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Distribution of Antibiotic Resistance and Antibiotic Resistant Genes in *Campylobacter jejuni* Isolated from Poultry in North West of Pakistan

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SBK, MAK, SF and IA designed the study. SBK, MAK, HU, SB and MM performed the experiments. SBK, MAK, SAK, FAK and NN analyzed the data and wrote the article.

Key words
Antibiotic resistance, Antimicrobial resistant genes, *Campylobacter jejuni*, Poultry meat, Zoonotic pathogens.

ABSTRACT

Campylobacter species are one of the most important food borne zoonotic pathogens. A total of 1260 poultry meat samples were collected from four different regions of Khyber Pakhtunkhwa province and processed for isolation of campylobacter species. A total of 182 (14%) *Campylobacter jejuni* were isolated using enrichment and plate media followed by confirmation through multiplex PCR. Isolates were tested for 15 antibiotics using disc diffusion method followed by detection of their respective antimicrobial resistant genes. Overall prevalence of *Campylobacter jejuni* was 14% being higher in Peshawar division (21%) followed by Bannu division (16%), Malakand division (13%) and Hazara division (8%). Over all highest antibiotic resistance was found against AMX (93%) followed by LIN (88%), AMP (86%), TET (82%), SXT (75), CHL (68%), CLR (65%), STR (50%), GEN (44%), OFX (27%), CIP (25%), LFX (13%) and AZM (11%) while the least resistance was found against GAT (8%) and CRO (9%). 90% isolates were found to have multiple drug resistance. As for as antibiotic resistant genes are concerned, the highest ARG was *blaTEM* (93%) followed by *tetA* (82%), sul2 (75%), *blaSHV* (72%), *tetC* (71%), *strA/strB* (50%), *sul1* (49%), *blaCMY2* and *aadA* (44%) while the least resistant gene was *aadb* (9%) followed by *sul3* (21%) and *aac(3)IV* (37%). About 92% isolates were found to have multiple drug resistance genes which is a matter of great concern from human public health perspective.

INTRODUCTION

Campylobacter is one of the most important pathogen implicated in food borne zoonoosis. The pathogen is world widely distributed and have been reported in different countries including European Union, USA and New Zeland (EFSA-ECDC 2015; CDC, 2017; Rapp *et al*., 2012). *Campylobacter jejuni* is the most important species responsible for human campylobacteriosisis while *Campylobacter coli* and *C. lari* are second and third responsible species (EFSA-ECDC, 2014). These organisms are fastidious, gram negative, bacilli, non spore forming, thermo tolerant, grow in microaerphilic conditions with a wide incubation period of 1-10 days (Gharst *et al*., 2013). Foods of animal origin are most commonly contaminated by this pathogen and the reason is these organism are

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commensals of GIT of different animals including cattle, buffalo, sheep, goat, swine and birds (Zhao *et al*., 2001; Bork and Petersen *et al*., 2005; Moran *et al*., 2009; Di Giannatale *et al*., 2010; Adzitey *et al*., 2012; Rejab *et al*., 2012; Wieczorek *et al*., 2013). Poultry meat is one of the most animal food source of this pathogen responsible for further transmission and cross contamination to other food items (Silva *et al*., 2011). Utilization of contaminated food items with this pathogen and under cooked meat have been reported for possible human illness. Gastrointestinal tract is mostly involved in human infection characterized by bloody diarrhea, vomiting, abdominal cramps and pyrexia. The disease may lead to further complications including Guillian barre syndrome, arthritis and Miller Fisher syndrome if not properly treated (WHO, 2017).

Antimicrobial resistance is a worldwide problem in all pathogens in general and in campylobacter species in particular. Unnecessary usage of antibiotics in animals feed particularly in poultry feed as a growth promoting factors is the main reason behind this AMR development. Besides this self medication/inappropriate usage of antibiotics in human illness are other contributing factors. Different mechanisms are involved in AMR development including biofilm formation, antibiotic resistant genes, plasmids and transposons.

Study on this pathogen in poultry is very scarce or very limited in KPK. To the best of our research and knowledge this is first study in Khyber Pakhtunkhwa province and therefore it was planned to find out the prevailing situation of AMR in campylobacter species in poultry meat along the supply chain.

MATERIALS AND METHODS

Samples collection

A total of 1260 poultry meat and tissue samples were collected and brought to laboratory under sterile condition. These meat samples were first cultured on preston campylobacter enrichment broth and then on Columbia blood agar under incubation temperature of 42°C for 48 h in microaerophilic atmosphere according to ISO standard. Identification was performed through colonial characteristics, microscopic morphology and rapid biochemical identification system (Oxoid, Basingstoke, UK). For extraction of genomic DNA from the bacterial isolates kit method was used (Omega Bio-Tek, USA). Species specific genes for campylobacter were targeted in genomic DNA through PCR. Specific primers, PCR amplifications and conditions are described in Table I.

Antimicrobial susceptibility testing (AST)

To check antibiotic susceptibility *Campylobacter* isolates were tested against 15 different antibiotics through disc diffusion method. For interpretation of the antibiotic susceptibility results standard guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Galni *et al.,* 2008). Following 15 different antibiotics were tested in the AST: Lincomycin (LIN, 2 µg), Azithromycin (AZM, 15 µg), Ampicillin (AMP, 10 µg), Suphamethoxazole+Trimethpram (SXT, 25 µg), Ciprofloxacin (CIP, 5ug), Gatifloxacin (GAT, 5ug), Ofloxacin (OFX, 5 µg), Levofloxacin (LVX, 5 µg), Clarithromycin (CLR, 15 µg), Chloramphenicol (CHL, 30 µg), Tetracycline (TET, 30 µg), Strptomycin (STR, 10 µg), Gentamycin (GEN, 10 µg), Amoxicillin (AMX, 20 µg), and Ceftriaxone (CRO, 30 µg). Multidrug resistance (MDR) strains (isolates resistant to three or more than three antibiotics) were determined.

Detection of antibiotic resistance genes (ARGs)

For detecting major resistance genes, a set of multiplex PCRs were used (Kozak *et al*., 2009). Major ARGs including b-lactamases (*blaCMY-2*, *blaTEM*, *blaSHV*), sulfonamides (*sul1*, *sul2* and *sul3*), gentamycin (*aac(3)IV*, *aadB*),

Table I.- Specific primers and PCR conditions for species specificity of *Campylobacter*.

Specific genes	Primers	Sequence of primers (5′-3′)	Size of products (bp)
C. jejuni 23S rRNA	23S F	TATACCGGTAAGGAGTGCTGGAG	650
	23S R	ATCAATTAACCTTCGAGCACCG	
C. fetus sapB2	CF F	GCAAATATAAATGTAAGCGGAGAG	435
	CF R	TGCAGCGGCCCCACCTAT	
C. upsaliensis glyA	CU F	AATTGAAACTCTTGCTATCC	204
	CU R	TCATACATTTTACCCGAGCT	
C. lari glyA	CL F	TAGAGAGATAGCAAAAGAGA	251
	CL R	TACACATAATAATCCCACCC	
C. coli glyA	CC F	GTAAAACCAAAGCTTATCGTG	126
	CC R	TCCAGCAATGTGTGCAATG	
C. jejuni hipO	CJ F	ACTTCTTTATTGCTTGCTGC	323
	CJ R	GCCACAACAAGTAAAGAAGC	

streptomycin (*strA/strB*, *aadA* and (*aac(3)IV*) and tetracycline [*tet(A)*, *tet(B)*, *tet(C)*] were targeted. These specific genes were targeted through specific primers. Details of the primers, PCRs amplification and conditions used are given in Table II.

Table II.- Zone of inhibition and concentrations of different antibiotics discs.

S. No	Antibiotics	Abbreviation	Disc content	Zone of inhibition (mm)		
				Sensitive	Intermediate	Resistance
1.	Azithromycin	AZM	15 µg	>18	14-17	<13
2.	Lincomycin	LIN	2 µg	>21	16-20	<15
3.	Ampicillin	AMP	10 µg	>17	14-16	<13
4.	Sulphamethoxazole + Trimethoprim	SXT	25 µg	>16	11-15	<10
5.	Ciprofloxacin	CIP	5 µg	>31	21-30	<20
6.	Gatifolxacin	GAT	5µg	>18	15-17	<14
7.	Ofloxacin	OFX	5 µg	>31	21-30	<20
8.	Levofloxacin	LVX	5 µg	>31	21-30	<20
9.	Clarithromycin	CLR	15 µg	>18	14-17	<13
10.	Chloramphenicol	CHL	30 µg	>18	13-17	<12
11.	Tetracyclin	TET	30 µg	>15	12-14	<11
12.	Strptomycin	STR	10 µg	>15	12-14	<11
13.	Gentamycin	GEN	10 µg	>15	13-14	<12
14.	Amoxicillin	AMX	20 µg	>17	14-16	<13
15.	Ceftriaxone	CRO	30 µg	> 23	20-22	<19

Table III.- Targeted antibiotic resistance genes, their primers and PCR conditions.

mPCR	Targeted genes	Primers	Sequence of primers	Annealing temp (°C)	Product size (bp)
1	*blaTM*	GKTEMF[d]	TTAACTGGCGAACTACTTAC	55	247
		GKTEMR[d]	GTCTATTTCGTTCATCCATA		
	blaSHV	SHV-F[j]	AGGATTGACTGCCTTTTTG	55	393
		SHV-R[j]	ATTTGCTGATTTCGCTCG		
	blaCMY-2	CMYF[d]	GACAGCCTCTTTCTCCACA	55	1000
		CMYR[d]	GGACACGAAGGCTACGTA		
2	*aadA*	4F[e]	GTGGATGGCGGCCTGAAGCC	63	525
		4R[e]	AATGCCCAGTCGGCAGCG		
	strA/strB	strA-F[f]	ATGGTGGACCCTAAAACTCT	63	893
		strB-R[f]	CGTCTAGGATCGAGACAAAG		
	aac(3)IV	aac4-L[g]	TGCTGGTCCACAGCTCCTTC	63	653
		aac4-R[g]	CGGATGCAGGAAGATCAA		
3	*aadB*	aadB-L[i]	GAGGAGTTGGACTATGGATT	55	208
		aadB-R[i]	CTTCATCGGCATAGTAAAAG		
4	*tet (A)*	TetA-L[c]	GGCGGTCTTCTTCATCATGC	63	502
		TetA-R[c]	CGGCAGGCAGAGCAAGTAGA		
	tet (B)	TetBGK-F2[m]	CGCCCAGTGCTGTTGTTGTC	63	173
		TetBGK-R2[m]	CGCGTTGAGAAGCTGAGGTG		
	tet (C)	TetC-L[c]	GCTGTAGGCATAGGCTTGGT	63	888
		TetC-R[c]	GCCGGAAGCGAGAAGAATCA		
5	*sul1*	sul1-F[b]	CGGCGTGGGCTACCTGAACG	66	433
		sul1-B[b]	GCCGATCGCGTGAAGTTCCG		
	Sul2	sulII-L[c]	CGGCATCGTCAACATAACCT	66	721
		sulII-R[c]	TGTGCGGATGAAGTCAGCTC		
	Sul3	sul3-GKa-F[d]	CAACGGAAGTGGGCGTTGTGGA	66	244
		sul3-GKa-R[d]	GCTGCACCAATTCGCTGAACG		

RESULTS

Prevalence of Campylobacter jejuni

Broiler meat samples (n=1260) were processed for detection of campylobacter species. All isolates were further confirmed through colony characteristics, morphology, biochemical testing and detection of their specific genes through PCR. The overall prevalence of *Camylobacter jejuni* was 14% being higher in Peshawar Division (21%) followed by Bannu division (16%), Malakand Division (13%) and Hazara Division (8%). A total of 182 isolates were obtained from four different regions. All the four regions are different in temperature and climatic condition.

Distribution of phenotypic antibiotic resistance

A total of 182 isolates were tested for 15 different antibiotics using disc diffusion method. Over all highest antibiotic resistance was found against AMX (93%) followed by LIN (88%), AMP (86%), TET (82%), SXT (75), CHL (68%), CLR (65%), STR (50%), GEN (44%), OFX (27%), CIP (25%), LFX (13%) and AZM (11%) while the least resistance was found against GAT (8%) and CRO(9%). There was a very obvious and crystal clear difference in distribution of antibiotic resistance in *Campylobacter jejuni* isolates from four different regions as shown in Table IV. 90% isolates were found to have multiple drug resistance.

Table IV.- Antibiotic resistance in *Campylobacter jejuni*.

S. No.	Antibiotics	No. of resistant isolates				
		Total n=182 (%)	Peshawar division n= 65(%)	Bannu division n= 50(%)	Malakand division n=42(%)	Hazara division n= 25(%)
1	LIN	160 (88)	50 (77)	45 (90)	35 (83)	25 (100)
2	AMX	170 (93)	62 (95)	48 (96)	37 (88)	23 (92)
3	TET	150 (82)	55 (84)	40 (80)	35 (83)	20 (80)
4	AMP	157 (86)	60 (92)	45 (90)	33 (78)	19 (76)
5	SXT	136 (75)	48 (74)	40 (80)	30 (71)	18 (72)
6	CHL	124 (68)	42 (65)	39 (78)	28 (67)	15 (60)
7	CLR	118 (65)	40 (61)	38 (76)	30 (71)	10 (40)
8	STR	91 (50)	25 (38)	24 (48)	32 (76)	10 (40)
9	GEN	80 (44)	32 (49)	21(42)	15 (36)	12 (48)
10	OFX	50 (27)	18 (28)	15 (30)	12 (28)	5 (20)
11	CIP	45 (25)	20 (31)	11 (22)	9 (21)	5 (20)
12	LFX	25 (13)	9 (14)	10 (20)	5 (11)	1(4)
13	AZM	20 (11)	8 (12)	7 (14)	5 (11)	0 (0)
14	CRO	15 (8)	6 (9)	5 (10)	4 (9)	0 (0)
15	GAT	10 (5)	5 (8)	4 (8)	1 (2)	0(0)

Table V.- Antibiotic resistant genes (ARGs) in *Campylobacter jejuni*.

ARGs	Total, n=182 (%)	Peshawar, n= 65(%)	Bannu, n= 50 (%)	Malakand, n=42(%)	Hazara, n= 25(%)
tetA	150 (82)	55 (85)	40 (80)	35 (83)	20 (80)
tetB	87 (48)	40 (61)	30 (60)	10 (24)	7 (25)
tetC	129 (71)	48 (74)	38 (76)	30 (71)	13 (52)
aadA	80 (44)	32 (49)	18 (36)	24 (57)	6 (24)
strA/strB	91 (50)	25 (38)	24 (48)	32 (76)	10 (40)
aac(3)IV	68 (37)	28 (43)	20 (40)	17 (40)	3 (12)
blaTEM	170 (93)	62 (95)	48 (96)	37 (88)	23 (92)
blaSHV	131 (72)	51 (78)	36 (72)	29 (69)	15 (60)
blaCMY-2	80 (44)	30 (46)	17 (34)	20 (48)	13 (52)
Sul1	90 (49)	29 (45)	23 (46)	18 (43)	20 (80)
Sul2	136 (75)	48 (74)	40 (80)	30 (71)	18 (72)
Sul3	38 (21)	12 (18)	17 (34)	10 (24)	1 (4)
aaddB	16 (9)	9 (14)	5 (10)	2 (5)	0 (0)

Distribution of antibiotic resistant genes (ARGs)

As for as antibiotic resistant genes are concerned, the highest ARG was *blaTEM* (93%) followed by *tetA* (82%), sul2 (75%), *blaSHV* (72%), *tetC* (71%), *strA/strB* (50%), *sul1* (49%), *blaCMY2* and *aadA* (44%) while the least resistant gene was *aadb* (9%) followed by *sul3* (21%) and *aac(3)IV* (37%). All the isolates from four different regions were found to have different distribution of resistant genes as shown in Table V. 92% isolates were found to have multiple antibiotic resistances.

DISCUSSION

Campylobacter species are among the most important food borne pathogens causing zoonosis. Mostly this infection is restricted to GIT in human but in severe cases it may lead to other severe syndromes. Different countries have reported different prevalence of campylobacter in poultry meat including 85% in Northern Ireland (Moran *et al*., 2009), 87% in Poland (Wieczorek *et al*., 2013), 20.8% in Estonia (Mäesaar *et al*., 2014), and 73-81% in Italy (Parisi *et al*, 2007; Pezzotti *et al*., 2003). These results are a bit higher and not consistent to our study and the reasons could be due to different climatic conditions, different slaughtering techniques, evisceration, and packing processing. Other reasons may due to different types of samples used.

Antibiotic resistance is one of the greatest threat to the world after infections. This study also described the prevailing situation of AMR in *Campylobacter jejuni*. Here are also different study reports from different countries describing different scenario of AMR in *Campylobacter*. Ledergerber *et al*. (2003) have reported 28.7% resistance to ciprofloxacin, 12.6% to tetracycline, 11.8% to sulphonamide, and 10.3% to ampicillin in a study conducted in Switzerland. Mattheus *et al*. (2012) have conducted a study in Belgium poultry in which he found resistance of *Campylobacter* species to AMP (47.4%), CIP (42.1%), Erythromycin (12.1%), GEN (25.6%), nalidixic acid (46.4%) and TET (45.3%). Miflin *et al*. (2007) have conducted a study on *Campylobacter jejuni* in Queensland region and found 18.4% resistance for tetracycline and 17.6% for ampicillin. Bester *et al*. (2008) have reported highest resistance for tetracycline (98.2%) and ceftriaxone (96.4%) in a study conducted in broiler in South Africa. Obeng *et al*. (2012) have observed extensive resistance of campylobacter to lincomycin (51-100%), ampicillin (33·3-60·2%) and tetracycline (5·6-40·7%). Wieczorek *et al*. (2018) conducted a study in Poland in poultry and found resistance to ciprofloxacin (92.5%), followed by nalidixic acid (88.9%) and tetracycline (68.4%). Another study conducted in Poland by Wysok *et al*. (2017), where he reported 52.7% resistance to ciprofloxacin, 56% to nalidixic acid and 61.3% to doxycycline.

Nguyen *et al*. (2016) have found high rate of resistance to nalidixic acid, tetracycline and ciprofloxacin of 77.4, 71.0 and 71.0%, respectively. Low resistance (25.8%) was detected for gentamicin and chloramphenicol. Gupta *et al*. (2004) have conducted a study on AMR in USA from 1998-2001. They observed that ciprofloxacin-resistant *Campylobacter* have increased from 13% to 19%. No increase was observed in erythromycin resistance which remains the same at 2% from 1998-2001. Senok *et al*. (2007) have discovered highest resistance of *Campylobacter* to CIP (88.8%) and 32.6% to TET in a study conducted in Kingdom of Bahrain. Similarly, a study conducted in China by Xia *et al*. (2010) have reported 98% resistance of *Campylobacter* to nalidixic acid, ciprofloxacin, enrofloxacin, tetracyclines and doxycycline. These studies are a clear indication of extensive AMR in *Campylobacter* around the world. The difference in the results could be due to different geographical locations, different climatic conditions and usage of different antibiotics in animal feeds.

To the best of our search, knowledge and understanding this is the first study that we conducted on the detection of ARGs in *Campylobacter* in Pakistan. Our study have reported the highest ARG *blaTEM* (93%) followed by *tetA* (82%), sul2 (75%), *blaSHV* (72%), *tetC* (71%), *strA/strB* (50%), *sul1* (49%), *blaCMY2* and *aadA* (44%) while the least resistant gene was *aadb* (9%) followed by *sul3* (21%) and *aac(3)IV* (37%) which is consistent to phenotypic data. Different countries have reported different ARGs in *Campylobacter*. Abdi-Hachesoo *et al*. (2014) have tested *Campylobacter* species for TET genes in Iran and found that 18% isolates were positive for TET (A) gene. Obeng *et al*. (2012) have found different antibiotic resistance genes including bla (OXA-61) (82·6-92·7%), cmeB (80·3-89%) and tet(O) (22·3-30·9%) in *C. coli* isolates from pigs, while *C. jejuni* from chickens were found to harbor bla(OXA-61) (59-65·4%) and tet(O) (19·2-40·7%). Similarly, Reddy and Zishiri (2017) have tested *Campylobacter* species for gyrA, bla_{OXA-61}, and TET genes. 68% isolates were found positive for tetO gene which was the most prevalent. Quinolone resistance was highly associated with gyrA genes. Gleisz *et al*. (2006) have tested campylobacter for AMR in Austria and found that 21% were resistant to tetracycline, 18% for AMP and 11% for STR and all isolates were found positive for *tetO* gene. Again results of ARGs are also in disagreement and possible reasons could be due to different geographical locations, usage of different antibiotics and testing of different targeted ARGs.

CONCLUSION

Campylobacter jejuni 90% have multiple drug resistance while more than 92% have multiple ARGs. This is an alarming situation of AMR in *Campylobacter jejuni* in Khyber Pakhtunkhwa province of the country which needs prompt attention of the concerned veterinary and public health authorities since the diseases is zoonotic that could pose potential health threat.

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Statement of conflict of interest

There is no conflict of interest.

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