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# MOLECULAR CHARACTERISATION AND VEROTOXIGENIC POTENTIALS OF ENTEROHAEMORRHAGIC *Escherichia coli* 0157:H7 ISOLATED FROM FERMENTED FRESH COW MILK (*Nunu*) SOLD IN SELECTED CITIES IN NIGERIA

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## ABSTRACT

*A total of 800 "Nunu" (a fermented fresh milk product) made up of 200 samples each from four major Nigerian cities, namely Abuja, Benin City, Lagos and Onitsha were investigated for the presence and prevalence rate of Enterohaemorrhagic Escherichia coli 0157:H7, using standard microbiological procedures on Sorbitol-MacConkey agar supplanted with cefixime and potassium tellurite (CT-SMCA). All non sorbitol fermenting isolates which were colourless on CT-SMCA, suspected to be Escherichia coli 0157:H7 were characterised serologically using Oxoid Latex agglutination kit and molecularly with PCR techniques. Tissue culture assay to determine the verotoxigenic potentials of all confirmed Escherichia coli 0157:H7 was done on Vero cells. Overall, E. coli was isolated from 268 (33.50%) samples, out of which 51(6.38%) were non sorbitol fermenting E. coli. Non sorbitol fermenters were isolated the most from samples collected in Lagos 17(8.50%) while Benin City recorded the lowest with 7(3.50%). Number of non sorbitol fermenting E. coli isolated from the survey was 31, 16, 58, and 43 from Abuja, Benin City, Lagos and Onitsha respectively. Of these, 9, 6, 17 and 21 were respectively confirmed as E. coli 0157:H7 with serological characterisation while molecular characterisation with PCR gave a varied result of 7, 5, 13, and 16 respectively. Tissue culture assay on Vero cells confirmed all the E. coli 0157:H7 isolates to be highly verotoxigenic. The findings from this study provide evidence of the presence of E. coli 0157:H7 in our food system as such, health authorities are advised on the need to properly monitor the production and sales of "Nunu" to prevent potential outbreak.*

**KEYWORDS**: *Escherichia coli* 0157:H7, *Verotoxigenic, Nunu, Fermented Milk*

## INTRODUCTION

Enterohaemorrhagic *Escherichia coli* 0157:H7 is an important food borne pathogen known to cause serious disease outbreaks worldwide. They are of considerable concern not only because of their increasing incidence worldwide, but also because of the severity of the infection they cause and their low infectious dose (Solomon *et al*., 2002.

Common complications arising from their infection include severe bloody

diarrhoea (haemorrhagic colitis), kidney failure (haemolytic uremic syndrome) and damage to the central nervous system (Nataro and Kaper 1998). This pathogen has caused severe public health and economic problems worldwide with numerous high-profile outbreaks and many sporadic cases.

Almost all of the outbreaks caused by enterohaemorrhagic *E. coli* 0157:H7 has been linked in one way or the other to food sources (Pradel *et al*., 2001, Solomon *et. al*., 2002, Hussein 2007).

Fermented fresh cow milk (nunu) which is one of such food produce that has been implicated as possible source of transmitting pathogenic microorganisms (Abdalla and El-Zubeir, 2006; Ekici *et al*., 2004; Adesiyun *et al*., 1995), is an opaque white to milky liquid food drink prepared and sold predominantly by wives of the nomadic Hausa/Fulani herdsmen who control over 80% of Nigeria's cattle production. It is a nutritiously rich food product whose consumption is known to transverse the Saharan tribes of West Africa sub-region extending to the inhabitants of the Mediterranean region and the Middle East (Ogbonna, 2011). Nunu is reported to be nutritionally rich, containing appreciable amount of essential amino acids, calcium, phosphorous and vitamins A, C, E and the B complex (Nebedum and Obiakor, 2007).

Although other investigators have analysed the microbiological quality of Nunu sold in Nigeria (Ogbonna, 2011; Obi and Ikenebomeh, 2007; Adesiyun *et al*., 1995), their reports have been of general nature and none have characterised the *E. coli* found in these reports to the sub strain level.

Consequent upon the above and the need to augment the scarce information on the organism in Nigeria, this research work was undertaken to isolate enterohaemorrhagic *E. coli* 0157:H7 from fermented fresh cow milk (nunu) sold in selected cities in Nigeria and thereafter characterize the isolates using both the popular serological methods used in Nigeria and the not very common molecular method with polymerase chain reaction (PCR) technique. Toxigenic potentials of all the isolates were also investigated on Vero cells to determine their verocytotoxicity.

## MATERIALS AND METHODS

Milk samples were collected randomly from different locations in four selected cities in Nigeria namely; Abuja, Benin City, Lagos and Onitsha. A total of 200 milk samples were collected from each city giving an overall total of 800 milk samples analysed. Sampling and screening was done between January, 2012 and December, 2012. Samples were collected in duplicates at point of sales in sterile plastic containers, labelled and transported to the laboratory for immediate analysis. All the samples were screened initially for *E. coli* from which all positive isolates were further screened for *E. coli* 0157:H7 using both serological method and molecular method with polymerase chain reaction (PCR) technique.

### *Isolation and identification of E. coli isolates*

The milk samples were homogenized in a sterile test tube by mixing with a sterile glass rod, thereafter 1 ml of the homogenate milk samples were suspended in 9 ml buffered peptone water. Serial dilutions of up to $10^{-5}$ were

then made and 1 ml of each plated on Eosin methylene blue (EMB) agar and incubated at 37°C for 24 h. Pure cultures of all colonies exhibiting typical dark to purple red colonies with metallic sheen which is characteristic of *E. coli* on EMB were then made in readiness for biochemical tests. Biochemical tests to confirm *E. coli* was done using the API 20E test strips and in accordance with the method described by Holt *et al.* (1994).

Identification and Characterisation of *E. coli* 0157:H7. Pure cultures of all positive *E. coli* were cultured on cefixim- tellurite sorbitol-MacConkey (CT-SMAC) agar using the method of Vernozy-Rozand (1997) and incubated at 37°C for 18 - 24 h. All non sorbitol fermenters which were colourless on CT-SMCA suspected to be colonies of *E coli* 0157:H7 were characterised serologically using *E. coli* 0157: H7 Latex agglutination test kit manufactured by Oxoid Ltd, UK. The tests were done according to manufacturer's instruction by emulsifying a colony of suspected isolates in a drop of antiserum on a clean test slide provided. Isolates suspected to be *E coli* 0157:H7 were recorded as those showing agglutination.

Molecular characterisation by PCR technique was done with primers manufactured by Primerdesign Ltd, UK, targeting the *stx 1* and *stx 2* genes according to method outlined by Blanco *et al.* (2003). DNA extraction was done according to Sambrook and Russell, (2001). The mixture for the amplification of *stx1* and *stx2* genes consisted of 2.5µl of PCR buffer (10mM Tris–HCl pH 9, 50mM KCl, and 0.1% Triton X-100), 2.5mM $MgCl_2$, 0.2mM of each dNTP, 1µM of each primer and 1.25U of Taq polymerase, in a final volume of 25µl. Amplification was done with a 96 well dual head Pelter thermocycler (DNA engine) model PTC-200. The amplification conditions consisted of an initial denaturation step at 94°C for 4 min, and 30 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (extension), and a final step at 72°C for 10 min. PCR products were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining in a UV trans-illuminator. All molecular work was done at the Lahor research and diagnostic Laboratory, Benin City.

***Tissue Culture Assay for Verotoxin production***

Preparation of extract for tissue culture assay was done by first inoculating pure cultures of all test isolates maintained on nutrient agar slants in 5ml Trypticase soy broth (TSB) and incubated overnight at 37°C. 500µl of the inoculated Trypticase soy broth was then transferred onto 5ml brain heart infusion broth (BHIB) and incubated at 37°C for 24 h. Extract filtrate was obtained by first centrifuging 1ml of the incubated BHIB at 4000xg using a Gemmyco centrifuge model PLC-025 to reduce debris and aid filtration. The supernatant was then passed through a 0.20µm pore-size titan two 30mm diameter nylon membrane syringe filter supplied by SMI-LabHut Ltd UK. Sterility check was done on all filtrate by plating an aliquot of the filtrate on MacConkey agar and incubated overnight at 37°C. Plates showing no growth were considered sterile.

The BHIB filtrates were screened for verocytotoxicity by using a modified method of Rahn *et al.* (1996). Filtrate was used to cause swelling, rounding or dissemination of vero cells prepared by

passaging on monolayer in 96 well micro titre plates. Vero cells for the cell culture assay was prepared by trysinizing with 1X trypsin – EDTA and seeded in 96 well flat bottom micro titre plate at 4 x $10^5$/well and using 1% Glasgow Minimum Essential Medium (GMEM) with addition of Hepes buffer as the growth medium . Growth medium was replaced with fresh GMEM after aspiration before cells were infected with filtrate. Undiluted 100µl of the filtrate was transferred to the first well of each plate containing the vero cells and 1:2 dilutions in each of the subsequent wells (i.e. 100µl, 50µl, 25µl, 12.5µl, 6.25µl and 3.125 µl) were then done. The plates were incubated at $37^0$C and examined for verotoxigenic activity after 3hrs, 6hrs, 12h 24h and 48h using an inverted microscope. Degree of verotoxigenic effect on each of the vero cells were noted and recorded after each examination. Picture of each well was taken before and after infection. Wells containing less than 25% cytotoxic effect after 48h end point were considered weak, greater than 25% but less than 50% fairly strong, greater than 50% but less than 75% strong, greater than 75% butless than 90% very strong and greater than 90% excellently strong. Tissue culture assay was done at the Morbilic and Related Virus Laboratory, National Veterinary Research Institute (NVRI), Vom, Jos Nigeria.

## RESULTS AND DISCUSSION

Enterohaemorrhagic *Escherichia coli* 0157:H7 is an important food borne pathogen known to cause serious disease outbreak worldwide. The isolation of the organism in any food source therefore, is usually of general public health importance, especially for the fact that disease outbreak caused by the organism usually result in significant medical situations and could lead to death (CDC, 2006; Chapman, 2000). *E. coli* 0157:H7 has previously been isolated from milk and milk products and have also been implicated as one of the major vehicle for the transmission of the organism to humans (Ferens and Horde, 2011; Baylis, 2009; Oliver *et al*., 2005). Result of the study shows that the organism was isolated from some of the samples examined in this investigation.

Overall, out of 800 samples analysed, *E. coli* was isolated from 268 samples giving a prevalence rate of 33.50% with Onitsha recording the highest (43.0%) and the lowest rate (24.0%) was recorded in Benin City (Table 1). Number and prevalence rates of sorbitol fermenting *E. coli* from the investigation, was 51(6.38%) overall. Lagos recorded the highest prevalence of sorbitol fermenting *E. coli* (8.50%) and Benin City the lowest with 3.50%. Basically, their inability to ferment Sorbitol in MacConkey agar is used as preliminary identification of *E. coli* 0157:H7 (Zedik *et al*., 1993).

Table 1: Prevalence and number of sorbitol fermenting and non – sorbitol fermenting *E. coli* isolated from fermented fresh milk

| City | No. of samples analysed | No. of samples with *E. coli* (%)** | No. of samples with sorbitol fermenting *E. coli* (%)** | No. of sorbitol fermenting *E. coli* isolated | No. of samples with non- sorbitol fermenting *E. coli* (%)** | No. of non-sorbitol fermenting *E. coli* isolated |
|---|---|---|---|---|---|---|
| Abuja | 200 | 53(26.50) | 42(21.0) | 93 | 11(5.50) | 31 |
| Benin City | 200 | 48(24.0) | 41(20.5) | 107 | 7(3.50) | 16 |
| Lagos | 200 | 81(40.50) | 64(32.0) | 139 | 17(8.50) | 58 |
| Onitsha | 200 | 86(43.0) | 70(35.0) | 164 | 16(8.00) | 43 |
| Total | 800 | 268(33.50) | 217(27.13) | 503 | 51(6.38) | 148 |

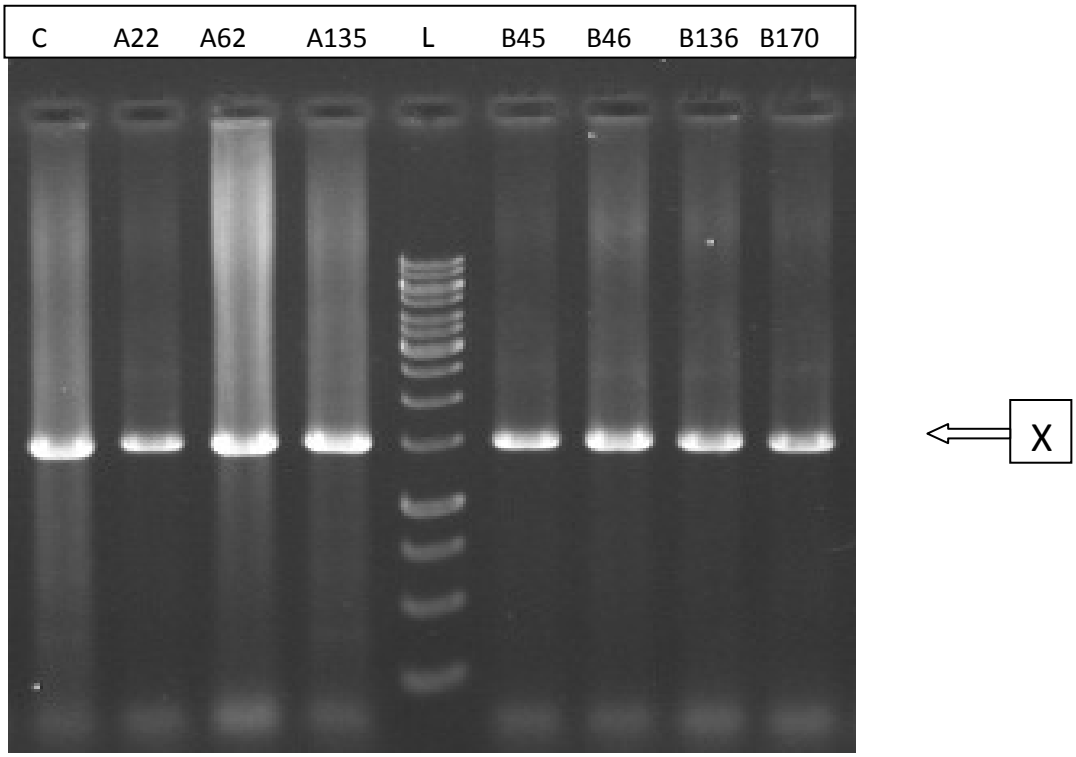
**(%) based on number of samples analysed

Table 2: Result of *E. coli* 0157:H7 characterised serologically and molecularly with PCR

| City | No. of non-sorbitol fermenting *E. coli* characterised | No. of Positive isolates with serology (%) | No. of positive isolates with PCR (%) | Difference btw result of Serology and PCR Characterised Isolates |
|---|---|---|---|---|
| Abuja | 31 | 9(29.03) | 7(22.58) | 2 |
| Benin City | 16 | 6(37.50) | 5(31.25) | 1 |
| Lagos | 58 | 17(29.31) | 13(22.41) | 4 |
| Onitsha | 43 | 21(48.84) | 16(37.21) | 5 |
| Total | 148 | 53(35.81) | 41(27.70) | 12 |

Traditional microbiological method of isolation and characterisation of food borne pathogens rely on conventional growth in culture media, followed by isolation, morphological, biochemical and serological identification. Recognition of surface antigens by antibodies resulted in the grouping of organisms based upon similarities of lipopolysacharides (LPS), capsule or flagella (Smith and Scotland, 1993). Detecting conserved traits such as fimbriae, toxins or invasion gene were often one of the first methods of pathogen identification (Whittam *et al*., 1993). However, recent developments of new analytical tools that require molecular typing of isolates have impacted profoundly on the specificity of identifying and proper characterisation of pathogenic microorganisms (Dinesh and Ambarish, 2009). Apart from the traditional isolation method on specific agar used and characterisation using serological techniques based on antigen antibody reactions,

Plate 1: *stx* Amplicons on gel electrophoresis. X is amplicon size 516, C is positive control, L is DNA ladder while the other wells are the test samples.




Molecular (PCR) technique that target specific gene(s) common to particular strain of microorganism was also used in characterising isolates in this survey. A comparison of the result obtained from characterising the non- sorbitol fermenters to confirm *E. coli* 0157:H7 using serology (latex agglutination) and molecular methods reveal that there was consistent difference in the result obtained from using the two methods as shown in Table 2 with the molecular method giving a lower prevalence rate than that confirmed with serology. Overall out of 148 non sorbitol fermenting *E. coli* characterised, 53 (35.81%) were positively confirmed serologically as *E. coli* 0157:H7 with a prevalence rate of 6.6% based on number of samples analysed; whereas 41(27.70%) was positively confirmed with PCR technique (plate 1) with a prevalence rate of 5.1%. The use of commercially available latex kits which is the most widely used method in Nigeria for confirming presence of *E. coli* 0157:H7 (Olorunshola *et al*., 2000; Ngbede *et al*., 2006; Dahiru *et al*., 2008 Enabulele and Uraih 2009), is known to have certain drawbacks which include the fact that antisera have been shown to cross react with other organisms antigen that might be present thereby giving false- positive result even with the most careful use of the kit according to manufacturer's instruction (Feng *et al*., 1996). The observed significant difference therefore between the two methods used is worthy of note as it has been shown severally that use of PCR gives consistent positive results that is almost 99% correct

(Vernozy-Rozand, 1997). Phenotypic variants have also been observed among *E. coli* 0157:H7 identified with antisera which were later characterised with other molecular methods (Feng *et al*., 1996).

Tissue culture assay using Vero cell is a method for the confirmation of verotoxin production in microorganisms. The profound sensitivity of Vero cells to shiga- toxins (STX) was first observed by Konowalchuk *et al*., (1977) and cytotoxicity to this cell line has remain a gold standard for confirming putative shiga- toxin producing isolates since Vero cells have a high concentration of globotriaosylceramide ($Gb_3$) and globotetraosylceramide ($Gb_4$) toxin-binding receptors in their plasma membranes and will detect all variants of verotoxins. *E. coli* 0157:H7 is a known shiga-toxin producing organism; however the mechanism of production whether chromosomally, phage or plasmid based is still a subject under serious investigation by scholars in the field. Plate 2 is the picture of the uninfected Vero cells while plates 3 and 4 show varying degree of typical cytotoxic effects of verotoxins on Vero cells.

Results of the verotoxin capability of isolates from the study(Table 3) indicate that all the non sorbitol fermenting *E. coli* showed some appreciable degree of cytotoxic effect with over half of those isolates confirmed as *E. coli* 0157:H7 with both serological and PCR techniques showing excellently strong (≥ 90%) cytotoxic effect on Vero cells. These results confirm the observation of other workers (Smith and Scotland, 1993; Slutsker *et al*., 1997; Al-Charrakh and Al-Muhana, 2010) that the organism is a vero-toxin producer. Vero or shiga toxins are the major virulence factor and defining characteristic of most Enterohaemorrhagic *E. coli (*EHEC). It is a potent cytotoxin that leads to serious medical conditions such as haemorrhagic colitis (bloody diarrhoea) and haemolytic uremic syndrome (HUS) which can lead to eventual death. The characteristics and cytotoxic effects of Vero toxins have been extensively reviewed by LeJeune, *et al*. (2006); Thorpe *et al*. (2002) and Sears and Kaper, (1996).

Milk and milk products of dairy cattle are known repository of food borne pathogens. Basically the presence of food borne pathogen in milk has been variously traced to direct contact with contaminated sources in the dairy farm environment, and to excretion from the udder of an infected animal (Oliver *et al.,* 2005). Although other ruminants such as sheep, goat and deer are known to harbour *E. coli* 0157:H7, cattle is recognised as the main reservoir of the organism (Doyle *et al*., 2006; Caprioli, 2005). Nunu is a fermented product produced directly from fresh milk obtained from cattle and is sold in the open market by wives of Fulani cattle herdsmen who have been variously observed from report of other workers (Aernan *et al.,* 2011; Ogbonna, 2011) on Nunu not to observe good hygienic practice in the milking, production and sales of the product. The product is observed to be widely consumed in Nigeria (especially by people of northern origin) unpasteurised and without further processing. Therefore the isolation of *E. coli* 0157:H7 from nunu is of significant public health importance. A recent report by Aernan *et al.,* 2011, stated that a family of seven died in the northern state of Zamfara after drinking the beverage in 2008.

Plate 2: Uninfected Vero Cells still intact

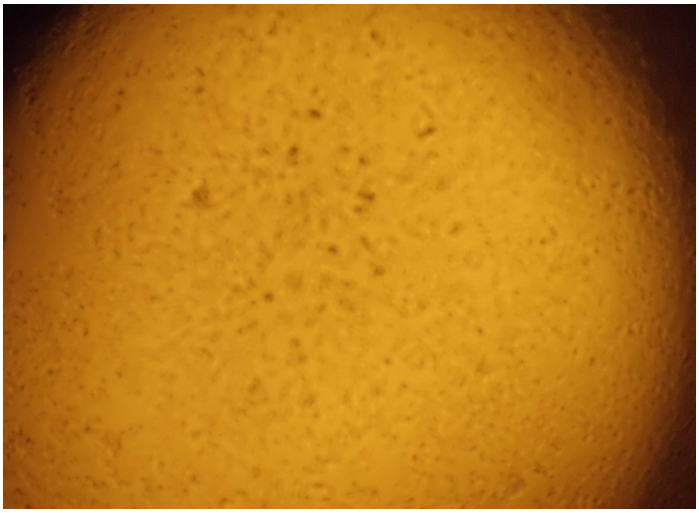


Plate 3: Infected Vero cells showing some degree of cytotoxic effect on it

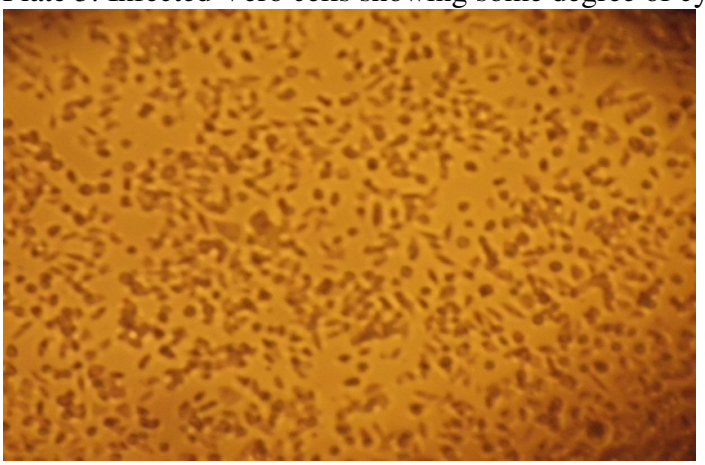


Plate 4: Infected Vero cells showing high degree of cytotoxic effect on it with the vero cells being detached and completely being eroded

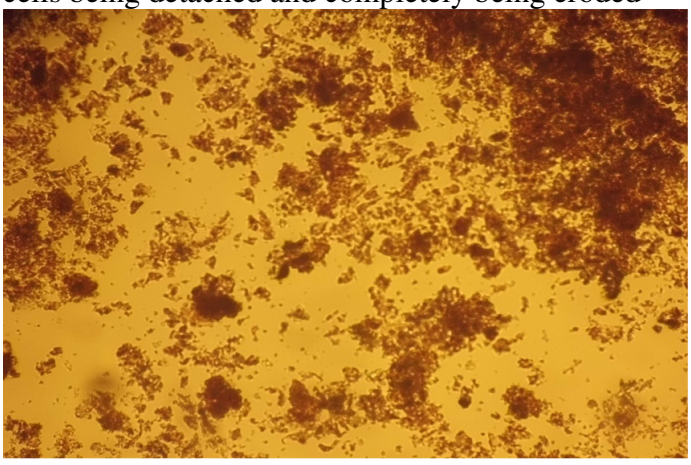


Table 3: Verotoxin potentials of non –sorbitol fermenting *E. coli,* PCR and serologically characterised *E. coli* 0157:H7 isolates from Milk

| City | Non-sorbitol fermenting *E. coli* tested | | | | | | Serologically confirmed *E. coli* | | | | | | PCR confirmed *E. coli* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. tested | No. of Isolates showing varying degree of verotoxigenicity | | | | | No. tested | No. of Isolates showing varying degree of verotoxigenicity | | | | | No. tested | No. of Isolates showing varying degree of verotoxigenicity | | | | |
| | | a | b | c | d | E | | a | b | c | d | E | | a | b | c | d | E |
| Abuja | 31 | Nil | 2 | 5 | 6 | 18 | 9 | Nil | 1 | Nil | 2 | 6 | 7 | Nil | Nil | 1 | 1 | 5 |
| Benin City | 16 | Nil | 1 | 1 | 4 | 10 | 6 | Nil | Nil | 1 | Nil | 5 | 5 | Nil | Nil | Nil | 1 | 4 |
| Lagos | 58 | Nil | 7 | 9 | 12 | 30 | 17 | Nil | Nil | Nil | 6 | 11 | 13 | Nil | Nil | Nil | 3 | 10 |
| Onitsha | 43 | Nil | 8 | 10 | 13 | 12 | 21 | Nil | 1 | 3 | 3 | 14 | 16 | Nil | Nil | 1 | 6 | 9 |
| Total | 148 | Nil | 18 | 25 | 35 | 70 | 53 | Nil | 2 | 4 | 11 | 36 | 41 | Nil | Nil | 2 | 11 | 28 |

Key: a = ≤ 25% (Weak Cytotoxic Effect); b = btw 26 and 50% (Strong Cytotoxic Effect);c = btw 51 and 75% (Very strong Cytotoxic Effect); d = btw 76 and 90% (Very very strong Cytotoxic Effect); e = ≥ 90% (Excellently strong Cytotoxic Effect)

The death may not be directly traced to the presence of the organism, however it is to be noted most often reported cases of bloody diarrhoea are hardly investigated for the presence of *E. coli* 0157:H7 as such detecting the organism is missed. Consequently, most local health centres where the patients go for treatment are not properly equipped to isolate and characterise such organisms hence the illness may not be attributed to the organism.

In conclusion, this study established the presence of *E. coli* 0157:H7 in nunu sold in Nigeria. Effort should therefore be made by local health authorities to properly monitor the way nunu is produced and sold and also put in place proper surveillance strategy aimed at identifying actual causative agents of food borne infections reported in the hospitals and especially those reported in the rural areas where these food products are mostly consumed.

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