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Plasmid Profile of Antibiotics Heteroresistant *Escherichia coli* Isolates from Diarrhoeic Children Attending Ahmadu Bello University Teaching Hospital, Shika, Zaria, Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author AS designed the study, wrote the protocol, managed the experimental processes and wrote the first draft of the study. Author PMD carried out the plasmid extraction of the heteroresistant E. coli isolates. Authors JAO, YKEI and HWI supervised all the experimental processes and contributed to the first draft of the study. All authors read and approved the final manuscript.

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ABSTRACT

In this study, 100 *Escherichia coli* (*E. coli*) isolates were obtained from the stool of 222 diarrhoeic children attending Ahmadu Bello University Teaching Hospital, Shika, Zaria. Out of the 100 isolates of *E. coli* obtained, five (5) were found to be heteroresistant to different antibiotics. The isolation and biochemical identification were performed using standard microbiological procedures. The antimicrobial susceptibility testing and the determination of the heteroresistance were performed using modified Bauer-Kirby sensitivity testing technique. The isolation of plasmid DNA was performed using the protocol described in the plasmid extraction kit (Thermo Scientific, UK).

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Multiplex PCR assay was performed to identify the different diarrhoeagenic groups of the antibiotics heteroresistant *E. coli* isolates. The results of antimicrobial susceptibility testing shows that 5 (100%) of the isolates are resistant to amoxicillin, 4 (80%) to trimethoprim/sulfamethoxazole, 2 (40%) to ciprofloxacin and chloramphenicol, 1(20%) to gentamicin and none was resistant to amoxicillin-clavulanic acid and ceftriaxone. Multiple antibiotic resistance (MAR) indexing of the E. coli isolates in this study showed that 80% have MAR index above 0.2, at such a MAR index, there is a significant level of misuse of these antibiotics within a particular environment. Among the five (5) heteroresistant *E. coli* isolates, 2 (40%) were found to be β-lactamase positive while 3 (60%) were found to be β-lactamase negative. The plasmid analysis showed that, four (4) of the five (5) E. coli isolates were found to harbor plasmids. However, there was no correlation between β-lactamase production and possession of plasmid by the antibiotics heteroresistant *E. coli* isolates. Following the Multiplex PCR assay, none of the antibiotics heteroresistant *E. coli* isolates harbor the virulence gene for any of the diarrhoeagenic *E. coli*.

Keywords: β-lactamase; heteroresistance; multiple antibiotic resistance index; plasmid.

1. INTRODUCTION

Heteroresistance is defined as resistance to certain antibiotics expressed by a subset of a microbial population that is generally considered to be susceptible to these antibiotics according to traditional *in vitro* susceptibility testing [1]. Heteroresistance is observed in cases of treatment failures [2] and also in a wide range of microbes [3] including *Staphylococcus aureus*, coagulase-negative staphylococci [4], *Acinetobacter baumannii* [5], *Mycobacterium tuberculosis* [6], *Streptococcus pneumoniae* [3], *Enterococcus faecium* [7], and *Cryptococcus neoformans* [8]. The frequency of heteroresistance, although this differs among species, is about one sub-clone in every 10^5–10^6 colonies, which roughly equals the normal rate of mutation [1]. Heteroresistance could be a tool for natural evolution to drug resistance, since it provides bacteria with an opportunity to explore the possibility of growth in the presence of antibiotics before acquisition of resistance by the major proportion of the microbial population [3]. Heteroresistance is considered to be a precursor stage, which may or may not lead to the emergence of a resistant strain. Although this phenomenon has been reproduced many times in the laboratory setting, its clinical significance is largely unknown. Bacterial plasmids serve as the scaffold on which are assembled arrays of antibiotic resistance genes, by transposition and site-specific recombination mechanisms [9]. Plasmids are circular, autonomously self replicating extrachromosomal DNA found in bacteria. Some extended spectrum β-lactamase enzymes such as CTX-M, SHV and TEM have been widely reported to be produced by *E. coli*, can hydrolyze a wide range of antibiotics and are mostly encoded by plasmids [10]. According to Daniel and Fabian [11], plasmids in particular have been implicated in the spread of antibiotic resistance genes. However, the selective pressures which favour plasmid-carried resistance genes have not been fully established. Antibiotic resistance is the ability of a bacterium or other microorganism to survive and reproduce in the presence of antibiotic doses that were previously thought effective against them. Transmission of resistance genes from normally more virulent pathogenic species to nonpathogenic organisms is very common with the animal and human intestinal tract micro flora [12].

2. METHODOLOGY

2.1 Study Population

The samples for this study were collected from Ahmadu Bello University Teaching Hospital, Zaria (including Institute of Child Health, Banzazzau), after ethical clearance (Ref No.: ABUTH/HREC/H02/2013). The samples were collected for a period of six months (November, 2013 to May, 2014) from children (both male and female) with incidents of diarrhoea under the age of five (5) years, after obtaining informed consent from their parents or their guardians/attendants.

2.2 Sample Collection and Treatment

The stool samples were cultured in a sterile nutrient broth within 2 to 4 hours of collection. About one gram of stool sample was suspended in a sterile nutrient broth (Oxoid, England) and incubated at 37ºC for 24 hours. The concentrated isolates from the nutrient broth were then used to inoculate MacConkey agar plate (Becton Dickson Microbiology Systems, USA) by streaking and incubated at 37ºC for 24

hours. Colonies that showed typical characteristics of *E. coli* morphology (pink-red) were subcultured into eosin-methylene blue agar (Micro Master, India), and incubated at 37ºC for 24 hours. The colonies that showed green metallic sheen, were further subjected to biochemical tests for confirmation.

2.3 Biochemical Tests

The biochemical tests (classical biochemical gallery) that were used to identify the *E. coli* include Indole production test, Methyl red test, Voges-Proskauer test and Citrate utilization test. Further biochemical test was carried out to confirm that the organism is *E. coli* using Eijkmann test [13]. This is the fermentation of lactose and gas production at 44.5ºC. The organism was inoculated into a sterile lactose broth (Oxoid, England) containing inverted Durham tube and incubated at 44.5ºC for 24 to 48 hours. The development of turbidity and gas production inside the Durham tube confirmed the presence of *E. coli.*

2.4 Antibacterial Susceptibility Testing

Antibiotic Susceptibility testing was performed by disc diffusion method using modified Bauer-Kirby sensitivity testing techniques [14]. A sterile swab was dipped into a standardized bacterial cell suspension at density of (0.5 McFarland) and used to evenly inoculate the entire surface of sterile Mueller-Hinton agar plate (Micro Master, India), after the agar surface has dried for about five minutes, the appropriate antibiotic discs were placed on it with a pair of sterilized forceps, the plates were allowed to stand at room temperature for 30 minutes before they were placed in a 37ºC incubator for 16-18 hours according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The diameters of inhibition zones were interpreted by referring to the reading table of Enterobacteria as recommended by the CLSI [15]. The antimicrobial agents that were used include trimethoprim/sulfamethoxazole (SXT, 25 µg), gentamicin (CN, 10 µg), ciprofloxacin (CIP, 5 µg), amoxicillin/clavulanic acid (AMC, 30 µg), Chloramphenicol (C, 30 µg), amoxicillin (AML, 10 µg) and ceftriaxone (CRO, 30 µg) (Oxoid, England).

2.5 Determination of Multiple Antibiotic Resistance (MAR) Index

MAR index is the number of antibiotic(s) to which the organism is resistant, divided by the total number of antibiotics tested [16]. The multiple antibiotic resistances (MAR) index was determined for each isolate as shown in the equation below.

MARI =

Number of Antibiotic (s) to which isolate was resistant/Total number of antibiotics tested

2.6 Determination of Resistance Category of the Isolates

The criteria described by Magiorakos et al. [17] were adopted to characterize the different patterns of resistance found among the *E. coli* isolated from diarrhoeic children. According to Magiorakos et al. [17]: multidrug-resistant (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, extensively drug-resistant (XDR) was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories), and pandrug-resistant (PDR) was defined as non-susceptibility to all agents in all antimicrobial categories.

2.7 Test for Detection of β-lactamases

The tube iodometric method as described by Catlin [18] was adopted.

Hydrolysis of penicillin yields penicilloic acid, which reduces iodine, decolourising starch-iodine complex. This reaction can be exploited to detect β-lactamase activity in tubes. Benzylpenicillin, 6 g/l in 0.1 M phosphate buffer pH 6.0, was distributed in 0.1 ml quantities in tubes. Bacterial colonies from agar were suspended in these solutions until they were heavily turbid (10^9 cfu/ml). The suspensions were held at room temperature for 30-60 min, and 20 µl of 1% (w/v) soluble starch in distilled water were added, followed by 20 µl of 2% (w/v) iodine in 53% (w/v) aqueous potassium iodide. β-lactamase activity was indicated by decolourisation of the iodine within 5 minutes [18].

2.8 Plasmid Analysis of the Heteroresistant *E. coli* Isolates

This analysis was carried out at Molecular Diagnostic Laboratory, Veterinary Teaching Hospital, Ahmadu Bello University, Zaria, Nigeria.

2.9 Extraction of Plasmid DNA

The plasmid extraction was carried out according to the protocol described by the manufacturer of the extraction kit (Thermo Scientific, UK). All isolates were inoculated into 5 ml Luria-Bertani liquid medium and incubated for 24 hrs at 37ºC. The culture was centrifuged at 10.000 rpm for 5 minutes, thereafter, the supernatant was discarded leaving the pellet. The pellet was further vortexed with little quantity of supernatant in a Stuart vortex mixer for 15 seconds. The pellets were then resuspended in 250 µl of the resuspension solution (50 mM glucose, 50mM Tris-Hcl, PH 8.0, 10 mM Ethylene diamine tetraacetic acid). The cell suspension was then transferred into a microcentrifuge tube. The bacteria was resuspended completely by vortexing until no cell clumps remain. The content was centrifuged for 2 minutes and the supernatant was then discarded using a pipette.

Thereafter, 250 µl of the lysis solution (20 mM NaOH, 1% Sodium dodecyl sulfate) was then added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. The content was centrifuged for 2 minutes and the supernatant was then discarded using a pippete. Then 350 µl of the neutralization solution (Potassium acetate) was added and shaken vigorously by inverting the tube. It was then centrifuged at 10.000 rpm for 5 minutes to pellet cell debris and chromosomal DNA. The supernatant was then transferred to the supplied GeneJET spin column by pipetting carefully to avoid disturbing or transferring the white precipitate. It was then centrifuged at 10.000 rpm for 1 minute and the flow through discarded. The column was then placed back into the same collection tube. Then 500 µl of wash solution (70% ethanol) was then added to the GeneJET spin column and then centrifuged for 1 minute at 10.000 rpm. The flow through was then discarded and the column placed back into the same collection tube. The wash procedure was repeated using 500 µl of the wash solution. The flow through was then discarded and centrifuged for an additional 1 minute to remove residual wash solution and to avoid residual ethanol in plasmid preparation. The GeneJET spin column was then transferred into a fresh 1.5 ml microcentrifuge tube and 50 µl of elution buffer was added to the center of the GeneJET spin column membrane to elute the plasmid DNA. This was then incubated at room temperature for 2 minutes and then spin at 10.000 rpm for 2 minutes. The pellet was then dried under vacuum for 3 minutes, re-suspended in 20 µl of TE buffer (10 mM Tris-Hcl, PH 8.0, 1 mM EDTA) for electrophoresis.

2.10 Identification of Diarrhoeagenic *E. coli* (DEC)

All isolates were inoculated into 5 ml Luria-Bertani liquid medium and incubated for 24 hrs at 37ºC. DNA extraction was carried out using the ZR Fungal/Bacterial DNA MiniPrep™ ZRD6005 (Zymo research, CA, USA).

A multiplex Polymerase Chain Reaction (PCR) technique was used to differentiate the diarrhoeagenic *Escherichia coli* pathotypes as described by Persson et al. [19]. PCRs were performed in a total reaction volume of 25 µl containing 1× PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM $(NH4)_2SO_4$, pH 8.3), 2.6 mM $MgCl_2$, 260 lM each of dATP, dCTP and dGTP, 520 lM dUTP, 0.15 U of UNG (Applied Biosystems, USA), 1.25 U of Taq polymerase (FastStart; Roche Diagnostics), and the 18 primers (Inqaba biotec, Pretoria, South Africa). These included a primer pair for the 16S rDNA gene as a positive internal PCR control (Table 1). Template volumes were 7 µl when PCRs were performed with extracted DNA from the *E. coli.*

Amplification conditions were comprised of 94ºC for 6 min (initial template denaturation), followed by 35 cycles at 94ºC for 50s (final denaturation), primer annealing at 57ºC for 40s, primer extension at 72ºC for 50s, and finally at 72ºC for 3 minutes (final extension). Amplicons were analysed by electrophoresis on agarose 1.5% w/v under standard conditions.

3. RESULTS

A total of five (5) heteroresistant *E. coli* isolates were obtained from children below five (5) years with diarrhoea attending Ahmadu Bello University Teaching Hospital, Shika-Zaria.

Table 2 shows the Multiple Antibiotic Resistance (MAR) Index, Resistance Categories and β-lactamase production of the Heteroresistant *E. coli* Isolates to eight (7) commonly used antibiotics in the treatment of diarrhoeal diseases in Zaria, Nigeria.

Table 1. Gene targets, primer sequences and Amplicon sizes for the multiplex PCR

Primer	Gene target	Virulence factor/gene	Sequence (5'-)	Pathotype	Amplicon size
StFh	*Human*	STIh	TTTCGCTCAGGATGCTAAACCAG	ETEC	151 bp
StRh	*estA*		CAGGATTACAACACAATTCACAGCAGTA		
StFp	*Porcine*	STIp	CTTTCCCCTCTTTTAGTCAGTCAACTG	ETEC	160 bp
StRp	*estA*		CAGGATTACAACAAAGTTCACAGCAG		
PS1	*eltA*	LTI	AAACCGGCTTTGTCAGATATGATGA	ETEC	479 bp
PS2			TGTGCTCAGATTCTGGGTCTCCT		
PS3	*vtx1*	VT1	GTTTGCAGTTGATGTCAGAGGGA	VTEC	260 bp
PS4			CAACGAATGGCGATTTATCTGC		
PS5	*Vtx2*	VT2	GCCTGTCGCCAGTTATCTGACA	VTEC	420 bp
PS6			GGAATGCAAATCAGTCGTCACTC		
PS7	*eae*	Intimin	GGYCAGCGTTTTTTCCTTCCTG	EPEC	377 bp
PS8			TCGTCACCARAGGAATCGGAG		
PS9	*ipaH*	IpaH	TTGACCGCCTTTCCGATACC	EIEC	647 bp
PS10			ATCCGCATCACCGCTCAGAC		
PS11	*aap*	Antiaggregation protein (dispersin)	CTTTTCTGGCATCTTGGGT	EAEC	232 bp
PS12			GTAACAACCCCTTTGGAAGT		
PS13	*16SrDNA*	16S rDNA	GGAGGCAGCAGTGGGGAATA	CTRL	1062 bp
PS14			TGACGGGCGGTGTGTACAAG		

These primer sequences identifies only five (5) E. coli pathotypes

R = A or G, Y = C or T, CTRL=CONTROL. Adopted from: Sheikh et al. [20], Persson et al. [19]

4. DISCUSSION AND CONCLUSION

In this study, a total of five (5) heteroresistant *E. coli* isolates were obtained from the stool of diarrhoea patients who were children below five (5) years attending Ahmadu Bello University Teaching Hospital, Shika, and Institute of Child Health, Banzazzau, an annex of Ahmadu Bello University Teaching Hospital, Shika-Zaria, Nigeria. The isolation and biochemical identification were performed using standard microbiological procedures. The role of antibiotics in the treatment of childhood diarrhoea is limited because of their limited benefits and potential side effects [21]. Yet, knowledge about antibiotic sensitivity pattern of these strains may be beneficial for hospital treatment in severe and complicated cases [22]. The antimicrobial susceptibility testing was performed using modified Bauer-Kirby disc diffusion technique [14]. Seven (7) commonly used antibiotics in the treatment of diarrhoeal diseases were selected which include trimethoprim / sulfamethoxazole, gentamicin, ciprofloxacin, amoxicillin/clavulanic acid, chloramphenicol, amoxicillin, and ceftriaxone. The results of antimicrobial susceptibility testing shows that 5 (100%) of the isolates were resistant to amoxicillin, 4 (80%) to trimethoprim/sulfamethoxazole, 2 (40%) to ciprofloxacin and chloramphenicol, 1 (20%) to gentamicin. No resistance was observed to amoxicillin-clavulanic acid and ceftriaxone. The high level of resistance of *E. coli* to amoxicillin, trimethoprim / sulfamethoxazole, and chloramphenicol may be due to the fact that these antibiotics have been in use for much longer time and also their oral route of administration is known to affect their rate of absorption into the blood stream [23], their resistance could also be due to the presence of factors such as plasmids [24]. The resistance observed could also be attributed to the low cost and irrational use of antibiotics for conditions that may not clinically indicate their use, over-the-counter sale of antibiotics in Pharmacies without prescription by authorized practitioners. Ceftriaxone, amoxicillin-clavulanic acid and ciprofloxacin are relatively expensive, due to their high cost of procurement, means these antibiotics have not been misused and hence are more effective compared to those that have been in use for quite a long time. It is worthy of note that ceftriaxone is probably less abused than other antibiotics because of its mode of administration (solely by injection) and its high cost of procurement. Gentamicin showed better activity than newer antibiotics with 80% of the *E. coli* isolates showing susceptibility to gentamicin, this may be due to its limited use [25]. The isolates exhibit heteroresistance to different antibiotics that include trimethoprim/ sulfamethoxazole, gentamicin, chloramphenicol, ciprofloxacin and amoxicillin. The genetic basis of heteroresistance remains obscure, although attempts to discover it have been made [26]. Heteroresistance can be a result of heterogeneous expression of antibiotic hydrolase genes and provides a heterogeneous selective advantage [27].

Multiple antibiotic resistances (MAR) indexing of the *E. coli* isolates in this study showed that 80% have MAR index above 0.2 (Table 2). At MARI above 0.2 there is a significant level of misuse of these antibiotics within a particular environment [28,16,29]. This increase bacterial drug resistance worldwide that exacerbate the diminishing number of new antimicrobial drugs in the pharmaceutical pipeline [30,31]; this will affect the worldwide escalation in both community-and hospital-acquired antimicrobial-resistant bacteria and hence, threatening the ability to effectively treat patients. Therefore, there is need to emphasize more on appropriate antimicrobial prescription, prudent infection control and new treatment alternatives [32].

Among the five (5) heteroresistant *E. coli* isolates, 2 (40%) were found to be β-lactamase positive while 3 (60%) were found to be β-lactamase negative (Table 2). These enzymes, most of which are called ESBLs, are divided into four main groups from A to D [33]. ESBL enzymes of CTX-M, TEM and SHV, from group A, have been widely reported to be produced by *E. coli* and are usually encoded by Plasmids [10,34].

Following plasmid analysis, four of the five *E. coli* isolates were found to harbor plasmids. Plasmid bands in Lanes 2, 3, 4, and 6 (Fig. 1) were found to have a molecular weight of 1650.24 bp, 1650.24 bp, 1653.237 bp and 1716.174 bp respectively. Resistance of *E. coli* to antibiotics were mostly plasmid mediated as reported by Cheesbrough [35]. Plasmids usually contain between 5 and 100 genes. Plasmids are not essential for normal bacterial growth and bacteria may lose or gain them without harm. They can, however, provide an advantage under certain environmental conditions. Bacterial plasmids serve as the scaffold on which are assembled arrays of antibiotic resistance genes, by transposition and site-specific recombination mechanisms [9].

The multiplex PCR assay as described by Persson et al. [19] was used in the identification of different diarrhoeagenic *E. coli* in this study. The assay reveals that, the antibiotics heteroresistant isolates did not harbor any diarrhoeagenic *E. coli* virulent gene (Fig. 2). The subjects employed in this study may be infected by other pathogens other than diarrhoeagenic *E. coli* since there are different pathogens that can cause diarrhoea in children, including other bacteria, viruses, and parasites. A typical example was obtained by some researchers in Saudi Arabia, which reported *Shigella* as having the highest incidence among other bacterial pathogens causing diarrhoeal disease in that country [36]. Various factors may account for such a difference. Antimicrobial therapy previous to sample collection might have been given in some cases where it was recommended, and it is known that this can reduce the percentage of bacterial enteropathogens isolation [37]. Thus, even taking into consideration the aforementioned study limitations, including the possible presence in the area of other enteropathogens that we did not test for, such as some diarrhoeagenic viruses, we must not underestimate the impact of malaria, which may account for an important percentage of the diarrhoea among children.

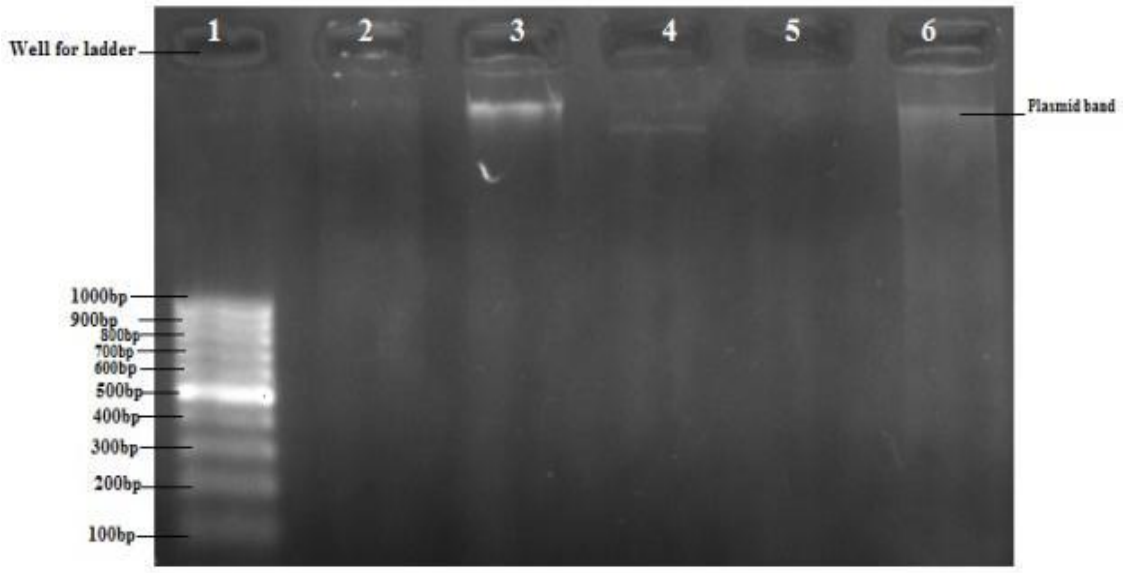

Fig. 1. Plasmid analysis on agarose (0.8%) gel electrophoresis for the five (5) antibiotic heteroresistant *E. coli*

Lanes 2, 3, 4, 5, 6 are for isolates 17, 28, 167, 180, and 198 respectively

Table 2. Multiple antibiotic resistance (mar) index, resistance categories and β-lactamase production among the antibiotic heteroresistant *E. coli* isolates

Isolate no	Resistance pattern	MAR index	Resistance category	β-lactamase production
17	SXT, CN, C, AML	0.6	MDR	-
28	SXT, CIP, AML	0.4	MDR	-
167	AML	0.1	NIL	+
180	SXT, CIP, AML	0.4	MDR	-
198	SXT, C, AML	0.4	MDR	+

SXT= trimethoprim/sulfamethoxazole, CN= gentamicin, CIP= ciprofloxacin, AMC= amoxicillin/clavulanic Acid, C= chloramphenicol, AML= amoxicillin, CRO= ceftriaxone, R= resistant, S= sensitive, I= intermediate, NIL= do not fall in either category of the classification, MDR= multidrug-resistant E. coli, + = Positive Reaction, - = Negative Reaction

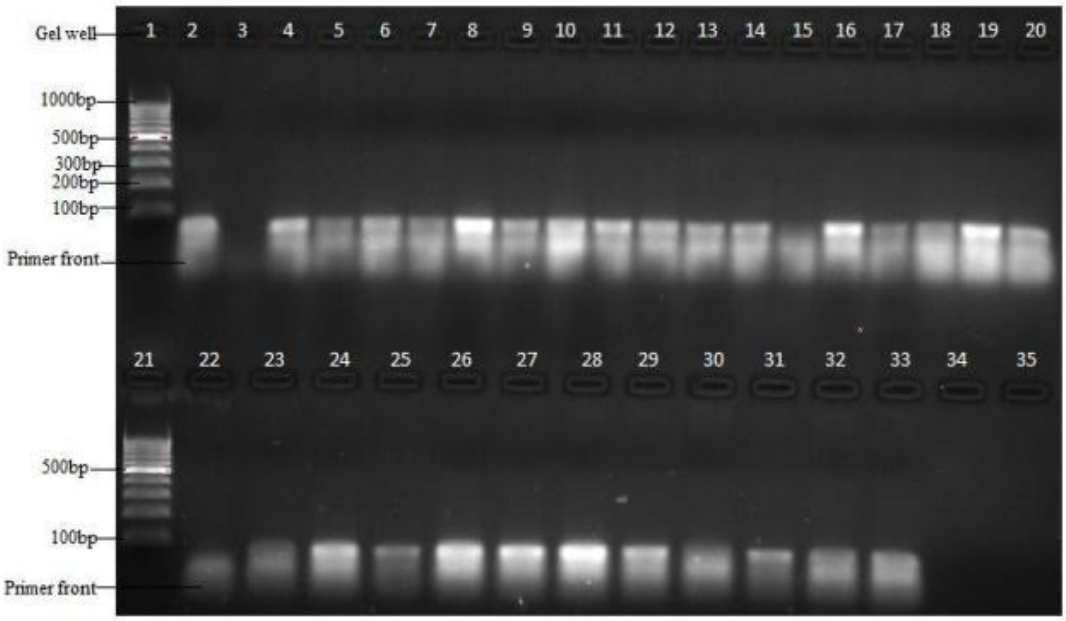

Fig. 2. Multiplex PCR on agarose (1.5%) gel electrophoresis for the identification of diarrhoeagenic *E. coli*

Lanes 2, 3, 4, 5, 6 are for isolates 17, 28, 167, 180, and 198 respectively

In conclusion, heteroresistance is increasingly recognized among clinical isolates, though it is not a new phenomenon. The clinical significance and therapeutic implications of heteroresistance are yet to be determined. More investigations on heteroresistant isolates are required.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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