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Molecular Identification of Virulence Genes of *Escherichia coli* Isolated from Cow Milk and Its Products in Abuja, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Shiga toxin-producing *Escherichia coli* have been identified as an emerging foodborne pathogen which portends serious risk to human health. Cow milk and its products are potential sources of shiga toxin-producing *Escherichia coli*. A relatively small number from the family of shiga toxin-producing *Escherichia coli* are pathogenic. It becomes necessary that Cow milk and milk products are regularly screened for the presence of virulence genes in microbes. The study aimed to genetically determine the presence of virulence genes that are characteristic of Enterohaemorrhagic *E. coli* in 600 milk samples. The *E. coli* isolates were recovered from the milk

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samples (n=35), biochemically examined and genetically screened for virulence genes by multiplex Polymerase Chain Reaction (PCR). The results of the molecular profiling revealed that (*stx2*) was detected in 17(60.7%), (*hlyA*) 11(39.3%) and *eae* genes 8(28.6%) of the *E. coli* isolates respectively, while (*stx1*) was not detected. The results indicated a high prevalence of virulent shiga toxin-producing *Escherichia coli* in the milk samples. Priority attention should be given to this microbe as it will demand stringent steps in the detection given that they are known to be rigorous in identification.

Keywords: Virulence; multiplex PCR; serotype; pathogens; Escherichia coli.

1. INTRODUCTION

Escherichia coli is a Gram-negative, lactose fermenting, facultative aerobic, rod-shaped and non-sporulating, flagellated bacterium in the family of *Enterobacteriaceae* [1,2,3,4,5,6]. The bacterium, *E. coli* is well recognized as a commensal inhabitant of the gastrointestinal tract [7,8] and considered as a reliable indicator of contamination by faeces [9], manure, soil, contaminated water and breach of hygiene in milking [10,11]. Strains of *E. coli* are characterized by their ability to produce shiga toxins and other bacterial toxins [12]. This attribute is definitive of the virulence factors of the class of *E. coli,* enteric pathogens known as shiga toxin-producing *E. coli* (STEC) or vero toxin-producing *E. coli* (VTEC). Transmission occurs through consumption of contaminated fruits and vegetables, undercooked meat, unpasteurized raw milk [5,13] or consumption of water directly or indirectly contaminated by faeces [14], direct or indirect contact with animals [15] or person to person contact [16,17]. The Enterohaemorrhagic *Escherichia coli* (EHEC), is a subgroup of shiga toxin-producing *E. coli* based on the clinical manifestations in humans [18]. Several virulence genes are associated with the pathogenicity of STEC, and genes encoding *E. coli* virulence factors are located either on plasmids on large genome regions called pathogenicity islands (10 to 200 kb), or on integrated bacteriophages [19] which can be moved by the mechanism of horizontal gene transfer. The genes encoding shiga toxins 1 and 2 (*stx1* and *stx2)* respectively are carried by lambdoid phages and can be integrated into the bacterial host genome in multiple copies [20] also known as phage-encoded cytotoxins. Shiga toxins are A1B5 toxins that halt protein synthesis in the host cells, a process that may lead to apoptotic cell death, and cause the vascular endothelial damage observed in patients with hemorrhagic colitis and the haemolytic uraemic syndrome (HUS) [2,21]. Shiga toxin *E. coli* capable of tightly attaching and causing attaching and effacing lesions on the intestinal epithelial cells is the intimin factor encoded by the *eae* gene (*E. coli* attachment and effacement). These are located in the chromosomal locus of enterocyte effacement (LEE), a pathogenicity island. The plasmid-encoded enterohaemolysin (*hlyA*) virulence gene is found in shiga toxin-producing *E. coli.* It is responsible for the lysis of the blood cells after the tissue damage and thus facilitating the proliferation of organisms [22]. Raw milk and dairy products are one of the main sources of transmission of the *E. coli* strains to humans [13]. Milk contamination is usually due to the faecal contamination by the pathogenic *E. coli* that colonize the gut of dairy animals which are subclinical and remain unnoticed. Personal and environmental hygiene during the milking process contribute to the risk of contamination of these ready-to-eat dairy products. The study aimed to determine the presence of virulence genes in shiga toxin-producing *E. coli* from Cow milk and its products.

2. MATERIALS AND METHODS

Six hundred (600) milk samples (Madara {153}, Kindrimo {160}, Nono {157} and Manshanu {130}) were randomly collected during the dry and wet seasons of the year between (December 2016 –August 2017), from the six Area Councils of Abuja (Nigeria) at various points. Samples were collected in sterile plastic containers in duplicates at point of sale. They were labeled and transported in the icebox to the laboratory for immediate analysis.

2.1 Bacteria Strain

Reference bacteria strains of *Escherichia coli* ATCC 25922, ATCC43888 and LMG 21766 were used in the study to serve as positive controls.

2.2 Isolation and Identification of *Escherichia coli*

Ten ml (10 ml) of milk samples were aseptically transferred into 90 ml of modified Tryptic Soy

Table 1. List of primers used in the characterization of virulence genes

Virulence factor	Primers	Sequence	Product size (bp)	Reference
Shiga Toxin 1	*stx1*f	GAAGAGTCCGTGGGATTACG	130	[24]
	*stx1*r	AGCGATGCAGCTATTA		
Shiga Toxin 2	*stx2*f	TTAACCACACCCACGGCAGT	346	[24]
	*stx2*r	GCTCTGGATGCATCTCTGGT		
Intimin	*eae*f	GCAAATTTAGGTGCGGGTCAGCGTT	494	[25]
	*eae*r	GGCTCAATTTGCTGAGACCACGGTT		
Enterohaemolysn	*hlyA*f	AGCTGCAAGTGCGGGTCTG	569	[25]
	*hlyA*r	TACGGGTTATGCCTGCAAGTTCAC		

The primers used in the amplification of stx1, stx2, eae and hlyA genes for the characterization of virulence genes in this study

broth (mTSBn) (Oxoid) supplemented with 20 mg/l novobiocin (Oxoid), homogenized for 2 mins in a stomacher (Lab Blender 400, Seward Medical, London, UK) and then incubated at 37°C for 18 hours as *E. coli* enrichment step [23]. A loopful of the enriched broth was streaked on the plate of Levine's Eosin Methylene blue agar (L-EMBA) (Oxoid). After overnight incubation at 37°C, at least 5 *E. coli*-like colonies were selected per plate. Presumptive colonies of *E. coli* (greenish metallic sheen appearance with dark purple centres) were Gram stained, biochemically identified using Microbact™ GNB 24E System Kit (Oxoid Limited, Basingstoke UK). Of the 600 milk samples, *E. coli* was isolated from 35 samples; (Madara {15}, Kindrimo {10}, Nono {6} and Manshanu {4}).

2.3 Molecular Detection

Genomic extraction: The genomic deoxyribonucleic acid (DNA) was extracted using ZR Genomic DNA™ Miniprep Kit (ZYMO Research Corp. USA) according to manufacturer's instructions. The extracted DNA samples were stored at -20°C ready for (PCR) molecular studies [25]. Multiplex PCR was carried out on 28 of the 35 *E. coli* isolates.

Master mix: The primers sets used in this study for the detection of virulence genes were *stx1* and *stx2* [24], *eae* and *hlyA* [25] as shown in Table 1. The PCR multiplex comprised of 5.0 µl DNA extract, 0.6 µM *hlyA*, 1.5 µM s*tx2*, 0.75 µM of *eae*, 0.25 µM of s*tx1* and 5.0 µl of 5 X Master Mix (NEW ENGLAND BIOLABS®) comprising 1 X Multiplex PCR Master Mix: 20 mM Tris-HCl (pH 8.9 @ 25°C), 50 mMKCl, 30 mM NH_4Cl, 2.5 mM $MgCl_2$, 100 units/ml Taq DNA Polymerase, 0.3 nmM each dNTP 3.2% glycerol, 0.08% IGEPAL® CA-630, 0.07% Tween® 20. The total volume of reaction mix was made up to 25 µl using nuclease free water.

Polymerase chain reaction: Amplification was performed in Gene Amp 9700 (Applied Biosystems) with initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 58°C for 40 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 8 minutes.

Electrophoresis: Five microliters of the PCR product was electrophoresed in agarose gel (1.5%) containing 5 µl of 10 mg/ml ethidium bromide at 80 V for 50 mins. Fifty (50) bp DNA marker (New England Biolabs®) was used as a molecular size marker. The genes were examined under U.V transilluminator and results documented using Gel Documentation System (BIO RAD).

3. RESULTS

The frequency of occurrence of the virulence genes in the *E. coli* strains from cow milk and its products is shown in Table 2. The *stx*2 gene was detected in (3, 23.1%) of the *E. coli* isolates from Madara, (4, 80%) from Kindrimo, (5, 100%) from Nono and Manshanu respectively. The *hlyA* gene was detected in (2, 15.4%) of the *E. coli* from Madara, (2, 40%) from Kindrimo, (4, 80%) from Nono and (3, 60%) from Manshanu. The *eae* gene was detected in (3, 60%) of the *E. coli* from Nono and (5, 100%) from Manshanu. The virulence gene *stx2* (17, 60.7%) was most prevalent in this study followed by *hlyA* with (11, 39.3%) and *eae* gene (8, 28.6%).

Fig. 1 shows that *stx2* gene (346 bp) was detected in *E. coli* strain represented in lanes 2, 3, 4, 5, 6, 7, 8, 10, 11 and 12; *eae* gene (494 bp) was detected in *E. coli* strain represented in lanes 3, 5, 7, 8, 15, 16 and 17; *hlyA* gene (569 bp) was detected in *E. coli* strain represented in lanes 2, 5, 6, 7, 8 and 17. Lane M:

Bench Top 50 bp DNA ladder (New England, BioLabs). Lane 15, 16 and 17 were positive controls (reference strains of ATCC 43888 and LMG 21766), lane 18 clinical samples and lane 19 negative control.

4. DISCUSSION

The present study revealed that the *E. coli* were positive for shiga-toxin genes (*stx*) in madara (raw cow milk), and the traditional dairy products (kindrimo, nono and manshanu). Among the two types of shiga-toxins, only shiga-toxin 2 (*stx2*) was detected while shiga toxin 1 (*stx1*) was not detected. Of the 28 *E. coli* isolates subjected to PCR assay, 17(60.7%) of the *E. coli* isolates expressed the *stx2* gene. This finding suggests that the milk samples were contaminated with STEC, a pathogenic *E. coli* capable of producing the shiga-toxins hence the name (STEC).

Table 2. Distribution of virulence genes present in the cow milk and its products

Types of samples	No examined	Virulence genes			
		***stx1* (%)**	***stx2* (%)**	***eae* (%)**	***hlyA* (%)**
Madara	*13*	0(0)	3(23.1)	0(0)	2(15.4)
Kindrimo	*5*	0(0)	4 (80)	0(0)	2(40)
Nono	*5*	0(0)	5(100)	3(60)	4(80)
Manshanu	*5*	0(0)	5(100)	5(100)	3(60)
Total	*28*	0(0)	17(60.7)	8(28.6)	11(39.3)
Controls					
ATCC43888		-	-	-	+
LMG 21766		-	-	-	+
CLINICAL		+	-	-	+

Key: += positive result, - = negative result, No= number, %= percentage, ATCC= American Type Culture Collection and LMG= Laboratory of Microbiology Gent (reference bacterial strains of E. coli), stx1 and stx2 = shiga-toxin genes, eae = intimin gene and hlyA = enterohaemolysin gene

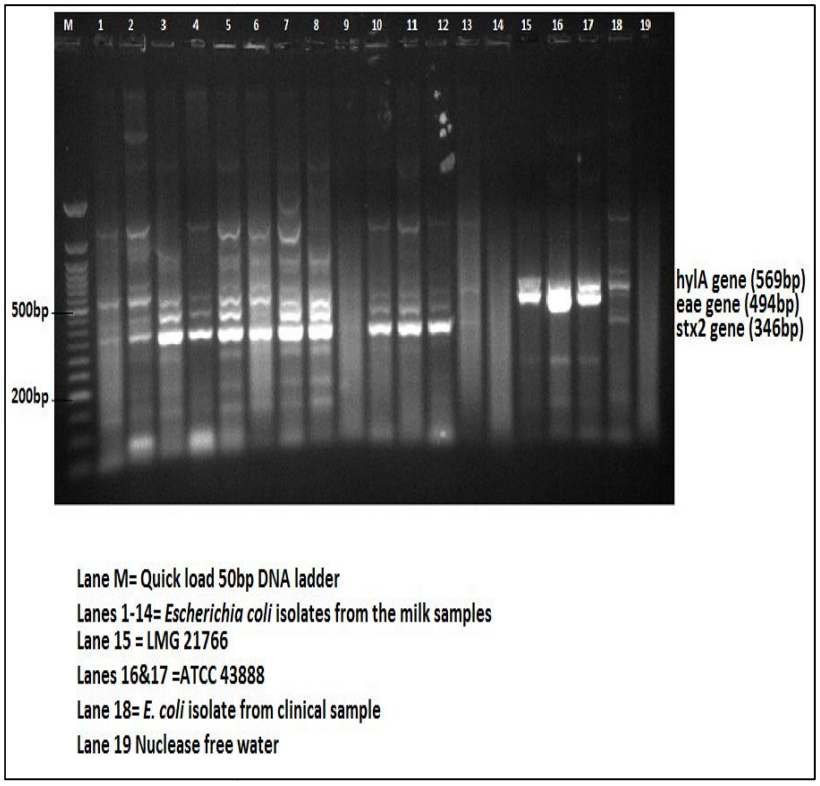

Fig. 1. Agarose gel electrophoresis of virulence genes by multiplex PCR

This is one of the most important virulence characteristics of STEC strains. The high contamination of milk and milk products by STEC could be attributed to the traditional methods of milking which increases the risk of transmission of the food pathogens into the milk samples, lack of storage facility to maintain the temperature of the milk products will increase the rate of survival and multiplication of pathogens in these milk samples, the unpasteurized milk product itself and the unhygienic status of the milkers. The result is consistent with the reports [26,27,28,29, 30,31] with the following prevalence (0.87%, 11.11%, 5.7%, 11.29%, 17.47%, 67.44%) at different places (Ontario, Belgium, Italy, Egypt, Iran, Saudi Arabia) respectively. Shiga-toxin 2 (*stx2*) in beef, 94.21% of STEC was reported in Saudi Arabia [31], 3.5% in Brazil [32] and 4.4% in Japan [33]. The variation in the distribution of the shiga-toxins at the different places could be as a result of the several factors such as geographical location, the season of the year, farm management practices, variation in sampling, variation in the type of samples evaluated, hygiene status and the methods of detection [30,34].

Escherichia coli from other sources (faecal, pork and poultry samples) that were positive for (*stx2*) gene have been reported in China [35], faecal samples in Bangladesh [36] with the prevalence of (40% and 50%) respectively. Shiga-like toxin *E. coli* strains could be detected alone, or in combination, either *stx1*, or *stx2* and s*tx2* variants toxins [27]. The combination of the two shiga-toxins (*stx1* and *stx2*) was observed in the report [37] from raw milk (madara) samples in South Africa. Momtaz et al. [13] reported that only shiga-toxin 1 (*stx1*) was detected in milk samples. In contrast, previous studies reported that none of the shiga-toxin genes was detected in the *E. coli* isolated from the milk sample that was screened by PCR [38]. It is possible that the *stx* genes are present in the samples but not expressed due to the isolation methodologies of the very scanty cells of STEC and *stx*-carrying phages [39,40]. In this study, the predominant virulence gene was *stx2* which have been reported to be more virulent than shiga-toxin1 (*stx1*) as it poses more health risk to humans through the consumption of the high-risk foods [37].

Moreover, *stx1* and *stx2* sequences are located on mobile genetic elements and might have different mobility patterns. Therefore, the transference of *stx*2 from non-pathogenic to pathogenic serotypes may happen more frequently [41]. It has been shown that accessory virulence factors such as intimin *eae*, and enterohaemolysin, *hlyA* genes are important in the pathogenicity of STEC [42]. In this study, the intimin *eae* gene was detected in 8(28.6%) of the *E. coli* isolates recovered from the milk samples. The finding is consistent with the reports [13,17,31,38,43] with prevalence (100%, 36%, 44.19%, 4.05-13.4%, 18.0%-24.3%) in these locations (Iran, South Africa, USA, Saudi Arabia) respectively. Al-zogibi et al. [31] reported *eae* gene of 10(58.82%) in *E. coli* isolates from raw beef, *E. coli* isolates from milk and dairy products in Iran were positive for *eae* genes [4], *E. coli* isolates from faecal samples of diarrheic calves [2] and chicken meat [44] were positive for *eae* gene in Iran. The variation of the prevalence of *eae* genes could be due to the geographical location, sample types and methods of detection of STEC positive for *eae* genes. This finding suggests that the presence of intimin *eae* gene in the isolates may increase the risk of infection in humans. It is considered as a marker for Enterohemorrhagic *Escherichia coli* [45]. The *eae* gene is capable of initiating pathogenesis in cell host by inducing attaching and effacing" (A/E) lesions, known to be a virulence factor of diarrhoea in children [46]. It has been reported that there is an association between STEC infection and the presence of the *eae* gene [47].

On the contrary, a previous study [30] reported that no *eae* gene was detected in the *E. coli* isolates from the milk and dairy samples in Iran. However, absence of *eae* gene does not completely eliminate virulence potentials in *E. coli*. Though some studies reported that *eae* gene is not essential in the pathogenicity in humans [48]. It was reported with evidence that etiologic agent STEC lacking *eae* gene was responsible for the outbreaks of human illness in the United States and Australia [49].

In this study enterohaemolysin *hlyA* gene was detected in 11 (39.3%) of *E. coli* isolates. These findings agree with the reports [17,37] in Iran and South Africa with prevalence (28.0%, and 2.4%) respectively.

The presence of *hlyA* gene expressed by the *E. coli* isolates from milk and dairy samples was reported in Iran [4,13]. This finding suggests that the *E. coli* producing-shiga toxin which expressed the enterohaemolysin gene (*hlyA*) is

potentially harmful pathogen in humans and its presence in food portends risk on public health. Studies have shown that enterohaemolysin production *hlyA* gene might have a direct association with the pathogenicity of a given STEC strain to cause more serious disease in humans [21].

5. CONCLUSION

The result of this study showed that shiga-toxin 2 (*stx2*), intimin (*eae*) and enterohaemolysin (*hlyA*) virulence genes were present in *E. coli* strains isolated from Cow milk and its products sold in Abuja (Nigeria). This indicates that the ready -to-eat dairy products are contaminated with virulent *E. coli* strains with potential to cause Enterohaemorrhagic food-borne diseases. It is important that standard hygiene practices are adopted at all stages of Cow milk processing.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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