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Antibiotic susceptibility profiling and virulence potential of *Campylobacter jejuni* isolates from different sources in Pakistan

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ABSTRACT

Objective: To determine antibiotic resistance patterns and virulence potential of *Campylobacter jejuni* (*C. jejuni*) isolates from clinical human diarrheal infections, cattle and healthy broilers. **Methods:** Antibiotic sensitivity patterns of *C. jejuni* isolates were determined by Kirby Bauer Disc Diffusion assay. These isolates were then subjected to virulence profiling for the detection of *map*A (membrane-associated protein), *cad*F (fibronectin binding protein), *wla*N (beta-1,3-galactosyltransferase) and *neu*AB (sialic acid biosynthesis gene). Further *C. jejuni* isolates were grouped by random amplification of polymorphic DNA (RAPD) profiling. **Results:** A total of 436 samples from poultry (n=88), cattle (n=216) and humans (n=132) from different locations were collected. Results revealed percentage of *C. jejuni* isolates were 35.2% (31/88), 25.0% (54/216) and 11.3% (15/132) among poultry, cattle and clinical human samples respectively. Antibiotic susceptibility results showed that similar resistance patterns to cephalothin was *ie*. 87.0%, 87.1% and 89%among humans, poultry and cattle respectively, followed by sulfamethoxazole+trimethoprim 40.0%, 38.7% and 31.0% in humans, poultry and cattle and Ampicillin 40%, 32% and 20% in humans, poultry and cattle respectively. Beta-lactamase activity was detected in 40.00% humans, 20.37% cattle and 32.25% in poultry *C. jejuni* isolates. *Cad*F and *map*A were present in all poultry, cattle and human *C. jejuni* isolates, *wla*N was not detected in any isolate and *neu*AB was found in 9/31 (36%) poultry isolates. RAPD profiling results suggested high diversity of *C. jejuni* isolates. **Conclusions:** Detection of multidrug resistant *C. jejuni* strains from poultry and cattle is alarming as they can be potential hazard to humans. Moreover, predominant association of virulence factors, *cad*F and *map*A (100 % each) in *C. jejuni* isolates from all sources and *neu*AB (36%) with poultry isolates suggest the potential source of transmission of diverse types of *C. jejuni* to humans.

1. Introduction

Campylobacter jejuni (*C. jejuni*) is an important food-born zoonotic pathogen, and one of the leading causes of human food borne illnesses (Campylobacteriosis) worldwide[1,2]. The most important source of transmission of this pathogen to humans is through contaminated animal products, especially poultry meat as well as direct contact with cattle shedding *C. jejuni*, or handling raw or undercooked poultry[3,4]. *Campylobacter* has been reported from broiler flocks in various European countries at the prevalence rates ranging from 38.1% to 79.2%[5,6]. Antibiotics play a vital role in human and veterinary medicine for treatment and prevention of infections but are also used as growth promoters in food animals[7]. Their increased use has resulted in the increased incidences of infection with enteric bacteria with higher levels of antibiotic resistance[8]. *Campylobacter* spp. has developed resistance to many clinically important antimicrobials, including fluoroquinolones (FQ) during the recent past[9–12]. It is believed that their transmission and spread are not only affected by the environmental and host factors, but also are influenced by the relative fitness of the drug-resistant

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organisms in the absence of selection pressure[13]. *Campylobacter* spp. has shown resistance to a large number of beta lactam antimicrobial agents. However, the behaviours of others, such as ampicillin and some of the expanded-spectrum cephalosporins, are variable and not very clearly defined[14].

The current study gives the perspective of distribution of multiple antibiotic resistance, beta lactamase activity and virulence attribution to *C. jejuni* isolates from clinical human diarrheal infections, cattle and healthy broilers sharing the environment with the humans from the heavily populated city of Rawalpindi, Pakistan and its suburbs. Furthermore, their random amplification of polymorphic DNA (RAPD) profiling was carried out for determining their diversity.

2. Materials and methods

2.1. Sampling

This study was carried out from December 2011 and December 2012. The samples were collected from poultry slaughter houses from one of the country's leading poultry producer city, cattle farms and human clinical diarrheal cases. A total of 436 samples collected consisting of 216 cattle faecal samples, 132 human clinical samples and 88 poultry samples. Samples were collected in sterile cotton swabs containing Carry-Blair medium and transported to Microbiology Laboratory of COMSATS Institute of Information Technology, Islamabad.

2.2. Culturing and isolation

The samples collected were streaked onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Oxoid, CM0739) containing CCDA selective supplement (Oxoid, SR0155). Samples were incubated in 2.5 litres airtight jar along with Campygen sachets (Oxoid, CN025A) to generate microaerophilic condition at 42 ℃ for 48-72 hours. Suspected *Campylobacter* colonies were subcultured on Muller Hinton Agar (Oxoid, CM0337) with addition of 5% sheep blood[15].

2.3. Biochemical identification

Positive growth of *Campylobacter* isolates was further subjected to standard biochemical tests consisting of oxidase, catalase, indoxyl acetate and hippurate. In the case of indoxyl-acetate test, change of colour from colourless to blue green indicative of the presence of *Campylobacter* spp. and in case of hippurate hydrolysis, development of blue/purple colour in hippurate solution indicated positive reaction for presence of *C. jejuni* with the production of hippuricase enzyme and clear or grey colouration indicate negative reaction for its presence. A positive test for both reactions was indicative of *C. jejuni*[16].

2.4. Molecular detection of C. jejuni

Bacterial DNA was obtained by whole-cell lysate method as described by Singh *et al*. Primers used for confirmation of *C. jejuni* by PCR were MDS-16S rRNA (targeting 16S RNA gene), hipO (Hippurate hydrolysis gene) as described in Table 1. PCR was performed as previously described[17,18]. Amplified PCR products were analyzed on 1.5% agarose gel stained with ethedium bromide.

2.5. Virulence typing

C. jejuni isolates were screened for the presence of virulence genes (Table 1). Primers were designed against *C. jejuni* adhesin, *cad*F (fibronectin binding protein) (400 bp) gene, *wla*N (putative beta-1,3-galactosyltransferase) (330 bp), *neu*AB (sialic acid biosynthesis gene) (755 bps) and *map*A (membrane-associated protein) (94 bps).

2.6. RAPD PCR

For RAPD analysis of *C. jejuni* OPA11 primer was used as described by Hernandez *et al*. Briefly, the reaction mixture was carried out in a total volume of 25 μL containing 40 ng total DNA of each strain, 1.36 pM primer), 1.6 U *Taq* DNA polymerase (Super *Taq*), 1.5 μL 500 mM $MgCl_2$, 0.7 μL 10 mM dNTPs in 1X PCR buffer (Fermentas). The PCR products were then separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Dendrogram was constructed using DendroUPGMA (genomes.urv.cat/UPGMA/) for RAPD PCR analysis.

2.7. Antimicrobial susceptibility profiling and beta-lactamase detection

Antibiotic susceptibility profiling was carried out using chloramphenicol C (30 μg), tetracycline (TE) (30 μg), streptomycin (S) (10 μg), ciprofloxacin (CIP) (5 μg), amoxicillin clavulenic (AMC) acid (30 μg), nalidixic acid (NA) (30 μg), erythromycin (E) (30 μg), gentamycin (CN) (10 μg), sulphomethoxazole + trimethoprim (SXT) (25 μg) and cephalothin (CEF), respectively (Oxoid, UK) as described by Gaudreau *et al*[19]. Analysis of zone diameter was done according to the CLSI (2010). Beta-lactamases were detected by use of Cefinase disks (BBL Microbiology Systems) as described by Lachance *et al*[20].

3. Results

3.1. PCR confirmation of Campylobacter isolates

Biochemically verified *C. jejuni* strains were further subjected to PCR using primers against conserved 16S rRNA (amplification product 857 bps) and *hip* gene (hippurate hydrolysis gene) (344 bps).

Table 1
Primers for identification and virulotyping of *C. jejuni*.

Primer	F/R	Sequence of primers	Annealing temperature	Product size	References
16Sr RNA	F	5`-ATCTAATGGCTTAACCATTAAC-3`	55 ℃	857 bp	[17]
	R	5`-GGACGGTAACTAGTTTAGTATT-3`			
hipo	F	5`-GACTTCGTGCAGATATGGATGCTT-3`	59 ℃	344 bp	[40]
	R	5`-GCTATAACTATCCGAAGAAGCCATCA-3`			
*map*A	F	5`-AAGCAATACCAGTGTCTAAAGTGC-3`	60 ℃	94 bp	[41]
	R	5`-GGTTTTGAAGCAAAGATTAAAGG-3`			
*cad*F	F	5`-TTGAAGGTAATTTAGATATG-3`	45 ℃	400 bp	[42]
	R	5`-CTAATACCTAAAGTTGAAAC-3`			
*neu*AB	F	5`-ATTATAGCCATTTGCTCACTTTG-3`	52 ℃	755 bp	[43]
	R	5`-AAAGCACCCTTAGTCGTACCTG-3`			
*wla*N	F	5′-TGCTGGGTATACAAAGGTTGTG-3′	60 ℃	330 bp	[44]
	R1	3′-AATTTTGGATATGGGTGGGG-5′			
	R2	3′-TTAAGAGCAAGATATGAAGGTG-5′			
OPA11		CAA TCG CCG T	36 ℃	varied	[38]

3.2. *C. jejuni* distribution pattern among various sources

Four hundred and thirty-six samples were analysed in this study. 100 out of a total of 436 samples were confirmed as *C. jejuni ie.*, the overall prevalence rate was 100/436 (22.93%). The isolation rate of *C. jejuni* was (n=31) 35.2%, (n=54) 25.0% and (n=15) 11.3% in poultry, cattle and humans, respectively.

3.3. Antimicrobial susceptibility profile

Antibiotic resistance profile of *C. jejuni* isolates from humans, cattle and poultry sources was determined using 10 antibiotics according to CLSI 2010. Comparison of antibiotic susceptibilities of *C. jejuni* isolates from different sources is shown in Table 2. Antibiotic susceptibility of the isolates revealed that resistance to cephalothin was the most common *ie.* 87.0%, 89.0% and 87.1%, followed by trimethoprim/sulfamethoxazole 40.0%, 38.7% and 31.0% and amoxicillin clavulenic acid 40%, 32% and 20% in human, cattle and poultry respectively (Figure 1). Multidrug resistance was also identified in strains from different sources (Table 3). Gentamicin was found to be the most sensitive antibiotic with resistance of 7%, 0% and 9% in humans, poultry and cattle isolates.

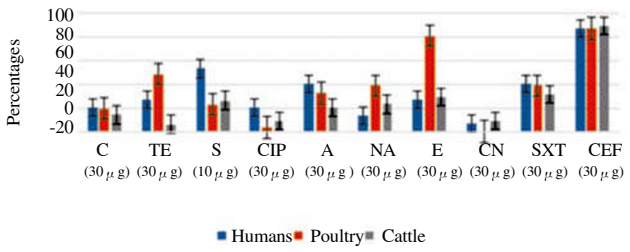

Figure 1. Prevalence of antibiotic resistance among different *C. jejuni* isolates of poultry, human and cattle.

Table 2
Percentages of antibiotic resistances of Campylobacter jejuni isolated from humans, poultry and cattle sources in Pakistan.

Antibiotics	Humans	Poultry	Cattle
Chloramphenicol (30 μg)	20	19.4	14
Tetracycline (30 μg)	27	48.39	6
Streptomycin (10 μg)	53	22.6	26
Ciprofloxacin (5 μg)	20	3.23	9
Ampicillin (30 μg)	40	32.26	20
Nalidixic acid (30 μg)	13	38.7	23
Erythromycin (30 μg)	27	80.6	29
Gentamycin (10 μg)	7	0.0	9
Sulphomethoxazole + Trimethoprin (25 μg)	40	38.7	31
Cephalothin (30 μg)	87	87.1	89

Beta-lactamase production was detected in 27 *C. jejuni* strains including 6 human, 10 poultry and 11 cattle strains. Thus, the overall frequency of beta lactamase producing strains in our study was 27/100 (27%).

3.4. RAPD Profiling

Analysis of *C. jejuni* isolates by RAPD profiling yielded 22 different banding profiles. Almost all the *C. jejuni* isolates were well dispersed among all clusters (Figure 2). However, five isolates did not produce any recognizable RAPD banding pattern.

3.5. Virulence typing

Virulence typing was performed using 4 genes as targets and results suggested that *cad*F and *map*A (adherence factors) were present in all isolates studied whereas *neu*AB (invasive factor) was found in 9 (36%) poultry samples only, whereas *wla*N (invasive factor) was not present in any of the isolates (Figure 3).

Table 3

Multidrug resistant strains from different sources.

Source	Number of isolates (%)	Multiple antibiotic resistance
Humans	5 (33.33%)	Streptomycin (S), ampicillin (A), sulphomethoxazole + trimethoprin (SXT), cephalothin (CEF)
Poultry	20 (64.51%)	Tetracycline (TE), ampicillin (A), nalidixic acid (NA), erythromycin (E), sulphomethoxazole + trimethoprin (SXT), cephalothin (CEF)
Cattle	28 (51.85%)	Streptomycin (S), erythromycin (E), cephalothin (CEF)

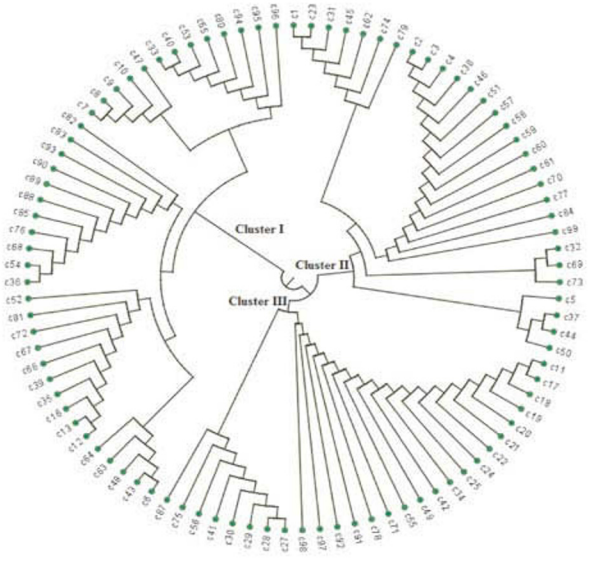

Figure 2. Dendrogram of RAPD profiles showing clusters of *C. jejuni* isolates.

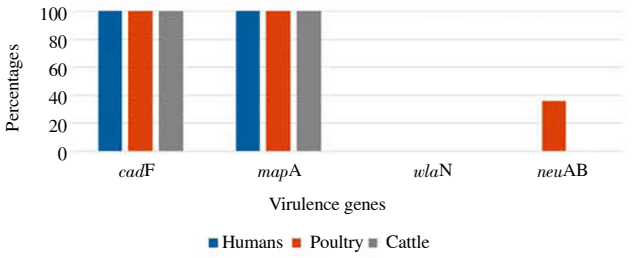

Figure 3. Prevalence of virulence genes in *C. jejuni* isolates from poultry, humans and cattle.

4. Discussion

The aim of this study was to assess the *C. jejuni* isolates obtained from different sources on the basis of antimicrobial resistance and thereafter screening them for virulence factors. The isolates were further characterized using RAPD analysis for possible relatedness. Little data is available from Pakistan to compare our data, previously isolation rates of *C. jejuni* from humans have been reported to be 29.5%[21], 12%[22] and 18%[23] and 21.5% *Campylobacter* spp. prevalence in food commodities[4]. While to our knowledge no reports are available for *C. jejuni* prevalence in poultry and cattle from Pakistan. The higher prevalence rates 100/436 (22.93%) of *C. jejuni* in this study are in agreement with reports from other countries[24–30]. Antibiotic susceptibility profile of *C. jejuni* isolates was determined using 10 antibiotics and compared among poultry, cattle and humans isolates. The results of antimicrobial susceptibility testing in this study indicate that the isolates were in general resistant to the tested antibiotics at rates ranging from 7% to 87% in clinical cases, up to 87.1% in poultry and 6% to 89% in cattle. Higher rate of resistance (80.6%) to erythromycin was seen among *C. jejuni* isolates from poultry. Since the ingestion of the infected poultry meat may account for most of human campylobacteriosis cases, this fact becomes more relevant to public health when seen in the context that Erythromycin is one of the commonly used drug for treatment of the patients. However, the frequency of resistance to ampicillin (40%), Tetracycline (27%) and gentamycin (7%) was comparable or lower than in the reports from most of the European countries[31,32]. Mostly tested isolates were susceptible to chloramphenicol and gentamycin. Among the isolates from different sources overall resistance rates were different. Tetracycline was listed as an alternative treatment for *Campylobacter gastroenteritis* in the past and they are widely used therapeutically and sub therapeutically as feed additives for livestock and poultry[33]. In our study, resistance to tetracycline (7%) was lower than previous reports[34–36]. The identification of multiple antibiotic resistant *C. jejuni* isolates from poultry, cattle and humans is alarming as such resistance strains may cause more prolonged or severe illness[37]. Further, 27 *C. jejuni* isolates of during the current study were B-lactamase producers. This is of significance as beta lactams are generally the first line of drugs for treating hospitalized cases. *Campylobacter* spp. are generally inherently resistant to many beta-lactams, however, there are variable reports of resistance to beta-lactams and some of the expanded spectrum cephalosporins but it is not clearly defined[14]. In our study 6 human diarrheal, 10 poultry and 11 cattle isolates were positive for resistance to B-lactams.

Virulence typing suggested that all isolates possess adherence property owing to the presence of *cad*F and *map*A genes, while 36% of only poultry *C. jejuni* isolates possess in addition invasive property attributable to the presence of *neu*AB implying their possibility of association with more severe disease. RAPD typing[38] results have shown the presence of 8 distinct types of *C. jejuni*. Despite some limitations, analysis of *Campylobacter* spp. isolates using RAPD has proved to be useful for preliminary characterization of strains[39] and the dendrogram constructed showed genetic diversity of isolates

from different sources. Three main clusters were clearly defined *ie.* clusters Ⅰ, Ⅱ and Ⅲ based on RAPD profiling. All invasive strains (strains positive for *neu*AB) were present in cluster I whereas all multidrug resistant and beta lactamase producing strains were randomly distributed in all clusters. Our study have shown that RAPD PCR assay can act as rapid and effective molecular tool, which can be used in any basic microbiology laboratory, for studying *C. jejuni* isolates from different sources and discriminating virulent strains.

This study analyses *C. jejuni* strains in Pakistani poultry, cattle and human diarrheal samples particularly with regard to their antibiotic resistance and virulence profiling. As compared with European surveillance programmes, the prevalence and antibiotic resistance of *C. jejuni* in Pakistan are not monitored and isolation of multiple antibiotic resistance *C. jejuni* from poultry and cattle during the current study serves as impetus for more elaborate studies regarding the prevalence and transmission patterns of *C. jejuni*.

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Conflict of interest statement

The authors declare no conflict of interest.

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