Virulence Factors, Plasmid Profiling and Curing analysis of Multi-drug Resistant *Staphylococcus aureus* and Coagulase negative *Staphylococcus* spp. isolated from Patients with Acute Otitis Media.

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ABSTRACT: Microbiological and molecular techniques were used to determine the virulence factors, plasmid profile and antibiotic susceptibility spectrum of Staphylococcus aureus and CON-Staphylococcus spp isolated from patients with acute otitis media attending University of Uyo Teaching Hospital, General Hospital, Ikot-Ekpene and Nekede Paediatric Hospital between January, 2009 and January, 2010. 42 (30.9%) Staphylococcus aureus and 21 (15.4%) CON Staphylococcus spp were isolated from the aural swab samples collected. Staphylococcus aureus produced 16 (38.1%), 22 (52.4%) and 4 (9.5%) of alpha, beta and gamma haemolysis, respectively, while CON-Staphylococcus spp. produced more of beta haemolysis than both alpha and gamma. The results showed that 14 (33.3%) Staphylococcus aureus and 9 (42.9%) of CON-Staphylococcus spp are beta-lactamase producer. The antibiotics susceptibility testing showed that 29 (69.0%), 26 (61.9%), 27 (64.3%), 28 (66.7%) and 29 (69.0%) of Staphylococcus aureus were sensitive to peni-cillin, ceftriazidime, cefoxitin, ciprofloxacin and levofloxacin, respectively. 12 (28.6%) of Staphylococcus aureus were resistant to streptomycin and iminipen, while about 45.2% - 50.0% were resistant to cephalothin and amoxicillin. CON-Staphylococcus spp also showed varied percentages of sensitivity ranging between 42.9% for streptomycin and 71.4% for moxifloxacin. The result also showed that 19.2 % of Staphylococcus aureus and 9.6% of CON-Staphylococcus spp. were resistant to more than eight antibiotics with (MAR) index ranging from 0.25 to 1.00 and 0.25 to 0.75 for Staphylococcus aureus and CON-Staphylococcus spp. respectively. The results obtained in this study are statistically significant ($p \leq 0.05$). Most of the Staphylococcus aureus and CON-Staphylococcus spp were cured of their plasmids showing that they are plasmid borne. Large molecular weight plasmids ranging from 23.13kbp to 50.0kbp were harboured by both Staphylococcus aureus and CON-Staphylococcus spp obtained from acute otitis media. However, continuous surveillance to monitor antimicrobial resistance and guide the antibacterial therapy is overwhelmingly necessary.

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INTRODUCTION

Otitis media is the inflammation of the middle ear due to pathogenic micro-organisms that are resident in the middle ear (Damoiseaux, 2005; Ekpo *et al*., 2009). Otitis media which may be acute otitis media (AOM), acute suppurative otitis media (ASOM) or chronic suppurative otitis media (CSOM) occurs in the area between the ear drum and the inner ear, including the Eustachian tube (Richard and Robert, 1996; Bluestone, 1998). Otitis media is prevalent among children because their eustachian tube is shorter, straighter, made up of more flaccid cartilage, more horizontal than adults and also they have not developed the same resistance to bacteria as found in adults (Bluestone and Klein, 2001; Ihsan *et al*., 2010). Research showed that 83% of

children will have at least one episode of AOM by the age of three years and this accounts for a large proportion of paediatric presentations to health care professionals and is the most common cause of hearing loss in children (Bluestone and Klein, 2001). The patients with acute otitis media (AOM) and chronic otitis media (COM) present the classic "earache", pain that is severe, continuous and is often accompanied by fever (39°C or more), possibly causing febrile seizures and can lead to insomnia for patients, mild to moderate hearing loss, loss of balanc e, unusual irritability, unresponsiveness to quiet soun ds, and draining of fluid in the ear.(Ehrlich *et al*., 200 2; Rovers *et al*., 2006). Staphylococci are Gram positive, facultative anaerobes, spherical bacteria in cluster with diameter ranging from 0.5 to 1.5 µm (Adejuwon *et al*., 2010; Brock and Frazier, 1996)**.** *Staphylococcus aureus*, a worldwide pathogen with its natural reservoir in human belongs to genus of the Micrococcaceae. It is recognized as one of the major causes of severe soft tissue infections, toxic shock syndrome (TSS) and as well as scalded skin syndrome in humans (Lowy, 1998; Weems, 2001). Over time, treatment of serious *S. aureus* infections can be challenging as the widespread use of antibiotics has led some *S. aureus* becoming more resistant to antibiotics (Archer, 1998; Akinjogunla *et al*., 2010). Recent development in the treatment of patients with otitis media include the evidence of the efficacy of antibiotics especially β-lactam antibiotics and newer topical quinolones such as ofloxacin and ciprofloxacin (Bearden and Danziger, 2001; Loy *et al*., 2002**).** The most common causes of bacterial resistance to β-lactam antibiotics are the production of β-lactamases, the presence of plasmid and mutation. Incidence of β-lactamase production in *Staphylococcus aureus* has consistently been reported to be over 80% in all parts of the world (Parker and Collier, 1990). Most developed countries have reported an increase in colonization and infection in hospitalized patients by CON-*Staphylococcus* spp. while there are scanty data on in fections caused by CON- *Staphylococcus* spp. in developing countries. The levels of antibiotic resistant infections in the developing world have increased steadily in the last few decades as a result of combination of microbial characteristics and the selective pressure of antimicrobial use (Blondeau and Tillotson, 2002). Microorganisms' mechanisms of overcoming the activities of antimicrobial agents include the production of structure-altering or inactivating enzymes (e.g. beta-lactamase or amino glycoside-modifying enzymes), alteration of penicillin-binding proteins or other cell-wall target sites, altered DNA gyrase targets, permeability mutations, active efflux and ribosomal modification (Gold and Moellering, 1996; Aaterson, 2001; Levy, 2002). Multidrug-resistant bacteria in both the hospital and community environment are important concern to the clinician, as it is the major cause of failure in the treatment of infectious diseases, increased morbidity, and mortality and the evolution of new pathogens (Hacker et al., 1997; Jones and Phaller, 1998).

Plasmids allow the movement of genetic material, including antimicrobial resistance genes between bacterial species and genera (Miranda *et al*., 2004). Plasmid profiles determination is the earliest DNA-based method used as serotype-specific reference patterns for detecting certain strain with possible variation in plasmid content which is very important in epidemiological studies. This study was carried out to determine the virulence factors, plasmid profile and curing analysis of multi-drug resistant *Staphylococcus aureus* and coagulase negative *Staphylococcus* spp. isolated from patients with acute otitis media.

MATERIALS AND METHODS

Middle-ear swabbed samples from 136 patients with acute otitis media attending University of Uyo Teaching Hospital, General Hospital, Ikot-Ekpene and Nekede Paediatric Hospital in Akwa Ibom, were collected from January, 2009 to January, 2010 under aseptic conditions and inoculated into broth cultures for 4-6hrs and later inoculated onto plates of Mannitol Salt Agar (MSA). The plates were incubated aerobically for 24 hrs at 37°C. After overnight incubation, the plates were examined for fermentation of mannitol indicated by colour change of the medium around each colony from red to yellow. The organisms on the positive plates were sub-cultured onto nutrient agar slants and further speciated by conventional laboratory techniques including Gram staining; catalase test, coagulase test, urease production, indole production, citrate

utilization and Voges-proskauer test and coagulase test and isolates that were Gram-positive cocci in cluster, indole negative**,** catalase positive, citrate positive and coagulase positive were considered as *Staphylococcus aureus* while the coagulase negative were considered as coagulase negative *Staphylococcus* spp (CON-*Staphylococcus* spp).

THE ANTIBIOTIC SUSCEPTIBILITY TESTING

The antibiotic susceptibility of the bacterial species isolated was performed on Muller-Hinton agar (MHA) (Merck) plates by disk diffusion method as described by the National Committee for Clinical Laboratory Standards with slight modification. 0.1 ml of each bacterial isolates was seeded into each of the Petri dishes containing Mueller-Hinton agar and were allowed to stand for 30 mins to enable the inoculated organisms to pre-diffuse. The commercially available discs containing the following antibiotics: Penicillin (PEN,10ug), streptomycin (STR,10ug), amoxicillin (AMY,10ug), iminipen (IMI,10 ug), ceftriaxone, (CEF,30ug), cephalothin (CEP,30ug), ceftazidime, (CAZ ,30ug), cefotaxime (CTX , 30ug), ofloxacin (OFL,5ug), ciprofloxacin (CIP, 5ug), levofloxacin (LEV, 5ug) and Moxifloxacin (MOX,5ug) (Oxoid, UK) were aseptically placed on the surfaces of the sensitivity agar plates with a sterile forceps and were incubated at 37°C over night. Zones of inhibition after incubation were observed and the diameters of inhibition zones were measured in millimeters. The interpretation of the measurement as sensitive and resistant was made according to the manufacturer's standard zone size interpretative table. The percentage resistance was calculated using the formula *PR=a/b×100, where 'PR'* was percentage resistance, '*a'* was the number of resistant isolates and '*b'* was the number of isolates tested with the antibiotic. The percentage sensitivity was calculated using the formula *PS=c/d×100, where 'PS'* was percentage sensitivity, '*c'* was the number of sensitive isolates and '*d'* was the number of isolates tested with the antibiotic.

DETERMINATION OF MULTIPLE ANTIBIOTIC RESISTANCE INDEX (MAR)

Multiple antibiotic resistance index (MAR) was determined using the formula MAR=*x/y,* where *x* was the number of antibiotics to which test isolate displayed resistance and *y* is the total number of antibiotics to which the test organism has been evaluated for sensitivity.

TEST FOR HEMOLYTIC ACTIVITY

The hemolytic activities of the bacterial species (*Staphylococcus aureus* and Coagulase negative *Staphylococcus* spp.) were identified by the presence of diffuse (α-hemolysis) or clear (β-hemolysis) halos around the colonies. A colony of each of the bacterial isolates was subcultured onto freshly prepared blood agar (nutrient agar containing human blood) plates incubated at 37^{o}C for 24 hours, after which the colonies were examined for hemolytic activity.

TEST FOR BETA LACTAMASE PRODUCTION

Beta-lactamase test was carried out using the Starch Paper Method (SPM) described by Odugbemi et al. (1977). Strips of starch paper about 4 – 6 cm were cut and sterilized using 70% ethanol, the strips were soaked for about 10 min in benzyl penicillin dissolved in phosphate buffer. The cut strips were then spread evenly on Petri dishes and about 18 – 24 hrs old cultures grown on Nutrient Agar were inoculated on the surface of the test starch paper and spread over an area of 2 -3 mm. The Petri dishes were incubated at 37^{o}C for 30 min then Gram's iodine solution was used to flood the plate and drained off immediately. The starch paper turns uniformly black within 30seconds of application. Colonies with decolourized zones are positive for beta-lactamase but colonies with black background show beta-lactamase negative.

PLASMID CURING EXPERIMENT:

Plasmid curing was carried out to determine in order to determine the location (plasmid-borne or chromosomal) of the drug resistance marker(s). The curing (elimination) of the resistant plasmids of the (*Staphylococcus aureus* and Coagulase negative *Staphylococcus* spp.) isolated was done using sub-inhibitory concentration of 0.10 mg/ml of acridine orange as described by Sheikh *et al*. (2003); Yah *et al* (2007); Akortha and Filgona (2009) with slight modification. Isolates *Staphylococcus aureus* and

Coagulase negative *Staphylococcus* spp. isolates were grown for 24hrs at 37°C in nutrient broth containing 0.10 mg/ml acridine orange. After 24hrs, the broth was agitated to homogenize the content and loopful of the broth medium were then subcultured onto Mueller Hinton Agar (MHA) plates and antibiotic sensitivity testing was carried out as previously described. Absence of zone of inhibition on Mueller Hinton agar was indicative of plasmids-mediated resistance (plasmid cured), while presence of zone of inhibition on Mueller Hinton agar was indicative of chromosome-mediated (plasmid not cured).

PLASMID PROFILING AND AGAR GEL ELECTROPHORESIS

Plasmid extraction was carried out using the method described by Ehrenfeld and Clewell.1987 wi th slight modification. Pure isolates were inoculated on MRS broth and incubated. The grown cells were harvested and suspended in 200µl of solution A (100mM glucose-50mM Tris hydrochloride (pH 8)-10mM EDTA) containing 10 mg of lysozyme per ml and 10µg/ml mutanolysin and incubated for 30 min at 37°C in an incubator. 400µl of freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH was added and the samples were mixed by inverting tubes. 300µl of a 30% potassium acetate solution (pH 4.8) was added and the samples were mixed by vortexing. After incubating on ice for 5 minutes, the debris was removed by a 5-minute centrifugation in a centrifuge (model 5415R; Eppendorf). The supernatant was removed and extracted once with a phenol-chloroform mixture (1:1) and precipitated with an equal volume of isopropanol. The plasmid DNA was then dissolved in 100µl of TE buffer. Electrophoresis of the DNA was carried out on a 0.8% agarose gel in a 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. Agarose gel was prepared by boiling 0.8g of agarose powder in 100mls of 0.5X TBE buffer. After boiling, the solution was allowed to cool and 10µl of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes and the comb was removed. 20µl of the plasmid DNA samples were then loaded into the wells after mixing with 2µl of bromophenol blue. A DNA molecular weight marker was also loaded into one of the wells. The gel was thereafter electrophoresed in a horizontal tank at a constant voltage of 60V for about 1 hour 30 minutes. After electrophoresis, plasmid DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light transilluminator and the photograph were taken using a digital camera. The DNA bands were matched with those for Lambda DNA Hind III digest molecular weight marker in the range 0.1 - 23.1kb. The approximate molecular weight of each plasmid was consequently obtained by extrapolation on graphical plots of molecular weight of marker against the distance traveled by the respective band

STATISTICAL ANALYSIS OF RESULTS

Frequencies and percentages were calculated for study variables. Chi-square ($\chi 2$) test was used to calculate probabilities and determine significance. A p-value of less than or equal to 0.05 was considered to be statistically significant ($p \leq 0.05$), while p-value more than 0.05 was considered to be statistically not significant (NS).

RESULTS AND DISCUSSIONS

The results of the morphological and biochemical characteristics of *Staphylococcus aureus* and CON-*Staphylococcus* spp. isolated from acute otitis media are shown in Table 1. The occurrence of the Bacterial spp. Isolated from 136 Patients with Acute Otitis Media are shown in Table 2. The virulence factors produced by *Staphylococcus aureus and* CON *Staphylococcus* spp**.** isolated are shown in Tables 3 and 4, with 16 (38.1%), 22 (52.4%) and 4 (9.5%) of *Staphylococcus aureus* producing alpha (diffuse haemolysis), beta (clear haemolysis) and gamma (absence of haemolysis), respectively, while CON-*Staphylococcus* spp**.** produced more of beta haemolysis than both alpha and gamma. The results showed that 14 (33.3%) *Staphylococcus aureus* are β-lactamase (βL) producer, while only 9 (42.9%) of CON-*Staphylococcus* spp produced β-lactamase (Table 4).

The antibiotic susceptibility testing data are shown in Table 5. Of the forty-two *Staphylococcus aureus* screened for susceptibility to the 12 antibiotics the results showed that 29 (69.0%), 26

(61.9%), 27 (64.3%), 28 (66.7%) and 29 (69.0%) of *Staphylococcus aureus* were sensitive to penicillin, ceftriazidime, cefoxitin, ciprofloxacin and levofloxacin, respectively. A total of 12 (28.6%) of *Staphylococcus aureus* were resistant to both streptomycin and iminipen, while about 45.2% - 50.0% were resistant to cephalothin and amoxicillin. The results of the antibiotic susceptibility profile of the twenty-one CON-*Staphylococcus* spp also showed varied percentages of sensitivity ranging between 42.9% for streptomycin and 71.4% for moxifloxacin. The resistant of CON-*Staphylococcus* spp to ciprofloxacin, levofloxacin, ceftriazidime and moxifloxacin were low compared to the result obtained when tested with streptomycin and iminipen (Table 5). The most effective antibiotic against *Staphylococcus aureus and* CON-*Staphylococcus* spp isolated from acute otitis media was moxifloxacin as only 26.0% of the bacteria were resistant to it. The multiple antibiotic resistance (MAR) indexes of the *Staphylococcus aureus* and CON-*Staphylococcus* spp are shown in Table 6. The antibiotic resistant *Staphylococcus aureus* have MAR index of 0.25 to 1.00, while the antibiotic resistant CON-*Staphylococcus* spp have MAR index of 0.25 to 0.75. The result showed that 19.2 % of *Staphylococcus aureus and* 9.6% of CON-*Staphylococcus* spp. were resistant to more than eight antibiotics. All the resistant *Staphylococcus aureus and CON-Staphylococcus* spp were subjected to plasmid-curing experiments using acridine orange and the results obtained showed that most of the strains lost their plasmids as a result of the cure by acridine (Mutagenic substance) (Tables 7 and 8). The plasmids molecular weights of both *Staphylococcus aureus and* CON-*Staphylococcus* spp ranged from 23.13kb to 50.0kb (Figures 1 and 2). The isolation of *Staphylococcus aureus* and coagulase negative *Staphylococcus* spp. in the middle ear of patients suffering from patient with acute otitis media in this research is in agreement with the report by Ekpo et al., 2009. The fermentation and growth of *Staphylococcus aureus and* CON-*Staphylococcus* spp on mannitol salt agar in this study could be attributed to its ability to grow on relatively high concentrations of sodium chloride, as contained in the medium (Nester et al., 1998). Pathogenicity of *Staphylococcus aureus* in acute otitis media are attributable to virulence factors such as coagulase and hemolysin produced by the organisms and the occurrence of this virulence factors in *Staphylococcus aureus* is in conformity with the reports of Nester et al., (1998). Geary et al, (1997) reported that coagulase negative *Staphylococcus* spp resistant to beta-lactam antibiotics produced beta-lactamase and this is in agreement with our finding as some of the coagulase negative *Staphylococcus* spp isolated are resistant to penicillin, iminipen, ceftriaxone, cephalothin, ceftazidime, and cefotaxime (beta-lactam antibiotics).

Resistant *S. aureus* was seen in clinical practice as early as the 1950s, by acquiring a plasmid that encodes the production of beta-lactamase enzymes causing resistance to beta-lactam antibiotics. The activities of antibiotics against *Staphylococcus aureus* and coagulase negative *Staphylococcus* spp. isolated from acute otitis media patients attending the three Hospitals showed the varied levels of multiple antibiotics resistance. There is wide variation in the use of antibiotics among the physicians of different nations from as low as 31% of cases of acute otitis media in Netherland to as high as 90% in Australia and United States (Delmar et al., 2003). The exceedingly increases and emergence of multidrug resistance pathogens in the developing countries can be attributed to indiscriminate use of antibiotics, complex socio-economic, behavioral antecedents and dissemination of drug-resistant pathogens in human medicine. (Okeke et al., 1999). Antibiotic resistance of pathogens typically causative of acute otitis media continues to increase as the emergence of multi-drug resistant strains especially *Staphylococcus aureus and* CON-*Staphylococcus* spp complicate the management of acute otitis media and increase the risk for treatment failure (Leibovitz, 2003).

Plasmid replication is inhibited by various agents especially acridine (acridine orange) that intercalates between the bases of DNA, without inhibiting the chromosomal DNA replication. In order to determine whether the observed multi drug resistance pattern in the isolates was plasmid or chromosomal mediated, the isolates were screened for the presence of conjugative plasmids using acridine orange and resultantly, some of the resistance markers were stably lost, the lost of resistance markers using acridine orange is line with

that of Yah et al (2007) and Akortha and Filgona (2009). Isolation of plasmids using agarose gel electrophoresis and observation under UV transilluminator showed the various bands for the *Staphylococcus aureus* and coagulase negative *Staphylococcus* spp. with the molecular weights of plasmids ranging from 23-50 Kbp .The molecular weights seemed to be strain specific rather than species specific. In conclusion, culture and sensitivity testing will be instrumental in the management of this infection and continuous surveillance to monitor antimicrobial resistance and guide the antibacterial therapy is overwhelmingly necessary.

Table 1: Morphological and Biochemical Tests of *Staphylococcus aureus and* CON- *Staphylococcus* spp.

Morphology / Biochemical Tests	*Staphylococcus aureus*	CON-*Staphylococcus* spp
Shape	Coccoid in cluster	Coccoid in cluster
Gram Staining	+ve	+ve
Mannitol	A	A
Sucrose	A	A
Maltose	A	A
Lactose	A	A
Galactose	A	A
Glucose	A	A
Catalase	+ve	+ve
Coagulase	+ve	-ve
Indole	-ve	-ve
Citrate	+ve	+ve
Methyl red	+ve	+ve
Voges-proskauer	-ve	-ve
Gelatin hydrolysis	-ve	-ve

Keys: +ve = positive; -ve = negative; A = Acid production

Table 2: Bacterial spp. Isolated From 136 Patients with Acute Otitis Media

Bacterial spp. isolated	Number of Occurrence	Percentage (%) of Occurrence
Staphylococcus aureus	42	30.9
CON *Staphylococcus* spp.	21	15.4
Total	63	46.3

$p \leq 0.05$

Table 3: Number of Occurrence and Types of Haemolysis Produced by *Staphylococcus aureus and* CON-*Staphylococcus* spp. Isolated from Patients with Acute Otitis Media

Bacterial spp.	Number of Occurrence	Types of Haemolysis α (%)	β (%)	γ (%)
Staphylococcus aureus	42	16 (38.1)	22 (52.4)	4 (9.52)
CON *Staphylococcus* spp.	21	6 (28.6)	8 (38.1)	7 (33.3)
TOTAL	63	22 (34.9)	30 (47.6)	11(17.5)

$p \leq 0.05$

Keys: α: alpha; β: beta; γ: gamma; CON: Coagulase negative.

Table 4: The Prevalence of Beta-Lactamase (βL) Producing *Staphylococcus aureus and* CON-*Staphylococcus* spp Isolated from Patients with Acute Otitis Media

Bacterial spp	Number of Occurrence	No / (%) of βL Producers	No / (%) of βL Non Producers
Staphylococcus aureus	42	14 (33.3)	28 (66.7)

CON *Staphylococcus* spp	21	9 (42.9)	12 (57.1)
TOTAL	63	23 (36.5)	40 (63.5)

p≤0.05

Table 5: Antibiotic Susceptibility Spectrum of Bacterial spp. Isolated from Acute Otitis Media

	Staphylococcus aureus (N=42)		CON-*Staphylococcus* spp (N=21)	
	Number /Percentage	Number /Percentage	Number /Percentage	Number /Percentage
Antibiotics Used	Sensitive	Resistant	Sensitive	Resistant
Penicillin	29 (69.0)	13 (31.0)	13 (61.9)	8 (38.1)
Streptomycin	30 (71.4)	12 (28.6)	9 (42.9)	12 (57.1)
Amoxicillin	21 (50.0)	21 (50.0)	10 (47.6)	11 (52.4)
Iminipen	30 (71.4)	12 (28.6)	9 (42.9)	12 (57.1)
Ceftriaxone	24 (57.1)	18 (42.9)	13 (61.9)	8 (38.1)
Cephalothin	23 (54.8)	19 (45.2)	11(52.4)	10 (47.6)
Ceftriazidime	26 (61.9)	16 (38.1)	14 (66.7)	7 (33.3)
Cefoxitin	27 (64.3)	15 (35.7)	13 (61.9)	8 (38.1)
Ofloxacin	23 (54.8)	19 (45.2)	11 (52.4)	10 (47.6)
Ciprofloxacin	28 (66.7)	14 (33.3)	14 (66.7)	7 (33.3)
Levofloxacin	29 (69.0)	13 (31.0)	14 (66.7)	7 (33.3)
Moxifloxacin	31 (73.8)	11 (26.2)	15 (71.4)	6 (28.6)

p≤0.05

Table 6: Multiple Antibiotic Resistance Index of Bacteria Isolated from Acute Otitis Media

Multiple Antibiotic Resistance Index (MAR)	*Staphylococcus aureus* Number / Percentage	CON-*Staphylococcus* spp. Number / Percentage
0.25	6 (14.3)	2 (9.5)
0.33	7 (16.7)	3 (14.3)
0.42	4 (9.52)	2 (9.5)
0.50	6 (14.3)	2 (9.5)
0.58	3 (7.1)	3 (14.3)
0.66	2 (4.8)	1 (4.8)
0.75	2 (4.8)	1 (4.8)
0.83	2 (4.8)	0 (0.0)
0.91	1 (2.4)	0 (0.0)
1.00	1 (2.4)	0 (0.0)

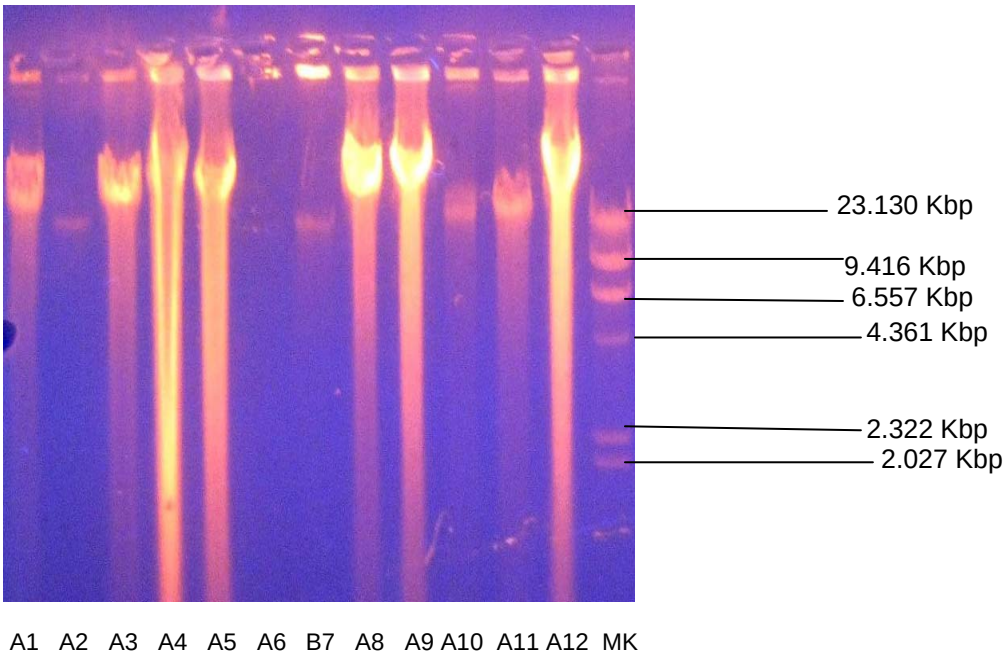

Figure 1: Agarose electrophoresis showing plasmid profile of *Staphylococcus aureus* isolated from acute otitis media: Line A1: (>23.13 kb), A2: (>23.13 kb), A3: (>23.13 kb); A4:(>23.13 kb); A5: (>23.13 kb); A6 (No plasmid); A7: (23.13 kb) ;A8: (>23.13 kb) ; A9: (>23.13 kb); A10: (23.13 kb); A11: (23.13 kb) A12: (>23.13 kb); MK: molecular weight marker (*Hind* III digest).

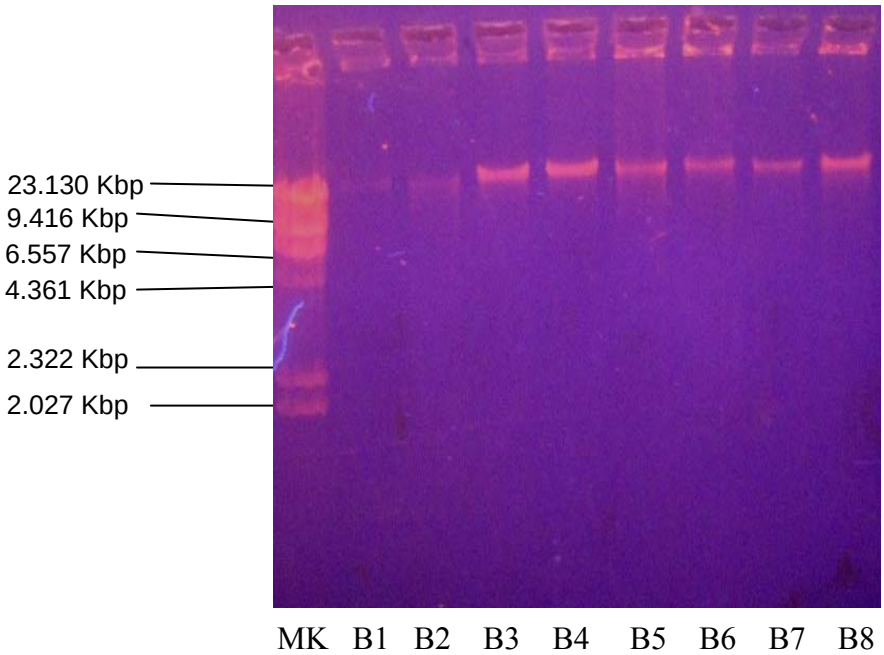

Figure 2: Agarose electrophoresis showing plasmid profile of CON-*Staphylococcus* spp. isolated from acute otitis media. MK: molecular weight marker (*Hind* III digest).Line B1: (No plasmid), B2: (23.13 kb), B3: (23.13 kb); B4 - B8: (>23.13 kb)

Table 7: Plasmid Curing Analysis of Resistant *Staphylococcus aureus* Isolated from Acute Otitis Media with Acridine Orange (0.1 mgml^{-1}).

Antibiotics Used	Number Resistant (Pre-curing)	Number /Percentage Cured	Number /Percentage Resistant (Post- curing)
Penicillin	13	8 (61.5)	5 (38.5)
Streptomycin	12	8 (66.7)	4 (33.3)
Amoxicillin	21	14 (66.7)	7 (33.3)
Iminipen	12	6 (50.0)	6 (50.0)
Ceftriaxone	18	13 (72.2)	5 (27.8)
Cephalothin	19	15 (78.9)	4 (21.1)
Ceftriazidime	16	12 (75.0)	4 (25.0)
Cefoxitin	15	9 (60.0)	6 (40.0)
Ofloxacin	19	14 (73.7)	5 (26.3)
Ciprofloxacin	14	10 (71.4)	4 (28.6)
Levofloxacin	13	9 (69.2)	4 (30.8)
Moxifloxacin	11	7 (63.6)	4 (36.4)

$p \leq 0.05$

Table 8: Plasmid Curing Analysis of Resistant CON-*Staphylococcus* spp Isolated from Acute Otitis Media with Acridine Orange (0.1 mgml^{-1}).

Antibiotics Used	Number Resistant (Pre-Curing)	Number / Percentage Cured	Number /Percentage Resistant (Post- curing)
Penicillin	8	6 (75.0)	2 (25.0)
Streptomycin	12	6 (50.0)	6 (50.0)
Amoxicillin	11	7 (63.6)	4 (36.4)
Iminipen	12	9 (75.0)	3 (25.0)
Ceftriaxone	8	7 (87.5)	1 (12.5)
Cephalothin	10	7 (70.0)	3 (30.0)
Ceftriazidime	7	4 (57.1)	3 (42.9)
Cefoxitin	8	5 (62.5)	3 (37.5)
Ofloxacin	10	8 (80.0)	2 (20.0)
Ciprofloxacin	7	6 (85.7)	1 (14.3)
Levofloxacin	7	5 (71.4)	2 (28.6)
Moxifloxacin	6	4 (66.7)	2 (33.3)

$p \leq 0.05$

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