



## Variation in antibiotic susceptibility and presence of type VI secretion system (T6SS) in *Campylobacter jejuni* isolates from various sources

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### ABSTRACT

*Campylobacter jejuni* is a major cause of infectious diarrhea in humans. The bacterium can be transmitted through contaminated poultry meat and waste water. We report the presence of *C. jejuni* from potential transmission sources including egg shells, poultry waste, waste water and migratory bird droppings with a prevalence rate of 78%, 66%, 86% and 70% respectively. Antibiotic resistance profile showed high number of isolates resistant to multiple antibiotics including 4<sup>th</sup> generation cephalosporins. *C. jejuni* isolates were further screened for presence of T6SS, an important virulence factor. None of the *C. jejuni* isolates from migratory birds carried a T6SS, whereas highest prevalence of T6SS isolates was observed in waste water samples, followed by poultry waste and egg shells. To determine virulence potential of the isolates, hemolytic activity of isolates was compared. Although variation in hemolytic potential between isolates from different sources was noted, higher hemolytic activity was observed for isolates possessing *hcp*, a T6SS gene. Furthermore, presence of T6SS affords the bacterium some survival advantage when compared to T6SS competent *Helicobacter pullorum* which occupies the same niche. Taken together our findings indicate that *C. jejuni* with T6SS have a fitness advantage increasing their isolation frequency from waste water and poultry waste.

### 1. Introduction

Infectious diarrheal diseases are of great concern throughout the world, as they cause considerable morbidity and mortality rates, especially in developing countries [1]. Many *Campylobacter* spp, especially *Campylobacter jejuni* and *Campylobacter coli* are the important source of foodborne illness in humans worldwide causing acute to chronic enteritis. Interaction and ingestion of poultry and poultry products is considered a threat for campylobacteriosis [2]. Poultry birds are raised for meat and egg production and the disease may be transmitted through egg laying birds to their egg and onto the chicks [3], as amplifiable *C. jejuni* DNA is present in eggshell and hatchery fluff [4]. Therefore, risk of exposure to poultry as well as its products, if not carefully handled can be a source of infection in humans. *C. jejuni* is equipped with a T6SS protein translocation system [5–7]. T6SS plays an important role in virulence contributing to cytotoxicity, hemolysis and colonization of host tissue [5]. Extensive use of antibiotics has resulted in multidrug resistance among *C. jejuni* [8]. Resistance to antibiotics is emerging rapidly among *Campylobacter* spp. due to promptly evolving genomes of the pathogens in the recent decades all over the world.

Inherent resistance in *C. jejuni* and *C. coli* is described against the penicillin, rifampicin, vancomycin, trimethoprim, sulfamethoxazole, and cephalosporins [9]. Resistance to some beta lactam antibiotics namely ampicillin and some of the expanded-spectrum cephalosporins in *Campylobacter* spp. have also been reported. These reports vary and are not very obviously elucidated [10]. In this study we have determined antibiotic resistance *C. jejuni* isolates from various sources including eggshells and migratory birds and screened T6SS effector Hcp to understand the possibility of transmission of more virulent strains to the host.

### 2. Material and methods

#### 2.1. Bacterial isolation and culture

Eggshells were collected from hatcheries and waste water, poultry waste and fecal samples of migratory birds namely Starling, Red wattle lapwing, Laura erekson, Wader, Seagull, Skimmers and Raman Raina from Rawalpindi, Islamabad and Swabi during September 2014 to January 2015. Eggshells and waste water were enriched in nutrient

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broth and incubated at 37 °C for 24 h and then streaked on mCCDA media (Oxoid, Basingstoke, UK) whereas fecal and poultry waste were streaked directly on mCCDA media (Oxoid, Basingstoke, UK) and incubation was carried out at 42 °C for 48 h under microaerophilic conditions with 5% oxygen and 10% available CO<sub>2</sub> provided by CampyGen sachets (Oxoid, Basingstoke, UK). Preliminary characterization of bacterial isolates via biochemical tests including oxidase, catalase, esterase and hippuricase were tested as described previously [11].

## 2.2. Molecular identification of the isolates

Samples that showed hippuricase activity were further identified by PCR using primers F5'GCCCAAAGCCCATCAAGCGGA'3 and R5'GCCCAAAGCCCATCAAGCGGA'3, amplifying housekeeping gene *gltA* (citrate synthase), for the identification of all isolates at molecular level. Reaction volume for PCR was 25 µL, that contained 3 µL DNA, 1X PCR buffer (Fermentas, Lithuania, UAB), 200 µM of each of dNTPs (Fermentas, Lithuania, UAB), 2.5 mM of MgCl<sub>2</sub> (Fermentas, Lithuania, UAB), 0.4 µM of each primer (Alpha DNA, Quebec, Canada) 0.25 U of Taq DNA polymerase (Fermentas, Lithuania, UAB), rest of the volume was attained with nuclease free water. PCR conditions were: DNA melting at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s and cooling at 4 °C for 10 min [7].

## 2.3. PCR based screening of T6SS

Primers *hcp* F5'CAAGCGGTGCATCTACTGAA'3 and *hcp* R5'TAAGCTTTGCGCTCTCTCCA'3 were used to screen conserved hallmark gene of T6SS, *hcp* (hemolysin co-regulated protein), to report presence or absence of this secretion system. A singleplex PCR was performed in a 25 µL reaction volume with 3 µL DNA, 1X PCR buffer (Fermentas, Lithuania, UAB), 200 µM of each of dNTPs (Fermentas, Lithuania, UAB), 2.5 mM of MgCl<sub>2</sub> (Fermentas, Lithuania, UAB), 0.4 µM of each primer (Alpha DNA, Quebec, Canada), 0.25 U of Taq DNA polymerase (Fermentas, Lithuania, UAB), rest of the volume was achieved with nuclease free water. *gltA* was added as an internal positive control at a concentration of 0.05 µM in each of the reaction mixtures to check for the PCR fidelity. PCR conditions were DNA melting at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s and cooling at 4 °C for 10 min [7].

## 2.4. Hemolysis assay

Freshly isolated human erythrocytes were used for the assay and obtained from healthy volunteers at the day of assay. Blood drawn in vacutainers was washed thrice in 9 volumes of PBS (phosphate buffer saline) through centrifugation for 5 min at 4000 rpm (25 °C). Finally, the pellet was suspended in PBS. Assay was performed as described earlier. Briefly, bacterial sample suspensions were made by taking growth from fresh plates in PBS and diluted to a concentration of 3 × 10<sup>9</sup> CFU per ml. Bacterial sample suspension and erythrocyte suspension were mixed in a ratio of 1:1. The mixture was centrifuged for 5 min at 5000 rpm (25 °C) to increase the interaction of erythrocytes with bacteria. Samples were then incubated for 4 h (42 °C) under microaerophilic conditions (85% N<sub>2</sub>, 10% CO<sub>2</sub> and 5% O<sub>2</sub>). After incubation pellet containing erythrocytes was re-suspended to liberate any lysed cells. The suspension was centrifuged for 5 min at 5000 rpm (25 °C) to pellet intact cells. Optical density of supernatant was determined at 540 nm using a nanospectrophotometer (Implen NanoPhotometer®P-Class P300).

Mean values of OD and standard error of the means were calculated and statistical analysis was performed using Student's T-test.

## 2.5. Bacterial growth competition assay

*C. jejuni* and *H. pullorum* strains were grown on mCCDA agar and Columbia blood agar plates supplemented with DENT (Oxoid, UK) supplement respectively. Bacterial colonies were dissolved in BHI broth and OD<sub>600</sub> adjusted to correspond to 2 × 10<sup>8</sup>CFU/ml. CFUs for *C. jejuni* and *H. pullorum* strains (with and without T6SS- where indicated) were then mixed together in a ratio of 1:1. The resulting bacterial suspension was plated onto BHI agar and incubated at 42 °C for 48 h under microaerophilic conditions (85% N<sub>2</sub>, 10% CO<sub>2</sub> and 5% O<sub>2</sub>). Individual isolates of *C. jejuni* and *H. pullorum* were also plated and stored under same conditions. After the completion of incubation time, colonies were harvested under aseptic conditions and dissolved in PBS. Serial dilutions were plated simultaneously on BHI agar containing DENT supplement (Selective for *H. pullorum*) and CCDA (Selective for *C. jejuni*) respectively. After 2 days, individual colonies were counted and CFU/ml was calculated.

## 2.6. Determination of antimicrobial susceptibility multidrug resistance pattern

Antimicrobial susceptibility testing was performed using a Kirby-Bauer disk diffusion method for sulphamethoxazole, tetracycline, erythromycin, chloramphenicol, imipenem, ciprofloxacin, nalidixic acid, streptomycin, tigecycline, gentamycin and cefotaxime. *C. jejuni* isolates exhibiting multiple drug resistance (MDR) was studied by the disc diffusion method as per recommendation of (CLSI Clinical and Laboratory Standards Institute, 2015). Pure culture of *C. jejuni* from the mCCDA agar (Oxoid, Basingstoke, UK) was streaked on Muller Hinton agar (Oxoid, Basingstoke, UK) and discs were placed and incubated under microaerophilic condition in airtight jars with CampyGen sachets (Oxoid, Basingstoke, UK) for 48 h at 42 °C.

## 3. Results

### 3.1. Prevalence of *C. jejuni* in various potential transmission sources

A total of 112 samples were collected from Islamabad and Rawalpindi (22) waste water, (30) poultry, (10) migratory birds (50) eggshell samples respectively. All isolates were positive for oxidase and catalase and out of 112 isolates, 85 were indoxyle acetate and hippurate positive and therefore designated *C. jejuni* spp. *jejuni*. Using specific PCR primers, 85 hippurate positive isolates were confirmed for *C. jejuni*. All isolates designated as *C. jejuni* tested positive for a housekeeping gene *gltA* specifically designed for *C. jejuni* and generated an amplicon size of 142 bp (Fig. 1a).

*C. jejuni* PCR screening of isolates showed that 78% of eggshell samples and 86% of waste water samples were positive for *C. jejuni*.

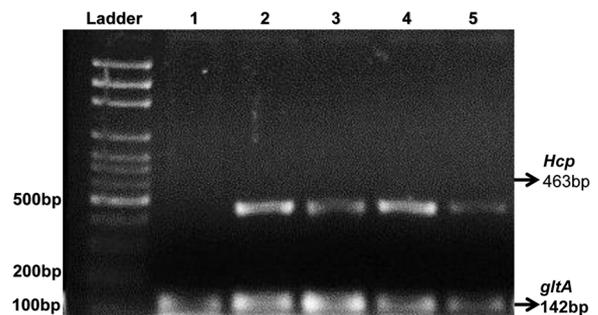


Fig. 1. Gel electrophoresis of *hcp* (haemolysin co-regulated protein) PCR for the identification of T6SS positive *C. jejuni* isolates, 1 kb ladder was used, Lane-1, *hcp* (haemolysin co-regulated protein) negative control, Lane-2-5, *C. jejuni* positive isolates. *gltA* housekeeping gene was used as a positive control for *C. jejuni*.

**Table 1**  
Sample collection and isolation frequency of *C. jejuni* from different sources.

Source	Total no of samples	Positive samples for <i>C. jejuni</i>	T6SS Positive
Eggshells	50	39	3
Migratory birds	10	7	0
Waste water	22	19	5
Poultry waste	30	20	4

Meanwhile 66% poultry tissue samples were positive and 70% migratory bird samples were positive for *C. jejuni*. Among the migratory bird droppings collected, starling from England, Red wattlebird from India, Laura Erikson from Siberia, Wadler from North America, Seagull from United Kingdom, Skimmer from South Asia, Africa and America, Raman Raina from South Asia, Africa and America were carriers for *C. jejuni*. Waste water samples tested from 22 different locations of Rawalpindi and Islamabad tested positive for *C. jejuni* include majority of the residential sectors lying in the east of Islamabad zero point in addition to some of the south western areas. While samples collected from western parts of Islamabad were free from *C. jejuni*. The highest prevalence of *C. jejuni* was observed in waste water, 86.34% (Table 1).

### 3.2. Identification of T6SS

Presence of a novel virulence factor, the T6SS was determined via screening for presence of *hcp* by PCR (Fig. 2). Out of 39 eggshell isolates tested, 3 were positive for T6SS (7.7%), whereas out of 20 poultry waste 4 were positive (20%). Out of 19 waste water samples, 5 were positive (26.3%), and none of the *C. jejuni* strains from migratory birds possessed *hcp* gene.

### 3.3. Hemolytic activity of isolates

All isolates tested induced erythrocyte lysis in infected erythrocytes compared to the uninfected control with marked variations in the hemolytic activity of strains from different sources (Fig. 3a). Highest hemolytic activity was recorded for the reference strain Cj-255 possessing a functional T6SS. This was followed by eggshell, waste water ( $p < 0.05$ ) and human fecal isolates ( $p < 0.005$ ). Furthermore, an increased trend in pooled hemolytic activity of T6SS positive isolates could be observed compared to T6SS (-) isolates (Fig. 3b) although this difference was not significant.

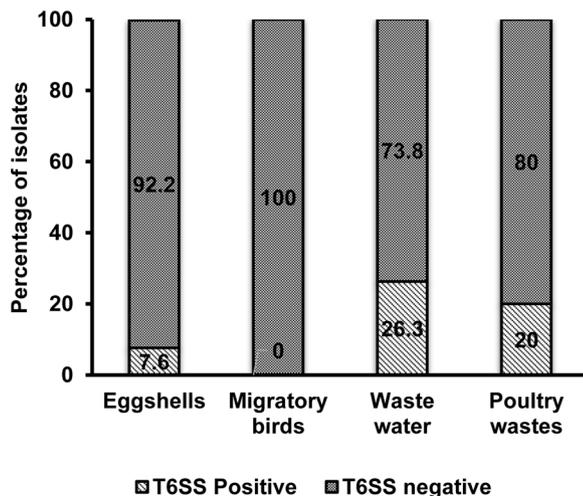


Fig. 2. Prevalence of T6SS positive isolates in different sources.

### 3.4. Bacterial growth competition

As both *C. jejuni* and *H. pullorum* share the same ecological niche, bacterial co-culture experiments were performed to determine the role of T6SS in the bactericidal activity of *C. jejuni* and effects of presence of *H. pullorum* on the growth of *C. jejuni*.

As seen in (Fig. 4) T6SS (+) *H. pullorum* completely inhibited the growth of *C. jejuni* T6SS (-). Whereas growth was restored in *C. jejuni* isolates possessing T6SS. In short, *H. pullorum* was able to outgrow *C. jejuni* but this effect was less pronounced in *C. jejuni* strain possessing T6SS.

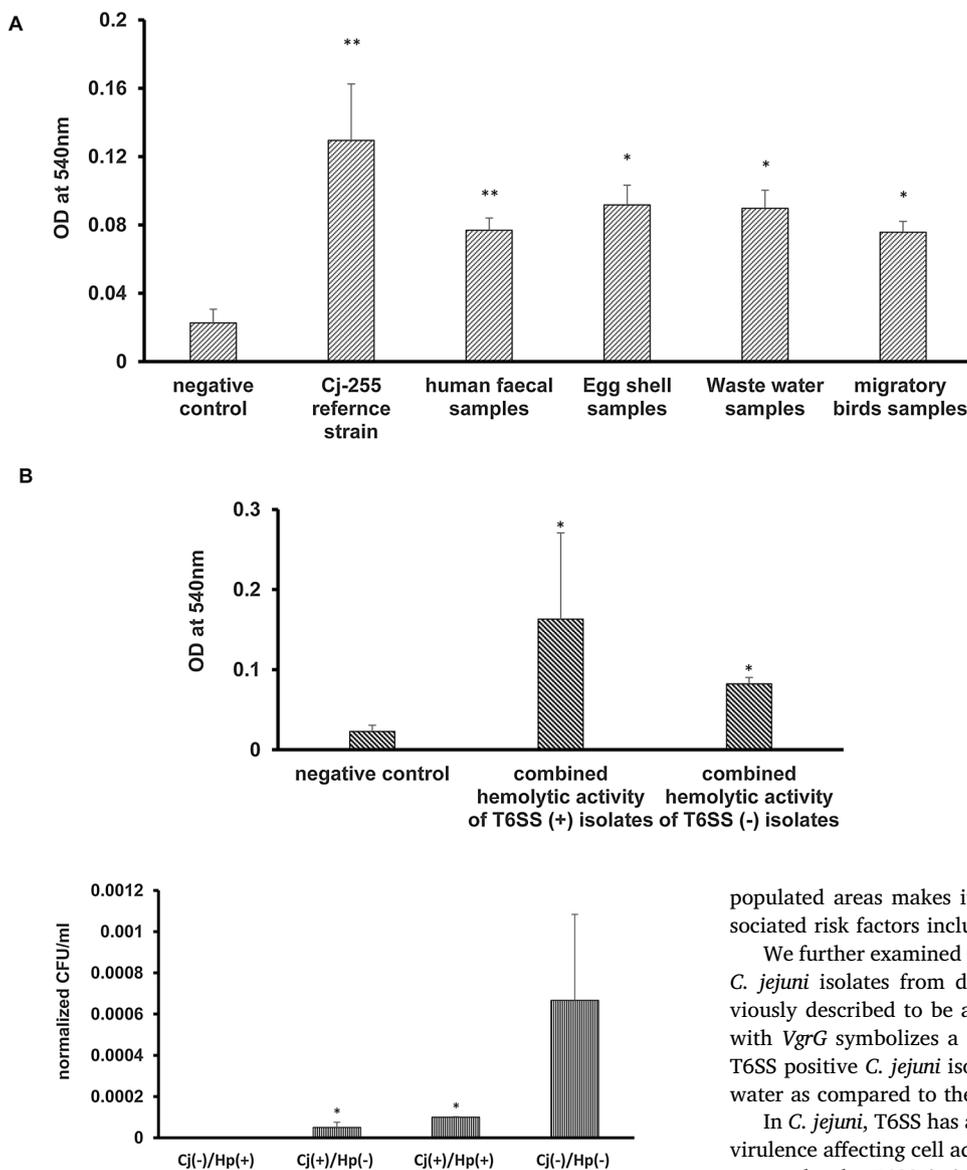
### 3.5. Antibiotic resistance

Antimicrobial susceptibility testing of *C. jejuni* isolates indicated random distribution of drug resistance among different sources (Fig. 3). *C. jejuni* eggshell isolates showed 45% resistance to Sulphamethoxazole, whereas 78.9% waste water isolates, 85% poultry waste isolates and 71.4% migratory bird fecal isolates were resistant to the antibiotic. Resistance to chloramphenicol was in 39% of eggshells isolates, 31.5% of waste water isolates, 50% of poultry waste samples, and 85.71% of migratory birds' fecal isolates. 100% resistance was observed against cefotaxime in almost all sources. *C. jejuni* isolated from waste water was sulphamethoxazole, 85%, tetracycline 95%, erythromycin 100%, chloramphenicol 50%, imipenem 20%, ciprofloxacin 70%, nalidixic acid 95%, streptomycin 95%, tigecycline 20%, gentamicin 75% and cefotaxime 100%. *C. jejuni* isolated from migratory birds showed high resistance against sulphamethoxazole 71.42%, tetracycline 42.85%, erythromycin 85.75%, chloramphenicol 85.71%, gentamicin 71.42%, cefotaxime 100%, ciprofloxacin 71.42%, nalidixic acid 57.14%, streptomycin 57.14%, whereas all isolates were susceptible to tigecycline and imipenem. Similarly, high resistance against erythromycin, tetracycline, streptomycin and nalidixic acid in waste water isolates and moderate resistance in isolates from remaining sources was observed. Gentamicin and ciprofloxacin resistance in a small number of isolates irrespective of source was observed, while most isolates showed least resistance to tigecycline and imipenem. It was interesting to observe that *C. jejuni* isolated from migratory birds' fecal sample were sensitive to both imipenem and tigecycline (Fig. 5).

## 4. Discussion

Poultry industry constitutes one fourth of the total meat production in Pakistan [12]. However with current farming practices, use of antimicrobials to cure and prevent disease and promote growth have been alarmingly high incurring a rise in multi-drug resistance in associated microbiota [13]. This is a pioneer study from Pakistan where we report *C. jejuni* prevalence in eggshells with a high prevalence rate of 78% compared to previous reports [14,15]. Since eggshells are used in the preparation of poultry feed as a calcium source, their role as a transmission source for *C. jejuni* to different poultry breeds may be underestimated. The prevalence of *C. jejuni* in poultry meat was 66.66% which is significantly higher than reports from other countries [16]. *C. jejuni* in poultry droppings was also detected and may be an additional source of infection spread in a flock as it may contaminate the bedding used in farms.

High prevalence of *C. jejuni* (70%) in migratory bird droppings was also observed. A similar study conducted in New Zealand revealed that the prevalence in migratory birds was 47% [17] with similar reports from Europe showing low prevalence of *Campylobacter* spp. in migratory birds [18,19]. The variations in the prevalence estimates could be attributed to variability in sample size, collection, age of fecal material and sensitivity of the culture techniques. Furthermore it has been reported that the seasonal variation may also play a crucial role in the variation observed as faeco-prevalence of *C. jejuni* is relatively higher during warmer months [17] and our samples being collected in the



**Fig. 4.** *C. jejuni* growth normalized to *H. pullorum*. CFU/ml of each was calculated and *C. jejuni* growth determined in presence of *H. pullorum*. Cj (+), Hcp (+) strains carrying T6SS and Cj (-), Hcp (-) T6SS lacking strains. Results are represented as mean of two independent experiments.

summer season. Shoreline-foraging birds feeding on invertebrates and opportunistic feeders are also most frequently infected by *Campylobacter* spp. [20]. Wild birds encounter the domesticated, farm birds and share their feed; leaving droppings in farm premises and in the feed as well. These birds being carriers of *Campylobacter* spp. can transmit *C. jejuni* and *C. coli* [21]. Migratory birds are known to spread *C. jejuni* through international borders [22]. Proliferation of countless other microorganisms harmful to vertebrates and poultry are also associated with migratory birds. Birds become more susceptible to pathogens and enhance their shedding rate as a result of stresses associated with migration [23]. The risk associated with the disease spread by these birds is confounded by presence of multi-drug resistance and virulence factors such as the T6SS. In this study *C. jejuni* was most prevalent in waste water, while similar reports from other regions show low prevalence of *C. jejuni* in waste water [24]. This indicates that Pakistan like many developing countries lacks proper planning such as lack of sanitation, lack of proper filtration of drinking water and mixing of waste water due to sewerage lines in drinking water may result in high prevalence. Moreover, the mushroom growth of poultry farms amid heavily

**Fig. 3.** a) Pooled hemolytic activity of isolates from different sources – human faecal, egg shell, waste water, migratory birds and *C. jejuni* strain Cj-255 was used as a reference and uninfected erythrocytes were used as a negative control. Results are represented as mean of two independent experiments with 2 replicates. \*p value = 0.05, \*\*p value = 0.005. b) Cumulative haemolytic activity of T6SS positive and negative isolates compared to uninfected cells. Results are represented as mean of two independent experiments with 2 replicates. \*p value = 0.05.

populated areas makes it difficult to control the dissemination of associated risk factors including hypervirulent *C. jejuni*.

We further examined the prevalence of T6SS virulence factor among *C. jejuni* isolates from different sources. *C. jejuni* Hcp has been previously described to be an important T6SS effector protein that along with VgrG symbolizes a functional T6SS [6]. According to our study, T6SS positive *C. jejuni* isolates are more common in poultry and waste water as compared to the other sources.

In *C. jejuni*, T6SS has a pleiotropic effect and is involved in bacterial virulence affecting cell adhesion, invasion and cytotoxic effects [25]. In our study, the T6SS (+) isolates showed a higher association with hemolytic activity. Hemolysin coregulated protein makes a channel of T6SS through which different proteins pass out of the bacterial cell into the cytosol of the infected cell, this may affect host cell membrane integrity as observed in earlier reports [26]. Other hemolysins may be present in these isolates which can contribute to the hemolysis observed for the strains lacking T6SS. However, to date few hemolysins have been identified in *C. jejuni*.

Presence of T6SS may reflect virulent forms of the bacterium that are predominant in poultry and waste water and there is possibility of acquisition of this virulence marker from environmental sources. T6SS positive *C. jejuni* hypervirulent strains may result in bloody diarrhea in humans through zoonosis [7]. However the transmission of these hypervirulent strains from water waste and poultry to humans with severe form of campylobacteriosis needs to be further validated by using source tracking markers as described previously [27].

To test whether presence of T6SS offers competitive growth advantage to bacterium, *C. jejuni* T6SS competent and deficient strains were co-cultured with *H. pullorum* a bacterium that occupies a similar physiological niche in chickens. Coincidentally putative genes of a T6SS have also been identified in most *H. pullorum* strains [28]. Although growth of *C. jejuni* was found to be limited by *H. pullorum*, T6SS lacking *C. jejuni* strains were not able to grow in the presence of the competing bacterium. Therefore it can be concluded that although *C. jejuni* T6SS is

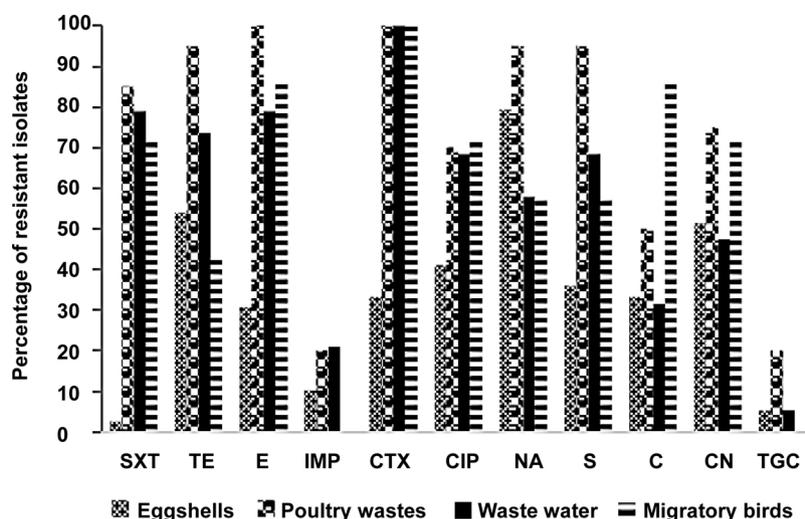


Fig. 5. Antibiogram of *C. jejuni* isolates from various sources. Sulphamethoxazole SXT, Tetracycline TE, Erythromycin E, Imepenem IMP, Cefotaxime CTX, Ciprofloxacin CIP, Nalidixic acid NA, Streptomycin S, Chloramphenicol C, Gentamycin CN, Tigecycline TGC.

not involved in direct bactericidal activity against *H. pullorum*, it provides the bacterium some competitive growth advantage as the strains lacking T6SS are not able to survive in a co-culture with *H. pullorum*.

This finding may have implications for poultry as *C. jejuni* is a commensal in poultry but *H. pullorum* in some cases is associated with pathology [29]. Interestingly, abundance of *Campylobacter* or *Helicobacter* species can drive changes in abundance and diversity of certain microbial taxa and impact the overall chicken microflora [29]. However, variations in growth kinetics of the bacteria may introduce some bias into our results. Presently, there is no reliable platform to predict the survival of any population in case of competition between bacterial populations of varying growth kinetics [30].

Growing antibiotic resistance among pathogenic bacteria poses a major threat for public health. A previous study conducted in Pakistan revealed the prevalence of antibiotic resistance in poultry revealed high tetracycline and erythromycin, resistance (Siddiqui et al., 2015). Another study performed in Spain has shown similarly high tetracycline but low erythromycin resistance and high ciprofloxacin resistance in contrast to our isolates, [31]. A study from the hunter region of New South Wales showed low resistance to tetracycline, nalidixic acid, ciprofloxacin, and erythromycin [32]. In our study resistance pattern observed in *C. jejuni* isolated from waste water showed high sulphamethoxazole, tetracycline, erythromycin, chloramphenicol, ciprofloxacin, cefotaxime, gentamycin, nalidixic acid and streptomycin resistance and low resistance against tigecycline. These results are in contrast to resistance pattern observed in Canada and Sweden from waste water *C. jejuni* isolates where ciprofloxacin, erythromycin and gentamicin resistance was absent, with low number of tetracycline resistance and 55% nalidixic acid resistant isolates [33,34]. However higher resistance trends against gentamicin, erythromycin and chloramphenicol from water isolates of *C. jejuni* in neighboring country India were observed with all isolates being resistant to cefotaxime [35]. Variations of drug resistance in isolates from different geographical regions may be indicative of climate affects and poultry rearing practices and regulations. In this study resistance to sulfamethoxazole, imepenem and tigecycline was reported for the first time in waste water isolates from Pakistan. Similarly, *C. jejuni* isolated from migratory birds showed high resistance against all antibiotics tested except tigecycline and imepenem. Chloramphenicol, erythromycin, gentamicin, and streptomycin susceptibility in *C. jejuni* isolates has been previously reported with ciprofloxacin and nalidixic acid resistance of 3.6% [36].

Quinolones (ciprofloxacin) and erythromycin are drugs of choice for *Campylobacteriosis* treatment. In this study high resistance rates in isolates from various sources has been observed ruling out effective

treatment with these drugs. However we recommend use of imepenem and tigecycline as alternative treatment option for treating drug resistant *Campylobacter* infections as low resistance against both drugs was observed. Multiple drug resistant (MDR) *C. jejuni* isolates are prevalent in poultry due to extensive and unnecessary use of antibiotics. From the current study it is suggested that prolonged use of antibiotics may result in the development of highly MDR *C. jejuni* strains which can be a major concern for the public health sector.

## 5. Conclusion

Our study is the first report from Pakistan of *C. jejuni* isolation from egg shells and migratory birds. High prevalence rates of the bacterium from poultry and waste water samples were observed. Hypervirulent strains containing T6SS effector *Hcp* was found in waste water and poultry waste. *Hcp* carrying strains demonstrated higher hemolytic activity and greater potential for survival in competition with *H. pullorum*, a bacterium sharing its niche.

The study also indicates high prevalence of multiple drug resistant *C. jejuni* in isolates which is an emerging problem in the region posing a serious threat to public health.

## Author contributions

Sobia Kayani, Vajeaha Aalam and Junaid Akhtar performed experimental work and contributed to the initial draft. Zobia Noreen contributed to experimental design; experimental work and final edit of manuscript. Dr. Sundus Javed and Dr. Fariha Masood contributed to the writing and editing of the manuscript. Prof. Dr. Habib Bokhari and Dr. Sundus Javed contributed to experimental design and the final edit.

## Declaration of Competing Interest

We declare no conflict of interest for this study.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

online version, at doi:<https://doi.org/10.1016/j.cimid.2019.101345>.

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