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Molecular Identification of Selected Multiple Antibiotic Resistance Bacteria Isolated from Poultry Droppings in Akure, Nigeria

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Abstract: Molecular techniques have the advantage that, they are rapid, less laborious, and more sensitive, specific and efficient compared to the conventional method of bacteria identification. This work aimed at Molecular Identification of Selected Multiple Antibiotic Resistance Bacteria Isolated from Poultry Droppings in Akure, Nigeria. Samples were plated on selective and differential media. Isolation and identification of bacteria were carried out using standard microbiological method, Antibiotics susceptibility test was determined by the disk diffusion method, Plasmid DNA extraction, profiling and curing, Genomic DNA extraction, Polymerase Chain Reaction (PCR), agarose gel electrophoresis, 16S rRNA gene sequence and phylogenetic analysis were carried out by standard method. Eight bacteria were selected; *Proteus mirabilis, Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella* sp. *Escherichia coli, Shigella* sp. and *Klebsiella pneumonia,* they were resistant to at least seven antibiotics. The result revealed that the plasmid size ranged from 8.60 to 128.00 kilobase pair (kbp) and the number of plasmid carried by bacteria ranged from 1 to 3 plasmids. Based on the 16s rRNA sequences, the following bacteria were identified *Salmonella enterica* subsp.*enterica* serovar Typhi str. CT18, *Proteus mirabilis* strain HI4320, *Pseudomonas fluorescens* SBW25, *Staphylococcus cohnii* subsp. cohnii strain 532 Contig16, *Salmonella enterica* subsp. *enterica* serovar Infantis, *Escherichia coli* str. K12 substr. DH10B, *Shigella flexneri* 2a str. 2457T and *Klebsiella pneumoniae* strain J1. The presence of multiple resistant bacteria and plasmid mediated resistance of human pathogenic bacteria isolated from poultry droppings in Akure is of great public health importance.

Keywords: Bacteria, Molecular Identification, Antibiotics, Plasmid

1. Introduction

In recent times, several foodborne pathogens of poultry origin have become important and a threat to public health. Surveillance studies have provided data and a better understanding into the existence and spread of foodborne pathogens. The survival of foodborne pathogens under a variety of environmental conditions warrants the development and use of efficient and reliable isolation, detection, differentiation, classification and/or typing techniques for their surveillance [1, 2].

In reality, increasing resistance levels among pathogenic bacteria are driven by antibiotic use in all sectors: in humans, in the community and in hospitals, on farms and in companion animals. Although resistance in human infections is mainly caused by human antibiotic use, for a range of bacteria, farm-animal use contributes significantly and for some infections is the main source of resistance. Antibiotic-resistant bacteria pass between humans, between animals and between humans and animals in both directions mostly through the food chain much more frequently than once realised. Copies of antibiotic-resistance genes can also move between bacteria, and this exchange can occur in the human gut, so in some cases the bacteria causing a human infection will not be of farm-animal origin, but the resistance will be [3, 4, 5].

Ever growing global migratory trend, rapid industrialization and extensive growth in poultry production

are thought to contribute the possible rapid dissemination of zoonotic pathogens posing public health a potential concern [6] and a big threat.

During research, it is of utmost importance to identify unknown bacteria if diversity is being studied. One of the main responsibilities is to determine the identity of pathogenic bacteria. Bacterial identification by diagnostic laboratories is based on phenotypic characteristics involves a few simple tests such as morphology, growth on various types of culture media and biochemical tests [7]. They are widely used and have the advantage that, they are cheaper, detect only viable bacteria, and yield isolates that can further be characterised and studied [1]. However, they are laborious, relatively slow and less efficient while Molecular techniques have also been widely used in surveillance, mutation and other genetic studies of foodborne pathogens to increase our understanding into the primary source of foodborne pathogens, source of infection and genetic diversity. Molecular techniques have the advantage that, they are rapid, less laborious, and more sensitive, specific and efficient compared to the conventional method [8, 9]. In developing countries where antibiotic resistance is a prime issue, data are least available [10, 11]. Thus, it became imperative to provide information on the molecular identity of multiple antibiotic resistant bacteria isolated from poultry droppings in Akure metropolis.

2. Materials and Methods

2.1. Sample Locations

Akure is the largest city and capital of Ondo State, located in south-west Nigeria. Akure lies about 70°15 north of the equator and 50°15 east Meridian. The city has a population of 588,000 which is 0.305% of Nigeria population based on 2006 population census; the people are of Yoruba ethnic group and are situated in the tropic rainforest. The city is a trade center for farmers where cocoa, bananas, palm oil, yams, cassava, corn, cotton and tobacco are mostly cultivated, the residents also engaged in various economic activities such as trading, transportation business, civil service and education. During this research, samples of poultry droppings were collected from nine (9) different locations in Akure metropolis.

2.2. Sample Collection

Six hundred and eighty four (684) samples of fresh poultry dung (layers and broilers) were collected from commercial poultry farms in nine (9) different locations (FUTA, Aba, Apatapiti, Ijoka, Oritaobele, Ado road, Ondo road, Alagbaka, and Lafe), while that of free range chicken was collected from chicken feeding ground at different locations in Akure, Ondo State, Nigeria. One gram of poultry dropping was collected in sterilised Mac Cartney bottle that contained peptone water and transported to Microbiology Research Laboratory of Federal University of Technology Akure within one hour of collection for bacteriological analysis. The

samples were collected between November 2015 and January 2016.

2.3. Isolation of Bacteria from Poultry Droppings

Bacteriological examinations were carried out using standard methods for aerobic bacteria (Brown, 2005). Sample collected in Mac Cartney bottle was gently shake and stirred with sterile glass rod until the dung mixed thoroughly, aliquot (1.0 ml) was transferred into the test tube containing 9.0 ml of sterile distilled water and diluted serially in one-tenth stepwise to 10^{-7} dilution factor and 1.0 ml each of dilution 10^{-5}, 10^{-6} and 10^{-7} was pure plated on Nutrient agar and some selective and differential media (Salmonella Shigella agar, Eosine Methyline Blue agar, MacConkey agar, Manitol salt agar and Cysteine Lactose Electrolyte Deficient agar), the plates were inverted and incubated aerobically at 37 °C for 24 hours after which the plates were examined for growth.

2.4. Biochemical Characterization

Biochemical characterisation and presumptive identification of isolates were carried out as described by [12].

2.5. Antibiotics Susceptibility Test

Antibiotics susceptibility test of all the isolates was determined by the disk diffusion method and interpreted as susceptible, intermediate and resistant as described by [13]. Gram negative pathogens were tested against the following antibiotics; Tetracycline (30μg), Ofloxacin (30μg), Gentamicin (20μg), Chloramphenicol (30μg), Augmentin (30μg), Ceftriazone (30μg), Nitrofuratoin (300μg), Cotrimoxazole (25μg), Ciprofloxacin (10μg) and Amoxicillin (30μg) while gram positive isolate were tested against Cotrimoxazole (25μg), Erythromycin (10μg), Gentamicin (20μg), Augmentin (30μg),Streptomycin (10μg), Cloxacilin (5μg) Tetracycline (30μg) and Chloramphenicol (30μg).

2.6. Plasmid DNA Extraction, Profile and Curing

Overnight growth of multidrug resistance bacteria broth culture was used for the plasmid isolation. TENS protocol describe by [14] was employed in plasmid extraction. 1.5ml of overnight culture was Spin for 1 minute in a micro-centrifuge to pellet cells. Followed by gentle decant of the supernatant leaving 50μl together with cell pellet and vortex mixed at high speed to re-suspend cells completely. 300μl of TENS was then added. An inverting tube was used to Mix for 3 times until the mixture becomes sticky. 150μl of 3.0M sodium acetate (pH 5.2) was then added to the preparation, followed by Vortex mixing. The preparation was spun for 5minutes in micro-centrifuge to pellet cell debris and chromosomal DNA and then the supernatant was transferred into a fresh tube; and mixed well with 900μl of ice-cold absolute ethanol. It was then spun again for 10 minutes to pellet plasmid DNA. (White pellet is observed) after which the supernatant was discarded; the pellet was rinsed twice with 1ml of 70% ethanol and dry pellet. Pellet was re-

suspend in 30μl of buffer or distilled water for further use. The extracted plasmid DNA was electrophoresized on 0.8% agarose gel stained with ethidium bromide in 10.0 ml Tris-acetate buffer and visualized by UV-transillumination according to Gamal, et al., (2014). (TENS composition: Tris 25mM, Ethyl-dimethyl tetra-amine; EDTA 10mM, Sodium hydroxide; NaOH 0.1M and Sodium dodecyl sulphate; SDS 0.5 %.). The controls used were molecular weight marker labeled M2 for examination of mega plasmids and molecular weight marker labeled M1 was used as control for evaluation of micro plasmids. The fragment bands observed were directly compared with the molecular weight marker bands. Plasmid curing was done by exposing the overnight grown culture at 37°C and 2% Sodium Dodecyl Sulphate (SDS). The isolate was further subjected to antibiotic sensitivity.

2.7. Molecular Identification of Multiple Antibiotic Resistant Bacteria

This test was carried out according to the method of [15], as follow:

(i) Genomic DNA extraction

1.5 ml of multidrug resistant bacterial broth culture was taken in centrifuge tube. Centrifuged at 10,000 rpm for 2 minutes and supernatant was discarded. To the pellet 1 ml of distilled water was added and dissolved the pellet completely. Again centrifuged at 10,000 rpm for 2 min, the procedure was repeated for two times. The supernatant was discarded and to the pellet 100μl of Tris EDTA buffer was added and dissolve the pellet completely in buffer. The supernatant containing the DNA were transferred to another tube and stored at -20°C. The concentration and purity of the extracted DNA was estimated using a Nanodrop spectrophotometer (Model: 752).

(ii) Polymerase Chain Reaction (PCR) amplification and purification of 16S rRNA gene

The 16S rRNA gene was amplified using primers 5'AGAGTTTGAT CCTGGCTCAG 3' and 5'GACGGGCRGTGWGTR CA 3' forward and reverse respectively. PCR mix contained 10X buffer, 100mM dNTPs, 2.52M MgCl2, 2U*Taq*DNA polymerase, 1μl of each (forward and reverse) primers, 2μl of genomic DNA and sterilized distilled water to make a final volume of 25 ml. *Taq*DNA polymerase initiates replication of DNA fragment by using nucleotide bases from dNTPs mixture (A, C, G, T). The PCR reaction included the following steps; initial denaturation of 2 min at 94°C (Pre heating) followed by 25 cycles were run on a thermal cycler, each comprising 1 min at 94°C (denaturation), 1 min at 94°C (annealing) and 1.5min at 94°C (extension), followed by a final extension of 10 min at 94°C for utilization of extra dNTPs in the PCR mixture.

(iii) Agarose gel electrophoresis

0.8g of agarose was weighted and taken in a 100 ml reagent bottle. To this 100 ml of 1X TBE buffer was added and it was heated in microwave oven till agarose melts. The agarose solution was then poured in a gel-casting unit assembled with appropriate comb and it was allowed to get polymerize. When the agarose polymerized, the comb was removed and the gel was kept in electrophoresis tank consisting of 1X TBE buffer. About 2 ml of isolated bacterial genomic DNA from PCR product was mixed with 2 ml of loading dye (bromophenol blue) and it was loaded in 0.8 % agarose gel. The gel was electrophoresed at 100 volts for about 30 minutes and it was observed on UV transilluminator.

(iv) 16S rRNA gene sequence, BLASTn and phylogenetic analysis

Identification of strain was done by 16S rRNA sequence analysis using sequencer, determined sequences were compared with sequences available in GeneBank, derived sequence aligned by Basic Local Alignment Search Tool (BLAST) algorithm, the highest S-ab value with identified species in the Sequence match search. Using the results received through BLASTn a phylogenetic tree is created using the BLASTn web-page.

3. Result

3.1. Bacterial Isolated from Poultry Droppings in Akure

Total number of one hundred and fifty seven (157) bacterial pathogens was isolated and based on biochemical characterization, they were identified as; *Escherichia coli, Klebsiella oxytoca, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris, Enterobacter aerogenes, Salmonella typhi, Salmonella* spp., *Shigella sonnei, Citrobacter freundii, Citrobacter diversus, Serratia marcescens, Pseudomonas aeruginosa, Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* and *Micrococcus luteus.*

Based on antibiotic resistance profile and medical importance, the following eight bacteria were selected for further study; *Proteus mirabilis, Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella* sp. *Escherichia coli, Shigella* sp. and *Klebsiella pneumonia.*

3.2. The Plasmid Size and Post Plasmid Curing Resistance Pattern of Selected Multiple Antibiotic Resistant Bacteria Isolated from Poultry Droppings

Plasmid DNA was extracted and subjected to gel-electrophoresis, different bands were observed and the sizes based on interpretation and post plasmid curing resistance patterns were recorded in Table 1. The result revealed that the plasmid size ranged from 8.60 to 128.00 kilobase pair (kbp) and the number of plasmid carried by bacteria ranged from 1 to 3 plasmids. Post plasmid curing antibiotic resistance pattern of the bacteria showed that the isolates are still resistant to at least two antibiotics.

Table 1. *The plasmid size and post plasmid curing resistance pattern of selected multiple antibiotic resistant bacteria isolated from poultry droppings.*

S/N	Bacterial Isolates	Plasmid size (kbp)	Source (Location)	Phenotypic Resistance Pattern	
				Resistance patterns before plasmid curing	Post plasmid curing resistance patterns
1	*Proteus mirabilis*	10.00	Broiler (D)	Aug Cro Nit Gen Amx Tet Cot	Cro Nit Cot
2	*Salmonella typhi*	24.00, 48.20, 70.00	Layer (D)	Aug Cro Nit Ofl Amx Cpx Tet Ch	Cro Tet Ch
3	*Pseudomonas aeruginosa*	8.60, 34.00, 70.00	Broiler (H)	Aug Cro Nit Gen Ofl Amx Cpx Tet Cot Ch	Cro Nit Gen Amx Ch
4	*Staphylococcus aureus*	20.00	Broiler (D)	Aug Cot Cxc Ery Gen Tet Ch	Ery Ch
5	*Salmonella* sp.	50.00, 67.00	Broiler (G)	Aug Cro Nit Amx Cpx Tet Cot Ch	Nit Ch
6	*Escherichia coli*	71.00, 128.00	Broiler (A)	Aug Cro Nit Gen Ofl Amx Cpx Tet Cot Ch	Cro Gen Tet Cot
7	*Shigella* sp.	20.00	Layer (A)	Aug Cro Nit Gen Amx Cpx Tet Cot Ch	Aug Cro Nit Ch
8	*Klebsiella pneumonia*	50.00, 67.00	Broiler (H)	Aug Cro Nit Ofl Amx Cpx Tet Cot Ch	Nit Tet Cot

KEY: AUG- Augmentin, CRO- Ceftriaxon, NIT- Nitrofuratoin, GEN- Gentamicin, OFL- Ofloxacin, AMX- Amoxicillin, CPX- Ciprofloxacin, TET- Tetracycline, COT- Cotrimoxazole, CH- Chloramphenicol, CXC- Cloxacillin, ERY- Erythromycin, A - FUTA, D - Ijoka, G - Alagbaka, H - Ondo road, kbp – kilobase pair

3.3. Molecular Identification of Selected Multiple Antibiotic Resistant Bacterial Isolates from Poultry Droppings in Akure

Molecular identification of multidrug resistant Bacterial isolates from poultry droppings is presented in Table 2. The length of amplified products was 1500 base pair. The sequence obtained was blasted in National Centre for Biotechnology Information (NCBI) database. Based on the 16s rRNA sequences, the following bacterium were confirmed *Salmonella enterica* subsp.*enterica* serovar Typhi str. CT18, *Proteus mirabilis* strain HI4320, *Pseudomonas fluorescens* SBW25, *Staphylococcus cohnii* subsp. cohnii strain 532 Contig16, *Salmonella enterica* subsp. *enterica* serovar Infantis, *Escherichia coli* str. K12 substr. DH10B, *Shigella flexneri* 2a str. 2457T and *Klebsiella pneumoniae* strain J1. The result also revealed a difference in cultural identification of *Staphylococcus cohnii* subsp. cohnii strain 532 Contig16, *Salmonella enterica* subsp. enterica serovar Infantis and *Pseudomonas fluorescens* SBW25.

Table 2. *Molecular identification of selected multiple antibiotic resistant bacterial isolates from poultry droppings in Akure.*

S/N	Biochemical Characterization of Selected Bacteria	16s rRNA sequence identification of Selected Bacteria	Max Identity score	Accession number
1	*Proteus mirabilis*	*Proteus mirabilis* strain HI4320	97%	NC010554.1
2	*Salmonella typhi*	*Salmonella enterica* subsp.enterica serovar Typhi str. CT18	100%	NC003198.1
3	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens* SBW25	92%	NC012660.1
4	*Staphylococcus aureus*	*Staphylococcus cohnii* subsp. cohnii strain 532 Contig16	99%	NZLATV01000012.1
5	*Salmonella* sp.	*Salmonella enterica* subsp. enterica serovar Infantis	80%	NZ LN649235.1
6	*Escherichia coli*	*Escherichia coli* str. K12 substr. DH10B	100%	NC010473.1
7	*Shigella* sp.	*Shigella flexneri* 2a str. 2457T	100%	NC004741.1
8	*Klebsiella pneumonia*	*Klebsiella pneumoniae* strain J1	99%	NZ CP013711.1

3.4. Phylogenetic Tree Based on the 16S rRNA Gene Sequence Comparisons

The DNA sequences were selected from the BLAST results and used as inputs for the phylogenetic tree reconstruction. The result of the sequence was aligned and Phylogenetic tree was constructed and given in Figure 1. The clustering pattern indicates the genetic and evolutionary relationship between eight bacterial species. Two clusters were generated; *Pseudomonas fluorescens, Shigella flexneri, Escherichia coli, Klebsiella pneumoniae* and *Salmonella enterica* subsp. *enterica* serovar Infantis are in cluster 1, *Staphylococcus cohnii, Salmonella enterica* subsp.*enterica* serovar Typhi and *Proteus mirabilis* are in cluster 2.
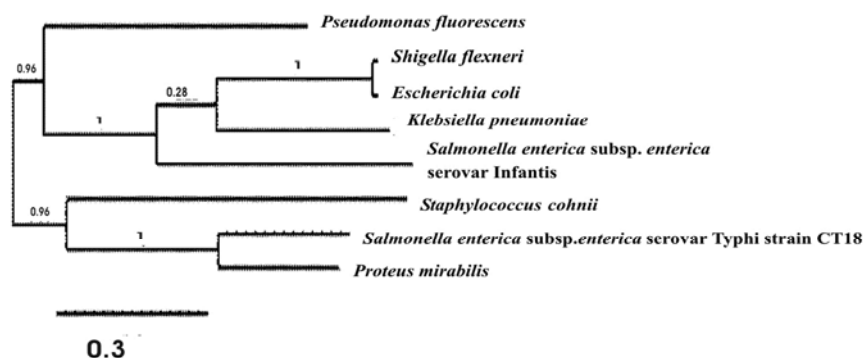
Figure 1. *A Phylogenetic tree showing the degree of similarity of 16S rRNA sequence of multidrug resistant bacterial isolates.*

4. Discussions

In this study, bacterial strains were resistant to multiple antibiotics and some had more than one plasmid. All bacterial isolates harbour low or heavy molecular weight plasmid while some had both low and heavy molecular weight plasmid and are multi-drug resistance. These results are in agreement with the earlier studies which revealed that plasmids with large and low molecular size are responsible for high resistance in bacteria to antibiotics [16]. Also post plasmid curing resistance pattern revealed that multiple antibiotic resistance observed in these bacteria are plasmid mediated, these was also reported by [17, 18, 19, 20, 21].

Worldwide, molecular approaches have emerged in clinical microbiology practices, molecular techniques have also been widely used in surveillance, mutation and other genetic studies of foodborne pathogens to increase our understanding into the primary source of foodborne pathogens, source of infection and genetic diversity. Molecular techniques have the advantage that, they are rapid, less laborious, and more sensitive, specific and efficient compared to the conventional method [8, 9]. Molecular method also provides insights into etiologies of infectious disease [22, 23] and appropriate antibiotic treatment [24]. PCR revealed that the molecular weight of the genomic DNA of sequenced bacteria is 1500bp. According to the 16S rDNA analyses, selected multiple antibiotic resistant isolates showed more than 80% similarity in the NCBI GenBank by BLASTn. Based on the BLASTn results the isolates confirmed are *Salmonella enterica* subsp.*enterica* serovar Typhi str. CT18, *Proteus mirabilis* strain HI4320, *Pseudomonas fluorescens* SBW25, *Staphylococcus cohnii* subsp. cohnii strain 532 Contig16, *Salmonella enterica* subsp. *enterica* serovar Infantis, *Escherichia coli* str. K12 substr. DH10B, *Shigella flexneri* 2a str. 2457T and *Klebsiella pneumoniae* strain J1. The result also revealed a difference in cultural identification of *Staphylococcus cohnii* subsp. cohnii strain 532 Contig16, *Salmonella enterica* subsp. enterica serovar Infantis and *Pseudomonas fluorescens* SBW25. This was also reported by [25], who reported differences in conventional method and molecular method of bacteria identification. However, the results of this work demonstrate clearly the interest and feasibility to introduce the 16S rDNA gene sequencing method in identification of human pathogenic bacteria of poultry origin in Akure, combination of conventional techniques and molecular approach will improve bacteriological diagnosis and allow specific and efficient identification of pathogenic bacteria and save human lives.

5. Conclusion

Multidrug resistant isolates habours plasmid which is of low or heavy molecular weight plasmid or both, resistance to ciprofloxacin and ofloxacin were found to be plasmid mediated. The molecular method of identification was found to be advantageous as bacteria were identified to strain level with the following bacteria of medical importance identified; *Proteus mirabilis* strain HI4320, *Salmonella enterica* subsp.*enterica* serovar Typhi strain CT18, *Pseudomonas fluorescens* SBW25, *Staphylococcus cohnii* subsp. cohnii strain 532 Contig16, *Salmonella enterica* subsp. *enterica* serovar Infantis, *Escherichia coli* str. K12 substrain DH10B, *Shigella flexneri* 2a strain 2457T and *Klebsiella pneumoniae* strain J1. Finally, antibiotic used in poultry may have led to the occurrence of multiple antibiotic resistant bacteria and occurrence of plasmid mediated multiple resistant antibiotics especially fluoroquinolones, in pathogenic bacteria of poultry origin in Akure is of great public health concern.

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