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Protease, Lipase, Ürease Activity in Biofilm Forming Strains of *Staphylococcus aureus*

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Abstract

The pathogenity of *S. aureus* strains are related with features like its adherence, various toxins, enzymes, structural and extracellular factors. In our study, the relationship between biofilm formation and lipase, protease, urease activity were investigated in *S. aureus* strains isolated from various clinical specimens sent to our microbiology laboratory. Congo red agar was used to detect biofilm production. The lipolytic activity of all strains was evaluated on Tween 20 agar. The proteolytic activity of the strains was evaluated by Skim Milk Agar. Christensen Urea agar was used to determine the urease activities of all strains. Slime factor and biofilm formation are pathogenity factors as well. 101 (57.7%) of 175 clinical isolates were negative for biofilm formation while 74 (42.3%) samples were positive according to phenotypic assessment of colony morphology on CRA. The relationship between biofilm formation and lipase, protease and urease activity of all the isolates are researched by using Spearman's correlation coefficient. There was an evident relation between biofilm formation with lipase activity (r=0.195, p=0.10) while protease (r=0.001, p=0.99) and urease (r=0.06, p=0.4) activity were not found related.

Keywords: *S. aureus*; Biofilm; Lipase; Protease; Ürease

Introduction

Staphylococcus aureus is a virulent pathogen that is currently the most common cause of infections in hospitalized patients [1]. The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors [2]. The structural characteristic of biofilms that has the greatest impact on the outcome of chronic bacterial infections, such as native valve endocarditis, is the tendency of individual microcolonies to break off and/or detach when their tensile strength is exceeded [3]. Urease is needed in the urea cycle and in the metabolism of amino acids to degrade urea to form CO_2 and NH_3 [4]. The resulting ammonium and/or ammonia (depending of the pH of the cells) is toxic for the host cells and might accumulate in and outside the bacterial cells [4,5]. Bacterial proteases secreted into an infected host may exhibit a wide range of pathogenic potentials. Staphylococci, in particular *Staphylococcus aureus*, are known to produce several extracellular proteases, including serine-, cysteine- and metalloenzymes [6,7]. Their insensitivity to most human plasma protease inhibitors and, even more, the ability to inactivate some of these make the proteases potentially harmful [6]. In our study, the presence of lipase, protease and urease enzymes in *S. aureus* strains isolated from various clinical specimens sent to our microbiology laboratory were investigated.

Materials and Methods

175 *S. aureus* strains isolated from various clinical specimens in patient with different diagnoses sent to the Microbiology Laboratory were included in the study at Hospital of Pamukkale University, Denizli in Turkey. This study is a laaboratory work, so that patients didn't follow up. In our study, the relationship between biofilm formation and lipase, protease , urease activity were investigated in *S. aureus* strains. As control strains, *S. epidermidis* ATCC 12228, which does not produce biofilm, *S. epidermidis* ATCC 35984, which produces strong biofilm, was used (Dieter Vancraeynest, Gent University, Belgium). Identification of strains was done by conventional methods. Samples were incubated at 37 °C for 24 hours with bloody agar. Considering colony morphology and gram staining, those with positive catalase and coagulase test were identified as *S. aureus* [8,9].

Investigation of Biofilm Formation

Congo red agar was used to detect biofilm production [10]. Congo reddish agar medium was prepared to contain 10 g of agar, 50 g of sucrose, 37 g of brain-heart infusion vial and 0.8 g of Congo red. Cultures made in such a way that a single colony fell on these mediums were incubated overnight at 37 °C, followed by incubation of the cultures for 48 hours at room temperature. *S. epidermidis* ATCC 12228, which does not produce biofilms, and *S. epidermidis* ATCC 35984, which produces strong biofilms, were used as controls.

Investigation of Lipolitic Activities

The lipolytic activity of the isolates was determined according to Sierra (1957) [11]. The experiments were carried out in The lipolytic activity of all strains was evaluated on Tween 20 agar (containing 10g peptone, 5g NaCl, 0.1g $CaCl_2$, 20g agar and 1ml Tween 20) per liter. Produced by incubation at 37 °C overnight in Brain heart medium (pH 7.5) containing 10g of peptone, 5g of yeast extract, 5g of NaCl, 1g of $K_2HPO_4.3H_2O$. Subsequently, strains diluted 1: 100 in Brain hart medium were inoculated into 20 µl of the wells opened with sterile glass pipette onto Tween 20 agar. The plates were evaluated after 72 hours incubation at 37 °C. The presence of lipolytic activity was detected around the inoculation by the appearance of halo formation, depending on whether the tween was a line-shaped precipitate [11].

Investigation of Proteolytic Activities

The proteolytic activity of the strains was evaluated by the agar plate method. For this, Skim Milk Agar (SMA) containing 1% skim milk, 1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar was used. 20 .mu.l of supernatant from 1:100 diluted samples were added to sterile diluted wells in SMA. Plates were assessed after being incubated for 48 hours at 37 °C. The presence of proteolytic activity was determined by the occurrence of opaque zones (halo formation) around the wells due to casein hydrolysis [6,7].

Urease Activity Investigation

Christensen Urea agar was used to determine the urease activities of all strains [12]. The 20 µl supernatant of the 1:100 diluted samples were taken grown on urea agar after being produced on Brain heart medium. The tubes were evaluated after 24 h incubation at 37 °C. Urea hydrolysis in this medium was shown by a change in color from the pale yellow of the fresh medium to an intense red-violet color.

SPSS Ver 10.0 was used for statistical analysis. Spearman's correlation coefficient was used to evaluate the relationship between biofilm production and enzyme activities of the working clinical isolates. The statistical error margin was accepted as 5%.

Results

Of the 175 *S. aureus* strains used in the study, 87 wound (49.7%), 25 blood culture (14.3%), 35 tracheal aspirate (20.0%), 10 sputum (5.7%), 8 catheter 4.6%) and 10 (5.7%) were isolated from various clinical specimens (eye, urine, nasal swab, etc.). Numbers and percentages of biofilm positive and negative specimens were shown on Congo red agar medium (Table 1).

	BİOFİLM	
Number of isolates n	Negative n (%)	Positive n (%)
Wound 87	34 (39.1)	53 (60.9)
Blood 25	17 (68.0)	8 (2.0)
Tracheal aspirate 35	31 (88.6)	4 (11.4)
Sputum 10	7 (70.0)	3 (30.0)
Catheter 8	7 (87.5)	1 (12.5)
Other 10	5 (50.0)	5 (50.0)
Total 175	101 (57.7)	74 (42.3)

Table 1: Number and percentage of biofilm positive and negative samples on Congo red agar medium according to the isolate as example

		BİOFİLM		
		Positive n(%)	**Negative n(%)**	**Total n(%)**
Lipase	Positive	68 (91.9)	78 (77.2)	146 (83.4)
	Negative	6 (8.1)	23 (22.8)	29 (16.6)
Protease	Positive	44 (59.5)	60 (59.4)	104 (59.4)
	Negative	30 (40.5)	41 (40.6)	71 (40.6)
Ürease	Positive	63 (85.1)	81 (80.2)	144 (82.3)
	Negative	11(14.9)	20 (19.8)	31 (17.7)

Table 2: Relationship between biofilm formation with lipase, protease and urease activity

The relationship between biofilm production and lipase, protease and urease activities of all isolates was investigated. Of the clinical isolates, 146 (83.4%) showed lipase activity whereas 29 (16.6%) did not have lipase activity. Lipase (+) was detected in 68 (91.9%) and lipase (-) was detected in 6 (8.1%) in 74 samples of biofilm positive in Congo red agar medium. In biofilm negative 101 samples, 78 (77.2%) were lipase (+) while 23 (22.8%) were lipase (-) (Table 2).

There was no relationship between biofilm production and urease activity of the isolates (r=0.06, p=0.4).

There was no correlation between biofilm production and proteolytic activity of the isolates (r=0.001, p=0.99).

There was a good correlation between lipase activity and biofilm production of the isolates (r=0.195, p=0.10).

144 (82.3%) of the clinical isolates showed urease activity, while 31 (17.7%) had no urease activity. Urease (+) was found in 63 (85.1%) and urease (-) was detected in 11 (14.9%) in 74 samples of biofilm positive in Congo red agar medium. Urease (+) was found in 81 (80.2%) and urease (-) was detected in 20 (19.8%) of the biofilm negative 101 samples (Table 2).

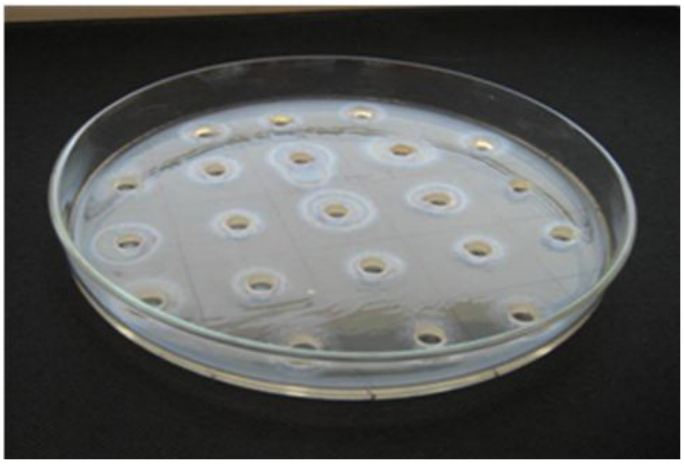

Figure 1: Lipolytic activity of *S. aureus* strains on Tween 20 agar

Of the 175 clinical isolates, 104 (59.4%) showed proteolytic activity whereas 71 (40.6%) showed no proteolytic activity. Protease (+) was detected in 44 (59.5%) and protease (-) was detected in 30 (40.5%) of the 74 samples in which biofilm was positive in Congo red agar medium. Protein (+) was found in 60 (59.4%) and protease (-) was detected in 41 (40.6%) of the biofilm negative 101 samples (Table 2).

Discussion

The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors. The structural characteristic of biofilms that has the greatest impact on the outcome of chronic bacterial infections, such as native valve endocarditis, is the tendency of individual microcolonies to break off and/or detach when their tensile strength is exceeded. Biofilm formation occurs through a series of steps which begins with initial attachment of planktonic bacteria to a solid surface that is present at the air-water/liquid interface. This step is followed by subsequent proliferation and accumulation of the cells in small multilayer cell clusters known as microcolonies. The microcolonies then further proliferate to form giant assemblages of cells enmeshed in an extracellular matrix, which covers entire surfaces, and protects its inhabitants from detrimental effects of all sorts [4-6]. A mature well established biofilm is not a static structure, rather it is highly dynamic in nature, where old cells are constantly being dispersed and new members being recruited for this surface-associated community to expand, at all times. The composition of the extracellular matrix is very difficult to ascertain and variable among different bacterial species and even within the same species under different environmental conditions [3]. Despite this fact exopolysaccharides are an essential component of virtually all biofilm structures, providing the necessary matrix in which the bacterial cells are initially embedded [7]. Bacterial cells have to protect themselves from a pH that is too low[13]. Bacterial cells have assumed that the urease activity determined contributes to the persistence of the bacterial cells in the biofilm by counteracting the low pH values caused by the production of lactic acid, acetic acid, and formic acid. Beenken et al. have also reported up-regulation of the urease operon in 7-day-old biofilms [5]. According to Resch et al. urease activity might be an important factor for keeping the biofilm alive [4]. Since excess ammonia would be toxic for the bacterial cells, they should have some mechanism of resistance against this chemical and should also have enzymes or other mechanisms to detoxify this compound. Bacterial proteases secreted into an infected host may exhibit a wide range of pathogenic potentials. Staphylococci, in particular *Staphylococcus aureus*, are known to produce several extracellular proteases, including serine-, cysteine- and metalloenzymes. Their insensitivity to most human plasma protease inhibitors and, even more, the ability to inactivate some of these make the proteases potentially harmful [6]. Bacterial protease is reported as a pathogenic factor. *S. aureus* has two enzymes, metallo-protease and serine protease. The distribution of both enzymes varies greatly between strains. Researchers report that the protease activity is a pathogenic factor. Urease activity is said to be important

in the maintenance of biofilm formation. Excess ammonium can be toxic in bacteria. This chemical can be protected by various enzymes and combined detoxification mechanisms. The bacterial cells in the biofilm protect themselves by up-regulation of the urease genes from the pH decreasing by the production of lactic acid, formic acid, which is the result of metabolism [12,13]. Bacteria living in biofilm are observed to have different patterns of gene expression when compared to planktonic bacteria [4]. This difference in gene expression results in the appearance of bacteria with phenotypically different properties from their planktonic analogues. The result of biofilms consisting of more than one cell type increases the chances of surviving bacteria to survive [2,5]. While the outer layers of the biofilm absorb the damage, time is taken to start the stress response in the inner layers. Species that can adapt more quickly to microvester changes due to food insufficiency can survive the attack and start to reproduce rapidly [3]. The most likely explanation is that the phenotypic changes of the biofilm bacteria have provided some protective properties for them [4]. Jongkon S and *et al.* 149 staphylococcal isolates from acne lesions were investigated for their virulence factors including lipase, protease, and biofilm formation [14]. Coagulase-negative staphylococci were demonstrated to present lipase and protease activities more often than coagulase-positive staphylococci.

In our study we found no correlation between biofilm formation and urease and protease activities, bacterial pathogenicity factors, and found a correlation between lipase and biofilm production. Although there are no previous studies in this area, these results suggest that the formation of lipase and biofilm, which play a role in settlement, may function together in pathogenic strains.

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