

Prevalence, Risk Factors Analysis and Antimicrobial Resistance Profile of Campylobacter Species Isolated from Food Animals in District Okara, Pakistan

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ABSTRACT

Campylobacter, one of the most prevalent bacteria responsible for gastroenteritis in human worldwide. **Campylobacter** infections result from ingestion of contaminated food and water depending upon susceptibility of individuals and virulence of infecting strain. The prevalence of these bacteria is highly diverse and present worldwide. Increase in antibiotic resistance among these bacteria is recognized as emerging public health problem. Current study is designed to analyze the risk factors related with **Campylobacteriosis**, prevalence in meat (chicken, beef and mutton) and antimicrobial resistance profiles of these bacteria from district Okara, Pakistan. The time span for lab work was from October 2020 to March 2021 including sampling and testing. A total of 300 swab samples were collected from poultry, beef and mutton. The ISO 10272-1:217(E) method for isolation through biochemical and molecular identification carried out. Disc diffusion method used for AMR calculation. The overall prevalence of campylobacter was only 2%, detected only in poultry meat out of 300 samples. Among the isolated strains, **Campylobacter** indicated highest resistance against Tetracycline 100%, followed by Cefotaxime, Tylocine 83%, Enrofloxacin 66%, Clindamycin and Nalidixic acid 50%, lowest resistance against Streptomycin and Doxycycline only 33% and 16% respectively. The significance relation was obtained for two variables that include eating during dealing meat and contact of excreta with meat. Prevalence of **Campylobacter** is very low as compared to study conducted earlier in Pakistan. The drug of choice for **Campylobacter** related infections is doxycycline and streptomycin. The results indicate that prevalence of **Campylobacter** is very low on meat in Okara region but higher microbial resistance.

Keywords: antimicrobial resistance, campylobacter, epidemiology, prevalence, risk factors.

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I. INTRODUCTION

Campylobacter is gram negative bacteria, producing infectious disease known as campylobacteriosis. Possible causes of infection include contaminated water and food along with direct interaction with carrier animals [1]. **Campylobacter** is first identified as common humanoid pathogen in 1970. Adults and children are equally experience bacteria enteritis by campylobacter infection that is second to salmonella in prevalence and almost parallel to Shigella [2]. The disease may be transferred from egg laying birds through their eggs and onto the chicks [3]. Raw poultry meat is considered as a major source for human campylobacteriosis [4]. Host factors may have significant characters in the pathogenesis of campylobacteriosis in human being [5].

Generally, **Campylobacter** resides in the intestine of warm-blooded animals, transmitting it to humans through contaminated food items [6]. **Campylobacter** species, including *C. coli*, *C. jejuni*, and *C. fetus* are commonly residing in the intestinal contents of bovine, their bile, gallbladder, bile ducts and liver [7]. **Campylobacter jejuni** is thermophile in nature, maximum growth at 42°C in 3–15% of oxygen and a CO₂ concentration of 2–10%. These adaptations enable this organism to reside in the intestine of warm blooded birds and many animals that represent as a reservoir for *C. jejuni* [8]. *C. jejuni* is highly vulnerable to stress as it lacks various stress bearing factors making it improbable to persist external to animal hosts [9]. Among these *C. jejuni* may produce leading bacterial source of reactive arthritis and Guillain-Barré syndrome [10]. Prevalence of **Campylobacter** has been increasing exponentially both in developed and developing countries.

At present, there are insufficient epidemiological documents to offer a precise calculation of the health consequences of the infectious illness in emerging countries, mainly in the Indian subcontinent [11]. Annually about 1.3 million individuals with a generally 14 persons per 100,000 of population are being affected by this organism [12].

Food poisoning and spread of antibiotic resistance among consumers are major hazards related to *Campylobacter* contamination. There is relation among use of antimicrobial drugs in food animals, emergence of antibiotic resistance in food related pathogens and transfer of these bacterium or their resistant genes to human via chain of food [13]. The drug of choice for campylobacteriosis is erythromycin and fluoroquinolones have been frequently used owing to their broad-spectrum activities against enteric pathogens [14]. Nearly 90% of deaths due to diarrhea arise among children below five years of age living in countries having low per capita income [15]. *Campylobacter* infections are self-limiting but Immuno-compromised or HIV Positive persons generally take antibiotics. This specie has inherent resistance to bacitracin, novobiocin, polymyxins and vancomycin, probably due to deficiency of suitable targets or little binding sites affinity for these drugs [16].

Major possible aspects for human *Campylobacteriosis* are mishandling of raw poultry and ingestion of undercooked meat [17]. The presence of Zinc oxide and nanoparticles (ZnO, NPs) in combination with chitosan led to increasing of chitosan effectiveness against *Campylobacter* isolates from broiler flocks and their environment [18]. Regulations for antimicrobials use are essential to control the growth and propagation of resistant bacteria and the genes programmed for this resistance particularly in poultry houses and farm animals [19].

II. MATERIAL AND METHODS

A. Study Area and Design

This study conducted in district Okara-Punjab Pakistan. Sample collected carried out at different retail shops and slaughter houses. Samples collected from whole carcass of poultry birds, swabbing Site=10cmx10cm=100cm² from flank and brisket regions in beef and swabbing Site= 5cm x 5cm= 25cm² from mutton available in retail shops and slaughter house. Convenient type of sampling performed.

B. Sample Collection and *Campylobacter* Isolation

This research was conducted according to recommended ISO protocols (ISO-10272-2006 and ISO 10272-2010). The sampling conducted according to meat standard committee of Australia [20]. Alkaline peptone water was used as transport media and preservation of swab samples. Swabs after sampling were placed in tubes containing transport medium and transported to laboratory in ice box for further processing. All samples retained cool in ice container box having almost 4°C temperature. Immediately after sampling, these conveyed to the laboratory for further treating within 4–8hours. Calculated amount from transport medium transferred to flask containing enrichment “Bolton Broth” supplemented with antibiotics in 1:10 ratio. So, 5ml of sample was transferred to flask containing 45ml of enrichment media. First incubation was provided for 4 hours

at 37°C, followed by 44 hours at 42°C under microaerophilic conditions. Anaerobic jar was used to provide required conditions through campy gas sachet.

C. Culturing

In order to protect colony from contamination and enhance *Campylobacter* recovery, combination of antimicrobial used, including vancomycin, cefoperazone, polymyxin B and amphotericin. Modified charcoal cefoperazone deoxycholate agar (mCCDA) used for culturing of campylobacter jejuni/coli in this study. Special environmental settings produce for growth of *Campylobacter* that include low oxygen (5%), high level of carbon dioxide (10%) and nitrogen (80–85%) with incubation at 42°C for 48 hours.

D. Biochemical Tests

Almost all *Campylobacter* strains were biochemically recognized by gram staining, oxidase, and catalase reactions; growth at 37°C and 42°C on blood containing agar along with Hippurate hydrolysis. The catalase test helps in the detection of the enzyme catalase in bacteria. Culture that showed positive results of biochemical testing were stored in medium containing 15% glycerin and 85% nutrient broth in autoclave Eppendorf tubes. Storage temperature was maintained at -20°C.

E. Molecular Identification

1) DNA extraction

DNA extracted from cultured bacteria using specific kit according to manufacturer's instructions. Extracted DNA stored at -20°C for further processing.

2) Primer selection

Three sets of primers for *Campylobacter* identification were used. One pair of primers targeting 16SrRNA gene to identify the genus of *Campylobacter*, second pair targeting mapA gene for *Campylobacter jejuni* and third primer was used to identify cueE gene for *Campylobacter coli*. Detail about all three primers regarding targeted gene, forward and reverse sequence and size of amplified fragments has shown in Table I.

TABLE I: THREE TYPES OF PRIMERS USED FOR MOLECULAR CONFIRMATION OF BACTERIA AT GENUS AND SPECIE LEVELS

Primers	Gene Targeted	Sequence 5'-3'	Size of amplified fragment
C. Genus	16SrRNA	F'(ATCTAATGGCTT AACCATTAAAC) R'(GGACGGTAAC TA GTTAGTATT)	857 bp
C. jejuni	mapA	F'(CTATTTTATTTT GAGTGCTTGTG) R'(GCTTTATTGCCA TTTGTTTTATTA)	589 bp
C. coli	ceuE	F'(AATTGAAAATTG CTCCAACATATG) R'(TGATTTTATTATT TGTAGCAGC)	462 bp

3) Visualization of PCR product

PCR products visualized by gel electrophoresis under UV light using 1.2% agarose gel. The amplification targets set

for gene according to need. DNA markers of Bionexus Hi-LoTM (Cat# BN2050, Bionexus, USA) were used as reference of bands sizes.

F. Antimicrobial Testing

Antimicrobial testing was carried out according to CLSI established protocol using disc diffusion technique. McFarland standard for preparation of isolates following spread onto Mueller-Hinton agar in a petri dish accomplished. Discs of commonly practiced antibiotics in human and animals in region of Okara examined for antimicrobial testing. Minimum inhibitory concentration values examined after 48 h according to EUCAST guidelines and result verified as whether organism is susceptible (S), intermediate susceptible (I) or resistance to specific antibiotic.

G. Risk Factors Analysis

A questionnaire was designed for risks factors analysis at slaughterhouse targeting the butchers. The transmission of any disease depends upon epidemiological triangle in which transmission chain consists of agent, host and environment [21].

II. RESULTS

A. Prevalence of Campylobacter

Samples from retail shops and slaughterhouses collected and cultured following mentioned protocols. After biochemical testing 38 isolates from cultures were confirmed positive for Campylobacter. Percentage of positive samples after biochemical tests was almost 12.7% among 300 samples. Among 38 detected as positive isolates after biochemical tests, 11 were from beef, 8 from mutton and remaining 19 from poultry meat. But only 6 isolates were declared positive for Campylobacter species out of 300 hundred samples through PCR, providing percentage of prevalence about 2% after molecular detection. Unfortunately, all 6 samples were from 19 poultry meat that declared positive after molecular confirmation. So, we proceed further with 100 samples from poultry only, as we had to calculate risks factors also. Prevalence of Campylobacter is very low as compared to study conducted earlier in Pakistan. Campylobacter species are often isolated from feces of animals where there is high prevalence.

B. Antimicrobial Resistance of Campylobacter Species

Only six isolates that were confirmed positive after biochemical testing were used for calculation of antimicrobial resistance profile. Eight discs of commonly used antibiotics in our region were used for this purpose that are tetracycline, cefotaxime, tylocine and enrofloxacin clindamycin, nalidixic acid, streptomycin and doxycycline. The overall percentage of antimicrobial resistance of Campylobacter species to different antibiotics varied from 16–100%. Among the isolated strains, Campylobacter showed highest resistance against Tetracycline 100% (6/6), followed by Cefotaxime and Tylocine 83%, Enrofloxacin 66%, Clindamycin and Nalidixic acid 50%, lowest resistance was observed against Streptomycin and Doxycycline that is only 33% and 16% (Table II). So, drug

of choice for campylobacter related infections is doxycycline and streptomycin. Graphical representation of resistance percentage for Campylobacter isolates against all these antibiotics has shown in Fig I. Multidrug resistant (MDR) bacteria are those that resistant to three or more than three antibiotics. This study also indicates that Campylobacter is Multidrug resistant. Irrational use of antibiotics in food animals establish resistant bacteria that transmit to public through meat.

TABLE II: AMR PROFILE OF CAMPYLOBACTER ISOLATES

Antibiotics	Resistance	Antibiotics	Resistance
Nalidixic Acid	3/6	Cefotaxime	5/6
NA-30	(50%)	CTX-30	(83%)
Clindamycin	3/6	Tylosin	5/6
CC-2	(50%)	Ty-30	(83%)
Enrofloxacin	4/6	Streptomycin	2/6
ENR-10	(66%)	S-10	(33%)
Doxycycline	1/6	Tetracycline	6/6
D-30	(16%)	TE-30	(100%)

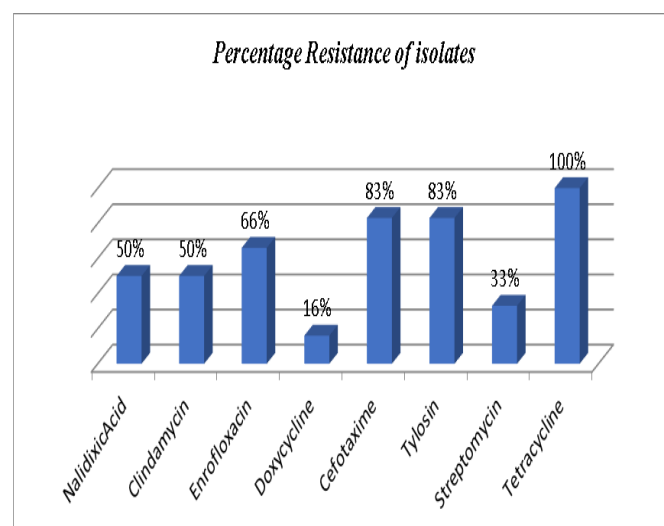


Fig. 1. Graphical representation of eight types of antibiotics used during AMR study.

C. Results of Risk Factors

For descriptive study a questionnaire was designed for risk factors analysis that was filled through questioning from butchers. These butchers belonged to different parts of district Okara. Response rate from butchers was hundred percent as questions were simple and short. All retailers were male (100%). The risk factors analysis was carried out through SPSS (IBM version 20) on basis of relation of positive results through PCR with all variables. The value of Chi-square, P-value and graph obtained through SPSS. These variables calculated through questionnaires from butchers. The significance relation was obtained for only two variables that include eating during dealing meat and contact of excreta with meat with P-value 0.046 and 0.041 respectively. The P-value <0.05 was taken as significant. So, these two factors may contribute in boosting the prevalence of Campylobacter in humans or butchers from food animals.

III. DISCUSSION

The aim of this study was to estimate the prevalence of *Campylobacter* on meat from retail shops at district Okara. The overall prevalence of *Campylobacter* species after molecular detection was only 2% out of 300 samples from chicken, beef and mutton. These results are not supported by former studies conducted in Pakistan. The other study conducted in Lahore which shows 29% prevalence in poultry, 18% in mutton and 15.5% in beef. Results from this study don't match with previous studies; reason of difference may be site of sampling. In previous studies sample taken from intestine that is site for high prevalence of this bacterium. This study conducted in winter season (October-February) and previous studies showed that prevalence of *Campylobacter* decreases in winter season [22], [23]. So, this factor may involve in low prevalence of *Campylobacter* in this study. This was first study conducted in district Okara, Punjab Pakistan, so there is low prevalence of *Campylobacter* on meat samples.

Among the isolated strains, the highest resistance was observed against Tetracycline 100%, Cefotaxime, Tylosine 83%, Enrofloxacin 66%, Clindamycin, Nalidixic acid 50% and the lowest resistance against Streptomycin and Doxycycline that is only 33% and 16%. So, drug of choice for *Campylobacter* related infections is doxycycline and streptomycin.

There would be many factors involve in incidence, trends and patterns of antimicrobial resistance in *Campylobacter*. Problem of antimicrobial resistance is found throughout the world especially in developing countries where health system is very weak to control undesirable situation. Infection from *Campylobacter* is also increasing due to improper hygienic measures, increase in resistant bacteria and travelling to those area where its prevalence is very high. Haphazardly antibiotics use in veterinary field also boosts prevalence of resistant bacteria.

Risks factors analysis was based on questionnaires. Different factors that may play role in causation or spread of *Campylobacteriosis* among butchers or in society were tried to calculate through questioning. Results were calculated using SPSS by finding "P value" and Chi-Square and outcome association with positivity of samples. There come two factors that related for positivity of samples or *Campylobacteriosis* in butchers were eating during meat handling and contact of excreta with meat.

Butchers should not eat during dealing with meat; they should wash their hands before or after dealing with meat properly, avoid older birds for slaughtering and hygienic slaughtering to avoid contact of excreta with meat may decrease possibility of *Campylobacteriosis*. It was observed that a single dirty cloth kept removing feces and blood from meat cutting surfaces. Dealing of currency was also carried by butcher itself, so there are maximum chances of spread of bacteria on meat through currency exchange. Only tap water was used for washing and cleansing purposes.

Poultry meat is reservoir for *Campylobacter* species but low prevalence of these bacteria was being observed that may be due to seasonal effects as our study conducted during winter season. High antimicrobial resistance was observed among isolates. It may be due to irrational use of

antibiotics both in animals and humans that results in rise of antimicrobial resistance, causing huge economic and health loses in public health and livestock sectors. Streptomycin and Doxycycline were labeled as drug of choice for *Campylobacteriosis* after AMR testing. Awareness about meat handling, meat-borne diseases and food safety measures was improper as observed through questionnaire. There is weak relation of knowledge, attitude and practices among butchers regarding hygienic measures. Contact of excreta with meat during slaughtering process was a major risk factor that was observed.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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