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Australian multicentre comparison of subtyping methods for the investigation of *Campylobacter* infection

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SUMMARY

In order to identify subtyping methods able to contribute to the surveillance or investigation of Australian *Campylobacter* infection, six genotypic and three phenotypic subtyping methods were evaluated on a collection of 84 clinical isolates collected over a 30-month period from one region in Australia. The aim was to compare the logistics of various subtyping methods and examine their ability to assist in finding outbreaks or common sources of sporadic infection. The genotypic subtyping methods used were sequencing of the short variable region of the *flaA* gene, two methods using restriction fragment length polymorphism (RFLP) of the *flaA* gene using either *Dde*I or *Eco*RI with *Pst*I, automated ribotyping, pulsed field gel electrophoresis and multilocus sequence typing. The phenotypic methods employed included Laboratory of Enteric Pathogens serotyping, Lior biotyping and antibiotic resistotyping. The level of agreement between subtyping results was determined. Phenotypic methods showed little agreement whereas genotypic typing methods showed a high level of agreement. Using the premise that five of the six genotypic typing methods were in agreement 15 genotypic groupings were identified. Sequencing of the short variable region of the *flaA* gene, RFLP of the *flaA* gene or automated ribotyping in conjunction with multilocus sequence typing best identified genotypic groupings. An alternative combination of RFLP of the *flaA* gene followed by ribotyping was equally satisfactory. RFLP of the *flaA* gene appeared to be suitable as a preliminary typing method based on ease of operation, equipment availability and cost.

INTRODUCTION

Campylobacter spp. are responsible for a large proportion of the foodborne bacterial enteritis in industrialized countries such as Australia, where in 2001 *Campylobacter* infection was the most commonly reported disease with a national rate of 125·2 cases/100 000 population [1]. Despite the rising number of clinical cases of campylobacterosis in many countries, outbreaks are unusual and the risk factors for contracting endemic *Campylobacter* infection are not fully understood [2]. In Australia, as in similar countries, the majority of cases of *Campylobacter* infection appear to be sporadic, their source is unknown, and outbreaks rarely occur [1]. Case-control studies show that the consumption of chicken is an important risk factor for *Campylobacter* infection, but other meats and foods, and contact with farm

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animals or pets are also implicated as risk factors [3]. Improved discrimination between subtypes and strains of *Campylobacter* spp. will enhance the laboratory support for epidemiological studies and surveillance of *Campylobacter* infection. Phenotypic typing methods have been used previously in epidemiological studies of *Campylobacter* infection [4–6]. The increasing availability of various molecular *Campylobacter* typing methods raises the possibility of using these in a larger number of laboratory centres instead of the previous phenotypic approach [6–9]. They have not been compared previously in an Australian or Western Pacific context.

Previous studies have analysed a smaller number of typing systems using Northern Hemisphere isolates, and have determined the level of discrimination using a highly diverse collection of isolates. This study attempted to identify the most suitable subtyping system for detection of previously unrecognized clusters of *Campylobacter* infection. We also aimed to determine which methods were sustainable in an Australian context by assessing the per-isolate cost. This study paved the way for future national studies by identifying potential problems with standardization of typing methods between laboratories and collation of their results for amalgamation in a single database.

The present study compared the performance of nine different typing systems on clinical *Campylobacter jejuni* isolates to identify the most suitable for laboratory identification of previously unsuspected case-clusters.

METHODS

Bacterial isolates

Eighty-four clinical isolates were included in this study. The isolates were collected over a 30-month period between January 1999 and July 2001 at the Hunter Area Pathology Service and are also part of a larger case-control study. The Hunter region has a population of 570 000 and includes urban, rural and semi-rural areas. No isolates were from a defined outbreak. Isolates were transported on chocolate agar under microaerobic conditions and maintained in 20% glycerol broth at −80 °C.

Laboratory methods

Diarrhoeal stool was cultured on charcoal blood-free agar with cefoperazone and amphotericin B (bioMérieux, Marcy-l'Etoile, Rhone, France). Plates were incubated microaerobically at 42 °C for 48 h. *Campylobacter* spp. were motile isolates with characteristic Gram-stain appearance and oxidase positivity. Isolates were stored at −80 °C in glycerol broth until analysed.

Species identification of each isolate was determined by hippurate hydrolysis and PCR targeted at *C. jejuni*-specific hippuricase and putative oxidoreductase genes as described previously [10–12]. There was complete agreement between tested hippurate and PCR species status. Template DNA for PCR was prepared using Instagene matrix as outlined in the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). PCR amplifications were performed in a 50 μl volume containing 10 mM Tris–HCl (pH 8·3), 1·5 mM $MgCl_2$, 50 mM KCl, 200 μM (each) dNTPs, 0·2 μM each primer, 1·25 U of *Taq* DNA polymerase (Roche Diagnostics, Castle Hill, Australia) and 1 μl of Instagene™ prepared DNA. All PCR experiments were performed on a PC-960G gradient thermal cycler or PC-960 and FTS thermal cyclers (Corbett Research, Sydney, Australia) and the amplification products were analysed on 1% agarose gels. This enabled classification of the isolates as either *C. jejuni* or non-*C. jejuni*.

Typing methods

Antimicrobial resistotyping (*AR*)

AR was performed by agar dilution methodology utilizing Mueller–Hinton agar with 5% lysed sheep blood in accordance with NCCLS methodology for *Helicobacter* spp. [13]. The inoculum was prepared from a 48-h blood agar culture as a saline suspension equivalent to a 2·0 McFarland standard. A replicator machine was used to inoculate the antibiotic plates. This technique places 1 μl of suspension per spot onto the agar dilution medium. Media was prepared containing doubled dilutions through a full range of concentrations for quinolone agents (nalidixic acid, norfloxacin and ciprofloxacin), tetracycline, ampicillin, gentamicin and macrolide agents (erythromycin, azithromycin, clarithromycin, roxithromycin). The inoculated plates were incubated microaerobically for 48 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration giving complete inhibition of visible growth on the plate. Interpretation of MIC levels was made with reference to accepted break-point values where available. *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used as quality-control strains.

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Multilocus sequence typing (*MLST*)

MLST was performed according to the method of Dingle *et al*. [14]. Briefly, *Campylobacter* isolates were subcultured from frozen stocks and DNA was extracted essentially according to the method of Pitcher *et al*. [15]. The seven loci were amplified and sequencing primers were used as described by Dingle *et al*. [14]. The amplification conditions were modified (denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min) with a total of 40 cycles performed. Amplification products were purified using the QIAquick® PCR Purification kit (Qiagen, Venlo, The Netherlands) and nucleotide sequences were determined using forward and reverse internal nested primers and the BigDye Terminator v.2 Ready Reaction Cycle Sequencing kit (Applied Biosystems). Unincorporated dye was removed with 75% (v/v) isopropanol, as per manufacturer's instructions, and the reaction products detected with the ABI Prism 3700 Capillary Sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned and assembled using the GeneBase computer program (Applied Maths, Kotrijk, Belgium). Sequences were assigned allele numbers and the isolates were assigned their sequence types by interrogation of the Campylobacter MLST database (http://campylobacter.mlst.net/). New sequences or allelic profiles were submitted to the database for assigning of new allele or sequence type numbers.

Pulse-field gel electrophoresis (*PFGE*)

PFGE was performed as previously described by Smith & Cantor [16]. Agarose plugs were prepared from a fresh culture on horse blood agar, organisms were prepared and washed in Pett IV (PIV) buffer (10 mM Tris–HCl, pH 7; 1 M NaCl) adjusted to 1·0–1·2 OD_{590}. The suspension was mixed with an equal volume of 2·4% low-melting-point agarose (Bio-Rad) then dispensed into plug moulds. One plug was placed into 1 ml ESP solution (0·5 M EDTA, pH 9·25; 1% Sarkosyl, 1 mg Proteinase K) and incubated overnight at 55 °C, then washed in 10 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8·0) containing 2 mM Pefabloc (Roche Diagnostics) for 2 h at 37 °C. There followed three washes in TE buffer for 20 min each then three washes in restriction endonuclease buffer of 15 min each. Restriction digestion was carried out with 20 U *Sma*I restriction endonuclease (New England Biolabs, Ipswich, MA, USA) overnight at 25 °C. PFGE using a Lambda ladder (Bio-Rad) as a standard was carried out using a CHEF Mapper (Bio-Rad) with a run time of 22·5 h at 12 °C. Switch times were 0·5–40 s with linear ramping.

Laboratory of Enteric Pathogens (*LEP*) *serotyping*

LEP serotyping was performed according to the method described by Frost *et al*. [4] with modification. This direct agglutination method employed absorbed antisera. Antisera were obtained from the Campylobacter Reference Unit, LEP, Central Public Health Laboratory, Colindale, UK. Each isolate was first tested with 12 antisera raised against 'common' serotypes. Only strains that did not react with these initial antisera were then tested with the full panel of 65 antisera. Reference antisera were diluted 40-fold and 25 μl aliquots were dispensed into U-bottomed microtitre trays. Direct agglutination was performed using growth harvested from 24–48 h plate culture, suspended in 1 ml of phosphate buffered saline (PBS) to produce a dense suspension which was heated at 100 °C for 30 min. The boiled suspension was added to 5 ml PBS to achieve opacity equivalent to McFarland Standard 4. Aliquots of 25 μl of the test suspension were added to the microtitre trays containing antisera. The trays were incubated at 50 °C in a moist atmosphere with gentle agitation in an orbital shaker and agglutination was read after 2 h. Isolates that did not agglutinate were incubated for a further 30 min and read again. Further titration of isolates agglutinating more than one standard antiserum was not undertaken.

Biotyping based on the Lior method

This was undertaken according to the method described by Lior [5]. Lior's biotyping scheme is based on rapid hippurate hydrolysis test, rapid H_2S production and a DNA hydrolysis test. The scheme was modified by replacing the H_2S test with gamma glutamyl amino peptidase and using a different DNA plate recipe (D. Wareing, Preston Public Health Laboratory, Preston, UK, personal communication).

Sequencing of the flaA *gene short variable region* (*SVR*–fla)

Typing by determination of the gene sequence (base positions 145–600) was performed according to the method described by Meinersmann *et al*. [17]. Briefly, a template was generated using primers described and the product was sequenced with a single pair of forward and reverse primers. Sequences were

assigned a type by submission to the website: http://outbreak.ceid.ox.ac.uk/campylobacter.

Restriction fragment length polymorphism (*RFLP*)

RFLP of the *flaA* gene (RFLP–*fla*) using the PCR product with a *Dde*I digestion (RFLP–*fla*–*Dde*I) or *Pst*I and *Eco*RI double digestion (RFLP–*fla*–*Pst*I/*Eco*RI), was based on the method described by Nachamkin *et al*. [18].

Ribotyping

Ribotyping was performed on the Automated RiboPrinter™ according to the manufacturer's (Qualicon, Wilmington, DE, USA) instructions using the restriction enzyme *Pst*I. In brief, *Pst*I-digested chromosomal DNA fragments were transferred from a gel to a membrane, denatured and visualized with a labelled ribosomal RNA probe.

Analysis

All experimental data were imported into a BioNumerics™ database. The gel images were imported as raw TIFF images. Numerical data were entered directly. The BioNumerics software was used to analyse banding patterns using the Dice band matching coefficient and UPGMA grouping method with a position tolerance of 1% and an optimization of 1%. The RFLP–*fla* patterns were divided into subtypes when the banding patterns were visually indistinguishable. Ribotypes were grouped into subtypes when the patterns were >90% similar and had no differing bands. Ribotype images were imported into BioNumerics from the gel image. Isolates typed by PFGE were grouped into subtypes when the patterns were >90% similar and had no different bands. The Tenover criteria [19] was not applied because isolates were collected over more than 1 year. SVR–*fla*, MLST, LEP serotypes and Lior biotype profiles were placed in a single subtype when the software found them to be identical using the categorical coefficient. Antimicrobial resistotypes were grouped into a subtype when >94% similar by the Euclidian distance coefficient.

A computer-generated dendrogram was created for each of the typing methods. Isolates were grouped into subtypes from the dendrograms according to the level of similarity mentioned above. Subtypes from banding patterns were visually checked. In some cases minor adjustments were made. In each typing method subtypes were numbered and the number of strains in each subtype counted in order to create the discrimination index (DI) [20]. The assigned subtype numbers of the different typing methods were used to sort the collection of isolates. 'Genotypic groupings' were created when five out of the six genotypic subtyping methods were in agreement. These genotypic groupings were analysed to determine two methods that in combination successfully delineated the assigned genotypic grouping and discriminated dissimilar strains.

Practical considerations of the subtyping methods

An algorithm was developed to allow comparison of the methods for speed, simplicity and cost. Participants determined the cost of typing methods by using a standard formula that included a labour cost and consumable cost component. This was based on an estimate of the time taken to prepare the specimen or isolate, perform the test, read, record, analyse and report the result. Labour costs were determined on a hourly rate for the minimum level of technician or scientist needed to perform the test from the annual salary taking into account annual leave, and public holidays. A 20% surcharge was added to cover departmental administration, clerical staff, cleaning, electricity and equipment depreciation. All calculations were based on performing optimal batch sizes for each method.

RESULTS

The 84 isolates included in this report were typed by all nine subtyping methods. The number of subtypes and the DI for all the subtyping methods are presented in Table 1. SVR–*fla* was the least discriminatory method among the genotypic subtyping methods and detected the least subtypes. SVR–*fla* had a DI of 0·87, RFLP–*fla* subtyping methods showed slightly higher DIs of 0·89 and 0·92 for the double digest and the single digest respectively. PFGE was the most discriminatory genotypic typing method with a DI of 0·97 followed by MLST with a DI of 0·96 and ribotyping with a DI of 0·95. Among the phenotypic subtyping methods serotyping had the highest DI of 0·90 followed by AR with a DI of 0·85. Biotyping by the Lior method with only four subtypes had the lowest DI of 0·58. The percentage of unique subtypes and the prevalence of the dominant subtypes are also presented in Table 1. Ribotyping results have been

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Table 1. *Results of the nine subtyping methods on the 84 isolates*

	SVR–*fla*	RFLP–*Pst*I/*Eco*RI	RFLP–*Dde*I	RT	MLST	PFGE	Sero-typing	Lior	AR
Number of subtypes	15	16	20	28	33	53	28	4	19
Discriminatory index	0·87	0·89	0·92	0·95	0·96	0·97	0·90	0·58	0·85
Percentage of unique subtypes	5%	7%	8%	12%	19%	50%	18%	1%	10%
Percentage of dominant type	30%	23%	16%	12%	13%	13%	25%	55%	33%
Percentage of 2nd dominant type	11%	17%	14%	10%	8%	7%	14%	35%	14%
Isolates/week	80	88	88	72	15	28	240	300	100
Complexity	Scientist	Tech	Tech	Scientist	Scientist	Tech	Tech	Tech	Tech
Ease of interpretation	+++	+	+	+++	+++	+	+++	+++	+
Cost/isolate	†	$18	$18.44	$125	$265	$64	†	†	$45
Availability	Yes	Yes	Yes	Yes*	Yes	Yes	Yes	Yes	Yes

SVR, Short variable region; RFLP, restriction fragment length polymorphism; RT, ribotyping; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; AR, antimicrobial resistotyping.
* Currently the automated RiboPrinter™ is only available at one public health laboratory.
† Costings for Lior biotyping, Laboratory of Enteric Pathogens (LEP) serotyping and SVR–*fla* were not available.
+++, Easy; +, difficult.

used to illustrate how the subtypes were generated from the dendrograms (Fig.). The designated subtype numbers of the nine typing exercises on the 84 isolates from sporadic campylobacteriosis cases are presented in Table 2.

There was very little correlation between subtypes within the phenotypic subtyping methods or between the phenotypic and genotypic subtyping methods. However, there was correlation between the six genotypic subtyping methods. Isolates that formed genotypic groupings with agreement in at least five of the six genotypic subtyping methods are placed together in Table 3. Fifteen such groupings were identified containing between two and eight isolates. Fifty isolates from the original 84 were, therefore, placed into one of the 15 genotypic groups (Table 3).

Within the genotypic groups the five methods most commonly in agreement were SVR–*fla*, RFLP–*fla*–*Dde*I, RFLP–*fla*–*Pst*I/*Eco*RI, ribotyping, and MLST. Three isolates typed by SVR–*fla* had a subtype that did not concur with their genotypic grouping. Two isolates had ribotyping that did not agree with their genotypic grouping. In one case the MLST subtype did not concur with its genotypic grouping and in 14 cases PFGE subtypes did not concur with their genotypic groupings. All isolates typed with both RFLP–*fla* methods had no different subtypes within the genotypic groupings. No subtyping method identified all of the genotypic groupings exclusively. The subtyping methods that delineated the genotypic groupings most successfully were MLST and ribotyping (Table 3).

Detecting the 15 genotypic groupings using a less discriminatory method in combination with a more discriminatory method

Investigation of the practical considerations of the various methods indicated that RFLP–*fla* typing was the cheapest method to perform, intermediate in the number of isolates that could be run within a week but regarded as difficult to interpret. MLST was regarded as easy to interpret but costly and with only a limited number of isolates that could be run within a week PFGE was intermediate in cost. Ribotyping was easy to perform but expensive (Table 1).

Table 4 shows that with SVR–*fla* as the first typing method and MLST as the second typing method all 15 genotypic groupings are revealed plus another six isolates that were not in the genotypic groupings and less four that were in the genotypic groupings. Another genotypic grouping of two isolates would have been added that other genotypic subtyping methods did not place. This resulted in 12 incorrectly placed isolates. Similarly other combinations of typing methods presented in Table 4 show how many isolates would be included in their genotypic grouping and how many would not be placed into their grouping. The combinations that resulted in the least number of incorrectly placed isolates were SVR–*fla*,

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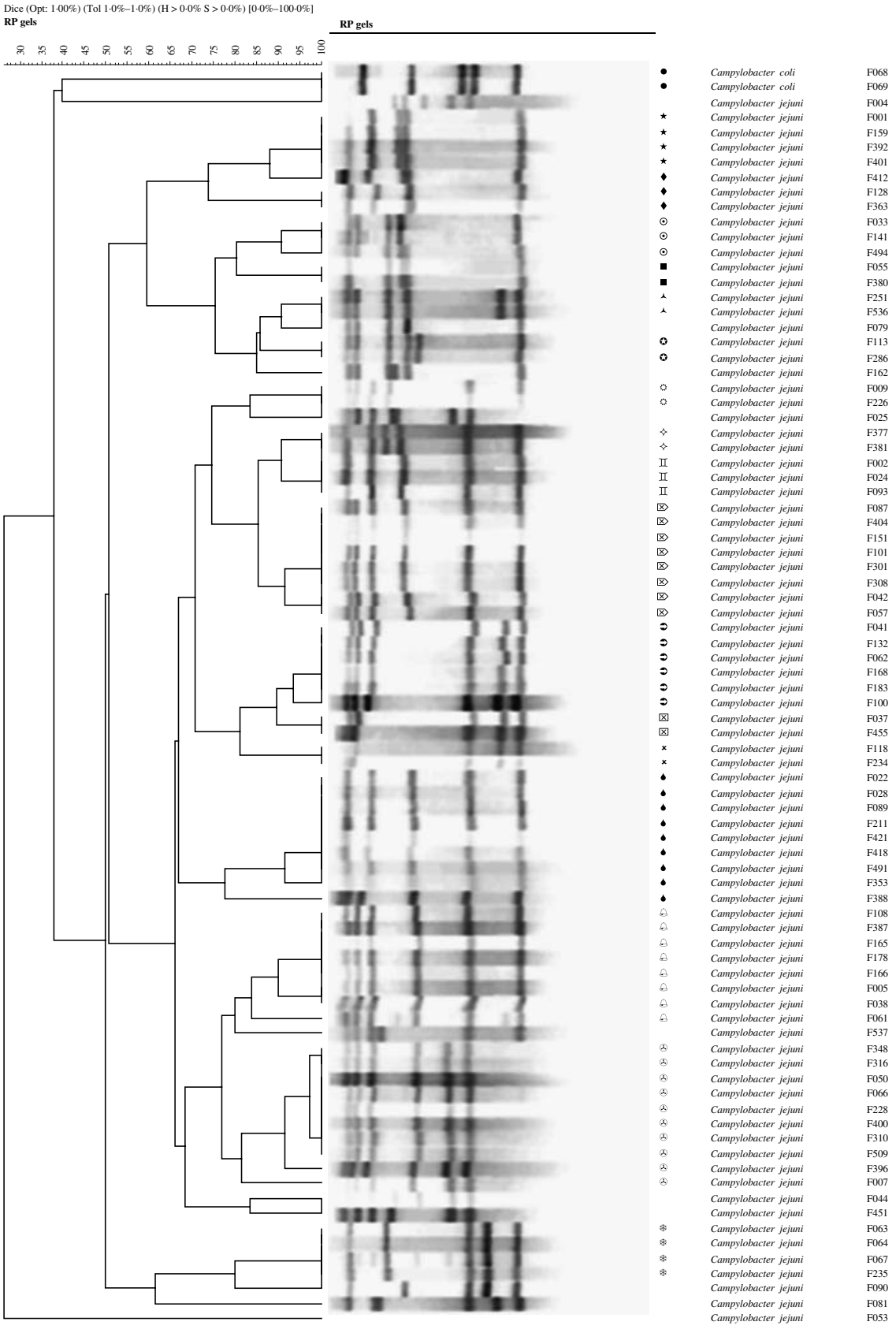

Fig. Dendrogram of the 84 isolates subtyped by ribotyping and grouped when the patterns were indistinguishable as indicated by the different symbols.

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RFLP–*fla* and ribotyping, followed by MLST or RFLP–*fla* followed by ribotyping. This analysis of the 84 isolates and the 15 genotypic groupings revealed those combinations of methods suitable for detecting genotypic groupings. This indicated that laboratories could use different genotypic methods for preliminary typing. The study also showed that the more discriminatory typing system agreed with or subdivided the groupings in the initial typing system.

Serotyping is frequently a primary typing tool and has been used in other studies to select isolates for subtyping comparison [9]. If PFGE or MLST were used to differentiate serotypes then six of the 15 genotypic groupings and 70% of the isolates in these genotypic groupings would have been seen.

Although there was a limited number of isolates some correlation could be seen between the genotypic groups and the identifiable risk exposures recorded for certain cases (Table 3). Group D cases occurred over the summer of 2000/2001 and were associated with exposure to eggs and fast food. Cases from groups F and O occurred continuously at low levels over the years 1999 to 2000 and had exposure to fast food as the common factor. Groups E and I cases occurred predominantly over 1999 and were linked by exposure to undercooked meat, seafood and restaurant food (OzFoodNet, personal communication).

DISCUSSION

While most *Campylobacter* subtyping studies have been performed in research settings, the aim of this study was to determine a typing method for regular use in public health reference laboratories. Our study, therefore, used a collection of *Campylobacter* isolates from public health investigations. The strength of this study was the number of different subtyping systems applied to the same set of isolates. This study also differed from others in that the isolates were collected systematically from clinical cases from the same geographical area over a 30-month period [5–7, 9].

The best typing methods for delineating genotypic groups were SVR–*fla*, RFLP–*fla*, or ribotyping followed by MLST, or RFLP–*fla* followed by ribotyping. It may be advantageous to use MLST and SVR–*fla* for typing because internationally recognized subtype numbers are available for these typing systems. Forming genotypic groupings based on the results of five or more of six subtyping methods is consistent with other work in this field [6, 7]. The discriminatory indices generated by typing methods used in our study were consistent with other work on *C. jejuni* [6]. Only three subtyping methods MLST, ribotyping and serotyping separated the two *C. coli* isolates from the *C. jejuni* isolates.

It has recently been reported that recombination events in *C. jejuni* are frequent [21] even in the absence of any selective pressure. Frequent recombination events between co-resident strains and intragenic alterations in the genome alter PFGE banding patterns but not MLST results [21]. MLST is reported to be a stable typing method suitable for bacterial populations which are weakly clonal [14]. No data is available on whether ribotyping results are affected by these recombination events in *Campylobacter*, although it is likely that ribotypes would be unaffected because the ribosomal RNA genes have been reported as stable [22]. The reliability of *flaA* typing has also been questioned because of possible recombination events in the *flaA* gene [23, 24]. The results of the present study did not support that view. RFLP–*fla* had no inconsistent subtype numbers within the genotypic groupings whereas SVR–*fla* had three. The stability of MLST, ribotyping, SVR–*fla* and RFLP–*fla* make these methods appropriate choices for a national typing system.

The genotypic markers appear to be more stable over time than phenotypic characteristics shown by the fact that 29 isolates within eight genotypic groupings had identical genotypic subtypes. Only two isolates had identical subtypes by all nine typing methods (F028 and F491), compared to 10 isolates with identical phenotypic subtypes in five genotypic groupings. Given the plasticity of the *Campylobacter* genome, this result may have been influenced by the progressive collection of our *Campylobacter* isolates over 30 months. RFLP–*fla* had no discordant subtype numbers within the genotypic groupings. Ribotyping, SVR–*fla* and MLST had occasional subtypes that did not agree with the genotypic grouping in which they were placed. This study found that serotyping did not group isolates into types that aligned with groups produced by other typing methods; a result that confirms the findings of another study that also showed disagreement between serotyping and other subtyping methods [25].

This study showed that a single typing method is not sufficient to delineate genotypic groupings of clinical *Campylobacter* isolates but may be able to identify time–space clusters or common source cases. Genotypic groupings were not delineated by any one typing method because overlapping subtype groups

Table 2. *Subtype numbers of the 84* Campylobacter *isolates generated from the BioNumerics dendrograms*

	Strain no.	SVR–*fla*	RFLP–*Pst*I/*Eco*RI	RFLP–*Dde*I	RT	MLST	PFGE	LEP serotype	Lior	AR
C. jejuni	F001	10	5	4	3	6	18	4	2	7
C. jejuni	F002	11	1	6	14	7	11	5	3	7
C. jejuni	F004	8	3	9	2	20	44	26	3	14
C. jejuni	F005	9	9	16	20	9	47	16	2	7
C. jejuni	F007	8	9	17	22	14	36	20	2	13
C. jejuni	F009	8	7	14	11	19	37	5	3	5
C. jejuni	F022	11	1	6	19	7	11	15	2	3
C. jejuni	F024	11	1	6	14	7	11	15	3	3
C. jejuni	F025	10	5	4	12	20	45	23	2	7
C. jejuni	F028	11	1	6	19	7	11	15	3	7
C. jejuni	F033	4	14	20	5	26	11	15	3	12
C. jejuni	F037	7	5	13	17	11	25	15	2	7
C. jejuni	F038	9	9	16	20	9	15	3	2	7
C. jejuni	F041	8	7	14	16	19	33	21	3	7
C. jejuni	F042	6	11	2	15	25	43	5	2	7
C. jejuni	F044	11	4	3	23	11	20	5	2	14
C. jejuni	F050	5	8	10	22	13	48	3	2	3
C. jejuni	F053	6	11	2	28	24	41	12	2	7
C. jejuni	F055	10	5	4	6	17	2	17	3	7
C. jejuni	F057	7	5	13	15	18	53	27	3	18
C. jejuni	F061	9	9	16	20	9	16	14	2	7
C. jejuni	F062	14	7	10	16	10	50	8	3	9
C. jejuni	F063	3	5	19	25	29	10	5	1	8
C. jejuni	F064	11	13	19	25	29	22	5	1	13
C. jejuni	F066	12	12	2	22	13	39	3	2	8
C. jejuni	F067	3	16	18	25	29	3	1	1	7
C. coli	F068	6	11	2	1	33	7	2	3	8
C. coli	F069	6	14	2	1	33	11	2	3	11
C. jejuni	F079	10	3	15	8	4	14	5	3	8
C. jejuni	F081	13	9	1	27	8	9	16	1	7
C. jejuni	F087	5	11	2	15	24	40	25	2	7
C. jejuni	F089	11	1	6	19	7	11	15	3	15
C. jejuni	F090	3	14	20	26	28	24	6	2	11
C. jejuni	F093	11	10	11	14	30	4	12	1	8
C. jejuni	F100	11	1	7	16	22	38	3	3	7
C. jejuni	F101	11	11	2	15	24	40	22	2	7
C. jejuni	F108	5	9	16	20	9	13	3	2	11
C. jejuni	F113	11	5	4	9	5	10	5	3	8
C. jejuni	F118	7	5	13	18	18	25	15	2	4
C. jejuni	F128	2	15	8	4	16	8	3	3	9
C. jejuni	F132	8	7	14	16	19	34	25	2	8
C. jejuni	F141	4	14	20	5	27	5	7	3	7
C. jejuni	F151	6	11	2	15	24	49	12	2	7
C. jejuni	F159	11	5	4	3	6	18	13	2	7
C. jejuni	F162	11	5	4	10	1	10	5	3	6
C. jejuni	F165	1	5	5	20	23	28	19	2	14
C. jejuni	F166	9	9	16	20	9	13	14	2	7
C. jejuni	F168	11	1	6	16	7	12	15	2	13
C. jejuni	F178	5	9	16	20	9	17	14	2	7
C. jejuni	F183	8	7	14	16	19	33	20	3	8
C. jejuni	F211	11	1	6	19	7	11	5	2	8
C. jejuni	F226	8	7	14	11	19	35	23	2	7
C. jejuni	F228	2	8	10	22	12	30	3	2	7
C. jejuni	F234	7	5	13	18	18	25	28	2	13
C. jejuni	F235	3	16	18	25	29	51	5	1	13

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Table 2 (*cont.*)

	Strain no.	SVR–*fla*	RFLP–*Pst*I/*Eco*RI	RFLP–*Dde*I	RT	MLST	PFGE	LEP serotype	Lior	AR
C. jejuni	F251	1	1	9	7	3	6	5	2	14
C. jejuni	F286	11	5	4	9	5	10	5	3	13
C. jejuni	F301	11	11	2	15	24	42	12	2	5
C. jejuni	F308	6	11	2	15	24	31	5	2	7
C. jejuni	F310	5	8	10	22	12	30	3	2	14
C. jejuni	F316	5	8	10	22	12	32	3	2	1
C. jejuni	F348	5	8	10	22	12	30	3	2	7
C. jejuni	F353	11	1	6	19	7	11	5	3	10
C. jejuni	F363	2	15	8	4	15	8	10	2	16
C. jejuni	F377	9	2	2	13	31	19	9	4	1
C. jejuni	F380	10	5	4	6	17	25	5	3	5
C. jejuni	F381	3	16	18	13	32	52	5	1	3
C. jejuni	F387	1	5	5	20	23	26	1	3	10
C. jejuni	F388	1	5	5	19	23	26	18	3	13
C. jejuni	F392	11	5	4	3	6	18	4	2	7
C. jejuni	F396	5	8	10	22	12	1	3	2	13
C. jejuni	F400	5	8	10	22	12	30	3	2	8
C. jejuni	F401	11	5	4	3	6	18	4	2	5
C. jejuni	F404	6	11	2	15	24	41	5	2	2
C. jejuni	F412	2	15	8	4	15	8	11	3	5
C. jejuni	F418	11	1	6	19	7	23	15	2	13
C. jejuni	F421	11	1	6	19	7	11	15	2	19
C. jejuni	F451	7	3	9	24	20	46	24	2	14
C. jejuni	F455	7	5	13	17	18	29	28	1	8
C. jejuni	F491	11	1	6	19	7	11	15	3	7
C. jejuni	F494	1	6	4	5	2	10	5	2	8
C. jejuni	F509	15	11	2	22	11	21	12	2	12
C. jejuni	F536	11	1	6	7	3	10	5	3	17
C. jejuni	F537	11	15	12	21	21	27	5	3	1

Abbreviations as in note to Table 1.

occurred in different genotypic groupings. Two methods did improve the delineation of isolate clusters but were insufficient to completely delineate the genotypic groupings. The subtypes generated by genotypic subtyping methods did not contradict each other but aligned with or subdivided the other subtyping methods.

The BioNumerics software performed well. The dendrograms were helpful in deciding on the grouping of subtypes and were important for typing systems that did not have an international numbering system. All typing exercises involving computerized band analysis with a program such as BioNumerics need to be standardized by consensual agreement. It is preferable to have at least three standards run on a gel and for the gels to be run for exactly the same length of time or for the same distance to provide better alignment of gel images from different laboratories. If this had occurred during our study, visual inspection of the gel images would have been unnecessary and the BioNumerics program could have been used to distinguish *Campylobacter* isolate groups and to calculate the discriminatory indices.

This study aimed to find a cost-effective and practical subtyping system for *Campylobacter* that can be used widely in public health laboratories throughout Australia. Analysis of results, speed, simplicity and cost indicated that using a cheaper method like RFLP–*fla* followed, if necessary, by MLST or ribotyping could be sufficiently accurate as a basis for future multicentre studies in Australia (Table 1). Although campylobacteriosis is the most reported foodborne disease in Australia, no subtyping method has proved epidemiologically useful in routine public health practice. This preliminary evaluation of *Campylobacter* typing methods used in Australian laboratories points the way to a more uniform approach to genotyping. However, the epidemiological

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Table 3. *Genotypic groupings formed on the basis of five out of the six genotypic subtyping methods showing agreement*

	Strain no.	SVR–*fla*	RFLP–*Pst*I/*Eco*RI	RFLP–*Dde*I	RT	MLST	PFGE	LEP serotype	Lior	AR
A	F388	1 (8)	5	5	19	23 (530)	26	18 (12, 37)	3	13 (ar)
	F165	1 (8)	5	5	20	23 (530)	28	19 (12)	2	14 (ar)
	F387	1 (8)	5	5	20	23 (530)	26	1 (23)	3	10 (ar)
B	F128	2 (11)	15	8	4	16 (527)	8	3 (18)	3	9 (a)
	F363	2 (11)	15	8	4	15 (5)	8	10 (3)	2	16 (t)
	F412	2 (11)	15	8	4	15 (5)	8	11 (60)	3	5 (art)
C	F067	3 (9)	16	18	25	29 (42)	3	1 (23)	1	7 (t)
	F235	3 (9)	16	18	25	29 (42)	51	5 (UT*)	1	13 (a)
D	F310	5 (20)	8	10	22	12 (528)	30	3 (18)	2	14 (zNIL)
	F316	5 (20)	8	10	22	12 (528)	32	3 (18)	2	1 (zNIL)
	F348	5 (20)	8	10	22	12 (528)	30	3 (18)	2	7 (zNIL)
	F396	5 (20)	8	10	22	12 (528)	1	3 (18)	2	13 (a)
	F400	5 (20)	8	10	22	12 (528)	30	3 (18)	2	8 (a)
	F228	2 (11)	8	10	22	12 (528)	30	3 (18)	2	7 (zNIL)
E	F108	5 (20)	9	16	20	9 (531)	13	3 (18)	2	11 (a)
	F178	5 (20)	9	16	20	9 (531)	17	14 (14, 50)	2	7 (a)
F	F151	6 (12)	11	2	15	24 (257)	49	12 (R†)	2	7 (zNIL)
	F308	6 (12)	11	2	15	24 (257)	31	5 (UT)	2	7 (zNIL)
	F404	6 (12)	11	2	15	24 (257)	41	5 (UT)	2	2 (ar)
	F053	6 (12)	11	2	28	24 (257)	41	12 (R)	2	7 (zNIL)
G	F118	7 (2)	5	13	18	18 (525)	25	15 (50)	2	4 (a)
	F234	7 (2)	5	13	18	18 (525)	25	28 (5, 50, 60, 62)	2	13 (a)
H	F041	8 (4)	7	14	16	19 (52)	33	21 (11, 13)	3	7 (ar)
	F132	8 (4)	7	14	16	19 (52)	34	25 (31)	2	8 (a)
	F183	8 (4)	7	14	16	19 (52)	33	20 (13)	3	8 (ar)
I	F005	9 (5)	9	16	20	9 (531)	47	16 (14)	2	7 (a)
	F038	9 (5)	9	16	20	9 (531)	15	3 (18)	2	7 (a)
	F061	9 (5)	9	16	20	9 (531)	16	14 (14, 50)	2	7 (a)
	F166	9 (5)	9	16	20	9 (531)	13	14 (14, 50)	2	7 (a)
J	F055	10 (10)	5	4	6	17 (524)	2	17 (37)	3	7 (a)
	F380	10 (10)	5	4	6	17 (524)	25	5 (UT)	3	5 (r)
K	F002	11 (1)	1	6	14	7 (48)	11	5 (UT)	3	7 (ar)
	F024	11 (1)	1	6	14	7 (48)	11	15 (50)	3	3 (ar)
L	F022	11 (1)	1	6	19	7 (48)	11	15 (50)	2	3 (art)
	F028	11 (1)	1	6	19	7 (48)	11	15 (50)	3	7 (ar)
	F089	11 (1)	1	6	19	7 (48)	11	15 (50)	3	15 (a)
	F211	11 (1)	1	6	19	7 (48)	11	5 (UT)	2	8 (ar)
	F353	11 (1)	1	6	19	7 (48)	11	5 (UT)	3	10 (a)
	F418	11 (1)	1	6	19	7 (48)	23	15 (50)	2	13 (ar)
	F421	11 (1)	1	6	19	7 (48)	11	15 (50)	2	19 (ar)
	F491	11 (1)	1	6	19	7 (48)	11	15 (50)	3	7 (ar)
M	F001	10 (10)	5	4	3	6 (227)	18	4 (4)	2	7 (zNIL)
	F159	11 (1)	5	4	3	6 (227)	18	13 (R)	2	7 (zNIL)
	F392	11 (1)	5	4	3	6 (227)	18	4 (4)	2	7 (ar)
	F401	11 (1)	5	4	3	6 (227)	18	4 (4)	2	5 (ar)
N	F113	11 (1)	5	4	9	5 (451)	10	5 (UT)	3	8 (ar)
	F286	11 (1)	5	4	9	5 (451)	10	5 (UT)	3	13 (a)
O	F101	11 (1)	11	2	15	24 (257)	40	22 (11)	2	7 (zNIL)
	F301	11 (1)	11	2	15	24 (257)	42	12 (R)	2	5 (ar)
	F087	5 (20)	11	2	15	24 (257)	40	25 (31)	2	7 (zNIL)

Abbreviations as in note to Table 1.
International codes are in parentheses following the BioNumerics group numberings.
SVR–*fla* sequence numbers from the website: http://outbreak.ceid.ox.ac.uk/campylobacter
Campylobacter MLST database at: http://campylobacter.mlst.net/
LEP serotype numbers with * UT, untypable; † R, rough colonies.
Resistotypes are denoted with a letter representing the antimicrobial to which the isolate is resistant. a, azithromycin; r, roxithromycin; t, tetracycline; c, ciprofloxacin; zNIL, not resistant [26].

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Table 4. *A comparison of combinations of two subtyping methods for delineation of the 15 genotypic groupings within the 84 isolates*

	First subtyping method			
	SVR–*fla*	RFLP–*fla*–*Dde*1	RFLP–*fla*–*Pst*I/*Eco*RI	RT
Second subtyping method				
MLST				
No of genotypic groupings	16	17	16	18
Isolates missing	4	1	1	3
No of non-conforming isolates*	12	10	8	11
PFGE				
No of genotypic groupings	10	11	11	9
Isolates missing	9	8	8	5
No of non-conforming isolates*	23	20	18	21
RT				
No. of genotypic groupings	20	19	16	
Isolates missing	3	1	2	
No. of non-conforming isolates*	15	11	6	

Abbreviations as in note to Table 1.

* Includes the number of isolates that would not be put into a genotypic grouping and the number that would be incorrectly placed into a grouping using two subtyping methods.

significance of these genotypic clusters has yet to be tested. The concordance between epidemiological data (case histories) and laboratory subtyping results will be evaluated in a successor study to include five Australian states. Analysis of the typing data using the BioNumerics software along with the associated case histories of patients should help OzFoodNet validate a uniform national typing approach for *Campylobacter*. Only when this further study has been completed will it be possible to establish whether the use of genotyping methods can assist public and environmental health measures to control *Campylobacter* infection in the Australian population.

APPENDIX. Australian Campylobacter Subtyping Study Group

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DECLARATION OF INTEREST

None.

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