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Antibiotic Resistance Profile and Phenotypic Detection of Beta-Lactamase-producers among Gram-negative Bacteria Isolated from the Gut of Household Cockroaches in and around University of Ibadan

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Cockroaches are carriers of numerous microorganisms. However, there is paucity of information on their role as potential reservoir for beta-lactamase producers.

Objectives: This research determined the antibiotics susceptibility profile of Beta-lactamase producing Gram-negative bacteria isolated from the gut of household cockroaches in selected locations in and around University of Ibadan, Oyo state.

Materials and Methods: Thirty Cockroaches from different locations in and around University of Ibadan were collected between June 2015 and March, 2016, and their intestinal homogenates cultured on different selective media for the isolation of bacteria. The isolates were identified using a combination of biochemical tests and 16S rRNA sequencing. Antibiotic susceptibility testing was done using the disc-diffusion technique and phenotypic detection of extended-spectrum beta-lactamase (ESBL), AmpC-beta-lactamase (AmpC) and Metallo-beta-lactamase (MBL) production was done using double-disc synergy, cefoxitin/cloxacillin and imipenem/EDTA double-disc methods respectively.

Results: A total number of 58 bacteria belonging to nine genera; Pseudomonas, Proteus, Klebsiella, Salmonella, Enterobacter, Escherichia, Serratia, Shigella and Raoultella were isolated. Higher percentage of the isolates exhibited resistance to erythromycin (90%), azithromycin (87.5%), amoxicillin (84.5%), ampicillin (74.1%), amoxicillin-clavulanic acid (74.1%), cefoxitin (67.2%) and chloramphenicol (54%), while lower percentage showed resistance to aztreonam (25%), ertapenem (13%), cefotaxime (8.6%), ceftazidime (10.3%), cefepime (5.2%), ciprofloxacin (3.5%), gentamicin (5.2%) and imipenem (0%). MDR phenotype was recorded among 82% of the isolates, 17.2% were positive for ESBL, 12% for AmpC and 13.8% for MBL production.

Conclusion: This study identified household cockroaches *Periplaneta americana*, as a potential reservoir for MDR and beta-lactamase-producing isolates.

Keywords: Susceptibility testing, ESBL, AmpC, MBL, Beta-Lactamase, MDR, Household cockroaches

INTRODUCTION

Cockroaches are insects of the order Blattodea some of which are associated with human environment (Beccaloni, 2014). One of the two species of cockroaches that has been reported by different authors to be widely distributed in Nigeria is the American cockroaches namely *Periplaneta americana* (Ajero *et al*., 2011; Etim *et al*., 2012; Akinjogunla *et al*., 2012; Tilahun *et al*., 2012).

The incidence of Cockroach infestation in restaurants, hospitals, warehouses, offices and within households, particularly the kitchen, have been reported to be very high in Nigeria (Omudu and Akosu, 2013; Braimah *et al*., 2015). The insects being nocturnal in nature are found in dark places within the households during the day hiding in opened and cracked sections of the wall, furniture, inside wardrobes and cupboards (Omudu and Akosu, 2013; Billah *et al*., 2015; Braimah *et al*., 2015). Sometimes they enter into cooking pots and other kitchen utensils if left opened. They feed on leftover food, cooked and uncooked food materials contaminating them with microorganisms on their body and in their faecal droppings especially those ready to eat food that do not require further processing before eating them (Xue *et al*., 2009). They are also found in other parts of the house including bedrooms (Etim *et al*., 2013).

Cockroaches are also found in garbage and sewage tanks, particularly in homes with pit latrine (Craczyk *et al*., 2005; Pai *et al*., 2005) making them a potential vector for pathogenic and non-pathogenic microorganisms from human origin (Graczyk *et al*., 2005; Pai *et al*., 2005; Vazirianzadeh *et al*. 2014). Studies have shown that most of the disease-causing bacteria of human origin can survive within the body system of cockroaches (Imamura *et al*., 2003), hence their presence in sewage and garbage elucidate their role as potential carriers of these human disease-causing agents (Vazirianzadeh *et al*., 2014).

Several authors have incriminated cockroaches as reservoir and transmitters of disease-causing microorganisms like *Enterobacter* spp., *Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Shigella* spp., *Campylobacter* spp. and *Salmonella* spp., especially those in the hospital environment (Craczyk *et al*., 2005; Tatfeng *et al*., 2005; Ukay *et al*., 2009; Tilahun *et al*., 2012; Vazirianzadeh *et al*., 2014). In addition, cockroaches have also been reported to carry microbial agents of leprosy, urinary tract infection (*Enterococcus* spp.), cholecystitis (*Helicobacter hepaticus*), plague (*Yersinia pestis*) and bacteremia, septic arthritis and peritonitis (*Oligella urethralis*) (Pat, 2006; Falsafi and Mahboubi, 2013).

Antibiotic resistance and its spread among bacterial isolates is a global problem and has been reported among bacterial isolates in both clinical and community settings (WHO, 2014). Cockroaches have been reported to harbour pathogenic and opportunistic pathogens that exhibited high level resistance to antibiotics, especially those used in life-threatening disease cases such as the cephalosporins and carbapenems (Fathpour *et al*., 2003; Bouamama *et al.*, 2010; Wannigama *et al*., 2014). Bacterial isolates exhibiting multidrug resistance phenotype have also been reported in cockroaches (Devi and Murray, 1991; Tetteh-Quarcoo *et al.*, 2013).

Resistance to beta-lactam antibiotics are known to be mediated mostly by beta-lactamase enzymes. The initial variants of these enzymes were active only on the penicillin class but the discovery of the third generation cephalosporin class help to overcome their destructive action (Paterson and Bonomo, 2005). The advent of extended-spectrum beta-lactamase, and metallo beta-lactamase enzymes among others which are known in addition to the penicillin class, to hydrolyze the third generation cephalosporins (Ceftazidime, cefotaxime, ceftriaxone) and the carbapenems (imipenem, ertapenem) respectively, created a serious problem for clinicians in the management of life-threatening infections caused by such bacteria habouring the enzymes (Paterson and Bonomo, 2005).

Currently, there is paucity of information on the various resistant determinants harboured by bacteria isolated from household cockroaches. This study therefore, aimed at using phenotypic techniques to determine multidrug resistance phenotype and the presence of common beta-lactamase enzymes among bacterial isolates from the gut of house-hold cockroaches in selected locations in and around University of Ibadan.

MATERIALS AND METHODS

Study Area

The study was carried out in University of Ibadan metropolis, Oyo state. The areas of choice were kitchen and toilets in residential apartments in and around the University of Ibadan.

Specimen Collection and Preparation for bacteria isolation

A total of 30 cockroaches, identified to be *Periplaneta americana*, through online file (Beccaloni, 2014), were collected from residential apartments using sticky rods and direct collection with sterile hand-gloves (Paul *et al*., 1992). They were introduced into separate sterile wide-mouth bottles aseptically and transported to the laboratory of the Department of Pharmaceutical Microbiology, University of Ibadan for freezing at 0^{o}C for 10–15min to anaesthetize them. Intestinal gut of each cockroach was prepared into samples using the method highlighted by Tetteh-Quarcoo *et al.* (2013)

Isolation and Identification of Gram-negative bacteria

All cockroach-intestinal samples were initially cultured on MacConkey agar by streaking so as to encourage the growth and isolation of Gram-negative bacteria. Bacteria colonies on the MacConkey agar were then transferred by streaking on some selective and differential media such as blood agar, cetrimide agar, Salmonella-Shigella Agar, Eosin Methylene Blue Agar for morphological and cultural identification. All cultured media were incubated at 37^oC for 24 hours aerobically in an incubator. The various distinct colonies were further identified using standard biochemical test and some difficult ones by 16SrRNA sequencing identification technique.

Bacteria DNA extraction for 16S rRNA amplification

Bacteria DNA was extracted using the boiling method as previously described by Mendonca *et al*. (2007) and PCR amplification of the 16S rRNA was done using a set of primer pair (27F-GAGAGTTTGATCCTGGCTCAG and 1492R-GGTTACCTTGTTACGACTT as described by Mateos *et al*. (2006). The PCR procedure for the amplification involved an initial denaturation step at 94°C for 2min, follow by 35 cycles of DNA denaturation at 94°C for 30s, primer annealing at 53°C for 30s, and primer extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 10min was added. With aid of a micropipette, 5µL aliquots of PCR products were analyzed by gel electrophoresis with 1% agarose gel (Bio-Rad). Gels were stained with ethidium bromide and visualized under a UV trans-illuminator to visualize the amplified 16S rRNA bands.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the isolates against selected antibiotics was carried out by disc-diffusion method on Mueller Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2011). Bacteria suspensions prepared from 24 hours culture in agar plates were diluted to concentration equivalent to 1.5×10^8 CFU/mL (0.5 McFarland standards) and with the aid of sterile cotton swabs, the inocula were spread on the set Mueller Hinton agar to give monolayer of the bacteria cells all over the agar surface. Using sterile forcep, antibiotic discs were placed at points equidistant from one another on the agar plates and then incubated after pre-diffusion period of 60minutes on the bench 37^o for 24 hours. The antibiotic discs used were purchased from Oxoid Ltd, UK and the discs were impregnated with the following antibiotics: ampicillin (10 µg), amoxicillin (30 µg), amoxicillin-clavulanic acid (20/10 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), cefepime (30 µg), erythromycin (15 µg), azithromycin (15 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), trimethoprim (5 µg), aztreonam (30 µg), imipenem (10 µg) and ertapenem (10 µg). Bacterial isolates that showed resistance to three or more classes of antibiotics were classified as multidrug resistant (MDR) strains as previously described by Magiorakos *et al*. (2012).

Phenotypic detection of production of selected Beta-Lactamases

Detection of Extended-Spectrum Beta-Lactamase (ESBL) Production

ESBL production was determined among the bacterial isolates that showed a zone diameter of ≤22 mm to ceftazidime (30 µg) and ≤27 mm cefotaxime (30 µg) in the susceptibility test. The double-disc synergy test (DDST) method was used to detect the ESBL production among the different bacterial isolates by diluting the suspension to 0.5 McFarland standard and then spreading over the surface of Mueller Hinton agar plates using sterile swab sticks. With the aid of a sterile Forceps, separate discs of ceftazidime (30 µg) and cefotaxime (30 µg) were placed 20 mm centre-to-centre around amoxicillin-clavulanic acid (20/10 µg) disc on the agar plates. The plates were then left for an hour on the bench and then incubated at 37^oC for 24 hours. ESBL production was inferred from those having the zone of inhibition around any of the two discs expanded ≥5 mm by the presence of clavulanic acid (CLSI, 2011).

Detection of AmpC Beta-Lactamases production

All the isolates that showed a zone diameter of ≤22 mm to ceftazidime, ≤27 mm to cefotaxime and ≤14 mm to cefoxitin but susceptible to the carbapenems were subjected to AmpC test by cefoxitin/cloxacillin double-disc method as described by Tan *et al*. (2009). The bacteria suspensions prepared from 24 hours culture in agar media plates were diluted to 0.5 McFarland standard and the inocula spread over Mueller Hinton agar plates as earlier described. Two cefoxitin discs, one inoculated with 200 µg of cloxacillin and the other without, were placed on the inoculated agar surface using sterile forcep. The plates were then left for an hour on the bench and then incubated at 37^oC for 24 hours. AmpC production was inferred from those having the zone of inhibition around the cloxacillin inoculated cefoxitin discs expanded ≥4 mm by the presence of the cloxacillin (Tan *et al*., 2009)

Detection of Metallo Beta-Lactamases (MBL) production

All bacterial isolates that showed resistance to ceftazidime, cefotaxime and the carbapenems were screened for the Metallo Beta-lactamase production using the methods described by Franklin *et al*. (2006). Briefly, bacteria suspensions prepared from 24 hours culture in agar media plates were diluted to 0.5 McFarland standard and the inocula spread over Mueller Hinton agar plates as earlier described. Two imipenem discs, one inoculated with 10 µL of 750 µg of EDTA solution and the other without, were placed on the inoculated agar surface using sterile forcep. The plates were then left for an hour on the bench and then incubated at 37^{o}C for 24 hours. MBL production was inferred from those having the zone of inhibition around the EDTA inoculated imipenem discs expanded >4 mm by the presence of the EDTA.

RESULTS

From the 30 cockroaches collected, a total number of 58 Gram-negative bacteria were isolated and identified. They include *Pseudomonas* spp., *Proteus* spp., *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp., *Escherichia coli, Serratia marcescens* and *Shigella* spp. The results of the 16S rRNA sequencing for the identification of some of the bacteria that their biochemical identification was inconclusive revealed the presence of *Enterobacter cloacae*, *Proteus vulgaris*, *Salmonella enterica*, *Pseudomonas orizyhabitans* and *Raoultella ornithnolytica* (Figure 1). The antibiotic susceptibility profile revealed that higher percentage of the isolates exhibited resistance to erythromycin (90%), azithromycin (87.5%), amoxicillin (84.5%), ampicillin (74.1%), amoxicillin-clavulanic acid (74.1%), cefoxitin (67.2%) and chloramphenicol (54%), while lower percentage showed resistance to aztreonam (25%), ertapenem (13%), cefotaxime (8.6%), ceftazidime (10.3%), cefepime (5.2%), ciprofloxacin (3.5%), gentamicin (5.2%) and imipenem (0%) (Figure 2). Multidrug resistance phenotype was recorded in >70% of the bacterial isolates in the genera Klebsiella, Proteus, Enterobacter, Salmonella, Shigella, Raoultella and Pseudomonas as they showed resistance to three or more classes of antibiotics (Table 1). Production of beta-lactamase enzymes such as the ESBLs, MBLs and AmpC was detected in 17.2%, 13.8% and 12% of the bacterial isolates respectively. Four (6.9%) of the isolates were co-producing ESBL and MBL, 3 (5.2%) co-producing ESBL and AmpC as well as AmpC and MBL and one (1.7%) co-producing the three beta-lactamse enzymes (Table 2).

DISCUSSION

Cockroaches are no doubt a serious house-hold pest in this and other parts of the world. Their continuous infestation of kitchens, toilets especially pit latrines and sewage tanks, bedrooms and other vital sections of the house, coming in contact with food items, kitchen utensils and clothing is of public health concern. In this study, the only specie of cockroach collected is the American cockroach suggesting that it is the commonest specie in Ibadan. Majority (83.3%) of the insect were collected from the kitchen area possibly because these insects tend to have access to left-over and raw food substances within the kitchen area more than any other section of the house. Apart from this, the kitchens are highly humid and warm enough for roaches' propagation. This is in agreement with the work of Wannigama *et al.* (2014) who reported the highest (67%) collection of cockroaches in the kitchen area of houses in Varanasi, India. Also, several other authors including Vazirianzadeh *et al.* (2014) in Iran and Malik *et al*. (2013) in Pakistan also supported this finding. In this study the Gram-negative bacilli isolated included *Pseudomonas* spp., *Proteus* spp., *Klebsiella* spp., *Salmonella* spp., *Escherichia coli, Serratia marcescens*, *Shigella* spp., *Pseudomonas orizyhabitans, Klesiella pneumoniae, Salmonella enterica, Proteus vulgaris* and *Raoultella ornithnolytica*. The bacterium *Raoultella ornithnolytica*, a Gram-negative encapsulated non motile aerobic bacillus belonging to the *Enterobacteriaceae* family, is not usually common in humans. The bacterium is usually found in aquatic environment as well as in fish and insects (Morais *et al*., 2009) but has been reported to cause infection in humans (Yalcin *et al*., 2011; Chun *et al*., 2015). Majority of these bacteria have also been similarly reported in cockroaches by other authors (Agbodaze and Owusu, 1989; Paul *et al*. 1992; Tachbele *et al*., 2006; Vazirianzadeh *et al.*, 2014). Vazirianzadeh *et al.* (2014) in their study on bacterial isolates from cockroaches collected in kitchen areas from domestic homes in Ahvaz South West Iran reported the presence of *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Escherichia coli*, *Salmonella* spp., *Proteus* spp., coagulase-negative *staphylococci*, *Serratia marcescens*, *Staphylococcus aureus*, and *Bacillus* species. In Bangladesh, *Salmonella*, *Shigella*, *S. aureus*, *B. cereus*, and *E. coli* were isolated from cockroaches from different houses (Paul *et al*. 1992). Tachbele *et al*. (2006) isolated *Salmonella* spp., *Shigella flexneri*, *Escherichia coli* O157, *Staphylococcus aureus*, and *Bacillus cereus* from hospitals and four restaurants in Addis Ababa, Ethiopia. In Ghana, Agbodaze and Owusu (1989) isolated various food-borne pathogens from

cockroaches collected from kitchens in some houses. These sets of bacteria are also commonly isolated in humans and animals (Pai *et al*., 2004; Craczyk *et al*., 2005). However, in view of the association of cockroaches with garbage, sewage, clothing and other materials used by humans, it will not be wrong to say that most of these bacteria isolated from the cockroaches are gotten from humans or animals either directly or indirectly going by the work of Fotedar *et al*. (1993) and Imamura *et al*. (2003). Survival of pathogens in experimentally infected cockroaches was reported by Fotedar *et al*. (1993) and Imamura *et al*. (2003). This suggests that bacteria from human can survive successfully in the body of cockroaches.

The result of the antibiotic susceptibility study revealed high level resistance to commonly used antibiotics such as erythromycin, azithromycin, ampicillin, amoxicillin and amoxicillin-clavulanic acid. Other stronger antibiotics like imipenem, ertapenem, cefepime recorded high level activity against the isolates in this study. This result is comparable with that of some other authors who recorded some level of resistance to various classes of antibiotics (Vazirianzadeh *et al*., 2014; Iboh *et al*., 2014; Malik *et al*., 2013). *Raoultella ornithinolytica* has been reported to be resistant to ampicillin but remain susceptible to cefotaxime and imipenem (Walckenaer *et al*., 2004), however, in this study, the bacterium was found to be resistant to cefotaxime but remain susceptible to imipenem as reported.

Multidrug resistance (MDR) is a global problem and thus carriers of bacteria exhibiting MDR phenotype are potential public health threat. In this study the level of MDR among the Gram-negative bacteria is very high (>80%) and this is of public health concern. Similar reports on carriage of MDR isolates by cockroaches have been published in previous studies (Devi and Murray 1991; Pai *et al*., 2005; Tetteh-Quarcoo *et al.,* 2013). Tetteh-Quarcoo *et al.* (2013) reported multiple drug resistance among the bacteria isolated from cockroaches at a tertiary hospital in Ghana, this ranged from 13.8% (*Escherichia coli*) to 41.1% (*Klebsiella pneumoniae*). Over 4% of cockroaches collected from hospitals, houses, animal sheds, grocery stores, and restaurants in India harboured multiple drug resistant *Salmonella* (Devi and Murray 1991). Pai *et al*. (2005) also reported bacterial strains from household-cockroaches exhibiting MDR phenotype.

Dissemination of beta-lactamase resistant determinants among bacterial isolates, especially the pathogenic or opportunistic pathogenic ones is a serious health concern. This study detected ESBL, MBL and AmpC beta-lactamase enzymes in 17.2%, 13.8% and 12% of the isolates respectively. Similar findings were observed and reported by Czajka *et al*. (2003) and Dugal and Fernandes (2011).

CONCLUSION

The persistence of cockroach infestation, particularly *Periplaneta americana*, in different homes in Ibadan is worrisome and of public health concern. This is not because of their destructive ability on textile and stationeries alone but because they are carriers of numerous Gram-negative bacteria habouring various resistant determinants and could be potential pathogens or opportunistic pathogens to humans. Hence, there is need for serious control measures against these vector pests within different homes so as to prevent them from spreading disease-causing multidrug resistant bacteria which may be difficult to treat and thus poses health threat to human life.

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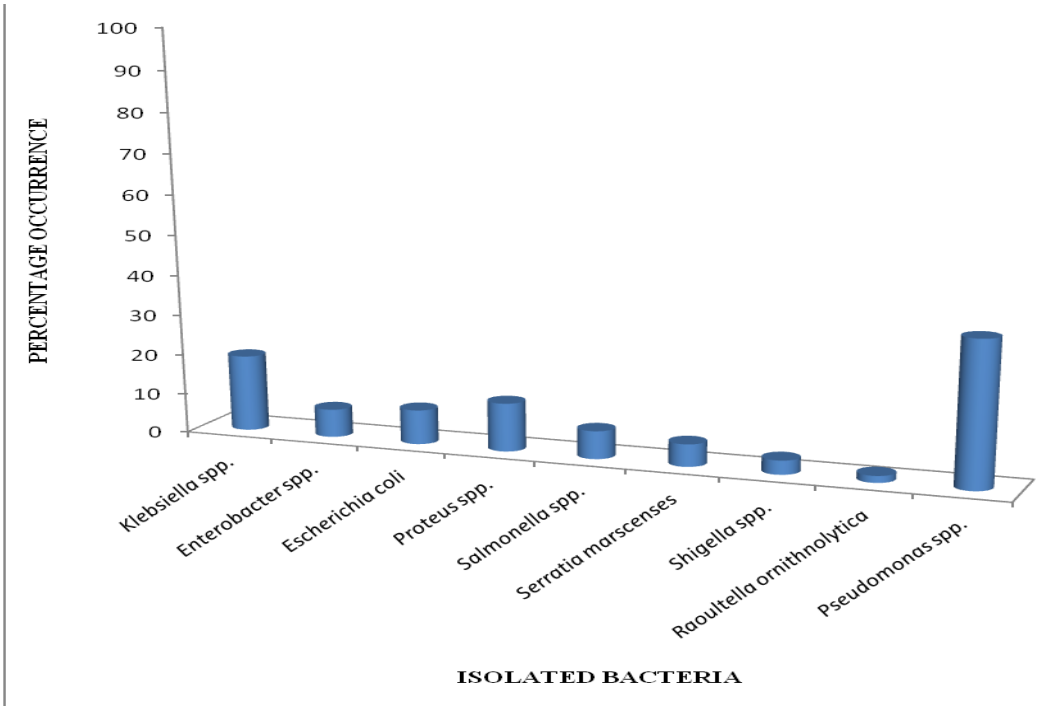

Figure 1: Percentage occurrence of Gram-negative bacteria genera from the gut of cockroaches

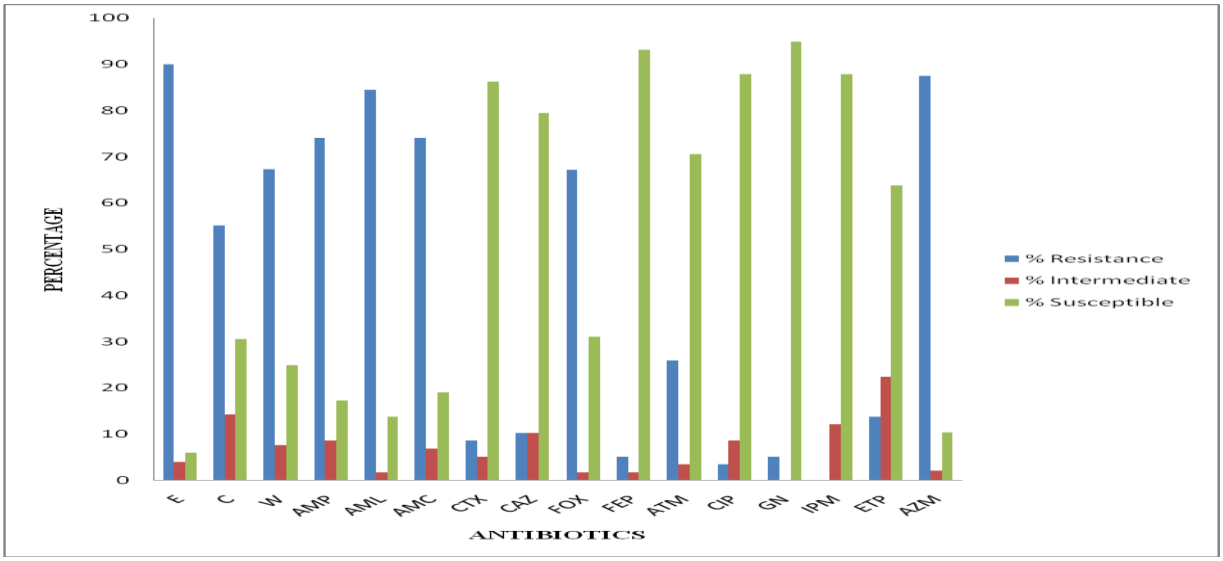

Figure 2: Percentage antibiotic susceptibility profile of bacteria isolated from cockroaches

KEYS: E= Erythromycin, W= Trimethroprim, C= Chloramphenicol, AMP= Ampicillin, AML= Amoxicillin, AMC= Amoxicillin-clavullanic CTX= Ceftriaxone, CAZ= Ceftazidime, FOX= Cefoxitin, FEP=Cefepime, ATM=Aztreonam, CIP=Ciprofloxacin, GN=Gentamicin, IPM= Imipenem, ETP= Ertapenem, AZM=Azithromycin.

Table 1: Percentage Resistance of each isolated bacterial genera to the tested antibiotics

Organism	Percentage (%) resistance																
	E	C	W	AMP	AML	AMC	CTX	CAZ	FOX	FEP	ATM	CIP	GN	IPM	ETP	AZI	%MDR
Klebsiella spp. (n=11)	89	50	75	82	100	64	27	9	55	0	18	0	0	0	18	67	73
Proteus spp. (n=7)	57	43	43	43	71	71	14	29	71	24	29	0	29	0	14	71	71
E. coli (n=5)	100	33	67	60	80	60	0	0	80	0	25	0	0	0	0	100	60
Enterobacter spp. (n=4)	75	67	75	50	50	50	0	0	75	0	25	0	0	0	50	75	75
Salmonella spp. (n=4)	100	33	67	25	75	75	0	50	50	0	25	0	0	0	0	75	75
Pseudomonas spp. (n=21)	95	79	75	90	100	90	0	14	76	4	33	10	4	0	ND	81	100
Serratia marcescens (n=3)	100	0	33	67	67	67	0	0	33	0	0	0	0	0	0	100	67
Shigella spp. (n=2)	100	50	50	100	100	50	0	0	50	0	50	0	0	0	50	100	100
Raoultella ornithinolytica (n=1)	100	0	100	100	100	100	100	0	0	0	0	0	0	0	0	100	100

KEYS: E= Erythromycin, W= Trimethroprim, C= Chloramphenicol, AMP= Ampicillin, AML= Amoxicillin, AMC= Amoxicillin-clavullanic CTX= Ceftriaxone, CAZ= Ceftazidime, FOX= Cefoxitin, FEP=Cefepime, ATM=Aztreonam, CIP=Ciprofloxacin, GN=Gentamicin, IPM= Imipenem, ETP= Ertapenem, AZM=Azithromycin, ND=Not Done.

Table 2: Phenotypic detection of beta-lactamase production among the bacterial isolates

S/N	Isolate ID	Isolate	ESBL	AmpC	MBL
1	U1A	Klebsiella sp.	+	ND	-
2	V2B	Klebsiella pneumoniae	-	ND	ND
3	JIAN	Proteus sp.	+	-	+
4	O2A	Shigella sp.	+	-	+
5	H2A	Proteus sp.	+	-	ND
6	G1	Klebsiella pneumoniae	-	+	+
7	M2C	Pseudomonas sp.	+	ND	+
8	PID	Pseudomonas sp.	+	+	+
9	U2N	Pseudomonas sp.	-	+	ND
10	W2A	Proteus sp.	+	+	ND
11	V1A	E. coli	+	ND	ND
12	B21	Pseudomonas sp.	-	-	ND
13	Y2AN	Proteus sp.	+	+	-
14	Z2A	Pseudomonas sp.	+	ND	ND
15	D21(B)	Enterobacter cloacae	ND	+	+
16	J2B	E. coli	ND	+	ND
17	AI	Pseudomonas sp.	ND	-	+
18	D1I	Pseudomonas sp.	ND	-	ND
19	E1I	Pseudomonas sp.	ND	-	ND
20	J2A	Proteus sp.	ND	ND	-
21	NIA	E. coli	ND	ND	-
22	L2BN	Proteus sp.	ND	ND	-
23	JIB	E. coli	ND	ND	-
24	P2C	Salmonella sp.	ND	ND	-
25	R2B	Enterobacter cloacae	ND	ND	+

KEYS: ND = Not done, + = beta-lactamase production, - = No beta-lactamase production

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