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Study of plasmid profile, susceptibility patterns of clinical *Staphylococcus aureus* isolated from patients with otitis media in Basrah

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Abstract

The present study was designed to investigate plasmid profile, the prevalence of multidrug resistant *Staphylococcus aureus*, which was done in Basrah, Iraq, during the period between September 2010 and January 2011. A total of seventy six Ear swab were collected from seventy six patients (32 male and 44 female) with chronic and acute suppurative otitis media (CSOM, ASOM) with or without discharges. Thirty five (46.02%) isolates diagnosed as *Staphylococcal spp* and twenty four (31.6%) as *S. aureus* isolates from (10 male and 14 female). *S. aureus* isolates tested for antibiotic susceptibility, high antibiotic resistance to Ampicillin, Carbenicillin, Amoxicillin, Nalidixic acid, and Ceftriaxone , mid resistance to Streptomycin , Erythromycin and Oxacillin, low resistance to Neomycin, Tetracycline, Kanamycin, Tobramycin ,Gentamicin, Clinamycin, Cephalexin, and sensitive to Chloramphenicol, Vancomycin, Ciprofloxacin and Rifampicin . *S.aureus* isolates showed multiple antibiotic resistance, Such that, two isolates resisted four types of antibiotics. Five isolates were resisted five types of antibiotics. three isolates resisted six types of antibiotics . Five isolates resisted seven types of antibiotics. Two isolates resisted eight types of antibiotics . Two isolates resisted nine types of antibiotics. Two isolates resisted ten types of antibiotics. Two isolates resisted eleven types of antibiotics. Only one isolate resisted fifteen types of antibiotics. The MAR index of the isolates ranged between 0.2 and 0.75. All isolates harbore Large molecular weight plasmids ranging from (21 – 22) Kbp .

Key words : *Staphylococcus aureus*, plasmid profile, otitis media, antibiotic resistance.

1. Introduction

Staphylococcus aureus is one of the most successful and adaptable human pathogens, it is also the most common colonizer of the skin and the nose, its remarkable ability to acquire antibiotic resistance has contributed to its emergence as an important pathogen in a variety of settings [1,2]. *S. aureus* caused of staphylococcal infections and is responsible for various diseases including: mild skin infections, (impetigo, folliculitis, Furuncle), invasive diseases (wound infections, osteomyelitis, bacteremia with metastatic complications), and toxin mediated diseases (food poisoning, Toxic Shock Syndrome (TSS) Scaled Skin Syndrome (SSS), etc). Infections are preceded by colonization. Common superficial infections include, carbuncles, impetigo, folliculitis cellulitis [3,4,5]. Nosocomial infections can be caused by a wide variety of pathogens, *S. aureus* is one of the common of both endemic and epidemic infections acquired in hospitals which result in substantial morbidity and mortality [6]. *S.aureus* was the commonest microorganism cultured in otitis media [7], causing 50% or more of hospital-acquired *S.aureus* infections in several countries [8,9]. Nasal carriage of *S. aureus* has been identified as a risk factor for community-acquired as well as nosocomial infections [10]. Multidrug-resistant strains of staphylococci have been reported with increasing frequency worldwide, including isolates that are resistant to methicillin, lincosamides, macrolides, aminoglycosides, fluoroquinolones, or combinations of these antibiotics [11]. Infections by *S.aureus* are often difficult to treat because of frequency of multiple antibiotic resistance of strains [12]. *S. aureus* has a proven ability to adapt to

the selective pressure of antibiotics [13]. At present, *Staphylococcal* resistance to antibiotic has been associated with resistant plasmids (R-plasmid) that have the ability to mediate the production of drug inactivated enzymes such as β -lactamase [14,15] and other functions [16,17]. The spread of resistance to antimicrobial agents in *S. aureus* is largely due to the acquisition of plasmids and/or transposons [18]. Plasmids allow the movement of genetic materials including antimicrobial resistance genes between bacterial species and genera, plasmid profiles determination is the earliest DNA based method used as serotype specific patterns for detecting certain strain with possible variation in plasmid content.

in epidemiological studies [19]. Otitis media is the infection associated with the inflammation of the middle ear due to pathogenic microorganisms that are resident in the middle ear and on skin [20,21]. In otitis media, the middle ear is usually affected as a result of colonization by pathogenic organisms which can ultimately result in deficiency or impaired hearing [23]. *Otitis media* differs in complication and this depends on the level of severity and the duration of the infection in relation to the causative organisms, hence, otitis media can be differentiated into chronic suppurative otitis media (CSOM) and acute suppurative otitis media (ASOM) [24] possibly causing febrile seizures and can lead to insomnia for patients, mild to moderate hearing loss, loss of balance, unusual irritability, unresponsiveness to quiet sounds, and draining of fluid in the ear.[23,25]

2. Materials And Methods

2.1. Bacteria

A total of seventy six (76) patients with otitis media were included in this study, diagnosis of otitis media was carried out under the supervision of the specialists of ENT. Microbiological investigation includes the identification of causative agents, antibiotic sensitivity and investigated plasmid profile. This study was carried out in Basrah General Hospital, out patients and in patients clinic, during the period from November 2010 to January 2011. Collected swabs were taken and cultured under sterile condition and transferred immediately to the laboratory by brain heart broth tubes, primary isolation on selective media to *Staphylococcus* (mannitol salt agar) at 37°C for 24-48 hr. then the identification and the biochemical characterization were carried out according to standard routine techniques [10]. Well isolated colonies were picked up and stored in nutrient agar slante at 4°C. The pure culture was made by picking single colonies from the stored isolation cultures.

2.2. Media and culture condations:

Mannitol salt agar, Muller Hinton agar, Blood agar, Nutrient agar, Nutrient broth, (Himedia, India) DNase agar, Brain-Heart infusion broth, Urease base agar, (Titan Biotech) were used in the isolation and identification. All media are sterilized by autoclave 121 under 15 lbs pressure for 15 min. other materials:

agarose (Bromega, USA) Lambda DNA, HindIII + EcoRI digested (Bioneer, Korea), NaOH, EDTA, lysozyme, glucose. High-Speed Plasmid Mini Kit, (Geneaid, UKAS), Tris-HCl, SDS, Sodium acetate and Boric acid are used. Bacterial culture was done at 37°C throughout the experiment.

2.3. Antibiotics discs :

Ampicillin 10 mg , Amoxicillin 25 mg ,Carbincillin 100 mg , Norfloxacin 10 mg , Ceftriaxon 30 mg , Cephalixin 30 mg , Tobramycin 10 mg , Neomycin 30 mg, Nalidixic acid 30 mg , Erythromycin 15 mg , Tetracycline 30mg , Ciprofloxacin 5 mg, Gentamicine 10 mg , Streptomycin 10 mg , Chloramphenicol 30 mg , Kanamycin 30 mg , Rifampicin 5 mg , Clindamycin 2 mg ,Vancomycin 30 mg (Bioanalyse), Oxacillin 1 mg (Oxoid.)

2.4. Plasmid DNA extraction

A total of 24 multidrug resistant isolates of *S. auerus* are selected for plasmid extraction. Plasmid DNA was extracted by a standard method then estimated by spectro- photometric [26].Plasmid extraction was carried out using the method : Pure isolates were inoculated on LB broth and incubated at37 for 18 hr. The grown cells were harvested and suspended in 200µl of solution A (100 mM glucose-50 mM Tris hydrochloride (pH 8)-10mM

EDTA) containing 10 mg of lysozyme per ml and incubating for 30 min at 37°C in an incubator. 400µl of freshly prepared 1% sodium dodecyl sulfate (SDS) in 0.2 N NaOH was added and the samples were mixed by inverting tubes then incubating for 10 min at 20 ° C. 300µl of a 30% Sodium acetate solution (pH 4.8) was added and the samples were mixed by inverting the tube 10 times. After incubating on ice for 5 minutes, the debris was removed by centrifugation (10.000 x g for 5-minute), and precipitated with an equal volume of Isopropanol plasmid DNA was dissolved in 50µl of TE buffer (containing RNaseA), incubating for 30 min at 37°C in an incubator and stored over night at 4°C before electrophoresis .

Plasmid NDA is isolated also by using High-Speed Plasmid Mini Kit. UKAS.

2.5. Electrophoresis analysis of the plasmid DNA

Agarose gel electrophoresis for plasmid DNA of multidrug resistant isolates of *S. auerus* was carried out on 1% agarose (Bromega ,USA), and ultraviolet light transilluminator at 60V for 1hr , 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 plasmid DNA bands were matched with those for Lambda DNA (HindIII + EcoRI digested) molecular weight marker in the range 0.1-22 Kbp (Bioneer. Korea) .

3. RESULTS

3.1. Isolation and Identification

A total of 76 aural swab collected from patients with otitis media, 35 bacterial isolates were grown on mannitol agar plates subjected to hemolytic test on blood agar ,gram staining , and catalase test in an attempt to screen the β-hemolytic , gram positive, and catalase positive strains and thirty strains were found to have such characters . These strains are subjected to various morphological and

biochemical tests . It was found only 24 (31.6%) isolates were identified as *Staphylococcus aureus* table (1) according to their growth morphology and biochemical reactions patterns such as coagulase, DNase and β-Galactosidase tests. in respect to cultural characteristics and colony morphology it was found that on blood agar the colonies are circular, golden yellow on nutrient agar, on microscopic

observation it was revealed that the cells were arranged in pair or short chain and

clusters, the isolates were coagulase, DNase and β -Galactosidase positive.

Table (1) Frequency of *S. aureus* , CONS and other bacteria from otitis media

No. of The samples	No. of Staphylococcal isolates			Others(%)
	Total (%)	<i>S aureus</i> (%)	CONS (%)	
76	35 (46.02%)	24 (31.58%)	11(14.47%)	41(53.94%)

3.2 Antibiotic susceptibility test :

All the twenty four *Staphylococcus aureus* isolates were tested in vitro to determine their antibiotic susceptibility patterns by antibiotic disc diffusion method .All isolates showed multiple antibiotic resistances to the antibiotics tested, the results showed in table (2). All isolates (100%) were resistant to Ampicillin ,Amoxicillin While, Carbenicillin showed resistance (95.8%), Nalidixic acid (87.5%), Ceftriaxone (75%), Streptomycin

(54.16%), Erythromycin, Tetracycline, Kanamycin and Neomycin (25%) , Oxacillin (41.67%), Tobramycin (29.16%), Gentamicin (20.83%), Clinamycin (8.33%) , Cephalexin (12.5%), and there was no resistance found to Chloramphenicol ,Vancomycin, Norfloxacin, Ciprofloxacin and Refampicin. These antibiotics the most effective against *S.aureus* isolated from acute otitis media .

Table (2): Susceptibility of clinical isolates of *S.aureus* to 20 diffirente antibiotics

Antibiotic	Abbrevative	Resistans	(%)	Intermediate	(%)	Sensitive	(%)
Ampicillin	AM	24	(100)	0	(0)	0	(0)
Amoxicillin	AX	24	(100)	0	(0)	0	(0)
Carbincillin	PY	23	(95.83)	1	(4.16)	0	(0)
Norfloxacin	NOR	0	(0.0)	7	(29.16)	17	(70.83)
Ceftriaxon	CRO	18	(75.0)	6	(25.00)	0	(0)
Cephalexin	CL	3	(12.5)	8	(33.33)	13	(54.16)
Tobramycin	TOB	7	(29.16)	2	(8.33)	15	(62.5)
Neomycin	N	6	(25.0)	15	(62.5)	3	(12.5)
Nalidixic acid	NA	21	(87.5)	2	(8.33)	1	(4.16)
Erythromycin	E	6	(25.0)	8	(33.33)	6	(25)
Tetracycline	TE	6	(25.0)	1	(4.16)	17	(70.83)
Ciprofloxacin	CIP	0	(0)	2	(8.33)	22	(91.66)
Gentamicine	CN	5	(20.83)	2	(8,33)	16	(66.66)
Streptomycin	S	13	(54.16)	9	(37.5)	2	(8.33)
Chloramphenicol	C	0	(0)	0	(0)	24	(100)
Kanamycin	K	6	(25.0)	7	(29.16)	11	(45.83)
Rifampicin	RA	0	(0)	1	(4.16)	23	(95.83)
Clindamycin	DA	2	(8.33)	10	(41.66)	12	(50)
Vancomycin	VA	0	(0)	0	(0)	24	(100)
Oxacillin	OX	10	(41.67)	0	(0)	14	(58.33)

3.3 Multiple antibiotic resistance :

All *Staphylococcus aureus* isolates showed multiple antibiotic resistance. Such that, two isolates resisted four types of antibiotics. Five isolates resisted five types of antibiotics. three isolates resisted six types of antibiotics. Five isolates resisted seven types of antibiotics. Two isolates resisted eight

types of antibiotics. Two isolates resisted nine types of antibiotics. Two isolates resisted ten types of antibiotics. Two isolates resisted eleven types of antibiotics .Only one isolate was resist fifteen types of antibiotics. (table 3) and (table 4)

Table (3) Antibiogram patterns to multiple antibiotic resistance isolates

multiblicity	Number of isolates	patterns of Antibiotic resistance
4	1	AM ,AX,PY,CRO
4	1	AM , AX , PY, NA
5	4	AM , AX, PY,NA,CRO
5	1	AM ,AX, NA ,CRO, TE
6	2	AM , AX, PY, CRO, NA,S
6	1	AM , AX , PY, CRO , TE , E
7	3	AM , AX , PY,CRO,NA, E,OX
7	2	AM , AX , PY, CRO , NA , S ,TE
8	2	AM , AX , PY, CRO, NA , S, CL ,OX
9	2	AM , AX , PY, NA , N, S , K , E , TOB
10	1	AM , AX , PY, NA ,N, S, K ,CN,TOB,OX
10	1	AM , AX , PY,CRO,S ,CN, TOB ,TE ,DA,OX
11	2	AM , AX , PY, NA , N, CN ,TOB , S, K , E ,OX
15	1	AM,AX,PY,CRO,NA,N,CL,TOB,S,E,K,CN,TE,DA,OX

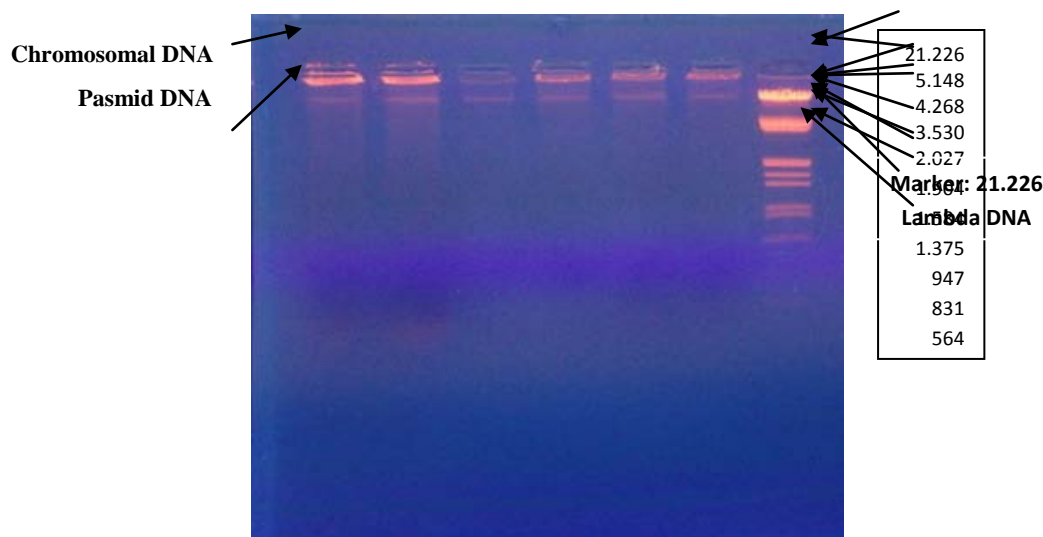
Table (4) Multiple antibiotic resistance index. (MAR Index)

NO.of isolates (%)	2 (8.3)	5 (20.8)	3 (12.5)	5 (20.8)	2 (8.3)	2 (8.3)	2 (8.3)	2 (8.3)	1 (4.6)
NO.of antibiotic	4	5	6	7	8	9	10	11	15
MAR Index	0.2	0.25	0.3	0.35	0.4	0.45	0.5	0.55	0.75

3.4 Electrophoretic analysis of the plasmid DNA and Plasmid profile :

Electrophoretic analysis of the plasmid DNA prepared was carried out by agarose gel Electrophoresis on 0.8% . 23(%95.83) isolates harbor a single plasmid DNA on basis of electrophoretic mobility on agarose gel. The molecular size of the plasmid DNA

was calculated to be (21-22Kbp) . Lambda DNA (HindIII + EcoRI digested) was used as marker DNA . The study results showed that 1 isolate non harbors any plasmid band which resisted four antibiotic (AM,AX,PY,CRO) .



Figure(1) plasmid profile of multidrug resistance *S. aureus* strains

4. Discussion

All swab samples were cultured on MSA plates. The results of isolation of *Staphylococcus aureus* isolates from patients with acute otitis media in this study was 24 isolate(%31). The results agreed with The studies done by [26,27,28,29,30,31]. *Staphylococcus aureus* was the most common agent in patients with otitis media and not approved [16,27,31]. 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 [29].

S. aureus resistance to ampicillin 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 [29]. The activities of antibiotics against *Staphylococcus aureus* that were isolated from acute otitis media patients in general Basrh Hospital showed the

varied levels of multiple antibiotics resistance. The exceedingly increases and emergence of multidrug resistance pathogens in the developing countries can be attributed to the indiscriminate use of antibiotics, complex socio-economic, behavioral antecedents and the dissemination of drug-resistant pathogens in human medicine [35]. Antibiotic resistance of pathogens typically causative of acute otitis media continues to increase as the emergence of multi-drug resistant strains especially *Staphylococcus aureus* complicate the management of acute otitis media and increase the risk for treatment failure [31]. The results of this study demonstrated that the majority of *Staphylococcus* strains that were isolated from otitis media patients showed a high level of sensitivity to Ciprofloxacin (91.22%), Norfloxacin (66.66%), and Clindamycin (50%) Most of these antibiotics are the third generation of antibiotics and are rare in use this prevents the organisms to develop resistance to them. The

development of moderate susceptibility to Erythromycin and Gentamycin despite the fact that they are not third generation antibiotics, so this indicates that they are not being abused or commonly prescribed. This study revealed that *S.aureus* resisted the commonly

prescribed antimicrobial agents such as Ampicillin, Carbincillin, Amoxicillin, Nalidixic acid and Ceftriaxon.

This study results agreed with [26,27,7,31,32] therefore it is not advisable to prescribe these agents to patients with otitis media caused by CPSA-induced infection in the studied area without first carrying out antibiotic sensitivity test on the isolate. Patients with antibiotic resistant CPSA pose a unique problem in terms of epidemiology of the disease as well as

5. Conclusions

This study revealed that otitis media in patients is highly prevalent caused by *S. aureus*, and there is the prevalence of antibiotic resistant strains which make a problem in treatment otitis media. Since otitis media is a nosocomial and community acquired infection, it is recommended that over crowding in the health institution should be avoided to reduce the spread of the infection within the hospital. *S.aureus* isolates are

its treatment [33]. Most of these patients may be asymptomatic carriers and could serve as source of infection to other susceptible population as well as other hospitals [34]. The isolation of plasmids using agarose gel electrophoresis and observation under UV transilluminator showed the single band for the *Staphylococcus aureus*. with the molecular weights of plasmids ranging from 21-22 Kbp, this results agreed with [31,32] Plasmid isolation procedure are based on the fact that plasmids usually occur in the covalently closed circular (supercoiled) ccc configuration within the host cells. After gentle cell lysis all intracellular macromolecules have to be eliminated whereas plasmid DNA is enriched and purified [18,20].

sensitive to Vancomycin by disc diffusion method, High percent of isolated *S. aureus* found to be resistant to Ampicillin and Amoxicillin that may be due to the irrational use of this antibiotic. The results obtained from electrophoresis analysis and fluorescence of bands under short wave ultraviolet light transilluminator showed that multidrug resistance mediated by plasmids.

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