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MOLECULAR CHARACTERIZATION OF EXTENDED SPECTRUM BETA LACTAMASES AND RESISTANCE TO GENTAMICIN AND QUINOLONES IN GRAM NEGATIVE BACTERIAL ISOLATES OF PERIPLANETA AMERICANA (COCKROACHES)

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ABSTRACT

The close association of cockroaches with man in his environment is a threat to public health due to their potential in the transmission of multi-drug resistant infectious agents. This study was therefore conducted to detect the presence of extended-spectrum beta lactamases (ESBLs) in Gram negative bacterial isolates of cockroaches. Two hundred (200) cockroaches were randomly collected and analyzed at the microbiology laboratory, Olabisi Onabanjo University. Isolated bacteria were tested for antibiotic susceptibility against eight clinically relevant antibiotics and Double Disc Synergy Test (DDST) for the confirmation of extended-spectrum beta-lactamase (ESBL) production. Existence of genes encoding ESBL production were identified using polymerase chain reaction (PCR). A total of 363 bacteria (208 (57) from the external surfaces and (155 (43) from the gut environment were recovered from the analyzed cockroaches. Twenty-six (26) of the forty (40) phenotypically confirmed ESBL organisms showed discrete amplification with the molecular technique. All isolates showed elevated trend of multi-drug resistance to the tested antibiotics with 12=46.2%, 11=42.3% and 3=11.5% of the 26 Gram negative isolates harboring one, two and three ESBL genes, respectively. The sulfhydryl variable (*SHV*) gene was the most significant with a percentage distribution of 69.2% of the total ESBL alleles suggesting cockroach as a vector for enteric bacterial infections. Findings also affirmed the reduced susceptibility of all the isolates to both gentamicin and quinolone antibiotics.

Keywords: Gram negative bacteria, Gentamicin, Quinolones, Periplaneta americana (cockroaches).

INTRODUCTION

Extended spectrum cephalosporin antibiotics have been used to treat variety of infections caused by Gram-negative bacteria since the 1980s. However, in recent times, a significant increase in the incidence of infections associated with extended spectrum beta lactamase (ESBL) producing organisms have been observed throughout the world (Gupta, 2007; Fatemeh *et al.,* 2012; Abhijit *et al.,* 2013; Majda *et al.,* 2013; Meeta *et al.*, 2013 and Rupinder *et al.,* 2013). These infections are due to the dissemination of different types of ESBL alleles (Michaela *et al.,* 2016), which are known to be transferred from one bacteria to the other with the aid of plasmid and transposons. These ESBLs have been grouped into class A, B, C and D with more than four hundred ESBLs caused by point mutation being documented (Bush and Jacoby, 2010; Isaiah *et al.*, 2011).

Today, different types of plasmid mediated extended spectrum beta lactamase alleles such as *SHV*-1 (named after the Sulfhydryl-variable active site), *CTX-M* (which preferentially hydrolyze cefotaxime over ceftazidime and also cefepime with high efficiency), *TEM*-1 (designated *Temoniera*) and *bla*CTX-*M*-15 have been described in many different genera of the family *Enterobacteriaceae* and *Pseudomonas aeroginosa*. In clinical strains, many ESBL encoding genes have been found to be located on plasmids of varying sizes (Gonullu *et al.,* 2008). Few studies which have done replicon types of these plasmids have established that majority of these plasmids are *IncFII* plasmids, either alone or in association with *Inc FIA* and *FIB* (Carattoli *et al.*, 2005; Carattoli, 2009).

One study had reported the presence of *IncFI* alone in one isolate in Turkey while other *Inc* groups like *IncI1*, *Inc*N have also been reported (Gonullu *et al.,* 2008). Most of these plasmids are conjugative, with conjugation frequency ranging between 10^{-2} and 10^{-7} and several multiple resistant genes. Several ESBL producing organisms have also been characterized in different foods including fish products (Franzetti *et al.,* 2001), raw and processed milk (Wiedmann *et al.,* 2000; Dogan and Boor, 2003) and even street vended foods (FAO, 2016).

Recently, the possibility of insect acting as a mechanical vector for the transmission of antibiotic resistant bacteria was documented (Rahuma *et al.,* 2005) and some of these insects are edible while a few others are microbiologically hazardous upon consumption (Abu-Ghannam and Crowley, 2006). Cockroaches, which are dorso-ventrally flattened insects are known for being abundantly notorious and an obnoxious non-biting pest (Mba and Kelly, 2003) have been reported to be on this planet earth for more than three hundred million years (Zurek and Schal, 2004). These insects are also known to be well adapted and include about 4500 species with *Periplaneta americana* and *Blatella germanica* being the most common species (Uneke, 2007).

Their nocturnal and filthy habits make them ideal carriers of various pathogenic microorganisms (Pai *et al.*, 2005; Blazar *et al.*, 2011), including *Staphylococcus aureus*, *Streptococcus spp.*, *Enterobacteriaceae*, *Pseudomonas aeruginosa* among other bacteria (Pai *et al.*, 2005; Fakoorziba *et al.*, 2010; Brown and Alhassan, 2015). The role of these insects in the epidemiology of different infections has been reported (Pai *et al.*, 2005; Fakoorziba *et al.*, 2010). In a report by Czajka *et al.* (2003), an outbreak of nosocomial disease due to extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in neonatal unit was attributed to infestation of the neonatal wards with cockroaches and the continuous presence of these cockroaches in every home are mainly due to complexity of building structures, furniture as well as the emergence of insecticide resistant strains (Zurek and Gorham, 2008).

There is a diverse collection of ESBL genes and Gram negative bacteria from different sources globally (Stefani *et al.,* 2014; Michaela *et al.,* 2016) and cockroaches are potential reservoir that could affect humans either by direct transmission of resistant strains through their droppings or body parts. This study was therefore aimed at

determining the presence of extended-spectrum beta lactamase producing Gram negative bacteria from cockroaches.

MATERIALS AND METHODS

Collection of cockroach samples

Prior to the commencement of the collection of cockroaches, visits were made to different places (houses, offices and shops) in Ago-Iwoye for awareness on the aim of this study and the importance of the study to the community and a total of two hundred (200) cockroaches were randomly collected from different parts of Ago Iwoye using sticky trap method and sterile hand gloves. A transparent cylindrical plastic container with an opening of 11 cm in diameter and a lid was used to trap the cockroaches. The inside of the container was lined with blotting paper smeared with petroleum jelly and the trap kept in the kitchen or other strategic parts of the house overnight, uncovered. The following day, live cockroach specimens were immediately transported to Microbiology Laboratory Unit of Olabisi Onabanjo University, Ago-Iwoye, Nigeria for analysis.

Cockroaches were studied for the presence of ESBL producing Gram negative bacteria at the Microbiology Department of the Olabisi Onabanjo University, Ago Iwoye (Coordinates 6° 57' 0" North, 3° 55' 0" East).

Isolation of bacteria from cockroach samples

The external surface of the cockroaches were prepared by suspending each cockroach into a sterile universal bottle containing 2 mL buffered peptone water and then shaken to dislodge organisms attached to it in order to produce a homogenate specimen, after which the samples were inoculated onto MacConkey Agar, Xylose Lysine Deoxycholate Agar, and Eosin Methylene Blue Agar and then incubated at 37°C aerobically for 24 hours. Also, prior to the isolation of bacterial isolates from the cockroaches gut, the cockroaches were aseptically removed from the container, decontaminated with 70% ethanol before being immobilized using absolute chloroform-soaked cotton wool. The cockroaches were thereafter placed into 5 ml of sterile normal saline tube to remove ethanol residues before being placed in a wax tray, fixed with pins and dissected under aseptic conditions. Cockroach gut was removed and kept in 2 ml buffered peptone water for 30 minutes to produce homogenate specimens (Bala and Sule, 2012). Precisely 0.1 ml of gut homogenate samples of each cockroach was separately inoculated onto MacConkey Agar, Xylose Lysine Deoxycholate Agar, and Eosin Methylene Blue Agar using spread plate method and then incubated at 37°C aerobically for 24 hours. (Sharawi *et al.*, 2019).

Sequencing of the hypervariable 16S rRNA gene of the isolated organisms

All the isolates obtained in this study were identified by sequencing the hyper variable 16S ribosomal RNA gene using the following primer pairs for forward and reverse sequence (5'-AGAGTTTGATCCTGGCTCAG3' and 5'-CCGTCAATTCACTTTAGAGTTT3'). The sequenced isolates were aligned in MEGA 7.0 bioinformatic software and then subjected to blasting in order to identify the organisms. The organisms with the highest bit score were chosen as the isolated organism while only Gram negative bacteria were used for subsequent analysis. All isolates were kept at -40°C until their analysis. (Frank *et al.*, 2001).

Purity plating and phenotypic detection of ESBLs

Culture purity was tested in MacConkey and Nutrient agar and suspensions were prepared to 0.5McFarland turbidity standard. (Brown and Alhassan, 2015). Suspensions were then inoculated onto Mueller-Hinton agar plates (Oxoid) for phenotypic study of the presence of ESBLs using double disk synergy test; the susceptibility to commonly used antibiotics was also investigated using the Clinical and Laboratory Standard Institute (CLSI) guidelines (2015). The Minimum Inhibitory Concentrations were also assessed and their values interpreted as recommended by EUCAST (2013) as follow isolates showing an inhibition zone size of <22mmwith ceftazidime (30ug), <25mm with ceftriaxone (30ug) and <27mm with cefotaxime (30ug) were identified as potential ESBL producers and short listed for

confirmation of ESBL production phenotypically using double disc synergy test (DDST). The outer membrane protein of both the ESBL and non ESBL isolates recovered from cockroaches were also studied. Reference strains *Klebsiella pneumoniae* ATCC 70063 and *Escherichia coli* ATCC 25922 were used in all assays as control.

Molecular determination of extended-spectrum beta lactamases

A single colony of each organism was inoculated into 500 µl of phosphate buffered saline (PBS) and mixed by vortexing. Cells from 2 ml of overnight culture were harvested by centrifugation at 13000 rpm for 5 minutes. The supernatant was discarded and cells suspended in 500 µl of phosphate buffered saline. This suspension was incubated for 10 minutes at 95°C to lyse the cells, and then centrifuged at full speed for 10 minutes to remove cellular debris. The supernatant, a template DNA in the PCR reaction was transferred into a new pre-labelled eppendorf tube by gentle aspiration using a micropipette. PCR amplification of *TEM*, *SHV* and *CTX-M* genes were performed as described previously using their specific primers (Table 1). For amplification, 2 µl of template DNA was added to a 18 µl mixture containing 4.0 µl Master mix (dNTPs, taq polymerase, buffer, $MgCl_2$), 0.2 µl of primer pair and 13.6 µl of sterile water). The reaction was performed in Simpliamp PCR system thermo cycler (Applied Biosystems, USA) under the following conditions: Initial denaturation at 94°C for 5 minutes followed by 30 cycles of 30 seconds denaturation at 94°C, 60 seconds annealing at 58°C, 90 seconds extension at 72°C, and a final extension at 72°C for 5 minutes. PCR products were detected with ethidium bromide fluorescence using the photo image system (BioRad, UK) after 45 minutes electrophoresis in 1.5 % TAE agarose gel done at 100 volts. Positive controls for *TEM*, *SHV* and *CTX-M* were used in every run (Kiratisin *et al.*, 2008).

Data Analysis and Presentation

Data were analyzed by using the Statistical Package for Social Sciences (SPSS) version 22. Frequency distributions were carried out for the analyses of bacteria from both external and gut surfaces, resistance profiles of bacteria, co-occurrence of ESBL genes and level of congruence between molecular and phenotypic detection of ESBL organisms. The mean zone of inhibition, range and standard deviation of the different variants of ESBL genes as well as the minimum inhibitory concentration that inhibited 90% and 50% of the isolates were done following standard recommended protocols. The relationship between the distribution of MIC values and the ESBL variants were calculated using analysis of variance (ANOVA), while the level of significance was set at $p<0.05$.

RESULTS

The identification of bacterial isolates from the external surfaces and gut of cockroaches is depicted in Table 2. In this table, a total of 363 bacteria (208 (57) from the external surfaces and (155 (43) from the gut environment were recovered from the analyzed cockroaches. These bacterial isolates belong to 20 different species for both external surfaces and gut environment. The most predominant of these bacterial isolates from both the external surfaces and gut was *Escherichia coli* having a prevalence rate of 43 (20.8%) and 24 (15.5%) respectively to depict a total percentage representation of 67 (18.5%) of the total isolates obtained.

Staphylococcus aureus, *Bacillus subtilis, Staphylococcus epidermidis*, and *Enterococcus faecalis* were the only Gram positive bacterial isolates recovered with a prevalence rate of 8 (3.8%), 14 (6.7%), 8 (3.8%) and 9 (4.3%) from external surfaces and 2 (1.3%), 6 (3.9%), 2 (1.3%) and 7 (4.5%) from gut environment respectively. Generally, Gram negative bacteria were more represented in both external surfaces and gut environment. The least isolated organisms were *Serratia marscencens* 3(1.4%) and *Citrobacter werkmanii* 3(1.4%) from the external surfaces while *Citrobacter freundii* 2 (1.3%) was the least isolated organism from cockroach gut environment. All the isolates recovered from the cockroach external surfaces were also recovered from the cockroach gut. Despite the almost equilibrium of the isolates of the external surfaces and the gut, the percentage

prevalence of the isolates from the external surfaces were found to be significantly higher than those from the gut environment (t_{value} = P<0.05).

All the tested bacterial isolates showed an elevated trend of antibiotic resistance but to varying degree of resistance (Table 3). *Klebsiella pneumoniae* was the most resistant Gram negative bacteria to both CAZ (88%) and CRX (75%) but the most susceptible to ciprofloxacin as shown by a relative low resistance pattern (38%). *Salmonella enterica* is the second most resistant to CAZ (85%) while *Klebsiella quasipneumoniae* showed the most susceptible pattern to CAZ (51%) and CRX (46%) except that *Shigella flexneri* also showed the same percentage of resistance as *Klebsiella quasipneumoniae* (46%). *Citrobacter werkmanii* was the most resistant to gentamicin (83%) and second most resistant to ciprofloxacin. *Escherichia coli* and *Shigella flexneri* occupied the second and the least most resistant to gentamicin. All the species of *Citrobacter freundii* were found to be resistant to CXM while approximately 85% of *Shigella flexneri* isolates followed closely in terms of resistance. The least resistant to these antibiotics was found to be *Klebsiella quasipneumoniae* (40%). *Citrobacter freundii* was also the most resistant to ofloxacin (83%) followed by *Shigella flexneri* and *Salmonella enterica* (77%). The susceptibility of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* were 79%, 75% and 39% to augmentin to represent the highest, higher and lowest resistant rate to the antibiotic. *Serratia ficaria* showed approximately 88% and 75% to both nitrofuratoin and ciprofloxacin. The least resistant isolates to nitrofuratoin and ciprofloxacin were *Klebsiella quasipneumoniae* and *Klebsiella pneumoniae* respectively (Table 3). Some of the ESBL isolates harbor more than one allele with the co-occurrence of *TEM* and *SHV* occupying 23.1%, *CTX-M* and *SHV* (15.4%) and only 3.85% of the isolates harbouring *CTX-M* and *TEM* genes (Table 4).

Of the 26 Gram negative isolates screened for ESBL genes, 12 (46.2%), 11 (42.3%) and 3 (11.5%) harboured one, two and three of the ESBL genes respectively (Table 5). The *SHV* gene was the most predominant in all the isolates with a percentage of 69.2% followed by *TEM* (50%) and *CTX-M* (46.2%) (Table 5). Out of the 36 isolates confirmed by the phenotypic technique to be ESBL producers, 11(30.5%) were found with no distinct amplification in a primer specific polymerase chain reaction to represent 69.5% percentage level of congruence between the two techniques (Table 6). The susceptibility of all the ESBL producing variants (*CTX-M, TEM* and *SHV*) increased in the presence of an ESBL inhibitor to affirm their ESBL producing attribute (Table 7). The resistance pattern of all the variants however, were found to be statistically indifferent as shown in their zones of inhibition (P>0.05) (Table 7).

Average percentages of non susceptible organism to ceftazidime and cefepime were found to be (6.4% and 6.8%) and (6.8% and 7.2%) for *CTX-M* and *TEM* respectively while for *SHV* variant, cefepime was found to be the most efficacious antibiotic followed by ceftazidime (Table 8). All variants however were found to be resistant to ampicillin and gentamicin with an elevated number of all the variants found to be resistant to ofloxacin (>70%) and ciprofloxacin (>68%) (Table 8). The MIC of ampicillin for all the ESBL variants was above 128μg/ml to depict the non effectiveness of this drug against the ESBL producing variants. This observation is the same for gentamicin as it reflects in their MIC values to this antibiotic (Table 9). However, a very high proportion of the ESBL isolates in ceftazidime had their MIC below 0.5 (81.9%, 63.4% and 69%) for *CTX-M*, *SHV* and *TEM* respectively to make *CTX-M* the most sensitive to this antibiotic. On the other hand, the *TEM* variant was the most sensitive to cefepime at < 0.5μg/ml (Table 9).

Table 1: ESBL specific primers

ESBL genes	Length	Sequence (5' – 3')
CTX-M F	27bp	5'ATGGTTAAAAAATCACTGGGYCAGTTC 3'
CTX-M R	30bp	5'TCACAAACCGTYGGTGACGATTTTAGCCGC 3'
SHV F	21bp	5'ACCTTTAAAGTAGTGCTCTGC 3'
SHV R	21bp	5'CACCATCCACTGCAGCAGCTG 3'
TEM F	22bp	5'ACAGCGGTAAGATCCTTGAGAG 3'
TEM R	21bp	5'GAAGCTAGAGTAAGTAGTTCG 3'

Table 2: Identification of bacteria from external surfaces and gut of cockroaches

Species	Cockroach external surfaces n (%)		
Cockroach gut flora n (%)	Total N (%)		
Staphylococcus aureus	8(3.8)	2(1.3)	10(pace 5.1)
Bacillus subtilis	14(6.7)	6(3.9)	20(10.6)
Escherichia coli	43(20.8)	24(15.5)	67(18.5)
Staphylococcus epidermidis	8(3.8)	2(1.3)	10(4.8)
Klebsiella quasipneumoniae	21(10.1)	14(9.0)	35(9.6)
Klebsiella rhinoscleromatis	27(12.9)	19(12.3)	46(12.7)
Proteus vulgaris	3(1.4)	11(7.1)	14(3.9)
Proteus mirabilis	7(3.4)	4(2.6)	11(3.0)
Salmonella typhimurium	10(4.8)	8(5.2)	18(4.9)
Salmonella enterica sub enterica	9(4.3)	4(2.6)	13(3.6)
Klebsiella pneumoniae	7(3.4)	9(5.8)	16(4.4)
Pseudomonas aeruginosa	12(5.8)	8(5.2)	20(5.5)
Serratia odorifera	4(1.9)	8(5.2)	12(3.3)
Serratia marscencens	3(1.4)	7(4.5)	10(2.8)
Serratia ficaria	5(2.4)	3(1.9)	8(2.2)
Shigella flexneri	4(1.9)	9(5.8)	13(3.6)
Klebsiella oxytoca	7(3.4)	5(3.2)	12(3.3)
Enterococcus faecalis	9(4.3)	7(4.5)	16(4.4)
Citrobacter freundii	4(1.9)	2(1.3)	6(1.7)
Citrobacter werkmanii	3(1.4)	3(1.9)	6(1.7)
Total	208(57)	155(43)	363(100)

Key: n- number of bacteria, %- percentage, N- total number

Table 3: Resistance profile of Bacteria isolated from *Periplaneta americana* (cockroaches)

Bacterial isolates	Resistance profile of Bacteria n (%)							
	CAZ	CRX	GEN	CXM	OFL	AUG	NIT	CPR
Staphylococcus aureus	7(70)	8(80)	6(60)	8(80)	6(60)	8(80)	7(70)	6(60)
Bacillus subtilis	12(60)	14(70)	9(45)	16(80)	8(40)	15(75)	10(50)	9(45)
Escherichia coli	56(84)	48(72)	51(76)	46(69)	47(70)	53(79)	44(66)	41(61)
Citrobacter werkmani	04(67)	03(50)	05(83)	04(67)	03(50)	04(67)	03(50)	04(67)
Staphylococcus epidermidis	08(67)	06(50)	07(58)	06(50)	05(42)	06(50)	06(50)	05(42)
Citrobacter freundii	04(67)	03(50)	03(50)	06(100)	05(83)	04(67)	03(50)	04(67)
Klebsiella quasipneumoniae	18(51)	16(46)	15(43)	14(40)	13(37)	14(40)	14(40)	17(49)
Klebsiella rhinoscleromatis	29(63)	23(50)	21(46)	20(43)	19(41)	21(46)	23(50)	20(43)
Proteus vulgaris	10(71)	08(57)	10(71)	08(57)	08(57)	06(43)	07(50)	09(64)
Proteus mirabilis	08(73)	08(73)	08(73)	06(55)	08(73)	07(64)	06(55)	07(64)
Enterococcus faecalis	10(63)	11(69)	10(63)	08(50)	07(44)	08(50)	10(63)	08(50)
Salmonella typhimurium	10(56)	10(56)	09(50)	10(56)	08(45)	07(39)	08(45)	08(45)
Klebsiella oxytoca	09(75)	06(50)	07(58)	06(50)	08(67)	08(67)	06(50)	07(58)
Salmonella enterica	11(85)	09(69)	07(54)	10(77)	10(77)	06(46)	08(62)	08(62)
*Klebsiella pneumonia*e	14(88)	12(75)	10(63)	10(63)	10(63)	11(69)	08(50)	06(38)
Serratia odorifera	08(67)	08(67)	06(50)	08(67)	08(67)	06(50)	09(75)	06(50)
Serratia marscencens	08(80)	06(60)	07(70)	08(80)	06(60)	07(70)	08(80)	05(50)
Serratia ficaria	06(75)	04(50)	06(75)	06(75)	06(75)	05(50)	07(88)	06(75)
Shigella flexneri	10(77)	06(46)	06(46)	11(85)	10(77)	08(62)	06(46)	06(46)

Key: CAZ- ceftazidime, CRX- ceftriaxone, GEN- gentamicin, CXM- cefotaxime, OFL- ofloxacin, AUG- augmentin, NIT- nitrofuratoin, CPR- ciprofloxacin, n- number of resistant isolate to a particular antibiotic, %- percentage of resistance to a particular antibiotic

Table 4: Co-occurence of ESBL genes in Gram negative isolates

ESBL genes	number	(%)	Isolatee
CTX-M and *TEM*	1	3.85	EC2
CTX-M and *SHV*	4	15.4	EC8,EC9,SM1,SM8,
TEM and *SHV*	6	23.1	EC 12-14,PV2,PV6,KL11
CTX-M, TEM and *SHV*	3	11.5	EC3,KL1,KL2

Key: EC- *Escherichia coli,* SM- *Serratia marscencens*, *Proteus mirabilis*,KL1- *Klebsiella quasipneumoniae*, KL2- *Klebsiella rhinoscleromatis,* KL11- *Klebsiella rhinoscleromatis,*

Table 5: Molecular detection of ESBL genes in Gram negative isolates

Isolate Laboratory code	ESBL types			
	CTX-M	*SHV*	*TEM*	REMARKS
EC1	-	-	+	GC
EC2	+	-	+	GA
EC3	+	+	+	GA
EC12	-	+	+	GA
EC13	-	+	+	GA
EC14	-	+	+	GA
KL1	+	+	+	GA
KL2	+	+	+	GA
PV6	-	+	+	GA
KL11	-	+	+	GA
SM7	-	-	+	GC
PV2	-	+	+	GA
PV3	-	-	+	GC
EC4	+	-	-	GC
EC5	+	-	-	GC
EC8	+	+	-	GA
EC9	+	+	-	GA
KL9	+	-	-	GC
SM1	+	+	-	GA
SM5	+	-	-	GC
SM8	+	+	-	GA
EC6	-	+	-	GC
EC10	-	+	-	GC
KL6	-	+	-	GC
KL10	-	+	-	GC
SM3	-	+	-	GC
N	12	18	13	

Key: GC- one gene, GA- two or three genes, EC- *Escherichia coli*, KL1- *Klebsiella quasipneumoniae*, KL2- *Klebsiella rhinoscleromatis,* PV- *Proteus vulgaris*, SM- *Serratia marscencens,* KL9- *Klebsiella rhinoscleromatis,* KL6- *Klebsiella quasipneumoniae,* KL10- *Klebsiella rhinoscleromatis*

Table 6: Characterization of ESBL producing organisms by Molecular and Phenotypic techniques

Isolates	Phenotypic Characterization	Molecular characterization	Level of congruence
EC1	+	+	100%
EC2	+	+	100%
EC3	+	+	100%
EC4	+	+	100%
EC5	+	+	100%
EC6	+	+	100%
EC7	+	-	50%
EC8	+	+	100%
EC9	+	+	100%
EC10	+	+	100%
EC11	+	-	50%
EC12	+	+	100%
EC13	+	+	100%
EC14	+	+	100%
EC15	+	-	50%
KL1	+	+	100%
KL2	+	+	100%
KL3	+	-	50%
KL4	+	-	50%
KL5	+	-	50%
KL6	+	-	50%
KL7	+	+	100%
KL8	+	+	100%
KL9	+	+	100%
KL10	+	+	100%
KL11	+	+	100%
SM1	+	-	50%
SM2	+	-	50%
SM3	+	+	100%
SM4	+	-	50%
SM5	+	+	100%
SM6	+	+	50%
SM7	+	-	100%
SM8	+	+	100%
PV1	+	-	50%
PV2	+	+	100%
PV3	+	+	100%
PV4	+	-	50%
PV5	+	-	50%
PV6	+	+	100%
Total	40	26	

Table 7: Mean zone of inhibition of different variants of ESBL producing organisms

	CAZ	CAZCA	CXM	CXMCA	CRX	CRXCA(mm)	ESBL TYPE
Mean	14.6	30.2	16.8	24.3	15.8	18.4	
SD	1.41	1.25	2.21	1.99	1.64	0.99	*CTX-M*
Range	11-16	17-33	14-20	22-31	1-20	6-28	
Mean	15.6	17.9	17.3	19.9	15.5	21.3	
SD	2.28	1.91	3.31	2.30	1.81	1.12	*TEM*
Range	11-18	19-23	13-21	19-28	12-20	18-31	
Mean	14.9	21.3	16.7	29.1	15.4	18.4	
SD	2.09	1.34	2.43	1.81	2.06	1.81	*SHV*
Range	11-22	18-33	10-21	15-36	1-27	7-36	

Key: CAZ- ceftazidime, CAZCA- ceftazidime + clavulanic acid, CXM- cefotaxime, CXMCA- cefotaxime + clavulanic acid, CRX- ceftriaxone, CRXCA- ceftriaxone + clavulanic acid, mm- millimeter

Table 8: MIC_{90}, MIC_{50} values and percentage resistance of ESBL producing organisms

Antibiotics assayed	*CTX-M* (n=12)			*SHV* (n=18)			*TEM* (n=13)		
	MIC50	MIC90	%NS	MIC50	MIC90	%NS	MIC50	MIC90	%NS
Ampicillin	>128	>128	100	>128	>128	100	>128	>128	100
Ceftazidime	<2	4	6.4	<4	16	10	4	8	6.8
Ceftriaxone	4	4	8	32	32	11	8	16	9
Gentamicin	>32	>32	100	>64	>64	100	>32	>32	100
Cefotaxime	32	64	40	64	64	42	32	64	40
Ofloxacin	<32	64	70.8	64	64	73	>64	>128	80.6
Augmentin	>16	>16	39	>32	>32	41	>32	>32	43
Nitrofurantoin	16	64	32	16	32	39	32	64	38
Ciprofloxacin	>4	>4	68	>16	>16	68	>8	>8	72
Cefepime	4	4	6.8	16	16	7.2	16	16	7.2

Table 9: Relationship between the distribution of MICvalues (µg/ml) and ESBL variants

Antibiotics	ESBL	<0.5	1	2	4	8	16	32	64	128	P_{value}
Ampicillin	*CTX-M*	0	0	0	0	0	0	0	0	100	
	SHV	0	0	0	0	0	0	0	0	100	0>0.05
	TEM	0	0	0	0	0	0	0	0	100	
Ceftazidime	*CTX-M*	81.9	3.1	0	11.3	0	0	4.7	0	0	
	SHV	63.4	0	14.6	0	2	0	3	9	8	0>0.05
	TEM	69	0	13	0	12	0	0	0	6	
Ceftriaxone	*CTX-M*	71	0	13	0	11	0	0	5	0	
	SHV	63	20	0	0	6	0	3	0	8	0>0.05
	TEM	58	16	0	14	0	2	0	10	0	
Gentamicin	*CTX-M*	0	0	0	0	0	0	0	0	100	
	SHV	0	0	0	0	0	0	0	0	100	0>0.05
	TEM	0	0	0	0	0	0	0	0	100	
Cefotaxime	*CTX-M*	25	0	12.5	0	12.5	0	0	50	0	
	SHV	64	0	16	0	12	0	0	8	0	0>0.05
	TEM	83	7	0	10	0	0	0	0	0	
Ofloxacin	*CTX-M*	67	13	0	20	0	0	0	0	0	
	SHV	29	11	0	40	0	20	0	0	0	0>0.05
	TEM	81	0	9	0	10	0	0	0	0	
Augmentin	*CTX-M*	24	0	6	1	0	0	0	0	0	
	SHV	69	0	16	0	0	0	0	0	0	0>0.05
	TEM	64	0	18	0	0	0	0	0	0	

Table 9: continued: Relationship between ESBL variants and distribution of their MIC values (µg/ml)

Antibiotics	ESBL	<0.5	1	2	4	8	16	32	64	128	P_{value}
Nitrofurantoin	*CTX-M*	63	0	21	0	15	0	0	0	0	
	SHV	71	0	17	0	11	0	9	2	0	0>0.05
	TEM	84	0	12	0	2	0	1	1	0	
Ciprofloxacin	*CTX-M*	91	0	3	0	3	0	3	0	0	
	SHV	84	0	16	0	0	0	0	0	0	0>0.05
	TEM	73	0	21	0	0	6	0	0	0	
Cefepime	*CTX-M*	63	0	12	0	8	0	7	0	0	
	SHV	74	0	20	0	6	0	0	0	0	0>0.05
	TEM	81	3	0	2	0	4	0	0	6	

DISCUSSION

The role of cockroaches as vector in the transmission of infectious agent has been well documented (Pai *et al.*, 2005; Al-marjani *et al.*, 2008). In this study, the bacterial contaminants of cockroaches were represented by bacterial genera belonging to *Staphylococcus* spp, *Bacillus* spp, *Escherichia coli, Klebsiella* spp, *Proteus* spp, *Salmonella* spp, *Pseudomonas* spp, *Serratia* spp, *Shigella* spp, *Enterococcus* spp and *Citrobacter* spp. This observation may not be unconnected to the fact that this important insect pest has close association with different wastes including garbages, sewage, sanitary waste among others, thus making them important carriers and transmitter of infectious agents including the multi drug resistant bacterial strains (Naher *et al.*, 2018). Of the bacterial contaminants of cockroaches, the most frequently identified were Gram negative bacilli, explicitly in the family *Enterobacteriaceae* (Chaichanawongsaroj *et al.*, 2004; Naher *et al.*, 2018). *Klebsiella* spp which were the mostly encountered bacterial isolates in cockroaches has been quoted in a related study as a major contaminant of insects (Kassiri *et al.*, 2014). The second most predominant in this study is *Escherichia coli* which corroborate the findings of other studies (Al-marjani *et al.*, 2008; Naher *et al.*, 2018). The findings of *Escherichia coli* from cockroaches is an indication that these insects have been in contact with human faeces contaminated materials and hence could be a health challenge to humans (Kassiri *et al.*, 2014) due to the possibility of these organisms causing infections including diarrhea, uremic syndrome, thrombocytopaenic purpura among others.

The isolation of higher rate (57%) of bacterial isolates from the external surfaces compared to gut (43%) of cockroach goes in agreement with other studies (Adeleke *et al.*, 2012; Naher *et al.*, 2018). In contrary to this observation, Tachbele *et al.* (2006) and Ejimadu *et al.* (2015) reported higher isolation of bacteria from gut of cockroaches rather than the external surfaces. The relative high external surface carriage rate may be related to filthy habits of cockroach which involves crawling and movement on different wastes thereby carrying microorganisms on their surface. The elevated trend of multi drug resistance to the different antibiotics tested observed in this study is a serious challenge that has negative consequences on infection control and hospital management of patients (Chandran *et al.*, 2008). This resistance was found to be higher in *Klebsiella* spp. followed by *Escherichia coli* and *Shigella flexneri* respectively. The finding is analogous to that documented by Reinert *et al.* (2007), where they documented high level of resistance to some of the most commonly prescribed antibiotics including extended spectrum cephalosporins, carbapenems and fluoroquinolones. The European Antibiotic Resistance Surveillance System report (2006) further buttress the continuous increase in resistance to third generation cephalosporins by both *Escherichia coli* and *Klebsiella* spp. The resistance of these organisms to third generation cephalosporins may be due to the production of extended spectrum beta lactamases which are known for hydrolyzing these antibiotics (Rawat and Nair, 2010). Some of the non ESBL producing isolates also showed some form of resistance to the third generation antibiotics and this may be attributed in part to lack of permeation of porins and AmpC beta lactamase production among other factors.

Of all the bacterial isolates recovered from cockroaches, forty (40) and twenty-six (26) were confirmed by both phenotypic and molecular methods respectively as ESBL producers. This observation is an indication that the phenotypic method may give a false positive reaction sometimes owing particularly to several factors including ineffective and expired disc coupled with non potency that could result from improper handling of this material (Williems *et al.*, 2013). However, the molecular method which involves the use of polymerase chain reaction in the detection of genes involved in the production of ESBL has been considered as the best method for such identification. This technique has also been reported to detect the existence of poorly or non-expressed genes and even those that are not detectable phenotypically (Diekema *et al.*, 2004).

Three types of ESBL were detected in this study, type *CTX-M*, *SHV* and *TEM*, with a predominance of *SHV* gene (69.2%). The predominance of *SHV* type of ESBL corroborate what was earlier reported by Lincopan *et al.* (2006) who stated that most of the resistance observed against the beta lactam and extended spectrum cephalosporins are as a result of the increased production of *SHV* type of ESBL for the past 20 years. This observation is contrary to what has been reported in Spain by Sabate *et al.* (2002), where they observed the predominance of *CTX-M* ESBL type. Rodriguez-Bano *et al.* (2004) also documented similar findings to that of Sabate *et al.* (2002). In Italy however, several researches also documented the *CTX-M* type of ESBL from various sources (Pagani *et al.*, 2003; Elderstein *et al.*, 2003; Munday *et al.*, 2004).

The *TEM* type of ESBL which was the second most predominant in this study has been well documented and are reported as still evolving in Europe and specific endemic clones have also been found. For example, *Salmonella* isolate with *TEM-52* in Spain. In the Middle East, all the strains of organisms screened for ESBL types were all found harbouring *TEM* type (Al-Agamy *et al.*, 2006). In Latin America, approximately 50% of the bacterial isolates harbour ESBL genes and the rate varies from 8% to 18% for *Enterobacteriaceae* (Winokur *et al.*, 2001). Also, *SHV-5* and *SHV-12* ESBLs have been found in South America as in other countries and the *TEM-10* enzyme observed in Argentina (Villegas *et al.*, 2008). In Nigeria at Ile-Ife, the prevalence of 32% was recorded for *blaTEM*, while *blaSHV* and blaCTX-*M*, were 32% and 36% respectively (Olowe *et al.*, 2012). The difference in the epidemiology of these ESBL types vary according to location as seen in their distribution patterns globally.

In conclusion, it can be ascertained that cockroaches are involved in the ubiquitous transmission of different types of ESBL alleles with the *SHV* type being the most predominance. All the isolates however showed reduced susceptibility to both gentamicin and quinolones antibiotics.

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