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 phagocytize 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 polymorphonuclear leukocytes 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, sepsis, meningitis, 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. Resistance against methicillin is encoded by the gene mecA which is located on a genomic island termed staphylococcal cassette chromosome mec (SCCmec), which itself has seven distinct types. SCCmec is a mobile genetic element that can be exchanged among different staphylococcal species. Microbes have genetic adjustability, which means that microorganisms 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.

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. 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. 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. 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).

Phagocytosis of microorganisms is a key element in host defences against bacterial infections. Two principal mechanisms of phagocytosis have been described, opsonin-dependent and opsonin-independent phagocytosis. 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. In opsonin-independent phagocytosis ligands present on the surfaces of microorganisms are directly recognized by Toll like receptors and scavenger receptors present on the membranes of phagocytes. These cells use an extraordinary array of oxygen-dependent and oxygen-independent microbialicidal weapons to destroy and remove infectious agents. Oxygen-dependent mechanisms involve production of reactive oxygen species (ROS), which can be microbialicidal, while oxygen-independent mechanisms rely on neutrophil functions, such as chemotaxis, phagocytosis, degranulation, release of lytic enzymes and bactericidal peptides.

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 °C 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 °C 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 °C. PMNs were isolated according to the protocol described above. The optical density (OD) of bacterial suspension was adjusted to 1.0 at 620 nm wavelength. An aliquot of control opsonin was diluted to 4% with HBSS. A 1:1 mix of 1 µl of opsonin plus 50 µl of PMN was added to one reaction tube and it was mixed by vortexing. Both tubes were incubated at 37 °C 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 °C. 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 1×10⁶ 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

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<thead>
<tr>
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<th>MRSA Mean±SD</th>
<th>MSSA Mean±SD</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Phagocytic Index</td>
<td>72.35±2.5</td>
<td>79.46±3.9</td>
<td>&lt;0.001</td>
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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. 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. 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. 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. 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. Like wise, Mekontso et al did not find any significant effect of S. aureus phenotype on neutrophil functions. 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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REFERENCES

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