IN VITRO PHAGOCYTOSIS OF METHICILLIN RESISTANT AND METHICILLIN SENSITIVE *STAPHYLOCOCCUS AUREUS* BY HUMAN POLYMORPHONUCLEAR LEUCOCYTES

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Background: *Staphylococcus aureus* is a gram positive bacterium that causes a number of diseases such as abscesses, infective endocarditis, septic arthritis, etc. It is acquiring resistance against many antibiotics like methicillin; therefore its control is becoming increasingly difficult. Peripheral blood phagocytes particularly polymorphonuclear leucocytes play an important role in the protective mechanisms against these organisms. Phagocytes interact with bacteria and phagocytose these microorganisms to kill them. **Methods:** Phenotypically different isolates of *Staphylococcus aureus* including methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA) were collected from various hospitals of Lahore, Pakistan. Fresh polymorphonuclaer leucocytes were obtained from healthy individuals by centrifugation using Ficol-Hypaque gradient combined with dextran sedimentation. Microbiological method was used for the determination of phagocytic index of phenotypic variants of *Staphylococcus aureus*. **Results:** A significant difference was observed between the phagocytic index of both bacterial groups. MSSA group showed the Mean±SD of 79.46%±3.9 while MRSA group showed 72.35%±2.5. **Conclusion:** Significant difference in phagocytic index indicates that it can be one of the mechanisms of MRSA to evade host immune system as compare to MSSA.

Keywords: MRSA, MSSA, Phagocytic index, Polymorphonuclear leucocytes

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a major gram positive pathogen linked with serious community and hospital-acquired diseases like abscesses, septicaemia, septic arthritis and infective endocarditis. Most of the *S. aureus* infections are caused by methicillin sensitive *Staphylococcus aureus* strains (MSSA) which are susceptible to many available anti-staphylococcal antibiotics but methicillin resistant *S. aureus* strains (MRSA) are involved in nosocomial and serious infections outbreaks. These MRSA strains show resistance to the wide range of antibiotics, thus minimizing treatment options to few drugs such as vancomycin and teicoplanin.[1,2] Resistance against methicillin is encoded by the gene *mecA* that is located on a genomic island termed staphylococcal cassette chromosome *mec* (SCC*mec*), which itself has seven distinct types. SCC*mec* is a mobile genetic element that can be exchanged among different staphylococcal species.[3,4] Microbes have genetic adjustability, which means that micro organisms have the ability to change themselves in to a resistant form under different environmental conditions. It is the selective pressure of antibiotics that assist the bacteria to survive and develop mechanisms to evade the killing action of antibiotics.

The treatment of several pathogens, including MRSA, is challenging. New solutions are needed to preserve the efficacy of current antibiotic range, to lower the overall risk of bacterial resistance and to successfully treat patients infected with resistant bacterial strains. Therefore various options for this purpose that can be used are development of new antibiotics to treat resistant organisms, vaccination to prevent infections, and the use of correct antibiotics. Proper use of antibiotics by all clinicians is imperative otherwise bacteria may eventually develop resistance against antibiotics. Appropriate use of antibiotic involves not only the selection of a 'targeted spectrum' antibiotic but its required dosage and duration as well.[5]

Innate immune system represents a highly conserved strategy against a wide array of bacterial, fungal, and viral pathogens. Activation of innate immune system results in an inflammatory response, which is essential to control infections rapidly before the infections spread to other parts of the body. It is now clear those cells of innate immune system contribute towards initiation and subsequently focus on ensuing adaptive immune responses.[6] Phagocytes are critical to the acute inflammatory response because they have the capacity to efficiently engulf and destroy a variety of pathogens. Phagocytes comprised of neutrophils, monocytes, macrophages, and eosinophils and they are also given the name of professional phagocytes.

Neutrophils (polymorphonuclear leukocytes) are important members of professional phagocytes. They are most numerous in circulation and these are usually the first cells to arrive at the site of inflammation. It is possibly the most significant cellular component of innate response during an acute infection.[7] Neutrophils are normally circulating in bloodstream (half life 7 hours) and migrate through tissues (2 to 3 days) and devote their short lifespan to

the process of immune surveillance.[8] However, during an infection, the lifespan of neutrophils is increased, and large numbers of neutrophils are rapidly recruited to the site of infection where they destroy invading pathogens. In this capacity, neutrophils serve as one of the body's first line of defence against infection. Immunity against staphylococcal diseases involve attachment of complement and opsonising antibodies to the bacterial surfaces, which leads to the activation of phagocytosis by polymorphonuclear leucocytes (PMNs).[9,10]

Phagocytosis of micro organisms is a key element in host defences against bacterial infections.[11] Two principal mechanisms of phagocytosis have been described, opsonin-dependent and opsonin-independent phagocytosis.[12,13] In opsonin-dependent phagocytosis, immunoglobulins or complement molecules bind to microorganisms, thereby promote adherence and ingestion via Fcγ or complement receptors which are present on phagocytic leukocytes.[12] In opsonin-independent phagocytosis ligands present on the surfaces of micro organisms are directly recognized by Toll like receptors and scavenger receptors present on the membranes of phagocytes.[13] These cells use an extraordinary array of oxygen-dependent and oxygen-independent microbicidal weapons to destroy and remove infectious agents.[14] Oxygen-dependent mechanisms involve production of reactive oxygen species (ROS), which can be microbicidal[15], while oxygen-independent mechanisms rely on neutrophil functions, such as chemotaxis, phagocytosis, degranulation, release of lytic enzymes and bactericidal peptides.[14]

There are not many studies on host defence mechanisms against different phenotypic strains particularly based upon antibiotic susceptibility; therefore the present study was designed to explore the differences in host defence mechanisms against different strains of *S. aureus*.

MATERIALS AND METHODS

Bacterial strains

Seventy-five bacterial isolates of *S. aureus* were collected from different hospitals of Lahore, Pakistan. It included 40 isolates of methicillin resistant *S. aureus* (MRSA) while 35 isolates were of methicillin sensitive *S. aureus* (MSSA). MRSA isolates comprised of 41 (82%) from Sheikh Zayed Hospital, 6 (12%) from Shaukat Khanum Hospital and 3 (6%) from Children Hospital, Lahore. For the MSSA group, 22 (44%) isolates were collected from Sheikh Zayed Hospital, 24 (48%) from Shaukat Khanum Hospital and 4 (8%) from Children Hospital**.** Single colonies were transferred from nutrient agar plates to brain heart infusion broth (BHI Oxoid, UK) and they were incubated overnight at 37 °C. These stains were maintained in brain heart infusion containing 25% glycerol (Sigma, St. Louis) at -20 °C.

Characterization of bacterial strains

All the bacterial strains were confirmed by growing them on mannitol salt agar (MSA Oxoid, UK) and catalase and DNase tests were also performed (DNase agar Oxoid, UK). Methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S aureus* (MSSA) strains were screened by using CHROM agar for MRSA (Beckton, Dickinson BBL).

Isolation of Neutrophils

Neutrophils were isolated by Ficoll-Hypaque dextran T-500 centrifugation. 5 ml of blood was collected in a centrifugation tube containing EDTA as anticoagulant. About 2.5 ml of Ficoll-Hypaque was added, mixed well and then centrifugation at 2000 rpm was done for 10 minutes. Plasma and buffy layer was discarded but the compact red cell layer was preserved. Equal volumes of HBSS (Sigma, St. Louis) and 6% dextran (Sigma, St. Louis) were added to the red cell layer. At 37 ºC it was incubated for 45 minutes. Supernatant was taken out from the settled RBC layer. Equal amount of HBSS was added. It was centrifuged at 1200 rpm for 10 minutes. After centrifugation, supernatant was removed while pellet was used. This step was repeated again. One ml of 1X lysing buffer was added to lyse RBC. It was incubated at 37 ºC for 1 to 5 minutes. Double amount of HBSS was added and it was spin at 1800 rpm for 10 minutes. The supernatant was removed and pellet was mixed gently. The same step of washing was repeated twice. The viability of cells was checked by mixing 100µl each of pellet and 0.4% trypan blue solution. Neubar's chamber was charged with the mixture and cells were counted using 40× objective.

Phagocytosis

One day before the scheduled assay, one vial from the stock of *S. aureus* was thawed and 10ml of brain heart infusion broth was inoculated with 5 µl of bacteria. It was incubated overnight at 37 ºC. PMNs were isolated according to the protocol described above. The optical density (OD) of bacterial suspension was adjusted to 1.0 at 620 ηm wavelength. An aliquot of control opsonin was thawed and maintained at 4 °C, until it was added to the reaction tubes. PMN-bacteria-opsonin reaction tubes were labelled and prepared as follows:

1. 500 µl of HBSS plus 50 µl of opsonin
2. 500 µl of PMN plus 50 µl of opsonin

0.1 ml of *S. aureus* (1.0 OD at 620 ηm) was added to each reaction tube and it was mixed by vortexing. Both tubes were incubated at 37 ºC in a shaking water bath for 20 minutes. After this, centrifugation was done for both the tubes at 500 rpm for 10 minutes. One ml of chilled HBSS was added. Tube

(A) was washed twice with chilled buffer. From the tube (B) supernatant was discarded and for the lysis of PMNs, 1ml of chilled sterile deionised water was added to it. One ml of 2X sterile normal saline was added to tube (B) and the volume in tube (A) was adjusted with HBSS. The lysate from tube (B) and control from tube (A) were transferred on two separate nutrient agar (Oxoid, UK) plates in a triplicate fashion and they were spread immediately with a glass spreader. The plates were incubated overnight at 37 ºC. Next day the colonies were counted on both the sets of plates and phagocytic index was determined.

RESULTS

Neutrophils

For every assay, neutrophils were freshly collected from a healthy individual. These neutrophils were >90% viable and their count was adjusted to 1×10^6 cells/ml.

Effect of antibiotic resistance on Phagocytosis

The range of phagocytic index for MSSA group was 70–86% with a mean of 79.45% while for MRSA group it was 68–77% with a mean of 72.35%. A significant difference was observed in phagocytic index between MSSA and MRSA groups (p<0.001) Mean±SD (79.46%±3.9 vs. 72.35%±2.5) respectively Table-1). It was noted that phagocytosis by neutrophils was more in MSSA group as compare to MRSA group.

Table-1: Mean phagocytic index of MRSA and MSSA group

	MRSA Mean±SD n =40	**MSSA Mean±SD** n= 35	***p*-value**
Phagocytic Index	72.35±2.5	79.46± 3.9	<0.001

DISCUSSION

In this study we compared phagocytosis of MRSA and MSSA by neutrophils. Microbiological method was used to find out the phagocytic index of MRSA and MSSA groups. Phagocytosis was made according to the prescribed method at 37°C in the presence of opsonin, i.e., normal human heat inactivated serum.[16] In contrast to our method Pramanik *et al* demonstrated more phagocytic activity in neutrophils at 38 °C and 39 °C but not at 37 °C.[17] According to them, in neutrophils the contractile element responsible for pseudopode formation is activated at higher temperature. We demonstrated methicillin sensitive *S. aureus* were more prone to be phagocytosed by PMNs as compare to methicillin resistant *S. aureus*. It suggests a mechanism by which MRSA can evade the host immune system.

Bukovsky *et al* indicated that the disintegrated form of *Staphylococcus aureus* has an inhibitory effect on non specific immune mechanisms of the host but it has a stimulatory effect on non specific immune mechanisms of mouse.[18] In contrast to our data, Peacock *et al* did not observe any difference during ingestion of multi-drug resistant MRSA and 3 antibiotic sensitive strains by human neutrophils which was done after 15, 30, 60 and 120 minutes of phagocytosis in the presence of fresh serum.[19] They found that more than 99% of bacteria were ingested after 60 minutes of incubation. Similarly, Jordens *et al* also could not detect the differences during ingestion of multi-drug resistant MRSA strains which were opsonised with fresh serum after 30 minutes by human neutrophils.[20] Like wise, Mekontso *et al* did not find any significant effect of *S. aureus* phenotype on neutrophil functions.[21] They demonstrated that the bactericidal activity of neutrophils against MRSA and MSSA during cardiac surgery.

Thus the present study provides an insight for the *in vitro* phagocytosis of phenotypically different forms of *S. aureus* by human polymorphonuclear leucocytes. It is indicated that the different strains of *S. aureus* based upon antibiotic sensitivity are phagocytosed differently by human PMNs. This suggests one of the ways by which antibiotic resistant *S. aureus* strains evade human immune mechanisms.

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