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PHENOTYPIC AND GENOTYPIC DETECTION OF COLISTIN RESISTANT *ESCHERICHIA COLI* FROM RAW BUFFALO MEAT IN PAKISTAN

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ABSTRACT

Buffalo meat has been considered as a rich and essential source of proteins for human health. Colistin is considered as the last treatment choice for gram negative bacteria. In this era of modern medicine, the incidence of colistin-resistant *Escherichia coli* in food, especially meat, has emerged as a significant threat to human health. The present study is planned to detect multidrug resistant *E. coli* from raw buffalo meat with an emphasis on phenotypic and genotypic detection of colistin resistant *E. coli*. For this purpose, fresh and chilled raw buffalo meat samples (n=300) were collected and processed for characterization of *E. coli.* Kirby-Bauer method was used to detect MDR status of *E. coli* isolates. The colistin broth disc elution test was employed to detect colistin resistance phenotypically while polymerase chain reaction was used to find out genotypic colistin resistance targeting the *mcr-1* and *mcr-2* genes. The results of the survey conducted revealed that among the total samples collected, 36% (108/300) buffalo meat samples were found positive for *E. coli.* The highest antibiotic resistance was observed (100%) against tetracycline followed by cefotaxime, cefepime (97.2%) and ciprofloxacin (94.4%). In total, 99 *E. coli* isolates were detected multidrug resistant and among them 60 (60.60%) isolates were found colistin resistant. The *mcr-1* gene was found in 44 (73.33%) colistin resistant isolates while none of the isolates was detected positive for *mcr-2* gene. The existence of phenotypic colistin resistance and *mcr-1* gene in *E. coli* isolates from raw buffalo meat is worrisome as this situation leaves us with no treatment options for gram negative pathogens leading the practitioners towards post-antibiotic era.

Keywords: *E. coli*, buffalo meat, colistin, multidrug resistant, mcr-1 gene

INTRODUCTION

Escherichia coli is among the prominent bacterial species responsible for variety of bacterial infections both in human beings and animals (Gallardo *et al*., 2017). The pathogenic strains of *E. coli* can cause various intestinal and extra

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intestinal infections in humans including neonatal meningitis, sepsis, hemolytic uremic syndrome and soft tissue infections (Ranjan *et al*., 2017). This bacterium has great adaptability to survive in external environments and spread among humans, animals and their products (Ewers *et al*., 2012). Various virotypes of *E. coli* are transmitted to human community primarily via consumption of contaminated raw milk, different types of meats and undercooked meat products (Sethulekshmi *et al*., 2016).

Meat and its products are valuable food commodities of animal origin due to their high protein, carbohydrates, vitamins and minerals profile. Meat contaminated with bacteria is considered as an important source of food borne illness because it has all the nutrition and factors favorable for efficient growth of pathogens (Wickramasinghe *et al*., 2017). The contamination of meat occurs during slaughtering of animals and direct/indirect contact with feces. The tools, equipments and cutting boards along with personnel and clothes also serve as source of contamination of meat (Adzitey, 2015).

Bacterial resistance to various antimicrobials can be transferred to human population when they consume meat and its products from farm animals containing resistant bacteria. Furthermore, animals and humans have the ability to contaminate food items and the surrounding environment with resistant bacteria. Similarly, the chances of spread of resistant bacteria in food chain, humans and animals from environment are also very often (Fair and Tor, 2014).

Colistin has been used for decades in veterinary practice for treating gastrointestinal tract infections caused by gram negative bacteria in cattle and poultry. Due to its toxic effects, it was rarely used clinically but in recent past it was reintroduced as a last therapeutic choice for multidrug resistant isolates belonging to family *Enterobacteriaceae* (Zajac *et al*., 2019). Along with fourth generation cephalosporins and quinolones, colistin is among the critically important antimicrobials and should be used for treating very severe human infections to preserve its effectiveness. The excessive use of colistin in veterinary and human clinical practice leads to the emergence of colistin resistance. The first mobile colistin resistance gene (*mcr-1*) was reported among humans, livestock and their meat during 2015 in China (Liu *et al*., 2016). After that several reports were also recorded from various corners of world showing *mcr-1* presence in broilers, farm environment, street food, food vendors and sewage samples (Islam *et al*., 2017; Dutta *et al*., 2020; Das *et al*., 2021). Among the plasmid encoded colistin resistance genes, the *mcr-1* and *mcr-2* have a potential of frequent dissemination (Mohsin *et al*., 2017). The presence of *mcr-1* gene leads to the increased frequency and dissemination of high-risk colistin-resistant pathogens in the food chain leading to a serious threat to food safety and public health (Ling *et al*., 2020).

Uptil now nine *mcr* genes (1-9) have been discovered along with some variants but *mcr-1* gene was found to be most prevalent. In Pakistan not a single study has been conducted to detect colistin resistance at molecular level in buffalo meat. Keeping in consideration the above-mentioned facts, the present study is designed to detect the multidrug resistant *E. coli* isolates harboring colistin resistant *mcr* genes from raw buffalo meat.

MATERIALS AND METHODS

Sample collection

A total of (n=300) raw buffalo meat samples were collected aseptically in sterile plastic bags and labeled properly with date, type, location and identification code. 25 grams of each fresh and chilled buffalo meat samples were collected in equal number (n=150) from local shops, butcheries and supermarkets. All the samples were collected with the consent of butchers and shopkeepers after explaining to them the purpose of study by qualified veterinarian. The samples were immediately shifted to Food Microbiology Laboratory in temperature-controlled boxes for further processing (Nawaz *et al*., 2021).

Isolation and identification of *Escherichia coli* from samples

All the samples were subjected to enrichment in 225 mL of sterile peptone water individually for 24 h at 37°C. Each enriched sample was streaked separately onto the surface of MacConkey's agar and Eosin methylene blue (EMB) agar followed by incubation aerobically at 37°C for 18 to 24 h. Pink colored colonies on MacConkey's agar and black colored colonies with metallic green shining appearance on EMB agar were processed further. The preliminary confirmation was done by Gram's staining technique and biochemical profiling of isolates. The preliminary identified *E. coli* isolates were preserved in 50% glycerol stock followed by storage at -20°C for further processing (Nawaz *et al*., 2019).

Molecular identification of *Escherichia coli* isolates

The preliminary identified isolates of *Escherichia coli* were subjected to molecular characterization using polymerase chain reaction targeting the *uid*A gene for confirmation. The genomic DNA of isolates was extracted using snap chill method from *E. coli* isolates (Nagappa *et al*., 2007). The primer sequence for *uid*A gene is mentioned in (Table 1). The thermal profile was adjusted 94°C for 5 minutes (Initial denaturing) followed by 35 cycles of (94ºC for 40 seconds, 55ºC for 40 seconds and 72ºC for 60 seconds) followed by final extension at 72 ºC for 10 minutes in (BioRad, USA) thermal cycler. The amplicons were analyzed by 1.5% agarose gel electrophoresis in Tris-Borate-EDTA (TBE) buffer (1x). The gel was visualized under a UV transilluminator (Slite 200W, Taiwan) and the images were documented (Nawaz *et al*., 2021).

Detection of antibiotic resistance profiling of *Escherichia coli* isolates

All the confirmed *E. coli* isolates were subjected to detection of antibiotic resistance by disc diffusion Kirby-Bauer method using commercially available antibiotic discs. Total eleven antibiotics from 6 different classes were used namely amikacin (30 µg), amoxycillin (30 µg), ampicillin (10 µg), cefepime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg) and tetracycline (10 µg). The results were interpreted as recommended by Clinical Laboratory Standards Institute (CLSI, 2020) guidelines. The isolates were included in multidrug resistant (MDR) category if they showed resistance to at least three different classes of antibiotics (Ilyas *et al*., 2016; Abdulhaq *et al*., 2020).

Phenotypic detection of colistin resistance in *Escherichia coli* isolates

The MDR *E. coli* isolates were further

tested for colistin resistance by colistin broth disk elution test. The test was performed and colistin MIC values were recorded visually according to the recommendations of (CLSI, 2020). The isolates having MIC ≤4 µg/mL were considered as colistin susceptible while having MIC ≥4 µg /mL was named as colistin resistant (Simner *et al*., 2019).

Genotypic detection of colistin resistance in *Escherichia coli* isolates

Colistin resistant isolates were proceeded for detection of mobile colistin resistance genes (*mcr-1* and *mcr-2*) by PCR using specific primer sets as shown in (Table 1). The thermal condition was optimized at 94°C for 15 minutes (Initial denaturing) followed by 25 cycles of (94ºC for 30 seconds, 58ºC for 90 seconds and 72ºC for 60 seconds) followed by final extension at 72ºC for 10 minutes in (BioRad, USA) thermal cycler. The amplicons were analyzed by 1.5% agarose gel electrophoresis (Rebelo *et al*., 2018).

Statistical analysis

The variables were presented in the form of percentages (%). The Chi square test was used to determine the association between variables by Stata 11 software (Stata Corp, USA). The value (P<0.05) was considered as significant.

RESULTS AND DISCUSSIONS

The results of the present study showed that out of the total 300 processed raw buffalo meat samples, 36% (66 fresh buffalo meat and 42 chilled buffalo meat) samples were found positive for *Escherichia coli* by microbiological, biochemical and molecular techniques. Based on sample type, the frequency of *E. coli* observed from fresh meat was found significantly higher at 66/150) in comparison to chilled beef samples 28% (42/150) at (P<0.05) as shown in (Figure 1 and 2A).

Antibiotic resistance rates in *Escherichia coli* isolates

The results showed that all the *E. coli* isolates (100%) were found resistant to tetracycline followed by cefepime, cefotaxime (97.2%) and ciprofloxacin (94.4%). The resistance pattern for ceftriaxone and amikacin was detected 88.8% and 81.5% respectively. In contrast, imipenem was the drug to which 50.9% of the isolates were found susceptible. Among the 108 *E. coli* isolates, 91.66% (n=99) were identified as multi drug resistant *E. coli* (MDR-EC) as shown in (Table 2).

Phenotypic colistin resistance in *Escherichia coli* isolates

All the (n=99) MDR-EC were tested for phenotypic colistin resistance by colistin broth disc elusion (CBDE) test and it was found that 60 (60.60%) isolates were colistin resistant phenotypically showing MIC ≥4 µg /mL. Among the total *E. coli* isolates, the phenotypic colistin resistance was found 55.55% (60/108).

Genotypic colistin resistance in *Escherichia coli* isolates

The isolates positive by CBDE were further processed for detection of mobile colistin resistant genes by PCR and the results revealed that out of 60 colistin resistant isolates, 44 (73.33%) were detected positive for *mcr-1* gene while no isolate was found positive for *mcr-2* gene (Figure 2B).

Escherichia coli infections in humans mostly occur due to the intake of contaminated food items existing in our food chain. Foods of animal origin such as meat and its products have played a

vital role in the transmission of *E. coli* infections to humans. Red meat from freshly slaughtered animals contains a high percentage of water and proteins providing a suitable environment for microbial growth (Datta *et al*., 2012). Antimicrobial resistant *E. coli* acts as a carrier for resistant determinants to other strains and other bacterial species (Rasheed *et al*., 2014). Colistin is widely used in veterinary practice as prophylaxis and growth promoter in food-producing animals (Liu *et al*., 2018). This excessive and indiscriminate use of colistin in animals is responsible for emergence of colistin-resistant bacteria which are transmitted to humans via food chain (Poirel *et al*., 2016).

In the present study (n=300) raw buffalo meat samples were processed and 36% among them were found positive for *E. coli* which is close to the findings of 35.40 % in Nepal (Saud *et al*., 2019), 38% in Ghana (Adzitey, 2020) and 42.34% in Japan (Nishino *et al*., 2017). In contrast an elevated frequency of *E. coli* was reported 47.6% in Pakistan (Rahman *et al*., 2019), 55.20% in Tanzania (Mgaya *et al*., 2021) and 61% in Pakistan (Shafiq *et al*., 2022). It was also observed that *E. coli* was prominently prevalent in fresh buffalo meat (44%) in contrast to chilled meat samples (28%). The variation in the results is due to the type of meat samples, contamination of collection sites and health status of slaughtered animals. The lack of quarantine facilities and improper hygienic measures during handling, transportation and processing of buffalo meat is responsible for its contamination (Adzitey, 2015).

The antibiotic susceptibility of *E. coli* isolates was performed against 11 antibiotics belonging to different classes and the results depicted highest resistance (100%) against tetracycline followed by (97.2%) for cefipime, cefotaxime and (94.4%) for ciprofloxacin which are in line with the previous findings in Pakistan, Nepal and China (Nawaz *et al*., 2021; Bista *et al*., 2020; Shafiq *et al*., 2019). Similarly, an elevated level of resistance was detected to ceftriaxone (88.8%), and amikacin (81.5%) among the isolates which resembles with the findings of various studies (Nawaz *et al*., 2021; Rahman *et al*., 2019). Among the 108 *E. coli* isolates, 91.66% (n=99) were found multi drug resistant. This increased frequency of MDR isolates among the meat samples was also reported 90% in China, 75% in Pakistan and 70% in Tanzania (Shafiq *et al*., 2019; Nawaz *et al*., 2021; Mgaya *et al*., 2021). The possible reasons behind such type of results are inopportune selection, indiscriminate use, underestimated dosage and incorrect duration of antibiotics usage.

This study reported 60.60% of the occurrence of colistin resistant *E. coli* among the MDR isolates on the basis of MIC ≥4 µg /mL. In recent past, the frequency of colistin resistant *E. coli* was reported 59% in Pakistan (Zulqarnain *et al*., 2021) as well as 71%, 73% and 88% in China and Bangladesh respectively (Shafiq *et al*., 2019; Wang *et al*., 2018; Uddin *et al*., 2022) among the poultry meat samples. The reason behind such an increasing trend of colistin resistance in the food animals is extensive use of colistin among food animals, especially in poultry as a growth promoter and last option for treatment against Gram negative bacteria. In addition, the counter sale of colistin without prescription of veterinarian or physician further aggravates the situation.

Recently, plasmid-mediated colistin resistant genes (*mcr-1 to 9*) have been extensively discovered throughout the globe. In this study the colistin resistant isolates of *E. coli* were also subjected to detection of *mcr-1 and 2* genes by PCR and results indicated that 73.33% colistin- resistant isolates were positive for *mcr-1* gene and not a

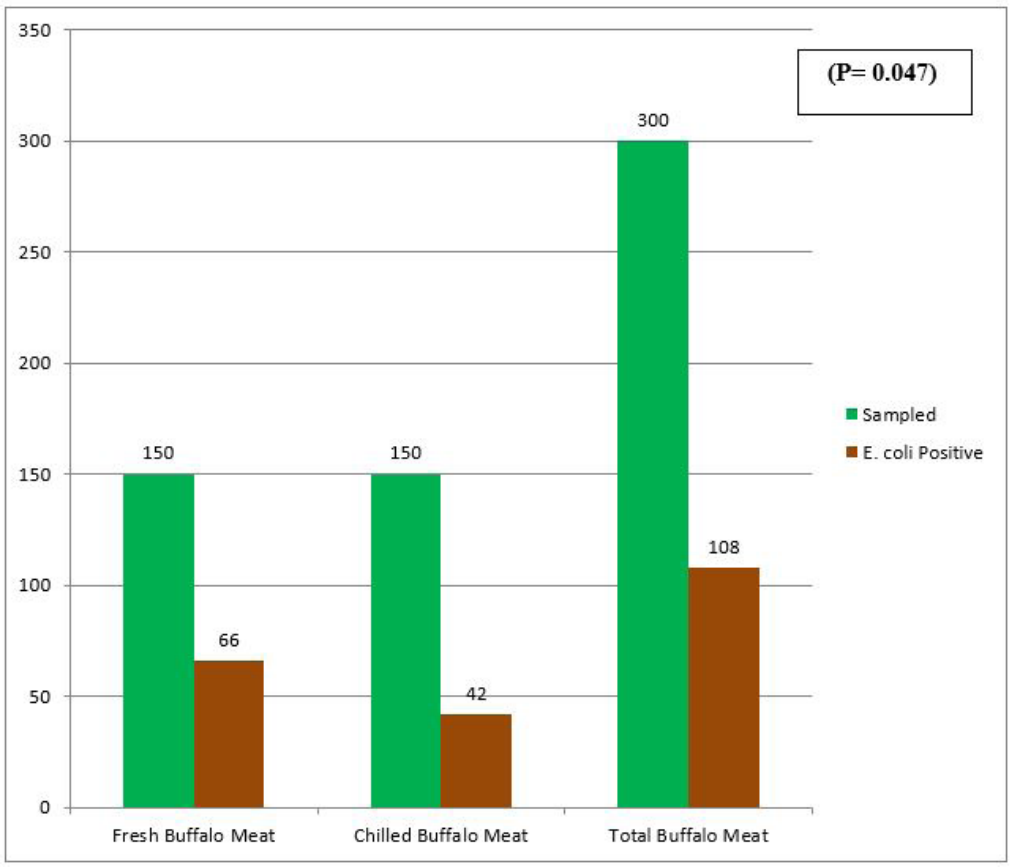

Figure 1. Frequency of *Escherichia coli* from fresh and chilled buffalo meat samples.

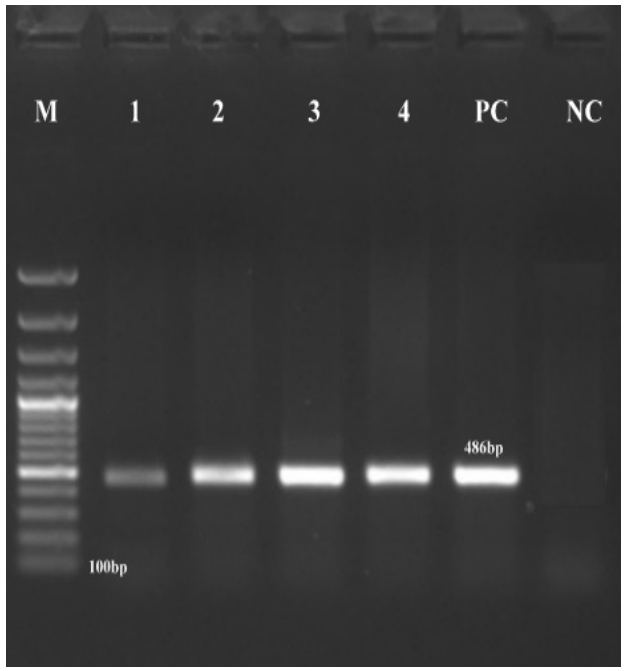

Figure 2A. Agarose gel electrophoresis showing PCR product of *uid*A gene of *Escherichia coli* having 486 bp band. M: 100bp Marker, 1to4: Positive-samples, PC: Positive- control, NC: Negative-control.

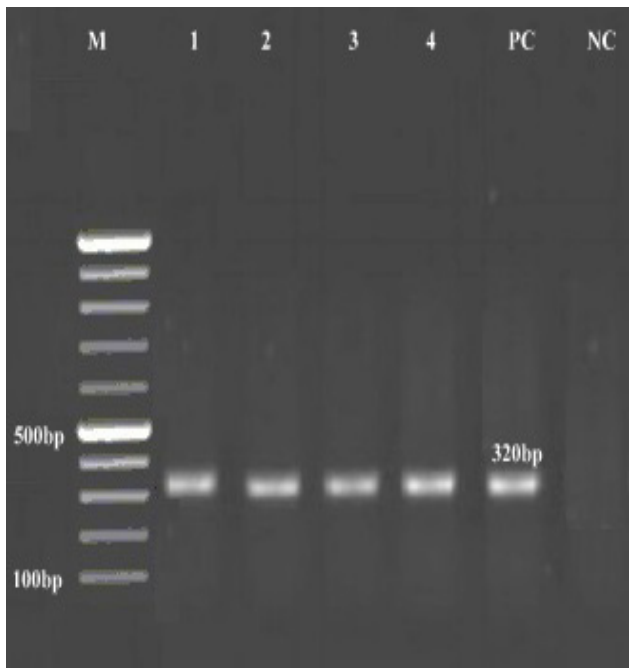

Figure 2B: Agarose gel electrophoresis showing PCR product of *mcr*1 gene having 320 bp band. M: 100bp Marker, 1to4: Positive-samples, PC: Positive-control, NC: Negative-control.

Table 1. Nucleotide sequences of primers used for PCR amplification.

Name of primer	Primer sequence	Product size	References
uidA-F	ATCACCGTGGTGACGCATGTCGC	486 bp	(Nawaz *et al*., 2021)
uidA-R	CACCACGATGCCATGTTCATCTGC		
mcr-1-F	AGTCCGTTTGTTCTTGTGGC	320 bp	Rebelo *et al*., 2018
mcr-1- R	AGATCCTTGGTCTCGGCTTG		
mcr-2-F	CAAGTGTGTTGGTCGCAGTT	715 bp	
mcr-2-R	TCTAGCCCGACAAGCATACC		

Table 2. Antibiotic susceptibility profiling of *Escherichia coli* isolates.

Name of antibiotic class	Name of antibiotic	Susceptibility pattern		
		Sensitive	Intermediate	Resistant
Aminoglycosides	Amikacin	06 (5.5%)	14 (12.9%)	88 (81.5%)
	Gentamicin	09 (8.3%)	18 (16.6%)	81 (75.0%)
Cephalosporins	Ceftriaxone	09 (8.3%)	03 (2.7%)	96 (88.8%)
	Cefotaxime	03 (2.7%)	00 (00%)	105 (97.2%)
	Cefepime	03 (2.7%)	00 (00%)	105 (97.2%)
Carbapenems	Imipenem	55 (50.9%)	30 (27.7%)	23 (21.2%)
	Meropenem	38 (35.2%)	47 (43.5%)	23 (21.2%)
Fluoroquinolones	Ciprofloxacin	06 (5.5%)	00 (0.0%)	102 (94.4%)
Penicillins	Amoxicillin	24 (22.2%)	35 (32.4%)	49 (45.3%)
	Ampiciliin	06 (5.5%)	18 (16.6%)	84 (77.7%)
Tetracyclines	Tetracycline	00 (0.0%)	00 (0.0%)	108 (100%)

single was found positive for *mcr-2* gene. Close findings were achieved by some recent studies (Shafiq *et al*., 2019; Uddin *et al*., 2022). On the other hand, much elevated (>80%) trend of *mcr-1*gene was observed in Japan (Nishino *et al*., 2017), in Nepal (Muktan *et al*., 2020) and in Pakistan (Javed *et al*., 2020). The existence of *mcr-1* gene among animal, clinical and environmental sources has also been reported in several Asian countries (Skov and Monnet, 2016). The *mcr-1* mediated colistin resistance can be transferred from animals to humans via food chain and represents a potential risk to public health. The results of this study along with the findings of previous studies, confirmed an astonishingly high rate of *mcr-1* in food animals and their meat is likely associated with the extensive and prolonged practice of colistin usage as a growth promoter.

CONCLUSION

The increasing trend of *mcr-1* carrying *E. coli* from raw buffalo meat in Pakistan is a threatening concern for both veterinary and human health. Food animals are responsible for the

transmission of antimicrobial resistance genes to humans, the environment and other animals. The low sanitary standards of butcher shops, unhygienic status of meat handlers and poor transportation of raw meat are the major contributing factors. There is a rapid need to incorporate hygienic practices among meat sellers and to control imprudent use of colistin in food animals to preclude the spread of resistance genes from food animals to human population.

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