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Extracellular Proteases Production in Methicillin Resistant *Staphylococcus aureus* Clinical Isolates

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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

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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 asymptomatically [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

2.1 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 ^{0}C) in case of any delay in processing. All the inoculated media with specific clinical samples were incubated at 37 ^{0}C for 24-48 hrs.

2.2 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].

2.3 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^{0}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 37ºC 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 Fig. 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 [Fig. 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 [Fig. 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.

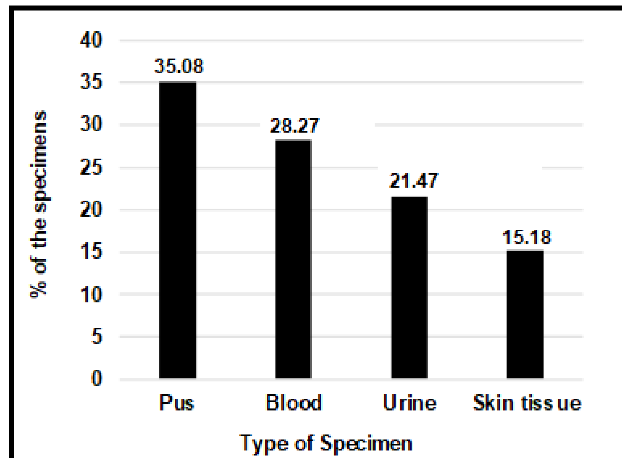

Fig. 1. Graph shows distribution of clinical samples according to the type of specimens

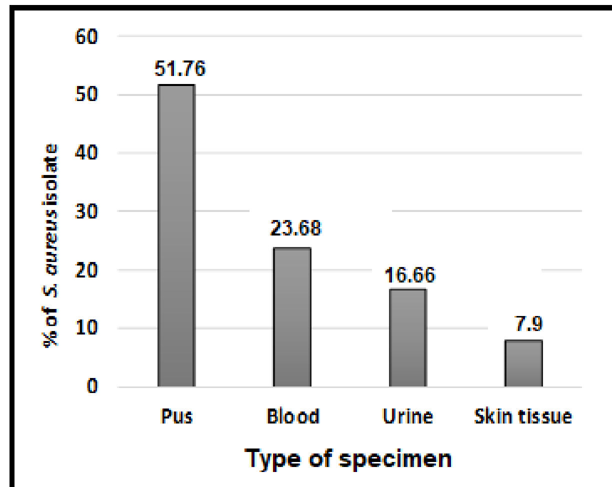

Fig. 2. Graph shows percentage distribution of *S. aureus* isolates recovered from different specimens

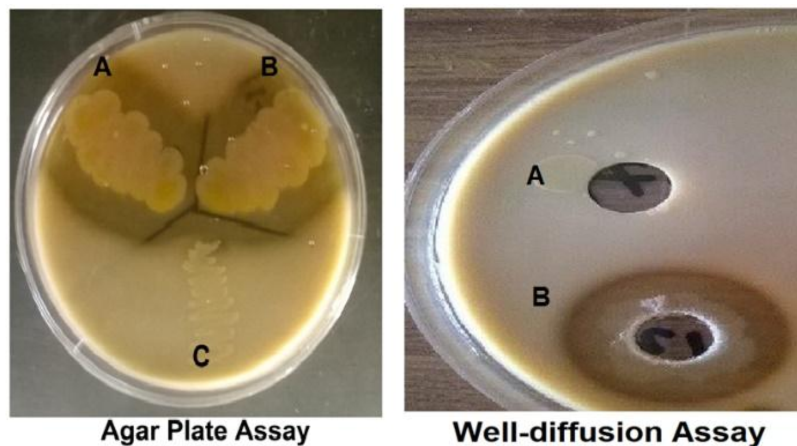

Fig. 3. Representative result of Extracellular protease production from clinical isolate of *S. aureus*

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=)	%
Pus	59	54	69.2
Blood	27	13	16.7
Urine	19	06	7.7
Skin tissue	09	05	6.4

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].

5. 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.

CONSENT AND ETHICAL APPROVAL

As per international standard or university standard guideline Patient's consent and ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kazar AJ, Abbas MK. A molecular study on Healthcare-associated *Staphylococcus aureus* MRSA (HA-MRSA) strains isolated from hospitalized patients with skin lesions in Iraq: 16S RNA sequencing and PCR detection of mecA and tst-1. Annals of the Romanian Society for Cell Biology. 2021;25(6):1329-1347.
2. Breuer K, Häussler S, Kapp A, Werfel T. *Staphylococcus aureus*: Colonizing features and influence of an antibacterial treatment in adults with atopic dermatitis. British Journal of Dermatology, 2002;(147):55-61. DOI:https://doi.org/10.1046/j.1365-2133.2002.04872.x
3. Li G, Wu S, Luo W, Su Y, Luan Y, Wang X. *Staphylococcus aureus* ST6-t701 isolates from food-poisoning outbreaks (2006–2013) in Xi'an, China. Foodborne pathogens and disease. 2015;12:203-206. DOI:https://doi.org/10.1089/fpd.2014.1850
4. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clinical microbiology reviews. 2015;28:603-661. DOI:10.1128/CMR.00134-14

5. Böttcher T, SA Sieber. β-lactones as specific inhibitors of ClpP attenuate the production of extracellular virulence factors of *Staphylococcus aureus*. Journal of the American Chemical Society. 2008;130:14400-14401.
DOI:https://doi.org/10.1021/ja8051365
6. Karlsson A, Arvidson S. Variation in extracellular protease production among clinical isolates of *Staphylococcus aureus* due to different levels of expression of the protease repressor sarA. Infection and immunity. 2002;70:4239-4246.
DOI:10.1128/IAI.70.8.4239-4246.2002
7. Ghssein G, Brutesco C, Ouerdane L, Fojcik C, Izaute A, Wang S, Richaud P. Biosynthesis of a broad-spectrum nicotianamine-like metallophore in *Staphylococcus aureus*. Science. 2016;352:1105-1109.
DOI:DOI: 10.1126/science.aaf1018
8. Shaw L, Golonka E, Potempa J, Foster SJ. The role and regulation of the extracellular proteases of *Staphylococcus aureus*. Microbiology. 2004;150:217-228.
DOI:https://doi.org/10.1099/mic.0.26634-0
9. Gimza BD, Jackson JK, Frey AM, Budny BG, Chaput D, Rizzo DN, Shaw LN. Unraveling the impact of secreted proteases on hypervirulence in *staphylococcus aureus*. Mbio. 2021;12(1):e03288-20.
10. Lindsay S, Oates A, Bourdillon K. The detrimental impact of extracellular bacterial proteases on wound healing. International wound journal. 2017;14:1237-1247.
DOI: https://doi.org/10.1111/iwj.12790
11. Overall CM, Blobel CP. In search of partners: Linking extracellular proteases to substrates. Nature reviews Molecular cell biology. 2007;8:245-257.
DOI:https://doi.org/10.1038/nrm2120
12. Boswihi SS, Udo EE. Methicillin-resistant *staphylococcus aureus*: An update on the epidemiology, treatment options and infection control. Current Medicine Research and Practice. 2018;8:18-24.
DOI:https://doi.org/10.1016/j.cmrp.2018.01.001
13. Goudarzi M, Goudarzi H, Sá AM Figueiredo, Udo EE, Fazeli M, Asadzadeh M, Seyedjavadi SS. Molecular characterization of methicillin resistant *Staphylococcus aureus* strains isolated from intensive care units in Iran: ST22-SCC mec IV/t790 emerges as the major clone. PloS one. 2016;11:e0155529.
DOI:https://doi.org/10.1371/journal.pone.0155529
14. Udo EE, Boswihi SS, Mathew B, Noronha B, Verghese T, Al-Jemaz A, Al Saqer F. Emergence of methicillin-resistant *staphylococcus aureus* belonging to clonal complex 15 (CC15-MRSA) in Kuwait Hospitals. Infection and Drug Resistance. 2020;13:617.
DOI: 10.2147/IDR.S237319
15. Haddad O, Merghni A, Elargoubi A, Rhim H, Kadri Y, Mastouri M. Comparative study of virulence factors among methicillin resistant *Staphylococcus aureus* clinical isolates. BMC Infectious diseases. 2018;18(1):1-8.
16. Naicker PR, Karayem K, Hoek KG, Harvey J, Wasserman E. Biofilm formation in invasive *Staphylococcus aureus* isolates is associated with the clonal lineage. Microbial pathogenesis. 2016;90:41-49.
17. Giersing BK, Dastgheyb SS, Modjarrad K, Moorthy V. Status of vaccine research and development of vaccines for *Staphylococcus aureus*. Vaccine. 2016;34:2962-2966.
DOI:https://doi.org/10.1016/j.vaccine.2016.03.110
18. Yunita MN, Effendi MH, Rahmaniar RP, Arifah S, Yanestria SM. Identification of spa gene for strain typing of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from nasal swab of dogs. Biochem. Cell. Arch. 2020;20(1):2999-3004.
19. Yin D, Guo Y, Li M, Wu W, Tang J, Liu Y, Hu F. Performance of VITEK 2, E-test, Kirby–Bauer disk diffusion, and modified Kirby–Bauer disk diffusion compared to reference broth microdilution for testing tigecycline susceptibility of carbapenem-resistant K. pneumoniae and A. baumannii in a multicenter study in China. European Journal of Clinical Microbiology & Infectious Diseases. 2021;40(6):1149-1154.
20. Yao H, Liu J, Jiang X, Chen F, Lu X, Zhang J. Analysis of the clinical effect of combined drug susceptibility to guide medication for carbapenem-resistant klebsiella pneumoniae patients based on the kirby–bauer disk diffusion method. Infection and Drug Resistance. 2021;14:79.

21. Pugazhendhi A, Michael D, Prakash D, Krishnamaurthy PP, Shanmuganathan R, Al-Dhabi NA, Kaliannan T. Antibiogram and plasmid profiling of beta-lactamase producing multi drug resistant *staphylococcus aureus* isolated from poultry litter. Journal of King Saud University-Science. 2020;32(6):2723-2727.
22. Olajuyigbe FM, Falade AM. Purification and partial characterization of serine alkaline metalloprotease from Bacillus brevis MWB-01. Bioresources and Bioprocessing. 2014;1:8. DOI:https://doi.org/10.1186/s40643-014-0008-6
23. Zdzalik M, Karim AY, Wolski K, Buda P, Wojcik K, Brueggemann S, Jonsson IM. Prevalence of genes encoding extracellular proteases in *Staphylococcus aureus*—important targets triggering immune response in vivo. FEMS Immunology and Medical Microbiology. 2012;66:220-229. DOI:https://doi.org/10.1111/j.1574-695X.2012.01005.x

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