Brief Report

DOI: 10.5582/bst.2012.v6.4.160

Lipase, protease, and biofilm as the major virulence factors in staphylococci isolated from acne lesions

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Summary **Staphylococci involve infections in association with a number of bacterial virulence factors. Extracellular enzymes play an important role in staphylococcal pathogenesis. In addition, biofilm is known to be associated with their virulence. In this study, 149 staphylococcal isolates from acne lesions were investigated for their virulence factors including lipase, protease, and biofilm formation. Coagulase-negative staphylococci were demonstrated to present lipase and protease activities more often than coagulase-positive staphylococci. A microtiter plate method (quantitative method) and a Congo red agar method (qualitative method) were comparatively employed to assess biofilm formation. In addition, biofilm forming ability was commonly detected in a coagulase-negative group (97.7%, microtiter plate method and 84.7%, Congo red agar method) more frequently than in coagulase-positive organisms (68.8%, microtiter plate method and 62.5%, Congo red agar method). This study clearly confirms an important role for biofilm in coagulase-negative staphylococci which is of serious concern as a considerable infectious agent in patients with acnes and implanted medical devices. The Congo red agar method proved to be an easy method to quickly detect biofilm producers. Sensitivity of the Congo red agar method was 85.54% and 68.18% and accuracy was 84.7% and 62.5% in coagulase-negative and coagulase-positive staphylococci, respectively, while specificity was 50% in both groups. The results clearly demonstrated that a higher percentage of coagulase-negative staphylococci isolated from acne lesions exhibited lipase and protease activities, as well as biofilm formation, than coagulase-positive staphylococci.**

Keywords: Biofilm, lipase, protease, staphylococci, acne

1. Introduction

Staphylococcus has been considered to be a major public health issue because it can cause both health-associated and community-associated infections, with considerable morbidity and even mortality. The infections are either minor such as infections of skin and soft tissues or more serious systemic infections including endocarditis, osteomyelitis, and septic shock syndrome (*1*). Staphylococcal infections are caused mainly by strains that have already colonized parts of the human body, making the colonized persons a reservoir for the spread of the organisms (*2*).

The organisms are one of the microorganisms commonly isolated from acne lesions (*3*). The skin environment contains lipids, polysaccharides, proteins, and a variety of mixed polymers. The skin microbiota produces a range of extracellular enzymes that degrade these polymers. Proteolytic, lipolytic, and esterolytic activities are frequently detected among various exo-enzymatic activities of staphylococci (*4,5*). The presence of oleic acid released from the hydrolysis of serum lipids by staphylococcal lipase could be pathologically important (*6*). On the other hand, protease is thought to be involved in host tissue invasion (*7*). This enzyme can interact with the host defense mechanisms and tissue components (*8*).

Additionally, the ability to form biofilm is one

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of the known staphylococcal virulence factors. The formation of biofilm involves two stages: (*i*) initial adhesion of bacterial cells to the host cell surface and (*ii*) the formation of a multilayered cell cluster surrounded by an extracellular polysaccharide matrix (*9*). The production of the exopolysaccharide matrix has been suggested to prevent the access of antibiotics to the bacterial cells embedded in the community (*10*). Treatment of staphylococcal infections is becoming difficult due to increased antibiotic resistance. Many reports indicated that antibiotic resistance in biofilm forming cells increased when compared to that in the planktonic growth phase (*11-13*). Consequently, there is a need to identify virulent strains by detecting their ability to produce biofilm. Detection of staphylococcal virulence factors may be a necessary step for prevention, control, and treatment of the infections. This may lead to more effective infection control practices, decreasing colonization, and development of vaccines and new or improved antimicrobial agents (*7*). Data on clinical isolates from various infection sites such as atopic dermatitis, conjunctiva, blood and infected devices have documented staphylococcal virulence factors including lipase, protease, and biofilm (*14-16*). In contrast, there has been limited information on virulence factors of staphylococci isolated from acne lesions.

In this study, a large collection of staphylococcal isolates from acne lesions was investigated for their enzyme activities including lipase and protease. In addition, biofilm formation, another important virulence factor, was assessed using a qualitative Congo red agar method, compared to a quantitative microtiter plate method used as the standard method.

2. Materials and Methods

2.1. *Bacterial isolates*

A total of 149 staphylococcal isolates from acne lesions were characterized according to Bergey's Manual of Systematic Bacteriology (*17*) including their characteristic macroscopic colony appearance, Gram-staining, production of acid from mannitol salt agar (Merck, Darmstadt, Germany), catalase activity and coagulase tests. They were further classified as 85 isolates of coagulase-negative and 64 isolates of coagulase-positive staphylococci. All cultures were maintained on tryticase soy broth (TSB, Difco, Becton Dickinson, Sparks, MD, USA) containing 20% glycerol (Vidhyasom, Bangkok, Thailand) at –80°C. *Staphylococcus aureus (S. aureus)* ATCC 25923 was included as a reference strain.

2.2. *Screening for lipase activity*

Nutrient Agar (NA, Difco) containing 1% tributyrin (Fluka, Buchs, Germany) was used to study lipolytic activity. The isolates were subcultured on tryticase soy agar (TSA, Difco) and incubated at 37°C for 24 h. They were inoculated on tributyrin agar plates and incubated at 37°C for 72 h. The presence of clear zones was taken as an indication of positive lipase activity.

2.3. *Screening for protease activity*

Nutrient agar supplemented with 2% casein (Sigma, Steinhelm, Germany) was used to screen for protease activity. The isolates were subcultured on TSA and incubated at 37°C for 24 h. They were inoculated on casein agar plates and incubated at 37°C for 24 h. The isolates producing opalescent zones around the colony were identified as protease positive.

2.4. *Detection of biofilm formation by the microtiter plate method*

Quantitative assessment of biofilm formation using the microtiter plate method was carried out according to the protocol (*18*). Briefly, an overnight culture grown in TSB supplemented with 0.25% glucose (Univar, Auckland, New Zealand) was adjusted to a McFarland standard No. 0.5 and diluted 100-fold. One hundred µL of bacterial suspension was transferred to a 96-well microtiter plate (Corning, New York, USA), 100 µL of TSB supplemented with 0.25% glucose was added, and incubated at 37°C for 24 h. After incubation, the broth was discarded from each well and washed twice with phosphate buffered saline to remove non-adherent cells. After airdrying, the wells were stained with 0.1% crystal violet (Merck) for 30 min, washed with water, and air dried before 200 µL of dimethyl sulfoxide (Merck) was added. The absorbance was measured at 595 nm with a microtiter plate reader (Bio Tek, Winooski, USA). All strains were classified into the following categories: non-adherent, weakly, moderately, and strongly adherent based on the optical density of bacterial films (*19*).

2.5. *Detection of biofilm formation by the Congo red agar method*

Qualitative evaluation of biofilm producers using the Congo red agar method to detect slime production was performed as previously described (*20*). The medium is comprised of brain heart infusion broth (Difco) 37 g/L, sucrose (Univar) 50 g/L, Congo red (Fluka) 0.8 g/L and agar (Merck) 10 g/L. Inoculated plates were incubated at 37°C for 24 h. Slime producing strains presented black colonies while non producing strains developed red colonies. The scale of colony color evaluation was assessed as follows: very black and black colonies were biofilm producing strains, almost black colonies were weak biofilm producers while very red to Bordeaux colonies were considered as non-biofilm producing

strains (*21,22*).

2.6. *Statistical analysis*

Parameters including sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the Congo red agar method were determined by the analysis of a 2 × 2 table. The microtiter plate method was used as the standard method.

3. Results and Discussion

Lipase and protease enzymes have been demonstrated in staphylococci associated with infection. About 17% of coagulase-negative staphylococci isolated from infectious processes of newborns revealed lipase activity (*22*). Other workers reported 97% of *S. aureus* from the colonized skin of patients with acute-phase atopic dermatitis exhibited protease activity (*14*). In this study, 100% of coagulase-negative (85 isolates) produced lipase enzymes. Of 64 coagulase-positive staphylococci, only 42 isolates (65.6%) were lipase positive. Fifty six (65.9%) coagulase-negative and 17 (26.6%) coagulase-positive staphylococci demonstrated protease activity. *S. aureus* ATCC 25923, a reference strain produced both lipase and protease enzymes. In the present study, coagulase-negative staphylococcal isolates produced both lipase and protease enzymes more frequently than the coagulase-positive group. The percentages of organisms with enzyme activities in our study focusing on staphylococci isolated from acne lesions are different from other reports. This may be due to the ability of staphylococci to produce enzymes depending on the environment and nutrition availability in different infectious sites. Furthermore, this report clearly indicated that staphylococci isolated from acne lesions are more common among lipase producers than protease producers. Lipolytic enzymes which are produced by *S. epidermidis* and possibly *S. aureus* could demonstrate that lipase enzymes are important to the organisms for their colonization and growth within the lipid-rich environment of the skin (*24*). The site of growth of staphylococci within the sebaceous unit is superficial (*25*). *S. epidermidis* which is coagulase-negative is prevalent in areas with many sebaceous follicles. The organism produces lipase whose activity may be involved in its virulence (*26*).

Production of lipase, a putative virulence determinant, was reported to be up-regulated in the biofilm mode of bacterial growth (*27*). Biofilm formation could be a characteristic of invasive staphylococcal isolates. In the analysis of 85 coagulase-negative staphylococci by the microtiter plate method (Figure 1A), 97.7% were biofilm formers and only 2.3% were non-biofilm producers. On the other hand, of 64 coagulase-positive staphylococci isolates, 68.8% exhibited biofilm formation while 31.2% could not produce biofilm. In the Congo red agar method, slime production was investigated. Similarly, 84.7% of coagulase-negative staphylococci were biofilm producing strains while 62.5% of coagulase-positive staphylococci were biofilm producers (Figure 1B). *S. aureus* ATCC 25923 was considered as a moderate biofilm former and biofilm producer by the microtiter plate method and Congo red agar method, respectively. The results demonstrated that both coagulase-negative and coagulase-positive staphylococci exhibited biofilm formation, but with greater frequency in the coagulase-negative group. Despite their low virulence, coagulase-negative staphylococci, particularly *S. epidermidis*, are well-adapted to adhere to smooth metal and plastic surfaces of foreign bodies such as vascular catheters, cardiac devices, and ventricular catheters (*28*). The results from other workers showed a high percentage of up to 90% of *S. epidermidis* isolated from infections associated with indwelling medical devices were biofilm producers. In contrast, a relatively lower percentage of biofilm producers were present in the skin, 35.7% and 64.3% were non-biofilm formers and weak biofilm producers, respectively (*29*). Similarly, only 32% from skin and nasal swabs showed biofilm production while 70% of invasive staphylococcal strains isolated from blood of patients hospitalized with peripheral intravenous devices exhibited biofilm forming ability (*22*). In contrast, this study demonstrated that up to 85% of staphylococci isolated from acne lesions were biofilm producers.

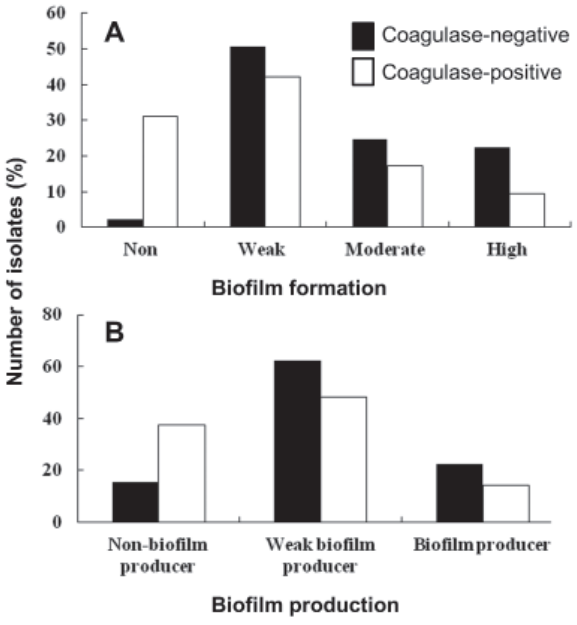

Figure 1. Quantitative screening of biofilm formation by microtiter plate method (A) and qualitative screening of slime production by Congo red agar method (B) from coagulase-negative staphylococci (■) and coagulase-positive staphylococci (□).

Table 1. Comparison of biofilm production of coagulase-negative staphylococci (*n* = 85) and coagulase-positive staphylococci (*n* = 64) by the microtiter plate method and Congo red agar method

Coagulase-negative staphylococci (*n* = 85)				Coagulase-positive staphylococci (*n* = 64)			
	Microtiter plate		Total		Microtiter plate		Total
	Positive	Negative			Positive	Negative	
Congo red agar				Congo red agar			
Positive	71	1	72	Positive	30	10	40
Negative	12	1	13	Negative	14	10	24
Total	83	2	85	Total	44	20	64

Table 2. Statistical evaluation of Congo red agar method for detection of biofilm formation in staphylococci using the microtiter plate method as the standard method

Staphylococci	Test characteristics (%)				
	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
Coagulase-negative (*n* = 85)	85.54	50.00	98.61	7.69	84.70
Coagulase-positive (*n* = 64)	68.18	50.00	75.00	41.66	62.50

The microtiter plate method was used as the standard method to evaluate the Congo red agar method. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated by analysis of a 2 × 2 table (Table 1). Sensitivities of the Congo red agar method were 85.54% and 68.18% for coagulase-negative and coagulase-positive staphylococci, respectively. Specificity of both staphylococci was 50% while accuracies were 84.70% and 62.50% for coagulase-negative and coagulase-positive staphylococci, respectively (Table 2). A positive predictive value was 98.61% for coagulase-negative staphylococci while the value for coagulase-positive staphylococci was 75%. On the other hand, negative predictive values were 7.69% and 41.66% for coagulase-negative and coagulase-positive staphylococci, respectively.

As sensitivity and accuracy in coagulase-negative staphylococci are much higher than those in the coagulase-positive group, it is suggested that the Congo red agar method is more suitable for the detection of coagulase-negative staphylococci which are capable of producing biofilm more readily than coagulase-positive organisms. This method is easy to perform and takes less time while the microtiter plate method is somewhat more sophisticated. However, the microtiter plate method is a quantitative tool for biofilm detection and can be used as a reliable technique since it can detect the process of biofilm formation, attachment to the surface or secreted extracellular polysaccharide production, while the Congo red agar method can only detect slime production which suggests biofilm formation. Additionally, the quantitative microtiter plate method is more efficient to categorize biofilm formation than the Congo red agar method which depends on chromatic evaluation. The variation from black to red colony color can sometimes result in a difficult decision for investigators.

In conclusion, our results indicate that coagulase-negative staphylococci isolated from acne lesions were demonstrated to present lipase and protease activities as well as biofilm formation more often than coagulase-positive staphylococci.

Acknowledgements

This research was supported by grants under the program Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program Thai Doctoral degree from the Office of the Higher Education Commission, Thailand and the National Research University Project of Thailand's Office of the Higher Education Commission.

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(Received January 30, 2012; Revised August 18, 2012; Accepted August 19, 2012)