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1. Department of Medical Microbiology, Faculty of Basic Clinical Sciences, Federal University of Health Sciences, Azare, Bauchi State, Nigeria

2. Department of Medical Microbiology, Ahmadu Bello University, Zaria, Kaduna State, Nigeria

3. Department of Medical Microbiology and Parasitology, Faculty of Basic Clinical Sciences, Usmanu Danfodiyo University Sokoto, Sokoto State, Nigeria

4. Center for Advance Medical Research and Training, Usmanu Danfodiyo University Sokoto, Sokoto State, Nigeria

5. Department of Medical Microbiology and Parasitology, University of Maiduguri, Maiduguri, Borno State, Nigeria

6. Department of Medical Microbiology and Parasitology, University of Ibadan, Ibadan, Oyo State, Nigeria

*Corresponding author's email:

yagubyahaya@gmail.com

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MOBILE COLISTIN RESISTANCE IN ENTEROBACTEREALES FROM CLINICAL ISOLATES AT A TERTIARY HOSPITAL IN NORTHWEST, NIGERIA: A CROSS-SECTIONAL STUDY

Abubakar Jibril ¹, Yahaya Yaqub ^{2*}, Mairo Usman Kadaura ¹, Sabitu Muhammad Zainu ³, Mustapha Umar Imam ⁴, Kellu Ali Bukar ⁵, Mohammed Yahaya ³, Rasheed Ajani Bakare ⁶

Abstract

Antimicrobial resistance (AMR) is an established global problem warranting reliance on antibiotics of last resort. Plasmid-mediated resistance to colistin has emerged and is spreading across the globe, especially among Enterobacterales. This study set out to detect colistin-resistance and other AMR in Enterobacterales from clinical isolates at a tertiary hospital in Nigeria. This was a hospital-based cross-sectional study at Usmanu Danfodiyo University Teaching Hospital Sokoto, Nigeria. Enterobacterales were isolated from different clinical samples, identified using the Analytical Profile Index 20E system, and subjected to antibiotic susceptibility testing. Colistin resistance was detected phenotypically using the Colistin Broth Dilution and Elution Method. Polymerase chain reaction was performed to detect *mcr* genes. A 12.5% (N=320) overall prevalence of colistin resistance was found and only *mcr*-2 was detected, which was found in an isolate of *Escherichia coli*. *Escherichia coli* (51.6%) and *Klebsiella pneumoniae* (29.1%) were the most common Enterobacterales implicated in all infections. More the 60% of the isolates were multidrug-resistant to co-trimoxazole, gentamicin, ceftazidime, and ceftriaxone. This study reports the first *mcr* gene from a clinical sample and a high prevalence of multidrug-resistant Enterobacterales infections in northwest Nigeria. This study call for urgent implementation antibiotic stewardship in our hospitals so as to limit the spread of antibiotic-resistant bacteria in our healthcare facilities.

Keywords: Colistin resistance, *mcr*-2 gene, Antimicrobial resistance, Enterobacterales, Surveillance studies, Nigeria

BACKGROUND

Antibiotic resistance is one of the most important public health issues worldwide. Severe infections due to multidrug-resistant bacteria, mainly carbapenem-resistant bacteria, in addition to lack of new antibiotics against Gram-negative pathogens, have led to a re-evaluation of old antibiotics.(1) In this light, colistin has gained clinical value as a last-line drug against serious bacterial infections, since it is effective against nearly all multidrug-resistant Gram-negative bacteria.(1)

Colistin (polymyxin E) is a polycationic peptide antimicrobial that was isolated from the Gram-positive organism *Bacillus polymyxa* and characterised in 1949.(2) Due to its significant nephrotoxicity and neurotoxicity, the clinical application of colistin in the treatment of human bacterial infections has been restricted.(2) Bacteria employ several means to protect themselves from adverse environmental stimuli, including exposure to cationic antimicrobial peptides, such as polymyxin B and colistin.(3) These strategies include alterations of their lipopolysaccharides (LPSs), which have overall negative charges and are the initial targets of polymyxins.(3) Unfortunately, colistin-resistant (Col-R) bacteria rapidly emerged owing to the improper use and abuse of colistin particularly in aquaculture.(4) Previously, resistance to colistin mostly involved chromosomal mutations.(4) However, since the first description of plasmid-borne resistance gene mobile colistin resistance-1

(mcr-1), the acquired resistance mechanism of colistin has received increased attention.(4)

In 2015, the first plasmid-mediated colistin resistance mechanism i.e. mcr-1 was identified in a strain of *Escherichia coli* isolated from a pig farm in China and presently, a total of 10 different variants (i.e. mcr-1 to mcr-10) have been described. These genes confer resistance by adding a phosphoethanolamine residue to lipid A in LPS and, more additionally, they are carried by mobile genetic elements so they have the ability to spread easily amongst different Gram-negative bacteria by horizontal gene transfer.(5) Carbapenem-resistant isolates acquiring mcr genes are an important threat to clinical practice as they further limit the already scarce treatment options, they are to be identified and contained promptly.(5) The ease with which MCR-family genes are horizontally transferred has seen them demonstrated world-wide, resulting in a variety of bacterial species showing increased colistin tolerance.(6) For serious infections caused by carbapenemase-producing Enterobacterales, the treatment options are restricted and rely on tigecycline and colistin either separately or in combination with other antibiotics.(7) Resistance against colistin has emerged worldwide threatening the efficacy of one of the last-resort antimicrobials that treat infections resulting from multidrug-resistant Gram-negative bacteria.(8) Thus, the global increase in carbapenemase-producing Enterobacterales has resulted in increased use of colistin with the inevitable risk of

emerging antibiotic resistance. This delicate balance between clinical necessity and prevention of resistance is further compromised by agricultural use of human antibiotics, where some countries have actively used colistin in animal production.(7)

Despite growing information on the global spread and distribution of *mcr* genes, more studies are needed to appreciate the prevalence of *mcr* mediated colistin resistance in African countries.(9) Available data shows that research from African countries on the prevalence, spread, and characteristics of a mobile colistin-resistant genes are limited. Most of the researches are from both animal and human studies .(9) To the best of the authors' knowledge, there has been no report from Sokoto State, Nigeria on the prevalence of *mcr* mediated colistin resistance. Thus, the exact epidemiology of the *mcr*-gene is unknown in this study area, indicating a need to conduct *mcr* mediated colistin resistance surveillance from human clinical samples. Therefore, this study set out to investigate the prevalence of mobile colistin-resistant genes as well as the antimicrobial susceptibility profile in Enterobacterales from clinical isolates at the Usmanu Danfodiyo University Teaching Hospital, Sokoto, Nigeria.

METHODS

Study Area

This study was carried out at Usmanu Danfodiyo University Teaching Hospital Sokoto (UDUTH), Sokoto state in the North-

western region of Nigeria. The hospital is a tertiary health centre with a 500-bed capacity. UDUTH Sokoto is a referral health facility and receives about 7,000 patients each year. The Medical Microbiology department of UDUTH Sokoto processes clinical samples received from within the hospital and other healthcare facilities within Sokoto metropolis.

Study design

This was a cross-sectional study to describe the molecular presence of *mcr* gene and antimicrobial susceptibility profiles of Enterobacterales isolated from clinical samples. It involved using clinical samples from adult patients with clinical diagnosis of infectious diseases e.g. blood stream infection, surgical site infection, abscess, urinary tract infection etc. The collected samples were processed at the Medical Microbiology department of UDUTH Sokoto.

Sample size calculation

The sample size required for this study was obtained using the formula according to Fischer formula: $n = (Z^2 pq) / (d^2)$. Where; *n* = the minimum sample size, *Z* = standard normal deviate, usually set at 1.96, *d* = level of precision set at 0.05, *p* = estimated prevalence of disease in the target population.

A prevalence of 28% for colistin resistance was used based on a similar study (10) $q = 1 - p$, therefore, for colistin resistance, $q = 1 - 0.28 = 0.72$. Therefore, the sample size of 320 was arrived at for this study via $n = (1.96)^2 \times 0.28 \times 0.72 / (0.05)$.

Inclusion Criterion

All clinical samples from patients that presented to UDUTH Sokoto with clinical diagnosis of infectious diseases and an established focus of infection.

Exclusion criterion

These consisted of clinical samples from patients who were in the above categories but are severely debilitated, on life-support or unconscious.

Sampling Technique

A consecutive sampling was used which involved recruiting every clinical samples from patients that fulfilled the inclusion criteria until the required sample size of 320 was achieved. Various clinical samples i.e. blood, urine, cerebrospinal fluid, stool, swabs, aspirate and pus were processed depending on the clinical diagnosis. The laboratory processing of the clinical samples was done between March and November 2023.

Ethical considerations

Before the commencement of the study, ethical approval (UDUTH/HREC/2022/1190/V2) was secured from the Ethics Committee of UDUTH Sokoto.

Bacterial isolation and screening

Samples were cultured on blood, chocolate and MacConkey agar. Following overnight incubation, the agar plates with obvious

growth were selected and the isolate were subjected to Gram staining. Isolates that were Gram-negative bacilli were subcultured onto nutrient agar, and later on oxidase test was performed on them. The oxidase test was performed by picking a colony of the isolate from the nutrient agar plate using a wooden stick and placing it on an oxidase strip (Oxoid, UK). Delayed appearance of purple discoloration was considered as negative and therefore a presumed Enterobacterales. Oxidase-negative Gram-negative bacilli (i.e. Enterobacterales) were the only isolates subjected to further analysis for this study.

Bacterial identification with Analytical Profile Index (Biomérieux) 20E system

Only bacterial isolate presumed to be members of Enterobacterales were subjected to the Analytical Profile Index (Biomérieux) 20E (API 20E) according to the manufacturer's instructions for identification as previously described by Maina *et al.*(11)

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was carried out on all the isolates to the following antibiotic agents in accordance with the clinical laboratory standard institute (CLSI 2022) document for disc diffusion: trimethoprim-sulfamethoxazole (i.e. cotrimoxazole) (1.25/23.75 µg), Meropenem (10 µg), Amikacin (30 µg), Amoxicillin/Clavulanic acid (30/3 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg),

Gentamicin (30 µg), Cefepime (30 µg), and Penicillin G (10IU). (12)

Quality control strains

Escherichia coli ATCC 25922 was the quality control strain used for this study.

Determination of Minimum Inhibitory Concentration to Colistin by Colistin Broth Dilution Elution

Colistin broth disk elution was performed as described previously by Nirmal *et al.*(12) Briefly, 10ug colistin disks was added to a test tube containing 1 or 10 ml CA-MHB (Remel, Lenexa, KS) to make 0ug/ml (0 disks in 10 ml), 1ug/ml (1 disk in 10 ml), 2 g/ml (2 disks in 10 ml), and 4 g/ml (4 disks in 10 ml) final concentrations. The tubes with the added disc were vortexed gently and the colistin was let to elute for a minimum of 30 minutes but no longer than 60min at room temperature. A 50uL of standardized inoculum was added to the control and 1,24ug/mL tubes to attain a final concentration of approximately 7.5×10^5 CFU/mL. Using a 10uL loop, a purity plate was made by sub culturing from the original inoculum tube to a blood agar plate. The tubes were capped tightly and each inoculated tube was vortexed on slow speed to mix. The cap was loosed slightly before incubation. The tubes and the plate were incubated at 33 to 35°C ambient air for 16-20hrs. The purity plate was examined to ensure inoculum is pure. The growth control tube will be examined, which demonstrate obvious turbidity for the test to be valid. The visualized on a blue light transilluminator.(4)(14)

MIC was read as the lowest concentration that completely inhibits growth of the test isolate. For Enterobacterales: ≥ 4 ug/mL resistance and ≤ 2 µg/mL = intermediate.(12)

Molecular Detection of Mobile Colistin Resistance Genes

This was carried out on all isolates of Enterobacterales that have been confirmed as colistin resistance by MIC. Nucleotide sequences of mcr genes (as shown in Table 1 below) i.e. mcr-1, mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8 and mcr-9 was collected from the previous published study.(4) Specificity of the designed primers was evaluated by in silico PCR against all mcr variant genes and also by BlastN analysis against the no redundant NCBI database. The primers were synthesized by Integrated DNA Technologies.(13) The ~~is~~ polymerase chain reaction was performed in a total volume of 25 µL containing 2 µL template DNA, 3 mM MgCl₂, 0.25 mM each deoxynucleoside triphosphate, 1 U of Taq polymerase (Invitrogen GmbH, Karlsruhe, Germany), 20 pmol of each primer; DNA thermal cycler, 2 min of initial denaturation at 94°C, 40 cycles of 94°C for 45 seconds, 52°C for 1 min and 72°C for 1 min each, and a final extension at 72°C for 10 mins. DNA fragments was analyzed by electrophoresis in a 1.5% agarose gel at 130 V for 25 min in 1X Tris-Borate EDTA (TBE) buffer containing ethidium bromide using 1100-bp DNA ladder (Promega, USA) as a size marker and

Table 1: Primers used in the Study

Primer name	Sequence (5'-3')	Target gene	Sequence
mcr1_205F	TCCAAAATGCCCTACAGACC	mcr 1	205
mcr1_205R	GCCACCACAGGCAGTAAAAAT		
mcr2_279F	CCTTTTGTGCTGATGGGTTT	mcr2	279
mcr2_279R	ATTTTGGAGCATGGTGGTGT		
mcr3_347F	CTTGCTGAACCAATCCCATT	mcr3	347
mcr3_347R	CCATCGTTCTCCTTCCAAAA		
mcr4_426F	CCATCGTTCTCCTTCCAAAA	mcr4	426
mcr4_426R	GATCCGAAGCTGTGTTCTG		
mcr5_522F	GGTTGGCCGAGAAGATAACA	mcr5	522
mcr5_522R	ATGTTGCCAGAAGGTCCAAC		
mcr6_252F	AGCTATGTCAATCCCGTGAT	mcr6	252
mcr6_252R	ATTGGCTAGGTTGTCAATC		
mcr7_791F	GTCAGTTACGCCATGCTCAA	mcr7	791
mcr7_791R	TTCTTGTCGCAGAACTGTGG		
mcr8_934F	AAACTGAACCCGGTACAACG	mcr8	934
mcr8_934R	GCCATAGCACCTCAACACCT		
mcr9_635F	GCGGTTGTAAAGGCGTATGT	mcr9	635
mcr9_635R	CAAATCGCGGTCAGGATTAT		

Primer sequences were adapted from Lui et al. 2020.(4)

RESULTS

Escherichia coli 165 (51.6%) was the most often isolated bacterium and this was followed by *Klebsiella pneumoniae* 93 (29.1%). The identity and distribution of other Enterobacterales found in this study are as shown below in Table 2.

Table 2: Distribution of Enterobacterales species isolated from clinical samples (N = 320).

S/No.	Enterobacterales	Number isolated	Frequency (%)
1	<i>Escherichia coli</i>	165	51.6
2	<i>Klebsiella pneumonia</i>	93	29.1
3	<i>Proteus mirabilis</i>	11	3.4
4	<i>Citrobacter freundii</i>	10	3.2
5	<i>Klebsiella oxytoca</i>	8	2.5
6	<i>Enterobacter cloacae</i>	7	2.2
7	<i>Serratia mercenscens</i>	5	1.6
8	<i>Proteus vulgaris</i>	4	1.3
9	<i>Salmonella enteritica</i> subsp. <i>Typhimurium</i>	3	0.9
10	<i>Citrobacter koseri</i>	3	0.9
11	<i>Serratia ficaria</i>	2	0.6
12	<i>Serratia fanticola</i>	2	0.6
13	<i>Serratia rubidaea</i>	3	0.9
14	<i>Erwinia spp</i>	2	0.6
15	<i>Shigella flexneri</i>	2	0.6
Total		320	100

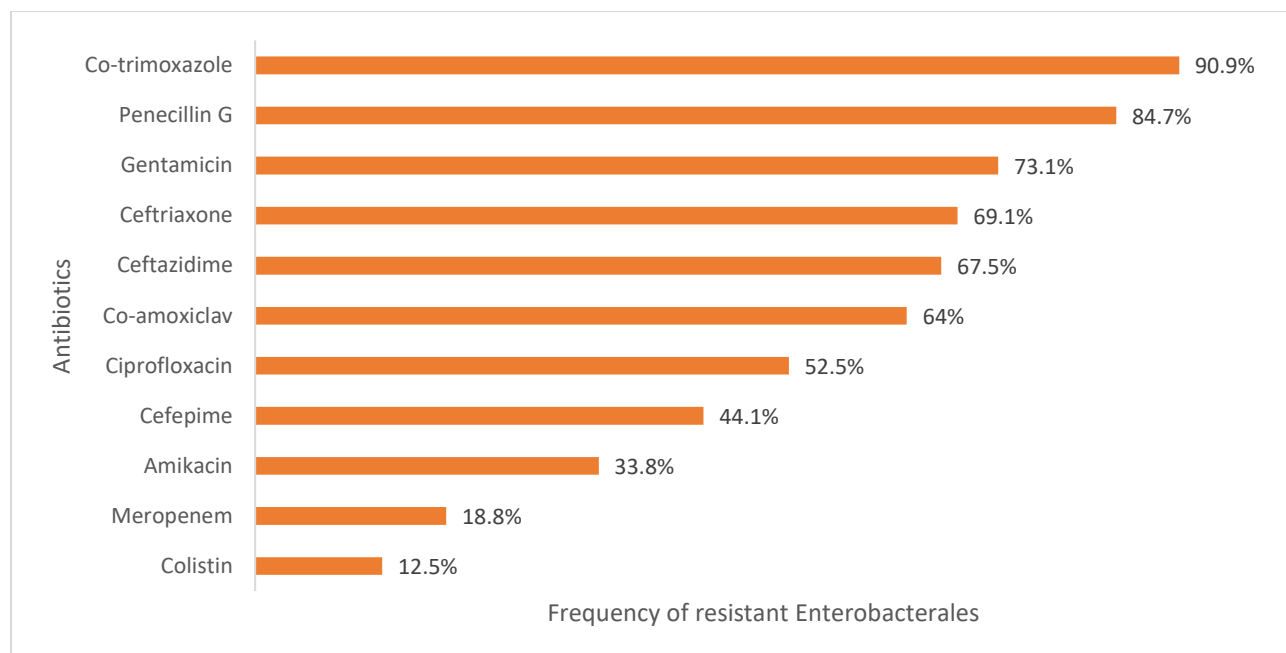


Figure 2: Antibiotic resistance pattern of the Enterobacteriales (N=320) from this study.

From Figure 2 above, the highest level of resistance was observed for Co-trimoxazole 291 (90.9%) followed by Penicillin G 271 (84.7%). Using the colistin broth dilution elution method, 40 (12.5%) of the 320 Enterobacteriales isolates were found to be colistin resistant phenotypically.

Out of the 40 phenotypically colistin resistant isolates of Enterobacteriales, only a single mcr-2 gene genotype was found of all the ten mcr genes (i.e. mcr 1-10), that were investigated for in this study as shown in Figure 3 (see Supplemental file).

DISCUSSION

In this study, 320 clinical isolates of Enterobacteriales were investigated for the presence of colistin resistance between March and November 2023. A 12.5% overall

prevalence of colistin resistance was found. Even though colistin is not used in our facility and the state in general for the treatment of infectious disease, the widespread use of the drug in the veterinary medicine, particularly poultry farming, may have increased the potential risk of the resistant variant being transferred from animals to humans. This study revealed a profound resistance profile towards the antibiotics investigated for, which reflects the anecdotal antibiotic choice in our setting, as shown in Figure 2. It is established that irrational use of other antibiotics like cephalosporins and carbapenems will put the bacteria under selective pressure and this could lead to this resistance. This resulted from poor infection prevention and control and antimicrobial stewardship practices in the healthcare environment. The widespread use of these antibiotics in both human and animal medicine may lead to the emergence and development of multigene resistance, which

can then spread across the bacterial community and ultimately causing public health problems. Our results is similar to what was reported from Egypt by Zafer *et al* (2019).(1) It is obvious that the prevalence of colistin resistance in this study is higher in comparison to the published data in 2018 by Olowa *et al*. Colistin resistance was detected in five isolates out of the sixty isolates that the 2018 study examined between July 2015 and June 2016, accounting for 8.3% of the cases.(15) Results from Enugu, Port Harcourt and Benue also disagrees with the findings of our study by reporting a higher prevalence.(16)(8)(9) A prevalence 0.4% has also been reported from a neighboring West Africa country, Senegal.(17) This is lower than what was reported from this study. Despite the fact colistin is not licensed to be used in many African countries and there is insufficient data that may indicate the actual level exposure to the medication in the continent, a high prevalence has also been reported from Tanzania(18) South Africa(19) and Tunisia(20). A prevalence of 0.67% have also been reported from Spain.(21) This is lower than what was reported from this study. Poor infection prevention and control and antimicrobial stewardship practices in our facilities, Nigeria and Sub-Saharan Africa may likely be the reason for the high prevalence from this study as compared to Spain. A pooled prevalence of 6.6% has also been reported from a systemic review and meta-analysis conducted by Narimisa *et al*.(22) A study by Bshabshe *et al*. from Saudi Arabia showed 34.5% resistance to colistin.(23) Higher prevalences has also

been reported from Thailand(24) and France.(25)

Africa, which consist of mostly of low and medium income countries (LMIC), is seeing a significant rise in transferable plasmid-mediated colistin resistance, with the prevalent genetic variant in human, animal, and environmental samples being *mcr-1*.(26) In this study however, mobile colistin resistance 2 (*mcr -2*) gene was responsible for colistin resistance in one (2.5 %) colistin resistant isolates. This falls within the global prevalence of 0.1–9.3% reported in a systematic review and meta-analysis by Elbediwi *et al*.(2) Other *mcr* genes (1 & 3 - 9) were not detected in this study. The inability to detect *mcr* genes from these isolates may be an evidence of other mechanisms of colistin resistance like chromosomally mediated or other novel plasmid mediated genes. Previously, *mcr-1*, *mcr-2*, *mcr-3*, and *mcr-8* has been reported from Africa.(27) The findings of this study is different from what was previously reported from Sokoto State, Nigeria in 2018 by Olowokere *et al*, that revealed none of the *mcr* gene was present.(28) This may be explained by the fact that *mcr* genes are spreading quickly and also, the larger sample size used in this study which may have increased the likelihood of detection. Most of the studies conducted in Nigeria that reported *mcr* genes were not strictly of human origin.(9) Otunokefor *et al* reported a prevalence of 8.6% for *mcr-1* which is falls within the global prevalence rate as found in this study.(8) The predominant genetic variant in human, animals as well as in the environmental

samples in Africa is *mcr-1*(27) but higher prevalence (i.e. above global prevalence rate) of 18.6% have been reported from Tanzania.(18) The prevalence of *mcr* genes have been reported in a higher than global prevalence rate from other part of the world particularly from china where *mcr* gene was first detected.(4)(29) Perhaps, the low prevalence of colistin resistance in Africa may be attributable to the unavailability of colistin for human use and/or lack of data from the region.(30) It is worthy of note however, that lower prevalence rates have also been reported from non-LMIC health systems like Italy and Spain.(20) (29)

Conclusion

As the world gradually returns to the pre-COVID 19 air-travel experience, the problem of antimicrobial resistance needs to be prioritized. This is necessary to control the spread of multidrug resistance bacteria (including *mcr* mediated) especially in LMIC, where the potential of outbreaks of difficult-to-treat infectious diseases in human and animal could be difficult to control. Mobile colistin resistance is a rapidly developing health issue of global concern with a potentially substantial impact on the Africa continent. This requires urgent institutionalization of aggressive health interventions to control the spread of *mcr* genes in Africa to avert this potential health catastrophe waiting to happen.

An important limitations of this study is that while it provides valuable insights into the prevalence and antibiotic resistance patterns of clinically isolated

Enterobacterales in the context of LMIC, generalization to other patient populations needs careful consideration. Also, no other antibiotic resistant gene was investigated for apart from the plasmid mediated colistin resistance genes (i.e. *mcr* 1-10), as such no molecular interrelatedness could be established. With the recent drive globally to incorporate more technologically advanced platforms like whole genome sequencing, diagnostic microbiology in sub-Saharan Africa will surely get a better appreciation of AMR (*mcr* included) by conducting more contemporary research which will guide the development and implementation of suitable policies.

Declarations

Authors' contributions: AJ, YY and MY conceived and implemented the study. AJ, YY, MUK, SMZ, MUI, KBA, MY and RAB designed the study and read and approved the final manuscript. AJ, YY, MUK, SMZ and MY were responsible for analysis and interpretation of data. AJ, YY, MUK, SMZ, MUI, KAB and MY drafted the manuscript. AJ, YY and MY are the guarantors of the paper.

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(UDUTH/HREC/2022/1190/V2). Informed consent to participate in the study was obtained from the parents or legal guardians of participants below the age of 16 years. Informed consent to participate in the study was obtained from participants aged 16 years and above.

Consent to Participate: Informed consent to participate in the study was obtained from the parents or legal guardians of participants below the age of 16 years. Informed consent to participate in the study was obtained from participants aged 16 years and above. This consent included permission for anonymous use of data for the purpose of publication.

Data availability: The data are provided in the article.

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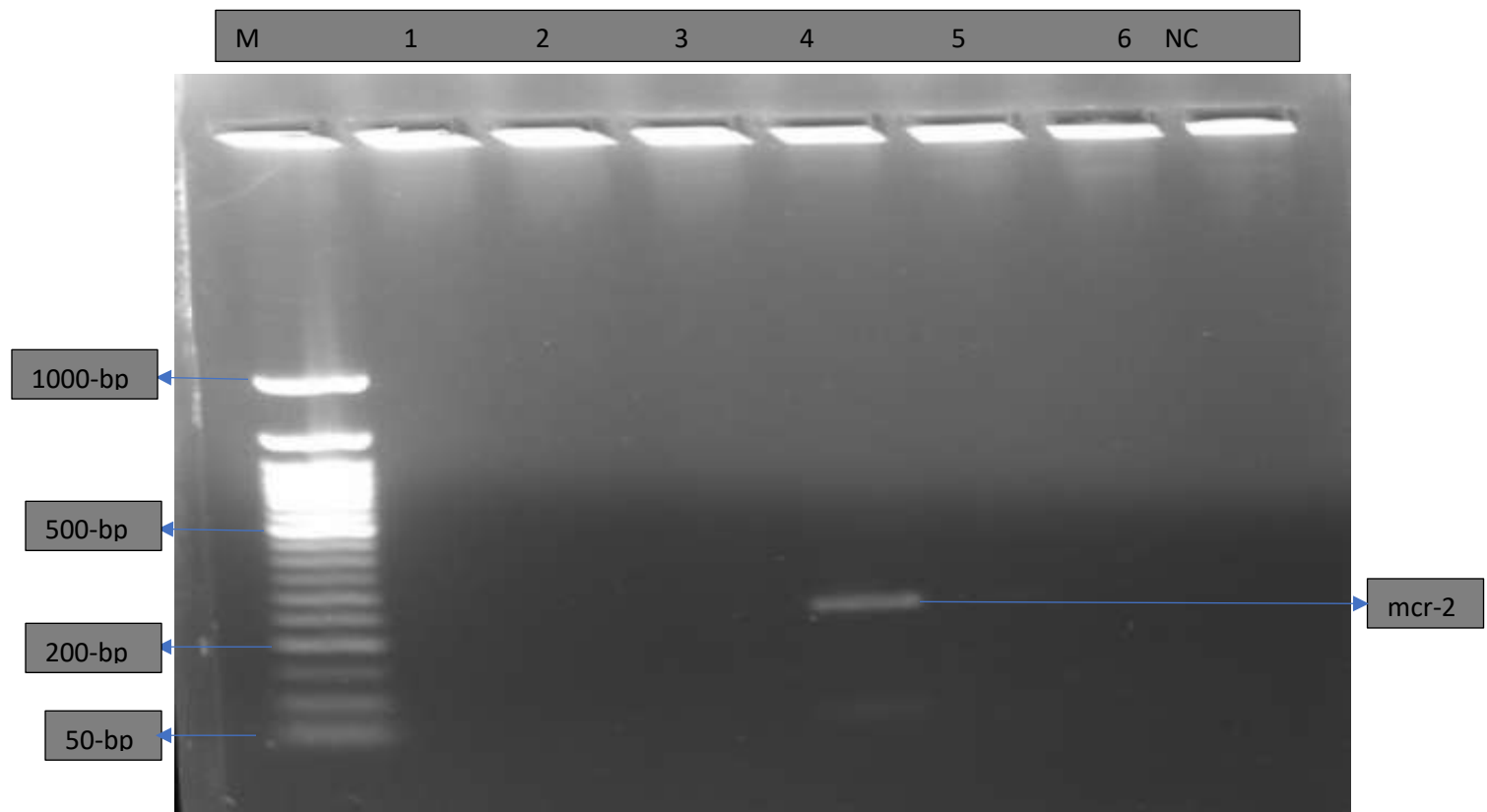
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Supplemental file



Note: M=Molecular Ladder, NC=Negative Control & bp=base pairs.

Figure 3: Gel electrophoresis showing mcr-2 gene positive *Escherichia coli* isolates in lane 4