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ANTIMICROBIAL RESISTANCE AMONG BACTERIAL STRAINS RECOVERED FROM NON-STERILE PHARMACEUTICAL PRODUCTS

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ABSTRACT: *Antibiotic resistance happens to be the main clinical and public health problem within the life time of most people living today, and the sources of antibiotic resistance bacteria is increasing in addition to the clinical and environmental sources. In this study, the presence of antibiotic resistance among bacterial strains previously recovered from non-sterile pharmaceutical products was evaluated using Kirby-Bauer disk diffusion method and PCR detection of antibiotic resistance genes; bla-SHV, bla-CTX-M, bla-TEM and class 1, 2 and 3 integrase genes. The bacterial strains were also screened for the presence of plasmids. The tested bacterial strains exhibit high-level resistance to sulphamethoxazole/trimethoprim (89.5%), amoxycillin/clavunanic (68.4%), and cefixime (52.6%). However, they were highly susceptible to norfloxacin (74%). Resistant bacterial strains habours one or more of the ESBL, integrase genes and plasmids. blaTEM, blaCTX, class 1 integron and plasmids were present in 10 (52.6%), 03 (15.8%), 09 (47.4%) and 11 (57.9%) of the tested bacterial strains respectively. Findings of the study have shown that contaminated non-sterile pharmaceutical products are a potential source for propagation and dissemination of resistant bacterial strains and genes in the environment.*

KEYWORDS: Antimicrobial Resistance, Non-Sterile Pharmaceutical Products, Plasmids, Environment.

INTRODUCTION

Pharmaceutical drugs of different forms are susceptible to microbial contamination during manufacturing, marketing, handling, and use (Oviasogie *et al*., 2015). Use of such kind of products may present potential health hazards to patients (Essam *et al*., 2013), may cause spoilage of the products or even convert the drugs to toxic products (Al-Charrakh, 2012; Ratajczak *et al*., 2015).

Moreover, contaminated pharmaceutical products may also harbour drug-resistant microbial strains, as was seen in *Bacillus* and *Staphylococcus* species recovered from oral and topical medicamentsshowing resistance to beta-lactam antibiotics, trimethoprim-sulfamethoxazole, ampicillin, amoxicillin, and tetracyclines (Al-Charrakh, 2012). Similarly, Daniyan and Sangodere, (2011) reported the isolation of multi-drug resistant *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* from pharmaceutical drug syrups preparations obtained from patent medicine stores in Minna metropolis, Nigeria. Moreover, the presence of multidrug-resistant bacteria *Escherichia coli* was isolated from different brands of paracetamol syrups marketed in Nigeria (Osungunna *et al.*, 2016).

Bacillus and *Klebsiella* species recovered from contaminated oral drugs in Tanzania were resistant to Augmentin and cloxacillin (Mugoyela and Mwambete, 2010). Furthermore, Fatema (2014) described the proliferation of drug-resistant bacteria such as *Klebsiella* spp., *Pseudomonas* spp. And *E. coli* within the pediatric drug samples tested in Bangladesh. Besides, Kabir and Hossain (2013) reported the presence of antibiotic-resistant *Escherichia coli* from vitamin B syrup samples in Bangladesh.

The presence of antibiotic-resistant bacteria in clinical and environmental sources have been widely reported. However, only a few studies have described the presence of antimicrobial resistance in bacteria isolated from pharmaceutical products with little or no studies on the molecular detection of antimicrobial resistance genes. The identification of antimicrobial resistance genes is essential for understanding the underlying mechanisms and the epidemiology of antimicrobial resistance (Zankari *et al.* 2012). This study, therefore, will examine the possibility of contaminated pharmaceuticals contributing to the problem of antimicrobial resistance, and it will highlight the possible role microbial contaminants play in the dissemination of antimicrobial resistance by studying the presence of resistance genes and plasmid profiling among the isolated microbial contaminants.

MATERIAL AND METHODS

Test Organisms

Nineteen (19) bacterial strains previously recovered as contaminants in non-sterile oral pharmaceutical formulations were employed for the study. On the basis of the phylogenetic analysis conducted, the isolates represent bacterial strains of the genus *Pseudomonas, Enterobacter, and Bacillus spp* whose sequences (16S rRNA gene partial sequences) were submitted to the NCBIGenBank, the sequences were accepted with accession numbers assigned.

Assessment of Antimicrobial Susceptibility Profile

Antimicrobial susceptibility of the bacterial isolates was determined using the Kirby-Bauer disk diffusion method on Mueller–Hinton agar plates, according to guidelines established by the National Committee for Clinical Laboratory Standards (CLSI, 2014). A panel of six (6) antibiotic discs representing atleast five (5) different classes were used. The antibiotics used were Sulphamethoxazole/Trimethoprim (25µg), Tetracycline (30µg), Norfloxacin (10µg), Cefepime (30µg), Cefixime (5µg) and Amoxycillin-Clavulanic Acid (30µg) Oxoid, UK. Multi-drug resistant strains were identified based on their resistance to at least three or more classes of antibiotics tested.

Molecular Detection of Drug-Resistant Genes

The presence of some antibiotic resistance genes such as β-lactamase resistance genes (*bla*-SHV, *bla*-CTX-M, *bla*-TEM) and class 1, 2 and 3 integrase genes was evaluated by polymerase chain reaction.

DNA Extraction

Genomic DNA was extracted from overnight cultures by picking a single, discrete colony of each organism and inoculated into a freshly prepared nutrient broth; the broth was incubated at 37°C for 12-14 hours. After the incubation period, the DNA was extracted using the Boiled lysis method (Lindsey *et al.,* 2017).

The concentration and purity of the extracted genomic DNA was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Uniplex PCR for the Detection of β-lactamase Resistance Genes

A 25 µl reaction mixture which consists of 12.5 µl of One Tag Quick-Load 2X Master Mix with standard buffer, 1µl each of the Forward and Reverse primers, 2 µl of the extracted DNA (DNA template), and then 8.5 µl of Nuclease-Free Water was prepared. A negative control, which consists of Master Mix, Primers and the Nuclease-Free Water, was used throughout the PCR assay. After this, the DNA amplification was conducted in a thermocycler (BIO-RAD, Applied Biosystems USA).

The forward and reverse primers used for the β-lactamase genes and the PCR conditions employed are presented in Table 1. After PCR amplification of the genes, agarose gel electrophoresis was carried out using 1.0% agarose, the PCR products were visualized using a Benchtop UV-Transilluminator (BioDoc-It 220 Imaging System, USA) and the targeted genes were identified based on their different sizes.

Table 1: Primers and PCR Conditions Used for the Detection of β-lactamase Genes

ESBL genes	Primer Sequence	Size	PCR Conditions
*bla*SHV	F-5'GCGAAAGCCAGCTGTCGGGC-3' R-5'GATTGGCGGCGCTGTTATCGC-3'	538bp	1min denaturation at 95°C, 30 cycles at 96°C for 30s, Annealing at 54°C for 30s, extension at 72°C for 30 s and a final extension at 72 °C for 10min.
*bla*CTX M	F-5' GTGCAGTACCAGTAAAGTTATGG-3' R-5'CGCAATATCATTGGTGGTGCC-3'	538bp	1min denaturation at 95°C, 30 cycles at 96°C for 30s, Annealing at 40°C for 30s, extension at 72°C for 30 s and final extension at 72°C for 10min.
*bla*TEM	F-5'AAAGATGCTGAAGATCA-3' R-5'TTTGGTATGGCTTCATTC-3'	425bp	1min denaturation at 95°C, 30 cycles at 96°C for 30s, Annealing at 44°C for 30s, extension at 72°C for 30 s and a final extension at 72°C for 10min.

(Ayodele et al., 2016)

Multiplex PCR for the Detection of Integrase Genes

The presence of class 1, 2 and 3 integrons among the bacterial strains was tested by multiplex polymerase chain reaction (PCR). The primers specific for selected Integrase genes included; Intl-1 (F-GGT CAA GGA TCT GGA TTT CG) and (R-ACA TGC GTG TAA ATC ATC GTC), Intl-2 (F-CAC GGA TAT GCG ACA AAA AGG) and (R-TGTA GCA AAC GAG TGA CGA AAT G) and Intl-3 (F-AGT GGG TGG CGA ATG AGT G) and (R-TGT TCT TGT ATCGGC AGG TG) with the following sizes 436bp, 788bp and 600bp respectively (Kargar, *et al.*, 2014). A 25 µl reaction mixture which consists of 12.5 µl of One Tag Quick-Load 2X Master Mix with standard buffer, 6 µl (1µl each) of the Forward and Reverse primers, 2 µl of the extracted DNA (DNA template), and then 4.5 µl of Nuclease-Free Water was prepared. A negative control, which consists of Master Mix, Primers and the Nuclease-Free Water, was used throughout the PCR assay.

Plasmid Profiling

The bacterial strains were screened for the presence of plasmids.in order to relate the number and size of plasmids with antibiotic resistance patterns of the bacterial strains. Plasmid DNA was extracted using the alkaline lysis method as described by Delaney *et al.* (2018) and Roy *et al.* (2018). Extracted plasmids were loaded together with 10µl loading dye and 5µl of 23kb marker. The gel was run on 1.0% agarose gel electrophoresis at 90V for 50 minutes. After this, the gels were visualized using a Benchtop UV-Transilluminator (BioDoc-It 220 Imaging System, USA).

RESULTS

The antimicrobial susceptibility profile among the contaminating bacterial strains showed varying susceptibility patterns to the tested antibiotics. The bacterial strains demonstrate high-level resistance to sulphamethoxazole/trimethoprim (89.5%) and showed high resistance against amoxicillin-clavunanic acid (68.4%), cefixime (52.6%), tetracycline (36.8%),and cefepime (31.6%) however, the organisms were significantly susceptible to norfloxacin (73.7%) with only 26.3% being resistant to the drug. The percentage resistance of the isolates to the tested antimicrobials is presented in Figure 1.0.

Of these, 14 (73.7%) of the isolates were identified as being Multi-Drug Resistant. The ESBL genes; *bla*TEM and *bla*CTX were present in 10 (52.6%) and 03 (15.8%) of the isolates respectively, while *bla*SHVgene was not detected. The combination of the two genes, *bla*TEM + *bla*CTX was observed in three (03) isolates, as shown in Table 2.

Moreover, result of the multiplex PCR for the detection of the integrase genes showed that 09 (47.4%) of the 19 bacterial strains were identified as being positive for class 1 integron, whereas, class 2 was positive in only two (10.5%) of the bacterial strains while class 3 integron was not absent in all the isolates. The occurrence of both class 1 and 2 integrons was observed in only one isolate while the combination of classes 1 and 3 was observed in three of the MDR isolates as presented in table 2.

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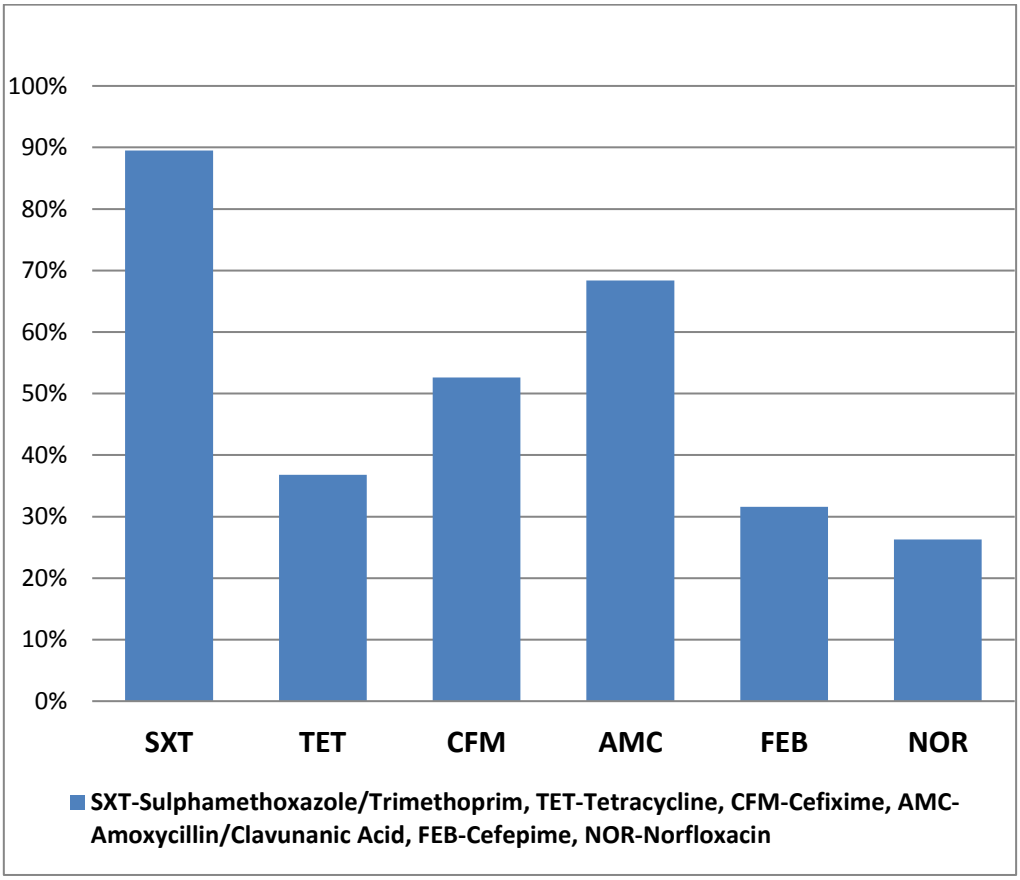

Figure 1: Percentage Resistance of the Bacterial Strains to the Tested Antibiotics

Table 2: Antibiotic Resistance Patterns and Distribution of β-lactamase and Integrase Gene Among the Bacterial Strains

Bacterial strains (Accession number)	Resistant phenotypes	bla TEM	bla CTX	Int-1	Int- 2
Pseudomonas aeruginosa strain MMM070 (MN620435)	SXT, AMC, FEB, CFM	+	-	+	+
Pseudomonas aeruginosa strain MMM221 (MN620436)	TET, SXT, AMC	+	-	+	-
Pseudomonas aeruginosa strain MMM232 (MN620437)	SXT, AMC, NOR, CFM	+	+	+	-
Pseudomonas aeruginosa strain MMM219 (MN620438)	TET, SXT, AMC, NOR,CFM	+	+	+	-
Pseudomonas aeruginosa strain MMM234 (MN620439)	SXT, AMC, NOR, FEB	+	-	+	-

Enterobacter spp strain MMM090 (MN620440)	TET, SXT, CFM	-	-	+	-
Enterobacter cloacae strain MMM060 (MN759461)	TET, SXT, AMC	-	-	-	-
Enterobacter cloacae strain MMM083 (MN620441)	AMC	-	+	-	-
Enterobacter cloacae strain MMM120 (MN620442)	TET, SXT, AMC, FEB	+	-	+	-
Enterobacter hormaechi strain MMM059 (MN620443)	SXT, NOR, FEB	+	-	-	-
Enterobacter spp strain MMM061 (MN620444)	SXT, AMC, FEB	-	-	-	+
Pluralibacter gergoviae strain MMM242 (MN620445)	SXT, AMC, NOR, CFM	+	-	+	-
Enterobacter spp strain MMM245 (MN620446)	TET, SXT	-	-	-	-
Pluralibacter gergoviae strain MMM246 (MN620447)	SXT	-	-	-	-
Sporosarcina luteola strain MMM055 (MN620448)	SXT, CFM, FEB	-	-	-	-
Bacillus cereus strain MMM008 (MN620449)	SXT, AMC, FEB	+	-	+	-
Bacillus cereus strain MMM022 (MN620450)	SXT, AMC, CFM	+	-	-	-
Bacillus subtilis strain MMM073 (MN620451)	TET, SXT, AMC, CFM	-	-	-	-
Bacillus subtilis strain MMM078 (MN620452)	CFM	-	-	-	-
	Total	**10 (52.6%)**	**03 (15.8%)**	**09 (47.4%)**	**02 (10.5%)**

SXT-Sulphamethoxazole/Trimethoprim, TET-Tetracycline, CFM-Cefixime, AMC-Amoxycillin/Clavunanic Acid, FEB-Cefepime, NOR-Norfloxacin.

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Plasmid profiling and antibiotic resistance patterns

The presence of plasmids was observed in 11 (57.9%) of the tested bacterial strains,carrying one plasmid, each with a molecular weight size of approximately 23 kb (Figure 2). Plasmid profiling and its relation with the antibiotic resistance patterns of the isolates are shown in table 3.

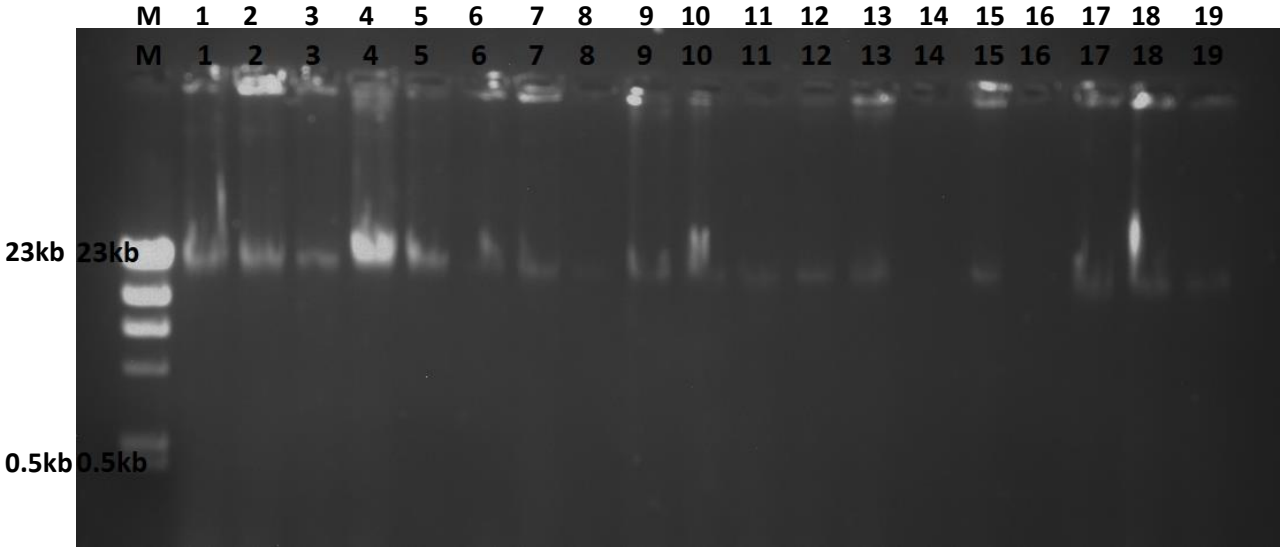

Figure 2: Agarose Gel Electrophoresis of Plasmid DNAshowing Plasmid Bands at Approx 23kb.

Lane M = Marker (0.5kb to 23kb)
Lanes 1, 2, 3, 4 and 5 = *Pseudomonas aeruginosa strains*
Lanes 6 and 7 = *Enterobacter cloacae* strains
Lane 14 = *Pluralibacter gergoviae* strain
Lane 17 = *Bacillus cereus* strain
Lane 18 = *Bacillus subtilis* strain

Table 3: Plasmid profiling and antibiotic resistance patterns among the bacterial *strains*

Bacterial strains (Accession number)	Resistant phenotypes	Plasmid	No./Size
Pseudomonas aeruginosa strain MMM070 (MN620435)	SXT, AMC, FEB, CFM	+	1 (23kb)
Pseudomonas aeruginosa strain MMM221 (MN620436)	TET, SXT, AMC	+	1 (23kb)
Pseudomonas aeruginosa strain MMM232 (MN620437)	SXT, AMC, NOR, CFM	+	1 (23kb)
Pseudomonas aeruginosa strain MMM219 (MN620438)	TET, SXT, AMC, NOR,CFM	+	1 (23kb)
Pseudomonas aeruginosa strain MMM234 (MN620439)	SXT, AMC, NOR, FEB	+	1 (23kb)
Enterobacter spp strain	TET, SXT, CFM	-	-

MMM090 (MN620440)				
Enterobacter cloacae	strain			
MMM060 (MN759461)		TET, SXT, AMC	+	1 (23kb)
Enterobacter cloacae	strain			
MMM083 (MN620441)		AMC	-	-
Enterobacter cloacae	strain			
MMM120 (MN620442)		TET, SXT, AMC, FEB	+	1 (23kb)
Enterobacter hormaechi	strain			
MMM059 (MN620443)		SXT, NOR, FEB	+	1 (23kb)
Enterobacter spp	strain			
MMM061 (MN620444)		SXT, AMC, FEB	-	-
Pluralibacter gergoviae	strain			
MMM242 (MN620445)		SXT, AMC, NOR, CFM	+	1 (23kb)
Enterobacter spp	strain			
MMM245 (MN620446)		TET, SXT	-	-
Pluralibacter gergoviae	strain			
MMM246 (MN620447)		SXT	-	-
Sporosarcina luteola	strain			
MMM055 (MN620448)		SXT, CFM, FEB	-	-
Bacillus cereus	strain			
MMM008 (MN620449)		SXT, AMC, FEB	+	1 (23kb)
Bacillus cereus	strain			
MMM022 (MN620450)		SXT, AMC, CFM	-	-
Bacillus subtilis	strain			
MMM073 (MN620451)		TET, SXT, AMC, CFM	+	1 (23kb)
Bacillus subtilis	strain			
MMM078 (MN620452)		CFM	-	-
		Total	**11 (57.9%)**	

SXT-Sulphamethoxazole/Trimethoprim, TET-Tetracycline, CFM-Cefixime, AMC-Amoxycillin/Clavunanic Acid, FEB-Cefepime, NOR-Norfloxacin.

DISCUSSION

In recent times, antibiotic resistance happens to be the main clinical and public health problem within the life time of most people living today (Essam *et al*., 2012), and the presence of antibiotic resistance bacteria residing from various sources is increasing.

As with other few studies of antimicrobial resistance from contaminated pharmaceutical products other than from clinical and environmental sources, this study has detected the presence of antimicrobial resistance among bacteria recovered from contaminated pharmaceutical products. This may indicate how widespread antibiotic resistance among bacteria isolated from different sources is, including those of clinical and environmental origin (Al-Charrakh, 2012). The bacterial contaminants exhibit a different level of resistance, including multidrug resistance to the various antibiotics tested. Most notably was the high rate of resistance to Sulphamethoxazole/Trimethoprim and resistance to the β-lactam antibiotics (amoxycillin/clavunanic, cefixime, and cefipime).

Resistance to β-lactam antibiotics was evident by the detection of ESBL genes among the tested bacterial strains. In this study, all the strains of *Pseudomonas aeruginosa, and some strains of Enterobacter, and Bacillus* possess at least one of the beta-lactamase resistance genes tested; with bla-TEM being the most abundant among the isolates. *bla*-TEM gene was the first described *bla* gene, previous studies have reported the gene as the most frequently detected compared to other *bla*genes (Luzzaro *et al*., 2006; Spanu *et al.,* 2002 and Adesoji *et al*., 2016). Resistance to β-lactam antibiotics is an increasing problem,and the production of ESBL genes is the most widespread mechanism of drug resistance, especially in Gram-negative bacilli. ESBLs have been most frequently reported in Enterobacteriaceae worldwide; however, the enzymes were also found in *Pseudomonas spp.* and *Acinetobacter baumannii* (Dallenne *et al.*, 2010).

Various integron classes habouring different integrase genes that are known to be associated with antibiotic resistance have been identified (Kargar *et al.*, 2014). In this study, the presence of the integrase genes has been detected, with class 1 integrons being the most abundant. Similar observation on the high prevalence of class 1 integron over the other classes was reported by other studies (Kargar *et al*., 2014, Adesoji *et al.*, 2016, Kheiri and Akhtari, 2016). In many Gram-negative bacteria including *Acinetobacter, Vibrio, Aeromonas, Proteus, Burkholderia, Alcaligenes, Campylobacter, Enterobacter, Citrobacter, Klebsiella, Mycobacterium, Pseudomonas, Serratia, Salmonella, Shigella, and Escherichia*, the presence of class 1 integrons have been reported (Kargar *et al.*, 2014).

Integrons allow the shuffling of smaller mobile elements called gene cassettes (Kheiri and Akhtari, 2016). These gene cassettes contain many genes that codes for different antibiotic resistance, hence, conferring multiple drug resistance traits to a single bacterium possessing it. Most of the genes responsible for resistance to aminoglycosides, β-lactams and sulpha drugs have been reported as gene cassettes in association with class 1 integron (Adesoji *et al*., 2016). This explains the reason for the high rate of resistance to sulphamethoxazole/trimethoprim, and the β-lactam antibiotics (amoxycillin/clavunanic acid, cefixime and cefipime) observed among the bacterial srains. More so, Kargar *et al.* (2014) detected a substantial correlation between class 1 integron and resistance to amikacin, gentamicin, chloramphenicol, ampicillin, tetracycline, nalidixic acid, and co-trimoxazole.

Plasmid profiling is essential in epidemiological surveillance of diseases and investigation of the antimicrobial resistance (Guo *et al.*, 2012). The presence of plasmids, as observed in this study, implies the possibility of the horizontal transfer of the beta-lactamase and integrase genes between different bacterial strains and subsequent dissemination of antimicrobial resistance factors in the environment.

This study could be the first report describing the presence of antimicrobial resistance genes among bacteria from non-sterile pharmaceutical oral drug preparations. This could add to the antimicrobial resistance data and improve the understanding of the epidemiology of antimicrobial resistance broadly.

CONCLUSION

This study had demonstrated that bacterial contaminants exhibit antimicrobial resistance and accordingly, contaminated non-sterile pharmaceutical products are a potential source for propagation and dissemination of resistant bacterial strains and genes in the environment.

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