111:H^- affected 341 people at a particular restaurant, although the specific source of infection was not identified (Oklahoma State Department of Health, 2009). In Alberta, Canada, non-O157 STEC serogroups O26, O103, O111 and O145 have been isolated among other non-O157 STEC serogroups (Pai et al., 1988). Non-O157 STEC serogroups were isolated from 29 out of 5415 (0.5 %) patients with diarrhoea with two HUS cases in this study, while the results from recent studies from Canada suggest that non-O157 STEC could be responsible for approximately 7–20 % of HUS cases (Johnson et al., 1996, Rowe et al., 1993). In addition, outbreaks caused by non-O157 STEC have occurred in Denmark (Ethelberg et al., 2009), France (Espié et al., 2006, King et al., 2010), Germany (Werber et al., 2002), Italy (Caprioli et al., 1994), and Norway (Schimmer et al., 2008) due to consumption of ruminant meat.

1.6.3 Annual incidence rates of STEC infections

Annual incidence of STEC infections are reported from a limited number of countries in the world. In the USA, STEC are estimated to cause > 265,000 infections per year with > 3600 hospitalizations and 30 deaths (Scallan et al., 2011). The incidence rate of STEC O157 infection was highest (2.62 per 100,000 population) at the start of surveillance for STEC O157 in 1996 in the USA (Crim et al., 2015). Since then the reported incidence of STEC O157 infection indicated a decreasing trend with a recorded incidence rate of 1.15 per 100,000 population in 2013. However, the incidence rate of non-O157 STEC increased in the USA from 0.19 per 100,000 population up to 1.18 per 100,000 population in 2013 since its surveillance began in the year 2000 (Crim et al., 2015). The concomitant increase in laboratory testing for non-O157 STEC has also contributed substantially to the increased reports of non-O157 infections. In Canada, the incidence of STEC O157 infection indicated a significant decline

from 3.8 cases per 100,000 population in 2002 to 1.39 cases per 100,000 population in 2012 (Public Health Agency of Canada, 2015). The incidence of STEC infections among the countries of the European Union indicated an increasing trend during 2006–2009 with the incidence rates ranging from 0.77 to 0.96 per 100,000 population (European Centre for Disease Prevention and Control, 2012). Ireland reported the highest incidence of STEC infection (4.41 per 100,000 population) in 2010 followed by Sweden (3.58 per 100,000 population) and Denmark (3.22 per 100,000 population) (European Centre for Disease Prevention and Control, 2012). In the UK, between 2009 and 2012, the crude incidence of STEC infection was 1.8 per 100,000 population per year (Byrne et al., 2015). However in the UK, the highest rate of STEC O157 infection has been reported in Scotland with an annual rate of 150–250 cases (Health Protection Scotland, 2010). In Australia, the incidence rate of STEC infection showed a slight increase during the 11 years from 2000–2010, with an annual incidence rate of 0.4 cases per 100,000 population (Vally et al., 2012). Overall, the incidence of STEC infections in Australia is lower in comparison to other developed countries such as USA. The annual incidence rate of STEC infection in New Zealand (4.1 cases per 100,000 population) is among one of the highest recorded (Institute of Environmental Science and Research Ltd, 2014) similar to the incidence of STEC infection observed in Ireland, Denmark, Sweden (European Centre for Disease Prevention and Control, 2014) and Scotland (Locking et al., 2014). One of the highest incidence of HUS in the world due to STEC infection is reported in Argentina with an annual incidence of 17 cases per 100,000 children younger than five years (Rivas et al., 2010). In contrast, the incidence of STEC infection is much lower in Hong Kong with a recorded incidence rate of 0–0.11 cases per 100,000 population between 1998 and 2013 (Centre for Health Protection, 2013).

1.7 Variation in disease severity of STEC infections

Severity of disease caused by pathogenic *E. coli* including STEC differs significantly between regions of one country as well as between different countries. This results from the variation of virulence that exists among the *E. coli* strains owing to their phenotypic and genotypic differences. The population genetics and epidemiology of STEC O157 has changed dramatically over the years resulting in different rates of hospitalisation and HUS incident among different regions of the world. For example, low rates of hospitalisation and HUS incidents were recorded in the large 1996 STEC O157 outbreak in Sakai city, Japan (Fukushima et al., 1999), while a high rate of both hospitalisation (> 50 %) and HUS (> 10 %) was recorded in the 2006 spinach outbreak in North America caused by STEC O157 (Centers for Disease Control and Prevention, 2006). In fact, the strain involved in the spinach outbreak and the strain involved in the Sakai outbreak were categorised by Manning et al. (2008) into two distinct clades, clade 8 and clade 1 respectively. In this study > 500 clinical strains of STEC O157 were separated into nine distinct clades based on variation of bacteriophages using a molecular subtyping method called 'Single Nucleotide Polymorphism' (SNP). SNP is widely used as a strain subtyping method, which can resolve variation to as much as a single nucleotide change in the genome of various STEC strains. Among the clades of STEC O157 identified, differences in the frequency and distribution of *stx* genes and clinical manifestations were observed (Manning et al., 2008). Greater virulence was observed in strains belonging to clade 8, which were found to frequently carry stx_2 and stx_{2c} and to be associated with HUS. Moreover, this study indicated that the disease caused by clade 8 strains has increased in frequency over the years, while the overall national prevalence of STEC O157 in the USA has decreased. Although previous studies have indicated a correlation between specific *stx* genes and disease, stx_2 and stx_{2c} in particular, greater virulence of clade 8 strains could not be attributed to the presence of both stx_2 and stx_{2c} genes, since not all clade 8 strains carried both genes.

In a similar manner to STEC O157, the population genetics as well as the epidemiology of non-O157 has also changed over the past years. The increase in non-O157 STEC outbreaks and sporadic cases and its geographical spread allows the diversification of non-O157 genotypes similar to the STEC O157, leading to the change in non-O157 epidemiology. In fact, between the year 2000 and 2005, the human disease caused by non-O157 showed a global increase of 60.5 % compared to STEC O157, which indicated only a 13 % increase (Anonymous, 2005). However, development of specific methods for the detection of non-O157 STEC over the years and the increase in testing and reporting its incidence could partly contribute to the increase observed in the disease caused by non-O157 STEC. Moreover the relative frequency of isolation of certain non-O157 serogroups from human disease has varied over the years. For example, as mentioned before, the most frequently isolated non-O157 STEC serotypes from patients with diarrhoea and HUS in Germany between 1994 and 1999 were O26:H11/H^- in contrast to serogroups O103 and O145, which were not initially detected but have emerged in the later years of the study (O103 since 1996 and O145 since 1997) (Bielaszewska and Karch, 2000). Compared to outbreaks caused by STEC O157, the variation in disease severity between the non-O157 outbreaks has not been studied extensively. However, the data on non-O157 outbreaks indicate that disease severity caused by a single non-O157 serogroup could vary between countries. For example, the STEC O111 outbreak observed in Australia in 1995 affected a high number of people resulting in 23 HUS cases and one death compared to other outbreaks of serogroup O111 observed in other countries such as the O111 outbreak occurred in Spain in the same year, which was less severe with only 13 people affected with no cases of HUS or deaths (Kasper et al., 2010). It is likely that variation of virulence determinants within a single serogroup of non-O157 STEC is causing the epidemiological differences as well as variation in the rates of hospitalisation and HUS as is observed with STEC O157.

The frequency and severity of STEC related disease has been shown to vary between countries and this has been attributed to genotypic and phenotypic differences between the STEC strains (Sahilah et al., 2010). Characterisation of STEC O157 and non-O157 populations is therefore important to establish the genetic diversity and clonal relatedness among STEC strains, as well as to detect emergence of highly virulent STEC strains, which may contribute to severe disease outbreaks.

1.8 STEC infections in Malaysia

In Malaysia, there are no reported outbreaks or sporadic cases of STEC infections caused by either O157 or non-O157 serogroups similar to most of the Asian countries including India, Hong Kong, Korea and Thailand. However, STEC O157 has been isolated from beef samples (Sukhumungoon et al., 2011) as well as clinical samples (Radu et al., 1996) from Malaysia. The lack of reported STEC infections in most Asian countries including Malaysia could be due to many factors: the absence of an adequate surveillance system and recording of disease caused by STEC; the absence of highly virulent STEC strains or the lack of virulent STEC strains transmission to humans due to differences in environmental factors, animal management practices and cooking practices. In Malaysia, there is a substancial deficit in the epidemiological data on STEC O157 and non-O157 serogroups in ruminanats. Since ruminanats are considered the major reservoir of STEC it is important to examine the presence or absence of STEC in ruminanats and analyse their characteristics to assess their virulence and potential threat to humans to understand the conditions for the lack of reported STEC infections in Malaysia.

The present study aimed to examine the presence of *E. coli* O157 and serogroups of *E. coli* non-O157 namely O26, O103, O11, O121, O145 and O45 in ruminant feces in Peninsular Malaysia, and further characterise the isolated strains of *E. coli* O157 and non-O157 based on their genetic diversity, presence of virulence factors and phenotypic properties. Determination

of prevalence and virulence characteristics of STEC serogroups in Malaysia would enable the assessment of potential virulent strains to humans or the lack there of. In addition, this study would aid in filling the existing knowledge gap on the epidemiology of STEC infections in Malaysia and how it influences the variation of STEC incidence as well as its disease severity between different geographical locations.

2.0 Research objectives

The overall aim of this project was to gain an understanding of the variation among *E. coli* O157 and non-O157 strains from ruminants in Malaysia and its influence on the differences in disease severity based on their pathogenicity, antibiotic resistance and survival under stress conditions.

The research objectives for this project were as follows:

(1) To isolate STEC O157 and non-O157 from ruminant feces in Malaysia and to assess their genotypic and phenotypic variation (Chapter 2).

(2) To determine antibiotic resistance among *E. coli* (both STEC and non-STEC) strains from ruminants in Malaysia (Chapter 3).

(3) To analyse *stx* phage insertion sites of non-O157 STEC from Australia and Malaysia (Chapter 4).

(4) To examine the effect of sub-lethal heat, cold and acid adaptation stress on the survival of *E. coli* under strong acidic conditions (Chapter 5).

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Devised the experimental design, conducted the experiment, analysed the results and drafted the manuscript	60

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Prof. Gary Dykes	Provided ideas and advice for the experiments and edited the manuscript	
Dr. Narelle Fegan	Provided ideas and advice for the experiments and edited the manuscript	
Dr. Charles Clarke	Provided ideas and advice for the experiments	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 30 September 2015
Main Supervisor's Signature	[redacted]	**Date** 30 September 2015

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 2

Characterisation of Shiga toxigenic *Escherichia coli* (STEC) O157 and non-O157 strains from ruminant feces in Malaysia

Article accepted for publication by Biomed Research International on February, 2015

(This chapter appears directly as accepted for publication)

Part of the results of this chapter was presented at the Australian Society for Microbiology (ASM) Annual Scientific Meeting (2013) as a poster

2.1 Introduction

Shiga toxin producing *E. coli* (STEC), a serologically diverse group of zoonotic pathogens, have emerged as one of the most virulent groups of bacteria associated with cases of food borne disease in humans (Gyles, 2007). STEC can cause a spectrum of diseases ranging from mild diarrhoea to severe bloody diarrhoea, called hemorrhagic colitis (HC), and even life-threatening sequelae such as hemolytic uremic syndrome (HUS). Patients with HUS were often diagnosed as having thrombotic thrombocytopenic purpura (TTP), although thrombotic microangiopathy is now considered a more accurate description of the condition associated with HUS caused by STEC (Tarr et al., 2005). Production of Shiga toxin (Stx) is considered as the major virulence factor of STEC (Gyles, 2007), which contributes to the development of HUS in humans (Tarr et al., 2005). Stx production alone is not sufficient for STEC to cause disease. Accessory virulence factors include a 34 kb chromosomal pathogenicity island called the 'locus for enterocyte effacement' (LEE) carrying several virulence associated genes, such as the attaching and effacing (*eaeA*) gene, and a large plasmid (60MDa) with an *ehxA* gene encoding an enterohemolysin. *EaeA* encodes an outer-membrane protein called intimin, which enables the intimate adherence of STEC to the intestinal epithelium of the host (Nataro and Kaper, 1998). The enterohemolysin protein is implicated in extracting iron from the blood released into the intestine (Law and Kelly, 1995).

The prototype STEC serotype is *E. coli* O157:H7 and its ability to cause HC and HUS in many regions and countries is well established. The pathogenic potential and public health significance of several non-O157 STEC serogroups, particularly O26, O103, O111, O121, O145 and O45 referred to as the 'big 6' non-O157 STEC serogroups (Bosilevac and Koohmaraie, 2012), has also been described in recent years due to their association with clinical HC and HUS in humans. In some geographical areas, such as in Europe, the disease

caused by non-O157 strains is significantly more common than that caused by O157:H7 (Bielaszewska et al., 1994, Caprioli and Tozzi, 1998).

Ruminants are considered an important source of both *E. coli* O157 and non-O157 with cattle being identified as the primary reservoir. Intestinal carriage of *E. coli* O157 and non-O157 in ruminants results in their fecal shedding and release into the environment. As a result infections of *E. coli* O157 and non-O157 can be transmitted to humans via the consumption of food and water contaminated by animal feces.

Data on *E. coli* O157 and non-O157 serotypes in ruminants is limited in countries of the tropical regions including Malaysia. In addition the data reported so far on *E. coli* O157 and non-O157 in ruminants from tropical countries other than Malaysia demonstrates substantial variation in their prevalence and virulence properties. In West Bengal, India, a total of 14 STEC O157 strains were obtained from two (2.0 %) slaughtered cattle feces samples and six (7.6 %) diarrhoeic calf feces samples (Manna et al., 2006). The majority of STEC O157 strains (85.7 %) obtained from this study carried *stx*$_2$ alone. STEC O157 was obtained from 0.6 % of cattle feces samples in Brazil, (Irino et al., 2005) where the majority of strains carried *ehxA* either with both *stx*$_1$ and *stx*$_2$ or *stx*$_2$ alone. The prevalence of *E. coli* O157 was found to be 1.3 % in cattle farms in central Mexico (Callaway et al., 2004). Non-O157 STEC was found in 17.9 % of cattle feces samples in Calcutta, India (Khan et al., 2002), in which *stx*$_1$ predominated. In another study in Brazil, non-O157 STEC was isolated from 5.8 % of calf feces samples (Leomil et al., 2003) where *stx*$_1$ was the dominant *stx* genotype observed.

Only three studies, which isolated STEC O157 from beef samples have to our knowledge been conducted in Malaysia (Apun et al., 2006, Radu et al., 1998, Sukhumungoon et al., 2011). Apart from a single study, which reported sporadic cases of STEC O157 infection among 14 % of patients presented with bloody diarrhoea at a local hospital in Kuala Lumpur, Malaysia (Radu et al., 1996), there are no other published reports of sporadic cases or outbreaks of STEC

O157 and non-O157 in the country. Although studies have demonstrated the presence of STEC O157 in foods of animal origin, the presence and characterisation of STEC O157 or non-O157 in ruminant feces from Malaysia has not yet been determined.

The aim of the present study was to examine ruminant feces samples for the presence of STEC O157 and the 'big 6' non-O157 STEC serogroups in Malaysia. The isolated strains of *E. coli* O157 and non-O157 were further characterised to determine their genetic diversity and presence of virulence factors to indicate the risk potential of these strains to public health.

2.2 Materials and methods

2.2.1 Sample collection and preparation

Samples were collected from six different ruminant farms in Peninsular Malaysia (Table 2.1). The geographical distribution of the six farms are depicted in Figure 2.1. Farms A, C, and F were small dairy cattle farms, while farm E was a small dairy farm consisting of cattle and goats. Farm B was also a dairy farm but with a larger number and diversity of ruminants consisting of cattle, buffaloes, goats and sheep. Farm D was a large beef cattle farm. A total of 136 fresh ruminant feces samples (~25 g each) from individual cattle, buffalo, sheep and goat were collected from the pen floors (over a period of six months) into sterile containers and were stored at 4°C on ice until processed in the lab on the same day. All feces samples collected were divided into two 10 g samples. One of the 10 g samples was used for enrichment and the other was used for long term storage in tryptone soya broth (TSB; Merck, Darmstadt, Germany) with 25 % glycerol at −70°C.

Table 2.1. Distribution of ruminant feces samples collected from farms A–F

Farm	Location	Ruminant feces samples				Total samples
		Cattle	Buffalo	Goat	Sheep	
A	Serdang	25	–	–	–	25
B	Kluang	9	20	7	8	44
C	Sentul	9	–	–	–	9
D	Gemas	24	–	–	–	24
E	Puchong	13	–	5	–	18
F	Lumut	16	–	–	–	16

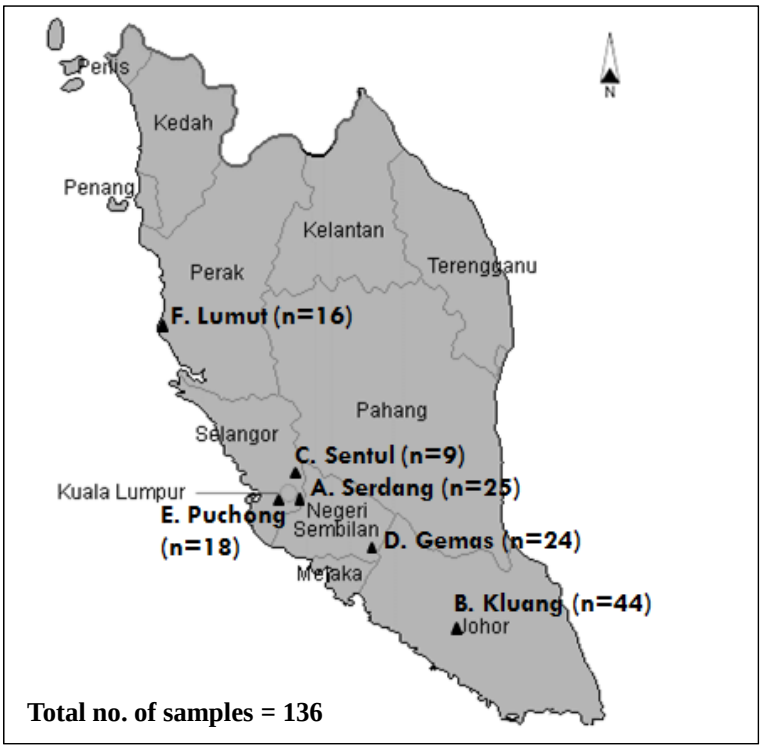


Figure 2.1. Geographical distribution of farms A–F in Peninsular Malaysia, from which the ruminant feces samples were collected.

2.2.2 Isolation and characterisation of *E. coli* O157

Each feces sample (10 g) was diluted 1/10 in buffered peptone water (BPW; Oxoid, Hampshire, UK) and homogenized for 30 s. Samples were incubated for 18 h at 37°C without agitation. Immunomagnetic separation (IMS) was performed using Dynabeads anti-*E. coli* O157 (Dynal, Oslo, Norway) according to the manufacturer's instructions. Resulting bead-bacteria complexes were spread on to sorbitol-MacConkey agar (SMAC; Oxoid, Hampshire, UK) and sorbitol-MacConkey agar containing the cefixime, tellurite supplement (CT-SMAC; Oxoid, Hampshire, UK) and incubated for 18 h at 37°C. A total of 10 presumptive *E. coli* O157 colonies per sample were serotyped using an *E. coli* O157 Latex Test Kit (Oxoid, Hampshire, UK). All strains agglutinating with the O157 antiserum were further characterised by polymerase chain reaction (PCR) to detect the presence of *rfbE*, *stx*$_1$, *stx*$_2$, *eaeA*, *ehxA* and *fliC* genes using primers and reaction conditions as previously described (Bai et al., 2010).

Characterisation of lineage-specific polymorphisms–6 (LSPA–6) of STEC O157 strains was performed using target amplification and capillary electrophoresis as described previously (Yang et al., 2004, Whitworth et al., 2010). An Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, California, USA) with a DS-33 matrix and GeneScan 600 LIZsize standard was used for capillary electrophoresis, while a Peak Scanner software (Version 1.0; Applied Biosystems, California, USA) was used to interpret amplicon sizes. LSPA–6 alleles were defined according to (Yang et al., 2004). Strains with LSPA–6 genotype 111111 or 211111 were classified as lineage I (LI) or lineage I/II (LI/II), respectively, while all other allele combinations were grouped as lineage II (LII) (Yang et al., 2004, Zhang et al., 2007).

Analysis of Shiga toxin encoding bacteriophage insertion sites (SBI) of STEC O157 strains was determined as previously described (Shringi et al., 2012b).

2.2.3 Detection, isolation and characterisation of non-O157 *E. coli*

Samples (10 g), which were initially stored at −70°C in TSB with 25 % glycerol were diluted 1/10 in BPW, homogenized for 30 s and incubated for 18 h at 37°C without agitation. DNA was extracted from 1 ml of the enriched sample using the Nucleospin Soli DNA extraction kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. A multiplex PCR was used to screen enrichments for the presence of STEC virulence genes *stx*$_1$, *stx*$_2$, *eae*A and *ehx*A using primers and reaction conditions as described by Paton and Paton (Paton and Paton, 1998) with several modifications. A reaction volume of 25 µl was used with 2 µl of DNA template and final concentration of 0.25 µM of each primer, 5 x Green GoTaq® Flexi Buffer (Promega, Madison, USA), 200 µM of dNTP, 2 mM of $MgCl_2$ and 1 unit of GoTaq® DNA polymerase (Promega, Madison, USA). The PCR products were separated by electrophoresis on a 2 % (wt/vol) agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized under UV light. Enriched samples positive for *stx* and *eaeA* by PCR were streaked on chromocult-TBX agar (Merck, Darmstadt, Germany) and coliformen agar enhanced selectivity (Merck, Darmstadt, Germany) and incubated overnight at 37°C. Following incubation, up to 50 *E. coli* colonies per sample were chosen based on colony morphology, extracted DNA and screened individually by multiplex PCR for the presence of *stx*$_1$, *stx*$_2$, *eae*A and *ehx*A as described above. Colonies that were positive for *stx* and *eae*A were then tested for the 'big 6' *E. coli* non-O157 serogroups by PCR using primers and conditions described previously (Bai et al., 2010, Paddock et al., 2012).

The enriched samples were also tested for the presence of genes specific to the 'big 6' *E. coli* non-O157 serogroups. Samples that tested positive by PCR for any of the target serogroups were subjected to IMS for O26, O111, O103 and O145 using Dynabeads (Dynal, Oslo, Norway) following the manufacturer's instructions. The bead-bacteria complexes formed during IMS of O26 were plated onto rhamnose MacConkey agar, while those of O111, O103

and O145 were plated onto chromocult-TBX agar and coliformen agar-enhanced selectivity, and incubated overnight at 37°C. Following incubation, ten presumptive colonies (per sample) based on colony morphology were subjected to serogroup specific PCR and those confirmed as a specific serogroup were tested by PCR for the presence of STEC virulence genes. Isolation of serogroups O45 and O121 was performed on enriched feces samples positive for STEC virulence markers, which were directly plated onto chromocult-TBX agar as described above.

2.2.4 Biochemical confirmation of *E. coli* strains

All the strains were biochemically identified as *E. coli* by citrate utilisation and indole production tests (Aslanzadeh, 2006).

2.2.5 Bacterial strains

The bacterial strains used as controls in this study are listed in Table 2.2.

2.2.6 Pulsed-field gel electrophoresis (PFGE)

PFGE using *Xba*I was performed on all *E. coli* O157 and non-O157 strains in a CHEF Mapper (Bio-Rad, California, USA) according to the standardized PulseNet protocol (Environmental Science and Research Ltd, 2013). Banding patterns were analysed using BioNumerics software, version 6.5 (Applied Maths BVBA, Sint-Martens-Latem, Belgium) following the PulseNet protocol.

2.2.7 Subtyping of *stx* and intimin (*eaeA*) genes of *E. coli* O157 and non-O157

The subtypes of *stx* and *eaeA* in strains carrying these markers were determined following previously published methods (Scheutz et al., 2012, Blanco et al., 2004a).

2.2.8 Detection of Shiga toxin expression

Stx expression by the STEC strains were determined according to the method adapted from Shringi et al. (Shringi et al., 2012a) using an ELISA kit (Premier EHEC, Meridian Bioscience,

Ohio, USA). Mitomycin C (Sigma Aldrich, Missouri, USA) was used at a final concentration of 0.5 µg/ml to induce Stx production. After induction, the cells were lysed using Polymixin B (Sigma Aldrich, Missouri, USA) at a final concentration of 0.5 mg/ml and incubated at 37°C for 1 h with rotary shaking (250 rpm). Polymixin B treated cultures were diluted 1:100 in sterile LB (Luria-Bertani) broth immediately followed by 1:2 dilution in sample diluent of the ELISA kit. Absorbance readings were obtained at wavelengths 450 nm and 630 nm using a Victor X microtiter plate reader (Perkin Elmer, Glen Waverley, Australia) and the results were displayed as the mean value of two independent biological replicates.

Table 2.2. Bacterial strains used in the study

Strain ID	Serogroup	Source	Country	Virulence traits
Sakai	O157	Radish sprouts	Japan	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
ATCC 43895	O157	Ground beef	USA	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
EC543a	O157	Cattle feces	Australia	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
EC6a	O157	Cattle feces	Australia	*stx*$_2$, *eaeA*, *ehxA*
1 UPM[a]	O157	Bovine milk	Malaysia	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
2 UPM[a]	O157	Bovine milk	Malaysia	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
3 UPM[a]	O157	Beef	Malaysia	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
4 UPM[a]	O157	Beef	Malaysia	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
MG1655 (*E. coli* K–12)	OR:H48:K-[b]	Laboratory strain	USA	none
EC3008a[c]	O26	Cattle feces	Australia	*eaeA*
EC3009a[c]	O45	Cattle feces	Australia	none
EC2998a[c]	O103	Cattle feces	Australia	none
EC3113a[c]	O111	Cattle feces	Australia	none
EC3111a[c]	O121	Cattle feces	Australia	none

[a]Provided by Prof. Son Radu at University Putra Malaysia (UPM).
[b]OR = O antigen rough strain, which does not produce the O antigen.
[c]*E. coli* non-O157 strains used as controls in the study, provided by Lesley Duffy at CSIRO, Brisbane, Australia.

2.3 Results

2.3.1 Presence of STEC O157 and virulence factors

STEC O157 was isolated from six (4.4 %) cattle feces samples, all of which were from farm A (Table 2.3). A total of 32 STEC O157 strains were obtained from six different cattle feces samples. The strains obtained were clustered into two different PFGE groups (at > 92 % similarity) with the majority of strains (28 strains from five different feces samples) belonging to one PFGE group and the remaining strains (four strains from a single feces sample) belonging to the other. All 32 STEC O157 strains were positive for the virulence factors *stx*$_{2,}$ *eaeA* and *ehxA* and also for *fliC* specific for the H7 antigen indicating they belong to the O157:H7 genotype. All samples from farms B–F were negative for the presence STEC O157.

LSPA–6 target amplification indicated that all the STEC O157:H7 strains collected from cattle feces samples in farm A belong to lineage II (Table 2.3). According to the SBI genotyping code, genotype SY2c was observed in all STEC O157:H7 strains collected from cattle feces samples in farm A indicating the association of *stx*$_{2c}$ with prophage insertion in the *sbcB* locus (Table 2.3).

In addition, all STEC O157:H7 strains obtained from UPM carried the virulence markers *stx*$_{1,}$ *stx*$_{2,}$ *eaeA* and *ehxA* and belonged to a single PFGE group (at > 92 % similarity) (dendogram indicating the PFGE band patterns of all the *E. coli* O157 strains obtained in this study is shown in Appendix 1). They were of lineage I and contained the SBI genotype WY12 indicating the association of *stx*$_1$ and *stx*$_{2a}$ with prophage insertion in the *yehV* and *wrbA* loci respectively.

Table 2.3. STEC O157 and non-O157 and their virulence profiles

STEC serogroup	Number of STEC+ samples (%)	Source	Number of strains	Virulence factors	Lineage	SBI profile
O157:H7[a]	6 (4.4 %)	Cattle feces	28[b]	*stx*$_{2c}$, *eaeA*-γ1, *ehxA*	II	SY2c
			4[b]	*stx*$_{2c}$, *eaeA*-γ1 *ehxA*	II	SY2c
non–O157[a] (unknown)	2 (1.5 %)	Cattle feces	1	*stx*$_{1a}$, *stx*$_{2a}$, *stx*$_{2c}$, *ehxA*	–	–
			1	*stx*$_1$	–	–

[a]Strains of STEC O157:H7 and non-O157 were only present in samples obtained from farm A. All samples from farms B–F were negative for STEC O157:H7 and non-O157 strains.
[b]In farm A, 28 of the STEC O157 strains belonged to one PFGE group (at > 92 % similarity) and the remaining four strains belonged to another PFGE group.
– Not applicable.

2.3.2 Presence of non-O157 STEC and virulence factors

In the initial PCR screen of the enriched samples, various combinations of virulence markers and genes for the target non-O157 serogroups were observed in all the farms except in farm F. Although samples in farm F were positive for different combinations of virulence markers, none of the samples were positive for any of the target non-O157 serogroups tested (Table 2.4). Overall, the combination of *stx* (either *stx*$_1$, *stx*$_2$ or both) and *eaeA* was present in 32.3 % (44 samples out of 136 samples), while the gene indicating the presence of serogroup O103 seemed to be predominant (44.1 % of samples) among all 136 samples.

Although the initial PCR screening of the enriched feces samples indicated a relatively high number of samples with the target genes for the virulence factors and non-O157 serogroups, only two samples (1.5 %) yielded non-O157 STEC strains (Table 2.3). Both of these were cattle feces samples collected from farm A, from which two non-O157 STEC strains (negative for

any of the ‘big 6’ non-O157 serogroups) were isolated, which belonged to two unique PFGE groups (dendogram indicating the PFGE band patterns of all the *E. coli* non-O157 strains obtained in this study is shown in Appendix 2). One of the two non-O157 STEC strains was positive for *stx*$_{1,}$ *stx*$_2$ and *ehxA* while the other strain was positive for *stx*$_1$ alone.

Table 2.4. Occurrence of target virulence factors and 'big 6' non-O157 serogroups in the initial PCR screen of the enriched samples from each farm (A–F)

Farm	No. of samples tested	Percent positive for virulence gene combinations[a]								Percent positive for serogroups[a]					
		stx$_1$, *stx*$_2$, *eaeA*, *ehxA*	*stx*$_1$, *stx*$_2$, *ehxA*	*stx*$_1$ or *stx*$_2$, *eaeA*, *ehxA*	*stx*$_1$, *ehxA*	*stx*$_2$, *ehxA*	*eaeA*, *ehxA*	*eaeA*	*stx*$_1$ or *stx*$_2$ alone	O111	O26	O121	O145	O45	O103
A	25	6.6	2.9	0	0	1.5	0	0	0	0	0	5.9	0	3.7	5.1
B	44	13.2	2.9	0	0	0	0	0	0	0.7	9.6	13.2	0	2.2	16.2
C	9	2.9	0	0	0	0.7	2.2	0.7	0	0	2.9	0.7	1.5	3.7	5.1
D	24	1.5	4.4	1.5	0	0	0.7	0	2.9	0	8.1	0	0.7	0.7	10.3
E	18	5.9	2.9	0.7	0	0	0	0	0	3.7	4.4	0.7	0.7	8.1	7.4
F	16	0	2.2	0	1.5	0	0	0	0	0	0	0	0	0	0
Total	136	30.1	15.4	2.2	1.5	2.2	2.9	0.7	2.9	4.4	25	20.6	2.9	18.4	44.1

[a]The percentage of samples positive were calculated by dividing the number of positive samples for each category in the initial PCR screen by the total number of samples (n=136) collected.

2.3.3 Characterisation of *E. coli* serogroups lacking *stx*

E. coli of the target serogroups (O157 and the 'big 6' non-O157) lacking *stx* but carrying other combinations of virulence markers were also isolated from ruminant feces samples (Table 2.5). These included *E. coli* of serogroups O157, O103 and O26, which were negative for any of the virulence markers, O157 which carried *eaeA* alone and O26 with *eaeA* and *ehxA*.

Table 2.5. Isolation and virulence profiles of *E. coli* O157 and 'big 6' *E. coli* non-O157 serogroups lacking *stx*

Serogroup	Farm	Source	No. of + samples	No. of strains tested	Virulence Factors	Intimin Subtype
O103	A	Cattle feces	3	3	none	–
O157	B	Cattle feces	1	2	*eaeA*	NT[a]
		Sheep feces	2	3	*eaeA* (1 strain) none (2 strains)	NT
		Buffalo feces	3	5	*eaeA*	NT
O26	B	Buffalo feces	2	5	*eaeA*, *ehxA* (2 strains) none (3 strains)	*eaeA*-β*1*
O103	B	Buffalo feces	1	7	none	–
O26	C	Cattle feces	2	11	none	–

[a]NT = non-typable.
–Not applicable.

2.3.4 *stx* and *eaeA* subtyping

All the STEC O157:H7 strains collected from cattle feces samples in farm A were positive for *stx*$_{2c}$ (Table 2.3), while all STEC O157:H7 strains from UPM were positive for *stx*$_{1a}$ and *stx*$_{2a}$. One of the non-O157 STEC isolates was positive for *stx*$_{1a}$, *stx*$_{2a}$ and *stx*$_{2c}$, while the other strain was positive for *stx*$_{1a}$ alone (Table 2.3). Overall, *stx*$_{2c}$ was the more prevalent genotype among the *stx*$_2$ positive strains.

Two different *eaeA* subtypes out of the seven *eaeA* variants (α1, α2, β1, β2, γ1, γ2/θ and ε) tested were present among the *eaeA*-positive strains of *E. coli* O157 and non-O157. The STEC O157:H7 strains were positive for *eaeA*-γ1 (Table 2.3) while the two O26 strains lacking *stx* were positive for *eaeA*-β1 (Table 2.5).

2.3.5 Shiga toxin production

Stx production of all the *stx*$_{2c}$ positive STEC O157:H7 strains collected from the cattle feces samples in farm A were below the level of detection. In contrast, all the *stx*$_{1a}$ and *stx*$_{2a}$-positive STEC O157:H7 strains obtained from UPM produced a high amount of Stx similar to the positive control STEC O157:H7 strains, ATCC 43895, EC543a and EC6a. Of the two non-O157 STEC strains, Stx production of the *stx*$_{1a}$ positive non-O157 strain was also below the level of detection. However, the *stx*$_{1a}$, *stx*$_{2a}$ and *stx*$_{2c}$-positive non-O157 STEC strain indicated a moderate amount of Stx production although lower than that observed for the UPM STEC O157:H7 strains.

2.4 Discussion

In Malaysia, no studies have been conducted so far to characterise STEC O157 or non-O157 in ruminant feces. Thus the goal of the present study was to gain insight on the virulence determinants of STEC O157 and non-O157 present in ruminant feces in Malaysia.

In this study STEC O157 was isolated from six (4.4 %) ruminant feces samples and non-O157 STEC was isolated from two (1.5 %) of the ruminant feces samples. Several other authors have also reported low isolation rates (less than 10 %) of STEC O157 and non-O157 in ruminant feces in tropical countries (Manna et al., 2006, Leomil et al., 2003). However, this study was not adequate to determine the prevalence of STEC O157 and non-O157 in Malaysia and thus, to obtain more comprehensive data on the prevalence of STEC O157 and non-O157 serogroups in Malaysia, sampling of a wider geographical area within Malaysia should be undertaken.

E. coli O157 populations have been shown to vary in their distribution among bovine and clinical sources due to their genotypic differences (Kim et al., 2001). LSPA–6 analysis, a simple multiplex PCR assay, categorizes *E. coli* O157 strains into three different genotypes referred to as, lineage I, lineage I/II and lineage II. Strains of lineage I and I/II are mostly associated with human clinical sources while lineage II strains are mostly associated with bovine sources (Yang et al., 2004). In this study, all the STEC O157:H7 strains belonged to lineage II in contrast to STEC O157 strains from countries such as Australia and USA where lineage I/II and lineage I predominates (Mellor et al., 2013). Interestingly, all the STEC O157:H7 strains from UPM were of lineage I indicating the presence of STEC O157 strains of both lineage I and II in bovine sources in Malaysia. STEC O157 strains of lineage II are shown to be less virulent and possibly impaired in their transmissibility to humans compared to lineage I or I/II (Leopold et al., 2009). The presence of STEC O157 strains of lineage II in ruminants in Malaysia from this study suggests that these strains could have less pathogenic potential in humans.

Pathogenic potential of STEC strains have also been shown to be associated with the presence of particular *stx* genotypes. *E. coli* strains carrying stx_1 or stx_{2c} are associated with low virulence potential compared to, those which carry stx_2 (stx_{2a}) (Kawano et al., 2008). In this study all the STEC O157:H7 strains obtained carried stx_{2c} indicating low virulence potential in humans

compared to the STEC O157:H7 strains from UPM with *stx*$_{1a}$ and *stx*$_{2a}$. One of the two non-O157 STEC strains of unknown serogroup with *stx*$_{1a,}$ *stx*$_{2a}$ and *stx*$_{2c}$ indicated a high pathogenic potential compared to the other isolate with *stx*$_{1a}$ alone.

Not all *E. coli* strains carrying *stx* produce Stx (Koitabashi et al., 2006). This was true for all *stx*$_{2c}$-positive STEC O157:H7 strains and one of the non-O157 strains positive for *stx*$_{1a}$ obtained in this study. In contrast the UPM STEC O157 strains produced Stx. Although the exact reasons for the discrepancy observed in Stx production of *stx*-positive *E. coli* strains from this study is not fully understood, previous studies have also identified *E. coli* strains positive for *stx* but negative for Stx production (Koitabashi et al., 2006, Koitabashi et al., 2008). In fact, the study by Koitabashi et al. (Koitabashi et al., 2008) suggested that *stx*$_2$-positive *E. coli* O157 strains that produce little or no Stx2 may be widely distributed in the Asian environment.

Particular *stx* genotypes of STEC O157 have been shown to be associated both with particular SBI genotypes and with their relative frequency of isolation from clinical and bovine sources (Shringi et al., 2012b). Clinical strains are generally characterized by the carriage of *stx*$_2$ and *stx*$_2$ -associated bacteriophage sequences adjacent to either *wrbA* or *argW* (SBI genotypes: WY12, AY2, ASY2, ASY22c), while bovine strains are characterized by carriage of *stx*$_{2c}$ and *stx*2c-associated bacteriophage sequences adjacent to *sbcB* (SBI genotypes: SY2c, SY12c, ASY12c). In agreement with these observations the STEC O157 strains obtained from cattle feces from this study carried *stx*$_{2c}$ with an occupied *sbcB* locus (SY2c). However, the STEC O157 from UPM, which were collected from bovine sources carried *stx*$_2$ and an occupied *wrbA* locus indicating characteristics of clinical strains.

All the STEC O157:H7 strains in this study and the STEC O157:H7 strains from UPM carried *eaeA*-γ1 as reported for *eaeA*-positive *E. coli* O157 in previous studies (Blanco et al., 2004a, Ramachandran et al., 2003). None of the *eaeA* positive non-STEC O157 could be subtyped using the primers for *eaeA* subtypes α1, α2, β1, β2, γ1, γ2/θ and ε. It is possible that these

strains belonged to other intimin subtypes such as δ/κ, ζ, η, ι, λ, μ, ν, which were not tested for in this study. The two *eaeA*-positive *E. coli* O26 strains carried *eaeA-β*1 similar to several other *E. coli* O26 strains previously associated with human STEC strains that cause HUS (Blanco et al., 2004a).

2.5 Conclusion

Despite the use of specific and sensitive methods of enrichment and IMS followed in this study to isolate STEC O157 and non-O157, it appears that the presence of both STEC O157 and non-O157 in ruminant feces was low (4.4 % and 1.5 % respectively). The *stx*$_{2c}$ carrying STEC O157:H7 strains of lineage II from this study suggests that these bacteria potentially represent a less pathogenic clone of STEC O157 in Malaysia. This together with the presence of STEC O157 and non-O157 in a small percentage of ruminants in this study, could contribute to the reasons for the lack of reported sporadic cases and outbreaks caused by STEC O157 in Malaysia. Similar to STEC O157, the low percentage of non-O157 STEC strains observed together with their low pathogenic potential indicated by the lack of *eaeA* and moderate to no Stx production suggests a low probability of causing disease in humans.

Chapter 3

Patterns of antibiotic resistance and resistance gene determinants in commensal *Escherichia coli* (*E. coli*) isolated from ruminants in Peninsular Malaysia

3.1 Introduction

Antibiotic resistance of bacterial strains are an increasing problem for the management of infectious diseases worldwide. In addition to their use in human medicine, antibiotic agents are extensively used in veterinary medicine for prophylaxis and metaphylaxis. They may also be incorporated into the feed of food-producing animals at sub-therapeutic doses for growth promotion in some geographical regions. Many of the antibiotic agents that are used to treat animals belong to the same families of antibiotic compounds used to treat disease in humans (Witte, 1998). When antibiotics are used to target pathogenic bacteria, a simultaneous selection pressure is exerted on other commensal enteric bacteria in both humans and animals encouraging the development of antibiotic resistance in these bacteria. The practise of antibiotic use in animals is believed to enhance the selection of resistant bacteria to a greater extent than the therapeutic use of antibiotics in clinical disease (van den Bogaard et al., 2001). Emergence of antibiotic resistance bacteria in food–producing animals poses a threat to food safety due to the risk of these bacteria entering the food chain and subsequent dissemination to humans.

E. coli is a commensal enteric organism found in humans as well as animals, and which is also commonly involved in opportunistic infections. In recent years, an increase of *E. coli* resistance to major classes of antibiotics used in the treatment of livestock and companion animals has been reported from surveillance studies conducted in different countries (Kadlec and Schwarz, 2008, Lanz et al., 2003). It has been suggested that the reservoir of antibiotic resistance determinants among commensal *E. coli* may substantially contribute to the spread of antibiotic resistance to pathogenic bacteria in animals and/or humans. In addition, the potential for spread of *E. coli* clones between humans and different animal hosts has been documented previously (Johnson et al., 2008). The presence and dissemination of antibiotic resistant *E. coli* clones is therefore of particular concern to both animal and public health alike.

The persistence of antibiotic resistance among bacteria has been shown to occur even in the absence of selective pressure. This highlights the importance of elements associated with antibiotic resistance. Horizontal gene transfer is considered as the major route of dissemination of antibiotic resistance genes among bacterial strains. The process of horizontal gene transfer is facilitated by mobile genetic elements such as plasmids, transposons and integrons. The significant role of integrons in the dissemination of antibiotic resistance in *E. coli* has been established in recent years (Guerra et al., 2003, Maidhof et al., 2002, Morabito et al., 2002, Singh et al., 2005). They are associated with conjugative broad-host-range plasmids and transposons and are frequently found on multidrug-resistant bacteria (Karczmarczyk et al., 2011a). Integrons are capable of capturing gene cassettes from the environment and incorporating them into the bacterial genome using site-specific recombination. Since their discovery, integrons have been found to be associated with genes encoding antibiotic resistance to most classes of antibiotics (Rowe-Magnus and Mazel, 2002). The backbone structure of an integron contains a 5' conserved region encoding the site specific recombinase generally known as the integrase (*intl*) and a variable region with integrated gene cassettes (Stokes and Hall, 1989). The 3' conserved region typically contains a *qacEΔ1* and a *sul1* gene encoding quaternary ammonium compound and sulphonamide resistance (Carattoli, 2001). Currently, integrons are categorised into five classes based on the type of integrase present i.e. class 1 integrase (*intl1*) defines class 1 integrons (Correia et al., 2003, Nield et al., 2001). Class 1 and class 2 integrons are shown to harbour resistance genes to many classes of antibiotics (Barlow et al., 2009) although class 1 integrons are identified as the most clinically relevant (Karczmarczyk et al., 2011a).

Data from the Institute of Medical research (IMR) in Malaysia indicates that there is an increase of antibiotic resistant *E. coli* in Malaysian hospitals (Health Action International Asia Pacific, 2013). Most reports from Malaysia document antibiotic-resistant *E. coli* in hospital settings

(Akter et al., 2012, Ho et al., 2012, Kor et al., 2013, Lim et al., 2009). Although antibiotic resistant *E. coli* in hospital environments clearly pose a threat to public health, the presence of antibiotic resistant *E. coli* colonising animals cannot be disregarded due to the significant role they play in dissemination of antibiotic resistance determinants among animals as well as humans. Currently, there is very limited data on antibiotic resistant *E. coli* and the genetic determinants underlying antibiotic resistance among food-producing ruminants in Malaysia. This study was conducted to characterise antibiotic resistance in a collection of *E. coli* isolated from food-producing ruminants from Peninsular Malaysia and to determine the genetic determinants of resistance and the potential risk of resistance determinant(s) transmission.

3.2 Materials and methods

3.2.1 Isolation of *E. coli*

E. coli were isolated from a total of 136 fresh ruminant feces samples (~25 g each), which were previously collected from six different ruminant farms in Peninsular Malaysia as described in Chapter 2. A 10 g sample from each fecal sample, which was stored in tryptone soy broth (TSB; Merck, Darmstadt, Germany) with 25 % glycerol at −70°C was first enriched by diluting 1/10 in buffered peptone water (BPW; Oxoid, Hampshire, UK). The samples were then homogenized for 30 s and incubated for 18 h at 37°C without agitation. Each enriched sample was directly streaked onto coliformen agar enhanced selectivity (Merck, Darmstadt, Germany) and incubated overnight at 37°C. Following incubation, one *E. coli* isolate per sample was chosen based on colony morphology and biochemically identified as *E. coli* by citrate utilization and indole production tests (Aslanzadeh, 2006). As such, 136 commensal *E. coli* strains were obtained from the 136 feces samples collected (one *E. coli* isolate per sample) to be used in the antibiotic susceptibility testing. In addition, another 17 distinct *E. coli* strains, which were characterised as either *E. coli* O157 or non-O157 as described in Chapter 2 were included in the antibiotic susceptibility testing. Among the 17 *E. coli* strains were two STEC

O157 strains and two non-O157 STEC strains. The details of the strains obtained are described in Table 3.1.

Table 3.1 Distribution of *E. coli* strains used in antibiotic susceptibility testing

Location	Ruminant sample (No. of samples, n)	No. of *E. coli* strains	No. *E. coli* O157 strains[a] (STEC/non-STEC)	No. *E. coli* non-O157 strains[a] (STEC/non-STEC)
Farm A	Cattle feces (n=25)	25	2 (STEC)	2 (STEC) 1 (non-STEC)
Farm B	Cattle feces (n=9)	9	1 (non-STEC)	none
	Buffalo feces (n=20)	20	none	6 (non-STEC)
	Goat feces (n=7)	7	none	none
	Sheep feces (n=8)	8	2 (non-STEC)	none
Farm C	Cattle feces (n=9)	9	none	1 (non-STEC)
Farm D	Cattle feces (n=24)	24	none	none
Farm E	Cattle feces (n=13)	13	none	none
	Goat feces (n=5)	5	none	none
Farm F	Cattle feces (n=16)	16	none	none
UPM[b]	Bovine milk (n=1)	none	1 (STEC)	none
	Beef (n=1)	none	1 (STEC)	none
Total no. of strains		136	7	10

[a]Characterised as *E. coli* O157 and non-O157 in Chapter 2.

[b]Provided by Prof. Son Radu at University Putra Malaysia and characterised in Chapter 2.

3.2.2 Resistance profiling of isolated *E. coli*

A collection of 153 *E. coli* isolates, which included those that were cultured from the 136 ruminant feces samples as described above and 17 isolates also collected from ruminant feces samples and characterised as either *E. coli* O157 or non-O157 in Chapter 2 were subjected to susceptibility testing using the standard Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute, 2013). The results were interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2013). In brief, a single *E. coli* colony was inoculated in to 10 ml TSB and incubated overnight at 37°C. Following incubation, all *E. coli* suspensions were adjusted to match the 0.5 McFarland turbidity standards in TSB and were plated on to Mueller-Hinton agar (Oxoid, Hampshire, UK) using a sterile cotton swab dipped in to the adjusted cell suspension. The plates were incubated at 37°C for 18 to 20 h. Susceptibility testing included a panel of 15 different antibiotic agents: amikacin (AK), 30µg; ampicillin (AMP), 10 µg; ciproflaxacin (CIP), 5 µg; cefuroxime sodium (CXM), 30 µg; cephalothin (KF), 30 µg; cefotaxime (CTX), 30 µg; ceftazidime (CAZ), 30 µg; chloramphenicol (C), 30 µg; gentamycin (CN), 10 µg; imipenem (IPM), 10 µg; kanamycin (K), 30 µg; nalidixic acid (NA), 30 µg; streptomycin (S), 10 µg; tetracycline (TE), 30 µg; trimethoprim (W), 5 µg. These antibiotic agents were chosen on the basis of their importance in treating human or animal *E. coli* infections and their use as feed additives to promote growth in animals as well as on the basis of their ability to provide diversity for representation of different antibiotic agent classes. All the discs were purchased from Oxoid (Hampshire, UK). *E. coli* ATCC 25922 was included as the quality control strain.

3.2.3 Detection of antibiotic resistance genes and class 1 and class 2 integrons

PCR assays (single) were used to detect the presence of antibiotic resistance genes and class 1 and class 2 integrons of the *E. coli* isolates. Total genomic DNA of each *E. coli* isolate was extracted using the boiling method (Radu et al., 2001) with a few modifications. Briefly a well

isolated *E. coli* colony from a tryptone soya agar (TSA; Oxoid, Hampshire, UK) plate was inoculated in to 1 ml of TSB and incubated for 18 h at 37°C. The resulting culture was centrifuged for 3 min at 13,000 rpm, supernatant discarded and the pellet re-suspended in 1 ml dH_2O. Following re-suspension, the culture was centrifuged again for 3 min at 13,000 rpm, supernatant discarded and the pellet re-suspended in 200 µl dH_2O. The cell suspension was subsequently boiled for 10 min at 100°C, centrifuged for 5 min at 13,000 rpm and the supernatant containing the genomic DNA was collected and stored at −20°C. The DNA concentration of each sample was measured using the infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland). Detection of antibiotic resistance markers and integron-associated genes was performed using the primers and gene targets listed in Table 3.2. The positive controls used for the detection of antibiotic resistance markers for ampicillin, cephalothin, chloramphenicol, kanamycin, nalidixic acid, streptomycin and tetracycline were kindly provided by Prof. Séamus Fanning from the University College Dublin, Ireland. Positive control DNA templates for the detection of resistance markers for trimethoprim were provided by Dr. Josée Harel and Garneau Philippe from the Université de Montréal, Québec, Canada, while the DNA templates for the detection of integron-associated genes were provided by Dr. Robert Barlow from CSIRO Food and Nutrition, Brisbane, Australia. All PCR reactions were performed as described previously (Karczmarczyk et al., 2011a) in a final volume of 25 µl consisting of 5 µl of 5 X Green GoTaq® Flexi Buffer (Promega, Madison, USA), 25 pmol of each primer, dNTP at a concentration of 200 µM, 1.5 mM of $MgCl_2$, 1U Taq DNA polymerase (Promega, Madison, USA), and 50 ng of genomic DNA. The PCR products were separated by electrophoresis on a 2 % (wt/vol) agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

Table 3.2. PCR primer characteristics

Antibiotic	Target gene	Primer direction	Nucleotide sequence (5'–3')	Annealing temp (°C)	Amplicon size (bp)	Reference
Ampicillin, Cephalothin	*ampC*	F	CCC CGC TTA TAG AGC AAC AA	53	634	(Feria et al., 2002)
		R	TCA ATG GTC GAC TTC ACA CC			
	bla$_{CMY-2}$	F	AAC ACA CTG ATT GCG TCT GAC	60	1226	(Perez–Perez and Hanson, 2002)
		R	CTG GGC CTC ATC GTC AGT TA			
	bla$_{OXA}$	F	TAT CTA CAG CAG CGC CAG TG	53	199	(Feria et al., 2002)
		R	CGC ATC AAA TGC CAT AAG TG			
	bla$_{SHV}$	F	TCA GCG AAA AAC ACC TTG	53	475	(M'Zali et al., 1996)
		R	TCC CGC AGA TAA ATC ACCA			
	bla$_{TEM}$	F	TAC GAT ACG GGA GGG CTT AC	53	716	(Belaaouaj et al., 1994)
		R	TTC CTG TTT TTG CTC ACC CA			
Chloramphenicol	*cat*	F	AGT TGC TCA ATG TAC CTA TAA CC	55	547	(Van et al., 2008)

		R	TTG TAA TTC ATT AAG CAT TCT GCC			
	cmlA	F	CCG CCA CGG TGT TGT TGT TAT C	59	698	(Keyes et al., 2000)
		R	CAC CTT GCC TGC CCA TCA TTA G			
	floR	F	TAT CTC CCT GTC GTT CCA G	53	399	(Keyes et al., 2000)
		R	AGA ACT CGC CGA TCA ATG			
Kanamycin	*aphA1*	F	ATG GGC TCG CGA TAA TGT C	53	600	(Maynard et al., 2003)
		R	CTC ACC GAG GCA GTT CCA T			
	aphA2	F	GAT TGA ACA AGA TGG ATT GC	53	347	(Travis et al., 2006)
		R	CCA TGA TGG ATA CTT TCT CG			
Nalidixic acid	*gyrA*	F	ACGTACTAGGCAATGACTGG	55	189	(Cavaco et al., 2008)
		R	AGAAGTCGCCGTCGATAGAAC			
	gyrB	F	CAGACTGCCAGGAACGCGAT	55	203	(Cavaco et al., 2008)
		R	AGCCAAGCGCGGTGATAAGC			
	parC	F	TGTATGCGATGTCTGAACTG	57	264	(Cavaco et al., 2008)

		R	CTCAATAGCAGCTCGGAATA			
	parE	F	TACCGAGCTGTTCCTTGTGG	55	266	(Cavaco et al., 2008)
		R	GGCAATGTGCAGACCATCAG			
Streptomycin	*aadA*	F	GTG GAT GGC GGC CTG AAG CC	60	525	(Madsen et al., 2000)
		R	AAT GCC CAG TCG GCA GCG			
	aadB	F	GAG GAG TTG GAC TAT GGA TT	53	208	(Travis et al., 2006)
		R	CTT CAT CGG CAT AGT AAA A			
	strA-strB	F	ATG GTG GAC CCT AAA ACT CT	60	893	(Tamang et al., 2012)
		R	CGT CTA GGA TCG AGA CAA AG			
Tetracycline	*tet*(A)	F	GCT ACA TCC TGC TTG CCT TC	55	210	(Ng et al., 2014)
		R	CAT AGA TCG CCG TGA AGA GG			
	tet(B)	F	TTG GTT AGG GGC AAG TTT TG	55	659	(Ng et al., 2014)
		R	GTA ATG GGC CAA TAA CAC CG			
	tet(C)	F	CTT GAG AGC CTT CAA CCC AG	55	418	(Ng et al., 2014)
		R	ATG GTC GTC ATC TAC CTG CC			
	tet(D)	F	AAA CCA TTA CGG CAT TCT GC	55	787	(Ng et al., 2014)

		R	GAC CGG ATA CAC CAT CCA TC			
	tet(E)	F	AAA CCA CAT CCT CCA TAC GC	55	278	(Ng et al., 2014)
		R	AAA TAG GCC ACA ACC GTC AG			
	tet(G)	F	GCT CGG TGG TAT CTC TGC TC	55	468	(Ng et al., 2014)
		R	AGC AAC AGA ATC GGG AAC AC			
Trimethoprim	*dhfr* I	F	AAGAATGGAGTTATCGGGAATG	50	391	(Maynard et al., 2003)
		R	GGGTAAAAACTGGCCTAAAATTG			
	dhfr V	F	CTGCAAAAGCGAAAAACGG	50	432	(Maynard et al., 2003)
		R	AGCAATAGTTAATGTTTGAGCTAAAG			
	dhfr VII	F	GGTAATGGCCCTGATATCCC	50	265	(Maynard et al., 2003)
		R	TGTAGATTTGACCGCCACC			
	dhfr IX	F	TCTAAACATGATTGTCGCTGTC	50	462	(Maynard et al., 2003)
		R	TTGTTTTCAGTAATGGTCGGG			
	dhfr XIII	F	CAGGTGAGCAGAAGATTTTT	50	294	(Maynard et al., 2003)
		R	CCTCAAAGGTTTGATGTACC			

Intl1	F	CAG TGG ACA TAA GCC TGT TC	59	160	(Koeleman et al., 2001)
	R	CCC GAG GCA TAG ACT GTA			
Class 1 gene cassette	F	GGC ATC CAA GCA GCA AGC	55	Variable	(Levesque et al., 1995)
	R	AAG CAG ACT TGA CCT GAT			
qacEΔ1	F	ATC GCA ATA GTT GGC GAA GT	53	250	(Sandvang et al., 1997)
	R	GAA GCT TTT GCC CAT GAA GC			
sul1	F	CGG CGT GGG CTA CCT GAA CG	66	433	(Kerrn et al., 2002)
	R	GCC GAT CGC GTG AAG TTC CG			
Intl2	F	CAC GGA TAT GCG ACA AAA AGG T	54	788	(Mazel et al., 2000)
	R	GTA GCA AAC GAG TGA CGA AAT G			

Class 2 gene cassette	F	CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA	62	Variable	(White et al., 2001)
	R	GAT GCC ATC GCA AGT ACG AG			

3.2.4 Screening for plasmid mediated resistance

Plasmids were extracted from all *E. coli* strains that indicated complete or intermediate resistance to any of the antibiotics tested. A single colony of each *E. coli* isolate was inoculated in to 5 ml Luria-Bertani (LB) broth (Oxoid, Hampshire, UK) and incubated overnight at 37°C. Plasmid DNA was purified from the overnight cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, USA) according to the manufacturer's instructions. The presence of the relevant antibiotic resistance gene(s) on the purified plasmid DNA was determined using PCR as described above.

3.2.5 Sequencing of integron gene cassettes and quinolone resistance genes

Gene cassettes from class 1 integrons and nalidixic acid (quinolone) resistance determinants *gyrA*, *gyrB*, *parC* and *parE* were amplified using the respective primer pairs and were purified from the agarose gel using Promega Wizard PCR and gel purification system (Promega, Madison, USA). Recovered amplicons were commercially sequenced (1st BASE, Selangor, Malaysia). Sequence similarity searches were carried out against sequences deposited in the current version of the GenBank database using the BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences obtained for the quinolone resistance determinants were compared with those previously reported for *gyrA* (GenBank accession no. X06373), *gyrB* (GenBank accession no. X04341), *parC* (GenBank accession no. CP011416.1) and *parE* (GenBank accession no. KJ136407.1).

3.2.6 Conjugation assays

A total of 25 *E. coli* strains carrying plasmid mediated resistance, which were selected based on their heterogeneous antibiotic resistance profiles as listed in Table 3.3 were used in the conjugation assays to determine the transfer of antibiotic resistance determinants. A rifampin-resistant, lactose-negative *E. coli* isolate 26R793 kindly provided by Prof. Séamus Fanning from the University College Dublin, Ireland was used as the recipient in all conjugation

experiments. Conjugation was performed through the process of broth mating as described previously (Karczmarczyk et al., 2011a). In brief, single colonies of the donor and recipient *E. coli* strains were grown in LB broth (Oxoid, Hampshire, UK) for 18 h at 37°C. Following incubation, equal volumes (5 ml each) of the donor and recipient cultures were mixed and incubated for 18 h at 37°C without agitation. The resulting transconjugants were selected on MacConkey agar (Oxoid, Hampshire, UK) supplemented with 100 µg/ml rifampin (Sigma-Aldrich, USA) together with either 50 µg/ml ampicillin, 20 µg/ml chloramphenicol, 50 µg/ml streptomycin, 30 µg/ml tetracycline or 50 µg/ml trimethoprim (Sigma-Aldrich, USA). Three presumptive conjugants from each antibiotic selection were chosen for further analysis of transconjugation. Phenotypic resistance to the respective antibiotics were tested as described under 3.2.2 followed by plasmid extraction of the antibiotic resistant strains to determine the transfer of respective antibiotic resistance determinants.

Table 3.3. Resistance profiles and resistance gene determinants of the transconjugants obtained during conjugation assays with the donor *E. coli* strains collected from the ruminant feces samples

Origin of *E. coli* strains	Strain no./origin	Resistance profile of donor	Resistance transferred to recipient/resistance gene(S) identified in transconjugants
Farm A	EC1/cattle feces	KF, S (I)[a]	S/*aadA*
	EC9/cattle feces	AMP, S (I)	AMP, S/*bla*$_{TEM}$, *aadA*
	EC10/cattle feces	AMP, KF (I)	AMP/*bla*$_{TEM}$
	EC14/cattle feces	S (I)	S/*aadA*, *strA-strB*
	EC15/cattle feces	TE, W (R)[b]	TE, W/*tet*(A), *dhfr* I & VII
	EC27/cattle feces	AMP, TE, S, W (R)	AMP, TE, S, W/*bla*$_{TEM}$, *tet*(A), *aadA* & *strA-strB*, *dhfr* V
Farm B	EC31/buffalo feces	S (I)	S/*aadA*
	EC36/buffalo feces	KF, S (I)	S/*aadA*
	EC37/buffalo feces	C, S (I); TE, W (R)	TE, S, W/*tet*(A), *aadA*, *dhfr* XIII

	EC67/sheep feces	AMP (I)	AMP/*bla*$_{TEM}$
	EC68/sheep feces	S (I); K, TE (R)	TE, S/*tet*(A), *aadA*
	EC72/sheep feces	AMP, KF (I)	AMP/*bla*$_{TEM}$
	EC76/buffalo feces	KF (I); AMP, TE, S, W (R)	AMP, TE, S, W/*bla*$_{TEM}$, *tet*(A), aadA, *strA*-s*trB*, *dhfr* V
Farm C	EC83/cattle feces	S (I); TE (R)	TE, S/*aadA*, *tet*(A)
	EC89/cattle feces	TE (R)	TE/*tet*(A)
Farm D	EC93/cattle feces	KF, S (I)	S/*aadA*
	EC100/cattle feces	S (I)	S/*aadA*
	EC109/cattle feces	KF (I); AMP, S, W (R)	AMP, S, W/*bla*$_{TEM}$, aadA & *strA*-*strB*, *dhfr* VII
Farm E	EC117/cattle feces	S (I), AMP, TE (R)	AMP, TE, S/*bla*$_{TEM}$, *tet*(A), *aadA*
	EC119/cattle feces	S (I)	S/*aadA*

	EC123/cattle feces	KF, S (I)	S/*aadA*
Farm F	EC135/cattle feces	KF, NA (I); C, K, TE, W (R)	TE, W/*tet*(A), *dhfr* V
	EC136/cattle feces	KF, S (I)	S/*aadA*
	EC140/cattle feces	AMP, KF, S (I)	AMP, S/bla_{TEM}, *aadA*
	EC148/cattle feces	S (I)	S/*aadA*

[a]Intermediate resistance to antibiotics
[b]Complete resistance to antibiotics

3.3 Results

3.3.1 Antibiotic susceptibility of the *E. coli* strains

Susceptibility testing of the 153 *E. coli* strains showed that resistance was most common against tetracycline (11/153, 7.2 %), followed by trimethoprim (6/153, 3.9 %), ampicillin (5/153, 3.3 %), streptomycin (3/153, 2.0 %), kanamycin (2/153, 1.3 %), chloramphenicol (1/153, 0.7 %) and nalidixic acid (1/153, 0.7 %) (Table 3.4). Almost two-thirds of the strains (62.0 %) showed intermediate susceptibility to different antibiotics. Among them, intermediate resistance was more frequently apparent against streptomycin (47/153, 30.7 %), followed by cephalothin (39/153, 25.5 %), and less frequently to ampicillin (7/153, 4.6 %), chloramphenicol and nalidixic acid (each 1/153, 0.7 %). Overall, 45.8 % of the *E. coli* strains (70/153), which included both STEC O157 strains, were sensitive to all the antibiotics used in the study. None of the 153 *E. coli* strains showed complete or intermediate resistance to aminoglycosides (amikacin and gentamicin), second generation cephalosporin (cefotaxime sodium), third generation cephalosporins (ceftazidime and cefuroxime), monobactam (imipenem) and fluoroquinolone (ciprofloxacin).

Strains that were resistant to two or more antibiotics belonging to different antibiotic groups were considered multi–antibiotic resistant. Multi-antibiotic resistance was observed in 5.9 % of the strains (9/153), which included one STEC non-O157 isolate and one non-STEC O103 isolate. Of these multi-antibiotic resistant strains all except one strain were resistant to tetracycline (8/9, 88.9 %). The second most common resistance was to trimethoprim (6/9, 66.7 %). The maximum number of antibiotic resistances observed in any one isolate was to four antibiotics, but very few strains showed this pattern of resistance (3/9, 33.3 %). No common resistance pattern was identified among any of the multi-antibiotic resistant strains.

3.3.2 Occurrence of resistance determinants

All the *E. coli* strains, which showed either complete resistance or intermediate resistance phenotypically to any of the antibiotics used in the study were tested for the presence of the relevant resistance genes. Of all the *tet* genes tested, *tet*(A) gene alone was detected in all *E. coli* strains (11/153, 7.2 %) phenotypically resistant to tetracycline (Table 3.4). The six strains phenotypically resistant to trimethoprim carried at least one dihydrofolate reductase (*dhfr*) gene, which mediates resistance to trimethoprim. D*hrf* V gene was detected in the majority of the *E. coli* strains (2.0 %) resistant to trimethoprim, while *dhfr* VII was the second highest (1.3 %) observed. Strains with either complete or intermediate resistance to ampicillin were positive for either one or both *ampC* and bla_{TEM} genes. However, majority of the strains carried both *ampC* and bla_{TEM} (8/153, 5.2 %) genes. The genetic markers observed in ampicillin resistant strains (*ampC* and bla_{TEM}) were also present in strains with intermediate resistance to cephalothin. Both *ampC* and bla_{TEM} genes were most commonly observed (33/153, 21.6 %) among all the strains with intermediate resistance to cephalothin. All of the 50 strains that were either completely or intermediately resistant to streptomycin carried *aadA* (32.7 %), and seven of them (4.6 %) were also positive for the *strA-strB* gene. Strains resistant to kanamycin simultaneously carried both *aphA1* and *aphA2* (2/153, 1.3 %) genes tested. The strains with either complete or intermediate resistance for chloramphenicol were negative for the *cat* gene usually observed among chloramphenicol resistant *E. coli* strains. However, the strain with complete resistance to chloramphenicol possessed the gene for the chloramphenicol-florfenicol transporter *floR* (0.7 %), while the strain with intermediate resistance possessed *cmlA* (0.7 %), conferring non-enzymatic resistance. Both strains with resistance to nalidixic acid (either complete or intermediate) carried *gyrA* (1.3 %), *gyrB* (1.3 %), *parC* (1.3 %) and *parE* (1.3 %) genes, which encode the subunits of the quinolone targets DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*).

The resistance determinants for tetracycline – *tet*(A), trimethoprim – *dhfr*, ampicillin and cephalothin – bla_{TEM}, streptomycin – *aadA* and *strA*–*strB*, and chloramphenicol – *floR* and *cmlA* were all detected on plasmid DNA.

Table 3.4. Occurrence of antibiotic resistance and the respective genetic determinants among the *E. coli* strains

Antibiotic group	Antibiotic Agent	No. (%) of resistant strains		Resistance determinant(s)	No. (%) of strains with resistant determinant
		Complete resistance	Intermediate resistance		
Aminoglycosides	Kanamycin	2 (1.3)	none	*aphA1* and *aphA2*	2 (1.3)
	Streptomycin	3 (2.0)	47 (30.7)	*aadA* alone	43 (28)
				aadB	none
				strA-strB alone	7 (4.6)
				aadA and *strA-strB*	50 (32.7)
Beta-lactams	Ampicillin (Penicillin)	5 (3.3)	7 (4.6)	*ampC* alone	2 (1.3)
				bla_{TEM} alone	2 (1.3)
				ampC and bla_{TEM}	8 (5.2)
				bla_{CMY-2}	none
				bla_{OXA}	none

				bla$_{SHV}$	
	Cephalothin (First generation cephalosporin)	none	39 (25.5)	*ampC* alone	5 (3.3)
				bla$_{TEM}$ alone	1 (0.7)
				ampC and *bla*$_{TEM}$	33 (21.7)
				bla$_{CMY-2}$	none
				bla$_{OXA}$	none
				bla$_{SHV}$	none
Quinolones	Nalidixic acid	1 (0.7)	1 (0.7)	*gyrA, gyrB, parC, parE*	2 (1.3)
Folate pathway inhibitors	Trimethoprim	6 (3.9)	none	*dhfr* I and *dhfr* VII	1 (0.7)
				dhfr V alone	3 (2.0)
				dhfr VII alone	1 (0.7)
				dhfr IX	none
				dhfr XIII alone	1 (0.7)

Phenicols	Chloramphenic-ol	1 (0.7)	1 (0.7)	*cat*	none
				cmlA alone	1 (0.7)
				floR alone	1 (0.7)
Tetracyclines	Tetracycline	11 (7.2)	none	*tet*(A) alone	11 (7.2)
				tet(B)	none
				tet(C)	none
				tet(D)	none
				tet(E)	none
				tet(G)	none

3.3.3 Sequence analysis of the resistant determinants for nalidixic acid (quinolone) resistance

Nucleotide sequence analysis of the *gyrA, gyrB, parC* and *parE* genes of the isolate with complete resistance and the isolate with intermediate resistance to nalidixic acid revealed that both strains contained a single amino acid substitution of Serine (Ser)-83 → Leucine in the quinolone resistance-determining region (QRDR) of the *gyrA* gene. Analysis of the *parE* gene of both strains indicated three amino acid substitutions of Serine (Ser)-425 → Valine (Val), Alanine (Ala)-426 → Cystine (Cys) and Leucine (Lys)-427 → Glutamine (Gln). None of the *gyrB* or *parC* genes indicated any amino acid substitutions.

3.3.4 Integrons and gene cassettes

A total of 65 *E. coli* strains (42.5 %) were determined to possess the class 1 integrase gene (*intl1*) located at the 5'–CS. The majority of the strains positive for the class 1 integron lacked the *qacEΔ1* and the *sul1* genes, located within the 3'–CS, while only two strains carrying the class 1 integron were positive for the *qacEΔ1* alone. PCR amplification of the regions flanking the gene cassettes of the class 1 integron yielded only two different gene cassettes of approximately 800 bp with antibiotic resistance determinants. Each of these gene cassettes was subjected to DNA sequencing. One of the 800 bp amplicons contained a single open reading frame (ORF) that was 100 % similar to a previously reported *dhfr* VII gene (GenBank accession no. KF534913.1). The remaining 800 bp amplicon contained a single ORF that was 99 % similar to a previously reported *dhfr* V gene (GenBank accession no. AJ419169.1). All other amplicons resulting from the PCR amplification of the class 1 gene cassette did not contain any antibiotic resistance determinants of interest. In addition, none of the *E. coli* strains carried the class 2 integrase gene (*intl2*).

3.3.5 Conjugal transfer of antibiotic resistance

Conjugal transfer of antibiotic resistance determinants was observed in all *E. coli* strains with plasmid mediated resistance to ampicillin (9/25, 36.0 %), streptomycin (19/25, 76.0 %), tetracycline (9/25, 36.0 %) and trimethoprim (6/25, 24.0 %) (Table 3.3). Transconjugation was not observed with strains carrying plasmid mediated resistance to chloramphenicol. Concomitant transfer of resistance to the four antibiotics: ampicillin, streptomycin, tetracycline and trimethoprim was observed in two (8.0 %) multi-antibiotic resistant strains. All transconjugants indicated the acquisition of the plasmid mediated antibiotic resistance determinants of the respective antibiotics.

3.4 Discussion

The continued use of antibiotic compounds for treatment and/or growth promotion in food animals increases the selective pressure for the emergence of antibiotic resistant bacteria. Studies have shown that resistant strains of animal origin are able to colonize or cause human infections (Aarestrup et al., 2008, Belanger et al., 2011, Collignon et al., 2009, Manges and Johnson, 2012). Antibiotic resistant commensal *E. coli* strains pose a significant threat to public health since they are found in a wide range of hosts, acquire resistance easily (Erb et al., 2007) and are capable of disseminating resistance determinants (Mazurek et al., 2013). Patterns of antibiotic resistance in *E. coli* vary in terms of geographical location (Jacob et al., 2008). In Malaysia, an increase in resistance to antibiotics such as ampicillin, cefotaxime, ceftazidime, ciprofloxacin and gentamycin has been reported from *E. coli* strains in hospital settings (Health Action International Asia Pacific, 2013). However, the extent of antibiotic resistance in Malaysia is not fully understood due to the lack of published data on antibiotic usage in animal husbandry in the country. This study examined the resistance patterns of commensal *E. coli* obtained from ruminants in Peninsular Malaysia to major antibiotics used in human and veterinary medicine as well as growth promotion in food animals. In addition, the respective

genetic determinants that correspond to their antibiotic resistance profiles were also determined to gain a better understanding of the underlying genetic mechanisms conferring antibiotic resistance.

Resistance to tetracycline was one of the most common resistance profiles identified among the *E. coli* strains in this study. It is not surprising because tetracycline has been widely used in therapy and to promote feed efficiency in animal systems since its approval in 1948 (McEwen and Fedorka-Cray, 2002). Tetracycline resistance was also commonly reported in coliforms from animals such as cattle and swine from Malaysia as well as from other countries (ALHaj et al., 2007, Lim et al., 2007, Tadesse et al., 2012). Of the six tetracycline resistance genes analysed by PCR, only *tet*(A) was observed in all the strains phenotypically resistant to tetracycline. This data supports previous findings, in which apart from *tet*(B), *tet*(A) was found to be particularly abundant among the *tet* genes implicated in tetracycline resistant *E. coli* strains cultured from animals (Karczmarczyk et al., 2011a). Tetracycline resistance genes are often plasmid mediated (Speer et al., 1992), which has facilitated their wide distribution. All the *tet*(A) genes of the tetracycline resistant *E. coli* strains from this study were also detected on conjugating plasmids indicating the ability for rapid dissemination.

Most of the *E. coli* strains, which were resistant to tetracycline were also commonly associated with either complete or intermediate resistance to streptomycin. Similarly to tetracycline, streptomycin has also been in use from the 1940s, and is commonly used in livestock. A high prevalence of resistance to streptomycin among commensal *E. coli* from this study was apparent and this is consistent with observations from previous studies on resistant phenotypes among commensal *E. coli* in food animals (Lim et al., 2007, Tadesse et al., 2012). PCR analysis of the genetic determinants underlying streptomycin resistance indicated that plasmid mediated *aadA* (32.7 %) was the most prevalent gene followed by *strA-strB* (4.6 %). This finding is consistent with earlier reports showing that these genes are commonly found in strains resistant

to streptomycin and/or other aminoglycoside drugs (Karczmarczyk et al., 2011a, Sunde and Norstrom, 2006). Conjugal transfer of streptomycin resistance determinants among the *E. coli* from this study indicates the risk of resistance transmission to human pathogenic bacteria.

In contrast to streptomycin, only a small percentage of *E. coli* strains were resistant to kanamycin (1.3 %), another aminoglycoside commonly used in veterinary medicine (Mora et al., 2005). A high prevalence of kanamycin resistance was apparent in *E. coli* strains obtained from animals as well as from other sources, which included clinical, marine, river and food samples in another study conducted in Malaysia (ALHaj et al., 2007). Kanamycin resistance of the strains in this study seemed to be mediated by plasmid borne *aphA1* and *aphA2* although *aphA2* is less frequently observed in kanamycin resistant strains in comparison to *aphA1* (Maynard et al., 2003, Travis et al., 2006).

β-lactams are among the most critically important antibiotics in veterinary medicine (Li et al., 2007). Among other β-lactams, ampicillin is an antibiotic, which has been in use in veterinary medicine since the early 1960s similar to tetracycline and streptomycin. Resistance to ampicillin is commonly observed among commensal *E. coli* from food animals. In this study, complete resistance to ampicillin was observed only in five *E. coli* strains (3.3 %) out of the 153 *E. coli* strains, while intermediate resistance was observed in seven *E. coli* strains (4.6 %). Although ampicillin is also commonly used in livestock in Malaysia (Health Action International Asia Pacific, 2013) it could be that these *E. coli* strains are not exposed to ampicillin as much as they have been exposed to tetracycline or streptomycin. The predominant mechanism of β-lactam resistance in Gram-negative bacteria such as *E. coli* is the production of β-lactamases, which are encoded chromosomally or on plasmids (Li et al., 2007). These β–lactamases include the AmpC-type β-lactamases encoded by chromosomal *ampC* or plasmid mediated genes such as bla_{CMY-2} as well as plasmid mediated cephalosporinases such as TEM and SHV encoded by bla_{TEM} and bla_{SHV}. In the present study the majority of the *E. coli* strains

with complete or intermediate resistance to ampicillin carried both *ampC* and plasmid–borne bla_{TEM} genes. In ampicillin resistant Gram–negative bacteria, plasmid encoded narrow-spectrum TEM have often been identified and are among the most prevalent β-lactamases reported (Li et al., 2007). Conjugal transfer of the bla_{TEM} gene was observed to confer resistance to ampicillin in the recipient *E. coli* isolate in this study indicating a direct link between the transfer of plasmid mediated bla_{TEM} and resistance to ampicillin. None of the ampicillin resistant strains were positive for the plasmid mediated AmpC-type β-lactamase bla_{CMY-2} and cephalosporinase bla_{SHV} or chromosomally encoded bla_{OXA}. Similarly to the ampicillin resistant strains, the majority of *E. coli* strains with intermediate resistance to cephalothin, a first generation cephalosporin, carried both *ampC* and plasmid-borne bla_{TEM} genes. The presence of the *ampC* gene alone was only observed in two strains with intermediate resistance to ampicillin and five strains with intermediate resistance to cephalothin. In *E. coli* the chromosomal *ampC* gene is normally expressed in low amounts due to a weak promoter and the presence of a transcriptional attenuator (Jaurin and Normark, 1983). Usually, such a low level of AmpC does not mediate resistance to amino-penicillins such as ampicillin or the narrow spectrum cepholosporins such as cephalothin. However, alterations in *ampC* promoter region have been observed in *E. coli* strains derived from animals or humans, leading to elevated production of AmpC that conferred significant resistance to penicillins, cephalosporins and monobactams (reviewed in Li et al., 2007). The reduced susceptibility to ampicillin of the *E. coli* strains carrying *ampC* alone in this study could most probably be attributed to the hyper-production of chromosomal AmpC.

Trimethoprim resistance was the second most common resistance identified among the *E. coli* strains of this study. Trimethoprim was first used to treat clinical disease in 1962 (Noall et al., 1962). Since then resistance to trimethoprim has been observed in *E. coli* strains of both human and animal origin. Several investigators have suggested that trimethoprim resistance is spread

from animals to humans (Amyes, 1986, Chirnside et al., 1985). However, the data on trimethoprim resistance among *E. coli* strains originating from animals is limited. One study conducted in Lithuania reported that the resistance to trimethoprim among *E. coli* isolated from cattle was 20 % (Seputiene et al., 2010). The trimethoprim resistance phenotype of the *E. coli* strains in this study was found to be associated with plasmid-borne, *dhfr* I (0.7 %), *dhrf* V (2.0%), *dhfr* VII (1.3 %), and *dhfr* XIII (0.7 %) genes. All four *dhfr* genes detected in the trimethoprim resistant *E. coli* strains in this study have been found among *E. coli* isolated previously, with a predominance of plasmid mediated *dhrf* V (Maynard et al., 2003). The presence of the *dhfr* genes on conjugative plasmids indicates the readily transfer of these genes among *E. coli* strains.

Chloramphenicol is a broad spectrum antibiotic that is effective against both Gram-positive and Gram-negative bacteria (Sorensen et al., 2003) with a history of veterinary use in all major food-producing animals. Its use has been banned in food-producing animals in several countries, including Malaysia, due to its suspected carcinogenicity and link with the development of aplastic anaemia in humans (Berendsen et al., 2010). Nevertheless, chloramphenicol resistant *E. coli* strains have been documented in various studies (Karczmarczyk et al., 2011a, Karczmarczyk et al., 2011b). It has been suggested that the detection of chloramphenicol resistance phenotypes in the absence of chloramphenicol use could be due to the use of plant material containing naturally occurring chloramphenicol as animal feed leading to subsequent transfer of chloramphenicol to animal tissues (Berendsen et al., 2010). In Malaysia, a high number of chloramphenicol resistant *E. coli* strains (75.7 %) have been isolated from different sources including animal samples, clinical samples (ALHaj et al., 2007) as well as from aquaculture and other environmental waters (Ng et al., 2014). In this study, only two *E. coli* strains (1.3 %) out of the 153 strains showed reduced susceptibility to chloramphenicol, one with complete resistance and the other with intermediate resistance.

The isolate with complete resistance to chloramphenicol carried *floR*, which is the second most commonly observed gene among chloramphenicol resistant strains after *cat* gene. The isolate with intermediate resistance carried *cmlA,* a resistance determinant less frequently observed among chloramphenicol resistant *E. coli* (Karczmarczyk et al., 2011b). The low level of resistance to chloramphenicol observed in this study could probably result from the different selective pressures encountered by the strains as well as the presence of the respective antibiotic resistance determinants on non-conjugative plasmids.

Quinolone resistance is usually chromosomally mediated (Li, 2005). The most frequent mechanism of resistance to quinolones in *E. coli* includes alterations in chromosomal genes that encode subunits of the quinolone targets DNA gyrase (in *gyrA* and *gyrB* genes) and topoisomerase IV (in *parC* and *parE*) (Everett et al., 1996, Piddock et al., 2003, Vila et al., 1996). These alterations mainly involve mutations located in the QRDR of the *gyrA* gene and its homologous region of the *parC* gene. In contrast, mutations in *gyrB* and *parE* genes are of minor importance and are rare contributors to quinolone resistance. In this study, both *E. coli* strains with reduced susceptibility to nalidixic acid carried all four chromosomal genes *gyrA*, *gyrB*, *parC* and *parE*. Nucleotide sequence analysis of the *gyrA* in both strains indicated amino acid substitutions at Ser-83 to Leu, which is commonly reported in literature (Saenz et al., 2003, Tamang et al., 2012) to be involved in quinolone resistance of *E. coli*. No amino acid substitutions were detected in the *gyrB* as well as *parC* gene although alterations in *parC* gene is also frequently identified to be responsible for quinolone resistance (Saenz et al., 2003, Tamang et al., 2012). Analysis of the *parE* gene of both nalidixic acid resistant strains indicated three amino acid substitutions at Ser-425, Ala-426 and Lys-427, although not commonly observed among quinolone resistant *E. coli*. Overall, amino acid substitutions observed in both *gyrA* and *parE* may have collectively contributed to the reduced susceptibility observed in the *E. coli* strains of this study.

Although few, the presence of the class 1 integrons associated with trimethoprim resistant determinants of the *E. coli* strains in this study is in agreement with previous studies (Karczmarczyk et al., 2011a, Lim et al., 2009). The different class 1 gene cassettes containing *dhfr* VII and *dhfr* V have been characterized in *E. coli* carrying the class 1 integrons and are widely distributed among the *Enterobacteriaceae* (Kadlec and Schwarz, 2008, Machado et al., 2005). The absence of *qacEΔ1* and *sul1* genes located within the 3'–CS among the majority of the class 1 integron carrying *E. coli* strains in this study is surprising. Nonetheless, several strains lacking this 3' conserved segment has been previously reported by others (Soufi et al., 2009). Although not reported in this study, class 2 integrons have been documented among *E. coli* strains from Malaysia (Ho et al., 2012, Lim et al., 2009).

3.5 Conclusion

The results of this study showed the occurrence of antibiotic resistance among commensal *E. coli* strains from food-producing ruminants in Malaysia. In addition, the presence of antibiotic resistance determinants associated with mobile genetic elements such as conjugative plasmids and integrons highlights the importance of the reservoir of commensal *E. coli* in food-producing animals, which contributes to the evolution and dissemination of antibiotic resistance. However, their contribution to resistance appeared to be directed against more traditional antibiotic compounds such as tetracycline, streptomycin and trimethoprim while complete susceptibility was observed to more modern antibiotics used in human medicine such as cefotaxime, ceftazidime, imipenem and ciprofloxacin, an indication of low risk to the public health. Nonetheless, given the high propensity of *E. coli* strains to spread antibiotic resistance genes, it is important to monitor the resistance patterns of *E. coli* associated with food-producing animals with time and in a wider geographical region.

Chapter 4

Genomic analysis of Shiga toxin encoding bacteriophage insertion sites (SBI) of non-O157 Shiga toxigenic *Escherichia coli* (STEC) from Australia and Malaysia

4. 1 Introduction

The pathogenic potential of Shiga toxigenic *Escherichia coli* (STEC) strains are largely determined by their ability to produce Shiga toxins (Stx), the most distinct virulence factor among STEC strains. The genes encoding *stx* lie within a highly diverse group of lysogenic bacteriophages, known as *stx* bacteriophages, in the bacterial chromosome (Allison, 2007, Muniesa et al., 2004b). The *stx* phages belong to the group of lamboid phages that carry a double stranded linear DNA genome and share similar physical morphologies consisting of an icosahedral head attached to a long non–contractile tail (Allison, 2007). Similarly to all temperate bacteriophages, the *stx* phage have two distinct lifestyles, lytic and lysogenic. During the lytic cycle, the phage infect the host, propagate within it and subsequently lyse the host. The new phage particles, which are released may go onto infect other susceptible host cells. During lysogeny, the temperate phage infects the host as in the lytic cycle but integrates its DNA into the host's genome without progression to lysis of the host cell. Once integrated, the bacteriophage is known as a prophage and the host, called a lysogen, reproduces normally copying the prophage and transmitting it to the daughter cells. Prophages often contribute to the pathogenic profile of the lysogen through introduction of novel virulence factors. In the case of *stx* phages they convey the ability to produce Stx in the lysogen (Allison, 2007).

Although a *stx* lysogen may stay stable for many bacterial generations, it may switch to its lytic phase often in the presence of DNA damage. *Stx* phage can also enter the lytic phase in the absence of a DNA damaging agent, a process referred to as spontaneous induction (Little and Mount, 1982). DNA damage could be caused by either physical agents, such as UV-radiation, or chemical agents, such as antibiotics including mitomycin C. The presence of DNA damage in the lysogen activates the bacterial protein RecA, which subsequently triggers bacterial DNA repair mechanisms, called the SOS pathway (Little and Mount, 1982). Once activated the RecA protein cleaves the phage cI, a repressor protein, which prevents the phage from entering the

lytic cycle and induces the prophage lytic cycle (Neely and Friedman, 1998, Shinagawa and Ito, 1973). Activation of the RecA protein is an indicator of phage progression to its lytic phase. Production and release of Stx from the lysogen coincides predominantly with the induction of the lytic cycle and lysis of the host cell. The process of Stx release in the presence of a DNA damaging agent forms the basis of phage induction in a laboratory setting.

Acquisition of *stx* bacteriophages not only confers the *stx* genotype to their host, it also contributes to the genomic diversity of STEC by having different genomic insertion sites (Ogura et al., 2009). To date five target *stx* phage integration sites of STEC O157 strains have been described: *wrbA*, which codes for a NAD(P)H dehydrogenase (quinone) (Patridge and Ferry, 2006); *yehV*, which codes for a transcriptional regulator (Yokoyama et al., 2000); *sbcB*, which produces an exonuclease (Ohnishi et al., 2002); *yecE*, whose function remains unknown (Recktenwald and Schmidt, 2002); and Z2577, which encodes an oxidoreductase (Koch et al., 2003). *Stx* phages conferring particular *stx* genotypes have been associated with specific insertion sites within the *E. coli* O157 genome (Manning et al., 2008, Mellor et al., 2012, Shaikh and Tarr, 2003). The *yehV* and *wrbA* loci have been described as *stx* prophage insertion sites for stx_1 and stx_2 in the USA hamburger-associated outbreak strain EDL933 (Perna et al., 2001) and the Sakai strain from the outbreak in Sakai city, Japan (Hayashi et al., 2001, Makino et al., 1999, Yokoyama et al., 2000). Characterisation of *stx* phage insertion sites among *E. coli* O157:H7 strains indicated the differential distribution of these *stx* phage insertion site genotypes among *E. coli* O157:H7 obtained from human and bovine hosts (Besser et al., 2007, Whitworth et al., 2010). The variation of chromosome insertion loci of *stx* phages within the *E. coli* O157 genome has been shown to contribute to the severity in clinical manifestation of the disease caused by different STEC serotypes (Ogura et al., 2009, Whitworth et al., 2010). Consequently, clinical strains of *E. coli* O157 are generally characterised by the carriage of stx_2 and stx_2-associated bacteriophage sequences adjacent to either *wrbA* or *argW*, while bovine

strains are characterised by carriage of *stx*$_{2c}$ and *stx*$_{2c}$-associated bacteriophage sequences adjacent to *sbcB* (Shringi et al., 2012b). In addition, *stx* phage insertion site genotypes of clinical *E. coli* O157 strains have been shown to differ significantly among strains obtained from different countries (Whitworth et al., 2010). Mellor et al. (2013) indicated that the Australian STEC O157 strains can be distinguished from Argentinean STEC O157 strains based on their unique SBI profiles. The *stx*$_1$ prophage inserts at the *argW* insertion site in Australian STEC O157 strains in comparison to the insertion of *stx*$_2$ prophage at the *argW* insertion site in Argentinean STEC O157 strains. *Stx*$_2$ prophage insertion at the *argW* site is frequently observed among highly virulent strains of STEC O157 (Shringi et al., 2012b).

A significant amount of data has been reported on the diversity of SBI among STEC O157 strains and the severity of infections caused by these different STEC O157 strains. Little, however, is known about SBI among the non-O157 STEC strains from bovine and clinical sources within and between countries. One study indicated the insertion of *stx*$_2$ phage within the *wrbA* locus of two *E. coli* O103:H25 strains recovered from patients in Norway (Sekse et al., 2008). Another study demonstrated the association of *stx*$_2$ phage in more than one locus, including *yecE* and *wrbA*, among STEC O26 strains implicated in human disease (Bielaszewska et al., 2007). An *stx*$_{2e}$-encoding bacteriophage frequently observed in *E. coli* from weaning pigs suffering from oedema disease has been shown to integrate into *yecE* in STEC ONT:H- (Recktenwald and Schmidt, 2002) strain isolated from humans. The data available are insufficient to draw conclusions on the distribution of SBI genotypes of non-O157 STEC strains among the bovine and clinical reservoir as well as between different countries or how the variation of SBI genotypes may affect the virulence of the non-O157 STEC strains. Identification of SBI in the non-O157 STEC population is therefore important to determine the distribution of different virulent clones of non-O157 STEC among sources of bovine and human origin within and between countries.

This study was conducted to investigate the *stx* phage insertion sites among a collection of previously characterised non-O157 STEC from Australia and Malaysia to determine the various *stx* phage insertion site genotypes present among these strains and how these genotypes relate to their potential virulence.

4.2 Materials and methods

4.2.1 Bacterial strains

The STEC O157 and non-O157 strains used in this study are summarised in Table 4.1. All strains were stored at −80°C in tryptone soy broth (TSB; Merck, Darmstadt, Germany) with 25 % glycerol. For resuscitation, all strains were inoculated in to 10 ml TSB and grown for 18 h at 37°C and subsequently streaked onto Luria-Bertani agar (LB; Oxoid, Hampshire, UK). DH5-α, an *E. coli* K-12 derivative strain, was used as the host for *stx* phage infections, and lysogen creation.

Table 4.1. Bacterial strains used in the study

Strain ID	Serogroup	Source	Country	Virulence traits
Sakai	O157	Radish sprouts	Japan	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
1 UPM[a]	O157	Bovine milk	Malaysia	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
3 UPM[a]	O157	Beef	Malaysia	*stx*$_1$, *stx*$_2$, *eaeA*, *ehxA*
DH5-α (*E. coli* K-12)		Laboratory strain	USA	none
EC 4	Non-O157[b]	Cattle feces	Malaysia	*stx*$_1$, *stx*$_2$, *ehxA*
EC3550a	O103	Cattle feces	Australia	*stx*$_2$, *ehxA*
EC3111a	O121	Sheep feces	Australia	*stx*$_1$, *stx*$_2$, *ehxA*

[a]Provided by Prof. Son Radu at University Putra Malaysia.
[b]Serogroup unknown but does not belong to serogroup O157.

4.2.2 Phage preparation

Phage lysates were prepared as described in Sim (2010). Single colonies from each STEC strain were obtained from LB agar plates and inoculated into 10 ml Luria-Bertani broth (LB; Oxoid,

Hampshire, UK). Each colony was grown for approximately 2 h at 37°C to the exponential phase (OD600 ~0.5). Mitomycin C (Sigma Aldrich, Missouri, USA) was added at a concentration of 0.5 µg/ml (Allison et al., 2003) or 1.0 µg/ml (Islam et al., 2012) to induce *stx* bacteriophages from the wild type STEC strains. The induced bacteriophages were amplified for another 6 h at 37°C. The resulting cultures were centrifuged at 4600 rpm for 10 min at 4°C and membrane filtered (Millipore; 0.22 µm pore diameter; Merck, Darmstadt, Germany). These putative phage-containing suspensions were analysed for the presence of *stx* using a previously published multiplex PCR protocol with primers for *stx*$_1$, *stx*$_2$, *eaeA* and *ehx*A (Paton and Paton, 1998). Those suspensions positive for *stx* were used to infect the DH5-α cells as described under 4.2.4. PCR analysis of all phage suspensions confirmed the presence of *stx*.

4.2.3 The standard dual layered agar dilution drop assay

The standard dual layered agar dilution drop assay was used to detect plaque formation by the induced *stx* phage (Kropinski et al., 2009). Briefly, a 100 µl aliquot of DH5-α cells was added to 5 ml LB agar supplemented with 10 mM $CaCl_2$ (Sigma Aldrich, Missouri, USA) and layered on top of LB agar. The phage suspension and its respective diluents were spotted on top of the top agar (Figure 4.1). The agar plates were incubated for 18 h at 37°C. Following incubation, single plaques, if present, were picked using a wide-bore pipette tip and transferred into a 1.5 ml Eppendorf tube containing 100 µl of sterile water. Single plaques were tested with a previously described multiplex PCR protocol with primers for *stx*$_1$, *stx*$_2$, *eaeA* and *ehx*A (Paton and Paton, 1998) to determine the presence of *stx*. PCR products were electrophoresed on 2 % (wt/vol) agarose gels, stained with ethidium bromide and viewed under UV light. Several modifications were made to the standard dual layered agar to obtain visible plaques formation of the *stx* phage, as described in Table 4.2.

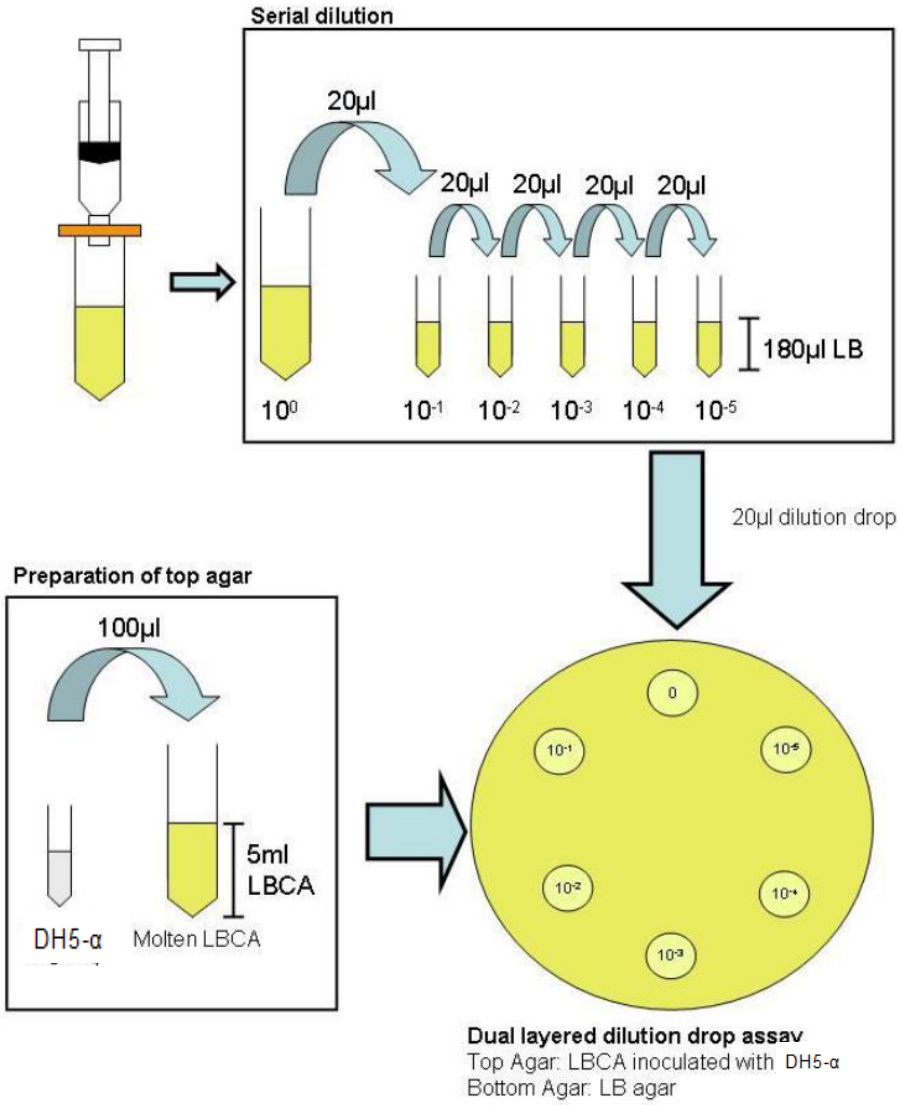


Figure 4.1. Dual layered agar dilution drop assay. Phage suspensions were filtered with a 0.22 µm membrane filter and serially diluted in LB. Aliquots of 20 µl of respective dilutions were spotted onto the dual layered agar and incubated at 37°C. Adapted from Sim (2010).

Table 4.2. Modifications of the standard dual layered agar dilution drop assay

Modification	Dual layered agar	
	Top agar (LB agar supplemented with 10 mM $CaCl_2$)	Bottom agar (LB agar alone)
Modification 1	Addition of 10 mM $MgCl_2$	None
Modification 2	Addition of mitomycin C at a final concentration of 1.5 µg/ml	None
Modification 3	None	Addition of ampicillin at a concentration of 2.5 µg/ml
Modification 4	Addition of 200 µl of DH5-α cells (2.5–3.0 at OD600) to 6 ml of top agar	None
Modification 5	Addition of 200 µl of DH5-α cells (2.5–3.0 at OD600) to 6 ml of top agar	Addition of ampicillin at a concentration of 2.5 µg/ml
Modification 6	Addition of 200 µl of DH5-α cells (2.5–3.0 at OD600) to 6 ml of top agar, 10 mM $MgCl_2$ and mitomycin C at a final concentration of 1.5 µg/ml	None
Modification 7	An aliquot of 100µl of the serially diluted phage lysate and 200 µl of DH5-α cells were mixed first and was incubated for 5–10 min at room temperature. The resulting culture was then mixed with 6 ml of top agar	None

Modification 8	An aliquot of 100µl of the serially diluted phage lysate and 200 µl of DH5-α cells were mixed first and was incubated for 5–10 min at room temperature. The resulting culture was then mixed with 6 ml of top agar	Addition of ampicillin at a concentration of 2.5 µg/ml
Modification 9	An aliquot of 100µl of the serially diluted phage lysate and 200 µl of DH5-α cells were mixed first and was incubated for 5–10 min at room temperature. The resulting culture was then mixed with 6 ml of top agar with 10 mM $MgCl_2$ and mitomycin C at a final concentration of 1.5 µg/ml	None
Modification 10	For full plate titrations, addition of 0.1 ml of phage lysate dilution to 1 ml of DH5-α cells followed by addition of 2 or 3 ml of the top agar	None
Modification 11	For full plate titrations, addition of 0.1 ml of phage lysate dilution to 1 ml of DH5-α cells followed by addition of 2 or 3 ml of the top agar	Addition of ampicillin at a concentration of 2.5 µg/ml
Modification 12	For full plate titrations, addition of 0.1 ml of phage lysate dilution to 1 ml of DH5-α cells followed by addition of 2 or 3 ml of the top agar with 10 mM $MgCl_2$ and mitomycin C at a final concentration of 1.5 µg/ml	None

4.2.4 Formation of *stx*-encoding DH5-α (*E. coli* K12) lysogen strains

Two separate assays (assay 1 and 2) were used for the formation of *stx*-encoding DH5-α (*E. coli* K12) lysogen strains to increase the probability of obtaining the *stx*-encoding lysogens.

Assay 1

Phage lysates positive for *stx* were used in the formation of *stx*-encoding DH5-α lysogen strains. A single colony of DH5-α was inoculated in to 10 ml of LB broth and incubated for 18 h at 37°C. First an aliquot of 3 ml of the phage–containing suspension obtained as described above was added to a 10 ml culture of the DH5-α cells (grown for 18 h at 37°C) and incubated for three days at 37°C. Different volumes (1 ml, 2 ml, 4 ml, or 5 ml) of the *stx* phage lysate from the STEC strains were then each mixed with different volumes (2 ml, 4 ml, 6 ml, or 8 ml) of the DH5-α cells (grown for 18 h at 37°C) according to Table 4.3 and were incubated for three days at 37°C. Following incubation, the cultures were directly streaked on to LB agar and left to grow for 18 h at 37°C. Single colonies of the presumptive *stx* lysogen were sampled and subjected to the above described multiplex PCR to determine the presence of *stx*. Strains positive for *stx* in the absence of additional virulence markers, *eaeA* and *ehx*A, of the donor STEC strains were determined to be *stx*-encoding *E. coli* K12 lysogen strains.

Table 4.3. Volumes of *stx* phage lysate and DH5-α cells used in lysogen formation assay 1

Volume of the *stx* phage lysate	Volume of the recipient DH5-α strain
1 ml	2 ml, 4 ml, 6 ml, 8 ml or 10 ml
2 ml	2 ml, 4 ml, 6 ml, 8 ml or 10 ml
3 ml	2 ml, 4 ml, 6 ml, 8 ml or 10 ml
4 ml	2 ml, 4 ml, 6 ml, 8 ml or 10 ml
5 ml	2 ml, 4 ml, 6 ml, 8 ml or 10 ml

Assay 2

An aliquot of 100µl of the phage lysate was added to 1 ml of recipient DH5-α cells grown to an optical density of 2.5–3.0 (at OD600) in the presence or absence of 10 mM $CaCl_2$. The cell culture was then incubated at 28°C for 50 min. Following incubation, the mixture was serially diluted with LB medium and plated onto LB agar plates. Plates were incubated for 18 h at 37°C. Single colonies of the presumptive *stx* lysogens were confirmed using multiplex PCR as described above.

4.3 Results

A total of seven STEC strains including three STEC O157 strains and three STEC non-O157 strains were used in the dual layered agar dilution drop assay for plaque formation as well as lysogen formation assays (assay 1 and assay 2) (Table 4.4). *Stx* phage were induced from the STEC strains using two different mitomycin C concentrations; 0.5 µg/ml and 1.0 µg/ml. Phage preparation of all STEC O157 strains and one of the STEC non-O157 strains (EC4) were performed by addition of mitomycin C at a concentration of 0.5 µg/ml. *Stx* phage could not be induced in the two STEC non–O157 strains, EC3550a and EC3111a using 0.5 µg/ml of mitomycin C. However, *stx* phage could be successfully induced from these two STEC strains using mitomycin C at a concentration of 1.0 µg/ml.

Table 4.4. Phage induction, plaque formation and lysogen formation of the STEC strains used in the study

Donor strain (serogroup)	Isolation of *stx* phage (added mitomycin C concentration – µg/ml)	Induced *stx* subtype	Recipient strain	Plaque formation in the dual layered agar dilution drop assay	Lysogen formation assay 1 & 2
Sakai (O157)	Positive (0.5)	*Stx*$_2$	DH5-α	Negative	Negative
1 UPM (O157)	Positive (0.5)	*Stx*$_1$	DH5-α	Negative	Negative
3 UPM (O157)	Positive (0.5)	*Stx*$_2$	DH5-α	Negative	Negative
EC 4 (non-O157)	Positive (0.5)	*Stx*$_2$	DH5-α	Negative	Negative
EC3550a (O103)	Positive (1.0)	*Stx*$_2$	DH5-α	Negative	Negative
EC3111a (O121)	Positive (1.0)	*Stx*$_2$	DH5-α	Negative	Negative

All STEC strains positive for *stx* in the phage lysate were used in the standard dual layered agar dilution drop assay to detect plaque formation by the induced *stx* phage. None of the *stx* phage induced from the STEC strains were able to form visible plaques in the standard dual layered agar dilution drop assay. Each STEC strain was subjected to all the modifications made in the standard dual layered agar dilution drop assay twice. However, none of the modifications made to the standard dual layered agar dilution drop assay were successful in creating visible plaques from any of the STEC strains.

All STEC strains positive for *stx* in the phage lysate were also included in the lysogen formation assays (assay 1 and 2) using the DH5-α cells as the recipient cells. In the first assay, mixing of different volumes of the *stx* phage lysate and the recipient DH5-α strain and incubation for three days at 37°C did not result in the formation of lysogens from any of the STEC strains.

Since lysogen formation was unsuccessful using the first assay, a second assay was conducted using *stx* phage lysates of all STEC strains to create lysogens in three separate attempts. Nonetheless lysogen formation was unsuccessful using the second assay from any of the STEC strains used.

4.4 Discussion

Bacteriophage plaque formation is a very complex process, which is governed by the concerted induction of multiple cellular pathways promoted by the expression of genes from the phage as well as the host (Islam et al., 2012). It is a very fundamental and important procedure for phage studies and phage isolation, purification and phenotype analysis to determine *stx* phage insertion sites among newly discovered STEC strains, which do not contain *stx* phage at the established insertion sites. The dual layered agar dilution drop assay is usually used to detect phage plaque formation and to determine phage concentration (Islam et al., 2012). While the examination of plaque-forming ability is an essential step for the characterisation of *stx* phage insertion sites in each phage, newly discovered phages do not always produce visible plaques in the standard dual layered agar dilution drop assay. Similarly, in this study visible plaque formation was not observed in the dual layered agar dilution drop assay conducted using *stx* phage lysates from different STEC strains. This has been identified to be a common problem among lamboid bacteriophages, which include *stx* prophages of STEC strains (Los et al., 2008, Makino et al., 1999).

Several modifications were included in the dual layered agar dilution drop assay to obtain visible plaques of *stx* phage induced from the STEC strains in this study. Among the modifications, addition of 1.5 µg/ml of mitomycin C to the top agar in addition to the10 mM $CaCl_2$ and/or $MgCl_2$ and addition of ampicillin at a concentration of 2.5 µg/ml to the bottom agar was included to increase the size of plaques. The presence of mitomycin C, a DNA damaging agent, in the top agar induces the SOS response in the host cells, which may prevent

the lysogenisation of *stx* phage and induce its lytic phase (Islam et al., 2012). As a result, the plaques are expected to become more visible on the dual layered agar dilution drop assay. According to Los et al. (2008) addition of ampicillin, a β-lactam antibiotic, at a low concentration of 2.5 µg/ml to the bottom agar would increase bacterial cell size by inhibiting the cell division process without decreasing the growth of the cell mass (Jay, 2000). Bigger cells would give rise to larger phage burst sizes (Abee and Wouters, 1999) and thus ampicillin treated cells would produce more virions leading to the production of larger plaques. Moreover, Islam et al. (2012) reported that the addition of Ca^{2+} and/or Mg^{2+} to the top agar of the dual layered agar dilution drop assay significantly improved the adsorption of Sp5, a Stx2 phage of the STEC O157 Sakai strain, to the host cells. It was suggested that the increased efficiency of phage adsorption to the host cell may allow a more efficient radial diffusion of the phage particles leading to visible plaque formation. Unfortunately, none of these modifications induced visible plaque formation of *stx* phage in the dual layered agar dilution drop assay conducted in this study. The *stx* phage concentration present in the *stx* phage lysates obtained from the STEC strains in this study may have been insufficient to infect the host cells and create visible plaques in the dual layered agar dilution drop assay. However, formation of visible plaques was required to measure the induced *stx* phage concentration of the phage suspension in the first place.

Lysogen formation by *stx* phages facilitates the isolation of a single *stx* phage from STEC strains, which may otherwise carry multiple *stx* and non-*stx* prophages. This also allows purification of phage DNA from a single bacteriophage. In addition, lysogen formation by *stx* phages confirms that the *stx* genes carried by the STEC strains are phage borne and that these prophages are capable of horizontal gene transfer. Although the process of lysogen formation was important in the identification of *stx* phage insertion sites, *stx* lysogens could not be formed using the STEC strains from this study. Lysogen formation from STEC strains has been

reported to be unsuccessful in other studies (Mellor et al., 2012, Muniesa et al., 2004a). Failure to form lysogens from STEC strains in this study may have resulted from *stx* phage progression to lytic phase more readily than the lysogenic phase. Although the *E. coli* strain DH5-α has been shown to form lysogens successfully in other studies, different recipient strains of *E. coli* apart from DH5-α may form lysogens more readily with the *stx* phages induced from the STEC strains of this study. Thus, the use of different recipient strains of *E. coli* in the lysogen formation assays from these *stx* phages should be examined in future studies. In addition, southern hybridization of dioxygenin-11-dUTP-labelled *stxA* to *xbaI* digested *E. coli* genomic DNA separated using pulsed-field gel electrophoresis is another method, by which *stx* phage insertion sites can be identified (Bielaszewska et al., 2007). This method could be used in further studies to identify *stx* phage insertion sites among the non-O157 STEC strains.

4.5 Conclusion

In conclusion, the effort to analyse *stx* phage insertion sites of the non-O157 STEC strains using lysogens was unsuccessful due to the inability of creating *stx* phage lysogens according to previously established methods. Further studies are required using different and improved methods and a variety of donor and recipient *E. coli* strains for the successful formation of lysogens in order to establish the *stx* phage insertion sites among the non-O157 STEC strains.

Chapter 5

Impact of sub-lethal heat, cold and acid adaptation stress on subsequent acid tolerance response of two multi-antibiotic resistant *Escherichia coli* (*E. coli*) strains isolated from Malaysia

5.1 Introduction

Bacteria often encounter a variety of stresses both in the environment and during infection of a host. These stresses include changes in temperature, pH, moisture content and water activity (Jay, 2000). The survival and growth of bacteria depends on these factors and manipulation of them has allowed the food industry to control growth of food-borne pathogens during food processing. Exposure to sub-lethal environmental stresses induces a stress response in both pathogenic and non-pathogenic bacteria, which can lead to subsequent resistance to a variety of environmental or processing parameters they may encounter, enabling them to survive under more severe conditions (Abee and Wouters, 1999, Law, 2000, Lin et al., 1996). The enhanced survival of bacteria following exposure to sub-lethal stress conditions has important implications for the existing control measures employed to minimize food borne pathogens, as these control measures may no longer be adequate to control the growth of bacteria in food products. For this reason the effect of sub-lethal stresses on the survival of bacteria should be considered in order to increase the efficacy of currently employed control measures.

Bacteria are exposed to changes in temperature during heating and refrigeration of foods, which are two important techniques used to preserve their quality and safety. Unfortunately, exposure of bacteria to high temperature conditions during insufficient cooking or reheating of foods and low temperature conditions during refrigeration can lead to heat and cold stress conditions respectively (Chang et al., 2009, Schlesinger, 1990). In response to a heat stress also called 'heat shock', bacteria produce proteins called heat shock proteins (HSPs) (Schlesinger, 1990), while cold shock proteins (CSPs) are produced in response to cold stress (Berry and Foegeding, 1997). These proteins serve as a protective mechanism to cope with heat and cold induced damage enabling them to survive the adverse conditions (Jones et al., 1987, Schlesinger, 1990).

Microbial inactivation by acids is another method commonly used to control the growth and survival of bacteria in food (Brown and Booth, 1991). Exposure of bacteria to moderate acidic

environments (pH 4.5 to 5.5) induces an adaptive tolerance response called the acid tolerance response (ATR) (Brown and Booth, 1991) in many bacteria leading to increased resistance to subsequent acid stresses (Chung et al., 2006). While the importance of gastric juice, which creates a strong acidic environment for bacteria is important in controlling food borne infections, enhanced survival under strong acidic conditions (pH 1.5 to 3.0) due to ATR may allow passage of enteric pathogens through the stomach decreasing the infective dose.

Adaptation of bacteria to one stress condition has been shown to enhance resistance to different stress conditions (Cheville et al., 1996). This phenomenon is referred to as cross-protection. Enhanced heat tolerance of bacteria following adaptation to acid stress and vice versa has been documented in previous studies (Leenanon and Drake, 2001, Rowe and Kirk, 2000, Wang and Doyle, 1998). *E. coli* have been shown to exhibit enhanced survival to heat or acid stress following sub-lethal environmental stresses (Juneja et al., 1998, Murano and Pierson, 1992). The nature and intensity of the stress response have been shown to vary widely between each strain of pathogenic and non-pathogenic *E. coli* (Benjamin and Datta, 1995, Duffy et al., 2000, Leenanon and Drake, 2001, McKellar and Knight, 1999). As such, a more complete understating of the stress response of different *E. coli* strains and its effect on subsequent resistance is required to assess and minimise the risk of food-borne illness. In addition, reports in the literature suggest that antibiotic resistant bacteria may display different resistance to food-related stresses such as acid and heat (Bacon et al., 2003). There is a lack of information on naturally antibiotic resistant bacteria and the effects of sub-lethal stresses on their physiological tolerance. This study was conducted to determine the effects of heat-shock and acid adaptation on post-stress acid tolerance of two naturally multi-antibiotic resistant *E. coli* strains, one *stx* positive and one *stx* negative.

5.2 Materials and methods

5.2.1 Bacterial strains and culture conditions

Two *E. coli* strains (one *stx* positive, EC27 and one *stx* negative, EC135), which were obtained from cattle feces samples collected from two different cattle farms in Peninsular Malaysia as described in Chapter 2 were used in all assays. Both *E. coli* strains were naturally multi-antibiotic resistant. EC27 was resistant to ampicillin, streptomycin, tetracycline and trimethoprim, while EC135 was resistant to chloramphenicol, kanamycin, tetracycline and trimethoprim. Stock cultures of both *E. coli* strains were stored in tryptone soya broth (TSB; Oxoid, Hampshire, UK) with 25 % glycerol at −80°C. Prior to use, cultures of both strains were grown for 18 h in TSB at 37°C and transferred to fresh medium on two consecutive days before use.

5.2.2 Inoculum preparation

Individual strains were cultured by inoculation of a loopful of bacteria obtained from a tryptone soya agar (TSA; Oxoid, Hampshire, UK) working culture plate in to 10 ml of fresh brain heart infusion (BHI; Oxoid, Hampshire, UK) broth and incubating for 18 h ± 2 h to the stationary phase (about 9 log cfu/ml) at 37°C. Cultured bacteria were diluted 1/10 in fresh BHI broth and were used in further assays (Wang and Doyle, 1998).

5.2.3 Determination of heat shock treatment temperature

The maximum growth temperature of the *E. coli* strains was established to determine the temperature, at which the heat shock treatment was to be conducted. To determine the maximum growth temperature of the strains, a single colony obtained from a TSA working culture plate was inoculated in to10 ml of fresh BHI broth and incubated for 18 ± 2 h at 37°C. Following incubation, 0.1 ml of the culture was added to 9.9 ml of fresh BHI broth, which was submerged in a shaking water bath (100 rpm) maintained at 45, 46, 47 or 48°C at an initial population of approximately 7 log cfu/ml. The viability of the *E. coli* strain was measured at

1/2 h intervals up to 3h by obtaining viable cell counts at each time point as described under 5.2.9. The incubation time of the *E. coli* cells were extended up to 48 h at each temperature and the absorbance reading of the cultures at 600 nm were obtained. Since the maximum growth temperature of the *E. coli* strains was determined to be 47°C (data not shown), heat shock treatment was performed at 48°C, a temperature higher than the maximum growth temperature.

5.2.4 Heat shock treatment followed by acid tolerance assay

E. coli cells (about 7 log cfu/ml) were heat shocked by holding cells at 48°C in a temperature controlled water bath with agitation (100 rpm) for 10 min (Wang and Doyle, 1998). Following heat–shock treatment, 0.1 ml of each culture was immediately used for acid tolerance assays as described under 5.2.8 to determine the effect of heat shock on the survival of *E. coli* cells under strong acidic environments (pH 2.5). *E. coli* cells without heat shock treatment were included as the non-heat shocked control.

5.2.5 Acid adaptation followed by acid tolerance assay

Fresh BHI broth was prepared, adjusted to pH 5 with concentrated HCl, distributed in 9.9 ml portions into universal bottles, and autoclaved for 15 min at 121°C. The pH of BHI broth in representative universal bottles was determined after sterilization to verify any changes in pH resulting from autoclaving the broth were ≤ 0.1 pH unit. For acid adaptation, an inoculum of 0.1 ml of bacterial culture (about 7 log cfu/ml) was added to the acidified BHI broth pre-warmed to 37°C and incubated for 1 h at 37°C (Wang and Doyle, 1998). Following incubation for 1 h at 37°C, 0.1 ml of the acid adapted cells were immediately used in the acid tolerance assays described under 5.2.8 to determine the effect of acid adaptation on the survival of *E. coli* cells under strong acidic environments (pH 2.5). *E. coli* cultures not exposed to the acid adaptation were included as the non-acid adapted control.

5.2.6 Acid adaptation–heat shock treatment followed by acid tolerance assay

E. coli cells were subjected to acid adaptation followed by heat shock treatment as described above prior to be used in the acid tolerance assay to determine the effect of acid adaptation and heat shock on the survival of *E. coli* cells under strong acidic environments (pH 2.5). *E. coli* cells subjected to heat shock treatment without the initial acid adaptation were used as the control cells.

5.2.7 Cold stress adaptation–heat shock treatment followed by acid tolerance assay

E. coli cells were cold-stored at 4°C in a temperature controlled water bath with agitation (100 rpm) for one week (Buncic and Avery, 1998). Following cold storage for one and seven days, 0.1 ml of each culture was immediately subjected to heat shock treatment (as described under 5.2.4) followed by the acid tolerance assay (described below) to determine the effect of cold storage and heat shock on the survival of *E. coli* cells under strong acidic environments (pH 2.5). Viable cell counts were performed following acid tolerance assay for the heat shocked cells after cold storage. *E. coli* cells subjected to heat shock without the initial cold shock treatment were included as the control cells.

5.2.8 Acid tolerance assay

Fresh BHI broth was prepared, adjusted to pH 2.5 with concentrated HCl, distributed in 9.9 ml portions into universal bottles, and autoclaved for 15 min at 121°C (Wang and Doyle, 1998). The pH of BHI broth in representative universal bottles was determined after sterilization to verify any changes in pH resulting from autoclaving the broth were ≤ 0.1 pH unit. All bottles were pre–warmed to 37°C in a water bath prior to use. An inoculum of 0.1 ml of each bacterial culture was immediately transferred to 9.9 ml of BHI broth (pH 2.5) following acid adaptation and/ or heat-shock treatments as described above. Viable cell counts were performed at 0, 2, 4, 6, 8, 10 and 20 min depending on the requirement of the assay.

5.2.9 Enumeration of viable *E. coli* cells

To enumerate the viable cells, the inoculated broth was serially diluted in phosphate-buffered saline (PBS; Oxoid, Hampshire, UK) and plated onto TSA in duplicate. All plates were incubated at 37°C, and colonies were counted after 48 h. A colony count of 300 cfu/ml (representing 30 colonies from 0.1ml of undiluted cell culture) was considered as the lower limit of enumeration. For all assays, the percent survival of the *E. coli* cells was obtained by dividing the population at each time point (0 to 10 min or 20 min) with the initial population (at 0* min) before exposure to the acidified broth (pH 2.5) in the acid tolerance assay, which represented 100% and converting to percent.

5.2.10 Statistical analysis

The mean value and standard deviation were calculated from the data obtained from three separate experiments. Data were analysed using an independent-samples t-test (IBM, SPPSS software, Version 20). Statistical significance was set up at $p < 0.05$.

5.3 Results

5.3.1 Effect of heat shock on subsequent acid tolerance

Heat shock treatment of strain EC27 at 48°C for 10 min reduced its initial cell population by approximately 0.2 log cfu/ml. However, heat shock treatment did not produce a significant difference ($p > 0.05$) in their survival in acidified BHI broth (pH 2.5) at any time point between 0 to 10 min as compared to the control cells without heat shock treatment (Figure 5.1). Initial exposure (0 min) to the acidified broth (pH 2.5) rapidly reduced the population of the heat shocked cells from both the heat shocked and control cells of EC27 to less than 50 %. Survival of both heat shocked and control *E. coli* cells in the acidified broth continued to reduce with time up to 10 min, at which the number of cells were lower than the limit of enumeration (< 300 cfu/ml). Heat shock treatment of strain EC135 at 48°C for 10 min reduced its initial cell population in 0.9 log cfu/ml. Similar to strain EC27, heat shock treatment of strain EC135 did

not indicate a significant difference ($p > 0.05$) in their survival in acidified BHI broth (pH 2.5) (Figure 5.2).

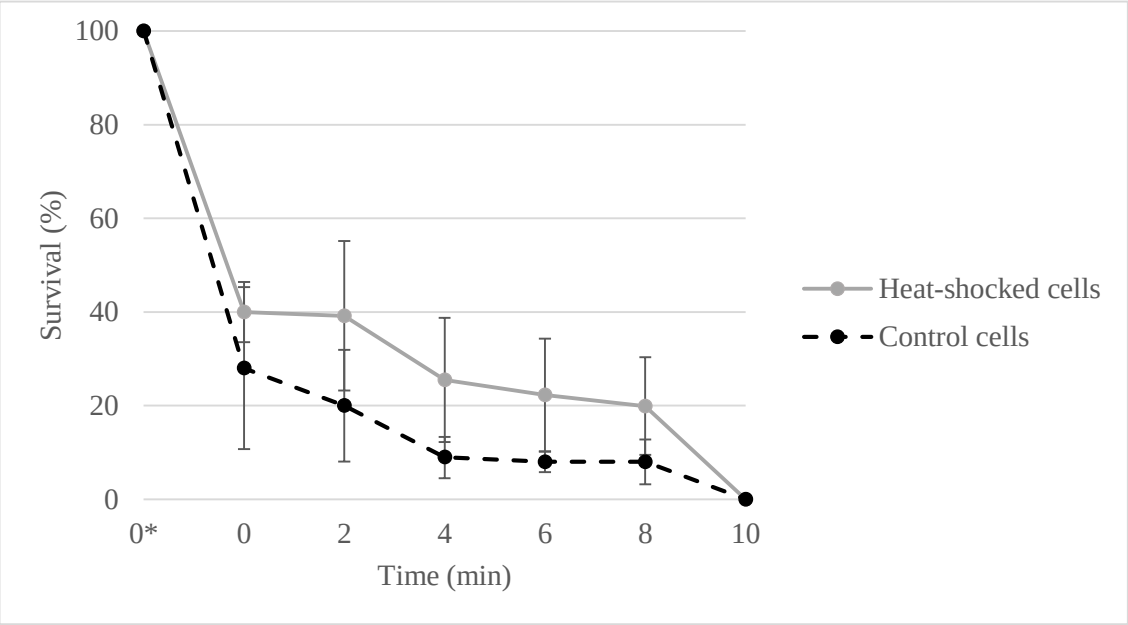


Figure 5.1. Effect of heat shock at 48°C for 10 min on the survival of *stx* positive *E. coli* strain (EC27) in acidified BHI broth at pH 2.5. Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC27 strain from 0 to 10 min in acidified broth (pH 2.5). Data were expressed as mean ± standard error from the three separate experiments.

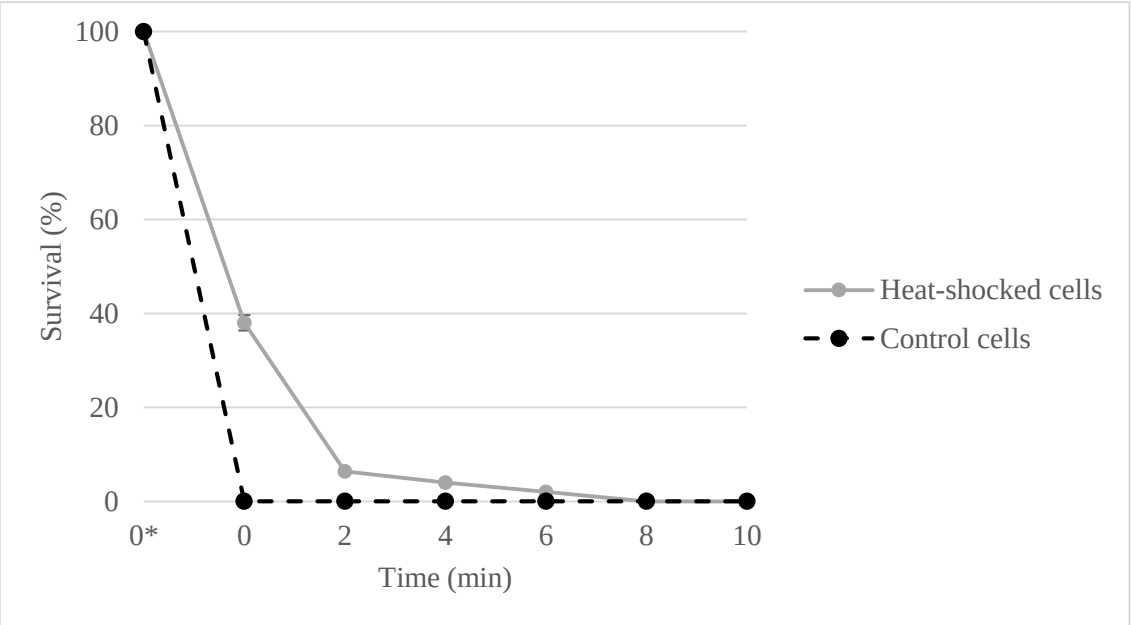


Figure 5.2. Effect of heat shock at 48°C for 10 min on the survival of *stx* negative *E. coli* strain (EC135) in acidified BHI broth at pH 2.5. Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC135 strain from 0 to 10 min in acidified broth (pH 2.5). Data were expressed as mean ± standard error from the three separate experiments.

5.3.2 Effect of acid adaptation on subsequent acid tolerance

Acid adaptation (at pH 5 for 1 h) of strain EC27 showed a 0.8 log cfu/ml reduction in its initial cell population. When exposed to the acidified broth at pH 2.5, survival of the non-acid adapted control cells of strain EC27 was significantly higher ($p < 0.05$) up to 6 min as compared to the acid adapted (at pH 5 for 1 h) cells (Figure 5.3). However, exposure of control cells to the acidified broth (pH 2.5) after 6 min did not show any significant difference ($p > 0.05$) in survival compared to the acid adapted cells. After 20 min of exposure to the acidified broth (pH 2.5), the number of control cells were less than the limit of enumeration (< 300 cfu/ml).

In the EC135 strain, acid adaptation (at pH 5 for 1 h) of cells reduced its initial cell population in approximately 0.1 log cfu/ml. Survival of the acid adapted (at pH 5 for 1 h) cells in the acidified BHI broth (pH 2.5) was significantly higher ($p < 0.05$) at 0 min in comparison to the

non-acid adapted control cells, which became less than the limit of enumeration (< 300 cfu/ml) upon exposure to the acidified broth at pH 2.5 (Figure 5.4). The acid adapted cells also were less than the limit of enumeration (< 300 cfu/ml) after 2 min of exposure to the BHI broth at pH 2.5.

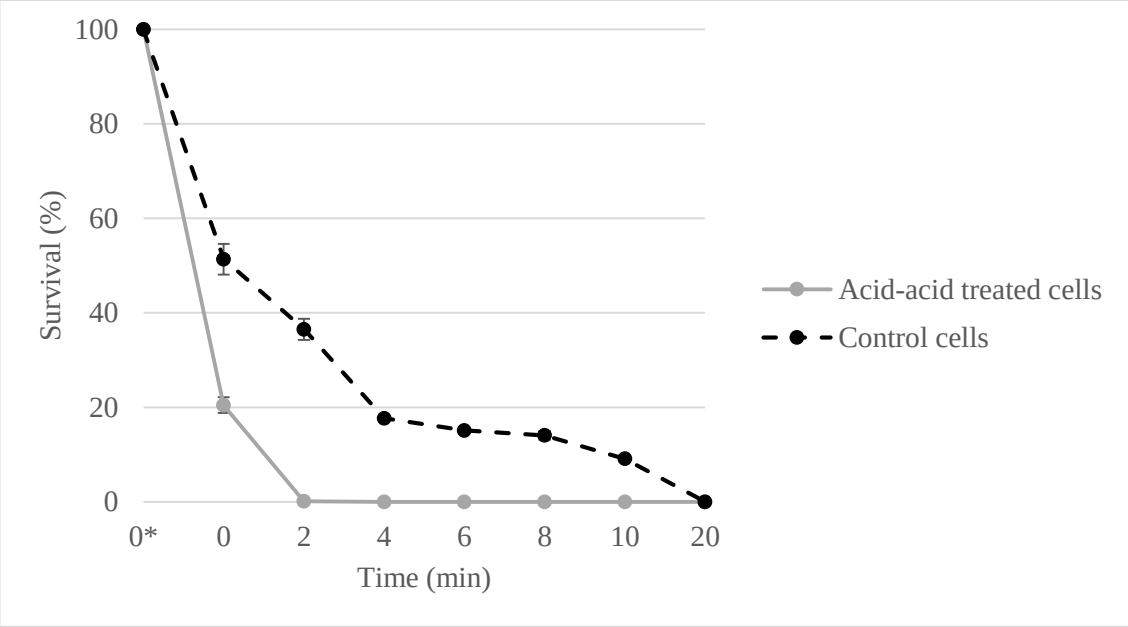


Figure 5.3. Effect of acid adaptation for 1 h in acidified BHI broth at pH 5 (with conc. HCl) on the survival of *stx* positive *E. coli* strain (EC27) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 20 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC27 strain from 0 to 20 min in acidified broth (pH 2.5). Data were expressed as mean ± standard errors from the three separate experiments.

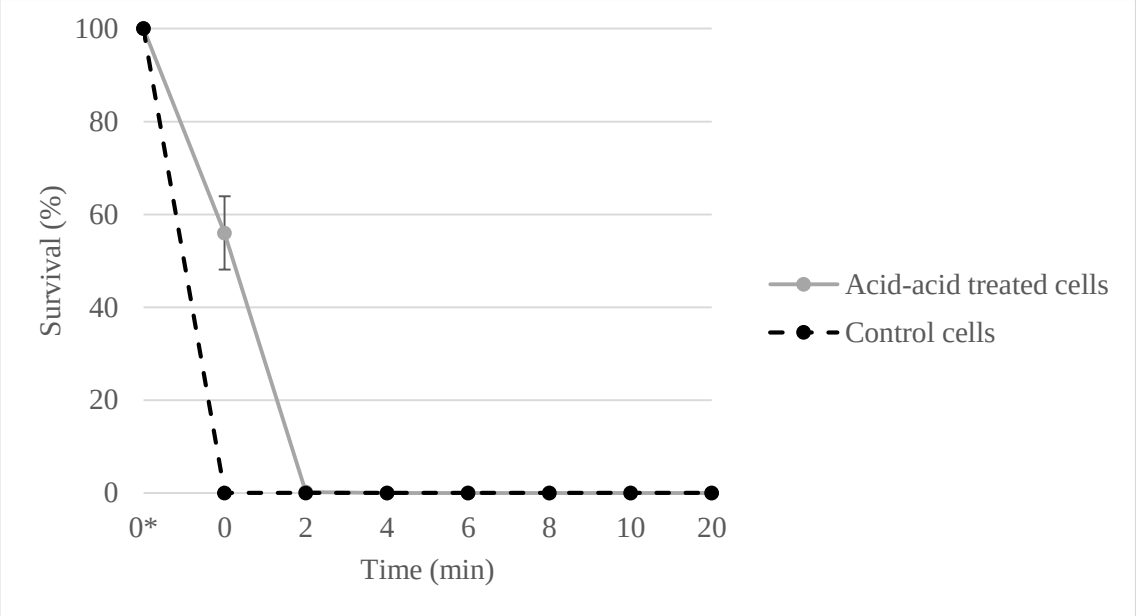


Figure 5.4. Effect of acid adaptation for 1 h in acidified BHI broth at pH 5 (with conc. HCl) on the survival of *stx* negative *E. coli* strain (EC135) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 20 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC135 strain from 0 to 20 min in acidified broth (pH 2.5). Data were expressed as mean ± standard error from the three separate experiments.

5.3.3 Effect of acid adaptation and heat shock treatment on subsequent acid tolerance

Acid adaptation (at pH 5 for 1 h) and subsequent heat shock treatment of strain EC 27 reduced its initial cell population in approximately 1.0 log cfu/ml. The number of control cells without the initial acid adaptation (at pH 5 for 1 h) declined approximately 0.3 log cfu/ml following heat shock treatment. Upon exposure to the acidified BHI broth at pH 2.5, the acid adapted (at pH 5 for 1 h) and heat shocked (at 48°C for 10 min) cells of strain EC27 were less than the limit of enumeration (< 300 cfu/ml) (Figure 5.5). In contrast, survival of the control cells, which were heat shocked at 48°C for 10 min without the initial acid adaptation (at pH 5 for 1 h), remained significantly higher ($p < 0.05$) at approximately 30 % when exposed to the acidified BHI broth (pH 2.5) at 0 min. The survival of the control cells decreased gradually and the

number of cells became less than the limit of enumeration (< 300 cfu/ml) after 10 min of exposure to the acidified BHI broth (pH 2.5).

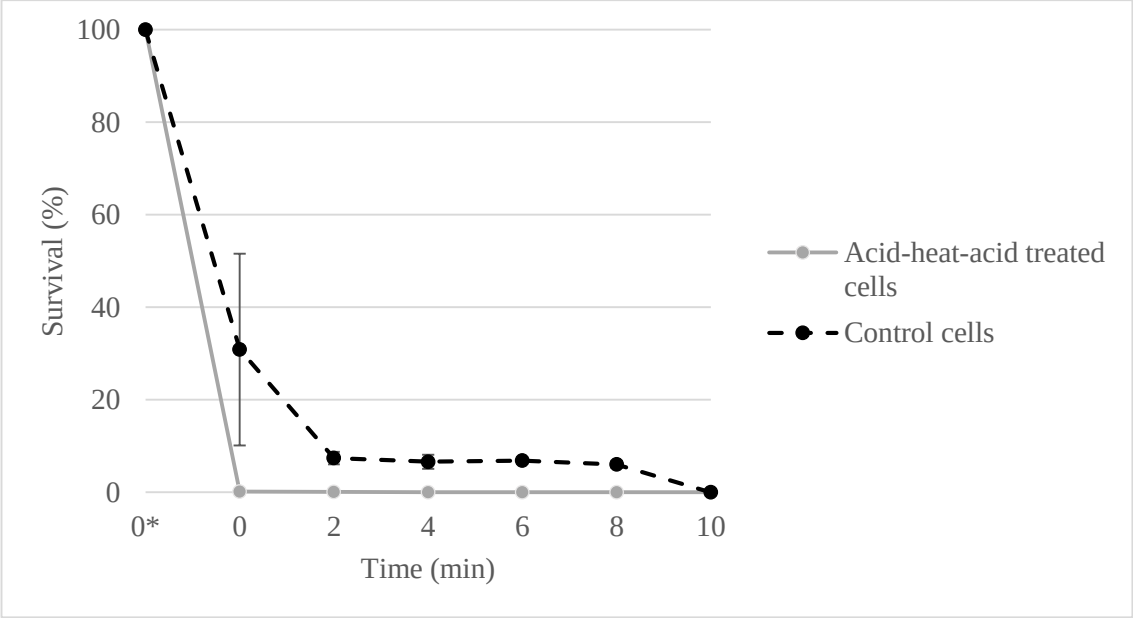


Figure 5.5. Effect of initial acid adaptation for 1 h in acidified BHI broth at pH 5 using conc. HCl on the survival of heat shocked (at 48°C for 10 min) *stx* positive *E. coli* strain (EC27) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC27 strain from 0 to 10 min in acidified broth (pH 2.5). Data were expressed as mean ± standard error from the three separate experiments.

The initial cell population of strain EC135 showed a reduction of approximately 1.3 log cfu/ml following acid adaptation (at pH 5 for 1 h) and heat shock treatment (48°C for 10 min), while the control cells without the initial acid adaptation showed only a reduction of 0.5 log cfu/ml following heat shock treatment. Similarly to the EC27 strain, the acid adapted (at pH 5 for 1 h) and heat shocked (48°C for 10 min) cells of strain EC135 became less than the limit of enumeration (< 300 cfu/ml) with the initial exposure (at 0 min) to the acidified BHI broth (pH 2.5) (Figure 5.6). The control cells without the initial acid adaptation at pH 5 remained largely

viable ($p < 0.05$) with a 40 % survival when exposed to the acidified BHI broth (pH 2.5) at 0 min. However, the number of control cells also became less than the limit of enumeration (< 300 cfu/ml) after 10 min of exposure to the acidified broth (pH 2.5).

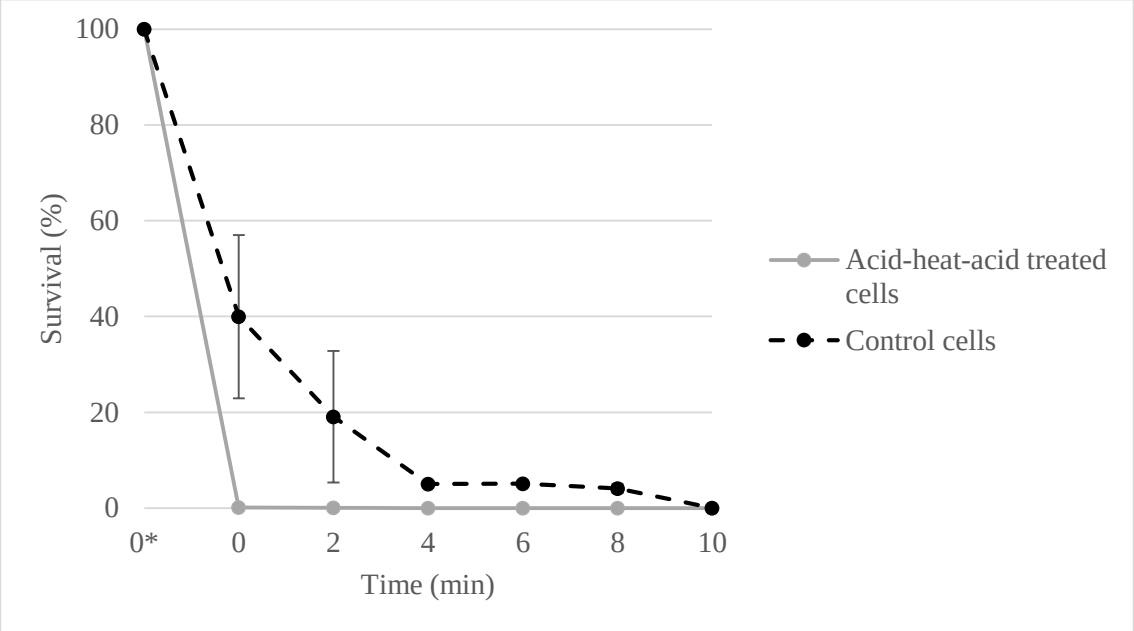


Figure 5.6. Effect of initial acid adaptation for 1 h in acidified BHI broth at pH 5 using conc. HCl on the survival of heat shocked (at 48°C for 10 min) *stx* negative *E. coli* strain (EC135) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC135 strain from 0 to 10 min in acidified broth (pH 2.5). Data were expressed as mean ± standard error from the three separate experiments.

5.3.4 Effect of cold-stress adaptation and heat shock treatment on subsequent acid tolerance

Cold storage of strains EC 27 and EC 135 at 4°C for 1 day did not change the initial cell populations although the heat shock treatment (48°C for 10 min) following cold storage (for 1 day) reduced the initial cell populations by approximately 1.0 log cfu/ml. The control cells of strain EC27 and EC135 without the initial cold-stress adaptation showed approximately 0.5 log cfu/ml and 0.7 log cfu/ml reductions respectively in their initial cell population following heat

shock treatment (48°C for 10 min). Exposure of the cells of strain EC27 cold-stored for 1 day at 4°C followed by heat shock treatment to the acidified BHI broth at pH 2.5 reduced the number of cells to less than the limit of enumeration (< 300 cfu/ml at 0 min) (Figure 5.7a). However, the survival of the control cells, which were heat shocked at 48°C for 10 min without the initial cold–stress adaptation remained significantly higher ($p < 0.05$) up to 8 min in the acidified BHI broth (pH 2.5) in comparison to the cold–stress adapted and heat shocked cells.

Seven days of cold storage of strains EC27 and EC135 at 4°C reduced their initial cell populations by approximately 1.0 log cfu/ml. Heat shock treatment (48°C for 10 min) following seven days of cold storage reduced the cell populations of both strains further in approximately 2 log cfu/ml. Overall, seven days of cold storage at 4°C followed by heat shock treatment (48°C for 10 min) reduced the initial cell populations of both strains by approximately 3 log cfu/ml. However, the control cells of strain EC27 and EC135 without the initial cold-stress adaptation for seven days showed approximately 0.4 log cfu/ml and 0.8 log cfu/ml reductions respectively in their initial cell population following heat shock treatment (48°C for 10 min). Exposure of strain EC27 to the acidified broth (pH 2.5) following cold storage for seven days followed by heat shock treatment did not enhance their survival in the acidified broth in comparison to the control cells. In fact, similar to cold storage of cells for one day, the number of cold-stored cells for seven days became less than the limit of enumeration (< 300 cfu/ml, at 0 min) immediately upon exposure to the acidified BHI broth at pH 2.5 (Figure 5.7b), while the control cells remained significantly viable ($p < 0.05$) up to 6 min in the acidified broth.

The number of cold-stress adapted cells of strain EC135 for both one day and seven days were less than the limit of enumeration (< 300 cfu/ml, at 0 min) immediately upon exposure to the acidified BHI broth at pH 2.5 (Figure 5.8a & 5.8b). In contrast, the control cells without the cold-stress adaptation indicated significant difference ($p > 0.05$) in their survival in the acidified BHI broth at pH 2.5 for up to 2 min.

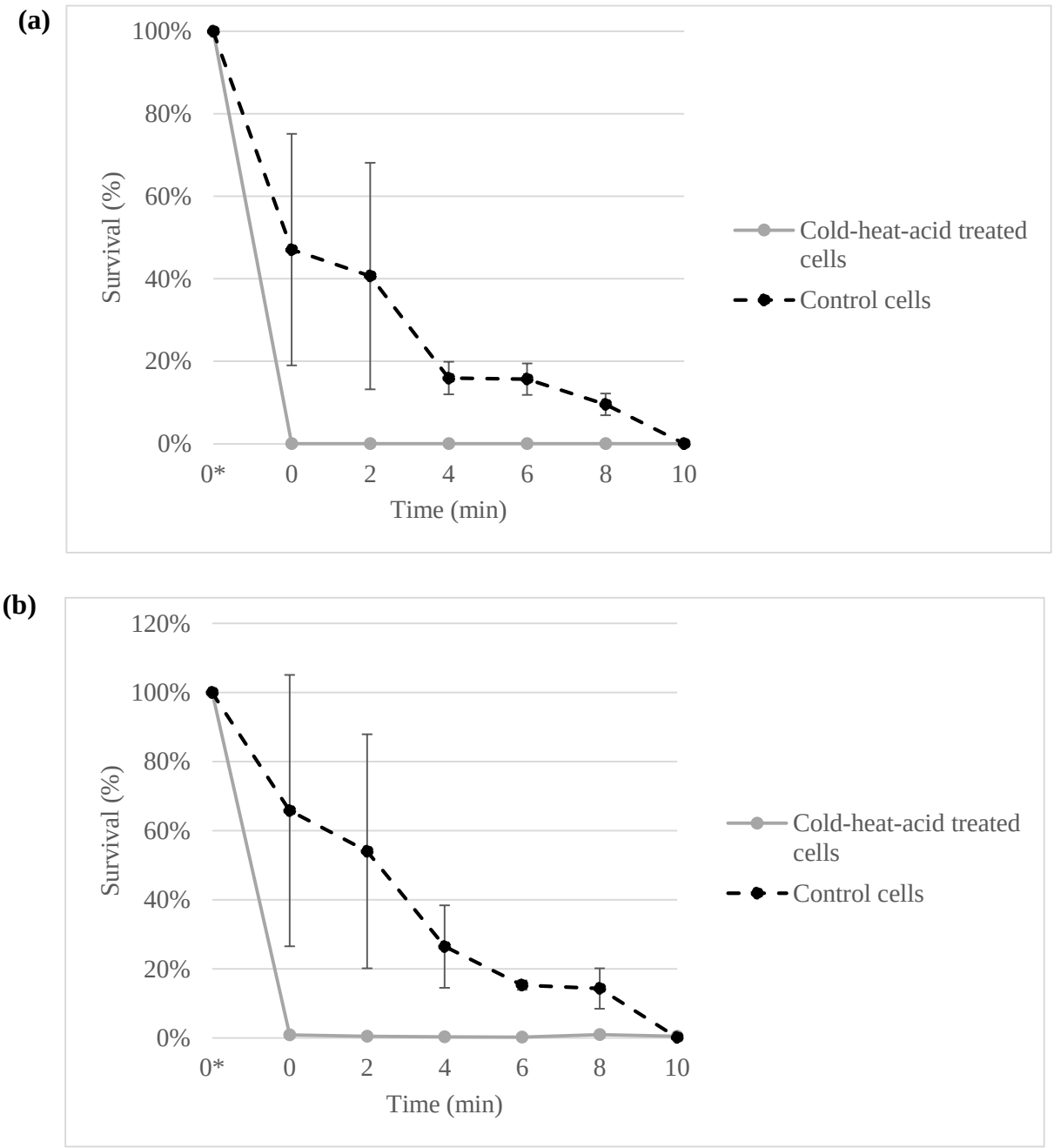


Figure 5.7. Effect of cold–stress adaptation at 4°Cfor (a) 1 day and (b) 7 days on the survival of heat shocked (at 48°C for 10 min) *stx* positive *E. coli* strain (EC27) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC27 strain from 0 to 10 min in acidified broth (pH 2.5). Data were expressed as mean ± standard error from the three separate experiments.

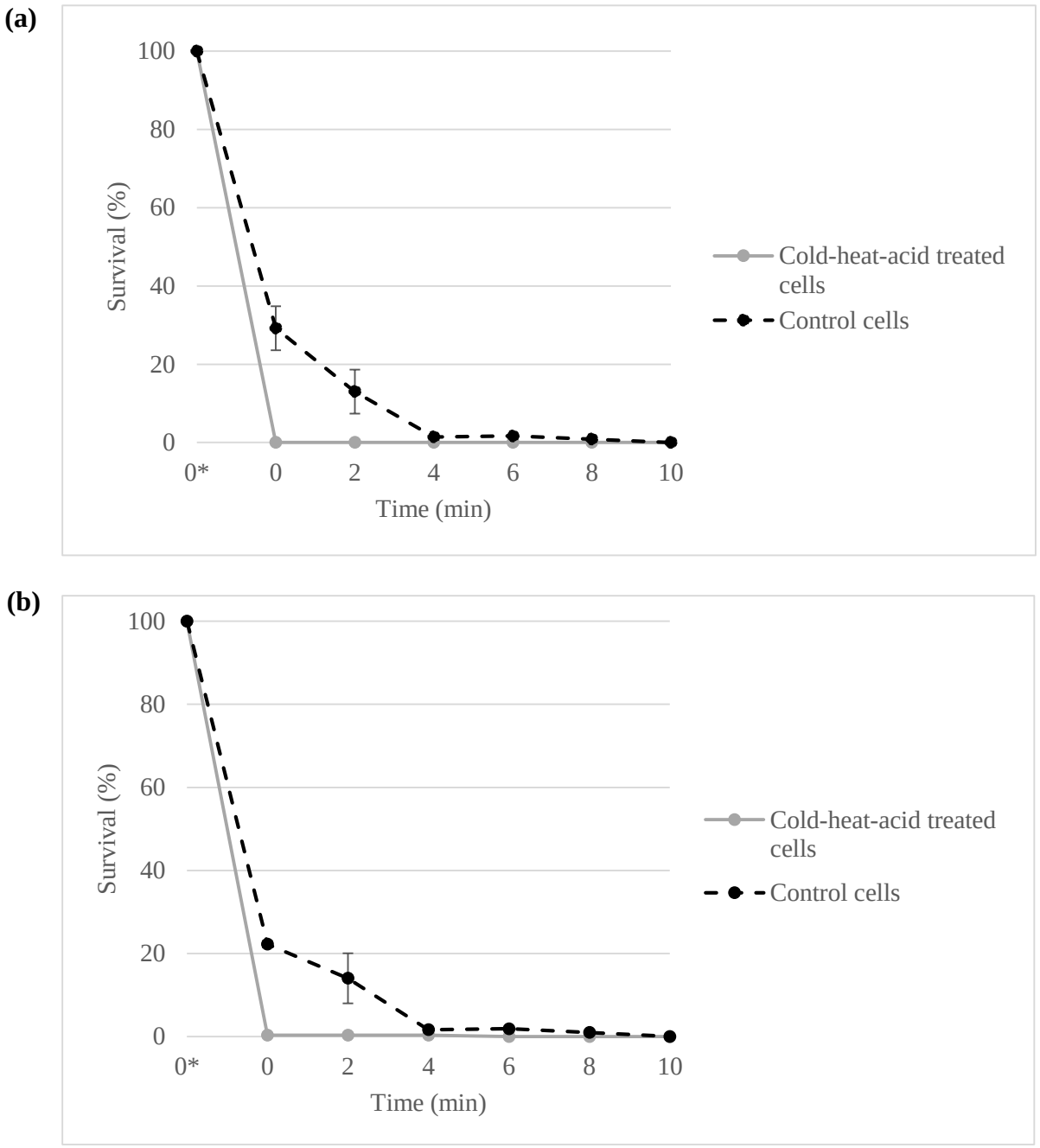


Figure 5.8. Effect of cold–stress adaptation at 4°Cfor (a) 1 day and (b) 7 days on the survival of heat shocked (at 48°C for 10 min) *stx* negative *E. coli* strain (EC135) in acidified BHI broth at pH 2.5 (with conc. HCl). Percent survival was obtained by dividing the surviving population at each time point (0 to 10 min) with the initial population (at 0* min, 100 %) before exposure to the acidified broth (pH 2.5) and expressing as a percentage. Percentage survival was obtained for exposure of the EC135 strain from 0 to 10 min in acidified broth (pH 2.5). Data were expressed as mean ± standard error from the three separate experiments.

5.4 Discussion

This study examined the survival of two naturally multi-antibiotic resistant *E. coli* strains, EC27 and EC135 under strong acid tolerance conditions, which mimics the acidic environment of the human stomach, following sub-lethal heat stress, cold stress and/or acid adaptation conditions. Although, most previous studies (Leenanon and Drake, 2001, Wang and Doyle, 1998) have shown that heat shock treatment of *E. coli* increases survival to subsequent strong acidic conditions, EC27 strain used in this study did not have enhanced survival after heat shock treatment as compared to the control cells without the heat shock treatment. Strain EC135 only exhibited a relatively weak tolerance to the strong acidic conditions (pH 2.5) even after the heat shock treatment. This contradicts the results obtained in other studies, in which heat shock treatment of *E. coli* cells at 48°C for 10 min resulted in increased survival in laboratory media acidified to pH 2.5 using concentrated HCl for up to 6 hours or even few days compared to the non-heat shocked cells (Wang and Doyle, 1998).

There is lack of data on the effect of cold storage of *E. coli* under food-related regimes especially when accompanied by exposure to common stress conditions before consumption of food and during their digestion. This study indicated that cold storage of the multi-antibiotic resistant *E. coli* strains at 4°C and subsequent heat shock treatment did not enhance their survival under strong acidic conditions encountered in the human stomach. In fact the survival of the cold stress-adapted EC27 and EC135 cells was significantly lower in acidified broth (pH 2.5) in comparison to the non-adapted cells. Increased heat sensitivity of cold stress-adapted cells at 4°C for seven days was observed in both *E. coli* strains of this study by the increased reduction of cell numbers after cold storage for seven days followed by heat shock treatment. Since the focus of the experiments of this study was to determine the effect of cold stress and heat shock on the acid tolerance of the cells, specific experiments were not conducted to fully analyse the effect of cold stress-adaptation on heat sensitivity of the cells. Similar findings were

reported in other studies where cold stress-adapted cells at 4°C showed increased heat sensitivity (Leyer and Johnson, 1993, Shen et al., 2011) as well as decreased acid tolerance (Elhanafi et al., 2004). The increased heat sensitivity of cold stress-adapted cells was suggested to be the result of accumulation of unsaturated fatty acids within the cell membranes leading to the reduction of their melting point (Semanchek and Golden, 1998).

Pre-exposure of *E. coli* cells to moderate acidic environments (pH 4.5–5.5) or acid adaptation has been shown to enhance their survival in subsequent strong acidic conditions (pH 1.5–3.0) in a number of reports (Arnold and Kaspar, 1995, Cheville et al., 1996). EC135 strain used in this study survived less than two minutes under strong acidic conditions (pH 2.5) following acid adaptation at pH 5. The data from the EC27 strain indicated that acid adaptation may have decreased its ability to survive in subsequent strong acidic conditions compared to the non-acid adapted control cells, which showed substantial survival for at least up to 6 min in pH 2.5 broth. A previous study indicated that mild injury in *E. coli* cells can occur in response to the use of HCl acid as an acidulant (Leyer et al., 1995). Acid adaptation of cells at pH 5 using HCl as the acidulant may have caused cell damage resulting in decreased survival of cell under more acidic conditions.

Both pathogenic and non-pathogenic bacteria may encounter several stress conditions in the environment and during food processing. As such, the effect of an initial acid adaptation on the survival of the two heat shocked *E. coli* strains in strong acid conditions (pH 2.5) was also examined. Other authors report acid adaptation induced significant cross-resistance to a wide range of stresses including heat in *E. coli* as well as other bacteria such as *Salmonella* (Arnold and Kaspar, 1995, Leyer and Johnson, 1993, Lee et al., 1995, Ryu and Beuchat, 1998). However, initial acid adaptation at pH 5 did not enhance the survival of the *E. coli* strains used in this study, which were heat shocked before exposure to the strong acidic conditions at pH 2.5. Although the exact effect of acid habituation has on subsequent heat shock treatment and

acid tolerance needs to be determined, mild injuries in *E. coli* cells, caused by the use of HCl acid as an acidulant (Leyer et al., 1995), and leakage of intracellular components commonly associated with heat–injured cells (Chang et al., 2009) reported in previous studies may together have rendered them vulnerable to the strong acidic environment.

Acquisition of antibiotic resistance may influence the behaviour of antibiotic resistant bacteria during typical stress conditions encountered by them. The behaviour of a naturally multi–antibiotic resistant *E. coli* strain was reported to be significantly different to the antibiotic sensitive strains when subjected to food stresses (acid and heat) (Duffy et al., 2006). This naturally multi-antibiotic resistant *E. coli* strain was observed to be significantly more heat sensitive during thermal inactivation studies conducted at 55°C and also to loose viability significantly faster than the other strains in moderate pH (Duffy et al., 2006). The authors of the study suggested that the differences in the stress responses observed between the multi–antibiotic resistant *E. coli* strain and others could be due to inter-strain differences among *E. coli*, which are currently well recognised (Durso et al., 2004, Kimmitt et al., 2000). In addition, they suggested that the poor survival of multi–antibiotic resistant *E. coli* may be linked to changes in stress response due to acquisition of antibiotic resistance. For example, mutations in the *RpoS* gene, a highly mutable gene, which required for the survival of *E. coli* at low pH (Waterman and Small, 1996) due to acquisition of antibiotic resistance may result in increased acid and heat sensitivity. Moreover, *slp*, another stress response gene responsible for the uptake of nutrients or medium constituents (Seoane and Levy, 1995) is reported to be downregulated by the multi-antibiotic resistance system (Price and St John, 2000).

With the growing reports of multi-antibiotic resistant strains, it is clear that further research is now needed on a larger number of multi-antibiotic resistant *E. coli* strains. Comprehensive data on the stress responses elicited by multi-antibiotic resistant *E. coli* strains allow assessment of whether pathogen survival under stress conditions is affected by the presence of multi-

antibiotic resistance genes. Comparison of these natural multi-antibiotic resistant *E. coli* strains to their antibiotic resistant gene knockout counterparts would significantly contribute to the understanding of whether the presence of multi-antibiotic resistance genes contributes to their survival under stress conditions. In addition, interaction with individual food components in food matrices as well as other microbiota may certainly impact stress responses of individual *E. coli* strains. Thus, effect of stress responses of *E. coli* in food should be studied to obtain a real-world overview on how *E. coli* react to sub-lethal environmental stresses although recovering target cells from the background microbiota presents a challenge. To obtain a complete view on the underlying mechanisms of stress responses elicited by these *E. coli* strains, the presence of genes responsible for stress response induction and their expression needs to be analysed.

5.5 Conclusion

In conclusion, this study shows that particular clones of *E. coli* can behave differently from each other when subjected to food-borne stresses. However, the degree of survival observed in the *E. coli* strains following exposure to sub-lethal stress environments in this study indicate that the current control measures employed in the food industry would be sufficient to control their growth despite the indication of high virulence owing to their multi-antibiotic resistance and/or presence of *stx*. As such, it can be said that the potential risk that these *E. coli* strains may pose on human health is relatively low.

Chapter 6

General Conclusion

E. coli are common inhabitants of the gastrointestinal tract of humans and animals, of which the majority are non–pathogenic commensals. However, pathogenic clones of *E. coli* exist, which have evolved as pathogens with the acquisition of virulence genes that enable them to cause a broad spectrum of intestinal and extra–intestinal diseases in humans. STEC is one of the most virulent group of clones among these pathogenic *E. coli* associated severe disease in humans. Ruminants are considered a major reservoir of these pathogens. In Malaysia, data on the prevalence and characterisation of *E. coli* O157 and the non-O157 serogroups in ruminants is limited.

The primary aim of this project was to characterise *E. coli* O157 and non-O157 strains obtained from food-producing ruminants in Malaysia to obtain an overview of their genotypic and phenotypic traits and the underlying virulence, based on their pathogenicity, antibiotic resistance and survival under stress conditions. To better understand the STEC epidemiology among ruminants in Malaysia the following questions were raised: (1) Do food-producing ruminants in Malaysia carry STEC and shed them in their feces? If STEC is present among ruminants in Malaysia, what are their genotypic and phenotypic characteristics and how do they relate to their virulence?; (2) Do the *E. coli* strains including STEC present among Malaysian ruminants carry antibiotic resistance? Are they capable of disseminating antibiotic resistance?; (3) What are the *stx* phage insertion sites of non-O157 STEC from Malaysia and Australia and how does the variation of these *stx* phage insertion sites affect their virulence?; (4) Can the *E. coli* strains including STEC among Malaysian ruminants survive under strong acidic conditions similar to what is encountered in the human stomach following exposure to sub-lethal heat, cold and acid adaptation stress conditions?

Do food-producing ruminants in Malaysia carry STEC and shed them in their feces? If STEC is present among ruminants in Malaysia, what are their genotypic and phenotypic characteristics and how do they relate to their virulence?

The results of the experiments and observations presented in Chapter 2 helped to understand the distribution of STEC serogroups among ruminanats in Malaysia to some extent. To isolate *E. coli* O157 and non-O157 strains, a total of 136 ruminant feces samples including cattle, buffalo, sheep and goat feces were collected from six different ruminant farms in Peninsular Malaysia as described in Chapter 2. A low prevalence of STEC O157:H7 (4.4 %) and non-O157 STEC (1.5 %) was observed among the ruminant feces samples collected in this study despite the use of specific and sensitive methods of enrichment and IMS. The existing data on STEC prevalence in tropical countries such as Brazil (Irino et al., 2005), India (Manna et al., 2006), Mexico (Callaway et al., 2004), and Thailand (Vuddhakul et al., 2000) also indicates a low prevalence (0–8.0 %) of STEC strains among ruminants. In contrast, a high prevalence (> 15.0 %) of STEC strains has been reported among ruminants from countries such as USA (Elder et al., 2000), UK (Chapman et al., 1997) and Japan (Kobayashi et al., 2001). The low prevalence of STEC strains observed especially in cattle feces may reflect a scarcity of these bacteria colonising the intestines of these animals. However, the infrequent nature of STEC occurrence in ruminant feces samples may not truly represent the prevalence of STEC due to many factors. These factors could be grouped into two major categories: animal-associated and nonanimal-associated. Animal-associated factors include age-dependent carriage of STEC, intermittent shedding of STEC, and extra-intestinal carriage (such as hides) of STEC with little correlation to fecal shedding of the bacteria (reviewed in Ferens and Hovde, 2011). Non-animal-associated factors include the differences in study design, sampling methods, isolation methods of STEC from samples, animal management practices (husbandry and farming systems) and environmental factors such as climate, season, and geographic location (reviewed

in Ferens and Hovde, 2011). In addition, the prevalence of STEC is probably underestimated in some tropical countries like Malaysia due to the lack of comprehensive data on STEC among ruminants. Thus far in Malaysia the study described in Chapter 2 solely reports the presence of STEC strains among food-producing ruminants in Malaysia. The reduced probability of detecting STEC strains in tropical countries may also reflect the small operations of ruminant farms typically present in these countries in comparison to the large farm operations in countries such as USA.

Genotypic and phenotypic characterisation of the STEC O157 and non-O157 strains obtained from this study suggested that the virulence potential of these strains to humans is probably low in comparison to STEC strains obtained from other studies (Blanco et al., 2004b, Mellor et al., 2012, Sharma et al., 2009). The absence of Stx production, which is a major virulence factor of STEC strains, carriage of *stx*$_{2c}$ and *stx*$_{2c}$-associated bacteriophage sequences adjacent to *sbcB* locus, and the characteristics of lineage II in the STEC O157 strains obtained from this study reflected their low virulence potential. Similarly, little or no production of Stx and the lack of *eaeA* responsible for the gastrointestinal attachment of the non-O157 STEC strains isolated from this study also suggests a low probability of causing disease in humans.

The lack of STEC strains with pathogenic potential in ruminant feces in this study does not implicate the absence of more virulent clones of STEC among ruminants in Malaysia. This was evident from the STEC strains from UPM characterised in this study, which were recovered from cow milk and beef. These STEC O157 strains represented a more virulent clone of STEC among bovine sources as was evident by their production of Stx, association of *stx*$_1$ and *stx*$_{2a}$ with prophage insertion in the *yehV* and *wrbA* loci, and characteristics of lineage I similar to clinical STEC strains. Stx producing STEC O157 strains were also recovered from beef samples in another study conducted in Malaysia (Radu et al., 1998). In addition, STEC strains can be introduced to the food chain through consumption of food products (such as beef)

routinely traded across international borders in the Southeast Asian countries. One study reported the presence of STEC O157:H7 among beef samples (11.1%) imported from Thailand to Malaysia (Sukhumungoon et al., 2011). These STEC strains were positive for the virulence factors *eaeA* and stx_2 important in the colonisation and infection of humans. Acquisition of *stx* phage such as phage carrying stx_{2a} from the environment could also lead to the emergence of virulent clones of STEC with high pathogenic potential in humans. In fact greater virulence was observed in STEC strains with the frequent carriage of stx_{2a} together with stx_{2c} (Manning et al., 2008), which were found to be associated with HUS. *Stx* phage lysogenisation of *E. coli* strains including commensal *E. coli* from the intestinal tract leading to Stx production through spontaneous induction has been reported in previous studies (Gamage et al., 2004, Iversen et al., 2015), and thus shows the probability of emergence of highly virulent STEC strains. This was also evident from the large outbreak caused by the enteroaggregative *E. coli* strain O104:H4 in Germany, which became a highly virulent strain with the acquisition of phage encoding production of Stx2 (Rasko et al., 2011).

Do the *E. coli* strains including STEC present among Malaysian ruminants carry antibiotic resistance? Are they capable of disseminating antibiotic resistance?

Antibiotic resistant commensal *E. coli* in food-producing animals are of major public health significance due to the risk of these bacteria entering the food chain and disseminating in humans. Chapter 3 describes the analysis of antibiotic susceptibility against 15 different antibiotics among a total of 153 *E. coli* strains recovered from the ruminant feces samples to determine the extent of antibiotic resistance and the potential risk of resistance determinant(s) among food–producing ruminants in Malaysia. Results of the study showed that resistance was most common against more traditional antibiotics such as tetracycline (7.2 %), trimethoprim (3.9 %), ampicillin (3.3 %), and streptomycin (2.0 %). It is not surprising that resistance to more traditional antibiotics (especially to tetracycline) was more commonly observed among

the *E. coli* strains of this study since these antibiotics have been in use in food animals for a long time (Health Action International Asia Pacific, 2013). In fact, resistance to tetracycline was most frequently observed among commensal *E. coli* of food–producing animals in other studies (ALHaj et al., 2007, Lim et al., 2007, Tadesse et al., 2012). However, resistance towards antibiotics such as tetracycline, streptomycin and ampicillin was relatively low in this study, in comparison to the frequency of resistance observed among commensal *E. coli* isolates obtained from food-producing ruminants in other countries (ALHaj et al., 2007, Lim et al., 2007, Tadesse et al., 2012). This may reflect the different levels of exposure to these antibiotics and the period of exposure between different farm settings. Previous studies have demonstrated a positive correlation between emergence and increase in antibiotic resistant bacteria with increase in antibiotics use (Alexander et al., 2008, DeFrancesco et al., 2004).

None of the *E. coli* isolates from this study showed complete or intermediate resistance to more modern antibiotics such as ciprofloxacin (fluoroquinolone), ceftazidime and cefuroxime (third generation cephalosporins) mainly employed to treat infections from Gram-negative bacteria in human medicine. Although this was a favourable finding, the number of farms sampled in this study was small and may not adequately represent the extent of antibiotic resistance among commensal *E. coli* in ruminants in Malaysia. Resistance to ciprofloxacin, cefotaxime and ceftazidime was reported among *E. coli* strains isolated from hospital settings in Malaysia (Ho et al., 2012, Kor et al., 2013, Lim et al., 2009). Studies conducted by Ho et al. (2012) and Lim et al. (2009) also reported a high number (46.0 % and 76.5 % respectively) of multi-antibiotic resistant *E. coli* from the hospital settings in comparison to the low number (5.9 %) of multi-antibiotic resistant *E. coli* strains observed in this study. The prevalence of antibiotic resistant *E. coli* to more modern antibiotics used in human medicine and the high number of multi-antibiotic resistant *E. coli* in hospital settings can be primarily explained by the repeated

exposure to these antibiotics resulting in an increased selective pressure in a hospital setting in comparison to a farm environment.

Among the genetic determinants of antibiotic resistance observed among the *E. coli* strains from this study, plasmid mediated resistance was apparent for tetracycline – *tet*(A), trimethoprim – *dhfr* I, V, VII, and XIII, ampicillin and cephalothin – bla_{TEM}, streptomycin – *aadA* and *strA–strB,* and chloramphenicol – *floR* and *cmlA.* Most importantly conjugation assays demonstrated the transfer of resistance to ampicillin, trimethoprim and streptomycin among *E. coli* strains. Among other mobile genetic elements mediating antibiotic resistance, class 1 integrons were present in 65 *E. coli* strains (42.5 %) from this study, which contained gene cassettes conferring resistance to trimethoprim (*dhfr* VII and *dhfr* V). *E. coli* are known to readily exchange genetic material among other *E. coli* as well as other bacterial species (Blake et al., 2003, Davison et al., 1999). The presence of antibiotic determinants on mobile genetic elements such as plasmids among the *E. coli* strains from this study highlights the significance of commensal *E. coli* as a reservoir of antibiotic resistance determinants and a potential source of resistance dissemination especially to pathogenic bacteria.

What are the *stx* phage insertion sites of non-O157 STEC from Malaysia and Australia and how does the variation of these *stx* phage insertion sites affect their virulence?

Experiments were conducted as described in Chapter 4 in an attempt to identify the *stx* phage insertion sites of non-O157 STEC from Malaysia and Australia. There is very little information available on the *stx* phage insertion sites of non-O157 STEC strains obtained from different sources as well as from different geographical locations world-wide. However, several *stx* phage insertion sites among STEC O157:H7 strains have been already established in previous studies (Koch et al., 2003, Ohnishi et al., 2002, Patridge and Ferry, 2006, Recktenwald and Schmidt, 2002, Yokoyama et al., 2000), and the differential distribution of these *stx* phage

insertion site genotypes among STEC O157:H7 obtained from human and bovine hosts has been identified (Besser et al., 2007, Whitworth et al., 2010). In addition, insertion of particular *stx* phages at specific loci within the *E. coli* O157 genome has shown to alter the severity of disease caused by different STEC O157 strains (Ogura et al., 2009, Whitworth et al., 2010). As a result, identification of *stx* phage insertion sites among STEC O157 strains has facilitated the categorisation of more harmful clinical STEC O157 strains from the less harmful bovine STEC O157 strains (Shringi et al., 2012b). Identification of *stx* phage insertion site distribution among the non-O157 STEC strains from different sources as well from different geographical locations would therefore significantly improve the characterisation of clinical non-O157 STEC strains that cause severe disease in humans.

As described in Chapter 4, phage lysates were obtained from the donor non-O157 STEC strains and were used in the lysogen formation assays to facilitate the study of *stx* phage insertion sites. Although phage lysates of these non-O157 STEC strains were confirmed to carry the *stx* gene through multiplex PCR, lysogenisation of the *stx* phages was unsuccessful using previously established methods and inclusion of several modifications to the assays. Several factors may have contributed to the lack of lysogen formation in this study such as insufficient *stx* phage concentration in the phage lysate, *stx* phage progression to lytic phase more readily than the lysogenic phase, and the use of a single recipient *E. coli* strain DH5-α for the lysogenic assays. Further studies must be designed to address the issues encountered during lysogen formation assays in order to establish *stx* phage insertion sites among the non-O157 STEC strains.

Can the *E. coli* strains including STEC among Malaysian ruminants survive under strong acidic conditions similar to what is encountered in the human stomach following exposure to sub-lethal heat, cold and acid adaptation stress conditions?

It is increasingly becoming apparent that *E. coli* utilizes complex networks to adapt to and survive the various stresses intended to control their growth in food production and preparation processes, and may actually develop long lasting tolerance to similar subsequent stresses. Interestingly exposure to certain stresses may also make these bacteria more resistant or sensitive to other stresses that are not applied at the time of the original stress (reviewed in Chapter 5). Exposure of pathogenic and non-pathogenic *E. coli* to sub-lethal stresses encountered during food processing conditions may significantly affect their survival under post-stress conditions.

The effect of acid adaptation, cold stress and/or heat stress on the survival of two multi-antibiotic resistant *E. coli* isolates, one positive and the other negative for *stx*, in subsequent acidic conditions, which mimics the acidic environment in the human stomach was investigated in Chapter 5. The results of this study showed that neither the heat shock treatment nor the acid adaptation of both multi-antibiotic resistant *E. coli* strains did not enhance their survival in subsequent strong acidic conditions (pH 2.5) in comparison to the control cells without the treatments. Majority of the studies on sub-lethal stress responses of *E. coli* report enhanced survival of *E. coli* cells in strong acidic conditions (pH 2.5) following heat shock treatment (Leenanon and Drake, 2001, Wang and Doyle, 1998) or acid adaptation (Arnold and Kaspar, 1995, Cheville et al., 1996). However, there is very limited data on the responses of antibiotic resistant *E. coli* to sub-lethal stress conditions. One study indicated increased heat sensitivity among naturally antibiotic resistant *E. coli* strains compared to the antibiotic sensitive strains (Duffy et al., 2006). Increased heat sensitivity could be a probable cause for the lack of enhanced survival following heat shock treatment observed in the two multi-antibiotic resistant

E. coli strains from this study. In addition, leakage of intracellular components have been shown to be commonly associated with heat-injured cells (Chang et al., 2009). Cells with increased heat sensitivity would be more vulnerable to injuries caused by heat shock treatment and subsequent acid stress in comparison to cells without heat shock treatment. Acid adaptation conditions also depends on the type of acidulant used (Chung et al., 2006) and the use of HCl may have caused cell damage, which has been reported in a previous study (Leyer et al., 1995) resulting in decreased survival of cells under more acidic conditions. However, acid adaptation of *E. coli* cells in laboratory media acidified with concentrated HCl for 1 h resulted in increased survival of the acid adapted cells under subsequent acid stress (pH 2.5) in comparison to the control cells without acid adaptation in one study (Wang and Doyle, 1998).

In practise, food-related sequential stresses such as those conditions encountered during cold storage, heating and acidification of food products before consumption and also acid stress conditions during digestion of food have not been studied sufficiently to obtain data on the survival of bacteria during food digestion following the sequential stress conditions. Acid adaptation of the multi-antibiotic resistant *E. coli* strains in this study followed by heat shock treatment did not enhance their survival under strong acidic conditions. Exposure of cells to HCl during acid adaptation and subsequent heat shock treatment may have resulted in cell injuries rendering them vulnerable to the strong acidic environment. The use of HCl for acid adaptation, although would expose bacteria to a low pH, has practical limitations since inorganic acids are not commonly used in food products. Bacteria are exposed to acidic conditions mostly during fermentation of foods. Acids are produced during food preparation by microflora naturally present in the food or added lactic acid-producing bacteria. Acid adaptation conditions during food fermentation can be simulated in a laboratory setting through the growth of bacteria in TSB containing > 0.75 % glucose (TSBG) (Buchanan and Edelson, 1996). The reduction of pH in the TSBG medium may better represent the acid adaptation

conditions and thus could be used in further experiments. When the multi-antibiotic resistant *E. coli* strains were exposed to cold stress at 4°C for a week followed by heat shock treatment, which mimics the cold storage of food and heating prior to consumption, decreased their survival in strong acidic conditions (similar to human gastric environment) in comparison to the cells without cold stress-adaptation. Although not specifically studied, cold stress-adaptation for seven days followed by heat shock treatment reduced cell numbers of both *E. coli* strains much more than the cold stress-adaptation alone. Increased heat sensitivity of cold stress-adapted cells was also reported in previous studies (Leyer and Johnson, 1993, Shen et al., 2011).

The degree of survival observed in the multi-antibiotic resistant *E. coli* strains following acid adaptation, cold stress and/or heat stress in this study showed that the current methods employed in the food industry would be effective to eliminate or limit the growth of these *E. coli* strains. In addition the study showed that particular strains of *E. coli* can behave differently from each other when subjected to food-borne stresses. In fact, the behaviour of a naturally multi-antibiotic resistant *E. coli* strains was reported to be significantly different to the antibiotic sensitive strains when subjected to food stresses such as acid and heat. It is important to understand that unlike pure cultures in laboratory media, foods are complex environments, in which a variety of microorganisms including spoilage and pathogenic bacteria coexist and compete for nutrients. Ideally all microecological factors involved in the processing of a specific food should be taken in to account to predict *E. coli* responses to stress. As such the survival of antibiotic resistant *E. coli* strains under sub-lethal stress conditions in foods such as beef should be studied in further experiments.

Implications of research

The results of this study indicated that ruminants in Malaysia are a potential source of STEC entry in to human food chain as well as STEC dissemination in the environment. Moreover, the results showed the significance of commensal *E. coli* in ruminants in Malaysia as a reservoir of antibiotic resistance determinants and a source of resistance dissemination. However, overall this study suggests that the *E. coli* O157 and non-O157 strains among ruminants in Malaysia may have limited impact on public health. This finding was supported by the low percentage of STEC O157 and non157 strains distributed among ruminants from this study and their low level of virulence observed (Chapter 2), which may contribute to the reasons for the lack of reported sporadic cases and outbreaks caused by STEC strains in Malaysia. In addition, the presence of antibiotic resistance against more traditional antibiotic agents and the lack of resistance towards more modern antibiotics as described in Chapter 3 indicates a relatively low zc health. The reduced survival of the multi-antibiotic resistant *E. coli* isolates under severe stress conditions observed in Chapter 5 of this study also indicates that the currently employed control measures in the food industry such as heating, acidification and cold storage seems to be sufficient to minimise the growth of these strains and thereby reduce the risk of food borne illness in humans.

Limitations of studies and future work

This study had several limitations. Only six ruminant farms were sampled in this study on a single visit to each farm. On each visit only ruminant feces samples were collected to determine the presence of STEC serogroups. To obtain a better understating on the STEC prevalence in Malaysia, sampling should be conducted on a large number of farms representative of ruminant farms within Malaysia in further studies. The intermittent shedding of STEC among ruminants requires several visits to a single ruminant farm and longitudinal studies to understand STEC

persistence among farm environments. In addition, samples should be obtained from various ruminant sources (feces samples, samples from extra-intestinal sites of ruminants, and food products originating from ruminants) and the associated environment in further studies in order to better assess prevalence of STEC serogroups in Malaysia and their variation and pathogenic potential towards humans.

Apart from the sampling of ruminants and the associated environment, clinical samples from diarrhoeal patients should also be included in further studies to determine STEC related disease, their virulence properties and characteristics, sources of infection, and strategies for the prevention of disease.

Given the high propensity of *E. coli* isolates to spread antibiotic resistance genes, it is important to monitor how the reservoir of antibiotic resistance determinants among the commensal *E. coli* contribute to the spread of these resistance determinants within the animal population as well as human hosts over time. As such, antibiotic resistance should be tested among a larger population of commensal *E. coli* strains representative of Malaysia to assess the risk of resistance dissemination to humans. On a farm level, antibiotic resistance of *E. coli* should be monitored among the farm animals, the farm environment and the farm workers to better understand the spread of antibiotic resistance within an animal farm. In addition, a national level monitoring system for risk analysis of antibiotic resistance in food-producing animals should be implemented in Malaysia. This would contribute to the understanding of broad prevalence of antibiotic resistance among farms as well as individuals on a farm.

Variation of *E. coli* stress responses among naturally antibiotic resistant strains should be further investigated using a large number of antibiotic resistant *E. coli* strains. To establish a link between the presence of antibiotic resistance and variation in stress response, studies should be conducted to examine the differences of stress responses between the resistant strains

and their resistance gene knockout counterparts. Nucleotide sequence analysis of genes should be conducted following resistance gene knockout studies in instances where acquisition of antibiotic resistance may have caused mutations in genes such as *RpoS*, which is considered as the global regulator of gene expression under various stress conditions. In addition, cellular levels of RpoS as well as other proteins downstream of RpoS expression, which are responsible for the regulation of stress responses can be monitored to obtain an overview of the differential expression of stress regulator genes among the antibiotic resistance and sensitive *E. coli* strains. Comprehensive data on the survival of antibiotic resistant bacteria under sub-lethal stress conditions encountered during food processing conditions would significantly contribute to the development and/or improvement of control strategies employed in the food industry to enhance the safety and quality of food products to meet consumer demands. Overall, data obtained from these studies would enhance the understanding of the variation of virulence among *E. coli* strains distributed among the food-producing animals in Malaysia and the potential risk towards the public health.

In summary, the findings from this study indicated that ruminants in Malaysia do carry STEC and shed them with their feces, which may allow the contamination of ruminant derived food products and the environment leading to STEC transmission to humans. However, the genotypic and phenotypic characteristics of the isolated STEC strains from this study indicated low level of virulence suggesting limited impact on public health. This study also showed that ruminants in Malaysia carry *E. coli* strains conferring antibiotic resistance and act as a significant reservoir of antibiotic resistance determinants with the propensity of antibiotic resistance dissemination among other ruminants, the environment and humans. However, the *E. coli* strains used in this study indicated a lack of antibiotic resistance towards more modern antibiotics such as cefotaxime, ceftazidime used in human medicine suggesting a relatively low risk to the public health. The results from this study further indicated that the two multi-

antibiotic resistant *E. coli* strains obtained from ruminant feces are highly susceptible to strong acidic conditions (similar to what is encountered in the human stomach) following exposure to sub-lethal heat, cold and acid adaptation stress conditions. High susceptibility of these *E. coli* strains to strong acidic environments such as that encountered in the human stomach suggests that the currently employed control measures in the food industry would be sufficient to minimise the growth of these strains and thereby reduce the risk of food borne illness in humans. Since the attempts to determine the *stx* phage insertion sites of non-O157 STEC from Malaysia and Australia in this study were unsuccessful, further research is required to determine these *stx* phage insertion sites and how the variation of these *stx* phage insertion sites affect their virulence. Overall, the results from this study suggested that the *E. coli* strains including STEC from ruminants in Malaysia have limited impact on public health. However, virulent STEC strains have been isolated from food products of bovine origin in Malaysia. Thus, the prevalence of such virulent strains among Malaysian ruminants and their potential to cause severe disease in humans should not be disregarded. Further research should be conducted using a large number of *E. coli* strains obtained from ruminant farms in Malaysia to obtain comprehensive data on the prevalence of virulent *E. coli* strains (including STEC) and their impact on public health.

References

AARESTRUP, F. M., WEGENER, H. C. & COLLIGNON, P. 2008. Resistance in bacteria of the food chain: epidemiology and control strategies. *Expert Rev Anti Infect Ther,* 6**,** 733-50.

ABEE, T. & WOUTERS, J. A. 1999. Microbial stress response in minimal processing. *Int J Food Microbiol,* 50**,** 65-91.

ADVISORY COMMITTEE ON THE MICROBIOLOGICAL SAFETY OF FOOD. 1995. Report on Verocytotoxin-producing *Escherichia coli* (VTEC).

ADZITEY, F., ALI, G. R. R., HUDA, N. & TING, S. L. 2013. Antibiotic resistance and plasmid profile of *Escherichia coli* isolated from ducks in Penang, Malaysia. *Int Food Res J,* 20**,** 1473-1478.

AHMED, S. 1997. An outbreak of *Escherichia coli* O157 in Central Scotland. *SUPPL. SCIH Weekly Rep.,* 13**,** 8.

AKTER, S. F. U., RANI, M. F. A., RAHMAN, J. A., NORDIN, M. S. S., S., AWANG, M. B., RATHOR, M. Y. & ARIS, M. A. B. M. 2012. Antimicrobial use and factors influencing prescribing in medical boards of tertiary care hospital in Malaysia. *Int J Sci, Environ and Technol,* 1**,** 274-284.

ALEXANDER, T. W., YANKE, L. J., TOPP, E., OLSON, M. E., READ, R. R., MORCK, D. W. & MCALLISTER, T. A. 2008. Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant *Escherichia coli* bacteria in feedlot cattle. *Appl Environ Microbiol,* 74**,** 4405-16.

ALHAJ, N., MARIANA, N. S., RAHA, A. R. & ISHAK, Z. 2007. Prevalance of antibiotic resistance among *Escherichia coli* from different sources in Malaysia. *Res J Pharmacol,* 1**,** 44-49.

ALLISON, H. E. 2007. Stx-phages: drivers and mediators of the evolution of STEC and STEC-like pathogens. *Future Microbiol,* 2**,** 165-74.

ALLISON, H. E., SERGEANT, M. J., JAMES, C. E., SAUNDERS, J. R., SMITH, D. L., SHARP, R. J., MARKS, T. S. & MCCARTHY, A. J. 2003. Immunity profiles of wild-type and recombinant shiga-like toxin-encoding bacteriophages and characterization of novel double lysogens. *Infect Immun,* 71**,** 3409-18.

AMYES, S. G. 1986. Epidemiology of trimethoprim resistance. *J Antimicrob Chemother,* 18 Suppl C**,** 215-21.

ANONYMOUS 2005. European Commission Annual Report 2005: surveillance of enteric pathogens in Europe and beyond; 1786/2002/EC. Brussels, Belgium: International surveillance network for the enteric infections-*Salmonella,* VTEC O157 and *Campylobacter*.

APUN, K., CHANG, P. P., SIM, E. H. U. & MICKY, V. 2006. Clonal diversity of *Escherichia coli* isolates from marketed beef in East Malaysia. *World J Microbiol & Biotech,* 22**,** 661-67.

ARNOLD, K. W. & KASPAR, C. W. 1995. Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. *Appl Environ Microbiol,* 61**,** 2037-9.

ASLANZADEH, J. 2006. Biochemical Profile-Based Microbial Identification Systems. *In:* TANG, Y. W. & STRATTON, C. W. (eds.) *Advanced Techniques in Diagnostic Microbiology.* New York: Springer.

BACON, R. T., SOFOS, J. N., KENDALL, P. A., BELK, K. E. & SMITH, G. C. 2003. Comparative analysis of acid resistance between susceptible and multi-antimicrobial-resistant *Salmonella* strains cultured under stationary-phase acid tolerance-inducing and noninducing conditions. *J Food Prot,* 66**,** 732-40.

BAI, J., SHI, X. & NAGARAJA, T. G. 2010. A multiplex PCR procedure for the detection of six major virulence genes in *Escherichia coli* O157:H7. *J Microbiol Methods,* 82**,** 85-9.

BARLOW, R. S., FEGAN, N. & GOBIUS, K. S. 2008. A comparison of antibiotic resistance integrons in cattle from separate beef meat production systems at slaughter. *J Appl Microbiol,* 104**,** 651-8.

BARLOW, R. S., FEGAN, N. & GOBIUS, K. S. 2009. Integron-containing bacteria in faeces of cattle from different production systems at slaughter. *J Appl Microbiol,* 107**,** 540-5.

BARLOW, R. S. & MELLOR, G. E. 2010. Prevalence of enterohemorrhagic *Escherichia coli* serotypes in Australian beef cattle. *Foodborne Pathog Dis,* 7**,** 1239-45.

BELAAOUAJ, A., LAPOUMEROULIE, C., CANICA, M. M., VEDEL, G., NEVOT, P., KRISHNAMOORTHY, R. & PAUL, G. 1994. Nucleotide sequences of the genes coding for the TEM-like beta-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). *FEMS Microbiol Lett,* 120**,** 75-80.

BELANGER, L., GARENAUX, A., HAREL, J., BOULIANNE, M., NADEAU, E. & DOZOIS, C. M. 2011. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic E. coli. *FEMS Immunol Med Microbiol,* 62**,** 1-10.

BELL, B. P., GOLDOFT, M., GRIFFIN, P. M., DAVIS, M. A., GORDON, D. C., TARR, P. I., BARTLESON, C. A., LEWIS, J. H., BARRETT, T. J., WELLS, J. G. & ET AL. 1994. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. *JAMA,* 272**,** 1349-53.

BENJAMIN, M. M. & DATTA, A. R. 1995. Acid tolerance of enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol,* 61**,** 1669-72.

BERENDSEN, B., STOLKER, L., DE JONG, J., NIELEN, M., TSERENDORJ, E., SODNOMDARJAA, R., CANNAVAN, A. & ELLIOTT, C. 2010. Evidence of natural occurrence of the banned antibiotic chloramphenicol in herbs and grass. *Anal Bioanal Chem,* 397**,** 1955-63.

BERRY, E. D. & FOEGEDING, P. M. 1997. Cold temperature adaptation and growth of microorganisms. *J Food Prot,* 60**,** 1583-1594.

BESSER, R. E., LETT, S. M., WEBER, J. T., DOYLE, M. P., BARRETT, T. J., WELLS, J. G. & GRIFFIN, P. M. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *JAMA,* 269**,** 2217-20.

BESSER, T. E., SHAIKH, N., HOLT, N. J., TARR, P. I., KONKEL, M. E., MALIK-KALE, P., WALSH, C. W., WHITTAM, T. S. & BONO, J. L. 2007. Greater diversity of Shiga toxin-encoding bacteriophage insertion sites among *Escherichia coli* O157:H7 isolates from cattle than in those from humans. *Appl Environ Microbiol,* 73**,** 671-9.

BIELASZEWSKA, M., JANDA, J., BLAHOVA, K., SRAMKOVA, L., HAVLIK, J. & POTUZNIK, V. 1994. Verocytotoxin-producing *Escherichia coli* in children with hemolytic uremic syndrome and diarrhea in the Czech Republic. *In:* KAMALI, M. A. & GOGLIO, A. G., eds. The 2nd International Symposium and Workshop on Verocytotoxin (Shiga-like toxin)-producing *Escherichia coli* infections, 1994 Bergamo, Italy. Amsterdam: Elsevier Science, 37-40.

BIELASZEWSKA, M. & KARCH, H. 2000. Non-O157:H7 Shiga toxin (verocytotoxin)-producing *Escherichia coli* strains: epidemiological significance and microbiological diagnosis. *World J Microbiol & Biotechnol,* 16**,** 711-718.

BIELASZEWSKA, M., PRAGER, R., KOCK, R., MELLMANN, A., ZHANG, W., TSCHAPE, H., TARR, P. I. & KARCH, H. 2007. Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Appl Environ Microbiol,* 73**,** 3144-50.

BLAKE, D. P., HUMPHRY, R. W., SCOTT, K. P., HILLMAN, K., FENLON, D. R. & LOW, J. C. 2003. Influence of tetracycline exposure on tetracycline resistance and the carriage of tetracycline resistance genes within commensal *Escherichia coli* populations. *J Appl Microbiol,* 94**,** 1087-97.

BLANCO, M., BLANCO, J. E., MORA, A., DAHBI, G., ALONSO, M. P., GONZALEZ, E. A., BERNARDEZ, M. I. & BLANCO, J. 2004a. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (eae-xi). *J Clin Microbiol,* 42**,** 645-51.

BLANCO, M., PADOLA, N. L., KRUGER, A., SANZ, M. E., BLANCO, J. E., GONZALEZ, E. A., DAHBI, G., MORA, A., BERNARDEZ, M. I., ETCHEVERRIA, A. I., ARROYO, G. H., LUCCHESI, P. M., PARMA, A. E. & BLANCO, J. 2004b. Virulence genes and intimin types of Shiga-toxin-producing *Escherichia coli* isolated from cattle and beef products in Argentina. *Int Microbiol,* 7**,** 269-76.

BOSILEVAC, J. M. & KOOHMARAIE, M. 2012. Predicting the presence of non-O157 Shiga toxin-producing *Escherichia coli* in ground beef by using molecular tests for Shiga toxins, intimin, and O serogroups. *Appl Environ Microbiol,* 78**,** 7152-5.

BROOKS, J. T., SOWERS, E. G., WELLS, J. G., GREENE, K. D., GRIFFIN, P. M., HOEKSTRA, R. M. & STROCKBINE, N. A. 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. *J Infect Dis,* 192**,** 1422-9.

BROWN, M. H. & BOOTH, I. R. 1991. Acidulants and low pH. *In:* RUSSEL, N. J. & GOULD, G. W. (eds.) *Food Preservastives.* Glasgow, Scotland: Blackie.

BUCHANAN, R. L. & EDELSON, S. G. 1996. Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells. *Appl Environ Microbiol,* 62**,** 4009-13.

BUNCIC, S. & AVERY, S. M. 1998. Effects of cold storage and heat-acid shocks on growth of verotoxin 2 production of *Escherichia coli* O157:H7. *Food Microbiol,* 15**,** 319-328.

BYRNE, L., JENKINS, C., LAUNDERS, N., ELSON, R. & ADAK, G. K. 2015. The epidemiology, microbiology and clinical impact of Shiga toxin-producing *Escherichia coli* in England, 2009-2012. *Epidemiol Infect,* 143**,** 3475-87.

CALLAWAY, T. R., ANDERSON, R. C., TELLEZ, G., ROSARIO, C., NAVA, G. M., ESLAVA, C., BLANCO, M. A., QUIROZ, M. A., OLGUIN, A., HERRADORA, M., EDRINGTON, T. S., GENOVESE, K. J., HARVEY, R. B. & NISBET, D. J. 2004. Prevalence of *Escherichia coli* O157 in cattle and swine in central Mexico. *J Food Prot,* 67**,** 2274-6.

CARATTOLI, A. 2001. Importance of integrons in the diffusion of resistance. *Vet Res,* 32**,** 243-59.

CAPRIOLI, A., LUZZI, I., ROSMINI, F., RESTI, C., EDEFONTI, A., PERFUMO, F., FARINA, C., GOGLIO, A., GIANVITI, A. & RIZZONI, G. 1994. Community-wide outbreak of hemolytic-uremic syndrome associated with non-O157 verocytotoxin-producing *Escherichia coli*. *J Infect Dis,* 169**,** 208-11.

CAPRIOLI, A., MORABITO, S., BRUGERE, H. & OSWALD, E. 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Vet Res,* 36**,** 289-311.

CAPRIOLI, A. & TOZZI, A. E. 1998. Epidemiology of Shiga toxin-producing *Escherichia coli* infections in continental Europe. *In:* KAPER, J. B. & O'BRIEN, A. D. (eds.) *Escherichia coli O157:H7 and other Shiga toxin-producing E. coli Strains.* Washington D.C.: American Society for Microbiology.

CAPRIOLI, A., TOZZI, A. E., RIZZONI, G. & KARCH, H. 1997. Non-O157 Shiga toxin-producing *Escherichia coli* infections in Europe. *Emerg Infect Dis,* 3**,** 578-9.

CAVACO, L. M., FRIMODT-MOLLER, N., HASMAN, H., GUARDABASSI, L., NIELSEN, L. & AARESTRUP, F. M. 2008. Prevalence of quinolone resistance mechanisms and associations to minimum inhibitory concentrations in quinolone-resistant *Escherichia* coli isolated from humans and swine in Denmark. *Microb Drug Resist,* 14**,** 163-9.

CENTERS FOR DISEASE CONTROL AND PREVENTION. 1995. *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami--Washington and California, 1994. *Morb Mortal Wkly Rep,* 44**,** 157-60.

CENTERS FOR DISEASE CONTROL AND PREVENTION. 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach--United States, September 2006. *Morb Mortal Wkly Rep,* 55**,** 1045-6.

CHANG, C. H., CHIANG, M. L. & CHOU, C. C. 2009. The effect of temperature and length of heat shock treatment on the thermal tolerance and cell leakage of *Cronobacter sakazakii* BCRC 13988. *Int J Food Microbiol,* 134**,** 184-9.

CHAPMAN, P. A., SIDDONS, C. A., GERDAN MALO, A. T. & HARKIN, M. A. 1997. A 1-year study of *Escherichia coli* O157 in cattle, sheep, pigs and poultry. *Epidemiol Infect,* 119**,** 245-50.

CHEVILLE, A. M., ARNOLD, K. W., BUCHRIESER, C., CHENG, C. M. & KASPAR, C. W. 1996. rpoS regulation of acid, heat, and salt tolerance in *Escherichia coli* O157:H7. *Appl Environ Microbiol,* 62**,** 1822-4.

CHIRNSIDE, E. D., EMMERSON, A. M. & SMITH, J. T. 1985. A follow-up survey of transferable, plasmid-encoded trimethoprim resistance in a general hospital (1975-1983). *J Antimicrob Chemother,* 16**,** 419-34.

CENTRE FOR HEALTH PROTECTION. 2013. Epidemiology, Prevention and Control of Shiga toxin-producing *Escherichia coli* Infection. 'Available:' http://www.chp.gov.hk/files/pdf/epidemiology_prevention_and_control_of_shiga_toxic_producing_escherichia_coli_infection.pdf.

CHUNG, H. J., BANG, W. & DRAKE, M. A. 2006. Stress Response of *Escherichia coli. Compr Rev Food Sci Food Saf,* 5**,** 52-64.

CLINICAL AND LABORATORY STANDARDS INSTITUTE. 2013. Performance Standards for Anitmicrobial Susceptibility Testing; Twenty-Third Informational Supplement, M100-S23. 3rd ed. Wayne, PA,: Clinical and Laboratory Standards Institute.

COLLIGNON, P., POWERS, J. H., CHILLER, T. M., AIDARA-KANE, A. & AARESTRUP, F. M. 2009. World Health Organization ranking of antimicrobials according to their importance in human medicine: A critical step for developing risk management strategies for the use of antimicrobials in food production animals. *Clin Infect Dis,* 49**,** 132-41.

CORREIA, M., BOAVIDA, F., GROSSO, F., SALGADO, M. J., LITO, L. M., CRISTINO, J. M., MENDO, S. & DUARTE, A. 2003. Molecular characterization of a new class 3 integron in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother,* 47**,** 2838-43.

COWDEN, J. M. 1997. Recent Outbreaks of *Escherichia coli* O157 in Scotland. *SUPPL. SCIH Weekly Rep.,* 13**,** 6-7.

COWDEN, J. M., AHMED, S., DONAGHY, M. & RILEY, A. 2001. Epidemiological investigation of the central Scotland outbreak of *Escherichia coli* O157 infection, November to December 1996. *Epidemiol Infect,* 126**,** 335-41.

CRIM, S. M., GRIFFIN, P. M., TAUXE, R., MARDER, E. P., GILLISS, D., CRONQUIST, A. B., CARTTER, M., TOBIN-D'ANGELO, M., BLYTHE, D., SMITH, K., LATHROP, S., ZANSKY, S., CIESLAK, P. R., DUNN, J., HOLT, K. G., WOLPERT, B., HENAO, O. L., CENTERS FOR DISEASE, C. & PREVENTION 2015. Preliminary incidence and trends of infection with pathogens transmitted commonly through food - Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2014. *MMWR Morb Mortal Wkly Rep,* 64**,** 495-9.

DEPARTMENT OF AGRICULTURE, FISHERIES AND FORESTRY – AUSTRALIA. 2007. Pilot Surveillance Program for Antimicriobial Resistance in Bacteria of Animal Origin Available: http://www.agriculture.gov.au/SiteCollectionDocuments/ag-food/food/food-safety/antimicrobial/AMR-pilot-survey-report.pdf [Accessed 26 Aug 2015].

DAVISON, J. 1999. Genetic exchange between bacteria in the environment. *Plasmid,* 42**,** 73-91.

DEFRANCESCO, K. A., COBBOLD, R. N., RICE, D. H., BESSER, T. E. & HANCOCK, D. D. 2004. Antimicrobial resistance of commensal *Escherichia coli* from dairy cattle associated with recent multi-resistant salmonellosis outbreaks. *Vet Microbiol,* 98**,** 55-61.

DESMARCHELIER, P. & FEGAN, N. 2003. Enteropathogenic *Escherichia coli*. *In:* HOCHING, A. D. (ed.) *Foodborne microorganisms of public health significance.* 6th ed. Sydney: Austrtalian Institute of Food Science and Technology (NSW Branch).

DUFFY, G., WALSH, C., BLAIR, I. S. & MCDOWELL, D. A. 2006. Survival of antibiotic resistant and antibiotic sensitive strains of *E. coli* O157 and *E. coli* O26 in food matrices. *Int J Food Microbiol,* 109**,** 179-86.

DUFFY, L. L., GRAU, F. H. & VANDERLINDE, P. B. 2000. Acid resistance of enterohaemorrhagic and generic *Escherichia coli* associated with foodborne disease and meat. *Int J Food Microbiol,* 60**,** 83-9.

DURSO, L. M., SMITH, D. & HUTKINS, R. W. 2004. Measurements of fitness and competition in commensal *Escherichia coli* and *E. coli* O157:H7 strains. *Appl Environ Microbiol,* 70**,** 6466-72.

EBLEN, D. R. 2006. Public Health Importance of Non-O157 Shiga Toxin-producing *Escherichia coli* (Non-O157 STEC) in the US Food Suppy. Available: http://www.fsis.usda.gov/wps/wcm/connect/9e41f955-a919-44fb-98b7-9e2218805ecf/STEC_101207.pdf?MOD=AJPERES [Accessed 22 Aug 2015].

ELDER, R. O., KEEN, J. E., SIRAGUSA, G. R., BARKOCY-GALLAGHER, G. A., KOOHMARAIE, M. & LAEGREID, W. W. 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc Natl Acad Sci U S A,* 97**,** 2999-3003.

ELHANAFI, D., LEENANON, B., BANG, W. & DRAKE, M. A. 2004. Impact of cold and cold-acid stress on poststress tolerance and virulence factor expression of *Escherichia coli* O157:H7. *J Food Prot,* 67**,** 19-26.

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL. 2014. Annual epidemiological report 2014 - food- and water-borne disease and zoonoses.

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL. 2012. Annual Epidemiological Report Reporting on 2010 Surveillance Data and 2011 Epidemic Intelligence Data.

ENVIRONMENTAL SCIENCE AND RESEARCH LTD. 2013. Standard operating procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*. 'Available:'

http://www.pulsenetinternational.org/assets/PulseNet/uploads/pfge/PNL05_Ec-Sal-ShigPFGEprotocol.pdf.
ERB, A., STURMER, T., MARRE, R. & BRENNER, H. 2007. Prevalence of antibiotic resistance in *Escherichia coli*: overview of geographical, temporal, and methodological variations. *Eur J Clin Microbiol Infect Dis,* 26**,** 83-90.
ESPIÉ, E., GRIMONT, F., VAILLANT, V., MONTET, M. P., CARLE, I., BAVAI, C., DE VALK, H. & VERNOZY-ROZAND, C. 2006. O148 Shiga toxin-producing Escherichia coli outbreak: microbiological investigation as a useful complement to epidemiological investigation. *Clin Microbiol Infect,* 12**,** 992-8.
ETHELBERG, S., OLSEN, K. E., SCHEUTZ, F., JENSEN, C., SCHIELLERUP, P., ENBERG, J., PETERSEN, A. M., OLESEN, B., GERNER-SMIDT, P. & MOLBAK, K. 2004. Virulence factors for hemolytic uremic syndrome, Denmark. *Emerg Infect Dis,* 10**,** 842-7.
ETHELBERG, S., SMITH, B., TORPDAHL, M., LISBY, M., BOEL, J., JENSEN, T., NIELSEN, E. M. & MOLBAK, K. 2009. Outbreak of non-O157 Shiga toxin-producing *Escherichia coli* infection from consumption of beef sausage. *Clin Infect Dis,* 48**,** e78-81.
EVERETT, M. J., JIN, Y. F., RICCI, V. & PIDDOCK, L. J. 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrob Agents Chemother,* 40**,** 2380-6.
FARMER, J. J., 3RD & DAVIS, B. R. 1985. H7 antiserum-sorbitol fermentation medium: a single tube screening medium for detecting *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *J Clin Microbiol,* 22**,** 620-5.
FOOD AND DRUG ADMINISTRATION − USA. 2010. National antimicrobial resistance monitoring system –enteric bacteria (NARMS): 2008 executive report. Available: http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm [Accessed 13 Feb 2012].
FEGAN, N., VANDERLINDE, P., HIGGS, G. & DESMARCHELIER, P. 2004. The prevalence and concentration of *Escherichia coli* O157 in faeces of cattle from different production systems at slaughter. *J Appl Microbiol,* 97**,** 362-70.
FENG, P., LAMPEL, K. A., KARCH, H. & WHITTAM, T. S. 1998. Genotypic and phenotypic changes in the emergence of *Escherichia coli* O157:H7. *J Infect Dis,* 177**,** 1750-3.
FERENS, W. A. & HOVDE, C. J. 2011. *Escherichia coli* O157:H7: animal reservoir and sources of human infection. *Foodborne Pathog Dis,* 8**,** 465-87.
FERIA, C., FERREIRA, E., CORREIA, J. D., GONCALVES, J. & CANICA, M. 2002. Patterns and mechanisms of resistance to beta-lactams and beta-lactamase inhibitors in uropathogenic *Escherichia coli* isolated from dogs in Portugal. *J Antimicrob Chemother,* 49**,** 77-85.
FERNANDEZ, D., IRINO, K., SANZ, M. E., PADOLA, N. L. & PARMA, A. E. 2010. Characterization of Shiga toxin-producing *Escherichia coli* isolated from dairy cows in Argentina. *Lett Appl Microbiol,* 51**,** 377-82.
FREMAUX, B., RAYNAUD, S., BEUTIN, L. & ROZAND, C. V. 2006. Dissemination and persistence of Shiga toxin-producing *Escherichia coli* (STEC) strains on French dairy farms. *Vet Microbiol,* 117**,** 180-91.
FUKUSHIMA, H., HASHIZUME, T., MORITA, Y., TANAKA, J., AZUMA, K., MIZUMOTO, Y., KANENO, M., MATSUURA, M., KONMA, K. & KITANI, T. 1999. Clinical experiences in Sakai City hospital during the massive outbreak of enterohemorrhagic *Escherichia coli* O157 infections in Sakai City, 1996. *Pediatr Int,* 41**,** 213-7.

FUKUSHIMA, H. & SEKI, R. 2004. High numbers of Shiga toxin-producing *Escherichia coli* found in bovine faeces collected at slaughter in Japan. *FEMS Microbiol Lett,* 238**,** 189-97.
GAMAGE, S. D., PATTON, A. K., HANSON, J. F. & WEISS, A. A. 2004. Diversity and host range of Shiga toxin-encoding phage. *Infect Immun,* 72**,** 7131-9.
GRIFFIN, P. M. & TAUXE, R. V. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev,* 13**,** 60-98.
GUERRA, B., JUNKER, E., SCHROETER, A., MALORNY, B., LEHMANN, S. & HELMUTH, R. 2003. Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *J Antimicrob Chemother,* 52**,** 489-92.
GUPTA, S., SONI, N. K., KAUR, P. & SOOD, D. K. 1992. Verocytopathic activity of *Escherichia coli* O157 & other 'O' serogroups isolated from patients of diarrhoea. *Indian J Med Res,* 95**,** 71-6.
GYLES, C. L. 2007. Shiga toxin-producing *Escherichia coli*: an overview. *J Anim Sci,* 85**,** E45-62.
HEALTH ACTION INTERNATIONAL ASIA PACIFIC. 2013. Antibiotic use and antibiotic resistance in food animals in Malaysia: a threat to human and animal health.
HARADA, K. & ASAI, T. 2010. Role of antimicrobial selective pressure and secondary factors on antimicrobial resistance prevalence in *Escherichia coli* from food-producing animals in Japan. *J Biomed Biotechnol,* 2010**,** 180682.
HAYASHI, T., MAKINO, K., OHNISHI, M., KUROKAWA, K., ISHII, K., YOKOYAMA, K., HAN, C. G., OHTSUBO, E., NAKAYAMA, K., MURATA, T., TANAKA, M., TOBE, T., IIDA, T., TAKAMI, H., HONDA, T., SASAKAWA, C., OGASAWARA, N., YASUNAGA, T., KUHARA, S., SHIBA, T., HATTORI, M. & SHINAGAWA, H. 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res,* 8**,** 11-22.
HEUVELINK, A. E., VAN DEN BIGGELAAR, F. L., ZWARTKRUIS-NAHUIS, J., HERBES, R. G., HUYBEN, R., NAGELKERKE, N., MELCHERS, W. J., MONNENS, L. A. & DE BOER, E. 1998. Occurrence of verocytotoxin-producing *Escherichia coli* O157 on Dutch dairy farms. *J Clin Microbiol,* 36**,** 3480-7.
HILBORN, E. D., MERMIN, J. H., MSHAR, P. A., HADLER, J. L., VOETSCH, A., WOJTKUNSKI, C., SWARTZ, M., MSHAR, R., LAMBERT-FAIR, M. A., FARRAR, J. A., GLYNN, M. K. & SLUTSKER, L. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch Intern Med,* 159**,** 1758-64.
HO, W. S., BALAN, G., PUTHUCHEARY, S., KONG, B. H., LIM, K. T., TAN, L. K., KOH, X. P., YEO, C. C. & THONG, K. L. 2012. Prevalence and characterization of multidrug-resistant and extended-spectrum beta-lactamase-producing *Escherichia coli* from pediatric wards of a Malaysian hospital. *Microb Drug Resist,* 18**,** 408-16.
HEALTH PROTECTION SCOTLAND. 2010. *E. coli* O157, Scotland, Annual Totals. Available: http://www.documents.hps.scot.nhs.uk/giz/10-year-tables/ecoli.pdf [Accessed 22 Aug 2015].
HUPPERTZ, H. I., BUSCH, D., SCHMIDT, H., ALEKSIC, S. & KARCH, H. 1996. Diarrhea in young children associated with *Escherichia coli* non-O157 organisms that produce Shiga-like toxin. *J Pediatr,* 128**,** 341-6.
INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS. 1996. Intestinally pathogenic *Escherichia coli*. *Microorganisms in food 5:*

Microbiological specifications of food pathogens. London: Blackie Academic and Professional.
INSTITUTE OF ENVIRONMENTAL SCIENCE AND RESEARCH LTD. 2015. Surveillance report - notifiable diseases in New Zealan: annual report 2014.
IRINO, K., KATO, M. A., VAZ, T. M., RAMOS, II, SOUZA, M. A., CRUZ, A. S., GOMES, T. A., VIEIRA, M. A. & GUTH, B. E. 2005. Serotypes and virulence markers of Shiga toxin-producing *Escherichia coli* (STEC) isolated from dairy cattle in Sao Paulo State, Brazil. *Vet Microbiol,* 105**,** 29-36.
IRSHAD, H., COOKSON, A. L., HOTTER, G., BESSER, T. E., ON, S. L. & FRENCH, N. P. 2012. Epidemiology of Shiga toxin-producing *Escherichia coli* O157 in very young calves in the North Island of New Zealand. *N Z Vet J,* 60**,** 21-6.
ISLAM, M. A., MONDOL, A. S., DE BOER, E., BEUMER, R. R., ZWIETERING, M. H., TALUKDER, K. A. & HEUVELINK, A. E. 2008. Prevalence and genetic characterization of shiga toxin-producing *Escherichia coli* isolates from slaughtered animals in Bangladesh. *Appl Environ Microbiol,* 74**,** 5414-21.
ISLAM, M. R., OGURA, Y., ASADULGHANI, M., OOKA, T., MURASE, K., GOTOH, Y. & HAYASHI, T. 2012. A sensitive and simple plaque formation method for the Stx2 phage of *Escherichia coli* O157:H7, which does not form plaques in the standard plating procedure. *Plasmid,* 67**,** 227-35.
IVERSEN, H., TM, L. A.-L., ASPHOLM, M., ARNESEN, L. P. & LINDBACK, T. 2015. Commensal *E. coli* Stx2 lysogens produce high levels of phages after spontaneous prophage induction. *Front Cell Infect Microbiol,* 5**,** 5.
JACOB, M. E., FOX, J. T., REINSTEIN, S. L. & NAGARAJA, T. G. 2008. Antimicrobial susceptibility of foodborne pathogens in organic or natural production systems: an overview. *Foodborne Pathog Dis,* 5**,** 721-30.
JAURIN, B. & NORMARK, S. 1983. Insertion of IS2 creates a novel ampC promoter in *Escherichia coli*. *Cell,* 32**,** 809-16.
JAY, J. M. 2000. *Modern Food Microbiology,* Gaithersburg, Md., Aspen Publishers Inc.
JOHNSEN, G., WASTESON, Y., HEIR, E., BERGET, O. I. & HERIKSTAD, H. 2001. *Escherichia coli* O157:H7 in faeces from cattle, sheep and pigs in the southwest part of Norway during 1998 and 1999. *Int J Food Microbiol,* 65**,** 193-200.
JOHNSON, J. R., CLABOTS, C. & KUSKOWSKI, M. A. 2008. Multiple-host sharing, long-term persistence, and virulence of *Escherichia coli* clones from human and animal household members. *J Clin Microbiol,* 46**,** 4078-82.
JOHNSON, R. P., CLARKE, R. C., WILSON, J. B., READ, S. C., RAHN, K., RENVICK, S. A., SANDHU, K. A., ALVES, D., KARMALI, M. A., LIOR, H., MCEWEN, S. A., SPIKA, J. S. & GYLES, C. L. 1996. Growing concerns and recent outbreaks involving non-O157:H7 serotypes of verotoxigenic *Escherichia coli. J Food Prot,* 10**,** 1112-1122.
JONES, P. G., VANBOGELEN, R. A. & NEIDHARDT, F. C. 1987. Induction of proteins in response to low temperature in *Escherichia coli*. *J Bacteriol,* 169**,** 2092-5.
JONES, T. H. 2012. Response of *Escherichia coli* to Environmental Stress. *In:* WONG, H. C. (ed.) *Stress Response of Foodborne Microorganisms.* New York: Nova Science Publishers Inc.
JORIS, M. A., PIERARD, D. & DE ZUTTER, L. 2011. Occurrence and virulence patterns of *E. coli* O26, O103, O111 and O145 in slaughter cattle. *Vet Microbiol,* 151**,** 418-21.
JUNEJA, V. K., KLEIN, P. G. & MARMER, B. S. 1998. Heat shock and thermotolerance of *Escherichia coli* O157:H7 in a model beef gravy system and ground beef. *J Appl Microbiol,* 84**,** 677-84.

KADLEC, K. & SCHWARZ, S. 2008. Analysis and distribution of class 1 and class 2 integrons and associated gene cassettes among *Escherichia coli* isolates from swine, horses, cats and dogs collected in the BfT-GermVet monitoring study. *J Antimicrob Chemother,* 62**,** 469-73.

KAPER, J. B., NATARO, J. P. & MOBLEY, H. L. 2004. Pathogenic *Escherichia coli*. *Nat Rev Microbiol,* 2**,** 123-40.

KARCH, H., BIELASZEWSKA, M., BITZAN, M. & SCHMIDT, H. 1999. Epidemiology and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Diagn Microbiol Infect Dis,* 34**,** 229-43.

KARCZMARCZYK, M., ABBOTT, Y., WALSH, C., LEONARD, N. & FANNING, S. 2011a. Characterization of multidrug-resistant *Escherichia coli* isolates from animals presenting at a university veterinary hospital. *Appl Environ Microbiol,* 77**,** 7104-12.

KARCZMARCZYK, M., WALSH, C., SLOWEY, R., LEONARD, N. & FANNING, S. 2011b. Molecular characterization of multidrug-resistant *Escherichia coli* isolates from Irish cattle farms. *Appl Environ Microbiol,* 77**,** 7121-7.

KARMALI, M. A. 1989. Infection by verocytotoxin-producing *Escherichia coli*. *Clin Microbiol Rev,* 2**,** 15-38.

KARMALI, M. A., STEELE, B. T., PETRIC, M. & LIM, C. 1983. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet,* 1**,** 619-20.

KASPER, C., DOYLE, M. E. & ARCHER, J. 2010. White paper on non-O157:H7 Shiga toxin producing *E. coli* from meat and non-meat sources [Online]. Available: http://www.namif.org/wp-content/uploads/08-402.pdf [Accessed 07 Jul 2015 2015].

KAUFFMANN, F. 1947. The serology of the coli group. *J Immunol,* 57**,** 71-100.

KAWANO, K., OKADA, M., HAGA, T., MAEDA, K. & GOTO, Y. 2008. Relationship between pathogenicity for humans and stx genotype in Shiga toxin-producing *Escherichia coli* serotype O157. *Eur J Clin Microbiol Infect Dis,* 27**,** 227-32.

KERRN, M. B., KLEMMENSEN, T., FRIMODT-MOLLER, N. & ESPERSEN, F. 2002. Susceptibility of Danish *Escherichia coli* strains isolated from urinary tract infections and bacteraemia, and distribution of sul genes conferring sulphonamide resistance. *J Antimicrob Chemother,* 50**,** 513-6.

KEYES, K., HUDSON, C., MAURER, J. J., THAYER, S., WHITE, D. G. & LEE, M. D. 2000. Detection of florfenicol resistance genes in *Escherichia coli* isolated from sick chickens. *Antimicrob Agents Chemother,* 44**,** 421-4.

KHAN, A., YAMASAKI, S., SATO, T., RAMAMURTHY, T., PAL, A., DATTA, S., CHOWDHURY, N. R., DAS, S. C., SIKDAR, A., TSUKAMOTO, T., BHATTACHARYA, S. K., TAKEDA, Y. & NAIR, G. B. 2002. Prevalence and genetic profiling of virulence determinants of non-O157 Shiga toxin-producing *Escherichia coli* isolated from cattle, beef, and humans, Calcutta, India. *Emerg Infect Dis,* 8**,** 54-62.

KIM, J., NIETFELDT, J., JU, J., WISE, J., FEGAN, N., DESMARCHELIER, P. & BENSON, A. K. 2001. Ancestral divergence, genome diversification, and phylogeographic variation in subpopulations of sorbitol-negative, beta-glucuronidase-negative enterohemorrhagic *Escherichia coli* O157. *J Bacteriol,* 183**,** 6885-97.

KIM, Y. B., OKUDA, J., MATSUMOTO, C., MORIGAKI, T., ASAI, N., WATANABE, H. & NISHIBUCHI, M. 1998. Isolation of an *Escherichia coli* O157:H7 strain producing Shiga toxin 1 but not Shiga toxin 2 from a patient with hemolytic uremic syndrome in Korea. *FEMS Microbiol Lett,* 166**,** 43-8.

KIMMITT, P. T., HARWOOD, C. R. & BARER, M. R. 2000. Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. *Emerg Infect Dis,* 6**,** 458-65.

KING, L. A., FILLIOL-TOUTAIN, I., MARIANI-KURKIDJIAN, P., VAILLANT, V., VERNOZY-ROZAND, C., GANET, S., PIHIER, N., NIAUDET, P. & DE VALK, H. 2010. Family outbreak of Shiga toxin-producing *Escherichia coli* O123:H-, France, 2009. *Emerg Infect Dis,* 16**,** 1491-3.

KOBAYASHI, H., SHIMADA, J., NAKAZAWA, M., MOROZUMI, T., POHJANVIRTA, T., PELKONEN, S. & YAMAMOTO, K. 2001. Prevalence and characteristics of shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. *Appl Environ Microbiol,* 67**,** 484-9.

KOCH, C., HERTWIG, S. & APPEL, B. 2003. Nucleotide sequence of the integration site of the temperate bacteriophage 6220, which carries the Shiga toxin gene stx(1ox3). *J Bacteriol,* 185**,** 6463-6.

KOELEMAN, J. G., STOOF, J., VAN DER BIJL, M. W., VANDENBROUCKE-GRAULS, C. M. & SAVELKOUL, P. H. 2001. Identification of epidemic strains of Acinetobacter baumannii by integrase gene PCR. *J Clin Microbiol,* 39**,** 8-13.

KOITABASHI, T., CUI, S., KAMRUZZAMAN, M. & NISHIBUCHI, M. 2008. Isolation and characterization of the Shiga toxin gene (stx)-bearing *Escherichia coli* O157 and non-O157 from retail meats in Shandong Province, China, and characterization of the O157-derived stx2 phages. *J Food Prot,* 71**,** 706-13.

KOITABASHI, T., VUDDHAKUL, V., RADU, S., MORIGAKI, T., ASAI, N., NAKAGUCHI, Y. & NISHIBUCHI, M. 2006. Genetic characterization of *Escherichia coli* O157: H7/- strains carrying the stx2 gene but not producing Shiga toxin 2. *Microbiol Immunol,* 50**,** 135-48.

KOR, S. B., CHOO, Q. C. & CHEW, C. H. 2013. New integron gene arrays from multiresistant clinical isolates of members of the *Enterobacteriaceae* and *Pseudomonas aeruginosa* from hospitals in Malaysia. *J Med Microbiol,* 62**,** 412-20.

KROPINSKI, A. M., MAZZOCCO, A., WADDELL, T. E., LINGOHR, E. & JOHNSON, R. P. 2009. Enumeration of bacteriophages by double agar overlay plaque assay. *Methods Mol Biol,* 501**,** 69-76.

LANZ, R., KUHNERT, P. & BOERLIN, P. 2003. Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. *Vet Microbiol,* 91**,** 73-84.

LAW, D. 2000. Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing E. coli. *J Appl Microbiol,* 88**,** 729-45.

LAW, D. & KELLY, J. 1995. Use of heme and hemoglobin by *Escherichia coli* O157 and other Shiga-like-toxin-producing *E. coli* serogroups. *Infect Immun,* 63**,** 700-2.

LEE, I. S., LIN, J., HALL, H. K., BEARSON, B. & FOSTER, J. W. 1995. The stationary-phase sigma factor sigma S (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. *Mol Microbiol,* 17**,** 155-67.

LEENANON, B. & DRAKE, M. A. 2001. Acid stress, starvation, and cold stress affect poststress behavior of *Escherichia coli* O157:H7 and nonpathogenic *Escherichia coli*. *J Food Prot,* 64**,** 970-4.

LEOMIL, L., AIDAR-UGRINOVICH, L., GUTH, B. E., IRINO, K., VETTORATO, M. P., ONUMA, D. L. & DE CASTRO, A. F. 2003. Frequency of Shiga toxin-producing *Escherichia coli* (STEC) isolates among diarrheic and non-diarrheic calves in Brazil. *Vet Microbiol,* 97**,** 103-9.

LEOPOLD, S. R., MAGRINI, V., HOLT, N. J., SHAIKH, N., MARDIS, E. R., CAGNO, J., OGURA, Y., IGUCHI, A., HAYASHI, T., MELLMANN, A., KARCH, H., BESSER,

T. E., SAWYER, S. A., WHITTAM, T. S. & TARR, P. I. 2009. A precise reconstruction of the emergence and constrained radiations of *Escherichia coli* O157 portrayed by backbone concatenomic analysis. *Proc Natl Acad Sci U S A,* 106**,** 8713-8.

LEVESQUE, C., PICHE, L., LAROSE, C. & ROY, P. H. 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother,* 39**,** 185-91.

LEVY, S. B. & MARSHALL, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med,* 10**,** S122-9.

LEYER, G. J. & JOHNSON, E. A. 1993. Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium*. *Appl Environ Microbiol,* 59**,** 1842-7.

LEYER, G. J., WANG, L. L. & JOHNSON, E. A. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. *Appl Environ Microbiol,* 61**,** 3752-5.

LI, X. Z. 2005. Quinolone resistance in bacteria: emphasis on plasmid-mediated mechanisms. *Int J Antimicrob Agents,* 25**,** 453-63.

LI, X. Z., MEHROTRA, M., GHIMIRE, S. & ADEWOYE, L. 2007. Beta-Lactam resistance and beta-lactamases in bacteria of animal origin. *Vet Microbiol,* 121**,** 197-214.

LIM, J. Y., YOON, J. & HOVDE, C. J. 2010. A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *J Microbiol Biotechnol,* 20**,** 5-14.

LIM, K. T., YASIN, R., YEO, C. C., PUTHUCHEARY, S. & THONG, K. L. 2009. Characterization of multidrug resistant ESBL-producing *Escherichia coli* isolates from hospitals in Malaysia. *J Biomed Biotechnol,* 2009**,** 165637.

LIM, S. K., LEE, H. S., NAM, H. M., CHO, Y. S., KIM, J. M., SONG, S. W., PARK, Y. H. & JUNG, S. C. 2007. Antimicrobial resistance observed in *Escherichia coli* strains isolated from fecal samples of cattle and pigs in Korea during 2003-2004. *Int J Food Microbiol,* 116**,** 283-6.

LIN, J., SMITH, M. P., CHAPIN, K. C., BAIK, H. S., BENNETT, G. N. & FOSTER, J. W. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol,* 62**,** 3094-100.

LITTLE, J. W. & MOUNT, D. W. 1982. The SOS regulatory system of *Escherichia coli*. *Cell,* 29**,** 11-22.

LOCKING, M., ALLISON, L., RAE, L. & HANSON, M. 2014. VTEC and HUS in Scotland, 2013: Enhanced Surveillance, Reference Laboratory and Clinical Reporting Data. *HPS Weekly Report,* 48**,** 257-63.

LOCKING, M. E., ALLISON, L. J., RAE, L. & HANSON, M. 2003. VTEC in Scotland 2002: Enhance Surveillance and Reference Laboratory Data. *SCIEH Weekly Rep.,* 37**,** 304-307.

LOPEZ, E. L., DIAZ, M., GRINSTEIN, S., DEVOTO, S., MENDILAHARZU, F., MURRAY, B. E., ASHKENAZI, S., RUBEGLIO, E., WOLOJ, M., VASQUEZ, M. & ET AL. 1989. Hemolytic uremic syndrome and diarrhea in Argentine children: the role of Shiga-like toxins. *J Infect Dis,* 160**,** 469-75.

LOS, J. M., GOLEC, P., WEGRZYN, G., WEGRZYN, A. & LOS, M. 2008. Simple method for plating *Escherichia coli* bacteriophages forming very small plaques or no plaques under standard conditions. *Appl Environ Microbiol,* 74**,** 5113-20.

M'ZALI, F. H., GASCOYNE-BINZI, D. M., HERITAGE, J. & HAWKEY, P. M. 1996. Detection of mutations conferring extended-spectrum activity on SHV beta-lactamases using polymerase chain reaction single strand conformational polymorphism (PCR-SSCP). *J Antimicrob Chemother,* 37**,** 797-802.

MACDONALD, I. A., GOULD, I. M. & CURNOW, J. 1996. Epidemiology of infection due to *Escherichia coli* O157: a 3-year prospective study. *Epidemiol Infect,* 116**,** 279-84.
MACHADO, E., CANTON, R., BAQUERO, F., GALAN, J. C., ROLLAN, A., PEIXE, L. & COQUE, T. M. 2005. Integron content of extended-spectrum-beta-lactamase-producing *Escherichia coli* strains over 12 years in a single hospital in Madrid, Spain. *Antimicrob Agents Chemother,* 49**,** 1823-9.
MADSEN, L., AARESTRUP, F. M. & OLSEN, J. E. 2000. Characterisation of streptomycin resistance determinants in Danish isolates of *Salmonella Typhimurium*. *Vet Microbiol,* 75**,** 73-82.
MAIDHOF, H., GUERRA, B., ABBAS, S., ELSHEIKHA, H. M., WHITTAM, T. S. & BEUTIN, L. 2002. A multiresistant clone of Shiga toxin-producing *Escherichia coli* O118:[H16] is spread in cattle and humans over different European countries. *Appl Environ Microbiol,* 68**,** 5834-42.
MAKINO, K., YOKOYAMA, K., KUBOTA, Y., YUTSUDO, C. H., KIMURA, S., KUROKAWA, K., ISHII, K., HATTORI, M., TATSUNO, I., ABE, H., IIDA, T., YAMAMOTO, K., ONISHI, M., HAYASHI, T., YASUNAGA, T., HONDA, T., SASAKAWA, C. & SHINAGAWA, H. 1999. Complete nucleotide sequence of the prophage VT2-Sakai carrying the verotoxin 2 genes of the enterohemorrhagic *Escherichia coli* O157:H7 derived from the Sakai outbreak. *Genes Genet Syst,* 74**,** 227-39.
MANGES, A. R. & JOHNSON, J. R. 2012. Food-borne origins of *Escherichia coli* causing extraintestinal infections. *Clin Infect Dis,* 55**,** 712-9.
MANNA, S. K., BRAHMANE, M. P., MANNA, C., BATABYAL, K. & DAS, R. 2006. Occurrence, virulence characteristics and antimicrobial resistance of *Escherichia coli* O157 in slaughtered cattle and diarrhoeic calves in West Bengal, India. *Lett Appl Microbiol,* 43**,** 405-9.
MANNING, S. D., MOTIWALA, A. S., SPRINGMAN, A. C., QI, W., LACHER, D. W., OUELLETTE, L. M., MLADONICKY, J. M., SOMSEL, P., RUDRIK, J. T., DIETRICH, S. E., ZHANG, W., SWAMINATHAN, B., ALLAND, D. & WHITTAM, T. S. 2008. Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. *Proc Natl Acad Sci U S A,* 105**,** 4868-73.
MARCH, S. B. & RATNAM, S. 1986. Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *J Clin Microbiol,* 23**,** 869-72.
MARTINEZ-GOMEZ, K., FLORES, N., CASTANEDA, H. M., MARTINEZ-BATALLAR, G., HERNANDEZ-CHAVEZ, G., RAMIREZ, O. T., GOSSET, G., ENCARNACION, S. & BOLIVAR, F. 2012. New insights into *Escherichia coli* metabolism: carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol. *Microb Cell Fact,* 11**,** 46.
MAYNARD, C., FAIRBROTHER, J. M., BEKAL, S., SANSCHAGRIN, F., LEVESQUE, R. C., BROUSSEAU, R., MASSON, L., LARIVIERE, S. & HAREL, J. 2003. Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. *Antimicrob Agents Chemother,* 47**,** 3214-21.
MAZEL, D., DYCHINCO, B., WEBB, V. A. & DAVIES, J. 2000. Antibiotic resistance in the ECOR collection: integrons and identification of a novel aad gene. *Antimicrob Agents Chemother,* 44**,** 1568-74.
MAZUREK, J., PUSZ, P., BOK, E., STOSIK, M. & BALDY-CHUDZIK, K. 2013. The phenotypic and genotypic characteristics of antibiotic resistance in *Escherichia coli*

populations isolated from farm animals with different exposure to antimicrobial agents. *Pol J Microbiol,* 62**,** 173-9.
MCEWEN, S. A. & FEDORKA-CRAY, P. J. 2002. Antimicrobial use and resistance in animals. *Clin Infect Dis,* 34 Suppl 3**,** S93-S106.
MCKELLAR, R. C. & KNIGHT, K. P. 1999. Growth and survival of various strains of enterohemorrhagic *Escherichia coli* in hydrochloric and acetic acid. *J Food Prot,* 62**,** 1466-9.
MEAD, P. S. & GRIFFIN, P. M. 1998. *Escherichia coli* O157:H7. *Lancet,* 352**,** 1207-12.
MELLOR, G. E., BESSER, T. E., DAVIS, M. A., BEAVIS, B., JUNG, W., SMITH, H. V., JENNISON, A. V., DOYLE, C. J., CHANDRY, P. S., GOBIUS, K. S. & FEGAN, N. 2013. Multilocus genotype analysis of *Escherichia coli* O157 isolates from Australia and the United States provides evidence of geographic divergence. *Appl Environ Microbiol,* 79**,** 5050-8.
MELLOR, G. E., SIM, E. M., BARLOW, R. S., D'ASTEK, B. A., GALLI, L., CHINEN, I., RIVAS, M. & GOBIUS, K. S. 2012. Phylogenetically related Argentinean and Australian *Escherichia coli* O157 isolates are distinguished by virulence clades and alternative Shiga toxin 1 and 2 prophages. *Appl Environ Microbiol,* 78**,** 4724-31.
MICHEL, P., WILSON, J. B., MARTIN, S. W., CLARKE, R. C., MCEWEN, S. A. & GYLES, C. L. 1998. A descriptive study of verocytotoxigenic *Escherichia coli* (VTEC) cases reported in Ontario, 1990-1994. *Can J Public Health,* 89**,** 253-7.
MILNES, A. S., SAYERS, A. R., STEWART, I., CLIFTON-HADLEY, F. A., DAVIES, R. H., NEWELL, D. G., COOK, A. J., EVANS, S. J., SMITH, R. P. & PAIBA, G. A. 2009. Factors related to the carriage of Verocytotoxigenic *E. coli, Salmonella,* thermophilic *Campylobacter* and *Yersinia enterocolitica* in cattle, sheep and pigs at slaughter. *Epidemiol Infect,* 137**,** 1135-48.
MINISTRY FOR PRIMARY INDUSTRIES – NEW ZEALAND. 2001. *Escherichia coli* O157: H7. Available: http://www.foodsafety.govt.nz/elibrary/industry/ Escherichia_Coli-Organism_Invades.pdf [Accessed 22 Aug 2015].
MOLINA, P. M., PARMA, A. E. & SANZ, M. E. 2003. Survival in acidic and alcoholic medium of Shiga toxin-producing *Escherichia coli* O157:H7 and non-O157:H7 isolated in Argentina. *BMC Microbiol,* 3**,** 17.
MONAGHAN, A., BYRNE, B., FANNING, S., SWEENEY, T., MCDOWELL, D. & BOLTON, D. J. 2011. Serotypes and virulence profiles of non-O157 Shiga toxin-producing *Escherichia coli* isolates from bovine farms. *Appl Environ Microbiol,* 77**,** 8662-8.
MORA, A., BLANCO, J. E., BLANCO, M., ALONSO, M. P., DHABI, G., ECHEITA, A., GONZALEZ, E. A., BERNARDEZ, M. I. & BLANCO, J. 2005. Antimicrobial resistance of Shiga toxin (verotoxin)-producing *Escherichia coli* O157:H7 and non-O157 strains isolated from humans, cattle, sheep and food in Spain. *Res Microbiol,* 156**,** 793-806.
MORABITO, S., TOZZOLI, R., CAPRIOLI, A., KARCH, H. & CARATTOLI, A. 2002. Detection and characterization of class 1 integrons in enterohemorrhagic *Escherichia coli*. *Microb Drug Resist,* 8**,** 85-91.
MUNIESA, M., BLANCO, J. E., DE SIMON, M., SERRA-MORENO, R., BLANCH, A. R. & JOFRE, J. 2004a. Diversity of stx2 converting bacteriophages induced from Shiga-toxin-producing *Escherichia coli* strains isolated from cattle. *Microbiology,* 150**,** 2959-71.
MUNIESA, M., GARCIA, A., MIRO, E., MIRELIS, B., PRATS, G., JOFRE, J. & NAVARRO, F. 2004b. Bacteriophages and diffusion of beta-lactamase genes. *Emerg Infect Dis,* 10**,** 1134-7.

MURANO, E. A. & PIERSON, M. D. 1992. Effect of Heat Shock and Growth Atmosphere on the Heat Resistance of *Escherichia coli.* O157:H7. *J Food Prot,* 55**,** 171-175.
MYAING, T. T., SALEHA, A. A., ARIFAH, A. K. & RAHA, A. R. 2005. Antibiotic Resistance and Plasmid Carriage among *Escherichia coli*. Isolates from Chicken Meat in Malaysia. *In:* MAKKAR, H. P. S. & VILJOEN, G. J. (eds.) *Applications of Gene-based Technologies for Improving Animal Production and Health in Developing Countries.* Springer.
NATARO, J. P. & KAPER, J. B. 1998. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev,* 11**,** 142-201.
NEELY, M. N. & FRIEDMAN, D. I. 1998. Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. *Mol Microbiol,* 28**,** 1255-67.
NEILL, M. A., TARR, P. I., TAYLOR, D. N. & TROFA, A. F. 1994. *Escherichia coli*. *In:* HUI, Y. H., GORHAM, J. R., MURELL, K. D. & CLIVER, D. O. (eds.) *Foodborne Disease Handbook.* New York: Marcel Decker Inc.
NG, K. H., SAMUEL, L., KATHLEEN, M. M., LEONG, S. S. & FELECIA, C. 2014. Distribution and Prevalance of Chloramphenicol-resistance gene in *Escherichia coli.* isolated from aquaculture and other environment. *Int Food Res J,* 21**,** 1321-1325.
NIELD, B. S., HOLMES, A. J., GILLINGS, M. R., RECCHIA, G. D., MABBUTT, B. C., NEVALAINEN, K. M. & STOKES, H. W. 2001. Recovery of new integron classes from environmental DNA. *FEMS Microbiol Lett,* 195**,** 59-65.
NOALL, E. W., SEWARDS, H. F. & WATERWORTH, P. M. 1962. Successful Treatment of a Case of Proteus Septicaemia. *Br Med J,* 2**,** 1101-2.
OGURA, Y., OOKA, T., IGUCHI, A., TOH, H., ASADULGHANI, M., OSHIMA, K., KODAMA, T., ABE, H., NAKAYAMA, K., KUROKAWA, K., TOBE, T., HATTORI, M. & HAYASHI, T. 2009. Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic *Escherichia coli*. *Proc Natl Acad Sci U S A,* 106**,** 17939-44.
OHNISHI, M., TERAJIMA, J., KUROKAWA, K., NAKAYAMA, K., MURATA, T., TAMURA, K., OGURA, Y., WATANABE, H. & HAYASHI, T. 2002. Genomic diversity of enterohemorrhagic *Escherichia coli* O157 revealed by whole genome PCR scanning. *Proc Natl Acad Sci U S A,* 99**,** 17043-8.
OJEDA, A., PRADO, V., MARTINEZ, J., ARELLANO, C., BORCZYK, A., JOHNSON, W., LIOR, H. & LEVINE, M. M. 1995. Sorbitol-negative phenotype among enterohemorrhagic *Escherichia coli* strains of different serotypes and from different sources. *J Clin Microbiol,* 33**,** 2199-201.
OKLAHOMA STATE DEPARTMENT OF HEALTH. 2009. Epidemiological Investigation of Restaurant Associated *Escherichia coli* O111:NM Outbreak- Mayes County, Oklahoma, 2008. Available: http://www.ok.gov/health2/documents/OOC-EcoliO111SummaryReport.pdf [Accessed 03 Aug 2015].
PADDOCK, Z., SHI, X., BAI, J. & NAGARAJA, T. G. 2012. Applicability of a multiplex PCR to detect O26, O45, O103, O111, O121, O145, and O157 serogroups of *Escherichia coli* in cattle feces. *Vet Microbiol,* 156**,** 381-8.
PADOLA, N. L., SANZ, M. E., BLANCO, J. E., BLANCO, M., BLANCO, J., ETCHEVERRIA, A. I., ARROYO, G. H., USERA, M. A. & PARMA, A. E. 2004. Serotypes and virulence genes of bovine Shigatoxigenic *Escherichia coli* (STEC) isolated from a feedlot in Argentina. *Vet Microbiol,* 100**,** 3-9.
PAI, C. H., AHMED, N., LIOR, H., JOHNSON, W. M., SIMS, H. V. & WOODS, D. E. 1988. Epidemiology of sporadic diarrhea due to verocytotoxin-producing *Escherichia coli*: a two-year prospective study. *J Infect Dis,* 157**,** 1054-7.

PATON, A. W. & PATON, J. C. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic *E. coli* hlyA, rfbO111, and rfbO157. *J Clin Microbiol,* 36**,** 598-602.

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**,** 1622-7.

PATRIDGE, E. V. & FERRY, J. G. 2006. WrbA from *Escherichia coli* and *Archaeoglobus fulgidus* is an NAD(P)H:quinone oxidoreductase. *J Bacteriol,* 188**,** 3498-506.

PENNINGTON, H. 2010. *Escherichia coli* O157. *Lancet,* 376**,** 1428-35.

PENNINGTON, T. H. 1998. Factors involved in recent outbreaks of *Escherichia coli* O157: H7 in Scotland and recommendations for its control. *Journal of Food Safety,* 18**,** 383-391.

PEREZ-PEREZ, F. J. & HANSON, N. D. 2002. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol,* 40**,** 2153-62.

PERNA, N. T., PLUNKETT, G., 3RD, BURLAND, V., MAU, B., GLASNER, J. D., ROSE, D. J., MAYHEW, G. F., EVANS, P. S., GREGOR, J., KIRKPATRICK, H. A., POSFAI, G., HACKETT, J., KLINK, S., BOUTIN, A., SHAO, Y., MILLER, L., GROTBECK, E. J., DAVIS, N. W., LIM, A., DIMALANTA, E. T., POTAMOUSIS, K. D., APODACA, J., ANANTHARAMAN, T. S., LIN, J., YEN, G., SCHWARTZ, D. C., WELCH, R. A. & BLATTNER, F. R. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature,* 409**,** 529-33.

PERSSON, S., OLSEN, K. E., ETHELBERG, S. & SCHEUTZ, F. 2007. Subtyping method for *Escherichia coli* shiga toxin (verocytotoxin) 2 variants and correlations to clinical manifestations. *J Clin Microbiol,* 45**,** 2020-4.

PIDDOCK, L. J., RICCI, V., PUMBWE, L., EVERETT, M. J. & GRIGGS, D. J. 2003. Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. *J Antimicrob Chemother,* 51**,** 19-26.

PRADEL, N., LIVRELLI, V., DE CHAMPS, C., PALCOUX, J. B., REYNAUD, A., SCHEUTZ, F., SIROT, J., JOLY, B. & FORESTIER, C. 2000. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. *J Clin Microbiol,* 38**,** 1023-31.

PRICE, G. P. & ST JOHN, A. C. 2000. Purification and analysis of expression of the stationary phase-inducible slp lipoprotein in *Escherichia coli*: role of the Mar system. *FEMS Microbiol Lett,* 193**,** 51-6.

PUBLIC HEALTH AGENCY OF CANADA. 2015. *E. coli*. 'Available:' http://www.phac-aspc.gc.ca/fs-sa/fs-fi/ecoli-eng.php#fig1 ['Accessed' 03 February 2016].

RADU, S., ABDUL MUTALIB, S., RUSUL, G., AHMAD, Z., MORIGAKI, T., ASAI, N., KIM, Y. B., OKUDA, J. & NISHIBUCHI, M. 1998. Detection of *Escherichia coli* O157:H7 in the beef marketed in Malaysia. *Appl Environ Microbiol,* 64**,** 1153-6.

RADU, S., ANSARY, A., RUSUL, G. & KARIM, M. I. 1996. Isolation of verotoxin-producing *Escherichia coli* associated with diarrhoea in Malaysia containing plasmids showing homology with biotinylated Shiga-like toxin DNA gene probes. *World J Microbiol Biotechnol,* 12**,** 243-6.

RADU, S., LING, O. W., RUSUL, G., KARIM, M. I. & NISHIBUCHI, M. 2001. Detection of *Escherichia coli* O157:H7 by multiplex PCR and their characterization by plasmid

profiling, antimicrobial resistance, RAPD and PFGE analyses. *J Microbiol Methods,* 46**,** 131-9.

RAMACHANDRAN, V., BRETT, K., HORNITZKY, M. A., DOWTON, M., BETTELHEIM, K. A., WALKER, M. J. & DJORDJEVIC, S. P. 2003. Distribution of intimin subtypes among *Escherichia coli* isolates from ruminant and human sources. *J Clin Microbiol,* 41**,** 5022-32.

RASKO, D. A., WEBSTER, D. R., SAHL, J. W., BASHIR, A., BOISEN, N., SCHEUTZ, F., PAXINOS, E. E., SEBRA, R., CHIN, C. S., ILIOPOULOS, D., KLAMMER, A., PELUSO, P., LEE, L., KISLYUK, A. O., BULLARD, J., KASARSKIS, A., WANG, S., EID, J., RANK, D., REDMAN, J. C., STEYERT, S. R., FRIMODT-MOLLER, J., STRUVE, C., PETERSEN, A. M., KROGFELT, K. A., NATARO, J. P., SCHADT, E. E. & WALDOR, M. K. 2011. Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany. *N Engl J Med,* 365**,** 709-17.

RECKTENWALD, J. & SCHMIDT, H. 2002. The nucleotide sequence of Shiga toxin (Stx) 2e-encoding phage phiP27 is not related to other Stx phage genomes, but the modular genetic structure is conserved. *Infect Immun,* 70**,** 1896-908.

REIMANN, H. P. & CLIVER, D. O. 1998. *Escherichia coli.* O157:H7. *In:* TOLLEFSON, L. (ed.) *The Veterinary Clinics of North America-Food Animal Practice: Microbial Foodborne Pathogens.* Philadelphia, PA: W.B. Saunders Co.

REY, J., BLANCO, J. E., BLANCO, M., MORA, A., DAHBI, G., ALONSO, J. M., HERMOSO, M., HERMOSO, J., ALONSO, M. P., USERA, M. A., GONZALEZ, E. A., BERNARDEZ, M. I. & BLANCO, J. 2003. Serotypes, phage types and virulence genes of shiga-producing *Escherichia coli* isolated from sheep in Spain. *Vet Microbiol,* 94**,** 47-56.

RILEY, L. W., REMIS, R. S., HELGERSON, S. D., MCGEE, H. B., WELLS, J. G., DAVIS, B. R., HEBERT, R. J., OLCOTT, E. S., JOHNSON, L. M., HARGRETT, N. T., BLAKE, P. A. & COHEN, M. L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med,* 308**,** 681-5.

RIVAS, M., MILIWEBSKY, E., CHINEN, I., ROLDAN, C. D., BALBI, L., GARCIA, B., FIORILLI, G., SOSA-ESTANI, S., KINCAID, J., RANGEL, J., GRIFFIN, P. M. & CASE-CONTROL STUDY, G. 2006. Characterization and epidemiologic subtyping of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic uremic syndrome and diarrhea cases in Argentina. *Foodborne Pathog Dis,* 3**,** 88-96.

RIVAS, M., PADOLA, N. L., LUCCHESI, P. M. A. & MASANA, M. 2010. Diarrheagenic *Escherichia coli* in Argentina. *In:* TORRES, A. G. T. (ed.) *Pathogenic Escherichia coli in Latin America.* Galveston, Texas: Bentham Science Publishers.

RODRIGUE, D. C., MAST, E. E., GREENE, K. D., DAVIS, J. P., HUTCHINSON, M. A., WELLS, J. G., BARRETT, T. J. & GRIFFIN, P. M. 1995. A university outbreak of *Escherichia coli* O157:H7 infections associated with roast beef and an unusually benign clinical course. *J Infect Dis,* 172**,** 1122-5.

ROWE-MAGNUS, D. A. & MAZEL, D. 2002. The role of integrons in antibiotic resistance gene capture. *Int J Med Microbiol,* 292**,** 115-25.

ROWE, M. T. & KIRK, R. B. 2000. Effect of nutrient starvation on the resistance of *Escherichia coli* O157:H7 to subsequent heat stress. *J Food Prot,* 63**,** 1745-8.

ROWE, P. C., ORRBINE, E., LIOR, H., WELLS, G. A. & MCLAINE, P. N. 1993. A prospective study of exposure to verotoxin-producing *Escherichia coli* among Canadian children with haemolytic uraemic syndrome. The CPKDRC co-investigators. *Epidemiol Infect,* 110**,** 1-7.

RYU, J. H. & BEUCHAT, L. R. 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int J Food Microbiol,* 45**,** 185-93.
SAENZ, Y., ZARAZAGA, M., BRINAS, L., RUIZ-LARREA, F. & TORRES, C. 2003. Mutations in gyrA and parC genes in nalidixic acid-resistant *Escherichia coli* strains from food products, humans and animals. *J Antimicrob Chemother,* 51**,** 1001-5.
SAHILAH, A. M., NOR' AISHAH, H., NORAIDA, I. & AZUHAIRI, A. A. 2010. Detection of Shiga toxin 1 and 2 (stx1 and stx2) genes in *Escherichia coli* O157:H7 isolated from retail beef in Malaysia by multiplex polymerase chain reaction (PCR). *Sains Malaysiana,* 39**,** 57-63.
SANDVANG, D., AARESTRUP, F. M. & JENSEN, L. B. 1997. Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol Lett,* 157**,** 177-81.
SANDVIG, K. & VAN DEURS, B. 1994. Endocytosis and intracellular sorting of ricin and Shiga toxin. *FEBS Lett,* 346**,** 99-102.
SASAKI, Y., TSUJIYAMA, Y., KUSUKAWA, M., MURAKAMI, M., KATAYAMA, S. & YAMADA, Y. 2011. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* O157 and O26 in beef farms. *Vet Microbiol,* 150**,** 140-5.
SCALLAN, E., HOEKSTRA, R. M., ANGULO, F. J., TAUXE, R. V., WIDDOWSON, M. A., ROY, S. L., JONES, J. L. & GRIFFIN, P. M. 2011. Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis,* 17**,** 7-15.
SCHEUTZ, F., TEEL, L. D., BEUTIN, L., PIERARD, D., BUVENS, G., KARCH, H., MELLMANN, A., CAPRIOLI, A., TOZZOLI, R., MORABITO, S., STROCKBINE, N. A., MELTON-CELSA, A. R., SANCHEZ, M., PERSSON, S. & O'BRIEN, A. D. 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J Clin Microbiol,* 50**,** 2951-63.
SCHIMMER, B., NYGARD, K., ERIKSEN, H. M., LASSEN, J., LINDSTEDT, B. A., BRANDAL, L. T., KAPPERUD, G. & AAVITSLAND, P. 2008. Outbreak of haemolytic uraemic syndrome in Norway caused by stx2-positive *Escherichia coli* O103:H25 traced to cured mutton sausages. *BMC Infect Dis,* 8**,** 41.
SCHLESINGER, M. J. 1990. Heat shock proteins. *J Biol Chem,* 265**,** 12111-4.
SEKSE, C., MUNIESA, M. & WASTESON, Y. 2008. Conserved Stx2 phages from *Escherichia coli* O103:H25 isolated from patients suffering from hemolytic uremic syndrome. *Foodborne Pathog Dis,* 5**,** 801-10.
SEMANCHEK, J. J. & GOLDEN, D. A. 1998. Influence of Growth Temperature on Inactivation and Injury of *Escherichia coli* O157:H7 by Heat, Acid, and Freezing. *J Food Prot,* 61**,** 395-401.
SEOANE, A. S. & LEVY, S. B. 1995. Identification of new genes regulated by the marRAB operon in *Escherichia coli*. *J Bacteriol,* 177**,** 530-5.
SEPUTIENE, V., POVILONIS, J., RUZAUSKAS, M., PAVILONIS, A. & SUZIEDELIENE, E. 2010. Prevalence of trimethoprim resistance genes in *Escherichia coli* isolates of human and animal origin in Lithuania. *J Med Microbiol,* 59**,** 315-22.
SHAIKH, N. & TARR, P. I. 2003. *Escherichia coli* O157:H7 Shiga toxin-encoding bacteriophages: integrations, excisions, truncations, and evolutionary implications. *J Bacteriol,* 185**,** 3596-605.
SHARMA, R., STANFORD, K., LOUIE, M., MUNNS, K., JOHN, S. J., ZHANG, Y., GANNON, V., CHUI, L., READ, R., TOPP, E. & MCALLISTER, T. 2009. *Escherichia coli* O157:H7 lineages in healthy beef and dairy cattle and clinical human cases in Alberta, Canada. *J Food Prot,* 72**,** 601-7.

SHEN, C., GEORNARAS, I., BELK, K. E., SMITH, G. C. & SOFOS, J. M. 2011. Thermal inactivation of acid, cold, heat, starvation and desiccation stress-adapted *Escherichia coli* O157:H7 in moisture-enhanced non-intact Beef. *J Food Prot,* 74**,** 531-538.
SHINAGAWA, H. & ITO, T. 1973. Inactivation of DNA-binding activity of repressor in extracts of lambda-lysogen treated with mitomycin C. *Mol Gen Genet,* 126**,** 103-10.
SHRINGI, S., GARCIA, A., LAHMERS, K. K., POTTER, K. A., MUTHUPALANI, S., SWENNES, A. G., HOVDE, C. J., CALL, D. R., FOX, J. G. & BESSER, T. E. 2012a. Differential virulence of clinical and bovine-biased enterohemorrhagic *Escherichia coli* O157:H7 genotypes in piglet and Dutch belted rabbit models. *Infect Immun,* 80**,** 369-80.
SHRINGI, S., SCHMIDT, C., KATHERINE, K., BRAYTON, K. A., HANCOCK, D. D. & BESSER, T. E. 2012b. Carriage of stx2a differentiates clinical and bovine-biased strains of *Escherichia coli* O157. *PLoS One,* 7**,** e51572.
SIEGLER, R. L. 1995. The hemolytic uremic syndrome. *Pediatr Clin North Am,* 42**,** 1505-29.
SIM, E. M. 2010. Comparative genomic analysis of Shiga toxin bacteriophages from Australian human and food source *Escherichia coli* O157. Bachelor of Scinece (Honours), University of Queensland.
SINGH, R., SCHROEDER, C. M., MENG, J., WHITE, D. G., MCDERMOTT, P. F., WAGNER, D. D., YANG, H., SIMJEE, S., DEBROY, C., WALKER, R. D. & ZHAO, S. 2005. Identification of antimicrobial resistance and class 1 integrons in Shiga toxin-producing *Escherichia coli* recovered from humans and food animals. *J Antimicrob Chemother,* 56**,** 216-9.
SLUTSKER, L., RIES, A. A., GREENE, K. D., WELLS, J. G., HUTWAGNER, L. & GRIFFIN, P. M. 1997. *Escherichia coli* O157:H7 diarrhea in the United States: clinical and epidemiologic features. *Ann Intern Med,* 126**,** 505-13.
SODHA, S. V., LYNCH, M., WANNEMUEHLER, K., LEEPER, M., MALAVET, M., SCHAFFZIN, J., CHEN, T., LANGER, A., GLENSHAW, M., HOEFER, D., DUMAS, N., LIND, L., IWAMOTO, M., AYERS, T., NGUYEN, T., BIGGERSTAFF, M., OLSON, C., SHETH, A. & BRADEN, C. 2011. Multistate outbreak of *Escherichia coli* O157:H7 infections associated with a national fast-food chain, 2006: a study incorporating epidemiological and food source traceback results. *Epidemiol Infect,* 139**,** 309-16.
SORENSEN, L. K., ELBAEK, T. H. & HANSEN, H. 2003. Determination of chloramphenicol in bovine milk by liquid chromatography/tandem mass spectrometry. *J AOAC Int,* 86**,** 703-6.
SOUFI, L., ABBASSI, M. S., SAENZ, Y., VINUE, L., SOMALO, S., ZARAZAGA, M., ABBAS, A., DBAYA, R., KHANFIR, L., BEN HASSEN, A., HAMMAMI, S. & TORRES, C. 2009. Prevalence and diversity of integrons and associated resistance genes in *Escherichia coli* isolates from poultry meat in Tunisia. *Foodborne Pathog Dis,* 6**,** 1067-73.
SPEER, B. S., SHOEMAKER, N. B. & SALYERS, A. A. 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clin Microbiol Rev,* 5**,** 387-99.
STOKES, H. W. & HALL, R. M. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol,* 3**,** 1669-83.
SUKHUMUNGOON, P., NAKAGUCHI, Y., INGVIYA, N., PRADUTKANCHANA, J., IWADE, Y., SETO, K., RADU, S., NISHIBUCHI, M. & VUDDHAKUL, V. 2011.

Investigation of stx2+, eae+ *Escherichia coli* O157:H7 in beef imported from Malaysia to Thailand. *Int Food Res J,* 18**,** 381-86.
SUMBALI, G. & MEHROTRA, R. S. 2009. *Principles of Microbiology,* New Delhi, Tata McGraw Hill Education Private Limited.
SUNDE, M. & NORSTROM, M. 2006. The prevalence of, associations between and conjugal transfer of antibiotic resistance genes in *Escherichia coli* isolated from Norwegian meat and meat products. *J Antimicrob Chemother,* 58**,** 741-7.
TADESSE, D. A., ZHAO, S., TONG, E., AYERS, S., SINGH, A., BARTHOLOMEW, M. J. & MCDERMOTT, P. F. 2012. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950-2002. *Emerg Infect Dis,* 18**,** 741-9.
TAMANG, M. D., NAM, H. M., CHAE, M. H., KIM, S. R., GURUNG, M., JANG, G. C., JUNG, S. C. & LIM, S. K. 2012. Prevalence of plasmid-mediated quinolone resistance determinants among *Escherichia coli* isolated from food animals in Korea. *Foodborne Pathog Dis,* 9**,** 1057-63.
TARR, P. I., GORDON, C. A. & CHANDLER, W. L. 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet,* 365**,** 1073-86.
TODAR, K. 2005a. Nutrition and Growth of Bacteria. *Todar's Online Textbook of Bacteriology* [Online]. Available: http://textbookofbacteriology.net/nutgro_2.html [Accessed 07 Jul 2015].
TODAR, K. 2005b. Pathogenic *E. coli*. *Todar's Online Textbook of Bacteriology* [Online]. Available: http://textbookofbacteriology.net/tuberculosis.html [Accessed 7 Jul 2015].
TRAVIS, R. M., GYLES, C. L., REID-SMITH, R., POPPE, C., MCEWEN, S. A., FRIENDSHIP, R., JANECKO, N. & BOERLIN, P. 2006. Chloramphenicol and kanamycin resistance among porcine *Escherichia coli* in Ontario. *J Antimicrob Chemother,* 58**,** 173-7.
VALLY, H., HALL, G., DYDA, A., RAUPACH, J., KNOPE, K., COMBS, B. & DESMARCHELIER, P. 2012. Epidemiology of Shiga toxin producing *Escherichia coli* in Australia, 2000-2010. *BMC Public Health,* 12**,** 63.
VAN DEN BOGAARD, A. E., LONDON, N., DRIESSEN, C. & STOBBERINGH, E. E. 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *J Antimicrob Chemother,* 47**,** 763-71.
VAN DONKERSGOED, J., BERG, J., POTTER, A., HANCOCK, D., BESSER, T., RICE, D., LEJEUNE, J. & KLASHINSKY, S. 2001. Environmental sources and transmission of *Escherichia coli* O157 in feedlot cattle. *Can Vet J,* 42**,** 714-20.
VAN, T. T., CHIN, J., CHAPMAN, T., TRAN, L. T. & COLOE, P. J. 2008. Safety of raw meat and shellfish in Vietnam: an analysis of *Escherichia coli* isolations for antibiotic resistance and virulence genes. *Int J Food Microbiol,* 124**,** 217-23.
VETERINARY ANTIBIOTIC USAGE AND RESISTANCE SURVEILLANCE WORKING GROUP. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN). Available: http:// www. wageningenur.nl/upload _mm/ 8/ d/3/941957e1-c924-47c6-bf31-7efbc5def9a8_MARAN2005.pdf [Accessed 26 Aug 2015].
VILA, J., RUIZ, J., GONI, P. & DE ANTA, M. T. 1996. Detection of mutations in parC in quinolone-resistant clinical isolates of *Escherichia coli*. *Antimicrob Agents Chemother,* 40**,** 491-3.
VON BAUM, H. & MARRE, R. 2005. Antimicrobial resistance of *Escherichia coli* and therapeutic implications. *Int J Med Microbiol,* 295**,** 503-11.
VU-KHAC, H. & CORNICK, N. A. 2008. Prevalence and genetic profiles of Shiga toxin-producing *Escherichia coli* strains isolated from buffaloes, cattle, and goats in central Vietnam. *Vet Microbiol,* 126**,** 356-63.

VUDDHAKUL, V., PATARARUNGRONG, N., PUNGRASAMEE, P., JITSURONG, S., MORIGAKI, T., ASAI, N. & NISHIBUCHI, M. 2000. Isolation and characterization of *Escherichia coli* O157 from retail beef and bovine feces in Thailand. *FEMS Microbiol Lett,* 182**,** 343-7.

WANG, G. & DOYLE, M. P. 1998. Heat shock response enhances acid tolerance of *Escherichia coli* O157:H7. *Lett Appl Microbiol,* 26**,** 31-4.

WANG, H., JING, H. Q., LI, H. W., NI, D. X., ZHAO, G. F., GU, L., YANG, J. C., SHI, Z. Y., LIU, G. Z., HU, X. S. & XU, J. G. 2004. [Epidemiological study on an outbreak caused by *E. coli* O157:H7 in Jiangsu province]. *Zhonghua Liu Xing Bing Xue Za Zhi,* 25**,** 938-40.

WATERMAN, S. R. & SMALL, P. L. 1996. Characterization of the acid resistance phenotype and rpoS alleles of shiga-like toxin-producing *Escherichia coli*. *Infect Immun,* 64**,** 2808-11.

WELLS, J. G., DAVIS, B. R., WACHSMUTH, I. K., RILEY, L. W., REMIS, R. S., SOKOLOW, R. & MORRIS, G. K. 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *J Clin Microbiol,* 18**,** 512-20.

WERBER, D., 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**,** 419-22.

WHITE, P. A., MCIVER, C. J. & RAWLINSON, W. D. 2001. Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob Agents Chemother,* 45**,** 2658-61.

WHITWORTH, J., ZHANG, Y., BONO, J., PLEYDELL, E., FRENCH, N. & BESSER, T. 2010. Diverse genetic markers concordantly identify bovine origin *Escherichia coli* O157 genotypes underrepresented in human disease. *Appl Environ Microbiol,* 76**,** 361-5.

WILLSHAW, G. A., CHEASTY, T., SMITH, H. R., O'BRIEN, S. J. & ADAK, G. K. 2001. Verocytotoxin-producing *Escherichia coli* (VTEC) O157 and other VTEC from human infections in England and Wales: 1995-1998. *J Med Microbiol,* 50**,** 135-42.

WITTE, W. 1998. Medical consequences of antibiotic use in agriculture. *Science,* 279**,** 996-7.

XU, J., CHENG, B., FENG, L., JING, H., YANG, J., ZHAO, G., WANG, H. & LI, H. 2002. [Serological investigations on patients with hemolytic uremic syndromes due to enterohemorrhagic *Escherichia coli* O157:H7 infection]. *Zhonghua Liu Xing Bing Xue Za Zhi,* 23**,** 114-8.

YAM, W. C., TSANG, D. N., QUE, T. L., PEIRIS, M., SETO, W. H. & YUEN, K. Y. 1998. A unique strain of *Escherichia coli* O157:H7 that produces low verocytotoxin levels not detected by use of a commercial enzyme immunoassay kit. *Clin Infect Dis,* 27**,** 905-6.

YANG, Z., KOVAR, J., KIM, J., NIETFELDT, J., SMITH, D. R., MOXLEY, R. A., OLSON, M. E., FEY, P. D. & BENSON, A. K. 2004. Identification of common subpopulations of non-sorbitol-fermenting, beta-glucuronidase-negative *Escherichia coli* O157:H7 from bovine production environments and human clinical samples. *Appl Environ Microbiol,* 70**,** 6846-54.

YOKOYAMA, K., MAKINO, K., KUBOTA, Y., WATANABE, M., KIMURA, S., YUTSUDO, C. H., KUROKAWA, K., ISHII, K., HATTORI, M., TATSUNO, I., ABE, H., YOH, M., IIDA, T., OHNISHI, M., HAYASHI, T., YASUNAGA, T., HONDA, T., SASAKAWA, C. & SHINAGAWA, H. 2000. Complete nucleotide sequence of the prophage VT1-Sakai carrying the Shiga toxin 1 genes of the

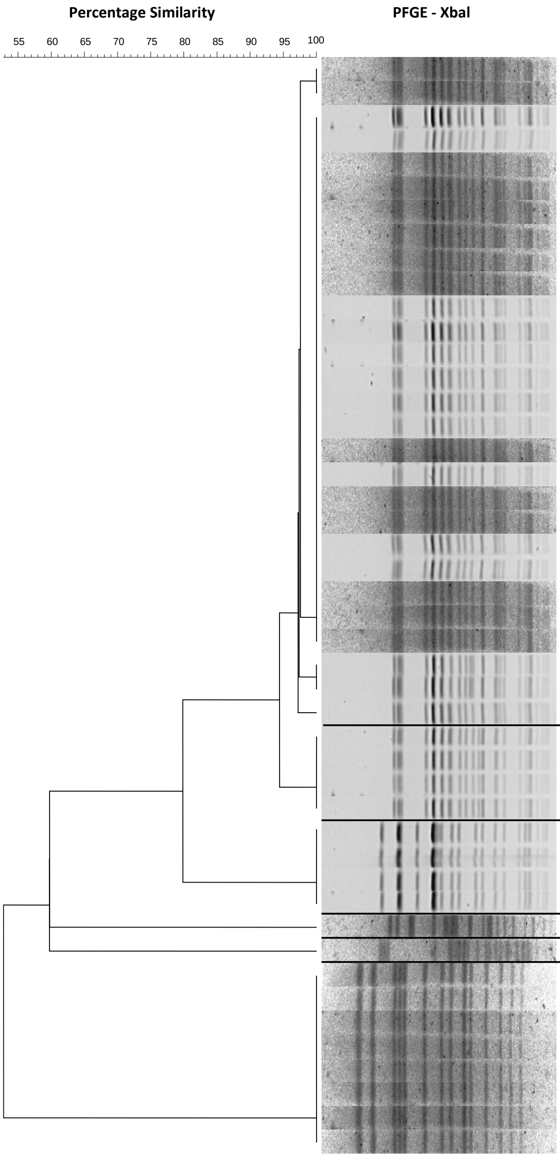
enterohemorrhagic *Escherichia coli* O157:H7 strain derived from the Sakai outbreak. *Gene,* 258**,** 127-39.

ZHANG, Y., LAING, C., STEELE, M., ZIEBELL, K., JOHNSON, R., BENSON, A. K., TABOADA, E. & GANNON, V. P. 2007. Genome evolution in major *Escherichia coli* O157:H7 lineages. *BMC Genomics,* 8**,** 121.

ZHAO, T., DOYLE, M. P., SHERE, J. & GARBER, L. 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. *Appl Environ Microbiol,* 61**,** 1290-3.

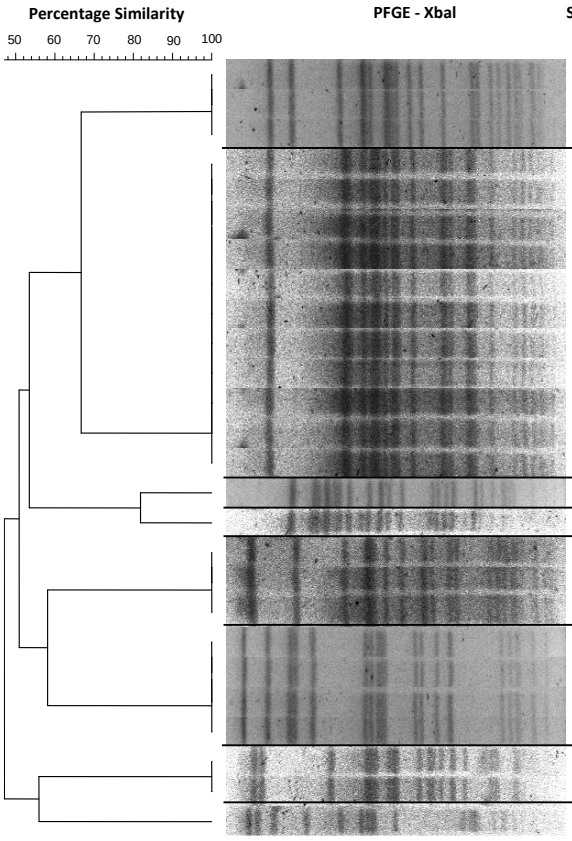
Appendices

Appendix 1. Dendogram indicating the PFGE band patterns of 46 *E. coli* O157 isolates. The designation of PFGE types was made by numbering the most frequent PFGE pattern as type 1, the next most frequent PFGE pattern as type 2 etc., that differ by a single DNA fragment. In addition, PFGE patterns were grouped in to clusters (Group I, II, III etc.) according to the percentage similarity and the numbers were assigned arbitrarily from top to bottom of the PFGE dendogram. The 46 *E. coli* O157 isolates were grouped into 6 clusters (I-VI) comprised of closely related PFGE types (more than 96% similarity).



Sample	Farm	Source	Serotype	PFGE type	PFGE Group	Virulence Factors
1	A	Cattle faeces	O157:H7	5	I	Stx_{2c}, *eaeA*, *ehxA*
2	A	Cattle faeces	O157:H7	5	I	Stx_{2c}, *eaeA*, *ehxA*
1	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
1	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
2	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
2	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
2	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
2	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
2	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
2	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
3	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
3	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
3	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
3	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
3	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
3	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
5	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
5	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
5	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
5	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
5	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
5	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
6	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
6	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
6	A	Cattle faeces	O157:H7	1	I	Stx_{2c}, *eaeA*, *ehxA*
1	A	Cattle faeces	O157:H7	6	I	Stx_{2c}, *eaeA*, *ehxA*
1	A	Cattle faeces	O157:H7	6	I	Stx_{2c}, *eaeA*, *ehxA*
3	A	Cattle faeces	O157:H7	7	I	Stx_{2c}, *eaeA*, *ehxA*
4	A	Cattle faeces	O157:H7	3	II	Stx_{2c}, *eaeA*, *ehxA*
4	A	Cattle faeces	O157:H7	3	II	Stx_{2c}, *eaeA*, *ehxA*
4	A	Cattle faeces	O157:H7	3	II	Stx_{2c}, *eaeA*, *ehxA*
4	A	Cattle faeces	O157:H7	3	II	Stx_{2c}, *eaeA*, *ehxA*
1	UPM	Bovine milk	O157:H7	4	III	Stx_{1a}, stx_{2a}, *eaeA*, *ehxA*
2	UPM	Bovine milk	O157:H7	4	III	Stx_{1a}, stx_{2a}, *eaeA*, *ehxA*
3	UPM	Beef	O157:H7	4	III	Stx_{1a}, stx_{2a}, *eaeA*, *ehxA*
4	UPM	Beef	O157:H7	4	III	Stx_{1a}, stx_{2a}, *eaeA*, *ehxA*
1	B	Sheep faeces	O157:H-	8	IV	None
2	B	Sheep faeces	O157:H-	9	V	None
3	B	Buffalo faeces	O157:H-	2	VI	*eaeA*
3	B	Buffalo faeces	O157:H-	2	VI	*eaeA*
4	B	Buffalo faeces	O157:H-	2	VI	*eaeA*
4	B	Buffalo faeces	O157:H-	2	VI	*eaeA*
5	B	Buffalo faeces	O157:H-	2	VI	*eaeA*
6	B	Cattle faeces	O157:H-	2	VI	*eaeA*
6	B	Cattle faeces	O157:H-	2	VI	*eaeA*
1	B	Sheep faeces	O157:H-	2	VI	*eaeA*

Appendix 2. Dendogram indicating the PFGE band patterns of 26 *E. coli* non-O157 isolates. The designation of PFGE types was made by numbering the most frequent PFGE pattern as type 1, the next most frequent PFGE pattern as type 2 etc., that differ by a single DNA fragment. In addition, PFGE patterns were grouped in to clusters (Group I, II, III etc.) according to the percentage similarity and the numbers were assigned arbitrarily from top to bottom of the PFGE dendogram. The 26 *E. coli* non-O157 isolates were divided into 8 groups (I-VIII) each containing closely related PFGE types (more than 90% similarity).



PFGE - Xbal	Sample	Farm	Sources	Serotype	PFGE type	PFGE Group	Virulence Factor
	1	B	Buffalo faeces	O103	3	I	None
	1	B	Buffalo faeces	O103	3	I	None
	1	B	Buffalo faeces	O103	3	I	None
	1	C	Cattle faeces	O26	1	II	None
	1	C	Cattle faeces	O26	1	II	None
	1	C	Cattle faeces	O26	1	II	None
	1	C	Cattle faeces	O26	1	II	None
	1	C	Cattle faeces	O26	1	II	None
	1	C	Cattle faeces	O26	1	II	None
	1	C	Cattle faeces	O26	1	II	None
	1	C	Cattle faeces	O26	1	II	None
	2	C	Cattle faeces	O26	1	II	None
	2	C	Cattle faeces	O26	1	II	None
	2	C	Cattle faeces	O26	1	II	None
	2	B	Buffalo faeces	O26	6	III	*EaeA, ehxA*
	2	B	Buffalo faeces	O26	7	IV	*EaeA, ehxA*
	1	A	Cattle faeces	O103	4	V	None
	2	A	Cattle faeces	O103	4	V	None
	3	A	Cattle faeces	O103	4	V	None
	1	B	Buffalo faeces	O103	2	VI	None
	1	B	Buffalo faeces	O103	2	VI	None
	1	B	Buffalo faeces	O103	2	VI	None
	1	B	Buffalo faeces	O103	2	VI	None
	3	B	Buffalo faeces	O26	5	VII	None
	3	B	Buffalo faeces	O26	5	VII	None
	3	B	Buffalo faeces	O26	8	VIII	None