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RESEARCH ARTICLE

Molecular Detection of Colistin Resistance Gene (MCR-1) in *E. coli* Isolated from Cloacal Swabs of Broilers

Muhammad Zulqarnain, Noreen Sarwar*, Aftab Ahmad Anjum, Sehrish Firyal[1], Tahir Yaqub and Masood Rabbani

Institute of Microbiology; [1]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan
*Corresponding author: noreen.sarwar@uvas.edu.pk

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ABSTRACT

The current emergence of antimicrobial resistance against colistin is a major challenge to treat multi drug resistant Gram negative bacteria. Broiler chickens can function as a source for dissemination of antimicrobial resistant strains to humans. In present study, 150 cloacal swabs of broiler birds, collected from retail shops in Lahore, were processed for isolation of *E. coli*. *E. coli* isolates (n=100) were tested for antimicrobial sensitivity to colistin sulfate using broth dilution method. The resistant isolates were tested by polymerase chain reaction for mcr-1 and mcr-2 genes of colistin resistance. The isolates, found resistant by broth dilution method, were also checked for pathogenicity by adding congo-red dye in nutrient agar. Phenotypically colistin resistant isolates were also tested for other antibiotics e.g. Amoxicilline (30ug), Cefepime (30ug), Gentamycin (10ug), Chloramphenicol (30mcg), Clindamycin (2ug), Ciprofloxacine (5ug), Norfloxacin (10ug), Amikacin (30ug), Lincomycin (10ug) and Streptomycin (10ug) by disc diffusion method. Out of 100 isolates, 59 showed resistance to colistin while taking MIC as 4mg/L. The colistin resistant *E. coli* also showed multidrug resistance against other antibiotics tested. Among 59 phenotypically colistin resistant isolates, only 7 isolates were possessing mcr-1 gene but none of the strains was observed bearing mcr-2 gene. About 15% (5/59) colistin resistant isolates were pathogenic by congo red dye test. The results indicated presence of mcr-1 harboring *E. coli* in commercial birds that can contribute in spread of mcr-1 gene.

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INTRODUCTION

Irrational use of drugs in veterinary side for treatment or prevention can lead to drug resistance in common food animals (Nguyen *et al.*, 2016). Colistin sulfate was banned in animal feed after colistin-resistant gene mcr-1 having *E. coli* were detected in human and animals. However, colistin has been extensively used to enhance growth in food animals specifically in poultry. *E. coli* is a major normal inhabitant of food animal GIT. Therefore play an important role in animal human environment (Shad and Shad, 2018). High prevalence of drug resistance isolates of *E. coli* may contaminate food chain and human animal environment. With timespan, treatment of animal and human can become challenging due to increased number of MDR (multi drug resistant) enterobactariacea. With the discovery of plasmid mediated drug resistant genes, *E. coli* pose to be great global threat. Mcr-1 gene was discovered in 2015 from farm animals of China (Liu *et al.*, 2016). Later on, different studies confirmed its prevalence internationally (Doumith *et al.*, 2016; Haenni *et al.*, 2016) along with mcr-2, mcr-3, and mcr-4 and mcr-5. Plasmid mediated colistin resistance genes mcr-1 and mcr-2 are having potential of dissemination (Mohsin *et al.*, 2017). After its emergence different studies were designed in other countries to investigate its spread in food born animals and their association with human and environment (Garcia-Graells *et al.*, 2018). Subsequently more colistin resistant genes (mcr-2 to mcr-5) were studied in different populations and different areas (Xavier *et al.*, 2016; Yin *et al.*, 2017). However, mcr-1 was observed to be more prevalent compared to other genes. Pathogenic *E.coli* containing mcr-1 gene have also been reported (Azam *et al.*, 2017) Few cases of mcr-1 gene containing isolates have also been reported from human patients (Quesada *et al.*, 2016; Mohsin *et al.*, 2017). As per density, burden of these

resistant genes is quite significant in poultry population of underdeveloped countries(Gharaibeh and Shatnawi, 2019). Colistin resistant *E.coli* having mcr-1 gene have been reported from different sources including food water and healthy humans also (Johura *et al.*, 2020).

A few studies in Pakistan have been conducted to investigate the present situation of mcr-1 genes in Gram-negative bacteria among poultry. In this study, we aimed to detect the mcr-1 and mcr-2 genes along with antibiotic profile in *E. coli* isolated from cloacal swabs of commercial broiler birds in Lahore.

MATERIALS AND METHODS

Isolation of *E. coli*: A total of 150 cloacal swabs were collected from broiler chickens at retail shops in Lahore and processed for isolation and identification of *E. coli*. Swab samples were inoculated into nutrient broth. Each culture was inoculated separately onto the surface of differential (MacConkey agar) and selective media (eosin methylene blue (EMB) agar). After incubation at 37ºC for 24 hours, growth and colony characteristics were observed. Pink colonies on MacConkey agar were inoculated onto the surface of EMB agar plate and incubated at 45ºC for 24 hours. Black colonies with green metallic sheen were subjected to inoculation on nutrient agar. After 24 hours incubation at 37ºC, cultures were stored in refrigerator for further processing.

The screening of *E. coli* isolates was done through microscopic examination and biochemical tests. Microscopically, Gram negative, non-spore forming rods were observed. Biochemical tests e.g. Triple sugar iron test, Voges Proskaur test, Methyl red test, Indole test, Oxidase and Catalase test were performed to confirm the identities of isolates (Akond *et al.*, 2009).

Resistance to colistin sulphate by broth dilution: The *E. coli* isolates were subjected to antibiotic sensitivity to colistin sulphate by micro broth dilution method. Stock solution of colistin sulfate was prepared as 1mg/ml. Different concentrations of colistin were tested while 4mg/L was selected as minimum inhibitory concentration (MIC) of resistant isolates. On the basis of difference in OD value at 0 times and after 24 hours of incubation, isolates were characterized resistant to a particular concentration of colistin sulfate (www. EUCAST.org).

Antibiotic Profiling in colistin resistant *E. coli:* *E. coli* isolates found resistant to colistin by broth dilution method were further tested against other antibiotics for antibiotic sensitivity by disc diffusion (Kirby-Bauer) method. Commercially available antibiotic disc including Amoxicillin (AUG) 30µg, Cefepime (FEP) 30µg, Gentamycin (CN) 10ug, Chlorphenicol (C) 30mcg, Clindamycin (DA) 2µg, Ciprofloxacin (CIP) 5µg, Norfloxacine (NOR) 10µg, Amikacin (AK) 30µg, Lincomycin (MY) 10µg and Streptomycin (S) 10µg were used. A suspension prepared from pure culture according to 0.5 Mac Farland turbidity standard was swabbed on plates prepared from Muller Hinton (MH) agar. Different antibiotic discs were implanted within 15 min of inoculation and plates were incubated at 37ºC for 18 to 24 hours. Following incubation, zones of inhibition (ZOI) were measured in millimeter (mm) and compared with CLSI standards to interpret susceptivity pattern.

Congo red dye test for pathogenic *E. coli*: Colistin resistant isolates were streaked on nutrient media having 0.3% congo-red dye and incubated at 37ºC for 72 hours. Pathogenic *E.coli* appeared as red colonies within 72 hours while nonpathogenic remained colorless (Sharma *et al.*, 2006).

PCR of mcr-1 and mcr-2 genes: The extracted DNA was analyzed by PCR for mcr-1 and mcr-2 genes. The colistin resistant *E. coli* isolates were inoculated in LB broth for 20 hours at 37ºC. Overnight culture was centrifuges for 5 min at 3000 rpm. Pallet of cells was re-suspended with 500ul of deionized water. Cells were heated at 95ºC for 10 min in water bath and cellular debris was removed via centrifugation for 5 min at 13000 rpm. Supernatant was collected and centrifuged at 14000 rpm for 5min. Pellet was mixed in T.E buffer and stored at -20ºC. The quantity of isolated DNA was measured by using Nano-drop. Primers and conditions for reaction were optimized accordingly (Irrgang *et al.*, 2016).

Isolates were tested by PCR for mcr-1, using primers F (5′-CGG TCA GTC CGT TTG TTC -3′) and R (5-CTT GGT CGG TCT GTA GGG -3′) (Liu *et al.*, 2016). Similarly, PCR was done for mcr-2, using mcr-2 F (5′-ATG ACA TCA CAT CAC TCT TGG-3′) and mcr-2 R (5′-TTA CTG GAT AAA TGC CGC GC-3′); cycling conditions consisted of 30 cycles of 95ºC for 15sec, 52ºC for 30 sec, 68ºC for 1 min, followed by 1 cycle of 72ºC for 5 min (Liassine *et al.*, 2016).

Gel Electrophoresis: Agarose gel 1.5% was used to observe amplified product. 4uL of PCR product was loaded in each well and 2uL molecular weight marker was used (DNA Leader of 100bp). Voltage of 110 volts was applied for 30 minutes and gel was viewed under UV light.

RESULTS

Identification of *E. coli*: A total of 150 cloacal swabs, collected from broiler birds at different retail shops, were subjected for growth of *E. coli* on differential and selective media. *E. coli* appeared as smooth, glossy and pink colored colonies on MacConkey Agar. On EMB agar, *E. coli* isolates produced green metallic sheen (Fig.1). Biochemical test showed *E. coli* isolate were positive for catalase, methyl red and indole tests while negative for oxidase and vogues prausker test. On TSI slant, acidic slant and acidic butt with gas were noticed. On the basis of physicochemical characteristics, a total of 100 isolates were recognized as *E. coli.*

Colistin sulphate resistant *E. coli*: The *E. coli* isolates were tested against colistin sulfate resistance by micro broth dilution method. Out of 100 isolates, 59 were found resistant to colistin sulphate while taking MIC value as 4mg/L.

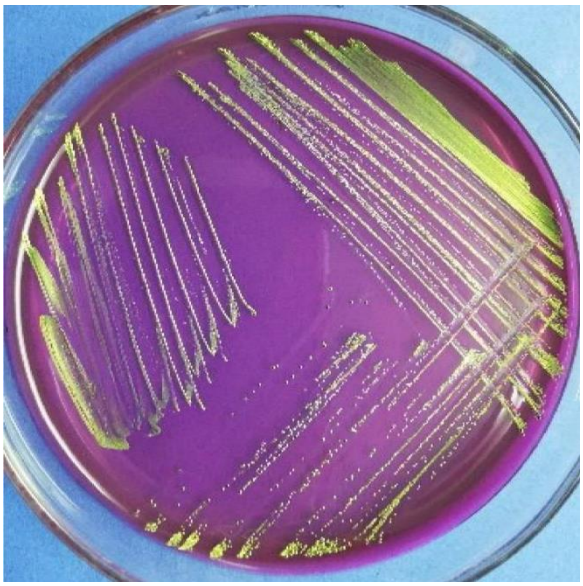

Fig. 1: Eosin Methylene Blue (EMB) agar plate; *E. coli* colonies with characteristic green metallic sheen.

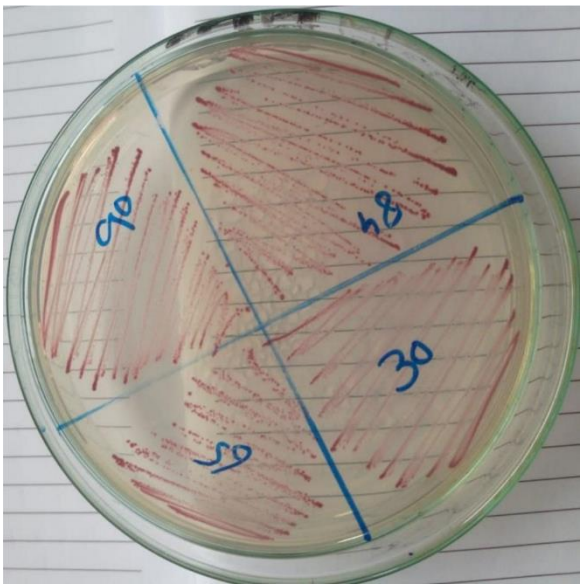

Fig. 2: Congo red dye test showed red growth of pathogenic *E. coli* in the presence of 0.3% Congo red dye.

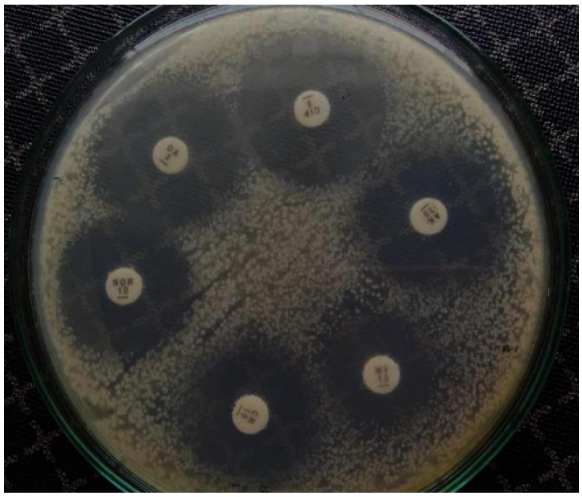

Fig. 3: Antibiotic sensitivity by Kirby-Bauer disc diffusion method with gentamycin (CN) 10ug, clindamycin (DA) 2µg, ciprofloxacin (CIP) 5µg, norfloxacine (NOR) 10µg, amikacin (AK) 30µg and lincomycin (MY) 10µg.

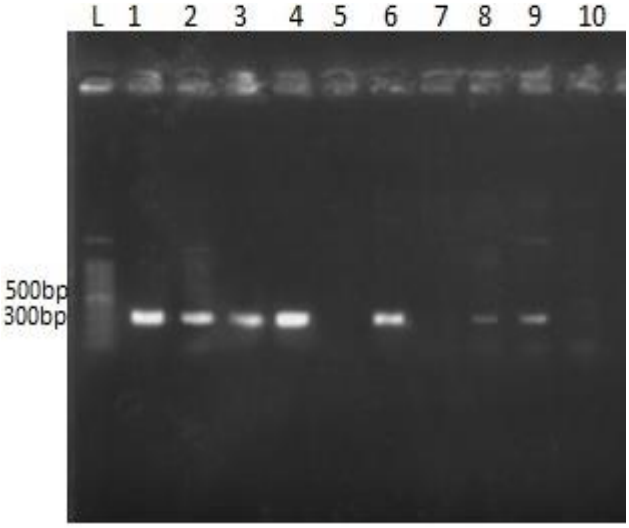

Fig. 4: Agarose gel electrophoresis showing PCR product for mcr-1 gene of *E. coli.* Lane L: 100 bp Ladder Lane 1,2,3,4, 6, 8, 9 showed 300bp amplicon of colistin resistant isolates

Table1: Antibiotic sensitivity pattern among colistin sulfate resistant *E. coli* isolates

Antibiotics	Conc	Zone of inhibition (mm)			Resistance observed %		
		S	I	R	S	I	R
Amikacine (AK)	30ug	≥64	17-63	≤16	0	83	17
Amoxicilline (AUG)	30ug	≥18	16-17	≤15	0	0	100
Clindamycine (DA)	2ug	≥18	16-17	≤15	35	0	65
Ciprofloxacine (CIP)	5ug	≥24	19-23	≤18	12	37	51
Chlorphenicol (C)	30mcg	≥18	13-17	≤12	59	39	2
Cefepime (FEP)	30ug	≥23	17-22	≤16	8	3	89
Gentamycine (CN)	10ug	≥23	NA	≥22	25	0	75
Lincomycine (MY)	10ug	≥18	15-17	≤14	18	5	74
Norfloxacine (NOR)	10ug	≥23	22-17	≤16	40	0	60
Streptomycine (S)	10ug	≥15	12-14	≤11	18	27	55

Detection of multidrug resistance in colistin sulphate resistant *E. coli:* The colistin resistance isolates (n=59) were tested against other antibiotic by disc diffusion method (Fig. 3). Among 59 colistin sulphate resistant isolates, 100 % (59/59) were resistant to amoxicillin, 17% (10/59) resistant to amikacin, 65% (38/59) resistant to Clindamycin, 51 % (30/59) resistance to ciprofloxacin, 60% (43/59) resistant to lincomycin and norfloxacin 89 % (53/59) resistant to cefepime, 75% (44/59) resistant to gentamycin, and 55% (33/59) resistant to streptomycin (Table 1). The results showed higher resistance to other antibiotics among the colistin resistant isolates. Among 59 colistin resistant *E. coli* isolates, 9% (6/59) were detected pathogenic via Congo red dye test (Fig. 2).

Detection of mcr-1 and mcr-2 genes: Out of 59 colistin resistant isolate of *E. coli*, only 7 isolates were positive for mcr-1gene. All the isolates were negative for mcr-2 gene. For mcr-1 positive isolates amplified product of 300bp was observed under UV light in 1.5% agar (Fig. 4).

DISCUSSION

Based on cultural and biochemical test, 100 isolates of *E. coli* were identified from 150 cloacal swabs. These *E. coli* isolates grew as circular, smooth and grayish white colonies on nutrient agar and black circular colonies with characteristic green metallic sheen on EMB agar. In a similar study cloacal swabs from poultry were processed for isolation and identification of different microorganisms. Among 20 positive samples, 5 (25%) *Salmonella* spp., 6 (30%) *E. coli.* 2 (10%) *Mycoplasma* spp., 4 (20%) *Staphylococci* spp. and 3 (15%) fungus were observed (Hakkani *et al.*, 2016). In another study cloacal swab samples from turkeys were used for isolation and identification of bacteria. On the basis phenotypic characteristics, it was found that 20.83% samples contained *Salmonella* spp., 18.76% samples were positive for *Salmonella* and *E. coli* and 8.33% samples showed no growth in subculture media (Roy *et al.*, 2017).

A couple of studies have reported the present status of mcr-1 genes harboring Gram-negative bacteria in domestic fowls. Colistin has been extensively used to enhance growth in food animals including poultry. After invention of mcr-1 gene from China, positive isolates were found in different continents and the responsible gene was also found in different species of food animals and highest prevalence was observed in broiler (Arcilla *et al.*, 2016). With detection of colistin resistance gene mcr-1, other correlated genes were also discovered that included mcr-2, mcr-3 genes. It was noticed that most of mcr-1 harboring isolates were also bearing correlated genes (Mohsin *et al.*, 2019; Zhang *et al.*, 2018).

In present study colistin sulphate resistant *E. coli* isolates (n=59) showed multidrug resistance to other antibiotics when tested by disc diffusion method of antibiotic sensitivity. About 100 % isolates were resistant to Amoxicillin, 89% isolates were resistant to Cefepime while 75% isolates showed resistant to Gentamycin. For Lincomycin, Norfloxacin and Streptomycin resistance was observed as 65, 74, 60 and 55% respectively. About 65% isolates were resistant to Amikacin and Clindamycin. In a relevant study, mcr bearing isolates were confirmed via PCR. After this colistin resistance isolates were subjected to other antimicrobial drugs that were commonly used to control infectious agents. It was found that most of colistin resistant isolates, having plasmid mediated gene mcr, were found resistant to most of other antimicrobial drugs too (El Garch *et al.*, 2018). A study has reported multidrug resistance in mcr-1 bearing isolates. It was found that all 13 mcr-1 containing *E. coli* strains were resistant to ampicillin and tetracycline while 12/13 strains were resistant to sulphamethoxazole-trimethoprium and erythromycin. 10/13 were resistant to nalidixic acid, 9/13 resistant to cephalothin and 8/13 resistant to gentamycin. 6/13 strains were resistant to azithromycin, ciprofloxacin and levofloxacin, 2/13 were resistant to chloramphenicol and 1/13 was resistant to aztreonam, cefixime and ceftriaxone (Johura *et al.*, 2020).

In this study it was found that only 7 isolates among 59 phenotypically colistin resistant *E. coli* isolates contained mcr-1 gene while none of them was having mcr-2 gene. A study in district Faisalabad has reported one isolate out of 10 bearing mcr-1 gene but none of the isolates was found positive for other genes (Azam *et al.*, 2017). In the Netherlands, a PCR based screening study, performed on fecal samples collected from healthy animals of different species involving broiler chicken and pigs, have reported 0.3% positive samples for mcr-1 gene (Veldman *et al.*, 2017). Another study have reported infrequent cases of mcr-1 containing isolates from 1970 to 2008 with marked increase from 5.2 to 30.0% of mcr-1 harboring isolates from 2009 to 2014 (Shen *et al.*, 2016). In Europe, a study have reported frequency of mcr-1 in Salmonella and *E.coli* as 0.1 and 0.7% while one of the isolates was having mcr-2 gene (El Garch *et al.*, 2018).

Conclusions: This study has reported the existence of pathogenic *E. coli* containing colistin resistance mcr-1 gene in the gut of healthy broiler chickens. These results highlighted that commercial broiler chicken quite likely be an important source of *mcr-1* harboring *E. coli* and therefore can contribute to dissemination of the *mcr-1* gene.

Authors contribution: NS conceived the idea and drafted the study. MZ executed the experiment and AA and SF analyzed and described the data. TY and MR critically revised the manuscript and approved for final submission.

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