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Highlights

- *Campylobacter* species are circulating in various type of meat i.e., Beef, mutton and chicken.
- High resistance was seen in our isolates.
- High resistance is a serious health issue and may pose a risk in human medicine and food safety

Prevalence and antimicrobial resistance patterns of *Campylobacter* spp. isolated from retail meat in Lahore, Pakistan

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ABSTRACT

Campylobacter spp. is a leading cause of gastroenteritis in humans. Contaminated food of animal origin is considered to be the common source. Some of these bacteria are multi-drug resistant, which results in treatment complications. Indiscriminate use of antimicrobial drugs has been suggested to be largely responsible for resistance in zoonotic pathogens including *Campylobacter*. This study was conducted to determine the prevalence and antimicrobial resistance pattern of *Campylobacter* isolated from meat of three different food animal species sold at retail shops in Lahore, Pakistan. A total of 125 *Campylobacter* were isolated and tested for antimicrobial resistance against nine commonly used antibiotics in veterinary and human medicine. The highest resistance was observed against enrofloxacin (79.2%) followed by tylosin (77.6%), ciprofloxacin and amoxicillin (71.2% each), colistin (69.6%), neomycin (32.8%), nalidixic acid (31.2%), gentamicin (25.6%) and doxycycline (8.8%). Most of the isolates (90.4%) were resistant to more than two antibiotics and were considered as multi-drug resistant bacteria. The results indicate that antibiotic resistant bacteria are prevalent in animal meat in Pakistan probably due to uncontrolled use of antibiotics in food animals, thus posing a threat to public health.

Keywords: *Campylobacter*, resistance, antibiotics, Lahore, Pakistan, beef, mutton, chicken, meat.

1. Introduction

Most cases of *Campylobacter* infection in humans are self-limiting and do not require antibiotic therapy. However, severe cases such as those in immune-compromised patients do need to be treated with antibiotics (Engberg, Aarestrup, Taylor, Gerner-Smidt, & Nachamkin, 2001; Gibreel, et al., 2004) such as fluoroquinolones and macrolides. For bacteremia caused by *Campylobacter*, aminoglycosides are often used (Alfredson & Korolik, 2007; Corcoran, Quinn, Cotter, Whyte, & Fanning, 2006; Lin, et al., 2007; Moore, et al., 2006; Payot, et al., 2006). Unfortunately, the presence of antibiotic resistance in bacterial pathogens is a serious public health concern throughout the world (Han, Jang, Choo, Heu, & Ryu, 2007; Hawkey & Jones, 2009; Isenbarger, et al., 2002). People infected with antibiotic resistant strains of *Campylobacter* are ill for a longer period of time and are more likely to be hospitalized (Gupta, et al., 2004). The success rate of treatment against *Campylobacter* infection is decreasing due to an increase in antibiotic resistance (Lehtopolku, et al., 2010). Unfortunately, information on antibiotic resistance in *Campylobacter* of animal origin in developing countries is not available (Osano & Arimi, 1999).

Irrational use of antibiotics for the treatment and control of infectious diseases in veterinary medicine is considered a key cause of development of antibiotic resistance in foodborne pathogens (Hoszowski & Wasyl, 2005). Most of the antibiotics used in human and animal medicine are similar and hence the use of antibiotics in animals poses a potentially serious risk to public health (Alfredson & Korolik, 2007; Hariharan, Sharma, Chikweto, Matthew, & DeAllie, 2009; Luangtongkum, et al., 2009). Recently, the prevalence of antibiotic resistance in foodborne pathogens has increased and has become a complex issue (Možina, Kurinčič, Klančnik, & Mavri, 2011).

Antibiotics are often used as growth promoters in food animals. In developing countries, large amounts of various antibiotics are used in domestic poultry for the control of infectious agents and for growth promotion. This may help select resistant strains and their subsequent transmission to humans via contaminated food (Hoszowski & Wasyl, 2005). In Pakistan, no data are available on the presence and antimicrobial properties of *Campylobacter* in humans and food animals. The aim of this study was to determine the prevalence and antimicrobial resistance patterns of *Campylobacter* in various meat sources (beef, mutton, and chicken) in Lahore, Pakistan.

2. Materials and Methods

2.1 Sampling

A total of 600 meat samples (200 each of beef, mutton, and chicken) were collected from retail meat shops from ten administrative divisions of Lahore district in Pakistan from September 2014 to February 2015. Most of the meat consumption in Pakistan is in Fall and Winter. From each division, 20 samples each of beef, mutton and chicken were collected. The samples were placed in an ice box, transported to the laboratory, and subjected to microbial analysis within 24 hrs. of collection.

2.2 Isolation and identification of *Campylobacter*

Isolation of *Campylobacter* was carried out according to the international organization for standardization ISO 10272-1:2006 (Moran, et al., 2009). Meat samples were placed in separate bags and homogenized in a stomacher for 2 minutes with buffered peptone water at 1/10 ratio of w/v. An aliquot (1 mL) of this homogenate was transferred to a tube containing 9 mL of Bolton broth for enrichment. The inoculated Bolton broth was incubated at 42˚C for 48 hrs under microaerophilic conditions using Campy Gas sachet (Gaspak EZ Campy Container BBL 260680) in an anaerobic jar. An aliquot from the enriched broth was streaked on plates of mCCDA agar (CM 0739 Oxoid, England) containing cefoperazone and amphotericin B (SR0155 Oxoid, England) followed by incubation at 42˚C for 48 hrs under microaerophilic conditions. Suspected colonies were lifted from plates and identified as

Campylobacter using Gram staining, motility, oxidase test, and latex agglutination (F46 Microgen, UK). These colonies were further streaked on fresh mCCDA plates for purification and DNA extraction. The purified isolates were also placed in 20% glycerol and stored at-80˚C for future use.

2.3 Speciation of *Campylobacter*

DNA was extracted from purified isolates using "QIAamp DNA Mini Kit" (Qiagen, cat# 51306, USA) according to manufacturer's instructions. The extracted DNA was stored at -20˚C until used. Multiplex PCR was carried out for confirmation and speciation of *Campylobacter.* Three sets of primers were used to identify *Campylobacter* spp: *C. jejuni,* and *C. coli* by targeting *16SrRNA*, *mapA* and *cueE* gene, respectively (Denis, et al., 1999; Gonzalez, Grant, Richardson, Park, & Collins, 1997; Linton, Lawson, Owen, & Stanley, 1997; Stucki, Frey, Nicolet, & Burnens, 1995). PCR amplification reaction was performed in 25µL mixture in a thermal cycler "T100" (BioRad USA). The PCR conditions for 35 cycles were: denaturation at 94˚C for 1 min, annealing at 48˚C for 1 min and extension at 72˚C for 1 min. The PCR products were visualized under UV light following by gel electrophoresis in 1.2 % agarose gel.

2.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility tests on 125 isolates were performed by the disc diffusion method (Bauer et al., 1966). Briefly, three to five well-isolated colonies were selected from the culture plate. The colonies were suspended in normal saline solution followed by adjustment of turbidity to 0.5 McFarland standard. A sterile cotton swab was dipped into the suspension and streaked on the entire surface of a Mueller–Hinton agar plate (Oxoid, England) containing 5% sheep blood. The inoculum was allowed to dry for 5 min followed by application of antibiotic discs and incubation at 42^{0}C for 48 hours under microaerophilic conditions. Stock cultures of *C. jejuni* (ATCC 33560) and *C. coli* (ATCC 33559) were used as reference strains. The diameters of the zones of inhibition were measured with a calliper and interpreted as recommended by Clinical and Laboratory Standards Institute Guidelines (CLSI, 2006). A total of nine antibiotics commonly used in veterinary and human practices were tested e.g., amoxicillin (10µg), ciprofloxacin (5µg), colistin (10µg), doxycycline (30µg), enrofloxacin (5µg), gentamicin (10µg), nalidixic acid (30µg), neomycin (30µg) and tylosin (30µg).

2.5 Statistical Analysis

Data were entered into a Microsoft Excel sheet for analysis and were analysed to obtain the numbers and percent of resistant and susceptible microorganisms using SPSS 20.0 statistical software.

3. Results

A total of 125 *Campylobacter* were isolated from the three meat sources of which 82 were *C. jejuni* and 43 were *C. coli*. Of the 200 beef samples, 31 (15.5%) were positive for *Campylobacter* while this number was 36 (18%) and 58 (29%) for mutton and chicken, respectively (Table 1). When tested for antimicrobial susceptibility, the highest resistance in all 125 isolates of *Campylobacter* spp. was against enrofloxacin (79.2%) followed by tylosin (77.6%), amoxicillin (71.2%), ciprofloxacin (71.2%), and colistin (69.6%). Most of the isolates (113 of 125 or 90.4%) were resistant to multiple antibiotics. The *C. jejuni* isolates (n=82) were highly resistant to enrofloxacin and tylosin (78%) followed by amoxicillin (72%), ciprofloxacin (68.3%), and colistin (67%). The rate of resistance against these five antibiotics was similar in *C. coli* isolates (n=43) too. The least resistance was against doxycycline (8.8% in *Campylobacter* spp.).

The overall resistance in *Campylobacter* isolates (n=31) from beef origin was the highest against ciprofloxacin 83.9% (26/31) followed by enrofloxacin 77.4% (24/31) and colistin 74.2% (23/31). Resistance against doxycycline, nalidixic acid and neomycin was low.

None of the *C. jejuni* (n=19) isolates from beef showed any resistance to doxycycline while 2 of 12 (16.7%) of *C. coli* isolates were resistant to this antibiotic (Table 2). The resistance of *C. coli* (n=12) to enrofloxacin, ciprofloxacin, and colistin was similar to that in *C. jejuni*. None of the *C. coli* isolates from beef was resistant to nalidixic acid.

Of the 36 isolates from mutton, 31 (86%) were resistant to tylosin followed by enrofloxcin (72.2%) and ciprofloxacin and colistin (66.7% each). Low resistance was observed against doxycycline 11% (4/36) and gentamicin 22% (8/36). Of the 36 mutton isolates, 25 and 11 were confirmed as *C. jejuni* and *C. coli*, respectively. In *C. jejuni*, the highest resistance was against tylosine (21/25 or 84%) followed by enrofloxacin (76%) and amoxicillin (72%). Only 8% (2/25) and 20% (5/25) of *C. jejuni* isolates were resistant to doxycycline and neomycin, respectively. For *C. coli,* the highest resistance was against tylosin (10/11 or 91%) and ciprofloxacin (72.7%) and the lowest resistance was against doxycycline and gentamicin 18% (2/11 each).

Of the 58 isolates from chicken meat, the highest resistance was against enrofloxacin (84%), tylosin (82%), and amoxicillin (82%). Five (8.6%) and 11 (19%) isolates were resistant to doxycycline and gentamicin, respectively. Most of the *C. jejuni* isolates from chicken were resistant to tylosin (81.6%), enrofloxacin (79%), and amoxicillin (79%). Only four (10.5%) and 10 (26.3%) isolates were resistant to doxycycline and gentamicin, respectively. Among *C. coli*, the highest resistance was observed in enrofloxacin (95%) followed by amoxicillin (90%), and tylosin (85%). Sixteen of 20 (80%) isolates were resistant to both ciprofloxacin and colistin and only one isolate each (5%) was resistant to gentamicin and doxycycline.

4. Discussion

The aim of this study was to estimate the burden of *Campylobacter* spp. in three different types of meat sold at retail shops in Lahore district of Pakistan. Another objective was to determine the antibiotic resistance of these isolates against commonly used antibiotics.

The percentage of *Campylobacter* species isolated from various sources of meat was; chicken (29%), mutton (18%) and beef (15.5%). In general, these results are similar to those reported previously e.g., the prevalence is greater in chickens than in other animals. For example, the overall prevalence of *Campylobacter* in Pakistan in 2007 was reported to be 48%, 11%, and 5% in poultry, beef, and mutton, respectively (Hussain, Shahid Mahmood, Akhtar, & Khan, 2007). In Turkey, these numbers were 50%, 22% and 11% in chicken, mutton and beef, respectively (Bostan, Aydin, & Ang, 2009). The results from Iran were different; the prevalence of *Campylobacter* was higher in mutton (12%) as compared to beef (2.4%) and camel meat (0.9%) (Rahimi, Ameri, & Kazemeini, 2010). The high number of *Campylobacter* in chicken in this study is accepted as normal, since chicken meat is considered a primary reservoir for this bacterium. Additionally, the traditional way of slaughtering of birds at retail shop and lack of hygienic measures during slaughtering is considered to be the potential risk factor in cross contamination. On the other hand, slaughtering of beef and mutton is carried out in established slaughterhouses with less chances for cross contamination.

The highest resistance in our study was found against enrofloxacin followed by tylosin, amoxicillin and ciprofloxacin. A study from Estonia showed similar results for enrofloxacin (73.3%) in *Campylobacter* isolates from poultry (Roasto, et al., 2007). The highest resistance in *Campylobacter* isolates from Spain, Poland and Latvia was also against macrolides (Kovaļenko, Roasto, Šantare, Bērziņš, & Hörman, 2014; Maćkiw, Korsak, Rzewuska, Tomczuk, & Rożynek, 2012). These results are comparable to a study on resistant *Campylobacter* isolates from layer chickens in two different farming systems in the United

States. Resistance against tylosin in conventional farming system was higher (34%) than organic farming system (25%). Additionally 66% and 46.3% of the isolates were resistant from CF-1 (Conventional Farm-1) and OF-1 (Organic Farm-1), respectively (Kassem, et al., 2017). . This is not surprising because tylosin has not been used as a growth promotor in poultry from January 1999 onwards in these countries (European Food Safety, 2010). Still this antibiotic is frequently used in broiler industry in Pakistan. In another study 38.7% of *Campylobacter* isolates from pigs, dairy and beef cattle were resistant to tylosin in Tanzania (Kashoma, et al., 2015). During 2007 to 2009 in northern Greece, *Campylobacter* spp. was found resistant to tetracycline, streptomycin, ciprofloxacin, and nalidixic acid and but they were susceptible to gentamicin and erythromycin (Lazou, Houf, Soultos, Dovas, & Iossifidou, 2014).

In our study, 71.2% of the isolates were resistant to ciprofloxacin. These results are similar to studies in Algiers and Poland where the resistance to this antibiotic was 83.7 and 66.3% isolates from broiler and chicken meat, respectively (Andrzejewska, Szczepańska, Śpica, & Klawe, 2015; Messad, Hamdi, Bouhamed, Ramdani-Bouguessa, & Tazir, 2014). The rise in prevalence of ciprofloxacin resistant *Campylobacter* in retail poultry meat has also been reported from Denmark (Andersen, et al., 2006), Spain, Germany, Italy, Holland and Austria (Maćkiw, et al., 2012). In Latvia and Estonia, 60% of *Campylobacters* isolated from slaughterhouses were resistant to ciprofloxacin (Kovaļenko, et al., 2014; Roasto, et al., 2007). The resistance to fluoroquinolones in *Campylobacter* is believed to be due to the use of this antibiotic for treatment purposes in food animals (Talsma, Goettsch, Nieste, Schrijnemakers, & Sprenger, 1999).

The resistance to nalidixic acid was not high perhaps because this antibiotic is not used extensively in veterinary practice in Pakistan. These results are different from those in Latvia and Estonia where 75.6% and 60% of the isolates from slaughterhouses were resistant, respectively. In Denmark, the resistance against nalidixic acid was high among the isolates from duck and turkey meat (12%) as compared to chicken meat (7.4%) (Andersen et al., 2006; Kovaļenko et al., 2014; Roasto et al., 2007). In Iran, 54% of the isolates from various meat types were resistant to nalidixic acid (Rahimi & Ameri, 2011). The authors suggested that this was due to the widespread use of antibiotics in prophylaxis and growth promotion (Rahimi & Ameri, 2011). All 19 (100%) *C. coli* isolates from ducks in Malaysia were resistant to nalidixic acid and norfloxacin while 79 (84%) and 75 (80%) of *C. jejuni* isolates were resistant to nalidixic acid and enrofloxacin, respectively (Adzitey, Rusul, Huda, Cogan, & Corry, 2012).

Overall resistance to gentamicin was low (25.6%) in our study, which could be related to the infrequent use of this antibiotic in poultry production because of its high cost. In Grenada, no resistance was seen against gentamicin in *Campylobacter* isolates from poultry (Hariharan, et al., 2009). Similarly, there was no resistance against gentamicin in isolates from small ruminants in Greece (Lazou, et al., 2014). The resistance to gentamicin in chicken isolates was low in Northern Ireland and Poland (6.3% and 5%, respectively). The low resistance may be attributed to the fact that gentamicin is rarely used in poultry as growth promotant. Gentamicin is used by subcutaneous or intramuscular administration which is labor intensive (Rodrigo, Adesiyun, Asgarali, & Swanston, 2007; Wilson, 2003).

The resistance against doxycycline was also low in this study; only 11 of 125 isolates were resistant. These results are in contrast to those from Poland, Spain, Latvia, Denmark and France where 79%-90% *Campylobacters* showed resistance to tetracycline. The difference in occurrence of antimicrobial resistance reflects different national practices (Andersen, et al., 2006; Kovaļenko, et al., 2014; Maćkiw, et al., 2012). Similarly, high resistance (70.6%) to tetracycline was observed in *Campylobacter* isolates from various meat types in Iran

(Talsma, et al., 1999), In Greece 48% of *Campylobacter* isolates from small ruminant slaughterhouses were resistant to tetracycline (Lazou, et al., 2014).

In the present study, resistance against enrofloxacin, amoxicillin and tylosin was high in isolates from chicken. Similarly, the highest level of resistance was in *Campylobacter* isolates from retail poultry meat in Denmark during 1996-2003 and it was against tetracycline, nalidixic acid and ciprofloxacin (Andersen, et al., 2006). In Latvia, during 2010, high resistance was against ciprofloxacin and nalidixic acid (Kovalenko K, 2014). The high resistance against antibiotics in this study is probably due to irrational use of antibiotic in poultry practice in Pakistan.

Our study indicated highest resistance against ciprofloxacin, enrofloxacin and colistin in isolates from beef origin. Other studies concluded that *Campylobacter* spp. isolated from cattle slaughterhouses were frequently resistant to nalidixic acid and ciprofloxacin (38.3%), followed by streptomycin (24.3%) and tetracycline (20.9%) in Poland (Wieczorek, Denis, Lynch, & Osek, 2013).

A total of 90.4% of the isolates were MDR (multi-drug resistant) from different sources of meat. All *Campylobacter* isolates (100%) from broiler farms and slaughterhouses were identified as MDR in Algiers (Messad, et al., 2014). In China, 86% of the isolates from poultry were MDR (Chen, et al., 2010), while in Latvia it was 67.2% (Kovaļenko, et al., 2014), in Trinidad 64% (Rodrigo, et al., 2007), in Korea 56.5% (Han, et al., 2007) and in Iran 44.9% (Ebrahim Rahimi & Ameri, 2011).

5. Conclusions

It is concluded that *Campylobacter* spp. are circulating in various meat sources in Pakistan. The high resistance seen in our isolates is a serious health issue and may pose a risk in human medicine and food safety. The irrational use of antibiotics in animal and human practices seems to be the major cause of increasing resistance against pathogenic bacteria like *Campylobacter*.

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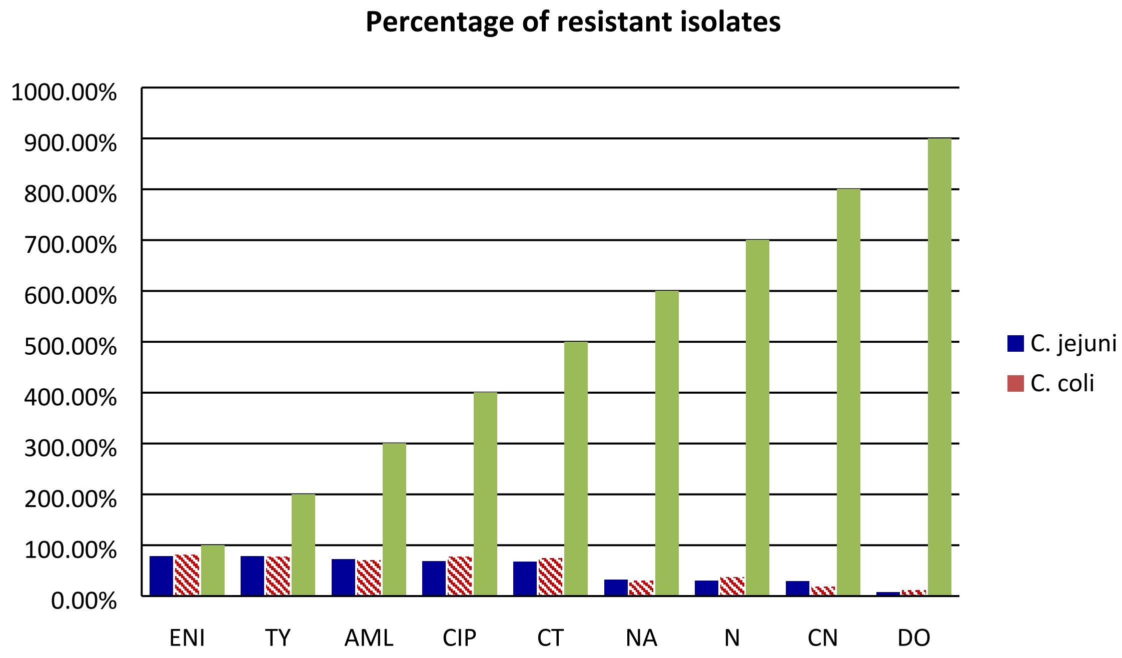

Fig. 1. ENI- Enrofloxacin; TY- Tylosine; AML- Amoxicillin; CIP- Ciprofloxacin; CT-Colistin; NA- Nalidixic Acid; N- Neomycin; CN-Gentmycin; DO-Doxycycline.

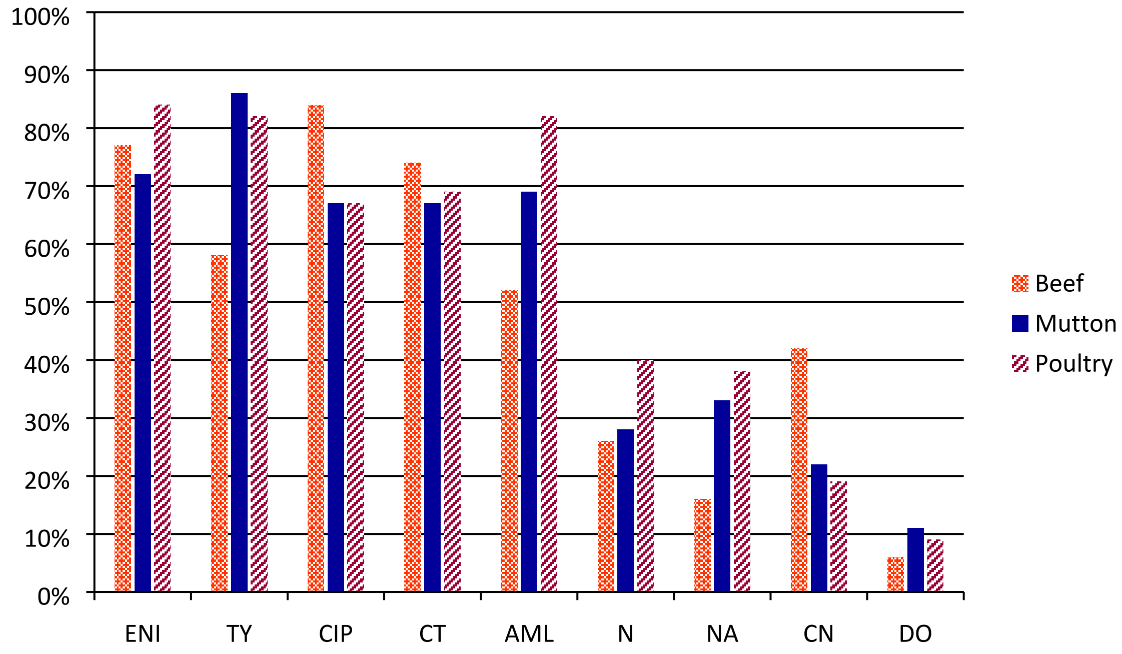

Fig. 2. Percentage of isolates from different sources of meat.
ENI- Enrofloxacin; TY- Tylosine; AML- Amoxicillin; CIP- Ciprofloxacin; CT- Colistin; NA- Nalidixic Acid; N- Neomycin; CN-Gentmycin; DO-Doxycycline.

Table 1
Antimicrobial resistance pattern of *Campylobacter* spp. isolated from various meat sources in Lahore, Pakistan.

Organism	Percent resistance to:								
	Enrofloxacin	Tylosin	Amoxicillin	Ciprofloxacin	Colistin	Nalidixic Acid	Neomycin	Gentamicin	Doxycycline
***C. jejuni* (n=82)**	64/82 (78%)	64/82 (78%)	59/82 (72%)	56/82 (68.3%)	55/82 (67.1%)	26/82 (31.7%)	25/82 (30.5%)	24/82 (29.3%)	6/82 (7.3%)
***C. coli* (n=43)**	35/43 (81.4%)	33/43 (76.7%)	30/43 (69.8%)	33/43 (76.7%)	32/43 (74.4%)	13/43 (30.2%)	16/43 (37.2%)	8/43 (18.6%)	5/43 (11.6%)
Total (n=125)	99/125 (79.2%)	97/125 (77.6%)	89/125 (71.2%)	89/125 (71.2%)	87/125 (69.6%)	39/125 (31.2%)	41/125 (32.8%)	32/125 (25.6%)	11/125 (8.8%)

Table 2
Antimicrobial resistance pattern of *Campylobacter* spp. isolated from beef, mutton and poultry meat in Lahore, Pakistan.

Meat origin		Percent resistant to:								
		Enrofloxacin	Tylosin	Ciprofloxacin	Colistin	Amoxicillin	Neomycin	Nalidixic Acid	Gentamicin	Doxycycline
Beef (n=31)	*C. jejuni* (n=19)	15/19 (78.9%)	12/19 (63.2%)	17/19 (89.4%)	14/19 (73.7%)	11/19 (57.9%)	5/19 (26.3%)	5/19 (26.3%)	8/19 (42.1%)	0
	C. coli (n=12)	9/12 (75%)	6/12 (50%)	9/12 (75%)	9/12 (75%)	5/12 (41.7%)	3/12 (25%)	0 (%)	5/12 (41.7%)	2/12 (16.7%)
	Total (n=31)	24/31 (77.4%)	18/31 (58.1%)	26/31 (83.9%)	23/31 (74.2%)	16/31 (51.6%)	8/31 (25.8%)	5/31 (16.1%)	13/31 (41.9%)	2/31 (6.5%)
Mutton (n=36)	*C. jejuni* (n=25)	19/25 (76%)	21/25 (84%)	16/25 (64%)	17/25 (68%)	18/25 (72%)	5/25 (20%)	8/25 (32%)	6/25 (24%)	2/25 (8%)
	C. coli (n=11)	7/11 (63.6%)	10/11 (90.9%)	8/11 (72.7%)	7/11 (63.6%)	7/11 (63.6%)	5/11 (54.5%)	4/11 (36.4%)	2/11 (18.2%)	2/11 (18.2%)
	Total (n=36)	26/36 (72.2%)	31/36 (86.1%)	24/36 (66.7%)	24/36 (66.7%)	25/36 (69.4%)	10/36 (27.8%)	12/36 (33.3%)	8/36 (22.2%)	4/36 (11.1%)
Poultry (n=58)	*C. jejuni* (n=38)	30/38 (78.9%)	31/38 (81.6%)	23/38 (60.5%)	24/38 (63.2%)	30/38 (78.9%)	15/38 (39.5%)	13/38 (34.2%)	10/38 (26.3%)	4/38 (10.5%)
	C. coli (n=20)	19/20 (95%)	17/20 (85%)	16/20 (80%)	16/20 (80%)	18/20 (90%)	8/20 (40%)	9/20 (45%)	1/20 (5%)	1/20 (5%)
	Total (n=58)	49/58 (84.5%)	48/58 (82.2%)	39/58 (67.2%)	40/58 (69%)	48/58 (82.2%)	23/58 (39.7%)	22/58 (37.9%)	11/58 (19%)	5/58 (8.6%)