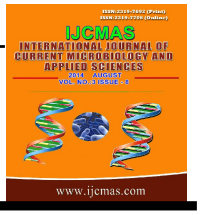
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 8 (2014) pp. 50-57**
http://www.ijcmas.com



Original Research Article

Demonstration of Virulence Markers and Methicillin Susceptibility of *Staphylococci* in various Clinical Isolates

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A B S T R A C T

The pathogenic potential of *Staphylococci* is associated with virulence factors mainly seen in *Staphylococcus aureus* but can also be expressed in coagulase negative staphylococci. This study identified some virulence factors in staphylococci isolated from various clinical isolates and also determined the relationship in the expression of these virulent factors in *S. aureus* and coagulase negative staphylococci (CONS) isolates with methicillin resistance. Production of coagulase, phosphatase, hemolysin, DNAse and slime formation were investigated on 100 staphylococci isolates using standard methods. The results showed that expression of virulence factors varied in isolates. The rate of expression was higher in *S. aureus* than in CONS although this difference was not statistically significant ($p>0.05$). Statistically significant correlation($p<0.05$) was observed between virulence markers haemolysin and slime formation in both coagulase positive and negative Staphylococci with methicillin resistance indicating these factors could help in suspecting methicillin resistance.

Keywords

Coagulase negative Staphylococci, MRSA, MSSA, virulence markers of Staphylococci.

Introduction

The staphylococci are a diverse group of bacteria that cause diseases ranging from minor skin infections to life-threatening bacteremia (Gill et al., 2005). *Staphylococcus aureus* has been recognized as a major human pathogen ever since Sir Alexander Ogston first proposed, in the 1880s, that it was the major cause of wound suppuration (Archer, 1998). The organism can produce an array of potential virulence factors such as alpha-, beta-,gamma- and delta-toxins, coagulase(Patel and Nowman, 1987) and slime formation. Virulence factors are required for colonization of host tissue and for protection against the host defense. Timely correct expression of the virulence factors is essential for the establishment and maintenance of an infection and represents a highly regulated process (Kullik et al.,1998). Biofilm producing *Staphylococci* frequently colonize catheters and medical devices and may cause foreign body related infections(Bose et al., 2009) enabling it to persist by evading host defenses and antimicrobials(Gordon

and Lowy, 2008). *S. aureus* strains associated with human infection have variable combinations of pathogenic determinants/virulence factors and either the presence or the expression of given combinations varies depending on the type of infection and genetic susceptibility of the affected host(Peacock et al., 2002).

Although virulence factors have been associated mainly with *S. aureus*, the coagulase negative staphylococci(CONS) isolated from clinical specimens have been reported to also express these virulence factors(Akinkunmi and Lamikanra, 2012). CONS are commonly isolated in clinical specimens and several species are recognized as important agents of nosocomial infections, especially in neonates (Turkyilmaz and Kaya, 2006) and have gained substantial interest as pathogens involved in nosocomial, particularly catheter-related infections(Otto, 2004).

The introduction of penicillin and beta-lactamase–stable penicillins, although dramatically improving the management of staphylococcal infection, have also contributed to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) strains(Diep et al., 2006). MRSA has become a progressively more important human pathogen since its initial description in 1961 and the first documented outbreak of infection in 1968(Davis et al., 2004). Numerous clinical studies have indicated, based on mortality rates, that methicillin-resistant Staphylococcus aureus (MRSA) strains are more virulent than methicillin-susceptible S. aureus (MSSA) strains(Rozgonyi et al., 2007).

Given the number and severity of *S. aureus* infections it is important to understand the nature and pathogenesis of infections and the current strategies available for therapy and prevention. Hence, the current study was done to demonstrate some virulence factors in coagulase positive(COPS) and coagulase negative staphylococcal(CONS) isolates from various clinical samples and their further correlation with methicillin susceptibility.

Materials and Methods

A total of 100 staphylococcal isolates from various clinical specimens like 42 form exudate, 40 from blood, 10 from sputum and 8 from urine were included in the study. The isolates subjected to the following tests to demonstrate the virulence markers.

Coagulase: Coagulase activity was determined by the method described by Quinn et al (1994). This test was performed as a Tube Coagulase test. Several colonies of each organism were mixed with 0.5 ml of citrated plasma in a sterile test tube. The tube was incubated at 37ºC and examined after 4 and 24 h. Clot formation at either reading was recorded as positive(Turkyilmaz and Kaya, 2006).

Phosphatase: 1% aqueous solution of sodium phenolphthalein diphosphate sterilized by filtration. 10 ml of this solution added to 1000ml of nutrient agar cooled to 50ºC and poured into slopes. Phenolphthalein diphosphate agar slopes inoculated and incubated overnight. A few drops of ammonia added. Test read positive when the colonies turn bright pink within a few minutes(Collee, 2007).

DNase(Deoxyribonuclease): This test was carried out by using commercially available DNase agar (Difco). Spot inoculation were done on the DNase agar and incubated at 37ºC. After incubation, 1 N HCl was poured on the agar. Clearing around the bacterial growth was evaluated as positive (Turkyilmaz and Kaya, 2006).

Hemolysis: Blood agar was prepared by adding 7% of sterile human blood aseptically to sterile nutrient agar which had been cooled to 45^{o}C and mixed thoroughly. To test for the production of haemolysin, the plates were streaked with loopfuls from bacterial cultures and incubated at 37^{o}C for 24 h. Clear zones around bacterial colonies indicated haemolysin production(Akinkunmi et al., 2012).

Slime Formation: The Congo Red Agar (CRA) method developed by Freeman was used in this study. The composition of medium was Brain Heart Infusion Broth (BHIB) 37 g/l, sucrose 50 g/l, agar 10 g/l and Congo red 0.8 g/l. Isolates which produced black colonies with dry crystalline consistency were regarded as slime positive, whereas those showing pink colonies were slime negative(Turkyilmaz and Kaya, 2006). Methicillin susceptibility was done by cefoxitin disc diffusion method as per CLSI guidelines.

Statistical analysis: Descriptive statistics was applied to calculate different variables in the table and arrange them in order. The crosstabs procedures applied for two-way and multi-way tables to know the association between various tests and measures. Chi-square test procedures used for tabulation. Results were statistically analyzed by using SPSS for Windows(version16.0).

Results and Discussion

Of the 100 Staphylococcal isolates, 90 were coagulase positive and 10 coagulase negative. Phosphatase was expressed by all. DNAse observed in 65(65%), hemolysis in 53(53%) and slime formation in 41(41%) isolates. Shown in Figures i-iv and Table 1.

Coagulase positive Staphylococci(COPS)

Coagulase positive Staphylococci(90) were isolated from 38 exudate ,36 blood,10 sputum and 6 urine samples. All the virulence markers expressed in exudate 12(31.4%),blood 17(47%),sputum 6(60%) and urine 3(50%) isolates. The distribution of virulence markers in various isolates among COPS shown in Table 2. All the haemolytic and slime forming isolates of sputum and urine were methicillin resistant. Correlation of methicillin resistance with various virulence markers among coagulase positive staphylococcal isolates is depicted in Table 3.

Coagulase negative Staphylococci(CONS)

Coagulase negative staphylococci isolates were 10(10%), comprised of 4 each from blood & exudate, 2 from urine. Phosphates was demonstrated in all the isolates. DNAse was not demonstrated in any CONS isolates. The distribution of other virulence factors among CONS is shown in Table 4. Methicillin resistance among coagulase negative staphylococcal isolates and their correlation with virulence markers is depicted in Table 5.

Among all the specimens, isolates expressing multiple virulence factors were found to be methicillin resistant. 6 isolates from sputum and 3 isolates from urine expressing all the five virulence factors were methicillin resistant. The given data shows higher expression of virulence markers among methicillin resistant isolates. Distribution of these virulence factors among clinical isolates of both COPS and CONS is shown in Table 6.

Statistical correlation was carried out between methicillin resistance and various virulence markers as shown in Table 7.

Statistical significance was not observed in Coagulase and DNAse markers as p value >0.05. Statistically significant correlation was observed between virulence markers haemolysin(p value-0.03) and slime formation(p value-0.001) in both coagulase positive and negative Staphylococci with methicillin resistance.

In this study, various virulence factors of Staphylococcal isolates from clinical specimens were demonstrated and their further correlation with methicillin susceptibility was observed. A number of biochemical activities are considered to contribute to the virulence of pathogenic staphylococci(Turkyilmaz and kaya, 2006). Coagulase activity, phosphatase, DNAse, hemolysis, and slime formation of the Staphylococcus spp. were regarded as pathogenicity criterions in laboratory.

In our study, coagulase was expressed in 90 isolates and phosphatase in all. Staphylococcal isolates were grouped into coagulase positive and negative for further study. DNAse was positive in 65(72%), Hemolysis in 50(55.5%) and slime formation in 38(42.2%) isolates among coagulase positive Staphylococci(90) when compared to coagulase negative(10) which did not exhibit DNAse but Hemolysis and Slime formation was observed in 3(30%)isolates each. Hence, a higher expression of virulence markers were seen in coagulase positive staphylococci. These results were parallel with other studies(Daghistani et al., 2000; Erganis et al., 1995).

Citak et al (2003) reported that 704 of 851 Staphylococci isolates from milk samples were S. aureus. These findings correlated with our study since 90 of 100 isolates were coagulase positive staphylococci. Damage to host cells is in part mediated by staphylococcal haemolysins, which contribute importantly to virulence in *S. aureus.* From the results, 55% of S. aureus and 30% of CONS strains produced haemolysin. Turkyilmaz and Kaya (2006) had earlier found a comparable rate of 58.9% in S. aureus while the rate for CONS (28.9%) was comparatively lower.

Testing for biofilm formation is another useful marker of the pathogenicity of staphylococci. This is because biofilm colonization by staphylococci facilitates infections that are often difficult to treat and therefore engender high morbidity and mortality(Weigel et al., 2007; Sauer et al., 2007). Many workers have reported that bacteria growing in a biofilm can be up to 1,500 times more resistant to germicides than the same bacteria growing in liquid culture(Weigel et al., 2007). 42% of COPS were positive for slime formation when compared to 30% of CONS which were in accordance with Akinkunmi et al.,2012 which found slime formation 36% in COPS and 32.8% in CONS.

The impact of methicillin resistance on the mortality of various infections remains controversial. In our study, 75 out of 100 Staphylococcal isolates were methicillin resistant, of which 68(91%) showed coagulase production. DNAse and hemolysis were each seen in 50(66.6%) and slime formation in 38(50%) isolates. Of the remaining 25 methicillin sensitive isolates, Coagulase, DNAse, hemolysis and slime formation were seen in 22(88%), 15(60%), 3(12%) and 3(12%) respectively. Multiple virulence factors were observed in the methicillin resistant isolates.

Table.1 Distribution of virulence factors among various clinical isolates

Virulence factors	No isolates
Coagulase	90
Phosphatase	100
DNAse	65
Hemolysis	53
Slime formation	41
Total	100

Table.2 Distribution various virulence markers in COPS isolated from various specimens

Isolates (no)	Haemolysin no(%)	DNAse no(%)	Slime formation no(%)
Exudate (38)	18(47%)	30(79%)	12(31%)
Blood (36)	21(58%)	23(64%)	17(47%)
Sputum (10)	7(70%)	8(80%)	6(60%)
Urine (6)	4(66%)	4(66%)	3(50%)

Table.3 Methicillin resistance in various virulence markers expressed in COPS isolates

Isolates (no)	Haemolysin no(%)	DNAse no(%)	Slime formation no(%)
Exudate (38)	16(89%)	23(77%)	10(83%)
Blood (36)	20(95%)	18(78%)	16(94%)
Sputum (10)	7(100%)	6(75%)	6(100%)
Urine (6)	4(100%)	3(75%)	3(100%)

Table.4 Distribution of virulence factors among CONS

Isolates(no)	Hemolysin no.(%)	Slime formation no.(%)
Exudate(4)	1(25%)	1(25%)
Blood(4)	1(25%)	2(50%)
Urine(2)	1(50%)	Nil

Table.5 Methicillin resistance in various virulence markers expressed in CONS isolates

Isolates	Hemolysin no.(%)	Slime formation no.(%)
Exudate(4)	1(100%)	1(100%)
Blood(4)	1(100%)	2(100%)
Urine(2)	1(100%)	Nil

Table.6 Distribution of virulence factors among various clinical isolates

Isolates (100)	*C + P + H + D + S no (%)	H + D + S no (%)	H + D no.(%)	D + S no. (%)	H + S no. (%)
Blood(40)	16(40%)	16(40%)	19(47%)	17(42%)	17(42%)
MR(31)	16(100%)	16(100%)	18(95%)	16(94%)	17(100%)
Exudate(42)	10(24%)	10(24%)	18(43%)	12(28%)	10(24%)
MR(30)	10(100%)	10(100%)	16(89%)	10(83%)	10(100%)
Sputum(10)	6(60%)	6(60%)	6(60%)	6(60%)	6(60%)
MR(7)	6(100%)	6(100%)	6(100%)	6(100%)	6(100%)
Urine(8)	3(37%)	3(37%)	3(37%)	3(37%)	3(37%)
MR(7)	3(100%)	3(100%)	3(100%)	3(100%)	3(100%)

*Abbreviations: MR-methicillin resistant, C-coagulase, P-phosphatase, H-hemolysis, D-DNAse-deoxyribonuclease, S- slime formation.

Table.7 Statistical correlation of virulence markers with methicillin resistance

Virulence markers	Coagulase	Hemolysis	DNAse	Slime formation
p value	0.708	0.03	0.807	0.001

Statistical analysis of the above data was carried out. Since phosphatase was expressed by all, it could not be considered as a significant virulence marker of methicillin resistance. Statistically significant p value<0.05 was seen in virulence factors hemolysis and slime formation when correlated with methicillin resistance.

Several studies have attempted to compare the outcome of nosocomially acquired MSSA and MRSA infections(Mekontso-Dessap et al., 2001). Three studies have observed similar mortality rates in patients who have MRSA and MSSA bacteremia(French et al., 1990; Marty et al., 1993; Mylotte et al., 1996) In contrast, 3 other studies have reported that methicillin resistance is a significant and independent risk factor for death in patients who have episodes of *S. aureus* bacteremia(Romero-vivas et al., 1995; Contemo et al., 1998; Moreia et al., 1998).

Some authors have observed a higher incidence of bloodstream infection with MRSA as compared with MSSA in humans(Rello et al., 1994) while Some have reported that nosocomial MRSA isolates produce significantly more antiphagocytic coagulase(Vaudaux and Waldyogel, 1979) than do methicillin-sensitive stains. Jordens et al., 1989 reported that enterotoxin A was produced by MRSA but not by MSSA. Therefore, differences in microbial virulence remain controversial, but they might have contributed to the results observed in the study.

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