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Prevalence of integrons in Enterobacteriaceae obtained from clinical samples

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Multi-drug resistant bacteria are a public health problem associated with high morbidity and mortality globally. This increasing drug resistance has been linked to gene exchange between bacteria. Integrons are gene exchange systems and are known to play a significant role in the acquisition and dissemination of antimicrobial resistance genes especially in Gram negative bacteria. Hence, this study aims to evaluate integrons in members of Enterobacteriaceae obtained from clinical samples. Forty-nine (49) isolates identified as *Escherichia coli* (45), *Proteus mirabilis* (2), *Shimwellia blattae* (1), and *Klebsiella pneumoniae* (1) were resistant to amoxicillin/clavulanate, cefuroxime, cefixime and ceftazidime while 43(87.76%), 45(91.84%), 46(93.88%) and 29(59.18%) of these strains were resistant to gentamicin, ofloxacin, ciprofloxacin and nitrofurantoin, respectively. Class 1 integrons were found in *E. coli* (18), *Klebsiella pneumoniae* (1) and *Proteus mirabilis* (1). This study revealed that large proportion of the strains studied were multi-drug resistant, and possessed integrons. Consequently, there is a need for proactive antibiotic surveillance system in both healthcare and community settings with a view to reducing the incidence and spread of antibiotic resistance genes between different species of bacteria.

Key words: Enterobacteriaceae, clinical samples integrons, multidrug resistance.

INTRODUCTION

Enterobacteriaceae is a large family of Gram-negative bacteria with rod shape, non-spore forming and capable of fermenting arrays of carbohydrates (Octavia et al., 2014). Clinical and community associated infections in humans have been caused by this group of bacteria especially *Klebsiella, Proteus*, *Citrobacter, Serratia*, *Escherichia, Enterobacter, Yersinia, Salmonella* and *Shigella* with 4.5 billion cases annually and 1.9 million deaths (Jarzab et al., 2011; Ye et al., 2018). Infections caused by this group of bacteria are preferably treated with broad beta-lactam antibiotics like carbapenems and cephalosporins (Khyade et al., 2018).

Currently, multi-drug resistant bacteria have become an increasing issue in healthcare system due to their ever increasing morbidity and mortality globally (Ye et al., 2018; Stephen and Kennedy, 2018; Nabti et al., 2019). Increasing drug resistance in Enterobacteriaceae has been a problem in clinical and community environments

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as a result of its attendant consequences. Arrays of different mechanisms have led to a spread of resistance genes in bacteria usually via horizontal gene transfer. This global dissemination and widespread of resistance genes among bacteria can threaten the therapeutic management of patients in the event of infections, thus, endangering the effectiveness of last resort antibiotics available. Resistance occurs intrinsically with time, but can be amplified quickly as a result of selective pressure ignited by inappropriate use or rather abuse of antibiotics (Morosini, 2017).

Microbial drug resistance will continue to be on the increase unless strict stewardship programs are established. Their burdens include prolonged hospitalization, recurrent infections, economic cost, and high mortality rate (Alemayehu et al., 2019).

The increasing drug resistance has been linked to gene exchange between bacteria occasioned by antibiotic pressure resulting from an excessive and unregulated use of these agents in various human applications (Ye et al., 2018). Multi-drug resistance although frequently acquired from healthcare settings can spread via chromosomal mutations and disseminated by extra chromosomal associated elements (such as plasmids, transposons, and integrons) acquired from other bacteria (Rezaee et al., 2011). Integrons are gene exchange systems and are known to play a significant role in the acquisition and dissemination of antimicrobial resistance genes especially in Gram negative bacteria (Domingues et al., 2012). Also, integrons are composed of integrase gene, the promoter and the attachment site (Rezaee et al., 2011). They are responsible for the integration and dissemination of resistance genes among the bacteria. Several classes of integrons have been described based on the amino acid sequences of respective integrase genes, but class 1 and 2 integrons are the most prevalent in MDR gram-negative bacteria which is associated with antibiotic treatment failure (Domingues et al., 2012; Deng et al., 2015; Hadi et al., 2018).

In Nigeria where the antibiotic surveillance system is at its infancy, and abuse of antibiotics eminent, there is therefore an urgent need to evaluate the extent of prevalence of this resistance determinant (integrons) in Enterobacteriaceae obtained from clinical setting as only pocket of reports in *Escherichia coli* and *Pseudomonas aeruginosa* had been investigated (Chah et al., 2010; Odumosu et al., 2013; Igbinosa and Obuekwe (2014); Adesoji et al., 2017; Odetoyin et al., 2018). Hence, this study was carried out to detect the prevalence of integrons in Enterobacteriaceae obtained from clinical samples.

MATERIALS AND METHODS

Sample collection

Three hundred and fifty-nine clinical samples (urine 104, stool 87, endocervical swab 86 and high vaginal swab 83) of patients submitted to Microbiology laboratory for normal routine services were collected within a six-month period (January to June 2019). The samples for microbiological analysis were transferred aseptically into a transport medium (Buffered peptone water, Oxoid LTD, Basingstoke, Hamshire, England) and transported to the Microbiology laboratory, for analysis. Samples were processed microbiologically within 48 h of collection on MacConkey and Eosin Methylene blue agar plates (EMB) (Biomark Laboratories, India) and incubated at 37°C. After 24 h, suspected *E. coli* strains showing green metallic sheen were purified and sub-cultured onto MacConkey sorbitol agar (MSA) (Biomark Laboratories, India) petri plates for the presumptive identification of *E. coli* 0157:H7. Colonies on MacConkey agar were also purified and stored in 40% glycerol at -20°C (Oladipo and Fajemilo, 2012; Moghaddam et al., 2015). Ethical clearance was authorized (BUHREC543/19).

DNA extraction

Qick-DNA™ miniprep plus kit (Zymo research, Biolab, USA) was used for the extraction. Briefly, physiological young culture samples of Enterobacteriaceae (200 µl) were added to micro tubes. An equal volume of biofluid cell buffer (Red) was added with the addition of 20 µl Proteinase K.

The contents contained in the tubes were thoroughly vortex for 10-15 s and then incubated at 55°C for 10 min. A volume of Genomic Binding Buffer (420 µl) was added to the digested samples and thoroughly vortex for 10-15 s. The mixtures were transferred to a Zymo-Spin™ IIC-XLR Column in collection tubes and centrifuged at ≥ 12,000 r.p.m. The collection tubes with the flow through were discarded. DNA Pre-Wash Buffer of 400 µl was added to the spin columns in a new collection tubes and centrifuged at ≥ 12,000 r.p.m. Exactly 700 µl g-DNA Wash Buffer was added to the spin columns and centrifuged at ≥ 12,000 r.p.m.

The collection tubes were discarded. g-DNA wash buffer of 200 µl was added to the spin columns and Centrifuge at ≥ 12,000 r.p.m. The collection tubes with the flow were discarded. The spin columns were transferred to a clean micro tube and exactly 50 µl of DNA elution buffer was added directly on the matrix. It was incubated for 5 min at room temperature, then centrifuged at maximum speed for 1 min to elute the DNA. The eluted DNA was used immediately for molecular-based applications.

Polymerase chain reaction (PCR) detection of *E. coli* and its shiga toxins by polymerase chain reaction

All isolates suspected of *E. coli* based on phenotypic screening were identified using specific primers targeting the *uid* gene and screened for O157:H7 strains. PCR mixture (25 µl) contained 12.5 µl solution of the master mix (New England Biolabs), 9.5 µl H_20, 0.5 µl 10 mM of each *uid* primers and 2.0 µl of DNA template. Amplification was carried out using miniPCR (USA) with the following thermal cycling profile: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30s, annealing as indicated in Table 1 for 30s and extension at 68°C for 30s and a final extension at 68°C for 5 min with period of 30 cycles. Amplicons were analysed by agarose gel electrophoresis.

Species barcoding

Seven representatives of isolates were selected for sequencing. Genomic DNA extracted above was quantified by NanoDROP 3300 spectrometer (Thermo Fisher Scientific Inc., USA). The quality of DNA was verified by 1.5 agarose gel electrophoresis prior to the PCR amplification reaction. The 16S rRNA of the bacteria was amplified using PCR with primers 341F 5'-CCTACGGGAGGCAGCAG3' and R806:5'GGACTACHVGGGTWTCTAAT-3'as described above. The

Table 1. Primers used for amplification of the integrase gene and its variable regions.

Primers	Sequence; 5'-3'	Genes	Amplicon size (bp)	Tm (°C)	References
hep35F	TGCGGGTYAARGATBTKGATTT	Int1,2,3	491	37	White et al. (2001)
hep36R	CARCACATGCGTRTARAT				
hep58F	TCATGGCTTGTTATGACTGT	Int1	Variable	46	White et al. (2001)
hep59R	GTAGGGCTTATTATGCACGC				
stx1F	ATAAATCGCCATTCGTTGACTAC	Stx1	180	51	Paton and Paton (1998)
stx1R	AGAACGCCCACTGAGATCATC				
stx2F	GGCACTGTCTGAAACTGCTCC	Stx2	255	52	Paton and Paton (1998)
stx2R	TCGCCAGTTATCTGACATTCTG				
uidA F	TGGTAATTACCGACGAAAACGGC	uidA	162	52	Godambe et al. (2017)
uidA R	ACGCGTGGTTACAGTCTTGCG				

Unidirectional sequence reads were performed by standard procedures and the contigs were assembled using bioedit (version 7.2.5.0) sequence program (Hall et al., 1999). The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the jukes-cantor method and are in the unit of the number of base substitutions per site (Jukes and Cantor, 1969). All positions containing gaps and missing data were eliminatory. Evolutionary analyses were conducted in molecular evolutionary genetics analysis 6.0 (MEGA6) (Tamura et al., 2013).

Susceptibility testing

Kirby-Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI, 2017) was applied. Briefly, a single colony of pure isolate was inoculated into a test tube containing 1 mL of nutrient broth (Oxoid, UK) and incubated overnight at 37°C. The overnight broth was then standardized to match 0.5 McFarland standard. A sterile swab stick was dipped in the standardized suspension and streaked over the surface of prepared Mueller Hilton agar plates (Oxoid LTD, Basingstoke, Hamshire, England). The antibiotic disc (Abtek Biologicals Limited Gram-negative discs); gentamicin (10 µg), ceftazidime (30 µg), cefturoxime (30 µg), nitrofurantoin (300 µg), ciprofloxacin (5 µg), amoxicillin/clavulanate (30 µg), ofloxacin (5 µg) and cefixime (5 µg) were placed on the agar surface maintaining a distance of 30 mm edge to edge. The plates were incubated at 37°C for 24 h. The clear zone of inhibition was measured with a ruler to the nearest diameter and results were interpreted in accordance with Clinical and Laboratory Standards Institute guidelines (2017).

Detection of integrons by PCR

The presence of integrons in Enterobacteriaceae isolates was determined by PCR using consensus primers (Hep35 and Hep36) as described elsewhere (Su et al., 2006). Amplification was carried out using miniPCR (USA) with the following thermal cycling profile: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 37°C for 30s and extension at 68°C for 30 s and a final extension at 68°C for 5 min for 30 cycles (Table 1). Amplicons were analyzed by blueGel agarose electrophoresis system (USA). Integrons were classified using restriction fragment length polymorphism (PCR-RFLP) polymerase chain reaction supplemented with gene-specific primers, while Class 1 integron was confirmed by Hep58 and Hep59 primers as described elsewhere (Rezaee et al., 2011).

RESULTS

Species identified and status of shiga toxins in *E. coli*

Three hundred and fifty-nine clinical samples were obtained, of which forty-nine Enterobacteriaceae comprising *E. coli* (45), *Proteus mirabilis* (2), *Shimwellia blattae* (1), and *Klebsiella pneumoniae* (1) were isolated from 36 (73.50%) female and 13 (26.50%) male subjects (Figure 1 and Table 2). Majority of the isolates were *E. coli* as confirmed by specific primer (Figure 2). However, *E. coli* O157:H7 strains were not detected in this study. Of the 49 isolates, 24 (48.98%), 17 (34.69%), 6 (12.24%) and 2(4.08%) were recovered from urine, stool, endocervical swab and high vaginal swab respectively (Table 2). All sequenced data were deposited in GenBank under the accession numbers MT271687-MT271693 and their phylogenetic relationship to those in GenBank was constructed (Figure 3).

Susceptibility pattern and integrons status of species encountered

All the twenty-four isolates (100.00%) obtained from urine were resistant to ceftazidime, cefuroxime, cefixime, amoxicillin/clavulanate, while 22(91.67%), 22(91.67%), 21(87.50%) and 16(66.67%) were found to be resistant to ofloxacin, ciprofloxacin, gentamicin, and nitrofurantoin respectively in the same urine sample. Also, isolates obtained from stool samples 17 (100.00%) were resistant to amoxicillin/clavulanate, cefixime, ceftazidime and cefuroxime while 16 (94.10%), 15 (88.23%), 14 (82.30%) and 7 (41.08%) were resistant to ciprofloxacin, ofloxacin gentamicin, and nitrofurantoin in that order. Likewise, isolates obtained from endocervical swab and high vaginal swab samples were equally resistant to most of

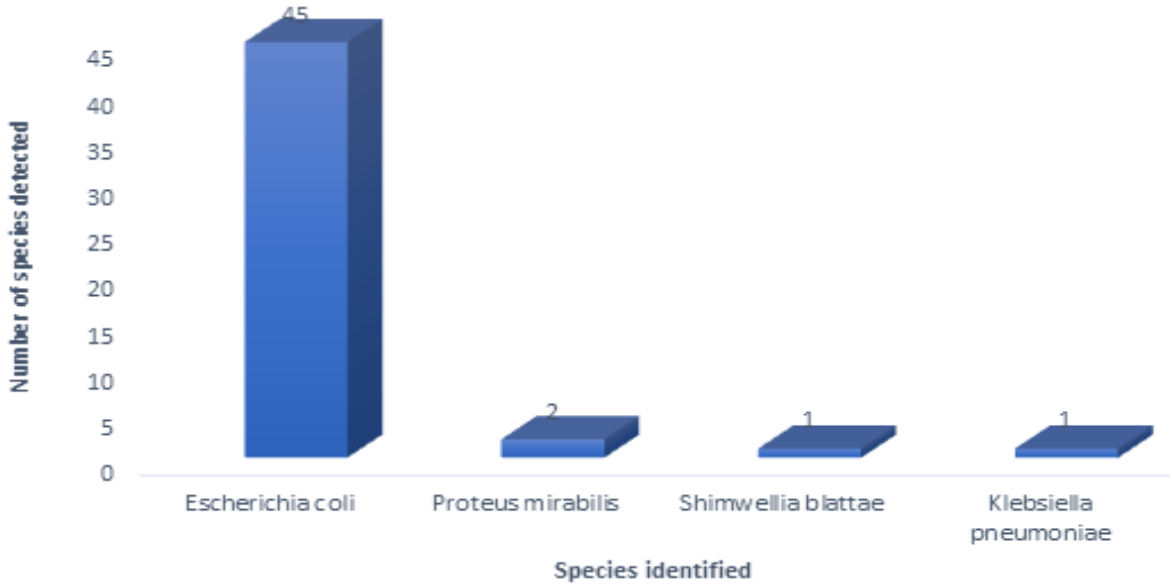

Figure 1. Distribution of species of Enterobacteriaceae obtained from clinical samples.

Table 2. Source distribution and target genes per source in studied isolates.

Sources	Number of clinical samples	Number of organisms isolated		*Integrase gene*		Class 1 integron		*E. coli* 0157:H7	
		No.	%	No.	%	No.	%	No.	%
Urine	80	24	48.98	7	29.17	7	29.17	x	0
Stool	70	17	34.69	6	35.29	6	35.29	x	0
Endocervical swab	80	6	12.24	5	83.33	5	83.33	x	0
High vaginal swab	79	2	4.08	2	100.00	2	100.00	x	0
Total	**309**	**49**	**100**	**20**					

X: Absence of target gene, no: number of target.

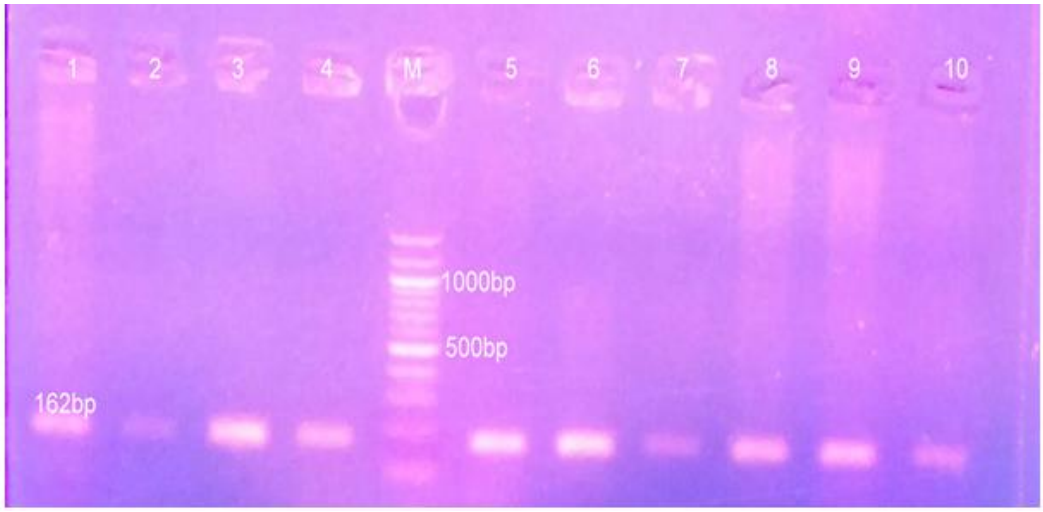

Figure 2. Electrophoregram of *E. coli* detection using uid primers. M: Molecular weight ladder (100 bp), known *E. coli* (control): 1, Isolates: 2-10.

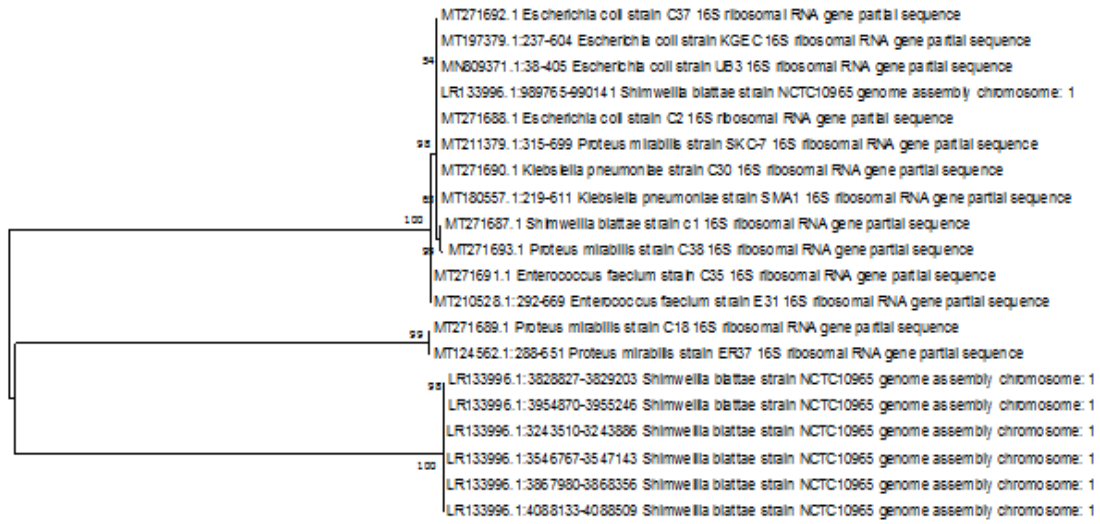

Figure 3. Phylogenetic tree illustrating the relationship between the isolates identified and their close relatives in NCBI. The evolutionary history was inferred using the Neighbour-Joining method and distances were computed using the Jukes-Cantor method. All the isolates were grouped into two clusters. Cluster: 1 E. coli C37, C2, K. pneumoniae C30, P. mirabilis C38. Cluster 2: P. mirabilis C18. Two species Enterococcus faecium were used to root tree.

the antibiotics (Figure 4). Multidrug resistance pattern showed that 29 (59.18%) of the isolates were resistant to all the antibiotics tested while 13 (26.53%), 5 (10.20%), 1 (2.04%) and 1 (2.04%) were resistant to seven, six, five and four antibiotics respectively (Figure 5). Class 1 integrons were found in 20 (40.82%) of the isolates. However, no class 2 and 3 integrons were detected in the isolates (Figure 6).

DISCUSSION

In this study, *E. coli*, *Shimwellia, Klebsiella*, *Enterobacter* and *Proteus* species were recovered from clinical samples. The family Enterobacteriaceae are usually found in the environment as well as the normal microbiota of the intestine in humans and other animals. The recovery of these species from urine, stool, endocervical swab and high vaginal swab is not surprising because members of this species remain harmlessly confined in some parts of the body. However, in weakened or immunosuppressed host, non-pathogenic strains can trigger infections that may be responsible for many illnesses in individuals and livestocks (Muhammad et al., 2011). The prevalence of Enterobacteriaceae in this work is comparable to a report by Malek et al. (2015). It is imperative to note that Enterobacteriaceae were recovered more in female than in male counterpart. This result was comparable to results obtained previously by other authors (Onyedibe et al., 2018; Ibrahim et al., 2018) in North Central Nigeria, and Saudi Arabia. The reason for high prevalence in the case of females may be attributable to the nature of their genitals which predispose them to faecal contamination when compared to their male counterpart whose relatively closed genitals prevent the establishment of pathogens.

Members of the family Enterobacteriaceae are frequently identified as etiological agents of nosocomial infections (Obeng-Nkrumah et al., 2013; Bouguenoun et al., 2016) and can cause various diseases, ranging from urinary tract infections (UTIs), pneumonia, wound infections, bloodstream infections, intestinal infections such as enteritis and diarrhea to central nervous system infections (Osman et al., 2018; Dougnon et al., 2020; Breijyeh et al., 2020).

In this study, *E. coli* was the most commonly isolated organism. This is consistent with results obtained by several authors (Tajbakhsh et al., 2015; Osman et al., 2018), but contrary to some other authors (Obeng-Nkrumah et al., 2013; Bouguenoun et al., 2016; Akbari et al., 2018). The variation may be attributable to the sample size used or species diversity in different study locations. Most of the Enterobacteriaceae detected in urine may be the primary cause of urinary tract infections. These bacteria adhere to vaginal epithelium cells, and also invade vaginal cells leading to infection (Brannon et al., 2020). Therefore, this might have accounted for their prevalence in the urine samples. Urinary tract infections are one of the most common bacterial infections caused by members of Enterobacteriaceae that affect humans in community and hospital settings which accounted for up to 88.0% cases (Park et al., 2017).

Colonization of *E. coli* in the vagina and cervix has been reported to cause a lot of diseases and illnesses to humans (Olowe et al., 2012; Kumari et al., 2016; Orish et al., 2016). In a study (Kumari et al., 2016), it was

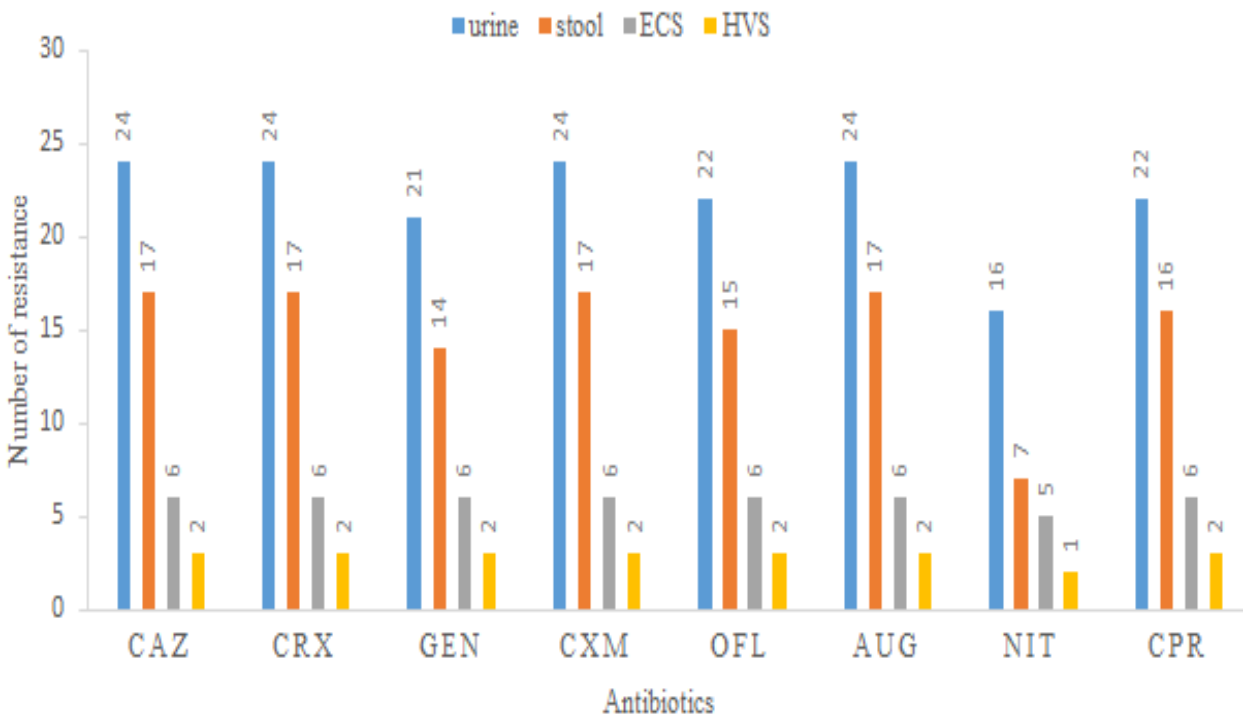

Figure 4. Resistance pattern of isolates against selected antibiotic classes. CAZ- Ceftazidime, CRX- Cefuroxime, GEN- Gentamicin, CXM- Cefixime, OFL- Ofloxacin, AUG- Amoxicillin/clavulanate, NIT- Nitrofurantoin, CPR-Ciprofloxacin

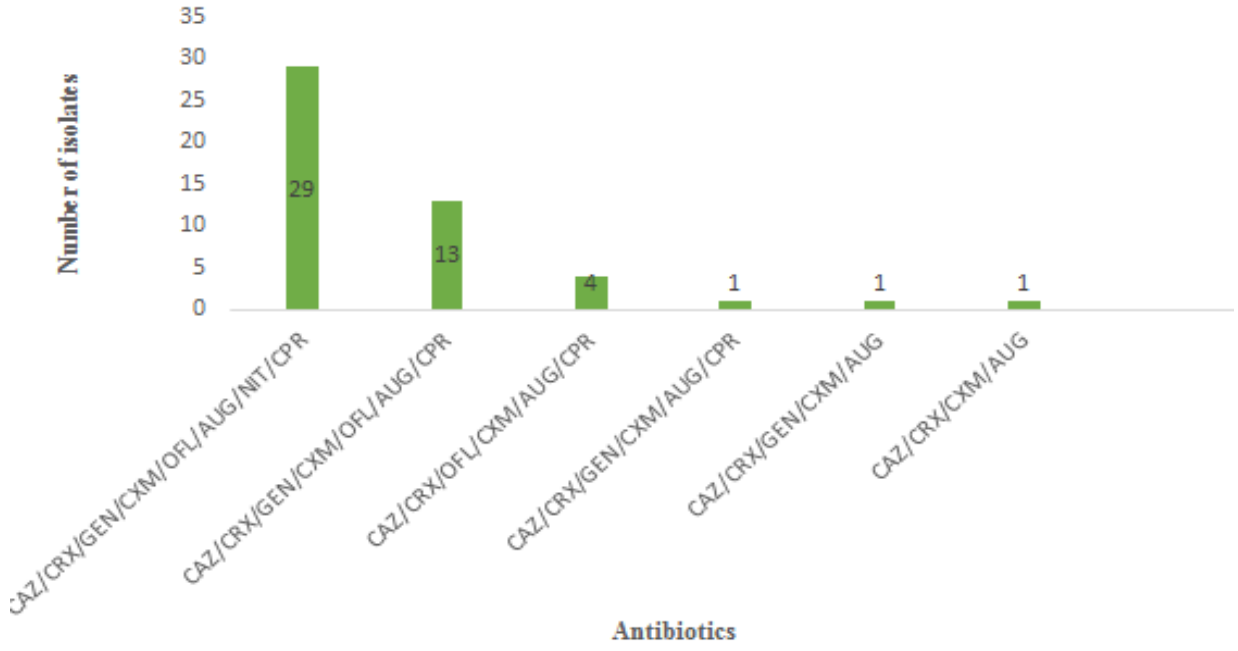

Figure 5. Multi-drug resistance profile of isolates obtained from clinical samples. CAZ- Ceftazidime, CRX- Cefuroxime, GEN- Gentamicin, CXM- Cefixime, OFL- Ofloxacin, AUG- Amoxicillin/clavulanate, NIT- Nitrofurantoin, CPR-Ciprofloxacin.

observed that the most predominant Gram-negative organisms responsible for pelvic inflammatory disease and infertility in women were *E. coli* and *Klebsiella*. In the same trend, studies amongst patients with suspected

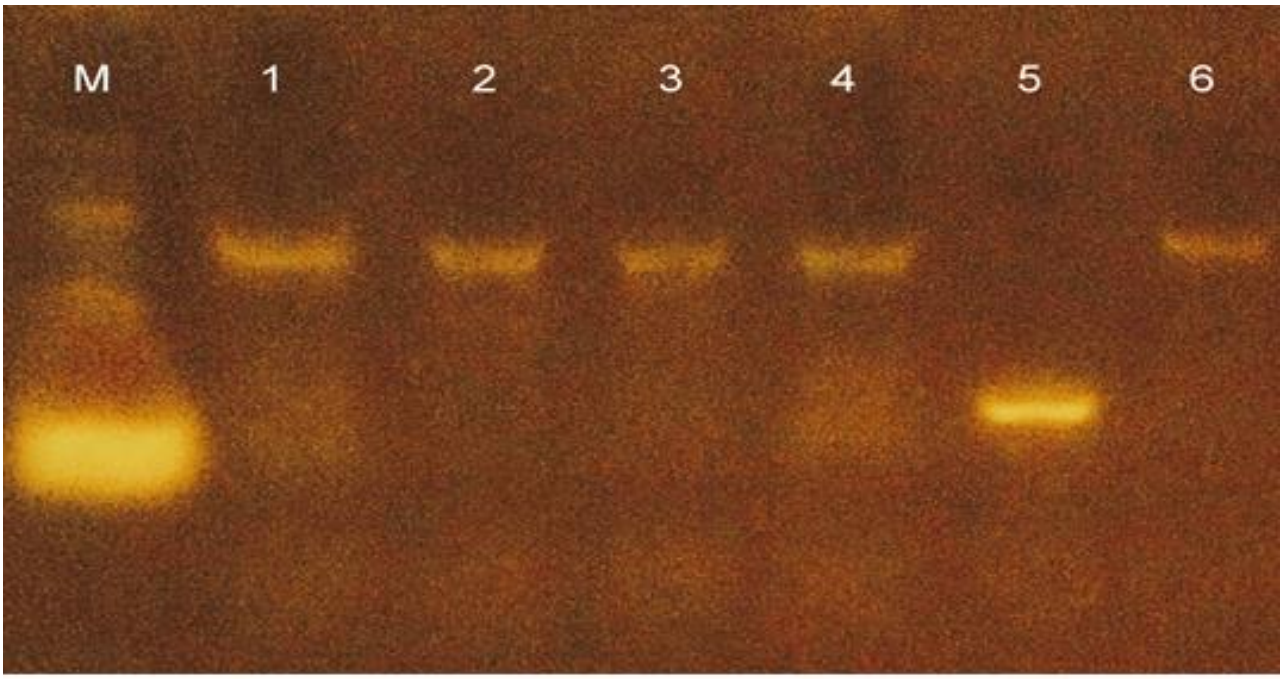

Figure 6. Electrophoregram of class 1 integron. M: Molecular Weight Marker (100bp), Isolates: 1-6.

pelvic inflammatory disease in Osogbo, Nigeria, revealed that 70% of female genitals were colonized by *E*. *coli* (Olowe et al., 2012). Thus we can say that the prevalence of *E*. *coli* over other species in the urine, high vaginal swab and endocervical swab could be an indication that the patients might have been suffering from either of the aforementioned infections.

High level resistance of isolates was observed against ceftazidime, cefuroxime, cefixime and amoxicillin/ clavulanate, ofloxacin, ciprofloxacin and gentamicin. This is in harmony as reported by Iliyasu et al. (2018). Most of the antibiotics we used have no bactericidal effect on the strains encountered. This observation is consistent with previous studies (Omololu-Aso et al., 2017; Ibrahim et al., 2019), but is in contrary to the report of other authors (Ogidi and Oyetayo, 2013; Waseem et al., 2015).

The high level of resistance may be attributed to antibiotic pressure in clinical settings. Kibret and Abera (2011) reported high level resistance of *E*. *coli* to amoxicillin (86.0%), but highly susceptible to nitrofurantoin (96.4%), norfloxacin (90.6%), ciprofloxacin (79.6%), erythromycin (89.4%) and (72.6%) tetracycline. The variation in resistance could be attributed to the different strains of bacteria encountered as well as different antibiotic pressures in the studied environments. In addition, antibiotic abuse associated with self-medication which often results in inadequate dosage could have contributed significantly to this resistance profile (Ezeamagu et al., 2018). Many factors affecting microbial resistance phenotype have been highlighted elsewhere (Corona and Martinez, 2013). The resistance of the isolates against nitrofurantoin was on the high side and is similar to Jafri et al. (2014) where (52.5%) of the organisms were resistant to the same antibiotics (Jafri et al., 2014). Nitrofurantoin is one of the most appropriate antibacterial agents for empirical therapy of UTIs because it is highly concentrated in the urine and it is administered orally. However, high level of resistance observed is a signal that in the nearest future treatment failure due to Enterobacteriaceae infections will be anticipated. Therefore, increasingly presence of these antibiotics in the clinical settings will result in rapid development of resistance (Munita and Arias, 2016; Tuem et al., 2018; Aslam et al., 2018).

The sale of medicines without a prescription is an important regulatory issue in the abuse of antibiotics. It has been reported that bacteria acquire resistance by horizontal gene transfer of mobile genetic elements and that high usage of the antibiotics influences the selection of existing resistance mechanisms (Stokes and Gillings, 2011). Multidrug resistance has serious implications for the empiric therapy of infections caused by bacteria such as *E*. *coli*, *Klebsiella*, *Enterobacter* and *Proteus* species especially those that harbour integrons.

Integrons play an important role in antibiotic resistance, and they are able to capture, integrate, and express those gene cassettes encoding antibiotic resistance (Park et al., 2018; Partidge et al., 2018). We found integrons belonging to class 1 in 40.81% of the isolates encountered while class 2 and 3 integrons were absent. Also, the prevalence rate of integrons is comparable to several studies (Chang et al., 2000; Essen-Zandbergen

et al., 2007; Japoni et al., 2008; Muhammad et al., 2011; Kor et al., 2013; Tuem et al., 2018; Ibrahim et al., 2019), but differed from results elsewhere (Daikos et al., 2007; Fuentes et al., 2013; Hadizadeh et al., 2017).

The variation could be attributed to geographical location and environment. Few studies in Nigeria have reported the presence of integrons in clinical and environmental isolates. Odetoyin et al. (2018) detected class 1 (31%) and class 2 (4%) integrons in faecal *E. coli* strains of mother-child pairs in Osun State, Nigeria. Class 1 integrons (57.4%) were also detected in *P. aeruginosa* isolated from clinical isolates in South-West Nigeria (Odumosu et al., 2013).

Adesoji et al. (2017) identified 27.3% class 1 integrons in multidrug-resistant *Pseudomonas* from water distribution systems in South-western, Nigeria. It is likely that integrons Class 1 are frequently detected among clinical isolates than environmental isolates in Nigeria. The presence of integrons has no association with the degree of resistance as observed in this work. Other authors (Dakic et al., 2007; Japoni et al., 2008) had a slight association in the degree of resistance although majority are not statistically significant in terms of resistance pattern.

Conclusion

It can be inferred from this work that a large proportion of the Enterobacteriaceae encountered were multi-drug resistant and possessed integrons. Consequently, there is a need for proactive antibiotic surveillance system in both healthcare and community settings with a view to reducing the incidence and spread of antibiotic resistance genes between different species of bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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