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BIOMOLECULAR IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS* AND SCREENING OF METHICILLIN RESISTANT STRAIN FROM PUS /WOUND SAMPLE IN TERTIARY CARE HOSPITAL QUETTA

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Abstract:

The aim of this study was the bio-molecular identification of Staphylococcus aureus and screening of methicillin resistant strain. To evaluate the study, 60 samples of pus and wound were collected from Bolan Medical Complex Hospital Quetta, Balochistan during period from September 2016 to December 2017. Out of 60 samples processed for isolation, ten (10) were found positive for staphylococcus aureus. Isolates were recognized by growth characteristic on selective media, Gram staining and different biochemical tests like oxidase, catalase, coagulase, motility, thermonuclease (DNase), haemolysis and mannitol fermentation and citrate utilization test. All the isolates found positive through biochemical tests also confirmed by PCR based on 16S rRNA and Nuc gene. In the present study antibiotic sensitivity and resistance pattern of Staphylococcus aureus was recorded to the following antibiotics; Tetracycline, Ciprofloxacin, Ceftriaxone, Vancomycin, Penicillin, Ampicillin, gentamicin, Cloxacillin, Methicillin and Novobiocin were 30%, 80%, 70%, 100%, 10%, 10%, 70%, 40%, 80% and 30% respectively. Two isolates which showed resistant to methicillin were also found resistant to all other antibiotics except Vancomycin.

Keyword: *MRSA, Staphylococcus aureus, antibiotic susceptibility, Biomolecular identification.*

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INTRODUCTION:

Staphylococcus aureus is a gram-positive coccus, adapted to colonize the human skin. It is 0.5 to 1.5 mm in diameter. It is non-motile, non-spore forming facultative anaerobes that produce catalase and coagulase enzymes. It is the major cause of bacteremia, pneumonia, myocarditis, acute endocarditis, pericarditis, osteomyelitis, encephalitis, meningitis, mastitis and scalded skin syndrome. In addition there is emergence of high rate of antimicrobial resistance such as methicillin resistant *S.aureus* (MRSA) which is an alarming situation [1]. MRSA is also among the most widespread nosocomial pathogen. The MRSA emergence as nosocomial agents due to extended hospital stay, unsystematic use of antibiotics, lack of awareness, and introduction of antibiotics before coming to the hospital [2].

Methicillin resistant *S. aureus* (MRSA) strains have colonized a gene that makes it resistant to nearly all beta lactam antibiotics. Such resistance is also observed in hospital associated MRSA. This organism also causes dangerous nosocomial diseases and its treatment has challenged the modern world [3]. Many PCR based molecular techniques were used for the right identification of *staphylococcus aureus*, the most widely and reliable method in use is by Nuc gene and 16S rRNA gene. This is the most important diagnostic way for the confirmation of *staphylococcus aureus* [4] Al. Reliable identification of *Staphylococcus aureus* is of major concern in clinical microbiological diagnostics. The widely effective and diagnostic taxonomic marker molecules are 16S rRNA[5] and the Nuc gene, which encodes thermonuclease, are widely used as a specific target for the identification of *Staphylococcus aureus* by PCR [6].

MATERIALS AND METHODS:

Bacterial Isolation: Present study was conducted from September 2016 to December 2017. A total of 60 samples were collected from Burn and wound section of Bolan medical college hospital Quetta (BMCH). All the sample were collected aseptically, transported in cool chain and cultured into Brain heart infusion (BHI) broth media and transferred to mannitol salt agar (MSA) for confirmation of Mannitol fermentation. All the inoculated samples were incubated overnight at 37^0Cas done by [7].

Identification of *Staphylococcus aureus* through Biochemical test: Identification of the clinical isolates were performed by growth characters on selective media and conventional biochemical tests, including Gram staining, oxidase, catalase, coagulase, motility, thermonuclease (DNase), hemolysis, mannitol fermentation tests and citrate utilization test [8].

Molecular identification

DNA isolation

Isolate of *Staphylococcus aureus* was cultured into 5 ml BHI broth and incubated at 37^0C for 24 h. DNA was isolated by CTAB (Cetyl-trimethyl-ammonium bromide) as performed by [9]. Isolated DNA was stored at -20°C.

PCR for 16S rRNA.

PCR approaches are being used routinely for the confirmation of *staphylococcus aureus*. All coagulase positive isolates were identified by species specific PCR (16S rRNA) and Multiplex PCR assay by using (16S rRNA and Nuc gene) (Table 4).

The following reaction mixture was added to each sample: PCR mixture was prepared as (2ul DNA, 2ul each primer (100 pmol), 1.5ul MgCl, 2.5 ul 10X PCR buffer, 0.5 ul dNTPs, 0.2 ul Taq polymerase. A total reaction of 25ul was made by adding PCR grade H_2O [12]. The amplification was performed in a Thermal Cycler (Eppendorf, Germany) beginning with an initial denaturation step at 94^0C for 2min followed by 35 cycles of 94^0C for 30 secs, 50^0C for 30sec, and 72^0C for 45 sec, ending with final extension step at 72^0C for 4 min.

Multiplex PCR for identification of *S. aureus*: Multiplex PCR was performed by using two primers (Nuc gene & 16S rRNA) (Table 4). The following reaction mixture was prepared for each sample. PCR mixture (2ul DNA, 2ul each primer (100 pmol), 1.5ul MgCl, 2.5ul 10X PCR buffer, 0.5uldNTPs, 0.2 ul Taq polymerase) and completed to 25ul volume by H_2O.

Amplification conditions were as follows: Initial denaturation at 94°C for 4min and then 35 cycles of denaturation at 94°C for 30 s, annealing at 57.5°C for 30 s, and extension at 72°C for 40s. Final extension cycle was performed at 70°C for 10 min [13] [14].

Gel electrophoresis of PCR products: 1 % agarose gel in 1X TAE buffer stained with 5 µl of ethedium bromide was prepared for electrophoresis. Electrophoresis was carried out in 1X TAE buffer at 120 V for 35 min. Molecular weight DNA ladder (100bp) was used for the confirmation of length of the amplified product [15]. Fig. 2 showing amplification by 16S rRNA and Fig. 3 showing multiplex PCR by using 16S rRNA and Nuc gene.

Antimicrobial Susceptibility testing:

Antimicrobial susceptibility testing was performed by

disk diffusion methods according to NCCLS guidelines [16]. Inhibition zones were measured in millimeter. All confirmed *S.aureus* were screened for methicillin resistance on Mueller Hinton agar supplemented with 4% NaCl. The 5ug Methicillin disc were also aseptically placed on the surface of the inoculated plates and incubated at 37°C for 24 hrs. The isolates were also checked against Tetracyclin (30ug), Ciprofloxacin (5ug), Ceftriaxone (30ug), Vancomycin (30ug), Penicillin (10ug), Ampicillin (30ug), Gentamincin (10ug), Cloxacillin (5ug) and Novobiocin (30ug) after incubation at 37^0C for 24hrs. The isolates were categorized as Resistant and Susceptible according to the zone of inhibition.

RESULTS AND DISCUSSION:

Staphylococcus aureus is the common cause of infection acquired during hospital stay and is consider as the causative agent of many infections such as skin infection, wound infection, bacteremia and sometime causes life endangering systemic infections, including endocarditis, sepsis [17][19]. *Staphylococcus aureus* is general cause of community and nosocomial acquired septicemia, about 260,000 of nosocomial infections annually are caused due to *staphylococcus aureus* [20] [21].
MRSA is a bacterium that has been found to be resistant to antibiotics such as Methicillin, Oxacillin, Penicillin and Amoxicillin [22]. The occurrence of *S. aureus* and MRSA is on the rise, resulting in increased incidences of hospital-acquired and community-acquired infections worldwide [23][24].

In the present study pus and wound sample were obtained from 60 patients. Among these 10 (16%) isolate was obtained on selective MSA media which were identified as *Staphylococcus aureus* by different biochemical test (Table 1). *Staphylococcus aureus*is gram positive, and showed Mannitol fermentation on Mannitol salt agar shown in (Fig. 1). Biochemical test performed are tabulated in (Table 1).

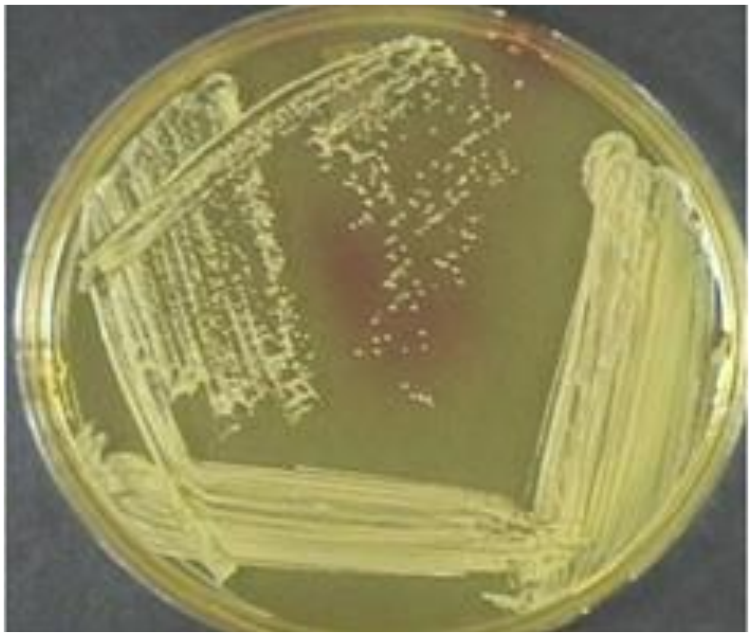

Fig. 1: *Staphylococcus aureus* on manitol salt agar

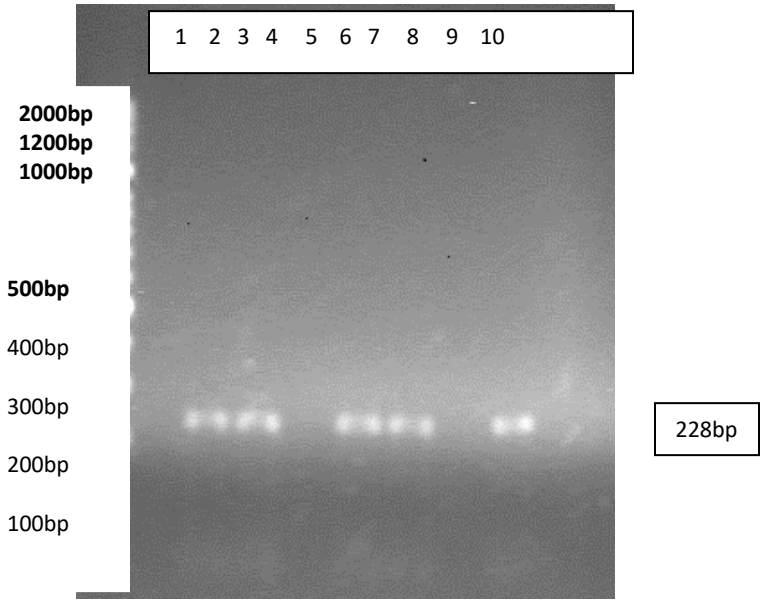

Fig 2. Agarose gel electrophoresis showing amplification of 228bp fragments of *S.aureus*. Lane 1: DNA ladders (100bp). Lane 2: Negative control, Lane 3: *S.aureus* positive control. Lanes 3, 6, 7 and 9,*S. aureus* isolates. Lane 5, 8, 10 negative sample for*S.aureus.*

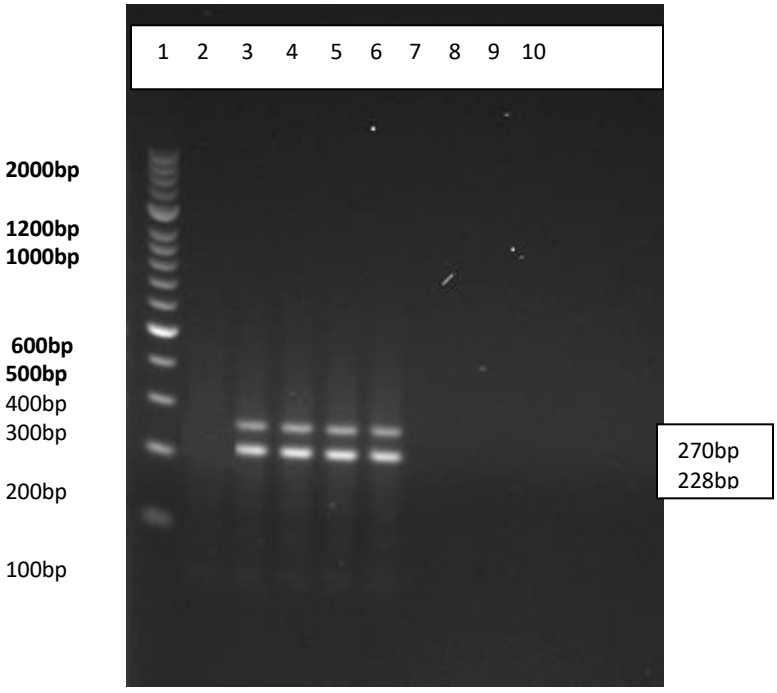

Fig 3. Agarose gel electrophoresis showing amplification of 228bp fragments of 16S rRNA.*S.aureus* and 270bp of Nuc gene. Lane 1: DNA ladder (100bp). Lane 2: negative control, Lane 3, 4*: S.aureus* positive control. Lane 5, 6: *S.aureus* positive control.

All the isolates in our study were also confirmed by PCR (Table 4) for the confirmation of 16S rRNA gene and Nuc gene. Multiplex PCR using 16S rRNA gene and Nuc gene was also used for the identification of *Staphylococcus aureus.* (Fig 3). Many researchers worked on phenotypic and genotypic identification of staphylococcus aureusby using 16S rRNA gene [25]. Similar Al–Alak, & Khadim Qassim (2016) also reported Molecular Identification of 16S rRNA gene for *Staphylococcus aureus* isolated from Wounds and Burns. Several other studies have also confirmed 16Sr RNA method by PCR for reliability and quick detection of *staphylococcus aureus.* [26]. The result of our study also matched with the study conducted in Iran by Malihe et al., (2011) who obtain the amplification of 16S rRNA gene for the identification of *Staphylococcus aurues* (n=126) isolates [27].

In the present study sensitivity pattern of *Staphylococcus aureus* to the following antibiotics; Tetracycline, Ciprofloxacin, Ceftriaxone, Vancomycin, Penicillin, Ampicillin, gentamicin, Cloxacillin, Methicillin and Novobiocin were 30%, 80%, 70%, 100%, 10%, 10%, 70%, 40%, 80% and 30% respectively. In this study two isolates which showed resistance to methicillin were also resistant to all other antibiotics except vancomycin. Similar finding was also reported by Sisirak*et al.,* (2010) which showed multiple drug resistance (MDR) against Penicillin, Ampicillin and Erythromycin, Gentamicin, Cloxacillin and Tetracyclin [28]. The prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in Pakistan range from 2-61%. MRSA is an increasing in number in large cities of Pakistan day by day.

CONCLUSION:

The current study represents the biomolecular identification of *staphylococcus aureus* and screening of methicillin resistant strain from pus and wound samples in tertiary care hospital Quetta Balochistan. From sixty samples 10 isolates of *staphylococcus aureus* confirmed through biochemical and PCR based techniques. The present study shows the resistance variability of *S. aureus* against different antibiotics. Among these 10 isolates two (n=2) which were methicillin resistant were also resistant to all antibiotics used in this study except Vancomycin. Furthermore, from our study it is also evaluated that high level of antibiotic abuse without prescription and lack of awareness enhanced development of resistance and commonly used antibiotic becomes completely ineffective in the treatment of *staphylococcus aureus.* It was further confirmed that the antibiotic resistance of *S. aureus* in today's times is rising problem.

Table1: Biochemical test for *S. aureus*

catalase	Oxidase	Citrate	Coagulase	Urease	MR	Oxidase	VP	Gelatin Liquifi	Indole
+ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve

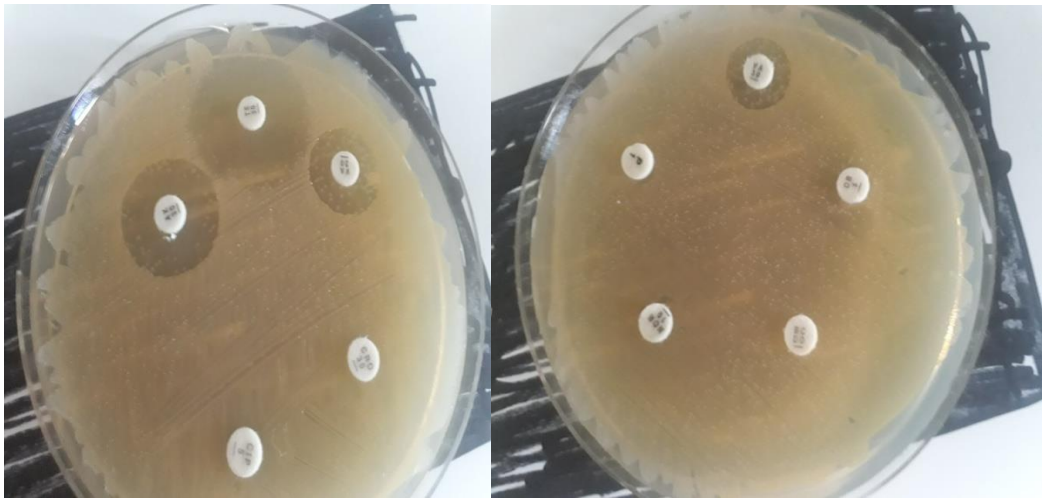

Fig4. Antibiotic Susceptibility test against 10 antibiotics.

Table2: Antibiotic sensitivity and resistance of S. aureus against different antibiotics

	Total isolate (n= 10)			
S.No	**Antibiotics/Code**	**Disk potency**	**Resistant**	**Sensitive**
1	Tetracyclin/ Te	30 ug	7 (70%)	3 (30%)
2	Ciprofloxacin/CIP	5 ug	2 (20%)	8 (80%)
3	Ceftriaxone/CRO	30 ug	3 (30%)	7 (70%)
4	Vancomycin/VA	30/ ug	-	10(100%)
5	Penicillin/P	10ug	9(90%)	1(10%)
6	Ampicillin/AM	30 ug	9(90%)	1(10%) -
7	Gentamcin/G	10ug	3 (30%)	7 (70%)
8	Cloxacillin/OB	5ug	6 (60%)	4 (40%)
9	Mehicillin/ME	5ug	2 (20%)	8 (80%)
10	Novobiocin/NO	30ug	7(70%)	3(30%)

Table 3: Antibiotic sensitivity of methicillin resistant S. aureus isolates against different antibiotics

	Total Methicillin resistant isolate (n= 2)			
S.No	**Antibiotics/Code**	**Disk potency**	**Resistant**	**Sensitive**
1	Tetracyclin/ Te	30 ug	10(100%)	-
2	Ciprofloxacin/CIP	5 ug	10(100%)	-
3	Ceftriaxone/CRO	30 ug	10(100%)	-
4	Vancomycin/VA	30/ ug	-	10(100%)
5	Penicillin/P	10ug	10(100%)	
6	Ampicillin/Am	30 ug	9(90%)	1(10%) -
7	Gentamcin/G	10ug	10(100%)	-
8	Cloxacillin/OB	5ug	10(100%)	-
9	Mehicillin/ME	5ug	10(100%)	-
10	Novobiocin/NO	30ug	8(80%)	2(20%)

Table4: Primer sequences of 16S rRNA, and NUC genes of *Staphylococcus aureus.*

Gene	Directon	Sequences (5'-3')	Amplicon Size	
16SrRNA	**F** **R**	GTA GGT GGC AAG CGT TAT CC CGCACATCAGCGTCAG	**228bp**	**[10]** Karmakar et al., 2016
Nuc	**F** **R**	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAA AGC	**270bp**	**[11]** (Monday and Bohach, 1999)

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