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Original Article

Whole genome sequencing analysis of non-O157 Shiga toxin-producing *Escherichia coli* in milk in Kwara State, Nigeria

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Abstract

Background: *Escherichia coli* is a major cause of poor microbial quality of milk, often resulting from unhygienic milk handling. Milk contamination poses public health concerns. Shiga toxin-producing *Escherichia coli* (STEC) strains in food products, particularly milk, is a critical concern for public health. Limited information exists on the detection of non-O157 *E. coli* pathotypes in milk sold by local processors in Nigeria. **Aims:** This study aimed to explore the diversity of non-O157 STEC isolates found in commercially available milk in Kwara State, Nigeria, to find the genetic diversity and potential risks associated with these strains. **Methods:** A subgroup of 18 representative non-O157 STEC isolated from milk samples (n=1225) was selected for whole genome sequencing (WGS) analysis. **Results:** Four novel sequence types (ST): ST398, ST540, ST1727, and ST9891 of non-O157 *E. coli* involving five serotypes: O176:H30, O176:H20, O8:H20, O21:H45, and O22:H7, carrying variable proportions of virulence factors, antimicrobial resistance genes, and plasmids, were identified. **Conclusion:** This investigation contributes valuable data to the ongoing efforts to ensure food safety and prevent the transmission of *E. coli* strains through dairy products. The findings have implications for public health policies and food quality standards in Kwara State, Nigeria. Improved hygienic practices during milk handling are recommended.

Key words: Food safety, Milk contamination, Nigeria, Non-O157 *Escherichia coli*, Whole-genome sequencing

Introduction

Shiga toxin-producing *Escherichia coli* (STEC), also known as verocytotoxigenic *E. coli* (VTEC) and enterohemorrhagic *E. coli* (EHEC), is a unique pathotype of *E. coli* that produces a Shiga toxin, which is related molecularly to the toxin expressed by *Shigella dysenteriae* type 1 (the Shiga bacillus). The most common illness from STEC strains is haemorrhagic colitis (Valilis *et al*., 2018). Non-O157 strains, including Shiga toxin-producing *Escherichia coli* (STEC) variants, have the potential to cause gastrointestinal illnesses characterized by symptoms such as diarrhoea, abdominal cramps, and in severe cases, kidney failure (haemolytic uremic syndrome or HUS) (USDA/FSIS, 2012). Estimates from the US Centres for Disease Control and Prevention (CDC) indicate that although non-O157 STEC strains are largely undetected, they outnumber O157 strains as causes of human illness (Hadler *et al*., 2011; Hale *et al*., 2012).

The virulence of non-O157 STEC is influenced by several factors, including specific virulence genes and serotypes impacting their pathogenicity (Mathusa *et al*., 2010; Monaghan *et al*., 2011). Thus, non-O157 STEC strains have gained clinical importance due to their ability to cause human infections. Their virulence profiles can vary, leading to differences in the severity of illnesses they cause (Hughes *et al*., 2006).

The microbiological safety of milk sold for human consumption is of serious public concern. The low- and middle-income countries bear the major burden of foodborne diseases (Grace *et al*., 2015), Nigeria inclusive. Africa experiences more hospitalization and death rates due to food safety issues than the rest of the world (Unnevehr and Hirschhorn, 2000). Nigeria has been reported to have the highest burden of foodborne illnesses in the West African sub-region (Havelaar *et al*., 2015). Food contamination by pathogens, especially *E. coli*, has a significant implication for public health and the food industry (Odetokun *et al*., 2018, 2021, 2022, 2023; Alhaji *et al*., 2019; Ghali-Mohammed *et al*., 2022, 2023). Non-O157 STEC can be found in milk and other food types (Mathusa *et al*., 2010; Mohammadi *et al*., 2013), leading to foodborne illnesses. Also, in Nigeria, few studies have identified non-O157 STEC in some food products and environment (Olowe *et al*., 2014;

Enabulele and Nwankiti, 2016; Ayoade *et al*., 2021; Fayemi *et al*., 2020); however, detailed genomic analysis revealing the specific sequence, serotypes, virulence, and antimicrobial resistant genes in Nigeria are scarce. This study seeks to provide insights into the genetic variations, virulence factors, and antibiotic resistance profiles of *E. coli* strains isolated from milk intended for human consumption in Kwara State, Nigeria, using whole genome sequencing techniques. This approach is essential for developing effective strategies to ensure the safety and quality of milk products consumed by the population of Kwara State.

Materials and Methods

E. coli isolates

A total of 548 and 677 vended milk samples (n=1225) were obtained from various markets over 12 months during the rainy and harmattan seasons, respectively in Kwara State, Nigeria. These samples were obtained in 11 markets across the three agroecological zones (North, Central, and South) of the State. Overall, 599 *E. coli* were isolated from the 1225 samples (prevalence = 48.9%), out of which 28 (n=2.3%) were non-O157 STEC strains isolated from milk samples obtained across the three agroecological zones in Kwara State. 18 of the 28 non-O157 STEC were randomly selected and subjected to genomic assays.

Whole genome sequencing assay

The United States Food and Drug Administration, College Park, Maryland, USA, received the non-O157 *E. coli* isolates on nutrient agar slants for whole genome sequencing. Following the manufacturer's re-commendations, bacterial DNA was extracted from overnight cultures using the DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, USA). 0.2 ng/µL of processed DNA was used to create sequencing libraries using the Nextera XT DNA library prep kit (Illumina, San Diego, CA, USA).

Following the instructions provided by the manufacturer, sequencing was done on the MiSeq Illumina instrument using the 500-cycle MiSeq reagent V2 kit (2 250 bp). The USFDA's GenomeTrakr database (Allard *et al*., 2016), which is housed under NCBI Pathogen Detection (NCBI Resource Coordinators, 2018), was promptly updated with the raw data (bioproject PRJNA186035). All *E. coli* strains' SRA accession numbers and assembly statistics are listed in Tables 1 and 2.

Analysis of the WGS result

The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) was used to annotate the genome after the genome assembly of raw data using SPAdes version 3.8 (Bankevich *et al*., 2012). The SeqSero version 1.0 software programme was used to predict the serotyping of *E. coli* by whole-genome sequencing (Zhang *et al*., 2015).

Resistance determinants from the ResFinder and PointFinder databases were located in assemblies using starAMR v. 0.4.0. A customised version of the PlasmidFinder database (https://github.com/StaPH-B/resistanceDetectionCDC) and abricate v. 0.8.10 (https://github.com/tseemann/abricate) were used to find the plasmid genes. The determinants identified, and the ResFinder and PointFinder drug keys created by the Centres for Disease Control and Prevention (https://github.com/StaPH-B/resistanceDetectionCDC) were used to assign predicted resistance phenotypes (Raufu *et al*., 2021).

Multilocus sequence typing (MLST) was performed using WGS data and the sequences of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*). The contig sequence files were added to the MLST database of the Centre for Genomic Epidemiology. To determine the sequence type (ST) of the isolates based on the set of alleles derived from the

Table 1: Serotype, MLST, mutation resistance, plasmid replicons, and virulence genes in *E. coli*

Strain name	Serotype	MLST	Plasmids	Virulence factors/toxins
CFSAN083654	O176:H30	ST540	*IncX1*	*gad*, *iss*
CFSAN083642	O8:H20	ST398	-	*gad*, *iss*
CFSAN083623	O176:H20	ST540	-	*gad*, *iss*
CFSAN083647	O8:H20	ST398	-	
CFSAN083635	O176:H30	ST540	*IncX1*	
CFSAN083633	O21:H45	ST398	*IncR*, *Col(pHAD28)*, *IncFIA(HI1)*	*capU*, *gad*, *iss*
CFSAN083637	O176:H30	ST540	*IncX1*	
CFSAN083630	O176:H30	ST540	*IncX1*	
CFSAN083650	O8:H20	ST398	*IncY*	
CFSAN083643	O176:H30	ST540	*IncX1*	
CFSAN083640	O8:H20	ST9891	*IncY*	
CFSAN083649	O176:H30	ST540	*IncX1*	
CFSAN083639	O8:H20	ST398	-	
CFSAN083648	O176:H30	ST540	*IncX1*	
CFSAN083628	O176:H30	ST540	*IncX1*	
CFSAN083652	O176:H30	ST540	*IncX1*	
CFSAN083624	O176:H30	ST540	*IncX1*	
CFSAN083622	O22:H7	ST1727	*Col(pHAD28)*	*gad*, *astA*, *ipfA*

MLST: Multilocus sequence types

Table 2: Antimicrobial resistance genes detected by *E. coli* isolates

Strain name	Serotype	AMG[a]	BL[b]	Sulphonamide	Tetracycline	Trimethoprim	Anticipated resistance
CFSAN083654	O176:H30	-	-	-	-	-	None
CFSAN083642	O8:H20	-	-	-	*tet(B)*	-	Tetracycline
CFSAN083623	O176:H20	-	-	-	*tet(B)*	-	Tetracycline
CFSAN083647	O8:H20	-	-	-	*tet(B)*	-	Tetracycline
CFSAN083635	O176:H30	-	-	-	-	-	None
CFSAN083633	O21:H45	*aph(3")-lb*, *aph(6)-ld*	bla_{TEM-1B}	*sul2*	*tet(A)*	*dfrA14*	Streptomycin, Ampicillin, Trimethoprim, Sulfisoxazole, Trimethoprim/Sulfamethoxazole, Tetracycline
CFSAN083637	O176:H30	-	-	-	-	-	None
CFSAN083630	O176:H30	-	$bla_{OXA-486}$	-	-	-	Ampicillin
CFSAN083650	O8:H20	-	bla_{TEM-1D}	-	*tet(B)*	-	Ampicillin, Tetracycline
CFSAN083643	O176:H30	-	-	-	-	-	None
CFSAN083640	O8:H20	-	bla_{TEM-1D}	-	*tet(B)*	-	Ampicillin, Tetracycline
CFSAN083649	O176:H30	-	-	-	-	-	None
CFSAN083639	O8:H20	-	-	-	*tet(B)*	-	Tetracycline
CFSAN083648	O176:H30	-	-	-	-	-	None
CFSAN083628	O176:H30	-	-	-	-	-	None
CFSAN083652	O176:H30	-	-	-	-	-	None
CFSAN083624	O176:H30	-	-	-	-	-	None
CFSAN083622	O22:H7	*aph(3")-lb*, *aph(6)-ld*	-	*sul2*	*tet(A)*	-	Streptomycin, Sulfisoxazole, Tetracycline

[a] Aminoglycoside, and [b] Beta-Lactam

seven loci above, data were collected in silico multilocus sequence typing (MLST) using the Centre for Genomic Epidemiology online tool (https://cge.cbs.dtu.dk/services/MLST/). The Public Health Agency of Canada's website (https://github.com/phac-nml/ecoli_vf.) was used to extract the virulence genes found in the genome. SNP phylogenies from the NCBI pathogens page (https://www.ncbi.nlm.nih.gov/pathogens/) were studied further to evaluate the genetic link between isolates (Raufu *et al*., 2021).

Results

Multilocus sequence typing and serotyping

The diversity of the non-O157 *E. coli* milk isolates was shown by the MLST and the serotyping data. Out of the 18 *E. coli* isolates, four sequence types (STs 398; n=5; 540; n=11; 891; n=1; 1727; n=1) were detected as shown in Table 1. Eleven (61.1%) isolates were ST540 of which 10 isolates belonged to the O176:H30 serotype and one was of the O176:H20 serotype. Five (27.8%) *E. coli* isolates belonged to ST398 of which four had the O8:H20 serotype and one had the O21:H45 serotype. One (5.6%) *E. coli* isolate each belonged to ST9891 and ST1727 with the O8:H2O and O22:H7 serotypes, respectively.

Virulence factors and plasmids

In the *E. coli* isolates, several virulence factors (VFs), plasmid replicons, toxin, and evasion genes were identified (Table 1). These VFs were found in five (27.8%) isolates, including the *gad*, *iss*, *capU*, *astA*, and *ipfA* genes. Plasmids detected in *E. coli* isolates included the *IncX1*, *IncY*, *IncR*, *Col(pHAD28)*, and *IncFIA(HI1).* Ten (55.6%) and 2 (11.1%) of the *E. coli* isolates carried the *IncX1* and *IncY/Col(pHAD28)* plasmids, respectively. One isolate each possessed the *IncR* and *IncFIA (HI1)* plasmids.

Antimicrobial resistance genes

Two (11.1%) *E. coli* isolates carried genes (*aph(3")-lb* and *aph(6)-ld*) encoding for aminoglycoside (Table 2). Four (22.2%) *E. coli* carried genes translating beta-lactam resistance enzymes, consisting to bla_{TEM-1D} (2/18), bla_{TEM-1B} (1/18), and $bla_{OXA-486}$ (1/18). Two *E. coli* isolates harbored the *sul2* gene encoding sulphonamides resistance. Only one (1/18) isolate carried the *dfrA14* gene encoding trimethoprim resistance enzyme. Most resistance genes were those coding tetracycline resistance enzymes *tet*(A) and *tet*(B) in a proportion of 2 (22.1%) and 6 (33.3%), respectively.

Discussion

These results showed that the dominating serotype O176:H3O of the ST540 is a major clade detected in vended milk in Kwara State, Nigeria. Previously, pathogenic *E. coli* of the ST540 has been isolated from urinary tract infections and bovine (Anes *et al*., 2020; Nüesch-Inderbinen *et al*., 2022). Also, *E. coli* of the ST398 has been detected in human and poultry environments in Nigeria (Aworh *et al*., 2021), while *E. coli* ST1727 was detected in meat samples in Ghana (Adzitey *et al*., 2020). More studies from other parts of the nation are required to understand better the epidemiology and public health significance of this serotype/ST.

These VFs, especially the long polar fimbriae (*ipfA*) found in serotype O22:H7 (ST1727), facilitate the bonding of *E. coli* with host cells or other *E. coli*. The *gad* gene was found in all isolates, while the *iss* gene, a virulence factor causing *E. coli* to evade by elevating the serum survival, was harboured by four of the five isolates. The *capU*, *astA*, and *ipfA* genes detected in this study promote toxin proliferation in *E. coli* isolates and are virulence factors commonly detected in enteroaggregative *E. coli* strains isolated in diarrheal patients, especially in children under the age of 5 years

(Ikumapayi *et al*., 2017; Sonda *et al*., 2018). The *gad*, *iss*, and *ipfA* genes, frequently detected in chicken, cow, and swine (Chen *et al*., 2018), are associated with pathogenic strains of *E. coli* (Bergholz *et al*., 2007; Solà-Ginés *et al*., 2015; Malik *et al*., 2017) and are responsible for causing colibacillosis outbreaks in animals (Solà-Ginés *et al*., 2015).

Plasmids in *E. coli* can carry genes that confer antibiotic resistance. This is of concern in both clinical and agricultural settings, including dairy farms, where *E. coli* with antibiotic-resistant plasmids can potentially impact food safety and public health (Li *et al*., 2019; Findlay *et al*., 2020). These plasmids are commonly detected in various sequence types of *E. coli* than reported in this study. For instance, these plasmids have been reported in swine, broiler, clinical bovine mastitis, real veal, and environmental *E. coli* isolates in previous studies (Tate *et al*., 2021; Bonvegna *et al*., 2022; Tofani *et al*., 2022; dos Santos Alves *et al*., 2023; Prendergast *et al*., 2023). The non-O157 *E. coli* strains isolated from milk in this study can be classified as pathogenic since the presence of these VFs is indicative of the potential of the *E. coli* serotypes to cause infection if the contaminated milk is consumed. The presence of these VFs, plasmid replicons, and immune evasion genes further emphasizes the ability of the non-O157 *E. coli* strains to spread infections to a wide range of hosts and environments (Sonda *et al*., 2018).

Other similar genes such as *aac(6')Ib-cr* codes for low-level ciprofloxacin resistance (and aminoglycoside resistance), have been identified in cattle, cattle attendants, and hospital settings (Madoshi *et al*., 2016; Sonda *et al*., 2018). The $bla_{TEM\text{-}1D}$ (2/18), $bla_{TEM\text{-}1B}$ (1/18), and $bla_{OXA\text{-}486}$ (1/18) detected in this study are comparable to isolates recovered from hospitals (Sonda *et al*., 2018). A different study reported a higher prevalence of *blaCTX-M-15* genes in *E. coli* isolates (Manyahi *et al*., 2017). The two *E. coli* strains carrying the *sul2* gene in this study suggest that such antibiotics must be monitored before widespread and complete resistance develops among various groups of cattle. A large number of *dfrA* genes were linked to *E. coli* isolates from cattle and human populations in Tanzania (Madoshi *et al*., 2016; Sonda *et al*., 2018) compared to only one milk *E. coli* isolate carrying this gene. This is comparable to the low levels of *dfrA* genes detected in *E. coli* from healthy high school students in Ghana and Nigeria (Labar *et al*., 2012). These findings suggested that humans may exhibit far higher levels of trimethoprim resistance than animals. The relatively large percentage of these tetracycline resistance-coding genes - *tet(A)* and *tet(B)* - found in *E. coli* suggests the potential for widespread tetracycline usage, abuse, and misuse in treating illness in animals.

A major limitation of this study is the WGS analysis of a limited number of *E. coli* isolates from only one major milk-producing state in Nigeria. These limitations may have affected the representativeness and generalizability of our findings. However, this study, to the best of our knowledge, is the first to apply the WGS analysis on non-O157 *E. coli* strains isolated from marketed milk.

In conclusion, this study on the diversity of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) isolates in marketed milk in Kwara State, Nigeria, using whole-genome sequencing analysis has yielded significant insights. The study revealed the presence of multiple STEC strains in commercially available milk, with some exhibiting alarming characteristics such as virulence factors and antimicrobial resistance genes. Specifically, identifying four new Sequence Types (ST) of non-O157 *E. coli* and their association with various serotypes highlights the genetic diversity of these pathogens. Furthermore, the study underscores the importance of addressing food safety concerns, particularly in the Nigerian dairy industry, to prevent the transmission of pathogenic *E. coli* strains. These findings have direct implications for public health policies and food quality standards in Kwara State, Nigeria. It emphasizes the need for improved hygienic practices during milk handling and processing to mitigate the risk of contamination and protect consumers from potential health hazards associated with STEC strains in milk. The results of this study are important in ensuring the safety of food products and demonstrate the importance of continued surveillance and monitoring of microbial quality in dairy products to safeguard public health.

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Conflict of interest

The authors declare no conflict of interest.

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