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COMPARISON OF THE MICROTITER PLATE METHOD AND THE CONGO RED AGAR TECHNIQUE IN THE DETERMINATION OF STAPHYLOCOCCAL BIOFILM

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Abstract

Coagulase-Negative Staphylococci are opportunistic pathogens that are commensal in human flora. One of the most important virulence factors known in the pathogenesis of infections of these bacteria is biofilm formation. The Microtiter Plate Method and The Congo Red Agar Technique are widely used to reveal biofilm formation. This study aims to compare human coagulase negative Staphylococcus spp. bacterial isolates, biofilm formations with the Microtiter Plate Method and Congo Red Agar Technique. In the study, it was concluded that 35 of 41 human coagulase negative staphylococcal isolates did not form biofilms according to the microtiter plate method, 6 isolates formed a weak biofilm, and none of the isolates formed a biofilm on the Congo Red Agar surface. It has been concluded that the results of the Microtiter Plate Method are more reliable, since the interpretation of the result in the Congo Red Agar Technique is difficult and subjective, based on observation. Since there are very few studies in the literature comparing the biofilm formation of coagulase negative staphylococci with the Microtiter Plate Method and the Congo Red Agar Technique, this study will be among the preliminary studies and will contribute to the literature.

Keywords: Biofilm, Coagulase-Negative Staphylococci, Congo Red Agar Technique, Microtiter Plate Method, Vagina

STAFİLOKOK BİYOFİLMİNİN BELİRLENMESİNDE MİKROTİTRE PLAKA YÖNTEMİ VE KONGO KIRMIZISI AGAR TEKNİĞİ'NİN KARŞILAŞTIRILMASI

Özet

Kolagülaz negatif stafiloloklar, insan florasında kommensal olarak yer alan fırsatçı patojenlerdir. Bu bakterilerin enfeksiyonlarının patogenezisinde bilinen en önemli virülans faktörlerinden biri biyofilm oluşumlarıdır. Biyofilm oluşumunun ortaya konmasında mikrotitre plaka yöntemi ve kongo kırmızısı agar tekniği yaygın bir şekilde kullanılmaktadır. Bu çalışmada, insan koagülaz negatif Staphylococcus spp. bakteri izolatlarının biyofilm oluşumlarının mikrotitre plaka yöntemi ve kongo kırmızısı agar tekniği ile karşılaştırılması amaçlanmıştır. Çalışmada, 41 insan koagülaz negatif stafilokok izolatından mikrotitre plaka yöntemine göre 35 tanesinin biyofilm oluşturmadığı, 6 izolatın zayıf derecede biyofilm oluşturduğu, kongo kırmızısı agar yüzeyinde ise hiçbir izolatın biyofilm oluşturmadığı sonucuna varılmıştır. Kongo kırmızısı agar tekniğinde sonucun yorumlanmasının güç ve gözleme dayalı olması sebebiyle, mikrotitre plaka yöntemi sonuçlarının daha güvenilir olduğu sonucuna varılmıştır. Literatürde koagülaz negatif stafilokokların biyofilm oluşumlarının mikrotitre plaka yöntemi ve kongo kırmızısı agar tekniğiyle karşılaştırıldığı neredeyse hiç çalışma olmaması sebebiyle, yapılan çalışma öncül çalışmalar arasında yer alıp, literatüre katkı sunacaktır.

Anahtar Kelimeler: Biyofilm, Koagülaz Negatif Stafilokok, Kongo Kırmızısı Agar Tekniği, Mikrotitre Plaka Yöntemi, Vajina

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1. Introduction

Biofilm is a collection of microorganisms that are aggregated by the adhesion of cells to a surface. Extracellular polymeric substances (extracellular polysaccharides, proteins, glycoproteins, and glycolipids), which these adherent cells generate on their own, are frequently embedded in these materials. In other words, the biofilm structure is covered with a matrix structure consisting of extracellular polymeric material produced by the microorganism itself [1].

Biofilm is of great clinical importance in determining antibiotic resistance, surface absorption of the drug, and the minimum inhibition concentrations required for effective treatment. Biofilm is characterized by

decreased susceptibility to antimicrobial agents [2, 3]. In the studies comparing the antibiotic susceptibilities of biofilm - forming microorganisms and planktonic microorganisms, it has been reported that the bacteria in the biofilm structure need concentrations up to 1000 times higher than planktonic bacteria against antibiotics [4, 5].

Biofilm infections have certain clinical characteristics wherever they occur in the human body. These infections develop gradually, and their symptoms usually appear over time. It is very difficult to eliminate the biofilm structure completely; however, the biofilm structure is fought by immunological host defence mechanisms such as phagocytic cells, T and B lymphocytes, and the body's normal flora. The cells in this structure release antigens that increase antibody production. However, the antibodies produced against the existing biofilm structure cannot kill the microorganisms in the biofilm and accumulate in the surrounding tissues. This may cause immune complex-related damage to the same tissues [2, 3].

One of the most important virulence factors known in the pathogenesis of staphylococcal infections is that these bacteria can produce a slime layer and form a biofilm [6]. Coagulase-Negative Staphylococci (CNS) are responsible for an estimated 1.7 million infections each year in the United States, resulting in 100,000 deaths, costing approximately $35-45 million annually [7, 8]. These bacteria are defined as the most common causes of biofilm-related infections by contaminating surgical instruments. CNS bacteria, especially *Staphylococcus epidermidis* and *S. haemolyticus*, pave the way for the formation of complex biofilms by contaminating medical instruments [8, 9]. Biofilm-forming CNS isolates are of great importance in modern medicine because they can have high levels of multiple antibiotic resistance [4]. Therefore, it is very difficult to keep CNS infections under control [10].

The Microtiter Plate Method, which is a quantitative method and the Congo Red Agar Technique, which is a qualitative method, in the phenotypic identification of biofilm-producing strains, are widely used [11]. In this study, it was aimed to compare the biofilm formations of human Coagulase-Negative *Staphylococcus* spp. bacterial isolates with the Microtiter Plate Method and Congo Red Agar Technique.

2. Material and Method

2.1. Material

2.1.1. Microorganisms used in the study

In the study, human vaginal *Staphylococcus* spp. bacterial isolates defined using the VITEK II test in the Microbiology Laboratory of Anadolu University Yunus Emre Vocational School of Health Services were used. Isolates were incubated at 37 °C for 24 hours under aerobic conditions in brain-heart infusion (BHI) agar medium (Merck) and were revived and taken to experiments.

2.2. Method

2.2.1. Determination of biofilm formations of isolates

2.2.1.1. Microtiter Plate Method

In order to determine the degree of biofilm formation of the isolates, the bacteria were incubated in BHI liquid medium (Merck) containing 2.5% glucose (Fluka) at 37 °C for 24 hours. After incubation, 200µL culture sample was inoculated on the 96 well-plate (NEST) and incubated at 37 °C for 24 hours. Then, the plates were emptied and each well was washed 3 times with sterile physiological saline. The washed wells were fixed with 200µL of 99% methanol (Merck) for 15 minutes. At the end of the period, the wells were emptied and left to dry. Each well was stained with 200µL 2% crystal violet (BESLAB) for 5 minutes. When the time was over, the wells were washed with sterile distilled water and dried. Then, 160µL of 33% glacial acetic acid (ISOLAB) was added to each well, plate reading was performed at 570 nm in the ELISA device (BioTek Epoch 2) and the optical density (OD) value of each isolate was recorded. As a result of reading, comments were made about biofilm formation according to OD values ((OD570 <0.120 (-), OD570 < 0.240 (+), OD570 < 0.500 (+ +), OD570 >0.500 (+ + +)). The tests were performed in 8 parallel, double repetitions [12].

2.2.1.2. Determination of biofilm formation in Congo Red Agar

The test bacteria were incubated in BHI agar medium (Merck) at 37 °C for 24 hours. After incubation, microorganism cultures were inoculated on the surface of Congo Red Agar (Merck). Then, the petri dishes (Isolab) were incubated at 35°C for 24 hours. After incubation, the isolates forming a black-colored colony were evaluated as biofilm positive, and the isolates forming a pink-red-colored colony were evaluated as biofilm negative [13].

3. Results and Discussion

3.1. Microtiter Plate Method

Table 1 shows the biofilm formation results of CNS isolates in 2.5% glucose medium by Microtiter Plate Method. Looking at the OD readings, OD value of 35 bacterial isolates (*Staphylococcus lugdunensis* 11K3, 11N3, 11Q7, 11K2, 12B5.1, 11N2.1, 7Q1.1 isolates, *S. haemolyticus* 7M10, 7Q2, 7N10, 7N3, 7M3, 7K10.1, 7B10, 7E5.1, 7Q3, 7M7, 7M8, 7M9, 7E6.1, 7K7, 7E9.1, 7B3.1, 7M6, 7M5, 7Q6 isolates, *S. epidermidis* 7Q5.1, 12K2.1, 7K9, 11Q8, 7N5 isolates, *S. lentus* 7B5, 10M1, 11N6 isolates, *S. hominis* ssp. *hominis* 7E10) was below 0.120, OD value of 6 isolates (*S. lugdunensis* 7Ç10 isolate, *S. epidermidis* 11K8 isolate, *S. lentus* 12N1, 11K1, 10M8 isolates, *S. caprae* 11N4 isolates) was observed to be between 0.121-0.240.

Table 1. Biofilm formation of CNS isolates in glucose medium by Microtiter Plate Method.

Bacterial isolate	(-)	(+)	(++)	(+++)
Staphylococcus lugdunensis	11K3, 11N3, 11Ç7, 11K2, 12B5.1, 11N2.1, 7Ç1.1 isolates	7Ç10 isolate		
Staphylococcus hemolyticus	7M10, 7Ç2, 7N10, 7N3, 7M3, 7K10.1, 7B10, 7E5.1, 7Ç3, 7M7, 7M8, 7M9, 7E6.1, 7K7, 7E9.1, 7B3.1, 7M6, 7M5, 7Ç6 isolates			
Staphylococcus epidermidis	7Ç5.1, 12K2.1, 7K9, 11Ç8, 7N5 isolates	11K8 isolate		
Staphylococcus lentus	7B5, 10M1, 11N6 isolates	12N1, 11K1, 10M8 isolate		
Staphylococcus hominis ssp. *hominis*	7 E10 isolate			
Staphylococcus caprae		11N4 isolate		
OD570<0,120 (-): no biofilm; OD570<0,240 (+): weak biofilm; OD570<0,500 (++): moderate biofilm; OD570>0,500 (+++): strong biofilm [12].				

3.2. Biofilm formation results in Congo Red Agar

As a result of the test, it was observed that all 41 *Staphylococcus* spp. isolates formed a pink colored colony in Congo Red Agar (Figure 1).

CNS bacteria are the third most common infectious agent on the earth and rank first as an infectious agent in prosthetic valve endocarditis [14]. These bacteria have recently been noted as important pathogens of hospital infections; they are the biggest cause of hospital-associated bacteremia and septicemia, causing morbidity and mortality, especially in immunocompromised individuals. In addition, CNS bacteria are defined as the biggest cause of late-onset neonatal bacteremia in neonatal intensive care units [4]. This shows how clinically important CNS bacteria are.

CNS bacteria are characterized by adhering to and colonizing the surfaces of biomaterials and forming biofilms in the polymer slime matrix structure they produce on their own. Many recent studies have focused on the slime production of these bacteria [15]. It has been reported that coagulase negative bacteremia occurs in the regions where CNS bacteria form biofilms after long-term intravenous catheter use, parenteral nutrition, and lack of attention to hand hygiene [16, 17].

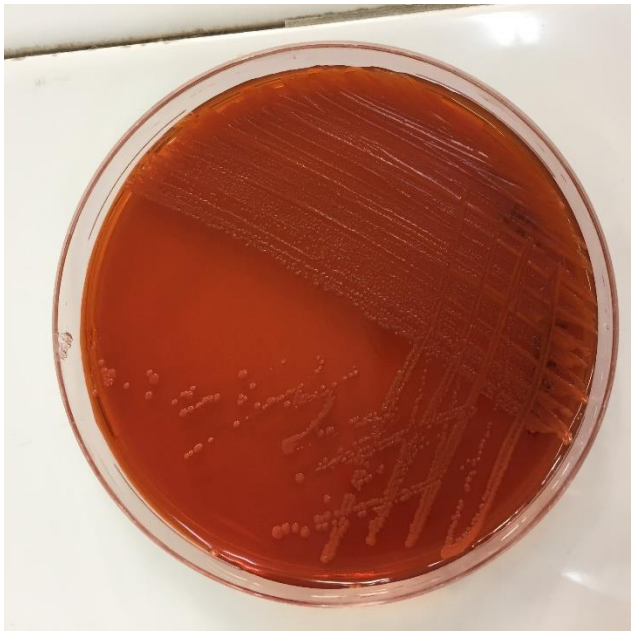

Figure 1. Negative biofilm formation on Congo Red Agar surface.

The biggest problem of foreign body infections such as intravenous catheters is the chronic persistence of infections due to the formation of biofilm on the surface of these objects [7, 8]. Studies have isolated *S. epidermidis*, S. *haemolyticus, S. warneri, S. xylosus, S. lugdunensis, S. hominis, S. saprophyticus, S. schleiferi*, and *S. chromogenes* CNS bacteria from human catheters [1]. Biofilm formation of bacteria is an important factor in determining the course of the disease; because these bacteria cause antibiotic drug resistance and make treatment difficult [18, 19]. Therefore, biofilm production has an important role in the pathogenesis of staphylococcal infections [6].

In the literature, the Microtiter Plate Method and the Congo Red Agar Technique are widely used to determine biofilm formation. In this study, these 2 tests were used to determine biofilm formation. The Microtiter Plate Method is a method developed instead of the test tube method, which is the first method used to macroscopically determine bacterial biofilm. The Microtiter Plate Technique is a quantitative method in which microorganisms grown in glucose medium are inoculated in a 96-well plate, the biofilm structure formed on the bottom of this plate is stained, and its optical density is measured spectrophotometrically [5, 11, 12].

The Congo Red Agar Technique is a test using a solid medium called Congo Red Agar. This method gives a result based on color change; colony formation in black color on the surface of the medium indicates the presence of biofilm, colony formation in pink-red color indicates the absence of biofilm. In addition, it allows for the direct analysis of the colonies and the identification of slime-forming strains (which appear as black colonies on the red agar) and non-slime-forming strains (red-coloured colonies). Since the Congo Red Agar Technique is based on subjective color change assessment, it does not give quantitative results [11].

4. Conclusion

In this study, it was concluded that according to the Microtiter Plate Method, 35 of 41 human CNS isolates (*Staphylococcus lugdunensis* 11K3, 11N3, 11Q7, 11K2, 12B5.1, 11N2.1, 7Q1.1 isolates, *S. hemolyticus* 7M10, 7Q2, 7N10, 7N3, 7M3, 7K10.1, 7B10, 7E5.1, 7Q3, 7M7, 7M8, 7M9, 7E6.1, 7K7, 7E9.1, 7B3.1, 7M6, 7M5, 7Q6 isolates, *S. epidermidis* 7Q5.1, 12K2.1, 7K9, 11Q8, 7N5 isolates, *S. lentus* 7B5, 10M1, 11N6 isolates, *S. hominis* ssp. *hominis* 7E10 isolate) did not form biofilm, 6 isolates (*S. lugdunensis* 7Ç10 isolate, *S. epidermidis* 11K8 isolate, *S. lentus* 12N1, 11K1, 10M8 isolates, S. caprae 11N4 isolate) form a weak biofilm, on the surface of Congo Red Agar, no isolate formed biofilm. In a study, it was reported that 9 of 25 human commensal CNS isolates were biofilm positive and 16 of them were biofilm negative by Microtiter Plate Method, 5 of them were biofilm positive and 20 of them were biofilm negative on the surface of Congo Red Agar [6]. In a study conducted by Melo et al. (2013), it was revealed that 93 of 94 *S. aureus* isolates were biofilm positive, 1 of them was biofilm negative in the Microtiter Plate Method, 80 of them were biofilm positive and 14 of them were biofilm negative on the Congo Red Agar surface [11].

In the studies conducted, Microtiter Plate Method was accepted as the gold standard in determining the biofilm formation of staphylococci, spectrophotometric measurement and quantitative data, higher sensitivity and reliability in the data compared to the Congo Red Agar Technique based on macroscopic interpretation and qualitative [6, 11,12, 20, 21, 22, 23]. In our study, it was also observed that the color formed on the Congo Red Agar surface was difficult to interpret and was based on subjective interpretation. Since the repeated results obtained in the Microtiter Plate Method are compatible with each other and are based on numerical data, it has been concluded that the Microtiter Plate Method is more reliable. There are very few studies in the literature comparing the biofilm formations of Coagulase-Negative Staphylococci between the Microtiter Plate Method and the Congo Red Agar Technique. Since there is no study on the biofilm formation of human vaginal Coagulase-Negative Staphylococci, this study will make an important contribution to the literature.

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