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COMPARATIVE ANALYSIS OF ANTIBIOTIC RESISTANCE AND R-PLASMIDS OF STAPHYLOCOCCUS AUREUS ISOLATES FROM HUMAN AND DOG SAMPLES

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ABSTRACT

Bacterial resistance to antibiotics constitutes a major cause of failure in the treatment of bacterial infections. The genetic exchange of plasmids containing antibiotic resistant determinants between bacteria is believed to play a critical role in the evolution of antibiotics resistant bacteria and this has been shown in *S. aureus*. This study was therefore carried out to investigate the nature of plasmids that determine antibiotic resistance in *Staphylococcus aureus* isolates from man and animal.

Thirty multiply drug resistant *S. aureus* isolates from a total of 147 apparently healthy humans and dogs, as well as from clinical cases were determined by antibiotic susceptibility test using the standard disc agar diffusion method. Plasmid isolation was carried out by the alkaline lysis method of Birnboim and Dolly. Electrophoresis as well as the transformation experiment was done.

The result showed that no particular sensitivity pattern or plasmid profile can be ascribed to either human or animal sources of isolates. Two isolates from a domestic dog and its owner (human) were observed to have identical plasmid profile and almost the same antibiogram. 23.130 kbp and 25.119 kbp plasmids that were responsible for amoxycilin resistance were transferred.

In conclusion, the genetic basis of antibiotic resistance by *S. aureus* in our locality was found to be partly plasmid mediated. Plasmid analysis, in conjunction with the antibiogram is valuable in differentiating multiple resistant *S. aureus*.
Furthermore, domestic pet animals were found to be reservoirs and potential risk factors in the transfer of multiply antibiotic resistant *S. aureus* and their R-plasmids to antibiotic susceptible *S. aureus* and other bacteria.

Key words:-Staphylococcus aureus, antibiotic resistant plasmid, plasmid profile, resistance pattern, ansformation.

INTRODUCTION

Bacterial resistance to antibiotics constitutes a major cause of failure in the treatment of infections by bacteria, and this is of concern, not only to clinicians, but also to medical scientists and the pharmaceutical industries (1). *S. aureus* is an important pathogen that is frequently found to be resistant to a large number of antibiotics (2, 3, 4). The genetic exchange of plasmids containing antibiotics resistant determinants between bacteria is believed to play a critical role in the evolution of antibiotic-resistant bacteria and this has been shown in *S. aureus* (1, 2, 5, 6).

A number of studies on the antibiotic resistance, carriage and infection of *Staphylococcus aureus* in both humans and animals have been conducted by researchers (2, 3, 7, 8, 9). However, only a limited number of studies have been conducted in Africa on the genetic basis of antibiotic resistance of bacteria, particularly on the animal strains of *Staphylococcus aureus.*

Molecular techniques are currently being used in differentiating strains of bacteria isolates and opinions vary as to the discriminatory power of the various types of these techniques (10, 11, 12,

13, 14, 15). Plasmid profile analysis has however been found by some authors as a molecular technique that is discriminatory enough in differentiating strains of *S. aureus* and other bacterial isolates (10, 13, 15, 16, 17, 22), while others have opined that no single technique was clearly superior to others for typing *S. aureus,* and that a combination of different techniques (both molecular and non-molecular) is sometimes necessary (7, 10, 14)

These observations necessitate and require further studies in our environment. This study was therefore conducted to analyze the antibiotic resistance and resistance plasmids of *Staphylococcus aureus* isolates from human and dog samples, in order to determine if there is any similarity or differences between animal and human strains of the isolates. Consequently the study will determine the discriminatory power of plasmid profile analysis in conjunction with antibiotic susceptibility pattern (antibiogram) in differentiating different strains of *Staphylococcus aureus.* The study will further demonstrate the potential transferability of resistance gene from *Staphylococcus aureus* to *Escherichia coli* with the aim of contributing to the knowledge of the genetic basis of antibiotic resistance by bacteria.

MATERIALS AND METHODS

Bacteria Isolates: A total of 30 multiple-resistant *Staphylococcus aureus* strains from human nasal colonisation (7 isolates), human ear colonisation (1 isolates), canine groin colonisation (4 isolates) and human clinical isolates (9) were subjected to plasmid extraction by modified alkaline lysis method of Birnboin and Dolly, 1979 (17). Multiple- resistance was defined as resistance to 4 or more antibiotics out of 8 different antibiotics the isolates were subjected to.

Colonisation isolates were obtained from samples colleted between July and December, 2006, from humans and dogs in Agege and Alimosho Local Government Areas of Lagos State, Nigeria; and inserted into Stuart's Transport medium pending culture on appropriate culture media. Clinical isolates were obtained from clinical samples collected within the same period, from two University Teaching Hospitals in Lagos State and Ogun State of Nigeria.

All *Staphylococcus aureus* isolates were identified culturally, microscopically and biochemically by standard methods according to Jawetz *et al*. 2002 (21).

Culture Media and Growth Conditions

The culture media used for isolation and preliminary identification of *Staphylococccus aureus* strains were Mannitiol Salt Agar (Britania) MacConkey agar (Biotec) and Blood Agar (Nutrient Agar, Biotec, supplemented with 5% expired human blood, HIV and Hepatitis B negative). Identified isolates were stored on Nutrient agar slopes at 4^0c.

Antibiograms

The identified *S. aureus* isolates and *Escherichia coli* K-12 transformants were subjected to antimicrobial susceptibility test using the Disc diffusion technique as described by Bauer *et al.* (1966). Antibiotic multidisc (Abtec, U.K.) for Gram positive bacteria consisting of Augmentin (30ug); Amoxycilin (5ug) Erythromycin (5ug), Tetracycline (10ug); Gentamycin (10ug); cotrimoxazole (25ug); Chloramphenicol (30ug) as well as single disc of methicillin (10 units) were used.

The multidisc plates of Mueller-Hinton Agar were incubated at 37^Oc for 24 hours while the methicillin disc plates were incubated at 30^Oc for 24 hours and antibiogram were evaluated by comparing zones of growth inhibition of the test isolates with that of antibiotic sensitive oxford strains of *Staphylococcus aureus* NCTC 6571 as control.

Plasmid isolation

Plasmid extraction was carried out by the modified version of the alkaline lysis method of Birnboin and Dolly (1979) as described by Zuccarelli *et al*. (1989)

The multiply resistant organisms were grown on Luria-Bartani (LB) agar and incubated at 37^Oc overnight. Each organism was harvested into 1.0ml of TE buffer in Eppendorf tubes and washed by vortexing and centrifugation at 12000g for 10 minutes. The supernatant was discarded, the sediment was vortexed to mix and suspended in 2.0ml to TE buffer containing 50ug/ml lysostaphin. This was incubated at

37^{O}c for 30mins and 0.4ml of 0.2M NaOH – 1% sodium dodecyl sulphate was added. The contents were mixed by inversion with hand and kept on ice for 10mins. After 10mins on ice 0.3ml of 3M potassium – 5M acetate was added and the lysate was cooled on ice for another 10mins. This mixture was centrifuged at 12,000 x g for 10mins and the supernatant transferred to another Eppendorf tube.

The supernatant was extracted once with buffered-saturated phenol and once with ether. The preparation was incubated with 2ul of heat-treated pancreatic RNAse A (10mg/ml Worthington Diagnostics, Freehold, N.J.) at 37^{O}c for 15mins. This was followed by extraction with 1.0ml of phenol-chloroform (1:1) through vortexing, centrifugation at 12,000 x g for 10mins and transfer of the upper layer into another eppendorf tube. The DNA content was precipitated with 1.0ml of absolute ethanol. The precipitate was collected by centrifugation, washed once with 1ml 70% ethanol by centrifugation. The supernatant ethanol was decanted and the DNA precipitate desiccated under vacuum. The dry precipitate was dissolved in 40ul of TE buffer, ready for agarose-gel electrophoresis.

Agarose Gel Electrophoresis

Electrophoresis of extracted plasmids was carried out in a horizontal agarose gel electrophorectic unit.

The agarose gel (0.8%) was prepared in Tris-Boric acid-EDTA (TBE) buffer, allowed to cool and 3 drops of ethidium bromide was added. The mixture was poured into the electrophoretic tank with the comb in place and allowed to set. The TBE buffer was poured into the tank until the appropriate mark was reached and the comb was removed to create the appropriate wells.

Using a micropipette, 20ul of each plasmid preparation, with added 0.2ul bromophenol blue dye (tracking dye) were applied into the horizontal well. Into the first well was added 20ul of DNA molecular Weight Marker(Roche Diagnostics, Germany; mol wt 0.12 – 23.1 kbp) to which 0.2ul of bromophenol blue had also been added as the standard. Electrophoresis was performed at 120v at room temperature for 2hrs. The electrophoretic bands were observed under a 302-nm transilluminator and then photographed onto Polaroid Gel cam instant camera through orange filter (Peca Products Inc. U.S.A.)

b. **Molecular Weight Estimation**

The size of each plasmid was estimated by taking measurement of the mobility of the plasmids as well as that of the standard plasmid. Log_{10} of molecular weight of the standard was plotted against the mobility of the standard. Molecular sizes of the *Staphylococcus aureus* plasmids were interpolated from the standard curve.

Gene Transfer Experiment

The method of Hanahan, 1983 (7) was used to transfer the resistant genes from *Staphylococcus aurues* isolates to strains of *Escherichia coli* K-12 devoid of plasmids

A single colony of recipient bacteria i.e. *Escherichia coli* k-12, from MacConkey agar plate was inoculated into fresh 5ml Mueller-Hinton broth and incubated at 37^{O}c overnight. Into 20ml of fresh broth (Mueller-Hinton) was added 0.5ml of the overnight culture and the mixture was incubated on a rotating shaker at 37^{O}c until cell density was 5 x 10^{7} cells per ml with an absorbance reading of 0.55 at 550nm.

The culture was chilled in ice for 10mins and centrifuged at 3,000 x g for 15mins at 4^{O}c. The supernatant was decounted and the cell pellet re-suspended in 10ml of ice cold 0.1M MgCl. This was centrifuged as before at 3,000g for 15mins and at 4^{O}c. The cell pellet was then suspended in 5ml of ice cold 0.1M CaCl, incubated on ice for 20mins and centrifuged as before. After centrifugation the cell were re-suspended in another 5ml ice cold 0.1M calcium chloride.

Two hundred microlitres (200ul) of competent cells were dispensed into labeled tubes. Into this was added 40ul of plasmid isolated from multiple resistant *Staphylococcus aureus* and the transformation mixture with experimental control (containing only competent cells) and sterility control (containing only plasmid DNA) were placed on ice for 30mins. The preparations were "heat shocked" by removing the tubes from the ice and placing them in a water bath, already set at 40^{O}c for 20 mins. The tubes were removed and placed back on ice for 5mins. Into

each tube, was added 1ml of Mueller Hinton broth and incubated at 37^{O}c for 1 hr.

After this incubation, 150ul of each of the tube content was plated into already prepared selective Luria-Bartani (LB) agar containing tetracycline (10ug/ml) and chloramphenicol (30ug/ml). The plate were incubated at 37^{O}c overnight, and examined for growth the following day.

RESULTS

Resistance Pattern/Plasmid Profile Analysis

A total of 30 multiply resistant *Staphylococcus aureus* isolates were screened for possession of plasmids, out of which 16 (53%) were found to harbor plasmids (fig. 1). The profile of the plasmids in conjunction with the resistance pattern of the corresponding isolates is as shown in table 1.

A total of 8, representing 26% of the total number (30) of multiply antibiotics resistant *S. aureus,* whose plasmid content were determined, were found to possess 3 or more plasmids. Single plasmid isolates were 3 (10%) while isolates with 2 plasmids were 5 (17%). Fourteen isolates (47%) were found to have no plasmids (table 2.)

The major plasmid sizes, based on the frequency of occurrence in different isolates, are found in human and canine isolates of different sources (table 3). This shows that the plasmid profile of isolates cuts across human and canine isolates of various sources, as no particular profile can be ascribed either to human or canine source alone. In the same vein, the resistance pattern of isolates cuts across human and canine sources as there were isolates from both sources having the same antibiotic resistance pattern (table 4). These groups of isolates were found to have different plasmid profile (table 4). On the other hand, 2 pairs isolates with same plasmid profile were found to have almost the same antibiotic resistance pattern, as there was only one antibiotic resistance difference in each of the 2 pairs of isolates. (table 5). One of the pair of isolates was a dog and its owner (human) cohabiting in the same residence while the other pair of isolates have no known relationship, and they were samples from human and nasal cavity (table 5).

Gene Transfer Result

The plasmid profile and the resistance pattern of donors and *Escherichia coli* k-12 transformant are shown in table 6 while fig. 2 is the electrophoretic mobility pattern of transformants.

Plasmid borne amoxycilin resistance of size 23.130 kbp and 25.1219 kbp was determined.

TABLE 1: PLASMID PROFILE AND RESISTANCE PATTERN OF PLASMID – CONTAINING ISOLATES.

Serial No	LAB NO	RESISTANCE PATTERN	PLASMID PROFILE (kbp)
1.	UK 1	Au, Am, Te, Me, Ge, Co	2.322
2	CN 'A' 2	Er, Me, Co, CI	0.282
3	CN23	Au, Am, Te, Me, CI	1.259
4	HN32	Au, Am, Te, Me, CI	25.119, 9.416
5	CN26	Au, Am, Te, Me, CI	25.119, 9.416
6	CN16	Au, Am, Te, Me, CI	23.130, 2.708
7	EAS1	Au, Am, Er, Te, Me, Ge, CI	23.130, 6.557
8	EK1	Au, Am, Er, Te, Me, Co	25.119, 1.259
9	HN8	Am, Te, Co, CI	23.130, 1.259, 0.560
10	HN27	Au, Am, Te, CI	23.130, 6.557, 4.361
11	HNE4	Au, Am, Er, Te, Me, Co	23.130, 1.259, 0.708
12	CNE2	Au, Am, Te, Me, Co	23.130, 1.259, 0.708
13	HE36	Au, Am, Er, Te, Me, Ge, Co, CI	23.130, 1.259, 5.623
14	CG22	Au, Am, Er, Te, Me, Co, CI	23.130, 1.259, 1.000
15	WK1	Au, Am, Te, Me, Co	23.130, 6.557, 6.310, 3.54
16	CG14	Au, Am, Te, Me	23.130, 6.557, 6.310, 2.322, 2.027

Au	=	Augumentin	Am	=	Amoxycillin	Er	=	Erythromycin
Te	=	Tetracycline	Me	=	Methicillin	Ge	=	Gentamycin
Co	=	Cotrimazole	Chl	=	Chloramphenicol			

TABLE 2: PLASMID DNA CONTENT OF STAPYLOCOCCUS AUREUS ISOLATES

Number of plasmids	Molecular weight Range	Number of Isolates
0	-	14 (47%)
1.	0.282-1.259	3 (10%)
2	0.708-25.119	5 (17%)
3	0.546-23.130	6 (20%)
4	3.548-23.130	1 (3%)
5	2.027-23.130	1 (3%)

TABLE 3: MAJOR PLASMID SIZES AND POSSESSING ISOLATE SOURCES.

Plasmids Size	No (%) of Possessing Isolates	Isolate Sources
25.119	3 (19%)	HN, CN, EK
23.130	10 (63%)	CN, EAS, WK, HN, HE, CG
9.416	3 (19%)	HN, CN, HE
6.557	4 (25%)	EAS. HN, WK, CG
6.310	2 (12.5%)	HN, CN
2.322	2 (12.5%)	CG, UK
1.259	6 (38%)	EK, CN, HN, CG
0.708	2 (12.5%)	HN, CN

HN = Human Nose CN = Canine Nose EK/EAS/WK/UK = Clinical Samples
HE = Human Ear CG = Canine Groin

TABLE 4: ISOLATES WITH SAME RESISTANCE BUT DIFFERENT PLASMID PROFILE

Isolates	Resistance Pattern	Plasmid Profile (kbp)	Host
CN23/EK1/HNE4	Au, Am, Er, Te,Me, Co	i. 1.259	Canine (colonisation)
		ii. 25.119, 1.259	Human (clinical)
		iii. 23.130, 1.259, 0.708	Human (colonisation)
HN32./CN16	Au, Am, Te, Me, CI	i. 25.119, 9.416	Human (colonisation)
		ii. 23.130, 2.708	Canine (colonisation)
CNE2/WK1	Au, Am,	i. 23.130, 1.259, 0.708	Human (colonisation)
	Te,Me, Co	ii. 23.130, 6.557, 6.310, 3.540	Canine (colonisation)
			Canine (colonisation)
			Canine (clinical)

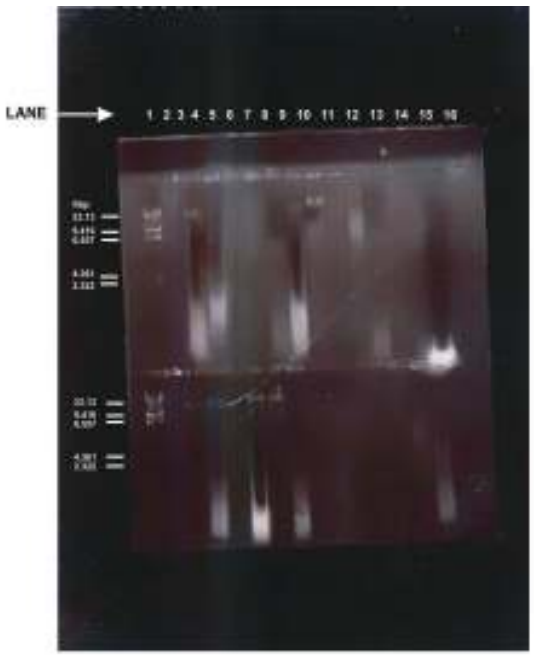
TABLE 5: IDENTICAL ISOLATES WITH SAME PLASMID PROFILE AND SIMILAR RESISTANCE PATTERN

Isolates	Plasmid Profile (kbp)	Resistance Pattern	Relationship	Basis for Identity
HNE4/CNE2	23.130, 1.259, 0.708	i. Au, Am, (Er), Te,Me, Co	Canine (Dog) and owner	Same plasmid profile and almost the same resistance pattern.
HN32./CN26	25.119, 9.416	i. Au, Am, (Te), Me, Co	No known relationship. Human and dog nasal carriage samples.	Same plasmid profile and almost the same resistance pattern

TABLE 6: PLASMID PROFILE AND RESISTANCE PATTERN OF DONORS & TRANSFORMANTS

Lab no	Resistance pattern of Donor	Plasmid profile of Donor (kbp)	Plasmid Transferred (kbp)	Resistance Pattern of Transformant
EAS1…(i)	Au, Am, Er, Te, Me, Ge, CI,	23.130, 6.567	23.130	Au, Am, Te, Er, CI,
ESA1…(ii)	Au, Am, Er, Te, Me, Ge, CI,	23.130, 6.567	23.130	Am, Te, Er, CI,
HN32…(i)	Au, Am, Te, Me, CI	25.119, 9.416	25.119	Au, Am, Te
HN32…(ii)	Au, Am, Te, Me, CI	25.119, 9.416	25.119	Au, Am, Te, CI
CN16…(i)	Au, Am, Te, Me, CI	23.130, 2.708	23.130	Au, Am, Te
CN16…(ii)	Au, Am, Te, Me, CI	23.130, 2.708	2.708, 23.130	Au, Am, Te, CI
HN8…(i)	Au, Am, Te, Co, CI	23.130, 2.159, 0.564	23.130, 1.259	Am, Te, CI
HN8…(ii)	Au, Am, Te, Co, CI	23.130, 2.159, 0.564	23.130	Am, Te, CI
CG22…(ii)	Am, Er, Te, Me, Co, CI	23.130, 1.259, 1.000	23.130, 1.259, 1.000	Am, Er, CI

(i) And (ii) are antibiotic selective plates for tetracycline and chloramphenicol respectively.



Upper Lanes: Lane 1: λ DNA (Standard of known molecular weight)
Lanes 2-16: Sample DNA

Lower Lanes: Lane 1: λ DNA (Standard of known molecular weight)
Lanes 2-16: Sample DNA

Fig. 1: Agarose Gel Electrophoretic Mobility Pattern of Plasmids

61(b)

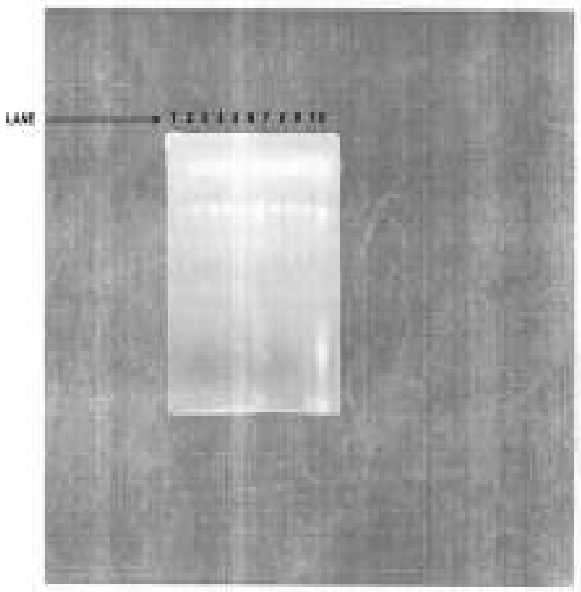


FIG. 2: Agarose Gel Electrophoretic Mobility Pattern of Transformants

Discussion

The resistance pattern and the plasmid profile of *S. aureus* isolates in this study revealed that the nature of resistance and the type of plasmids possessed cut across human and animal isolates of different sources. This observation is in conformity with findings of Whittam *et al.* 1989 (19) in their investigation of the genetic relationships among *Escherichia coli* isolates causing urinary tract infection in humans and animals. In the said work, they found that the independent isolates collected from humans and animals (dogs and cats) in separate geographical regions, share similar genotypes as identified by electrophoretic type; and they suggested that many cases of urogenital disease (in man and animal) may be caused by a small number of uropathogenic clones. In the same vein, the findings in this study, suggested that the various plasmid types (based on their sizes in terms of molecular weight) are widely distributed in humans and animals. This is also consistent with the findings that resistance pattern cuts across human and animal isolates of *S. aureus* in this study.

However, there was no consistency in relationship between resistance pattern and the corresponding plasmid profile of isolates. This findings also agrees with that of other workers who investigated the occurrences of multiple resistance and R-Plasmids in *Enterobacteriaceae* isolates from developing countries (11, 18, 19). Nevertheless, in this study while there existed isolates with the same resistance pattern but different plasmid profile, 2 pairs of isolates with the same plasmid profile also have similar (almost the same) resistance pattern.

One of these pairs of isolates were from nasal

swab of canine household pet (dog) and its owner (man) of the same residence, while the other is also from nasal cavity of man and animal, but of no known relationship. Hollis *et al.,* 1988 (7) has earlier suggested that family members may serve as reservoir for nosocomial infection with MRSA when a familiar carriage of MRSA and subsequent infection of a premature neonate was established. In their study, the *S. aureus* strains were characterized by antibiogram, plasmid analysis and genomic

DNA typing and the isolates were found to be identical by all 3 techniques (7). Other workers have also differently attested to the discriminatory power of plasmid profile analysis in conjunction with other non-molecular techniques like the antibiotic resistance pattern (antibiogram) in differentiating strains of S. aureus and other bacteria isolates.

The findings of this study support plasmid profile analysis and antibiogram as epidemiological investigating tool and extends the findings of Hollis *et al.* , (7) by suggesting, subject to further investigation (because of statistical insufficiency) that domestic pets are most probably a risk factor in transfer of both community acquired and nosocomial infections as well as R-plasmids.

The result of transformation conducted identified a 23.130kbp and a 25.119 kbp plasmid as coding for amoxycillin resistance and this conforms with the findings of other workers (5, 10, 11). Although the result does not conclusively established the plasmid size coding for tetracycline or chloramphenicol resistance and further work is necessary to elucidate this, the existence of transformant in this study confirms the genetic basis of antibiotic resistance of *S. aureus* in our locality of being partly plasmid mediated and also probably chromosomal or transposon mediated, as no plasmid was detected in 47% of the multiple antibiotic resistance isolates whose plasmid content were investigated.

In conclusion, plasmid profile analysis in conjunction with the antibiogram is valuable in differentiating multiple resistant *S. aureus.* Furthermore, the combination of the findings of this study and other earlier studies suggested the transferability of multiple resistant *S. aureus* and their R-Plasmids, not only from man to man but also from domestic animal to man and vice versa.

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