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Comparative effect of biofilm and ESBL production on antibiotic susceptibility of bacteria isolates from *Clarias gariepinus*

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ABSTRACT

Sixty six bacteria isolates from three organs (skin, gut and gills) of Clarias gariepinus, African catfish, were tested for the production of biofilm and extended spectrum beta-lactamase (ESBL) using conventional methods. Positive isolates for the production of either or both factors were subjected to antibiotic susceptibility test. All bacteria producing either or both factors were multidrug resistant (MDR). The fluoroquinolones (ofloxacin and ciprofloxacin) were highly potent against all isolates at 100%, closely followed by gentamycin at 85.1%. However, resistance were recorded at high rates to augmentin, cefixime, cefuroxime and ceftazidime at 100%, 89.6%, 85.1% and 74.6% respectively. A test of statistical significance on the effect of either or both factors in the number of drugs resisted using t-test at $p<.05$ reveal that there is no significant difference between the effects of ESBL, biofilm and ESBL-biofilm production among the isolates. However, a higher mean was observed among biofilm producers compared to ESBL and ESBL-biofilm.

Keywords: resistance, biofilm, ESBL, fluoroquinolones

INTRODUCTION

Biofilm is a densely packed multicellular communities of microorganisms attached irreversibly to a surface or interface. These micro-colonies may enclose communities of bacterial cells that may be composed of one or more species, and depending on the species involved; the micro-colony may be composed of 10 – 25% of cells and 75 – 90% of extracellular polymeric substances (EPS) matrix [15]. Biofilm formation begins with the adhesion of microbes to surfaces (be it biotic or abiotic) and subsequent processes established the microorganisms in an irreversible adhesion [26]. The advantages of biofilm are numerous to bacteria, especially in regards to protection from antibiotics, disinfectants and dynamic environments [15]. Biofilms are also extraordinarily resistant to phagocytosis, which makes their eradication from living hosts difficult [6, 23]. Antibiotic and immune response to biofilm producers rarely resolve the effects of biofilms on living hosts [9, 25], and may even cause immune complex damage to the surrounding tissues [17]. In human medicine, bacteria in biofilms have been reported to cause therapy resistance, recurrent and chronic nosocomial infections [37], while in veterinary medicine, a host of biofilm formers have been reported to resist very potent antibiotics either in combinations or singly.

In the mid-1980s, a new group of enzymes, the extended-spectrum β-lactamases (ESBLs), was detected (first detected in 1979) [34]. ESBLs are beta-lactamases that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam. Over the years, resistance to cephalosporins among members of enterobacteriaceae has increased mainly due to the spreading of Extended-spectrum β-Lactamases (ESBL) [5, 29, 38-39]. This resistance increases morbidity and mortality in infected individuals by hampering the adequate provision of effective chemotherapy therefore making treatment more costly [1, 14]. The production of ESBL can be plasmid-mediated or chromosomal in origin. Plasmid-oriented ESBLs are often acquired by transfer of genetic-

related information from one organism to another and it often codes for resistance determinants to other antimicrobial agents; hence, multidrug resistance (MDR) is expected of ESBL-producing isolates [3, 4]. However, most of these isolates have been reported susceptible to cephamycins, cabapenems and related compounds [30]. Many reports have been made on effects of ESBL production in drug susceptibility especially among enterobacteria and from different clinical sources [3, 4, 12, 18, 24, 31, 40], as well as animal feed [42].

This study examines the effects of biofilm, ESBL and a combination of biofilm-ESBL production among isolates in drug susceptibility.

MATERIALS AND METHODS

Sample Analysis

Each of the specimens was dissected aseptically to remove the gut, gills and skin. Each organ was placed in sterile beaker containing 5ml sterile distilled water and vigorously shaken to allow the content to dissociate in water. For bacteria count, 1ml was taken and serially diluted to 10^5 from which pour plate method was carried out using nutrient agar. After incubation at 37^{o}C for 24 hours, counts were taken and expressed in colony forming units (CFU) per milliliter (ml). One (1) ml of the original suspension was streaked on the surfaces of freshly prepared Eosin Methylene blue agar (EMB), Trypticase soy agar (TSA), and Macconkey agar (MAC) respectively. The plates were incubated aerobically at 37˚C for 24hours and representative colonies emerging from the plates were grouped according to their cultural characteristics, purified by repeated sub–culturing and maintained on appropriate agar slants as stock culture. All isolates were characterized using standard microbiological and biochemical tests [10]. Bacterial isolates were identified with the help of Bergey's Manual of Determinative Bacteriology and online Gideon Informatics (1994-2015) [20].

Biofilm Detection

Biofilm production in isolates was detection using the Congo Red Agar (CRA) method [19].

Extended spectrum beta-lactamase test

The double disc synergy test (DDST) [43] was employed. Organisms to be tested were spread on Mueller-Hinton Agar plates using sterile swab sticks and allowed to absorb. Three antimicrobial disks (ceftriaxone 30µg, amoxicillin/clavulanic acid 30 µg and ceftazidime 30 µg) were placed 25mm apart, with amoxicillin/clavulanic acid in the middle, using sterile forceps. The plates were incubated aerobically at 37^o C and later the zones of inhibition were measured and interpreted according to Clinical and Laboratory Standards Institute [43]. The test is positive if, after 24-hour incubation, the zone of inhibition in between the disks is enhanced by ≥ 5mm, giving a dome/egg shape.

Antibiotic susceptibility test

All the isolated organisms were tested for antibiotic susceptibility by Kirby-Bauer disc diffusion method on Mueller-Hinton agar. This was carried out by making an even spread of 0.5 McFarland standard suspension of the pure isolates on prepared Mueller-Hinton agar using sterile swab sticks and aseptic placement of the antibiotics discs using sterile forceps. The plates were incubated aerobically at 37˚C for 24 hours after which the zones of inhibition were measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI) [43]. Antibiotics used are Augmentin (30µg), Ofloxacin (5µg), Gentamicin (10µg), Nalidixic acid (30µg), Nitrofurantoin (200µg), Amoxycillin (25µg), Tetracycline (25µg) for gram negative isolates and Augmentin (30µg), Cotrimoxazole (25µg), Erythromycin (5µg), Gentamicin (10µg), Streptomycin (10µg), Tetracycline (10µg) and Chloramphenicol (10µg) for gram positive isolates.

RESULTS AND DISCUSSION

A total of sixty three isolates were isolated, characterized and identified from the different organs of the fishes. Details of the bacterial isolates are listed in Table 1. *Escherichia coli* had the highest occurrence, 17 (25.8%) followed by *Klebsiella oxytoca*, 10 (15.2%); both having the highest number of isolates producing biofilm, ESBL and both.

Table 1. Frequency of bacterial isolates per organ

Isolated bacteria	Fish Organs		
	Gut (%)	Skin (%)	Gills (%)
Escherichia coli	6 (28.5)	5 (22.7)	6 (25)
Klebsiella oxytoca	5 (23.7)	3 (13.7)	2 (8.3)
Proteus vulgaris	3 (14.3)	0 (0)	1 (4.2)
Enterobacter aerogenes	1 (4.8)	0 (0)	1 (4.2)
Shigella sonnei	1 (4.8)	1 (4.5)	1 (4.2)
Enterobacter cloacae	1(4.8)	1 (4.5)	0 (0)
Shigella flexneri	3 (14.3)	2 (9.2)	3 (12.4)
Prevotella pallens	0 (0)	2 (9.2)	1 (4.2)
Chromobacterium violaceum	0 (0)	1 (4.5)	0 (0)
Providencia rettgeri	0 (0)	2 (9.2)	2 (8.3)
Porphyromonas macacae	0 (0)	1 (4.5)	0 (0)
Pantoea agglomerans	0 (0)	1 (4.5)	1 (4.2)
Pseudomonas oryzihabitans	0 (0)	1 (4.5)	0 (0)
Chryseobacterium indologenes	0 (0)	0 (0)	1 (4.2)
Erwinia chrysanthemi	0 (0)	0 (0)	1 (4.2)
Citrobacter koseri	0 (0)	0 (0)	3 (12.4)
Rhodococcus gordoniae	1 (4.8)	0 (0)	0 (0)
Kytococcus schroeteri	0 (0)	1 (4.5)	0 (0)
Luteococcus sanguinis	0 (0)	1 (4.5)	1 (4.2)

In all isolates, 49 (74.2%) produced biofilms on CRA, 18 (27.3%) produced ESBL, while 13 (19.7%) produced both biofilm and ESBL (Table 2). The least number of drugs resisted in all categories were three, and a maximum of six. Bacteria isolates and the numbers of drugs resisted are detailed in Table 3.

Table 2. Frequency of bacterial isolates per factor

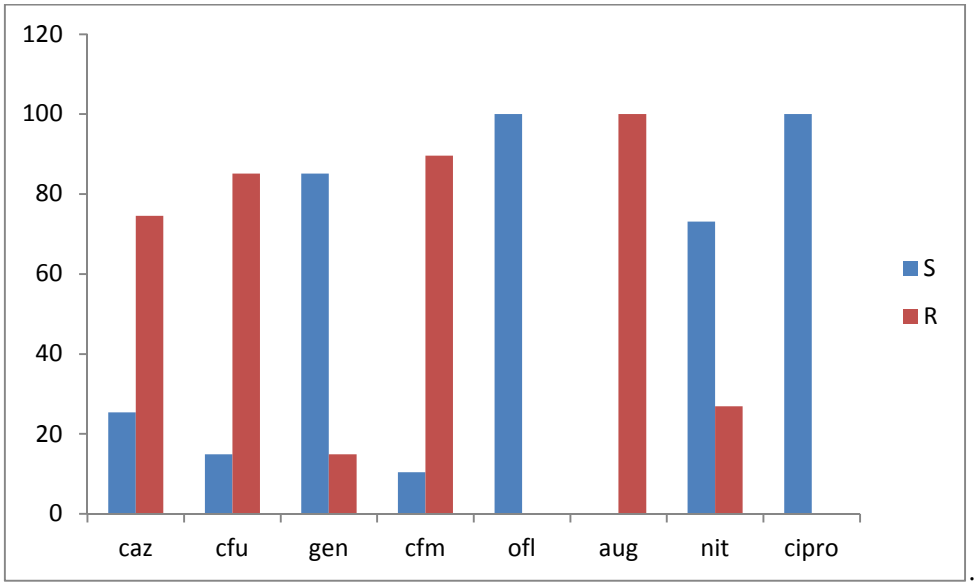
Isolated bacteria	Factor		
	Biofilm	ESBL	Biofilm-ESBL
Escherichia coli	15	5	4
Klebsiella oxytoca	9	3	2
Proteus vulgaris	3	2	2
Enterobacter aerogenes	1	0	0
Shigella sonnei	1	2	1
Enterobacter cloacae	2	1	1
Shigella flexneri	6	0	0
Prevotella pallens	1	0	0
Providencia rettgeri	4	1	1
Porphyromonas macacae	1	0	0
Pantoea agglomerans	2	1	1
Chryseobacterium indologenes	1	0	0
Erwinia chrysanthemi	1	0	0
Rhodococcus gordoniae	1	1	1
Kytococcus schroeteri	0	1	1
Luteococcus sanguinis	1	1	0

Table 3. Number of drugs resisted per factor

Isolated bacteria	Drugs resisted per factor		
	Biofilm alone	ESBL alone	Biofilm-ESBL
Escherichia coli	3	3	3
Klebsiella oxytoca	6	4	5
Proteus vulgaris	0	6	3
Enterobacter aerogenes	0	4	0
Shigella sonnei	5	0	4
Enterobacter cloacae	0	3	5
Shigella flexneri	0	4	0
Prevotella pallens	0	4	0
Providencia rettgeri	0	5	4
Porphyromonas macacae	0	5	0
Pantoea agglomerans	0	4	3
Chryseobacterium indologenes	0	4	0
Erwinia chrysanthemi	0	6	0
Rhodococcus gordoniae	0	0	3
Kytococcus schroeteri	4	0	0
Luteococcus sanguinis	3	3	0

The highest number of drugs resisted (6 drugs) was observed in *K. oxytoca* (producing ESBL alone); *P. vulgaris* and *E. chrysanthemi* (producing biofilm alone). However, *E. cloacae and K. oxytoca* resisted the highest number of drugs (5 drugs) while producing both biofilm and ESBL factors.

Antibiotic susceptibility test showed the fluoroquinolones as the best drug against all isolates at 100%, followed closely by gentamycin at 85.1%. Low potency against all isolates was observed with augmentin, cefixime, cefuroxime and ceftazidime (Figure 1).



Legend: S-sensitive, R-resistant, caz-ceftazidime, cfu-cefuroxime, gen-gentamycin, cfm-cefixime, ofl-ofloxacin, aug-augmentin, nit-nitrofurantoin, cipro-ciprofloxacin

Figure 1. Percentage susceptibility of isolates

Statistical analyses reveal no significant difference among the means of drugs resisted by isolates producing either or both of the factors at $p< .05$. However, a higher mean of drugs resisted was seen in biofilm producers.

A boxplot of the effect of both ESBL and biofilm production in relation to drug resistance is plotted.

The ability of bacteria to form biofilms helps the bacterium to survive in hostile environments within the host and is considered to be responsible for chronic or persistent infections [13]. Several studies have shown that the formation of slime and biofilms by organisms causing catheter-associated and nosocomial infections is associated with the presence of the icaA and icaD genes [7-8, 41]. A total of forty nine (74.2%) bacteria isolates were detected as biofilm producers using Congo red agar method. Jain and Agarwal [22] evaluated the phenotypic Congo Red Agar and microplate test in biofilm detection and concluded that both tests demonstrated good sensitivity and specificity in the detection of microorganisms that produced biofilms. Of high importance to food industry are biofilms as they occur on various food contact surfaces like stainless steel, rubber, glass, conveyor belts etc. Many pathogenic biofilm formers have been reported as common contaminants in food industries [16, 27-28, 32, 35] and in human medicine, bacteria in biofilms have been reported to cause therapy resistance, recurrent and chronic nosocomial infections [37], while in veterinary medicine, a host of biofilm formers have been reported to resist very potent antibiotics either in combinations or singly.

The increasing resistance to broad spectrum cephalosporins amongst enterobacteria especially *E. coli*, *Salmonella* and *Klebsiella* species predominantly due to the production of ESBLs have been reported from different countries [5, 11, 24, 31, 39]. In Nigeria, many reports on ESBL isolates from clinical diagnosis are available from different researchers and in different parts of the country: Kano, Benin, Lagos and Enugu [2, 3, 18, 21, 40]. This study reports a total of 18 (27.3%) ESBL isolates. These isolates are often in the environment and sometimes through human and animal agencies; they contaminate foods; plants, animals and their products. Plasmids responsible for ESBL production in bacteria have also been reported to carry genes responsible for resistance to other drug classes, therefore antibacterial drug options in the treatment of patients infected by ESBL-producing isolates are very limited [36].

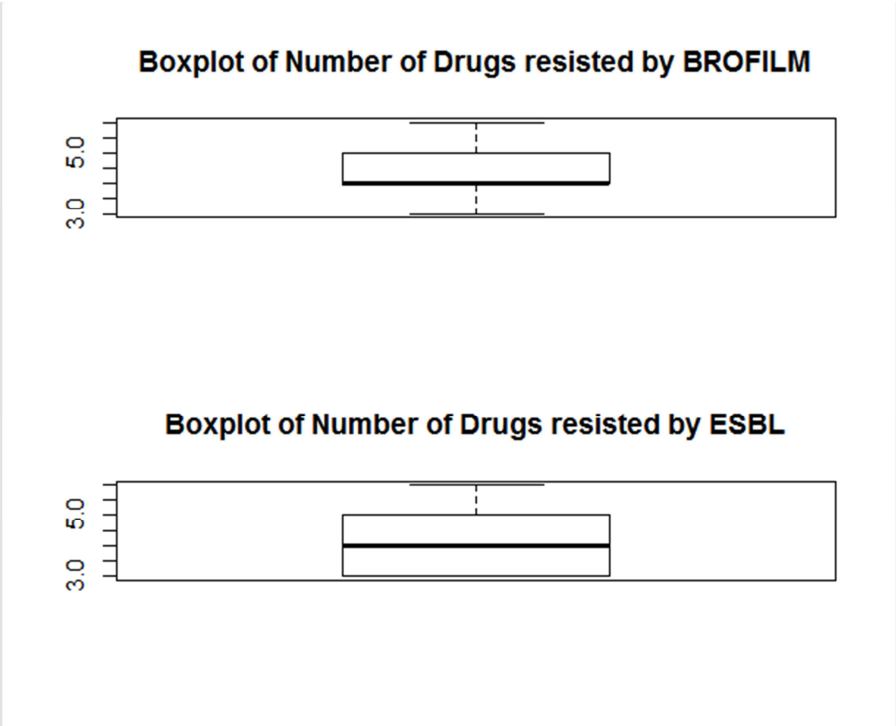


Figure 2. Boxplot chart

CONCLUSION

This study re-enact the importance of bacterial and antimicrobial surveillance especially from animal food and their products for food safety. A necessary and important monitoring of use of antimicrobials in animal feed and veterinary medicine will help safeguard multidrug resistance factors in bacterial isolates.

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