Short variable regions flaA gene (SVR-flaA) diversity of multidrug resistant Campylobacter strains from poultry and poultry meat in India

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Abstract

Human gastrointestinal infections caused by *Campylobacter* species is the second most important foodborne illness after Salmonellosis worldwide. Poultry are the largest reservoir for *Campylobacter* organisms. In the present study the short variable region of flagellin gene (SVR-*flaA*) typing was carried out to determine the variation among the circulating strains of *C. jejuni* and *C. coli*. The *Campylobacter jejuni* and *C. coli* isolated from poultry birds and poultry meat were screened for the presence of virulence determinants like *cadF*, *flaA*, *cdtB*, and *wlaN* gene. The screening for *wlaN* gene is crucial in view of fact that *C jejuni* strains that elicit Guillain Barre's (GB) syndrome in humans carry *wlaN* gene. Out of the 200 samples comprising of poultry meat and cloacal swabs, 21.5% of samples were found positive for *Campylobacter* spp, 2.5% for *C. jejuni*, and 19% for *C. coli*. The *cadF*, *flaA*, *cdtB* virulence genes were detected in all strains of *Campylobacter* isolated in the present study. The presence of the *wlaN* gene in the *C jejuni* strains isolated in the present study may pose a public health threat with long term human health implications. The SVR-*flaA* typing of *Campylobacter* strains revealed that *Campylobacter* coli *flaA* sequence OL471375 is a new strain with a novel allele type 1675 and peptide sequence 5 which stands deposited in pubMLST database for *Campylobacter*. The other *flaA*-SVR gene sequences identified in this study were OL471369, OL471370, OL471371, OL471372, OL471373, and OL471374. Variable Ddel-RFLP patterns of *Campylobacter* strains in the present study ranging from 100-1000bp were observed. Antimicrobial profiling of the strains revealed 100% resistance to ciprofloxacin (CIP), ampicillin (AMP), penicillin (PCN) nalidixic acid (NAL) whereas resistance to Tetracycline (TET) was 57.1%, 57.1% for erythromycin (Ery) 28% for amoxicillin (AMX) and enrofloxacin (ENFX) and 85% for amikacin (AMK). The high degree of resistance to fluoroquinolones is important in view of fluoroquinolones being drugs of choice for treatment of human *Campylobacter* infections.

INTRODUCTION

*Campylobacter* is a leading foodborne bacterial pathogen linked with the consumption of animal source food products worldwide (Li et al., 2019). The *Campylobacter* infections caused by consumption of contaminated poultry meat and products represent about 50–70% of the global *Campylobacter* infection burden (Seliwiorstow et al., 2015). It is the most common cause of bacterial gastroenteritis responsible for 400–500 million cases of diarrhoea each year (Ruiz-Palacios., 2007). Although diarrhoea is the most common clinical manifestation of infection in humans, 30% of cases with a preceding history of *Campylobacter* infection may develop Guillain Barre syndrome (GBS), an acute demyelinating disease of the peripheral nervous system, within 1–3 weeks after the onset of diarrheal illness. The molecular mimicry hypothesis between glycosylated lipopoligosaccharides and gangliosides on neuronal cells results in the onset of GBS. Other sequelae like Miller- Fisher syndrome, Reiter’s syndrome, and irritable bowel syndrome have also been reported following *Campylobacter* gastroenteritis (Mangen et al., 2015).

The two species most frequently associated with human disease are *Campylobacter jejuni* and *Campylobacter coli* which together account for more than 95% of human infections. In Europe, foodborne campylobacteriosis is most frequently a notified disease since 2005, whereas in USA it is the second
most occurring bacterial illness in humans preceded by the infection due to *Salmonella* spp. The data pertaining to low- and middle-income countries although very scant is still suggestive of an increase in rate of infection by *Campylobacter* in humans. Although several preventive measures like assessment of management risk along the chain “farm-to-table,” and better understanding of pathogenesis have been put in place to prevent this foodborne infection, *Campylobacter* infections continue to show an increasing trend around the globe.

The major human enteric pathogens of human *Campylobacter* infection are divided into three groups which include *C. jejuni subsp. jejuni* (*Cjj*), *C. jejuni subsp. doyley* (*Cjd*), *C. coli* and and the minor pathogens include *C. concisus, C. upsaliensis, C. lari*, and *C. hyointestinalis*. The major veterinary pathogens are *C. fetus subsp. venerealis* (*Cfv*) and *C. fetus subsp. fetus* (*Cff*) (Igwaran and Okoh, 2019). *C. jejuni* is the principal pathogen causing enteric infections in comparison to other *Campylobacter* species (Epps et al., 2013).

Poultry birds are the largest reservoir of *Campylobacter* spp and the droppings of birds infected with *Campylobacter* contain $10^5$ to $10^8$CFU/g (Epps et al., 2013). Experimental studies in poultry have revealed that the infected birds excrete *C. jejuni*, from the second or third week (Acuff et al., 1982) of infection and develop weight loss, gut damage, and impaired intestinal permeability (Awad et al., 2018). Potential sources of flock infection include old litter, untreated drinking water, other farm animals, domestic pets, wildlife species, insects, equipment transport vehicles, and farm workers. The wild birds (Passeriformes, Columbiformes, and Anseriformes) in non-bird proof poultry farms may also be the source of transmission of *C. jejuni* to poultry.

The frequent inter and intraspecies genetic recombination leads to genetic diversity among *C. jejuni* isolates. Several molecular subtyping methods with enhanced discriminatory power have been used to investigate the epidemiology of *C. jejuni* (Wieczorek et al., 2017). The SVR-*flaA* typing method has been reported to be useful and convenient cost effective sequence based tool employed in epidemiological studies involving a small number of isolates (Moore et al., 2005). The next-generation sequencing, whole genome sequencing methods possess increased discriminatory power over other methods. However, such methods are not cost effective in low-income countries. The sequencing of PCR-amplified short variable regions (SVRs) of the flagellin-encoding (*flaA*) gene located between 450 and 600bp is a helpful and economical tool for *Campylobacter* genotyping (Harrington et al., 1997). The *flaA*-SVR nucleotide alleles sequences are available in the pubMLST *Campylobacter* database, with open access to all the *flaA*-SVR types isolated worldwide. The SVR-*flaA* typing provides sufficient discrimination for its use as a subtyping method for *C. jejuni* due to the fact that some of the regions of the flagellin encoding A gene are genetically stable over a long period thereby helping in molecular typing and differentiation of isolates(Manning et al., 2003).

Fluoroquinolones such as ciprofloxacin and macrolides like erythromycin are drugs of choice to treat *Campylobacter* infections in humans. In the recent years a direct correlation between indiscriminate
fluoroquinolone use in poultry and an increase in antimicrobial resistance of *Campylobacter* isolates in humans has been observed (Garcia-Migura et al., 2014).

The information pertaining to SVR-*flaA* types of *Campylobacter* circulating in poultry, poultry meat or products in Jammu & Kashmir is not available in pubMLST *Campylobacter* database union. The present study aimed to determine the occurrence of campylobacter infection in poultry and poultry meat, virulence profile, circulating *flaA* types with the ultimate goal of assessing the potential risk to human population.

**MATERIALS AND METHODS**

**Sample Collection**

Sampling methodology and isolation of *C. jejuni* from the poultry and poultry meat was carried out as described previously by (Wieczorek et al., 2018). Briefly, a total of 200 samples comprising of 86 cloacal swabs, 50 enteritis samples from Government poultry farms (Hariparbat and Broiler farm at Faculty of Veterinary Sciences & Animal Husbandry SKUAST-K), and 64 fresh poultry meat samples (poultry wings and drumsticks) from local retail vendors around Srinagar and Ganderbal districts of Kashmir valley were collected. The samples were collected in sterile vials in transport media and transported to the laboratory on ice and processed within 24 hours of collection.

**Sample Processing, Bacterial Strains isolation, Phenotypic Characterization and Molecular typing**

The primary enrichment of the samples was carried under microaerophilic conditions at 42°C for 48 hrs in Campy-thioglycollate broth (Hi-Media) supplemented with Blaser-Wang supplement (Hi-Media). The enriched samples in duplicates were cultured on Campylobacter agar base medium (Hi-Media) supplemented with Blaser-Wang (Hi-Media), 10% defibrinated sheep blood and on modified Cefaperazone Charcoal Agar (mCCDA) (Hi-Media) supplemented with CCDA supplement (Hi-Media) and subsequently incubated under microaerophilic (campygen gas pack,Oxoid) at 42°C for 24-48hrs. The primary identification of suspected colonies was based on colony morphology, gram staining, and biochemical tests (catalase test, oxidase test, and Hippurate hydrolysis).

**Bacterial DNA Extraction & PCR assays**

DNA was extracted using the Wizard DNA extraction kit (Promega). The isolates were confirmed by genus-specific primer (16SrRNA) of *Campylobacter* spp and multiplex PCR using species-specific primers of *C. jejuni* and *C. coli* as per the protocol of (Denis et al., 1999). All the PCR assays in the present study were performed in 25µl reaction volume in the Mastercycler gradient (Eppendorf, Germany). The reaction mixture comprised of 3.0µl template DNA, 12.5 µl of GoTaq® Green Master Mix, 0.5µM concentration of each primer, and sterile nuclease-free water (NFW) to make up the volume. Sterile water was incorporated as the negative control. The *C. jejuni* and *C. coli* positive controls were kindly provided by Prof. Linda Van
der Graff, Department of Infectious Disease and Immunology, Faculty of Veterinary Medicine, Utrecht University, Netherland. The cyclic conditions comprised of initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing specific to the primer pair for 60sec, and extension at 72°C for 60sec, followed by a final extension at 72°C for 10 min. The virulence gene profile of C. jejuni and C. coli strains for cadF, cdtB and wlaN genes was determined. The primers, annealing temperatures, target genes of the current study are depicted in Table 1. The amplified PCR products were visualized in 1.5% agarose gel in tris acetate EDTA (TAE) buffer, 0.5µg/ml ethidium bromide (EtBr) and visualized under ultraviolet illumination and documented using Gel Documentation System (UHV, Imaging System).

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA (Genus specific)</td>
<td>F-GGTTAAGTCCCGCAACGAGCCGC R-GGCTGATCTACGATTACTAGCGAT</td>
<td>50</td>
<td>283</td>
<td>Leite et al., (1996)</td>
</tr>
<tr>
<td>mapA (C.jejuni)</td>
<td>F-CTATTTTTTGGGTTGCTTGTG R- GCTTTATTTGCCATTTTGGTTATTATA</td>
<td>52</td>
<td>589</td>
<td>Denis et al., (1999)</td>
</tr>
<tr>
<td>ceuE (C.coli)</td>
<td>F-AATTGAAAAATGCTCAAATATG R- TGATTTTATTATTTGTAGCAGCG</td>
<td>52</td>
<td>462</td>
<td>-do-</td>
</tr>
<tr>
<td>cdtB</td>
<td>F-CAGAAAGCAATGGAGTGTGT R- AGCTAAAAGCGGTGGAGTAT</td>
<td>52</td>
<td>620</td>
<td>Hickey et al., (2000)</td>
</tr>
<tr>
<td>cadF</td>
<td>F-TTGAAGGTAATTTAGATATG R-CTAATACCTAAAGTTGAAAC</td>
<td>45</td>
<td>400</td>
<td>Konkel et al., (1999)</td>
</tr>
<tr>
<td>wlaN</td>
<td>F-TTAAGAGCAAGATGATGTTG R-CCATTTGAATTGATATTTTG</td>
<td>46</td>
<td>672</td>
<td>Linton et al., (2000)</td>
</tr>
<tr>
<td>flaA</td>
<td>F5’GGATTTCTGATTAACAAATGCTGC3’ R5’CTGTAATCTTTAAAACATTGGG3’</td>
<td>52</td>
<td>1728</td>
<td>(Nachamkin et al., 1993)</td>
</tr>
</tbody>
</table>

**Short Variable Region- fla A (SVR flaA) typing**
Short variable region (SVR) typing of *flaA* gene of *C. jejuni* and *C. coli* strains was determined using the primer sequence listed in Table 1. The cyclic conditions for *flaA* typing consisted of initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C-30 sec, annealing at 46°C-30 sec, and extension at 72°C-30 sec, followed by a final extension at 72°C for 5 min and visualization in 1.5% agarose gel. The PCR products were purified using the QIA quick PCR Purification Kit (Qiagen). The cloning of the products was done by ligating them to pGEM-T Easy Vector using a PCR cloning kit (pGEM®-T Easy Vector Systems. Promega). The ligation reaction mixture (10µl) comprised of 5µl of 2X rapid ligation buffer, 1µl of pGEMT Easy Vector (50ng), 1µl T4 DNA Ligase (3 Weiss units/µl), 2µl of purified PCR product and sterile nuclease-free water. The ligation reaction mixture was incubated for 1 hour at room temperature. The DH5α competent cells were prepared using 0.1M of CaCl₂ solution. The transformation was carried out by adding 2.5µl of ligation mixture to 100µl of competent cells. 100µl of transformed cells were pipetted out and poured on LB agar plates containing ampicillin (100µg/ml), X-gal (40mg/ml), and IPTG (100mM). The plates were incubated at 37°C overnight. The white coloured colonies were screened for the presence of insert using colony PCR. The plasmids were extracted using a plasmid extraction kit (PureYield™ Plasmid Miniprep System). The sanger sequencing of cloned plasmids was done at AgriGenome Labs Pvt. Ltd, (Kerala, India) using. The *flaA* sequences were submitted to Genbank and one novel peptide sequence was deposited in pubMLST database for *Campylobacter* ([https://pubmlst.org/pubmlstcampylobacter_isolates](https://pubmlst.org/pubmlstcampylobacter_isolates)). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 7 nucleotide sequences. A total of 402 positions were identified in the final dataset. Evolutionary analyses were conducted in MEGA X.

**Restriction Fragment Length Polymorphism (RFLP)**

The *flaA* gene (1728 bp) of *Campylobacter* strains was amplified using primer sequence given in Table 1. The cyclic conditions used were initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 92°C-30 sec, annealing at 52°C-1.5 min and extension at 72°C-2.5 min, and final extension at 72°C-5 min. The amplified *flaA* gene products (1728 bp) were digested using HpyF3I: Ddel (ThermoScientific™) restriction enzyme as per the protocol of Nachamkin *et al.*, (1993). The digestion mixture consisted of, 1µl of 4U Ddel, 2µl of 10X Restriction buffer, 10µl of PCR product, and the final volume was made with nuclease free water and incubated at 37°C for 16 hrs and the products were resolved on 2% agarose gel.

**Antimicrobial Resistance Study**

The antimicrobial profile was determined using the standard disc diffusion method on Muller-Hinton agar containing 5% defibrinated sheep’s blood. The panel of antibiotic discs included penicillin(10mcg), amoxicillin(10µg), ampicillin-sulbactam(10/10mcg), ampicillin (10mcg), amikacin(30mcg),...
tetracycline (30 µg), ciprofloxacin (5 mcg), enrofloxacin (5 mcg), and erythromycin (15 mcg). From a pure culture of 18–24 h of incubation, a bacterial suspension with an opacity of 0.5 McFarland was prepared and diluted 1:10. After inoculation by swab on Mueller–Hinton agar containing 5% fresh sheep blood (Hi Media India), and application of antibiotic discs (Hi Media India), the plates were incubated in a microaerophilic atmosphere for 24 h at 37°C. The interpretation of the test was done based on M-45; Clinical and Laboratory Standards Institute (Beilei-Ge et al., 2013).

RESULTS

The burden of Campylobacter infection in poultry and poultry meat was 21.5% of which C. jejuni was 2.5% and C. coli 19% (Fig. 1). The broiler meat samples (poultry wings and drumsticks) carried the highest percentage (26.5%) of Campylobacter spp followed by cloacal swabs (24.4%) and enteric samples (10%). The cloacal swab samples from the broiler production unit of Hariparbat farm recorded higher incidence (53.8%) of Campylobacter infection in comparison to other poultry rearing unit of the same farm (50%) followed by FVSc & A.H poultry farm (13.11%). The retail broiler meat vendors from Ganderbal area had higher Campylobacter load (36%) in broiler meat samples than those from Srinagar area (20.5%).

Among the various virulence factors that play a major role in Campylobacter pathogenesis cdtB, cadF were detected in all Campylobacter strains of the study. wlaN gene was detected in all the C jejuni strains (Fig. 2). In the present study of the twelve isolates obtained seven (07) flaA-SVR(CAMP1255) types have been identified and one new C coli sequence unique to this geographical region has been identified. The flaA-SVR sequence type that have been identified in present study are 62, 33, 78, 79, 72, and 44. The most common sequence type identified among the strains of C jejuni and C coli is 33. The distribution of most prevalent flaA-SVR genotypes of the present study is depicted in Table 2. The analysis of this new flaA sequence type of C coli strain (0L471375) indicative of a new strain has not been reported previously in pubMLST Campylobacter database. This sequence type has been assigned fla-nucleotide allele 1675, (peptide sequence 5) in the database. The sequence analysis for flaA gene in pubMLST Campylobacter database revealed that four C. coli strains isolated in present study that is, JKP-A (OL471369), JKP-E(OL471373), JKP-F(OL471374), and JKP-G(OL471375) exhibited the highest homology (> 95%) to allele type 62, 33, 33 and 78, respectively whereas C. jejuni strains JKP-B (OL471370), JKP-C (OL471371) and JKP-D (OL471372) had higher homology with allele types 79, 72 and 44, respectively reported in pubMLST Campylobacter database. In present study fla A C coli sequence types OL471372 and OL471373 are more closely related to the OL471371 sequence type than OL471374. The partial cds of the flaA type OL471369 is more closer to fla A sequence types C25 and C44 of C coli strains reported from other areas of the globe than to the fla A sequence types OL471375 and OL471373 of the present study (Fig. 3). The whole genome sequences of Campylobacter coli strains available in pubMLST Campylobacter database depicts that the OL471369 sequence type is closely related to Campylobacter coli strain AR-0420 chromosome complete genome. The OL471369 flaA gene partial cds of Campylobacter coli strain is more closely related to flaA of C jejuni strain FDAARGOS_422 chromosome(complete genome), C jejuni strain CFSAN032806 chromosome (complete genome), C jejuni
subsp. jejuni NCTC 11168-GSv (complete genome) and C. jejuni strain CS0048 flagellin A ( flaA) gene, partial cds (Fig. 3). Among the Campylobacter strains of this study the flaA gene, partial cds of OL471375 and OL471373 of C. coli strains are more closely related to the OL471374 flaA gene, partial cds sequence of C. jejuni strain isolated in the present study.

Table 2

<table>
<thead>
<tr>
<th>Isolate Name (Gen Bank Accession No)</th>
<th>Species</th>
<th>Source</th>
<th>Match Sequence Similarity (&gt;95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JKP-A (OL471369)</td>
<td>C. coli</td>
<td>Cloacal swab</td>
<td>CAMP1255(flaA):62</td>
</tr>
<tr>
<td>JKP-B (OL471370)</td>
<td>C. jejuni</td>
<td>Broiler meat</td>
<td>CAMP1255(flaA):79</td>
</tr>
<tr>
<td>JKP-C (OL471371)</td>
<td>C. jejuni</td>
<td>Broiler meat</td>
<td>CAMP1255(flaA):72</td>
</tr>
<tr>
<td>JKP-D (OL471372)</td>
<td>C. jejuni</td>
<td>Cloacal swab</td>
<td>CAMP1255(flaA):44</td>
</tr>
<tr>
<td>JKP-E (OL471373)</td>
<td>C. coli</td>
<td>Cloacal swab</td>
<td>CAMP1255(flaA):33</td>
</tr>
<tr>
<td>JKP-F (OL471374)</td>
<td>C. coli</td>
<td>Cloacal swab</td>
<td>CAMP1255(flaA):33</td>
</tr>
<tr>
<td>JKP-G (OL471375)</td>
<td>C. coli</td>
<td>Cloacal swab</td>
<td>CAMP1255(flaA):78</td>
</tr>
</tbody>
</table>

In present study 1728bp PCR amplified flaA gene depicted three different Ddel-RFLP patterns with band size ranging from 100-1000bp, indicative of strain variation among the Campylobacter isolates (Fig. 4). The results are in agreement with SVR– flaA typing of the present study.

A high degree of resistance of Campylobacter strains in the present study to ciprofloxacin (CIP), ampicillin (AMP), penicillin (PCN) nalidixic acid (NAL) was observed. The resistance to ciprofloxacin was (85%), both tetracycline (TET) and erythromycin was 57.1%, 85% for amikacin (AMK), 28% for amoxicillin (AMX), and enrofloxacin (ENFX). All the strains of present study were completely resistant to ampicillin (100%), penicillin (100%).

DISCUSSION

The flagellin gene locus of Campylobacter is composed of two genes flaA and flaB (Khoshbakht et al., 2015). The short variable region (SVR) of the flaA gene has been used by workers for molecular typing of Campylobacter spp. The SVR flaA typing method of Campylobacter has discriminatory power with the sequence data and genotypic information stored publicly in an accessible pubMLST database. The SVR-flaA gene variability among the strains in the present study may be attributed to the high variability of the Campylobacter genome caused by its instability (Wieczorek et al., 2019). The flaA flagellar gene has been reported to undergo spontaneous mutations during host infections which may have a crucial role in molecular variation(Guerry 2007). Sequence type 33 of the pubMLST Campylobacter database was the most common sequence identified among C. jejuni and C. coli strains. The genetic diversity of
Campylobacter isolates using flaA-SVR typing has been demonstrated by other workers (Wassenar et al., 2009, Magnusson et al., 2011, Sing and Kwon, 2013, Gomes et al., 2016).

In the present study out of the seven (07) flaA-SVR types identified, one sequence is unique to its origin. The sequences identified were 62, 33, 78, 79, 72, and 44. A similar genetic diversity in SVR region of the flaA gene of Campylobacter spp has been reported by several workers (Wieczorek et al., 2019, Wassenaar et al., 2009 and Corcoran et al., 2006). Corcoran et al., (2006) reported SVR-flaA allele types 325, 66, 343, 78, 427, 8, and 410 in the poultry population of Ireland. Wieczorek et al., (2019) observed the presence of 16, 54, 36, 287, 78, 14, 49, 100 SVR-flaA allele types in meat and faeces of poultry in Poland. Wassenaar et al., (2009) reported a total of 67 allele types in poultry from three European regions. The common allele types found in poultry were 36, 8, 70, 21, 32, and 34.

The flaA gene is has also been found suitable for restriction fragment length polymorphism (RFLP) analysis (Wassenaar et al., 2000). In view of good discriminatory power, the restriction enzyme DdeI, has been preferred for typing Campylobacter spp (Ghorbalanizadgan et al., 2016). The DdeI-RFLP pattern in the present study revealed three different patterns between 100-1000bp indicative of strain variation among the Campylobacter isolates from Kashmir. Yadav et al., (2018) reported 15 different DdeI-RFLP patterns in Campylobacter isolates ranging from 200 to 1100bp.

The 64 broiler meat samples screened in the present study, 17(26.5%) were positive for Campylobacter spp. of which 3 (17.64%) were positive for C. jejuni and 14 (82.35%) for C. coli. Sinulingga et al., (2020) reported an occurrence of 68.9% (C.jejuni) and 17.2% (C. coli) from poultry meat samples collected from wet markets and supermarkets in Malaysia. The cad F gene product adhesin and fibronectin-binding proteins help Campylobacter to adhere to the extracellular matrix of intestinal cells (Khoshbakht et al., 2013). Cdt blocks G2/M phase of eukaryotic cells prior to cell division, inducing cytoplasmic distension ultimately leading to cell death (Reddy et al., 2103). Glycosyl transferase a product of wlaN, glycosylates the LOS of C. jejuni. The glycosylated LOS mimics the structure of glycans and gangliosides receptors present in neuronal cells of humans. The immunoglobins produced in response to the C. jejuni epitopes cross react with human neurons resulting in Guillain-Barre syndrome (GBS) neuropathy. The presence of wlaN gene in all C. jejuni strains isolated in presents study is indicative of a potential human health hazard. Datta et al., (2013) reported presence of this gene in 23.8% and 4.7% of broiler meat and broiler faeces, respectively. Wieczorek et al., (2018) reported a 13.7% presence of the wlaN gene, in C. jejuni isolates from chicken faeces and 12.5% from poultry meat. However, since only three C jejuni strains are reported in the present study, the percentage of the wlaN gene in C jejuni strains in present study cannot be a true representative of the distribution of this gene in poultry here. Nevertheless, its presence in C. jejuni strains in present study is of concern given the zoonotic potential of this organism.

The study of resistance patterns of antimicrobials and molecular diversity of Campylobacter isolates is important for the treatment and control of campylobacteriosis. The indiscriminate use of antibiotics in the poultry sector has led to the emergence of resistant strains of Campylobacter spp in humans. In the present study high degree of resistance observed in Campylobacter strains to ampicillin, penicillin,
amikacin, and fluoroquinolones like ciprofloxacin. The resistance of *Campylobacter* to enrofloxacin (28%) was less in comparison to other antibiotics which has been also been reported earlier (Koenraadi *et al.*, 1995). Gharbi *et al.*, (2018) has reported highest resistance of *Campylobacter* isolates to tetracycline (100%) and erythromycin (100%) followed by ciprofloxacin (99.2%), ampicillin (61.4%) and aminoglycosides (12.9%) antibiotics. This increase in resistance during the last decade is important in view of the fact that fluoroquinolones are still the first choice for the treatment of Campylobacteriosis. The increase in antimicrobial resistance calls for reducing antimicrobial use in poultry and incorporation of specific procedures to reduce chicken carcass contamination by *Campylobacters* spp.

**Declarations**

**Acknowledgments**

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**Author Contributions**

Sabia Qureshi conceived the idea, Saima Iqbal, Shaheen Farooq, Zahid Kashoo, Mahrukh Hafiz, Maliha Gulzar executed different parts of the work from isolation, detection, RFLP, SVR-Fla typing. Data analysis and interpretation was carried out by Arif Pandit, Muddasir Sharief Banday, Mohd Altaf Bhat. Manuscript editing review was done by Md Isfaquol Hussain, Pervaiz Dar, Gulzar Badroo, Faheem Ud Din, and Junaid Mehraj.

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**Data availability**

The data that supports the finding of this study are available with the corresponding author which can be made available on request.

**Ethical approval**

The study did not require any experimentation on animals or human subject

**Consent to participate**: not applicable

**Consent to publish**: Not applicable

**Competing Interests**

The authors declared that they have no competing interests.
References


Figures
Figure 1

Multiplex PCR for *C. jejuni* and *C. coli*.

**Lane J:** Positive control of *Campylobacter jejuni*

**Lane N:** Negative control

**Lane 1-2:** Isolates positive for *Campylobacter jejuni* (589bp)

**Lane M:** 100bp ladder

**Lane C:** Positive control of *Campylobacter coli*

**Lane N:** Negative control

**Lane A-E:** Isolates positive for *Campylobacter coli* (462)
Figure 2

PCR for \textit{wlaN} gene of \textit{Campylobacter jejuni}.

\textbf{Lane P}: Positive control

\textbf{Lane M}: 100bp ladder

\textbf{Lane1-3}: Isolates positive for \textit{wlaN} gene(672bp)

\textbf{Lane N}: Negative control
Figure 3

Molecular phylogenetic analysis of C. jejuni and C. coli isolates by Maximum Likelihood method
Figure 4

*DdeI* digestion of *flaA* gene of *Campylobacter* isolates

**Lane M:** 100bp ladder

**Lane 1-3:** Digestion pattern of *flaA* gene PCR product