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PII: S2213-7165(21)00136-3
DOI: https://doi.org/10.1016/j.jgar.2021.04.030
Reference: JGAR 1586

To appear in: *Journal of Global Antimicrobial Resistance*

Received date: 28 January 2021
Revised date: 7 April 2021
Accepted date: 22 April 2021

Please cite this article as: Anyanwu M.U. , Marrollo R. , Paolucci M. , Brovarone F. , Nardini P. , Chah K.F. , Shoyinka S.V.O. , Carretto E , Isolation and characterization of colistin-resistant Enterobacterales from chickens in Southeast Nigeria, *Journal of Global Antimicrobial Resistance* (2021), doi: https://doi.org/10.1016/j.jgar.2021.04.030

Isolation and characterization of colistin-resistant Enterobacterales from chickens in Southeast Nigeria

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RUNNING TITLE: colistin-resistant Enterobacterales in Nigeria

KEYWORDS: antimicrobial resistance, Enterobacterales, Nigeria, *mcr*-1, mobile colistin resistance, *mcr*-1.22

HIGHLIGHTS

- Demonstration of *mcr*-1 circulation in poultry birds in a wide area of Southeast Nigeria.
- New allelic variant (*mcr*-1.22) detected.
- Among 22 *mcr*-1 positive *E. coli*, MLST distinguished 11 fingerprints, three of them new.
- Urgent need for educational projects and strategies to regulate colistin use in Nigeria.

ABSTRACT

Background – The resistance to colistin mediated by mobile elements had a broad impact worldwide. There is an intensified call for epidemiological surveillance of *mcr* in different reservoirs to preserve colistin for future generations. In Nigeria, the poultry industry is a key livestock sector. This study was undertaken to screen putative colistin-resistant Enterobacterales from poultry birds in Southeast Nigeria and determine the genetic relatedness of *mcr*-harboring isolates.

Methods – Faecal and cloacal swab samples (n=785) were collected from chickens in 17 farms located in three contiguous states in Southeast Nigeria between March and November 2018. After selective cultures, colistin-resistant Enterobacterales were isolated. On these isolates, confirmation of colistin resistance, antibiotic susceptibility tests, molecular detection of genes *mcr*-1 to *mcr*-10, strain typing by multilocus sequence typing (MLST), and randomly amplified polymorphic DNA (RAPD) were

carried out. A questionnaire was distributed to investigate the chicken farm caretakers' knowledge and colistin use.

Results – Forty-five (5.7%) out of the 785 samples evaluated were positive for 48 colistin-resistant Enterobacterales, and among them, 23 harbored the *mcr*-1 gene (22 *E. coli*, 1 *Klebsiella pneumoniae*). In two *E.coli* isolates, a new allelic variant (*mcr*-1.22) was detected. RAPD allowed for the identification of 11 different fingerprints. MLST also revealed 11 STs, three of them new.

Conclusion – *Mcr* significantly spread in poultry birds of Southeast Nigeria, which poses worrisome risks to veterinary and human health. Strategies to prevent the indiscriminate use of colistin in farms should be quickly adopted before colistin resistance becomes a huge global health issue.

1.0 - INTRODUCTION

The global spread of multi-drug resistant Gram-negative bacteria (GNB) has led to a significant limitation in the therapeutic options available. Colistin (CST) has been considered the last option to treat severe infections caused by multi-drug resistant (MDR) *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and carbapenem-resistant Enterobacterales [1].

Until recently, CST resistance was always thought to be chromosomally encoded and mutationally acquired, allowing vertical transmission only, and thus, by its very nature, rare and self-limiting [2]. A plasmid-mediated *mcr*-1 gene, encoding for a phosphoethanolamine transferase, was recently described [3], and this mechanism of

resistance was quickly reported worldwide [4]. In recent years, a growing number of *mcr* genes (namely, from *mcr*-2 to *mcr*-10) have been characterized, allowing to postulate a rapid evolution of the *mcr* family under selective pressures, which raise global health concerns [5].

There is an intensified call for epidemiological surveillance of *mcr* in different reservoirs to devise effective strategies for curbing the spread of superbugs and preserving CST for future generations [6].

Sub-Saharan Africa is a hotspot for the development and spread of resistant microorganisms because the countries are major culprits engaging in practices that stimulate antimicrobial resistance [7]. Poultry and their products are important reservoirs of *mcr*-harboring Enterobacterales worldwide, including in Africa [8].

In Nigeria, the poultry industry is a key livestock sector, providing 25% of animal protein and huge employment opportunities for the burgeoning population, but the industry has been constrained by invasive intestinal infections/diseases involving MDR or extensively-drug resistant (XDR) GNB [9]. There are many reports on the isolation of extended-spectrum beta-lactamases (ESBL) and AmpC-producing Enterobacterales from poultry and poultry meat in Nigeria [10, 11].

Thus, detailed surveillance of *mcr* in the Nigerian livestock industry is urgently warranted. This study was undertaken to screen putative colistin-resistant Enterobacterales (CST-r-E) from poultry birds at farms in Southeast of Nigeria (SEN), by evaluation of phenotypic antimicrobial susceptibility, detection of *mcr* genes (*mcr*-1 to *mcr*-10), also determining the genetic relatedness of *mcr*-harboring isolates.

2.0 – MATERIALS AND METHODS

2.1 – Collection of microbial isolates

Nonduplicate fecal and cloacal swab samples from chickens (broilers and layers) were collected from March to November 2018 in three contiguous states in SEN: Enugu (ENU), Ebonyi (EBO), and Anambra (ANA) (Figure 1). Seventeen poultry farms (stocking capacity at these sites: 200-1000 birds) were sampled by convenience in ENU (7 farms), EBO (5), and ANA (5). From each farm, 5% of the total flock were randomly selected.

Samples were transported in ice packs and processed on the day of collection in the Veterinary Microbiology Laboratory, Department of Veterinary Pathology and Microbiology, University of Nigeria. Swabs were inoculated on selective MacConkey agar (MCA, Oxoid, UK), containing 4 µg/mL colistin sulfate (Sigma-Aldrich, St. Louis, USA) and incubated at 37°C for 18-24 hours in ambient air. A single lactose-fermenting colony per morphotype per sample was then picked up and subcultured on selective MCA and incubated at 37°C for 18-24 hours. The isolates were later identified through phenotypical tests such as citrate reaction, growth on triple sugar iron, and eosin methylene blue agar.

The isolates grown on MCA with morphology suggestive for *Klebsiella* species (lactose-fermenting, mucoid, large shiny and dark pink) or *Escherichia coli* (lactose-fermenting, dry, donut shaped, dark pink) were selected and inoculated on nutrient agar slant and stored at 4°C until their shipment to the reference laboratory (the Microbiology

Laboratory of the IRCCS Arcispedale Santa Maria Nuova, Reggio Emilia, Italy). At their arrival, the isolates were subcultured on Columbia blood agar (CBA) with 5% sheep blood (Becton Dickinson, USA) and MCA (Becton Dickinson, USA), and pure cultures were identified through the MALDI-ToF technology (Bruker Daltonics, USA) using the protocols recommended by the manufacturer's.

2.2 – Colistin resistance and antimicrobial susceptibility testing (AST)

Minimal inhibitory concentrations (MICs) of colistin for the different isolates were evaluated by using a broth microdilution-based method, the ComASP™ Colistin (Liofilchem, Italy) as previously described [1]. The quality control *E. coli* ATCC-25922^T was used as a control for all susceptibility testing.

Strains identified as susceptible to CST were tested for a second time, subculturing the primary agar slant using a selective enrichment procedure. Briefly, 20 µl of 0.5 McFarland bacterial suspension were inoculated into 5 mL of Trypticase Soy Broth (Becton Dickinson, USA), containing a 10 µg colistin disk (Oxoid Thermo Fisher, USA) put into the broth 60 minutes before. These cultures were incubated overnight at 36°C (ambient air), and one drop further subcultured on CBA and MCA (incubation: 18-20 hours, 36°C, ambient air). All the colonies grown were the identified using MALDI-ToF, and their colistin MIC was determined using the ComASP™ Colistin (Liofilchem, Italy). If the isolates were susceptible to CST, they were discarded from the study.

Isolates with MICs ≥2 µg/mL were considered resistant to CST and stored at -80°C in microbeads (Microbank™, Pro-Lab Diagnostics, USA) for further analyses.

Antimicrobial susceptibility tests were performed on all the CST resistant isolates using the automated instrument Phoenix 100™ (Becton Dickinson, USA), according to the manufacturer's recommendations.

2.3 – DNA extraction

Whole genomic DNA was extracted using DNeasy UltraClean Microbial kit™ (Qiagen, Germany) according to the manufacturer's instructions, and then diluted to obtain a final concentration of 40 ng/µL. The extracted DNA was stored at -20°C for further uses.

2.4 – Determining *mcr* presence and allelic variants

The presence of *mcr* genes from *mcr*-1 to *mcr*-5 was investigated using the multiplex-PCR as described in Rebelo et al. [12], while *mcr*-6 to *mcr*-9 were studied using the multiplex-PCR described in Borowiak [13]. *Mcr*-10 was examined by using a PCR based on the paper of Xu et al. [14]. The protocols are detailed in the supplemental material ("Supplemental material – Material and methods").

To determine the allelic variant of the *mcr*-1 gene, all isolates found positive by PCR underwent further amplification using the primers CLR5-int-F1 and CLR5-int-R1. The primers and the amplification protocols are described in detail in the supplemental material ("Supplemental material – Material and methods"). The PCR products were then purified through an enzymatic method using exonuclease I (*Exo*I) and shrimp alkaline phosphatase (SAP), both manufactured by New England Biolabs, USA.

The purified amplicons were finally sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit™ (ThermoFisher, USA). The resulting sequences were assembled and compared to known *mcr*-1 allelic variants using BioEdit, version 7.2.6 [15].

2.5 – RAPD analyses

RAPD experiments were performed on the *mcr*-1 positive *E. coli*. The primers NP2, NP3, NP4, and NP5 were used, as previously described [16]. Two *E. coli*, previously isolated from clinical samples in the reference laboratory, were included as controls for the discriminatory power of the method. Two profiles were considered different if they showed a single band of diverse size [17]; the bands' intensity was also considered discriminant, if double compared with others at the same molecular weight.

Patterns were analyzed by both naked eyes and the software PyElph 1.4 [18].

2.6 – MLST analysis

It was performed on the *mcr*-1 positive *E. coli* according to the EnteroBase protocols that use seven house-keeping genes as in (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html) and detailed in the supplemental material ("Supplemental material – Material and methods").

The PCR products of the seven different amplifications were then purified using the enzymatic method described above.

The amplification primer pairs were then used for sequencing, as previously described but with specific extension conditions; 60°C for 4 min (primers: *gyrB*, *icd*, *mdh*) or 55°C for 4 minutes (primers *adk*, *fumC*, *purA*, *recA*). The sequences obtained were assembled and analyzed using the software BioEdit, version 7.2.6 [15]. The allelic

profile and subsequent sequence types were determined using the EnteroBase and the Center for Genomic Epidemiology websites (https://cge.cbs.dtu.dk/services/MLST-2.0).

2.7 – Chicken farm caretakers' knowledge of CST use

To investigate the caretakers' awareness and knowledge about the use of CST in their farms, they were asked to complete a survey questionnaire (12 items) about the CST use during their farming practice. It was structured with both open-ended and closed-ended questions and focused on CST. The questionnaire is available as supplemental material ("Supplemental material – Questionnaire").

2.8 – Data analysis

The results of the various tests were entered in a Microsoft Excel™ file. Data on the occurrence of CST resistance, *mcr*-1-carrying Enterobacterales were exported to SPSS (version 15.0) and Graphpad Prism statistical package (version 8.3.1) for analysis. Frequencies, percentages, and 95% Confidence Interval (CI95) of variables were calculated as appropriate. Chi-square ($\chi 2$) was used to determine the possible association between variables and the awareness/knowledge and use of CST by chicken farm caretakers.

3.0 – RESULTS

3.1 – Microbial isolates, colistin resistance and antimicrobial susceptibility testing

A total of 785 birds (498 broilers and 287 layers) were evaluated (ENU, n = 265; EBO, n = 260; and ANA, n = 260).

Seventy-five strains of Enterobacterales isolated from 45 samples (5.7%) were sent to the reference laboratory on agar slants. They were isolated from all the three states, but with a variable proportion from the farms in different locations: 7/7 (100%) in ENU, 4/5 (80%) in ANA, and 3/5 (60%) in EBO states, respectively (Table 1). The isolation rate of CST-r-E was significantly different among the location ($\chi 2 = 22.38$; $P < 0.0001$), being significantly higher in ENU compared with the other locations.

The 45 samples yielded 48 CST-r-E, comprising 32 *E. coli*, 12 *K. pneumoniae* and 4 *Enterobacter cloacae* complex. The distribution of the isolates among the states is shown in Table 1.

The MIC values of colistin for the 48 CST-r-E are presented in Table 2.

Forty-one (91.1%) of the 45 samples yielding CST-r-E were collected from broilers, whereas 4 (8.9%) from layers. The carriage of CST-r-E was therefore significantly different between broilers and layers ($p<0.001$).

Susceptibility tests demonstrate that the *E. cloacae* complex isolates did not express AmpC but were resistant to cotrimoxazole (all the isolates) and to fluoroquinolones, gentamicin and tobramycin (3 out of the four strains). For *K. pneumoniae*, no ESBL or carbapenemase-producing strains were detected. Half of the isolates showed a wild-type antibiotype (susceptible to all the antibiotics except for the intrinsically resistant drugs); the others were resistant to cotrimoxazole (6 out of the 12 strains), fluoroquinolones (4/12), gentamicin and tobramycin (3/12).

Finally, among the 32 *E. coli* tested, none was resistant to carbapenems. Three were ESBL-producers. All the isolates showed various resistance traits: to aminopenicillins

(30 out of the 32 strains), gentamicin and tobramycin (26/32), fluoroquinolones (24/32), and cotrimoxazole (24/32).

3.2 – *Mcr* gene detection

The *mcr*-1 gene was detected in 23 strains (47,9%) out of the 48 CST-r-E analyzed, comprising 22 (68.8%) of the 32 *E. coli* and 1 (8,3%) of 12 *K. pneumoniae* (Table 1). No *mcr*-1 genes were found in *E. cloacae*. Thus, our results demonstrate a *mcr*-1 carriage rate of 2.9% (23/785 isolates) across the Nigerian farming sites sampled. Among the 22 *mcr*-1 positive *E. coli*, the *mcr*-1.1 variant was identified in 20 isolates (90.9%), while a novel *mcr*-1 variant (*mcr*-1.22) was detected in two (8.7%) (Table 1).

The sequence of novel *mcr*-1.22, deposited in GenBank with the accession number MN017134 (https://www.ncbi.nlm.nih.gov/nuccore/MN017134), showed a single nucleotide mutation in position 1277, with thymine instead of cytosine. This nonsynonymous substitution (1277C>T) resulted in the replacement of serine with phenylalanine coding S426F.

None of the CST-r-E was found to harbor *mcr*-2 to *mcr*-10 genes.

Eleven (52.6%) out of 19, 8/9 (88.9%) and 3/4 (75%) CST-r *E. coli* isolates from ENU, ANA and EBO states, respectively, harbored *mcr*-1. The *mcr*-1-positive *E. coli* were isolated from 6 out of the 7 farms sampled in ENU, from 4 out of the 5 farms in ANA, and from 3 out of the 5 farms in EBO, respectively (Table 1). The *mcr*-1 positive *K. pneumoniae* isolate was detected in ENU state. There was no association ($\chi 2 = 1.835$; $p = 0.3995$) between the occurrence of *mcr*-1 positive isolates and the state of sample origin.

3.3 – MLST and RAPD analyses

MLST allowed distinguishing 11 different STs. Eight were known (ST-398, ST-1286, ST-34, ST-48, ST-155, ST-226, ST-6836 and ST-746), and 3 were new (Table 3, supplemental material as "Supplemental material – MLST table").

For *rec*A, two new allelic variants were detected. The analysis performed using the database of the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/MLST-2.0), demonstrated that the closest allelic sequence to those detected was the #6.

In our isolates, three new sequence types were also determined. Again, according to the results obtained using the database of the Center for Genomic Epidemiology, the nearest STs are ST-4542 (for isolates EC-49 and KL-37), ST-168 (for EC-160) and ST-656 (for EC-29 and EC-540).

ST-1286 was the most common sequence type, with a frequency of 31.8% (7/22) followed by ST-34 (3/22, 13.6%). The ST-656, ST-4542 and ST-746 were represented by 2 (9.1%) strains each. Six of these STs: ST-155, ST-226, ST-168, ST-6836, ST-48 and ST-398 were represented by singletons (4.5%).

There was an overlap of ST-656, ST-1286, ST-34 and ST-746 among the states (Figure 2). More than one strain from the same farm belonged to ST-4542 and ST-1286 in ENU and ANA states, respectively.

Twenty of the *E. coli* strains belonged to six known clonal complexes (CC), whereas two strains belonged to undetermined CC. The CC most represented was CC-10, which encompasses 15 strains (68.1%). One strain each (4.5%) belonged to CC-398, CC-155,

CC-165, CC-168 and CC-226. The sequence type distribution among the states is shown in Figure 2.

The MLST results were in agreement with those obtained with RAPD analysis: regarding this technique, all the primers used were able to discriminate among the isolates, resulting in different fingerprints when comparing the control strains and the microorganisms analyzed. NP3 and NP4 were the most efficient in producing highly discriminatory profiles (generation of many bands). The patterns generated by NP4 are shown in Figure 3: 11 different fingerprints (A to K) can be recognized. The strain clusterization was the same as for MLST.

The isolates with the allelic variant *mcr*-1.22 clustered together, having the RAPD pattern J (figure 3) and the new MLST close to ST-4542, which possesses two new allelic variants for *rec*A.

3.4 – Chicken farm caretakers' awareness and use of CST in SEN

Demographically, all the 17 farm caretakers were male, and 12 of them (70.6%) possessed basic secondary school qualifications, 4 (23.5%) were diploma holders, while 1 (5.9%) was a Doctor of Veterinary Medicine. About two-thirds of the farmers had no information about CST (64.7%), and almost all (94.1%) did not know about the CST mechanism of action. There was no association ($p > 0.05$) between knowledge about CST/CST resistance and educational qualification, as well as location.

Concerning practices that can facilitate CST resistance, close to two-thirds (64.7%) of the caretakers reported that drug sellers recommended CST-containing materials used on their farms. The majority of the caretakers (88.2%) said that they use the CST-

supplemented feed on their farm for treating intestinal infections (82.3%) and administer CST-based drugs in birds' drinking water (94.1%). The 88,2% of the farmers declared that they still used CST-based drugs, irrespective of whether, following the previous usage, the birds recovered (64.7%) or died (23.5%). However, more than one-third of the farmers (47.1%) reported using CST-based drugs for prophylaxis. More worrisome, some of them (23.5%) reported that CST-based drugs previously used in their farms are no longer effective.

4.0 – DISCUSSION

Colistin is one of the few last-line antibiotics used in treating deadly infections. Thus, CST resistance in GNB is a global public health crisis needing urgent surveillance in diverse ecological niches to determine the occurrence and devise strategies for solving this global menace. In this study, CST resistance in Enterobacterales colonizing chickens at farm level in SEN was determined. Prior studies in Nigeria, performed before the discovery of mobile resistance and not focused on CST resistance, did not detect CST resistance among enterobacterial isolates from chickens [19]. Recently, Authors reported the presence and the circulation of *mcr* genes in Nigeria [20, 21].

The occurrence of CST-r-E in the study samples is 5.7% (45 out of the 785 samples analyzed), indicating that CST-r-E colonizes a sizeable percentage of chickens in SEN. These results imply that CST resistance has emerged in Nigeria's poultry industry, mainly among broilers (41 out of the 45 CST-r samples), which have unrestricted access

to antimicrobial supplemented feed and water), compared with layers, which are fed twice daily in all the farms sampled.

As stated by some authors, the development of CST resistance may correlate with the frequent/habitual use of CST in the poultry sector [22]. It could be postulated that this resistance trait might have been spreading in Nigeria a long time ago. Veterinary drug formulations containing CST, e.g., Keproceryl®, which contains vitamins and CST (imported from Europe) has been in use in Nigeria for more than 30 years. The delivery of similar products may have exposed the birds to a sub-therapeutic concentration of CST, which was demonstrated to select CST resistance [23, 24], exerting a selective pressure in the poultry sector in SEN.

There is currently no enforced regulation on the importation, marketing, sales, and use of antimicrobials in Nigeria. In this report, we analyzed the farmers' knowledge about CST resistance using a questionnaire. The data collected and detailed in the supplemental materials highlighted that only 35,3% of the farmers knew CST, and only one of them (a veterinarian) in depth. These findings and observations call for immediate attention because of the possible impact of CST resistance on public health. The use of colistin for prophylaxis, metaphylaxis, and growth promotion in livestock production (especially poultry and pigs) is responsible for mobilizing *mcr* in Gram-negative bacteria [25]. Strategies to prevent indiscriminate use of CST in farms should be adopted, and farmers should receive adequate training before CST resistance becomes a huge global health issue.

The occurrence of CST-r-E in the present study cannot be easily compared with data published in other experiences from other countries, because the prevalence is very heterogeneous due to variation in sample type and size, and methods used for primary isolation of the CST resistant organisms, as well as health status and rate of colonization of the birds, and usage of antimicrobial agents in the study areas [26]. Reported prevalence ranged from 0% in chicken reported by El-Garch et al. in Europe, to 52.4% in chicken meat samples analyzed in Brazil [27-31].

In our experience, *mcr*-1 was detected in 23 out of the 48 CST-r isolates, suggesting that the gene is widely spread among Enterobacterales colonizing chickens in SEN. Higher *mcr*-1 detection rates among CST-r-E were observed in Italy (88.1%) [32], whereas lower detection rates were reported from China (1.1%) [33] and Romania (12%) [34]. Regarding *mcr* diffusion among different bacteria, also in our setting, *mcr*-1 spread widely among *E. coli* colonizing chickens. Only 8,3% CST-r *K. pneumoniae* carried *mcr* genes, which suggested a suboptimal transmission of this plasmid to this genus. *E. coli* possesses the greatest propensity for the acquisition of *mcr*-1, and this result is consistent with previous studies [33, 35].

The detection of a novel allelic variant in the present study (*mcr*-1.22), detected in two different isolates from farm #4 in Enugu State, may suggest that the poultry sector could act as a reservoir for *mcr*-1 variants and a potential source for dissemination. The emergence of new allelic variants (their appearance, stabilization, and replication among different hosts, even if in the absence of increasing bacterial virulence or resistance) should be of concern for public health, especially in countries such as

Nigeria, where antimicrobial agents, including the critically-important ones like CST, are used unregulated [36, 37].

In this study, none of the CST-r isolates harbored *mcr*-2 to *mcr*-10, unlike what has been reported in CST-r-E from poultry elsewhere [13, 38-40], probably because those genes are restricted in their distribution in terms of bacterial host species or region [13]. Alternatively, resistance mechanisms not mediated by plasmids, could be postulated.

Regarding the genetic similarity among the isolates, the MLST showed that *mcr*-1-positive *E. coli* in this study belonged to four singletons, four CCs, and two undetermined CCs, thus demonstrating that there was a large genetic diversity among the organisms isolated in different farms and states. Despite this diversity, STs of CC-10 were repeatedly identified among different isolates from farms in the three states, suggesting the successful spread of this particular lineage in SEN. In this study, the dominance of CC-10 meant successful clonal dissemination of this CC in poultry in SEN. Other studies have shown that most *mcr*-1-positive *E. coli* from poultry belong to CC-10 [25, 28].

The overlapping of some STs (ST-656, ST-1286, ST-34, and ST-746) among states may suggest that *mcr*-1-carrying *E. coli* from different farms are clonally-related, probably emerging from similar lineages. Cross-contamination of the farms with the strains through vectors such as flies, human carriers, or poultry birds may explain this finding. It is also possible that birds were colonized by the overlapped clonal strains which

originated from the same parent stocks or hatcheries (as day-old-chicks), previously contaminated [41].

Interestingly, the two *mcr*-1.22 carrying *E. coli* belonged to CCs not yet on the Enterobase website, showing a new allelic variant for *rec*A. Unfortunately, we could not have a definition of the new CCs, since the sequences' data are processed by Enterobase only if provided by using next-generation sequencing methods.

The RAPD fingerprints confirmed the high genetic diversity of *mcr*-1 positive *E. coli*. The wide variety of RAPD clusters (11) demonstrates the dissemination of *mcr*-1 via plasmids [27]. The RAPD profiles agreed with the MLST, showing an excellent discriminatory power of the primers selected.

The present study has as a significant limitation the lack of a WGS approach. This did not allow an in-depth investigation of our isolates, making impossible the extensive characterization of the STs (mostly new STs), the study of the genetic backbone, as well as the plasmid type of the isolates. Moreover, the strain selection was initially focused only to *Escherichia coli* and *Klebsiella pneumoniae*, which were the microorganisms most frequently harboring *mcr*-1 or *mcr*-3. Recently discovered mcr genes have been often isolated in Enterobacterales belonging to other genera (e.g., mcr-10 in Enterobacter species), and therefore our study may underestimate the epidemiology of these genes. Finally, the questionnaire could have been improved, including more general questions about bacterial resistance and on the knowledge of how resistant isolates are generated and spread, and not being focused only on CST.

Except for 3 *E. coli* isolates, which were ESBL positive, the CST-r-E isolated in our setting did not express other important antimicrobial resistance traits. It would be desirable that this favorable situation is preserved, e.g., through the establishment of antibacterial stewardship programs. If this is not urgently done, the possible emergence of enterobacteria coproducing *mcr*, ESBL, AmpC and/or carbapenemases would definitely result in difficult-to-treat diseases capable of causing outrageously high economic losses in animal and public sectors in Nigeria.

In conclusion, chickens raised in SEN are potential reservoirs of CST-r-E. The new STs and CCs found in our settings could be new *E. coli* lineages, capable of spreading *mcr* genes, including novel ones, posing worrisome risks to the health of the birds, other animals, and the public. The food animal sector is a potential source for disseminating superbugs to the human-environmental ecosystem in Nigeria. Individuals who make direct contact with these birds, such as the caretakers/handlers, veterinarians, slaughterhouse personnel, and meat sellers, are at greater risk of acquiring these organisms and then transferring them to their households/the public. Further studies involving human isolates are needed.

ACKNOWLEDGMENTS

The Authors would like to thank Jonathan Tyrrell and Timothy Walsh for their support and their keep believing in them. Thanks to Jon for his suggestions and for the revision of the Material and Methods section.

AUTHOR CONTRIBUTIONS

AMU conceived and designed the study, collected samples, performed the microbiological analyses in Nigeria and wrote the manuscript draft; PM and NP discussed and revised the manuscript; MR performed the microbiological and molecular analyses and elaborated the results; BF performed the molecular analyses; CKF and SSVO evaluated the results and discussed the findings, also working on the manuscript draft; CE conceived the study, analyzed and discussed the results, and finalized the final version of the manuscript.

FUNDING

This research did not receive external funding.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not required

CONFLICTS OF INTEREST

All the authors declare no conflict of interest.

REFERENCES

1. Carretto E, Brovarone F, Russello G, Nardini P, El-Bouseary MM, Aboklaish AF, et al. Clinical validation of SensiTest Colistin, a broth microdilution-based method to evaluate colistin MICs. J Clin Microbiol 2018;56. https://doi.org/10.1128/JCM.01523-17

2. Landman D, Georgescu C, Martin DA, Quale J. Polymyxins revisited. Clin Microbiol Rev 2008;21:449-65. https://doi.org/10.1128/CMR.00006-08

3. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, 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;16:161-8. https://doi.org/10.1016/S1473-3099(15)00424-7

4. Schwarz S and Johnson AP. Transferable resistance to colistin: a new but old threat. J Antimicrob Chemother 2016;71:2066-70. https://doi.org/10.1093/jac/dkw274

5. Li Y, Dai X, Zeng J, Gao Y, Zhang Z, Zhang L. Characterization of the global distribution and diversified plasmid reservoirs of the colistin resistance gene *mcr*-9. Sci Rep 2020;10:8113. https://doi.org/10.1038/s41598-020-65106-w

6. Al-Tawfiq JA, Laxminarayan R, Mendelson M. How should we respond to the emergence of plasmid-mediated colistin resistance in humans and animals? Int J Infect Dis 2017;54:77-84. https://doi.org/10.1016/j.ijid.2016.11.415

7. Kariuki S and Dougan G. Antibacterial resistance in sub-Saharan Africa: an underestimated emergency. Ann N Y Acad Sci 2014;1323:43-55. https://doi.org/10.1111/nyas.12380

8. Apostolakos I and Piccirillo A. A review on the current situation and challenges of colistin resistance in poultry production. Avian Pathol 2018;47:546-58. https://doi.org/10.1080/03079457.2018.1524573

9. Fagbamila IO, Barco L, Mancin M, Kwaga J, Ngulukun SS, Zavagnin P, et al. *Salmonella* serovars and their distribution in Nigerian commercial chicken layer

farms. PLoS One 2017;12:e0173097. https://doi.org/10.1371/journal.pone.0173097

10. Chah KF, Ugwu IC, Okpala A, Adamu KY, Alonso CA, Ceballos S, et al. Detection and molecular characterisation of extended-spectrum beta-lactamase-producing enteric bacteria from pigs and chickens in Nsukka, Nigeria. J Glob Antimicrob Resist 2018;15:36-40. https://doi.org/10.1016/j.jgar.2018.06.002

11. Ojo OE, Schwarz S, Michael GB. Detection and characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* from chicken production chains in Nigeria. Vet Microbiol 2016;194:62-8. https://doi.org/10.1016/j.vetmic.2016.04.022

12. Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, et al. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5 for surveillance purposes. Euro Surveill 2018;23. https://doi.org/10.2807/1560-7917.ES.2018.23.6.17-00672

13. Borowiak M, Baumann B, Fischer J, Thomas K, Deneke C, Hammerl JA, et al. Development of a novel *mcr*-6 to *mcr*-9 multiplex PCR and assessment of *mcr*-1 to *mcr*-9 occurrence in colistin-resistant *Salmonella enterica* isolates from environment, feed, animals and food (2011-2018) in Germany. Front Microbiol 2020;11:80. https://doi.org/10.3389/fmicb.2020.00080

14. Xu T, Zhang C, Ji Y, Song J, Liu Y, Guo Y, et al. Identification of *mcr*-10 carried by self-transmissible plasmids and chromosome in *Enterobacter roggenkampii*

strains isolated from hospital sewage water. Environ Pollut 2021;268:115706. https://doi.org/10.1016/j.envpol.2020.115706

15. Hall TA. BioEdit: a user friendly biological sequence editor and analysis program for Windows 95/98/NT. Nucl. Acids Sysmp. Ser. 1999;41.

16. Barbarini D, Russello G, Brovarone F, Capatti C, Colla R, Perilli M, et al. Evaluation of carbapenem-resistant Enterobacteriaceae in an Italian setting: report from the trench. Infect Genet Evol 2015;30:8-14. https://doi.org/10.1016/j.meegid.2014.11.025

17. van Belkum A, Tassios PT, Dijkshoorn L, Haeggman S, Cookson B, Fry NK, et al. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect 2007;13 Suppl 3:1-46. https://doi.org/10.1111/j.1469-0691.2007.01786.x

18. Pavel AB and Vasile CI. PyElph - a software tool for gel images analysis and phylogenetics. BMC Bioinformatics 2012;13:9. https://doi.org/10.1186/1471-2105-13-9

19. Adelowo OO, Fagade OE, Agerso Y. Antibiotic resistance and resistance genes in *Escherichia coli* from poultry farms, southwest Nigeria. J Infect Dev Ctries 2014;8:1103-12. https://doi.org/10.3855/jidc.4222

20. Ngbede EO, Poudel A, Kalalah A, Yang Y, Adekanmbi F, Adikwu AA, et al. Identification of mobile colistin resistance genes (*mcr*-1.1, *mcr*-5 and *mcr*-8.1) in Enterobacteriaceae and *Alcaligenes faecalis* of human and animal origin, Nigeria. Int J Antimicrob Agents 2020;56:106108. https://doi.org/10.1016/j.ijantimicag.2020.106108

21. 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;23:401-5. https://doi.org/10.4314/jasem.v23i3.5

22. Kim S, Woo JH, Kim N, Kim MH, Kim SY, Son JH, et al. Characterization of chromosome-mediated colistin resistance in *Escherichia coli* isolates from livestock in Korea. Infect Drug Resist 2019;12:3291-9. https://doi.org/10.2147/IDR.S225383

23. Founou LL, Founou RC, Essack SY. Antibiotic resistance in the food chain: a developing country-perspective. Front Microbiol 2016;7:1881. https://doi.org/10.3389/fmicb.2016.01881

24. Sharafi T and Ardebili A. Plastic binding feature of polymyxins: the effect on MIC susceptibility measurements. Infect Drug Resist 2019;12:2649-53. https://doi.org/10.2147/IDR.S219130

25. Liu Y and Liu JH. Monitoring colistin resistance in food animals, an urgent threat. Expert Rev Anti Infect Ther 2018;16:443-6. https://doi.org/10.1080/14787210.2018.1481749

26. Gelbicova T, Barakova A, Florianova M, Jamborova I, Zelendova M, Pospisilova L, et al. Dissemination and comparison of genetic determinants of *mcr*-mediated colistin resistance in Enterobacteriaceae via retailed raw meat products. Front Microbiol 2019;10:2824. https://doi.org/10.3389/fmicb.2019.02824

27. El Garch F, de Jong A, Bertrand X, Hocquet D, Sauget M. *Mcr*-1-like detection in commensal *Escherichia coli* and *Salmonella* spp. from food-producing animals at slaughter in Europe. Vet Microbiol 2018;213:42-6. https://doi.org/10.1016/j.vetmic.2017.11.014

28. Elbediwi M, Li Y, Paudyal N, Pan H, Li X, Xie S, et al. Global burden of colistin-resistant bacteria: mobilized colistin resistance genes study (1980-2018). Microorganisms 2019;7. https://doi.org/10.3390/microorganisms7100461

29. Fernandes MR, Moura Q, Sartori L, Silva KC, Cunha MP, Esposito F, et al. Silent dissemination of colistin-resistant *Escherichia coli* in South America could contribute to the global spread of the *mcr*-1 gene. Euro Surveill 2016;21. https://doi.org/10.2807/1560-7917.ES.2016.21.17.30214

30. Kurekci C, Aydin M, Nalbantoglu OU, Gundogdu A. First report of *Escherichia coli* carrying the mobile colistin resistance gene *mcr*-1 in Turkey. J Glob Antimicrob Resist 2018;15:169-70. https://doi.org/10.1016/j.jgar.2018.09.013

31. Pishnian Z, Haeili M, Feizi A. Prevalence and molecular determinants of colistin resistance among commensal Enterobacteriaceae isolated from poultry in North-West of Iran. Gut Pathog 2019;11:2. https://doi.org/10.1186/s13099-019-0282-0

32. Alba P, Leekitcharoenphon P, Franco A, Feltrin F, Ianzano A, Caprioli A, et al. Molecular epidemiology of *mcr*-encoded colistin resistance in Enterobacteriaceae from food-producing animals in Italy revealed through the EU Harmonized Antimicrobial Resistance Monitoring. Front Microbiol 2018;9:1217. https://doi.org/10.3389/fmicb.2018.01217

33. Wang X, Liu Y, Qi X, Wang R, Jin L, Zhao M, et al. Molecular epidemiology of colistin-resistant Enterobacteriaceae in inpatient and avian isolates from China: high prevalence of *mcr*-negative Klebsiella pneumoniae. Int J Antimicrob Agents 2017;50:536-41. https://doi.org/10.1016/j.ijantimicag.2017.05.009

34. Maciuca IE, Cummins ML, Cozma AP, Rimbu CM, Guguianu E, Panzaru C, et al. Genetic Features of *mcr*-1 mediated colistin resistance in CMY-2-producing *Escherichia coli* from Romanian poultry. Front Microbiol 2019;10:2267. https://doi.org/10.3389/fmicb.2019.02267

35. Forde BM, Zowawi HM, Harris PNA, Roberts L, Ibrahim E, Shaikh N, et al. Discovery of *mcr*-1-mediated colistin resistance in a highly virulent *Escherichia coli* lineage. mSphere 2018;3. https://doi.org/10.1128/mSphere.00486-18

36. Musa BM, Imam H, Lendel A, Abdulkadir I, Gumi HS, Aliyu MH, et al. The burden of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Nigeria: a systematic review and meta-analysis. Trans R Soc Trop Med Hyg 2020;114:241-8. https://doi.org/10.1093/trstmh/trz125

37. Oloso NO, Fagbo S, Garbati M, Olonitola SO, Awosanya EJ, Aworh MK, et al. Antimicrobial resistance in food animals and the environment in Nigeria: a review. Int J Environ Res Public Health 2018;15. https://doi.org/10.3390/ijerph15061284

38. Borowiak M, Hammerl JA, Deneke C, Fischer J, Szabo I, Malorny B. Characterization of *mcr*-5-harboring *Salmonella enterica* subsp. *enterica* serovar typhimurium isolates from animal and food origin in Germany. Antimicrob Agents Chemother 2019;63. https://doi.org/10.1128/AAC.00063-19

39. Sobur MA, Ievy S, Haque ZF, Nahar A, Zaman SB, Rahman MT. Emergence of colistin-resistant *Escherichia coli* in poultry, house flies, and pond water in Mymensingh, Bangladesh. J Adv Vet Anim Res 2019;6:50-3. https://doi.org/10.5455/javar.2019.f311

40. Wang X, Zhai W, Li J, Liu D, Zhang Q, Shen Z, 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. https://doi.org/10.1128/AAC.02106-17

41. Vinueza-Burgos C, Ortega-Paredes D, Narvaez C, De Zutter L, Zurita J. Characterization of cefotaxime resistant *Escherichia coli* isolated from broiler farms in Ecuador. PLoS One 2019;14:e0207567. https://doi.org/10.1371/journal.pone.0207567

Table 1 – Study synopsis

	ENUGU	ANAMBRA	EBONYI	TOTAL
Farms included (nr.)	7	5	5	17
Farms with isolation of CST-r (nr, %)	7 (100%)	4 (80%)	3 (60%)	14 (82%)
Samples collected (nr)	265	260	260	785
Broilers	160	160	150	470
Layers	105	100	110	315
N. positive samples (%)	29 (10.9%)	12 (4.6%)	4 (1.5%)	45 (5.7%)
Broilers	27	10	4	41
Layers	2	2	0	4
N. isolates resistant to Colistin	31	12	5	48
E. coli	19	9	4	32
K. pneumoniae	11	1	0	12
E.cloacae	1	2	1	4
N. *mcr*-1.1 positive isolates	10	8	3	21
E.coli	9	8	3	20
K.pneumoniae	1	0	0	1
N. *mcr*-1.22 positive isolates	2	0	0	2
E. coli	2	0	0	2

Table 2 - MIC values of the isolates showed to be resistant to colistin

	Colistin MIC values (µg/ml)			
	4	**8**	**16**	**≥16**
Enterobacter cloacae complex	0	0	0	4
Escherichia coli	20	10	4	0
Klebsiella pneumoniae	1	0	4	7

Table 3 – Molecular characterization of the *mcr*-positive *E. coli* isolated in the study

Location (nr. of strains)	Source	Strain ID	RAPD cluster	MLST	Clonal complex
Enugu (11)	1-1	KL13	C	ST34	10
	1-1	EC11-A	B	ST1286	10
	1-1	EC15	D	ST155	155†
	1-2	EC18	F	ST226	226†
	1-3	EC19	B	ST1286	10
	1-3	EC29	G	ST656*	10
	1-4	KL37‡	J	ST4542*	ND
	1-4	EC49‡	J	ST4542*	ND
	1-5	EC160	E	ST168*	168†
	1-6	EC100	A	ST398	398†
	1-6	EC131-A	C	ST34	10
Anambra (8)	2-1	EC377	H	ST6836	165
	2-2	EC400	I	ST746	10
	2-3	EC540	G	ST656*	10
	2-3	EC602	B	ST1286	10
	2-3	EC603	B	ST1286	10
	2-3	EC605	K	ST48	10
	2-3	EC601	B	ST1286	10
	2-5	EC612	B	ST1286	10
Ebonyi (3)	3-1	EC700	K	ST746	10
	3-2	EC734	C	ST34	10
	3-5	EC791	B	ST1286	10

Legenda - * new sequence types: it is shown the ST that has the nearest correspondence according to the Center for Genomic Epidemiology; † = singleton; ǂ = *mcr*-1.22-carrying strain; ND = not determined

Figure 1 – Geographical setting of the study. The red line defines the Southeast region of Nigeria. The blu points indicate the three States where the study was performed.

Figure 2 – Geographical distribution of the 11 STs of the 22 *mcr*-positive *E. coli*

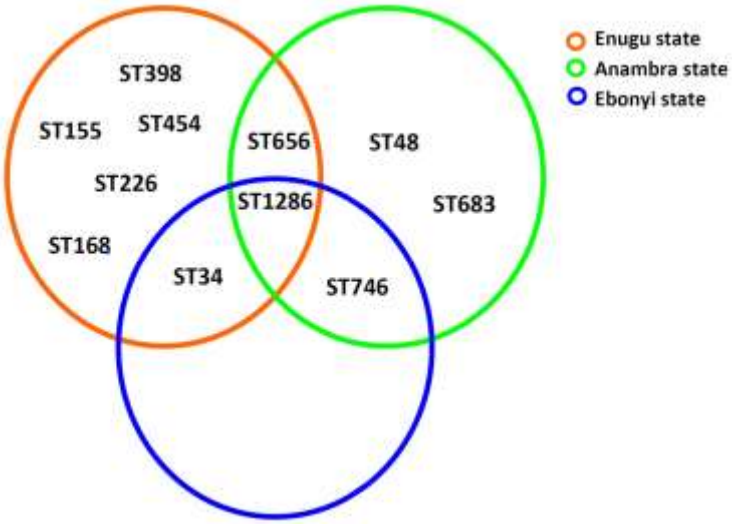

Figure 3 – RAPD fingerprinting obtained with primer NP4. M = molecular weight markers. Left side, 100-3000 bp (bright bands at 500bp and 1000bp); right side, 100-600 bp (more bright, band at 400bp). IC1 and IC2, isolates used as internal controls. C-, negative control. In the different wells, the analyzed isolates and, bottom, the fingerprint differentiation (as capital letters).