Original Research Article

RESISTANT GENES OF MICROBES ASSOCIATED WITH ABATTOIR WASTES

A B S T R A C T

Antimicrobial drug resistance has become prominent as a universal health threat. This has been studied not only in humans but food animals as well. Many genes located on the chromosomal DNA of bacteria have been linked with drug resistance. It is therefore crucial that its occurrence in abattoirs where these animals are slaughtered be studied. This study was thus aimed at identifying some resistance genes in microbes isolated from abattoir ecologies. One hundred and eighty (180) samples consisting of service water, waste blood, wastewater, soil and faecal matter collected from Iwofe, Rumuodumaya and Trans-Amadi abattoirs within a period of one year were used in this study. Processing of the samples was done using standard microbiological protocols and the antibiotics sensitivity profile of the isolated bacteria determined using popularly consumed antibiotics. The presence of Beta-lactamase genes was checked for in the multidrug resistant isolates after they had been identified using molecular biological technique. Two strains of *Escherichia coli* had the *bla*CTX-M gene, *Pseudomonas* sp. strain 6174 had the *bla*SHV and *bla*TEM genes, *Bacillus amyloliquefaciens* had the *bla*SHV gene, *Bacillus flexus* had the *bla*TEM genes, *Staphylococcus aureus* had *bla*SHV and *bla*TEM genes. *bla*SHV, *blaCTX-M* and *bla*TEM genes were found in most of the Gram negative bacteria as *Klebsiella* sp. strain EIKU11 showed prominence of all three β-lactamase genes. This brings to light that some microbes in abattoir environments possess the β-lactamase genes which enable these microorganisms exhibit multidrug resistance, thereby making treatment resulting from them difficult. Adequate sanitary measures should thus be carried out to reduce the spread of these organisms to humans.

Keywords: β-lactamases, multidrug resistance, abattoirs, chromosomal DNA, Microorganisms

Introduction

In nature, antibiotics are used in the protection of human and animal health or as food additives in enhancing animal growth rate. However, most of these antibiotics are excreted from the animals to the environment. This exposes the aquatic environment to impacts of the antibiotic remnants including antibiotic resistance ([1]; [2]). This is largely due to the failure of regulatory agencies in controlling drug use, as such these drugs are obtained over-the-counter without the supervision of prescription by a licensed veterinary doctor ([3]; [4]). The risk involved is the possibility of transmission of the resultant resistant bacteria from the environment to humans either through indirect or direct contact [5]. According to previous studies, domestic animals and environments are huge reservoirs of antibiotic resistant bacteria and resistant genes which can be transferred to humans directly from animals to humans, indirectly through food or due to the deposition of animal wastes on land ([6]; [7]; [8]; [9]). Often times, antibiotics are administered to animals in order to treat infections, enhance their performance or enhance growth [10].

Antibiotics resistance however, refers to the mechanism by which microorganisms become resistant to an antibiotic which include degrading of the antimicrobial substance, modification of the chemical structure of the antibiotic, over-secretion of the target enzyme, obtaining alternate pathways to those drugs that can inhibit or cause changes in the bacterial cell permeability restricting the access of the antimicrobial agent to the target site, active removal of the antibiotic from the bacterial cell and remodeling of the target for the antibiotic [11]. Genes associated with microbial antibiotic resistance has been found in different environments at quantities higher than those present before antibiotics were mass-produced [12]. These antibiotic resistance genes (ARGs) are unique contaminants in that, they are of biological origin and can be transferred through genetic processes into different types of organism regardless of distance. Also, their source(s) could be agriculture, wastewater treatment plants or animal farms [13]. Although antibiotic resistant β-lactamases only became popular when

clinical resistance surfaced, spontaneous mutation alone cannot be implicated in the prevalence and spread of microbial resistance to modern antibiotics ([14; 15]).

The emergence and selection of resistance in bacteria from animals subjected to antibiotic regimens suggests that after the introduction of veterinary antibiotics, the resistance in pathogenic and faecal bacteria has increased [10]. More often, these animals fall ill, they are treated with antibiotics and in some cases, they pick up the antibiotics during open grazing which exposes them to antibiotic resistant microorganisms [16]. Antibiotic resistance among microorganisms isolated from cows has been studied especially to tetracycline, neomycin, virginiamycin and tylosin [17]. The use of antimicrobials in agricultural animals causes metabolic disturbance, affecting various biochemical processes and pathways including nitrogen excretion and protein synthesis. This disturbance in the intestinal tract can both negatively affect the animal and result in the selection or emergence of Antibiotic Resistant Organisms (AROs) in the affected species. In this case, the impact on the microbiome is the result of veterinary antibiotic use [18]. Research has shown that an estimated 50-90% of administered drugs to farm animals are excreted un-metabolized or as metabolic intermediates into the environment, which although are inactive and may be transformed to active forms in the environment increasing the risk of drug resistance [19].

According to Lin *et al* [20] multiple mechanisms for antibiotic resistance exists which can be coded for by either single or multiple genes. These mechanisms start first with random mutation in an organism which later spreads to other organisms through the process of gene transfer [13]. Horizontal gene transfer (HGT) occurs through transduction, transformation and conjugation [21]. Conjugation takes place when DNA transferred to a cell through direct cell contact or through a multi-protein conjugative complex [22]. Transformation occurs by the take up of exogenous DNA by an organism while bacterial transduction takes place when a bacteriophage injects a DNA into a bacterial cell ([23]; [24]). Either of these mechanisms aids gene transfer from the environmental gene pool consisting of genetic information that can be reached by more than one species of bacteria [25].

Resistance to extended Spectrum β-lactam drugs such as ceftriaxone, cefotaxime and Ceftazidime which are 3rd generation Cephalosporins by Enterobacteria especially, has been reported as a result of the promiscuous use of these antibiotics [26]. However, in recent time, resistance to these drugs has been reported owing to the secretion of extended spectrum beta-lactamases in Enterobacteria ([27]; [26]). The secretion of Extended-Spectrum Beta-Lactamases (B-LACTAMASEs) as an antibiotic resistance mechanism to third generation Cephalosporins, is frequent among bacteria of the Enterobacteriaceae family such as *Escherichia coli* and *Klebsiella pneumoniae* [28]. Microorganisms which are ESBL resistant are often resistant to antibiotics of phenicols, aminoglycosides, potentiated sulfonamides and fluoroquinolone class ([29]; [26]). Subsequently, β-lactamase resistant bacteria have also been termed multi-drug resistant microbes. Infections caused by these β-lactamase-resistant bacteria thus have limited therapeutic options as the bacteria exhibit multidrug resistance [30].

In the past, bacteria producing β-lactamase was only found in humans; however recently, they have been observed in livestock which has led to monitoring researches carried out on animals [31]. These observations have led to the assumption that livestock especially food-producing animals may be reservoirs of infection sources of β-lactamase-producing bacteria [29]. β-lactamase-resistant bacteria residing as commensals in the intestinal tract of food-producing animals may be deposited in the environment leading to the spread of β-lactam resistance encoding genes. This study was thus aimed at determining the presence of some resistant genes in microorganisms isolated from abattoir wastes in Port Harcourt as there exists paucity of data on this.

Materials and Methods

Description of Study area

Samples for this study were collected from abattoirs within Port Harcourt which is one of Nigeria's busiest and most populous cities. The abattoirs are located at Rumuodumaya, Iwofe and Trans-Amadi. The Trans-Amadi abattoir is Port Harcourt's largest abattoir. Its effluents are drained into the Okpoka Creek. The Creek passes through Woji, Oginigba and Azubiae communities where activities such as dredging, bathing, fishing, disposal of excreta, swimming and navigation are carried out; these abattoir effluents thus affect the activities carried out along this Creek. It is located at latitude 007 2.303E and longitude 04 48.442 N. Rumuodumaya abattoir is located at longitude 04 '52' 48.0 N and latitude 7'58'20.0 E. The Iwofe abattoir has only existed for less than three years and is located at latitude 4 59'14.0N and longitude 7 16' 12.0 E. Inhabitants of these areas are mainly traders, artisans, civil servants, fishermen and farmers. Table 1 shows the GPS coordinates of the sampling points while figure 1 is a map showing the sampling locations. The samples were collected within one year covering both dry and wet seasons.

Table 1: Sampling points, GPS Coordinates and Types of Samples

Sampling stations	Sampling points	Sampling coordinates		Samples
		Northing	Easting	
Iwofe abattoir	1	004° 48.598′	006° 58.517′	Blood
	2	004° 48.592′	006° 58.501′	Soil
	3	004° 48.601′	006° 58. 525′	Water
	4	004° 48.594′	006° 58.518′	Faecal matter
	5	004° 48.598′	006° 58.517′	Waste water
Rumuodumaya abattoir	1	004° 52.118′	006° 59.580′	Blood
	2	004° 52.102′	006° 59.571′	Soil
	3	004° 52.124′	006° 59. 602′	Water
	4	004° 52.120′	006° 59.582′	Faecal matter
	5	004° 52.118′	006° 59.580′	Waste water
Trans-Amadi abattoir	1	004° 48.442′	007° 02.303′	Blood
	2	004° 48.434′	007° 02.293′	Soil
	3	004° 48.456′	007° 02.319′	Water
	4	004° 48.444′	007° 02.301′	Faecal matter
	5	004° 48.442′	007° 02.303′	Waste water

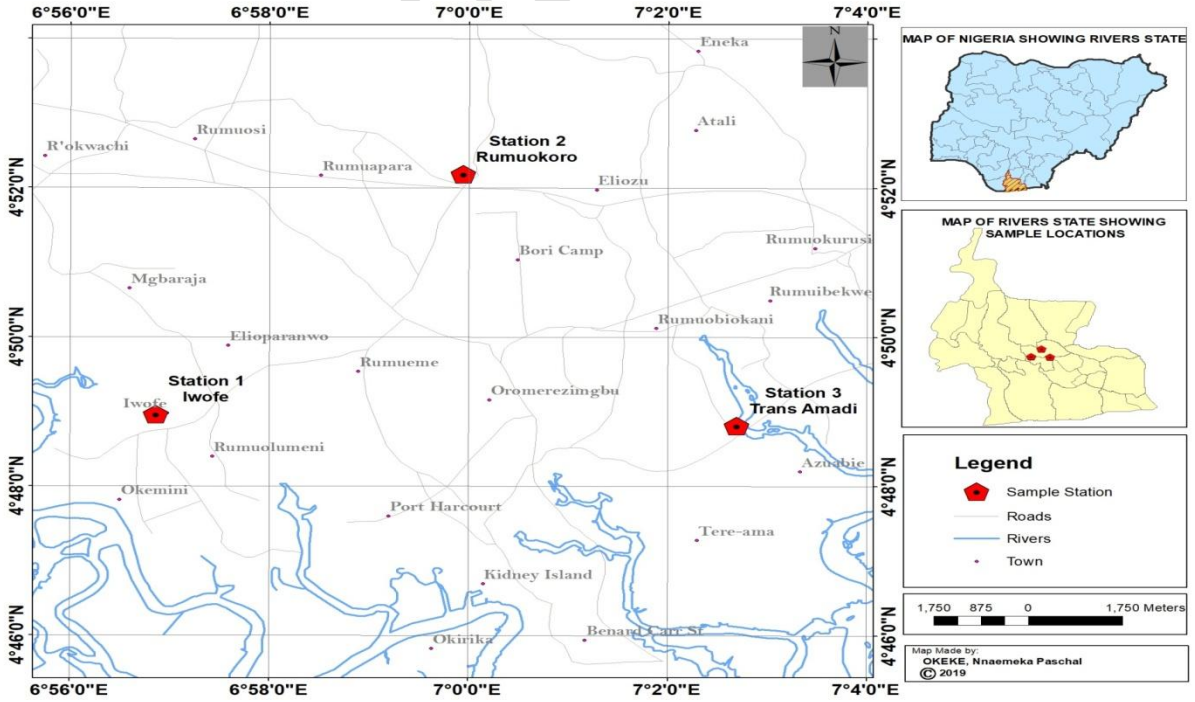

Figure 1: Map of Port Harcourt Metropolis showing the sampling points

Sample collection

Water samples used in servicing the abattoir was collected using sterile containers. The taps were allowed to run for 30 seconds from the water source before the samples were collected. The cow faecal matter was scooped from the intestine of the animal using sterile spatula and put in sterile sample bottles while waste blood samples from the cow was collected with sterile syringes as it gushed out through the vein during slaughtering. Blood for physicochemical analyses were collected using sterile 1 litre sample bottles. Wastewater samples were collected after the cow carcasses were washed using one litre sterile sample containers while composite soil samples (500 g) were collected in sterile sample bottles with the aid of a hand soil auger at 0-15cm depth.

Microbiological analyses

The samples were analyzed using standard microbiological procedures [32]. One milliliter of the water sample was added to 9 ml of saline solution and then a 10-fold serial dilution was done. The process was repeated for the blood samples. For the faecal samples, 1g was added to pre-sterilized and cooled 5 ml bacteriological peptone before serial dilution was carried out. Water samples were diluted to 10^{-3}, blood to 10^{-3}, wastewater, soil and faecal to 10^{-6}. These dilutions were determined after a pretest was carried out to determine the dilution at which the counts will be less than 300. Aliquots (0.1 ml) of various dilutions were transferred to prepared, cooled and surface-dried Nutrient agar plates.

Antibiogram of Bacterial isolates

The antibiotic sensitivity profile of the isolates was determined using the disc diffusion method. Multiple antibiotics sensitivity discs containing eight different antibiotics including Gentamicin (10µg), Ceftriaxone (30 µg), Erythromycin(5 µg), Cloxacillin (5 µg), Ofloxacin (5 µg), Augmentin (30 µg), Ceftazidime (30 µg), Cefuroxime (30 µg), Nitrofurantoin (300 µg), Cefixime (5 µg) and Ciprofloxacin (5 µg). on each ring were used. Mueller-Hinton agar was used as the culture medium for the tests [32]. The zones of inhibition were measured using a graduated metre rule, recorded in millimetres and the values compared with the CLSI standards to determine the level of resistance or susceptibility of the isolates to the antibiotics [33]

Molecular Identification

Molecular identification of the isolates that exhibited multiple antibiotics resistance was done using the Polymerase chain Reaction (PCR) to determine the 16S rRNA sequence. This was done by first extracting the DNA, carrying out the PCR and sequencing of the amplified DNA (amplicon).

Extraction (Boiling Method) and Quantification of DNA

An 18-hour old culture of the bacterial isolate was transferred to Luria Bertani (LB) and incubated overnight. From this overnight broth, 5 ml was transferred to an Eppendorf tube and spun for 3 minutes at 14000 rpm. The sediment which was made up of the cells were resuspended in 1 ml of normal saline and heated for 20 mins at 95 °C. This heated suspension was further cooled on ice and spun in a centrifuge at 14 rpm for 3 minutes. The supernatant which contained the DNA was transferred to a 1.5 ml microcentrifuge tube and kept at 20 °C pending further reactions [34]. Using a Nanodrop 1000 spectrophotometer, the extracted genomic DNA was quantified.

Amplification of 16S rRNA

Using the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') primers on an ABI 9700 Applied Biosystems Thermal cycler, the 16S rRNA region of the rRNA of the bacterial isolates were amplified. A final volume of 50 µl was used for 35 cycles in the reaction process. The PCR mix was made up of 0.4 nM of primers, X2 Dream taq Master mix (Inqaba, South Africa) and the template which was the extracted DNA (50 ng). The Master mix was made up of Magnesium Chloride ($MgCl_2$), taq DNA polymerase and dNTPs (Deoxyribonucleotides). The conditions for the reaction were Initial denaturation at 95 °C for 5 minutes, denaturation at 95 °C for 30 seconds, annealing at 52 °C for 30 seconds, extension at 72 °C for 30 seconds and final extension

at 72 °C for 5 minutes. Resolution of the product was done on a 1% agarose gel for 15 minutes and viewed on a UV transilluminator. Sequencing of the amplified DNA was using the BigDye Terminator kit on a 3510 ABI sequencer (Inqaba Biotechnogical, Pretoria, South Africa).

Detection of Resistance Genes

Using the SHV F: 5' CGCCTGTGTATTATCTCCCT-3' and SHV R: 5'-CGAGTAGTCCACCAGATCCT-3' primers, *bla*SHV genes from the isolates were amplified on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 µl for 35 cycles. The resultant product was resolved on a 2 % agarose gel at 90V for 25 minutes and visualized on a UV transilluminator for a 281 bp product size. The same procedure was carried out but CTX-MF: 5'-CGCTTTGCGATGTGCAG-3' and CTX-MR: 5'-ACCGCGATATCGTTGGT-3' primers were used for CTX-M gene and TEMF: 5'-ATGAGTATTCAACATTTCCGTG-3' and TEMR: 5'-TTACCAATGCTTAATCAGTGAG-3' primers for *bla*TEM gene [35]. Sizes of resolved products were 281 bp, 550 bp and 850 bp for *bla*SHV, *bla*CTX-M and *bla*TEM, respectively.

Results

The results of the multiple drug sensitivity test for the Gram negative isolates are presented in Figure 2, showing that the isolates exibited 100% resistance to Cefuroxime and a corresponding 100% susceptibilty to Ofloxacin. The Gram positive isolates exihibited 100% susceptibility to Ofloxacin and 100% resistance to Ceftazidime, Cefuroxime and Augmentin (Figure 3). Table 2 shows the microbes that exihibited multidrug resistance and the drugs were resistant to. *Bacillus amyloliquefaciens, Bacillus flexus* and *Proteus mirabilis* were resistant to Ceftazidime, Cefuroxime and Augmentin. Plates 1, 2 and 3 shows the agarose gel electroporesis for the amplified *bla*CTX-M, *bla*SHV and *bla*TEM genes respectively. The occurrence of resistance genes among the isolates is presented in table 3. *Escherichia coli* strain 2017C-4109 had the *bla*CTX-M gene, *Pseudomonas* sp. strain 6174 had the *bla*SHV and *bla*TEM genes, *Bacillus amyloliquefaciens* had the *bla*SHV gene, *B. flexus* had the *bla*TEM genes, *Staphylococcus aureus* had *bla*SHV and *bla*TEM genes, *Proteus mirabilis* had the *bla*CTX-M and *bla*TEM genes while *Klebsiella* sp strain EIKU11 possessed all three resistance genes.

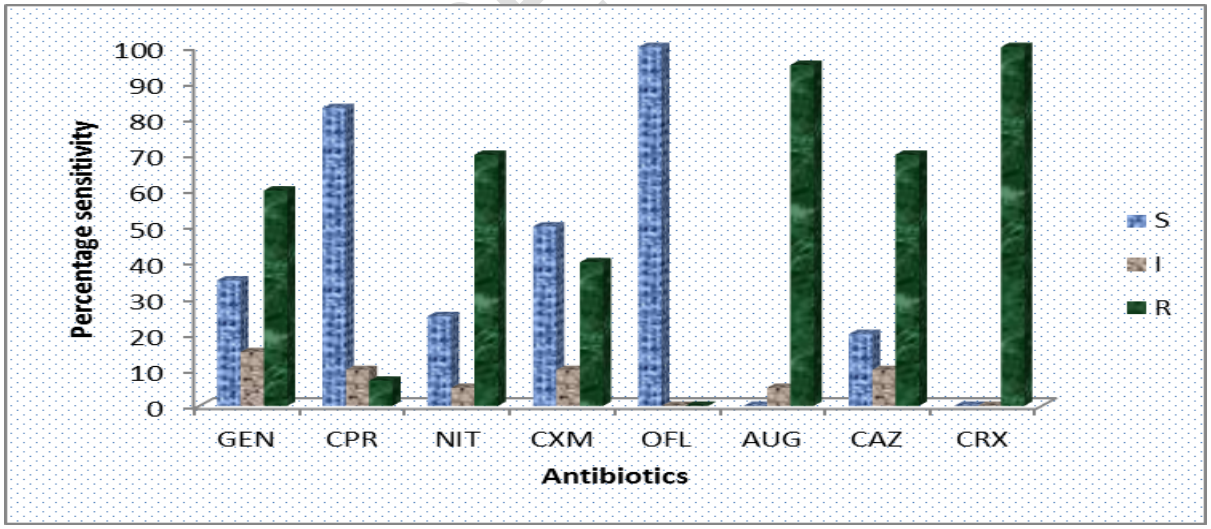

Figure 2: Multiple drug sensitivity test for the Gram negative isolates

Key: S - sensitive, I - Intermediate, R- Resistant, GEN- Gentamicin (10µg), OFL- Ofloxacin (5 µg), AUG- Augmentin (30 µg), CAZ- Ceftazidime (30 µg), CRX-Cefuroxime (30 µg), NIT- Nitrofurantoin (300 µg), CXM- Cefixime (5 µg) and CPR-Ciprofloxacin (5 µg)

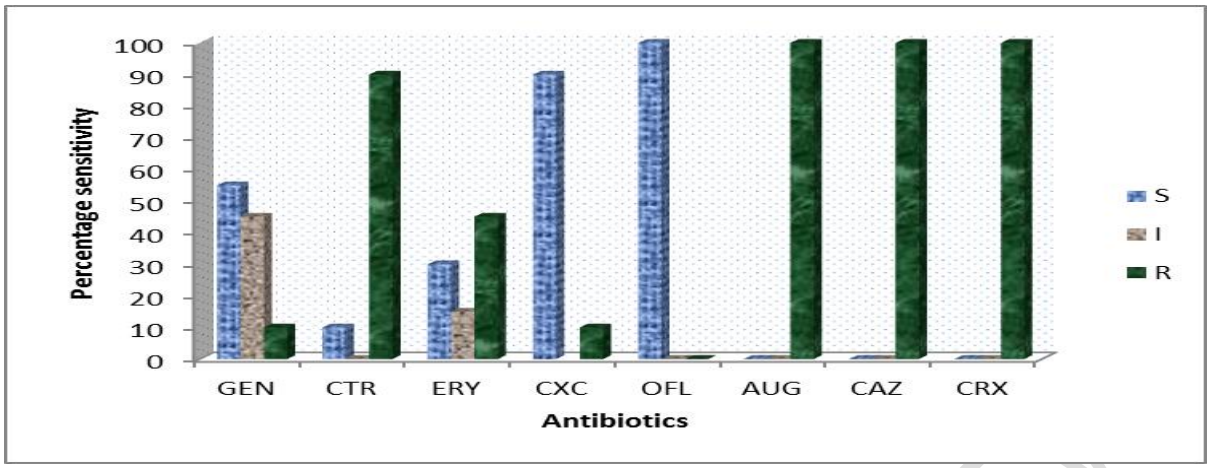

Figure 3: Multiple drug sensitivity test for the Gram positive isolates

Key: S - Sensitive, I - Intermediate, R- Resistant, GEN- Gentamicin (10µg), OFL- Ofloxacin (5 µg), AUG- Augmentin (30 µg), CAZ- Ceftazidime (30 µg), CRX-Cefuroxime (30 µg), NIT- Nitrofurantoin (300 µg), CXM- Cefixime (5 µg) and CPR-Ciprofloxacin (5 µg)

Table 2: Resistance pattern of bacterial isolates

Bacteria	Antibiotics
***Escherichia coli* strain 2017C-4109**	CAZ
***Klebsiella pneumoniae* strain K20**	CAZ
***Bacillus amyloliquefaciens* strain WU-12**	CAZ, CRX, AUG
***Pseudomonas* sp. strain 6174**	CAZ
***Escherichia coli* strain SAMA_EC**	CAZ
***Bacillus flexus* isolate Murraya**	CAZ, CRX, AUG
***Klebsiella* sp. strain EIKU11**	CAZ
***Proteus mirabilis* strain 46X4**	CAZ
***Staphylococcus aureus* strain NCIM2654**	CAZ, CRX, AUG

Key: CAZ- Ceftazidime (30 µg), CRX-Cefuroxime (30 µg), AUG- Augmentin (30 µg)

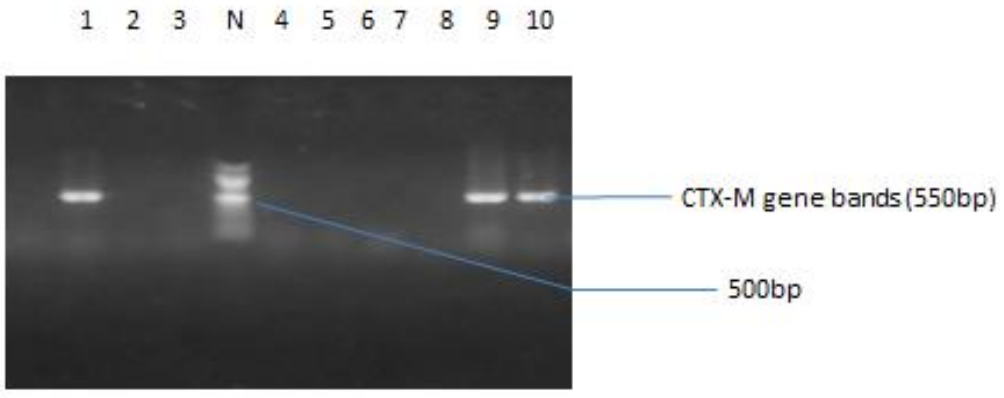

Plate 1: Agarose gel electrophoresis of *bla*CTX-M (560bp) gene of the bacterial isolates. Lanes 1, 9 and 10 sowed *bla*CTX-M (550 bp). Lane L represents a 100bp molecular ladder.

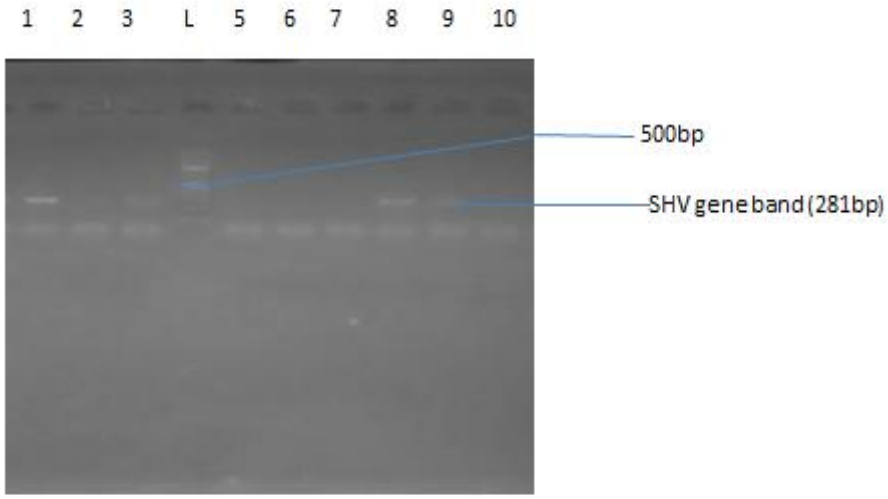

Plate 2: Agarose gel electrophoresis of *bla*SHV gene of the bacterial isolates. Lanes 1,3,8 and 9 showed *bla*SHV (281) bands. Lane L represents a 100bp molecular ladder.

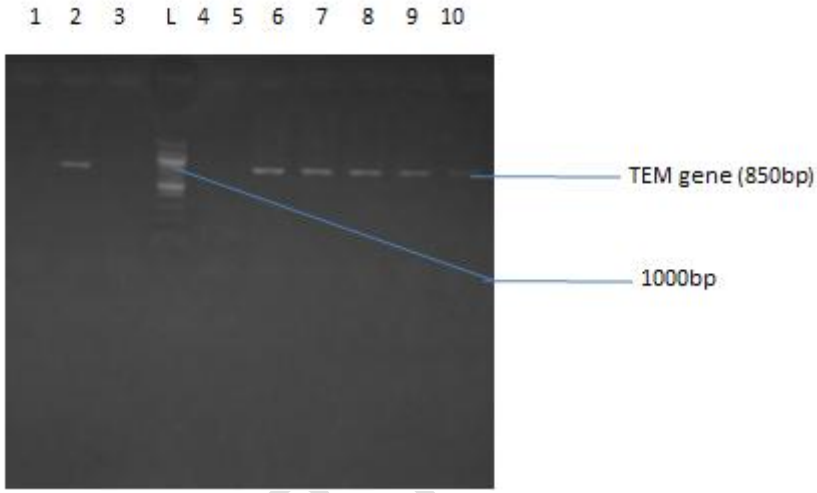

Plate 3: Agarose gel electrophoresis of *bla*TEM (960bp) gene of the bacterial isolates. Lanes 2,5-11 represent *bla*TEM gene bands Lane L represents a 100bp molecular ladder.

Table 3: Occurrence of Resistance genes among the bacterial isolates

Isolate	*bla*CTX-M	*bla*SHV	*bla*TEM
Escherichia coli strain 2017C-4109	+	+	-
Pseudomonas sp. strain 6174	-	+	+
Bacillus amyloliquefaciens strain WU-12	-	+	-
Klebsiella pneumoniae strain K20	-	-	-
Klebsiella pneumoniae strain K20	-	-	
Bacillus flexus isolate *Murraya*	-	-	+
Escherichia coli strain SAMA_EC	-	-	+
Staphylococcus aureus strain NCIM2654	-	+	+
Klebsiella sp. strain EIKU11	+	+	+
Proteus mirabilis strain 46X4	+	-	+

Discussion

Microorganisms isolated in this study were of the genus *Staphylococcus, Pseudomonas, Klebsiella, Proteus* and *Bacillus.* Similar organisms had been isolated from abattoir environments by some authors [36-39]. These microorganisms have been implicated as pathogens of various infections including diarrhoea, bacteremia, dysentery, urinary tract infections in humans which makes them of public health importance [40]). This is especially important as several persons visit these abattoirs daily to purchase meat and its products and are thus predisposed to the health risks associated with

these organisms to humans. Multiple antibiotics sensitivity testing of the bacterial isolates showed they exhibited a high level of susceptibility to Ofloxacin (a quinolone) and a corresponding resistance to β-lactam drugs including Ceftaxidime, Cefuroxime and Augmentin. Drugs with lower Minimum Inhibitory Concentrations (MICs) such as Ofloxacin and Cloxacillin exhibited lower inhibitory activity against the isolates compared with those with higher MICs such as Augmentin which may be attributed to the frequent use of these antimicrobial drugs for treatment of infections. Also, this trend has been reported by Adesoji *et al.* [41] and Harrison and Bratcher [42], who studied the susceptibility of some microorganisms from abattoir sources to some drugs including Augmentin and cefuroxime. This poses a threat as treatment against infections caused by these organisms becomes difficult or may take a longer time to respond against the causative agent [39]. Microbial drug resistance has been reported to be linked with mechanisms such as inappropriate use of the drugs, widespread use of antibiotics in the production of animal feeds, its use in treatment of infections and for prophylaxis, excretion of metabolized or non-metabolized administered drugs by animals into the environment which undergo transformation into their active forms ([43]; [19]; [44], [45]).

The resistance genes checked for in this study were *bla*SHV and *bla*TEM which are genes coding for the production of β-lactamases- enzymes able to deactivate penicillin drugs and *bla*CTX-M gene coding for the inactivation of extended spectrum β-lactam drugs, which are 3rd and 4th generation cephalosporins [26]. The presence of these genes in enteric bacteria such as *E. coli* and *Klebsiella* sp. in food-producing livestock such as cows, poses a public health risk as these organisms are discharged into the environment. Spread of resistance genes through horizontal gene transfer to human pathogens may occur, thereby complicating antibacterial therapy when infection occurs [27]. Production of β-lactamase has been noted as the commonest mechanism of resistance to 3rd and 4th generation cephalosporins among enteric bacteria [28]. Studies on animal faecal samples in Ado-Ekiti, Nigeria, revealed that *bla*CTX-M, *bla*SHV and *bla*TEM were detected in the isolated chromosomal DNA of *E. coli* [46]. Detection of TEM gene was also reported by Igbinosa and Obuekwe [47] in abattoir samples. *bla*TEM and *bla*CTX-M have been isolated from abattoir samples as observed in this study [48]. These animals may thus, become reservoirs of β-lactamases thereby contributing to the increased rate of antibiotic resistance.

Conclusion

This study recorded the presence of *bla*CTX-M, *bla*SHV and *bla*TEM genes that code for antibiotic resistance in microorganisms isolated from abattoir environments. These microorganisms exhibited multi-drug resistance to popularly consumed antibiotics including Augmentin and Ceftazidime and Cefuroxime. Spread of microorganisms carrying these genes can be reduced by carrying out adequate sanitary conditions in abattoir environments. From the results obtained in this study, antibiotics resistant bacteria are widespread as nearly all the isolated microorganisms were resistant to most of the antibiotics for which they were tested for. This may be due to either the intrinsic resistance of many microorganisms to antibiotics or acquired resistance of the organisms enabled by the transfer of resistance of drug resistance plasmids among members of the isolates. Since antibiotics in animal feed promote animal growth, improved efficiency of feed conversion to body weight, and may also affect disease prophylaxis among the confined microbes in such animals and their subsequent impact on human health, it has increased its indiscriminate use [48].

Ethical: NA
Consent: NA

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