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Prevalence of multidrug resistant *Staphylococcus aureus* in GonoShastho Nagar Hospital, Dhaka, Bangladesh

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Antibiotics are used in the prevention of different kinds of infectious diseases, but now-a-days, multidrug resistant strain of pathogenic bacteria is increasing due to the excess or misuse of antibiotics. These antibiotic resistant bacteria have become a great problem for the big population of Bangladesh. In this study, seven clinical samples were screened from about 250 patients having upper respiratory infections at Gonoshasthya Nagar Hospital, Dhanmondi, Dhaka. The isolated *S. aureus* was screened for their antibiotic resistance profiles. In this study, eleven antimicrobial drugs were used. The objective of this study was to check the sensitivity and resistance ability of *S. aureus* in different antibiotic concentrations and growth rate at room temperature after isolation, purification and characterization because infectious diseases could play a great role in human health in developing countries due to multidrug resistance activity of bacteria. *S. aureus* was found to be resistant to eight commonly used antibiotics and concentration of the organism was reasonably high in the urine sample followed by others. In room temperature, it grows continuously for about 10 h without changing the growth media and then the growth rate is decreased gradually with a small stationary phase. In future study, it could be helpful to find out more information on the relationship between this multidrug resistance microorganism and antibiotics with the solution of *S. aureus* related diseases.

Key words: *Staphylococcus aureus,* antibiotic, drug resistant.

INTRODUCTION

Many decades after the first patients were treated with antibiotics, bacteria became resistant and a substantial clinical problem. In developing countries where sanitation is still poor, antibiotics reduce the morbidity and mortality caused by food-borne pathogens. Therefore, the number of infections is increasing gradually with medical cost

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(Ventola, 2015). It may create a diverse array of life-threatening infections and capable of adapting to different environmental conditions. Therefore, *Staphylococcus aureus* can be considered as a greater concern pathogen (Lowy, 2003; Chamber, 2005; Onanuga and Temedie, 2011). *S. aureus* has become resistant to penicillin due to the production of lactamases, enzymes that destroy the antibiotic by hydrolysing the lactam ring and this quickly reduced the usefulness of penicillin in serious staphylococcal infections, especially among hospitalized patients in whom resistant strains were frequently found before they spread to the community (Drawz and Bonomo, 2010; Ventola, 2015). These community-acquired strains are now uniformly resistant to all lactam antibiotics and show cross-resistance to other antimicrobial agents (Chambers and Deleo, 2009). Antibiotic resistance level of a community or hospital can be predicted by these impor-tant measures; the proportion of resistant organisms introduced from outside the population, the extensive use of antimicrobial agents and the proportion that is spread from person to person (Onanuga et al., 2005).

In the treatment of infectious diseases, antibiotic resistance is one of the most serious threats which are world wide spread at present. The microbial population can survive by genetic mutation, expression of a latent resistance gene and acquisition of genes with resistance determinants and become threat to hosts. In a recent review, researchers pointed out that indiscriminate use of antibiotics within human population and veterinary medicine lead to the development of resistance to antibiotics by the pathogens and there is an urgent need for alternative medicine to control these organisms (Costelloe et al., 2010).

It is now well established that drug resistance arises from spontaneous or induced genetic mutations or by horizontal gene transfer from other bacteria (Schroeder et al., 2017). Drug resistance plasmids in pathogenic bacteria can play a critical role in designing antibiotic therapy to community. These plasmids have been named as drug resistance or R plasmids. A plasmid may carry only one marker or more than one marker. When plasmids carry multiple markers, they are called multiple drug resistance markers or MDRs (Huang et al., 2012). Multidrug-resistant *S. aureus* has become a major community-acquired (CA) pathogen, with enhanced virulence and transmission characteristics that influence infections in the community (Otter and French, 2009; Davies and Davies, 2010). Antibiotics resistance in the environment is increasing due to environmental pollution with broad-spectrum antibiotics (Larsson and Fick, 2009).

The World Health Organization reported that inappropriate use of antibiotics in animal contributes to the emergence and spread of antibiotic-resistant bacteria (Ventola, 2015). The use of antibiotics in food animals cause bacteria resistance to antibiotics used in humans, and these might spread via the food to humans and cause human infection. The actual risk seems small, and there might be drawback to human and animal health. The low dosages used for growth improvement are an unquantified hazard. Although, some antibiotics are used both in animals and humans, most of the resistance problem in humans has arisen from human use (Phillips et al., 2004). Due to horizontal gene transfer and mutations in the pathogen genome, antibiotics resistance can occur. The mechanism of antibiotics resistance in bacteria is transferred from organism to organism and is risky to the environment (Wintersdorff et al., 2016). Wintersdorff et al. (2016) also proposed that antibiotic is inactivated by certain enzyme; target site of the antibiotic is altered, metabolic pathway of the organism may be altered and reduced amount of drug may be accumulated in the cell. It has so far been discovered that almost all the common pathogens are responsible for human diseases and may be resistance to the popularly used antibiotics.

During the last few decades, human civilization observed not only phenomenal increase in new drugs to combat MDRs but also new terminologies to describe various new organisms (Chambers and Deleo, 2009). Biswas et al. (2014a) carried out research on the extent and pattern of antibiotics use in Bangladesh. They showed that ampicillin, penicillin, tetracycline, sulfonamides chloramphenicol and oxytetracycline were the most commonly used antibiotics in Bangladesh. In the rural area of Bangladesh, antibiotics are consumed more frequently than any other single class of drug, and 28% of the people's expenditure at pharmacies for drugs is on antibiotics (Biswas et al., 2014b). This is comparable with estimates in other areas where infectious diseases are prevalent (Biswas et al., 2014b).

Tremendous use of antibiotic by the physicians and general public lead to the development of severe drug resistance phenomena in Bangladesh. A time has come when study of drug resistance has to be done on individual organism. This study focuses on the characterization of drug resistance of *S. aureus* by determining the pattern of drug resistance exhibited by it. The main goal is to find out the resistance and sensitive property of *S. aureus* to antibiotics and the growth rate of the organism at room temperature.

MATERIALS AND METHODS

Sample collection and bacterial culture conditions

Routine method and materials were used in this study in the diagnostic laboratory. The chemicals used in this study were purchased from Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO, USA). The chemicals used were analytical grade. The Samples collected from about 250 patients attending an outdoor-patient department of GonoShastho Nagar Hospital. Samples were collected and inserted into leak proof labelled containers. Then nutrient broth was incubated for six hours. Colonies were isolated from nutrient agar plate using serial dilution technique. Few colonies were gram stained and examined under oil emulsion lenses. 5 ml. of venous bloods were collected by using a

dry sterilized one-time syringe in aseptic condition. Blood was taken into blood culture bottle which contains 50 ml media containing peptone, dextrose, sodium succinate, sodium lactate, gelatin, sodium carbonate and blue tetrazolium. The change of colour indicates the growth of organism. Pour plate and streak culture were used for the isolation of bacteria. The nutrient agar media was used for initial isolation of the organism.

Staphylococcus aureus count

S. aureus counts were carried out on nutrient agar media by spread plate technique. The samples were transferred into 1 ml sterile saline and then it was grown in a volume of 5 ml nutrient broth. The colonies were then grown on nutrient agar plates overnight. The colonies in each plate were counted and converted to standard value and were expressed in CFU/100 ml (Weinrich et al., 2009).

Drug resistance assay

Antibiotic resistant pattern was detected by the disc diffusion techniques (Ruan, 2013). Minimum inhibitory concentration of antibiotics was determined by using 30, 50, 75 and 100 µg concentration of antibiotic for selective pathogenic strain.

Agar disk-diffusion method

Agar disk-diffusion testing developed in 1940 (Heatley, 1944), is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. In this study, this method was performed according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) for bacteria testing (CLSI, 2012, 2004). Although not all fastidious bacteria can be tested accurately by this method, the standardization has been made to test certain fastidious bacterial pathogens like *Streptococci, Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, using specific culture media, various incubation conditions and interpretive criteria for inhibition zones (CLSI, 2012). In this well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6 mm in diameter), containing the test microorganism at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions. The diameters of inhibition growth zones are measured after 24 h inoculation with 30°C.

The methods described in Bergey's manual were used for the identification of organism (Emerson et al., 2008). The biochemical methods which were used in this study are given below: Catalase test, coagulase test, oxidase test, indole test, Voges-proskauer (VP) test, Simmon's citrate and motility indole urea (MIU) test.

Catalase test

The enzyme catalase is capable of decomposing hydrogen peroxide into water and molecular oxygen. For these reason, 24 h old bacterial culture was inoculated into T1N1 Broth (Tryptone Salt Broth) tube and incubated overnight at 37°C. After incubation, 1.0 ml of 3% hydrogen peroxide solution was added to each of the tubes and examined immediately after 5 min. Production of bubbles indicated positive result (Mustafa, 2014).

Oxidase test

For oxidase test, bacteria were inoculated in nutrient agar media and incubated at 37°C for 48 h. Then, a portion of the colony was taken with a sterile platinum-loop and rubbed on a strip of a filter paper impregnated with freshly prepared solution of 1% tetra-methy-p-phenylenediamine di-hydrochloride. Positive test was indicated by production of dark purple colour within 10 s (El-Hadedy and El-Nour, 2012).

Coagulase test

Coagulase causes plasma to clot by converting fibrinogen to fibrin. For coagulase test, a drop of distilled water was placed on two separate slides. A colony of the test organism was emulsified in each of the drops to make two thick suspensions. A loop of plasma was added to one of the suspensions and mixed gently. Clumping of the organisms within 10 s indicated positive coagulase test (Sperber and Tatini, 1975).

Indole test

Indole is produced by the action of bacteria on the amino acid tryptophan. For indole test, 24 h old culture was inoculated in T_1 N_1 broth tube and incubated at 37°C. After incubation, 0.5 ml Kovac's reagent was added and vigorously shaken for one minute. A red colour in the reagent layer indicated indole production (El-Hadedy and El-Nour, 2012).

Voges-Proskauer (VP) test

Methyl red-Voges Proskauer (MR-VP) media was inoculated with 24 h old bacteria culture and then incubated at 37°C for 24 h. After incubation, 0.6 ml of 5% naphtha solution was added into each tube, followed by 0.2 ml KOH aqueous solution. The tubes were then shaken vigorously for 1 to 2 min. Formation of eosin pink colour indicated positive result (Baird-Parker, 1963).

Motility indole urea (MIU) test

The tubes containing MIU medium 10 ml were inoculated by stabbing the medium to a depth of 5 mm and indole paper strips (soaked with lead acetate, which was soaked again in a solution p-dimethyl benzaldehyde 5 g, methanol 50 ml, O-phosphoric acid 10 ml) were inserted between the cotton-plug and the tubes. The tubes were then incubated at 37°C for 24 h. Motile organisms dispersed through the medium, which become turbid, growth of non-motile organisms, were confined to the stab. Positive test of urease activity was indicated by pink colour of the medium and indole production by pink colour of the indole paper strip (Abdel-Shafi et al., 2013).

Simmon's citrate test

Tubes of Simon's citrate agar (Appendix) were used for this test. The cultures were inoculated in the Simon's citrate agar tube media and incubated at 37°C for 24 h. The positive test was indicated by change of colour from green to blue (Korithoski et al., 2005).

Statistical analysis

Each experiment was repeated twice or thrice. The results are reproducible in each experiment.

RESULTS

S. aureus count

The result shows that urine blood and pus samples had higher number of organism as compared to other samples

Table 1. *Staphylococcus aureus* count in different samples.

Sample	*S. aureus* count/100 ml
Urine sample	3.1×10^{54}
Pus sample	2.0×10^{54}
Blood sample	2.3×10^{54}
Sputum sample	3×10^{4}
H.V.S sample	1×10^{4}
Ear swab	1×10^{4}
Throat swab	1×10^{4}

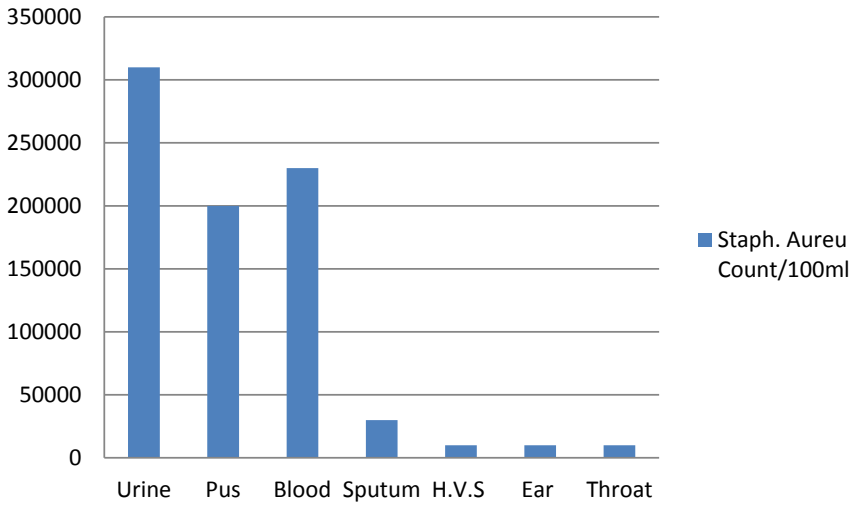

Figure 1. *S. aureus* count/100 ml in different samples.

used in this experiment. The count of *S. aureus* from various samples are shown in Table 1 and Figure 1.

Antibiotic resistance and sensitivity determination

The concentrations of antibiotics were used in this study for minimum inhibitory concentration (MIC) test. The guidelines followed were published by the Clinical and Laboratory Standards Institute (CLSI, 2012, 2004). The results were same for 30 µg/ml, 50 µg/ml and 75 µg/ml concentrations whereas 100 µg/ml concentration had been shown some variations in result. *S. aureus* could not tolerate 100 µg/ml concentration of Amoxicillin and Erythromycin. So Amoxicillin and Erythromycin were sensitive against *S. aureus* which has been summarized in Tables 2 and 3, respectively. The result has been represented in Figure 2.

Growth rate of *S. aureus*

The growth rate of *S. aureus* was determined under laboratory condition. This organism was aerobic; therefore, it was grown well in a shaker incubator. For about 14 h, the organism was grown in low concentration of antibiotics so that the organism does not lose the antibiotic resistance property. The initial absorbance was kept high so that the lag time is reduced but due to inefficient aeration, the lag phase could not be reduced substantially. The lag phase is about 5 h which is very long for an aerobic culture (Figure 3). Mean generation time was about two hours. The early log phase which was monitored for 4 h indicate that the early log phase started after 5 h of growth of the organism. Usually, maximum DNA synthesis takes place in the early log phase. It is expected that plasmid DNA synthesis would also be increased during this period. Late log phase starts after 9 h of growth of the organism. After then, the stationary phase started and the decline phase also occurred approximately after 12 h.

DISCUSSION

Patients used in this study are from lower income group who could not afford clinician's service always and as such they used self-medication. They used antibiotics randomly and in most cases without prescription.

Table 2. Antibiotic resistance and sensitivity determination for 30, 50 and 75 µg/ml concentration.

Isolate no.	Amoxicillin	Erythromycin	Cloxacillin	Cephalexin	Cipro-floxacin	Gentamycin	Ceftriaxone	Cloramphenicol	Ofloxacin	Kanamycin	Azithromycin
Urine	+	+	+	+	+	-	+	+	+	-	-
Pus	+	+	+	+	+	-	+	+	+	-	-
Blood	+	+	+	+	+	-	+	+	+	-	-
Sputum	+	+	+	+	+	-	+	+	+	-	-
H.V.S	+	+	+	+	+	-	+	+	+	-	-
Ear	+	+	+	+	+	-	+	+	+	-	-
Throat	+	+	+	+	+	-	+	+	+	-	-

+ = Resistant, - = sensitive.

Table 3. Antibiotic resistance and sensitivity determination for 100 µg/ml concentration.

Isolate no.	Amoxicillin	Erythromycin	Cloxacillin	Cephalexin	Ciprofloxacin	Gentamycin	Ceftriaxone	Cloramphenicol	Ofloxacin	Kanamycin	Azithromycin
Urine	-	-	+	+	+	-	+	+	+	-	-
Pus	-	-	+	+	+	-	+	+	+	-	-
Blood	-	-	+	+	+	-	+	+	+	-	-
Sputum	-	-	+	+	+	-	+	+	+	-	-
H.V.S	-	-	+	+	+	-	+	+	+	-	-
Ear	-	-	+	+	+	-	+	+	+	-	-
Throat	-	-	+	+	+	-	+	+	+	-	-

+ = Resistant, - = sensitive.

Concentration of the organism was reasonably high in the urine sample followed by blood and pus. Antibiotic resistance pattern of the organism was determined and it was found that the organism is resistant to a number of antibiotics but it is sensitive to a number of broad spectrum antibiotics (Davies and Davies, 2010; Kumar et al., 2013).

The results presented in Tables 1 to 3 show that the antibiotic resistance is very much valid because the organism can tolerate very high level of antibiotics in the medium. Growth profile of the organism was determined using an incubator. The culture was shaken by hands frequently. As a result, proper air supply to the medium could not be maintained. The organism showed long lag phase and delayed log phase. The lag phase was about 5 h and the mean generation time was about 2 h. The duration of late log phase was consistent with the condition of growth. Stationary and decay phases were fairly long. Confluent lysis of the organism was observed during stationary and decay phases. The cell lysis might have occurred due to induction of certain lysogenic phases into lytic one due to non-availability of oxygen. In other words, oxygen stress in the growth medium triggered the lytic cycle of the phage. This has initiated the cell lysis.

The above mentioned phenomena are worth investigation. The presence of or induction of lytic cycle may be used for autolysis of drug resistant microorganisms. A new therapy called the phage therapy may be used to control the drug resistant *S. aureus*. In this connection, the phage may be isolated by mutation and its characteristics may be studied. If it is not a lytic phage, it may be converted to lytic status. *S. aureus* is *a* worldwide pathogen which causes different types of infections of skin and soft-tissue (Tong et al., 2015), and it is continuously developing resistance to many antibiotics in the developing countries (Davies and Davies, 2010). The result of the

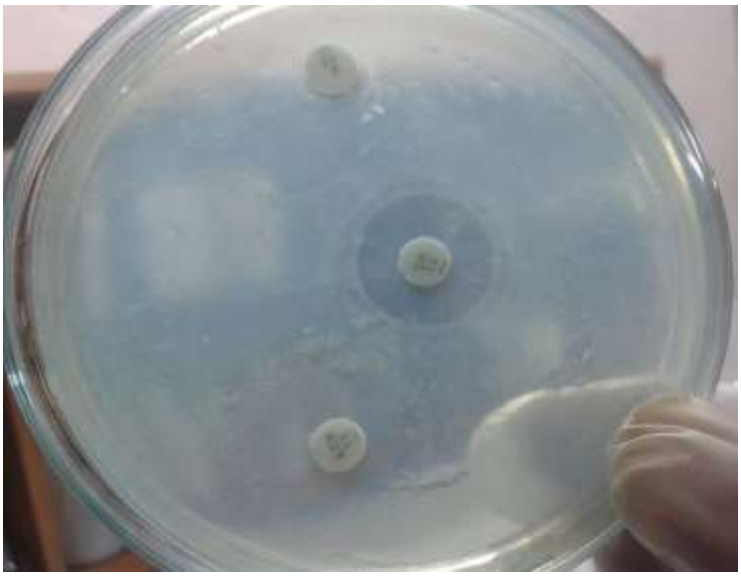

Figure 2. Sensitivity of Gentamycin against *S. aureus* in nutrient agar media.

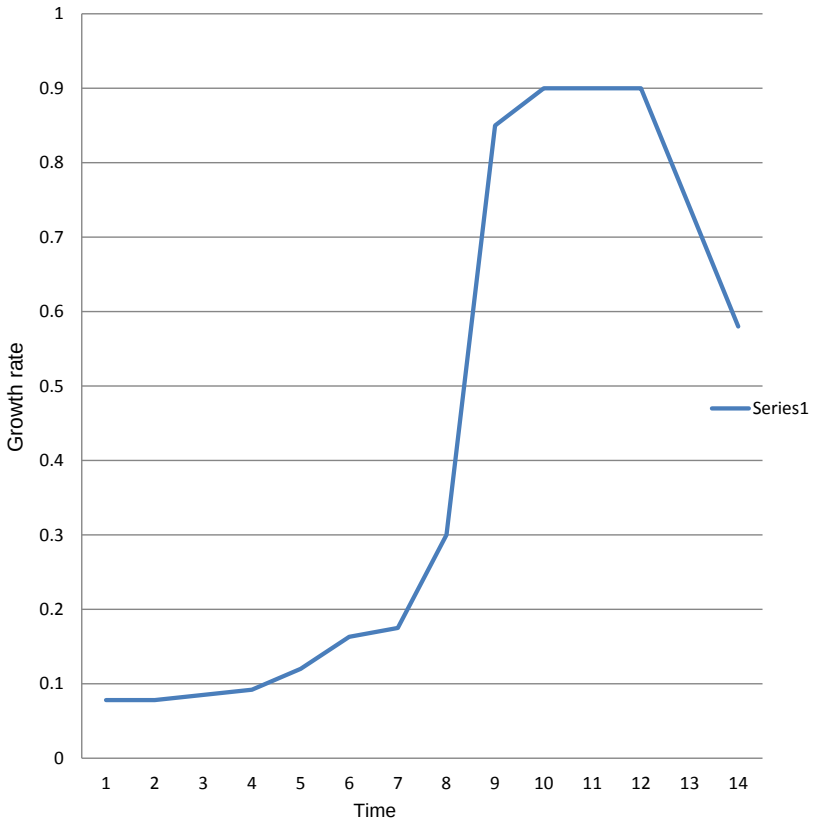

Figure 3. Growth rate of *S. aureus* in LB broth at 37°C temperature.

thickening of the cell wall has been reported by some authors (Hyo et al., 2013). These were however not determined in this work. Most of the isolates were highly susceptible to gentamicin, ofloxacin, ciprofloxacin, pefloxacin and sparfloxacin, which are in agreement with previous reports (Olayinka et al., 2010). Antimicrobial

resistant bacteria remain in high rate in the healthy members of the community (Kumar et al., 2013). The society is presently characterized by inappropriate prescription, unethical dispensing and indiscriminate use of antibiotics. Thus, most of the antibiotics are going to lose the battle against resistant organisms. Therefore, it is important for health professionals to carry out effective measures (including trainings) to promote rational use of antibiotics.

In this study, it was shown that *S. aureus* was resistant to eight antibiotics in 30, 50 and 75 µg/ml antibiotic concentrations but some variation has been shown in 100 µg/ml concentration. Therefore, as a result it is clear that *S. aureus* has multidrug resistance activity. It is hugely present in urine, pus, blood and normally present in other samples. In the laboratory condition S. *aureus* has been grown around 10 h and then the growth rate is decreased gradually with a small stationary phase between late log phase and decline phase.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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