

## **Original Research Article**

### **EXTRACELLULAR PROTEASES PRODUCTION IN METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) ISOLATES**

#### **ABSTRACT**

Methicillin resistant *Staphylococcus aureus* (MRSA) bacteria are responsible for wide range of infections, while the treatment of such infections has become a challenge for public health. Moreover, the production of extracellular proteases by these pathogens has recently been considered as a major virulence factor as the staphylococcal proteases can inactivate and cleave several important host proteins, including elastin, proteinase inhibitors and heavy chains of all human immunoglobulins. The present study was carried out to isolate and identify MRSA strains from various clinical samples (pus, blood, urine and skin) followed by screening for the production of extracellular proteolytic enzymes. The identification of the clinical isolates was achieved by microscopic and specific biochemical methods. MRSA strains were identified by Kirby-Bauer disc diffusion method. The extracellular protease activity was detected using casein medium by agar plate and well-diffusion bioassay methods. A total of 114 MRSA clinical isolates were isolated and pure cultured from different (n=119) clinical specimens (pus, blood, urine and skin). It was observed that 51.7% (n=59) MRSA isolates were recovered from pus samples, while 23.7% (n=27), 16.7% (n=19) and 7.9% (n=09), were obtained from blood, urine and skin samples, respectively. The enzymatic analysis of the MRSA isolates showed that 68.4% of them were highly positive for the production of the extracellular protease enzymes. Extracellular protease production was frequent in the clinical isolates of MRSA suggesting a critical role of the production of extracellular proteases in pathogenesis of MRSA in humans.

**Comment [AP1]:** It is not clearly written whether authors assessed the protease production only in MRSA or all isolates of *S. aureus*.

**Comment [AP2]:** Address the comments in the main body of manuscript and change Abstract accordingly.

**Keywords:** Casein medium, Clinical specimens, Skin infections, Staphylococcal proteases

### 1. Introduction:

*Staphylococcus aureus* is a Gram-positive bacterium normally found as commensal and attached with approximately 20-30% of the human population asymptotically [1]. However, several types of skin and soft tissue infections, abscesses, respiratory tract infections such as sinusitis, and food poisoning caused by *S. aureus* bacterial strains have been reported worldwide [2, 3, 4]. It has been shown that *S. aureus* causes a variety of infections through the production of a plethora virulence factors [5]. Among the virulence factors, extracellular enzymes including proteases are of great importance because these can inactivate immune defence system in humans.

There are many proteases discovered so far and the most of them are coded by chromosomal genes. The genes for the production of extracellular proteases have also been discovered in clinical isolates of *S. aureus* bacterial strains [6]. *S. aureus* excrete several types of proteins or enzymes, extracellularly which include nucleases, and proteases such as metalloprotease (autolysin), serine protease, cysteine proteases (staphopains) [7, 8]. The extracellular proteolytic enzymes obtained by *S. aureus* bacterial strains can degrade protease-inhibitors found in humans and in result the deregulation of human proteins which consequently leads to connective tissue degradation, occurs. However, all strains of *S. aureus* do not produce same extracellular protease. Although the production of extracellular proteases have been reported decades ago, its involvement in pathogenicity as a major virulence factor of *S. aureus* has recently been

**Comment [AP3]:** Consider changing the term "attached"

**Comment [AP4]:** Consider changing the term

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elucidated [9, 10]. The function of protease in the development of an infection can be species specific and is crucial to understand before using protease production as drug target [11].

**Comment [AP8]:** Difficult to understand, consider rephrasing

The development of antibiotic resistance in *S. aureus* bacterial strains isolated from clinical specimens has made this bacterium medically challenging. More specifically, MRSA is one of the current public health problems worldwide [12, 13, 14]. Therefore, current scenario demands alternative treatment or vaccine against *S. aureus*, which is unfortunately, has not been approved despite of much research and development done so far [15].

The present study has revealed the screening of pathogenic *S. aureus* strains isolated from various clinical samples for the production of extracellular proteases. This study will highly be beneficial to design a pathway to overcome infections caused by several drug resistant *S. aureus* strains particularly MRSA producing extracellular proteases as virulence factors.

**Comment [AP9]:** This line should tell the reader about the objectives of this research, not what the study revealed.

## 2. Materials and Methods

### Sample Collection:

Clinical samples (Pus, blood, tissues, and urine specimens) were collected from the patients visiting different government and private institutions and general clinics of the Hyderabad city. All the samples were processed for the isolation of bacteria particularly *S. aureus* species within three hours of the collection period. Exclusion criteria included samples collected before antibiotic therapy. The majority of pus samples were collected using sterile swabs along with transport medium, while sterile syringes were also used for the collection of pus samples depending on the volume of the pus appeared. Special care was taken while collecting pus from abscesses, wounds, or other sites to avoid contamination of the specimen with commensal

**Comment [AP10]:** Is this sentence implying that the specimen was collected after the antibiotic therapy? If so, why?

organisms from the skin. Samples from skin were collected using the sterile cotton-swabs. The infected area was cleansed carefully to avoid any contamination. Sterile cotton swabs were rubbed on the skin site to ensure collection of skin samples. Blood samples were collected under aseptic conditions. A total of 05-10 ml of the blood sample was collected using sterile syringe. Blood sample was immediately transferred to blood culture (BACTEC) bottle. Urine samples were collected using sterile wide mouth containers. Preferably, early morning urine samples were collected. Urine samples were processed within 2-hours of the collection or refrigerated in case of any delay in processing.

**Comment [AP11]:** Hyphens needed here?

**Comment [AP12]:** At what temperature?

**Comment [AP13]:** Incubation condition is not mentioned.

### Identification of *S. aureus*

Clinical isolates of *S. aureus* were identified using differential and selective media. Colonies appearing yellow on mannitol salt agar (MSA) media accompanied with the change of color of MSA were presumably identified as *S. aureus*. The isolates were further identified by microscopic examination and standard biochemical tests such as coagulase and catalase test [16]. Antibiotic resistant strains particularly methicillin resistant *Staphylococcus aureus* (MRSA) strains were identified by Kirby-Bauer disc diffusion method [17, 18, 19].

**Comment [AP14]:** Are these just two biochemical tests sufficient to differentiate *S. aureus* from other species of *Staphylococcus*?

**Comment [AP15]:** Which antibiotic was used to screen for MRSA? Which guideline did the researcher follow for the antibiotic susceptibility test?

**Comment [AP16]:** Did authors use any Positive/Negative control?

### Screening for Extracellular Protease

All the identified clinical isolates of *S. aureus* were screened for the production of extracellular protease enzyme. Casein agar medium and skim agar were used to investigate the production of protease enzyme. Casein agar medium is generally used to detect hydrolytic microorganisms that produce enzyme protease. Casein medium contained 1% casein and other nutrients [20]. Screening for the production of protease enzyme from *S. aureus* was performed by two different

**Comment [AP17]:** Are these two media same or different? if same, rephrase. if different, why authors later mentioned they used "casein agar" only?

methods using the casein agar medium; (i) agar plate assay and (ii) well-diffusion assay. In agar plate assay, the production of the extracellular protease was detected qualitatively. Clear zone of casein hydrolysis around the bacterial growth after incubation was an indication of extracellular protease production. In well-diffusion assay, the production of extracellular enzymes was measured quantitatively. In this assay, wells of about 6 mm in diameter were punched in casein-agar and up to 65  $\mu$ l of the sterile cell free supernatants of the *S. aureus* culture were pipetted in to each well. All the plates were incubated for 24-48 h at 37°C and analyzed for clear zones of casein hydrolysis.

**Comment [AP18]:** Earlier in this same paragraph, they said that they used casein agar and skim agar, but here they used only casein agar?? See the preceding comment above!

**Comment [AP19]:** Incubation temperature and duration?

### 3. Results

A total of 119 different clinical samples were collected from the suspected patients visiting different diagnostic centers located at the Hyderabad city. Sample distribution according to the type of specimen is shown in **Figure 1**. It was further identified that 114 clinical samples were found with *S. aureus* bacterial strains, therefore pure cultures of the identified *S. aureus* isolates were obtained and included in the present study for further investigations. The data revealed that the highest ratio of *S. aureus* clinical isolates belonged to pus specimens followed by blood, urine and skin tissue specimens, respectively (**Figure 2**).

**Comment [AP20]:** ?

**Comment [AP21]:** Consider rephrasing.

Antibiotic sensitivity test showed that majority of the isolated *S. aureus* cultures were oxacillin resistant thus were considered as MRSA isolates. It was observed that 68.4% (n=78) of the *S. aureus* clinical isolates in this study were positive for the production of extracellular protease, which is revealed by both, agar plate and well diffusion bioassays (**Figure 3**). Whereas 31.6% (n=36) of the isolates were unable to produce the enzyme evidenced by the negative result from

**Comment [AP22]:** How many are the "majority"? Give the exact number.

**Comment [AP23]:** The "Methods" section says that authors employed the disc-diffusion method to screen MRSA, and here authors are using oxacillin to screen MRSA! CLSI suggests the "broth microdilution or agar dilution" method while screening MRSA (mecA mediated) with oxacillin (see CLSI 2020, M100). And the authors have not mentioned the "incubation condition." That must be explicitly mentioned. The authors have not mentioned the  $\mu$ g content of the antibiotics.

**Comment [AP24]:** Are these 78 isolates out of total *S. aureus* or out of total MRSA?

agar plate assay. Moreover, a comparative analysis of the protease production by *S. aureus* isolates of the different type of specimens was done. The data of this study showed that the *S. aureus* bacterial strains obtained from pus were more frequent in the production of extracellular proteases 69.2% of the total *S. aureus* isolates. However, the frequency of *S. aureus* isolates from blood, urine and skin were less frequent for the extracellular protease activity (Table 1), suggesting that the type of infection or localization of the pathogen may affect the activity of the extracellular proteolytic enzymes from *S. aureus* clinical bacterial isolates.

Comment [AP25]: Rewrite.

Comment [AP26]: Italics?

#### 4. Discussions

Methicillin resistance in *S. aureus* is now global challenge. In this study, it was revealed that large numbers of *S. aureus* strains causing different infections in human have acquired resistance to oxacillin and become MRSA. The present study further investigated the 114 MRSA isolates for the production of extracellular proteases. The data revealed that large number of *S. aureus* (MRSA) clinical bacterial isolates can produce extracellular proteolytic enzymes. Furthermore, the frequency of the production of such enzymes is much higher among those isolates which cause skin and soft tissue infections, wound infections since the higher percentage of the isolates of pus specimen were showing the production of extracellular proteases *in vitro*. Consequently, 68.4% of the total isolates was highly positive to produce extracellular protease enzymes in both agar plate and well-diffusion bioassays. High protease activity shown by these pathogenic bacterial isolates indicates that they may have a critical role in virulence using protease as a major virulence factor.

Comment [AP27]: This statement is beyond the objective and scope of this research.

Comment [AP28]: This has been already presented in the Result section, no need to repeat the data in the Discussion section.

Comment [AP29]: This style of writing is confusing. If you are talking exclusively about MRSA, just write MRSA. If you're referring to all *S. aureus* isolates, be sure to provide that information.

Although a previous study has reported that no correlation between identified gene patterns of clinical isolates of *S. aureus* with specific infections was established [21], the present study has observed a link between the type of infection and frequency of extracellular proteases production. The findings of the present study can be supported by a previous study [6], which has observed that the genes for the production of extracellular proteases are found in both extracellular protease positive as well as negative isolates of *S. aureus*, suggesting that some regulating host cell factor may be involved in the lack of protease production [6].

**Comment [AP30]:** This whole paragraph needs to be re-written. The comparison with other research is obscure.

**Comment [AP31]:** HOW this study "observed the link between the type of infection and frequency of extracellular proteases"? I see no experiments or statistical measures applied to verify this statement.

## Conclusion

In conclusion, the present study has reported *in vitro*, high frequency of extracellular protease enzymes among the clinical isolates of *S. aureus* bacteria that are methicillin resistant and indicates a critical role of extracellular proteases enzymes in pathogenicity of *S. aureus* bacterial species.

**Comment [AP32]:** This cannot be concluded from this research.

## References

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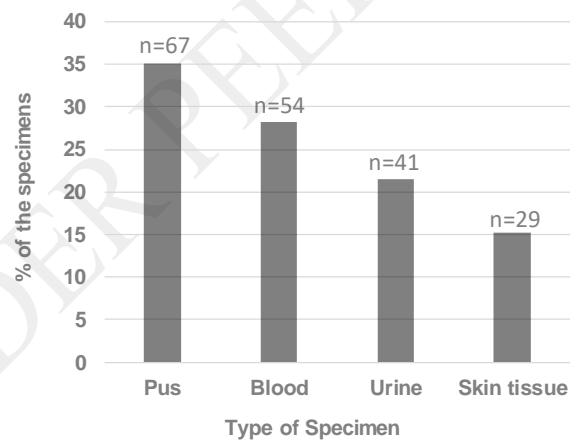
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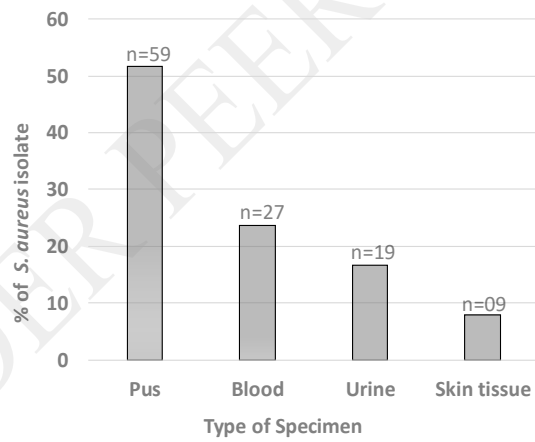
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### Legends of the figures

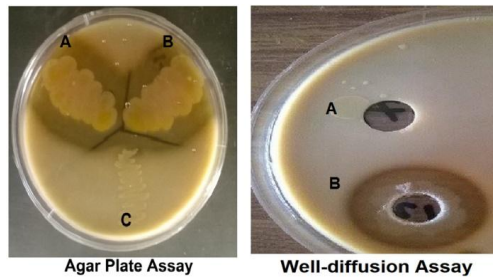
**Figure 1** Graph shows distribution of clinical samples according to the type of specimens



**Figure 2** Graph shows percentage distribution of *S. aureus* isolates recovered from different specimens



**Figure 3** Representative result of Extracellular protease production from clinical isolate of *S. aureus*



**Table 1** Frequency of *S. aureus* isolated producing extracellular proteases

Type of specimens	Number of <i>S. aureus</i> isolates tested	Number of isolates positive for the production of extracellular proteases	
		(n=)	%
<b>Pus</b>	59	54	69.2
<b>Blood</b>	27	13	16.7
<b>Urine</b>	19	06	7.7
<b>Skin tissue</b>	09	05	6.4