Molecular Characterization of *mcr* genes and class1 integron gene (*int1*) associated with colistin resistance *E. coli* isolated from diarrheal cases and environment samples in Basrah province

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ABSTRACT

Aims: This study aimed to determine whether mcr genes are present among E. coli that detect class 1 integronintegrase gene (int1) from these sources and to evaluate the prevalence of E. coli that is resistant to E. coli from human and diarrheal cows as well as from environmental samples in Basrah province.

Methodology and results: Firstly, the colistin resistance *Escherichia coli* was diagnosed phenotypically and genetically. The results were revealed that a total of 53(100%) colistin resistance suspected bacterial isolates were identified by using a conventional PCR assay for the presence of the *uidA* gene. Additionally, all colistin resistance *E.coli* isolates were used for determining a colistin-resistance genes *mcr*-1 to 5 by multiplex PCR. Wherein a (83.33%) of isolates from cattle and (100%) from human and environment were found to have a mcr-3. Additionally, none of the isolates contained mcr-1, mcr-2, mcr-4, or mcr-5. Subsequently, the class 1 integron gene was detecting in isolates that carries colistin resistance genes (*mcr*). The results shown that 48(100%) of *E.coli* isolates were having *IntI*1.
Conclusion, significance and impact of the study: In concluded, The appearance of mcr-3 and its coexistence with other integron genes, such as class 1 integron gene (intI1), in isolates of *E.coli* which collected from diarrheal cases and even from environmental samples in Basrah province represent a concern not only to the animals trade but also to public health.

***Key words*:** mcr genes, int1 gene, colistin resistance *Escherichia coli*

1. INTRODUCTION

Escherichia coli is a widely dispersed genus of Gram-negative bacteria that can colonies and persist in humans, warm-blooded animals, and abiotic settings (Kaper et al., 2004; Tenaillon *et al.,*2010). However, some pathovars of *E.coli*, plays a significant role in causing gastrointestinal illnesses and is a major factor in sporadic outbreaks of diarrhea affecting both humans and animals particularly in developing countries (Khudaier *et al.,*2012; Kareem and Khudaier, 2016; Othman, 2018; Abd Al Wahid and Abd Al-Abbas, 2019; Abdulkaliq and Othman, 2024) serious gastrointestinal disorders and a variety of extra-intestinal infections in both people and animals (Aqeela et al.,2018;Sharp *et al.,*2014). Furthermore, *E. coli* is a leading cause of bovine mastitis (BM), newborn calf diarrhoea (NCD), and avian colibacillosis (AC), resulting in significant economic losses. Colistin is a polymyxin medication that was once thought to be a last resort for treating infections caused by members of E.

coli and other members of the MDR Enterobacteriaceae family. Because they represent a serious risk to both human and animal health, colistin-resistant bacteria are important on a global scale (Li et al., 2017). Several mobile colistin resistance variants have been discovered in numerous countries across the five continents since the initial report of mcr. As of right now, ten distinct mcr genes and their numerous variations have been identified in bacterial isolates from the environment, animals, people, food, and poultry farms across the globe (Huang et al.,2021). Colistin has been utilized in livestock feed for many years to enhance growth and manage intestinal infections. It serves as an effective remedy for diarrhea induced by *E.coli*, a condition that is rare in poultry, yet it is employed as a growth enhancer (Kumar et al.,2020). The discovery of mobile colistin genes resistance or mcr that carried on on plasmids raises concerns due to the rapid transmission of colistin genes resistance amongst bacterial populations, in which three distinct mcr genes included:mcr-1, mcr-2, and mcr-3 have been first discovered on plasmids in Enterobacteriaceae family (Hernández *et al.,*2017; Randall et al.,2018), The mcr-4 and mcr-5 genes are among the additional genes that were later added (Zhang et al.,2018; Hallenberg et al.,2019) The aim of this research was to assess the prevalence of E. coli that is resistant to E. coli from diarrheal cows and human and from environment samples in Basrah province and for determining whether mcr genes are existing among *E.coli* with detect class 1 integron integrase gene (int1) from these sources.

2. MATERIALS AND METHODS

2.1. Sample collection and bacterial cultivation :

The collection was conducted during the period from September to December 2023. Two hundred and fifty samples, one hundred from animal and human diarrhoea and fifty from the environment, were collected and tested for identifying of *Escherichia coli*. The collected sample was streaked onto selective and differential agar plates, such as MacConkey agar and Eosin Methylene Blue (EMB) agar, to help isolate and distinguish enteric bacteria. Bacterial colonies of various morphologies (size, shape, color texture) are visible on the agar plates following incubation. These colonies reflect the many bacterial species present in the sample.

2.2. Phenotypic characterization of colistin-resistant *E. coli.*

All probable *E.coli* isolates from cattle, human, and environmental samples were tested for colistin resistance. A 10µl aliquot of the dilution then applied to MacConkey agar supplied with 4 mg/L colistin (Sigma, St Louis, MO). Subsequently for selecting the presumptive colistin-resistant *E.coli*, the agar culture then incubated 24 hours at 37°C (Dang et al.,2020).

3. Molecular diagnosis of colistin resistant *E. coli* isolates using the *UidA.*

The bacterial DNA from the stored broth was extracted utilizing the Bosphore® Bacterial DNA Extraction Spin Kit (Anatolia, Turkey). This method relies on the separation of nucleic acids through a silica membrane column, facilitating the extraction and purification of bacterial nucleic acids (Shalal et al., 2022). The conventional PCR was used to identified the uidA gene that codding for β-glucuronidase enzyme, that is present in all *E.coli* species. To amplify the uidA gene, specific set of primer was used with following sequence (F:5-CCAAAAG CCAG ACAGAGT-3) and (R:5- GCACAGCA CTTCAAA GAG-3). The PCR reaction tube contained 12.5 µl of hot start premix, 1 µl of each primer (10 pmol), and 4 µl of template DNA. Nuclease-free water was then added to achieve a final volume of 25 µl. The PCR amplification was carried out utilizing a PCR system, commencing with a pre-PCR heating phase at 95°C for a duration of 5 minutes. This was succeeded by 35 cycles, each comprising 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C. A concluding extension was performed for 5 minutes at 72°C (Moyo et al., 2007). The amplified product size was confirmed using a red safe DNA solution on a 2% agarose gel electrophoresis.

4. Multiplex PCR to detect genes of colistin-resistance (*mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5*) in *E. coli*

A specific sets of primers (Table 1) are used to detect the mcr-1, mcr-2, mcr-3, mcr-4, and mcr-5 genes through using multiplex PCR (Rebelo et al., 2018; Borowiak et al., 2017). The PCR reaction tube contained five primer pairs, 5.5µl of nuclease-free water, and 2µl of DNA template of identified *E.coli*. The reactions of PCR were included the initial denaturation step started with 94°C for a duration of 15 minutes, followed by 35 cycles that included denaturation at 94°C for 30 seconds, annealing at 58°C for 90 seconds, and extension at 72°C for 60 seconds. The procedure concluded with a final extension at 72°C for 10 minutes. using a PCR thermal cycler. For electrophoresis 1.5% agarose gel was used and the results was analyzed by checked the gel under UV light.

5. Detection of class1 integron (*int1*) gene for colistin resistance by conventional PCR.

The class 1 integron (intI1) gene was identified by utilizing a specific primer : INT1-F 5'-CTC CCG CAC GAT GAT CGT-3' and INT1-R 5'-TTG CGT GAG CGC ATA CGC-3'(Nguyet et al.,2022). The amplification steps of PCR was performed starting with an initial denaturation step at 94 °C for 3 minutes. This was succeeded by 25 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 90 seconds, and extension at 72 °C for 60 seconds. The process concluded with a final extension at 72 °C for 5 minutes. Subsequently, the last extension was carried out at 72 °C for five minutes. The PCR band with 450bp were analyzed by using 1.5% agarose gel electrophoresis.

Table 1. Primers utilized in multiplex PCR for the identification of colistin resistance genes.

Primer name	Sequence (5-3)	Target gene	Size (bp)	Ref.
mcr1 fa mcr1 ra	AGTCCGTTTGTTCTTGTGGC AGATCCTTGGTCTCGGCTTG	mcr-1	320	Rebelo *et al.,* 2018
mcr2 f mcr2 r	CAAGTGTGTTGGTCGCAGTT TCTAGCCCGACAAGCATACC	mcr-2	715	Rebelo *et al.,* 2018
mcr3 f mcr3 r	AAATAAAAATTGTTCCGCTTATG AATGGAGATCCCCGTTTTT	mcr-3	929	Rebelo *et al.,* 2018
Mcr4f Mcr4 r	TCACTTTCATCACTGCGTTG TTGGTCCATGACTACCAATG	mcr-4	1116	Rebelo *et al.,* 2018
Mcr5 f Mcr5 r	ATGCGGTTGTCTGCATTTATC TCATTGTGGTTGTCCTTTTCTG	mcr-5	1644	Borowiak *et al.* (2017)

6. RESULTS

6.1 Bacterial Isolation and Identification

The results showed that (80%) of cattle samples, (40%) human samples and (60%) of environment samples were gave positive results based on culturing on MacConkey and Eosin Methylene Blue agar (Table 2) .

6.2. Phenotypic characterization of colistin-resistant *E.coli.*

All the suspected *E.coli* isolates from cattle, human and environmental samples were screening for colistin-resistant by Streaking it on MacConkey medium containing 4% colistin , For 24 hours, the agar plate was incubated at 37°C in order to select for presumed colistin-resistant *E.coli*. The results showed that (37.5%), (30%) and (36.6%) of cattle, human and environmental samples were colistin resistant respectively as demonstrated in Table 3 and Figure 1.

6.3. Molecular diagnosis of colistin resistant *E. coli* isolates using the *UidA*:

A total of fifty three colistin resistance suspected bacterial isolates were identified. For this purpose the conventional PCR was used for detect the presence of the β-glucuronidase enzyme-encoding *uidA* gene, which is present in all E. coli species. All the isolates (100%) showing positive results (Table 4). The size of the uidA gene band was 623 bp, and the gene was represented by a single band in the corresponding region of the DNA ladder (Figure 2).

Table 2: Numbers and percentages of animal, human and environmental that based on culturing on MacConkey agar ,Eosin methylene blue agar.

Sample Type	Agar Type	Growth NO.	Growth (%)
Cattle	MacConkey agar	80/100	80%
	Eosin-methylene blue	80 /100	80%
Human	MacConkey agar	40/100	40%
	Eosin-methylene blue	40/100	40%
Environment	MacConkey agar	30/50	60%
	Eosin-methylene blue	30/50	60%
Total		250	

Table 3: Number of colistin resistant from suspected *E.coli* isolates**.**

Sample type	No. of suspected *E.coli* isolates	No. of colistin-resistant *E. coli*
Cattle	80	30
Human	40	12
Environment	30	11
Total	150	53

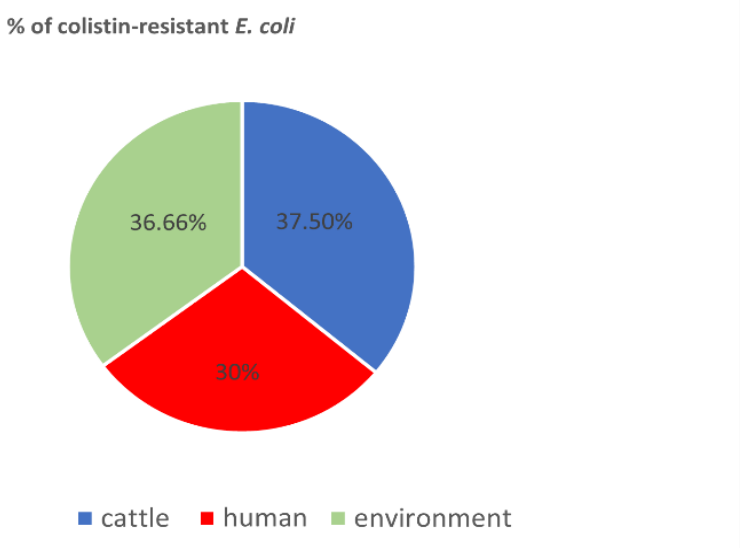

Figure 1: Percentage of colistin resistant *E. coli*

Table (4) Number and percentages of the positive results of *E. coli* uidA gene

Gene	Isolates source	No (%)positive of *E. coli*	Total(%)
UidA	Cattle	30(100%)	53/53(100%)
	Human	12(100%)	
	Environment	11(100%)	

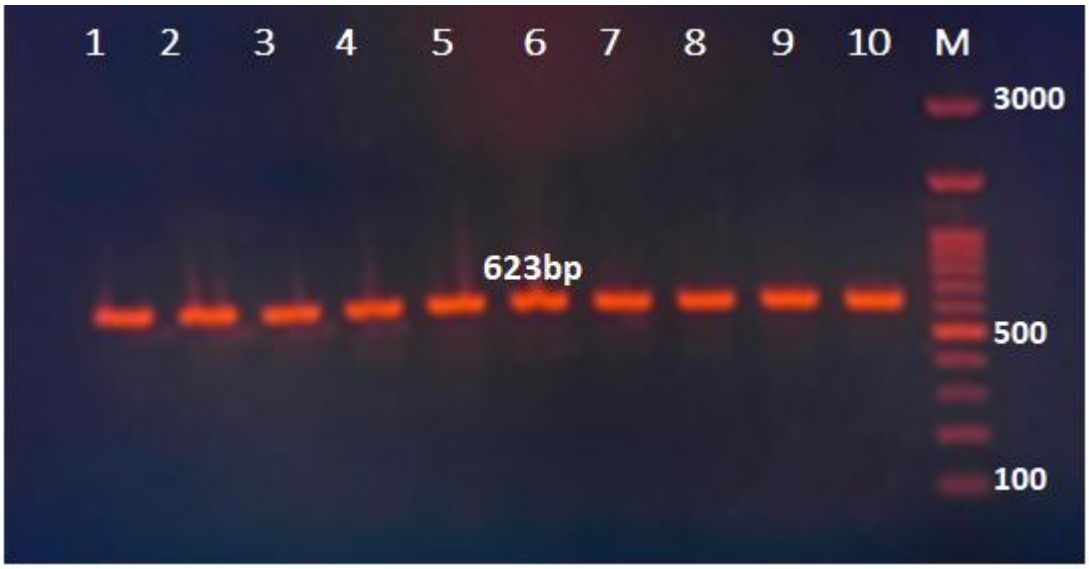

Figure 2: PCR products of the uidA gene of *E. coli*. The size of the PCR product is 623bp. M: Marker DNA ladder (100bp-3000bp).

Multiplex PCR to detect colistin-resistance (*mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5*) genes in *E. coli*

A total of 53 *E. coli* that phenotypically colistin resistant were used to determine a colistin-resistance gene mcr(1-5) by multiplex PCR. Wherein a (83.33%) of isolates from cattle and (100%) from human and environment were found to have a mcr-3. Additionally, none of the isolates contained mcr-1, mcr-2, mcr-4, or mcr-5 (Table 5), (Figure 3,4 & 5).

Detection of class 1 integron (*intI1*) gene for colistin resistance – by conventional PCR.

Table 6 was revealed the results of class 1 integron gene detecting in isolates that carries colistin resistance genes (*mcr*). The results shown that 48(100%) of *E.coli* isolates were having *IntI1.* Furthermore, clear bands of the expected size (929bp) were observed and reflected the *intI*1gene detecting in different examined isolates (Figure 3,4 & 5).

Table 5: Number and percentage of *mcr-3* gene in positive *E.coli* isolates.

Gene	Isolates source	+ve No. of *mcr-3* in *E. coli*	(%) of *mcr-3* in *E. coli*	Total
Mcr-3	Cattle	25/30	83.33%	48/53
	Human	12/12	100%	
	Environment	11/11	100%	

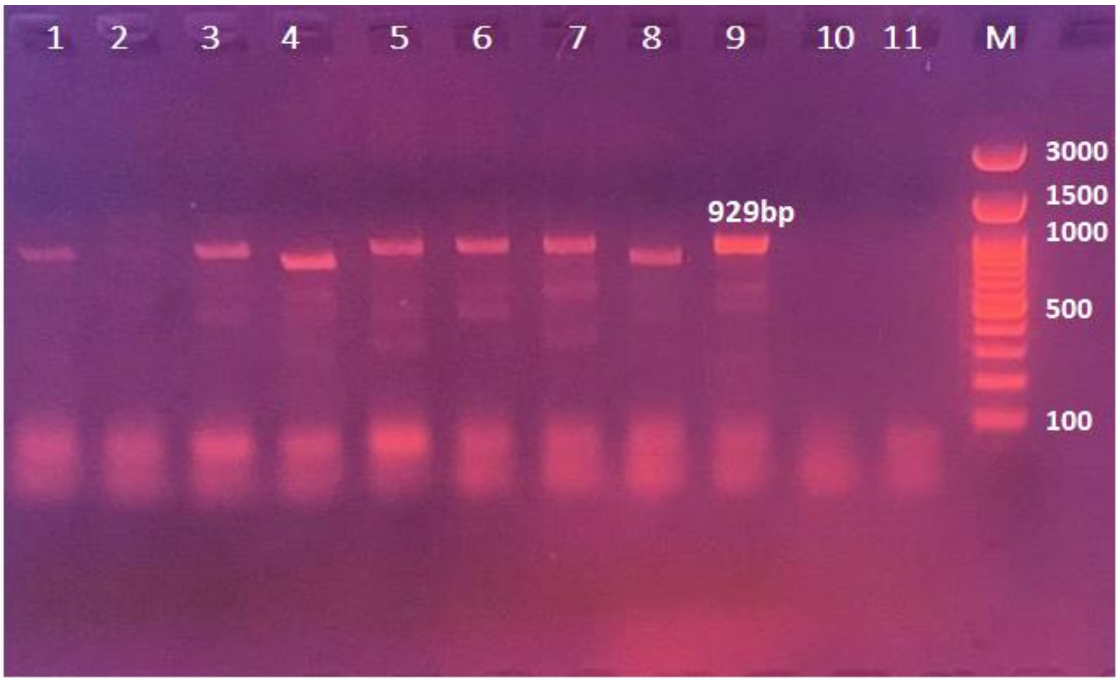

Figure 3: Multiplex PCR results in1.5% Agarose gel electrophoresis for detecting the colistin resistance (mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5) genes in *E.coli* isolated from cows samples. M: Molecular weight marker (100bp-3000bp), Lanes 1,2&10: Negative result. Lanes 3-9 &11: Positive results of mcr-3 gene (929bp).

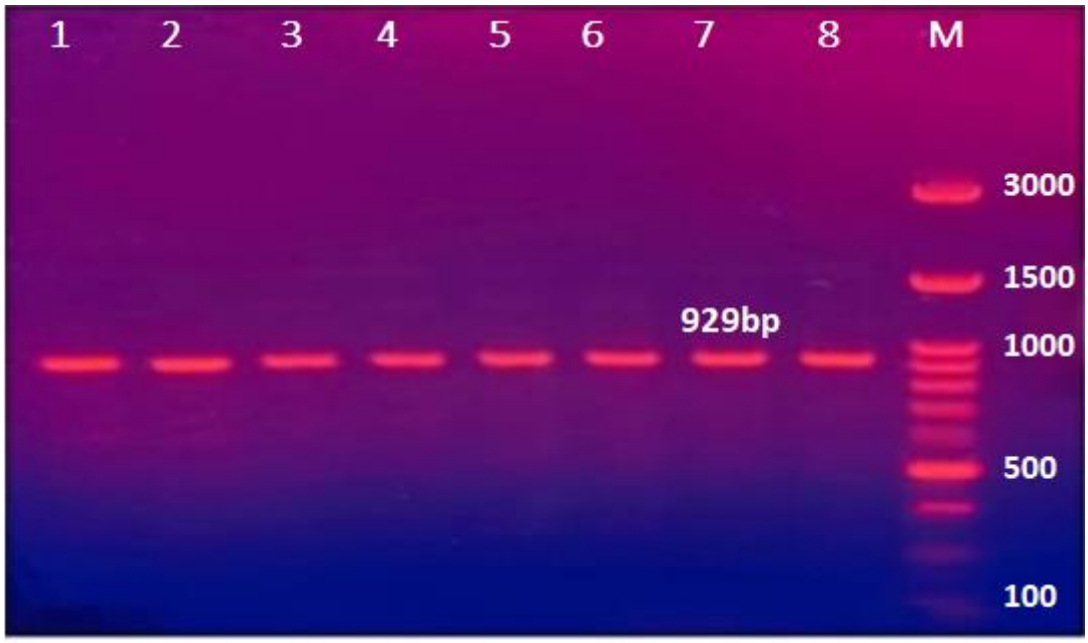

Figure 4: Multiplex PCR results in1.5% Agarose gel electrophoresis for detecting the colistin resistance (mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5) genes in *E.coli* isolated from human samples. M: Molecular weight marker (100bp-3000bp), Lanes 1-8: Positive results of mcr-3 gene (929bp).

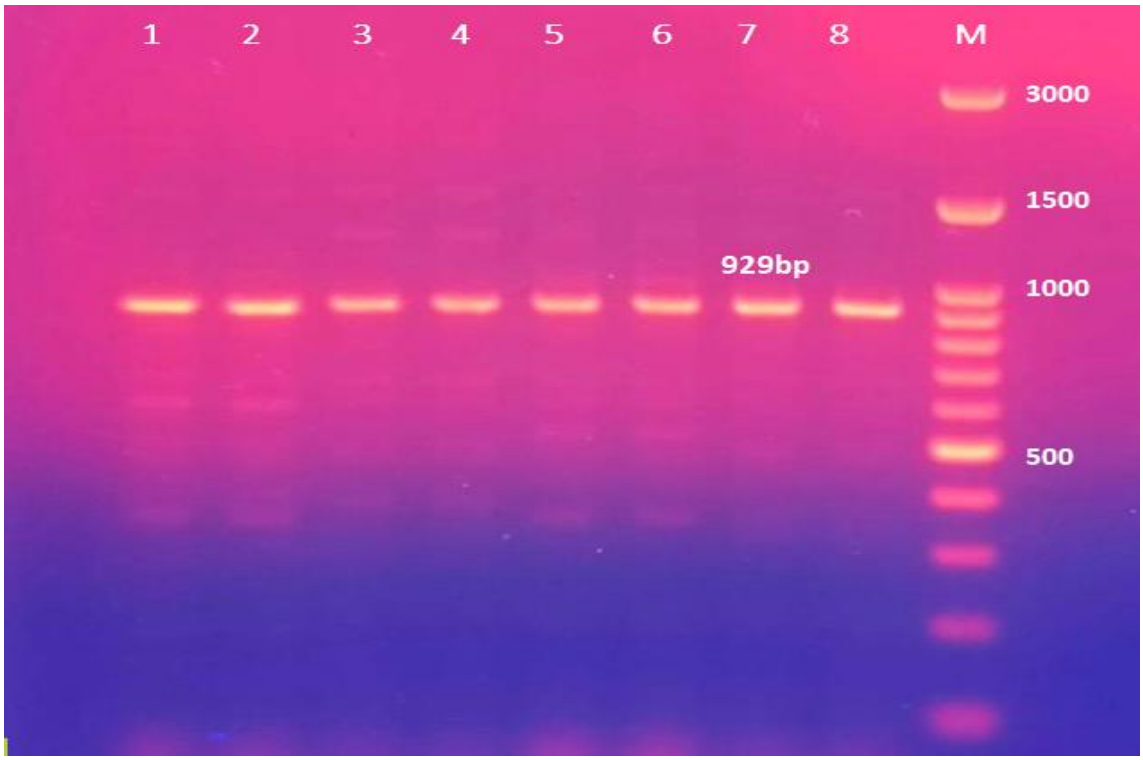

Figure 5: Multiplex PCR results in1.5% Agarose gel electrophoresis for detecting the colistin resistance (mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5) genes in *E.coli* isolated from environment samples. M: Molecular weight marker (100bp-3000bp), Lanes 1-8: Positive results of mcr-3 gene (929bp).

Table 6: The percentage of class 1 integron (*int1*) gene for colistin resistance *E .coli.*

Gene	Isolates source	+ve No (%)	Total
class 1 integron gene (*intI*1)	Cattle	25 (100%)	48(100%)
	Human	12 (100%)	
	Environment	11 (100%)	

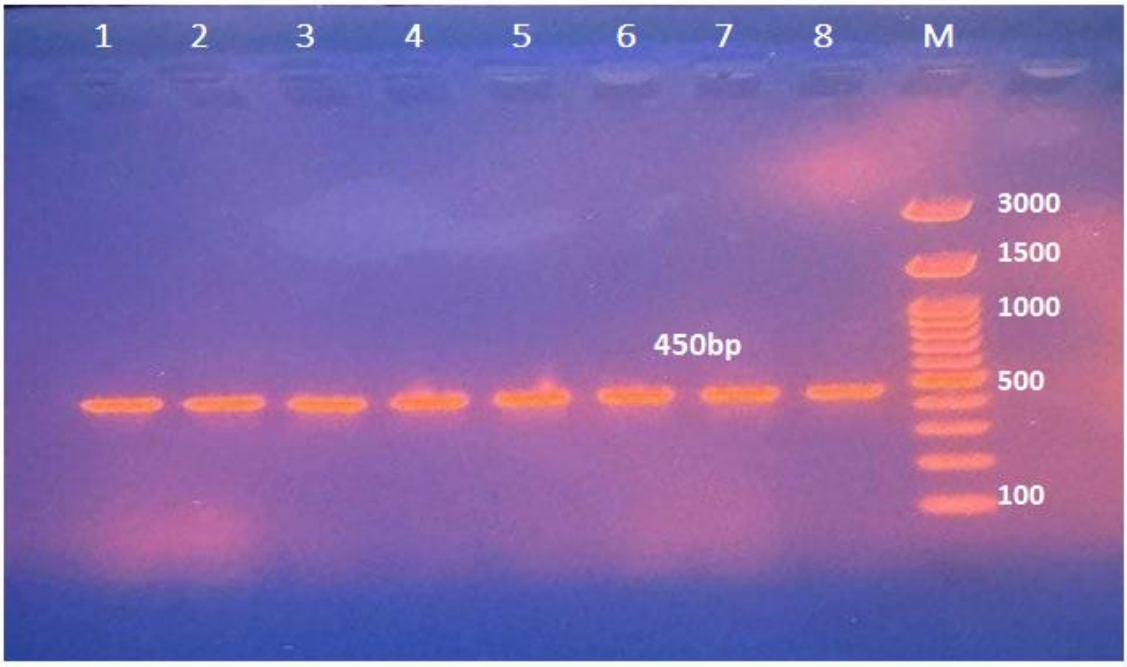

Figure 6: Conventional PCR results of *IntI* 1 gene (450 bp) on 2% agarose gel electrophoresis which detected in *E.coli* isolated from cattle. M: molecular marker (100bp), Lanes 1-11: positive samples.

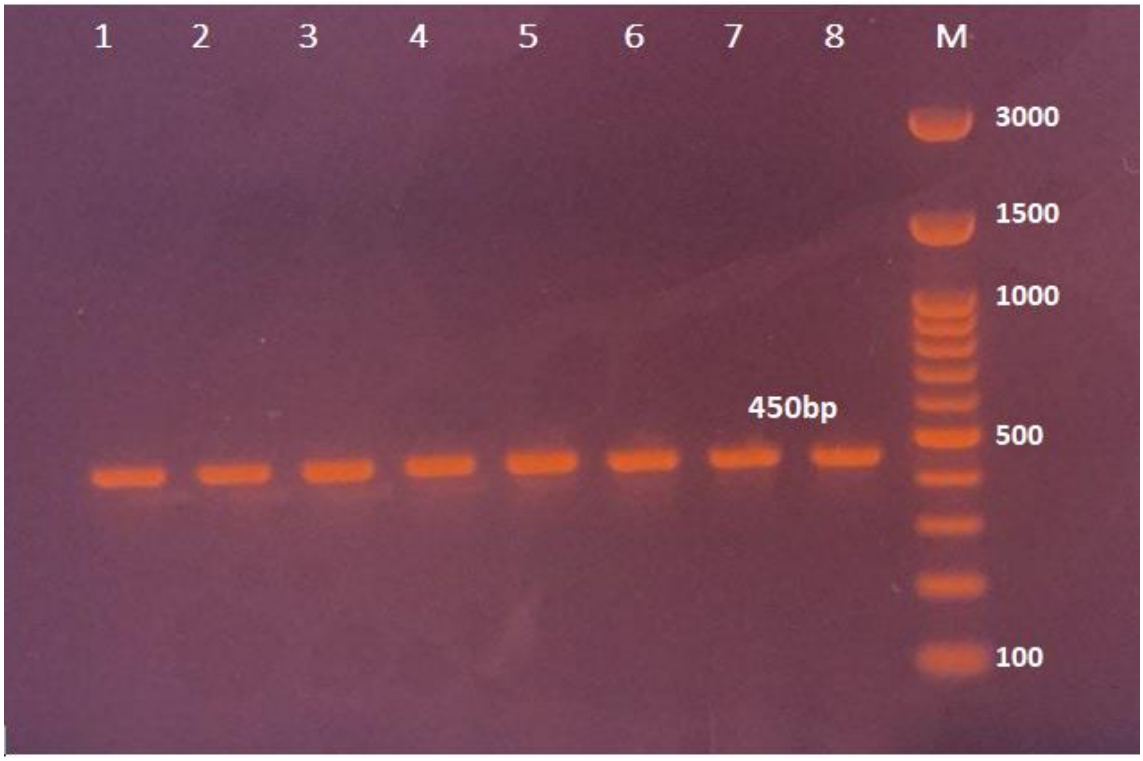

Figure 7: Conventional PCR results of *IntI* 1 gene (450 bp) on 2% agarose gel electrophoresis which detected in *E.coli* isolated from human. M: molecular marker (100bp), Lanes 1-11: positive samples.

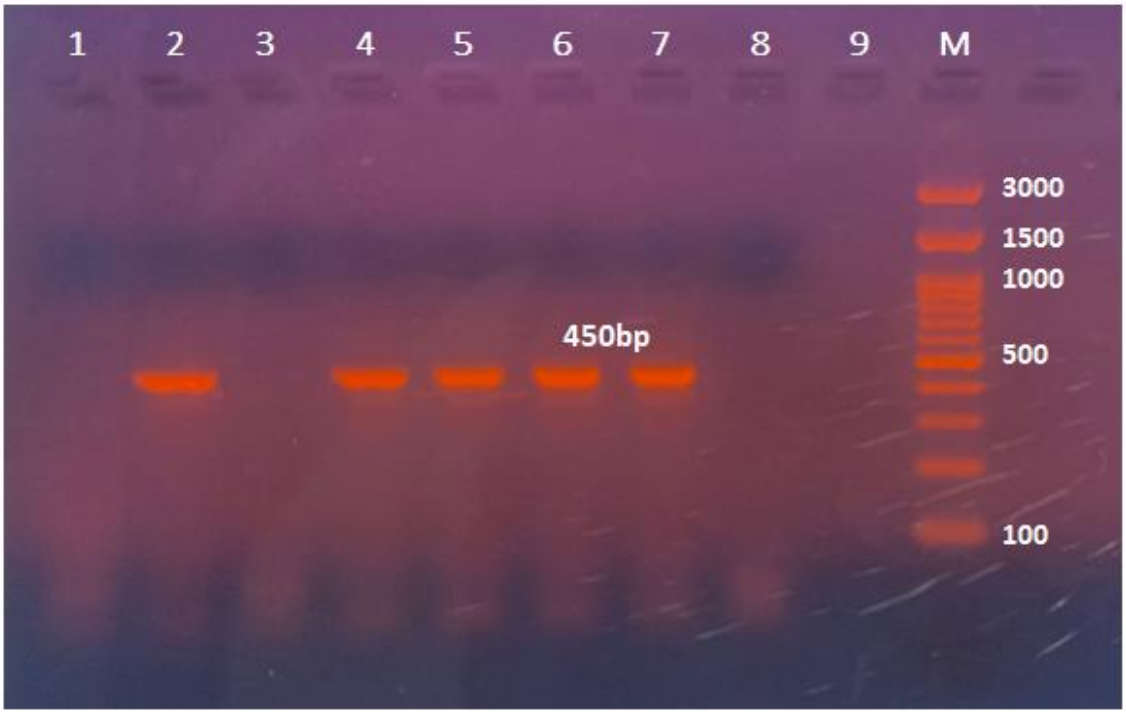

Figure 8: Conventional PCR results of *IntI* 1 gene (450 bp) on 2% agarose gel electrophoresis which detected in *E.coli* isolated from environment. M: molecular marker (100bp), Lanes 2 & 4-7: positive samples. Lanes 1,3,8&9: negative samples

7. DISCUSSION

E.coli is primarily transmitted to humans from animals via contaminated food sources (Barlaam et al.,2019). *E.coli* originating from zoonotic sources can lead to severe foodborne illnesses or gastrointestinal infections (Lazarus et al., 2015; Falgenhauer et al., 2019), The colonization of human flora may lead to urinary tract infections or septicemia. Numerous animal-derived foods have been associated with these illnesses, raising significant concerns. The application of antimicrobial therapy in humans could mitigate the severity of these diseases (Falgenhauer et al.,2019; World Organisation for Animal Health, 2019) and animals (World Organisation for Animal Health, 2019; Clifford et al.,2018). The rise of antimicrobial-resistant Enterobacteriaceae has increasingly become a significant public health concern in various regions across the globe (Moyo *et al.*,2007; Ahmed *et al.*,2019; Islam *et al.,* 2016). Antimicrobial agents that are frequently approved for the treatment of bacterial infections exhibit a weakened effectiveness (Wasyl et al.,2015; Iwamoto et al.,2017) leading to treatment failure (Tribble, 2017). In the present study, A total of two hundred and fifty samples, one hundred from animals and human's diarrhea and fifty from environment are collected. All samples that identified based on morphological, biochemical, and molecular techniques to identify colistin-resistant *E. coli* isolates and to detect mcr

colistin resistance genes. and class 1 integronintegrase gene (*int1*). The results showed that (80%) cattle samples , (40%) human samples and (60%) environment samples gave positive results, these results are consistent with the results of (Mequanint *et. al* 2022). Among the total samples analyzed, suspected isolates of *E.coli* were detected in 74 (38.3%) of the samples based on their morphological characteristics. This included 54 (37.8%) samples from children experiencing diarrhea and 20 (40%) samples from calves with diarrhea. The Phenotypic characterization of colistin-resistant E.*coli* results were showed that (37.5%), (30%) and (36.6%) of cattle, human and environmental samples were colistin resistant respectively, The elevated rates of resistance observed are likely attributable to the extensive application or inappropriate use of these antibiotics in veterinary practices. This has led to the proliferation of antimicrobial-resistant bacteria, which pose a risk of infection to humans and play a role in the emergence of acquired infectious diseases (Rahaman *et al.*,2014, Niaz, 2016). In the present study multiplex PCR was used to identify the colistin-resistance genes mcr(1-5) in all 53 isolates of *E.coli* that phenotypically colistin resistant. According to our results 83.33% of the isolates were from cows, and 100% from human and environment were found to have *mcr-3*. Additionally, none of these isolates contained mcr-1, mcr-2, mcr-4, or mcr-5. In their investigation, Luong et al. (2022) found that 28(75.7%) isolates, had the mcr gene. The mcr-1 and mcr-3 genes were found in these 28 mcr-positive *E.coli*, but the mcr-2 to mcr-10 genes were not. Additionally the results of current study were In accordance with a previous study conducted by García et al. (2018), it was observed that three mcr-positive isolates exhibited sensitivity to colistin, whereas six out of 143 colistin-resistant isolates did not possess mcr genes. The inactivity of mcr may be attributed to the insertion of (1.7Kb IS1294b) element into mcr-18. It is noteworthy that there was one isolate that was mcr-negative yet resistant to colistin. An alternative mechanism for colistin resistance may exist that does not rely on the mcr gene. This potential mechanism warrants further investigation. Furthermore, the current study identified the presence of the class 1 integron gene in colistin-resistant isolates that also harbored the mcr gene. In which 25 (100%) samples from cattle, 12(100%) samples from human and 11(100%) samples from the environment were found to have the *intI*1 gene. This result was in line with the result of (Halaji et al., 2020) who found a significant variables that contribute to *E.coli* and development of antibiotic resistance, especially in connection with colistin resistance. Moreover, the study shows that these integrons aid in the acquisition and spread of resistance genes, resulting in strains that are multidrug-resistant (MDR). Furthermore, the connection between the colistin resistance mcr genes and class1 integrons was hardly studied by (Habibi et al., 2018; Al-Hammadi et al., 2020),in both studies the occurrence of class 1 integrons were found to be highly prevalent in MDR *E.coli* in clinical conditions. There were also notable correlations between integron presence and

resistance to several antibiotics, indicating a possible connection to colistin resistance as well.

7. CONCLUSION

In concluded, The appearance of mcr-3 and its coexistence with other integron genes, such as integron belonged to class 1 (intI1) gene, in isolates of *E.coli* which collected from diarrheal cases and even from environmental samples in Basrah province represent a concern not only to the animals trade but also to public health.

CONFLICT OF INTEREST

The authors state declare that the research was no conflicting of interest

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