

## **Original Research Article**

### **EXTRACELLULAR PROTEASES PRODUCTION IN METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS **CLINICAL** 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 using **cefoxitin antibiotic discs**. 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=191)** 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.

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

## 1. Introduction:

*Staphylococcus aureus* is a Gram-positive bacterium normally found as commensal and associated 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 produced by *S. aureus* bacterial strains can degrade protease-inhibitors found in humans and cause deregulation of human proteins which consequently lead to connective tissue degradation. However, all strains of *S. aureus* do not produce same extracellular protease. Although the production of extracellular proteases had been reported decades ago, its

involvement in pathogenicity as a major virulence factor of *S. aureus* has recently been elucidated [9, 10]. The function of protease in the development of an infection can be species specific and is crucial to target protease production using specific drugs [11].

The development of antibiotic resistance in *S. aureus* bacterial strains isolated from clinical specimens has made this bacterium medically challenging. More specifically, methicillin resistant *Staphylococcus aureus* (MRSA) is one of the current public health problems worldwide [12, 13, 14]. MRSA bacterial strains cause variety of infections such as osteomyelitis, pneumonia, endocarditis, septicaemia and skin and soft tissue lesions [15, 16]. MRSA has been regarded as a major health threat worldwide with increasing rates of morbidity and mortality [15].

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 [17].

The present study was carried out to screen pathogenic MRSA clinical isolates 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.

## 2. Materials and Methods

### Collection and processing of clinical samples:

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. The patients with current antibiotic therapy were excluded. 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 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 (2-8 °C) in case of any delay in processing. All the inoculated media with specific clinical samples were incubated at 37 °C for 24-48 hrs.

### **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 [18].

Antibiotic resistant strains particularly methicillin resistant *Staphylococcus aureus* (MRSA) strains were identified by Kirby-Bauer disc diffusion method using cefoxitin antibiotic discs [19, 20, 21].

### Screening for Extracellular Protease

All the identified clinical isolates of MRSA strains were screened for the production of extracellular protease enzyme. Casein agar medium was 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 [22]. Screening for the production of protease enzyme from *S. aureus* was performed by two different 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 at 37°C for 2-3 days 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 µl 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.

### 3. Results

A total of 191 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. Over all, 114 bacterial strains were identified as MRSA

bacterial strains based on morphological and biochemical characteristics and growth on MSA agar. The bacterial strains were found highly resistant to cefoxitin thus were considered as MRSA isolates. All the pure cultures of the identified MRSA clinical strains were included in the present study for further investigations. The data revealed that the highest ratio of MRSA clinical isolates belonged to pus specimens followed by blood, urine and skin tissue specimens, respectively (Figure 2).

It was observed that 68.4% (n=78) of the MRSA 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 agar plate assay. Moreover, a comparative analysis of the protease production by MRSA clinical isolates of the different type of specimens was done. The results of this study showed the highest production of the extracellular protease (69.2%) among the MRSA strains isolated from pus samples. However, the frequency of the 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.

#### 4. Discussions

Methicillin resistance in *S. aureus* is now global challenge. The data revealed that large numbers of *S. aureus* 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.

Although, a previous study has reported that no correlation between identified gene patterns of clinical isolates of *S. aureus* with specific infections was established [23], however the present study suggests there may be a possible link between the type of infection and frequency of extracellular proteases production. Further, 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].

### 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 possible role of extracellular proteases in pathogenicity of *S. aureus* bacterial species. Further in detailed molecular research could be of value to confirm role of extracellular proteases in development of infections in MRSA pathogenic bacteria.

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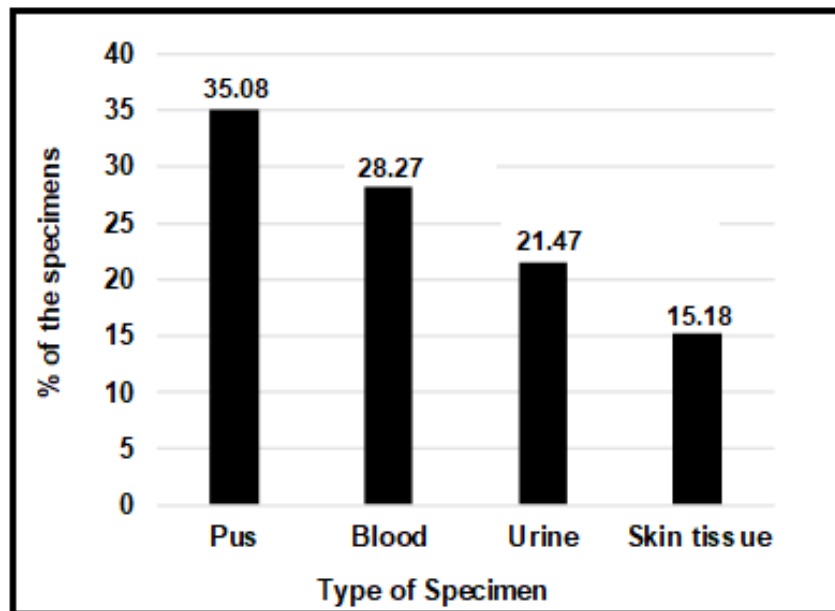
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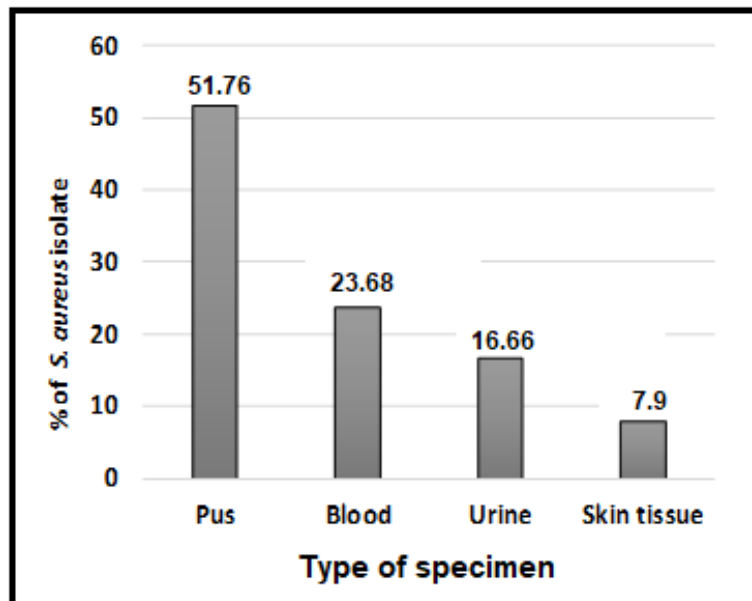
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### Legends of the figures

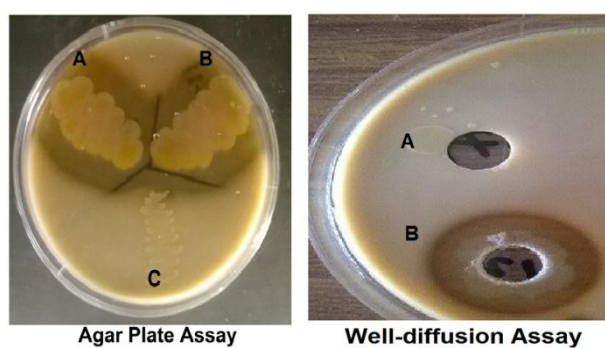
Figure 1 Graph shows distribution of clinical samples according to the type of specimens



**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 MRSA clinical isolates producing extracellular proteases**

Type of specimens	Number of 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