Research Article

Comparison of Virulence Factors in Clinical Isolates and Standard Strain of *Staphylococcus aureus*

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Accepted on: 04-09-2015; **Finalized on:** 30-09-2015.

ABSTRACT

Staphylococcus aureus is an important human bacterial pathogen causing wide range of diseases both by nosocomial and community-acquired infections. The organism produces a number of virulent toxins and proteins which are responsible for serious infections damaging host cells and tissues thereby involving in pathogenesis. Due to the continuous use of antibiotics, the organism show resistant to most of the available antibiotics and thereby creating a major problem and threaten to human and public health. In this present study, six *S. aureus* strains and clinical isolates (S1, S2, C1, C2, S2 Mut and MTCC 737) were compared for the antibiotics susceptibility test in which all the strains were found to be resistant to one of the most important antibiotics-vancomycin. Plasmid curing analysis was done to know if the antibiotic resistance was plasmid-mediated or chromosome-mediated. The result showed the presence of zone of inhibition even after treating the strains with acridine orange thus inferring it as chromosome mediated. The strains were assayed for the activity of three important virulence factors produced by the organism such as protease, lipase and biofilms by plate assays which have specific roles in causing disease in host. The results showed that among the six tested strains the clinical isolate C1 and MTCC strain were found to be more virulent since all the three studied virulence factors lipase, protease and biofilm were produced in them when compared to other strains S1, S2 C2 and S2 mut.

Keywords: *Staphylococcus aureus,* Virulence factors, lipase, protease, biofilms, Antibiotic susceptibility test.

INTRODUCTION

Staphylococcus aureus is a major human pathogen causing a wide spectrum of nosocomial and community-associated infections with high morbidity and mortality.[1] The organism is responsible for a multitude of diseases ranging from mild and requiring no treatment boil, impetigo contagiosa, ecthyma, carbuncle, and furuncle) to severe and potentially fatal (pneumonia, endocarditis, meningitis, toxic shock syndrome).[2-4] The success of *S. aureus* as a human pathogen is the result of the wide variety of virulence factors that the organism possesses and the immense genome plasticity that assists it in adapting to an array of chemotherapeutic agents available.[5] The pathogenicity of the organism is due to the coordinated action of large number of virulence factors including surface adhesive proteins that promote colonization of host tissue, invasions that promote the bacterial spread, surface factors that help overcome phagocytic engulfment, intracellular enzymes that help overcome oxidative stress and a core of secreted proteins responsible for colonization and infection.[6] The natural ecological niches of this species are the nasal cavity and the skin of warm-blooded animals.[7] Because of its importance and high prevalence in nosocomial infections, *S. aureus* is one of the most studied Gram positive pathogen. *S. aureus* can cause infection when there is a break in the skin or mucous membrane that grants it access to the surrounding tissues.[8,9] It is well known that the organism produces various extracellular active substances, such as coagulase, hemolysins, nuclease, acid phosphatase, lipase, proteases, fibrinolysin, enterotoxins, and toxic shock syndrome toxin. These active substances are thought to contribute to the pathogenicity of the organism.[10] *In vitro*, during early growth phase, surface proteins, functioning mainly as adhesion factors, are expressed. As growth progresses towards the late exponential phase, synthesis of these factors is suppressed and the production of various extracellular proteins intensifies. The transition is regulated by several global regulators of which the best characterized are agr (accessory gene regulator) and sar (staphylococcal accessory regulator).

Treatment of serious *Staphylococcus aureus* infections can be challenging, and the associated mortality rate remains 20% to 25% despite the availability of highly active antimicrobial drugs.[11] *S. aureus* colonizes the nares, axillae, vagina and damaged skin surfaces.[12] Approximately 60% of women harbour this organism intermittently at one or more body sites.[13] Studies have shown that 7-25% of women harbour toxin-producing S. aureus.[14] Persons colonised with *S. aureus* strains are at increased risk of becoming infected with these strains.[1,15]

Staphylococcal lipases are produced by pathogenic members of the genus, i.e., *S. aureus* and *S. epidermidis*. Lipolytic activity of Staphylococci has been extensively studied. This activity is responsible for the release of considerable amounts of fatty acids, particularly octadecenoic acid, by *Staphylococcus aureus* in human plasma.[16] The enzymes catalyzing this reaction are lipases and phospholipids which are secreted into the medium. The enzyme is important for the bacterial nutrition. It promotes intestinal spreading of the bacteria.

Furthermore, lipase interferes with the phagocytosis of the infectious lipase-producing *S. aureus* cells by host granulocytes, thus indicating a direct involvement of lipase in pathogenesis.[17] Also, free fatty acids, the end products of lipolytic activity, are known to impair several immune system functions.

In vitro studies have shown that staphylococcal proteases can cleave and degrade a number of important antibacterial and matrix proteins including the heavy chains of all human immunoglobulin classes, plasma proteinase inhibitor, and elastin[18-21] indicating that they are important virulence factors.

Biofilms are defined as an assemblage of microbial cells that are irreversibly associated with a surface and enclosed in a matrix primarily of extracellular polysaccharides substances (EPS).[22] The polysaccharide covering the biofilm is called the glycocalyx (slime) and is involved in number of important functions such as protecting the bacterial surface against chemical and mechanical damages. Biofilm formation is influenced by a number of factors among which, the most important is synthesis of the polysaccharide intercellular adhesion (PIA) by the organism.[23,24]

Virulence factors of pathogenic bacteria (adhesins, toxins, invasins, protein secretion systems, iron uptake systems, and others) may be encoded on chromosomal DNA, bacteriophage DNA, plasmids, or transposons[25] in particular regions of the prokaryotic genome termed pathogenicity islands (PAIs). Understanding of the common themes in microbial pathogenicity is essential to recognize the microbial virulence in order to develop novel vaccines and other therapeutic agents for the treatment and prevention of infectious diseases.

Here, we investigated and compared six *S. aureus* strains for the production of extracellular lipase, protease and biofilms which were considered important in causing infections in host.

MATERIALS AND METHODS

Strains and culture conditions

The clinical strains used in this study were obtained from St. John's medical college and Hospital, Bangalore and the reference strain was MTCC 737 strain. The strains were inoculated in Tryptic Soy broth (TSB) and were plated on Tryptic Soy agar media. The individual colonies of each strain were grown in TSB and stored as glycerol stock at -80˚C.

The S2 mut strain was S2 strain mutated by treating 2ml of *S. aureus* culture in Tryptic Soy broth (TSB) with 4ml of ethyl methane sulphonate (EMS) incubated at 37°C for 10 minutes. It was then centrifuged and the cells were washed with Sodium thio sulphate so as to stop the mutation occurring reaction. The cells were added with 5ml of nutrient broth and incubated at 37°C for 18-24 hrs. It was then plated on Nutrient agar and used for further study.[26]

The antibiotic susceptibility test

The antibiotic susceptibility of the bacterial species isolated was performed on Muller-Hinton agar (MHA) (Merck) plates by disk diffusion method[27] as described by the National Committee for Clinical Laboratory Standards with slight modification (NCCLS).[28] 0.1 ml of each bacterial isolate was seeded into each of the Petri dishes containing Mueller-Hinton agar and were allowed to stand for 30 minutes to enable the inoculated organisms to prediffuse. The commercially available discs containing the following antibiotics (Sigma): Penicillin, Amoxicillin, Tetracycline, Gentamicin, Erythromycin, Norfloxacin, Amikacin, Vancomycin, Nalidixic acid and Chloramphenicol, 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 zones were measured in millimeters. The interpretation of the measurement as sensitive, intermediate and resistant was made according to the manufacturer's standard zone size interpretative table.

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.[29]

Plasmid curing analysis

Staphylococcus aureus strains 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 sub cultured 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).[29]

Determination of biofilm production

The method developed by Freeman[30] was used in this study. The composition of medium (CRA) was brain heart infusion broth (BHI) 37 g/l, sucrose 50 g/l, agar 10 g/l, and Congo Red 0.8 g/l.

The Congo Red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121˚C for 15 min and was added when the agar had cooled to 55˚C. Plates were inoculated and incubated aerobically at 37˚C for 24 h.

Isolates that produced black colonies with dry crystalline consistency were regarded as slime positive, whereas those showing pink colonies were slime negative.

Extracellular lipase activity

Rhodamine B and olive oil based specific plate assay was used to evaluate the extracellular lipase production by clinical isolates of *S. aureus*.[31] Modified nutrient broth containing sodium chloride 4 g/l and agar 10 g/l was prepared and pH adjusted to 7.0 prior to autoclaving at 121˚C, 15 psi for 15 min. 1 mg/ml stock of rhodamine B was prepared in distilled water and sterilized by filtration. To the growth medium (cooled to 60˚C) was added olive oil (2.5% w/v) along with rhodamine B stock solution (0.001% w/v) with vigorous stirring and emulsified by mixing for 1 min.

The growth medium was then allowed to stand for 10 min at 60˚C to reduce foaming after which 20 ml of medium was poured into sterile petridish. To quantify lipase activity, 3-mm-diameter holes were punched into the agar and filled with 10 µl of cell-free culture supernatant. Lipase-producing strains were identified by irradiating plates with UV light at 350 nm.

Upon irradiating with UV light lipase producers began to show fluorescence after 16 h. With continuing incubation time fluorescent halos were formed around the colonies of lipase producing strains.

The final zone diameters were recorded after incubation for 48 h at 37˚C. The logarithmic of lipase activity is linearly related to zone diameter.

Extracellular protease activity

Protease activity was determined on casein agar plates following the procedure described by Bjorklind.[32] The production of protease could be confirmed by a clear zone or a broad zone of precipitation around the bacterial streak**.**

Briefly, Casein (1%) and Nutrient agar was autoclaved separately in conical flasks and cooled and mixed well. The media was poured in petriplates and after solidification of the medium colonies of each isolates were streaked on the surface of the agar. They were incubated overnight for 48 hrs at 37˚C.

The results were indicated as positive for protease when they showed clear zone around the streak of colonies.

RESULTS

The antibiotic susceptibility test results given in Table.1 showed that the highest zone of inhibition size was found in S1 and C2 strains for Penicillin, MTCC for Amoxicillin and Norfloxacin.

The lowest zone of inhibition was found for Nalidixic acid in all the test strains which were resistant. All the samples were sensitive to Tetracycline and Chloramphenicol and resistant to Vancomycin and Nalidixic acid. The S1 was sensitive to Gentamicin, Penicillin, Amoxicillin, Erythromycin, and Norfloxacin and intermediate to Amikacin. The S2 was sensitive to Gentamicin, and intermediate to Erythromycin and Amikacin, and resistant to Amoxicillin, Penicillin and Norfloxacin. The C1 was sensitive to Amikacin and intermediate to Gentamicin and Erythromycin and resistant to Amoxicillin, Penicillin and Norfloxacin. The C2 was sensitive to Erythromycin and Penicillin and intermediate to Gentamicin and Amikacin and resistant to Amoxicillin and Norfloxacin. The MTCC reference strain was sensitive to Gentamicin, Amoxicillin, Erythromycin, Norfloxacin and Amikacin and resistant to Penicillin antibiotic. Thus all the test strains were resistant to Nalidixic acid and Vancomycin and S2, C1, C2 showed resistance to Amoxicillin and Norfloxacin and S2 and C1 showed resistance to Penicillin also which were considered difficult to treat infections caused by the strains.

Table 1: Overall comparison of antibiotic susceptibility test of six *S. aureus* strains

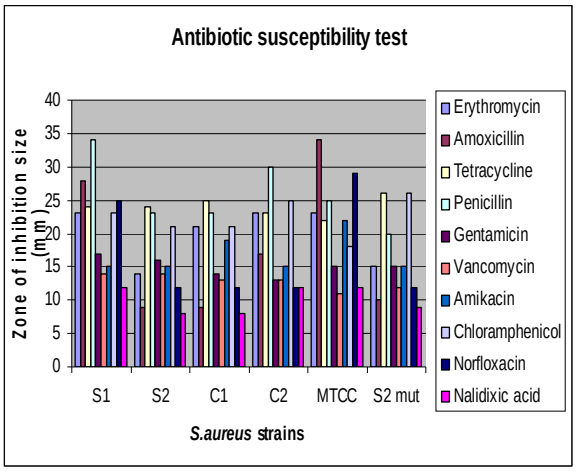

Table 2: Multiple antibiotic-drug Resistance (MAR)

S. aureus strains	MAR
S1	2/10
S2	5/10
C1	5/10
C2	4/10
MTCC	3/10
S2mut	5/10

The Table.2 representing the results of multiple antibiotic drug resistance analysis showed that S2, C1 and S2 mut were resistant to 5 antibiotics which were higher when compared to the other test strains in which S1 was resistant to 2 and C2 to 4 and MTCC to 3 antibiotics. Multiple antibiotic drug resistance in these strains might reduce the chance of number of available antibiotics for the treatment of *S. aureus* infections.

The resistance in these strains may be due to the inactivation of antibiotics as a result of structural modification by enzymatic action, prevention of access to target by altering the outer membrane permeability, alteration of the antibiotic target size, efflux pump which pumps out the antibiotic, and target enzyme bypass or over production.

Table 3: Plasmid curing analysis (Diameter of zone of inhibition in mm)

Antibiotics	S1		S2		C1		C2		MTCC	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Erythromycin	23	23	14	16	21	21	23	24	23	23
Amoxicillin	28	30	9	26	9	11	17	26	34	34
Tetracycline	24	24	24	24	25	27	23	24	22	22
Penicillin	34	34	23	26	23	24	30	32	25	34
Gentamycin	17	18	16	17	14	17	13	15	15	16
Vancomycin	14	14	14	15	13	14	13	14	11	13
Amikacin	15	17	15	16	19	20	15	15	22	24
Chloramphenicol	23	24	21	21	21	23	25	26	18	19
Norfloxacin	25	26	12	12	12	14	12	13	29	30
Nalidixic acid	12	15	8	10	8	9	12	13	12	13

Plasmid replication is inhibited by various agents especially 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 plasmid curing analysis was performed in all the strains with the above mentioned antibiotics in which acridine orange was used as curing agent. The results were given in the Table 3 showing the presence and increased zone of inhibition sizes in all the strains. The genes encoding resistance to antibiotics could be located on the plasmid or chromosome. Thus, when the strains were treated with acridine orange used for curing plasmids, the zone of inhibition was present as the resistance was due to chromosome mediated.

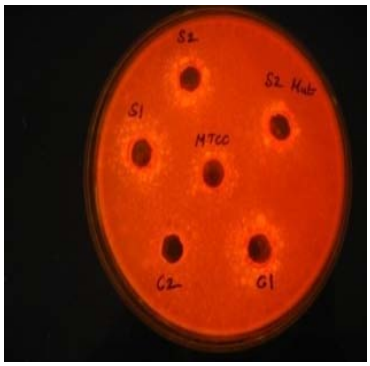

Figure 1: Lipase activity of *S.aureus* strains. The presence of fluorescent halos around the wells filled with *S. aureus* supernatants (S1, S2, C1, MTCC, S2 mut). Absence of lipase production in S2.

The *S. aureus* strains which were assayed for the presence of the three important virulence factors were considered important to be involved in colonization and invasion of host tissues. The fatty acid releasing Lipase enzyme of *S. aureus* was produced by all the test samples except C2. Protease which is an important enzyme acting as a virulent protein was produced by S1, C1 and MTCC whereas it was absent in S2, S2mut and C2 strains when performed with Casein agar plate assay. Biofilm of *S. aureus* which is involved in adhering of host tissues and materials and devices was produced by all the test strains except S1. In this study, C1 and MTCC were considered to be more virulent when compared to others since protease, lipase and biofilm were produced by these strains. The results were shown in the figures Fig.1, Fig.2 and Fig.3. The overall production of the studied virulence factors were given in the Table. 4.

Figure 2: Protease activity of *S. aureus* strains. Presence of clear zone around the streak of *S. aureus* strain S1 was indicated as positive for protease production. S2 and S2mut were negative for protease activity.

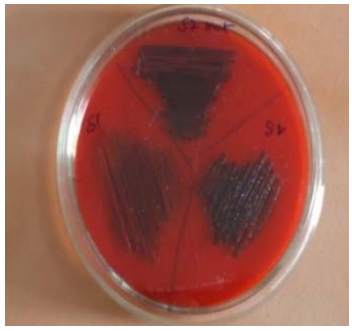

Figure 3: Biofilm formation of *S. aureus* isolates. S2 and S2mut were positive for biofilm formation whereas S1 was biofilm negative.

Table 4: Lipase, protease and biofilm production in test *S.aureus* strains

S. No.	Strains	Lipase	Protease	Biofilm
1	S1	+	+	-
2	S2	+	-	+
3	C1	+	+	+
4	C2	-	-	+
5	MTCC	+	+	+
6	S2 Mut	+	-	+

DISCUSSION

Lipase is an important lipolytic enzyme of *S. aureus* that contributes significantly to the pathogenesis of staphylococcal infection.[33] The fact that isolates from deep infections are generally lipase positive suggests that lipase plays an important role in tissue invasion.[34] A variety of assays have been used to assess lipase activity in *S. aureus.*[36-39] Lipases along with other proteins help bacteria degrade the host components thereby playing a role in virulence. Staphylococcal lipases are known to possess broad specificity for triglyceride molecules such as ester bond to oleic acid, palmitic acid and stearic acid which are enriched in human serum.[39,40] Like other bacterial lipases, the staphylococcal lipase plays a significant role in the bacterial lipid metabolism and their involvement in pathogenic processes.[41]

Extracellular staphylococcal proteases which are a class of proteolytic enzymes are instrumental in establishment and dissemination of *S. aureus* by breaking tissue proteins like collagen, myoglobin and fibrin indicating that they are important virulence factors. Recent reports suggest that proteases also play a role in the transition of *S. aureus* cells from an adhesive to an invasive phenotype by degrading bacterial cell surface proteins such as fibronectin binding protein and protein A.[42] They form a complex interactive network of components with pleiotropic roles in the pathogenesis.[43] There are different classes of extracellular staphylococcal proteases with respect to tissue organization. Aureolysin is for modulation of immunogenic reactions, Staphopain A and B help in tissue invasion, ulceration and sepsis, while V8 protease is involved in interference with host defense by inactivation of plasma serpins and immunoglobulin degradation.[42] Another major function of extracytoplasmic proteases is to protect the cells against the effects of toxic peptides.[44]

It has also been found that cross interaction between lipase 2 and sortase A plays an important role in biofilm formation by *S. aureus*.[45] Biofilm helps them to survive hostile conditions within host and is considered to be responsible for chronic or persistent infections.[46]

In this study, all the test strains except C2 produced two out of the three virulent factors and C1 and reference MTCC strain produced all the three. C1 and MTCC were found to be more virulent when compared to other strains since all the three studied virulence factors were produced in them. Thus, they might involve in the colonization and survival in host tissues and degrading it by involving in pathogenesis.

The important fact to be noted in the results was lipase enzyme was produced in all the strains except C2 and biofilm was produced in all the test strains except S1. This infers that *S. aureus*, which is an important human pathogen produce these virulence factors frequently in almost all the strains; but protease was produced by half of the tested strains (S1, C1, MTCC-positive; S2, C2, S2 mut=negative). This inferred that protease enzyme production was due to difference in the expression of Protease Repressor Sar A.[47] As mentioned earlier with references, the presence of lipase, protease and biofilms play an important role in colonization and attachment and degrading host tissues so as to get nutrition and survive well in host producing serious infections and disease.

As a result of plasmid curing for the 5 clinical isolates, the antibiotics which were resistant in all the strains lost their resistance and became sensitive except with Nalidixic acid. Also, in sensitive strains the zone of inhibition sizes increased after curing with acridine orange. This demonstrated that the antibiotic resistant genes are chromosomal encoded rather than plasmid encoded.

CONCLUSION

In this study, antibiotic susceptibility test revealed that all the strains were vancomycin-resistant strains and also S2, C1 and C2 were Amoxicillin and Norfloxacin resistant strains and S2 and C1 were resistant to penicillin. Plasmid curing analysis inferred that the strains which possessed zone of inhibition even after treatment with acridine orange was not plasmid cured. Thus the antibiotic resistance was due to chromosome mediated. The test clinical isolates and the reference strain were tested for the presence of three virulence factors namely lipase, protease and biofilm. The biofilm was produced in all the strains except S1, lipase was produced in all the strains except C2 and protease was produced in all except S2, C2 and S2 mut. From this, we could conclude that C1 and MTCC which were clinical isolate and MTCC reference strains respectively were more virulent when compared with others as these two strains produced all the three studied virulence factors.

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Source of Support: Nil, **Conflict of Interest:** None.