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OA did the laboratory work and literature search; JBA (of blessed memory) read and edited the manuscript; OSO designed and provided guidance on the molecular aspect. CMZW improved on some of the methods and also read the manuscript.

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Antibiotic Resistance Profile and Detection of TEM Genes in *E. coli* Strains Isolated from Some Food Samples

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Abstract:

The study aimed to determine the antibiotic resistance profile using disc diffusion test and to assess the presence of TEM genes from twelve *E.coli* strains isolated from some food samples (smoked fish, 'zoborodo' and grilled meat, 'suya'). Multidrug resistance was found in approximately 67% of the total *E. coli* strains with four patterns; AMP, KF, CPD, SXT, TE; AMP, KF, CPD, CRO, TE; AMP, KF, CPD, SXT, TE, AMC and AMP, KF, CPD, CRO, SXT, TE, AMC. All strains were susceptible to amikacin and ciprofloxacin. MAR index analysis reveals that eleven out of twelve *E. coli* strains (91.7%) had high MAR index greater than 0.2. The *E. coli* strains were analyzed by PCR amplification for the presence of bla_{TEM}, using primers and conditions described in the literature. They are typically encoded by plasmids that can be exchanged readily between bacterial species. Eight *E. coli* strains (67%) were found to possess the TEM gene. Seven of the eight (87.5%) were resistant to cefpodoxime and /or ceftriaxone in the antibiotic sensitivity disc diffusion test (DDT) of the strains. The remaining one was found to be susceptible to all the antibiotics studied, yet harbouring a TEM gene. This could be due to inoculum effect and substrate specificity which may render the enzyme in an un-induced state at the time of the DDT. This creates a major challenge in laboratory routine susceptibility tests. Three of the *E.coli* strains (Ze_2, Sye_2& Sye_6) were all resistant to cefpodoxime, yet do not harbor TEM genes. Other beta-lactamase genes that were not included in this study may have been responsible for the resistance. *E. coli* strain $SFe_{15,}$ was not resistant to any of the 3rd generation cephalosporins and does not harbor TEM genes. This result minimally shows that the environment is markedly burdened and therefore resistance to commonly used antibiotics is inevitable.

INTRODUCTION

Foodborne illnesses are an important challenge to public health and cause significant economic problems in many countries. *Escherichia coli* infection is a disease that can be transmitted directly or indirectly between animals and humans. It is common in developing countries (like Nigeria) because of the poverty level and lack of education, because of the prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory systems, lack of financial resources to invest in safer equipment, and lack of education for food handlers, coupled with very low awareness of the general public (Tadesse *et al.,* 2018). *E. coli*, a surrogate marker employed to reflect the hygienic quality of food, is abundant in the human gastrointestinal tract and also serves as a reservoir or carrier of antibiotic-resistant genes. It has also been found to show increasing resistance to different antibiotics as well as to third-generation cephalosporins (Ayandele *et al.,* 2020).

Antibiotic resistance (AR) has become an issue of global interest. This microbial resistance is not a new phenomenon since all microorganisms have an inherent capacity to resist some antibiotics. However, the rapid surge in the development and spread of AR is the main cause of concern (Agyare *et al.,* 2018). Antibiotic resistance is therefore a threat to clinicians and pharmaceutical industries, research institutes, and the public at large. The World Health Organization (WHO) lists antibiotic resistance as one of the most significant threats to global health, food security, and development today (Kuti *et al.,* 2018). Antibiotic resistance genes are not confined to the clinic; instead, they are widely prevalent in different bacterial populations in the environments (Peterson and Kaur, 2018). Research has shown that the emergence and spread of these drug-resistant genes can limit therapeutic options and are characterized with increased morbidities and mortalities, prolong hospital stays, and cost massive economic loss (Sah *et al.,* 2021). Authors from several countries have reported an increase in the resistance rate of *E. coli* strains to numerous antibiotics (Nguyen *et al.,* 2020). The presence of antibiotic-resistance genes in *E. coli* is a serious worldwide public health concern. This is because *E. coli* is not only a common constituent of intestinal microbiota but also an important indicator of feacal contamination of aquatic environments. Its presence in food generally indicates direct or indirect feacal contamination. The substantial number of *E. coli* in foods suggests a general lack of cleanliness in handling and improper storage. Most antibiotic-resistant *E. coli* strains enter the aquatic ecosystem and subsequently food chain through various anthropogenic activities, discharge from livestock, poultry production, hospital and municipal wastewaters (Singh *et al.,* 2018). Antibiotic-resistant bacteria and antibiotic-resistant genes can easily spread at each stage of the food production chain, hence the term from 'farm to fork' and can cause infections in humans (Founou *et al.,* 2016). Plasmids are extrachromosomal genetic material found in most bacteria. They play a key role in the evolution of bacterial antibiotic resistance disseminating resistance genes among the most worrisome pathogens (Milian, 2018). Antibiotic resistance (AR) has led to the recognition of the emergence of multidrug-resistant organisms which in turn has given rise to increased mortality and economic burden, and Nigeria is no exception to the challenges faced due to AMR (Aworh *et al.,* 2019). High prevalence of multidrug resistance indicates a serious need for surveillance and planning of effective interventions to reduce multidrug resistance in such pathogens. Multiple antibiotic resistance (MAR) indexing has been shown to be a cost-effective and valid method of bacteria source tracking. MAR in bacteria is most commonly associated with the presence of plasmids that contain one or more resistance genes, each encoding a single antibiotic resistance phenotype (Bhuvaneshwari, 2017).

TEM β-lactamases were the first beta-lactamase genes found in Gram-negative bacteria. They are specifically transferred by plasmids, and more than 200 subtypes have been identified, mainly encoding enzymes that hydrolyze penicillin and first-generation cephalosporins (Xiao *et al.,* 2019). Bacteria acquire resistance to antibiotics through two principal routes:

chromosomal mutation and the acquisition of mobile genetic elements such as plasmids by horizontal gene transfer (Millan, 2018). The present study was undertaken to evaluate the presence of multidrug resistance, multiple antibiotic resistance index, and characterization of TEM-β-lactamase -genes from *Escherichia coli* isolated from some ready-to-eat foods.

MATERIALS AND METHODS

Isolation and identification of bacterial strains

Twelve strains of *E. coli* were isolated from some ready-to-eat foods (smoked fish, processed roasted meat ('suya'), and zoborodo. They were identified using some traditional methods and confirmed with Microgen Gram-negative identification kit as stated in previous literature (Adenaike *et al.,* 2013).

Antibiotics Sensitivity Testing

Antibiotic sensitivity testing of the *E. coli* isolates was carried out using Kirby–Bauer disk diffusion method. Nine single antibiotic discs were firmly placed on sterile Mueller Hinton agar (MHA) plates seeded with the tested strains, standardized to 0.5 McFarland's turbidity standard and incubated at 37°C for 24 h. Diameter of zones of inhibition were measured to the nearest millimetre and reported in accordance with the antimicrobial susceptibility breakpoint of CLSI (Tsaku *et al.,* 2019). The nine antibiotics used were Ampicillin 10µg, Cephalothin 30µg, Cefpodoxime 10µg, Ceftriaxone 30µg, Ciprofloxacin 5µg, Trimethoprim-sulfamethoxazole 25µg, Tetracycline 30µg, Amikacin 30µg and Amoxicillin/clavulanic acid 25µg (Oxoid Ltd., Basingstoke, Hampshire, England). A standard strain *E. coli* ATCC 25922 was used as quality control (Parussolo *et al.,* 2019). Multidrug resistance (MDR) was taken as resistance to four or more of the antibiotics tested (Adenaike *et al.,* 2013).

Determination of multiple antibiotic resistance (MAR) index

MAR index was calculated as a /b, where a = number of antibiotics organism was resistant to; b = the total number of antibiotics to which the organism was exposed (Afunwa *et al.,* 2020).

Plasmid DNA extraction

E. coli strains were subcultured overnight in Luria-Bertani broth (Merck, Germany) and plasmid DNA was extracted using Zyppy™ Plasmid Miniprep Kit (Inqaba Biotech, South Africa) according to manufacturer's instructions, briefly,100µl of 7X lysis buffer (blue)was added to about 600µl of overnight culture in a 1.5ml microcentrifuge and mixed thoroughly by inverting the tube 4-6 times. (After this, next step is preceded within 2minutes). (After addition of 7X Lysis buffer, complete lysis is indicated by colour change from opaque to clear blue). About 350µl of cold Neutralization Buffer (Yellow, Containing RNase), was added and mixed thoroughly. Complete neutralization was ensured by inverting samples for about 2-3 times. (The sample turned yellow when neutralization was completed and a yellowish precipitate was formed). Centrifugation at 15,000g for 3min. followed and precipitates were washed again with 400µl of Wash buffer (containing ethanol) and further centrifuged for 1minute. The column was transferred into a clean1.5ml microcentrifuge tube followed by addition of 30µl Elution buffer (10 mMTris-HCl, pH 8.5 and 0.1 mM EDTA) directly to the column matrix and allowed to stand for one minute at room temperature. Centrifugation was carried out for 30sec. to elute the plasmid DNA (Protocols, 2016).

Polymerase Chain Reaction

To amplify the sequences of TEM β-lactamase genes, PCR was carried out with the primer sets as described by Iseghohi et al. (2020) with slight modifications. Reactions were performed in a GeneAmp PCR system 2400 (Perkin-Elmer) in 20µl reaction mixtures containing 10 µl Premix with non-interfering dye (consisting of Taq DNA polymerase, dNTPs, $MgCl_2$, reaction buffer, PCR stabilizer and enchancer at optimer

concentrations). The oligonucleotide primer concentration (i.e. forward and reversed) added was 0.5µl, while template concentration added was 1.0µl. The PCR conditions used were 35 cycles of amplification at a denaturation temperature of 94°C for 3mins (first cycle only), subsequently 94°C for 45s, an annealing temperature of 51°C for 30s, and an extension temperature 72°C for one min. Followed by a final extension at 72°C for 1min.

Table 1. Oligonucleotide primer used for detection of β-lactamase TEM genes (Mohammed *et al.,* 2016).

Primer	Melting Temperature (°C)	Nucleotide Sequence (5'-3')	References (GenBank number)	Expected Amplicon Size (bp)
TEM-F TEM-R	56.2 50.1	TTTCGTGTCGCCCTTATTCC ATCGTTGTCAGAAGTAAGTTGG	AB282997	403

Key: F-Forward R-Reversed

Agarose gel electrophoresis

Ten microliters (10µl) of PCR products were loaded into wells of 1.0% agarose gel containing ethidium bromide. A molecular size marker, Perfect 1,000bp DNA ladder- SibGene, was run on both sides with PCR products. Electrophoresis was carried out in Tris Acetate EDTA (TAE) buffer (1x contains; Tris 40mM, Acetic Acid 20Mm, EDTA 1Mm, pH 8.0; BIOLAND SCIENTIFIC LLC) containing ethidium bromide (20 ml of 50 X TAE and 4.0 µl of 10 µg/ml ethidium bromide per litre) at 90 V for 40min. Plasmids were viewed on a U/V transilluminator and photographs taken using a gel documenting machine (Gel doc 2000; BIO-RAD). Plasmid sizes were assessed and estimated from the molecular sizes of the DNA ladder against their migration distance (Lee *et al.,* 2012).

Plasmid DNA Sequencing

In order to confirm and characterize the TEM-lactamase genes detected in PCR assays, DNA sequence analysis of the PCR amplicons was performed (Ghasemi *et al.,* 2013). Briefly, sequencing of the purified PCR products were performed with the Dye Terminator Cycle sequencing (DTCS) Quick start kit using the sequencer CEO 2000 XL DNA analysis system (Beckman Coulter, USA).

RESULTS AND DISCUSSION

Antibiotic resistance profile of *E. coli* strains used in this study is shown in Table 2. For the multidrug resistance strains, eight (8) out of 12 (66.7%) tested strains are resistance to four or more antibiotics; three *E. coli* strains (25%) are referred to as non-multidrug because they were resistance to less than four antibiotics. One strain (8.3%) was susceptible to all the antibiotics. This is presented in Figure 1.

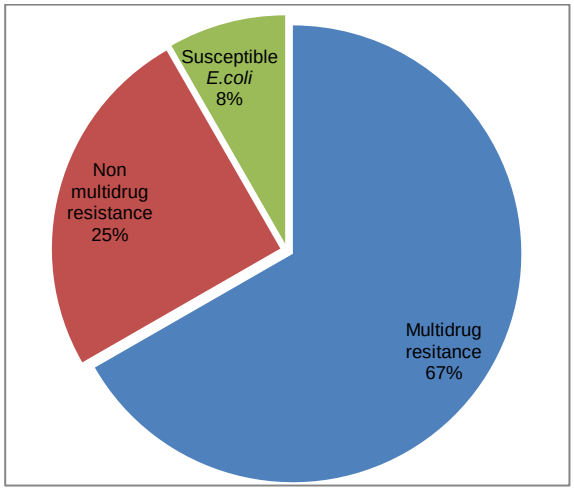


Fig. 1. Percentage population of the different categories of antibiotic sensitivities in the *E.coli* strains.

Table 2. Phenotypic profile of *E. coli* strains used in the study.

S/N	Isolate	Identification of the isolate	Source of isolate	Antibiotic resistant Pattern	No. of antibiotics *E. coli* showed resistance	Detection of TEM gene
1.	Sfe15	*E.coli*	Smoked fish	AMP, KF, TE	3	Nil
2.	Sfe16	*E.coli*	Smoked fish	NIL	Nil	TEM
3.	Ze11	*E.coli*	Zoborodo	AMP,KF,CPD,CRO,TE	5	TEM
4.	Ze17	*E.coli*	Zoborodo	AMP, KF,CPD, SXT, TE	5	TEM
5.	Ze2	*E.coli*	Zoborodo	AMP, KF, CPD	3	NIL
6.	Sye1	*E.coli*	Suya	AMP, KF,CPD, SXT, TE,AMC	6	TEM
7.	Sye8	*E.coli*	Suya	AMP, KF,CPD, CRO, TE	5	TEM
8.	Sye10	*E.coli*	Suya	AMP, KF,CPD, SXT, TE	5	TEM
9.	Sye17	*E.coli*	Suya	AMP,KF,CPD,CRO,SXT,TE,AMC	7	TEM
10.	Sye2	*E.coli*	Suya	AMP, KF,CPD, SXT, TE	5	Nil
11.	Sye 6	*E.coli*	Suya	AMP, KF,CPD, SXT, TE	5	Nil
12.	Sye9	*E.coli*	Suya	AMP, KF, CPD	3	TEM

Key: Sye -*E.coli* from suya; SFe- *E.coli* from smoked fish; Ze- *E.coli* from zoborodo; AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin; AMC-Amoxicillin-clavulanic acid (Augmentin)

The eight multidrug resistant *E.coli* demonstrated four resistance patterns as shown in Table 2. The antibiotic resistance pattern of AMP, KF, CPD, SXT, TE was found in four strains; AMP, KF,CPD, CRO,TE was exhibited by two strains of *E. coli* while AMP, KF, CPD, SXT, TE, AMC and AMP, KF,CPD, CRO, SXT, TE, AMC were observed in just one *E. coli* strain each.

It should be noted that out of six *E. coli* strains isolated from 'suya' processed meat; five strains, 83.3% exhibited multidrug resistance. The use of antibiotics in Agriculture and human treatments will continue to increase especially in regions where people have less knowledge in it negative effects. The use of antibiotic is a main driver of selection pressure that contributes to resistance (Adzitey *et al.,* 2015). The continuous use of antibiotics in food animal production is cited as a major determinant for carriage of antibiotic resistant bacteria in food animals. A proportion of antibiotic classes are used in both animals and humans, creating the need to monitor the spread of resistant bacteria from animals to humans at all stages of the transmission pathway (Dsani *et al.,* 2020).

Antibiotic resistance is a natural phenomenon that occurs when microorganisms are exposed to antibiotic drugs. Under the selective pressure of antibiotics, susceptible bacteria are killed or inhibited, while bacteria that are naturally (or intrinsically) resistant or that have acquired antibiotic-resistant traits have a greater chance to survive and multiply. Not only the overuse of

antibiotics but also the inappropriate use (inappropriate choices, inadequate dosing, poor adherence to treatment guidelines) contribute to the increase of antibiotic resistance (Prestinaci *et al.,* 2015).

On the overall, eight out of the twelve *E. coli* strains (approx. 67%) showed multi-drug resistance with resistance to four or more antibiotics. This is slightly higher than a study on *Pseudomonas aeruginosa* isolated from fish with 55.5% MDR (Algammal *et al.,* 2020). However, lower that 79.2% MDR *E. coli* obtained in a study on poultry workers stool analysis (Aworh *et al.,* 2019). Also, 78.8% MDR *E. coli* were obtained in a study in Tanzania (Sonola *et al.,* 2021), while 92.2% MDR *E. coli* were obtained from a hospital study in Sudan (Ibrahim *et al.,* 2012). Infections caused by multidrug-resistant (MDR) organisms are associated with increased mortality compared to those caused by susceptible bacteria and they carry an important economic burden. This situation is worsened by a paucity of a robust antibiotic pipeline, resulting in the emergence of infections that are almost untreatable and leaving clinicians with no reliable alternatives to treat infected patients (Munita and Arias, 2016).

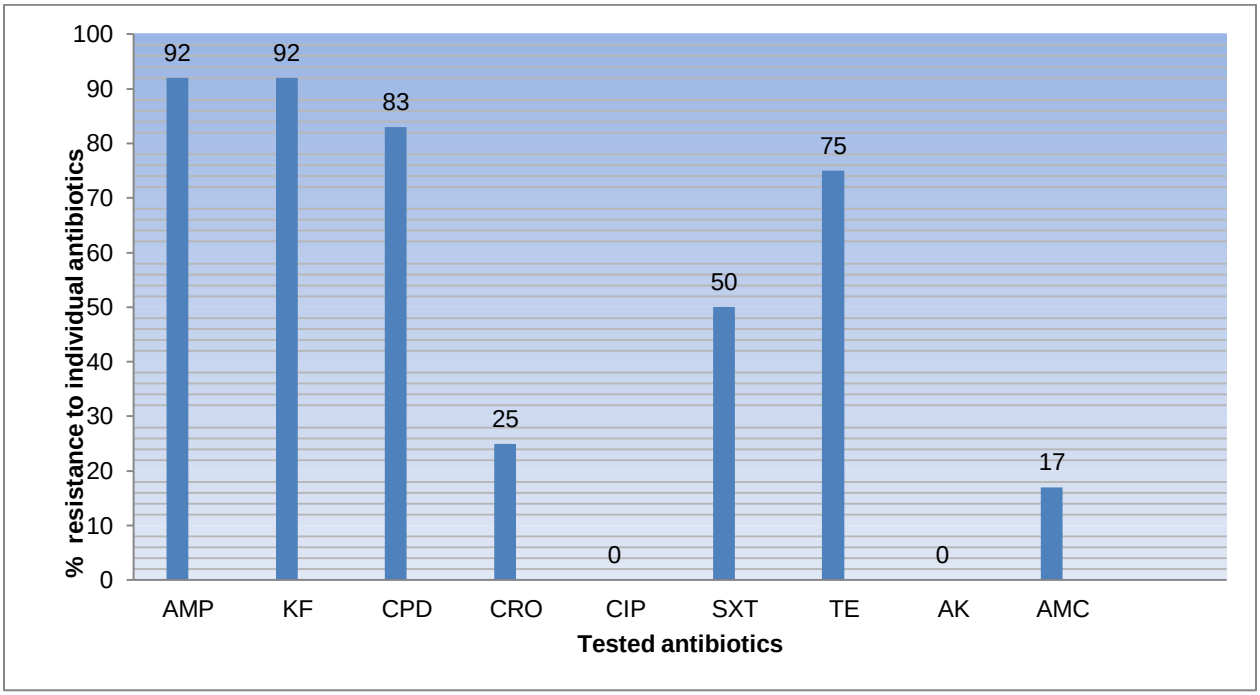


Fig. 2. *E. coli* isolates showing resistance trend to the tested antibiotics.

Key: AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin; AMC-Amoxicillin-clavulanic acid (Augmentin).

Antibiotic resistance in *E. coli*, is on the rise all over the world. In Figure 2, there was a remarkable increase in ampicillin and cephalothin resistance (92%). Reports from other researchers had also indicated *E. coli* isolates' resistance to ampicillin and cephalothin (Tadesse *et al.,* 2018). This is followed by resistant to cefpodoxime (83%) and agrees but a little higher than results obtained from the study on some uropathogenic *E. coli* strains where resistance to ampicillin, cephalothin and cefpodoxime were 88.4%, 74% and 67% respectively (Raeispour and Ranjbar, 2018). Resistance to tetracycline was observed to be

equally high (75%). This is much higher than the tetracycline resistance (45%) observed in a similar study carried out in Ghana. Tetracyclines are commonly used for therapy in humans and livestock and for growth promotion in intensive farming systems through feed in gut bacteria of livestock in such settings may contribute to the observed rates of tetracycline resistance in *E. coli* isolated from meat products, other ready to eat foods and the environment (Dsani *et al.,* 2020).

Increased resistance to commonly used antibiotics is a cause for concern. Our study revealed resistance to co--trimethoprim (SXT) (50%) to be reduced to a study on *Salmonella* with resistance to SXT with 69.38% (Peruzy *et al.,* 2020). The emerging co-trimoxazole resistance is of serious concern, as it is the preferred drug for many Gram-negative bacteria. The most common mechanism of resistance to co-trimoxazole is the acquisition of plasmid-mediated, variant diaminopyrimidine folate reductase enzymes (Odonkor and Addo, 2018). There is reduced resistance of 25% and 17% to ceftriaxone (CRO) and augumentin (AMC) respectively. Notable on a study on *Staphylococcus aureus* with resistance to CRO and AMC being 10.3% and 28% respectively (Onyeka *et al.,* 2020). This is also worrisome because CRO is a 3rd generation cephalosporin. Third generation cephalosporins are used frequently for empirical therapy for patients with suspected Gram-negative bacteremia acquired in the community. Third generation cephalosporin resistance leaves us with only limited options for treating patients with Gram-negative bacteremia, and is considered the treatment of choice (Park, 2014). Cephalosporins belong to the β-lactam class of antibiotics and are presently the most commonly used antibiotics to treat gram negative bacilli infection. *E. coli* strains can become resistant to beta lactam antibiotics by producing extended spectrum beta lactamase (ESBL), which is a plasmid-mediated β-lactamase that is capable of hydrolysing and inactivating b-lactams such as cephalosporins and monobactams (Wu *et al.,* 2021). Reported frequencies of resistance to amoxycillin–clavulanic acid (augumentin) in *E. coli* have varied considerably, between the 5-40% across the globe in past years (Xiao *et al.,* 2019).

In this study resistance to amoxicillin-clavulanic acid was 17%. Clavulanic acid broadens the spectrum of amoxicillin, although clavulanic acid displays limited antibacterial activity. There is however, evidence that clavulanic acid increases the activity of β-lactams by mechanisms other than the inhibition of β-lactamases. Due to these properties of clavulanic acid, amoxicillin/ clavulanic acid is generally regarded as a drug with a broader spectrum and higher selective potential than amoxicillin (Espinosa-Gongora *et al.,* 2020). Hence, the name Augmentin, for the augmented powers that clavulanate confers to amoxicillin. The remarkable degree of resistance to many drugs represents public health hazard due to the fact that foodborne outbreaks would be difficult to treat and this pool of MDR *E. coli* in food supply represents a reservoir for communicable resistant genes (Raeispour and Ranjbar, 2018). All the *E. coli* strains were susceptible (100%) to amikacin (AK) and ciprofloxacin giving 0% resistance. Several studies have reported excellent susceptibilities of bacteria to amikacin and other aminoglycoside. More than 90% of E*. coli* strains collected in a study in a Chinesse hospital were susceptible to amikacin (Kuti *et al.,* 2018) while 82. 6% susceptibility was obtained in another study in Iran (Pirouzi *et al.,* 2020). Amikacin and ciprofloxacin have been found to be most effective against *E. coli* and may therefore be used in the treatment of *E. coli* infections.

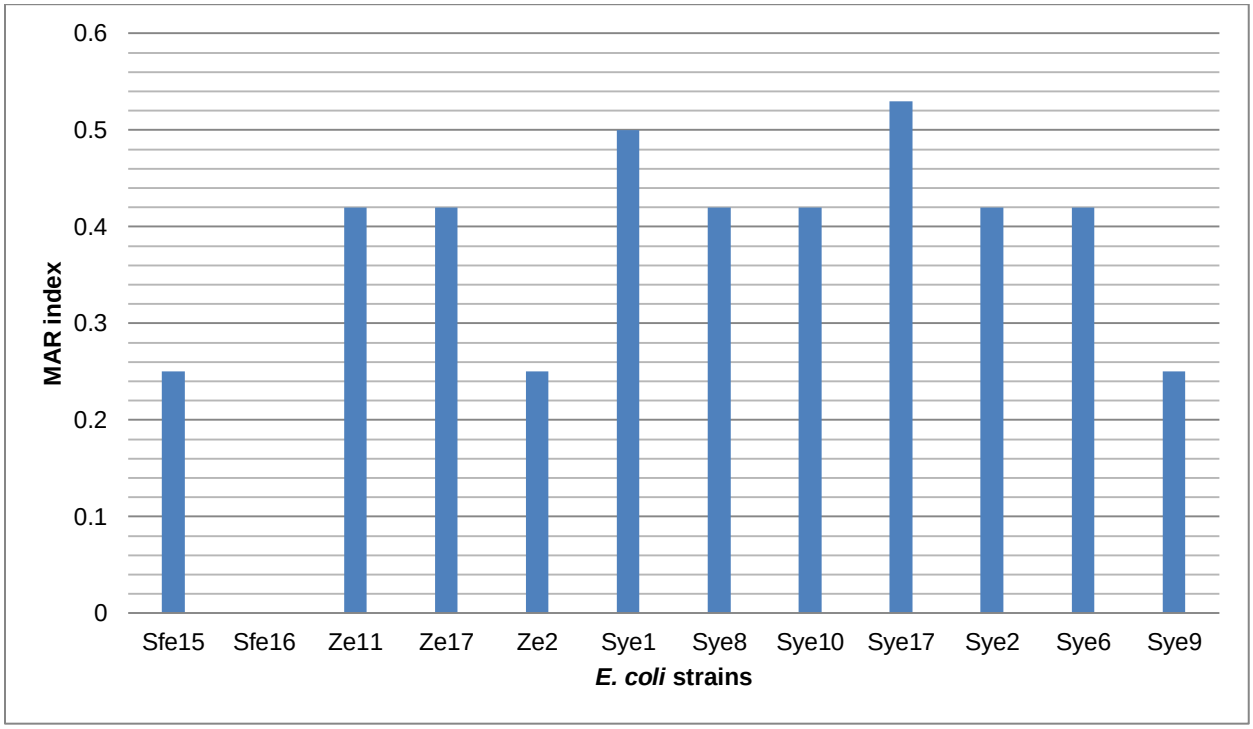


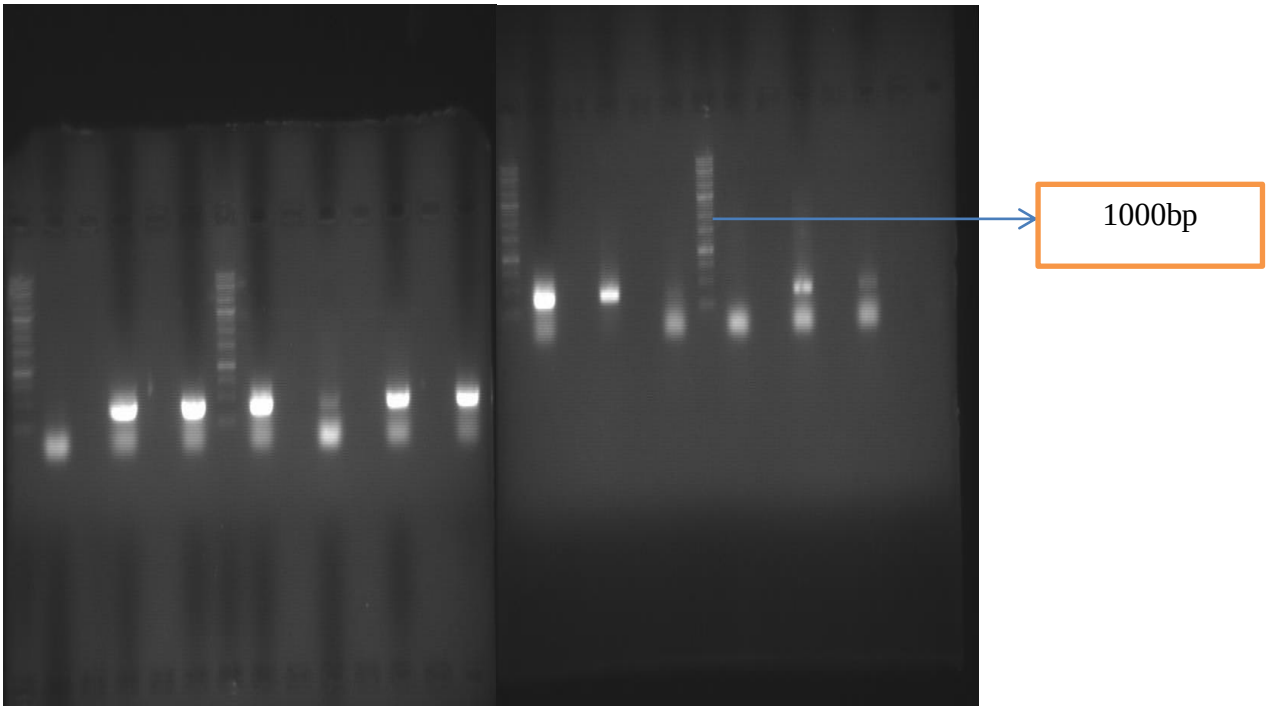
Fig. 3. Multiple Antibiotic Resistance (MAR) index of *E. coli* strains used in the study.

Key: Sfe---*E.coli* strain isolated from smoked fish; Ze--- *E.coli* strain isolated from zoborodo; Sye--- *E.coli* strain isolated from 'suya', processed meat.

Multiple antibiotic resistance (MAR) index of the *E. coli* strains is presented in Figure 3. The MAR index of an isolate is defined as *a/b*, where *a* represents the number of antibiotics to which the isolate was resistant and *b* represents the number of antibiotics to which the isolate was subjected. The MAR index analysis reveals that eleven out of twelve *E. coli* strains (91.7%) had high MAR index value (>0.2). It has been reported that bacteria originating from an environment where several antibiotics are used usually produce MAR index greater than 0.2 (Odonkor and Addo, 2018). This is one great and negative impact of urbanization on antibiotic resistance levels. MAR indexing below 0.2 (0.0) is found in only one organism (Sfe16) which was susceptible to all the tested antibiotics.

TEM gene was detected in 8 (66.7%) *E. coli* strains (Figure 4).

Table 2 shows the antibiotic resistance profile of the *E. coli* strains as obtained by using disc diffusion test (DDT) vis a vis the presence of TEM gene. One isolate Sfe16 that was susceptible to all the antibiotics in the disc diffusion test (DDT) was however found to harbour a TEM gene. This discrepancy in the DDT and PCR could be due to inoculum effect and substrate specificity which may render the enzyme in an un-induced state at the time of testing with DDT. This creates a major challenge in laboratory routine susceptibility tests.



(a) (b)

Fig.4. Gel electrophoresis of TEM amplicons.

M-1000bp DNA Marker

C-Negative Control

It should be noted that three *E. coli* strains that were resistant to cefpodoxime but not ceftriaxone (3rd generation cephalosporins) did not contain the TEM gene, other β-lactamase genes may be responsible for the resistance. The isolates may also have more than one bla_{TEM} genes present, and the amplification only detected a single genotype. This is because, if multiple bla_{TEM} genes are present, the predominant one will preferentially amplify.

CONCLUSION

E. coli has become a potential source of foodborne illness due to the presence and possible transfer of plasmid mediated antibiotic-resistant genes. In addition to this, the need to regulate the administration of antibiotic usage in humans and animals cannot be overemphasized.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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