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The *mcr-1* gene transformationthrough conjugation assay in avian pathogenic *E. coli* from Pakistan

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Abstract

Escherichia coli are the normal flora of both human and animal intestinal tract. But still it is one of the most frequent members of the Enterobacteriaceae linked with extra intestinal infections. Similarly, global propagation of multidrug resistant (MDR) *E. coli* in food chains is a threat not only to animal health but also to human health. Colistin as the last resort antimicrobial in the arsenal of antibiotics. But in recent past, plasmid mediated colistin resistant gene has been detected in humans, livestock and on other retail meat products. The poultry industry is an important component of Pakistan gross domestic product. So, the aim of the study is to access the potential ability of mcr-1 resistant gene to transfer to other bacteria through conjugation. Biochemical and morphological identification was followed molecular identification. Therefore, DNA was extracted by DNA kit. The isolated mcr-1 bearing strains *E. coli* 79 was used to transformed mcr-1 gene to the donor *E. coli* J53, via conjugation. *E. coli* 79 isolate was used as donors, and *E. coli* J53 (resistant to sodium-azide) was used as the recipient strain. In result, the mcr-1 gene transformation was confirmed phenotypically and through PCR. In conclusion, the presence of mcr-1 gene in poultry isolated strains and their transformation potential was mad e the last resort colistin on stack. Therefore, strict policies need to impose on the over use of the antimicrobial in animals.

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Introduction

Over the last two decades poultry trade rise globally, and poultry meat among the top to fulfill the demand of animal protein. Statically extracted data shows that worldwide poultry meat export among different countries was 8.79 million tons in 2000, which were almost doubled to reach 94% increase from the total exported in the previous 15 years earlies (Johnson *et al.*, 2015). In Pakistan, poultry industry is playing very crucial role in reducing the gap of demand and supply of animal protein. While, commercially poultry farming was started in the 1960s and rapid growth was observed in a few decades. The flourishing of this sector was the result of attractive policies of the government and the persistency of the poultry investor groups. The most encouraging from the government was declare this sector exempt of the sale and income tax along with import duties for several years (Sadiq 2004).

Overall, poultry production is one of the most dynamic and most systematic sectors in Pakistan contributing about 26.8% meat production, 5.76 in agriculture sector and 1.26 is the overall GDP for the last several years. Furthermore, it has been the emerging source of employment for more than 1.5 million people (Hussain *et al.*, 2015). The family of Enterobacteriaceae consist of *Escherichia. coli* which the most predominant commensal digestive tracts organism of warm-blooded animals including humans, on the same time the crucial pathogen. It is very beneficial as commensal organism to their host, but also the reason of different diseases in both animals and humans (Dhama *et al.* 2013, Valentin *et al.* 2014).

Human enteric illness was mostly associated with those pathogens that originate from animal and transfer both directly and indirectly to humans. Moreover, the horizontal gene transfer facilitates the spread of resistance genes in and between various bacteria (Calistri *et al.*, 2013; Parmley *et al.*, 2013). Extensive antibiotic use is the most important reason of developing or emerging of antibiotic resistance microbes both in the animal and human site. It's not only pathogenic bacteria become resistant but also make strong impact on the normal flora of these organism or populations (Bilinski *et al.*, 2012; Servin 2005).

Antibiotics has saved countless lives in the human history and is considered as the most revolutionized drug in the history of medicine (Cohen 2000, Yoneyama and Katsumata 2006). Until now, more than 10000 different antibiotics have been discovered. In which, 75% of them are produced by the actinomycete bacteria and co-incidentally by the genus “streptococcus” of this group produced 75% of the total antibiotic production (Hamaki *et al.,* 2005, Ikeda *et al.*, 2003, Martens and Demain 2017). Additionally, antibiotics which is used in the agriculture are the same or almost of the same composition to the clinical antibiotics (McEwen and Fedorka-Cray 2002). But, unfortunately the extensive use of this wonder drug is accompanied by resistant microbes. That’s why medical experts predict, “this overuse of antibiotics will lead us to the pre-antibiotic era” (Gilbert 2014, Liu B. and Pop 2009, Piddock 2012, Spellberg and Ventola 2015).

Every year millions of kilograms of antibiotics are produced globally and are used for the precautions of humans, animals and agriculture. They kill the susceptible bacteria and leave the resistant one. Here, the question arises that how the bacteria get resistant? It is because most of the drug resistant genes can easily be shared and transferred between different and among the same species with mobile genetic elements such as bacterial eating viruses(bacteriophage), extra circular and genomic DNA (plasmid) and other mobile elements like, transposons and the naked DNA (Levy 2005, Levy and Marshall 2004, Mellon *et al.*, 2001).

However, colistin (polymyxin E) has considered as last choice against the gram-negative pathogenic bacteria (Baron *et al.* 2016, Schwarz and Johnson 2016). Nevertheless, it seems that these bacteria develop the mechanism to bypass the influence of colistin (Baron *et al.* 2016, Gao *et al.*, 2016, Liu Yi-

Yun *et al.* 2016, Schwarz and Johnson 2016).

Material and methods

Sample collection

National Reference lab for Poultry Diseases (NRLPD) has a well-established surveillance system in coordination with provincial livestock departments in Pakistan. These provincial departments collect and submit samples for analysis at NRLPD on regular basis. For this study, the isolated positive mcr-1 positive *E. coli* was received through the surveillance system was analyzed for AMR (Antimicrobial resistance) in poultry.

Isolation and identification of E. coli

A loopful from liver were inoculated onto the autoclaved nutrient broth and incubated at 37°C for 24 hours. Thereafter, the Loopfuls from the broth were streaked onto EMB (Eosin methylene blue) and On MA (MacConkey's agar) plates agar plates and incubated for 24 h at 37°C. The suspected colony was picked up and streaked on blood agar for morphological and biochemical identification (Amer *et al.*, 2018, Quinn *et al.*, 2002). However, the strains were further confirmed by using API 20E kits (biome rieux, Durham, NC).

Gram staining and colony morphology

Gram-negative bacteria (*E. coli*) was studied based on size (large, moderate, small), colony shape (round, irregular), colony color (green-metallic sheen, pink) and colony margins and elevation (concave, convex, raised). Colony of such characteristics was further examined by gram staining.

Catalase test

This test is used to differentiate *E. coli* (catalase positive) from non-catalase (streptococcus) species. The test was performed to detect the presence of catalase enzyme in the organism by using hydrogen peroxide (H_2O_2). A drop of 3% hydrogen peroxide was placed on clean, sterile glass slide. A colony was picked with the help of sterile toothpick and mixed with H_2O_2 drop. Bubble production indicates positive result. Catalase enzyme converts H_2O_2 into O_2 and H_2O. Therefore, no bubble formation was indicative of catalase negative organism (Bertrand *et al.,* 2002, Dezfulian *et al.*, 2010).

Motility test by hanging drop method

This test is performed to check if bacteria are motile by means of flagella. As non-motile bacteria do not possess flagella. While *E. coli*is motile organism.

Analytical profile index (API20E)

API 20E is series of biochemical tests for the identification and differentiation of the members of Enterobacteriaceae family. It consists of plastic strips having twenty mini test chambers and has chemically defined composition for each test. The procedure adopted was previously mentioned (Robinson *et al.*,1995).

Colistin susceptibility testing

Colistin susceptibility testing of the isolated *E. coli* was conducted by using Kirby-Bauer disk diffusion method (Boyen *et al.*, 2010). The test was performed according to the guideline of clinical and Laboratory standard Institute (CLSI, 2017). Muller Hinton agar was prepared in accordance of manufacturer's instructions (Oxiod, UK). Control strains used in this study was*E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (Jones *et al.*, 2005). Then for more confirmation MIC (minimum inhibitory concentration) was also performed (Table 2).

DNA Extraction and determination of mcr-1 gene

Genomic DNA extraction was done by using bacteremia DNA isolation Kit (Qiagen, Germantown, MD). Furthermore, molecular confirmation of conjugation assay, PCR conditions was optimized to amplify the mcr-1 gene in both *E. coli* 79 and J53 strains (Table 1). For this purpose, a known pair of primer of 200bp of the Forward 5'-AAATCAGCCAAACCTATCCC -3'and reverse primer 5'- CGTATCATAGACCGT GCCAT -3' were selected (Table 2.0), previously described (Zhang *et al.* 2017).

DNA was analyzed by gel electrophoresis

After completion of PCR reaction, PCR product was

analyzed by gel electrophoresis. In first well, a DNA marker (Biolabs 100bp ladder) was also loaded. The gel was then run for 50 minutes of 90V in 1X TBE buffer BIO RAD gel electrophoresis tank. After the defined time, gel was observed under ultraviolet trans-illuminator to visualize bands. Photograph of PCR bands was saved in BIO RAD gel documentation system (Bintvihok *et al.* 2016).

Conjugation assay

We did conjugation experiment to determine the mcr1 positive isolates (*E. coli* 79) and could confirm the transfer of mcr1 gene to wild type *E. coli* J53 as previously described (Potter *et al.* 2018). The presence of mcr1 gene on plasmid was already confirmed by PCR (Zhang *et al.* 2017).

Tryptic soya broth was prepared according to the manufacturer's instructions (Sigma Aldrich, St Louis, MO, USA). Both donor (*E. coli* 79) and recipient (*E. coli)* strains were separately suspended in TS broth. The strains were diluted to 0.005 OD600. After dilution, 100 µl of donor strain (*E. coli* 79) was suspended in the 100 µl of recipient strains (11 ratio) and diluted up to 5000 µl with TS broth. Now the Co-cultures were incubated at 37°C for 24 hours. On next day 50 µl of co-cultures were suspended on agar plats (MacConkey agar) contained sodium azide and colistin (5mg/ml). The liquid co-culture was spread with glass beads on agar plates and was incubated at 37°C for 18-24 hours. However, individual trans-conjugants colonies were suspended in TS broth supplemented with colistin (5mg/ml) and was incubated at 37°C on shaker of 220 rpm (Potter *et al.*, 2018).

Results

Biochemical characterization of the bacterial isolates

While, upon culturing dark convex and small size colonies with a green metallic sheen appeared on EMB (eosin methylene blue) (Fig. 1a). while, flat, pink colonies with a surrounding darker pink area were seen on MA (MacConkey Agar) (Fig.1b). Further, the isolated samples were observed under microscope after differential staining (Gram staining).

Table 1. Scheme for total volume of PCR reaction for mcr-1 gene amplification.

PCR Reaction components	Stock concentration	Final concentrations	Volume per Reaction
PCR Master Mix	2X	1X	12.5 µl
Forward Primers Each	100 pmol/µl	2 pmol/µl	0.5 µl x 9 = 4.5 µl
Reverse Primers Each	100 pmol/µl	2 pmol/µl	0.5 µl x 9 = 4.5 µl
Nuclease Free Water	-	-	1.5 µl
Template DNA	-	-	2 µl
Total PCR volume			25 µl

In addition, other biochemical analysis was done through Catalase test, rapid bubble formation after addition of few drops of hydrogen peroxide on the inoculum. The identification codes (ID) of API20E (Analytical profile index) and motility tests using hanging drop method also given further biochemical identification (Fig. 4).

Molecular identification

DNA was isolated using Bacteremia genomic DNA extraction kit (Qiagen, Germantown, MD, USA) and PCR conditions were optimized to amplify the mcr-1 gene in both *E. coli* 79 and J53 strains (Table 1). While DNA quantity was checked by Qubit machine (Table 2).

Antibiotic Profiling of reveled E. coli from glycerol stock

To assess the effect of ARG burden on phenotypic antibiotic resistance, the well-known Kirby-Bauer Disk Diffusion method and Clinical Laboratory and Standards Institute "CLSI" (2018), interpretative criteria for Enterobacteriaceae was used for analysis.

Table 2. *E-coli* 79 and j53 activity against various test.

Serial number	Isolates	Colistin disk (10 µg)	Colistin MIC value (ug/ml	API 20E code	DNA Quantity measure by qubit machine	mcr-1 primer	Putative resistant determinant
1	*E. coli*-79	Positive	4	5044572	17.0ug/ml	Forward 5'AAATCAGCCAAACCTATCC C 3 Reverse primer 5'-CGTATCATAGACCGT GCCAT -3	mcr-1
2	J53	Negative	Nil		28.0ug/ml	-	Negative
3	*E. coli* ATCC 25922	Negative	Nil		21.0ug/ml		Negative

Conjugation assay

The confirmed mcr-1 gene was transferred from strain *E. coli* 79, to the same, *E. coli* J53, via conjugation. *E. coli* 79 isolate were used as donors, and *E. coli* J53 (resistant to sodium-azide) was used as the recipient strain (Table 2). Transfer of the mcr-1 plasmid to *E. coli* J53 was successful only for strains harboring both mcr-1 and *(E. coli* 79), on mixed growth plate and while no growth was observed on J53controlplate (Fig. 2). Therefore, mcr-1 was likely located on a plasmid, not was chromosomally encoded in strain *E. coli* 79 and was confirmed by PCR amplification (Fig.3).

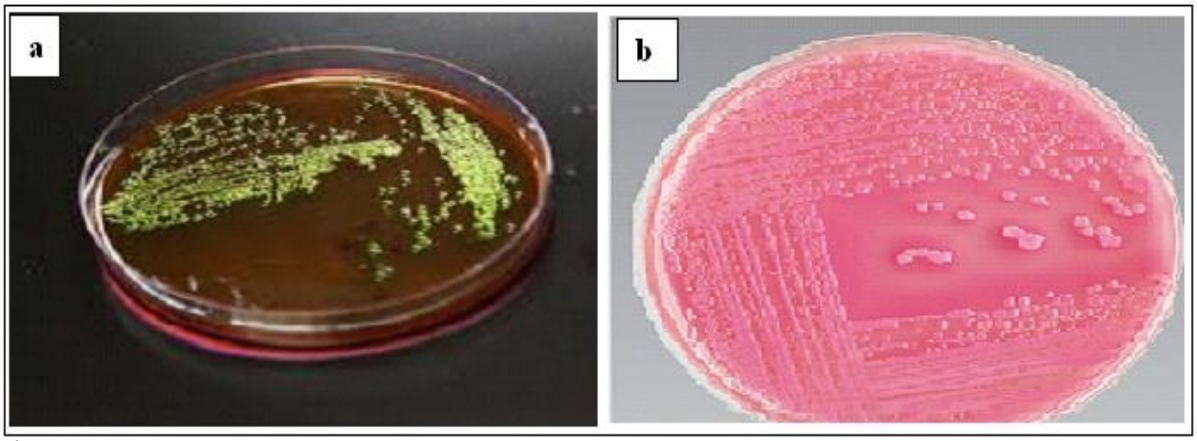

Fig. 1. Colonies of *E. coli* growth on EMB (a) and MA(b).

Discussion

The poultry industry is an important component of Pakistan gross domestic product**.** However, the Pakistani poultry industry faces several environmental conditions that threaten continued economic output, livestock health, and human health *(Hussain et al., 2015). While,* the prevalence of mcr-1 gene increased in Pakistan poultry is also a great threat. which was successfully employed in previous studies (Gutiérrez *et al.*, 2019). In addition, the transability of these mcr-1 gene on plasmid was studied by conjugation assay. Therefore, the isolated *E. coli* strain was confirmed through Gram stanning and selective media (Fig 1). while for more confirmation some additional biochemical tests were performed such as API (Fig 4).

The similar results were reported by Robinson *et al.*, (1995), through these biochemical tests. Biochemical and morphological identification was followed by more advanced and authentic molecular identification techniques. However, DNA extraction was the initial step for most of molecular identification and characterization techniques. DNA was isolated using Bacteremia genomic DNA extraction kit (Qiagen, Germantown, MD, USA) and quantity was checked by Qubit machine (Table 4). Our result was consistent with (Potter *et al.*, 2018).

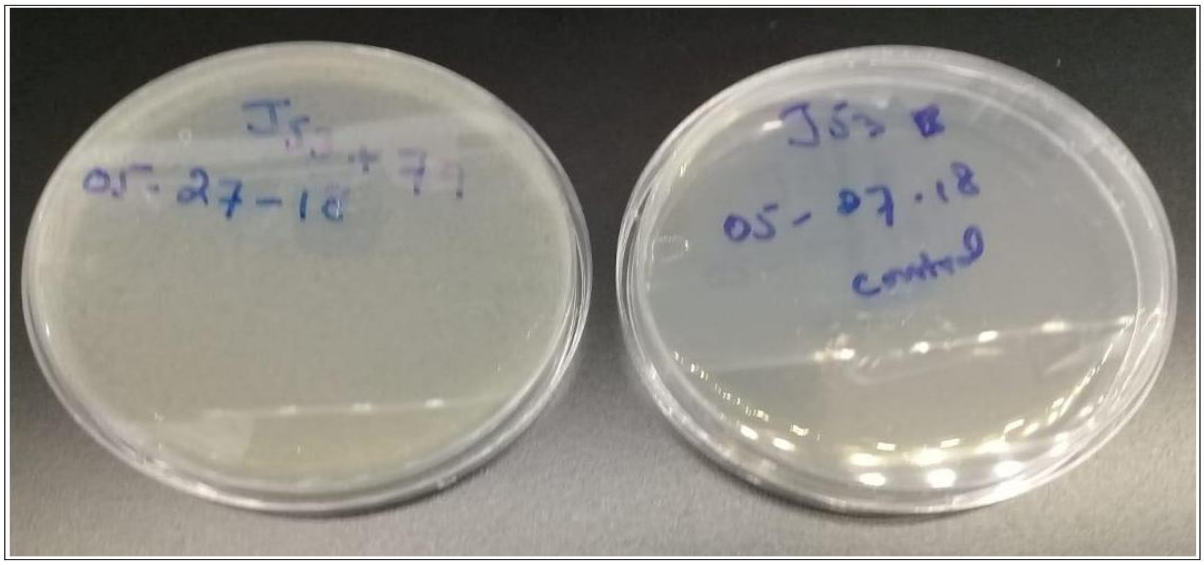

Fig. 2. Mixed growth of j53 (recipient) and 79 (Donor) *E. coli.*

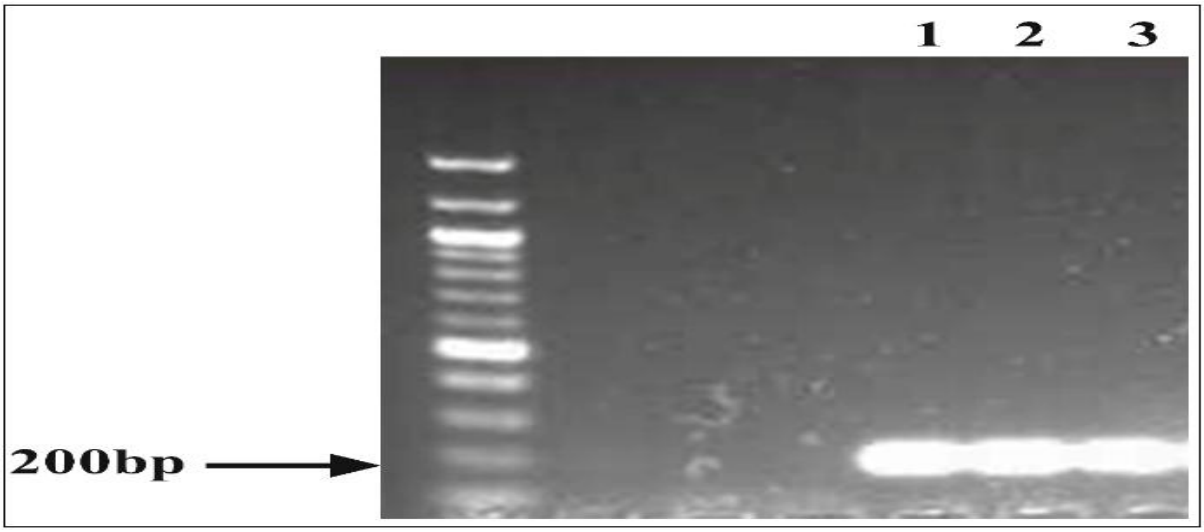

Fig. 3. Gel Electrophoresis image show DNA amplification of mcr1 gene of 200bp in five of the *E. coli* isolates including recipient J53 (1) and donor *E. coli* 79(2) and (3) Control strain represent.

Pakistani poultry farms were the reason of high prevalence of plasmid-mediated mcr-1 genes in these strains. Now it is confirmed through several studies that spread of colistin resistance gene in gram negative bacteria, specifically in Enterobacteriaceae are via mcr-1 bearing plasmids. These plasmids were isolated from human fecal samples, animals' products and environment (Matamoros *et al.*, 2017). Therefore, the transability of these mcr-1 gene on plasmid was studied by conjugation assay, for this, *E. coli* J53 strain sodium azide resistant strain and colistin susceptible was selected as recipient strain. While, *E. coli*-79 mcr-1 positive strain was used as donor strain. (Fig 2). Besides this, the recipient strain having mcr-1 gene was confirmed through conventional PCR using specific primer (Table 2). Similarly, study conducted by Zhang *et al.*, (2017) has also used the same primer for the confirmation of mcr-1 gene, so our finding was complete agreement with his finding.

More specifically, the pattern of susceptibility to other antibiotics of J53-mcr-1 was not changed, indicating the transfer of only mcr-1 gene. Similar finding was obtained in previous study conducted in Canada and Argentina (Tijet *et al.* 2017) and Chile (Gutiérrez *et al.,* 2019). In which the resultant transformant was resitant to colistin. Which was agrreement to the finding of our results.

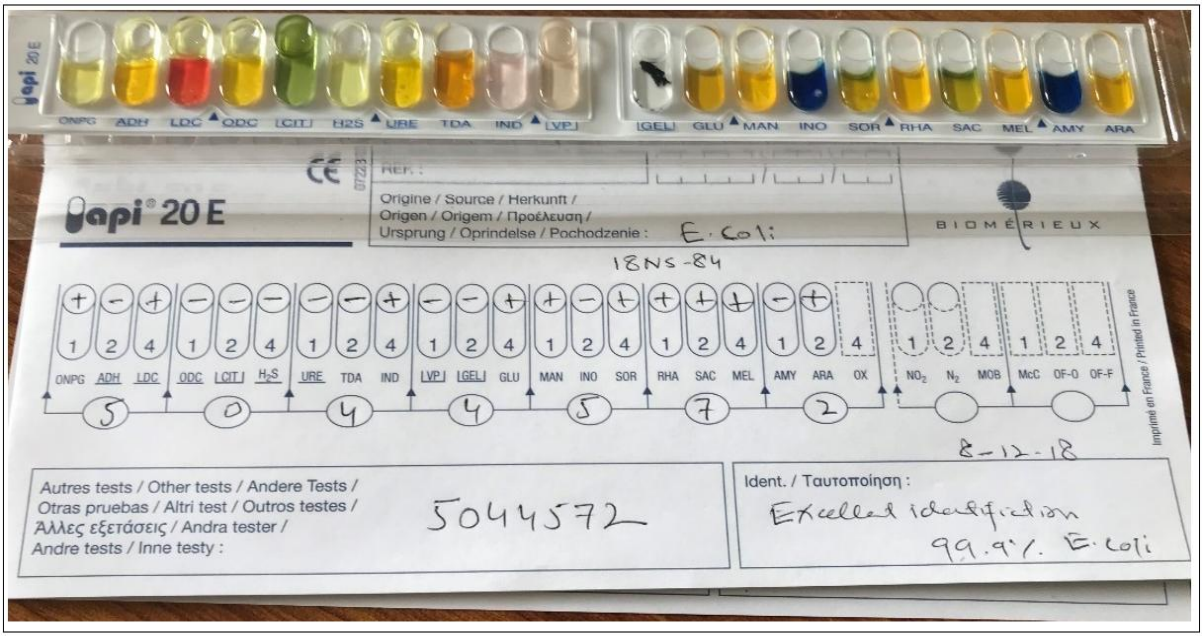

Fig. 4. Analytical profile index of isolated *E. coli* (79).

Conclusion

In conclusion, the increasing use of colistin in Pakistani poultry farms was the reason of high prevalence of plasmid-mediated mcr-1 genes in *E. coli.* Now, it is confirmed through several studies that spread of colistin resistance gene in gram negative bacteria, specifically in Enterobacteriaceae are via mcr-1 bearing plasmids.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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