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Periplaneta americana: Antibiotic Susceptibility Profiles of Associated Biofilm-Forming Bacteria

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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: The high resistance attributed to biofilms can result in recurrence and persistence of infections with attendant consequences of increased morbidity and mortality rates, increased cost of treatment as well as length of hospital stay of the patient.

Objectives: This study aimed at examining the biofilm-forming capacity of bacterial isolates from the external body of cockroaches (*Periplaneta americana*) and their susceptibility to selected commonly used antibiotics.

Methods: Bacterial isolates associated with seventy (70) cockroaches were isolated, identified and characterized using morphology and conventional biochemical tests. The biofilm-forming capacity of the isolates was evaluated using the Congo red agar (CRA) method. The antibiotic susceptibility profiles of the CRA-positive isolates to selected commonly used antibiotics were evaluated using the Kirby-Bauer disc diffusion method.

Results: Of the one hundred and four (104) isolates, *Bacillus subtilis* was the predominant bacterial species (77.9%) while the least was *Salmonella typhi* (1.0%). However, 42% of the isolates showed tendency to form biofilms. The susceptibility study revealed that gentamicin was active against both Gram-positive and negative biofilm-formers.

Conclusion: This study concluded that cockroaches (*Periplaneta americana*) can habour some bacterial species capable of forming biofilms which may adversely affect public health.

Keywords: Biofilm; antibiotic; Antibiotic susceptibility; *Periplaneta americana*

INTRODUCTION

Recently, the existence and ubiquity of *Periplanata americana*, a common species of cockroach, has become one of the leading public health threat (Ojiezeh and Ogundipe, 2015). Aside their ability to habour pathogenic bacteria, they have also served as intermediate hosts for pathogenic helminthes, and to carry helminth eggs, viruses, protozoa and fungi affecting man and other vertebrate animals (Tatang et al., 2017). Their potential as a health hazard to man is borne out of their filthy habits, indiscriminate diet, feeding mechanisms and morphology as well as their free movement from place to place (Adenusi et al., 2018). Indeed, occasional contact of cockroaches with surfaces is sufficient to foster the spread of bacteria (Xue et al., 2009).

Cockroaches have been found to be naturally contaminated with about 40 different species of bacteria that are pathogenic to vertebrates including *E. coli*, *S. aureus*, *Ps. aeruginosa*, *Salmonella* spp, *Streptococcus faecalis*, *Clostridium perfringens*, *Proteus mirabilis*, *Proteus vulgaris* and *Klebsiella* spp, among others (Mpuchane et al., 2006, Vahabi et al., 2007). All these organisms, however, differ in their virulence.

One important virulent habit often displayed is the formation of biofilm which has been responsible for reoccurrence and persistence of associated infections (Lebeaux et al., 2014). Biofilms often display reduced susceptibility to antimicrobial agents which may result in increased rate of mortality and morbidity, increased cost of treatment and increased length of hospital stay by the patients (Moscoso et al., 2009; Penesyan et al., 2020). It therefore follows that biofilm formation should not be encouraged in the management of any infection.

However, while information about different species of organisms associated with cockroaches as well as their susceptibility to different antibiotics abounds in literature, information about the capacity of those organisms to form biofilm is missing. This study therefore aimed at (i) isolating and characterizing different bacterial species associated with the body surface of cockroach (ii) evaluating the isolates for their ability to form biofilm and (iii) determining the antibiotic susceptibility profiles of the biofilm-formers among the isolates to commonly used antibiotics.

METHODOLOGY

Materials

Isolation and characterization of bacterial isolates

Bacterial isolates used for this study were obtained from the external body surface of cockroaches collected from various locations such as toilets and bathrooms, dumpsites, kitchens, drainage pipes, broken waste disposal systems in residential areas in Ile-Ife, Osun State Nigeria, using the modified method of Feleke et al. (2016). Briefly, the cockroaches used were first immobilized and killed with insecticides, and the dead cockroaches were then picked with a forceps into universal bottles and transported to Pharmaceutical Microbiology Laboratory for microbiological analysis. Sterile Normal saline was poured into the universal bottles to submerge the cockroaches and then mixed using a vortex mixer. A flamed platinum loop was used to transfer a loopful from the universal bottle onto nutrient agar plates and then streaked. The streaked agar plates were then incubated at 37 ^{o}C for 24 hours. The discrete colonies were then identified using conventional biochemical tests and the identified bacterial isolates then stored on agar slopes and kept inside refrigerator until needed for further work.

Evaluation of bacterial isolates for ability to form biofilm

This was done as described by Freeman *et al*. (1989). Congo red stain (0.8 g/L) prepared as concentrated aqueous solution and sterilized by autoclaving at 121 °C for 15 min. was added to sterile Brain Heart Infusion agar containing Brain Heart Infusion (BHI) broth (HiMedia, India) (37 g/L) and agar number 1(HiMedia, India) (10 g/L)] supplemented with sucrose (5 g/L) at 55 °C. The isolated bacteria were streaked on the CRA plates and incubated aerobically at 37 °C for 24 h. Formation of biofilm was indicated by distinct black colonies with a dry crystalline consistency.

Antibiotic Susceptibility Test

All isolates capable of forming biofilm as detected by CRA method were evaluated for antibiotic susceptibility using the Kirby-Bauer disc diffusion technique as exemplified in the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2021). The antibiotic discs used for the CRA-positive Gram-positive isolates include: gentamicin (10µg), ampiclox (30µg), zinnacef (20µg), amoxicillin (30µg), Ceftriaxone (25µg), ciprofloxacin (10µg), streptomycin (30µg), septrin(30µg), erythromycin (10µg), while septrin (30µg), chloramphenicol (30µg), sparfloxacin (10µg), ciprofloxacin (10µg), amoxycillin (30µg), augmentin (10µg), gentamicin (10µg), ofloxacin (10µg) and streptomycin (30µg) were used against the CRA-positive Gram-negative isolates.

McFarland standard equivalent of 0.5 (A_{625} nm = 0.09) was prepared by inoculating 10 mL of sterile distilled water with five colonies of the test bacterium and rigorously mixed with a spin mixer. The turbidity of the resulting suspension was visually compared and adjusted to match the turbidity of 0.5 McFarland standard prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4). The final suspension was evenly spread on the surface of Mueller–Hinton agar using a sterile swab stick. Selected antibiotic discs were then pressed onto the surface of the agar using a pair of sterile forceps following 20 minutes incubation at 37 °C for acclimatization and growth of the inocula. All plates were incubated at 37 °C for 18 h after a 30-minute refrigeration at 4°C to ensure sufficient diffusion of antibiotics. *Escherichia coli* ATCC 25922 was used as control strain. The diameters of inhibition zones were measured in millimeters and interpreted according to the CLSI manual.

RESULTS AND DISCUSSION

The distribution of the bacterial isolates from the study is as shown in Table 1. *Bacillus subtilis* is the predominant species (77.9%) while *Salmonella typi* is the least species (1.0%). The percentage distribution of the ability of the isolates to form biofilm as detected by the congo red agar (CRA) method is as shown in Table 2. Out of the 42 isolates that were CRA-positive, 76.19% are *B. subtilis* and 2.38% are *Proteus mirabilis.* However, none of *Staphylococcus aureus* and *Salmonella typhi* shows the capacity to form biofilm.

Table 1: Distribution of bacterial isolates associated with external body of cockroaches

Organism	Number of organisms	Percentage distribution (%)
Bacillus subtilis	81	77.9
Proteus vulgaris	11	10.6
Klebsiella oxytoca	6	5.8
Proteus mirabilis	3	2.9
Staph aureus	2	1.9
Salmonella typhi	1	1.0
Total	104	100

Table 2: Percentage Distribution of the CRA-positive bacterial species associated with external body of cockroaches

S/N	Bacterial species	Number of CRA-positive (n = 42)	%	Number of CRA-negative (n = 62)	%
1.	*Bacillus subtilis*	32	76.19	49	79.03
2.	*Proteus vulgaris*	4	9.52	7	11.29
3.	*Klebsiella oxytoca*	5	11.90	1	1.61
4.	*Proteus mirabilis*	1	2.38	2	3.22
5.	*Staph aureus*	-	-	2	3.22
6.	*Salmonella typhi*	-	-	1	1.61
	Total	**42**	**100**	**62**	**100**

The percentage distribution of the resistance profiles of the CRA-positive *B. subtilis* isolates from the study is as shown in Table 3. All the CRA-positive *B. subtilis* are susceptible to gentamycin and ciprofloxacin. However, resistance to the beta-lactam antibiotics (ampiclox and amoxycillin) used in the study is high, 94 and 91%, respectively. From Table 4, the resistance profiles of different CRA-positive Gram-negative species differ. However, all the CRA-positive Gram-negative species are susceptible to gentamicin

Table 3: Percentage distribution of antibiotic resistance profiles of CRA-positive *Bacillus subtilis* isolates associated with external body of cockroaches

Antibiotics	CRA-positive *B. subtilis* (n = 32)
Gentamicin	-
Ampiclox	94
Zinacef	44
Amoxycillin	91
Rocephin	19
Ciprofloxacin	-
Streptomycin	19
Septrin	19
Erythromycin	72

Table 4: Percentage distribution of antibiotic resistance profiles of CRA-positive Gram-negative bacterial species associated with external body of cockroaches

Antibiotics	CRA-positive *P. vulgaris* (n = 4)	CRA-positive *P. mirabilis* (n = 1)	CRA-positive *K. oxytoca* (n = 5)
Gentamicin	-	-	-
Septrin	25	100	20
Ofloxacin	-	-	20
Chloramphenicol	25	100	-
Ciprofloxacin	25	-	20
Streptomycin	-	100	40
Augmentin	100	100	100
Amoxycillin	-	-	20
Sparfloxacin	25	100	-

DISCUSSION

Several bacterial species that have been implicated in one form of disease or the other have been isolated from the body surface of cockroaches. For instance, *Mycobacterium leprae* and leprosy; *Shigella paradysenteriae* and diarrhea in children; *Pseudomonas aeruginosa* and urinary tract infections; *Staphylococcus aureus* and boils and abscesses; *Escherichia coli* and infections of urogenitals and intestine; *Salmonella typhirium* and *Clostridium perfringens* and food poisoning; and *Salmonella typhosa* and typhoid fever (Donkor, 2020). In this study, one hundred and four (104) bacterial species were isolated from 70 cockroaches as shown in Table 1. The fact that *Bacillus subtilis* was the predominant bacterial isolate in this study is consistent with the report of Isaac et al. (2014) Similarly, isolation of other species of bacteria as found in this study is consistent with the reports of previous researchers (Pai, 2013; Feleke et al., 2016). Predominance of *B. subtilis* as found in this study attests to the filthy feeding habits of cockroaches which involve feeding on garbage as well as sewage as *B. subtilis* is a soil-dwelling, non-pathogenic, Gram-positive bacterium that is believed to be a commensal species of the human gastrointestinal tract (Hong et al., 2009). Notwithstanding the non-pathogenicity of *B. subtilis*, there have been reports of its association with some infections as endocarditis, pneumonia, bacteremia and septicemia. It has also been implicated in several cases of food poisoning (Apetroaie-Constantin et al., 2009). Apart from the difference in pathogenicity of different bacterial isolates associated with cockroach surfaces, they also differ in their virulence. One such virulence habit often displayed is the formation of biofilm. Biofilms are characterized by (i) attachment to biotic or abiotic surfaces to form bacterial communities (ii) being embedded in self-produced exopolymeric matrix (iii) high resistance to antimicrobial agents (iv) high resistance to host immune clearance (Flemming and Wingender, 2010; Chen and Wen, 2011). However, association between biofilm formation and bacterial persistence has been reported (Balaban et al., 2004). In this study, *B. subtilis* displayed the highest capacity to form biofilm (Table 2). It has been reported that *Bacillus subtilis* has the capacity to choose at the individual cell level between biofilm formation and flagellum-mediated swimming motility with bacterial cells in a population expressing genes required for biofilm formation or genes required for swimming motility but not both. The decision to form either biofilm or free swimming cells by *B. subtilis* is mediated by a bistable switch controls called "bet hedging" that ensures that subpopulations of bacteria continue to grow as conditions change and/or become unfavorable (Ryan-Payseur and Freitag, 2018). The various processes and stages involved in biofilm formation by *B. subtilis* have been described (Gingichashvili et al., 2017; Ryan-Payseur and Freitag, 2018; Arnaouteli et al., 2021).

One important attribute of biofilm that encourages their persistence is the reduced susceptibility to antimicrobials including antibiotics, disinfectants and antiseptics (Stewart, 2015). It has been postulated that the antibiotic concentrations required to inhibit or kill bacteria in biofilms may be from 100- to 1000-fold greater than those required to inhibit or kill planktonically grown strains (Sedlacek and Walker, 2007). In this study, the CRA-positive *B. subtilis* displayed varying degrees of resistance to different commonly used antibiotics. Higher resistance was observed for the beta-lactam antibiotics namely: ampiclox and amoxycillin, used in the study. However, all the CRA-positive *B. subtilis* were susceptible to the aminoglycoside (gentamicin) and flouroquinolone (ciprofloxacin) used in the study (Table 3). On the other hand, while all the CRA-positive *P. vulgaris*, *P. mirabilis* and *K. oxytoca* displayed different degrees of resistance to selected antibiotics used in the study, all the 3 species were however susceptible to gentamicin (Table 4). While

gentamicin acts by binding to the 16s rRNA at the 30s ribosomal subunit, disturbing the translation of mRNA and thus leading to the formation of truncated or non-functional proteins, ciprofloxacin acts by inhibiting DNA replication through the inhibition of bacterial DNA topoisomerase and DNA-gyrase (Walsh, 2000). However, while resistance to gentamicin can be by any or combination of (1) enzymatic modification and inactivation of the aminoglycoside, mediated by aminoglycoside acetyltransferases, nucleotidyltransferases, or phosphotransferases and commonly observed across Gram-positive and -negative bacteria, (2) increased efflux; (3) decreased permeability; and (4) modifications of the 30S ribosomal subunit that interferes with binding of the aminoglycosides (Doi *et al*., 2016), resistance to ciprofloxacin can occur through any or combinations of (i) protection of the target site (ii) over-expression of efflux pumps that prevent the drug from being accumulated in the cell, and (iii) changes in genes encoding the target site (DNA gyrase and topoisomerase IV) (Acheampong et al., 2019).

CONCLUSION

This study concluded that cockroaches (*Periplaneta americana*) can habour some bacterial species capable of forming biofilms which may lead to the persistence of associated infections with attendant consequences of increased rate of morbidity and mortality, increased cost of treatment and increased length of hospital stay.

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