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The comparison of methods used for the detection of biofilm formation that cause antibiotic resistance of Staphylococcus epidermidis and Staphylococcus aureus

Staphylococcus epidermidis ve Staphylococcus aureus antibiyotik direncine sebep olan biyofilm oluşumunun belirlenmesi için kullanılan metotların kıyaslanması

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Abstract

Aim: This study was performed to compare microtiter plate method, tube method ve Congo red agar screening methods used for the detection of biofilm formation by Staphylococcus spp. such as Staphylococcus aureus and Staphylococcus epidermidisof which treatment can be impossible and hard, and infections such as host and indwelling device-associated infections can be recurrent.

Materials and Methods: In this study 121 isolates were used and microtiter plate method was used as gold standard method. Sensitivity, specificity, positive predictive value and negative predictive value parameters were calculated.

Results: The sensitivity, specificity of tube methodand Congo red agar methods were 97%, 100% and 87%, 94% respectively.

Conclusion: This study revealed that tube method is more reliable method than Congo red agarmethod.Tube method can be recognized as themain screening method for the identification of biofilm producer bacteria in the laboratories. By the usage of reliable biofilm detection method, wrong diagnoses and recurrent infections can be prevented.

Keywords: Biofilm formation, Staphylococcus epidermidis, Staphylococcus aureus, microtiter plate method, tube method, Congo red agar method

Öz

Amaç: Bu çalışma, tedavileri imkansız ve zor olan, konak ve yabancı cisim ilişkili infeksiyonlar gibi tekrarlayabilen infeksiyonları olan Staphylococcus aureus and Staphylococcus epidermidis gibi Stafilokoklar tarafından oluşturulan biyofilmin belirlenmesinde kullanılan mikrotitre plak metodu, tüp metotve Kongo kırmızısı agar metodutarama metotlarını kıyaslamak için gerçekleştirildi.

Gereç ve Yöntemler: Bu çalışmada, 121 izolat kullanıldı ve altın standart metot olarak mikrotitre plak metodukullanıldı. Sensitivite, spesifisite, pozitif tahmin değeri venegatiftahmin değeri parametreleri hesaplandı.

Bulgular: Tüp metotve Kongo kırmızısı agar metotlarının sensitivite, spesifisiteleri, sırasıyla %97, %100 ve %87, %94' dür.

Tartışma: Bu çalışma tüp metodun, Kongo kırmızısı agar metottan daha güvenilir olduğunu gösterdi. Tüp metot, laboratuvarlarda biyofilm oluşturan bakteri identifikasyonu için ana tarama testi olarak tavsiye edilebilir. Güvenilir biyofilm belirleme metodunun kullanımı ile yanlış tanılar ve tekrarlayan infeksiyonlar önlenebilir.

Anahtar kelimeler: Biyofilm oluşturma, Staphylococcus epidermidis, Staphylococcus aureus, mikrotitre plak metodu, tüp metot, Kongo kırmızısı agar metodu

Introduction

Staphylococcus is a main cause of nosocomial and environmental infections. Staphylococcus have gained attention due to its ability to produce biofilm that cause biofilm-associated infections and responsibility of one-half of prosthetic device-associated infections [1]. Staphylococcus epidermidis (S. epidermidis) and Staphylococcus aureus (S. aureus) cause indwelling device-related infections. Staphylococcus aureus cause host infections such as osteomyelitis [2], septic arthritis [3], ocular infections, endocarditis, chronic wound infections, and chronic rhinosinusitis [4].

Biofilm formation that begins with the adherence of the bacteria to a surface continues with the aggregation formed by cell-cell adhesion [5]. Adherence of bacteria is mediated by surface adhesins such as surface protein G (SasG) [5] and fibronectin binding proteins (FnbA and FnbB) of S. aureus[6]. Aggregation that is mediated by the synthesis of either polysaccharide intercellular adhesion/ poly-N-acetylglucosamine (PIA/PNAG) [6, 7] is formed in cell clusters till multi-layer structured biofilms formed.

Biofilm has an important role in the pathogenesis of staphylococcal infections. The bacteria within the biofilm resist antibiotics, antimicrobials and immune system [4]. Biofilms are produced on the outer and inner surfaces of indwelling medical devices such as prosthetic heart valves, intravenous catheters and stents [8], on the host tissues such as heart valves (endocarditis) [4], teeth [9], in the middle ear of patients with otitis media [10], in the lungs of patients with cystic fibrosis (CF) (chronic bronchopneumonia) [11],in chronic osteomyelitis and prosthetic joint infections [2,3,12], in chronic wounds and in chronic rhinosinusitis [4] by bacteria.

There are different biofilm detection methods [13-18]. To reduce probability in detection of false negatives biofilm screening methods must be tested and compared with each other. In this study tube method and Congo red agar methods were compared according to microtiter plate method as a gold standard.

The aim of this study is to compare microtiter plate method, tube method ve Congo red agar screening methods used for the detection of biofilm formation by Staphylococcus aureus and Staphylococcus epidermidis of which treatment can be impossible and hard, and infections such as host and indwelling device-associated infections can be recurrent.

Materials and Methods

The Bacteria. 121 isolates of Staphylococcus which were used as test microorganisms were obtained from Abant Izzet Baysal University, Faculty of Medicine, Medical Microbiology Laboratory, Bolu; Turkey. All of the isolates were identified as S. epidermidis and S. aureus according to colonial and microscopic morphology, positive catalase for both, negative and positive coagulase, respectively. All isolates were tested for biofilm production in triplicates.

Congo red agar method (CRA). Strains of Staphylococcuswere inoculated on Congo red agar media (CRA) (Merck TM) as described by Freeman et al. (1989) to identify whether strains were biofilm producer or not (15). The CRA medium was constructed by mixing 0.8 g of Congo red and 36 g of sucrose (Sigma, Missouri, EUA) to 37g/L of BHI (Oxoid, Basingstoke, Hampshire, England). After incubation period that was 24 h at 37°C, morphology of staphylococcal colonies that undergone to

different colours were differentiated as biofilm producers or not. Black colonies with a dry crystalline consistency indicated biofilm producers, whereas colonies retained pink were non-biofilm producers.

Tube method (TM). The biofilm formation of Staphylococcus was also detected by this method described by Christensen et al. (1985). The Staphylococcus strains was inoculated in polystyrene test tube which contained tryptic soy broth (TSB) and incubated at 24 h at 37°C [19]. The sessile Staphylococci of which biofilms formed on the walls of polystyrene test tube were stained with saphranin for 1 hour, after planktonic cells were discharged by washing twice with phosphate-buffered saline (PBS). Then, saphranin stained polystyrene test tube was washed twice with PBS to discharge saphranin stain. After air drying of test tube process, the occurence of visible film lined the walls and the bottom of the tube indicates biofilm production[19]. These visible films that were measured spectrophotometrically at 540 nm by a microplate reader (Thermo Instruments TM) rated as 1 (weak/non biofilm producers), 2 (intermediate biofilm producers) and 3 (high/strong biofilm producers). The studies were repeated in triplicates.

Microtiter plate method (MtP). 200 µl of bacterial suspension of which optical density (OD) had adjusted to approximately 0.600 by a spectrophotometer (Hitachi TM) earlier was inoculated into 96-well flat-bottomed sterile polystyrene microplate (LP Italiana SPA TM) which contained TSB. Uninoculated wells containing sterile TSB were used as controls. Microplates incubated at 24 h at 37°C. The Sessile Staphylococci of which biofilms formed on the walls of wells of microplate were stained with saphranin for 1 hour, after planktonic cells in wells of microplate had discharged by washing twice with PBS(pH 7.2) and wells had dried at 60 °C for 1 h. Then, saphranin stained wells of microplates were washed twice with PBS to discharge saphranin stain. After air drying process of wells of microplate, biofilms lined the walls of the microplate were measured spectrophotometrically at 540 nm by a microplate reader (Thermo Instruments TM) The studies were repeated in triplicates. Uninoculated wells containing sterile TSB used as blanks. The blank absorbance values were used to identify whether biofilm formation of Staphylococcus strains exist or not. The strains producing biofilm higher than blank corrected mean absorbance value of 0.05 were considered as weak biofilm producers, and if the value was higher than 0.10 and 0.20, it was revealed intermediate and stronger/higher biofilm producer, respectively. The biofilm production studies of each strain were repeated in triplicates. The cut-off value (ODc) that was calculated for gaining the better results is constructed by three standard deviations (SD) that are higher than the mean OD of the negative control. The ODc was calculated according to the given formula; (3×SD of negative control) + the mean OD of negative control = ODc. TheODc value was calculated for each microplate separately. The negative value stated as zero indicated that bacterium strain tested was a non-biofilm producer, whereas positive value revealed that bacterium strain tested was a biofilm producer. According to the biofilm production, the staphylococcal strains were categorized into not only such groups [20];0 (non-biofilm producer), 1 or + (weak biofilm producer), 2 or ++ (intermediate biofilm producer) and 3 or +++ (strong biofilm producer), but also such groups that depend on OD values; $OD \leq ODc$ (non-biofilm producer), $ODc< OD \leq 2 \times ODc$ (weak biofilm producer), $2 \times ODc < OD \leq 4 \times ODc$ (intermediate biofilm producer) and $4 \times ODc < OD$(strong biofilm producer) [21].

Statistical analysis

MtP method was considered as gold standard for this study and compared with data of TM and CRA methods. Sensitivity, specificity, positive predictive value and negative predictive valueparameters were calculated. True positives were biofilm producers by MtP, TMand CRA methods whereas true negatives were non-biofilm producers by MtP, TM and CRA methods.

False positives meaned that MtP method indicated strains as non-biofilm producers whereas TM and CRA methods indicated that strains as biofilm producers. False negatives meaned that MtP method indicated strains as biofilm producer whereas TM and CRA methods indicated that strains as non-biofilm producers.

Results

Among 121 isolates, 54% and 46% isolates were found to be S. epidermidis and S. aureus, respectively. In this study, 121 isolates were used to compare three biofilm screening methods and MtP method was used as gold standard method. 62 (51%) and 59 (49%) of strains were identified as biofilm producers and non-biofilm producers by the MtP method, respectively (Table 1). 62 (51%) and48 (40%)

isolates were identified as biofilm producers, 59 (49%) and 73(60%) isolates were identified as non-biofilm producers by TM and CRA, respectively (Table 1).

Tablo 1. The detection of biofilm production by MtP, TM and CRA methods

	Biofilm formation	
	Positive **(weak, intermediate, strong)**	**Negative** **(non)**
MtP	62 (51%)	59 (49%)
TM	62 (51%)	59 (49%)
CRA	48 (40%)	73(60%)

Among 121 isolates,13 (11%) and 14 (12%) isolates were identified as strong/high biofilm producers, 25 (21%) and 34 (28%) isolates were identified as intermediate biofilm producers, isolates were identified as 83 (69%) and 73 (60%) weak or non biofilm producers by TM and CRA, respectively (Table 2).

Table 2. The categorization of biofilm production

Biofilm formation	MtP	TM	CRA	n
Strong/high	13 (11%)	13 (11%)	14 (12%)	121
Intermediate	26 (21%)	25 (21%)	34 (28%)	
Weak/non	82 (68%)	83 (69%)	73 (60%)	
Weak	23 (19%)	24 (20%)	0	
Non	59 (49%)	59 (49%)	73 (60%)	

Any false positive and false negative result was not determined by TM (Table 3). False negative results of 14 (19%) were determined by CRA (Table 3). Any false positive result wasn't determined by CRA (Table 3). 59(100%) of isolates that were actually negative according to MtP method identified as true negative results by both TM and CRA methods. 62 (100%) and 48 (77%) isolates that were actually positive according to MtP method identified as true positive results by TM and CRA, respectively.

Table 3. The evaluation of positive and negative results

	TM	CRA
True positive (TP)	62 (100%)	48(77%)
False negative (FN)	0	14 (19%)
False positive (FP)	0	0
True negative (TN)	59 (100%)	59 (100%)

When the results of CRA, TM and MtP methods were compared according to MtP method as a gold standard, the results revealed that the sensitivity and the specificity of the CRA and TMtests were 77% and 100%, 100% and 100%, respectively (Table 4). The sensitivity of CRA method remained low.

Based on a negative predictive value (NPV), 81% and 100% of nonbiofilm producer strains that were actually negative by MtP were revealed as negative by the CRA and TM method, respectively. Based on positive predictive value (PPV), 100% of biofilm producers strains that were actually positive by MtP were revealed as positive by both of CRA and TM (Table 4).

Table 4. Parameters of Tube method and Congo red agar methods against Staphylococcus isolates

Screening methods	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
TM	100%	100%	100%	100%
CRA	77%	100%	100%	81%

Discussion

All of the methods were effective to detect biofilm production of S. epidermidis and S. aureus due to acceptable sensitivity and specificity. The sensitivity and the specificity of TM were 100%. This result revealed that correlation was foundbetween the results of the TM and MtP methods. The sensitivity and the specificity of CRA were 77% and 100%. False positive results of 14 (19%) were determined by CRA. The TM represented higher sensitivity than CRA method to detect biofilm formation of S. epidermidis and S. aureus. These results of TM and CRA methods revealed that TM was better than CRA method in the biofilm detection, due to its' higher sensitivity, and any false positive and negative results of CRA was determined. In the laboratories, TM method can be an alternative to MtP method and TM must be recommended to detect biofilm formation of S. epidermidis and S. aureus rather than CRA method.

De Castro Melo et al. (2003) indicated that 28.6% of isolates that were actually negative identified as true negative results, and 100% of isolates that were actually positive identified as true positive results by CRA method. They also compared the results of CRA and MtP methods using the MtP method as a gold standard. They indicated that the sensitivity and the specificityof the CRA test were 86% and 100%, respectively[22].These results suggest that the

CRA method can be used to detect biofilm production by S. aureus. But the resultsof molecular analysis to compare CRA and MtP methods indicated that MtP method was more sensitive [22]. Jain and Agarwal (2009) identified biofilm formation of Staphylococci by CRA and MtP methods, using the MtP as a gold standard. They indicated that the sensitivity and specificity of CRA method against S. aureus biofilm were 90.63% and 90.6% respectively[23].

Mathur et al. (2006) indicated that 53.9% of isolates were biofilmproducers, and 46% of isolates were non-biofilm producersaccording totissue culture plate method (TCP) [25]. Ruzicka et al. (2004) showed that 79 (53.7%) and 64 (43.5%) of S. epidermidis isolates wereidentified as biofilm producers by TM and CRA method, respectively. They indicated that TM was better to identify biofilm formation than CRA [26]. Baqai et al. (2008) indicated that 75% of uropathogens were identified as biofilm producers by TM [27]. Knobloch et al. (2002) indicated that 11 isolates were biofilm producers and 99 isolates were non-biofilm producers according to CRA method. Sensitivity (11%), specificity (92%) of CRA method were very low. 62 isolates were found to be false negative and 3 isolates were false positive. CRA method was not recommended in their study sinceonly 3.8% of S. aureus isolates were identified as biofilm producers by CRA method compared to TCP that identified 57.1% of S. aureus isolates as biofilm producers [28].Hassan et al. (2011) determined that 70 (64.7%) of isolates were biofilm producer, and 40 (36.3%) of isolates were non or weak biofilm producers according to TCP method (MtP) [4]. Hassan et al. (2011) determined that 49% isolates were biofilm producer, and 51% isolates were non-biofilm producers. False positive results of three isolatesand false negative results of 19 isolates were determined by TM. They determined the sensitivity and specificity of TM as 73%, and 92.5%, respectively. They indicated that there was a correlation between TCP and TM to identify strong biofilm producers [24]. TM was not suggestedas general biofilm screening method, in accordance with other studies [14, 25].Kumar Gupta et al. (2013) studied CRA and MTP methods to detect biofilm formation of Staphylococcus. They determined that MtP was more specific than CRA method [2]. Dhanawade et al. (2010), demonstrated that MtP and CRA methods were correlated with the molecular analysis[30].

By the usage of reliable biofilm detection method, wrong diagnoses and recurrent infections can be prevented.

Conflict of Interest

There is no financial or personal relationship which can cause a conflict of interest regarding this article.

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