

**Determination of the Antibiogram, Extended Beta-Lactamase Production, and Metallo-beta-lactamase of *E. coli* O157:H7 Isolated from Pigs and Periwinkles sold in Rivers State**

**Abstract**

The emergence of multi-drug resistant and Extended Beta-lactamase producing *E. coli* O157:H7 is a general public health problem. Food samples of animal origin are undoubtedly the reservoirs of MDR which are in most cases transmitted to humans. The presence of extended beta-lactamase and metallo-beta-lactamase producing *E. coli* O157:H7 was investigated amongst food samples and pig dung. A total of 240 samples comprising pig dung, pork and periwinkles were collected from three different locations in Port Harcourt, Rivers State, Nigeria. Samples were analyzed for the presence of *E. coli* O157:H7 using standard microbiological methods. The antibiotic susceptibility pattern and the production of ESBL, MBL and AmpC was analyzed using standard methods. The results of the antibiotics susceptibility test of *E. coli* O157: H7 isolated from various samples revealed that out of the 16 *E. coli* O157: H7 isolated from the samples, the percentage resistance to Tetracycline, Ciprofloxacin, Ampicillin, Cotrimoxazole, Chloramphenicol, Amikacin, Ceftrizole, Gentamycin, Streptomycin and Nalidixic was 62.5, 18.7, 100, 43.7, 50, 87.5, 87.5, 31.2, 50 and 56.3 %, respectively. While the antibiotics susceptibility pattern for *E. coli* O157 revealed that out of the 35 *E. coli* O157 isolated from the samples, the percentage resistance to Tetracycline, Ciprofloxacin, Ampicillin, Cotrimoxazole, Chloramphenicol, Amikacin, Ceftrizole, Gentamycin, Streptomycin and Nalidixic was 60, 22.9, 57.1, 42.9, 34.3, 60, 71.4, 42.9, 68.6 and 45.7 %, respectively. The results of the *E. coli* O157:H7 with Extended  $\beta$ -Lactamase, Metallo Beta-Lactamase and Amp C  $\beta$ -Lactamase Production revealed that out of the thirty-five isolates screened phenotypically only two (25.5%) were positive for ESBL, five (62.5%) for MBL while 1 (12.5%) was positive for AmpC. Thus, the food samples do not only harbour enteropathogenic *E. coli* but are reservoirs of beta-lactamases resistant genes which conferred multidrug resistance to the isolates. These food samples and dungs could serve as vehicles for the transmission of these resistant genes in the food chain.

*Keywords: Extended Beta-Lactamase, Metallo-beta-lactamase, E. coli O157:H7, Pigs, Periwinkles*

**Introduction**

In poor and developing countries where there is little or no attention to environmental hygiene, foodborne infections are the main cause of morbidity and mortality (Ugwu *et al.*, 2018). Food animals especially pigs and poultry act as one of the major reservoirs of antimicrobial-resistant (AMR) organisms and determinants due to the overuse/misuse of antibiotics in farms to prevent and reduce the risk of infection (Landers *et al.*, 2012). In a previous study, it was reported that food products of animal origin play a key role in the dissemination of antibiotic resistance since antibiotics are used in the rearing of farm animals, and the antibiotic residues in these animals tend to cause the emergence of resistant bacteria via selective pressure (Ugwu *et al.*, 2018). Also, another vital factor that has promoted the emergence of multidrug-resistant (MDR) bacteria in aquatic fauna is the direct or indirect discharge of wastes through land-based anthropogenic

activities into water bodies and the rapid dissemination of extended-spectrum  $\beta$ -lactamase-producing Enterobacteriaceae in marine coastal ecosystems is troublesome due to the presence of enterobacteria species especially *E. coli* which inhabits the gut of fishes (Hassen *et al.*, 2020) and other benthic fauna as commensal bacteria.

The enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is amongst the food borne pathogenic *E. coli* that causes disease in humans. (Bell 2000; Leelaporn *et al.*, 2003; Marnizume *et al.*, 2005; Sugiyama *et al.*, 2005). *E. coli* O157:H7 is an emerging public health concern in most countries of the world (Sugiyama, 2005) and it is an important cause of foodborne human disease. Since the first reported case of *E. coli* O157:H7 in 1982 in USA, out breaks and sporadic cases of disease due to this organism have also had a fairly wide geographic distribution in the Africa continent (Isibor *et al.*, 2012). *E. coli* O157:H7 is highly virulent with a low infections dose such that an inoculum of fewer than 10-100 CFU in food and water is sufficient to cause infection compared to over one million CFU/ml for other pathogenic *E. coli* (Greig, 2007). The production of extended-spectrum beta-lactamase (ESBL) and metallo-beta-lactamases (MBLs) especially by pathogenic *E. coli* is well documented.

The extended-spectrum beta-lactamase (ESBL) is an enzyme that confers resistance to most Gram-negative bacterial isolates against beta-lactam antibiotics (penicillin, cephalosporins and monobactam aztreonam) and these ESBL producing microbes belong to the Enterobacteriaceae family of bacteria which could be home to different antibiotic resistance determinants thereby leading to difficulty in treating infections caused by these pathogens (Chishimba *et al.*, 2016). Similarly, metallo-beta-lactamases (MBLs) are beta-lactamase enzymes that are mostly associated with pathogenic Gram-negative microbes, mostly in *E. coli* species and the clinical infections with MBL-producing isolates are known to cause higher morbidity and mortality (Fazlul *et al.*, 2019). Previous study has also reported frequent resistance to many antimicrobial agents (such as gentamicin, fluoroquinolones, and trimethoprim-sulfamethoxazole) that are mostly recommended for the treatment of infections caused by the ESBL pathogens (Rodriguez-Bano *et al.*, 2008). The World Health Organization has listed the ESBL-producing *E. coli* as the most critical AMR pathogen to human health and a major public health concern (Kanokudom *et al.*, 2021). Bacterial isolates which are known ESBL producers possess complex epidemiology and the commonest bacteria known are *E. coli* and *K. pneumoniae* whose reservoirs are mostly in

the environment (soil and water), wild animals, farm animals, food, and pets (Carattoli, 2008). It has also been reported that antibiotic resistance genes could be transferred among bacteria of varying taxonomic groups and that the transmission of resistant microbes from animals to humans is well established. Sibhghatulla *et al.* (2015) in a previous study reported that ESBLs are generally transmissible  $\beta$ -lactamases which are encoded and expressed by genes that can be exchanged between bacteria. To the best of our knowledge, there is paucity of information in the detection or isolation of ESBL and MBLs *E. coli* O157:H7 associated with pigs and periwinkles. Thus, this current study seeks to provide useful information which could serve as epidemiological tool in control/prevention of outbreaks of *E. coli* O157:H7 infections.

## **Materials and Methods**

### **Collection of Sample**

Fresh pork and periwinkle samples were bought from retailed markets in three geographical areas (Romokoro, Rumuji and Oginigba) all in Rivers State. This was replicated twenty times, to obtain a sample size of 60 for each while 20 faecal samples from pig were collected randomly in piggery farms from the same geographical areas in Rivers State, and also repeated twenty times (5-10 droppings per sampling point). The faecal samples were collected from the rectum of the animals using sterile arm-length gloves and place inside a sterile sample container. A total of 240 samples was generated and all samples were quickly transported to the Microbiology laboratory of the Department of Microbiology, University of Port Harcourt for immediate analysis.

### **Extraction of periwinkles**

The unpasteurized periwinkles (in the shell) were washed by scrubbing the shell with a sterile stiff brush under running water, rinsed and water allowed to drain off the shell. The raw periwinkles were shucked without subjecting the periwinkle to any heat treatment in order to effectively assess the microbiological qualities of this sample. In view of this, the raw periwinkle samples were shucked based on the methods of Reynolds and Wood (1959). In this method, a metal hammer was used in cracking the periwinkle shell on an improvised sterile anvil while the periwinkle flesh was individually extracted from the broken shell using forceps (Odu,1989). The extracted meat was placed into sterile beaker and consequently analyzed. Prior to cracking of the shell, the hammer and forceps were sterilized by cleaning with 70% ethanol before dipping in

absolute ethanol and flamed in a Bunsen burner. While the improvised anvil was washed with mild soap solution, rinsed with distilled water, wrapped in aluminum foil and oven dried. This was subsequently sterilized by dry heat using electrical oven (Unitemp, Greenfield, Oldham England) at 160°C for 1hour 30 minutes. After which, twenty-five grams (25g) of extracted meat and liquor sample was homogenized in 225ml sterile trypticase soy broth (Oxoid UK) supplemented with Novobiocin (2µg/ml) and Cefixime (50ng/ml) using Lab stomacher blender 400. The homogenate was incubated at 37 °C for 8hrs.

### **Pasteurized chunked periwinkles samples**

Similarly, the periwinkles used as the pasteurized samples were first washed by scrubbing the shell with a sterile stiff brush under running water. Twenty-five grams (25g) of the Pasteurized chunked sample was homogenized in 225mls of sterile trypticase soy broth (Oxoid UK) supplemented with Novobiocin (2µg/ml) and Cefixime (50ng/ml) using Lab stomacher blender 400. The homogenate was incubated at 37 °C for 8hrs.

### **Preparation of Fresh Pork Samples; FDA method**

Fresh pork samples were prepared using the FDA method (BAM, 1998). In this method, twenty-five grams (25g) of the pork sample was homogenized in 225mls of trypticase soy broth (Oxoid UK) supplemented with Novobiocin (2µg/ml) and Cefixime (50ng/ml) using Lab stomacher blender 400. The homogenate was incubated at 37°C for 8hrs. While the dungs were prepared by transferring 10g of dung sample into 250ml conical flasks containing sterile 90 ml trypticase soy broth (Oxoid UK) supplemented with Novobiocin (2µg/ml) and Cefixime (50ng/ml), it was homogenized by shaking gentle for 5mins. The homogenate was incubated at 37 °C for 8hrs

### **Isolation of *E. coli* O157:H7**

Tenfold serial dilutions were made of the pre-enriched samples of unpasteurized and pasteurized periwinkles, pork and pig dung in trypticase soy broth using 2% peptone water. After which, 0.1ml from each dilution of respective samples was plated on prepared labelled Sorbitol MacConkey Agar (SMAC) supplemented with Cefixime (0.05mg/ml) and Potassium Tellurite (2.5mg/ml) in duplicates. The plates were incubated at 37°C for 24hrs. After 24hrs, the non-sorbitol fermenting colonies (colorless or grayish colonies) presumptively identified as *E. coli*

O157:H7 were collected and sub-cultured for purification on CT-SMAC medium, and stored on Trypticase Soy with 1% yeast extract Agar slant for preservation.

### **Confirmation of Presumptive *E. coli* O157:H7 Isolates**

#### **Slide Agglutination Test for O157 Test Procedure**

Forty micrograms (40µl) of sterile normal saline were placed on three circles of the reaction card and labeled accordingly (test, negative control, positive control), the mixing stick was used to obtain a small amount of the 18hrs old 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 (red color (Remel, Oxoid) was placed in one circle; a drop of the O157 positive control latex was dropped on the second circle, and a drop of O157 negative control latex on the third circle. The contents of the circles were mixed together, spreading the latex over the entire area of the circle. The card was rocked slowly for 30 seconds and observed for agglutination. The presence of agglutination (clumping) indicated a positive result while absence of agglutination indicated negative result for O157.

#### **Slide Agglutination Test for H7 Test Procedure**

Forty micrograms (40µl) of sterile normal saline were placed on three circles of the reaction card and labeled accordingly (test, negative control, positive control), 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 H7 test latex (blue (Remel, Oxoid) was placed in one circle; a drop of the H7 positive control latex was dropped on the second circle, and a drop of H7 negative control latex on the third circle. The contents of the circles were mixed together, spreading the latex over the entire area of the circle. The card was rocked slowly for 30 secs and observed for agglutination. The presence of agglutination indicated a positive result while absent of agglutination indicated negative result for H7 (Nataro and Kasper ,1998).

### **Antibiotic Sensitivity Screening of Test Isolates Using Standard Disk Diffusion Test**

#### **Preparation of Inoculum**

Ten milliliters (10ml) of sterile Nutrient broth were prepared; a loopful of the test isolate (18hrs) was collected and inoculated into the broth to make a suspension. The suspension was adjusted

to achieve a turbidity equivalent to a 0.5 McFarland standard (suspension contains approximately  $1 \times 10^8$  CFU/ml), this was performed visually by using adequate light to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black line (CLSI, 2012).

### **Disk Diffusion Test**

The antibiotics susceptibility test was evaluated using the disk diffusion technique as described by Wemedo and Robinson (2018). In this method, a sterile swab stick which was immersed in the standardized inoculum was swabbed evenly on freshly prepared Mueller-Hinton agar plate which were allowed to dry for 3 minutes (Robinson and Korobe, 2019). After which, disks containing antibiotics was placed on the surface of the dried agar plates and incubated at 37°C for 18-24 hours in the incubator. After incubation, zone diameters were measured using a graduated rule in mm and were interpreted according to CLSI (2012).

### **Determination of Extended Spectrum Beta Lactamase**

This was also carried out as described by CLSI (2012). Muller Hinton agar plates were inoculated with the suspension of the standard inoculum of the test organisms, it was left for 3-5mins before disks containing 30µg of Aztreonam, Ceftazidime and ceftriazone 15mm (edge to edge) from an Amoxicillin–Clavulanic acid disk (20µg and 10µg, respectively) were placed on each of the inoculated plates. The plates were incubated at 37°C for 18-24hrs. An enhanced zone of inhibition between any one of the beta lactams disks and the disk containing Clavulanic acid is interpreted as evidence for the presence of an ESBL.

### **Determination of Metallo Beta-Lactamase Production**

This was done as described by CLSI (2012). In this method, Muller Hinton agar plates were prepared and inoculated with the standardized inoculum of the test isolate. Plates were left for 3-5mins for diffusion. After which, antibiotic disks containing Meropenem and Meropenem + EDTA. The meropenem + EDTA disc was prepared by adding 10µl of 0.5 M EDTA solution to the imipenem discs (Fazlul *et al.*, 2019). The discs were placed on each of the inoculated plates 25-30 mm apart. The plates were incubated at 37°C for 18-24hrs (Barua *et al.*, 2013). The plates were observed for growth or zone of inhibition after incubation. An increase in zone size of  $\geq 7$

around Meropenem + EDTA than Meropenem alone is taken as positive test for metallo  $\beta$  lactamase production (Fazlul *et al.*, 2019).

### **Determination of Amp C $\beta$ –Lactamase Production**

Muller Hinton agar plates were inoculated with the suspension of the standard inoculum of the test organisms and left for 3-5mins before disks containing 30 $\mu$ g of Cefoxitin and another containing 40 $\mu$ g of Boronic acid were placed on each of the inoculated plates 20-25mm apart. The plates were incubated at 37 °C for 18hrs. 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 is considered an Amp C producer (Barua *et al.*, 2013).

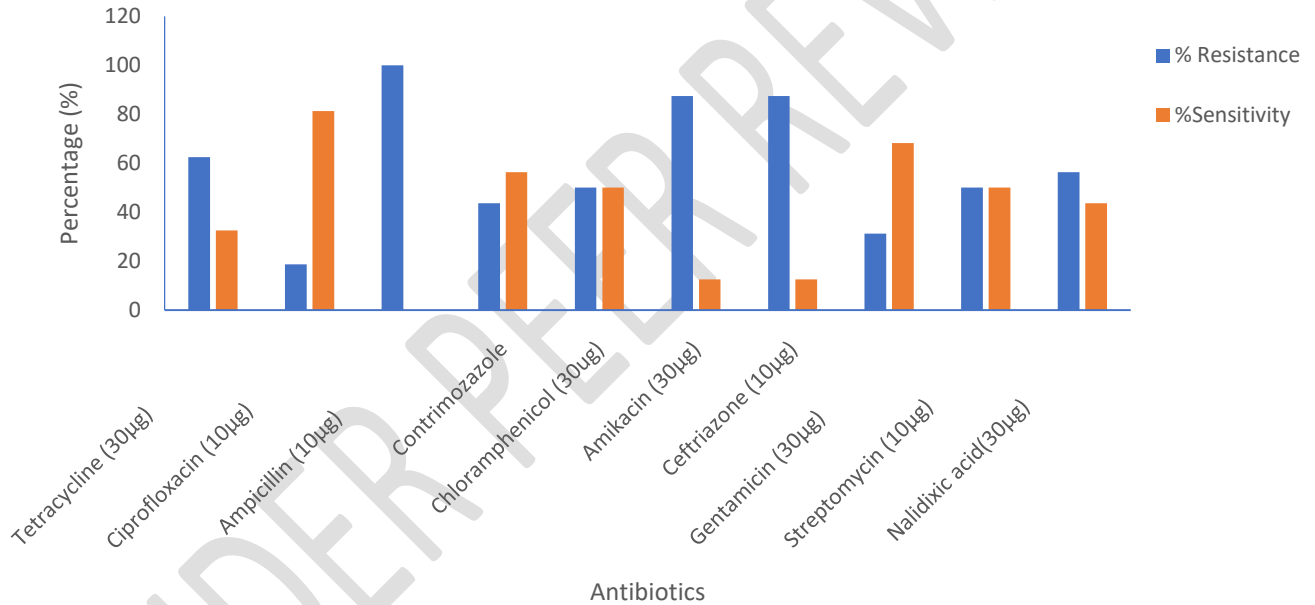
### **Results**

In this study, a total of 240 samples from pasteurized and unpasteurized periwinkle, pork and faecal samples from pig were analyzed for the presence of ESBL and MSBL genes in *E. coli* O157:H7. Results of the antibiotics susceptibility test of *E. coli* O157: H7 isolated from various samples is presented in fig 1. The result revealed that out of the 16 *E. coli* O157: H7 isolated from the samples, the percentage resistance to Tetracycline, Ciprofloxacin, Ampicillin, Cotrimoxazole, Chloramphenicol, Amikacin, Cefetrizole, Gentamycin, Streptomycin and Nalidixic was 62.5, 18.7, 100, 43.7, 50, 87.5, 87.5, 31.2, 50 and 56.3 %, respectively. While the level of sensitivity of the antibiotics in same order was 32.5, 18.3, 0, 56.3, 50, 12.5, 12.5, 68.3, 50 and 43.7 %.

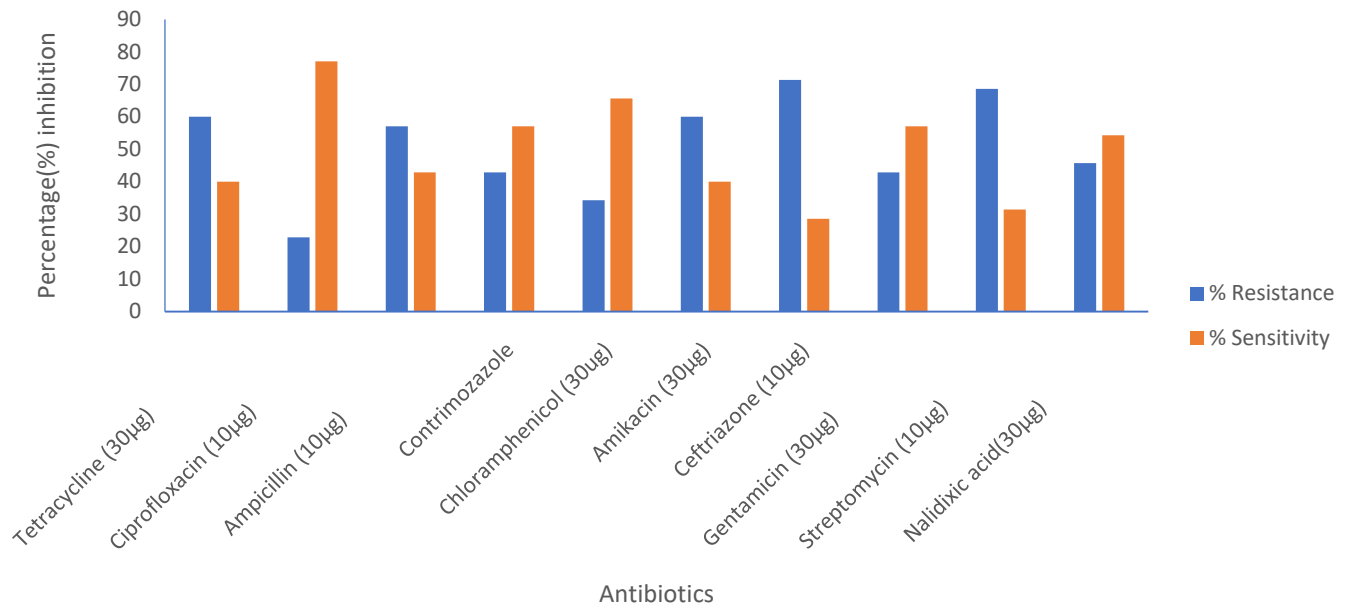
Results of the antibiotics susceptibility test for *E. coli* O157 isolated from various samples is presented in fig 1. The result revealed that out of the 35 *E. coli* O157 isolated from the samples, the percentage resistance to Tetracycline, Ciprofloxacin, Ampicillin, Cotrimoxazole, Chloramphenicol, Amikacin, Cefetrizole, Gentamycin, Streptomycin and Nalidixic was 60, 22.9, 57.1, 42.9, 34.3, 60, 71.4, 42.9, 68.6, 45.7 %, respectively.

The results of the *E. coli* O157:H7 with Extended  $\beta$ –Lactamase, Metallo Beta-Lactamase and Amp C  $\beta$ –Lactamase Production is presented in Table 1. Out of the thirty-five (35) isolates of *E. coli* screened phenotypically for ESBL, MBL and AmpC production, only two (25.5%) isolates were positive for ESBL, five (62.5%) for MBL while 1 (12.5%) was positive AmpC production.

Thus, amongst the isolates tested, two were resistant to Aztreonam, Ceftazidime, ceftriazone and clavulanic acid, five were resistant to meropenem while only one isolate was resistant to cefoxitin antibiotics. The result also showed that the enzymes which aid in the resistance were not evenly distributed across the various samples. For instance, the ESBL were only detected in *E. coli* isolated from pig dungs and pasteurized periwinkles, AmpC was only detected in pork while MBL were only detected in Pig dung, unpasteurized periwinkle, pasteurized periwinkle and Pork.



**Fig. 1: Antibiotics susceptibility pattern of strains of *E. coli* O157:H7 isolated from various samples (n=16)**



**Fig. 2: Antibiotics susceptibility pattern of strains of *E. coli* O157 isolated from the various samples (n= 35)**

**Table 1: Numbers of *E. coli* O157:H7 isolates positive for ESBLs, MBLs and AmpC production**

Source of Isolate	No. with ESBL (%)	No. with MBL (%)	No. Amp C (%)
Rumuokoro			
Pig dung	1	1	0
u. periwinkle	0	1	0
Oginigba			
U. periwinkle	0	1	0
Pig dung	0	1	0

Rumuiji			
P. periwinkle	1	1	0
Pork	0	1	1
Total	2 (25%)	5 (62.5)	1(12.5)

Keys: *ESBL* = extended beta-lactamase; *MBL*= metallo-beta-lactamase, *AmpC*= cefoxitin resistance; *U*= unpasteurized, *P*= pasteurized

## Discussion

Antimicrobial resistance has become a great public health concern due to the inability of pathogens to respond to treatment especially to drugs which they had been sensitive to. The WHO has reported that antimicrobial resistance (AMR) is common in many countries (Mahmoodi *et al.*, 2020). The antimicrobial susceptibility tests of both the *E. coli* O157 and O157:H7 showed high presence of multidrug resistant. Multidrug resistant *E. coli* which agreed with the findings of this current study has been reported by previous studies (Hassen *et al.*, 2020; Mahmoodi *et al.*, 2020; Ugwu *et al.*, 2018). High rate of AMR amongst food products has been ascribed to the continuous use of antimicrobial agents as food supplements in rearing of animals. According to Chishimba *et al.* (2016) food products serves as vehicles and reservoirs in the spread of AMR, and the desire to optimize animal production has led to indiscriminate use of antibiotics in Africa. In Nigeria, the use of antibiotics as supplements in food has increased especially with increased animal farms and most of these farms use the antibiotics indiscriminately without proper guidance on the required dosage. This could explain the high level of antimicrobial resistance observed in this study. Also, the indiscriminate dumping of refuse or antimicrobials in water bodies could also cause an increase in AMR. This agreed with Hassen *et al.* (2020). In this current study, the percentage of *E. coli* O157:H7 with ESBL, MBL and AmpC production were 25, 62.5 and 12.5%, respectively. Ugwu *et al.* (2018) in a previous study reported that 12% (7 isolates) of *E. coli* were positive for the production of ESBL out of the 45% (27 isolates) screened while Chishimba *et al.* (2016) in a previous study reported a total of 20.1% (77 isolates) out of 384 *E. coli* were confirmed to be ESBL-producing isolates. Also,

the findings in this current study do not agree with the findings of Ismail *et al.* (2016) who reported 80% ESBL producing *E. coli* from food recovered from animal origins in Brazil. ESBL production has been attributed to the high use of antibiotics and this has resulted to an increased trend of resistance to commonly used antibiotics such as ampicillin, cotrimoxazole, gentamicin, erythromycin, tetracycline, and third generation cephalosporins (Reich *et al.*, 2013). The presence of these enzymes (ESBL, MBL and AmpC) has been reported to confer resistance against beta-lactam antibiotics especially the microbes belonging to *Enterobacteriaceae* family. The antimicrobial susceptibility tests revealed that most of the antibiotics with high resistance were the beta-lactam antibiotics. Thus, this resistance could be attributed to the production of beta-lactamases which enhanced the inactivation of the beta-lactam ring as well as conferring resistance to other antibiotics (Sibhghatulla *et al.*, 2015). The antimicrobial susceptibility showed that five of the *E. coli* were resistant against Meropenem and this could be due to the presence of the MBL genes. According to Cornaglia *et al.* (2011), metallo beta-lactamase has been reported to enhance resistance against carbapenems and this is an increasing public problem especially since these genes can be transferred to other organisms in the environment. Contrary to the 10% strains of MBL producing *E. coli* reported by Ugwu *et al.* (2018) this current study recorded higher MBL producing *E. coli* isolates. Resistance to cefoxitin by the isolates were also observed and amongst the isolates, only one (12.5%) was AmpC producer unlike the 20% of *E. coli* producing AmpC isolated from anal region of cows which was reported in a previous study carried out in Ebonyi State, Nigeria (Malini and Aditi, 2016).

## **Conclusion**

Generally, this study revealed that food samples especially periwinkles, pork and pig dung are reservoirs for enteropathogenic *E. coli* which are multidrug resistant. The level of antimicrobial resistance including the presence of ESBL, MBL and AmpC producing *E. coli* in these food samples calls for public health attention. Thus, proper processing of food samples before consumption is highly recommended and the indiscriminate use of antibiotics on food samples or its disposal of antibiotics in the environment should be regulated.

**COMPETING INTERESTS DISCLAIMER:**

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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