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Isolation and Identification of Antibiotic-Susceptible Bacteria from Abattoir Effluent in Port Harcourt, Rivers State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Abattoir environment can become a significant reservoir for antibiotic resistance bacteria, particularly in abattoirs that do not treat their waste before discharge. This study seeks to verify the antibiotics susceptibility profile of bacteria isolated from abattoir wastewater in Port Harcourt, River State, Nigeria. The bacteria were isolated from the wastewater using a standard plating technique. The antibiotics sensitivity pattern of all bacterial isolates was determined by the Kirby-Bauer disk

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diffusion method. Isolates with high multiple antibiotic resistance (MAR) index (≥0.5) were screened for genes for pathogenicity (icaC, adhesion gene) and antibiotic resistance (QnrA gene). The abattoir wastewaters were contaminated by bacteria resistant to no less than two of the antibiotics experimented with (MAR index range, 0.2-1.0). The isolates were identified as *Acinetobacter brisouii*, *Bacillus altitudinis*, *Bacillus stratosphericus* and *Priestia flexa*. Among these isolates, 2(50.0%) harboured the QnrA gene while 3(75.0%) harboured the icaC gene. The findings underscore the importance of abattoir wastewater as an environmental flashpoint for antibiotic resistance. Detection of bacteria with multiple antibiotic resistance in abattoir wastewater would inform cautious use of antibiotics, to check the spread of antibiotic resistance emanating from this source.

Keywords: Abattoir wastewater; antibiotic resistance; pathogenicity.

1. INTRODUCTION

Abattoirs constantly produce large amounts of wastewater, particularly from washing operations. Abattoir wastewater contains a variety of organic pollutants including microorganisms dispelled from the visceral skin of slaughtered animals [1]. The presence of microorganisms in abattoir wastewater, particularly those with a strong propensity to cause infectious diseases, is a serious public health hazard [2].

In third-world countries, owing to reasons of poverty and ignorance, abattoir waste gets dumped indiscriminately into the environment. Many people are not aware of how the effluent from abattoirs contributes to environmental degradation and affects the health of flora and fauna. If abattoir waste is spilt into the environment, enteric pathogens and excessive nutrients could find their way to surface waters, groundwater, and soil [3].

Abattoir wastewater contains a high concentration of antibiotics, heavy metals and organic pollutants which can create an environment conducive to the development and spread of antibiotic-resistant bacteria [4]. Several studies have informed on the instances of pathogenic bacteria in abattoir wastewater, including WHO priority bacteria which are designated opportunistic bacterial pathogens with widespread antimicrobial resistance, namely *Klebsiella pneumoniae*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, *Enterobacter* spp and *Staphylococcus aureus* [5]. The presence of antibiotic-resistant bacteria in abattoir wastewater is a growing public health concern as it increases the risk of transmission of infectious diseases.

Microbiological quality assessment reports on abattoir wastewater have consistently revealed the presence of classical and opportunistic pathogens including *Streptococcus* sp., *Staphylococcus* sp., *Bacillus* sp., *Escherichia coli*, *Enterobacter* sp., *Klebsiella* sp., *Shigella* sp., *Pseudomonas* sp. and *Salmonella* sp. [6-8]. Olawale et al. [9] investigated the antibiotic susceptibility profiles of bacteria isolated from abattoir wastewater in Ilorin, Nigeria. The study isolated various Gram-negative and positive bacteria species, belonging to the families Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae, Staphylococcaceae and Streptococcaceae. The results revealed high rates of resistance to commonly used antibiotics such as ampicillin, sulfamethoxazole and tetracycline, The study emphasized the need for strict antibiotic stewardship practices to prevent the dissemination of antibiotic-resistant Gram-negative bacteria. Akpan et al. [10] investigated the antibiotic susceptibility patterns of Gram-negative bacteria (*Escherichia coli*, *Enterobacter* sp., *Klebsiella* sp., *Shigella* sp., *Pseudomonas* sp. and *Salmonella* sp.) isolated from abattoir wastewater in Abeokuta, Nigeria, and reported that a substantial proportion were multidrug resistant.

Though antibiotics can be enhanced by human activities, they can also be driven by environmental factors. Heavy metals in the environment, for instance, are known to increase antibiotic resistance in bacteria [11]. Zhang et al. [12] isolated a strain of *Bacillus flexus* from an oil reservoir that was resistant to ampicillin, erythromycin, fosfomycin, fosmidomycin, gentamicin, teicoplanin, tetracycline and vancomycin. Shivaji et al. [13] reported that a strain of *Bacillus pumilus* isolated from air samples at high altitudes was resistant to ampicillin, ciprofloxacin, lincomycin and novobiocin.

According to Abia et al. [4] wastewater is a significant environmental reservoir for antibiotic resistance and the problem is much of an issue in Africa, because of the indiscriminate use of antibiotics, the derisory state of sanitation and the prevalent practice of not treating wastewater before discharge into the environment. Abattoir wastewater ought to be treated before discharge into the environment to avert adverse altering in microbiological properties. When the wastewater is laden with pathogenic and antibiotic-resistant microorganisms, the spread of disease and antibiotic resistance is heightened. This study seeks to isolate and identify antibiotic-susceptible bacteria from abattoir effluent in Port Harcourt Rivers State.

2. MATERIALS AND METHODS

2.1 Wastewater Collection from Abattoirs

Wastewater was garnered from discharge points of Chokocho abattoir, Port Harcourt, Rivers State, Nigeria, in sterile 2.0-litre sample vials. The samples were collected in the morning when slaughtering activity was at its peak. The samples were quickly shipped in an ice chest to the Microbiology Laboratory, University of Port Harcourt, for isolation of bacteria.

2.2 Isolation and Characterisation of Bacterial Isolates

Bacteria present in the wastewater samples were isolated using pour plate technique. The following bacteriological media were used: nutrient agar, MacConkey agar salmonella-shigella agar, thiosulphate citrate bile salt agar, eosin methylene blue agar and Cetrimide agar.

Distinct colonies on agar plates were sub-cultured in nutrient agar to plate out pure isolates. Isolates were characterized through Gram staining and a battery biochemical test, as described by Aneje [14].

2.3 Bacterial Antibiotics Susceptibility Testing

The antibiotics sensitivity pattern of all bacterial isolates was determined by the Kirby-Bauer disk diffusion method described by Anele et al. [15] on Mueller Hinton (MH) agar.

The antibiotics used are Amoxycillin/Clavulinate (AU) 30 µg, Amoxicillin (AML) 30 µg, Ampiclox (APX) 20 µg, Ampicillin (APX) 10 µg, Cefalexin (CEP) 10 µg, Chloramphenicol (CH) 30 µg, Ciprofloxacin (CPX) 5 µg, Cotrimoxazole (SXT) 30 µg, Erthromycin (E) 15 µg, Gentamicin (CN)10 µg, Levofloxacin (LEV) 20 µg, Nalidixic Acid (NA) 30 µg, Norfloxacin (NB) 10 µg, Ofloxacin (OFX) 5 µg, Reflacine (PEF) 10 µg, Rifampicin (RD) 20 µg and Streptomycin (S) 30 µg.

Sterile MH agar was dispensed into sterile Petri dishes and allowed to solidify. Afterwards, 0.1ml of the bacteria isolates agar plates using a sterilised hockey stick. Then multiple antibiotic discs were placed firmly on the agar plates using sterilized forceps. Plates were incubated at 35 ± 2^0C for 24 hours.

The zone of growth impedance observed was measured to the nearest millimetre, recorded and interpreted as sensitive, intermediate or resistant for each of the assayed antimicrobial agents, based on EUCAST 2019 categories [16].

Multiple antibiotic resistance (MAR) index (the ratio of antibiotics an organism was resistant to in comparison to all antibiotics was exposed to) was calculated for each isolate [9].

2.4 Structural and Functional Genes Amplification and Sequencing

Isolates were ascertained based on their 16S rRNA sequences following genomic DNA amplification (ABI 9700 Applied Biosystems thermal cycler) using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers.

Amplification of icaC genes from the selected isolates was done using primers icaCF: 5'-TAACTTTAGGCGCATATGTTTT -3' and icaCR: 5'- TTCCAGTTAGGCTGGTATTG -3' primers.

The QnrA gene from isolates was amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers.

DNA sequencing was carried out using an Applied Biosystems (Foster City, CA, USA) automatic DNA sequencer (ABI PRISM 3130 x 1 Genetic Analyzer) and an Applied Biosystem Big Dye (ver. 3.1) kit.

3. RESULTS

3.1 Antibiotic Susceptibility Profile of Bacteria

Table 1 shows the antibiotic sensitivity pattern of Gram-positive bacterial isolates obtained from Wastewater samples. All isolates showed resistance to rifampicin and levofloxacin, and are resistant to two or more of the antibiotics. MAR index range was 0.2-0.5.

Table 2 shows the antibiotic sensitivity pattern of Gram-negative bacterial isolates obtained from Wastewater. All the isolates were resistant to ampicillin. All the isolates were resistant to at least two antibiotics (MAR index range 0.2-1.0). *Acinetobacter* sp. was resistant to all antibiotics experimented with

3.2 Molecular Identification

Analysis of 16s rRNA gene sequence placed the isolates within the *Bacillus, Priestia* and *Acinetobacter* sp., and revealed a close relatedness to*, Acinetobacter brisouii* strain AB859735 (99.8%), *Bacillus altitudinis* strain CP099861(99.9%)*, Bacillus stratosphericus* strain ON878108 (99.7%) and *Priestia flexa* strain ON838176 (99.8%) (Fig. 1).

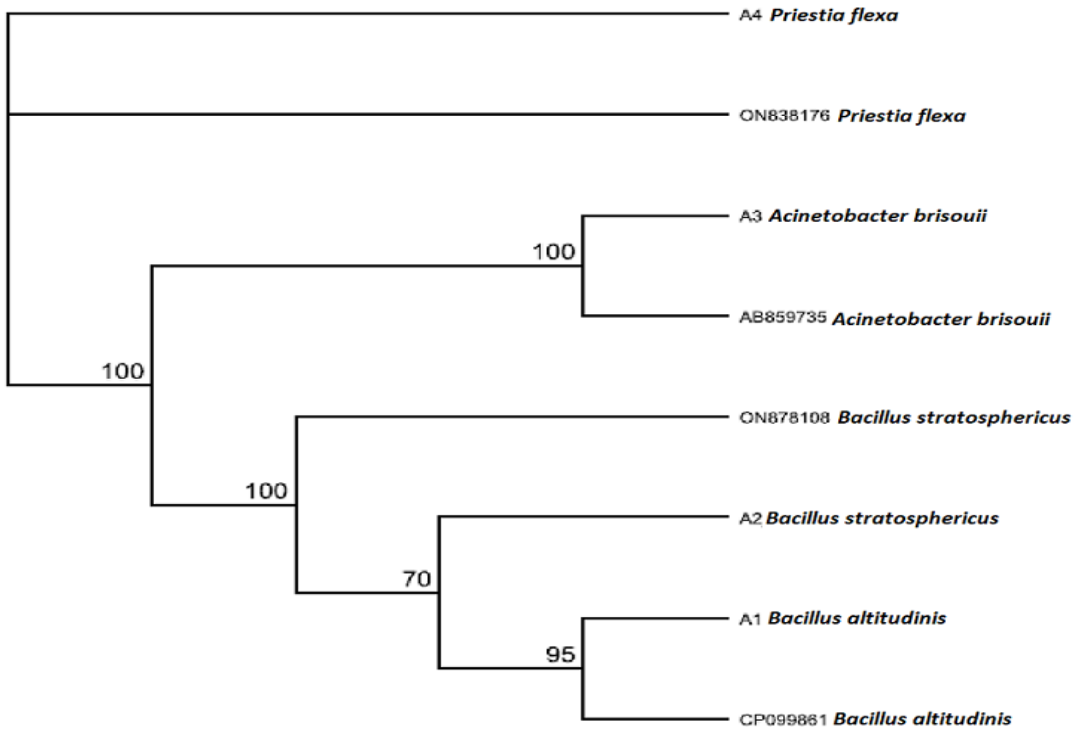

Fig. 1. Phylogenetic tree showing the evolutionary distance between the bacteria isolated from abattoir wastewater

Table 1. Antibiotic sensitivity pattern of Gram-positive bacteria

Bacteria	Antibiotic/ Diameter of zone of inhibition (mm)										MAR index
	CPX	NB	CN	AML	S	E	RD	CH	APX	LEV	
Staphylococcus sp.	15(S)	9(I)	18(S)	11(I)	16(S)	16(S)	2(R)	19(S)	15(S)	4(R)	0.2
Bacillus sp.	12(I)	4(R)	17(S)	8(R)	3(R)	18(S)	1(R)	18(S)	14(I)	2(R)	0.5
Priestia sp.	3(R)	15(S)	2(R)	5(R)	17(S)	12(I)	6(R)	4(R)	10(I)	1(R)	0.6
Bacillus sp.	10(I)	1(R)	18(S)	8 (R)	17(S)	9(R)	3 R)	0 (R)	5 (R)	1(R)	0.7

Keys: R=Resistance, I= Intermediate, S= Sensitive (EUCAST, 2019)
CPX= Ciprofloxacin, NB= Norfloxacin, CN=Gentamycin, AML=Amoxicillin, S= Streptomycin, E= Erythromycin, RD= Rifampicin, CH= Chloramphenicol, APX= Ampiclox, LEV=Levofloxacin

Table 2. Antibiotic sensitivity pattern of Gram-negative bacterial

Bacteria	Antibiotic/ Diameter of zone of inhibition (mm)										AMR index
	OFX	PEF	CPX	AU	CN	S	CEP	NA	SXT	PN	
E. coli	17(S)	15(S)	15(S)	18 S)	18(S)	17(S)	0(R)	0(R)	4(R)	0(R)	0.4
Shigella sp.	15(S)	18(S)	15(S)	14(R)	15(S)	17(S)	15(S)	19(S)	6 (R)	5(R)	0.3
Acinetobacter sp.	6(R)	8(R)	10(R)	9(R)	7(R)	6(R)	11(R)	6(R)	5(R)	3(R)	1.0
Enterobacter sp.	4(R)	6(R)	11(I)	14(I)	16(S)	18(S)	15(S)	8(R)	15(S)	2(R)	0.4
Salmonella sp.	15(S)	5(R)	10(I)	17(S)	15(S)	6(R)	4(R)	15(S)	18(S)	5(R)	0.4
Pseudomonas sp.	11(I)	14(I)	10(I)	7(R)	15(S)	14(I)	10(I)	15(S)	13(I)	6(R)	0.2

Keys: R=Resistance, I= Intermediate, S= Sensitive (EUCAST, 2019)
OFX= = Ofloxacin 5 µg, PEF= Reflacine, CPX=Ciprofloxacin, AU= Amoxycillin/Clavulinate, CN=Gentamycin, S= Streptomycin, CEP= Cefalexin, NA= Nalidixic Acid, SXT= Cotrimoxazole, PN= Ampicillin

Table 3. Distribution of virulence and resistance genes among the bacterial isolates

Organism	Accession Number	QnrA	icaC
Acinetobacter brisouii	OR462205	+	+
Priestia flexa	OR462206	-	+
Bacillus stratosphericus	OR462207	-	+
Bacillus altitudinis	OR462208	+	-
Total		*2(50.0%)*	*3(75.0%)*

3.3 Resistance and Virulence Genes

The distribution of resistance and virulence genes among the bacterial isolates revealed that 4 bacteria 2(50.0%) harboured the resistance gene QnrA while 3(75.0%) had the icaC gene for adhesion.

The bacterial isolates harbouring the resistance gene QnrA are *A. brisouii* and *B. altitudinis* while *P. flexa, A. brisouii* and *B. stratosphericus*. harboured icaC gene for adhesion.

4. DISCUSSION

4.1 Antibiotic Sensitivity Pattern

The bacteria isolated from wastewater in this study are opportunistic pathogens. Results for antibiotics sensitivity pattern of Gram-positive bacterial isolates obtained from wastewater samples show that all isolates showed resistance to rifampicin and levofloxacin, and are resistant to two or more of the antibiotics. Results for antibiotics sensitivity pattern of Gram-negative bacterial isolates obtained from Wastewater show that all the isolates were resistant to ampicillin. All isolates were resistant to at least two of the antibiotics tested.

In the present study, *B. stratosphericus* were resistant to norfloxacin, amoxicillin, erythromycin, rifampicin, chloramphenicol, ampiclox, levofloxacin, rifampicin and levofloxacin. Antibiotic resistance in *Bacillus* sp. from clinical and environmental samples has been severally reported. Sundaramanickam [17] reported resistance of *B. pumilus* isolated from shrimp to 14 antibiotics including norfloxacin, amoxicillin, erythromycin, rifampicin, chloramphenicol, ampiclox, levofloxacin and levofloxacin. György [18] reported that *B. stratosphericus* SALKÖ, isolated from commercially available spice were resistant to amoxiclav, azithromycin, oxacillin, penicillin G and rifampicin. Zhang et al. [12] isolated a strain of *B. flexus* from an oil reservoir that showed resistance to ampicillin, erythromycin, fosfomycin, fosmidomycin, gentamicin, teicoplanin, tetracycline and vancomycin.

Acinetobacter brisouii isolated from an abattoir wastewater in the present study was resistant to reflacine, ciprofloxacin, amoxycillin/clavulinate, streptomycin, cefalexin, nalidixic acid, ofloxacin, gentamycin, nalidixic acid, cotrimoxazole, streptomycin and ampicillin. *A. baumannii* is a well-known pathogenic *Acinetobacter* species, but it is not the only member of that species that is resistant to commonly used antibiotics against Gram-negative bacteria. Antibiotic resistance has

been reported in non- *Acinetobacter* species such as *A. brisouii* [19,20].

The isolate *P. flexa* was resistant to ciprofloxacin, gentamycin, amoxicillin, streptomycin, rifampicin, chloramphenicol and levofloxacin. Deswal [21] reported a strain of *P. flexa* isolated from human faeces that was resistant to cefixime, clavulanic acid/ceftazidime, nafallin, methicillin, trimethoprim, kanamycin and nalidixic.

Modern animal husbandry requires the administration of antibiotics for disease control and improved yield [22]. Thus, abattoirs are considered a significant source from where antibiotics enter the environment [23]. Disemboweling of animals at the abattoir releases antibiotics in quantities that accumulate over time. Microorganisms exposed to sublethal doses of antibiotics in the abattoir environment develop resistance to the antibiotics. This perhaps explains the resistance of the bacterial isolates to multiple antibiotics in the present study. Gene transfer increases mobility of acquired genes picked up by bacteria in the environment [4,22,24]. This worsens the problem of the presence of antibiotic resistant microorganisms in wastewater which can serve as a reservoir antibiotic resistance gene [25]. Microorganism harbouring such genes can easily find their way into the human body, especially in underdeveloped countries like Nigeria where assess to clean water is still a challenge, and wastewater rarely receives any form of treatment before being discharged into the environment.

The presence of multidrug resistant strains of bacterial isolates in abattoir wastewater is a pointer it could act as a reservoir and conduit for the propagation of antibiotic resistance in the environment. A similar view was expressed in the study by Ogbonna and Azuonwu [2] in which multidrug resistant strains of bacteria were isolated from abattoir effluents. This is a source of concern because of the growing trend of antibiotic resistance globally.

4.2 Resistance and Virulence Genes

Bacterial isolates in wastewater samples were identified based on their 16S rRNA genes to be closely related to *Bacillus* sp*., B. pumilus, P. flexa* and *A. brisouii*. These isolates were also analysed to ascertain if they are carriers of resistance and virulence genes. The bacterial isolates harbouring the resistance gene QnrA are *A. brisouii* and *B. altitudinis* while *P. flexa, A. brisouii* and *B. stratosphericus* harboured icaC gene for adhesion. Sokolov [26] detected icaC in Staphylococcus species but not in Bacillus. The icaC gene is part of icaABCD cluster under the control ica operon, known for the synthesis of polysaccharide intercellular adhesin (PIA), a major component of the extracellular matrix necessary for biofilm formation by bacteria [27,28]. Biofilm contributes to microbial pathogenicity as adhesion to surfaces is an important pathogenic factor and also enhances antibiotic resistance by their impermeable nature [27,29]

Bacillus altitudinis and *A. brisouii* harboured QnrA gene. The presence of QnrA gene has been associated with decreased susceptibility to quinolones in bacterial species. *Acinetobacter brisouii* was resistant to levofloxacin, ofloxacin, ciprofloxacin and nalidixic acid, while *B. altitudinis* was resistant to levofloxacin and norfloxacin. The QnrA gene is a plasmid-mediated quinolone resistance gene that encodes for a protein that protects the DNA gyrase and topoisomerase IV, from the effect of quinolone antibiotics [30].

Treatment of wastewater can reduce microbial load and improve the physiochemical properties of abattoir wastewater [31]. This would help in checking the spread of antibiotic resistance. In addition, changing therapeutic intervention for infectious disease by using alternatives to conventional antibiotics such as by using antimicrobial peptides, phage therapy and natural products, could also help to check the progression of antibiotic resistance [25].

5. CONCLUSION

Four bacteria were isolated from the abattoir wastewater. The isolates (*Acinetobacter brisouii*, *Bacillus altitudinis, Bacillus stratosphericus* and *Priestia flexa*) were multidrug resistant. The bacterial isolates, *A. brisouii* and *B. altitudinis* harboured the antibiotic resistance gene QnrA, while *P. flexa, A. brisouii* and *B. stratosphericus*. harboured icaC gene for adhesion. The findings underscore the importance of abattoir wastewater as an environmental flashpoint for antibiotic resistance. Detection of bacteria with multiple antibiotics resistance in abattoir wastewater would inform cautious use of antibiotics, to check the spread of antibiotic resistance emanating from this source.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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