Differential typing of Campylobacter
Differential typing of *Campylobacter*

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Campylobacter continues to be the major cause of human gastrointestinal illness in Australia, with rates of infection approximately double those for Salmonella spp. each year. Worldwide, it is now recognised that there are multiple non-poultry potential sources for transmission of Campylobacter into the human population. While undercooked poultry products continue to be accepted as a significant exposure method, there is now a better appreciation that other exposure methods exist and play a role in the overall picture of human campylobacteriosis.

The objective of this project was to validate and apply a DNA-based typing scheme which allows the allocation of strains of Campylobacter to types that are host specific and/or non-host specific. In turn this will increase our capacity to associate isolates with specific hosts, resulting in improved epidemiological investigations.

This publication describes the validation and application of single nucleotide polymorphism (SNP) typing to over 500 Campylobacter isolates originating from six different host species, humans, chicken, dairy cattle, feedlot cattle, dogs and cats.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to RIRDC’s diverse range of over 2000 research publications and it forms part of our Chicken Meat R&D program, which aims to support increased sustainability and profitability in the chicken meat industry.

RIRDC’s publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation
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The output of this project is due largely to the high level technical skills and dedicated work of Jan-Maree Hewitson.

The SNP, Clustered, regularly interspaced short palindromic repeats (CRISPR), Binary typing and \textit{flaA} (High Resolution Melt) HRM work on \textit{C. jejuni} described in Chapter 1 is part of a PhD study being undertaken by Shreema Merchant at the Queensland University of Technology, under the supervision of Professor Phil Giffard and Dr Flavia Huygens.

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## Abbreviations

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AFLP</td>
<td>Automated Fragment Length Polymorphism</td>
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<td>ARI</td>
<td>Animal Research Institute</td>
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<tr>
<td>AS</td>
<td>allele-specific</td>
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<td>BT</td>
<td>binary type</td>
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<td>CARMA</td>
<td><em>Campylobacter</em> Risk Management and Assessment</td>
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<td>CDC</td>
<td>Centre for Disease Control</td>
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<tr>
<td>CRISPR</td>
<td>Clustered, regularly interspaced short palindromic repeats</td>
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<tr>
<td>CRC</td>
<td>Cooperative Research Centre</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>D</td>
<td>Simpson’s index of diversity</td>
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<tr>
<td>HRM</td>
<td>High Resolution Melt</td>
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<tr>
<td>Min/s</td>
<td>minute/minutes</td>
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<tr>
<td>MLST</td>
<td>Multilocus Sequence Typing</td>
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<tr>
<td>NSW</td>
<td>New South Wales</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PFGE</td>
<td>Pulsed Field Gel Electrophoresis</td>
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<tr>
<td>PHRANA</td>
<td>Progressive Hierarchical Resolving Assays using Nucleotides</td>
</tr>
<tr>
<td>QPI&amp;F</td>
<td>Queensland Primary Industries and Fisheries</td>
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<tr>
<td>QLD</td>
<td>Queensland</td>
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<tr>
<td>RIRDC</td>
<td>Rural Industries Research and Development Corporation</td>
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<tr>
<td>RSPCA</td>
<td>Royal Society of the Protection of Animals</td>
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<tr>
<td>QMRA</td>
<td>quantitative microbial risk assessment</td>
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<tr>
<td>Sec/secs</td>
<td>second/seconds</td>
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<tr>
<td>SA</td>
<td>South Australia</td>
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<tr>
<td>SEQ</td>
<td>South East Queensland</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
<td>ST</td>
<td>sequence type</td>
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<tr>
<td>SVR</td>
<td>Short Variable Region</td>
</tr>
<tr>
<td>UPGMA</td>
<td>unweighted pair group method with averages</td>
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<tr>
<td>VIC</td>
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Executive Summary

What the report is about

This report describes the validation and application of a DNA-based typing scheme to over 500 Campylobacter isolates originating from six different host species: humans, chicken, dairy cattle, feedlot cattle, dogs and cats.

Who is the report targeted at?

This report is targeted at chicken meat processing companies, food safety professionals and food safety regulators.

Background

Campylobacter is the major cause of human gastrointestinal illness in Australia, with rates of infection approximately double those for Salmonella spp. each year. While poultry are a significant source of these infections, there is a considerable body of evidence which indicates that there are other sources, for example raw milk and pets.

Some types of C. jejuni/coli appear host-specific (meaning that some types occur only in chickens) while other types can be found in multiple hosts (e.g. in both chickens and humans). There is a general agreement that the definitive method for typing C. jejuni/coli is Multilocus Sequence Typing (MLST). The high cost of MLST has recently been overcome by the use of a combination of kinetic polymerase chain reaction (PCR) and interrogative data analysis that provides the power of conventional MLST but at a much lower cost and with a more rapid response time. This new technology is called Single Nucleotide Polymorphism (SNP) analysis.

Aims/objectives

1. Establish the technique of SNP analysis for typing of C. jejuni/coli at Queensland Primary Industries and Fisheries (QPI&F)
2. Extend the current QPI&F Campylobacter collection to include poultry isolates from other research groups and industry, and to obtain non-poultry isolates from sources such as diary cattle, pets and humans
3. Establish SNP types of the extended QPI&F Campylobacter collection
4. Compare and contrast the SNP types of the poultry C. jejuni with the non-poultry types
5. Compare and contrast the capacity of SNP typing with pulsed field gel electrophoresis (PFGE) and flaA typing
6. Establish an ongoing typing technique that will function as a national reference service on a user pays basis.

Methods used

In the initial section of the work the following genotyping techniques were used:

- SNP typing – based on seven house-keeping genes derived from MLST
- PFGE typing – whole genome restriction and electrophoresis
- Binary typing – analysis of the presence/absence of nine binary genes
• CRISPR typing – analysis of repetitive short palindromic sequences

• *flaA* HRM typing – High Resolution Melt (HRM) analysis of the short variable region of *flaA* gene.

The techniques were applied to a well characterised set of 88 *C. jejuni* isolates and nine *C. coli* isolates.

In the second section of the study, SNP typing was applied to 577 *C. jejuni* isolates. The isolates consisted of:

• 32 chicken faecal/caecal isolates from epidemiological studies (1999-2003)
• 36 chicken factory isolates (2008)
• 76 chicken factory isolates (2005-2006)
• 77 chicken caecal isolates collected from a national survey (2003-2004)
• 93 dairy cattle isolates (2006-2008)
• 123 feedlot cattle isolates (2006-2008)
• 39 dog and eight cat isolates
• 46 human isolates (2000)
• 47 human isolates (2008).

**Results/key findings**

In the first part of the study, we were able to genotype *C. jejuni* isolates by a number of different methods. We have shown that PFGE can provide a definitive result when required, e.g. in an outbreak situation. However, as PFGE is technically demanding and lacks portability between research groups, it is our view that SNP typing is the preferred method. SNP typing is relatively easy to implement and is directly related to MLST, hence it is easy to compare results with other researchers from around the world. Therefore, we suggest that the most efficient method of genotyping *C. jejuni* isolates would be to do an initial screen with SNP typing and then follow up with either PFGE or full MLST on a smaller subset of isolates if an indisputable answer is required.

In the second part of our study, we applied SNP typing to 577 *C. jejuni* isolates from six different host species. Our results show that some genotypes are associated with multiple host species whereas other genotypes are predominantly associated with limited host species. As an example, SNP type 44 was a genotype found only in humans, dogs and cats. SNP typing has also shown subtle differences in genotype distribution. SNP type 5 was associated with dairy cattle while SNP type 13 was associated with feedlot cattle. Some preliminary data from our studies indicates that regional and company influences may play a role in the genotype distribution of *Campylobacter* isolates in poultry and further investigation of this would be worthwhile.

Overall, SNP typing has been shown to be a convenient first line tool for screening *Campylobacter* isolates. It is user friendly, easily transportable between research groups, is relatively cheap and has the advantage that it is directly linked to MLST. Unlike PFGE, SNP typing lends itself to robotics for sample preparation and assay set up.
Implications for relevant stakeholders

- SNP typing is an effective method for genotyping *C. jejuni*, including the capacity to recognise host associations

- Some *Campylobacter* genotypes show host specificity, which should be considered when investigating outbreaks of campylobacteriosis in humans

- The genotyping results clearly show that there are sources other than poultry meat associated with human campylobacteriosis

- There is some evidence of regional and company influences on the genotypes present in chickens.

Recommendations

A number of recommendations for food safety professionals, regulators and the industry research body have come from this study.

- SNP typing should be considered as a front line typing method when investigating outbreaks of campylobacteriosis or when looking for host associations with particular genotypes of *Campylobacter*

- Pets need to be considered as a source of *C. jejuni* for humans

- The preliminary evidence uncovered in this study of regional and company influences on *Campylobacter* genotypes in chickens requires further research.
Introduction

_Campylobacter jejuni_ is the most frequently reported cause of gastrointestinal disease in many developed countries, including the USA, the UK and Australia. In Australia, the highest age-specific rate is in zero to four year old children (188 cases per 100,000 population) (Anon. 2001). The incidence in developing countries is higher by several orders of magnitude, particularly in the very young (40,000 per 100,000 in children under five years old) (Oberhelman and Taylor 2000).

Human illness caused by _C. jejuni_ or _Campylobacter coli_ primarily involves acute diarrhoea of varying severity, which may last from two to ten days. The onset of diarrhoea may be preceded by fever, headache and dizziness (Skirrow and Blaser 2000). A distinguishing feature of _Campylobacter_ enteritis is the severity of the accompanying abdominal pain, which may be misdiagnosed as acute appendicitis (Skirrow and Blaser 2000).

The vast majority of _Campylobacter_ infections occur as sporadic cases that are not associated with recognised outbreaks. This is a probable explanation for why _Campylobacter_ has escaped the notoriety associated with other food-borne pathogens such as _E. coli_ 0157 or _Salmonella_ (outbreaks associated with these latter organisms have previously attracted media attention). The sporadic nature also means that most outbreaks are never traced to a specific source (Corry and Atabay 2001). The infectious dose for humans is approximately 500 organisms (Robinson 1981). As a consequence of this low infectious dose, errors in food handling may result in human infection, even though _campylobacters_ will not grow readily on foods at room temperature (Doyle and Jones 1992) and thorough cooking will eliminate campylobacters (Blankenship and Craven 1982).

When common source outbreaks of campylobacteriosis do occur, they are usually associated with the consumption of either contaminated water or unpasteurised milk (Skirrow and Blaser 2000). A percentage of sporadic cases can be attributed to contact with pets, especially puppies or kittens with _Campylobacter_ diarrhoea (Skirrow 1991). The generally propagated view is that raw or undercooked poultry products are the major source of human _Campylobacter_ infections, with this view based on studies in the 1980s for example, Deming _et al._ (1987) and Oosterom _et al._ (1984).

This traditional acceptance of the primary role of chicken products in the transmission of _Campylobacter_ into the human population has been challenged in a number of recent studies. Studies in the 1990s with the existing molecular typing techniques available at the time indicated that a significant proportion of _Campylobacter_ from humans were not of poultry origin (Koenraad _et al._ 1995; Hudson _et al._ 1999) and that some originated from cattle (On _et al._ 1998).

There is clearly a need for an Australian-based capacity for rapid, DNA-based typing techniques on _Campylobacter jejuni/coli_ to support the Australian poultry industry. While the various State Health Departments are developing or have developed this type of capacity, there is no matching capacity that can service the specific needs of the poultry industry.

**Molecular Typing of Campylobacter jejuni/coli**

An abundance of molecular typing methods for _C. jejuni/coli_ have been reported in the literature (Wassenaar and Newell 2000). It is beyond the scope of this introduction to provide a full description of the various molecular typing methods. Basic descriptions of the various techniques are provided in the review by Wassenaar and Newell (2000).

The commonly applied methods for the molecular typing of _C. jejuni/coli_ are _flaA_ (or _flaB_) gene PCR-based typing (with some use of sequencing rather than typing) and Pulsed Field Gel Electrophoresis (PFGE) (Wassenaar and Newell 2000). The two emerging techniques are Multilocus Sequence Typing (MLST) and Automated Fragment Length Polymorphism (AFLP) (Wassenaar and Newell 2000).
While PFGE has been a “gold standard” because of adoption by the Centre for Disease Control (CDC) in the USA (Ribot et al. 2001), there are a number of reports of the power of AFLP (Schouls et al. 2003; Siemer et al. 2004) and MLST (see below).

MLST is already in use in clinical typing applications in Australia (Mickan et al. 2007). The method has been used for the study of population and global epidemiology (Dingle et al. 2005) as well as for the identification of clones of Campylobacter associated with particular animal hosts and thus the sources of human infections (Colles et al. 2003b). MLST has been used to show that cattle harbour a clone of Campylobacter commonly associated with human disease, suggesting the importance of cattle as a source of human infection in the UK (Fox et al. 2003). MLST was used in a UK study which concluded that wild birds represent an ultimate source of human infection with certain types being amplified in chickens (Colles et al. 2003a). Of particular relevance is the adaption of MLST to the more cost-effective and rapid detection technology of single nucleotide polymorphisms (SNPs) – detected by such methods as real time PCR (Best et al. 2004).

Overall, there are considerable strengths and advantages associated with typing by MLST – the method gives a high resolution and has been shown to be useful for most pathogens including C. jejuni/coli (Dingle et al. 2005). As the basis of MLST is gene sequences, the data from many different laboratories can be directly and easily compared and also easily shared (Dingle et al. 2005). There is a major website (http://pubmlst.org/campylobacter/) which allows sharing of data and comparison of results.

**Epidemiology of human outbreaks of campylobacteriosis**

A large risk assessment and risk management project has been undertaken in the Netherlands. This large study, named CARMA (Campylobacter Risk Management and Assessment) draws heavily on statistical modelling methods and quantitative microbial risk assessment (QMRA) (Havelaar et al. 2003). In one of the detailed aspects of this large overall study, the relative importance of transmission routes of Campylobacter into the human population was studied by exposure assessment methods (Evers et al. 2003). While limited by a lack of detailed information (a common problem when attempting to determine the source of food-borne diseases), this study found that, for the Dutch situation, the top five transmission routes for campylobacteriosis were attendance at a city farm (direct animal exposure), raw, non-filleted chicken (food), raw milk (food), goats & sheep (direct animal exposure) and chickens (direct animal exposure) (Evers et al. 2003). Hence, this study found that almost two thirds of the exposure was due to direct animal contact (city farms etc.) while only one third of the exposure was associated with food. Whether this relative high importance of city farms would apply in Australia is not clear. Nevertheless, the study highlights that food transmission routes are not the only transmission route for campylobacteriosis and direct animal exposure may have a much greater role than previously appreciated.

Recent classical risk-factor analysis of human Campylobacter infections have continued to reflect that non-poultry sources can be important. In Scotland, the major identified risks were overseas travel (not for young children), consumption of raw milk, consumption of water from private supplies, and ownership of a pet (Smith-Palmer and Cowden 2003). A similar study in Finland concluded that water-associated risk factors (swimming and drinking water from wells) must be considered along with the more widely recognised risk factor of under-cooked poultry meat (Schonberg-Norio et al. 2004).

These findings of alternative sources of human Campylobacter infection need to be considered in light of other studies that continue to show connections between poultry meat and human Campylobacter infections. In particular, there have been studies using a range of molecular typing methods that link poultry and human isolates, for example in Ireland (Canty et al. 2003), Denmark (Nielsen et al. 2003), Hungary (Damjanova et al. 2003) and Germany (Luber et al. 2003).
To summarise the current literature, there is now a greater recognition that there are multiple sources for transmission of Campylobacter into the human population. While under-cooked poultry products continue to be accepted as a significant exposure method, there is now a better appreciation that other exposure methods exist and play a role in the overall picture of human campylobacteriosis.

**MLST and SNP analysis**

The drawback with MLST is that the method is expensive, requiring DNA sequencing of at least seven different genes. As noted above, there are now options of applying MLST in a manner that involves a significant cost reduction for example the use of single-nucleotide polymorphism (SNP) analysis to identify MLST ST-21 of C. jejuni/coli (Best et al. 2004).

Researchers at the Queensland node of the Cooperative Research Centre (CRC) for Diagnostics have also proceeded along the track of adapting SNP analysis to existing MLST databases. The power of this approach has been set out in a recent publication from this research group (Robertson et al. 2004). This approach requires an existing database for C. jejuni/coli. The MLST data-base is freely available on the Internet (http://pubmlst.org/campylobacter/).

The CRC group have developed a computer program that allows known genetic diversity (in this case, multilocus sequence types) to be recognised by detecting single nucleotide changes, rather than the conventional approach of full DNA sequencing. This interrogative data analysis approach means that it is possible to allocate a field isolate to a particular MLST type by establishing the nucleotide present at number of key positions, rather than establishing the identity of around 3,000 nucleotides (the conventional MLST approach). The CRC group have further progressed the methodology by developing robust kinetic PCR technology for establishing the identity of these key nucleotides (Robertson et al. 2004; Price et al. 2006b).

A similar SNP-based typing scheme, based on Progressive Hierarchical Resolving Assays using Nucleotides (PHRANA) has recently been proposed for Bacillus anthracis (Keim et al. 2004). Keim et al. (2004) indicate that this type of approach allows both a deeply rooted phylogenetic approach as well as powerful high resolution discrimination of closely related isolates.

This concept of both deeply rooted phylogeny as well as powerful high resolution discrimination is highly relevant to the particular needs that the poultry industry has with regards to the typing of Campylobacter. The industry needs to integrate a method which has the capacity to function at a high level (host association) as well as fine resolution (movement of strains between birds in a flock, between sheds on a farm and so on).
Summary

This brief overview of relevant literature has confirmed the impact of molecular typing on our understanding of the epidemiology of \textit{C. jejuni/coli} in both animals and humans. When the results of these modern typing techniques are combined with conventional epidemiological and modelling approaches, there are a number of points that emerge:

A) Molecular typing methods – if applied with rigour and on large sets of strains – generally recognise the same subtypes/clones within a \textit{C. jejuni/coli} collection

B) The molecular typing methods are showing that multiple types of \textit{C. jejuni/coli} exist within chickens and that not all of these types are found in humans

C) MLST has significant technical advantages over the other available typing techniques

D) SNP analysis – done via kinetic PCR and supported by effective interrogative data analysis – offers a very rapid, robust and practical means of applying MLST to the routine typing of field isolates of \textit{C. jejuni/coli}

E) Epidemiological studies, while continuing to show a connection between poultry meat and human campylobacteriosis, are showing that a number of other risk factors exist.

There is now the scientific justification for the establishment of a rigorous molecular typing technique (MLST performed by SNP analysis) to the routine typing of \textit{C. jejuni/coli}. The use of this typing approach will provide significant benefits to the poultry industry.
Objectives

1. Establish the technique of SNP analysis for typing of *C. jejuni/coli* at QPI&F

2. Extend the current QPI&F *Campylobacter* collection to include poultry isolates from other research groups and industry and to obtain non-poultry isolates from sources such as dairy cattle, pets and humans

3. Establish SNP types of the extended QPI&F *Campylobacter* collection

4. Compare and contrast the SNP types of the poultry *C. jejuni* with the non-poultry types

5. Compare and contrast the capacity of SNP typing with pulsed field gel electrophoresis and *fla*A typing

6. Establish an ongoing typing technique that will function as a national reference service on a user pays basis.
Methodology

Bacteriology

Faecal samples

Samples were held on ice during transport to the laboratory. All faecal samples were streaked directly onto Karmali *Campylobacter* Agar Base (Oxoid CM935, Oxoid, Melbourne) containing *Campylobacter* Selective Supplement (Oxoid SR167E), and incubated at 42°C for 48 hours in a tri-gas incubator in an atmosphere of 85% N₂, 10% CO₂ and 5% O₂. On the basis of colony morphology, agar plates were recorded as having negative (non-*Campylobacter*) growth or suspect *Campylobacter* growth. All isolates showing suspect colony morphology were examined by phase contrast microscopy for typical *Campylobacter* motility. One presumptive *Campylobacter* isolate from each positive faecal sample was subcultured to Sheep Blood Agar (BBL Blood Agar Base, Becton Dickinson with 5% sheep blood) and incubated as above for further characterisation.

Characterisation and storage of isolates

All presumptive *Campylobacter* isolates were identified to species level using real-time PCR detection of the *mapA* (for *C. jejuni*) or the *ceuE* (for *C. coli*) regions using a modification of the original methods (Stucki et al. 1995; Gonzalez et al. 1997) as described by Price et al. (2006a).

Isolates were stored as viable cultures at -70°C in Brucella broth with 10% glycerol. As well purified DNA was prepared using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems) and the extract stored at -20°C.

SNP Typing

SNP typing was carried out essentially as described by Price et al (2006b).

As previously described, highly informative, generalised *C. jejuni* SNPs identified using the ‘Minimum SNPs’ software package (Robertson et al. 2004) were used. The inputs for the Minimum SNPs software were alleles and corresponding STs from the *C. jejuni* MLST database (http://pubmlst.org/campylobacter/).

Using Primer Express version 2.0 software (Applied Biosystems) and the CLUSTALX version 1.8 alignment tool, both allele-specific (AS) and consensus primers were designed. All primers were designed around minimal potential for primer-dimer formation and a calculated melting temperature (T_m) of 59.0°C. The primer sequences used for interrogating the seven SNPs were as listed by Price et al. (2006b) with one exception. The exception was that the glyA267-G primer was modified to 5’ TGAGGAAATGGACTTGGTTGC 3’.

A Rotor-Gene 3000 and later a Rotor-Gene 6000 real-time PCR apparatus (Corbett Life Science) were used to perform the PCR assays. The assays were set up using a robotic liquid handling system. Each reaction contained 5 pmol of each primer, 2 µl DNA, 1 x Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and distilled water to a total volume of 10 µl. Cycle conditions were 95 °C for 2 mins, followed by 40 cycles of (95°C for 1 secs; combined annealing and extension at 61°C for 10 secs). Negative controls containing H₂O in place of the DNA template were used for each primer set. For each reaction, all available MLST alleles were tested in duplicate.

Essentially the SNP calling criteria were as described by Price et al. (2006b).
**Pulsed Field Gel Electrophoresis (PFGE)**

PFGE analysis was performed essentially as described by the PulseNet USA (2004).

In brief, *Campylobacter* isolates were prepared and digested with *SmaI* and run under standard PulseNet conditions. *Salmonella* Braenderup strain H9812 digested with *XbaI* was run as a size standard. Patterns were clustered using BioNumerics v4.5 (Applied Maths, Ghent, Belgium). Matching and dendrogram UPGMA (unweighted pair group method with averages) analysis of the PFGE patterns was performed using the Dice coefficient with an optimisation of 0.5% and a position tolerance of 1.5%. From the dendrogram generated, numerical PFGE types were arbitrarily allocated to each pattern showing <90% similarity to any other pattern. PFGE types showing greater than 90% similarity were designated as subsets and were designated with a small case alphabetical letter, for example PFGE type 21a.

**DNA Sequencing**

MLST determination was performed according to Dingle *et al.* (2001).

Prior to sequencing PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Clifton Hill, Australia), according to the manufacturer’s instructions. Between 10 and 40 ng of template DNA was mixed with relevant sequencing primer at a final concentration of 9.6 pmol in a 12 µl reaction. Sequencing was carried out at the Australian Genomic Research Facility, Brisbane, using a protocol of 96°C for 1 min, 96°C for 10 secs, 50°C for 5 secs and 60°C for 4 mins on the AB3730SL platform. Sequencing results were analysed using Sequencher v4.8 (Genereresearch, Arundel, Australia) software.

**Binary typing**

Binary typing was done using the genes selected by Price *et al.* (2006a). The nine genes targeted were:

- *Cj0269* possible lipoprotein
- *Cj0265c* putative periplasmic protein
- *Cj0178* putative outer membrane receptor
- *Cj0299* putative periplasmic beta-lactamase
- *Cj1319* putative nucleotide sugar dehydratase
- *Cj0265c* putative periplasmic protein
- *Cj1723c* putative periplasmic protein
- *Cj0008* hypothetical protein
- *Cj0486* putative sugar transporter.

The application of these PCRs generated results of presence (P), absence (A) or intermediate (I) for each gene. The intermediate category was allocated to genes giving results that were close to the results of the control. This meant that each strain was allocated a sequence of nine digits such as IAPAPAAAP. Arbitrarily each unique eight letter profile was identified as a Binary type (BT).
**CRISPR HRM**

High resolution DNA melt (HRM) curve analysis of the clustered, regularly interspaced short palindromic repeats (CRISPRs) and the allocation of CRISPR HRM types was done essentially as described by Price *et al.* (2007).

**flaA HRM**

*flaA* HRM was done as described by Merchant-Patel *et al.* (2009). In brief, real-time PCR and HRM were performed on the Rotor-Gene 6000 (Corbett Life Sciences). DNA was amplified using FLA4F and FLA625RU primers (Meinersmann *et al.* 1997) and under PCR amplification conditions of: 95°C for 2 mins, 40 cycles of 95°C for 30 secs, 59°C for 20 secs and 72°C for 45 secs, 72°C for 2 mins and 50°C for 2 mins. The amplified DNA was then subjected to HRM with 0.05°C increments in temperature ranging from 72°C to 84°C and the fluorescence reading was recorded at each step to obtain a *flaA* Short Variable Region (SVR) HRM type for each isolate. The reference isolate NCTC11168 was included in each run as a control for inter-run variability.
Chapter 1: Comparison of Typing Methods

Introduction

A study was conducted on a subset of carefully selected Campylobacter isolates derived from chickens to validate the different typing techniques. The isolates were subjected to a total of five new genotyping methods in addition to the original flaA RFLP typing that was used to select the isolates. The approach of using multiple typing techniques allowed us to determine if any or all of these new genotyping techniques were effective at resolving isolates from chickens. In addition we can analyse if the methods alone or in combination have sufficient resolving power to detect epidemiological linkage, and to compare the genotypes of the chicken isolates to genotypes derived from other host species such as humans, cattle and companion animals.

The laboratory work in this chapter was largely completed by our colleagues at Queensland University of Technology, in particular PhD Student Shreema Merchant. The exception is the PFGE and full MLST typing which was completed by QPl&F researchers.

Material and Methods

All isolates were obtained from chicken faecal samples, except one C. jejuni isolate that was obtained from darkling beetle larvae, during ongoing epidemiological studies based in South East Queensland (SEQ) as part of previous RIRDC-funded projects DAQ-245A (Miflin 2001) and DAQ-282A (Templeton and Miflin 2005). The isolates were initially shown to be C. jejuni or C. coli by PCR (Linton et al. 1997) and were later confirmed by real-time PCR detection of mapA (for C. jejuni) or the ceuE (for C. coli) regions (Price et al. 2006a).

C. jejuni

The first investigation was to evaluate the genotyping methods with C. jejuni. The study was designed to contain three groups of isolates:

- Group 1 – a collection of 32 isolates, each representing a different flaA RFLP type
- Group 2 – consisted of three groups of 10 isolates with the same flaA RFLP type collected on the same day from one farm within one shed (flaA types I, VIII and XXVI)
- Group 3 – a collection of 26 isolates with the same flaA RFLP type (flaA type I) but having no known epidemiological connection and selected to be as diverse as possible, that is, from different farms, different processing companies and different sampling dates.

The identity of the isolates was blinded from the researchers until the new genotyping methods were complete.

All three groups were subjected to the following genotyping techniques:

- SNP typing – based on seven house-keeping genes derived from MLST
- PFGE typing – whole genome restriction and electrophoresis
- Binary typing – analysis of the presence/absence of nine binary genes
- CRISPR typing – analysis of repetitive short palindromic sequences
- flaA HRM typing – HRM analysis of the short variable region of flaA gene.
**C. coli**

The second investigation was to evaluate genotyping methods for *C. coli* isolates. Initially nine *C. coli* isolates were subjected to the following techniques:

- SNP typing – based on seven house-keeping genes derived from MLST
- PFGE typing – whole genome restriction and electrophoresis
- Binary typing – analysis of the presence/absence of nine binary genes
- *flaA* HRM typing – HRM analysis of the short variable region of *flaA* gene

After this initial application a further 160 *C. coli* isolates were selected to be screened further by SNP typing only. These isolates included chicken caecal and chicken factory isolates as well as pig, feedlot cattle and dairy cattle faecal isolates. In the context of this study chicken factory isolates were obtained from whole bird rinses. Details of the chicken, feedlot cattle and dairy cattle isolates are provided in Chapter 2. The pig faecal isolates were obtained from Professor Mary Barton (University of South Australia, Adelaide).

Four of the above *C. coli* isolates were subjected to full MLST essentially as described by Dingle *et al.* (2001). The four isolates selected included two isolates from a NSW chicken company (same farm on same day), one isolate from a Victorian chicken company and one isolate from feedlot cattle in Queensland collected during 2006.

**Results**

**C. jejuni**

The results from the five genotyping methods for Group 1, *flaA* master types, are presented in Table 1.1 below. The isolates were selected to represent 32 distinct isolates based on *flaA* RFLP typing.

SNP typing resolved the isolates into 21 distinct SNP types with seven SNP types having multiple isolates. In all cases the isolates within a single multi-isolate SNP type were differentiated from each other by *flaA* RFLP typing and by at least one other typing technique for each distinct group.

For PFGE typing, isolates that showed a similarity of >90% were regarded as sharing a PFGE type. Figure 1.1 shows the dendrogram of the PFGE patterns of these *flaA* type master isolates. PFGE typing resolved the isolates into 30 distinct types. There were only two types that contained more than one isolate, PFGE types 23 and 42. In both groups the two isolates were differentiated from each other by both *flaA* RFLP and Binary typing. CRISPR typing separated one of these multiple isolates PFGE types (type 42) while SNP typing did not subdivide either PFGE type.

Binary typing resolved the isolates into 22 distinct types. In all cases the isolates within the groups were differentiated by both *flaA* HRM and PFGE.

CRISPR typing resolved the isolates into 27 distinct types. In all cases *flaA* HRM was the only typing method that could differentiate isolates within all the CRISPR types. The remaining methods did achieve some differentiation within the CRISPR types. For example, CRISPR type 1 (the largest CRISPR type with five isolates) consisted of four SNP types, four PFGE types and four Binary types.

*flaA* HRM resolved the isolates into 31 distinct types. On one occasion two isolates were grouped together, *flaA* HRM type 21. On this occasion SNP, PFGE and CRISPR typing all differentiated between the two isolates.
Table 1.1  List of 32 *C. jejuni* flaA master types indicating origin, epidemiological information and additional genotyping results using SNP, PFGE, Binary, CRISPR and flaA HRM

<table>
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<th>Farm Code</th>
<th>Date Collected</th>
<th>Company</th>
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<th>SNP Type</th>
<th>Predicted MLST</th>
<th>PFGE Type</th>
<th>Binary Type</th>
<th>CRISPR Type</th>
<th>flaA HRM Type</th>
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</table>

\(^a\) All strains were obtained from chicken faecal samples except that isolate C1302 was obtained from darkling beetle larvae.

\(^b\) As described by Miflin et al. (2001) and Templeton and Miflin (2005)

\(^c\) SNP types, PFGE types, Binary types, CRISPR types and \(flaA\) HRM types all allocated as described in text.

\(^d\) As described by Price et al. (2006b).
The results from the five genotyping methods for Group 2, the collection of 30 isolates with three subsets of 10 isolates which are epidemiologically linked, are presented in Table 1.2.

All five typing methods resolved the isolates into the three distinct subsets and there was no variation within each group.

Figure 1.2 shows the dendrogram of the PFGE patterns of the three subsets of 10 isolates. PFGE typing resolved the isolates into three distinct groups and showed 100% similarity within each group.

**Figure 1.1** Dendrogram showing the PFGE patterns of 32 *C. jejuni flaA* type master isolates
Table 1.2  List of 30 *C. jejuni* isolates\(^a\) consisting of three groups of 10 isolates with the same *flaA* RFLP type collected on the same day from one farm within one shed

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<th>Source Code</th>
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<th>SNP Type(^c)</th>
<th>PFGE Type(^c)</th>
<th>Binary Type(^c)</th>
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<th><em>flaA</em> HRM Type(^c)</th>
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\(^a\)All strains were obtained from chicken faecal samples
\(^b\)As described by Miflin *et al.* (2001) and Templeton and Miflin (2005)
\(^c\)SNP types, PFGE types, Binary types, CRISPR types and *flaA* HRM types all allocated as described in text
Figure 1.2  Dendrogram showing PFGE patterns of 30 C. jejuni isolates which consisted of three groups of 10 isolates with the same flaA RFLP type collected on the same day from one farm within one shed (flaA types I, VIII and XXVI)

The results for the five genotyping methods for Group 3, flaA type I isolates collected over time are presented in Table 1.3. This is a collection of 26 isolates with the same flaA RFLP type (flaA type I) that have no known epidemiological connection and were selected to be as diverse as possible, that is from different farms and different processing companies.

In this dataset two pairs of isolates were shown to be identical to each other by all typing methods. One pair consisted of isolates C495 and C586 while the other pair was C656 and C729.

SNP typing resolved the isolates into 10 distinct types, with seven of the 10 types being a single isolate. One type, SNP type 37, had two isolates in the group with these being differentiated by PFGE and Binary typing. Of the remaining two groups, the SNP type 2 grouping had nine isolates and included the identical pair C495 and C586. The remaining isolates in the SNP type 2 grouping were all differentiated from each other by PFGE, while the remaining typing methods recognised between two
to three types within the grouping. The dendrogram of the PFGE patterns (Figure 1.3) shows that all PFGE pattern types within the SNP type 2 grouping formed a cluster with a minimum similarity of 60%. The SNP type 5 grouping had eight isolates and included the identical pair C656 and C729. The remaining isolates in the SNP type 5 grouping were differentiated from each other by PFGE except for isolate L176 which shared the same PFGE type as the identical pair C656 and C729. In fact, the only typing method to separate L176 from C656 and C729 was CRISPR typing. The dendrogram of PFGE patterns (Figure 1.3) shows that all the PFGE patterns present in the SNP type 5 grouping had a similarity of 70% or higher. Within the eight isolates in SNP type 5, the other typing methods recognised six CRISPR types, four Binary types and two flaA HRM types.

PFGE typing resolved the isolates into 22 distinct types, with 20 of the 22 groups comprising a single isolate. One group, PFGE type 25 which consisted of the identical pair of isolates C656 and C729 were not able to be differentiated by any other method. The other group, PFGE type 7 consisted of four isolates, two (L176 and C1307) which were differentiated by one of the other four methods, CRISPR and SNP respectively. The remaining two isolates were one of the identical pairs, C656 and C729.

Binary typing resolved the isolates into 11 distinct groups, with seven groups consisting of a single isolate. There were two groups consisting of two isolates in each, both of which could be differentiated from the other in the group, one by PFGE typing and one by CRISPR typing. One group, Binary type 21 consisted of six isolates and contained the identical pair C656 and C729. The remaining four isolates were differentiated by either SNP or CRISPR typing. The remaining group, Binary type 30 consisted of nine isolates and contained the identical pair C495 and C586. The remaining isolates in the group were differentiated by PFGE typing.

CRISPR typing resolved the isolates into 13 distinct groups, with nine groups consisting of a single isolate. One group, CRISPR type 6 contained three isolates and all were differentiated by PFGE typing. CRISPR type 2 group consisted of four isolates and all were differentiated by PFGE typing. CRISPR type 9 group consisted of five isolates and contained the identical pair C656 and C729. The remaining three isolates in the group were differentiated by SNP typing. CRISPR type 10 consisted of five isolates and included the identical pair C495 and C586. The remaining three isolates in this group could be differentiated by PFGE.

flaA HRM resolved the isolates into five groups, with three groups consisting of a single isolate. flaA HRM type 1 consisted of four isolates all of which were differentiated from each other by PFGE typing only. The flaA HRM type 2 consisted of 19 isolates and included both pairs of identical isolates. The remaining isolates in the groups were differentiated by PFGE typing except for L176 and C1306 which were differentiated by SNP, Binary and CRISPR typing methods.
Table 1.3  List of 26 *C. jejuni* flaA type I isolates\(^a\) collected over time indicating origin, epidemiological information and additional genotyping results using SNP, PFGE, Binary, CRISPR and *flaA* HRM

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\(^a\) All strains were obtained from chicken faecal samples

\(^b\) As described by Miflin et al. (2001) and Templeton and Miflin (2005)

\(^c\) SNP types, PFGE types, Binary types, CRISPR types and *flaA* HRM types all allocated as described in text

\(^d\) As described by Price et al. (2006b)
Figure 1.3  Dendrogram showing PFGE patterns of 26 C. jejuni flaA type I isolates collected over time. The groups that were allocated to SNP types 2 and 5 are marked. PFGE type 6 (isolate P2) and PFGE type 1a (isolate C734) both lie within the cluster of SNP type 5 but are SNP type 34 and SNP type 4, respectively. PFGE type 27 (isolate C628) lies within the cluster of SNP type 2 but was SNP type 36.

C. coli

The results from the four genotyping methods for the initial nine C. coli isolates are presented in Table 1.4 below. The isolates were selected to represent the C. coli flaA type masters that were obtained directly from chickens.

SNP typing resolved the isolates into four distinct SNP types. In all cases the isolates within a single SNP type were differentiated from each other by all other methods except Binary typing.

For PFGE typing, isolates that showed a similarity of >90% were regarded as sharing a PFGE type. PFGE typing resolved the C. coli isolates into nine distinct types, all consisting of individual isolates. Figure 1.4 shows the dendrogram of the PFGE patterns for 63 flaA type master isolates including both C. jejuni and C. coli.

Binary typing resolved the nine C. coli isolates into four distinct types. In all cases the isolates within the groups were differentiated by flaA RFLP, flaA HRM and PFGE.

flaA HRM resolved the isolates into eight distinct types. In one instance two isolates were grouped together, flaA HRM type 42. On this occasion flaA RFLP, SNP and PFGE typing all differentiated between the two isolates.
Table 1.4  List of 9 *C. coli* flaA master types indicating origin\(^a\), epidemiological information and additional genotyping results using SNP, PFGE, Binary and flaA HRM

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\(^a\) All strains were obtained from chicken faecal samples except that isolate C1309 was obtained from a darkling beetle  
\(^b\) As described by Miflin *et al.* (2001) and Templeton and Miflin (2005)  
\(^c\) SNP types, PFGE types, Binary types and flaA HRM types all allocated as described in text  
\(^d\) As described by Price *et al.* (2006b)
Figure 1.4 Dendrogram showing PFGE patterns for 63 flaA type master isolates including both C. jejuni and C. coli (marked with *). C. coli isolates are clustered at the top of the dendrogram except for three C. jejuni flaA types (XL, XLI and XLII) that were also included in the cluster.
The additional 160 *C. coli* isolates were all shown to be the same SNP type, a new SNP type that had not been seen before, detailed results are not shown.

Full MLST was carried out on four of these isolates and the results are summarised in Table 1.5 below.

Table 1.5  Summary of MLST results of four *C. coli* isolates

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<th>glnA</th>
<th>gltA</th>
<th>glyA</th>
<th>pgmA</th>
<th>tktA</th>
<th>uncA</th>
<th>Sequence Type</th>
<th>Clonal Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1101</td>
<td>33</td>
<td>39</td>
<td>30</td>
<td>79</td>
<td>112</td>
<td>47</td>
<td>17</td>
<td>ST-966</td>
<td>ST-828</td>
</tr>
<tr>
<td>1102</td>
<td>33</td>
<td>39</td>
<td>30</td>
<td>79</td>
<td>112</td>
<td>47</td>
<td>17</td>
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<td>113</td>
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<td>ST-3263</td>
<td>-</td>
</tr>
<tr>
<td>314</td>
<td>33</td>
<td>39</td>
<td>66</td>
<td>174</td>
<td>104</td>
<td>43</td>
<td>41</td>
<td>ST-1181</td>
<td>ST-828</td>
</tr>
</tbody>
</table>

The two isolates from the NSW poultry processor (1101 and 1102) were exactly the same by MLST. However, the poultry isolates from NSW were shown to be different to those from the Victorian poultry processor and different again from the Queensland feedlot isolate.

**Discussion**

The aim of this study was to validate five genotyping techniques for their ability to differentiate chicken isolates of *C. jejuni* and *C. coli*, with these isolates being originally selected on the basis of *flaA* RFLP typing. In addition we analysed if the methods, alone or in combination, would have sufficient resolving power to detect epidemiological linkages.

The *Campylobacter* isolates were carefully selected from an extensive culture collection that was established during epidemiological studies of the SEQ chicken industry over the past eight years. Previously strains were characterised by *flaA* RFLP typing and this, together with the detailed epidemiological information that accompanies each isolate, has created a unique collection for use in comparing typing techniques.

Analysis of Group 1, a collection of 32 *C. jejuni* *flaA* type master isolates showed that of the five genotyping methods selected, *flaA* HRM and PFGE were more discriminatory than SNP, Binary and CRISPR typing. As expected that *flaA* HRM gave comparable results to *flaA* RFLP as the two methods are based on the same *flaA* gene. In contrast, PFGE is based on an enzymatic digest of the whole genome and the subsequent patterns are not necessarily linked to any particular set of genes. Hence changes in PFGE patterns can arise due to minor genetic changes that are not linked to specific, critical genes.

Analysis of Group 2, the three subsets of 10 isolates with the same *flaA* RFLP type collected on a single day from one shed on a farm, were held together in their respective groups by all typing methods.

Analysis of Group 3, a collection of 26 *C. jejuni* isolates with the same *flaA* RFLP type (*flaA* type I) but having no known epidemiological connection and selected to be as diverse as possible, showed that PFGE was more discriminatory than any other genotyping technique applied.

Overall these genotyping results show that PFGE is the most discriminatory technique of those applied in our studies. The fact that PFGE provides a greater discrimination than *flaA* RFLP has been reported elsewhere (Stanley et al. 1995; Santesteban et al. 1996; Fitzgerald et al. 2001). One of the difficulties associated with both these methods is the exchange of results between research groups. It has been reported that whilst comparison of PFGE patterns may work very well within one laboratory it is difficult to compare results with others due to variations in restriction enzymes and electrophoresis.
conditions used (de Boer et al. 2000; Wassenaar and Newell 2000). In addition, PFGE is expensive, time consuming and not suitable for handling large numbers of isolates.

Of the other genotyping techniques evaluated in our study, SNP, Binary and CRISPR typing, comparable discrimination of the isolates was achieved. However, of the three methods SNP typing has the added advantage of being derived from the MLST database, therefore allowing possible sequence types which can be deduced from a SNP type (Price et al. 2006b).

An analysis of the genotyping techniques was also carried on a collection of nine C. coli isolates. This collection was smaller than the C. jejuni collection due to the lower prevalence of C. coli isolates that was observed in chickens during our epidemiological studies (Miflin 2001; Templeton and Miflin 2005). Initial application of four genotyping techniques, SNP, PFGE, Binary and flaA HRM typing, initially showed some promise of diversity. Therefore a larger, more diverse collection of C. coli isolates was selected from a range of host species to screen further by SNP typing alone. Unexpectedly, no diversity at all was observed in the second set of C. coli isolates. As a result, a subset of four isolates was carefully selected to further interrogate by full MLST.

Analysis of the four isolates by full MLST proved that indeed two of the isolates were identical, as expected, but were shown to be different from the remaining two isolates which were also different from each other. Further analysis of the DNA sequence at the SNP site proved that the real-time PCR was correctly identifying the single nucleotide that forms the basis of the polymorphism at this SNP site. Therefore it was clear that the SNP typing was performing correctly but the SNPs were not as informative for C. coli isolates as they are for C. jejuni isolates.

The lack of informative power for SNP typing of C. coli isolates using the current primers could be due to the fact that C. jejuni and C. coli are quite distinct organisms. There is adequate evidence to support this suggestion. For example, our PFGE analysis showed that C. jejuni and C. coli form two distinct separate clusters (Figure 1.4). A similar finding has been reported by Yan et al. (1991). Further evidence of the difference between C. jejuni and C. coli is that for full MLST, distinctly different primers are used for C. jejuni and C. coli (Dingle et al. 2005). Indeed, where MLST analysis by radial neighbour-joining trees is performed, there is a total separation of C. jejuni and C. coli (Dingle et al. 2005). Overall, the development of SNP typing for C. coli isolates would require a return to the initial data analysis stage and the development of more informative SNP primers specific for C. coli.

It is disappointing that SNP typing as currently performed is not useful for C. coli. However, there are a number of reports that indicate C. coli cause only 10% of human infection (Gillespie et al. 2002). In addition our studies have shown that C. coli contribute only a minor proportion of the Campylobacter population of poultry in Australia (Miflin 2001).

In summary, we have shown that we can genotype C. jejuni isolates by a number of different methods, at both the individual gene level and at the genome level. Our studies have shown that PFGE can provide a definitive result when required, e.g. in an outbreak situation. However, due to the difficulties with PFGE that have been discussed above, it is our view that SNP typing is the method of choice.

SNP typing is relatively easy to implement and is directly related to MLST, hence it is easy to compare results with other researchers from around the world. Therefore, we suggest that the most efficient method of genotyping C. jejuni isolates would be to conduct an initial screen with SNP typing and then follow up with either PFGE or full MLST on a smaller subset of isolates if an indisputable answer is required.

In particular, when conducting large-scale investigations to determine population structure or host association, it is our view that SNP typing is the optimal method. SNP typing is technically easy, relatively cheap and generates data that can be easily shared between laboratories. In addition, SNP types can be linked back to MLST, a technique that is widely accepted as providing the most comprehensive overview of bacterial population structures.
Chapter 2: Application of SNP Typing

Introduction

A study was conducted to collect and use SNP typing to compare isolates of C. jejuni originating from six different host species: humans, chicken, feedlot cattle, dairy cattle, dogs and cats.

Material and Methods

South East Queensland (SEQ) Chicken Factory Isolates - 2008

Throughout 2008 Campylobacter isolates were collected from two major SEQ processing operations. The isolates were sourced from routine quality control testing done by the companies. There was no special collection of isolates. Company A isolates were obtained from whole carcass rinses collected after chilling. Company B isolates were obtained from both whole carcass rinses after chilling and rinses from a range of cuts. The isolates were added to our culture collection as described previously in the Methodologies sub-section of this report entitled “Characterisation and Storage of isolates”.

Chicken Factory Isolates – 2005 and 2006

Chicken factory Campylobacter isolates were obtained from Food Science Australia and were collected as part of RIRDC funded projects. The isolates were a subset from the original study and represented those available after revival from storage. Four factories were included in our studies, two from New South Wales (NSW) and one each from Victoria (VIC) and Queensland (QLD). The isolates were added to our culture collection as described previously in the Methodologies sub-section of this report entitled “Characterisation and Storage of isolates”.

National Antibiotic Resistance Survey Isolates – 2003 and 2004

Chicken Campylobacter were obtained from the caeca of randomly selected birds at slaughter. The selection of the processing plants was a formal process designed to reflect national trends. Plants were based in NSW, QLD, South Australia (SA) and VIC. The isolates were added to our culture collection as described previously in the Methodologies sub-section of this report entitled “Characterisation and Storage of isolates”. Full details of the isolates are available from the formal report (DAFF 2007).

Dairy Cattle Isolates – 2006 and 2008

Four dairy farms were selected for the study in SEQ. On each study farm opportunistic sampling was done while the herds were in the paddock grazing after milking. Whilst a formal sampling plan was not used, every effort was made to collect samples throughout the whole herd and not just from a small subset of animals. Following observation of an animal defecating, a small sample was collected and transported back to the laboratory on ice. Samples were processed on the day of collection as described previously in the Methodologies sub-sections of this report entitled “Faecal Samples” and “Characterisation and Storage of isolates”.

All four farms were sampled in 2006 and then again in 2008.

Feedlot Cattle Isolates – 2006 and 2008

In total, five commercial feedlots surrounding Brisbane were sampled - three in 2006 and four in 2008, with two of the feedlots being sampled in both years. On each feedlot opportunistic sampling was done. Whilst a formal sampling plan was not used, every effort was made to collect samples throughout the whole herd and not just a small subset of animals. Following observation of an animal defecating, a small sample was collected and transported back to the laboratory on ice. Samples were
processed on the day of collection as described previously in the Methodologies sub-sections of this report entitled “Faecal Samples” and “Characterisation and Storage of isolates”.

Farms A, B and C were sampled in 2006. Farms B, C, D and E were sampled in 2008.

**Dog and Cat Isolates**

Dog and cat faecal samples were collected from a number of different sources for this study. Firstly, dog and cat owners at the Animal Research Institute (ARI) were given kits and asked to collect a fresh faecal sample. Samples were processed on the day of collection as described previously in the Methodologies sub-sections of this report entitled “Faecal Samples” and “Characterisation and Storage of isolates”.

Isolates were obtained from Dr Mary Barton’s culture collection in SA. These isolates were of a historical nature and therefore no data was available with the isolates. The isolates were added to our culture collection as described previously in the Methodologies sub-section of this report entitled “Characterisation and Storage of isolates”.

Isolates were obtained from a private veterinary pathology laboratory, IDEXX at South Brisbane. Isolates were collected from routine submissions with varying amounts of data. The isolates were added to our culture collection as described previously in the Methodologies sub-section of this report entitled “Characterisation and Storage of isolates”.

The final set of *Campylobacter* isolates were obtained from the University of Queensland Veterinary School. The isolates were originally obtained during a one year survey of dogs at the Royal Society of the Protection of Animals (RSPCA) in Brisbane, from both healthy and diarrhoeic animals. The isolates were added to our culture collection as described previously in the Methodologies sub-section of this report entitled “Characterisation and Storage of isolates”.

**Human Isolates – 2000 and 2008**

Human *Campylobacter* isolates in 2000 were obtained from the Queensland Health Department. The isolates were collected from sporadic cases of gastroenteritis with data on age and sex of the patient included. The isolates were added to our culture collection as described previously in the Methodologies sub-section of this report entitled “Characterisation and Storage of isolates”.

Human *Campylobacter* isolates in 2008 were obtained from Sullivan and Nicholaides Pathology Laboratories in Brisbane, through the University of Queensland Veterinary School. The isolates were collected from sporadic cases of gastroenteritis but with no additional data available. The isolates were added to our culture collection as described previously in the Methodologies sub-section of this report entitled “Characterisation and Storage of isolates”.

**SNP Typing**

All isolates were subjected to SNP typing as described previously in the Methodologies sub-section of this report entitled “SNP Typing”.

**Statistical Analysis**

Pearson’s chi-square test was used to test for association between SNP types and host species. A two-way frequency table of host species by SNP type was created, and the observed number of isolates from each host within the SNP type was compared with that expected on the assumption of independence of the row and column categories. The expected value was obtained by assuming that the isolates from each host are distributed among the SNP types according to the proportion of the total number of isolates assigned to each SNP type. For example, since 2.4% of the data set constitutes
SNP Type 1, the assumption is that 2.4% of the isolates from each host species would be part of the SNP Type 1 Group.

This analysis was also done to compare host species with the predicted MLST clonal complex.

**Results**

**Host Specific Studies**

*South East Queensland (SEQ) Chicken Factory Isolates - 2008*

The SNP typing results from 36 chicken factory isolates collected during 2008 are summarised in Table 2.1 below. Detailed results are presented in the Appendix (Table 2A.1).

<table>
<thead>
<tr>
<th>SNP Type</th>
<th>1</th>
<th>5</th>
<th>6</th>
<th>11</th>
<th>12</th>
<th>15</th>
<th>19</th>
<th>20</th>
<th>44</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Company A</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>Company B</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
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<td>3</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>10</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

A total of 15 isolates were collected by Company A with five different SNP types and 21 isolates were collected by Company B with eight different SNP types. SNP types 5, 15, 19 and 20 were common to both companies while SNP type 12 was unique to Company A and SNP types 1, 6, 11 and 44 were unique to Company B.

*Chicken Factory Isolates – 2005 and 2006*

The SNP typing results from 76 chicken isolates collected during 2005 and 2006 are presented in the Appendix (Table 2A.2). Twenty isolates were obtained from Company B, 19 were SNP type 11 and one isolate was SNP type 20. Nine isolates were obtained from Company C, seven were SNP type 20 while there was one isolate of SNP type 11 and 29. One isolate was obtained from Company D and was SNP type 17.

Company E yielded 46 of the 76 isolates in this collection. Eight isolates were obtained in 2005, two were SNP type 11 and six were SNP type 15. 38 isolates were obtained in 2006 and all were SNP type 15.

*National Antibiotic Resistance Survey Isolates – 2003 and 2004*

The SNP typing results for 77 *C. jejuni* isolates collected during 2003 and 2004 are summarised in Table 2.2 below. Detailed results are presented in the Appendix (Table 2A.3). Forty nine (~63%) of the 77 isolates collected in the survey were from NSW, where SNP types 1, 5, 11, 12, 14, 15, 22, 28, 36, 39 and 41 were observed. SNP types 15 and 36 were the most common, constituting 30 of the 49 isolates from NSW, with SNP type 36 being exclusively isolated in NSW. Thirteen (~17 %) of the 77 isolates were from QLD, with SNP types 5, 11, 14, 15, 18, 20, 21 and 22 being observed. Twelve (~16%) of isolates were collected in VIC where SNP Types 2, 11, 12, 14, 17 and 20 were observed. Three (~4%) of the 77 isolates were from SA where SNP Types 3 and 6 were found exclusively.
Table 2.2  Summary of genotyping of 77 C. jejuni chicken caecal isolates collected in 2003 and 2004

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>6</th>
<th>11</th>
<th>12</th>
<th>14</th>
<th>15</th>
<th>17</th>
<th>18</th>
<th>20</th>
<th>21</th>
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<td>1</td>
<td>2</td>
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<td>2</td>
<td>1</td>
<td>14</td>
<td>2</td>
<td>1</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QLD</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>1</td>
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<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>SA</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>VIC</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>3</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Total</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>9</td>
<td>17</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>14</td>
<td>2</td>
<td>1</td>
<td>77</td>
</tr>
</tbody>
</table>

Overall chicken results

The distribution of genotypes of 221 C. jejuni chicken isolates that include both the chicken isolates from this chapter and the chicken isolates from Table 1.1 in Chapter 1, are presented below in Figure 2.1.

![Figure 2.1 Percentage frequency of SNP Types for 221 C. jejuni chicken isolates](image)

SNP type 15 shows the highest frequency amongst the chickens isolates, constituting 33% of the total. The next most frequent SNP type is SNP type 11 (13.6%) followed by SNP type 20 (8.1%), SNP type 36 (6.3%) and SNP type 12 (5.3%). The remaining SNP types all fell below 5% frequency.

Dairy Cattle

The SNP typing results from 93 C. jejuni dairy cattle isolates collected during 2006 and 2008 are summarised in Table 2.3 below. Detailed results are presented in the Appendix (Table 2A.4).
Table 2.3  Summary of genotyping of dairy cattle isolates

<table>
<thead>
<tr>
<th>SNP type</th>
<th>Year Collected</th>
<th>Farm Code</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>4</td>
<td>2006</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>1</td>
<td>-</td>
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<tr>
<td>5</td>
<td>2006</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>2006</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>2006</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>2006</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>39</td>
<td>2006</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total No. of Isolates(^a)</td>
<td>13</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Total No. of Genotypes(^a)</td>
<td>6</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^a\) Total includes single isolates that have not been shown in the table

The total number of isolates and genotypes present on each farm are summarised in Table 2.3 above. It is important to note that Farm D had more *C. jejuni* isolates than any other farm. Despite these high numbers being present, Farm D had noticeably limited diversity compared to the other three farms in the study. Farm D had a dominance of SNP type 13 in 2008. Farm C had three SNP types, 9, 13 and 39 in common from 2006 to 2008. Farm D had of two SNP types, 5 and 9 in common from 2006 to 2008. Farm A had SNP type 4, in common from 2006 to 2008. Overall in 2006, SNP type 5 was the dominant genotype present on three farms. Overall in 2008 there was a shift to SNP type 13 as the dominant genotype, present on two farms but with Farm D yielding the majority of isolates.

**Feedlot Cattle**

The SNP typing results from 123 feedlot cattle isolates collected during 2006 and 2008 are summarised in Table 2.4 below. Detailed results are presented in the Appendix (Table 2A.5).

The total number of isolates and genotypes present on each farm are summarised in Table 2.4 below. It is important to note that on the two farms (B and C) that were sampled in both years of the study, a similar diversity was observed between the two farms (7 genotypes in 54 isolates for Farm B and 5 genotypes in 34 isolates for Farm C) over two years. Farm B had two SNP types, 4 and 13, in common from 2006 to 2008. Farm C had four SNP types, 4, 9, 13 and 39, in common from 2006 to 2008. Overall, SNP type 13 was the dominant genotype present in both years of the study representing approximately 50% of the isolates in each year.
Table 2.4  Summary of genotyping of feedlot cattle isolates

<table>
<thead>
<tr>
<th>SNP type</th>
<th>Year Collected</th>
<th>Farm Code</th>
<th>Total</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>A B C D E</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2006</td>
<td>2 10 1 NT NT</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>NT 5 1 - 3</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>2006</td>
<td>4 - - NT NT</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>NT 2 - 5 1</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>2006</td>
<td>- 1 - NT NT</td>
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<td>1</td>
</tr>
<tr>
<td>9</td>
<td>2006</td>
<td>- - 5 NT NT</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>NT 2 3 3 -</td>
<td>8</td>
</tr>
<tr>
<td>13</td>
<td>2006</td>
<td>11 11 6 NT NT</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>2008</td>
<td>NT 20 12 - 1</td>
<td>32</td>
</tr>
<tr>
<td>33</td>
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<td>40</td>
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<td></td>
<td>2008</td>
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<td>5</td>
</tr>
<tr>
<td>Total No. of Isolates</td>
<td>17 54 34 9 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total No. of Genotypes</td>
<td>3 7 5 3 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Dogs and Cats**

The distribution of SNP typing results for 39 dog and eight cat isolates are summarised in Figure 2.2 below. Detailed results are presented in the Appendix (Table 2A.6). It is important to note that 50% of the isolates are SNP type 44 and this SNP type was only isolated in QLD. In addition, SNP types 1, 4, 6, 13 and 20 were only found in QLD. SNP types 8, 11, 15 and 16 were found in both QLD and SA, while SNP types 42 and 43 were only found in SA.
Figure 2.2 Distribution of SNP Types for dog and cat isolates in SA and QLD

**Human Isolates**

The distribution of SNP types from 46 human isolates collected in 2000 and 47 human isolates collected in 2008 is presented in Figure 2.3 below. Detailed results are presented in the Appendix (Tables 2A.7 and 2A.8).

![Figure 2.3 Distribution of human isolates from 2000 (black bars) and 2008 (white bars)](image-url)
SNP type 44 is the most common SNP type observed in both years of the study, representing 35% of isolates in 2000 and 21% in 2008. SNP type 12 is also common to both years of the study, representing 15% of isolates in 2000 and 8.5% in 2008. SNP types 6 and 15 were more common in 2008 each representing 12.8% if the total isolates.

The percentage frequency of SNP types from 93 human isolates collected in 2000 and 2008 are summarised in Figure 2.4 below. Detailed results are presented in the Appendix (Tables 2A.7 and 2A.8). It is important to note that 35% of the isolates are SNP type 44, with the next most frequent SNP type present being type 12 and representing 15% of isolates. The only other SNP types to occur with more than 5% frequency were SNP types 15 (8.6%), 6 (6.5%) and 21 (5.4%).

![Figure 2.4 Overall percentage frequency of SNP types for human isolates](image)

**Comparison of Genotypes Between Host Species**

An overall view of the number of genotypes for each host species is presented in Table 2.5 below. The 221 chicken isolates shown include 189 from the present chapter and the 32 isolates presented in Table 1.1 in Chapter 1.

It is worth noting that the least amount of diversity was observed in the feedlot cattle. The diversity observed in the humans, dog and cat isolates and the faecal/caecal chicken isolates was similar, whilst the diversity observed in the chicken faecal/caecal isolates was greater than that observed in the chicken factory isolates. In addition, the diversity observed in the dairy cattle was greater than that in the feedlot cattle.
Table 2.5 Summary of the number of genotypes and isolates present for each host species

<table>
<thead>
<tr>
<th></th>
<th>No. of Genotypes</th>
<th>No. of Isolates</th>
<th>Ratio of Genotypes to Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>26</td>
<td>93</td>
<td>3.6</td>
</tr>
<tr>
<td>Chicken - Total</td>
<td>28</td>
<td>221</td>
<td>7.9</td>
</tr>
<tr>
<td>- Feecal and Caecele</td>
<td>26</td>
<td>109</td>
<td>4.2</td>
</tr>
<tr>
<td>- Factory</td>
<td>11</td>
<td>112</td>
<td>10.2</td>
</tr>
<tr>
<td>Feedlot Cattle</td>
<td>8</td>
<td>123</td>
<td>15.4</td>
</tr>
<tr>
<td>Dairy Cattle</td>
<td>14</td>
<td>93</td>
<td>6.6</td>
</tr>
<tr>
<td>Dogs &amp; Cats</td>
<td>12</td>
<td>47</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Figure 2.5 below shows the distribution of genotypes across the host species in this chapter and includes the chicken isolates from Table 1.1 in Chapter 1. A key point to note in this comparison is that where a genotype has at least 10 isolates present in the group, there are multiple hosts associated except for SNP type 36, which is solely associated with chickens. It should also be noted that SNP types 4, 9 and 13 have an association with cattle while SNP type 15 is associated with chickens. SNP type 44 is associated with humans as well as dogs and cats.

**Association between SNP Type and Host Species**

The distribution of isolates, both expected and observed, from the various host species in the 14 most common SNP types found in our study is shown in Figure 2.6 below. Our statistical analysis allowed a formal recognition of when a SNP type was present in a host at a frequency that significantly exceeded the expected frequency based on the total collection. There are highly significant associations (P < 0.001) for SNP type 13 and feedlot cattle isolates, SNP Type 15 and chicken isolates, as well as SNP type 44 with dog and cat isolates. There are significant associations (P < 0.01) for SNP type 4 and feedlot cattle isolates, SNP type 5 and dairy cattle isolates, as well as SNP type 44 with human isolates.

**Association between Predicted MLST Clonal Complexes and Host Species**

The distribution of isolates, both expected and observed, from the various host species in the 10 most common predicted MLST clonal complexes found in our study is shown in Figure 2.7 below. There are highly significant associations (P < 0.001) for clonal complex ST-61 and feedlot cattle, as well as clonal complex ST-534 or ST-52 and chicken. There is a significant association (P < 0.01) for clonal complex ST-21 and dairy cattle.
Figure 2.5 Comparison of genotypes found in different host species

<table>
<thead>
<tr>
<th>SNP Type</th>
<th>Dogs &amp; Cats</th>
<th>Dairy Cattle</th>
<th>Feedlot Cattle</th>
<th>Chicken</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No. of Isolates
Figure 2.6 Continued
Figure 2.6 Observed (black bars) and expected (white bars) numbers of isolates from each source within 14 of the most common SNP types observed in our studies. Sources showing a significant association (P < 0.01) are indicated with *. Sources showing a highly significant association (P < 0.001) are indicated with **.
Figure 2.7 Observed (black bars) and expected (white bars) numbers of isolates from each source within 10 of the most common predicted MLST clonal complexes observed in our studies. Sources showing a significant association (P < 0.01) are indicated with *. Sources showing a highly significant association (P < 0.001) are indicated with **.
Discussion

The aim of this study was to apply SNP typing to a collection of *C. jejuni* from a wide range of host species that included human, chicken, feedlot cattle, dairy cattle, dogs and cats. The study included 577 *C. jejuni* isolates with the following composition: 16% human, 38.3% chicken, 21.3% feedlot cattle, 16.1% dairy cattle and 8.1% dogs and cats. The study compared isolates from the different host species in an attempt to identify potential overlaps between the host species and to investigate if there was any host specificity amongst the *C. jejuni* strains.

In looking at possible host specificity we limited our analysis to SNP types that were reasonably represented in the collection. Therefore SNP types that had less than 10 isolates in the grouping (Figure 2.5) were not analysed further. Among the *C. jejuni* isolates, SNP types 4, 5, 6 and 39 were associated with multiple host species, in reasonable numbers (Figure 2.6). SNP types 4 and 5 correspond to MLST clonal complex ST-21, a complex which has been shown in overseas studies to have a multiple host association (Manning *et al.* 2003; Sheppard *et al.* 2009). SNP type 6 corresponds to MLST clonal complexes 45 or 403. A study in the UK by Dingle *et al.* (2002) reported that ST-45 complex was isolated from multiple species but there was a predominance of human and chicken isolates for this complex whereas ST-403 was isolated only from humans, beef and lamb, but not chickens. This is an example of where SNP typing is not completely predictive of MLST and consideration of the host species of the isolate is needed.

SNP types 1, 12, 14, 15 and 44 were clearly associated with only two host species in reasonable numbers in our collection of *C. jejuni* isolates (Figure 2.6). SNP types 1, 12, 14 and 15 were shown to be associated with humans and chickens whereas SNP type 44 was clearly associated with humans as well as dogs and cats. SNP type 1 corresponds to MLST clonal complex ST-353, SNP type 12 to clonal complex ST-52, SNP type 14 to clonal complex ST-257 and SNP type 15 to clonal complex ST-354 or 52. All of these clonal complexes have been reported in overseas studies to be clearly associated with human and poultry except for clonal complex ST-52 which is also associated with cattle and sheep as well as humans and poultry (Dingle *et al.* 2002; Sheppard *et al.* 2009). SNP type 44 is the only SNP type in our study clearly associated with humans as well as dogs and cats. Unfortunately at this stage, none of these isolates have been subjected to full MLST so there is no knowledge of sequence type or clonal complex association. Clearly, full MLST analysis on representative isolates of this SNP type is required in the future.

SNP types 9, 11, 13, 20 and 36 were predominantly associated with only one host in reasonable numbers in our collection of *C. jejuni* isolates (Figure 2.6). SNP types 9 and 13 were shown to be associated with cattle and corresponded to clonal complexes ST-42 and ST-61 respectively. These results are in line with overseas findings that clonal complexes ST-42 and ST-61 mainly comprised isolates associated with humans, cattle and sheep (Dingle *et al.* 2002; Manning *et al.* 2003; Sheppard *et al.* 2009). SNP types 11, 20 and 36 were predominantly associated with chickens, with SNP type 36 being only isolated from chickens in our study. SNP type 11 corresponds to clonal complex ST-443, a clonal complex that has been strongly associated with humans and chickens in overseas studies (Dingle *et al.* 2002; Sheppard *et al.* 2009). SNP type 20 corresponds to clonal complex ST-61 or to sequence type 531, which is not assigned to a clonal complex. As there is data to support the fact that clonal complex ST-61 is associated with cattle, there is clearly a need to carry out full MLST to determine if this group of chicken isolates in our study is more like sequence type 531. The MLST database contains only two isolates belonging to sequence type 531, both are human isolates, one from Australia and one from England. Clearly a representative chicken isolate from our collection needs to be added to the database. SNP type 36 is the only SNP type in our study that is solely associated with chickens and no other host species. As with SNP type 44, there is a clear need to perform full MLST on representative isolates.

In addition to the above observations, formal statistical analysis was carried out to test for significant associations between SNP types and host species, as well as between clonal complexes and host species. At the SNP type level significant associations were observed between SNP type 4 and feedlot cattle, SNP type 5 and dairy cattle, SNP type 13 and feedlot cattle. SNP type 44 was significantly
associated with humans as well as dogs and cats, although the association was more significant with
dogs and cats than humans. At the clonal complex level, a significant association was observed between
clonal complex ST-21 and dairy cattle. Highly significant associations were observed between clonal
complex ST-61 and feedlot cattle, as well as between clonal complex ST-354 or ST-52 and chickens.
Manning et al. (2003) used a similar statistical method to seek host specificity association with clonal
complexes. As in the current study, Manning et al. (2003) found a significant association of clonal
complex ST-61 with cattle. However, Manning et al. (2003) did not find any significant host
association for clonal complex ST-21, clonal complex ST-354 or clonal complex ST-52.

There are two possible explanations for the differences between significant host associations between
our study and that of Manning et al. (2003). Firstly, there may be a true difference between the United
Kingdom and Australia in terms of clonal complex distribution amongst host species. We feel that this
is an unlikely explanation. While there may be differences between the two countries there is nothing
that would explain a complete change in distribution of clonal complexes. The second, and we feel
more likely explanation, is the difference in makeup of isolates for each study. Our study contains a
reasonably even distribution of isolates from each host species with chickens being the largest group
and representing 38% of isolates. In contrast, the Manning et al. (2003) study has a less even
distribution of isolates with human isolates representing 61% of the isolates studied.

A clear difference between our study and the majority of overseas studies is the separation of feedlot
cattle and dairy cattle, and the inclusion of both sets of data in our study. Overseas studies have either
only included beef cattle (Dingle et al. 2002) or not defined the type of cattle in their study (Manning et
al. 2003; Sheppard et al. 2009). This is of importance as we found a difference between the two types
of cattle. As stated above, we observed significant associations between SNP type 4 with dairy cattle
and SNP type 5 with feedlot cattle. For this reason we would recommend that future studies on host
associations of C. jejuni should make the distinction between feedlot cattle and dairy cattle.

When looking more closely at individual host species, we were able to analyse some of our data in
relation to the company and/or the region that the isolates were collected from. For example, in the SEQ
Chicken Factory Isolates collected in 2008, some of the initial findings showed that whilst both
companies had multiple genotypes present there was also preliminary evidence that genotypes may be
unique to or dominant in one company. This was again emphasised in the Chicken Factory Isolates
collected in 2005 and 2006 where one company was dominated with the one SNP type over an 18
month period and this same SNP type was not found in any other company in the study. When
analysing the National Antibiotic Resistance Survey Isolates we found preliminary evidence which
showed that the region the isolates are collected from may also influence the genotypes present, with 11
of the 18 genotypes present being observed in only one region during the study.

In addition, we observed that while one region may have several genotypes present, it may be
dominated by one or a few genotypes. For example, in the NSW region we observed the presence of 11
genotypes and only two genotypes were represented in reasonable numbers. Similar findings were
observed for dairy cattle and feedlot cattle in terms of farm-genotype links, and for dogs and cats in
terms of geographical-genotypes links. Overall there is evidence of associations between genotypes and
farm/companies as well as linkages between genotypes and geographical regions. This preliminary
evidence would suggest that a larger study on the effect of regional and company influences on the
genotype distribution in chickens would be worthwhile.

When looking more closely at the source of chicken isolates, in our studies we observed a marked
difference in the genotypic diversity observed between faecal/caecal isolates compared with the
factory/abattoir isolates. The faecal/caecal isolates yielded a new genotype every four isolates collected
whereas the factory/abattoir isolate yielded a new genotype only every 10 isolates collected. Possible
explanations for this difference in diversity could be the stress of processing (scalding, spin chilling) or
the ability of isolates to attach to the chicken meat, resulting in a selection for only a limited number of
genotypes to carry through onto the finished product. The influence of factory processing on the genetic
diversity of Campylobacter from overseas studies is divided. Two studies agree with our findings that
the diversity of genotypes present after processing is reduced (Newell et al. 2001; Hiett et al. 2002) whereas a study by Lindmark et al. (2006) found the opposite.

In summary, we have applied SNP typing to 577 C. jejuni isolates across six different host species. Our results show that some genotypes are associated with multiple host species whereas other genotypes are predominantly associated with limited host species, such as SNP type 44 which is clearly a genotype found in humans, dogs and cats. SNP typing has also shown subtle differences in genotype distribution such as those observed in dairy cattle and feedlot cattle. Some preliminary data from our studies indicates that regional and company influences may play a role in the genotype distribution of Campylobacter isolates in poultry and further investigation of this would be worthwhile. Overall, SNP typing has been shown to be a convenient first line tool for screening Campylobacter isolates. It is user friendly, easily transportable between research groups, is relatively cheap and has the advantage that it is directly linked to MLST. Unlike PFGE, SNP typing lends itself to robotics for sample preparation and assay set up.
Implications

This study has a number of important implications:

- There are a range of genotypes of *Campylobacter* that are host-associated but these hosts are not chicken
- Pets need to be considered as a potential reservoir of *Campylobacter* genotypes that are associated with human campylobacteriosis
- SNP typing is an effective method for genotyping *C. jejuni*, including the capacity to recognise host associations
- There is some evidence of regional and company influences on the genotypes present in chickens.
Recommendations

This report provides novel information of importance to food safety professionals and the chicken industry in general. The novel information includes an understanding of the power and capacity of SNP typing. Formal publication and conference papers are planned to ensure the information reaches the relevant target groups (food safety professionals and the chicken industry).

The study has provided preliminary evidence of the impact of regional and company influences on the genotypes of Campylobacter present in chicken. Further research on this issue is required.
### Appendices

#### Table 2A.1 List of 36 *C. jejuni* chicken factory isolates collected during 2008 in SEQ

<table>
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<th>Study Code</th>
<th>SNP Type</th>
<th>Predicted MLST</th>
<th>Company</th>
<th>Date Collected</th>
</tr>
</thead>
<tbody>
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<td>ST-5 or 527</td>
<td>ST-353</td>
<td>B</td>
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<td>A</td>
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<td>ST-21</td>
<td>B</td>
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<td>ST-21</td>
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* As described by Price *et al.* (2006b)
Table 2A.2 List of 76 C. jejuni chicken factory isolates during 2005 and 2006

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<th>Study Code</th>
<th>SNP Type</th>
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<th>Company</th>
<th>Collection Point in Factory</th>
<th>Date Collected</th>
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<tbody>
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<td>ST-51 ST-443</td>
<td>B</td>
<td>after bleed</td>
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<tr>
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<td>after bleed</td>
<td>14/07/2005</td>
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<td>ST-51 ST-443</td>
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*a As described by Price et al. (2006b)
Table 2A.3 List of 77 *C. jejuni* chicken caecal isolates collected for a national antibiotic resistance survey during 2003 and 2004

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\* As described by Price et al. (2006b)
### Table 2A.6 List of 39 *C. jejuni* dog and eight *C. jejuni* cat isolates

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* As described by Price *et al.* (2006b)
Table 2A.7 List of 46 *C. jejuni* human isolates collected in Queensland during 2000

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<sup>a</sup> As described by Price et al. (2006b)
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Differential typing of Campylobacter

By Jillian Templeton

Pub. No. 14/030

Campylobacter continues to be the major cause of human gastrointestinal illness in Australia, with rates of infection approximately double those for Salmonella spp. each year.

Worldwide, it is now recognised that there are multiple non-poultry potential sources for transmission of Campylobacter into the human population. While undercooked poultry products continue to be accepted as a significant exposure method, there is now a better appreciation that other exposure methods exist and play a role in the overall picture of human campylobacteriosis.

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