



PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF MDR ISOLATES OF *STAPHYLOCOCCUS AUREUS* FROM BLOOD AND PUS IN HIMACHAL PRADESH, INDIA

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ABSTRACT

Drug resistance in *Staphylococcus aureus* with variable epidemiology has become a serious public health problem worldwide. To study the phenotype prevalent in this region (Himachal Pradesh) and to choose the appropriate antibiotic therapy regimen both phenotypic and genotypic characterization of the given isolates were performed in the present work. A total 25 isolates (20 of pus and 5 of blood) were obtained from IGMC Shimla and their antibiotic resistance profiling was done. 100% resistance was obtained in case of tetracycline and 48% for oxacillin. Vancomycin, netilmycin, teicoplanin and linezolid, showed 100% sensitivity where as in case of clindamycin 88% sensitivity was seen as three of the isolates were found to be intermediate. Genotypic characterization of both MSSA and MRSA strains (5 each) was done for the presence of tet 38 gene by PCR. The results of PCR revealed all the 10 isolates carrying tet 38 gene with amplicon size of 1353 base pair irrespective of whether the isolates were MSSA or MRSA.

KEYWORDS: *S. aureus*, MRSA, VRSA, PCR, tet38 gene, Antibiotic susceptibility

INTRODUCTION

Staphylococcus aureus is a facultative anaerobic, Gram positive coccus which appears as grape like cluster when viewed under microscope and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates.¹ It is frequently part of skin flora and found in nose and on skin. Beside this bacterial colonization is common in certain areas in the body namely-axilla, umbilicus, perineal region, and mammary folds. *S. aureus* is one of the main pathogens responsible for a number of infections in hospitals settings, with a considerable morbidity and mortality.²⁻⁵ *S. aureus* may be acquired from different sources like patients and hospital staff mainly through their hands and also from their normal flora. The common disease caused by *S. aureus* are various types of infections including staphylococcal pneumonia, polyneuropathy, toxic shock syndrome, scalded skin syndrome etc^{6,7,8} and oral infections⁹.

An extensive and indiscriminate use of antibiotics has resulted in increased resistance among *S. aureus* clinical isolates¹⁰. Methicillin resistant *S. aureus* has already become endemic worldwide. In some areas, more than 95% of *S. aureus* isolates are now resistant to penicillin or ampicillin and more than 50% have developed resistance to methicillin¹¹. Over the last 20 years, the incidence of both hospital and community acquired *S. aureus* infections have increased and antibiotic treatment is increasingly being hampered by the spread of strains resistant to multiple drugs including penicillin, methicillin, clindamycin, erythromycin and tetracycline etc^{10,12,13}. Vancomycin is the terminal antibiotic of choice for highly drug resistant strains¹⁴. Tetracycline, clindamycin and cotrimoxazole are drugs exhibiting high efficacy against *S. aureus*^{15,16} but linezolid, teicoplanin and netilmycin are also some of the drugs given in such cases^{17,18,19}. Due to the selection of vancomycin, as the only treatment option now, the emergence of vancomycin resistance (VRSA) has been increasing which seriously threatens the most important treatment option available²⁰. Vancomycin resistance was found to be unstable and expressed at very low level in the absence of vancomycin

pressure and this may be the reason why these strains are hard to detect clinically²¹.

There appear to be significant variable in the epidemiology and prevalence of drug resistant *S. aureus* in different parts of the world and even in different region of the country. Also there has been scarcity of data on the drug resistance in clinical isolates of *S. aureus*, particularly in Himachal Pradesh. Hence the constant monitoring of these strains is essential in order to control their spread in hospital environment and transmission to the community. Therefore in this study we endeavor to identify such life-threatening drug resistant strains from Himachal Pradesh and the drug selected are specifically those which are known to have better efficacy in case of *S. aureus* infections.

MATERIALS AND METHODS

IRB/ EC Approval

The experimental protocol was approved by institutional ethical committee of Shoolini University, Solan India under Registration Number: SUIEC/11/13.

Chemicals and reagents

Chemicals and reagents of analytical grade of Hi Media and Genaxy were used.

Collection and processing

25 isolates (20 of pus and 5 of blood) were procured from IGMC, Shimla in 40% glycerol stock solution. Collected isolates were streaked on nutrient agar plates and were incubated for 24 hrs at 37°C.

Phenotypic characterization

Identification of *S. aureus* isolates was based on growth and fermentation on mannitol salt agar and its colonial morphology on nutrient agar. Other tests performed were gram staining (Gram positive cocci in clusters) and biochemical tests including catalase, coagulase, MR test, VP test, Alkaline Phosphatase, ONPG, Urease, Arginine utilization, Mannitol, Sucrose, Lactose, Arabinose, Raffinose, Trehalose and Maltose. All the biochemical tests except catalase, coagulase and MR⁴ were done by using KB004 HiStaph™ Identification kit.

Antibiotic susceptibility test

In vitro sensitivity of *S. aureus* strains (25) to 7 antibiotics [Vancomycin (30 mcg), Linezolid (30mcg), Teicoplanin (30 mcg), Clindamycin (2 mcg), Netilmycin (30 mcg), Tetracycline (30 mcg), Oxacillin (1 mcg)] was determined using Bauer-Kirby disc diffusion assay modified by Clinical Laboratory Standards Institute guidelines.²² All tests were performed on Mueller-Hinton agar supplemented with 4% NaCl.

Genotypic characterization

DNA isolation

DNA from cultured bacteria was isolated by using protocol from Wilson and Walker, 2010.²³ 10 isolates, 5 of MSSA and 5 of MRSA were chosen for further genotypic characterization by PCR randomly. Bacterial culture was grown in nutrient broth for overnight (12-14hr). Overnight grown bacterial culture was centrifuged at 12000 rpm for 2 min to pellet down the cells. Supernatant was discarded without disturbing the cell pellet and extraction was carried out following the protocol.

PCR assay

The following oligonucleotides were used in PCR amplification:

F 5'CCCGGATCCGTATGGGTATTTTAGGTGTA3'

R 5'CCCGGATCCCGTGTATATAATCTCTTTA3'

which amplified a 1353-bp fragment of the tet38 gene. PCR amplification was done according to Yanpeng Ding et al., 2008²⁴ with little modifications. The cycle followed for amplification was as: 94°C for 15 min, followed by 40 cycles of 95 °C for 15s, 55 °C for 15s, and 72 °C for 15 s. Amplicons were loaded onto 1.5% Agarose Gel containing 1 µg/ml ethidium bromide.

RESULTS

Phenotypic characterization

Traditional analysis of 25 samples collected from IGMC, Shimla (20 of pus and 5 of blood) were carried out using bacterial isolation and biochemical identification. Results revealed the presence of Gram positive, non-spore forming cocci, arranged in form of grapes or in irregular clusters. The colonies were circular, smooth and glistening. Biochemically; they were catalase, coagulase positive and mannitol fermenter which proved to be *S. aureus*. Other biochemical tests were done by using KB004 HiStaph™ Identification kit and isolates were positive for mannitol, VP, MR, Alkaline Phosphatase, sucrose, lactose, trehalose, maltose, weakly positive for urease, arginine utilization and negative for ONPG, arabinose and raffinose.

Antibiotic susceptibility test

Antibiotic sensitivity test was carried out using eight antibiotic disks (HiMedia). Resistant pattern of the isolates were found to be highly variable. 100% resistance was shown for tetracycline and oxacillin. Clindamycin exhibited 88% sensitivity and 12% of the isolates were intermediate for it whereas linezolid, teicoplanin, netilmycin and vancomycin showed 100% sensitivity respectively (Table 1, Fig. 1).

Genotypic characterization

10 isolates (P₄, P₆, P₈, P₁₀, P₁₅, P₁₇, P₁₈, P₁₉, B₃, B₄) 5 MSSA and 5 MRSA were chosen for further genotypic characterization by PCR randomly. Out of isolates showed amplicon of 1353 bp, indicating the presence of Tet38 gene (Fig. 2).

DISCUSSION

MRSA has emerged as a very significant killer over the last few decades with variable epidemiology prevalence in different parts of the world. Constant monitoring of these

strains is essential in order to control their spread in the hospital environment and transmission to the community. Both phenotypic and genotypic characterization of the given isolates was performed in the present work to study the phenotype prevalent in this region (Himachal Pradesh) and to choose the appropriate antibiotic therapy regimen. A total of 25 isolates (20 of pus and 5 of blood) were obtained from IGMC, Shimla. Isolates were identified with the help of gram staining first and all were found to be gram +ve. On biochemical analysis they were identified to be positive for coagulase, catalase, mannitol, VP, MR, Alkaline Phosphatase, sucrose, lactose, trehalose, maltose, weakly positive for urease, arginine utilization and negative for ONPG, arabinose and raffinose which confirms the isolates belonging to genus *Staphylococcus* and sp. *aureus*. As *S. aureus* shows golden yellow colonies on MSA they were streaked on the media confirming the sp. *aureus*.

In this study resistant profiling of the isolates were done by using 7 antibiotics tetracycline (30 mcg), oxacillin (1mcg), clindamycin (2mcg), netilmycin (30 mcg), linezolid (30 mcg), teicoplanin (30mcg) & vancomycin (5 mcg). 100% resistance was observed against tetracycline and 48 % resistance against oxacillin. So this seems to be the case of tetracycline resistant *S. aureus* and tetracycline resistant MRSA phenotype. This in accordance with various studies which reports high level of tetracycline resistance in clinical isolates of *S. aureus*. Tetracycline resistance was prevalent in MRSA in Bulgaria and Turkey and also common in England and Wales before the mid 1990s. Tetracycline is the second most common resistant phenotype in MRSA strains isolated in Poland. Schmitza et al. have investigated the tetracycline susceptibility of 3052 *S. aureus* isolates collected from 25 university hospital in the Europe. They also determined the distribution of the four tetracycline resistant genes in 600 tetracycline resistant *S. aureus* isolates, 400 MRSA and 200 MSSA *S. aureus* isolates. They found there was an obvious relationship between oxacillin resistance and resistance to tetracycline 32(Schmitza et al., 2012). Across Europe the rate of tetracycline resistance concomitant with methicillin resistance has been reported to be as high as 57.1%. Conversely, in the recent British Society for Antimicrobial Chemotherapy study, MSSA and MRSA isolates from the United Kingdom and Ireland showed rates of tetracycline resistance of 4.3% and 1.5%, respectively. In North America, the rates of occurrence of tetracycline resistance among MRSA isolates from the United States and Canada were recently reported to be 15.6% and 14.8%, respectively, whereas in Latin America and the western Pacific the rates were considerably higher, exceeding 60%.

Vancomycin is the drug of last resort for highly drug resistant *S. aureus* and this fact is very well supported by present study. Many studies have shown 100% sensitivity of *S. aureus* against vancomycin. Drugs other than vancomycin which have equally good susceptibility profile are linezolid, netilmycin and teicoplanin. The fact is confirmed by the results obtained i.e. 100% sensitivity was obtained for the above mentioned drugs in this case also. There is rarely any report of resistance against linezolid. Clindamycin is also drug of choice against *S. aureus* related infections but in this study its activity was not found comparable with other drugs i.e. 88% sensitivity was seen and 12% strains were found intermediate. The present study therefore support or authenticate the efficacy of vancomycin, linezolid, netilmycin, teicoplanin and clindamycin for empirical treatment of serious *S. aureus* infections. Another important

observation of this study is that tetracycline resistance was present in all isolates (pus and blood) irrespective of the sources of clinical samples.

The resistance of tetracycline is due to active efflux mechanism. One such gene present on chromosome that confers resistance to tetracycline by active efflux is tet 38. It is a chromosomal gene and over expression of tet 38 gene led to a 32- fold increase in resistant to tetracycline . Tet 38 is regulated by Mgr A, a transcript regulator and an indirect repressor which increase its transcription and tetracycline MIC by 32 folds. In the present study the genotypic characterization of 5 MRSA and 5 MSSA isolates were done for the presence of tet 38 gene by PCR. The results revealed all isolates carrying tet 38 gene with amplicon size of 1353 bp. In a study done by Truong-Bolduc et al it was noticed that Tet 38 efflux pump shares 46% similarity with Tet K in their study had noticed that Tet 38 protein carried on plasmid in *S. aureus* efflux pump confers resistance to tetracycline and shares 46% similarity with the tetracycline Tet K protein carried on plasmids in *S. aureus*. Since tet 38 is a newly characterized gene, not much reports about its expression, in *S. aureus* (tet resistant phenotype), are available. More extensive studies are therefore needs to be carried out in this regard which can throw some light on the importance of gene in tet resistant *S. aureus*.

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REFERENCES

1. Ryan KJ, Ray CG. Sherris Medical Microbiology: An Introduction to Infectious Diseases". McGraw Hill, 2004.
2. Deurenberg RH, Stobberingh EE. The evolution of *S.aureus*. Infect Genet Evol 2008; 8: 747-863.
3. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli, Y. Comparison of mortality associated with methicillin sensitive *S.aureus* bacteremia meta analysis. Clin Infect Dis 2003; 36: 53-59.
4. Engemann JJ, Carmeli Y, Cosgroove SE, Flower VG, Bronstein MZ, Trivette SL, et al. Adverse clinical and economic outcomes attributable to Methicillin resistance among patient with *S.aureus* surgical site infection. Clin Infect Dis 2003; 36: 592-598.
5. Ho KM, Robinson JO. Risk factors and outcomes of methicillin resistance *S. aureus* bacteraemia in a critically ill patients: A case control study. Anaesth Intensive Care 2009; 37: 457-463.
6. Mark DE, Sheila S, Elliot KF. Necrotizing pneumonia caused by community acquired MRSA: An increasing cause of "mayhem in the lung". Emergency Radiol 2009;16(2): 159.

7. Hida K, Yanai, S, Shimizu K, Yamamoto H, Konishi Y, Takeda J. A case of liver abscess with secondary brain and lung abscess in a healthy man. J Japan Surg Assoc 2005; 66: 1990-3.
8. Yves LL, Florence B, Gautier M. *S. aureus* and food poisoning. Gen Mol Res 2003 ;56 (1): 63-76.
9. Smith AJ, Jackson MS, Bagg J. The ecology of *Staphylococcus* species in the oral cavity. J Med Micro 2001;50: 940- 946.
10. Tiwari K, Sen R. Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary care hospital from northern part of India. BMC Infectious Disease 2006; 6:156.
11. Brumfitt W, Hamilton-Miller J. The world wide problem of methicillin-resistant *Staphylococcus aureus*. Drugs Exp Clin Res 1990; 16: 205-214.
12. Rijnders M, Deurenberg RH, Boumans M, Hoogkamp-Korstanje M, Beisser P, Stobberingh EE. Antibiotic resistance of *Staphylococcus aureus* from ICUs in the Netherlands 1996 to 2006. Crit Care 2009; 13(1).
13. Deotale V, Mendiratta DK, Raut U, Narang P. Inducible clindamycin resistance in *Staphylococcus aureus* isolated from clinical samples. Ind J Med Micro 2010; 28(2): 124-126.
14. Rubeena H, Maleeha A, Shahbaz A. Incidence of Methicillin resistant *Staphylococcus aureus* in blood culture isolates a retrospective study. Ann King Edward Med Coll 2001; 7(4): 264-266.
15. Trzcinski K, Cooper BS, Hryniewicz W , Dowson CG. Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother 2000; 45: 763-770.
16. Kery L, Plante L, Steven NL, David RA, William AC, Michael J. Activities of clindamycin, daptomycin, doxycycline, linezolid, trimethoprim-sulfamethoxazole, and vancomycin against Community-Associated Methicillin-Resistant *Staphylococcus aureus* with inducible clindamycin resistance in murine thigh infection and in vitro pharmacodynamic models. American society for Antimicrob Chemother 2008; 52(6): 2156-2162.
17. Borgia S, Shenoy S, Vidyasaxmi K, Dhanshree B. Netilmicin resistance in *S. aureus* Natl Med. J India 1999; 12: 192-192.
18. Kumar R., Yadav BR, Singh RS. Genetic Determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitis crossbred cattle. Curr Microbiol 2010; 60: 379-386.
19. Rao BN, Parbhakar, T. Prevalence and antimicrobial susceptibility of MRSA in and around Visakhapatnam, Andhra Pradesh. JPBMS 2011; 4(3): 1-5.
20. Murray PR. Manual of clinical microbiology, 8th ed. ASM Press, Washington DC., 2003.
21. Bozdogan B, Esel D, Whitener C, Browne FA, Appelbaum PC. Antibacterial susceptibility of a vancomycin-resistant *Staphylococcus aureus* strain isolated at the Hershey Medical Center. J Antimicrob Chemo 2002; 52: 864-868.
22. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 16th information supplement (M100-S16). Clinical and Laboratory Standards Institute, Wayne, 2006.
23. Wilson K, Walker J. Isolation and separation of nucleic acid", Principle and techniques of biochemistry and molecular biology, Edt. 7, 2010; p. 164-165.
24. Yanpeng D, Yoshikuni O, Jean C, Hooper C. Nor B, an efflux pump in *S. aureus* strain MW2, contributes to bacterial fitness in abscess. J bacterio 2008; 190(21): 7123-7129.

S.No	Isolates	Antibiotics													
		N(30 mcg)		Tei(30 mcg)		LZ(30 mcg)		Cd (2 mcg)		V(5mcg)		Tet (30 mcg)		Ox (1mcg)	
		IZD (mm)	R/S/I	IZD (mm)	R/S/I	IZD (mm)	R/S/I	IZD (mm)	R/S/I	IZD (mm)	R/S/I	IZD (mm)	R/S/I	IZD	R/S/I
1	P ₁	21	S	24	S	30	S	31	S	18	S	11	R	10	R
2	P ₂	23	S	21	S	25	S	29	S	16	S	10	R	20	S
3	P ₃	27	S	20	S	27	S	24	S	18	S	No Zone	R	10	R
4	P ₄	25	S	18	S	26	S	17	I	15	S	No Zone	R	22	S
5	P ₅	26	S	23	S	24	S	32	S	17	S	No Zone	R	<10	R
6	P ₆	19	S	22	S	27	S	26	S	19	S	No Zone	R	24	S
7	P ₇	24	S	19	S	28	S	28	S	16	S	No Zone	R	10	R
8	P ₈	28	S	20	S	28	S	19	I	15	S	No Zone	R	23	S
9	P ₉	21	S	21	S	27	S	31	S	15	S	10	R	<10	R
10	P ₁₀	20	S	21	S	29	S	32	S	16	S	10	R	21	S
11	P ₁₁	22	S	24	S	31	S	25	S	17	S	10	R	<10	R
12	P ₁₂	17	S	19	S	29	S	19	I	15	S	<10	R	10	R
13	P ₁₃	27	S	23	S	28	S	29	S	16	S	No Zone	R	24	S
14	P ₁₄	26	S	26	S	27	S	24	S	19	S	No Zone	R	<10	R
15	P ₁₅	23	S	22	S	30	S	26	S	15	S	No Zone	R	26	S
16	P ₁₆	29	S	16	S	32	S	28	S	18	S	No Zone	R	<10	R
17	P ₁₇	24	S	15	S	29	S	26	S	15	S	<10	R	22	S
18	P ₁₈	24	S	14	S	31	S	27	S	15	S	No Zone	R	29	S
19	P ₁₉	24	S	15	S	28	S	25	S	17	S	<10	R	23	S
20	P ₂₀	24	S	14	S	26	S	28	S	16	S	<10	R	<10	R
21	B ₁	21	S	15	S	26	S	26	S	17	S	No Zone	R	21	S
22	B ₂	27	S	15	S	26	S	32	S	15	S	No Zone	R	<10	R
23	B ₃	24	S	14	S	28	S	27	S	19	S	10	R	20	S
24	B ₄	28	S	19	S	32	S	31	S	15	S	No Zone	R	23	S
25	B ₅	28	S	22	S	30	S	26	S	16	S	10	R	10	R
26	6571	27	S	20	S	31	S	29	S	18	S	24	S	25	S

Table1: Antibiotic susceptibility of samples Pus P₁-P₂₀ and Blood B₁-B₅ IZD-inhibition zone diameter, R-resistant, I-intermediate, S-sensitive, N-Netilmicin, Tei- Teicoplanin, LZ-Linezolid, Cd-Clindamycin, V-Vancomycin, Tet-Tetracycline, Ox-oxacillin.

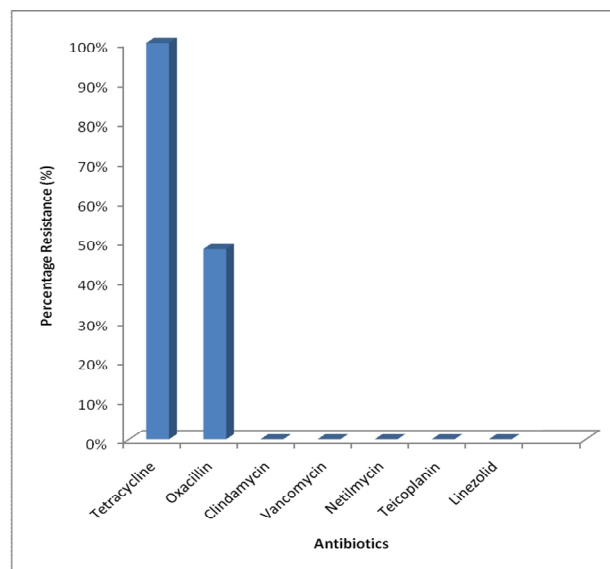


Fig. 1: Resistant Pattern of *S. aureus* against various antibiotics

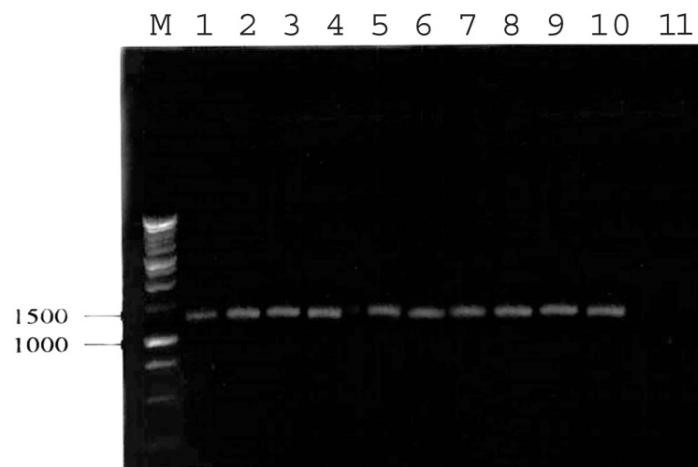


Fig. 2: Amplification products of *S. aureus* Tet 38 gene by PCR. Lane 1: 2000 bp ladder, Lane 1, 2, 3, 4, 5, 6, 7, 8, 9 & 10 all were Tet 38 positive samples. Reference strain no. 6571 in lane 11 was used as negative control.

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