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Detection of Antimicrobial Resistance by Phenotypic and Genotypic Methods in *E. coli* Isolated from Diarrhea of Cattle Calves in Mhow, Indore

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ABSTRACT

Background: Neonatal calf diarrhoea, a complex illness, continues to be the leading cause of neonatal calf deaths. The complex pathophysiology of infectious calf diarrhoea results in severe losses to the dairy sector, either directly *via* mortality or indirectly through the costs of prophylaxis and slowed growth in infected calves. Neonatal calf diarrhoea during the first month of life causes between 80 to 85 per cent of all deaths in India.

Methods: A total 137 cattle calve diarrhea samples were collected from calves that show clinical signs of diarrhea. Swabs of the rectum were taken directly from diarrhoeic calves and collected in sterile test tubes. They were then immediately transported to the laboratory for the isolation of *E. coli.*

Result: A total 64 *E. coli* isolates were detected and identified from samples *via* biochemical and molecular assays employing PCR targeting the *E. coli* specific 16s rRNA gene. The result of antibiotic sensitivity of 64 isolated strains of *E. coli* to 15 antimicrobial drugs shows highest sensitivity towards antibiotic gentamycin (68.75%) followed by sulfadiazine and co-trimoxazole (39.06), ciprofloxacin (32.81%) and highest resistance against ampicillin (87.5%) followed by ceftriaxone (82.813%), ceftazidime plus clav (81.25%). There is the variation in the expression of the AMR genes in the isolated *E. coli* strains. The detection rate of AMR gene *blaTEM* was highest 90% and that of *aadA* was the lowest (0%). The other AMR genes *blaCTX-M, blaSHV, sul1* and *tetA* showing 50%, 20%, 50% and 30%.

Key words: Antibiotic resistance, Calve diarrhea, *E. coli.*

INTRODUCTION

Around the world, newborn calves are a significant source of animal production for either meat or breeding. In various nations, diarrhoea is one of the extremely prevalent disease syndromes in newborn calves and this can have detrimental effects on both the economy and animal welfare (Africa Union, 2008). Despite decades of research, neonatal calf diarrhoea, a complex illness, continues to be the leading cause of neonatal calf deaths (Uhde *et al*., 2008). The complex pathophysiology of infectious calf diarrhoea results in severe losses to the dairy sector, either directly *via* mortality or indirectly through the costs of prophylaxis and slowed growth in infected calves (Radostits, 2007). The non-infectious causes (predisposing variables) (flaws or gaps in management-inadequate nourishment, exposure to a harsh environment, neglect of the newborn calf, or a combination of these) and infectious causes interact in a variety of ways (Blanchard, 2012; Cho and Yoon, 2014). Neonatal calf diarrhoea during the first month of life causes between 80-85 per cent of all deaths in India (Singh *et al*., 2009). According to several studies, *E. coli* is the main cause of diarrhoea, hemorrhagic colitis and dysentery in weak, undernourished, unhealthy and immunosuppressed calves (Ellaithi, 2004 and Mohamed *et al*., 2007).

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MATERIALS AND METHODS

Study area

This study was conducted from July 2020 to the end of June 2021. Neonatal cattle calves of different ages (1 day-4 months old) examined clinically for diarrhea on dairy farms in and around Mhow, Indore, Madhya Pradesh, India. On the old Mumbai-Agra Street, 23 kilometers south of Indore city, Mhow is toward Mumbai. In 2003, the Madhya Pradesh government changed the town's name to Dr. Ambedkar Nagar, Mhow, Madhya Pradesh, India, has a latitude of 22.552437 degrees and a longitude of 75.756531 degrees. In Mhow, the average annual temperature is 25.1°C. The rainfall here is around 1126 mm per year. This region is home to a large number of organized and unorganized dairy farms that provide the town and its neighbours' residents with milk and milk-related products. Depending on the scale of production, these dairy farms either have local or crossbred animals.

Study population

Animals included in this investigation were cattle calves under 4 months of age with clinical diarrhoea, showing symptoms of systemic sickness (such as impaired suckling reflex, poor appetite, fever and dehydration) and pasty-watery feces. A total 137 diarrhea samples were collected from calves that show clinical signs of diarrhea. Swabs of the rectum were taken directly from diarrhoeic calves and stored in sterile test tubes and transported to the laboratory of the department of Veterinary Microbiology in the college of Veterinary Science and Animal Husbandry in Mhow for processing.

Isolation and identification of *E. coli*

The sample was inoculated in BHI broth and incubated aerobically at 37°C for 24 hours, followed by streaking on MacConkey agar plate and EMB for selective isolation of *E. coli*. The plates were incubated aerobically at 37°C for 24 hours. Bacteria producing pink colony on MacConkey agar and characteristic purple-black colonies with dark centre with metallic sheen on EMB agar suggestive of *E. coli* (Fig 1) and (Fig 2). Following the guidelines outlined in Quinn *et al.* (2002), conventional bacteriological methods were used to identify probable *E. coli* colonies. As a result, IMViC assays, such as indole, methyl red, Voges Proskauer and citrate utilization, were used to preliminary characterize *E. coli* isolates.

DNA extraction

The isolates were inoculated in Brain heart infusion broth (Hi Media, Mumbai) and incubating at 37°C for 12-18 hours. Extraction of DNA was done using readymade kit (GenElute™ Bacterial Genomic DNA Kit, Sigma) as per the manufacturer's instructions. The extracted DNA was stored at -20°C till further use.

Molecular identification of *E. coli* isolates using polymerase chain reaction (PCR)

16S rRNA is a highly conserved gene that was chosen for the molecular detection for *E. coli* isolates according to Sobur *et al*. (2019) (Table 1). DNA amplification was accomplished in a total 25 µL mixture volume, including 2 µL of DNA template, 12.5 µL of Master mix (SIGMA life science), 1 µL of 16S rRNA forward and reverse primers (20 pmol/µL) and 8.5 µL PCR-grade nuclease free water (SIGMA life science). Samples were subjected to initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s) and elongation (72°C, 2 min). These cycles were followed by a final elongation step (7 min at 72°C).

Antimicrobial susceptibility tests

Isolates were screened for resistance to antibiotics by a disk diffusion method. Bacteria were suspended in saline to the density of a McFarland No. 2 standard, diluted 1:20 and streaked by the method of Bauer *et al*. (1966) on Mueller-Hinton agar and incubated for 24 hours at 37°C. Characterization of strains as sensitive, intermediate or resistant was based on the manufacturer's instructions (Hi-media). Fifteen antibiotics like Ampicillin (AMS) 10 µg, Cefotaxime (CTX) 30 µg, Enrofloxacin (Ex) 10 µg, Gentamycin (G) 10 µg, Azethromycin (AZM) 15 µg, Ceftrixone (Ci) 10 µg, Ciprofloxacin (CIP) 5 µg, Colistin (CL) 10 µg, Co-trimoxazole (COT) 25 µg, Ceftazidime plus Clav (CAC) 30 µg, Aztreonam (AT) 30 µg, Nitrofurantoin (NF) 100 µg, Streptomycin (S) 10 µg, Sulphadiazine (SZ) 100 µg, Tetracycline (TE) 10 µg were used in this investigation.

Identification of antibiotic resistance genes

All *E. coli* strains were analysed for six antibiotic resistance genes *blaTEM* (betalactams), *blaCTX-M* (Cephalosporin), *blaSHV* (betalactams), *tetA* (tetracycline), *sul1* (sulfonamides) and *aadA* (streptomycin) by PCR. PCR were used to investigate the target genes of the *E. coli* isolates. Lists of primers used and the amplified products are presented in Table 1. All reactions were carried out on a PCR thermocycler (Applied Biosystems). Amplification of DNA was conducted in a total volume of 25 µl, including 2 µL of DNA template, 12.5 µL of Master mix (SIGMA life science), 1 µL of forward and reverse primers (20 pmol/µL) each and 8.5 µL PCR-grade nuclease free water (SIGMA life science). The PCR were carried out as per specific cyclic conditions with different genes as per the Table 2. The amplified products were separated by electrophoresis through 1.5% agarose (wt/vol), stained with 0.5 µg/mL ethidium bromide, visualized under UV illumination, imaged with a Gel Doc 1000 fluorescent imaging system (Uvitech).

RESULTS AND DISCUSSION

When diseased calves were examined clinically, it was discovered that they had varying degrees of profuse watery diarrhoea as well as varying degrees of dehydration, which could be seen in their decreased skin elasticity, sunken eyes and inability to stand. The *E. coli* strains were diverse and varied, with several different virulence factors. The presence

of a large molecular weight plasmid harbouring several genes involved in disease was the cause of the heterogeneity. This plasmid contributes to the pathotypes heterogeneity by facilitating the acquisition or deletion of virulence genes. Variation in resistance and sensitivity per cent has been found in various reports. Following a bacterial analysis, A total 64 *E. coli* isolates were detected and identified from 137 diarrheic samples via biochemical and molecular assays employing PCR targeting the *E. coli* specific 16s rRNA gene (Plate 1). Walid *et al.* (2020) and Mousa and Abo Shama (2021) also used 16S rRNA to confirm the *E. coli* genotypically. Over all, *Escherichia coli* (*E. coli)* appeared to be an important causative agent either alone or in combination with other bacteria in the etiology of calf diarrhea and were isolated with the highest frequency from diarrhoeic calves in our study. In accordance with our findings, numerous researchers have also reported a significant prevalence of *E. coli* strains in cases of infectious diarrhea in calves. (Nguyen *et al*., 2011 and Shahrani *et al*., 2014).

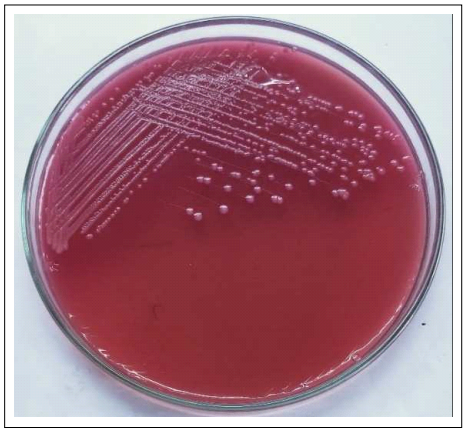


Fig 1: Pink colonies of *E. coli* on MacConkey agar.

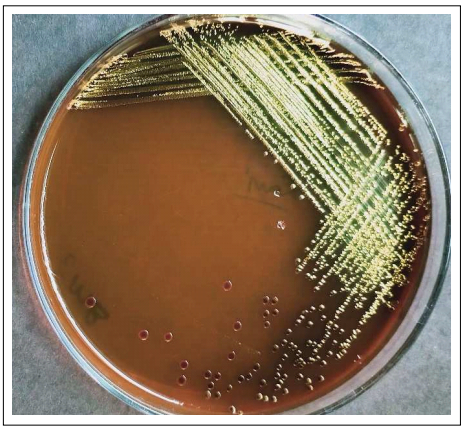


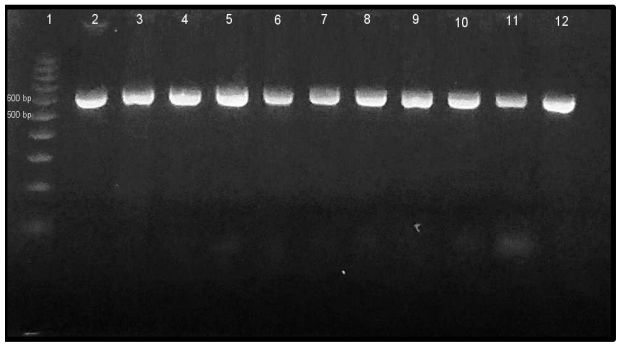
Fig 2: *E. coli* on EMB agar showing metallic sheen.

The results of Antibiotic sensitivity test of *E. coli* isolates showed variable percentage of sensitivity and resistance to the different antibiotics Fig 3. The result of antibiotic sensitivity of 64 isolated strains of *E. coli* to 15 antimicrobial drugs are presented in Fig 4. The highest sensitivity was attributed towards antibiotic gentamycin (68.75%) followed by sulfadiazine and co-trimoxazole (39.06), ciprofloxacin (32.81%), Azithromycin (31.25%), Tetracycline (25%), Nitrofurantoin (21.87%), Aztreonam and streptomycin (20.31), cefotaxime (12.5%), Ceftizidime plus clav (7.81%), ceftriaxone (6.25%), enrofloxacin (4.687%), Ampicillin (3.125%) and colistin (0%). In present study gentamycin showed the highest sensitivity**.** In accordance with our findings, numerous scientists have also reported a sensitivity to gentamycin The Hemashenpagam *et al.* (2008) recorded 75% and Kirkan *et al.* (2018) recorded 89% which is very close to our percent. Whereas lower sensitivity *viz*. 62.5%, 60.2% was recorded by Abdeen *et al.* (2019) and Cengiz and Adiguzel (2020), respectively.

The highest resistance was recorded against ampicillin (87.5%), ceftriaxone (82.813%), ceftazidime plus clav

Table 1: Details of primers used for PCR reactions.

Target gene	Primer sequence	Product size (bp)	Reference
16S rRNA	F: GACCTCGGTTTAGTTCACAGA	585	Sobur *et al*., 2019
	R: CACACGCTGACGCTGACCA		
*bla*CTX-M	F: GTGCAGTACCAGTAAAGTTATGG	538	Adesoji *et al*., 2015
	R: CGCAATATCATTGGTGGTGCC		
*bla*SHV	F: TCGGGCCGCGTAGGCATGAT	628	Gundran *et al*., 2020
	R: AGCAGGGCGACAATCCCGCG		
*bla*TEM	F: TTGGGTGCACGAGTGGGTTA	506	Gundran *et al*., 2020
	R: TAATTGTTGCCGGGAAGCTA		
*Sul*1	F: TTCGGCATTCTGAATCTCAC	822	Shehata *et al*., 2016
	R: ATGATCTAACCCTCGGTCTC		
*tet*A	F: GTCAAACCCAACATACCCC	887	Van *et al*., 2008
	R: GAAGGCAAGCAGGATGTAG		
aadA	F: TGATTTGCTGGTTACGGTGAC	284	Van *et al*., 2008
	R: CCGTACGCATACTGGCTTTGC		



Lane 1: 100 bp ladder.
Lane 2: Positive control.
Lane 3-12: Positive sample.

Plate 1: Results of PCR Showing 16 S rRNA gene of *E.coli*.

(81.25%), colistin (76.56%), enrofloxacin (75%), Azethromycin (68.75%), cefotaxime (67.18%), tetracycline (62.5%), aztreonam (57.81%), sulphadiazine (48.438%), Nitrofurantoin (46.875%), co-trimoxazole (43.75%), streptomycin (37.5%), ciprofloxacin (29.688%) and gentamycin (12.5%).

E. coli showed variable percent of resistance against Ampicillin by many researchers viz. Hemashenpagam *et al.* (2008) (66.66%), Abdeen *et al.* (2019) (81.25%), Manjushree *et al.* (2019) (84%), Adeladlew *et al.* (2020) (100%) and Sobhy *et al.* (2020) (54.5%). In contrast of present finding Kadam (2018) (60%), Sumedha *et al.* (2019) (66%), Srivani *et al.* (2020) (100%), Algammal *et al.* (2020) (78.5%) and Merera *et al.* (2020) (73.85%) reported higher sensitivity against Ampicillin. To compare the phenotypic resistance the isolates were subjected to detection of six different

Table 2: Thermocyclic conditions for different AMR genes.

Step	*blaTEM*	*blaCTX-M*	*blaSHV*	*sul1*	*tetA*	*aadA*
Initial denaturation	95°C	95°C	95°C	94°C	94°C	95°C
Denaturation	94°C	96°C	94°C	94°C	94°C	94°C
Annealing	60°C	52°C	64°C	54°C	60°C	58°C
Extension	72°C	72°C	72°C	72°C	72°C	72°C
Final extension	72°C	72°C	72°C	72°C	72°C	72°C

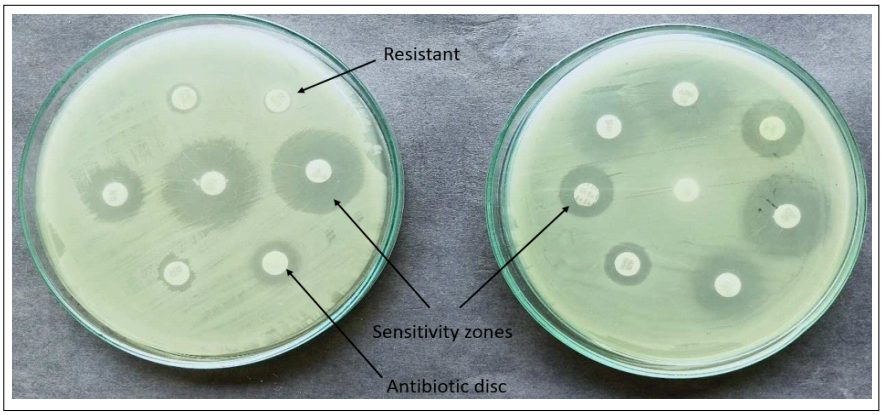


Fig 3: Results of antibiotic sensitivity test.

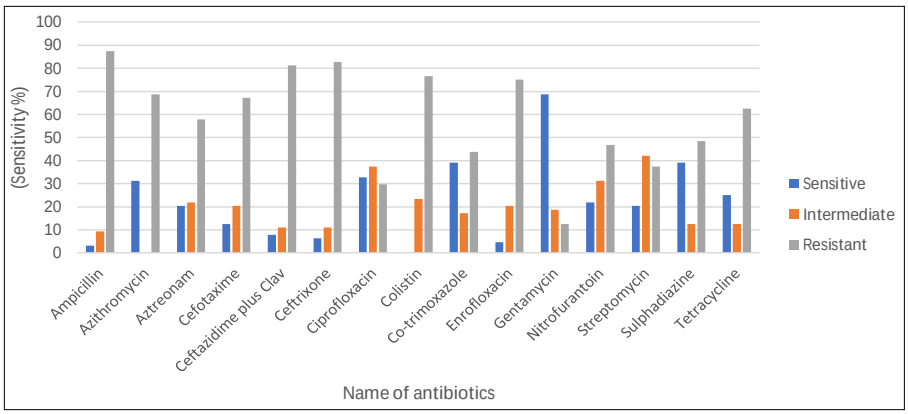
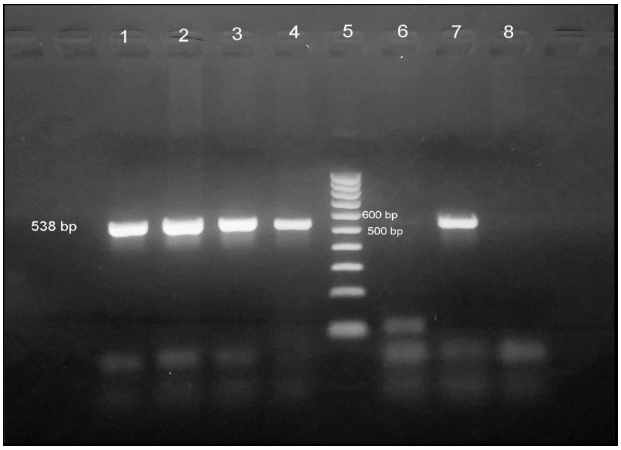
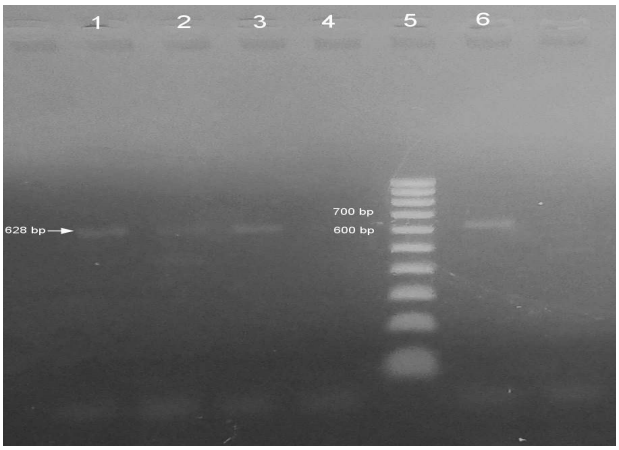


Fig 4: Sensitivity of *E. coli* isolates to different antimicrobial agents.



Lane 5: 100 bp ladder.
Lane 4: Positive control.
Lane 8: Negative control.
Lane 1,2,3 and 7: Positive samples (Size 538 bp).
Lane 6: Negative sample.

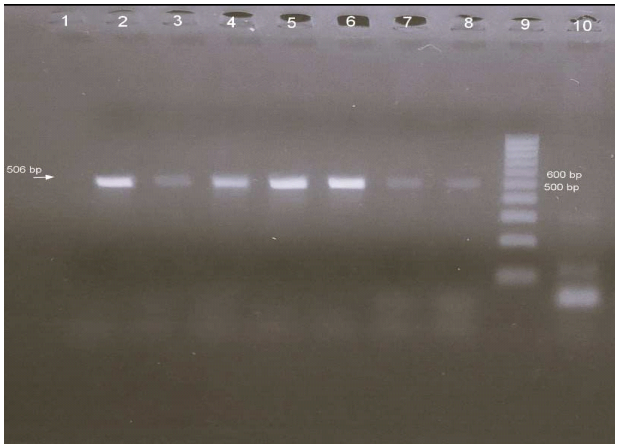
Plate 2: Results of PCR showing *bla CTX-M* gene of *E.coli.*



Lane 5: 100 bp ladder.
Lane 6: Positive control.
Lane 7: Negative control.
Lane 4: Negative sample.
Lane 1,2 and 3: Positive samples (Size 628 bp).

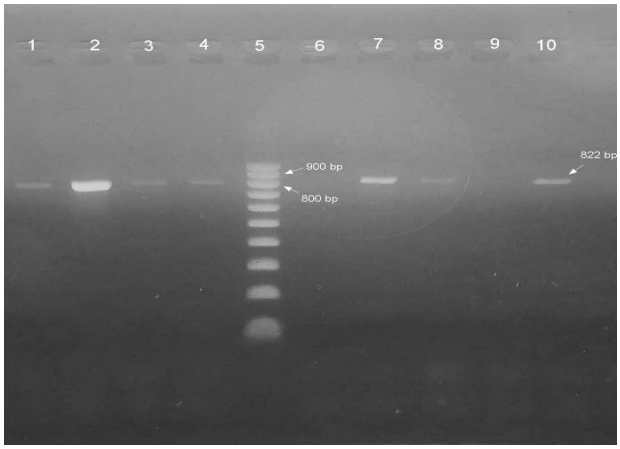
Plate 3: Results of PCR sowing *blaSHV* gene of *E.coli.*

antibiotic resistance gene belonging to 4 different antibiotic groups presented in Table 2. There is the variation in the expression of the AMR genes in the *E. coli.* The detection rate of AMR gene *blaTEM* was highest 90% and that of *aadA* was the lowest (0%). The other AMR genes *blaCTX-M, blaSHV, sul1*and *tetA* showing 50%, 20%, 50% and 30% (Plate 2-6). Similarly, many scientists studied the AMR gens in isolated *E. coli.* Jiang and Zhang (2013), El Bably *et al.* (2016) and Abdeen *et al.* (2019) detected *blaTEM* gene in 84.6%, 80% and 100%, isolates respectively, which is in close agreement with the present research and, however lower percent of *blaTEM* gene was detected by Algammal *et al.* (2020) and Khawaskar *et al.* (2022) reported 21.5% and 8.3% respectively. El Bably *et al.* (2016) and Yue *et al.* (2021) reported the *blaSHV* gene in 33.3% and 18.5%, respectively which is close to the present detection level. Abdeen *et al.* (2019), Khawaskar *et al.* (2022) reported lower per cent of *blaCTX-M* 31.25% and 28.3%, respectively. However, Esmaeel and Naseer (2017) reported gene in 80%, higher as compared to the present findings. Abdulgayeid *et al.* (2015) and El Bably *et al.* (2016) reported 60% and 50% isolates to be positive for *sul1* gene, respectively which is



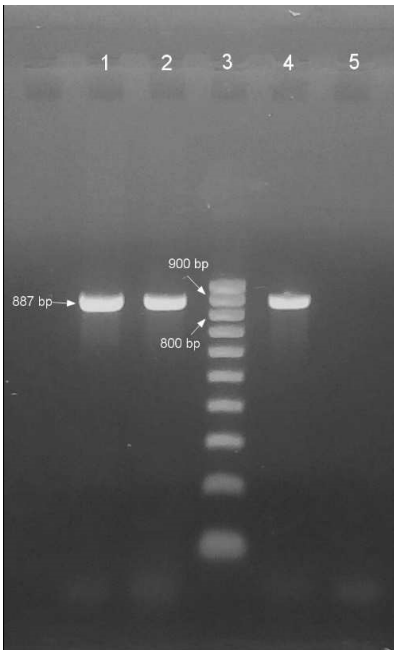
Lane 9: 100 bp ladder.
Lane 2: Positive control.
Lane 1: Negative control.
Lane 3-8: Positive sample (Size 506 bp).
Lane 10: Negative sample.

Plate 4: Results of PCR showing *blaTEM* gene of *E.coli.*



Lane 5: 100 bp ladder.
Lane 2: Positive control.
Lane 6: Negative control.
Lane 1,3,4,7,8 and 10: Positive sample (Size 822 bp).
Lane 9: Negative sample.

Plate 5: Results of PCR showing *Sul*1 gene of *E.coli.*



Lane 3: 100 bp ladder.
Lane 4: Positive control.
Lane 5: Negative control.
Lane 1-2: Positive sample (Size 887 bp).

Plate 6: Results of PCR Showing *tetA* gene of *E.coli.*

very similar to the present findings but higher percent of *sul1* gene 74%, 81.25% and 100%, was recorded by Jiang and Zhang (2013), Abdeen *et al.* (2019) and El sayed *et al.* (2020), respectively. In contrast to present finding very higher percentage was *tetA* gene reported by Abdeen *et al.* (2019), Abdulgayeid *et al.* (2015), El Bably *et al.* (2016), Yue *et al.* (2021) and El sayed *et al.* (2020) reported 93.75%, 100%, 50%, 96.3% and 100%, respectively. In contrast to findings of present study Jiang and Zhang (2013) and Yue *et al.* (2021) reported 73.3% and 70.4% *aadA* gene in isolated *E. coli*. A great variation in gene detection percentage in *E. coli* was reported by different scientists. These differences might be partly explained by differences in geographic location, the complexity of the healthcare institutions involved, the exposure to healthcare environments, the usage of antibiotics and antibiotic stewardship practices.

CONCLUSION

The intensity and incidence of the disease are regulated by several variables that interact dynamically over time since diarrhoea is a complicated condition that includes various bacteria, among these members of Enterobacteriaceae family specially *E. coli* and *Salmonella* are major pathogens. Antimicrobial resistance in bacteria is frequently linked to various resistant genes, transmission mechanisms and reservoirs of antibiotic resistance among various species. In present study high level of antibiotic resistance is reported which is one of the major issue globally.

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Conflict of interest

The authors declare that there is no conflict of interest.

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