Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Campylobacter species in dogs and cats and significance to public health in New Zealand

A thesis in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy in Veterinary Science
at Massey University, Palmerston North,
New Zealand,

by

Krunoslav Bojanić

2016

Massey University
^{m}EpiLab
Institute of Veterinary, Animal & Biomedical Science
Palmerston North, New Zealand

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

^{m}EpiLab ivabs

Abstract

Campylobacter spp. are a major cause of bacterial gastroenteritis in people in the developed world, including New Zealand. Many sources and transmission routes exist, as these bacteria are common in animals and the environment. *C. jejuni* is most frequently associated with poultry whereas *C. upsaliensis* and *C. helveticus* with dogs and cats, respectively. Published data on *Campylobacter* in dogs and cats in New Zealand and on the pathogenic potential of *C. upsaliensis* and *C. helveticus* are very limited. This thesis investigated the prevalence of *Campylobacter* spp. in household dogs and cats in Manawatu region, New Zealand, and in raw meat pet food commercially available in Palmerston North, New Zealand. Five *Campylobacter* spp. were isolated and the prevalence rates were significantly influenced by the culture methods used. *C. upsaliensis* and *C. helveticus* were most frequently detected from dogs and cats, respectively and *C. jejuni* in pet food samples. An expanded panel of culture methods was used to screen working farm dogs and their home-kill raw meat diet in Manawatu. This study reported three *Campylobacter* spp. and *Helicobacter winghamensis* as being isolated from dogs for the first time. The culture methods were again shown to bias the prevalence estimates. The isolates of *C. upsaliensis* and *C. helveticus* from the household pets study and *C. hyointestinalis* from locally farmed deer were used in a study to investigate the analytical sensitivity in spiked human clinical faecal samples using the ProSpecT^{TM} *Campylobacter* Microplate Assay test that was developed for detection of *C. jejuni/coli*. The results showed the ability of the test to detect all three species and showed the influence of bacterial dose, faecal consistency and of the individual faecal samples on the test results. Further studies investigated the pathogenic potential of *C. upsaliensis* and *C. helveticus* in comparison to *C. jejuni* using an insect model of disease, *Galleria mellonella*, and whole-genome analyses, respectively. The results of the survival analysis in the *G. mellonella* study indicated that *C. upsaliensis* and *C. helveticus* have pathogenic potential, but to a lesser extent than *C. jejuni.* Additionally, several variables of experimental design were shown to significantly influence estimates of hazard rates in survival analysis. Whole genome analyses also showed indications of the pathogenic potential of *C. upsaliensis* and *C. helveticus* relative to *C. jejuni,* and how it varies between and within species in association with the core and accessory genomes, functional gene content profiles, and documented and predicted pathogenic proteins. This thesis has furthered our understanding of the epidemiology, detection, and pathogenicity of *Campylobacter* spp. in dogs, cats and humans, and confirmed raw meat animal food as a potential source of *Campylobacter* spp. for both people and animals.

Acknowledgements

Foremost I would like to thank my supervisors Dr Els Acke, Dr Anne Midwinter and Dr Patrick Biggs for their great support and guidance throughout the PhD and without whom this thesis would not had been written. I am very grateful to my collaborators Dr Wendi Roe, Mrs Lynn Rogers and, especially Dr Jonathan Marshall, for their help and teaching and to the ^{m}EpiLab and AgResearch staff. A special thank you to Prof Boyd Jones for support in many ways to keep me and the projects going. I am indebted to the funders of the research projects, and to the Massey University for financial support with the Doctoral Scholarship, the Joan Berry Fellowship, and the Travel grants awarded.

I would like to specially thank my parents, mum Karmen and dad Boris, and all my family for all the love, and encouragement they have always given me. Diana and Steve, Anne and Hamish, Raewyn and John, and last but not least Ljubica and Jakša, have all stepped in as my “kiwi” and “PhD” mums and dads in this path too. Throughout this PhD I was surrounded by my many old and also new friends I made along the way that supported my endeavours and enriched my life with kindness, laughter and adventures. Huge thanks and hugs to Taž, RaŠtRkAnCi, Pat, Zoe, Anja, Kerrie, Lorelle, Paul, Kate and Fred, Lintonian and Cliftonian flatties, and office(s) roomies. Special thanks to my best friend Mirna Barbić to whom I promise a third road-trip of Aotearoa.

This work is dedicated to dogs and cats.

Publications

- **Zoonoses and Public Health** (*in press*) 'Isolation of *Campylobacter* spp. from client-owned dogs and cats, and retail raw meat pet food in the Manawatu, New Zealand' K Bojanić, AC Midwinter, JC Marshall, LE Rogers, PJ Biggs, E Acke
- **Journal of Microbiological Methods (2016 Vol. 127, p236-241)** 'Analytical sensitivity of the ProSpecT® Campylobacter Microplate Assay for detection of *C. upsaliensis*, *C. helveticus* and *C. hyointestinalis* in spiked human clinical faecal samples' K Bojanić, AC Midwinter, JC Marshall, LE Rogers, PJ Biggs, E Acke
- **CHRO conference 2015** (Rotorua, New Zealand) Research Abstract 'Pathogenicity of *Campylobacter jejuni*, *C. upsaliensis* and *C. helveticus* in the invertebrate disease model *Galleria mellonella*' K Bojanić, AC Midwinter, PJ Biggs, JC Marshall, E Acke
- **CHRO conference 2015** (Rotorua, New Zealand) Poster Abstract 'Diagnosis of campylobacteriosis in humans from the Manawatu, New Zealand' K Bojanić, A Nohra, LE Rogers, J Delores-Main, JC Marshall, PJ Biggs, AC Midwinter, E Acke
- **ECVIM 2015** (Lisbon, Portugal) Poster abstract 'Pathogenicity investigation of *Campylobacter jejuni*, *C. upsaliensis* and *C. helveticus* isolated from dogs and cats using *Galleria mellonella* larvae' K Bojanić, AC Midwinter, PJ Biggs, J Marshall, E Acke

- **ECVIM 2014** (Mainz, Germany) Poster abstract 'Whole-genome analyses of *Campylobacter upsaliensis* and *C. helveticus* isolated from dogs and cats and *in silico* investigation of pathogenic potential' K Bojanić, AC Midwinter, PJ Biggs, NP French, E Acke
- **NZVA conference 2014** (Hamilton, NZ) Invited Research lecture 'Canine diarrhoea – *Campylobacter* conundrum' K Bojanic, AC Midwinter, PJ Biggs, E Acke
- **CHRO conference 2013** (Aberdeen, UK) Research abstract 'Comparison of six culture protocols for isolation of *Campylobacter* spp. from faecal and meat samples' K Bojanic, AC Midwinter, L Rogers, PJ Biggs, E Acke
- **NZVA conference 2013** (Palmerston North, New Zealand) Research abstract '*Salmonella*, *E. coli* and *Campylobacter* spp. in Working Farm Dogs in New Zealand and their Home-Kill Diets' K Bojanic, AC Midwinter, PJ Biggs, J Benschop, N Cave, E Acke
- **WSAVA conference 2013** (Auckland, New Zealand) **Winner of WSAVA Global One Health Congress Recognition Prize** Research Abstract '*Campylobacter* spp. in dogs and cats in New Zealand' K Bojanic, AC Midwinter, E Kwan, PJ Biggs, E Acke
- **International Sheep and Beef Veterinary Congress 2013** (Rotorua, New Zealand) Research presentation '*Salmonella*, *E. coli* and *Campylobacter* spp. in Working Farm Dogs in New Zealand and their Home-Kill Diets' K Bojanic, AC Midwinter, PJ Biggs, J Benschop, N Cave, E Acke

- **Infectious Disease Research Centre Science Symposium 2012** (Palmerston North, New Zealand) Research Poster ‘ProSpecT *Campylobacter* Microplate Assay: first results but second thoughts?’ K Bojanic, AC Midwinter, J Marshall, L Rogers, PJ Biggs, E Acke
- **ECVIM 2012** (Maastricht, The Netherlands) Research Abstract ‘ProSpecT Elisa in the diagnosis of *Campylobacter* spp. infections’ K Bojanic, AC Midwinter, L Rogers, PJ Biggs, E Acke

Table of contents

1. Introduction 18

1.1. General background 18

1.2. Thesis structure and format 21

2. Literature review 24

2.1. *Campylobacter* 24

2.1.1. Historical overview 24

2.1.2. Taxonomy and microbiology 26

2.1.3. Sources of Campylobacter species 34

2.1.4. Detection and identification methods 36

2.1.5. Pathobiology 53

2.1.6. Typing of *Campylobacter* species 61

2.2. Epidemiology and public health 68

2.2.1. Epidemiology in humans 68

2.2.2. Epidemiology in dogs and cats 77

2.3. Research aims 90

3. Isolation of *Campylobacter* spp. from client-owned dogs and cats, and retail raw meat pet food in the Manawatu, New Zealand 94

3.1. Impacts 95

3.2. Summary 95

3.3. Introduction 96

3.4. Materials and methods 98

3.4.1. Study design 98

3.4.2. Bacterial culture 98

3.4.3. Isolate identification and genotyping 99

3.4.4. Statistical analysis 100

3.5. Results 101

3.5.1. Dogs and cats 101

3.5.2. Raw pet food products 104

3.5.3. Culture methods 105

3.5.4. MLST of *C. jejuni* isolates 105
3.6. Discussion 108

4. Isolation of emerging *Campylobacter* species in working farm dogs and their frozen home-kill raw meat diets 116
4.1. Highlights 117
4.2. Abstract 117
4.3. Introduction 118
4.4. Materials and methods 120
4.4.1. Study design 120
4.4.2. *Campylobacter* isolation 121
4.4.3. *Campylobacter* identification and typing 123
4.4.4. Statistical analysis 124
4.5. Results 124
4.6. Discussion 126

5. Variation in the limit-of-detection of the ProSpecT *Campylobacter* Microplate enzyme immunoassay in stools spiked with emerging *Campylobacter* species 132
5.1. Highlights 133
5.2. Abstract 133
5.3. Introduction 134
5.4. Materials and Methods 136
5.4.1. Isolates 136
5.4.2. Patient samples 136
5.4.3. EIA testing 137
5.4.4. Quantification of bacterial loads 138
5.4.5. Statistical analysis 138
5.5. Results 139
5.6. Discussion 144
5.7. Conclusions 148

6. Comparison of the pathogenic potential of emerging *Campylobacter* spp. using larvae of *Galleria mellonella* as an infection model 152
6.1. Abstract 153

6.2. Introduction 154
6.3. Results 155
6.4. Discussion 166
6.5. Concluding remarks 176
6.6. Materials and methods 177
6.6.1. Strains and cultures 177
6.6.2. *Galleria mellonella* assays 180
6.6.3. *Campylobacter* inocula 181
6.6.4. Histopathology 182
6.6.5. Statistical analysis 183
7. Whole Genome Comparison of *Campylobacter upsaliensis*, *C. helveticus* and *C. jejuni* 186
7.1. Abstract 187
7.2. Introduction 188
7.3. Materials and methods 190
7.3.1. Bacterial genomes sources 190
7.3.2. Genomic DNA preparation 190
7.3.3. Genome assembly, curation and annotation 191
7.3.4. Comparative genomics 192
7.3.5. Statistical analysis 193
7.4. Results and Discussion 193
7.5. Conclusions and limitations 228
8. General discussion and future research 232
9. References 251
10. Appendix 296

List of abbreviations

General

CC	Clonal Complex
COG	Cluster of Orthologous Groups
CoxPH	Cox proportional hazard
Ctrl	Control larvae
DALY	Disability-adjusted life year
DNA	Deoxyribonucleic acid
EIA	antigen test / ProSpecT® Campylobacter Microplate Assay
Fig.	Figure
HL	High bacterial inoculum load
ID	Identity number
KM	Kaplan-Meier
LL	Low bacterial inoculum load
LOD	Limit of detection
ML	Medium bacterial inoculum load
MLST	Multilocus sequence typing
NAAT	Nucleic acid-based test
PBS	Phosphate buffered saline
PBS-ctrl	Phosphate buffered saline-inoculated larvae control
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rMLST	Ribosomal multilocus sequence typing
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
spp.	Species
SSF	Semi-solid faeces
ST	Sequence type
VBNC	Viable but non culturable
WF	Watery faeces

COG-specific functional groups

A	RNA processing and modification
B	Chromatin structure and dynamics
C	Energy production and conversion
D	Cell cycle control, cell division, chromosome partitioning
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
G	Carbohydrate transport and metabolism
H	Coenzyme transport and metabolism
I	Lipid transport and metabolism
J	Translation, ribosomal structure and biogenesis
K	Transcription
L	Replication, recombination and repair
M	Cell wall/membrane/envelope biogenesis
N	Cell motility
O	Posttranslational modification, protein turnover, chaperones
P	Inorganic ion transport and metabolism
Q	Secondary metabolites biosynthesis, transport and catabolism
R	General function prediction only
S	Function unknown
T	Signal transduction mechanisms
U	Intracellular trafficking, secretion, and vesicular transport
V	Defense mechanisms
W	Extracellular structures
X	Mobilome: prophages, transposons
Y	Nuclear structure
Z	Cytoskeleton

List of Tables

Table 2.1. Identified sources of *Campylobacter* spp.[a] and association with reported diseases in animals and humans. 27
Table 2.2. Phenotypic characteristics[a] of selected[b] *Campylobacter* species. 33
Table 2.3. Composition of some commonly[a] used culture broths for isolation of *Campylobacter* species. 41
Table 2.4. Composition of some commonly[a] used culture media for isolation of *Campylobacter* species. 43
Table 2.5. Diagnostic performance characteristics[a] of some commonly used faecal antigen tests for *Campylobacter* spp. in humans. 47
Table 2.6. Summary of results obtained with each of 25 *Campylobacter* strains representing 15 taxa examined with assays established in individual laboratories for various *Campylobacter* spp. The tests used (and their taxonomic range and original description, where relevant) in each laboratory is given. Sensitivity and specificity values for each test were calculated with respect to each assays taxonomic range. 50
Table 2.7. Reported prevalence rates[a] of *Campylobacter* spp. in dogs and cats. 79
Table 3.1. Prevalence of isolated and PCR confirmed *Campylobacter* spp. from client-owned pets and retail raw pet food diets in the Manawatu, New Zealand. 103
Table 4.1. Number of positive working farm dogs[1] from Manawatu, New Zealand (N = 50) using seven culture methods and isolates identified by PCR. 125
Table 5.1. Limit-of-detection of ProspecT™ *Campylobacter* Microplate Assay in human stools spiked with three *Campylobacter* species. 140
Table 6.1. Distribution of histopathology scores* in larvae infected with *Campylobacter* spp. and the uninfected control larvae groups. 164
Table 6.2. Details of *Campylobacter* species isolates used in the study. 179
Table 7.1. Number of unique gene clusters (and total genes) in Clusters of Orthologous Groups by genome compartments of *Campylobacter* species. ... 204
Table 7.2. Average number of genes[1] in three *Campylobacter* spp.[2] (42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus*) by COG functional groups and genome compartments. 208
Table 7.3. Presence of toxin-antitoxin modules in *Campylobacter* spp. genomes.. 215
Table 7.4. Average number of predicted pathogenic proteins[a] in three *Campylobacter* spp.[2] (42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus*) by COG functional groups and genome compartments. 221
Supplemental Table 3.1. PCR assays used on isolates from dogs, cats and retail raw meat pet food products. 297
Supplemental Table 7.1. List of strains and related metadata of *Campylobacter* spp. genomes used in the study. 299
Supplemental Table 7.2. Number of strains (and number of genes) in *Campylobacter* spp. for genes documented to be involved or associated with pathogenicity of *C. jejuni*. 305
Supplemental Table 7.3. Number of predicted pathogenic proteins (and % proportion from total predictions) in genome compartments of *Campylobacter* species. 324

List of Figures

Fig. 2.1. Scanning electron micrograph of *Campylobacter jejuni*. 26
Fig. 2.2. *Campylobacter* colonies on mCCDA medium. 31
Fig. 2.3. Pathogenic mechanisms of *Campylobacter* infection. 55
Fig. 2.4. Neighbour-joining phylogenetic tree of the genus *Campylobacter* based on partial *rpoB* gene sequences. *E. coli* was used as an outgroup. Bootstrap values of 500 simulations are indicated at major branches. Bar, 2% divergence..... 66
Fig. 2.5. Framework showing sources of information and modelling approaches for the transmission of zoonotic diseases, including campylobacteriosis. Note the terms reservoir, pathway, exposure and risk factor are used here for illustrative purposes, to show how various levels of data disaggregation and refinement can be incorporated into different models for informing decision making. 73
Fig. 2.6. The incidence and prevalence of campylobacteriosis caused by *C. jejuni*/*C. coli* world wide. 75
Fig. 3.1. Occurrence of *C. jejuni* MLST types observed in pets and raw meat pet food diets from Manawatu, New Zealand, across five sources from the ^{m}EpiLab database (N = 1,176). The horizontal axis shows sequence types (ST) isolated in this study from food only products (black bars), from pets only (light grey) and from both food and pets (dark grey) and their occurrence within the five sources (Other primarily denotes wild birds and to a lesser extent other wild animals and companion animals) on the vertical axiis..... 107
Fig. 5.1. Model predicted probabilities of obtaining a positive result using ProSpecT^{TM} *Campylobacter* Microplate Assay in human clinical stools spiked with *Campylobacter* species. The line represents point estimates of predicted probabilities, the dark shaded areas 95% CI based on variation of the fixed effects (species, faecal consistency and bacterial concentrations) and the light shaded areas 95% CI with the addition of the random effect (faecal sample ID). Points at 0 and 100% represent negative and positive results of the raw data respectively. 144
Fig. 6.1. Kaplan-Meier survival curves of larvae (n = 4,273) inoculated with three 100-fold dilutions of *Campylobacter* species. 157
Fig. 6.2. Kaplan-Meier survival curves of larvae inoculated with *Campylobacter* spp. in different environmental conditions according to *in vitro* growth requirements. 158
Fig. 6.3. Kaplan-Meier survival curves of larvae inoculated with temperature-inactivated whole-cells and cellular components, and secreted products of *Campylobacter* species. 163
Fig. 7.1. Genome features of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus*. 195
Fig. 7.2. Venn diagram of number of Clusters of Orthologous Groups (COG) shared between 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* genomes. 198
Fig. 7.3. Number of gene copies in Clusters of Orthologous Groups (COG) functional groups between 42 *C. jejuni*, 33 *C. upsaliensis* and 9 *C. helveticus* genomes. 201
Fig. 7.4. Heat map of the number of gene copies in Clusters of Orthologous Groups (COG) functional groups between the strains of three *Campylobacter* species. Number of gene copies has been square root transformed. 202

Fig. 7.5. Pan-genome and core genome rarefaction curves of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* genomes. 205
Fig. 7. 6. Distribution of the number of genes per strain in the pan-genome of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups. 209
Fig. 7.7. Distribution of the number of core genes per strain in 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups. 210
Fig. 7.8. Distribution of the number of accessory genes per strain in 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups. 211
Fig. 7.9. Distribution of the number of MP3 predicted pathogenic proteins per strain in the pan-genome of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups. 222
Fig. 7.10. Distribution of the number of MP3 predicted pathogenic proteins per strain in the core genome of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups. 223
Fig. 7.11. Distribution of the number of MP3 predicted pathogenic proteins per strain in the accessory genome of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups. 224
Fig. 7.12. Histogram of gene cluster distribution size by OrthoMCL core genome analysis of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* genomes. ... 225
Fig. 7.13. NeighborNet visualized in SplitsTree for the 52 rMLST genes in 84 genomes of *Campylobacter* species. Alignment of 20,779 amino acids in length with 6,525 (31.4%) variable sites excluding the *rpmD* gene that is lacking in the Campylobacterales order; Cj, Cu, and Ch denote *C. jejuni*, *C. upsaliensis* and *C. helveticus* respectively. 227
Supplemental Fig. 4.1. Occurrence of *Campylobacter jejuni* MLST types isolated in working farm dogs from Manawatu, New Zealand, across all sources from the ^{m}EpiLab database (N = 950). 298
Supplemental Fig. 7.1. Cumulative contig length plots for 42 *C. jejuni* genome assemblies. 325
Supplemental Fig. 7.2. Cumulative contig length plots for 33 *C. upsaliensis* genome assemblies. 326
Supplemental Fig. 7.3. Cumulative contig length plots for nine *C. helveticus* genome assemblies. 327
Supplemental Fig. 7.4. Distribution of the number of unique Clusters of Orthologous Groups (COG) by functional groups per strain in 42 *C. jejuni* (257 unique COGs), 33 *C. upsaliensis* (169 unique COGs) and nine *C. helveticus* (49 unique COGs) isolates**.** *Campylobacter* spp. respresents the number per strain of 1,111 COGs shared between the three species. 328
Supplemental Fig. 7.5. Distribution of the number of singleton genes per strain in 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups**.** 329
Supplemental Fig. 7.6. Agreement between MP3 and PathogenFinder predictions of pathogenic proteins of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* genomes. 330

Supplemental Fig. 7.7. Distribution of the number of MP3 predicted pathogenic proteins per strain in singletons of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups. 331

Supplemental Fig. 7.8. NeighborNet visualised in SplitsTree for the amino acid sequences of 182 core genes in 84 genomes of *Campylobacter* species. Only genes of same length were used and had an alignment of 45,949 amino acids in length with 9,840 (21.42%) variable sites. Cj, Cu, and Ch denote *C. jejuni*, *C. upsaliensis* and *C. helveticus*, respectively. 332

Supplemental Fig. 7.9. NeighborNet visualised in SplitsTree for the amino acid sequences of 649 core genes in 84 genomes of *Campylobacter* species. The genes included are those that have a length range of 20% in the cluster and had an alignment of 197,053 amino acids in length with 59,486 (30.19%) variable sites. Cj, Cu, and Ch denote *C. jejuni*, *C. upsaliensis* and *C. helveticus*, respectively. 333