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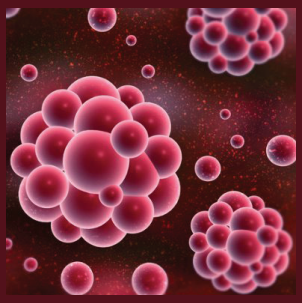
Research Article: Antibiotics Resistant Genes and Plasmid curing of Multidrug Resistant Clinical Bacterial Isolates from the Three Geographical Regions of Ondo State, Nigeria



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Antibiotics Resistant Genes and Plasmid curing of Multidrug Resistant Clinical Bacterial Isolates from the Three Geographical Regions of Ondo State, Nigeria

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ABSTRACT

This study shows the characteristics of multidrug resistant clinical isolates analyzed for their antibiotic resistant genes in laboratory. Genomic DNA was then extracted from each sample using Column-pure Bacteria Genomic DNA Isolation kit. Similarly, plasmid profile, plasmid curing and the antibiotic susceptibility profile of thirty two (32) cultured multidrug resistant clinical isolates were analyzed for their antibiotic resistant genes. The bacterial species tested are *Acinetobacter haemolyticus, Budricia aquatic, Burkhoderia cepacia, Citrobacter gilleric, Enterobacter gergoriac,Enterobacter agglomerane, Escherichia coli, Escherichia hermamii, Klebsiella oxytoca, Klebsiella ornithinolytica, Klebsiella terrigena, Pseudomonas aeruginosa, Pseudomonas fluorescens, Staphylococcus spp., Staphylococcus aureus* and coagulase –ve, *Staphylococcus* spp. Nutrient agar and Mueller Hinton Agar were used routinely during the study. The genomic test done on the thirty two (32) multidrug resistant strains did not show any resistant gene. But, the molecular plasmid profile conducted shows that all the isolates possess plasmid. Plasmid curing done reduces the antibiotics resistance nature of the isolates. This shows that the resistance of the isolates were plasmid mediated. The re-assessment of the antibiotic susceptibility profile of the clinical bacterial isolates tested confirmed this. It was also clarified in this study that most of the selected resistant bacterial strains isolated from hospitals were susceptible to medicinal plants such as *Moringa oleifera and Andrographis peniculata* tested on them. This implies that the use of appropriate medicinal plants can be optimized for pharmaceutical purpose to overcome infections caused by these aetiologic agents.

Keywords: Antibiotics resistant genes; Clinical bacterial isolates; Genomic analysis; Nigeria; Plasmid curing

Introduction

Antibiotic resistance has increasingly created health threat with related ecological implications around the world. This has resulted to resistance to commonly used antibiotics which were earlier effective in the treatment of various infections thus limiting their usage. Antibiotic resistant organisms have been isolated from health facilities and aquaculture sources in different parts of the world (Agoba *et al.*, 2017). According to Levy (2002), a major feature contributing to the dissemination of antibiotic resistance is the ability of the resistance genes to move into other bacteria by a variety of genetic means. One transfer mechanism is by plasmids, extrachromosomal elements that can move genes between bacteria of vastly different evolutionary backgrounds, including transfer between Gram-positive and Gram-negative bacteria. There are bacteriophages that can deliver chromosomal- or plasmid-associated resistance genes to a new bacterial host. Finally, naked DNA, released from dead bacteria, can be picked up and incorporated into new strains. The last mechanism, called transformation, is documented in the emergence of resistance among pneumococci and *Haemophilus* spp. Not all organisms have all three mechanisms, but each one helps to amplify the resistance determinant within the microbial world (Levy, 2002).

The use of antimicrobial agents is important to curtail various infectious diseases ravaging our societies. There are some complexities because of multi drug resistant organisms. Concern about antibiotic resistance and its transmission to human pathogens is important because these resistant bacteria may colonize the human intestinal tract as well as related tissues and may contribute resistance genes to

human endogenous flora. The episomal transfer of resistance factor between the intestinal pathogens may lead to evolution of drug resistant bacterial strains in human being which is of public health importance (Sahoo *et al.*, 2012).

Medicinal plants are widely used for various purposes in traditional medicine in many developing nations (Mwitari *et al.*, 2013). This implies that almost three-quarters of the world's population is reliant on these natural products (Petrovska, 2012) as tested in this article.. According to the World Health Organization, the functional and structural units of such plants contain chemical compounds that are used for therapeutic reasons or in the control/treatment of disease conditions. Alkaloids, terpenoids, essential oils, and phenolic compounds such as flavonoids and phenolic acids are examples of chemical/bioactive components, often known as phytochemicals. Antioxidant, anticancer, antimicrobial, anti-inflammatory, anti-allergic, antimutagenic, antiviral, antithrombotic, and vasodilatory properties have all been reported for phytochemicals (Proestos *et al.* 2013). Details about their architecture and origins, as well as evidence of their functionality, are being compiled regularly (Gibbons *et al.*, 2004; Copping and Duke, 2007; Stavri *et al.*, 2007).

The abortifacient properties of Moringa oleifera leaf extract amongst other medicinal plants was also shown to be 100% abortifacient at a dose equivalent to 175 mg/Kg of starting dry material in a study by Paul *et al.*, (2019), to test the plant's anti-reproductive potential. These antibacterial activities of medicinal plants are critical because they have the potential to combat increasing microbial strain resistance mechanisms (Dantas *et al.*, 2008). The clinical isolates have been shown to possess multidrug resistance to various antibiotics tested in first phase of this research (Ajayi *et al.*, 2020). This study helps to determine the type of resistant genes possessed by bacterial isolates from health facilities in designated area of Nigeria and their characteristics as well as the determination of plasmids possessed in conferment of antibiotic resistance. This is done with the aim of bringing lasting solution to these problems in health management systems.

Materials and Methods

Sample collection.

Fifty (50) Agar slants in bijou bottles were submitted to each of the twelve (12) selected hospitals namely State specialist hospitals at Ikare, Ondo, Okitipupa and Akure; for the collection of clinical isolates. Others are the Federal Medical Center, Owo and the Mother and Child hospital Ondo. The collection was done once in a month for three consecutive months.

Purification/ Subculture of Isolates.

All isolates collected were purified by sub-culturing them on freshly prepared Blood Agar and MacConkey agar plates. After incubation at 37^0C for 24hrs, the isolates were stored at 4^0C until further analysis.

Identification of isolates.

This was done through observation of isolate's morphology on agar plates, staining procedures (Gram's stain, spore staining) biochemical tests (catalase, oxidase, sugar fermentation) and use of API -20E Test kit as described by Cowan and Steel (1993) and Cheesbrough (2006). The bacteria used are *Acinetobacter haemolyticus, Budricia aquatic, Burkhoderia cepacia, Citrobacter gilleric, Enterobacter gergoriac,Enterobacter agglomerane, Escherichia coli, Escherichia hermamii, Klebsiella oxytoca, Klebsiella ornithinolytica, Klebsiella terrigena, Pseudomonas aeruginosa, Pseudomonas fluorescens. Staphylococcus spp., Staphylococcus aureus and Coagulase* negative Staphylococcus.

Plasmid profile, Plasmid curing and Susceptibility test:

Thirty two cultured multidrug resistant clinical isolates were analyzed for their antibiotic resistant gene during the study in laboratory using nutrient agar routinely. The microorganisms were subcultured in peptone water for 18 hours. Genomic DNA was then extracted from each sample using Column-pure Bacteria Genomic DNA Isolation kit. Plasmid curing of resistant isolates were done according to the method described by Tomoeda *et al.*, (1968). Bacteria were cultured onto peptone water and cured of plasmid by the addition of 2 drops of arginine red to each of the culture and incubated for 24 hours. Sensitivity was done on Muller Hilton agar. Disc diffusion technique described by CLSI, (2016) was employed.

Extraction of Genomic DNA from Bacteria

• 2mls of bacteria suspension from the overnight broth culture was aseptically transferred into a sterile eppendorf tube.

• It was spun at 6000xg (8000rpm) for 5 minutes at room temperature. The supernatant was discarded and the pellet (cells) was suspended in 200µl of cold Tris EDTA buffer (TE Buffer)

• 400µl of digestion solution was added to the 200µl of samples gotten from step 1. After mixing well, 3µl of Proteinase K solution (2mg/150microlitre) was added to the sample. It was incubated in water bath for 5minutes.

• 260µl of 100% ethanol was added to the sample from step 2. The mixture was applied onto a column-pure spin column that is placed into a collection tube. It was spun at 8000xg (10000rpm) for 2 minutes.

• The flow through in the collection tube was discarded and 500µl of wash solution was added. It was spinned again at 8000x g (10000rpm) for 2 minutes. (The whole step was carried out twice)

• The flow through was discarded and the sample was spun again for additional one minute to remove the residual amounts of wash solution

• The spin column was removed from the collection tube and placed in a clean Eppendorf tube. 45µl of elution buffer was added into the center part of the membrane in the column. It was incubated at 50°C for 2minutes to increase the DNA recovery yield.

• It was spined at 8000 x g (10000rpm) for 2 minutes to elute DNA from the column.

• Aliquots of purified genomic DNA were then preserved at -20°C.

• The DNA quality was measured by UV absorption at A260.

• The DNA template was then stored at -20 degrees

Primer dilution

The list of primers used for this study is listed in the table below. The Primers were supplied in a concentrated form.

The primers were then subsequently diluted in a 2/98 dilution as the working dilution.

Table 1: Primer sequences used for multiplex polymerase chain reaction

Name	Sequence	Band Size
blaTEM:	TEM-F, 5-ATGAG-TATTCAACATTTC-CG-3, and TEM-R, 5-CTGA-CAGTTACCAAT-GCTTA-3	190
blaSHV:	Sequence SHV-F, 5-GGTTATGCGT-TATATTCGCC-3, and SHVR, 5- TTAG-CGTTGCCAGT-GCTC-3	286
blaCTX-M:	CTX-MU1, 5-ATGT-GCAGYACCAGTA-ARGT-3, and CTX-MU2, 5-TGGG-TRAARTARGTSAC-CAGA-3	178

Table 2: Multiplex polymerase chain reaction

Components	Volume
Template DNA	2µl
F	2µl
R	2µl
F	2µl
R	2µl
F	2µl
R	2µl
2x PCR Taq Plus MasterMix/ with dye	25µl
Nuclease free water	To make up to 50

Key: F- Forward R - Reverse

The PCR tubes containing prepared samples were incubated in a thermal cycler at 94°C for 3mins to completely denature the templates. Thirty five (35) cycles of PCR amplifications was performed as follows;
- Denature: 94°C for 30sec
- Anneal: 55°C for 30sec
- Extend: 72°C for 1 min.

The mixture was incubated for an additional 5 minutes at 72°C and later maintained at 4°C.
The samples were stored at -20°C. The amplification products were analyzed by agarose gel electrophoresis and visualize by ethidium bromide staining.

Plant extracts and their antimicrobial activities
The modified Agar disc diffusion method of Mwitari *et al.*, (2013) and CLSI (2016) were used to test the ability of the various extracts to inhibit bacterial growth. The samples shown below were used for antimicrobial screening in this study.
Sample A *Andrographis peniculata*, and,
Sample B *Moringa oleifera*

Two different replicates of antimicrobial controls were used for each comparative analysis. A positive control, Ofloxacine was used in the microbial activities presented in first set of the assay. In antimicrobial analysis, the dilution ratio in 100mg/mL were as stated here;
• 1g of *Andrographis peniculata* (Ethanolic extracts) was dissolved in 10ml solvent containing 2.5ml of DMSO and 7.5ml of Water.
• 1g of *Moringa oleifera* (Ethanolic extracts) was dissolved in 10ml solvent containing 2.5ml of DMSO and 7.5ml of Water.

RESULT
This study shows the plasmid profile, plasmid curing and the antibiotic susceptibility profile of thirty two (32) cultured multidrug resistant clinical isolates from the three geographical regions of Ondo State, Nigeria determined for their antibiotic resistant gene. The genomic test done on the thirty two (32) multidrug resistant strains did not show any resistant gene (Fig 1, Fig 2 and Table 4). Nevertheless, the labels of clinical bacterial isolates analyzed that have multiple resistance to antibiotics are shown in Table 4. But, the molecular plasmid profile conducted shows that all the isolates possess plasmid. Plasmid curing done reduces the antibiotics resistance nature of the isolates. This shows that the clinical isolates were plasmid mediated. Table 4 and Fig. 4 shows the antibiotic susceptibility profile of clinical bacterial isolates tested to confirm this. Sample of antibiogram of tested clinical bacterial strains are shown in Fig. 3 in this regard. Table 5 shows that most of the selected resistant bacterial strains isolated from hospitals were susceptible to medicinal plants such as Andrographis peniculata and Moringa olioferatested on them. This implies that the use of appropriate medicinal plants can be administered as alternative therapy to overcome infections caused by these aetiologic agents.

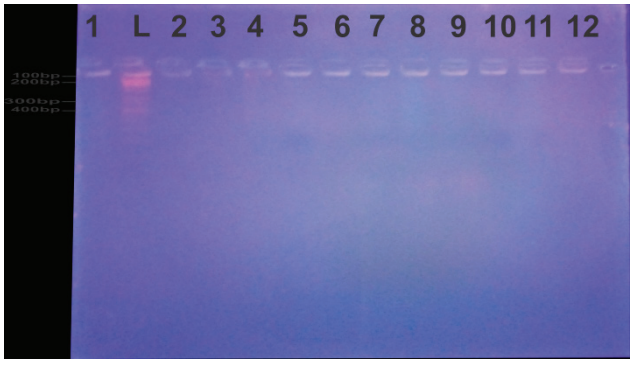

Fig. 1: Fingerprint of samples 1 to 12 (Table 3)

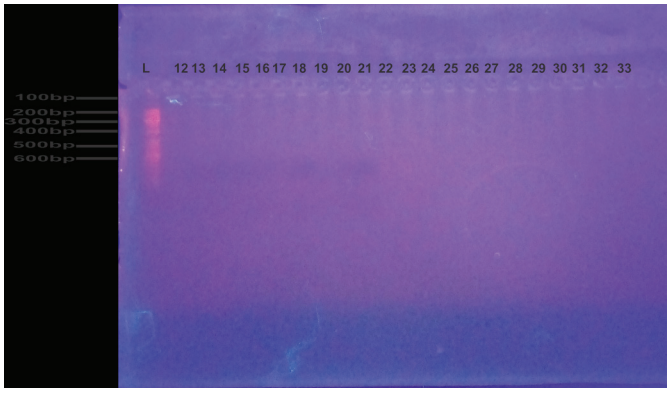

Fig. 2: Fingerprint of samples 12 to 33 (Table 3)

Table 3: Labels of clinical bacterial isolates analyzed that have multiple resistance to antibiotics

Lab. labelling	Site labelling	Bacterial species (Isolates)
1	OKI 26	*Staphylococcus spp.*
2	T 23	*Klebsiella oxytoca*
3	T 30	*Klebsiella oxytoca*
4	OKI 18	*Klebsiella ornithinolytica*
5	OWO 28	*Escherichia coli*
6	IK 24	*Escherichia coli*
7	OWO 5	*Pseudomonas aeruginosa*
8	IK 9	*Escherichia coli*
9	AK 18	*Enterobacter agglomerane*
10	T 12	*Budricia aquatica*
11	AK 16	*Escherichia coli*
12	OKI 5	*Staphylococcus aureus*
13	AKR 17	*Escherichia coli*
14	T 28	*Escherichia coli*
15	T 29	*Escherichia coli*
16	T 18	*Klebsiella oxytoca*
17	OWO 9	*Klebsiella terrigena*
18	OWO 16	*Escherichia hermamii*
19	OWO 13	*Burkhoderia cepacia*
20	OWO 6	*Klebsiella terrigena*
21	T 11	*Citrobacter gilleric*
22	T 32	*Escherichia coli*
23	OWO 15	*Pseudomonas aeruginosa*
24	T 7	*Eschericha coli*
25	OWO 21 B	*Staphylococcus aureus*
26	IK 14	*Escherichia coli*
27	OKI 10	*Acinetobacter haemolyticus*
28	IK 13	*Klebsiella ornithinolytica*
29	IK 27	*Budricia aquatic*
30	OWO 22	*Pseudo fluorescens*
31	OWO 30	*Enterobacter gergoriac*
32	AKR 7	*Staphylococcus sp (coagulase –ve)*

Table 4: Antibiotic susceptibility profile of clinical bacterial isolates after plasmid curing

Isolates	Antibiotics susceptibility test zones of inhibition (in mm)							
	CAZ	CRX	GEN	CXM	OFL	AUG	NIT	CPR
Klebsiella oxytoca	21	5	22	No inhibition	23	3	6	31
Klebsiella ornithino-lytica	11	4	6	4	7	6	17	6
Entero-bacter agglomer-ane	18	17	19	18	5	6	15	7
Escherichia coli	11	6	4	5	6	5	19	8

Isolates	Antibiotics susceptibility test zones of inhibition (in mm)							
Staphylococcus aureus	6	6	5	6	8	6	6	7
Escherichia coli		No inhibition	No inhibition	25	No inhibition	No inhibition	No inhibition	15
Escherichia coli	11	5	5	12	6	5	19	No inhibition
Escherichia coli	No inhibition	5	4	No inhibition	No inhibition	No inhibition	10	4
Klebsiella oxytoca	4	7	4	No inhibition	4	3	11	5
Klebsiella terrigena	6	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	3	No inhibition
Citrobacter gilleric	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
Escherichia coli	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	4	12	No inhibition
Escherichia coli	10	9	12	11	10	7	22	6
Enterobacter gergoriac	12	11	7	6	20	No inhibition	12	17

Legend: CAZ – Ceftazidime AUG – Augmentin CPX – Cefuroxime CPR – Ciprofloxacin
GEN – Gentamicin NIT – Nitrofurantion OFL – Ofloxacin

Table 5: Antimicrobial activities of *Andrographis peniculata* ethanolic extracts on multidrug resistant isolates

S/N	Isolates	Antimicrobial agents concentrations and zones of inhibition (in mm)			
		A, 100mg/mL	B, 50mg/mL	C, 25mg/mL	D, 12.5mg/mL
1.	*Staphylococcus aureus*	13	9	-	-
2.	*Acinetobacter baumannii*	10	9	-	-
3.	*Klebsiella pneumoniae*	17	15	12	10
4.	*Bacillus cereus*	10	9	9	9
5.	*Escherichia coli*	10	9	9	-
6.	*Salmonella typhi*	15	12	1	-
7.	*Proteus mirabilis*	10	-	-	-
8.	*Pseudomonas aeruginosa*	9	-	-	-
9.	*Salmonella pullorum*	17	15	10	8
10.	*Candida albicans*	10	9	9	8

Table 6: Antimicrobial activities of *Moringa oleiofera* ethanolic extracts on multidrug resistant isolates

NAME	Control – Ofloxacin	100mg/mL
Staphylococcus aureus	15	9
Acinetobacter baumannii	21	-
Klebsiella pneumoniae	28	-
Bacillus cereus	28	11
Escherichia coli	24	10
Salmonella typhi	18	-
Proteus mirabilis	31	-
Pseudomonas aeruginosa	29	-
Salmonella pullorum	16	-
Candida albicans	12	-

DISCUSSION

This study shows the multidrug resistant nature of clinical bacterial isolates recovered from the three geographical regions of Ondo State, Nigeria studied for this purpose. The clinical bacterial isolates used for this study (Fig 1 and Fig 2) have been shown to possess plasmid and multidrug resistance to various antibiotics tested in first phase of this research as published (Ajayi *et al.*,, 2020). Most of the resistant bacterial strains were susceptible to most antibiotics they were originally resistant to after curing. This infers that the resistances of the clinical strains to antibiotics are plasmid mediated. According to Trevors (1985), since many plasmids cannot be cured (refractory), it should be noted that some plasmids are stable and can be maintained through successive generations by being portioned to each daughter cell during cell division. This allows each cell to receive at least one plasmid copy (Trevors, 1985). Chromosomal or plasmid DNA resistance genes can be transferred from one bacterium to another by in vivo gene manipulation which includes conjugation, transduction, and transformation as reported by Ajaz *et al.,* (2004). For this reason, some of the commonly used antibiotics may become ineffective. Hence, the need for alternative or more intensive therapy.

Some medicinal plants such as *Andrographis peniculata* and *Moringa oleifera* were also tested to assess their efficacy against the isolates and possible source of overcoming antibiotics resistance. This activity corroborates the previous studies by Paul *et al.,* (2019) and *Shinga et al.,* (2003) respectively, whereby a plant growth-boosting hormone of the cytokinin type has been discovered in an ethanolic extract of *M. oleifera. M. oleifera* leaf extract has been demonstrated to block the peripheral conversion of thyroxine (T4) to tri-iodothyronine (T3) in female rats, suggesting that it could be utilized to treat hyperthyroidism. However, the effect was not seen in male rats, according to this study. This is synonymous with other plant activities discovered by previous investigators. Similarly, report of Shinga *et al.,* (2003) shows the antimicrobial activities of *Andrographis peniculata* based on its combined effect of the isolated arabinogalactan proteins and andrographolides components which enhance its activity.

Moringa leaves have been shown to provide nutritional benefit according to Paul *et al.* (2019) and other investigators (van Vuuren, 2008; Ajayiand Fadeyi, 2015; Dangana *et al.,* 2016; Anyanwu and Okoye 2017). This study helps to clarify that resistance of the clinical bacterial strains examined are plasmid mediated and that most of the selected resistant bacterial strains isolated from hospitals were susceptible to medicinal plants such as *Moringa oleifera* and *Andrographis peniculata* tested on them. This implies that the use of appropriate medicinal plants can be optimized and incorporated into our pharmaceutical drugs to serve as alternative therapy and improve the efficiency of medications to overcome infections caused by these aetiologic agents.

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