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## **Study of *Staphylococcus aureus* from Clinical Samples in Savar, Bangladesh**

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### **ABSTRACT**

In this study, clinical samples were collected and characterized by culture techniques, antibiotic-resistance patterns, hemolysis and susceptibility to herbal agents. A total of 50 specimens of pus, urine, throat swab and sputum were collected to study *Staphylococcus aureus*. Eight of the isolates showed positive coagulation test and produced characteristic results for *Staphylococcus aureus* in culture media and biochemical tests. Results of antibiotics sensitivity test revealed the sensitivity of these isolates to Penicillin G, Oxacillin, Erythromycin, Gentamycin, Amikacin, Methicillin, Cotrimoxazole, Ciprofloxacin, and Erythromycin. However, 2  $\beta$ -hemolytic isolates showed development of spontaneous resistance to 30  $\mu\text{g mL}^{-1}$  of Penicillin and 15  $\mu\text{g mL}^{-1}$  of Tetracycline. They were also resistant to traditionally known products such as *Ocimum sanctum* (Tulshi), *Agadiracta indica* (Neem) and *Curcuma longa* (Turmeric). One of the  $\beta$ -hemolytic isolate was resistant to action of fresh animal sera, indicating potential pathogenicity and resistance against the concentration of phenyl used in routine disinfection practices, which implies possibility of nosocomial transmission. Nevertheless, these two isolates were moderately sensitive to phenyl at 1:10 and 1:20 concentration. In conclusion, the clinical isolates of *S. aureus* in a Bangladeshi community are resistant to penicillin G, tetracycline, traditional herbal agents as well as certain concentration of disinfectants, implying risk of possible transmission.

**Key words:** *Staphylococcus aureus*, clinical samples, Bangladesh, beta-hemolytic strain, resistance to traditional therapy

### **INTRODUCTION**

*Staphylococcus aureus* (*S. aureus*) is the focus of attention in medical bacteriology in the twenty first century because of its diverse pathological features and drug resistance. According to the report of the Journal of the American Medical Association, more people in the United States die due to invasive Methicillin resistant *Staphylococcus aureus* than HIV/AIDS (Klevens *et al.*, 2007). *S. aureus* is a member of the normal flora in humans, but ability of some *S. aureus* strains to produce coagulase,  $\beta$ -lactamase, exotoxin, leukocidin, toxic shock syndrome toxin-1 and enterotoxin can turn them virulent (Brooks *et al.*, 2001). This Gram-positive coccus is responsible for endocarditis, food poisoning, scalded skin syndrome, toxic shock syndrome, scarlet fever and sepsis with suppuration in any organ. Hospital-acquired methicillin resistant *S. aureus* is emerging in Asia (Jean and Hsueh, 2011) and is a genuine threat to public health. Asian population depends

on both allopathic and traditional herbal medicine; therefore different herbal medicines are often advised by local practitioners. Pavaraj *et al.* (2011) reported efficacy of *Clitoria ternatea* Linn. and *Achyranthes aspera* on pathogenic *S. aureus* isolated from urinary tract infection and Oladunmoye (2007) demonstrated inhibitory effects *Mirabilis jalapa* extracts.

This research aimed to find out distribution of *Staphylococcus aureus* among people living in Savar, a suburban area in the outskirts of Dhaka, Bangladesh. There had been reports of Methicillin-Resistant *Staphylococcus aureus* (MRSA) infection among Bangladeshi patients (Hossain *et al.*, 2003; Haq *et al.*, 2005). This study tried to determine if MRSA infection occurs in Savar community and the possibilities of diseases from pathogenic *S. aureus* in this community.

## MATERIALS AND METHODS

**Collection of specimens:** Bacterial samples of pus, urine, sputum and throat swab from 50 patients were collected aseptically from outdoor of Pathological Laboratory of Gonoshasthya Shamajvittik (Public Health Community) Medical College and Hospital and Lab Zone Diagnostic Center situated in Savar, Dhaka, Bangladesh. The samples were collected from patients of different ages and sexes for several diseases like cough, fever, wounds, abscess, tonsillitis etc. The clinical symptoms were recorded. This study was conducted from April, 2010 to November, 2010.

**Isolation and identification of *Staphylococcus aureus*:** Specimens were inoculated on to Mannitol Salt Agar (MSA) agar (Oxoid). Following overnight incubation, bright yellow colonies were subjected to coagulase test, Gram staining and catalase tests (Local supplier). All the primary screened strains were subjected to various morphological and biochemical tests to ensure their identity (Holt *et al.*, 2004). The biochemical tests included oxidase (Nanhjing, China) test, Motility Indole Ornithine (MIO) (Oxoid), Methyl Red-Voges Proskeur (MR-VP) (Hi-care, India), lipid hydrolysis, starch hydrolysis and gelatinase (Oxoid) test.

**Haemolysis test:** Coagulase positive samples were subjected to hemolysis test on blood agar with defibrinated sheep blood.  $\beta$ -hemolytic bacteria were isolated and purified.

***In vitro* antibiotic susceptibility test:** All the 8 *S. aureus* strains were tested *in vitro* to determine their antibiotic susceptibility pattern (Table 1) by antibiotic disc-diffusion method (Brantner *et al.*, 1994) on Muller-Hinton agar (Oxoid). Used commercial discs of Amikacin (30  $\mu$ g), Ciprofloxacin (5  $\mu$ g), Cotrimoxazole (25  $\mu$ g), Erythromycin (15  $\mu$ g), Oxacillin (1  $\mu$ g), Penicillin G (10 units), Tetracyclin (30  $\mu$ g), methicillin (10  $\mu$ g) and Gentamycin (10  $\mu$ g) were from Oxoid (USA).

Table 1: Biochemical and cultural characteristics of the isolates

Isolate No.	$\gamma$ Reaction	Coagulase	Catalase	Oxidase	TSI	NO <sub>3</sub> Lipid			Starch		Gelatinase	Hemolysis	
						MR	VP	reduction	hydrolysis	hydrolysis			
238	+	+	+	-	A	-	+	-	+	+	-	+	$\alpha$
301	+	+	+	-	A	-	+	-	+	+	-	+	$\alpha$
368	+	+	+	-	A	-	+	-	+	+	-	+	$\beta$
704	+	+	+	-	A	-	+	-	+	+	-	+	$\alpha$
756	+	+	+	-	A	-	+	-	+	+	-	+	$\alpha$
761	+	+	+	-	A	-	+	-	+	+	-	+	$\beta$
1346	+	+	+	-	A	-	+	-	+	+	-	+	$\alpha$
1416	+	+	+	-	A	-	+	-	+	+	-	+	$\alpha$

+: Positive reaction, -: Negative reaction, A: Acid production,  $\alpha$ :  $\alpha$  hemolysin,  $\beta$ :  $\beta$  hemolysin

**In vitro susceptibility to human serum:** Five milliliter human blood was collected from young healthy donors and the serum was separated by centrifuging whole blood for 2 min at 1000 rpm. Then, 150  $\mu\text{L}$  of serum was added to 150  $\mu\text{L}$  ( $10^5$  cells) of bacterial culture in 0.85% NaCl solution and incubated at  $37^\circ\text{C}$  for 1 h. The positive control for this test contained 50  $\mu\text{g mL}^{-1}$  Ciprofloxacin and negative control contained heat-inactivated serum with  $10^5$  cells  $\mu\text{L}^{-1}$  of *S. aureus* isolates.

Then 25  $\mu\text{L}$  mixtures were drop-plated on MSA and incubated overnight. The plates were examined for growth next day (Ehrenkranz *et al.*, 1971).

**In vitro disinfectant susceptibility test:** The  $\beta$ -hemolytic strains were subjected to *in vitro* disinfectant susceptibility test by serial dilution method. Different dilutions of Dettol (1:5, 1:10, 1:20); Savlon (1:80, 1:90, 1:100); Phenyl (10:25, 9:25, 7:25) and Phenol (1:80, 1:90, 1:100) were used in the test. A loopful of  $\beta$ -hemolytic *S. aureus* was inoculated into the different dilution of the disinfectant tubes containing 3 mL of the disinfectant. one drop of suspension was added to the nutrient agar plate at intervals of 5, 10 and 15 min. Growth was observed after overnight incubation.

**Action of traditional plant extracts:** The  $\beta$ -hemolytic strain was selected to determine the activities of traditional plant extracts by hole-plate diffusion method (HPD) (Biswas *et al.*, 2002) Aqueous extracts of 3 traditional plants, namely *Ocimum sanctum* (Tulshi), *Agadiracta indica* (Neem) and *Curcuma longa* (Turmeric) were used in the test. In case of Tulshi and Neem, aqueous extracts were prepared using plant leaves and distilled water, with an extract yield of 10.38 and 10.62%, respectively, whereas aqueous extract of Turmeric (*Curcuma longa*) was prepared using roots of the plant with an extract yield of 11.75%.

Certain amount of products were weighed and heated under reflux condenser for 15 min with continuous stirring. Then, the extract was covered with aluminum foil pack and allowed to cool. Sterile cotton swab was dipped into a well mixed saline test culture and was streaked on the nutrient agar plate to ensure a heavy growth over the entire surface. An 8 mm core of MHA agar was removed from seeded plate. Three wells were aseptically filled up with 50  $\mu\text{L}$  of each plant extract. After incubation, the plates were examined and the diameter of the zones of complete inhibition was measured in mm.

## RESULTS

**The isolates from clinical samples:** 8 specimens produced yellow colonies on MSA agar typical of *S. aureus*. 4 of them (Isolate No. 238, 368, 301 and 756) were collected from Pus samples, 2 from urine samples (Isolate No. 1346 and 1416), 1 from Throat swab (Isolate 761) and 1 from sputum sample (Isolate 704).

**Identification and assessment of pathogenic potential:** The primary biochemical tests showed typical reactions for *S. aureus*. Most importantly, two isolates (368 and 761) were  $\beta$ -hemolytic, which constitute 25% of the total isolates (Table 2).

**In vitro susceptibility to human serum:** The activity of human serum is evidenced by decrease of colony forming units mL (CFU mL) from more than 400 CFU mL (TNTC) to less than 300 CFU mL $^{-1}$ . If the isolate produces  $>400$  CFU mL $^{-1}$  after action of serum, then it is not susceptible

Table 2: Susceptibility of *S. aureus* isolates to the action of human serum

Isolate No.	238	368	301	756	761	704	1346	1416
Activity of serum	-	+	-	+	-	-	-	-
CFU (25 µL)	TNTC	215	TNTC	20	TNTC	TNTC	TNTC	TNTC

(TNTC) > 300 colonies per 25 µL drop

Table 3: Disinfectant susceptibility test

		Growth in subcultures (min)					
		Isolate 761			Isolate 368		
Disinfectant	Dilution	5	10	15	5	10	15
Dettol	1:5	-	-	-	-	-	-
	1:10	-	-	-	-	-	-
	1:20	-	-	-	-	-	-
Phenol	1:80	-	-	-	-	-	-
	1:90	-	-	-	-	-	-
	1:100	-	-	-	-	-	-
Phenyl	1:5	-	-	-	-	-	-
	1:10	+	+	+	-	-	-
	1:20	+	+	+	-	-	-
Savlon	1:80	-	-	-	-	-	-
	1:90	-	-	-	-	-	-
	1:100	-	-	-	-	-	-

+: Present ; -: Absent

Table 4: Antibiotic susceptibility pattern of the isolates

Isolate	Gentamycin	Methicillin	Amikacin	Ciprofloxacin	Cotrimoxazole	Erythromycin	Oxacillin	Penicillin G	Tetracyclin
238	S	S	R	S	S	R	R	S	R
368	S	S	S	S	S	S	S	R	R
301	S	S	S	S	S	S	R	R	S
756	S	S	S	R	S	S	S	S	S
761	S	S	R	R	R	S	S	R	R
704	R	S	S	S	R	S	S	S	S
1346	S	S	S	R	S	S	S	R	S
1416	R	S	S	S	R	S	S	S	S

S: Sensitive, R: Resistant

to the inhibitory action of antibody and complement proteins in serum. If <300 CFU mL<sup>-1</sup> is found after serum exposure, the isolate is being inhibited by serum complements (Mold *et al.*, 1981; Weinrauch *et al.*, 1996). The results are presented in (Table 3) . Fresh human serum from apparently healthy volunteers could inhibit six out of 8 (75%) of the *S. aureus*. The isolate 761 was resistant to the action of fresh serum *in vitro*, indicating possible mechanism of protection against complement action. This is one important aspect in pathogenicity.

**In vitro disinfectant susceptibility test:** Susceptibility of the β- hemolytic isolates to commonly used disinfectants is presented in (Table 4). Isolate 761 was resistant to 1:10 and 1:20 dilutions of phenyl. Isolates 368 and 761 were sensitive to the other tested concentrations of Dettol (1:5, 1:10, 1:20); Savlon (1:80, 1:90, 1:100); Phenyl (1:5) and Phenol (1:80, 1:90, 1:100).

**Action of traditional plant extracts:** The coagulase positive and  $\beta$ -hemolytic *S. aureus* were shown to be fully resistant to the natural extracts of 10.32, 10.62 and 11.75% of Turmeric, Neem and Tulshi, respectively.

**In vitro antibiotic susceptibility test:** The antibiotic susceptibility profile of isolated *S. aureus* strains is Amikacin (75%), Ciprofloxacin (62%), Cotrimoxazole (62%), Erythromycin (88%), Oxacillin (75%), Penicillin (50%), Tetracycline (62%), Methicillin (100%), Gentamycin (75%). The  $\beta$ -hemolytic strains were resistant to 15 g mL<sup>-1</sup> tetracycline and 30 g mL<sup>-1</sup> penicillin concentration but were sensitive to oxacillin.

## DISCUSSION

Darmstadt *et al.* (2009) reported that 40% of neonatal bacteria in Bangladesh is caused by *Staphylococcus aureus*. Struelens *et al.* (1991) had previously shown that *S. aureus* is responsible for nontyphoid bacteremia in Bangladeshi population. Lqbal *et al.* (1999) from Dhaka had reported presence of community-acquired MRSA in Bangladesh. This study did not find MRSA in the Savar population because no isolate in this study was resistant to methicillin and oxacillin. The antibiotic resistance profile in our study could be compared to reports from Sudan (Saeed and Ahmed, 2009) and Saudi Arabia (Abulreesh and Organji, 2011), where multi-drug resistance is prevalent in clinical and non-clinical samples, respectively without methicillin resistance. But there are studies indicating alarming proportion of vancomycin-intermediate *S. aureus* from Kerala, India (Jeshina and Surekha, 2009). The isolates from patients are coagulase positive and only two are  $\beta$ -hemolytic, meaning these two are more likely to be pathogenic. One isolate (761) is resistant to the action of serum proteins. This causes concern because there are a number of proteins in serum that provide non-specific immunity to pathogens. The complements and C-reactive proteins are the major players in opsonization of bacteria in blood (Mold *et al.*, 1981). If a strain is resistant to fresh serum in-vitro experiments then it might contain virulence factors to progress pathogenesis in vivo. This information is particularly interesting to us because cold plasma can kill *S. aureus* by damaging surface structures and plasma, serum and blood products are potential candidates in anti-bacterial therapy instead of antibiotics (Weinrauch *et al.*, 1996). Isolate 761 is also resistant to 1:10 and 1:20 concentrations of Phenyl, which indicates possibility of hospital acquired infection. Enough information is not available on hospital acquired infection in Bangladesh. The effect of traditionally used plant extracts were checked on the  $\beta$ -hemolytic isolates. Turmeric (*Curcuma longa*) has therapeutic use in alternative medicine and recently the biochemical basis of its antibacterial effect has been unraveled (Rai *et al.*, 2008). Curcumin is the active ingredient in turmeric that has been shown to stop *S. aureus*. Margo or neem (*Azadiracta indica*) contains nimbidin, which can prevent bacterial and viral infections (Joshi *et al.*, 2009; Sittiwet *et al.*, 2008). Tulsi (*Ocimum sanctum*) was included because the most important  $\beta$ -hemolytic isolate (761) was isolated from throat swab of a patient who experienced typical symptoms of throat sore. Such cases were also reported from Iran (Nikakhlagh *et al.*, 2011; Pavaraj *et al.*, 2011). In such conditions, Bangladeshi patients often take the aqueous extract of tulsi. The active ingredient of tulsi has not been well characterized. None of the three plant extracts could inhibit the  $\beta$ -hemolytic isolates in vitro. Therefore, patients require highly specific antibiotic for recovery from severe clinical conditions of *S. aureus* infections (Aboulmagd *et al.*, 2011). Sittiwet *et al.* (2008) reported even MRSA strains could be inhibited by crude extracts of *Malvastrum coromandelinum* Garke. Detailed study could reveal the molecular mechanism by which alkaloids inhibit *S. aureus*.

For future direction, a long-term study on community-acquired and hospital-acquired *S. aureus* distribution in the defined population might be planned with emphasis on age/sex and nutritional status of the patients. Quick and easy detection methods can also be established.

## CONCLUSION

The isolation of a  $\beta$ -hemolytic *S. aureus* with potential virulence factors like resistance to human serum *in vitro*, resistance to 1:10 and 1:20 dilutions of phenyl and to aqueous extracts of *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa* indicate necessity of investigation into the pathogenic features and proper inhibitory agents to control possible infection and disease.

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