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Antimicrobial susceptibility of clinical isolates of *Campylobacter jejuni* from New South Wales, Australia

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ABSTRACT

Objectives: The aim of this study was to investigate the prevalence of resistance to commonly used antimicrobials in *Campylobacter jejuni* isolates from clinical faecal samples in New South Wales (NSW), Australia.

Methods: A total of 117 *C. jejuni* isolates from human faecal samples from regional and metropolitan NSW were examined for antimicrobial resistance.

Results: Of the 117 isolates tested, 15.4% were resistant to ampicillin, 5.1% to tetracycline and 13.7% to ciprofloxacin. Most of the isolates were susceptible to erythromycin, except for three that showed intermediate resistance. Furthermore, 9.4% of isolates were resistant (or intermediate-resistant) to more than one antimicrobial agent. Isolates that were resistant to ampicillin and tetracycline harboured the *bla*$_{\text{OXA-61}}$ and *tet*(O) genes, respectively. A mutation in the *gyrA* gene, resulting in the T86I substitution, was identified in the majority of ciprofloxacin-resistant isolates.

Conclusion: The data obtained in the current study demonstrate that the majority of *C. jejuni* isolates evaluated were susceptible to one or more antimicrobials tested. Apart from three isolates that demonstrated intermediate resistance, all of the isolates were susceptible to erythromycin, which is the drug of choice for treating *Campylobacter* infections.

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1. Introduction

Campylobacteriosis is one of the most commonly reported zoonoses in many parts of the world, including Europe, and is the most common foodborne notifiable disease in Australia and New Zealand [1–4].

The most common *Campylobacter* spp. causing human infections are *Campylobacter jejuni* and, to a lesser extent, *Campylobacter coli* [5]. Although human campylobacteriosis is generally a self-limiting disease with typical enteric symptoms such as vomiting, fever, diarrhoea and abdominal pain [6–8], more serious sequelae such as reactive arthritis and Guillain–Barré syndrome may occur in rare cases [9]. For immunocompromised individuals, infants and the elderly, infection may result in severe cases of prolonged enteritis and septicaemia for which antimicrobial therapy is required [10]. Typically, treatment for these patients is with fluoroquinolone or macrolide antimicrobials. In the last few decades, antimicrobial-resistant/multidrug-resistant (MDR) *C. jejuni* have been increasingly identified as a significant issue and have been associated with treatment failures in some countries [11–13].

In Australia, there is limited information on antimicrobial resistance in human *C. jejuni* isolates, with some data published on fluoroquinolone and macrolide resistance, showing that fluoroquinolone resistance is more common in *Campylobacter* spp. infections acquired overseas than in locally acquired infections [13]. Other antimicrobials against which resistance in *C. jejuni* has been reported include tetracycline and ampicillin [11,12].

The aim of this study was to investigate the prevalence of resistance to commonly used antimicrobials in *C. jejuni* isolates from clinical faecal samples in New South Wales (NSW), Australia. The genes associated with the observed antimicrobial resistance from these isolates were partially characterised by PCR and sequencing.

2. Materials and methods

2.1. Samples

This study was performed on 117 *C. jejuni* isolates from human faecal samples obtained from Westmead Hospital (Sydney) and

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Wagga Wagga Base Hospital (Wagga Wagga) in NSW, Australia. Of the 117 isolates tested in this study, 106 were from Sydney and 11 were from Wagga Wagga.

It was not possible to collect equal an number of isolates from each location as *C. jejuni* isolates from clinical samples were not routinely stored long-term in the Wagga Wagga laboratory, hence only 11 samples from this laboratory were available at the time that this study was undertaken. Due to the low representation of isolates from Wagga Wagga, isolates from the two study locations were treated as one sample set in the subsequent analyses.

2.2. Sample collection, and isolation and identification of Campylobacter species

Initial isolation of *Campylobacter* strains was performed using human diarrhoeal faecal samples in the Sydney and Wagga Wagga hospital laboratories. Isolates putatively identified as *C. jejuni* by the laboratories using standard biochemical tests were included in the current study. Isolates were collected in microfuge tubes containing nutrient broth supplemented with 20% glycerol and were transferred to Charles Sturt University (Wagga Wagga, NSW, Australia) on ice within 7 h of collection and were processed immediately.

On receipt at Charles Sturt University, isolates were immediately inoculated onto blood-free *Campylobacter* selective medium supplemented with cefoperazone, amphotericin B and teicoplanin (Thermo Fisher Scientific Australia Pty Ltd., SA, Australia) and were incubated for 48 h at 42 °C in an anaerobic jar under microaerobic conditions provided by gas-generating sachets containing 5% O_2, 10% CO_2 and 85% N_2 (Campy-Gen^{TM}; Thermo Fisher Scientific Australia Pty Ltd.).

2.3. Antimicrobial susceptibility testing by disk diffusion

The activity of selected antimicrobials against the 117 *C. jejuni* isolates was determined using the Kirby–Bauer disk diffusion method by measuring the inhibition zone diameter. Bacterial plates were inoculated and incubated following the standard procedure as previously described [14,15]. An organism was classified as susceptible, intermediate or resistant according to the zone of inhibition of bacterial growth in the proximity of a disk impregnated with the antimicrobial of interest. All disks were purchased from Oxoid Ltd. (Basingstoke, UK). Each strain was evaluated for resistance to four antimicrobial agents (with their respective concentrations): ampicillin, 10 μg; tetracycline, 30 μg; erythromycin, 15 μg; and ciprofloxacin, 5 μg. Clinical and Laboratory Standards Institute (CLSI) guidelines for the disk diffusion technique were followed for reading the susceptibility pattern [15].

Susceptible *C. jejuni* NCTC 11351, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as control strains for antimicrobial susceptibility testing.

2.4. DNA extraction

C. jejuni colonies were harvested from the blood-free agar plates and genomic DNA (gDNA) was extracted using a QIAGEN DNeasy Blood and Tissue Kit (QIAGEN Pty Ltd., Chadstone, VIC, Australia) according to the manufacturer's instructions.

2.5. PCR amplification of antimicrobial resistance genes

Conventional PCR was utilised for amplification of selected genes from the extracted gDNA using previously published primer sets (Table 1). Initially, the identity of isolates was confirmed as *C. jejuni* by targeted amplification of fragments of the *Campylobacter* genus-specific 16S rRNA gene and *C. jejuni*-specific *mapA* gene (Table 1).

All resistant and intermediate-resistant *C. jejuni* isolates were tested for reactivity with the relevant PCR assay to amplify fragments of one of the genes *bla*$_{OXA-61}$, *tet*(O), *gyrA* and *aphA3-1* depending upon whether they were resistant/intermediate-resistant to ampicillin, tetracycline, ciprofloxacin or erythromycin, respectively. These genes were selected on the basis of previous studies on antimicrobial resistance in *C. jejuni* [10,18–21]. The PCR assays were run together with the susceptible *C. jejuni* control strain NCTC 11168.

All reactions contained 10 μL of 2× Go*Taq*® Green Master Mix (Promega Corp., Madison, WI), 400 μM dNTP mix, 3 mM $MgCl_2$ and 0.5 μM of the oligonucleotide pairs for each amplicon as shown in Table 1. The following cycling parameters were used: 5 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C at 1 min.

2.6. Sequencing of PCR products

Purified PCR products were directly sequenced using Sanger dideoxy technology with the amplification primers shown in Table 1 (Australian Equine Genetics Research Centre sequencing service, University of Queensland, QLD, Australia) and the sequences were compared with the GenBank databases using the BLAST suite of programs [22]. Multiple sequence alignments were performed in

Table 1

Oligonucleotides amplifying the 16S rRNA and *mapA* genes used to confirm the identity of the isolates as *Campylobacter jejuni*, and the oligonucleotide pairs targeting selected regions of genes associated with antimicrobial resistance.

Gene	Antimicrobial resistance	Primer name	Primer sequence (5′ → 3′)	PCR amplicon size (bp)	Reference
16S rRNA[a]	NA	C412	GGATGACACTTTTCGGAGC	816	[30]
		C1228	CATTGTAGCAGGTGTGTC		
mapA[b]	NA	MDmapA1	CTATTTTATTTTTGAGTGCTTGTG	589	[16]
		MDmapA2	GCTTTATTTGCCATTGTTTTATTA		
bla$_{OXA-61}$	Ampicillin	BlaOXA_61_F	AGAGTATAATACAAGCG	372	[18]
		BlaOXA_61_R	TAGTGAGTTGTCAAGCC		
tet(O)	Tetracycline	tetO_F	GCGTTTTGTTTATGTGCG	559	[12,17]
		tetO_R	ATAGACAACCCGACAGAAG		
gyrA	Ciprofloxacin	GZgyrA5_F	ATTTTTAGCAAAGATTCTGAT	673	[31]
		GZgyrA6_R	CCATAAATTATTCCACCTGT		
aphA3-1	Erythromycin	aphA_3_1_F	TGCGTAAAAGATACGGAAG	701	[18]
		aphA_3_1_R	CAATCAGGCTTGATCCCC		

NA, not applicable.

[a] For genus-level identification of *Campylobacter* spp.

[b] For species-level identification of *C. jejuni*.

ClustalW2, using MEGA6 and BioEdit Sequence Alignment Editor software (http://www.ebi.ac.uk/Tools/msa/clustalo/).

3. Results

3.1. Identification of isolates

The identity of all *C. jejuni* isolates used in this study was confirmed by successful PCR amplification of the 16S rRNA and *mapA* gene products (data not shown).

3.2. Antimicrobial susceptibility of isolates

The results of antimicrobial susceptibility testing of the *C. jejuni* isolates to ampicillin, tetracycline, ciprofloxacin and erythromycin by the disk diffusion method are shown in Table 2.

All of the control isolates (*C. jejuni* NCTC 11351, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922) were susceptible to the antimicrobials used in this study. Of the 117 *C. jejuni* isolates tested, the highest levels of resistance were to ampicillin (15.4%) and ciprofloxacin (13.7%). Some isolates demonstrated intermediate resistance to ampicillin (3.4%) and some others to ciprofloxacin (4.3%). All isolates were susceptible to erythromycin, except for three isolates (2.6%) that exhibited intermediate resistance. Several isolates were resistant (5.1%) or showed intermediate resistance (1.7%) to tetracycline (Table 2).

Multidrug resistance (including intermediate resistance) was detected in 11 isolates (9.4%), with 6 of these isolates demonstrating resistance to two antimicrobials (Table 3). Four of these isolates were resistant to ampicillin and ciprofloxacin, one was resistant to ampicillin and tetracycline, and one demonstrated resistance to ciprofloxacin and tetracycline. Isolates resistant to more than one antimicrobial were considered to be MDR (Table 3).

3.3. Characterisation of the bla$_{OXA-61}$ gene ampicillin-resistant C. jejuni *isolates*

PCR was used to determine whether the *bla*$_{\text{OXA-61}}$ gene, encoding the class D β-lactamase OXA-61, was present in the ampicillin-resistant and intermediate-resistant *C. jejuni* isolates. All ampicillin-resistant and intermediate-resistant isolates yielded an amplicon of the expected size. Comparison of the nucleotide sequences of the amplicons showed minimal variation, with >98% identity to the reference sequence. Comparison of amino acid translations of the PCR amplicons revealed substitutions in seven isolates compared with the reference sequence (GenBank accession no. **WP_002872036**). Some examples of substitutions include nucleotide point mutations such as I138L (isolate H34) and F144L (isolate H152), and an N129D substitution in isolate H259. The resistant isolate H34 was the most divergent, with three amino acid substitutions (F112S, Q125P and I138L).

Table 3
Campylobacter jejuni isolates exhibiting resistance (R) and/or intermediate resistance (I) to more than one antimicrobial agent.[a]

Isolate	Phenotype			
	Ampicillin	Tetracycline	Ciprofloxacin	Erythromycin
H78	R		R	
H79			I	I
H83	R		R	
H106	I		R	I
H134	I	R		
H152	R		R	
H159	R		R	
H232		R	R	
H243	R	R		
H246			I	I
H259	I	I		

[a] All isolates exhibiting a multidrug-resistant phenotype were from Sydney, NSW.

3.4. Characterisation of the tet(O) gene in tetracycline-resistant C. jejuni *isolates*

PCR was used to determine whether the *tet*(O) gene, which encodes the tetracycline resistance protein O [Tet(O)], was present in the tetracycline-resistant and intermediate-resistant isolates identified in this study. Target amplicons were successfully amplified and sequenced from seven of the isolates. The partial *tet*(O) gene sequences were compared with a corresponding sequence from the tetracycline-resistant *C. jejuni* strain 81-176 (GenBank accession no. **WP_011187228**) and the level of nucleotide sequence identity was >98.2%.

Comparison of the Tet(O) translation from the PCR amplicons identified conservative and non-conservative amino acid substitutions in five of the isolates characterised. Conservative amino acid substitutions N299K and E412K were identified in one isolate (H134), whereas three isolates (H96, H99 and H259) had conservative substitutions I331M and I346L. The most divergent isolate (intermediate isolate H259) had six amino acid substitutions (Y295C, S304P, I331M, I346L, S355P and C372G).

3.5. Characterisation of the gyrA gene in ciprofloxacin-resistant C. jejuni *isolates*

Mutations in the *gyrA* gene encoding the DNA gyrase subunit A (GyrA) were investigated in strains that showed resistance or intermediate resistance to ciprofloxacin. Of the 21 ciprofloxacin-resistant and intermediate-resistant isolates identified in this study, the *gyrA* amplicon was amplified and sequenced from 20 isolates (gDNA from 1 intermediate-resistant isolate could not be obtained). Comparison of the nucleotide sequences of the *gyrA* gene segments showed minimal variation, with 97.6–98.9% identity to the reference sequence (GenBank accession no. **BAC57595**). Of the ciprofloxacin-resistant and intermediate-

Table 2
Antimicrobial susceptibility determined by the disk diffusion method of *Campylobacter jejuni* isolates ($n = 117$) to ampicillin, tetracycline, ciprofloxacin and erythromycin.

Antimicrobial agent	Antimicrobial susceptibility of isolates					
	Susceptible		Intermediate		Resistant	
	n	% (95% CI)	*n*	% (95% CI)	*n*	% (95% CI)
Ampicillin (10 μg)	95	81.2 (72.3–87.4)	4	3.4 (1.7–9.2)	18	15.4 (8.4–22.7)
Tetracycline (30 μg)	109	93.2 (89.1–98.3)	2[a]	1.7 (0–5.0)	6	5.1 (0.8–8.4)
Ciprofloxacin (5 μg)	96	82.1 (73.1–89.1)	5[a]	4.3 (1.7–10.9)	16	13.7 (7.6–21.0)
Erythromycin (15 μg)	114	97.4 (94.1–100)	3[a]	2.6 (0–5.9)	0	0

CI, confidence interval.

[a] DNA could not be isolated from five isolates; tetracycline-intermediate-resistant ($n = 1$); ciprofloxacin-intermediate-resistant ($n = 1$); and erythromycin-intermediate-resistant ($n = 3$). Hence, information on the sequences of antimicrobial resistance genes was not available for these isolates.

resistant isolates sequenced, 16 contained a previously reported amino acid substitution linked to ciprofloxacin resistance in GyrA [21]. The amino acid substitution was due to a single nucleotide change in codon 86 (ACA → ATA) of the *gyrA* gene resulting in the amino acid substitution T86I. In two of the ciprofloxacin-resistant isolates (H85 and H154), additional conservative substitutions were observed with H48Y and I115L detected, respectively. The resistant isolate H157 was the most divergent strain with five non-conservative amino acid substitutions (R79G, S105G, N111D, A120G and S152I). The T86I substitution was not identified in one ciprofloxacin-resistant isolate (H157) or in three isolates (H246, H255 and H258) with intermediate resistance to ciprofloxacin.

3.6. Characterisation of the aphA3-1 *gene in erythromycin-resistant* C. jejuni *isolates*

Whilst no isolates were resistant to erythromycin, three isolates exhibited intermediate resistance. Attempts to isolate DNA from these isolates were unsuccessful and therefore PCR and sequencing analysis of the *aphA3-1* gene was not possible.

4. Discussion

This study identified resistance in *C. jejuni* isolates from NSW to the commonly used antimicrobials ampicillin, tetracycline and ciprofloxacin. Similarly, in recent years many studies have reported on resistance to clinically important antimicrobials in *C. jejuni* and *C. coli* isolates [23–27]. The level of antimicrobial resistance found in the current study is, however, different to that reported in a previous study undertaken in NSW [13]. The study by Sharma et al. [13] was conducted in the Hunter Region of NSW, which was not an area included in the current study, and reported ampicillin resistance at 64% (15.4% in the current study), tetracycline resistance at 11.2% (5.1% in current study) and ciprofloxacin resistance at 2.9%. One reason for the differences in the percentages of resistant isolates in the two studies could be that the data generated by Sharma et al. relate to cases of campylobacteriosis from 1999–2001, which is >10 years before the current study was conducted, and some infections in that study were also known to have been acquired overseas [13].

High levels of ampicillin resistance have been reported previously in human and chicken *Campylobacter* spp. isolates [19,28]. Similarly, tetracycline resistance rates also appear to be variable, with a higher rate than that seen in this study reported in a previous study conducted in NSW, Australia [13]. High levels of tetracycline resistance (14.4–99.3%) have also been reported in other countries [14,28,29]. Based upon published data, it is reasonable to conclude that the level of antimicrobial resistance is variable depending on when the study was conducted and the cohort being studied and could possibly be related to the use of that antimicrobial in that population [21].

Broadly speaking, development of resistance to an antimicrobial can occur by two key pathways, namely gene acquisition or gene mutation. Acquisition would typically involve the transfer of mobile genetic elements, such as plasmids or transposons, from a resistant strain to a susceptible strain. The resistance genes examined in this study that typically fall in this category are *bla*$_{\text{OXA-61}}$ and *tet*(O).

Ampicillin-resistant and intermediate-resistant isolates in this study contained the *bla*$_{\text{OXA-61}}$ gene encoding OXA-61, a class D β-lactamase that has been identified in *Campylobacter* spp. and members of other bacterial genera such as *Fusobacterium*, *Acinetobacter* and *Pseudomonas* [19]. The tetracycline-resistant/intermediate-resistant isolates contained *tet*(O) encoding the Tet(O) protein, which inhibits the action of tetracycline. Several mutations/deletions were detected in the *bla*$_{\text{OXA-61}}$ and *tet*(O) genes in some isolates, however it is unclear what effect, if any, this had on the functional properties of the protein as these isolates were all resistant to the relevant antimicrobials. Some of these mutations have been reported previously, whilst others have not [26,27,30].

Resistance to fluoroquinolones in *Campylobacter* spp., whilst associated with many single amino acid substitutions, is predominantly associated with a nucleotide mutation in the *gyrA* gene leading to a T86I substitution in the expressed protein [31,32]. In the current study, this substitution was detected in most ciprofloxacin-resistant isolates. Isolate H157, which lacked the T86I substitution, had the most divergent GyrA. Further studies are required to determine whether any other substitutions detected in H157 and/or other mutations/deletions in parts of the *gyrA* gene in this isolate that were not sequenced in this study play a role in conferring resistance to fluoroquinolones. Other substitutions in GyrA, such as P104T and P104S substitutions, have been previously reported in ciprofloxacin-resistant *C. jejuni* isolates from Austria and Germany, however these were not detected in the current study [21,33]. The emergence of the variant efflux pump RE-CmeABC has been reported to promote the emergence of fluoroquinolone-resistant mutants under selection pressure with antimicrobials, together with *gyrA* mutations, reportedly conferring high-level resistance to fluoroquinolone [34]. Therefore, further investigations are required to determine the role that genes/mutations could play in conferring resistance to ciprofloxacin in this isolate. Other point mutations associated with resistance/intermediate resistance to ciprofloxacin have been reported by others but were not detected in the isolates in the current study [21,32,33].

In this study, all tested isolates except for three (which demonstrated intermediate resistance) were susceptible to erythromycin. Low or no resistance to this antimicrobial has been reported in other studies suggesting that *C. jejuni* generally remains susceptible to this antimicrobial, which is currently the drug of choice to treat *Campylobacter* infections [13,35–37]. However, it is essential to continue monitoring erythromycin susceptibility of Australian strains of *C. jejuni* to ensure early detection of emerging resistance.

The value in characterising the specific sequences of antimicrobial resistance genes and their products is the potential to follow the movement of specific strains from one population to another. This has been proposed previously and would be particularly useful when investigating outbreaks involving travel-associated campylobacteriosis, as there is evidence to show that there is not only genetic diversity between *C. jejuni* strains from different countries but also a difference in the antimicrobial susceptibility profiles of these strains in humans and animals [38].

The data in the current study demonstrate that the majority of isolates were susceptible to one or more antimicrobials tested. However, it is important to continue to monitor strains of *C. jejuni* for antimicrobial resistance, and future studies should be undertaken on a larger number of samples, from regional and metropolitan areas, and conducted over a longer period, to further investigate the prevalence of antimicrobial resistance in *C. jejuni*. It is widely accepted that antimicrobial resistance in foodborne diseases such as campylobacteriosis may be linked to the use of antimicrobials in food-producing animals [35,36,38,39]. Therefore, further studies should include not only additional human isolates but also isolates from farm animals and environmental sources as this could be useful in identifying the source of antimicrobial resistance. Knowledge of the source of resistant *C. jejuni* isolates is important in order to develop measures to prevent the emergence of MDR *C. jejuni* in the future.

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Competing interests

None declared.

Ethical approval

Not required.

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