

**Antibiotic Resistance and Beta-Lactamase Genes Detection among  
Extended Spectrum Beta-Lactamase (ESBL)-Producing  
*Escherichia coli* and *Salmonella* Species Isolated from Cockroaches  
(*Periplaneta americana*) in Abakaliki, South-East Nigeria**

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**Abstract:** Infections caused by *Escherichia coli* and *Salmonella* species contribute significantly to reasons for seeking medical care especially in developing countries. Moreover, multidrug resistant and ESBL-producing strains of these organisms are now serious problem of public health concern. Arthropods like cockroaches which live in filthy environments and in close association with man could serve as vehicles through which these pathogens can be transmitted to human population. This study was aimed at isolating *Escherichia coli* and *Salmonella* species from cockroaches infesting hospital and non-hospital environments with a view to determine the antibiotic resistance profile and the presence of *bla*<sup>CTX-M</sup>, *bla*<sup>TEM</sup> and *bla*<sup>SHV</sup> genes among ESBL-producing strains. The external body washes and gut homogenates of 517 adult cockroaches (*Periplaneta americana*) caught from a tertiary hospital and residential homes in Abakaliki, Ebonyi state, South-East Nigeria were cultured to isolate *Escherichia coli* and *Salmonella* species employing standard microbiological techniques. Phenotypic ESBL production and antibiotic susceptibility were determined by the Modified Double Disc Synergy Test (MDDST) and Kirby-Bauer disc diffusion technique respectively. The presence of *bla*<sup>CTX-M</sup>, *bla*<sup>TEM</sup> and *bla*<sup>SHV</sup> genes was determined by polymerase chain reaction using specific primers. Culture of the specimens yielded 101 isolates of *Escherichia coli* and 21 isolates of *Salmonella* species. Phenotypic ESBL-production was detected in 16 (15.8 %) and 4 (19.0 %) of *Escherichia coli* and *Salmonella* species respectively. Result of antibiotic susceptibility test showed that greater percentage of the isolates were resistant to the antibiotics tested. PCR analysis revealed that 17 (85.0 %) of the ESBL-producers were positive for *bla*<sup>TEM</sup>, 15 (75.0 %) for *bla*<sup>SHV</sup>, while 12 (60.0 %) were positive for *bla*<sup>CTX-M</sup>. The detection of *bla*<sup>CTX-M</sup>, *bla*<sup>TEM</sup> and *bla*<sup>SHV</sup> genes in pests like cockroaches reveals the diverse and complex nature of the reservoirs of organisms that harbor genes encoding for ESBLs thus suggesting the possible role of these insects in the dissemination of multidrug resistant bacteria among human population both in the hospital and community settings.

**Key words:** Cockroaches • *Escherichia coli* • *Salmonella* Species • Antibiotic Resistance • ESBLs • *bla*<sup>CTX-M</sup> • *bla*<sup>TEM</sup> • *bla*<sup>SHV</sup>

## INTRODUCTION

Cockroaches are typically tropical insects; however, they can survive in subtropical and cooler climates so

long as they remain indoors or are closely associated with humans [1]. Cockroaches are important carriers of pathogens due to their unsanitary lifestyle. They breed and forage in sewer systems, septic tank areas, garbage

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bins and latrine pits prior to contacting human food thus raising concerns of deleterious health consequences for humans [2-4]. From these unsanitary areas, they can enter urban structures such as hospitals and residential homes where they could possibly transmit microorganisms leading to a multitude of human infections [5]. It is estimated that in developing countries, above 25% of hospitalized patients acquire nosocomial infections [6] the sources of which apart from patients, medical personnel, visitors, hospital equipment and devices, can be arthropods inhabiting hospitals [7]. Multidrug resistant (MDR) and extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* especially *Escherichia coli* and *Salmonella* have caused a major concern in several countries, being frequently implicated in life threatening human infections [8-10]. Bacteria harbouring ESBLs confer significant resistance to penicillins, narrow and extended spectrum cephalosporins and monobactams, but they are inhibited by clavulanic acid [11]. These plasmid-mediated enzymes also frequently show resistance to aminoglycoside, sulphamethoxazole-trimethoprim and quinolones [12]. The TEM and SHV beta lactamases were more prevalent than the CTX-M enzymes in the 1980s and 1990s, mainly restricted to outbreaks in hospitals particularly in the intensive care units, involving members of *Enterobacteriaceae* [12]. The CTX-M enzymes though discovered in 1989, took over a decade before they caught global attention. They are wide spread in both hospitals and in the community and are most often detected in *Escherichia coli* [13]. The CTX-M-type extended spectrum beta-lactamases (ESBLs) unlike those of TEM and SHV did not originate by base modifications of native plasmid genes but by incorporation of chromosomal beta-lactamase genes from the environmental organisms, *Kluyvera* species into mobile genetic elements [14]. The first TEM-type beta-lactamase that exhibited ESBL phenotype was TEM-3, subsequently over one thousand variants of TEM-1 have been reported. Unlike TEM-1, there are relatively few derivatives of SHV-1, however majority of these derivatives exhibit ESBL phenotype [15]. Greater number of SHV-type ESBLs is found in strains of *Klebsiella pneumoniae*; however they have equally been reported in other members of *Enterobacteriaceae* and *Pseudomonas aeruginosa* [16]. Isolation of ESBL-producing *Enterobacteriaceae* in wildlife, farm and domestic animals and their possible role in the persistence and dissemination of resistant bacterial species to human population have been widely reported [9, 17-20]. In contrast, there is still paucity of information on ESBL-producing organisms from insect species particularly synantropes like cockroaches. This study

therefore investigated the antibiotic resistance profile and the presence of *bla*<sup>CTX-M</sup>, *bla*<sup>TEM</sup> and *bla*<sup>SHV</sup> genes among ESBL-producing *Escherichia coli* and *Salmonella* species isolated from cockroaches infesting a tertiary hospital and residential homes in Abakaliki, South-East Nigeria.

## MATERIALS AND METHODS

**Sample Collection and Processing:** A total of 517 *P. Americana* adult cockroaches comprising 137 cockroaches from hospital environment and 380 cockroaches from residential homes were collected using the off-target insecticide spray technique. The cockroaches were picked with gloved hands, placed in sterile and labeled universal containers and transported to the Microbiology Laboratory Unit of Ebonyi State University, Abakaliki, Nigeria for analysis. The external body washes and gut homogenates of the cockroaches were used for analysis. The external body wash was prepared by adding 5 ml of sterile normal saline into sterile universal containers with one cockroach each and then shaken to dislodge organisms attached to it [21]. The cockroaches were then removed aseptically from the containers, decontaminated with 70 % alcohol, allowed to air-dry before placing into 5 ml of sterile normal saline in a tube to remove ethanol residues [22]. The gut homogenates were prepared by dissecting the alimentary duct under a dissecting microscope to dislodge its content which was subsequently suspended in 5 ml of sterile normal saline [21, 22].

**Culture and Identification of Bacteria:** Precisely 1 ml of external body washes and gut suspension of each cockroach were separately inoculated into 10 ml of Selenite-F broth (Oxoid, UK). Two loopful of each inoculum were further inoculated onto Deoxycholate Citrate Agar, MacConkey Agar and Eosin Methylene Blue agar (Oxoid, UK). All inoculated media were incubated at 37°C for 24 hours. Subcultures from Selenite – F broth were further made onto DCA and incubated further at 37°C for 24 hours [23, 24]. Suspect bacteria colonies were identified using standard microbiological techniques [25-27].

**Screening and Detection of ESBL-Positive Isolates:** Isolates resistant to one or more of the third generation cephalosporins were considered potential ESBL-producers and were further tested for the production of the ESBL phenotypically using the modified double disc synergy test, MDDST [18]. An overnight broth culture of

test isolates were diluted in sterile physiological saline and adjusted to 0.5 MacFarland standard. These were then inoculated onto Mueller-Hinton agar (Oxoid, UK) using sterile swabs. Amoxycillin-clavulanate disc (20/10 µg) was placed in the middle of the plate with 30 µg discs of ceftriaxone, ceftazidime (30µg), cefotaxime(30µg) (placed 15 mm away from the center disc) and cefepime (30µg) (placed 20 mm away from center disc). The plates were incubated overnight at 37°C. Extension of the inhibition zone around the four discs towards amoxicillin/clavulanic acid disc indicates a positive result [28].

**Antibacterial Sensitivity Testing:** *In-vitro* antibiotic susceptibility of ESBL-producing *Escherichia coli* and *Salmonella* was determined by the standard Kirby-Bauer disc diffusion technique using Oxoid discs; cefoxitin (30 µg), imipenem (10 µg), sulphamethoxazole-trimethoprim (25 µg), chloramphenicol (30 µg), gentamicin (10 µg) and ciprofloxacin (5 µg) [29]. Few colonies of each test isolate were suspended in sterile normal saline to match 0.5 MacFarland turbidity standards. Precisely 0.1 ml of the suspension was then evenly spread on Mueller-Hinton agar. The inoculated plates were allowed to air-dry prior to the placing of the discs [30, 31]. The diameters of the inhibition zones were measured in millimeters (mm) using a ruler after 24 hours' incubation at 37°C. The interpretation of the inhibition zones as susceptible or resistant was according to standard guidelines [32].

**DNA Extraction and PCR Analysis of ESBL Genes:** This was done at the Biotechnology Development and Research Center, Ebonyi State University, Abakaliki Nigeria. Plasmid DNA of the ESBL-producing isolates was extracted using ZR Plasmid Miniprep Kit (Zymo Research Corporation, USA) according to manufacturer's instructions. The presence of *bla*CTX-M, *bla*TEM and *bla*SHV genes was investigated by PCR using the primer pairs shown in Table 1. PCR amplification was performed in volume of 25 µl which consisted of 2.0 µL 100 ng DNA, 2.5 µL of 1 x buffer (Bioline, USA), 1.5 µl of 50 mM MgCl<sub>2</sub>, 2.0 µl of 2.5 mM dNTPs and 0.2µl of 500 U Taq DNA polymerase (Bioline for *bla*TEM and *bla*SHV; Promega, USA for *bla*CTX-M), 1.0µl of each 10 mM primer pair and 14.8 µl of DEPC treated water (Invitrogen Corporation) all in a 0.2 ml PCR tube. The PCR cycling profile used for the reaction consists of an initial step at 96 °C for 5 minutes (*bla*TEM and *bla*SHV) & 7 minutes (*bla*CTX-M), 35 cycles of 94°C for 30 seconds, 58°C (*bla*TEM and *bla*SHV) & 60°C (*bla*CTX-M) for 1 minute and 72°C for 1 minute and a 10 minute final extension at 72°C. Precisely 10 µl of

amplified PCR products of each test isolate along with appropriate DNA standards (250 bp for *bla*TEM and *bla*SHV and 100 bp for *bla*CTX-M) were separated on 1.5 % agarose gel for 2 hours at 80 volts, stained with ethidium bromide and the resulting bands were visualized under ultraviolet light. The visual output was documented using photo imager (Fotodyne Incorporated, Foto/Analyst Express, Japan).

Table 1: Primers used for the detection of *bla*CTX-M, *bla*TEM and *bla*SHV genes in *Escherichia coli* and *Salmonella*

Genes	Primer Sequence	Expected Size (bp)	Reference
TEM-F	ATGAGTATTCAACATTTC	867	20, 44
TEM-R	CTGACAGTTACCAATGCTTA		
SHV-F	GGTTATGCGTTATATTCGCC	867	20, 44
SHV-R	TTAGCGTTGCCAGTGCTC		
CTX-M-MU1	ATGTGCAGYACCAGTAARGT	593	18, 44
CTX-M-MU2	TGGGTRAARTARGTSACCAGA		

## RESULTS

A total of 101 *Escherichia coli* and 21 *Salmonella* species were recovered from the 517 cockroach samples analyzed. The results obtained from MDDST revealed 16 (15.8 %) of the *Escherichia coli* and 4 (19.0 %) of the *Salmonella* species to be positive for phenotypic ESBL production (Tables 2 and 3) respectively. As presented in Table 4, the percentage of ESBL-producing *Escherichia coli* resistant to each of the antibiotics tested include 87.5 % (Sulphamethoxazole-trimethoprim), 68.8 % (Imipenem and gentamicin), 62.5 % (Cefoxitin), 56.6 % (Ciprofloxacin) and 50.0 % (Chloramphenicol). All the ESBL-producing *Salmonella* (100 %) were resistant to cefoxitin, imipenem and sulphamethoxazole-trimethoprim, 75 % were resistant to chloramphenicol and gentamicin while 50 % was resistant to ciprofloxacin. Agarose gel electrophoresis of the PCR products for *bla*CTX-M, *bla*TEM and *bla*SHV genes is shown in Figs. 1-3 respectively. Frequency of occurrence of the genes among the 20 ESBL-producers were; 12 (60.0 %) for *bla*CTX-M, 17 (85.0 %) for *bla*TEM and 15 (75.0 %) for *bla*SHV. Co-existence patterns observed were; *bla*CTX-M + *bla*TEM + *bla*SHV in 6 of *Escherichia coli* and 1 of *Salmonella*, *bla*CTX-M + *bla*TEM in 1 of *Escherichia coli* and 2 of *Salmonella*, *bla*CTX-M + *bla*SHV in 1 of *Escherichia coli* while *bla*TEM + *bla*SHV was observed in 6 of *Escherichia coli* and 1 of *Salmonella* species. In addition, *bla*CTX-M alone was detected in 1 of the *Escherichia coli*. One of the isolates (*Escherichia coli*) was found not to harbor any of the genes investigated.

Table 2: Distribution of ESBL-producing *Escherichia coli* and beta-lactamase genes in sample collection sites

Sample sites	Number positive for ESBL (%)	Number Positive for CTX-M (%)	Number Positive for TEM (%)	Number Positive for SHV (%)
Hospital (n= 24)	7 (29.2)	6(25)	5(20.8)	5(20.8)
Residential homes (n= 77)	9(11.7)	3(3.9)	8(10.4)	8(10.4)
Total (101)	16(40.9)	9(28.9)	13(31.2)	13(31.2)

Table 3: Distribution of ESBL-producing *Salmonella* species and beta-lactamase genes in sample collection sites

Sample sites	Number positive for ESBL	Number Positive for CTX-M	Number Positive for TEM	Number Positive for SHV
Hospital (n= 4)	1(25)	Nil	1(25)	Nil
Residential homes (n= 17)	3(17.6)	3(17.6)	3(17.6)	2(11.8)
Total (21)	4(42.6)	3(17.6)	4(42.6)	2(11.8)

Table 4: Antimicrobial resistance pattern of ESBL-producing *Escherichia coli* and *Salmonella* species

Antibiotics	<i>Escherichia coli</i> (%)	<i>Salmonella</i> species (%)
Cefoxitin	62.5	100
Imipenem	68.8	100
Sulphamethoxazole-trimethoprim	87.5	100
Chloramphenicol	50.0	75.0
Gentamicin	68.8	75.0
Ciprofloxacin	56.6	50.0

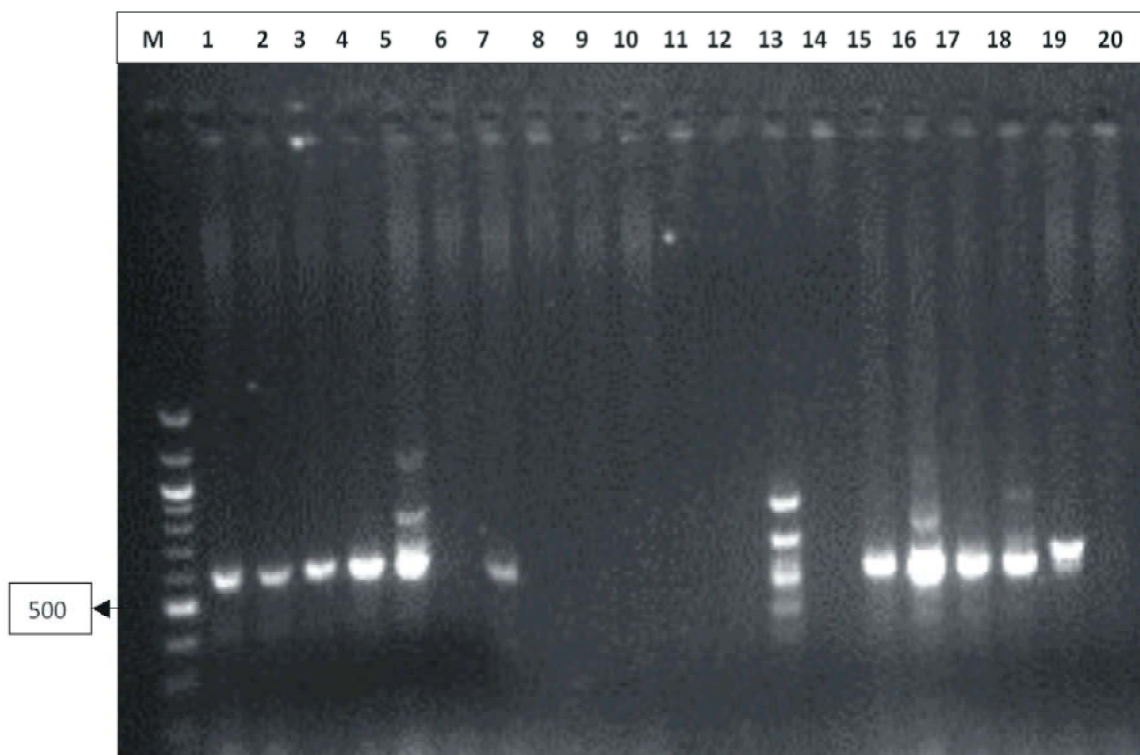


Fig. 1: Gel Electrophoresis of PCR Products of CTX-M Genes

M = 100 base pair DNA ladder

Lanes 1 – 16 = ESBL-producing *Escherichia coli*

Lanes 17 – 20 = ESBL-producing *Salmonella* species

Molecular weight of positive control = 1000kb

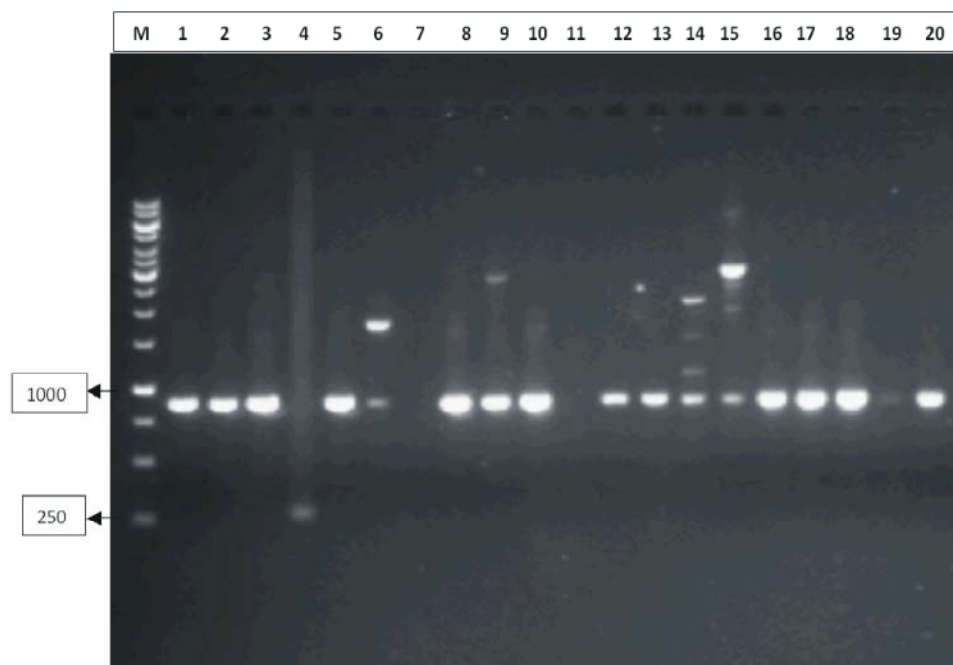


Fig. 2: Gel Electrophoresis of PCR Products of TEM Gene

M = 250 base pair DNA ladder

Lanes 1 – 16 = ESBL-producing *Escherichia coli*

Lanes 17 – 20 = ESBL-producing *Salmonella* species

Molecular weight of positive control = 1000kb

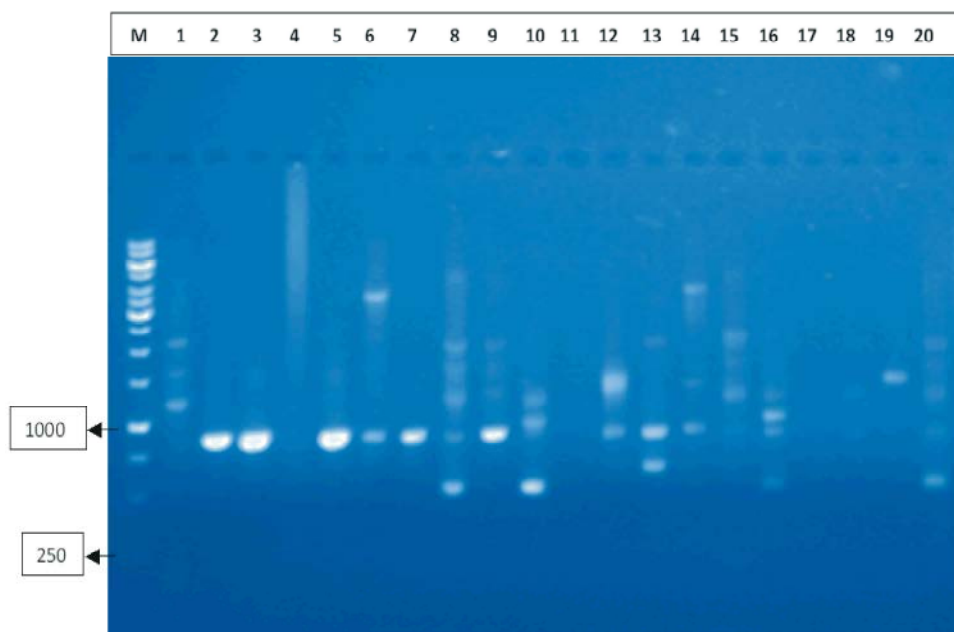


Fig. 3: Gel Electrophoresis of PCR Products of SHV Gene

M = 250 base pair DNA ladder

Lanes 1 – 16 = ESBL-producing *Escherichia coli*

Lanes 17 – 20 = ESBL-producing *Salmonella* species

Molecular weight of positive control = 1000kb

## DISCUSSION

Cockroaches like wildlife are not known to be exposed to antimicrobial agents but can acquire antimicrobial resistant-bacteria through contact with humans, animals and the environment [17]. In this study, phenotypic ESBLs activity was detected in 20 (16.4 %) out of the total number of 122 isolates from the cockroaches comprising of 101 *Escherichia coli* and 21 *Salmonella* species. Similar findings have been observed and reported [33, 34]. Once a clinical isolate is identified as ESBL-producer, all cephalosporins are usually avoided as therapy, thus making therapeutic options for ESBL-producing bacteria pathogen very limited. There was relatively low percentage of ESBL-producing *Escherichia coli* (56 %) and *Salmonella* (50 %) that were resistant to ciprofloxacin as observed in this study, this contradicts reports that a strong association exists between quinolone resistance and ESBL production [35-37]. Moderate resistance to the fluoroquinolones corroborating our findings has been reported from clinical isolates [38, 39]. The high percentage of ESBL-producing *Escherichia coli* (68.8 %) and *Salmonella* (100%) that were resistant to imipenem (Carbapenem) in this study is quite worrisome and does not agree with reports from many clinical studies that carbapenems are still very efficient in the treatment of serious infections caused by ESBL-producing organisms and are therefore specifically reserved for this purpose [40-43]. Most of these reports recorded zero percent resistance to the carbapenems; however a similar concern over increasing resistance to the carbapenems among ESBL-isolates has been raised in a study which reported a resistant rate of 37.5 % [39]. The resistance pattern of the isolates to cefoxitin (62.5 & 100 %), sulphamethoxazole-trimethoprim (87.5 % and 100 %), gentamicin (68.8 & 75 %) and chloramphenicol (50 & 75 %) for *Escherichia coli* and *Salmonella* respectively simulates reports from clinical studies [39-41]. Very low resistance to amoxycillin-clavulanic acid (9.7 %) has however been reported [38]. Subsequent PCR analysis of the ESBL-positive isolates led to the detection of *bla*TEM in 17 (85%), *bla*SHV in 15(75%) and *bla*CTX-M in 12 (60%). This suggests that *bla*TEM and *bla*SHV still contribute significantly to ESBL-mediated resistance thereby contradicting earlier report [14] that TEM and SHV ESBL-types are usually displaced or over shadowed consequent to the penetration of *bla*CTX-M-type ESBLs into a particular region. A lower prevalence of *bla*TEM (8.3%) and higher prevalence of *bla*CTX-M (83.3 %) have earlier been observed in cockroach isolates elsewhere [44]. In this study, *bla*CTX-M+*bla*TEM+*bla*SHV co-existed in 7 (35%) of

ESBL-producing isolates, *bla*CTX-M + *bla*TEM in 3 (15%), *bla*CTX-M + *bla*SHV in 1 (5%) and *bla*TEM+ *bla*SHV in 7 (35%). Co- existence of beta-lactamase genes in isolates from cockroaches similar to our findings has been reported [44]. The phenotypic ESBL activity exhibited by the isolate in which none of the genes investigated was detected could be due to the presence of other beta-lactamase genes encoding ESBLs.

## CONCLUSIONS

This study revealed cockroaches (*Periplaneta americana*) as reservoirs for ESBL-producing *Escherichia coli* and *Salmonella* species. TEM gene was more prevalent followed by SHV and CTX-M genes. The isolates exhibited significant resistance to the antibiotics tested; this highlights the negative impact of increasing and inappropriate use of antimicrobial agents both in human and veterinary medicine in our environment. Ciprofloxacin may be useful for treating infections due to ESBL-producing organisms when they are susceptible to the drug *in-vitro*.

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