Final report: FDI / 236 /2005
Enhancing Surveillance of Potentially Foodborne Enteric Diseases in New Zealand:
Human Campylobacteriosis in the Manawatu: Project extension incorporating additional poultry sources

prepared by Professor Nigel French
and the Molecular Epidemiology and Veterinary Public Health Group

19th October 2009

Hopkirk Institute
Institute of Veterinary, Animal and Biomedical Sciences
College of Sciences
Massey University
New Zealand
Email - N.P.French@massey.ac.nz
Phone - +64 (06) 356 9099 extn 81188
Fax - +64 (06) 350 5714

Contents

1 Introduction **4**

2 Methods **5**

2.1 Data collection 5

2.1.1 Human faecal samples 5

2.1.2 Duck, turkey and end-of-lay meat breeder (also known as "spent hen") sample size and selection 5

2.1.3 Other food and environmental samples 5

2.1.4 Laboratory methods 6

2.1.4.1 Sample preparation and culture techniques 6

2.1.4.2 Species confirmation by PCR 6

2.1.4.3 Multilocus sequence typing 7

2.2 Data analysis 8

2.2.1 Enumeration of *Campylobacter* on poultry carcases 8

2.2.2 Minimum spanning trees 8

2.2.3 Source attribution modelling 8

2.2.3.1 Proportional Similarity Index 8

2.2.3.2 Dutch model 9

2.2.3.3 Modified Hald model 9

2.2.3.4 Island model 10

3 Results **11**

3.1 Sample details 11

3.2 Prevalence and level of contamination of ducks, turkeys and end-of-lay meat breeders 11

3.2.1 Crude prevalence 11

3.2.2 Modelling of duck, turkey and end-of-lay meat breeder carcases: prevalence and counts for positive carcases 12

3.3 Prevalence and level of contamination of chicken carcases 16

3.3.1 Modelling of chicken carcases: prevalence and counts for positive carcases 16

3.4 Comparison between MLST genotypes found in human cases and poultry . 19

3.4.1 MLST genotypes isolated from ducks, turkeys and end-of-lay meat breeders 19

3.4.2 Comparing MLST genotypes in humans with all poultry sources in the Manawatu 21

3.4.3 Distribution of human MLST genotypes in the Manawatu 23

3.5 Source association using proportional similarity and attribution modelling . 23

3.5.1 Proportional similarity index 23
3.5.2 Dutch, Island and Modified Hald model 25

4 Discussion and concluding comments **29**

5 Acknowledgements **29**

Bibliography **29**

List of Figures **32**

List of Tables **33**

1 Introduction

Recent NZFSA-funded studies showed a strong correlation between *Campylobacter jejuni* populations isolated from fresh broiler chickens and those isolated from human clinical cases in New Zealand [13, 11, 12]. This work was conducted between March 2005 and Feb 2008 in the Manawatu, Christchurch and Auckland, and supported previous studies indicating that broiler chickens were the primary food source responsible for human campylobacteriosis in this country. This led to the national control policy targeting broiler poultry in New Zealand [1]. To date the focus of all New Zealand studies has been on fresh broiler poultry on sale in supermarkets, and few studies have explored alternative poultry as sources of human infection. Approximately 95% of poultry consumption in New Zealand is chicken meat, with turkey, duck, and roasting fowl making up the remaining 5%. Although consumption of these alternative poultry sources is relatively low, evidence from other countries suggests that fresh duck and turkey carcases may be highly likely to be contaminated with *Campylobacter* [3, 2, 14, 18] and may therefore represent a source of human infection disproportionate to their relative market share.

In order to develop a more comprehensive picture of the contribution of *Campylobacter* from fresh retail poultry to human disease in New Zealand, and explore transmission cycles within and between these sources, studies of carriage of *Campylobacter* in end-of-lay meat breeders (also known as "spent hens"), ducks and turkeys were conducted. This study examined not only the probability of carcases being contaminated with *Campylobacter*, but also the level of contamination and genotypes of *C. jejuni* present. Using recently developed modelling approaches [11, 12, 20], data from these sources was then included in an analysis of the contribution of multiple food and environmental sources to estimate how many cases may be attributable to poultry sources, including non-broiler meat.

This report describes an extension of NZFSA-funded studies carried out between 2005 and 2008, and should be read in conjunction with the 2008 report "Enhancing surveillance of potentially foodborne enteric diseases in New Zealand: Human campylobacteriosis in the Manawatu" [13]. The earlier report describes the methodology in detail, including the sampling strategies, laboratory methods and analytical tools.

2 Methods

C. jejuni were isolated from human clinical cases, and food and environmental sources over a four-year period between March 2005 and July 2009 in the Manawatu. The study described in this report sampled additional poultry sources between December 2008 and May 2009. Both the probability of carcase contamination and levels of contamination with *Campylobacter* spp. were estimated for these sources and *C. jejuni* isolates were genotyped using multilocus sequence typing (MLST). Genotype distributions were compared using four methods, of which three were model-based tools designed to estimate the number of human cases attributable to each source, with an estimate of uncertainty.

2.1 Data collection

2.1.1 Human faecal samples

Human specimens submitted to MedLab Central, Palmerston North that were positive for *Campylobacter* by ELISA (ProSpecT®, Remel, USA) were sent to the Hopkirk Molecular Epidemiology laboratory over the 4 year period 1st March 2005 to 31st July 2009. Faecal swabs were made using Amies Charcoal transport swabs (Copan, Italy).

2.1.2 Duck, turkey and end-of-lay meat breeder (also known as "spent hen") sample size and selection

We aimed to source 12 samples from each source (duck, turkey and end-of-lay meat breeder) per month between December 2008 and May 2009 (depending on availability) providing a maximum sample size of 72 for each source. With this sample size a prevalence of 50% would be detected with confidence intervals of 39-61%. We anticipated a prevalence of between 30% and 70% for ducks and turkeys [2, 14, 18] and similar prevalence estimates for end-of-lay breeders . We estimated this would provide approximately 144 isolates for multilocus sequence typing.

All end-of-lay meat breeders were sourced directly from supplier Z and were delivered directly from the processing plant. Turkeys from two suppliers were either purchased when available from supermarkets in Palmerston North (supplier Z) or sourced directly from the supplier (supplier X). Ducks were either sourced directly from the supplier (supplier X) or purchased from supermarkets in Palmerston North and Fielding.

2.1.3 Other food and environmental samples

Full details of the sampling protocols for all other food and environmental sources, including broiler chicken meat, are described elsewhere [13, 12].

2.1.4 Laboratory methods

Samples were cultured for *Campylobacter* spp. in a microaerophillic chamber using standard methods. Isolates were then confirmed as *C. jejuni* by PCR, and genotyped using multilocus sequence typing.

2.1.4.1 Sample preparation and culture techniques Human faecal swabs were cultured on modified Cefoperazone Charcoal Deoxycholate agar (mCCDA) plates (Fort Richard, Auckland) and in Bolton Broth (Lab M, Bury, England) and incubated at 42°C in a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2) for 2 days. A single colony resembling *Campylobacter* species was subcultured to Blood Agar (BA) (Fort Richard, Auckland) and incubated microaerobically at 42°C for 2 days before DNA preparations were made. Cultures were frozen at -80°C in Glycerol Broth (Difco, USA).

End-of-lay meat breeders, chickens and ducks were washed and massaged in 200 ml of Buffered Peptone Water (BPW) (Difco, USA) in stomacher bags (Seward, England) or autoclave bags. The turkeys were washed in 400 ml of BPW in autoclave bags. The wash was centrifuged (10,000 rpm, 6°C, 35 mins, Sorvall RC5B for chickens and ducks, 8,500 rpm, 6°C, 45 mins for turkeys) and the resultant pellet resuspended in 5 ml of BPW. Approximately 3 ml of the resuspended pellet was added to 90 ml of Boltons broth which was incubated at 42 °C microaerobically for 2 days. After incubation the broth was subcultured onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Fort Richard, Auckland) and incubated microaerobically at 42 °C for 2 days. Single colonies resembling *Campylobacter* species was subcultured to BA and incubated microaerobically at 42 °C for 2 days before DNA preparations were made. Cultures were frozen at -80 °C.

Presumptive *Campylobacter* spp. in the wash and resuspended pellet were plated onto mCCDA using a Wasp Spiral Plater (Don Whitley Scientific, UK) for counting. Duplicate mCCDA plates were inoculated with 50μl (spiral plater) or 1ml (spread plate) aliquots of wash or 100μl (spiral plater) aliquots of resuspended wash pellet. The plates were incubated microaerobically at 42°C for 2 days. Colonies were counted manually or by using a plate reader (aCOLyte, Synbiosis, England).

2.1.4.2 Species confirmation by PCR DNA was extracted from freshly grown cultures by boiling for 10 min in the presence of 2% Chelex (Biorad), followed by centrifugation to remove both cell debris and the Chelex, which inhibits PCR. The supernatant, containing the nucleic acids, was transferred to a fresh tube and used for amplification PCR and MLST. The isolates of *Campylobacter* were speciated by multiplex PCR to detect genes associated with either *C. jejuni* or *C. coli*. The mapA gene was shown to be found only in *C. jejuni* [17], so primers MapA-F (5'-CTTGGCTTGAAATTTGCTTG-3') and MapA-R (5'-GCTTGGTGCGGATTGTAAA-3') were designed to target this gene for its identification. Detection of *C. coli* was performed using primers (COL3 and MDCOL2)[6]. These

two sets of primers were combined into one PCR reaction (multiplex PCR) for the simultaneous identification of the two species of *Campylobacter*. Amplification was performed in a 20 μl reaction containing 1 unit of Platinum Taq Polymerase (Invitrogen), 100 μM of each dNTP, 200 nM of each primer (MapA-F, MapA-R, COL3, and MDCOL2), and 1.5 mM MgCl2. The reactions were carried out in an Applied Biosystems 9700 Thermocycler by heating the sample to 96°C for 2 mins, followed by 38 cycles of 96°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, with a final extension of 72°C for 2 mins. The PCR products were visualised by subjecting a 5μl aliquot to electrophoresis in a 1% agarose gel in TBE buffer, which was then stained with ethidium bromide and exposed to UV light. The presence of a 603bp product indicated *C. jejuni* while a 462bp one indicated *C. coli.*

2.1.4.3 Multilocus sequence typing After speciation, MLST of *C. jejuni* isolates was performed using seven house-keeping genes: *asp*A (aspartase A), *gln*A (glutamine synthase), *glt*A (citrate synthase), *gly*A (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase) and *unc*A (ATP synthase alpha subunit) based on the method as outlined by Dingle et al., 2001[7]. Each amplification reaction comprised 2μl of the DNA preparation, 5 pmoles of both forward and reverse amplification primers, 12.5μl of ABI 2x AmpliTaq Gold PCR Mastermix and water to make up to a total volume of 25μl. Amplification was performed on a Corbett Palm Cycler under the following conditions: Initial denaturation was for 15 mins at 94°C followed by 35 cycles of 94°C denaturation for 30 sec, 50 °C annealing for 30 sec and 72°C extension for 90 sec. Final extension was for 72°C for 7 mins. PCR products were precipitated with 25μl 20% PEGS/2.5 M NaCl solution, washed with 80% EtOH, dried and taken up in 12μl H_2O and screened on agarose gels. Sequencing reactions were performed using 2μl of PCR product, 3.2pmoles primer, 2μl ABI BigDye, 2μl of x5 BigDye buffer and water to a total volume of 10μl. Reaction were performed under the following conditions. Initial denaturation at 96°C for 3 min. then 25 cycles of 96°C for 15 sec, 50°C for 15 sec and 60°C for 4min. Sequenced products were precipitated with 0.1M Na acetate/78% EtOH solution, washed with 70%EtOH, dried and taken up in 12μl H_2O and the sequence read at ESR, Kenepuru, on an ABI 3130XL automated DNA sequencer using ABI BigDye v3.1 (Applied Biosystems) following the manufacturer's instructions. Sequence data were collated by Dr Phil Carter at ESR, and alleles assigned using the Campylobacter PubMLST database (http://pubmlst.org/campylobacter/). Novel alleles and sequence types were submitted for allele and ST designation as appropriate and alleles that did not give clear results were re-amplified and sequenced using primers sets published by Miller et al., (2005)[10] using the same protocol as above.

2.2 Data analysis

2.2.1 Enumeration of *Campylobacter* on poultry carcases

The aim was to estimate both the proportion of carcases positive and the levels of *Campylobacter* present on positive carcases. The method of analysis employs a novel application of recently developed statistical tools for the analysis of count data where there are a large proportion of zeros, and several replicates at the sample level. Full details of the model are provided elsewhere [13]. The output from these models is presented as a series of graphs describing the probability of a carcase containing *Campylobacter*, by supplier and by quarter, and the estimated number of viable *Campylobacter* on positive carcases - again by supplier and quarter. This method ensures that all the individual replicate counts for each sample are analysed appropriately.

2.2.2 Minimum spanning trees

Minimum spanning trees (MSTs) are a graphical tool available in the BioNumerics software (Applied Maths; http://applied-maths.com/bionumerics/bionumerics.htm). The technique uses a clustering algorithm, designed for use with MLST data. In the analysis presented here, MSTs are a convenient way of representing the distribution and diversity of sequence types (STs) amongst the difference sources, whereby each ST is represented as a pie chart. The size of the circle indicates the number of isolates, and each coloured segment represents the proportion from each source.

2.2.3 Source attribution modelling

Four methods were compared to assess the relative contribution of each source to the burden of human infections in the Manawatu. The first (Proportional Similarity Index) simply assesses the area of overlap of the genotype distributions from each source with that of the human genotype distribution. The other three techniques estimate the number of human cases attributable to each source using models (Dutch, Hald and Island models) that are based on different underlying assumptions.

2.2.3.1 Proportional Similarity Index The proportional similarity index (PS) is an objective and simple estimate of the area of intersection between two frequency distributions [15]. In this context, the PS estimates the similarity between the frequency distributions of STs of each source and the distribution of STs amongst human cases. The values for PS range from 1, for the highest possible similarity, to 0 for distributions with no common types.

$$\mathrm{PS} = 1 - 0.5 \sum_i |p_i - q_i|$$

where $|p_i - q_i|$ is the modulus of the difference in the relative frequency of MLST genotype i in source p compared to source q.

Bootstrap confidence intervals for this measure were estimated based on the approach of Garrett et al. [8].

2.2.3.2 Dutch model The Dutch method compares the number of reported human cases caused by a particular bacterial subtype with the relative occurrence of that subtype in each source. The number of reported cases per subtype and source is estimated by:

$$\lambda_{ij} = \frac{p_{ij}}{\sum_j p_{ij}} x_i,$$

where p_{ij} = relative occurrence of bacterial subtype i in source j,
x_i = estimated number of human cases of type i per year,
λ_{ij} = expected no. of cases / year of type i from source j.

A summation across subtypes gives the total number of cases from source j, denoted by λ_j:

$$\lambda_j = \sum_i \lambda_{ij}.$$

The method of Garret et al. [8] was extended to provide bootstrap confidence intervals for the Dutch model.

2.2.3.3 Modified Hald model We modified the Bayesian risk assessment model originally developed to quantify the contribution of different food sources to the number of human cases of salmonellosis in Denmark [9, 11]. The original model compares the number of human cases caused by different 'types' with their prevalence in different food sources, weighted by the amount of food source consumed. This model is a further development of the frequentist model described in section 2.2.3.2 and requires a heterogeneous distribution of some types among animal and food sources. Like the Dutch model, this approach compares the number of human cases caused by different bacterial subtypes with their prevalence in different food sources. However, by using a Bayesian approach, the Hald model can explicitly include and quantify the uncertainty surrounding each of the parameters. In our study the Hald model was adapted to overcome some of the problems associated overparameterisation and to incorporate uncertainty in the prevalence matrix. Further, the food consumption terms was removed to enable the inclusion of environmental sources of campylobacteriosis.

The core of the modified Hald model is the model equation $\lambda_{ij} = p_{ij} q_i a_j$ where

- p_{ij} is the prevalence of type i in source j,

- q_i is the bacteria ST dependent factor for sequence type i,
- a_j is the food source dependent factor for food source j.

Full details of the model are provided in [11].

2.2.3.4 Island model This method [19] is based on coalescent models, which are different from classical phylogenetic methods in their explicit considerations of the genealogical history of sampled alleles [16]. This approach is fundamentally different from the Dutch and Hald models in that it explicitly models the genealogy of all isolates, using their allelic profiles and taking into account the relatedness of STs.

Island models were first proposed by Wright, 1931 [21] and are models of gene flow derived from population genetics. The technique devised by Wilson et al 2008 [19] reconstructs the genealogical history of the isolates, based on their allelic profiles, and estimates mutation and recombination rates, as well as the 'migration' rates from each source into the human 'Island'. It is these migration rates that are used to estimate the relative contribution from each source. Importantly this technique has one major advantage over the other methods; it can assign human cases that have no identified reservoir in the animal or environmental reservoirs.

3 Results

3.1 Sample details

Table 1 shows the number of samples processed between December 2008 and June 2009. The supply of spent hens was sporadic: 12 were available in March 2009 and then three batches of 12 were available in May 2009 at the end of weeks 1, 2 and 3. Turkeys were sourced from two suppliers (X and Z), although turkeys from supplier Z were only available in December.

Table 1: Number of samples from each source and supplier by month of sampling.

		Month of sampling							
Species	Supplier	Dec08	Jan09	Feb09	Mar09	Apr09	May09	Jun09	Total
Duck	X			6	6	6	6		24
Duck	Y	15	12	6	3	6	6	3	51
EOL breeder	Z				12		36		48
Turkey	X	3		12	12	12	12		51
Turkey	Z	12							12
Total	All	30	12	24	33	24	60	3	186

Of the turkey carcases, 83.6 and 75.0% from suppliers X and Z respectively were wrapped with the wrapping still intact. In contrast only 37.5 and 45.1% of the wrappings on duck carcases from suppliers X and Y respectively were intact. None of the end-of-lay meat breeder carcases were wrapped.

3.2 Prevalence and level of contamination of ducks, turkeys and end-of-lay meat breeders

In this section we provide crude summary estimates of prevalence and then use Bayesian model based techniques to estimate probabilities of contamination and levels of contamination conditional on carcases being positive, with 95% credible intervals.

3.2.1 Crude prevalence

With the exception of turkeys from Supplier Z , over 90% of carcases from all sources were positive for presumptive *Campylobacter*. Table 2 provides a breakdown of the number of samples and percentage positive for all sources.

Table 2: Percentage of carcases positive by supplier

		Percent positive presumptive *Campylobacter* spp.			
Supplier	Species	Negative	Positive	Number sampled	% positive
Supplier X	Duck		24	24	100.0
Supplier Y	Duck	2	49	51	96.1
Supplier Z	EOL breeder		48	48	100.0
Supplier X	Turkey	5	46	51	90.2
Supplier Z	Turkey	6	6	12	50.0
Total		13	173	186	93.0

3.2.2 Modelling of duck, turkey and end-of-lay meat breeder carcases: prevalence and counts for positive carcases

Simple histograms of the crude count data are shown in Figure 1. Figure 2 shows the estimated probability of contamination with associated credible intervals for each source and quarter. All sources in all quarters were estimated to have a high (>90%) proability of contamination. The estimated levels of contamination are provided in Figure 3 and Table 3. The median number of *Campylobacter* spp. isolated from ducks and turkeys ranged from 2.6 to 3.4 logs whereas the median number isolated from end-of-lay meat breeders was slightly lower at 2.1 logs.

Table 3: Presumptive counts of *Campylobacter* spp. on positive carcases by supplier and quarter. Medians with 2.5 and 97.5th centiles of the log counts are provided

		Centiles of count distribution (+ve carcases) in logs					
		Quarter 1 2009			Quarter 2 2009		
Species	Supplier	2.5th	Median	97.5th	2.5th	Median	97.5th
Duck	X	1.82	2.80	4.08	2.27	3.49	4.74
Duck	Y	1.95	3.43	4.96	1.98	3.14	4.72
EOL breeder	Z	0.91	2.13	3.08	1.56	2.61	3.77
Turkey	X	1.55	2.62	4.59	1.02	2.22	3.29

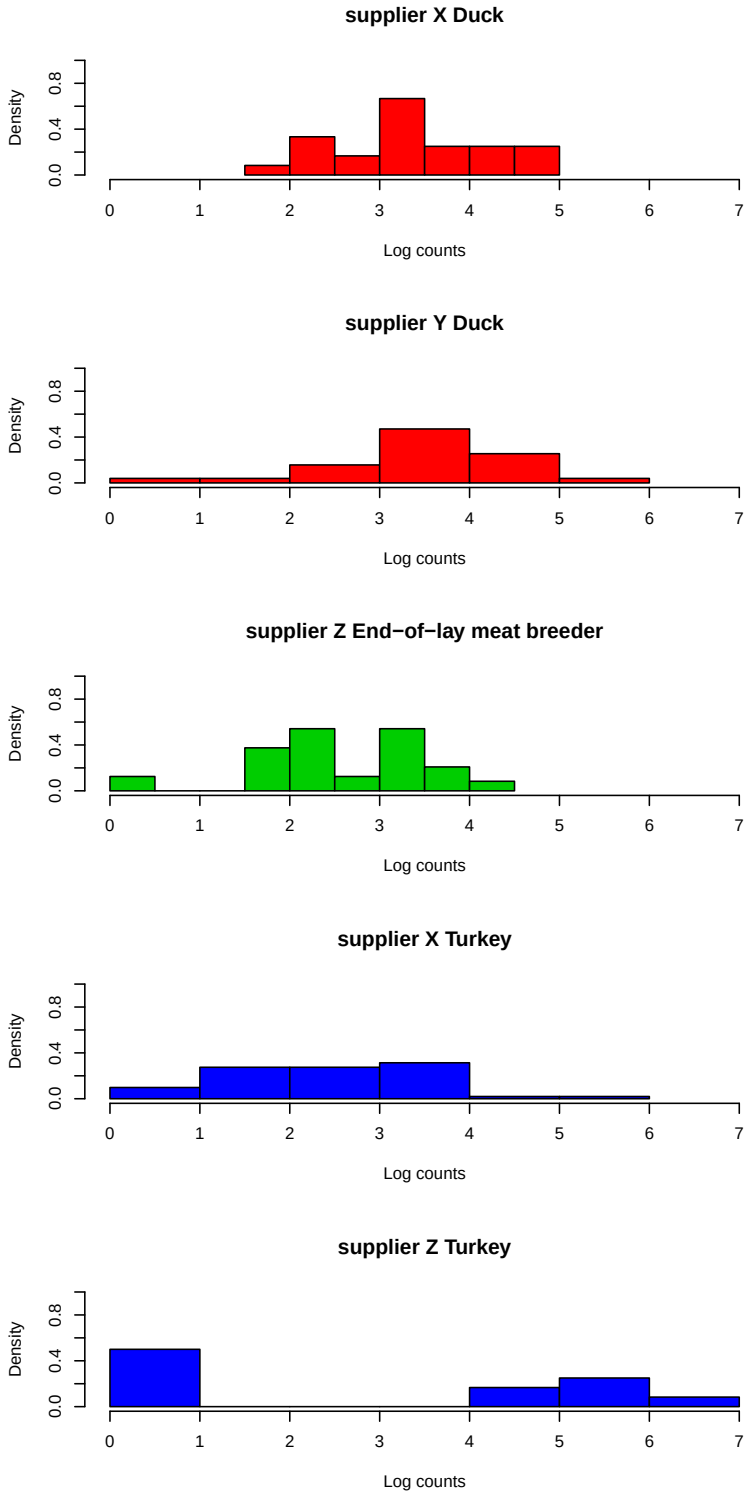

Figure 1: *Campylobacter* on poultry carcases by quarter: histogram of contamination for each supplier

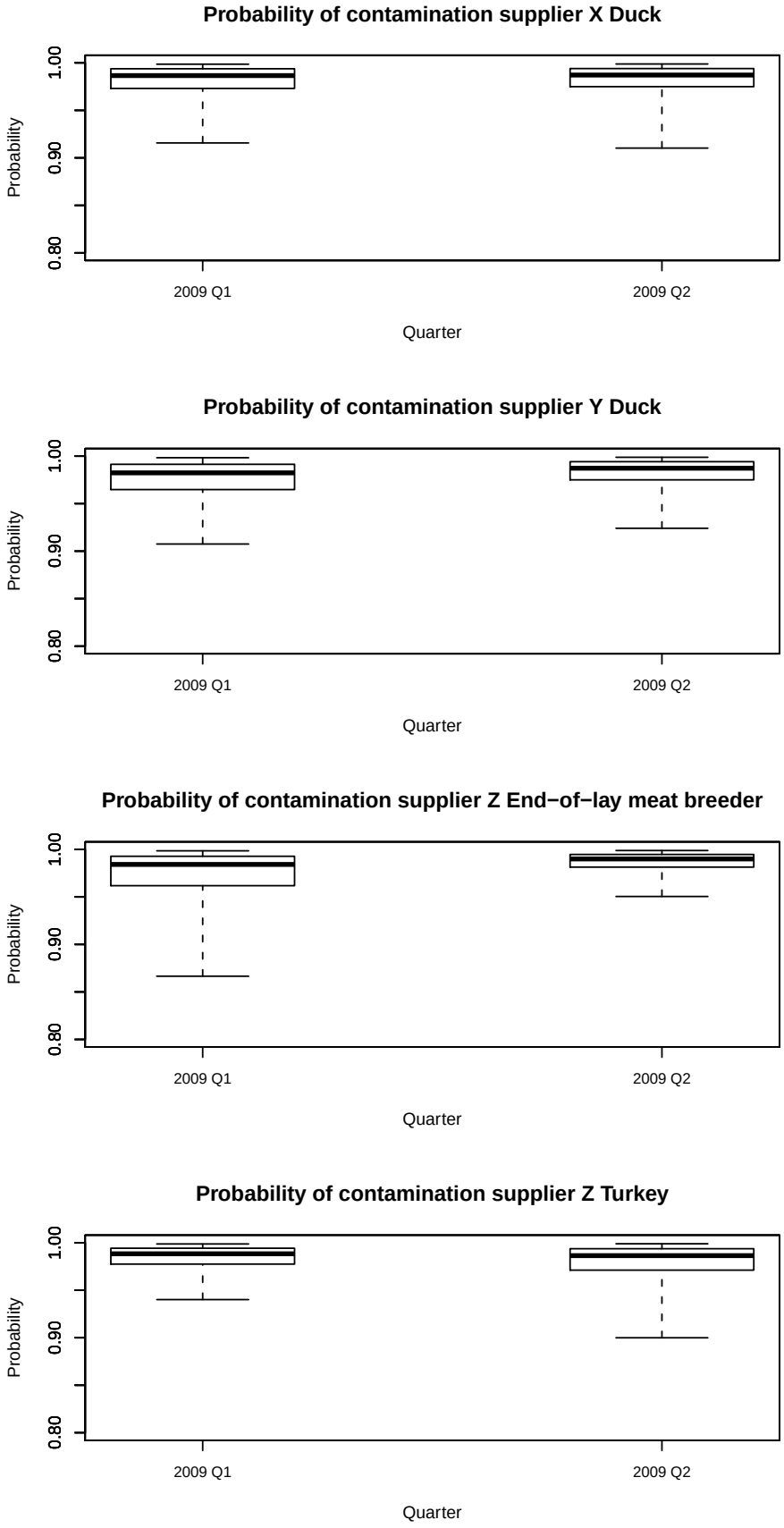

Figure 2: *Campylobacter* on duck, end-of-lay meat breeder and turkey carcases by quarter: probability of contamination for each supplier

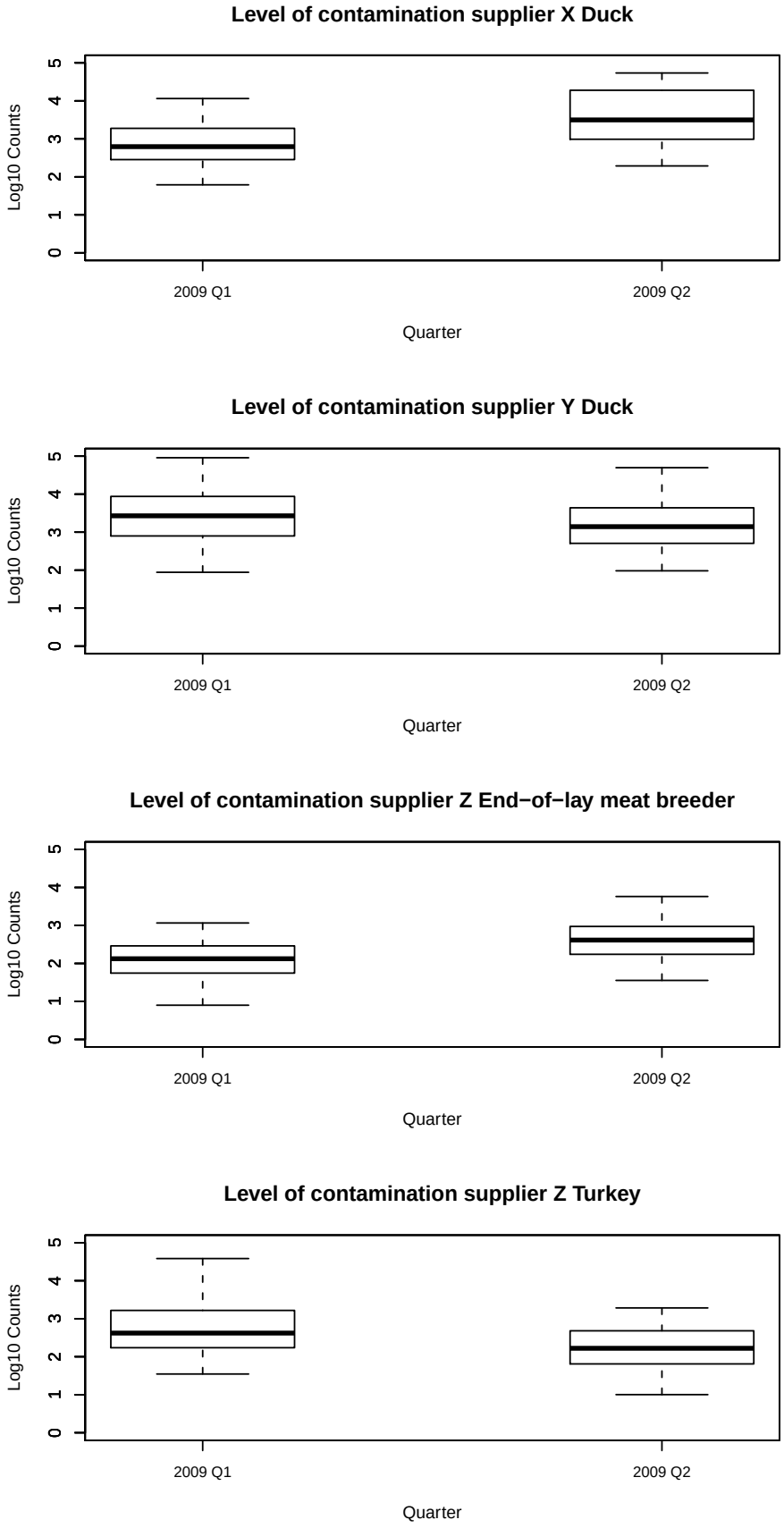

Figure 3: *Campylobacter* on duck, end-of-lay meat breeder and turkey carcases by quarter: level of contamination on positive carcases for each supplier

3.3 Prevalence and level of contamination of chicken carcases

The following section is intended to provide an update of the previously reported prevalence and level of contamination of chicken carcases provided in the 2008 report [13]

3.3.1 Modelling of chicken carcases: prevalence and counts for positive carcases

The estimated proportion of positive carcases (Figure 4), and the counts conditional on being positive (Figure 5), are shown for each supplier and each quarter from October 2006 June 2009. There is a moderately high probability of contamination for all suppliers throughout the study period, with over 80% of carcases from supplier A being positive in all quarters with the exception of the fourth quarter of 2006 and the first two quarters of 2008, and over 60% of carcases were positive for most suppliers in most quarters (Figure 4). The level of contamination for supplier A appeared to decline steadily from 2006 to the third quarter of 2008 and then increase for the next two quarters, albeit with wide credible intervals. Supplier B showed a moderate decline from the second quarter of 2007 to the end of 2008 and supplier C showed highly variable counts throughout the study period. Generally, the counts were higher for supplier A compared to supplier B throughout the study period.

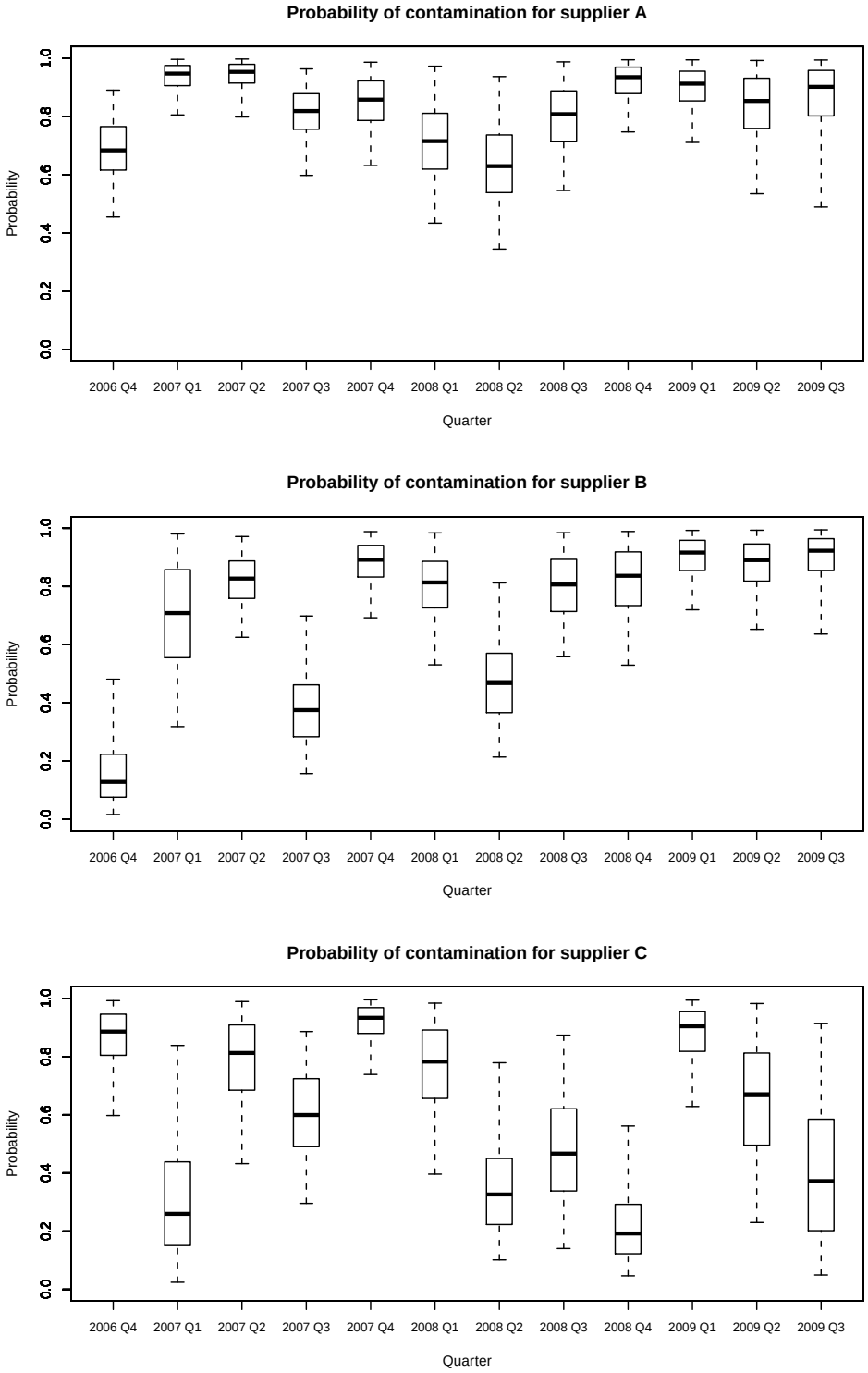

Figure 4: *Campylobacter* on chicken carcases by quarter: probability of contamination for each supplier

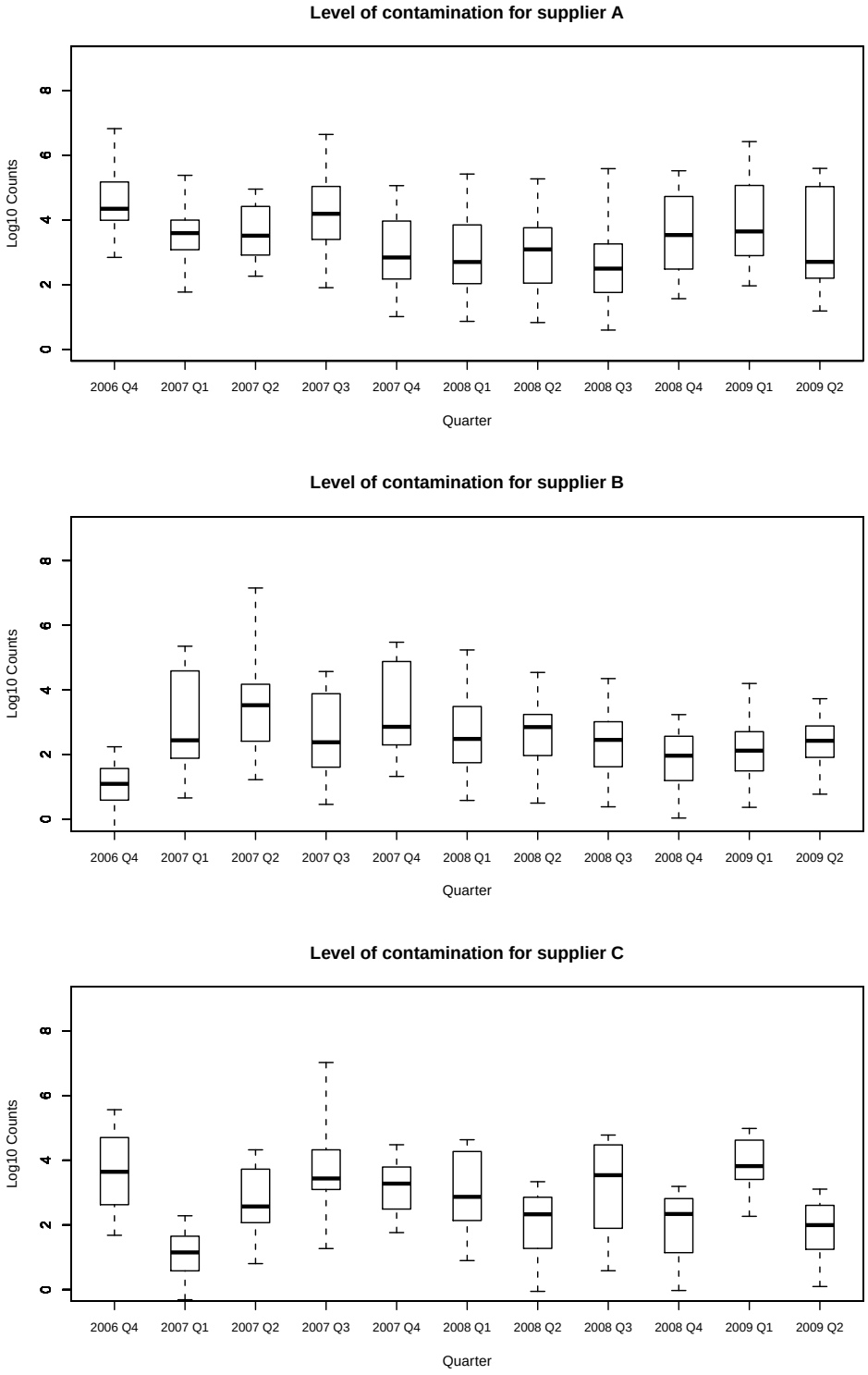

Figure 5: *Campylobacter* on chicken carcases by quarter: level of contamination on positive carcases for each supplier

3.4 Comparison between MLST genotypes found in human cases and poultry

In this section the distribution of MLST genotypes found in human cases in the Manawatu is compared with all poultry and, more specifically, with the genotypes isolated from the non-broiler chicken sources.

3.4.1 MLST genotypes isolated from ducks, turkeys and end-of-lay meat breeders

A total of 120 isolates were assigned to clonal complexes (CCs), many belonged to new STs that have not yet been assigned an ST number on the Oxford PubMLST database. Table 4 shows the distribution of CCs by poultry species. Most evident are the associations between ducks and CC 692, turkeys and CC 1034 and end-of-lay meat breeders and CC 45. Clonal complexes 1034 and 692 are closely related to each other and both have been associated with non-broiler poultry in other countries [5, 4]. The breakdown of complete profiles (N=92 to date) is provided in Table 5. Over a half of all STs isolated were unique to this study, with many new alleles identified.

Table 4: The distribution of MLST clonal complexes amongst ducks, turkeys and end-of-lay meat breeders

	Clonal complex										
	21	45	48	52	177	353	692	828	1034	1275	Totals
Duck supplier X		1					3		3		7
Duck supplier Y	3	1			1		45	1			51
EOL breeder supplier Z	8	15	4	7		2				2	38
Turkey supplier X	3					2		2	13		20
Turkey supplier Z		3						1			4
Grand Total	14	20	4	7	1	4	48	4	16	2	120

Table 5: The distribution of MLST sequence types amongst ducks, turkeys and end-of-lay meat breeders (EOL)

MLST		Allelic profile							Species			
ST	CC	*aspA*	*glnA*	*glt*	*gly*	*pgm*	*tkt*	*uncA*	Duck	EOL	Turkey	Tot
25	45	4	7	10	1	1	7	1		1		1
45	45	4	7	10	4	1	7	1	1	12	1	14
52	52	9	25	2	10	22	3	6		5		5
53	21	2	1	21	3	2	1	5		1		1
190	21	2	1	5	3	2	3	5	1		1	2
356	353	14	17	5	2	11	3	6		2		2
474	48	2	4	1	2	2	1	5		3		3
538	45	4	7	10	4	42	25	1		1		1
583	45	4	7	10	4	42	51	1		1	1	2
1581	828	129	66	30	82	189	47	17			1	1
1595	828	33	38	30	79	104	43	17			1	1
1911	353	7	84	5	10	119	178	26			1	1
3093	21	2	1	21	3	2	3	5		2		2
3230	828	33	39	30	322	104	85	17	1			1
3725	692	37	1	57	26	107	29	35	6			6
3960	692	8	1	57	26	74	29	35	3			3
3962	692	2	131	57	26	107	29	35	1			1
4035	1034	84	61	29	368	127	29	23			1	1
u1225	1275	27	33	22	49	NEW	7	31		2		2
u1301a	692	2	146	57	26	126	29	35	7			7
u2584b	692	2	1	57	26	107	29	35	1			1
u2584a	692	2	1	57	28	74	29	35	1			1
u3264	1034	84	61	29	64	127	29	23			1	1
u3725	692	37	1	57	26	74	24	35	1			1
u4035	1034	84	61	29	26	127	29	23			2	2
u690e	692	2	52	57	NEW	74	29	6	1			1
u690a	692	8	52	57	26	74	29	NEW	7			7
u690b	692	2	52	57	26	74	29	6	7			7
u690c	692	2	52	57	26	74	29	35	1			1
u690d	692	2	52	57	NEW	74	29	6	1			1
u692b	692	37	1	57	26	74	29	23	1			1
u692a	692	37	52	57	26	74	29	23	1			1
u92	692	2	NEW	57	26	NEW	61	23	1			1
u980	692	22	146	29	26	NEW	25	23	2			2
u991c	1034	84	61	57	64	107	29	23			5	5
u991a	692	37	7	57	26	107	29	23	1			1
u991b	1034	NEW	61	120	64	NEW	29	23	1			1
Total									47	30	15	92

3.4.2 Comparing MLST genotypes in humans with all poultry sources in the Manawatu

The distribution of STs amongst human clinical cases and all poultry is shown in a Minimum Spanning Tree in Figure 6. Each circle represents a sequence type (ST) and the size of the circle is proportional to the number of isolates of that ST and the area of each sector is proportional to the number of isolates from each source (i.e. a pie chart). The relatedness between each ST is also captured by their spatial proximity to each other; circles closer together are more closely related than circles further apart. The majority of isolates from ducks (yellow) and turkeys (red) form distinct clusters that are not associated with human disease. This is in contrast to the isolates from chicken sources which are closely related to the human isolates. The end-of-lay meat breeder isolates belong to a small number of STs that group more closely with the chicken isolates than the duck and turkey isolates (e.g. ST 45 and ST 52).

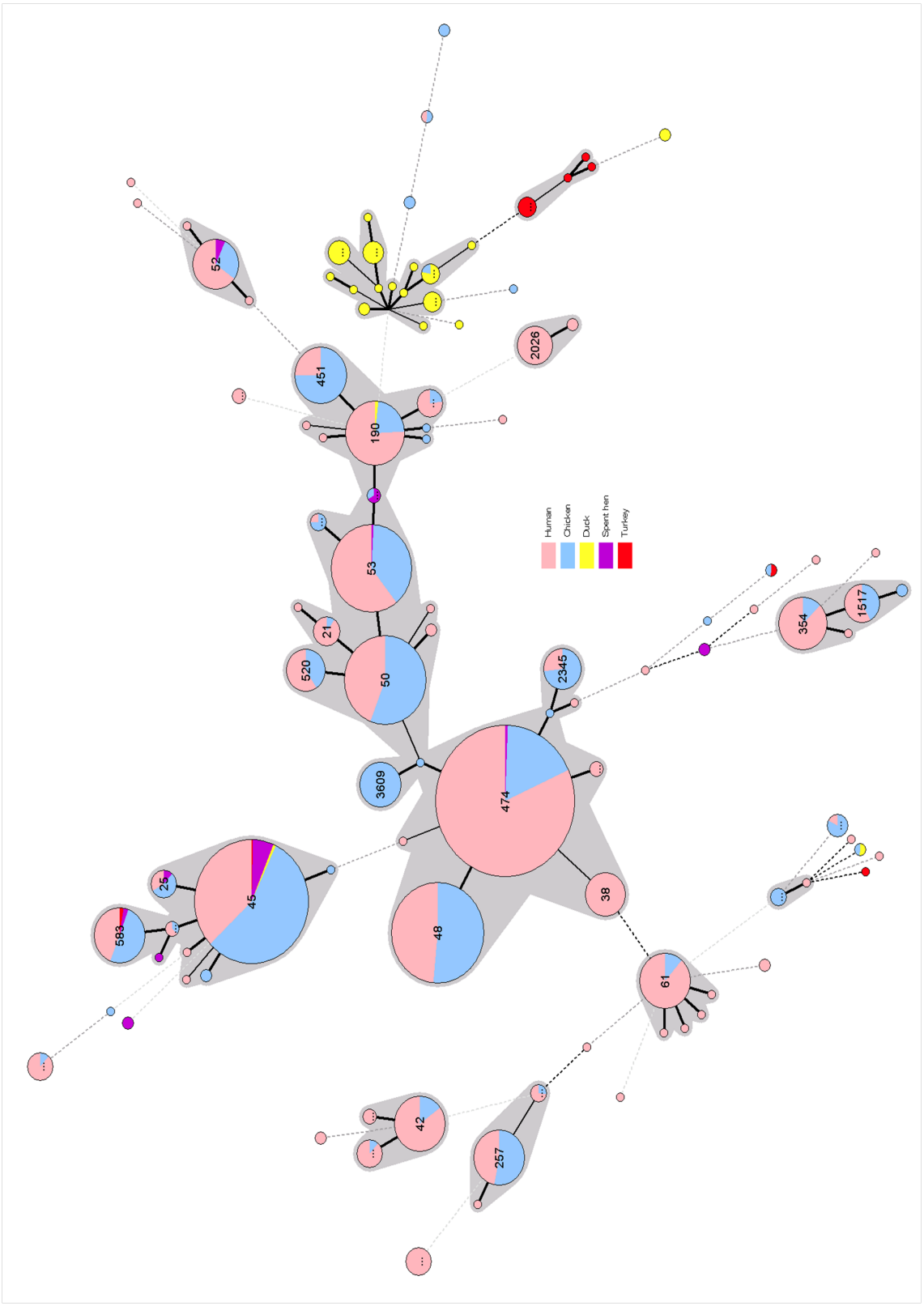

Figure 6: Sequence types from all human cases and poultry between 2005 and 2009. End-of-lay meat breeders are referred to as spent hens in the figure legend.

3.4.3 Distribution of human MLST genotypes in the Manawatu

All human STs isolated from the Manawatu are shown in Figure 7. Although many of these have also been isolated from chicken sources as shown in Figure 6, only three were present as single isolates in duck and turkey samples (ST 45, ST 190 and ST 583).

3.5 Source association using proportional similarity and attribution modelling

In this section four different methods are used to examine the relationship between human cases and all sources sampled, including ducks, turkeys and end-of-lay meat breeders. First we consider the simple proportional similarity index and then provide more formal estimates of the number of human cases attributable to each source using three model-based approaches (Dutch, modified Hald and Island models).

3.5.1 Proportional similarity index

This technique provides a measure of the degree of similarity between the STs identified with each source and the human cases. The analysis reveals a very low level of similarity between human isolates and those isolated from ducks and turkeys. In contrast Table 6 shows a strong similarity between the STs found in chicken supplier A and the human cases (PSI = 0.54). The two other chicken suppliers and cattle and sheep all have similar PSIs, (0.37, 0.39, 0.34 and 0.38 for poultry suppliers B and C and sheep and cattle respectively). The fact that the confidence intervals for chicken supplier A do not overlap with any other sources suggest this value is significantly different from all other sources. The lowest PSIs were those estimated for duck (0.04) and turkey (0.08) sources, confirming that the STs found in these sources are generally different from those found in humans, and they are significantly more dissimilar to the human STs than those from all other sources, with the exception of wild birds. The PS index for end-of-lay meat breeders (0.24) was higher than that for ducks and turkeys, possibly reflecting similar transmission routes to the other chicken sources.

Sequence type and clonal complex		Human case data		Allelic profile							Duck/turkey
ST	CC	Number cases	% Human cases	ASP	GLN	GLT	GY	PGM	TKT	UNC	Host
474	48	189	28.3	2	4	1	2	2	1	5	
45	45	56	8.4	4	7	10	4	1	7	1	D T
48	48	51	7.6	2	4	1	2	7	1	5	
53	21	43	6.4	2	1	21	3	2	1	5	
50	21	36	5.4	2	1	12	3	2	1	5	
190	21	32	4.8	2	1	5	3	2	3	5	D
354	354	26	3.9	8	10	2	2	11	12	6	
61	61	26	3.9	1	4	2	2	6	3	17	
42	42	22	3.3	1	2	3	4	5	9	3	
52	52	19	2.8	9	25	2	10	22	3	6	
38	48	18	2.7	2	4	2	2	6	1	5	
2026	403	14	2.1	10	1	16	19	10	5	7	
257	257	14	2.1	9	2	4	62	4	5	6	
583	45	13	1.9	4	7	10	4	42	51	1	T
436	U/A	11	1.6	7	21	5	62	4	61	44	
520	21	9	1.3	2	1	12	88	2	1	5	
21	21	8	1.2	2	1	1	3	2	1	5	
451	21	8	1.2	2	1	2	3	2	3	5	
677	677	7	1.0	10	81	50	99	120	76	52	
3676	42	6	0.9	1	307	3	4	5	9	3	
1517	354	5	0.7	8	10	149	2	11	12	6	
422	21	5	0.7	2	1	5	3	2	5	5	
2345	206	4	0.6	2	4	5	2	2	5	5	
403	403	3	0.4	10	27	16	19	10	5	7	
137	45	2	0.3	4	7	10	4	42	7	1	
2350	48	2	0.3	2	4	1	2	2	5	5	
3711	257	2	0.3	1	2	4	62	4	5	17	
3712	362	2	0.3	1	2	215	4	90	24	8	
3715	21	2	0.3	2	1	12	3	357	1	5	
3793	61	2	0.3	1	4	2	2	446	3	17	
658	658	2	0.3	2	4	2	4	19	3	6	
1457	U/A	1	0.1	2	165	73	147	220	190	104	
1581	U/A	1	0.1	129	66	30	82	189	47	17	
1707	607	1	0.1	9	2	5	2	11	3	1	
2140	574	1	0.1	9	53	2	53	11	3	3	
219	61	1	0.1	1	4	2	2	6	3	1	
2219	45	1	0.1	10	7	10	4	1	7	1	
2343	48	1	0.1	2	4	5	2	10	1	5	
2391	1034	1	0.1	2	15	4	48	360	25	23	
25	45	1	0.1	4	7	10	1	1	7	1	
3072	828	1	0.1	33	39	30	82	104	173	68	
3222	U/A	1	0.1	33	283	44	82	189	44	17	
3538	U/A	1	0.1	47	2	4	2	6	5	17	
3610	21	1	0.1	2	1	5	88	2	11	5	
3713	21	1	0.1	2	1	12	3	11	1	6	
3717	21	1	0.1	2	1	21	3	62	1	5	
3718	48	1	0.1	2	4	1	4	1	1	5	
3720	49	1	0.1	3	1	5	10	11	11	6	
3727	45	1	0.1	48	7	10	4	183	7	1	
3728	U/A	1	0.1	166	2	1	10	17	3	1	
3784	354	1	0.1	8	315	2	2	11	12	6	
3792	257	1	0.1	9	316	4	62	4	5	6	
459	42	1	0.1	1	2	3	3	5	9	3	
5	353	1	0.1	7	2	5	2	10	3	6	
51	443	1	0.1	7	17	2	15	23	3	12	
578	61	1	0.1	1	4	2	2	2	3	17	
829	828	1	0.1	33	39	30	82	113	43	17	
U/A	U/A	1	0.1	2	1	1	NEW	2	1	5	
U/A	U/A	1	0.1	2	1	6	3	2	3	5	

Figure 7: Sequence types isolated from human cases in the Manawatu between 1/3/05 and 31/7/09

Table 6: The Proportional Similarity index for each source compared to the distribution of human genotypes, with 95% bootstrapped confidence intervals. Higher values indicate a strong similarity between the STs identified in the source and the human cases.

Comparison source	Proportional Similarity index	Lower 95% CI	Upper 95% CI
Chicken supplier A	0.54	0.48	0.58
Chicken supplier B	0.37	0.31	0.40
Chicken supplier C	0.39	0.31	0.42
Cattle	0.38	0.32	0.42
Sheep	0.34	0.28	0.38
Duck	0.04	0.00	0.10
Turkey	0.08	0.00	0.11
EOL breeder	0.24	0.13	0.34
Wild bird	0.10	0.07	0.12
Environmental water	0.22	0.14	0.24

3.5.2 Dutch, Island and Modified Hald model

Estimates of source attribution provided by the Dutch, modified Hald and Island models are shown in Table 7 and Figure 8. All models show similar relative attributions for each source, with the highest attribution assigned to chicken supplier A , and consistently low attribution values for duck and turkey sources. The estimate for end-of-lay meat breeders is higher for the Dutch model compared to the modified Hald and Island models, although the confidence intervals are wide. Table 8 and Figure 9 show the attribution estimates for models including all three chicken sources combined into a single chicken source.

Table 7: Source attribution for human cases in the Manawatu including chicken (three suppliers), ducks, turkeys and end-of-lay meat breeders as potential sources. Mean values are provided with 95% confidence and credible intervals.

	Dutch model			modified Hald model			Island model		
	Mean%	2.5%	97.5%	Mean%	2.5%	97.5%	Mean%	2.5%	97.5%
Chicken sup. A	26.0	21.3	32.9	40.6	7.0	66.1	57.6	50.6	65.2
Chicken sup. B	12.7	10.6	15.3	13.1	1.3	30.6	11.2	6.2	15.8
Chicken sup. C	10.4	7.4	13.7	9.0	0.4	29.2	3.2	0.3	7.0
Cattle	16.1	12.8	19.4	12.3	0.4	36.3	19.6	12.9	26.5
Sheep	12.1	10.2	14.9	16.4	1.3	37.8	3.9	0.2	9.6
Dog/Cat	4.3	2.3	5.7	1.4	0.0	5.5	1.0	0.0	4.0
Duck	0.7	0.0	1.9	1.0	0.0	3.8	0.2	0.0	0.9
Turkey	0.9	0.0	2.0	1.3	0.0	4.8	0.7	0.0	3.0
EOL breeder	8.9	2.7	14.5	2.1	0.0	8.3	0.6	0.0	2.2
Wild bird	2.1	1.1	3.1	1.5	0.0	5.9	1.6	0.1	3.5
Env. water	5.8	2.5	8.6	1.4	0.0	5.6	0.4	0.0	1.3

Table 8: Source attribution for human cases in the Manawatu including chicken (combined sources) ducks, turkeys and end-of-lay meat breeders as potential sources. Mean values are provided with 95% confidence and credible intervals.

	Dutch model			modified Hald model			Island model		
	Mean%	2.5%	97.5%	Mean%	2.5%	97.5%	Mean%	2.5%	97.5%
Chicken	34.0	29.2	42.6	68.6	43.7	85.0	69.2	63.3	75.0
Cattle	20.5	16.3	25.8	13.0	0.4	36.6	24.4	18.1	30.5
Sheep	15.2	12.6	19.2	11.8	0.6	30.6	1.9	0.1	6.3
Dog/Cat	4.9	2.4	6.5	1.2	0.0	4.8	1.1	0.0	3.9
Duck	0.9	0.0	2.3	1.1	0.0	4.5	0.2	0.0	0.8
Turkey	1.2	0.0	2.5	0.9	0.0	3.8	0.8	0.0	3.2
EOL breeder	13.6	3.3	20.8	1.5	0.0	6.5	0.6	0.0	2.5
Wild bird	2.6	1.5	4.1	1.0	0.0	4.3	1.4	0.0	3.3
Env. Water	7.1	3.0	10.9	0.9	0.0	3.9	0.4	0.0	1.4

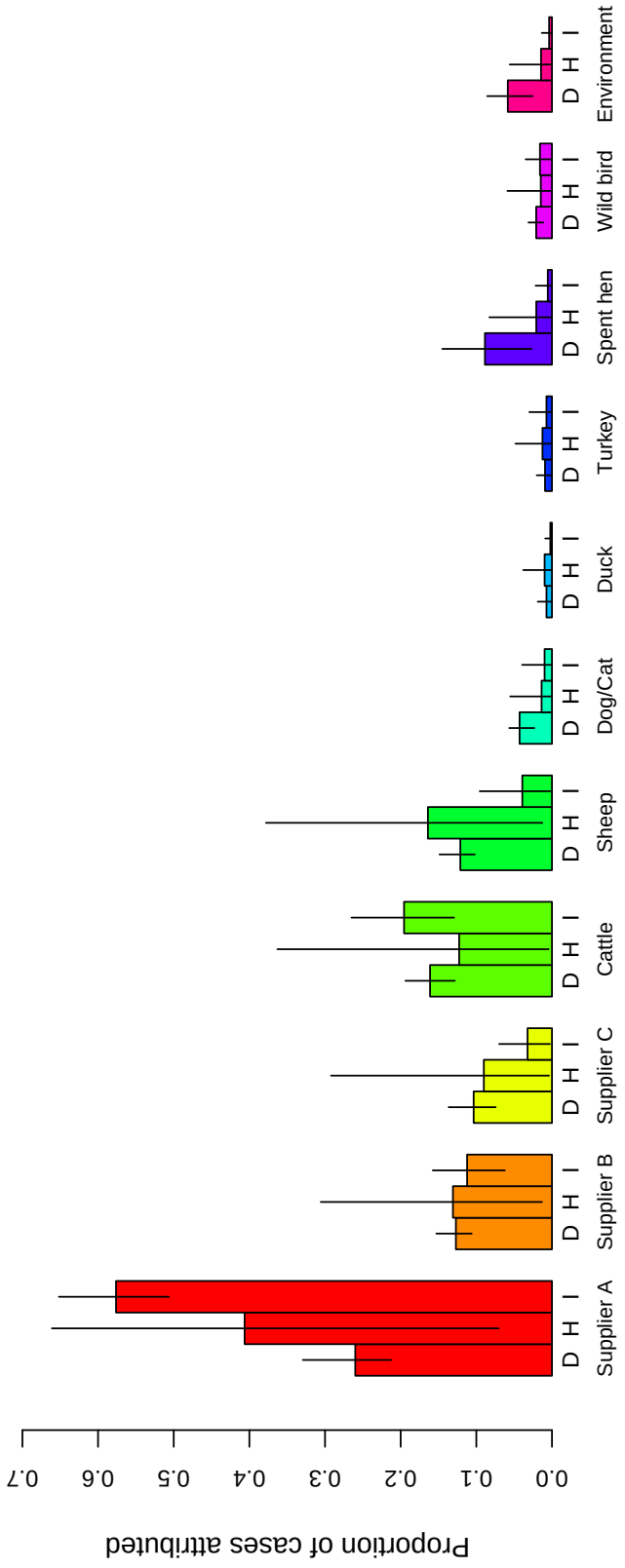

Figure 8: Source attribution for human cases in the Manawatu including three chicken meat sources (labelled suppliers A-C), ducks, turkeys and end-of-lay meat breeders (spent hens) as potential sources. Error bars represent 95% confidence or credible intervals

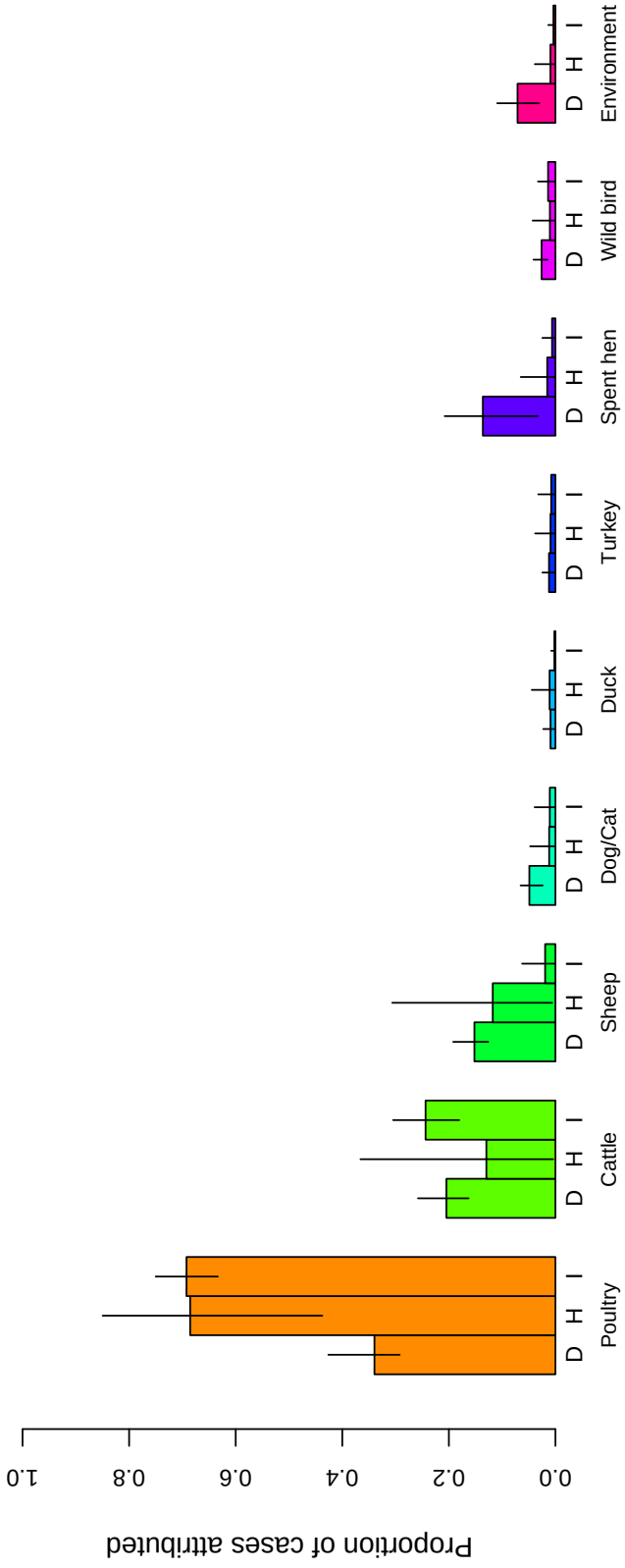

Figure 9: Source attribution for human cases in the Manawatu including chicken (combined three sources labelled "poultry"), ducks, turkeys and end-of-lay meat breeders (spent hens) as potential sources. Error bars represent 95% confidence or credible intervals

4 Discussion and concluding comments

This study provides additional information on the carriage of *Campylobacter* spp. in non broiler poultry sources in New Zealand, as well as an update on the recent trends of carriage in broiler chickens. *Campylobacter* spp. were present on most duck and turkey carcases examined, in similar numbers to those found on broiler chickens. However, the genotypes of *C. jejuni* isolated from these sources were not commonly found in humans. Source attribution models therefore indicated a very low contribution to human infection from these sources. This may be due to these genotypes displaying lower pathogenicity but, given the relatively low consumption of these poultry sources, it is more likely that the low human case attribution merely reflects a lower exposure.

5 Acknowledgements

This work was funded by the New Zealand Food Safety Authority and was done in collaboration with ESR Ltd, Kenepuru and MidCentral Public Health Services. We acknowledge the following individuals who contributed to the writing of this report: Dr Daniel Wilson (University of Chicago, formerly Lancaster University, UK), Dr Simon Spencer, Dr Anne Midwinter, Dr Julie Collins-Emerson, Dr Jonathan Marshall, Petra Mullner and Tui Shadbolt. The work was carried out by the above and Rebecca Pattison, Rukhshana Akhter, Errol Kwan, Lynn Rogers, Isabel Li, Jim Learmonth, Anthony Pita, Sarah Vaughan, (Massey Molecular Epidemiology group, Hopkirk Institute), Dr Phil Carter, Ruth Pirie and Dr Tecklok Wong (ESR), Dr Grant Hotter (AgResearch). We also acknowledge the following individuals and organisations for their contribution to the study: Palmerston North Hospital, MidCentral PHS, MedLab Central, Graham McBride (NIWA), Poultry Industry, Professor Mike Hedley, Dr Geoff Jones, Dr Alasdair Nobel, Professor Martin Hazelton, Dr Michael Baker, Diane Richardson, Horizons Regional Council, and the farmers in the Manwatu who allowed us to sample cattle and sheep.

References

[1] Anon. New Zealand Food Safety Authority Campylobacter Risk Management Strategy: http://www.nzfsa.govt.nz/foodborne-illness/campylobacter/strategy.htm. 2007.

[2] J. Arsenault, A. Letellier, S. Quessy, J. P. Morin, and M. Boulianne. Prevalence and risk factors for Salmonella and *Campylobacter* spp. carcass contamination in turkeys slaughtered in Quebec, Canada. *J Food Prot*, 70(6):1350–9, 2007.

[3] J. Arsenault, A. Letellier, S. Quessy, V. Normand, and M. Boulianne. Prevalence and risk factors for *Salmonella* spp. and *Campylobacter* spp. caecal colonization in broiler

chicken and turkey flocks slaughtered in Quebec, Canada. *Prev Vet Med*, 81(4):250–64, 2007.

[4] F. M. Colles, K. E. Dingle, A. J. Cody, and M. C. Maiden. Comparison of Campylobacter populations in wild geese with those in starlings and free-range poultry on the same farm. *Appl Environ Microbiol*, 74(11):3583–90, 2008.

[5] F. M. Colles, T. A. Jones, N. D. McCarthy, S. K. Sheppard, A. J. Cody, K. E. Dingle, M. S. Dawkins, and M. C. Maiden. Campylobacter infection of broiler chickens in a free-range environment. *Environ Microbiol*, 10(8):2042–50, 2008.

[6] M. Denis, J. Refregier-Petton, M. J. Laisney, G. Ermel, and G. Salvat. *Campylobacter* contamination in French chicken production from farm to consumers. Use of a PCR assay for detection and identification of *Campylobacter jejuni* and *C. coli*. *J Appl Microbiol*, 91(2):255–67, Aug 2001.

[7] K. E. Dingle, F. M. Colles, D. R. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. Willems, R. Urwin, and M. C. Maiden. Multilocus sequence typing system for *Campylobacter jejuni*. *J Clin Microbiol*, 39(1):14–23, Jan 2001.

[8] N. Garrett, M. L. Devane, J. A. Hudson, C. Nicol, A. Ball, J. D. Klena, P. Scholes, M. G. Baker, B. J. Gilpin, and M. G. Savill. Statistical comparison of *Campylobacter jejuni* subtypes from human cases and environmental sources. *J Appl Microbiol*, 103(6):2113–21, Dec 2007.

[9] T. Hald, D. Vose, H. C. Wegener, and T. Koupeev. A Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. *Risk Anal*, 24(1):255–69, Feb 2004.

[10] W. G. Miller, B. M. Pearson, J. M. Wells, C. T. Parker, V. V. Kapitonov, and R. E. Mandrell. Diversity within the *Campylobacter jejuni* type I restriction-modification loci. *Microbiology*, 151(Pt 2):337–51, Feb 2005.

[11] P. Mullner, G. Jones, A. Noble, S. E. Spencer, S. Hathaway, and N. P. French. Source attribution of food-borne zoonoses in New Zealand: a modified Hald model. *Risk Anal*, 29(7):970–84, 2009.

[12] P Mullner, SEF Spencer, DJ Wilson, G Jones, AD Noble, AC Midwinter, JM Collins-Emerson, P Carter, S Hathaway, and NP French. Assigning the source of human campylobacteriosis in New Zealand: A comparative genetic and epidemiological approach. *Infect Genet Evol*, in press, 2009.

[13] N. French, and the Molecular Epidemiology and Veterinary Public Health Group. Enhancing surveillance of potentially foodborne enteric diseases in New Zealand: Human

campylobacteriosis in the Manawatu: http://www.nzfsa.govt.nz/science/research-projects/Campy_Attribution_Manawatu.pdf, 2008.

[14] J. A. Ridsdale, H. I. Atabay, and J. E. L. Corry. Prevalence of campylobacters and arcobacters in ducks at the abattoir. *Journal of Applied Microbiology*, 85(3):567–573, 1998.

[15] O. Rosef, G. Kapperud, S. Lauwers, and B. Gondrosen. Serotyping of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter laridis* from domestic and wild animals. *Appl Environ Microbiol*, 49(6):1507–10, Jun 1985.

[16] N. A. Rosenberg and M. Nordborg. Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. *Nat Rev Genet*, 3(5):380–90, may 2002.

[17] U. Stucki, J. Frey, J. Nicolet, and A. P. Burnens. Identification of *Campylobacter jejuni* on the basis of a species-specific gene that encodes a membrane protein. *J Clin Microbiol*, 33(4):855–9, Apr 1995.

[18] P. Whyte, K. McGill, D. Cowley, R. H. Madden, L. Moran, P. Scates, C. Carroll, A. O'Leary, S. Fanning, J. D. Collins, E. McNamara, J. E. Moore, and M. Cormican. Occurrence of Campylobacter in retail foods in Ireland. *Int J Food Microbiol*, 95(2):111–8, 2004.

[19] D. J. Wilson, E. Gabriel, A. J. Leatherbarrow, J. Cheesbrough, S. Gee, E. Bolton, A. Fox, P. Fearnhead, C. A. Hart, and P. J. Diggle. Tracing the source of campylobacteriosis. *PLoS Genet*, 4(9):e1000203, 2008.

[20] D. L. Wilson, J. A. Bell, V. B. Young, S. R. Wilder, L. S. Mansfield, and J. E. Linz. Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. *Microbiology*, 149(Pt 12):3603–15, Dec 2003.

[21] S. Wright. Evolution in Mendelian Populations. *Genetics*, 16(2):97 – 159, 1931.

List of Figures

1 *Campylobacter* on poultry carcases by quarter: histogram of contamination for each supplier 13
2 *Campylobacter* on duck, end-of-lay meat breeder and turkey carcases by quarter: probability of contamination for each supplier 14
3 *Campylobacter* on duck, end-of-lay meat breeder and turkey carcases by quarter: level of contamination on positive carcases for each supplier 15
4 *Campylobacter* on chicken carcases by quarter: probability of contamination for each supplier 17
5 *Campylobacter* on chicken carcases by quarter: level of contamination on positive carcases for each supplier 18
6 Sequence types from all human cases and poultry between 2005 and 2009. End-of-lay meat breeders are referred to as spent hens in the figure legend. 22
7 Sequence types isolated from human cases in the Manawatu between 1/3/05 and 31/7/09 24
8 Source attribution for human cases in the Manawatu including three chicken meat sources (labelled suppliers A-C), ducks, turkeys and end-of-lay meat breeders (spent hens) as potential sources. Error bars represent 95% confidence or credible intervals 27
9 Source attribution for human cases in the Manawatu including chicken (combined three sources labelled "poultry"), ducks, turkeys and end-of-lay meat breeders (spent hens) as potential sources. Error bars represent 95% confidence or credible intervals 28

List of Tables

1 Number of samples from each source and supplier by month of sampling. . 11
2 Percentage of carcases positive by supplier 12
3 Presumptive counts of *Campylobacter* spp. on positive carcases by supplier and quarter. Medians with 2.5 and 97.5th centiles of the log counts are provided 12
4 The distribution of MLST clonal complexes amongst ducks, turkeys and end-of-lay meat breeders 19
5 The distribution of MLST sequence types amongst ducks, turkeys and end-of-lay meat breeders (EOL) 20
6 The Proportional Similarity index for each source compared to the distribution of human genotypes, with 95% bootstrapped confidence intervals. Higher values indicate a strong similarity between the STs identified in the source and the human cases. 25
7 Source attribution for human cases in the Manawatu including chicken (three suppliers), ducks, turkeys and end-of-lay meat breeders as potential sources. Mean values are provided with 95% confidence and credible intervals. 25
8 Source attribution for human cases in the Manawatu including chicken (combined sources) ducks, turkeys and end-of-lay meat breeders as potential sources. Mean values are provided with 95% confidence and credible intervals. 26