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FEMS Microbiology Letters 216 (2002) 201–209

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Speciating *Campylobacter jejuni* and *Campylobacter coli* isolates from poultry and humans using six PCR-based assays

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Received 30 May 2002; received in revised form 12 September 2002; accepted 15 September 2002

First published online 12 October 2002

Abstract

Six previously published polymerase chain reaction (PCR) assays each targeting different genes were used to speciate 116 isolates previously identified as *Campylobacter jejuni* using routine microbiological techniques. Of the 116 isolates, 84 were of poultry origin and 32 of human origin. The six PCR assays confirmed the species identities of 31 of 32 (97%) human isolates and 56 of 84 (67%) poultry isolates as *C. jejuni*. Twenty eight of 84 (33%) poultry isolates were identified as *Campylobacter coli* and the remaining human isolate was tentatively identified as *Campylobacter upsaliensis* based on the degree of similarity of 16S rRNA gene sequences. Four of six published PCR assays showed 100% concordance in their ability to speciate 113 of the 116 (97.4%) isolates; two assays failed to generate a PCR product with four to 10 isolates. A *C. coli*-specific PCR identified all 28 hippuricase gene (*hipO*)-negative poultry isolates as *C. coli* although three isolates confirmed to be *C. jejuni* by the remaining five assays were also positive in this assay. A PCR–restriction fragment length polymorphism assay based on the 16S rRNA gene was developed, which contrary to the results of the six PCR-based assays, identified 28 of 29 *hipO*-negative isolates as *C. jejuni*. DNA sequence analysis of 16S rRNA genes from four *hipO*-negative poultry isolates showed they were almost identical to the *C. jejuni* type strain 16S rRNA sequences ATCC43431 and ATCC33560 indicating that assays reliant on 16S rRNA sequence may not be suitable for the differentiation of these two species.

Keywords: Diagnosis; Epidemiology; *Campylobacter jejuni*; *Campylobacter coli*

1. Introduction

Campylobacter spp. are recognised as a major cause of human enterocolitis [11,22]. The two most common species associated with enteritis are *C. jejuni* and *C. coli*, of which *C. jejuni* is responsible for the majority (80–90%) of infections [18]. *Campylobacter* spp. can be found in the intestinal tract of a wide variety of wild and domesticated animals without obvious clinical signs of disease. The consumption of contaminated food, water and unpasteurised milk and direct contact with infected animals represent confirmed routes of transmission to humans. Undercooked poultry represents one of the most common sources of human *Campylobacter* infection [8].

Campylobacters are fastidious, poorly hydrolyse a range of sugars and possess few biochemical characteristics that can reliably be used to distinguish between species [2,3]. Differentiation of *C. jejuni* from other *Campylobacter* spp. has relied on its ability to hydrolyse hippurate [22], although about 10% of *C. jejuni* isolates do not hydrolyse this metabolite. Genetic diversity within *C. jejuni* and *C. coli* has been well established and it has been suggested that this reflects the ubiquitous nature of these organisms in the environment [22]. Since large outbreaks of *Campylobacter* enterocolitis are rare and cases are often sporadic, it would be useful to have reliable typing methods to trace sources of infection and routes of transmission. Although several polymerase chain reaction (PCR)-based assays have been developed to facilitate the differentiation of *C. jejuni* from *C. coli* [6,7,9,10,13,15,19,23], the ability of some of these assays to accurately speciate a collection of *Campylobacter* isolates of veterinary and human origin has not been evaluated. In this study we examined the

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0378-1097/02/$22.00
PII: S0378-1097(02)01016-9

ability of six published PCR-based assays to identify 84 *Campylobacter* isolates cultured from a range of poultry species collected from 13 poultry processing plants across New South Wales, Australia and 32 clinical isolates from human infections from two hospitals in New South Wales, Australia. In addition we developed and evaluated a PCR–RFLP (restriction fragment length polymorphism) assay based on the 16S rRNA gene to speciate this collection.

2. Materials and methods

2.1. Bacterial strains

One hundred and sixteen isolates were used in this study. Eighty four isolates identified as *C. jejuni* and sourced from a range of poultry types were supplied by the Elizabeth Macarthur Agricultural Institute, Menangle, Australia. Nineteen human isolates previously identified as *C. jejuni* were supplied by the Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, Sydney, Australia. Another 13 isolates also previously identified as *C. jejuni* were supplied by the John Hunter Hospital (JHH), Newcastle, Australia. The origin of each isolate is listed in Table 1.

2.2. Identification of C. jejuni isolates

All isolates were cultured on Skirrow's agar (Oxoid, SR69) and sheep blood agar (Oxoid, CM854) in a microaerophilic atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at 37°C for 48 h. Isolate identification was based on colony morphology (low, flat, greyish, finely granular and translucent colonies that may spread and swarm or 1–2 mm in diameter, raised, convex, smooth and glistening colonies with an entire edge), typical microscopic appearance in carbol fuchsin-stained smears (small tightly coiled spirals) [2] and positive motility, oxidase, catalase [3] and hippurate hydrolysis tests. Hippurate hydrolysis was determined using hippurate-containing tablets (Rosco Diagnostica, Taastrup, Denmark) used according to the manufacturer's instructions. *Streptococcus agalactiae* and *Erysipelothrix rhusiopathiae* were used as positive and negative controls respectively for the hippurate hydrolysis test.

2.3. DNA extraction

DNA was extracted by one of two methods. Purified whole cell DNA was extracted as described previously [4]. Crude whole cell DNA was extracted using InstaGene™ matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, an isolated bacterial colony was resuspended in 1 ml of sterile, pure water (milli Q), centrifuged at 11 000 × *g* for 1 min and the supernatant was removed. InstaGene™ matrix (200 μl) was added to the pellet, vortexed briefly and incubated at 56°C for 30 min. The solution was vortexed for 10 s and heated to 100°C for 8 min, briefly vortexed and centrifuged at 11 000 × *g* for 150 s after which 1 μl was used for PCR.

2.4. PCR amplification

For previously published PCR assays, the sources of the primers and reaction conditions used in this study are listed in Table 2. Primers CJ16SF and CJ16SR which amplify a 1426-bp fragment of the 16S rRNA gene from different *Campylobacter* spp. were designed based upon conserved regions of the 16S rRNA gene sequences from 15 different species of *Campylobacter* including *C. jejuni* strains ATCC33560, CCUG11284, and CCUG24567 (accession numbers M59298, L04315, and L14630 respectively), *C. coli* strains ATCC3359 and CCUG11283 (acces-

Table 1
Bacterial strains used in this study

Strain No.	Source	Location	No. of strains
ICPMR	Institute Of Clinical Pathology and Medical Research	Sydney, central NSW	19
JHH	John Hunter hospital	Newcastle, central NSW	13
98/C053	Domestic poultry	Beresfield, NSW	14
98/C719	Domestic poultry	Lisarow, central NSW	13
98/E600	Domestic poultry	Girraween, NSW	9
98/E599	Domestic poultry	Girraween, NSW	9
99/2933	Domestic poultry	Cardiff, central NSW	1
99/6120	Domestic poultry	Moorebank, central NSW	1
98/E003	Chinese silky	Baerami, northern NSW	6
98/E066	Corn-fed chickens	Tamworth, NSW	10
99/3577	Ducks	Shanes Park, northern NSW	3
99/3912	Spatchcock	Bringelly, central NSW	10
99/6027	Quail	Kemps Creek, central NSW	5
99/6454	Quail	Box Hill, central NSW	1
99/A778	Jewish kosher chickens	Austral, central NSW	2

Table 2
Oligonucleotide primers and PCR programmes used in this study

Primer pair	Target gene	Primer sequence (5′–3′)	Reaction temp. (°C)			Product size (bp)	References
			denaturation	annealing	extension		
HIP400F	*hipO*	GAAGAGGGTTTGGGTGGTG	94 (60 s)[a]	66 (60 s)	72 (60 s), 25 cycles	735	[13]
HIP1134R		AGCTAGCTTCGCATAATAACTTG					
C1	Putative oxidoreductase gene (PID 6967888)	CAAATAAAGTTTAGAGGTAGAATGT	94 (60 s)	49 (60 s)	72 (60 s), 25 cycles	160	[9,23]
C4		GGATAAGCACTAGCTAGCTGAT					
CJ16SF	16S rRNA gene	GCCTAATACATGCAAGTCG	94 (60 s)	55 (60 s)	72 (60 s), 25 cycles	1426[b]	This study
CJ16SR		GATTCCACTGTGGACGGT					
CC18F	Putative aspartokinase gene and flanking ORF	GGTATGATTTCTACAAAGCGAG	94 (60 s)	60 (60 s)	72 (60 s), 25 cycles	500	[13]
CC519R		ATAAAAGACTATCGTCGCGTG					
CJAG48-1L	*mapA*	ATGTTTAAAAAATTTTTG	94 (60 s)	43 (60 s)	72 (60 s), 35 cycles	641	[19]
CJAG48-1R		AAGTTCAGAGATTAAACTAG					
THERM1	23S rRNA gene	TATTCCAATACCAACATTAGT	94 (60 s)	56 (60 s)	72 (60 s), 35 cycles	491[b]	[6,7]
THERM4		CTTCGCTAATGCTAACCC					
BO4263	Unknown	AGAACACGCGGACCTATATA	94 (45 s)	60 (45 s)	72 (45 s), 35 cycles	256[b]	[10]
BO4264		CGATGCATCCAGGTAATGTAT					

[a]The value in parentheses is the reaction time.
[b]This is the size of the product before digestion.

sion numbers M59073 and L04312 respectively), *Campylobacter hyointestinalis* strains ATCC35217 and NADC2006 (accession numbers M65010 and M65009), *Campylobacter showae* strains CCUG3054 (accession number L06974), *Campylobacter rectus* strain CCUG19168 (accession number L06973), *Campylobacter concisus* strain FDC288 (accession number L06977), *Campylobacter curvus* strain C10Etoh (accession number L06976), *Campylobacter hominis* strain NCTC13146 (accession number AJ251584), *Campylobacter lari* strain CCUG23947 (accession number L04316), *Campylobacter fetus* subsp. *fetus* strain ATCC27374 (accession number M65012), *C. fetus* subsp. *venerialis* strain ATCC19438 (accession number M65011), *Campylobacter helveticus* strain NCTC12470 (accession number U03022), and *Campylobacter upsaliensis* strain CCUG14913T (accession number L14628). The 1426-bp fragment of the 16S rRNA gene was used as a target for RFLP analyses to differentiate between the thermophilic *Campylobacter* spp. 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 10 ng of genomic DNA (or 1 μl of InstaGene™ prepared DNA) except for the amplification of the *hipO* and putative aspartokinase genes described by Linton et al. [13] where 20 mM Tris–HCl (pH 8.3) and 2.5 mM $MgCl_2$ was used. All PCR experiments were performed on a PC-960G gradient thermal cycler or PC-960 and FTS thermal cyclers (Corbett Research, Australia). Fragments were analysed on 1% agarose for the *hipO* [13], putative oxidoreductase gene [9], the *C. coli*-specific putative *aspA* and flanking sequence [13] and *mapA* [19] PCRs. RFLP fragments were analysed on 0.7% agarose gels for the 16S rRNA assay and on 2% agarose gels for assays described by Fermer and Engvall, [7] and Jackson et al. [10]. All gels were electrophoresed in 0.5× Tris–borate EDTA buffer, visualised with ethidium bromide and recorded electronically using a GelDoc 1000 (Bio-Rad, Richmond, CA, USA).

2.5. RFLP analyses

Amplification products (1426 bp) from the 16S rRNA gene PCR developed for this study were digested separately with *Rsa*I and *Cfo*I (37°C, 16 h). Digestion products were separated on 2.5% agarose, stained with ethidium bromide and visualised as described above. Products amplified using PCRs developed by Fermer et al., [7] and Jackson et al. [10] were digested (37°C, 16 h) with *Alu*I and *Dra*I respectively.

2.6. 16S rRNA gene sequencing

16S rRNA gene sequencing was performed as described previously [12]. Sequencing was done using the dRhod-

Table 3
Summary of PCR results for avian *Campylobacter* spp.

Strain ID	*hipO* PCR	Harmon PCR	Aspartokinase PCR	*mapA* PCR	Fermer PCR–RFLP	Jackson PCR–RFLP
98/CO53/1[a]	−	−	+	−	*C. coli*	*C. coli*
98/CO53/2[a]	−	−	+	−	*C. coli*	*C. coli*
98/CO53/3[a]	+	+	−	+	*C. jejuni*	*C. jejuni*
98/CO53/4[a]	−	−	+	−	*C. coli*	*C. coli*
98/CO53/5[a]	−	−	+	−	*C. coli*	*C. coli*
98/CO53/6[a]	−	−	+	−	*C. coli*	*C. coli*
98/CO53/7[a]	+	+	−	+	*C. jejuni*	*C. jejuni*
98/CO53/8	+	+	−	+	*C. jejuni*	*C. jejuni*
98/CO53/9[a]	+	+	−	+	*C. jejuni*	*C. jejuni*
98/CO53/10[a]	+	+	−	+	*C. jejuni*	*C. jejuni*
98/CO53/11[a]	−	−	+	−	*C. coli*	*C. coli*
98/CO53/12[a]	−	−	+	−	*C. coli*	*C. coli*
98/CO53/13[a]	−	−	+	−	*C. coli*	*C. coli*
98/CO53/14[a]	−	−	+	−	*C. coli*	*C. coli*
98/C719/1	−	−	+	−	*C. coli*	*C. coli*
98/C719/3	−	−	+	−	*C. coli*	*C. coli*
98/C719/4	+	+	−	+	*C. jejuni*	*C. jejuni*
98/C719/5	−	−	+	−	*C. coli*	*C. coli*
98/C719/6	−	−	+	−	*C. coli*	*C. coli*
98/C719/8	+	+	−	+	*C. jejuni*	*C. jejuni*
98/C719/9	−	−	+	−	*C. coli*	*C. coli*
98/C719/10	−	−	+	−	*C. coli*	*C. coli*
98/C719/11	+	+	−	+	*C. jejuni*	*C. jejuni*
98/C719/12	+	+	−	+	*C. jejuni*	*C. jejuni*
98/C719/13	+	+	−	+	*C. jejuni*	*C. jejuni*
98/C719/14	+	+	−	+	*C. jejuni*	*C. jejuni*
98/C719/15	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E600/1	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E600/2	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E600/4	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E600/5	+	+	−	−	NP	*C. jejuni*
98/E600/6	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E600/7	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E600/8	+	+	−	+	NP	*C. jejuni*
98/E600/9[a]	−	−	+	−	*C. coli*	*C. coli*
98/E600/10	−	−	+	−	*C. coli*	*C. coli*
98/E599/1	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E599/2	−	−	+	−	*C. coli*	*C. coli*
98/E599/3	−	−	+	−	*C. coli*	*C. coli*
98/E599/4	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E599/5[a]	−	−	+	−	*C. coli*	*C. coli*
98/E599/6[a]	−	−	+	−	*C. coli*	*C. coli*
98/E599/7	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E599/8	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E599/10	+	+	+	−	*C. jejuni*	*C. jejuni*
99/6120/3	+	+	−	+	*C. jejuni*	*C. jejuni*
98/2933/7	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E003/1	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E003/2	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E003/3	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E003/4	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E003/5	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E003/6	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E066/1[a]	−	−	+	−	*C. coli*	*C. coli*
98/E066/2	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E066/3[a]	−	−	+	−	*C. coli*	*C. coli*
98/E066/4	−	−	+	−	*C. coli*	*C. coli*
98/E066/5	−	−	+	−	*C. coli*	*C. coli*
98/E066/6	−	−	+	−	*C. coli*	*C. coli*
98/E066/7	−	−	+	−	*C. coli*	*C. coli*
98/E066/8	+	+	−	+	*C. jejuni*	*C. jejuni*
98/E066/9[a]	−	−	+	−	*C. coli*	*C. coli*
98/E066/10	+	+	−	+	*C. jejuni*	*C. jejuni*

Table 3 (*Continued*).

Strain ID	*hipO* PCR	Harmon PCR	Aspartokinase PCR	*mapA* PCR	Fermer PCR–RFLP	Jackson PCR–RFLP
99/3912/1	+	+	−	+	NP	*C. jejuni*
99/3912/2	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3912/3	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3912/4	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3912/5	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3912/6	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3912/7	+	+	+	−	*C. jejuni*	*C. jejuni*
99/3912/8	+	+	−	−	*C. jejuni*	*C. jejuni*
99/3912/9	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3912/10	+	+	−	+	*C. jejuni*	*C. jejuni*
99/A778/7	+	+	−	+	*C. jejuni*	*C. jejuni*
99/A778/8	+	+	−	+	*C. jejuni*	*C. jejuni*
99/6454/3	+	+	−	+	NP	*C. jejuni*
99/6027/1	+	+	−	+	ND	*C. jejuni*
99/6027/3	+	+	−	+	NP	*C. jejuni*
99/6027/4	+	+	−	+	NP	*C. jejuni*
99/6027/5	+	+	−	+	NP	*C. jejuni*
99/6027/8	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3577/1	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3577/3	+	+	−	+	*C. jejuni*	*C. jejuni*
99/3577/9	+	+	−	+	*C. jejuni*	*C. jejuni*

NP: No product. ND: Not determined

[a]Isolates used in Southern hybridisation experiments with the 735-bp DIG-labelled *hipO* fragment.

amine Dye Terminator kit (Applied Biosystems, Foster City, CA, USA) and run on an AB310 automated sequencer (Applied Biosystems). Sequences were edited on both strands using Sequencher™ software (GeneCodes, Ann Arbor, MI, USA) and the resulting proof-read sequences were compared using the BLAST algorithm [1].

2.7. Southern hybridisation

*Cfo*I digested DNA (37°C, 16 h) from a random selection of 14 of 28 *hipO* PCR-negative *Campylobacter* isolates (Table 3) were electrophoretically separated using 0.7% agarose and transferred to nylon Hybond H^+ membrane (Amersham, Little Chalfont, UK) using standard procedures [17]. DNA was fixed to the membrane using UV light and the membrane was probed using a DIG-labelled 735-bp fragment of the *hipO* gene amplified using primers described by Linton et al. [13]. Membranes were washed twice under stringent conditions (2× SSC, 0.1% SDS at 68°C, 15 min) as recommended by the manufacturer (Roche Diagnostics, Castle Hill, Australia) prior to exposure to light-sensitive film.

2.8. Nucleotide accession numbers

16S rRNA gene sequences representing *Campylobacter* isolates 98/C053/1, 98/C053/2, 98/C053/11, 98/C053/12, ICPMR6, ICPMR11, 98/E599/10 and 98/E600/5 have been deposited in the EMBL database with the accession numbers AF372093, AF372094, AF372095, AF372096, AF393205, AF393204, AF393203 and AF393202 respectively.

3. Results

3.1. Identification of bacterial isolates

One hundred and sixteen isolates comprising 84 from various poultry sources and 32 clinical isolates of human origin were putatively identified as *C. jejuni* based on morphological characteristics and biochemical reactions. Repeat tests using the hippurate assay suggested that some isolates unreliably hydrolysed hippurate indicating their species identity was questionable. Linton et al. [13] described a PCR for the amplification of a portion of the hippuricase (*hipO*) gene. Using this PCR we confirmed that 56 of 84 (67%) poultry isolates and 31 of 32 (97%) human isolates contained the *hipO* gene; the remaining 29 isolates failed as template for amplification of the 735 bp fragment (Tables 3 and 4). Southern hybridisation analyses of 14 of 28 (50%) *hipO*-negative poultry isolates using a DIG-labelled 735 bp fragment of the *hipO* gene confirmed that these isolates did not possess *hipO*; the remaining *hipO* negative isolates were not tested (Table 3; data not shown). These data indicated that 31 of 32 human isolates and 56 of 84 poultry isolates were *C. jejuni*. The 29 *hipO*-negative isolates could be other *Campylobacter* species or represent *hipO*-negative *C. jejuni* [14,20] and further analyses were required to determine their species identities.

3.2. 16S rRNA PCR–RFLP and gene sequence analyses

RFLP analysis of the 16S rRNA gene has been used successfully to identify a wide range of bacterial species [21]. Alignment of five 16S rRNA genes deposited in pub-

Table 4
Summary of PCR results for *C. jejuni* isolates from human sources

Strain ID	*hipO* PCR	Harmon PCR	Aspartokinase PCR	*mapA* PCR	Fermer PCR–RFLP	Jackson PCR–RFLP
ICPMR/1	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/2	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/3	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/4	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/5	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/6	–	–	–	–	ND	ND
ICPMR/7	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/8	+	+	–	+	NP	*C. jejuni*
ICPMR/9	+	+	–	+	NP	*C. jejuni*
ICPMR/10	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/11	+	+	+	+	ND	*C. jejuni*
ICPMR/12	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/13	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/14	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/15	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/16	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/17	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/18	+	+	–	+	*C. jejuni*	*C. jejuni*
ICPMR/19	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/1	+	+	–	+	NP	*C. jejuni*
JHH/2	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/3	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/5	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/9	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/15	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/17	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/18	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/20	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/21	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/22	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/23	+	+	–	+	*C. jejuni*	*C. jejuni*
JHH/26	+	+	–	+	*C. jejuni*	*C. jejuni*

NP: No product. ND: Not determined.

lic databases for *C. jejuni* strains ATCC33560, CCUG11284, CCUG24567 (accession numbers M59298, L04315 and L14630 respectively) and *C. coli* strains ATCC3359 and CCUG11283 (accession numbers M59073 and L04312 respectively) showed that the sequences shared greater than 99.5% sequence similarity within each of these species and 98% similarity between each of these species (data not shown). However, there was sufficient sequence variation to develop an RFLP assay to differentiate between *Campylobacter* species. An assay was developed with the primary aim of determining the species identities of the 29 *hipO*-negative isolates. PCR primers (Table 2) designed to conserved regions at the 5′- and 3′-end of 16S rRNA genes from a variety of *Campylobacter* spp. were designed and used to amplify a 1426-bp fragment from 35 isolates including all 29 *hipO*-negative; six *hipO*-positive isolates were included as *C. jejuni*-positive controls. The remaining 87 *hipO*-positive isolates were not examined in this assay as they were considered to be *C. jejuni* based on a positive *hipO* PCR result and other morphological characteristics.

Digestion of PCR-amplified 16S rRNA genes from *C. jejuni* and *C. coli* with *Rsa*I and *Cfo*I generated profiles capable of differentiating these two species (Fig. 1A). The six *hipO*-positive isolates (*C. jejuni* positive control isolates) generated a RFLP profile with both *Rsa*I (510, 419, 336, 77, 54, 14 bp) and *Cfo*I (363, 353, 264, 220, 184, 63 bp) that was consistent with a *C. jejuni* 16S rRNA gene sequence although fragments less than 100 bp could not be resolved (Fig. 1A). Control *C. coli* strains ATCC3359 and LMG 6440 generated RFLP profiles with *Cfo*I (757, 363, 264, 63 bp) and *Rsa*I (510, 419, 405, 77 bp) that were consistent with predicted cleavage sites determined from 16S rRNA gene sequences of *C. coli* strains ATCC3359 and CCUG11283 (accession numbers M59073 and L04312 respectively (Fig. 1A). RFLP profiles representative of the 28 of 29 *hipO*-negative *Campylobacter* isolates surprisingly were consistent with *C. jejuni* 16S rRNA gene sequences of strains ATCC33560, CCUG11284, CCUG24567; we were unable to amplify a 1426-bp fragment from isolate ICPRM/6. These data suggested that these 28 *hipO*-negative isolates possessed 16S rRNA gene(s) typical of *C. jejuni*.

The 16S rRNA gene sequences derived from the four *hipO*-negative isolates 98/C053/1, 98/C053/2, 98/C053/11, 98/C053/12 (EMBL accession numbers AF372093, AF372094, AF372095, AF372096 respectively) showed 99.7% (three mismatches in 1060 bp) identity to the *C.*

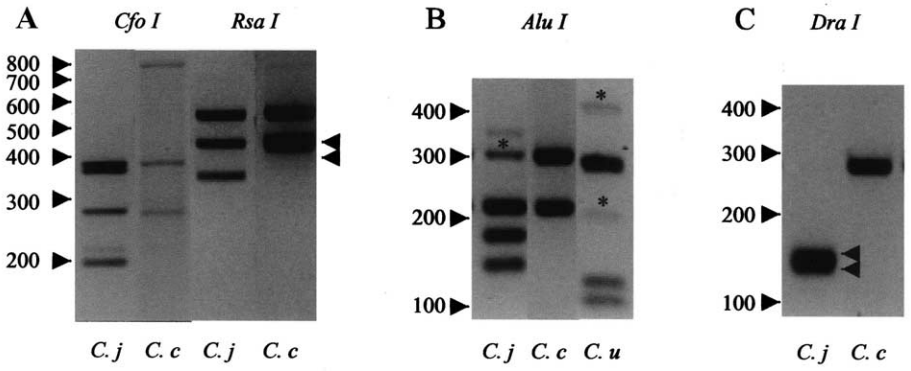

Fig. 1. Agarose gel electrophoresis of representative RFLPs used to type *Campylobacter* spp. A: 16S rRNA gene fragments amplified using primers CJ16SF and CJ16SR from DNA isolated from *C. jejuni* (*C. j*) and *C. coli* (*C. c*) separately digested with *Cfo*I and *Rsa*I. B: RFLP analysis of 491-bp fragments of the 23S rRNA gene amplified from *C. jejuni* (*C. j*), *C. coli* (*C. c*) and *C. upsaliensis* (*C. u*) and digested with *Alu*I. C: RFLP analysis of a 256-bp fragment amplified from *C. jejuni* (*C. j*) and *C. coli* (*C. c*) and digested with *Dra*I. Asterisks indicate partially digested DNA fragments. Molecular mass markers (bp) are indicated and arrowheads denote doublets.

jejuni type strain NCTC 11351T sequence (accession number AF372091). In comparison, the four 16S rRNA gene sequences showed 98.2% (19 mismatches in 1060 bp) sequence identity with the *C. coli* type strain LMG 6440 sequence (accession number AF372092). Based on 16S rRNA gene sequence analysis the *hipO*-negative isolates were identified as *C. jejuni* confirming the findings of the 16S rRNA PCR–RFLP analysis described above (data not shown). 16S rRNA gene sequence analyses (AF393205) also showed that the *hipO*-negative human strain (ICPMR/6) possessed a 16S rRNA gene sequence with greatest identity (99.8%, three mismatches in 1341 bp) to *C. upsaliensis* type strain CCUG14913T (accession number L14628; see later).

3.3. Molecular analyses of Campylobacter isolates

Several PCR-based assays have been developed to differentiate between *C. jejuni* and *C. coli* [6,7,9,10,13,15,19,23]. A *C. jejuni*-specific PCR described by Harmon et al. [9] was reported to amplify a 160-bp fragment of an unidentified gene. Alignment of primer sequences with the *C. jejuni* genome sequence [16] enabled us to identify the target gene as a 160-bp fragment of a putative oxidoreductase subunit gene (PID 6967888; Cj0414). Amplification of a 160-bp portion of the putative oxidoreductase occurred among 31 of 32 human isolates and 56 of 84 poultry isolates (Tables 3 and 4) in a pattern that matched exactly the pattern generated using the *hipO* PCR further confirming that these isolates were *C. jejuni*. None of the *hipO* PCR negative isolates were template for amplification of this fragment further adding to the difficulty in speciating these isolates and contradicting the results of 16S rRNA gene sequence analyses.

3.4. Amplification of C. coli-specific gene sequences

Linton et al. [13] described the development of a PCR assay that amplifies a 500-bp fragment of a *C. coli*-specific fragment containing the 3′-end of a putative aspartokinase gene and a downstream short open reading frame (ORF) encoding a gene of unknown function. A 500-bp fragment was amplified in only one of 32 (ICPMR/11) human isolates (Table 4). A 500-bp fragment was amplified from 30 poultry isolates (Table 3). Twenty-eight of these isolates were negative for both the *hipO* and putative oxidoreductase genes consistent with a *C. coli* genotype (Table 3). Two poultry isolates (98/E599/10 and 99/3912/7) were positive for the *hipO* and putative oxidoreductase genes (Table 3) as was human isolate ICPMR11 (Table 4). The species identities of isolates 98/E599/10, 99/3912/7 and ICPMR/11 could not be confirmed without further analyses. 16S rRNA gene sequence analysis of isolates 98/E599/10 and ICPMR11 showed greatest identity to *C. jejuni* type strain NCTC11351T (99.8%, three mismatches in 1341 bp).

3.5. Amplification of the C. jejuni-specific mapA gene

Stucki et al. [19] cloned and characterised a *C. jejuni*-specific membrane protein (*MAPA*) and showed that amplification of a 641-bp fragment of the *mapA* gene could reliably differentiate *C. jejuni* from other *Campylobacter* species. A 641-bp fragment of *mapA* was amplified from 31 of 32 human isolates, a result consistent with the *hipO* and putative oxidoreductase PCR assays (Table 4). The *mapA* fragment was amplified from 52 of 84 poultry isolates (Table 3). With the exception of a single human isolate (ICPMR/11), all human and poultry isolates positive for the *mapA* PCR were negative in the *C. coli*-specific PCR described by Linton et al. [13] (Tables 3 and 4). All 52 *mapA*-positive poultry isolates were also positive for the *hipO* and putative oxidoreductase genes confirming these isolates as *C. jejuni* (Table 3). Four isolates (98/E600/5, 98/E599/10, 99/3912/7, 99/3912/8), all *mapA*-negative, were positive for both the *hipO* and oxidoreductase genes; two of these (98/E599/10, 99/3912/7) were positive in the *C. coli*-specific PCR. These four isolates were considered

to be atypical *Campylobacter* isolates and required further analyses to confirm their species identity. 16S rRNA gene sequences of isolates 98/E600/5 and 98/E599/10 showed 99.9% (one mismatch in 1341) and 99.8% (three mismatches in 1341) identity respectively to *C. jejuni* type strains ATCC43431 and NCTC11351T.

3.6. Identification of C. jejuni, C. coli, C. upsaliensis and C. lari based on RFLP analyses of the 23S rRNA gene

Fermer and Engvall [7] described primers capable of amplifying a 491-bp fragment of the 23S rRNA gene of these four *Campylobacter* spp. RFLP analysis using the restriction endonucleases *Alu*I and *Tsp*509I was reported to generate specific restriction patterns for each species. RFLP analyses of the 491-bp fragment identified 27 of 32 human isolates as *C. jejuni* (Fig. 1B); the PCR failed to amplify a product from three isolates (ICPMR/8, ICPMR/9 and JHH1) (Table 4). The results for human isolates ICPMR/6 and ICPMR/11 were inconclusive. RFLP analyses of the poultry isolates identified 48 of 84 (57%) isolates as *C. jejuni*; seven isolates failed as a template for amplification of the 491-bp fragment and one isolate (99/6027/1) showed inconclusive results (Table 3).

3.7. Identification of C. jejuni, C. coli and C. upsaliensis based on RFLP analyses of a novel gene

Jackson et al. [10] described a PCR assay that amplifies a 256-bp fragment of an ORF immediately downstream of a novel two-component regulator gene from the thermophilic *Campylobacter* spp. *C. jejuni*, *C. coli* and *C. upsaliensis*. Digestion of the 256-bp fragment with restriction endonucleases *Alu*I, *Dde*I and *Dra*I was sufficient to differentiate between these three *Campylobacter* species [10]. RFLP analyses of the 256 bp fragment identified 31 of 32 human *Campylobacter* isolates as *C. jejuni* (Fig. 1C). Isolate ICPMR6 (negative for the *hipO*, oxidoreductase, *mapA* PCRs and the *C. coli*-specific PCR) could not be accurately characterised using this assay. 16S rRNA gene sequence analysis of isolate ICPMR/6 showed greatest identity to *C. upsaliensis* type strain CCUG14913T. RFLP analyses of the 84 poultry isolates showed that 56 were *C. jejuni*. Of these, 52 (93%) were PCR-positive for the *hipO*, *mapA* and oxidoreductase genes and negative in the *C. coli*-specific PCR. The remaining four isolates (98/E600/5, 98/E599/10, 99/3912/7, 99/3912/8) appeared to be *mapA*-negative *C. jejuni*; two of these (98/E599/10, 99/3912/7) were positive for the *C. coli*-specific gene fragment.

4. Discussion

Several PCR-based assays have been developed to determine the species identity of *Campylobacter* spp. although we were unable to find any reports comparing the accuracy of these assays on a geographically diverse collection of *Campylobacter* isolates of poultry and human origin. We used a panel of six published PCR assays to speciate a collection of 116 *Campylobacter* isolates which had been previously identified as *C. jejuni* based on their ability to hydrolyse hippurate. We confirmed that 87 of 116 isolates were *C. jejuni*; 28 of the remaining 29 isolates were shown to be *C. coli*. One isolate (ICPMR/6) could not be identified using any of the published assays but had a 16S rRNA gene sequence with highest sequence identity to the *C. upsaliensis* type strain. However, the PCR-RFLP assays of Fermer and Engvall [7] and Jackson et al. [10] could not confirm this tentative species identification. PCR assays that amplified a putative oxidoreductase [9] and *hipO* [13] and a PCR–RFLP assay developed by Jackson et al. [10] each confirmed that 56 of 84 poultry and 31 of 32 human isolates were *C. jejuni* (100% concordance). The assay of Stucki et al. [19] identified 52 of 84 poultry and 31 of 32 human isolates as *C. jejuni*: four poultry isolates (98/E600/5, 98/E599/10, 99/3912/7 and 8) confirmed to be *C. jejuni* based on the collective results of a minimum of three other molecular assays were negative in this assay. The inability to amplify a PCR product with these four isolates may reflect minor sequence variations in either one or both primer sites or the presence of PCR inhibitors in the whole cell DNA preparations. The PCR–RFLP assays described by Fermer and Engvall [7] was the most unreliable assay as it did not generate a PCR amplicon for 10 isolates and a further three isolates generated RFLP patterns that could not be typed. The *C. coli*-specific PCR [13] accurately identified all 28 *C. coli* isolates. However, three *C. jejuni* isolates (two poultry and one human) were positive with this assay suggesting that the *C. coli*-specific PCR of Linton et al., [13] should not be the sole assay used to confirm a *C. coli* species designation.

Although *C. jejuni* is the only *Campylobacter* species that hydrolyses hippurate Totten et al. [20] described hippurate-negative *C. jejuni* isolates and recommended that hippurate hydrolysis should not be used as the sole criterion for differentiating thermophilic *Campylobacter* spp. In this study, we observed that results of hippurate hydrolysis were not always reproducible and that weakly positive reactions were open to interpretation and varied in intensity depending upon inoculant size. A recent study by Engvall et al. (2002) [5] reported that 52 of 174 *Campylobacter* isolates recovered from different domestic and wild animals and which had initially been identified as either *C. jejuni*, *C. coli*, *C. lari* or *C. upsaliensis* were either incorrect or unreliable. Our data and those of Engvall et al. [5] suggest that phenotypic and biochemical assays are unreliable for speciating Campylobacters, particularly those recovered from non-human sources.

Campylobacter spp. are common inhabitants of a range of domesticated meat-producing animals and are a common cause of gastrointestinal disease in humans. We demonstrated that the hippurate hydrolysis test was particu-

larly unreliable since 29 *hipO*-negative isolates, particularly those of poultry origin were positive in this biochemical test. Furthermore, the 16S rRNA RFLP assay developed in this study could not confirm a *C. coli* species designation for any of the 28 *hipO*-negative poultry isolates. RFLP profiles consistently showed patterns consistent with a *C. jejuni* 16S rRNA gene sequence. This was confirmed when DNA sequence analysis of 16S rRNA genes from four isolates (98/C053/1, 2, 11, 12) identified to be *C. coli* using six different PCR-based assays showed sequences with highest identity to 16S rRNA genes of the *C. jejuni* type strain. These data suggest that there is insufficient sequence variation in the 16S rRNA gene to discriminate among this group of closely related bacteria.

Acknowledgements

We thank Dr John Ferguson from the John Hunter Hospital, Newcastle, New South Wales, Australia and Dr Chiew from the Institute for Clinical Pathology and Medical Research, Westmead, New South Wales, Australia for providing human *C. jejuni* isolates. We thank Mr Stuart King from NSW Agriculture, Tocal, New South Wales, Australia for providing the poultry from which the *Campylobacter* isolates were cultured. We are also grateful to the staff in the Bacteriology Unit at the Regional Veterinary Laboratory, Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales, Australia for their assistance in the culturing of *Campylobacter* isolates.

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