

Prevalence and Antibiotic Susceptibility Pattern of Enterohemorrhagic *Escherichia coli* O157:H7 isolated from various samples obtained from three different areas of Rivers State, Nigeria

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Abstract: Prevalence and antibiotic susceptibility pattern of enterohemorrhagic *Escherichia coli* O157:H7 isolated from various samples obtained from three different areas of Port Harcourt, Rivers State, Nigeria were investigated. Two hundred and forty (240) samples of fresh beef, dung, cabbages and carrots were obtained from three different areas of Port Harcourt viz: Rumuokoro, Rumuji and Oginigba. Isolates were obtained on Sorbitol-MacConkey Agar (SMAC) supplemented with cefixime and potassium tellurite following pre-enrichment on Triptycase Soy Broth supplemented with novobiocin and cefixime and incubated at 37°C for 24h. Following routine biochemical tests, confirmation was carried out with the Wellcotex *E. coli* O157:H7 specific antiserum (Oxoid, UK). Both *E. coli* O157 and *E. coli* O157:H7 strains were isolated depending on the sample and sample site. Determination of beta lactamase enzyme production ability of the isolates revealed that most isolates produced Metallo β-lactamase enzyme while none of them produced Extended Spectrum β-lactamase enzyme (ESBL). The antibiotic susceptibility screening revealed that Nitrofurantoin, Cefotaxime, Gentamicin and Ciprofloxacin were effective against the isolates obtained with the isolates being most sensitive to Ciprofloxacin. Due to its public health importance, although the prevalence level was very low, only the presence of one positive sample can serve as a source of a major chain of events. It is therefore very important to develop proper sanitary hygiene within and outside the home, vegetable farms and slaughter houses. The need for proper education of farm workers by the relevant government agencies cannot be overemphasized.

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1. Introduction

E. coli includes a very large population of bacteria that exhibit a very high degree of both genetic and phenotypic diversity. Studies have shown that *E. coli* remains one of the most diverse bacterial species with only about 20% of the genome common to all strains (Lukjancenko *et al.*, 2010). Different strains are often host-specific making it possible to determine the source of fecal contamination in samples (Feng *et al.*, 2002). For instance, knowing the *E. coli* strains present in cow dung allows a researcher to make assumptions about the origin or source of a particular contamination.

Although a normal flora of the gut of humans, some strains develop traits that can be harmful to the host. In the developing world, these virulent strains typically cause a bout of diarrhoea that is unpleasant in healthy adults and is often lethal to children (Nataro and Kaper, 1998). More virulent strains such as O157:H7 cause serious illness or death in the elderly, the very young or the immune compromised (Hudault *et al.*, 2001; Nataro and Kaper, 1998).

Currently, there are six main classes of diarrheagenic *E. coli* based on their pathogenic

features and distinct virulence determinants. These include the enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusively adherent *E. coli* (DAEC) (Nataro and Kaper, 1998).

Studies have shown that among the diarrheagenic *E. coli* strains, STEC strains are distinguished by their ability to cause complications that are severe and life-threatening, such as haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Karch *et al.*, 2005). Most people infected with STEC O157 usually develop watery diarrhoea, often bloody diarrhoea, abdominal cramps and hemorrhagic colitis (HC) may also occur. STEC strains that cause HC and HUS are also called EHEC. Most illnesses end on their own within 7 days while some last longer and can be more severe. Rarely, some develop the more severe infection, Hemolytic Uremic Syndrome (HUS), which is a type of kidney failure that begins as the diarrhoea is improving. HUS can occur in people of any age but children under 5years, the

elderly and immunocompromised individuals are more likely to suffer from the most severe complications (Thorpe, 2004; Duffy *et al.*, 2006, Razzaq, 2006; Walch *et al.*, 2006; Centre for Disease Control and Prevention, 2012).

In the fall of 1991, an *E. coli* O157:H7 outbreak in Southern Massachusetts provided an opportunity to study and identify the transmission of the pathogen via an unlikely vehicle. From the study, it was revealed that *E. coli* O157:H7 likely to cause severe infections can be transmitted through fresh pressed unpreserved apple cider. Thorough washing and brushing of the apples before pressing and preservation of the cider with sodium benzoate can reduce the risk of transmission (Besser *et al.*, 1993).

In 1994, there was a multistate outbreak of *Escherichia coli* O157:H7 in Washington. The largest reported outbreak resulted from errors in meat processing and cooking. Infection was associated with eating hamburgers at a fast food chain in 10 days before the symptoms began (Bell *et al.*, 1994). In 1996, another multistate outbreak of *Escherichia coli* O157:H7 infection associated with the consumption of Mesclun lettuce was reported in Connecticut and Illinois. The implicated lettuce was traced to a single grower-processor, where cattle, a known *E. coli* O157:H7 reservoir, were found near the lettuce fields (Hilborn *et al.*, 1999).

In May 2011, an *E. coli* strain, *Escherichia coli* O104:H4, was the subject of a bacterial outbreak that began in Germany. The outbreak started when several people in Germany were infected with EHEC bacteria, leading to HUS, a medical emergency that requires urgent treatment. The outbreak did not only concern Germany but 11 other countries including regions in North America. On 30 June 2011, the German Federal Institute for Risk Assessment, a federal fully legal entity under public law of the Federal Republic of Germany, an institute within the German Federal Ministry of food, Agriculture and Consumer Protection announced that seeds of fenugreek from Egypt were likely the cause of the EHEC outbreak (Federal Institute for Risk Assessment, 2011). Knowing that *E. coli* O157:H7 is host specific and has the ability to infect foods, vegetables and food products, it is important to investigate its presence in various samples of interest.

2. Materials And Methods

2.1. Sample Collection

The samples were obtained from different sources in three different markets/areas (Rumuokoro, Rumuji and Oginigba) of Rivers State. For each of the four sample types, twenty were collected per market/area (that is, 60 samples per sample type). The beef samples were purchased from retailers in

local markets, placed in sterile plastic bags and appropriately labelled. The cow dung samples were collected directly from the rectum of cow using sterile arm-length gloves placed in sterile sample collection bottles and appropriately labelled. The carrots were purchased from retailers at local markets or shops, placed in sterile plastic bags and appropriately labelled. The cabbages were purchased from retailers at local markets or shops, placed in sterile plastic bags and appropriately labelled. After collection, samples were held at 4°C in an ice box and immediately transported to the laboratory for analyses. The samples were analysed at not more than 2h on arrival.

2.2. Isolation of Enterohemorrhagic *E. coli* O157:H7

Twenty-five grams (25g) of each sample was homogenised, using Lab Stomacher blender 400, in 225ml of Triptycase Soy Broth supplemented with novobiocin (2µg/ml) and cefixime (50µg/ml) and incubated at 37°C for 24h. A tenfold serial dilution of the pre-enriched samples was made using Peptone Water. From the dilution for use, 0.1ml aliquot dilution was plated onto Sorbitol-MacConkey Agar (SMAC) supplemented with cefixime (0.05mg/ml) and potassium tellurite (2.5mg/ml) and seeded plates incubated at 37°C for 24h.

2.3. Serological Confirmation Of *Escherichia coli* O157:H7

Confirmation of isolates was by slide agglutination using Wellcotex *E. coli* O157:H7 specific antiserum, Oxoid, UK. (Ateba and Mbewe, 2011 modified). Forty micrograms (40µl) of sterile normal saline was placed on four circles of the reaction card and labelled accordingly. The mixing stick was used to obtain a small amount of the test isolates and emulsified in the saline with the flat end of the sticks and the sticks were discarded. A drop of the O157 test latex (Wellcolex REF 30959601) was placed in one circle; a drop of the O157 positive control latex was dropped on the second circle, a drop of H7 test latex on the third circle and H7 positive control latex on the fourth. The contents of the individual circles were mixed together in each circle, spreading the latex over the entire area of the circle. The card was rocked slowly for 30secs and observed for agglutination. The presence of agglutination (clumping) indicated a positive result while absence of agglutination indicated negative result for either O157 and/or H7 antigens as was applicable.

2.4. Determination Of Enzyme Production

2.4.1. Determination of Extended Spectrum Beta Lactamase (ESBL) Production

Mueller Hinton agar plates were seeded with the standard inoculum suspensions of the test isolates and

allowed to stand for 15mins for diffusion. Antibiotic disks containing 30 μ g of Aztreonam, Cefazidime and Ceftriaxone were placed 15mm from an Amoxicillin-Clavulanic acid disk (20 and 10 μ g respectively) on the seeded medium and incubated at 37°C for 18-24h. An enhanced zone of inhibition between any of the β -lactam disks and the disk containing clavulanic acid was interpreted as an evidence for the presence of an ESBL (Akujobi *et al.*, 2008).

2.4.2. Determination of AmpC Beta Lactamase Production

Mueller Hinton agar plates were seeded with the standard inoculum suspensions of the test isolate and allowed to stand for 15mins for diffusion. Antibiotic disks containing 30 μ g of Cefoxitin and another containing 30 μ g of Cefoxitin and 40 μ g of Boronic acid were placed on the seeded medium. An organism demonstrating a zone diameter around the disk containing Cefoxitin and Boronic acid \geq 5mm than the zone diameter around the disk containing Cefoxitin alone was considered an AmpC producer. (Akujobi *et al.*, 2010).

2.4.3. Determination of Metallo Beta Lactamase Production

Mueller Hinton agar plates were seeded with the standard inoculum suspensions of the test isolate and allowed to stand for 15mins for diffusion. Antibiotic disks containing Meropenem, Meropenem+EDTA, Imipenem and Imipenem+EDTA were placed on each of the seeded plates. The plates were incubated for 18-24h at 37°C and examined for growth inhibition. An increase in zone size of \geq 7mm around imipenem+EDTA and/or meropenem+EDTA disk than meropenem or imipenem alone is taken as a positive test for metallo β -lactamase production. (Akujobi *et al.*, 2010).

2.5. Antibiotic Sensitivity Screening

2.5.1. Inoculum Preparation

Ten millilitres (10mls) of sterile Nutrient broth was prepared. A loopful of the test isolate (18h) was collected and inoculated into the sterile broth to make a suspension. To achieve a turbidity equivalent to a McFarland standard, which contains approximately 1×10^8 CFU/ml, adequate light was visually used to compare the inoculum tube and the 0.5 McFarland standards against a white card with a contrasting black line.

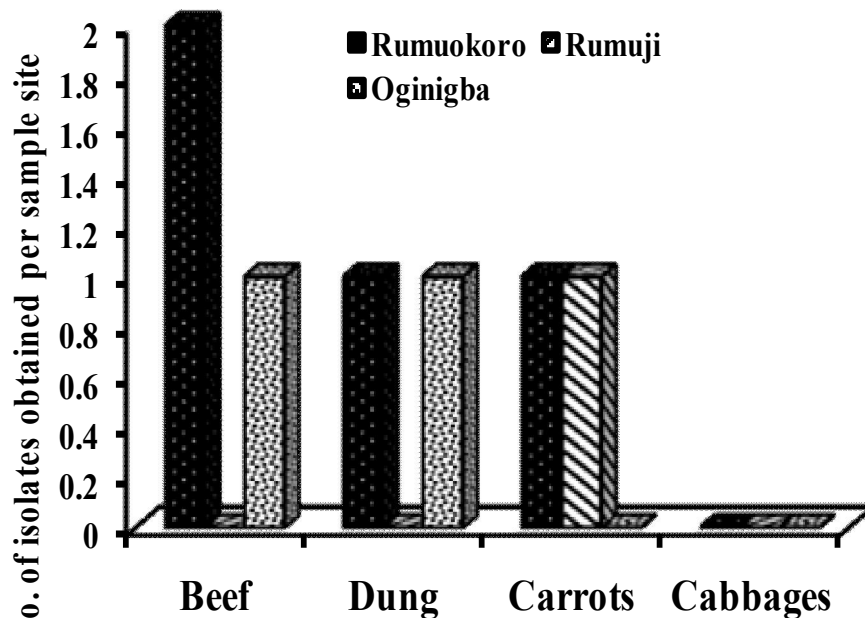


Figure 1: Distribution of *E. coli* 0157 isolated from various samples in three different areas

2.5.2. Antibiotic Susceptibility Screening Using Disk Diffusion Test

Fifteen minutes after adjusting the turbidity of the inoculum suspension, a sterile swab stick was

dipped into the adjusted suspension and the dry surface of the Mueller-Hinton agar plate seeded with the test organism by streaking the swab over the entire surface of the sterile agar. This was repeated

twice to ensure an even distribution of the test isolate. The seeded plate was then allowed to stand for 15mins afterwards, and then the antibiotic disks were placed on seeded plates. The antimicrobial agents tested include Ceftazidime, Cefuroxime, Gentamycin, Ciprofloxacin, Floxacin, Amoxycillin/ Clavulanate, Nitrofurantoin and Ampicillin. The plates were inspected for growth after 24h incubation at 37°C and the diameters of the zones of inhibition carefully

measured then translated into susceptible, intermediate or resistant categories as is applicable. The presence of a growth inhibition zone larger than the established breakpoint diameter is an indication of susceptibility to that agent (British Society for Antimicrobial Chemotherapy, 2012).

3. Result

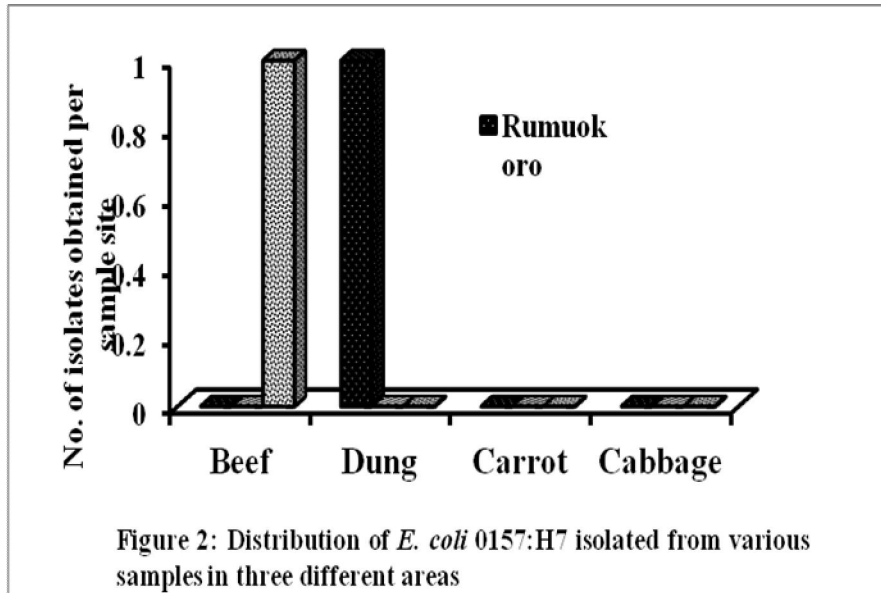


Figure 2: Distribution of *E. coli* 0157:H7 isolated from various samples in three different areas

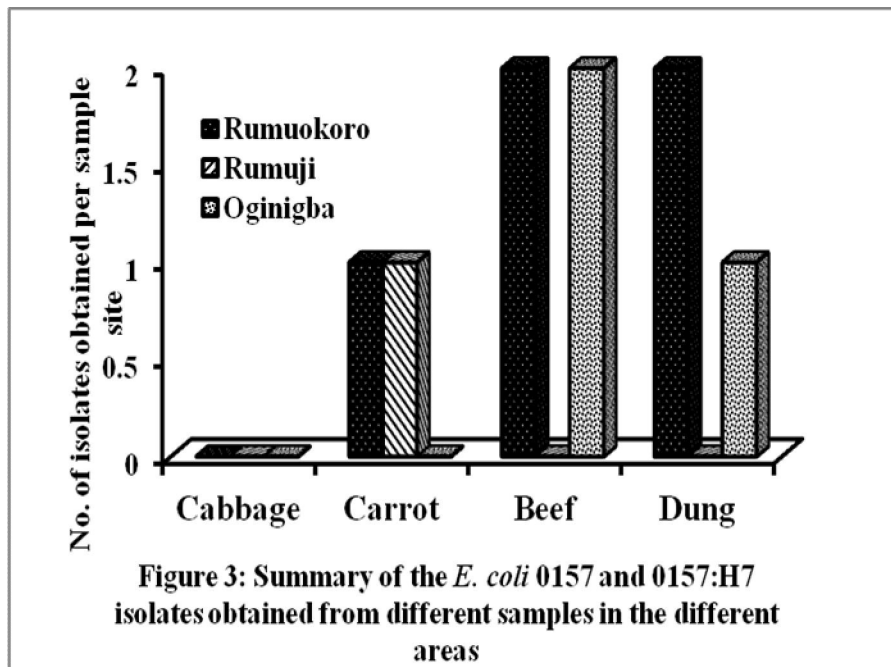


Figure 3: Summary of the *E. coli* 0157 and 0157:H7 isolates obtained from different samples in the different areas

Samples were collected from tree locations in Port Harcourt, Rivers State, Nigeria. The locations are Rumuokoro, Rumuji and Oginigba areas of Port

Harcourt. There were variations in the distribution of isolates made in the different locations. Beef samples from Rumuokoro yielded the highest number of

E.coli 0157 isolates but no isolate of *E.coli* 0157:H7 strain was recovered. Beef sample from Rumuji yielded neither *E.coli* 0157 nor *E.coli* 0157:H7 strains while the beef samples Oginigba yielded equal numbers of both strains. Also, the cow dung samples from Rumuokoro yielded equal numbers of both strains of *E.coli*. There was no isolate from dung samples of Rumuji. Equally, there was no isolate of any of the strains from cabbage samples collected from Oginigba while carrot samples from Rumuji and Rumuokoro yielded equal numbers of *E.coli* 0157 strain. These are presented in figures 1 and 2. There was no isolation of *E.coli* 0157:H7 from carrot

samples. Generally, the highest numbers of isolates were obtained from beef and dung samples of Rumuokoro and Oginigba (Figure 3).

The strains were tested for the production of Extended Spectrum β -Lactamase (ESBL), AmpC β -lactamase and Metallo β -lactamase enzymes. Among the *E.coli* 0157:H7 isolates, only cow dung isolates were positive for the production of AmpC and Metallo β -lactamase enzymes. The number of isolates that produced AmpC β -lactamase enzyme were higher than those that produced Metallo β -lactamase enzyme (Figure 4).

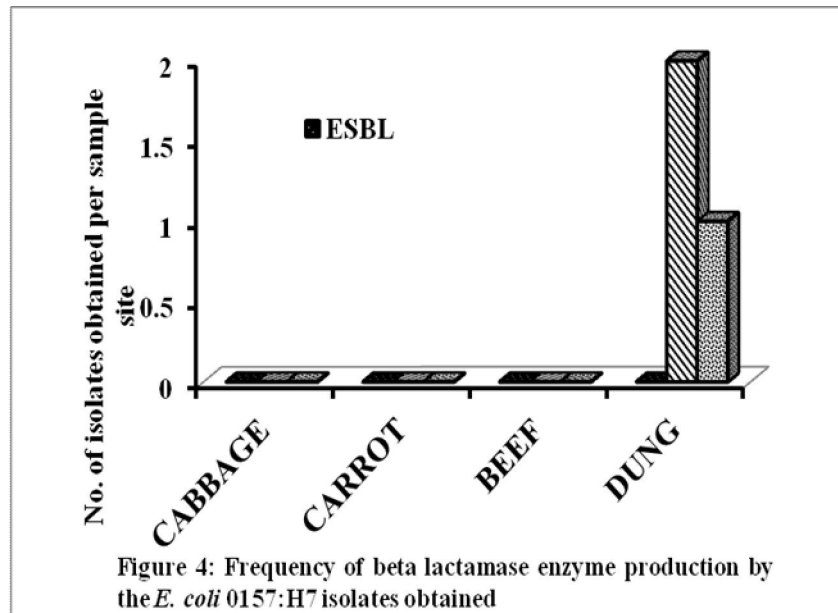
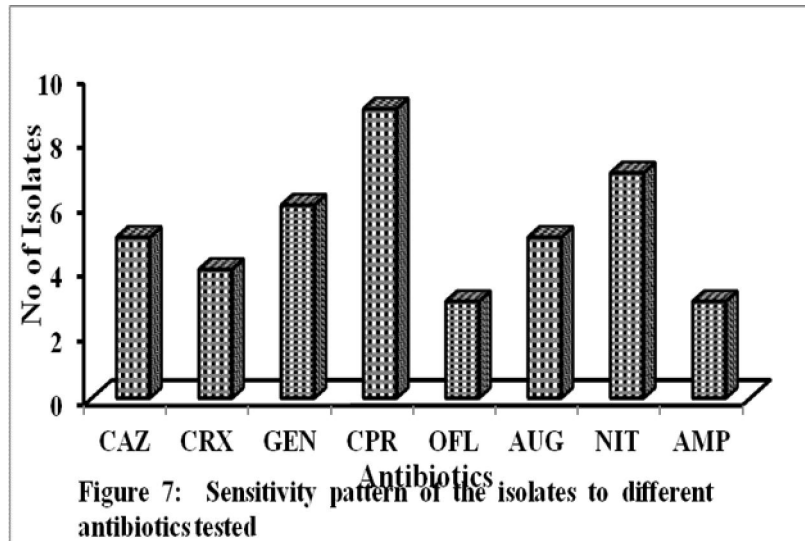
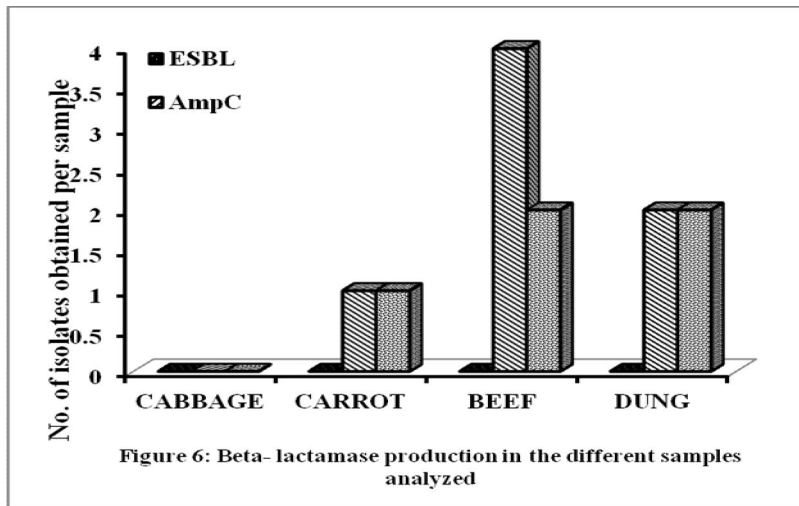
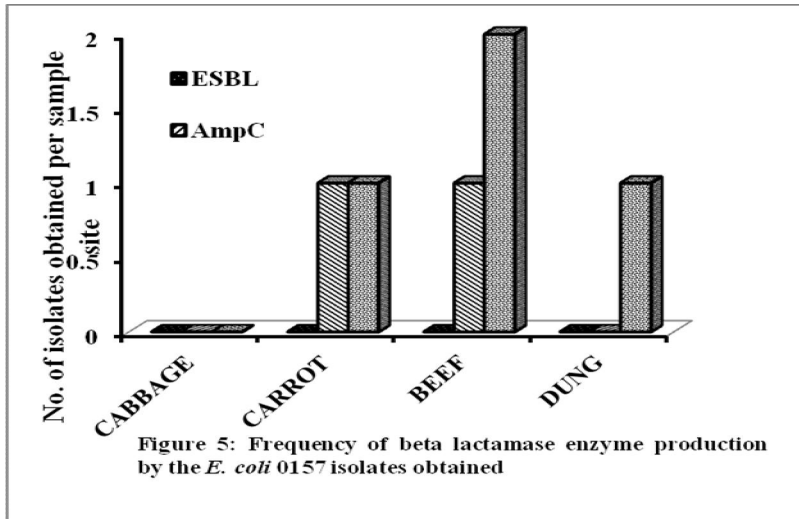


Figure 5 shows the frequency of β -lactamase enzyme production by *E.coli* 0157 isolates. The result shows that more isolates from beef samples produced Metallo β -lactamase enzyme. ESBL was not produced by any isolate while equal numbers of isolates from carrot and dung samples produced AmpC and Metallo β -lactamase enzymes respectively. Considering the production of the enzymes by both isolates from various samples, it was discovered that the highest number of isolates from beef samples produced AmpC and Metallo β -lactamase enzymes. Equal numbers of isolates from carrot samples produced AmpC and metallo β -lactamase enzyme. Also equal numbers of isolates from dung samples produced AmpC and metallo β -lactamase enzymes but the numbers of isolates that produced these enzymes in dung samples were higher than those that

produced the enzymes in carrot samples as depicted in figure 6.

The isolates had varying degrees of sensitivities to the various antibiotics tested. Most of the isolates were susceptible to ciprofloxacin antibiotic (Figure 7). Most of the isolates were resistant to ampicillin and ofloxacin antibiotics. The second highest level of susceptibility was observed in nitrofurantoin antibiotic. The highest percentage sensitivity to the antibiotics in both strains was observed in their sensitivity to ciprofloxacin, *E.coli* 0157 showed higher percentage antibiotic sensitivity than *E.coli* 0157:H7 (Figure 8). *E.coli* 0157:H7 strain showed higher percentage antibiotic sensitivity in nitrofurantoin, ampicillin, augmentin, cefuroxime and ceftazidime while *E.coli* 0157 strain showed higher percentage antibiotic sensitivity to gentamicin and ciprofloxacin only.



KEY: CAZ-Ceftazidime; CRX- Cefuroxime; GEN- Gentamicin; CPR- Ciprofloxacin
 OFL- Ofloxacin; AUG- Augmentin; NIT-Nitrofurantoin; AMP-Ampicillin

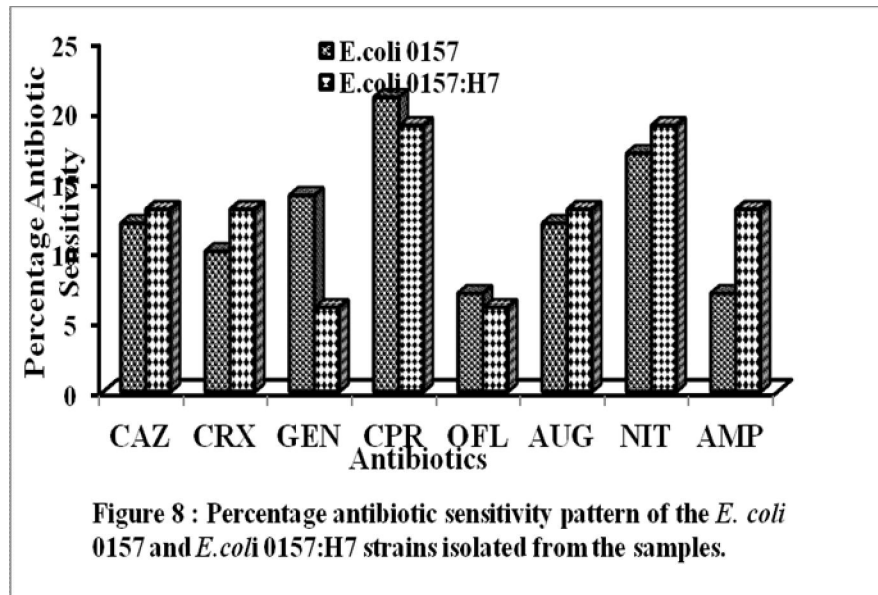


Figure 8 : Percentage antibiotic sensitivity pattern of the *E. coli* 0157 and *E. coli* 0157:H7 strains isolated from the samples.

KEY: CAZ-Ceftazidime; CRX- Cefuroxime; GEN- Gentamicin; CPR- Ciprofloxacin
OFL- Ofloxacin; AUG- Augmentin; NIT-Nitrofurantoin; AMP-Ampicillin

4. Discussion

Due to the health implications of the microorganism of interest, the extent to which the isolates can contaminate foods and food products cannot be overemphasized. The microorganisms from a normal and healthy animal can contaminate the slaughtering environment, cutting materials and even the personnel during the production and processing. If good hygiene is not properly observed, the pathogens may also be transferred to the food products, especially at sale points (Bouvet *et al.*, 2001; Tutenel *et al.*, 2003; Yilmaz *et al.*, 2006).

In this study, *E. coli* 0157:H7 was successfully isolated from the cow dung and beef samples tested. *E. coli* 0157 was isolated from a carrot sample while neither *E. coli* 0157 nor 0157:H7 was isolated from the cabbage samples tested.

The mere presence of *E. coli* 0157:H7 and *E. coli* 0157 isolates in the dung samples analyzed is a cause for alarm because it reveals the presence of the STEC in the gastrointestinal tract of the ruminant which may be transferred by cross contamination to beef cuts and other materials that may come in contact with the dung. Furthermore, studies have also revealed that *E. coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation which allows genetic material spread horizontally through an existing population. It is this process that can lead to the spreading of the gene encoding shiga toxin from *Shigella* to *E. coli* 0157:H7 carried by a bacteriophage as reported by Brussow *et al.*, (2004). It is therefore imperative to ensure that even one cell of this pathogen is not

transferred to foods or food products so as to avoid a transfer of virulent genes to previously non-toxicogenic bacteria because just one cell has the ability to transfer plasmids to surrounding populations of microorganisms.

The fresh beef samples from Rumuokoro and Oginigba had varying numbers each of *E. coli* 0157 and 0157:H7 while Rumuji had neither of the strains. Under normal circumstances, meat is sterile prior to the exsanguinations and slaughtering of animals. It is therefore most likely that the isolates obtained from the fresh beef were introduced from the gastrointestinal tract, cow dung on the slaughter floor or poor handling practices during the slaughtering and exsanguinations of cattle. Furthermore, there is also the possibility of transferring the isolates during the transportation of the beef as beef cuts from an uncontaminated batch may be mixed with those from a contaminated lot. As such, contamination ensues.

With a high potential for food borne transmission to humans, STEC are mainly commensal bacteria in animals (Caprioli *et al.*, 2005). The predominant reservoir of STEC are ruminants, predominantly cattle, and beef products serve as one of the most important sources of food-borne STEC transmission (Tsuji *et al.*, 2002; Caprioli *et al.*, 2005; Maruzumi *et al.*, 2005) as was supported by the result in a study showing that almost all STEC isolates obtained in a study involving different samples were recovered from ground beef (Ateba and Mbewe, 2011). Other researchers around the world have also studied the contamination of beef by STEC. A recent study by Samapdour *et al.* in the United States reported STEC

in 3.5% of 1750 retail ground beef samples obtained from stores in Seattle, WA (Samadpour *et al.*, 2006). Other studies on STEC revealed 4% in beef samples in France (Pradel *et al.*, 2000), 3% in raw beef samples in Australia, 1.75% of minced beef samples in Switzerland (Fantelli and Stephan, 2001) and 1.5% of beef samples in Korea (Lee *et al.*, 2009).

It is worthy of note that the demand for beef in Nigeria is quite high and as such, a possible contamination of beef is a cause for serious concern. Although the results of studies carried out worldwide to show the prevalence of *E. coli* O157:H7 in beef varies, it would be fair to suggest that the prevalence of *E. coli* O157:H7 in animals and meats sold by meat vendors in the markets largely depends on the hygienic conditions of the farms that reared the animals, the equipment used in the slaughterhouses, the surrounding environments during slaughter and the personnel involved in meat processing.

The Rumuokoro market is densely populated with a carnal flowing beside and used water from the slaughter flows back into the water body. After slaughtering, beef cuts, primal and sections are transferred by carriers using wheel barrows, trucks or bikes to the meat vendors at different sale points within and/or outside the market. An originally sterile beef cut, if mixed with a contaminated batch will get contaminated. Also, the meat vendors at the markets neither wash their aprons daily nor do they package the beef cuts and as such there could be an easy and repeated transfer of the pathogen from one beef cut to another. The beef cuts are repeatedly touched by either the meat vendors or possible buyers hence exposing the beef cuts to more contamination. The meat vendors sometimes have to serve more than one person at a time; using the butchers' knife to cut a bought part into smaller cuts and using the same knife for another cutting can result in a possible transfer of the pathogen to a previously 'pathogen-free' cut. The underlying issue of poor hygiene and handling practices is therefore a cause for concern and meats bought must be adequately cooked to reduce the risk of a possible infection in humans.

The study revealed the presence of *E. coli* O157 strain in Rumuokoro and Rumuji carrot samples tested while the Oginigba sample had none. From the results obtained in this study, the pathogens may have been transferred in the market or at the vegetable purchasing point. Another possibility is that the contamination may have come from the farm land by contaminated manure, the water used for washing the vegetables prior to display on the market tables or during transportation to the market.

The cabbage samples obtained from the three different areas and analyzed did not have the pathogens of interest. Inability to isolate the pathogen

may have been due to the nature of the cabbage, which consists of clusters of stiff vegetables superimposed one over the other in compact layers giving it a round or globular shape, and the fact that only the inner layers are consumed. Although the cabbages tested were not contaminated, contamination may have been on the surface of the cabbage and as such removal of the outer leaves and subsequent washing may get rid of any remaining microorganisms. It is, however, still very important to observe good sanitary hygiene on farms, during harvesting, handling and packaging to ensure that the vegetable is not contaminated at any point.

The beta lactamase production ability of the isolates obtained from the study was also determined. Increasing resistance to third-generation cephalosporins is predominantly due to the production of extended-spectrum beta lactamases. These plasmid mediated enzymes mostly evolved via point mutations of the classical TEM-1 and SHV-1 beta lactamases but other groups are increasingly predominant, notably the CTX-M types, which evolved via the escape and mutation of chromosomal beta lactamases from *Kluyvera* spp (BSAC, 2012). In this study, none of the isolates obtained were able to produce the extended beta-lactamase enzyme but produced the metallo and AmpC beta lactamase enzyme although at varying capabilities. The cow dung isolates were also observed to produce the beta lactamases more than the other isolates obtained from other sources.

Antibiotic susceptibility pattern of the isolates obtained in this study was also carried out. Studies have shown that in recent times, *E. coli* strains have evolved the ability to withstand antibiotics and as such there is a need to check the susceptibility pattern of the isolates obtained to regular antibiotics routinely used in hospitals. From the antibiotic sensitivity pattern in this study, it was observed that different isolates responded differently to the antibiotics used. However, the study revealed that all the isolates were most sensitive to Ciprofloxacin and the highest resistance was observed in Ampicillin. The sensitivity of a particular isolate to an antibiotic may be due to the host specific nature of the isolate or the position of its virulence gene.

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