Journal of Medical Laboratory Science, 2022; 32 (2): 14-24
http://jomls.org; info@jomls.org **Eriamiatoe *et al***
http://doi.org/10.5281/zenodo.7195883

Phenotypic Detection of Mobilized Colistin Resistance Among Clinical Isolates in University of Benin Teaching Hospital, Benin City, Nigeria

Richard Etinosa Eriamiatoe[1], Eghe Ikponmwosa Onaiwu[1,†], Obiora Donatus Aguh[1], Eghonghon Isivbakhome Dedekuma[1], Angela Akugbe Eghiomon[1], Nosakhare Lawrence Idemudia[1], Ephraim Ehidiamen Ibadin[1], Christopher Aye Egbe[1, 2], Nana Atinuke Anogie[1], Isaac Ohiorenuan Igbarumah[1, 3], Osagioduwa Wuraola Fapohunda[4] and Richard Omoregie[1, 5,*]

[1]Medical Microbiology Division, Medical Laboratory Services, University of Benin Teaching Hospital, Benin City, Nigeria, [2]Anti-retroviral Laboratory Unit, Medical Microbiology Division, Medical Laboratory Services, University of Benin Teaching Hospital, Benin, Nigeria, [3]Molecular Virology Laboratory Unit, Medical Microbiology Division, Medical Laboratory Services, University of Benin Teaching Hospital, Benin City, Nigeria, [4]Medical Microbiology Department, Irrua Specialist Teaching Hospital, Irrua, Nigeria and [5]School of Medical Laboratory Sciences, University of Benin Teaching Hospital, Benin City, Nigeria

ABSTRACT

Background: Mobilized colistin resistance (MCR) is a public health problem resulting in limited therapeutic options. **Objective:** This study aims to phenotypically detect mobilized colistin resistance (MCR)-mediated resistance in a setting where colistin is not used. **Method:** The combined disk test (CDT) technique and minimum inhibitory concentration (MIC) were used to screen a total of 238 isolates for the existence of MCR producers. **Results:** Of the 238 isolates, 47 (19.75%) were CDT positive, and 37 out of the 47 had MIC >2mg/L and were referred to as MCR producers. Age, gender, type of specimen and the wards/clinics the isolates were recovered from did not significantly affect the prevalence of MCR producers ($p>0.05$). Type of isolate significantly ($p=0.0184$) affected the prevalence of MCR producers. **Conclusion:** This study underscores a high prevalence [15.55% (37/238)] of phenotypically detected MCR-mediated colistin resistance in a resource-poor setting were colistin is not used.

Key words: Mobilized colistin resistance; Phenotypic; Tertiary Hospital; Benin City; Nigeria
†Deceased
***Corresponding author: richyomos@yahoo.com, richard.omoregie@ubth.org; Tel: +2348105543053; ORCID: 0000-0002-8601-159X**
Authors' contributions: This work was conducted and approved in collaboration between all the authors who take responsibility for its intellectual contents, accuracy and integrity. All authors designed the study; RO, REE, EIO, ODA, EID, AAE, NLI, EEI, CAE, NAA and IOI wrote the protocol; RO, REE, EIO, ODA, EID, AAE, NLI, EEI and OWF contributed in literature search; All authors did the experiments; RO, EEI, NLI, REE, NAA and IOI did statistical analysis; All authors drafted the manuscript; RO and CAE supervised the study; RO Wrote the final manuscript; All authors proofread the manuscript.

Received: October/25, 2021; **Accepted**: May/24, 2022; **Published**: June/30, 2022.
Citation: Eriamiatoe RE, Onaiwu EI, Aguh OD, Dedekuma EI, Eghiomon AA, Idemudia NL, Ibadin EE, Egbe CA, Anogie NA, Igbarumah IO, Fapohunda OW, Omoregie R. Phenotypic Detection of Mobilized Colistin Resistance Among Clinical Isolates in University of Benin Teaching Hospital, Benin City, Nigeria. **J Med Lab Sci, 2022; 32 (2): 14-24**

INTRODUCTION

Multidrug-resistant organisms have been labeled as "a serious threat to public health" by the Centers for Disease Control and Prevention (CDC) and as "one of the three greatest threats to human health," by the World Health Organization.[1] Emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria, as well as the lack of novel agents against these pathogens, have led to the reintroduction of colistin, an old and valuable antibiotic as a last-resort treatment option.[2, 3] However, resistance to colistin has emerged, and this resistance could either be intrinsic or acquired.[4] Acquired plasmid-mediated resistance caused by the presence of mobilized colistin resistance (*mcr*) genes in animals and humans was first reported in China.[5] The discovery of this gene changed the scenario of resistance to polymyxins (colistin), as this gene could be promptly disseminated among Gram negative bacilli becoming a major global concern for public health.[6] The *mcr* genes are currently distributed worldwide.[7, 8]

The *mcr* gene results in the synthesis of MCR protein which is a member of the phosphoethanolamine transferase family.[6] The *mcr* gene (or protein) mediates the addition of phosphoethanolamine to lipid A which results in increased net charge of modified lipid A and reduces the affinity of positively charged polymyxins, leading to resistance.[5, 6] As at now 10 *mcr* variants (*mcr-1* to *mcr-10*) have been reported.[9, 10] Thirteen *mcr-1* subtypes (*mcr-1.1* to *mcr-1.13*) as well as numerous subtypes for other variants have also been reported.[6]

The extensive use of colistin in the animal production industry is recognized as one of the reasons for the emergence and dissemination of the plasmid-borne colistin resistance gene (*mcr*), thus, the dissemination of *mcr* genes is different between countries.[8] The presence of plasmid-mediated colistin resistance has been reported in Nigeria[11, 12], albeit, none has been reported from our institution. The presence of *mcr-1* genes in animals was traced to 1980 while that for humans' dates back to 2008, indicating that *mcr-1* gene has existed unidentified for a long time in enteric bacteria.[6, 13, 14] Therefore, in our institution where colistin is not used (Institutional antibiotic policy), there may already exist plasmid-mediated colistin resistance.

The use of minimum inhibitory concentration (MIC) to detect colistin resistance does not differentiate between plasmid- and chromosomal-mediated resistance.[6] Molecular techniques to detect and sequence the *mcr* gene are the gold standard for detecting plasmid-mediated resistance.[6] These methods are not within the reach of resource-poor countries. However, a number of phenotypic tests have been developed for the detection of MCR producers.[15-17] Recent structural studies have revealed that the active site of MCR-1 phosphoethanolamine transferase contains zinc.[6] Thus, the use of ethylenediamine tetra-acetic acid (EDTA) to chelate the zinc will result in increased detection and could represent a strategy for phenotypic detection of *mcr* genes. A number of phenotypic tests to detect *mcr* genes based on EDTA exists.[6] A combination of one of these phenotypic methods and determination of MIC in a calcium-enhanced environment, as calcium has been reported to be optimal for the detection of MCR producers[18], will be helpful in phenotypically detecting *mcr*-mediated colistin resistance in resource-poor settings. Against this background, this study aims to phenotypically detect *mcr*-mediated colistin resistance in a tertiary health facility where colistin is not used.

METHODS

Bacterial isolates

A total of 238 consecutive and non-repetitive Gram-negative bacteria recovered from clinical specimens between the months of February and April 2020 in the Medical Microbiology Laboratory, University of Benin Teaching Hospital, Benin City, Nigeria, were used for this study. The isolates were identified using Microbact 24E (Oxoid, England). Information on the specimens, age and gender of patients, and ward/clinics the isolates were obtained from laboratory records.

Phenotypic screening for MCR-producer

Phenotypic screening for MCR-producers was done using a modification of the combined-disk test (CDT) previously described[15,19]. Briefly, test organisms were emulsified in sterile water and the turbidity matched with 0.5 McFarland standard. Once matched, a sterile cotton-wool swab was dipped in the organism suspension and excess liquid was removed by turning the swab on the side of the test tube. The entire surface of Mueller-Hinton agar plate (Titan Biotech Limited, India) was seeded by swabbing in three directions with the swab. Two 10µg colistin disk (in-house prepared) were placed on the surface of the seeded plate, and 10µL of 100mM EDTA solution added to one of the colistin disks. The colistin used for the disk preparation was colistin sulphate (Pantex, Holland). The plates were incubated overnight at 37^0C. A positive MCR-producer was inferred if the zone diameter of disk with colistin and EDTA was ≥3mm than that of the disk with colistin only. A negative control organism – *Escherichia coli* ATCC 25922, was included in the experiment

Minimum inhibitory concentration in calcium-enhanced Mueller-Hinton broth

Minimum inhibitory concentration in calcium-enhanced Mueller-Hinton broth (CE-MHB) for all isolates that were positive for the CDT was utilized to verify MCR-producing isolates phenotypically. The MIC of the CDT-positive isolates was determined using macrobroth dilution test. The CE-MHB (MHB was a product of TM Media [Titan Biotech Limited, India]) was prepared as previously described[18] except that calcium chloride anhydrous (Guangdong Guanghua Sci-Tech Co., Ltd, China) was used instead of calcium chloride dihydrate. Colistin sulphate (Pantex, Holland) was diluted in the CE-MHB to cover the range 0.25 to 32mg/L. An inoculum size of 5 x 10^5 cfu/mL of each test isolate was used to challenge each concentration of colistin in the CE-MHB. The inoculated tubes were incubated at 37^0C for 18 – 24 hours. The MIC was read visually as the least concentration without growth (turbidity). The EUCAST[20] breakpoint was used for interpretation of the MIC results: isolates with colistin MICs of ≤2 mg/L were categorized as susceptible, and those with MICs of >2 mg/L were categorized as resistant. A negative control organism – *Escherichia coli* ATCC 25922, was included in the experiment.

An isolate was inferred to be a phenotypic MCR-producer if it was positive for CDT and have an MIC >2mg/L.

Statistical analysis

The data obtained was analyzed with Chi square (X^2) test using the statistical software INSTAT® (Graph Pad Software Inc, La Jolla, CA, USA). Statistical significance was set at $p < 0.05$.

RESULTS

Of the 238 Gram negative bacilli used in this study, 47(19.8%) were positive for CDT while 37 (78.7%) of the CDT-positive isolates had MIC >2mg/L. Thus, a prevalence of 15.6% of phenotypically verified MCR was obtained in this study (Table 1). Although higher prevalence of MCR isolates was recovered from males, there was no significant difference in the prevalence of MCR between genders. Similarly, age of the patients from whom the isolates were recovered from showed no association with MCR (p=0.1042; Table 2)
Isolates recovered from sputum had the least prevalence of MCR while isolates from blood culture and tracheal swabs had the highest prevalence of MCR (50% each). However, type of specimens from which the isolates were recovered did not significantly (p= 0.2594) affect the prevalence of MCR (Table 2). Isolates recovered from Burns, Gynecology, Oncology and Ophthalmic wards did not harbor MCR producers. Isolates recovered from Intensive care unit (ICU) had the highest prevalence of colistin resistance mediated by *mcr* genes, albeit, the ward/clinics that the isolates were recovered from showed no association with MCR producers (p=0.6173; Table 2).
The only isolate of *Moraxella* species, *Pseudomonas fluorescens*-25 and *Vibrio vulnificus* were MCR positive. Among isolates of *Salmonella* subspecies 3B, 66.7% were MCR positive. The type of isolates used significantly (p=0.0184) affected the prevalence of MCR producers (Table 2).

Table 1: Prevalence of phenotypically confirmed MCR resistance

No. of isolates	No. positive for CDT (%)*	No. with MIC (%)*	
		≤2 mg/L	>2 mg/L
238	47 (19.8)	10 (4.2)	37 (15.6)

*= percentage is of 238

Table 2: Distribution of MCR resistance

Characteristics	No. tested	No. with MCR	P value

		resistance (%)	
Gender			0.6156
Male	110	19 (17.3)	
Female	128	18 (14.1)	
Age (years)			0.1042
≤1 – 10	45	9 (20.0)	
11 – 20	21	4 (19.1)	
21 – 30	36	4 (11.1)	
31 – 40	44	10 (22.7)	
41 – 50	14	1 (7.1)	
51 – 60	22	3 (13.6)	
61 – 70	28	0 (0.0)*	
71 – 80	17	2 (11.8)	
81 – 90	9	2 (22.2)	
91 – 100	2	2 (100.0)	
Specimens			0.2594
Aspirate	9	0 (0.0)*	
Blood culture	4	2 (50.0)	
Catheter tip	10	2 (20.0)	
Cerebrospinal fluid	1	0 (0.0)*	
Corneal scrapping	1	0 (0.0)*	
Ear swab	18	2 (11.1)	
Endo-cervical swab	7	2 (28.6)	
Eye swab	1	0 (0.0)*	
High vaginal swab	1	0 (0.0)*	
Sputum	10	1 (10.0)	
Stool	10	3 (30.0)	
Throat swab	1	0 (0.0)*	
Tracheal swab	2	1 (50.0)	
Urine	97	11 (11.3)	
Wound swab	66	13 (19.7)	
Ward/Clinics			0.6173
Adult emergency units	18	1 (5.6)	
Burns ward	2	0 (0.0)*	
Children emergency unit	15	4 (26.7)	
Geriatric ward	9	1 (11.1)	
Gynecology ward	10	0 (0.0)*	
Intensive care unit	11	4 (36.4)	
Labour ward	11	1 (9.1)	
Maternity ward	8	1 (12.5)	
Medical ward	24	5 (20.8)	
Neonatal intensive care unit	4	1 (25.0)	

Neurosurgical ward	10	3 (30.0)	
Oncology ward	2	0 (0.0)*	
Ophthalmic ward	1	0 (0.0)*	
Out-patient departments	69	9 (13.0)	
Paediatric ward	17	3 (17.7)	
Surgical wards	27	4 (14.8)	
Isolates			0.0184
Citrobacter amalonaticus	2	0(0.0)*	
Citrobacter freundii	1	0(0.0)*	
Citrobacter youngae	1	0(0.0)*	
Cronobacter sakazakii	2	1 (50.0)	
Enterobacter aerogenes	5	0(0.0)*	
Enterobacter agglomerans complex	1	0(0.0)*	
Enterobacter cloacae	10	2 (20.0)	
Enterobacter hormaechei	1	0(0.0)*	
Escherichia coli	75	11 (14.8)	
Hafnia alvei	3	0(0.0)*	
Klebsiella oxytoca	10	3 (30.0)	
Klebsiella pneumoniae	23	1 (4.4)	
Klebsiella terrigena	4	1 (25.0)	
Morganella morganii ssp. morganii	3	0(0.0)*	
Proteus mirabilis	10	0(0.0)*	
Proteus vulgaris	2	1 (50.0)	
Providencia stuartii	3	1 (33.3)	
Raoultella ornithinolytica	6	0(0.0)*	
Salmonella ssp. 3B	3	2 (66.7)	
Serratia fonticola	2	0(0.0)*	
Serratia liquefaciens complex	1	0(0.0)*	
Serratia marcescens	1	0(0.0)*	
Serratia odorifera biogp 1	1	0(0.0)*	
Acinetobacter baumannii	2	1 (50.0)	
Acinetobacter haemolyticus	1	0(0.0)*	

Aeromonas caviae	3	0(0.0)*
Aeromonas hydrophila	3	0(0.0)*
Burkholderia pseudomallei	2	0(0.0)*
Moraxella species	1	1 (100.0)
Pseudomonas aeruginosa	52	10 (19.2)
Pseudomonas fluorescens-25	1	1 (100.0)
Pseudomonas stutzeri	2	0(0.0)*
Vibrio vulnificus	1	1 (100.0)

*Not used in statistical analysis

DISCUSSION

The CDT method has been validated to have a sensitivity of 96.7% and a specificity of 89.6% for *E. coli* only.[15] This may explain why not all 47 CDT-positive isolates were resistant to colistin. It is plausible that the 10 colistin-susceptible isolates (MIC ≤2mg/L) may harbor *mcr* genes as previously reported.[21] However, the limited specificity mentioned above may indicate a false positivity for *mcr* gene. The use of a higher concentration of calcium (200mg/L) in MHB was reported to efficiently discriminate between colistin-susceptible and colistin-resistant isolates.[18] This type of calcium-enhanced medium was used for this study. Molecular detection and sequencing of the *mcr* genes is needed to verify if the colistin-susceptible isolates possess *mcr* genes. A combination of both CDT and MIC in a calcium-enhanced medium in this study showed that 15.6% of the 238 isolates were phenotypically MCR producers. This prevalence is high compared to a range of 0.9% to 1.4% reported in China[22], and 0.26% reported in Italy.[23] This is concerning since colistin is not currently utilized at our institution, and this discovery may mean that colistin will not be used to treat resistant Gram negative bacterial infections brought on by bacteria that are multidrug resistant and that produce extended spectrum -lactamases, ampC -lactamases, and carbapenemases, all of which have been documented in our institution.[24] The prevalence of MCR producers among clinical *E. coli* isolates in this study (14.8%) is lower than 23.08% reported in Port Harcourt, Nigeria.[12] It is important to note that these referred studies investigated only *E. coli* isolates whereas all other Gram negative bacterial isolates were considered in this study. Use of β-lactam antibiotics and fluoroquinolones have been reported among the risk factors for colistin resistance.[25, 26] In Nigeria, both human and animal usage of antibiotics is uncontrolled, and the over-the-counter sale of antibiotics without a prescription is commonplace.[27-30] Also, extended–spectrum cephalosporins and fluoroquinolones are widely used as broad–spectrum antibiotics and remain the drugs of choice to treat infections caused by various Gram negative pathogens.[31] This may explain the findings in this study.

In this investigation, the frequency of MCR producers was somewhat greater in males than in females (p=0.6156). The chance of contracting an illness that is mcr-1 positive

has been linked to male gender.[26] In a recent study, univariate analysis, rather than multivariate analysis, revealed that the prevalence of MCR producers was considerably greater in females than in males.[23] This shows that there are contradicting findings regarding how gender affects MCR prevalence. This study's conclusion that age had no discernible impact on the prevalence of MCR producers is consistent with earlier studies.[23, 26]

Although isolates recovered from blood cultures and tracheal swabs had the highest prevalence of MCR producers, type of specimen did not significantly ($p>0.05$) affect their prevalence – a finding that agrees with a previous report.[26] Similarly, the wards/clinics attended by the patients from whom the isolates were recovered did not affect the prevalence of MCR producers ($p=0.6173$), though isolates from ICU had the highest prevalence of MCR producers. This is surprising for an institution where colistin is not used.

Plasmid-mediated colistin resistance has previously been observed in all the isolates that were positive of MCR production in this study except *Proteus vulgaris*. *Proteus* species are intrinsically resistant to colistin.[18] However, the presence of *mcr-1* and *mcr-3* have recently been detected in *Proteus mirabilis*.[32, 33] This agrees with the finding in this study and molecular analysis will be needed to confirm and detect the *mcr* variants in the *mcr*-positive *Proteus vulgaris* in this study, as it is also possible that the positive CDT result for *P. vulgaris* may be false positive.

Limitations

The non-availability of molecular techniques was a limitation in this study as it would have confirmed the presence and type of mcr gene(s) in our isolates. The use of broth macrodilution method (BMAD) for MIC in this study as against broth microdilution recommended by EUCAST is also limitation. However, BMAD has been shown to have very good agreement with BMD as the methods only differ in volume of growth medium and sterile glass tubes (for BMAD) as against tray (for BMD)[34-37]. MIC in CE-MHB has not been validated for bacterial isolates except *E. coli*. It is also important to note that CDT method used in this study has only been used for *E. coli* and *Pseudomonas aeruginosa*.

CONCLUSION

This study underscores a high prevalence of phenotypically determined *mcr*-mediated colistin resistance in a resource-poor setting were colistin is not used. It is recommended that molecular techniques be employed in order to ascertain the true prevalence due to MCR producers. Measures to combat the spread of this type of resistance are advocated.

REFERENCES

1. Thaden JT, Lewis SS, Hazen KC, et al. Rising rates of carbapenem-resistant *Enterobacteriaceae* in community hospitals: a mixed-methods review of epidemiology and microbiology practices in a network of community hospitals in the southeastern United States. *Infect Control Hosp Epidemiol.* 2014; *35* (8): 978 – 983.
2. Baron S, Hadjadj L, Rolain J-M, et al. Molecular mechanisms of polymyxin resistance: knowns and unknowns. *Int J Antimicrob Agents.* 2016; 48(6):583–591
3. Li R, Xie M, Zhang J, et al. Genetic characterization of mcr-1-bearing plasmids to depict molecular mechanisms underlying

dissemination of the colistin resistance determinant. *J. Antimicrob. Chemother*. 2017; 72, 393–401.
4. Aghapour Z, Gholizadeh P, Ganbarov K, et al. Molecular mechanisms related to colistin resistance in Enterobacteriaceae. *Infect Drug Resist* 2019; 12: 965–975
5. Liu YY, Wang Y, Walsh TR, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016; 16:161–168.
6. Dalmolin T V, de Lima-Morales D, Barth, AL. Plasmid-mediated Colistin Resistance: What Do We Know? *J. Infectiology* 2018; 1(2): 16 – 22
7. Nordmann P, Poirel L. Plasmid-mediated colistin resistance: an additional antibiotic resistance menace. *Clin Microbiol Infect* 2016; 22: 398–400
8. Luo Q, Wang Y, Xiao Y. Prevalence and transmission of mobilized colistin resistance (mcr) gene in bacteria common to animals and humans. *Biosafety and Health* 2020; 2: 71 – 78
9. Anyanwu MU, Jaja I. F, Nwobi OC. Occurrence and characteristics of mobile colistin resistance (*mcr*) gene-containing isolates from the environment: a review. *Int. J. Environ. Res. Publ. Health* 2020;17:1028. doi: 10.3390/ijerph17031028
10. Wang C, Feng Y, Liu L, et al. Identification of novel mobile colistin resistance gene *mcr-10*. *Emerg.Microbes Infect*. 2020; 9, 508–516.
11. Olowe OA, Olowe RA, Oluremi AS, et al. A novel report of colistin-resistant *Escherichia coli* carrying mcr-1 gene from animal and human faecal samples in Nigeria. *Pan Afr J Life Sci* 2018; 1: 7 – 10
12. Otokunefor K, Tamunokuro E, Amadi A. Molecular detection of mobilized colistin resistance (*mcr-1*) gene in *Escherichia coli* isolates from Port Harcourt, Nigeria. *J. Appl. Sci. Environ. Manage* 2019; 23(3): 401 – 405
13. Shen Z, Wang Y, Shen Y, et al. Early emergence of mcr-1 in Escherichia coli from food-producing animals. *Lancet Infect Dis*. 2016; 16 (3): 293
14. Skov RL, Monnet DL. Plasmid-mediated colistin resistance (mcr-1 gene): three months later, the story unfolds. *Euro Surveill.* 2016; 21(9):30155. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.9.30155
15. Esposito F, Fernandez MR, Lopes R, et al. Detection of colistin resistant MCR-1-positive *Escherichia coli* by use of assays based on inhibition by EDTA and zeta potential. *J Clin Microbial* 2017; 55: 3454 – 3465
16. Coppin M, Connately A, Antonelli A, et al. A simple phenotypic method for screening of MCR-1-mediated colistin resistance. *Clin Microbiol Infect* 2018; 24: 201.e1 - e201.e3
17. Gonzales Escalante E, Yauri Condor K, Di Conza JA, et al. (2020). Phenotypic detection of plasmid-mediated colistin resistance in *Enterobacteriaceae*. *J Clin Microbiol* 2020; 58:e01555-19.

https://doi.org/10.1128/JCM.01555-19.

18. Gwozdzinski K, Azarderakhsh S, Imirzalioglu C, et al. An improved medium for colistin susceptibility testing. *J Clin Microbiol* 2018; 56:e01950-17. https://doi.org/10.1128/JCM.01950-17.
19. Abd El-Baky RM, Masoud SM, Mohamed DS, et al. Prevalence and some possible mechanisms of colistin resistance among multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa*. *Infect Drug Resist* 2020;13: 323–332
20. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version10.0. 2020. http://www.eucast.org
21. Terveer EM, Nijhuis RHT, Crobach MJT, et al. Prevalence of colistin resistance gene (mcr1) containing Enterobacteriaceae in feces of patients attending a tertiary care hospital and detection of a mcr-1 containing, colistin susceptible E. coli. PLoS ONE 2017; 12(6): e0178598. https://doi.org/10.1371/journal.pone.0178598
22. Jiang Y, Zhang Y, Lu J, et al. Clinical relevance and plasmid dynamics of *mcr-1*-positive *Escherichia coli* in China: a multicentre case-control and molecular epidemiological study. *Lancet Microbe* 2020; 1: e24–33
23. Principe L, Piazza A, Mauri C, et al. Multicenter prospective study on the prevalence of colistin resistance in *Escherichia coli*: relevance of *mcr-1*-positive clinical isolates in Lombardy, Northern Italy. *Infect Drug Resist* 2018; **11:** 377–85
24. Ibadin EE, Omoregie R, Igbarumah IO, et al. Prevalence of extended spectrum β-lactamase, AmpC β-lactamase and metallo-β-lactamase among Gram negative bacilli recovered from clinical specimens in Benin City, Nigeria. *Int J Enteric Pathog.* 2017; 5(3): 85 – 91
25. Zarkotou O, Pournaras S, Voulgari E, et al. Risk factors and outcomes associated with acquisition of colistin-resistant KPC-producing *Klebsiella pneumoniae*: a matched case-control study. *J Clin Microbiol.* 2010;48(6):2271-2274
26. Wang Y, Tian GB, Zhang R, et al. Prevalence, risk factors, outcomes, and molecular epidemiology of *mcr-1*-positive Enterobacteriaceae in patients and healthy adults from China: an epidemiological and clinical study. *Lancet Infect Dis* 2017; **17:** 390–99.
27. Okeke IN, Lamikaran A, Edelman R. Socio-economic and behavioural factors leading to acquired bacterial resistance to antibiotics in developing countries. *Emerg Infect Dis* 1999; 5 (1): 18 – 27.
28. Omoregie R, Eghafona NO. Urinary tract infection among asymptomatic HIV patients in Benin City. Nigeria. *Br J. Biomed Sci.* 2009; 166 (4):190 – 193.
29. Ogbolu DO. Impact of ESBLs and CREs – the Nigerian experience. *APUA News Lett*. 2013; 31 (2): 15 – 16
30. Nigeria Centre for Disease and Control (NCDC). Antimicrobial Use and Resistance in Nigeria: situation analysis and recommendations. 2017. Available at:

https://www.ncdc.gov.ng/themes/common/docs/protocols/56_1510840387.pdf

31. Ogbolu DO, Daini OA, Ogunledun A, et al. High levels of multidrug resistance in clinical isolates of Gram negative pathogens from Nigeria. *Int J. Antimicrob Agents* 2011; 37 (1): 62 – 66.
32. Wang X, Zhai W, Li J, et al. Presence of an *mcr-3* variant in *Aeromonas caviae, Proteus mirabilis*, and *Escherichia coli* from one domestic duck. *Antimicrob Agents Chemother* 2018; 62:e02106-17. https://doi.org/10.1128/AAC.02106-17.
33. Hmede Z, Kassem, II. First report of the plasmid-borne colistin resistance gene (mcr-1) in *Proteus mirabilis* isolated from a toddler in non-clinical settings. IDCases 2019; 18: e00651
34. Hindler JA, Humphries RM. Colistin MIC variability by method for contemporary clinical isolates of multidrug resistant Gram-negative bacilli. *J Clin Microbiol* 2013; 51:1678 –1684.
35. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 10th ed. M7-A10. Clinical and Laboratory Standards Institute, Wayne, PA. 2015.
36. Richter SS, Karichu J, Otiso J, et al. Evaluation of Sensititre broth microdilution plate for determining the susceptibility of carbapenem-resistant *Klebsiella pneumoniae* to polymyxins. *Diagn Microbiol Infect Dis* 2018; 91:89–92.
37. Ezadi F, Ardebili A, Mirnejad R. Antimicrobial susceptibility testing for polymyxins: challenges, issues, and recommendations. J Clin Microbiol 2019; 57:e01390-18. https://doi.org/10.1128/JCM.01390-18.