AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

The Analysis of Virulence Factors and B-LACTAMASE Production In Clinical Isolates of *Staphylococcus Aureus*

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ABSTRACT

Staphylococcus aureus is associate with variety of clinical infections in various communities and healthcare institutions both in developed and developing countries. It produces extracellular enzymes like lipase, protease and lecithinase that degrade lipid, proteins present in the skin environment. Study was conducted to associated virulence factors in clinical isolates of *S. aureus* and to evaluate the incidence of β lactamase production. It comprised 271 clinical samples (pus from surgical, burn, ulcer, abscess and sputum from hospitals of Allahabad. *S. aureus* was isolated and identified by conventional culture followed by biochemical tests including coagulase test. β-lactamase test and antibiotic susceptibility tests were performed for all isolates. Among 59 isolates of *S. aureus*, coagulase, carotenoid pigment, haemolysis, lipase, lecithinase and protease activity was examined to be in 59 (100%), 55(93.22%), 51 (86.44%), 23 (39.98%), 25 (42.37%) and 19 (32.2%) respectively. Secretion of lipase, protease and lecithinase were insignificantly related to each other as well as bacterial isolates from different sample type. Regarding 43 (72.89%) penicillin resistant isolates, 31(72.09%) were β-lactamase producers and of 20 (33.89%) methicillin resistant *S. aureus* (MRSA), 14 (70%) were β-lactamase producer. β-lactamase production was analyzed to be statistically significant for penicillin but not quite significant for methicillin resistant *S. aureus* ($\chi 2$=24.30, P>0.05 for penicillin and $\chi 2$=3.7, P=0.054 for methicillin). Positive correlation occurs between β-lactamase production and resistance to other antibiotics included in the study (R=0.97).It seems no variation in the liberation of virulence detecting enzymes among *S. aureus.* Even if penicillin resistance majorly results due to β-lactamase, methicillin resistance shares some other mechanism of resistance.

Keywords: *S. aureus*, virulence factors, β-lactamase, penicillin and methicillin

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Received 25 February 2014, Accepted 07 March 2014

Please cite this article in press as: Chaudhari K *et al* The Analysis of Virulence Factors and B-Lactamase Production In Clinical Isolates of *Staphylococcus Aureus.* American Journal of PharmTech Research 2014.

INTRODUCTION

S. aureus, mere normal microbial flora of skin and nasopharynx has become one of leading aetiological agent of bacterial infection. Majority of human population are carrier which helps its opportunistic entrance to inner tissue and organs. It causes various types of disease including Staphylococcal toxic shock syndrome. More commonly being the causal factor of hospital as well as community acquired infection around the globe, it has over burdened the clinicians and therapeutics [1].

S. aureus produces various types of extracellular enzymes besides toxins and carotenoid pigment. The enzymes are hyluronidase, staphylokinase, coagulase (free and bound), proteases, lipases, lecithinase, nucleases, etc., and toxins are haemolysin, leucocidin, exfoliatin toxin, toxic shock syndrome to toxin [2]. Some of these enzymes are proved to be supportive for pathogenesis directly whereas others are not fully participating in invasion. They may be basically related with bacterial metabolism and indirectly helping in virulence.

Penicillin was the first antibiotic to be discovered. It lead the chemotherapeutic world to antibiotic era. Bacterial infections were terrific in pre-antibiotic era, among that one of the common pathogen was *S. aureus* as the mortality rate raised by 80% and more than 70% patients showed metastatic infections[3]. Penicillin put strong effort in treatment of staphylococcal infection until resistance strain developed. Within a couple of decade after it was identified, resistance against the antibiotic prominently spread over hospital acquired infections along with community acquired infections [4].

Bondi and Dietz[5] explained the cleavage of β-lactam ring by β-lactamase that accounts for penicillin resistance. It is inductive extracellular enzyme, in presence of penicillin as substrate. Besides this, mutation in target site *of* penicillin binding protein is also the reason for resistance. Because of identical structure of β-lactam antibiotics and peptidoglycan monomer, PBPs bind covalently with penicillin via catalytic serine residues at its active site. As a result, enzyme for peptidoglycan synthesis gets inactivated. Cell wall inhibition causes death. More likely, alteration in specific binding site cannot recognize the antibiotic molecule to get bonded and resistance occurs [6].

Present research was objected to analyze the production of enzymes (lipase, lecithinase and protease) that help the organism to spread through tissue along with the incidence β-lactamase producers. Penicillinase enzymes producing strains were correlated with isolates resistant to various antibiotics tested.

MATERIALS AND METHODS

Site for sample collection was Leprosy mission Leprosy Mission Hospital and hospital under the affiliation of Motilal Nehru Medical College, Allahabad. A total of 271 clinical samples that included pus swabs from burn, surgical wound, ulcer and skin in addition to sputum. *S. aureus* was isolated by growth on selective media (mannitol salt agar) and identified by biochemical tests finally confirmed with slide and tube coagulase, acetoin production, anaerobic mannitol fermenting test. All the isolates were grown on 5% sheep blood agar to observe for haemolysis. Further they were checked for production of protease, lipase and lecithinase. All the bacteriological media, egg yolk emulsion and antibiotics used were Himedia product.

Lipase test:

It was done in nutrient agar medium supplemented with 1% tween 80. Bacteria was streaked in the medium and observed for opalescent zone around the colony after 48 hours [7].

Protease test:

All the isolates were cultured on milk agar for 24hrs at 37°C. They were examined for the hydrolysis of casein and formation of clear zone around the growth line.

Lecithinase test:

It was performed on 10% egg yolk emulsion agar. Organism was inoculated & incubated at 37°C. After 48 hours, result was noted if any opalescent zone was formed around growth area [7].

β-lactamase test:

The test was done by iodometric method as explained in Devapriya *et al.,*[8]. Penicillin G solution of 6000 µg/ml was prepared by dissolving in 0.05M potassium phosphate buffer with PH 6.0. 0.5ml penicillin solution was aliquoted in tubes then a loopful of culture was mixed and incubated for 1hour at room temperature. After incubation 3-4 drops of 1% starch solution was added followed by 2-3 drops of iodine solution. Blue colour appeared. Further tube was rotated for 1 min and left for colour change. Persistence of blue colour was noted to be negative whereas decolourization of blue solution to colourless was positive for β-lactamase.

Antibiotic susceptibility test:

Antibiotic resistance-pattern was studied by disc agar diffusion (DAD) test[9]. The suspension of organism with concentration resembling 0.5 Mc Farland solution was swabbed uniformly on Mueller Hinton Agar. Four antibiotic disc were placed on one plate and incubated for 24 hrs. Inhibition zones were measured and categorized into Sensitive, Intermediate and Resistance according to CLSI chart. The data recorded during the course of study was analyzed by 'Analysis

of Variance', 'Chi Square' and 'Karl Pearson's Coefficient of Correlation'. Graph Pad online software was used in calculating P-value of statistical test results.

RESULTS AND DISCUSSION

Among 59 isolates of *S. aureus,* coagulase, carotenoid pigment, haemolysis, lipase, lecithinase and protease activity was examined to be in 59 (100%), 55(93.22%), 51 (86.44%), 23 (39.98%), 25 (42.37%) and 19 (32.2%) respectively. Figure 1 illustrates the pigmentation and haemolysis shown by the isolates. Similarly, table 1explains the sample wise enzyme production. ANOVA test for secretion of lipase, protease and lecithinase was found to be insignificant. These three soluble enzymes acting as spreading factor was found to be equally produced by all isolates ($F_{enzymes}$=0.048) also the bacteria isolated from different sample type made no difference with the enzyme liberation (F_{sample}=2,02).

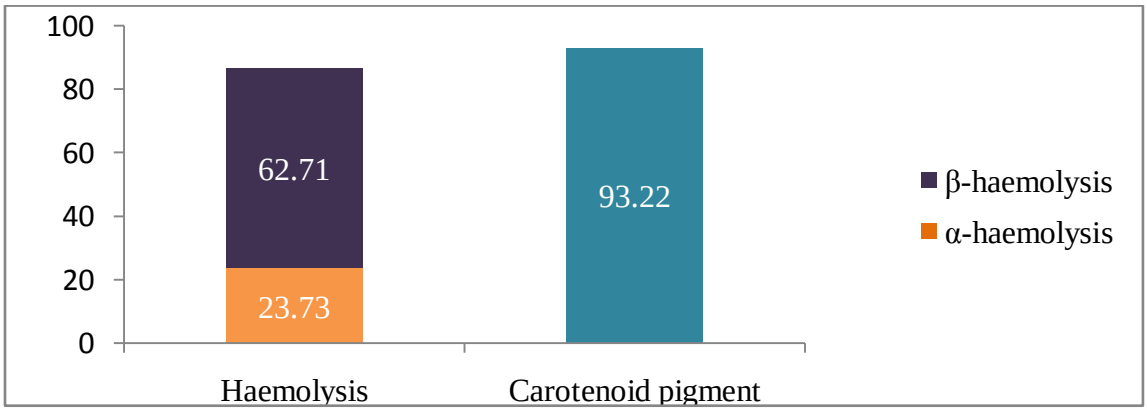

Figure 1-Percentage of isolates producing types of haemolysin and pigment

Table1: Sample-wise distribution of enzyme production by isolates

Sample type	Isolates	Lipase	Lecithinase	Protease
Surgical	10	6	5	3
Burn	2	1	1	1
Ulcer	36	13	15	12
Skin abscess	7	3	2	3
Sputum	4	-	2	-
Total	59	23 (39.98%)	25 (42.37%)	19 (32.2%)

$F_{enzymes}$=0.048 with d.f. 2,8 (P= 0.95); F_{sample}=2.02 with d.f. 4,8 (P=0.18). Analysis of above data by applying two way ANOVA showed statistically insignificant.

Out of total strains, 43(72.89%) isolates were penicillin resistant among which 31(72.09%) were β-lactamase producers (table 2). Chi-square analysis was shown to be significant (X^2 cal= 24.3, P-value < 0.05). Table 3 mentions 20 (33.89%) MRSA with 14 (70%) of β-lactamase producing strains. Result was analyzed to be statistically insignificant (X^2 cal =3.7, P-value=0.054). Frequency of β-lactamase producing strains among all resistant isolates to each antibiotics is

illustrated in figure 2. Positive correlation exists between β-lactamase production and resistant to antibiotics included.

Table 2: β-lactamase producer among penicillin resistant and penicillin sensitive strains

Isolates	β-lactamase producer	β-lactamase non producer	Total
Penicillin resistant	31	12	43
Penicillin sensitive	-	16	16
Total	31	28	59

X^2 cal= 24.3, P-value < 0.05, Statistically significant association occurs between β-lactamase producer among penicillin resistant.

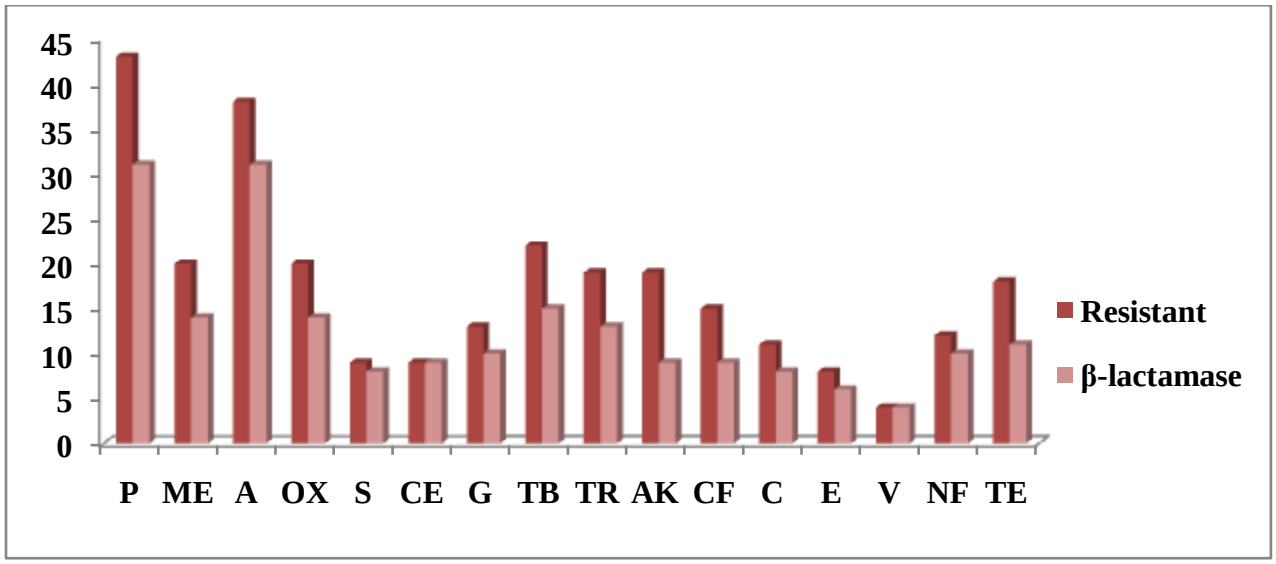

Figure 2- Frequency of β-lactamase producers among resistant isolates to individual antibiotics

Indications: P (Penicillin), ME (Methicillin), A (Ampicillin), OX (Oxacillin), S (Streptomycin), CE (Cefotaxime), G (Gentamycin), TB (Tobramycin), TR (Trimethoprim), AK (Amikacin), CF (Cipr ofloxacin), C (Chloramphenicol), E (Erythromycin), V (vancomycin), NF (Nitrofurantoin), TE (Tetracycline)

R=0.97, Probable error (P.E) = 0.01, Since r > 6P.E, correlation coefficient is significant.

Versatility nature has distinguished it as superbug. All the strains of *S. aureus* necessaryly do not follow the same way of pathogenesis. Some strains are α-toxin producer while others are β-haemolytic and even some are non-haemolytic. It is not only confined to haemolysis [10]. There are other various structural factors, invasions and enzymes that all the strains could not produce still they are virulent. It clues that if some strains are capable of releasing a type of spreading factor yet others will be producing different invasions depending on type of infection it cause. In general they produce lipolytic, proteolytic, nuclease enzymes [11].

Coagulase test was taken as confirmatory isolates were so all the isolates were checked for it. Though carotenoid pigment is important factor for identification, only 55(93.22%) formed yellow colonies and rest 4 isolates formed white colonies on nutrient agar. Similarly haemolysis, lipase, lecithinase and protease activity along with pigment production was examined to be on 51 (86.44%), 23 (39.98%), 25 (42.37%) and 19 (32.2%) of the total isolates. Figure 1and table 1 illustrates release of toxin and soluble enzymes by strains from different samples in elaborated way. Few other researchers have also demonstrates the similar findings [7, 12].

Among the 59 isolates of *S. aureus* obtained from the clinical samples, 31(52.54%) were β-lactamase producers. The result similar to that obtained by Akindele *et al.*,[13] but it exceeded the data (33.8%) of Oncul *et al.* [14] and that (33.3%) of Akinjogunla and Enabulele [10]. Most of the researches have investigated the β-lactamase production rate to be more than 50% which supports the present study. Variation shown regarding this may be due to the different sample type taken from various clinical setting.

Antibiotic susceptibility pattern shows 43 (72.89%) penicillin resistant and 33.89% MRSA. Only 31 (72.09%) of penicillin resistant and 14 (70%) of MRSA were β-lactamase producers, rest of resistant isolates did not produced the enzyme. As mentioned earlier, non β-lactamase producer are resistant either due to conformational change of penicillin receptors i.e. penicillin binding protein (PBPs) or its overproduction. Bacterial cell wall synthesis and the role of PBPs in its synthesis is a very good target for drugs of selective toxicity because the metabolic pathways and enzymes are unique to bacteria. However, modification in target site of PBPs or formation of low affinity PBPs leads resistance. Sometimes even overproduction of these protein help in resistance. Its abundant availability may not interfere bonding with modular pieces of peptidoglycan. Bacterial cell wall synthesis is completed without hindrance showing no effect of penicillin used [15].

Methicillin, semisynthetic penicillinase-resistant penicillin was introduced to combat the nuisance causing penicillin resistant *S. aureus*. It stands for the result that all penicillinase producing isolates were not MRSA, still 14 (70%) of MRSA produced the enzyme [10,16] Staphylococcal β-lactamases are narrow-spectrum penicillinases with relatively poor activity against semisynthetic antistaphylococcal penicillins i.e methicillin.

The present research shows positive correlation between β-lactamase production and and resistance to other antibiotics tested. Supportive fact behind this is the plasmid containing transposable element carry additional resistance genes for other antimicrobial in addition to the gene for β-lactamase [5]. The phenotypic expression of genes responsible for penicillin's may lead

to the expression of other antimicrobial resistance genes present in the same plasmid. Another possible cause may be the transfer of plasmid carrying resistance genes for corresponding antibiotics.

Table3: β-lactamase producing isolates among MRSA and MSSA

Isolates	β-lactamase producer	β-lactamase non producer	Total
MRSA	14	6	20
MSSA	17	22	39
Total	31	28	59

X^2 cal=3.7, Since X^2 cal < X^2 tab at 5% level of significant and 1 d.f but X^2 cal > X^2 tab at 10%, MRSA is not quite significantly related with β-lactamase production (P-value=0.054).

CONCLUSION

As most isolates were related to skin and soft tissue infection, there was no variation in the liberation of virulence detecting enzymes. Further these enzymes should be detected in strains from other clinical samples like blood, urine, CSF and their role in individual disease related pathogenesis. The result clearify that majority of *S. aureus* are able to produce penicillinase in presence of penicillin as the enzyme is inductive type but does not account for all the β-lactam resistance. It is the leading cause for penicillin resistance rather than modification in penicillin binding receptors. Unlikely, for methicillin resistance, formation of PBP2a plays major role than β-lactamase production. Moreover, the enzyme accounts for β-lactam resistance along with other antibiotics, so plasmid carrying the gene that encodes the enzyme should be analysed for presence of genes encoding the resistant antibiotics.

ACKNOWLEDGEMENT

We want to express our gratitude toward all the faculties and staffs of Department of Microbiology and Fermentation Technology, SHIATS for their help to accomplish the work and hospitals for providing samples.

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