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OCCURRENCE OF TET GENE-BEARING ANTIMICROBIAL-RESISTANT *ESCHERICHIA COLI* FROM DAIRY FARMS IN NIGERIA

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ABSTRACT

This study focused on antibiotic resistance genes as emerging contaminants with potential global human health implications. Intensive livestock farming has been identified as a major contributor to the spread of resistant bacteria and genes. The study examined antimicrobial-resistant *Escherichia coli* and tetracycline-resistant genes in raw milk from commercial dairy farms in Kano State. Out of 300 registered farms, 54 (18 %) were purposively sampled for the study. A total of 313 milk samples were collected and processed through enrichment and inoculation on selective media for *Escherichia coli* isolation. The antibiogram pattern of the isolated *Escherichia coli* strains was assessed using the disk diffusion method. The results revealed resistance to various antimicrobial agents, with no resistance to quinolones but high resistance to ampicillin (100 %), erythromycin (73.3 %), and tetracycline (46.7 %), among others. The multiplex polymerase chain reaction was conducted on all *Escherichia coli* isolates to detect tet genes (tet A, B, C, D, and M), and one isolate carried the tet M resistance gene, while six (40 %) others carried the tet A resistance gene. The study concludes that a significant proportion of the cultured *Escherichia coli* strains were resistant to one or more tested antibiotics, indicating a potential public health threat associated with *Escherichia coli* contamination in raw milk. We recommend implementing robust regulatory policies governing the use and sales of antimicrobials in animal production. Furthermore, we suggest further investigation into other resistant genes that these isolates might carry to better understand the extent of antibiotic resistance in the region.

Key words: antimicrobial resistance; dairy cows; *Escherichia coli*; raw milk

INTRODUCTION

Nigeria ranks amongst the fastest-growing populations in the world, with estimates indicating that the country's population will be above 350 million by the year 2050 [15]. Currently, the country is the most populous in Africa [30]. Meeting the food demand of such a large population requires accelerating food production including intensification of both arable and food animal production [7]. Intensive livestock and fish production favour the highest level of use of chemical and veterinary drugs to combat

and prevent diseases, improve factors of production, and in the management of stress [2]. Several studies have shown a close association between intensive veterinary drug use and the emergence and spread of antimicrobial-resistant pathogens and the occurrence of their residues in edible animal products [13]. Antimicrobial Resistance (AMR) is a major global health challenge of the 21st century [3]. Antibiotic resistance genes (ARGs) are emerging contaminants posing a potential worldwide human health risk [31]. Intensive livestock farming has been identified as a major factor in the dissemination of resistant bacteria and antimicrobial resistance genes (ARGs) [8].

Many interventions in the livestock and food production sector in developing countries like Nigeria sought to transform smallholder operations into intensive ventures [18]. One of the intervention programs that were able to boost local productivity and farmer's capacity, is the Federal Government of Nigeria Commercial Dairy Project, evidently increasing the output of milk and milk products in regions where the program operates [12]. The operations programs were characterized by inappropriate and indiscriminate use of veterinary drugs, in addition to poor drug use regulations and control in the country [10]. These factors are well-recognized enablers of the emergence and spread of resistant pathogens and the dissemination of ARGs coding for resistance amongst animals, potentially zoonotic, and environmental bacteria [28]. The contributions of these enhanced dairy production initiatives in the spread of resistant pathogens and ARGs in Nigeria have not been studied [29]. However, local literature abounds with reports of contamination of milk and milk products with pathogenic and often zoonotic [25]. Milk and milk products can be contaminated with antimicrobial-resistant bacteria or resistant genes from the environment, and during processing, handling, transportation, and storage [20].

The occurrence of antimicrobial-resistant bacteria and antimicrobial-resistant genes in milk compromises the quality of the product, a situation that will affect the marketability of the product and its wholesomeness [32]. The incidence of antimicrobial-resistant bacteria and ARGs in the dairy supply chain is of great concern [6]. This will defeat the aim of the intervention given that infection with the resistant pathogen is bound to increase the cost of production and can make it difficult for the farmers to repay their loans and access further grants, in addition to other socio-economic costs.

Recently in 2019, the country began implementing a National Action Plan against AMR modelled along the recommendations of the Global Action Plan that offers a One Health platform for combating AMR in human and animal health. Sustained surveillance at all fronts of livestock, poultry, and fish production is essential for detecting the emergence and trend of resistance in priority zoonotic pathogens. Most of the information on AMR from animal food production systems is from poultry production, and a limited number of reports [1, 23] described the occurrence of important reservoirs of antibiotic-resistant genes in Nigerian poultry production environments. They have also provided evidence of the spread of such genes from poultry farms to human populations via manure and water [21]. This study provides insight into the problem of antimicrobial resistance to *Escherichia coli* in dairy production in one of the major milk-producing regions of Nigeria [5].

MATERIALS AND METHODS

Sampling procedure/sample collection

This was a cross-sectional study and purposive sampling was conducted. Three hundred commercial dairy farms were registered under the commercial dairy project by the Kano State Agricultural and Rural Development Authority (KNARDA). Eighteen percent (18 %) of the total dairy-registered farms were sampled giving a total of fifty-four (54) farms. This was based on a pilot study that was earlier conducted. A systematic random sample was employed in each farm visited, whereby every 3rd cow in the milking parlour or milking arena was sampled. The animals were sampled according to how they were arranged in the milking parlour sometimes the milking sequence of the farmers was followed. Hand milking procedure was used to extract milk from all four quarters of the udder with the exception of two farms that employed the machine milking process. The udder was thoroughly cleaned and decontaminated with clean water and soap. Also, the hands of the milker were washed with chlorhexidine soap. The udder and milker's hands were then dried properly with a sanitary towel and each cow was restrained properly in order to collect the milk sample. The milk was collected from all four quarters of the mammary glands. Approximately 25 ml of raw milk was collected from each quarter

in a sterile sample bottle giving a total of 100 ml per cow. Twenty-five percent of the milking cows were sampled from each farm. The milk samples collected were stored on ice packs and transported to the Department of Veterinary Public Health and Preventive Medicine Laboratory, Ahmadu Bello University Zaria, where they were analysed 4–6 hours after collection.

Isolation and identification of *E. coli* from milk

Enrichment of sample

Lauryl sulphate tryptose (LST) broth containing MUG (4-methylumbelliferyl-B-D-glucuronide) was used for the enrichment. It was done according to Manafi [22]. One millilitre (1 ml) of the milk sample was dispensed into 5 ml of the broth (LST) in test tubes containing Durham's tubes and incubated for 24 hours at 37 °C and observed for fermentation/gas formation in the test tubes. This was evident by the gas that was trapped at the top of the Durham's tubes which pushed the media down.

Inoculation and incubation of samples

Exactly 0.1 ml of the incubated sample in broth was streaked on eosin methylene blue (EMB) agar, with a sterile inoculating loop, and incubated at 37 °C for 24 hours. Greenish metallic sheen growth was suggestive of *E. coli* which was inoculated on nutrient slants for further biochemical tests.

Conventional biochemical test

Suspected *E. coli* isolates on EMB were further screened by means of five biochemical tests, namely: Simmon citrate, urea, triple iron sugar (TSI), sulphate, indole, motility (SIM), methyl red (MR), and Voges-Proskeur (VP). Various reactions of the tests such as colour change, motility, and gas formation were used to interpret results as either positive or negative after a 24-hour incubation. These tests were carried out as described by Gbarakoro et al. [16].

Identification of *E. coli* by Microbact GNB Kit 12E

Commercially available biochemical test strip Microbact GNB 12E (Oxoid, UK) was used according to the manufacturer's instructions to confirm isolates as *E. coli* based on the result of the biochemical tests. Using the software, the results of individual tests were automatically evaluated and the resulting identity of the organism and its percentage probability were recorded.

Determination of antibiotic susceptibility of *E. coli* isolates

The antibiotic susceptibility profile of each isolate was determined using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) protocol [14]. Colonies (4–5) of the test isolates from overnight cultures on EMB plates were picked and emulsified in sterile normal saline. The turbidity of the suspension was adjusted to match 0.5 MacFarland's standard. Ten (10) µl of the suspension was then dispensed and spread on Mueller-Hinton agar plates to create a uniform lawn. The pre-inoculated plates were used for the antibiotics disc diffusion test.

Extraction of DNA

DNA extraction was done as described by Higgins et al. [17]. Briefly, the tubes containing the cultures were centrifuged at a medium speed (1,000 × g) on a tabletop centrifuge to pellet the cells. All but 100 µl of the supernatant was discarded, and the pellet was re-suspended. Aliquots were removed and processed as described below. Colonies of *E. coli* on EMB plates were removed with a sterile plastic loop and vortexed in 100-µl volumes of sterile water in a microcentrifuge tube to dislodge the bacteria. The sample was then centrifuged, the supernatant was discarded, and the pellet was re-suspended in 30 µl of sterile water and processed. A 30-µl sample was suspended in 200 µl of Instagene matrix and vortexed, followed by heating at 56 °C for 15 min. The samples were vortexed again and heated at 100 °C for 8 min and then centrifuged to pellet the matrix. Aliquots of 10 and 20 µl (the recommended amount) were used as templates for PCR.

Extraction with the Isocode paper was done according to the manufacturer's instructions. Briefly, 10-µl aliquots of bacterial cultures were spotted directly onto 8-mm-diameter disks of the paper and DNA was eluted in a 100-µl volume of sterile water, with 10 to 20 µl used as the template for PCR.

1. PCR analysis of 16S r RNA genes for *E. coli* identification

After the extraction of DNA, *E. coli* was identified by PCR using gene 16S r RNA. A control strain was used: *E. coli* ATCC 25922, accession number x80724. The detection of gene 16S r RNA in *E. coli* was done using the following primers:

Primers: F: TGA CGTTA CCCGCA GAAGAA, R: CTCCAA TCC GGACT ACGACG (Size of the product 832bp).

2. Detection of tetracycline (tet) resistance genes

For those *E. coli* isolates found to be resistant to tetracycline, polymerase chain reaction (Multiplex PCR) was used to detect five different types of tet genes – tet A, tet B, tet C, tet D, and tet M commonly reported in *E. coli* [9, 26].

The PCR assay was performed in 50 µl volume containing 0.5 µg of extracted DNA as template, 2.5 µl PCR buffer mix, 300 µM deoxynucleoside triphosphate (dNTPs), primers (see primers sequences in Table 1) reported by N g et al. [24] and $MgCl_2$ were optimized for each multiplexed primer group. The reaction was carried out with an amplification thermal cycler (Applied Biosystem 9700) in repeated cycles of initial denaturation at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 1 minute, primer annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute 30 seconds, and final extension/elongation at 72 °C for 10 minutes.

Agarose gel electrophoresis

The PCR products were analysed by gel electrophoresis using 1.5 % agarose (1st base agarose biotechnology grade) in TAE buffer, which was subjected to 80 volts for 50 minutes. The bands were stained with Ethidium bromide (1 $\mu g.ml^{-1}$) for 5 minutes and visualized under translumination. Product sizes were determined by comparing them with the 100 bp ladder.

RESULTS

The isolation of *E. coli* from milk samples

Escherichia coli was isolated from 55 (17.57 %) out of the 313 milk samples collected and tested. Out of the 55 *E. coli* isolates identified using the conventional biochemical test, only 15 (27.27 %) were confirmed to be *E. coli* using the Microbact 12 E kit (Fig. 1).

Antibiotic resistance pattern of *Escherichia coli* isolates

The antibiogram of 15 isolates confirmed by the Microbact 12 E kit is shown in Table 2 along with their respective resistance pattern. None of the *E. coli* isolates was resistant to the quinolones (nalidixic acid and ciprofloxacin). All 15 *E. coli* isolates showed resistance to ampicillin (100 %), while 11 (73.3 %) were resistant to the macrolide (erythromycin) and 2 (13.3 %) of the *E. coli* isolates were resistant to nitrofuran (nitrofurantoin) and cefixime (cephalosporin). Also, 5 (33.3 %) of the *E. coli* isolates were resistant to amoxycillin+clavulanic acid (synthetic penicillin) and co-trimoxazole (sulpha drug). Also, 4 (26.7 %) of *E. coli* isolates were resistant to gentamicin and 6 (40 %) were resistant to kanamycin (aminoglycosides), 7 (46.7 %) of the *E. coli* isolates were resistant to tetracycline (Fig. 2).

Table 1. Tetracycline resistance PCR primers

Plasmid	Tetracycline resistance genes	PCR Primer sequence 5'–3'	Expected amplicon size (bp)
pSl18	tet(A)	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210
pRT11	tet(B)	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659
pBR322	tet(C)	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418
pSl106	tet(D)	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787
pJ13	tet(M)	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406

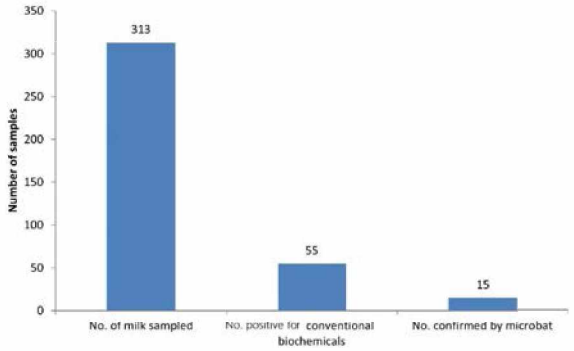

Fig. 1. Screening of *E. coli* isolates from milk samples

Table 2. Antibiotic resistance patterns of *E. coli* isolates from milk of dairy cows in Kano state

Isolate ID	Resistance profile
AA	AMP, TE, K, E
AB	AMP, AMC
AC	AMP,TE, E
BA	AMP, AMC, SXT, K, E, C
CA	AMP, AMC, TE, K, CN, E
DA	AMP, AMC, CFM, TE, SXT, K, E, F
DB	AMP, K, CN, E
DC	AMP, CN, E
DE	AMP
EA	AMP, AMC, SXT, E
EB	AMP, SXT, E
EC	AMP, AMC, TE, E
FA	AMP, CN
FB	AMP, TE
GA	AMP, CFM, TE, SXT, K, E, C

AMP – Ampicilin; TE – Tetracycline; K – Kanamycin; E – Erythromycin; AMC – Amoxycillin + Clavulanic acid; SXT – Co-trimoxazole; C – Chloramphenicol; CN – Gentamicin; CFM – Cefixime–F–Nitrofurantoin

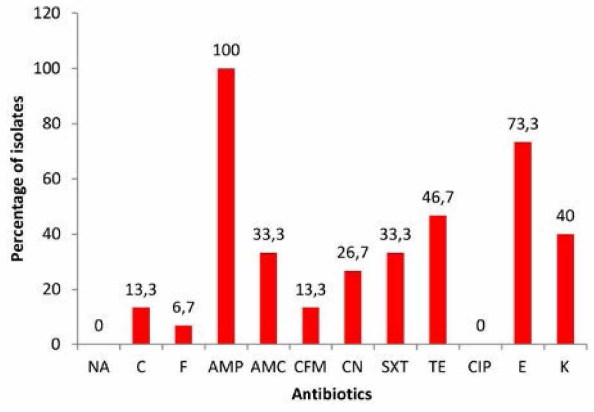

Fig. 2. Percentage resistance of *E. coli* isolates to various antibiotics

AMP – Ampicilin; TE – Tetracycline; K – Kanamycin; E – Erythromycin; AMC – Amoxycillin + Clavulanic acid; SXT – Cotrimoxazole; C – Chloramphenicol; CN – Gentamicin; CFM – Cefixime–F–Nitrofurantoin

Escherichia coli isolates and multiple antibiotic resistance indices

The antibiograms were used to calculate the multiple antibiotic resistance indices, 73.3 % of the *E. coli* isolates had MAR (multiple antibiotic resistance) values greater than 0.2. The MAR indices of the bacterial isolates are displayed in Figure 3; MAR was calculated by dividing the total number of antibiotics to which an isolate was resistant by the total number of antibiotics tested (12).

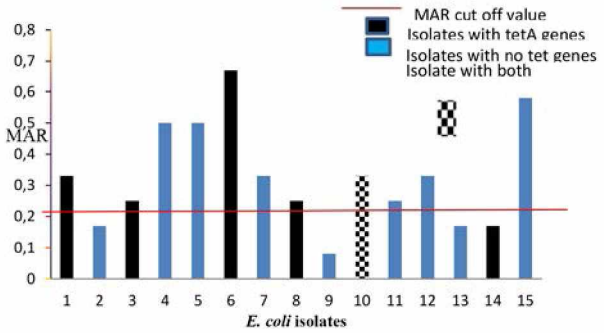

Fig. 3. Multiple antibiotic resistance index of the *E. coli* isolates.

Multiplex PCR for determination of tetracycline resistance genes

The agarose gel electrophoresis of PCR products from the 15 *E. coli* isolated by Microbact 12 E kit is shown in Fig. 4. The PCR result showed that 6 (40 %) of the isolates (AA, BA, CA, DA, EC, GA) were carrying *tet* A gene and the isolate EC carried both *tet* M and *tet* A genes.

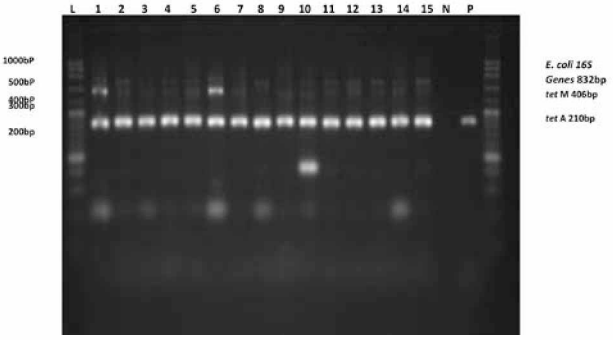

Fig. 4. Multiplex PCR result displaying *tet* M (406 bp) at lane 10, *tet* A (210 bp) at lanes 1, 3, 6, 8, 10, and 14, and *E. coli* 16S RNA genes.

L represents the 100 bp ladder; N = Negative control; P = Positive control.

Note: Lane1 = AA; 2 = AB; 3 = AC; 4 = BA; 5 = CA; 6 = DA; 7 = DB; 8 = DC; 9 = DE; 10 = EA; 11 = EB; 12 = EC; 13 = FA; 14 = FB; 15 = GA

DISCUSSION

This study revealed a low occurrence of *E. coli* in milk samples from dairy farms in Kano state, Nigeria. All the *E. coli* isolates from Microbact 12E displayed resistance to more than one type of antibiotics on the antibiotic disc diffusion test.

Most of the *E. coli* isolates had Multiple Antibiotic Resistance Index (MAR) greater than 0.2, this suggests that they were isolated from an environment where antibiotics are probably abused or frequently used. The resistance pattern varied between and within different classes of antibiotics. None of the *E. coli* isolates were resistant to any of the quinolones tested (nalidixic acid and ciprofloxacin). Colignon and McEwen [11] attributed low-level resistance of quinolones to low usage in livestock therapy in Nigeria as due to their relatively high cost they are not readily available compared to other classes of antibiotics. Testing showed that 13.3 % of the isolates were resistant to chloramphenicol (a synthetic antibiotic) and cefixime (a cephalosporin), which implied resistance spread to broad-spectrum antibiotics and a new generation antibiotic which will make the treatment difficult. Thirty-three point three percent (33.3 %) of the isolates were resistant to amoxicillin+clavulanic acid (a synthetic penicillin) and co-trimoxazole (a sulpha drug), this resistance pattern most likely was due to the integron system of a multidrug resistance mechanism. In most cases of sulpha drug resistance, integron systems are involved [19]. Furthermore, 26.7 % and 40 % of the isolates were resistant to gentamicin and kanamycin (aminoglycosides) respectively. Resistance to tetracycline was 46.7 % and all of the isolates were resistant to ampicillin (a synthetic penicillin) in this case there is the likelihood that the efflux pump system of drug resistance is involved as it gives room for resistance to different classes of drugs at the same time. Similar high-level resistance to ampicillin by *E.coli* isolated from milk was reported by [4].

The PCR result showed that 6 (40 %) of the isolates carried *tet* A gene which codes for efflux pump and EC isolate carried *tet* M gene which codes for both efflux pump and ribosomal protection resistance mechanisms (see Fig. I) and this supports the multidrug resistance displayed by most of the *E. coli* isolates as these two resistance mechanisms give room for the multidrug resistance. Isolate EC displayed multiple resistances on the disc diffusion test. The *tet* A and *tet* M genes are the most common resistance mechanisms of *E. coli* to tetracyclines as reported by Sheykhsaran et al. [27] and the findings of this research suggest that *tet* A and *tet* M are the predominant resistance genes of the *E.coli* isolates in the study area.

CONCLUSIONS

This study shows that raw milk from commercial dairy farms in Kano State had an occurrence of multi-drug resistant *E. coli* from 15 milk samples (4.79 %) and this is a major public health threat. Out of the 15 *E. coli* that were isolated and identified, 14 were resistant to more than one antibiotic from the disc diffusion test, and 73.3 % of the *E. coli* isolates had MAR index greater than 0.2.

The study further revealed that some of the *E. coli* isolates carried genes that code for both efflux pumps and ribosomal protection resistance mechanisms.

We recommend that the farmers should be enlightened on the proper hygienic practices of milking and handling milk. Also, there should be a good regulatory policy by the government on the use and sales of antimicrobials in animal production in the country. There is a need to further detect other resistant genes apart from *tet* genes as the isolates could be carrying them.

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