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RESEARCH ARTICLE

The evaluation of biofilm production by *Streptococcus equi zooepidemicus* isolates

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Streptococcus equi zooepidemicus izolatlarının biofilm üretiminin değerlendirilmesi

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Öz

Amaç: *Streptococcus equi zooepidemicus* izolatlarının biofilm üretme kapasitelerinin ölçülmesi amaçlandı.

Gereç ve Yöntem: Selçuk Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı kültür koleksiyonundan sağlanan kırk adet *Streptococcus equi zooepidemicus* izolatı Kongo kırmızısı agar, Kristal moru boyama ve taramalı electron mikroskobu (TEM) olmak üzere 3 farklı yöntem ile biofilm oluşturma yönünden değerlendirildi.

Bulgular: Kongo kırmızısı agar ile Kristal moru boyama sonuçları arasında uyumsuzluk vardı. Otuz bir pozitif suşun 19'u Kongo kırmızısı agarda kuvvetli pozitif verirken 30'u Kristal moru boyama ile kuvvetli pozitif bulundu. Biofilm üretimi TEM ile doğrulandı.

Öneri: Ülkede at hekimliği hizmeti veren klinisyenlerin, *Streptococcus equi zooepidemicus* etkenlerinin in vitro şartlarda biofilm oluşturabileceğinin farkında olmaları önerilir.

Anahtar kelimeler: *Streptococcus equi zooepidemicus*, biofilm.

Abstract

Aim: The purpose of this study is to measure biofim production capabilities of *Streptococcus equi zooepidemicus* isolates.

Materials and Methods: Forty one horse strains of *Streptococcus equi zooepidemicus* from the laboratory stock collection of Microbiology Department of the Veterinary Faculty, Selçuk University were evaluated by the biofilm formation using three different methods: Congo red agar, crystal violet staining method and scanning electron microscopy (SEM).

Results: Incompatibility between the results obtained from Congo red agar and crystal violet staining methods was detected. Nineteen out of 31 positive strains gave strong positive on Congo red agar while 30 of them were considered as strongly biofilm producing strains in crystal violet staining method. The biofilm production was confirmed and the biofilm layers were detected by using the SEM microscope.

Conclusion: Equine clinicians in this Country should be aware of that *Streptococcus equi zooepidemicus* isolates are capable of forming biofilm in vitro.

Keywords: *Streptococcus equi zooepidemicus*, biofilm.

Introduction

The Lancefield group C member the *Streptococcus equi* subspecies *zooepidemicus* is a commensal organism of the tonsil and nasopharyngeal mucosa of the equine. This species may opportunistically start respiratory infections such as purulent rhinitis, bronchitis and pneumonia in foals and donkeys of different ages. In older horses that have been under heat or transportation stress, it may also cause acute hemorrhagic pneumonia (Sellon and Long 2013, Filioussis and Karavanis 2019). In terms of veterinary public issues this agent needs to be focused since it may even be transmitted from dogs to human leading a severe disease as evidenced recently (Zahlanie et al 2019).

Biofilm is a factor that may contribute to infections and disease development. Day by day facing infections related to biofilm is becoming a bigger problem. The negative impact of biofilm can be seen in health and industrial sector as well. During infection the formation of biofilm layer prevents the contact of immune cells with the invading bacterial populations. It also helps the microorganisms to resist extreme heat, pH, UV and other environmental conditions. Inside the intestine, the biofilm formation on the epithelial surface protects the pathogens and allows them to thrive without any outside disturbance (Çiftçi 2005, Lindsay and Von Holy 2006).

According to some publications, around 80% of all infections some of which are difficult to eradicate involve biofilm productions. Biofilms are formed as a sessile of bacterial communities. These bacterial cells are attached irreversibly to each other and embedded to a substratum. This is facilitated by the bacterial production of extracellular polymeric substances that make up the matrix that surrounds and contains the bacterial population. This structure allows the communication among bacteria by some chemotactic particles in a process called quorum sensing. Many factors contribute to the formation of biofilm. The availability of nutrients, surface adhesins, bacterial motility and chemostatics are examples of these factors (Hassan et al 2011).

The presence of bacterial populations within a biofilm makes it remain exposed to antibiotics longer. This would enable the selection of more drug resistant populations and make the future application of treatment more and more difficult even with the high concentration (Wilcox et al 2001, Di Bonaventura et al 2004).

The bacterial biofilm layers formation is widely distributed in nature and it is the base for the development of various infectious diseases and resistance genes exchange among bacteria. The interventional and consistent techniques used in medical applications increase the occurrence rate of biofilm based infections. Gram positive and gram negative bacteria along with fungi are increasingly seen forming biofilm on various medical instruments such as catheter, artificial heart valves and many other plastic tools and this contributes to the increasing hospital infections (Monzón et al 2001, Donlan 2002). A high degree of genetic variability in *Streptococcus equi* subspecies *zooepidemicus* isolates from horses in Italy has also been reported by a recent study (Preziuso et al 2020).

The detection of the microorganism's ability to form biofilm can be assessed by several ways. Congo red agar is one of the rapid ways for the detection of biofilm in bacteria. Bacterial isolates are streak cultured on brain heart agar supplemented with sucrose and Congo red dye. The formation of blackish colonies after incubation represents the strains with the ability to form biofilm (Cotter et al 2009).

The degree of biofilm can be measured by using the optical density of the stained biofilm. Pure bacterial isolates are cultured in a sugar containing bacterial broth in a sterile micro plate wells. After incubation, the formed biofilm is washed and dried. After washing, crystal violet is then used for staining. The stained biofilm is then solubilized in a solution containing methanol and acetic acid which is then read in optical density reader (Moore 2009).

Material and Methods

Forty one *Streptococcus zooepidemicus* isolates were assessed by their abilities of biofilm formation in this study. First of three methods used in this study was the Congo red agar method. The Congo red agar formulation was prepared according to a previously conducted study (Kaisera et al 2013). According to the producer (LAB), 49 gram of brain heart infusion agar powder was added to 1 liter of distilled water and mixed well. 0.8 gram of Congo red dye is then added and mixed for one minute before autoclaving for at 121°C for 15 minutes. Fifty gram of sucrose was dissolved in 100 ml of sterile distilled water. It was then added to the cooled BHI after it has passed through 0.2 micrometer filter. The agar then was poured to sterile petri dishes and left to solidify. The organisms under evaluation were cultured and incubated for 48 hours in 37°C in an aerobic incubator. The formation of black colonies with a black shadow after 24 hours was considered as strong positive. The black colonies without a blackening of the surrounding formed after 48 hours were also considered positive but weak. The red and transparent colonies were considered biofilm negative. *Streptococcus zooepidemicus* ATCC 35246 was used as a positive control along with Staphylococcus epidermidis.

The tissue culture plate method was used for the evaluation of the biofilm formation by bacteria. The strains that have given positive results on Congo red agar are cultured in 5 ml of

Tryptic Soy Broth (TSB) at 37°c for one night. Homogenization of the broth is required. Ten µl of the culture was taken and diluted with 990 µl of fresh TSB. After mixing, 100 µl of one sample was added six times to a sterile micro plate wells along with the negative and positive control. In this study the *Streptococcus zooepidemicus* ATCC 35246 was used as a standard strain along with the positive control Staphylococcus epidermidis. It was covered and incubated for 48 hours to allow the biofilm formation. After incubation, the cultures were washed 4 times using PBS (phosphate buffered solution) and left to dry for 1 hour in 60 °C. The bacterial strains that have had produced biofilm were seen adhered to on the walls and bottom of the wells. It was then fixed and ready for staining by Hucker's crystal violet solution. Hundred µl of Hucker crystal violet solution was added to each well and left for two minutes for staining. The stain was then washed with distilled water to remove the excess stain and left to dry. After it was dried, a solution containing %10 methanol and %7.5 acetic acid was added to the wells. It was then shacked for 2 minutes in order to solubilize the biofilm in the liquid. The micro plates were then read on 563 nm absorbance to evaluate the amount of biofilm formed in the wells.

The scanning electron microscope was used for the detection of biofilm in order to confirm the ability of positive strains to produce biofilm. The scanning electron microscope (SEM) is an advanced technology that allows highly accurate detection of variety of structure at the micro level and allows the production of photographs of very small structures with high resolution. Four positive isolates were tested. They had given strong biofilm according to the results of culture plate method. The four isolates were cultured in TSB with 1% glucose in small glass petri dishes. Sterile square shape (1 cm2) pieces of glass were immersed in the broth. The cultures were incubated for 48 hours in 37°C. The glass pieces were washed with PBS and then air dried. The test was done by the scanning electron microscope ZEISS in the Center of Advanced Technology in Selçuk University.

This study was approved by the Ethics Committee of Veterinary Faculty on Experimental Animals (SUVDAMEK) (Decision Date & No: 22.11.2018, 2018/162).

Results

Only 31 of the 41 strains gave positive results on Congo red agar. Nineteen of them were considered strong positive. The degree of biofilm production of all the Congo red positive strains along with the negative and the positive control was tested by measuring the optical density. The ATCC 35246 was used as a standard strain. Each sample was tested six times and the average was taken. The average was used for the calculation of the biofilm degree according to the cutoff value. The strain with an optical density more than twice the cutoff value was considered as strongly positive. The strains were categorized as weakly Positive if their OD was between the cutoff and 2 times this number. The strain is considered Negative if the optical density was below the cutoff value. Thirty of the strains were considered strong positive.

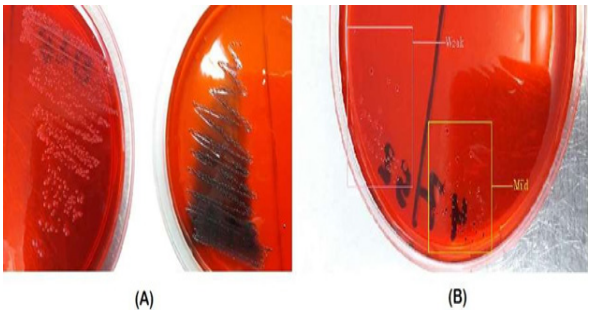

Figure 1 Biofilm detection by Congo Red Agar Method. (A)The red colonies on the left agar plate represent the biofilm negative organism. The positive black colonies with blackened surrounding on the right represent organisms with strong ability to form biofilm. (B)The strain is considered weak when only few black colonies appear. It's considered mild when most of the colonies are black with no shadow on the agar surface.

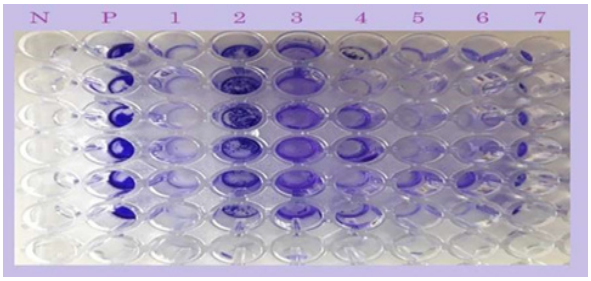

Figure 2 Evaluation of the biofilm degree by Crystal Violet Staining Method. The result of staining the biofilm formed on the bottom and walls of the wells

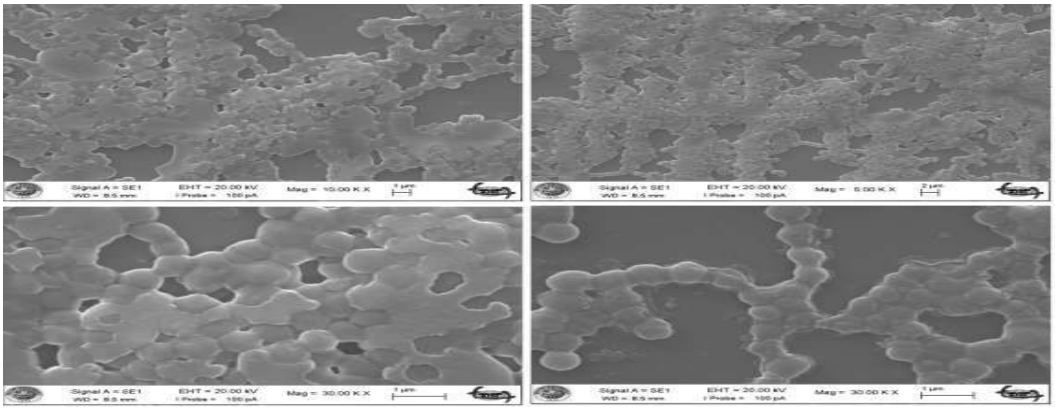

Figure 3 Confirmation of Biofilm Production by SEM. The scanning and detection of the biofilm formed by Streptococcus zooepidemicus isolates. The photographs were taken in different magnifications provided by SEM. The formation of different layers and the cells clamping in slime is very clear.

Four positive strains were selected in order to confirm the presence of biofilm by using the scanning electron microscope (SEM). The all 4 tested isolates were confirmed to be a biofilm producing strains. The SEM revealed the bacterial clamps and the formation of multiple layers. This represents the biofilm matrix that facilitates the adherence of the cells to the glass surface that was seen clearly in this test.

Discussion

In this study Congo red agar and crystal violet staining methods were used. These are the most commonly used methods for the evaluation of the biofilm production (Moore 2009, Kaisera et al 2013). As noticed, the results were incompatible. The results of the Congo red agar method showed that the majority of the strains had produced biofilm. The isolates that were considered as biofilm producing strains were tested by crystal violet method. The calculations showed that the majority of the strains (n=31; 75.61%) were strong positive while only 19 of them (61.29%) formed blackening on the Congo red agar surface and considered strong positive. Crystal violet method would be considered more accurate as this method depends on calculating the average of the optical density of each repeatedly grown bacterial strain. One more reason that would support the idea of crystal violet staining method being more accurate than the Congo red agar method is that the evaluation of the biofilm production and degree in Congo red method depends on the colony observation only while the crystal violet method is more standardized and depends on calculations. In this study there was a need for the results confirmation by more accurate method. The formation of biofilm can be observed and seen in the laboratory and in nature on different surfaces as some adherent materials (Lindsay and Holy 2006). The scanning electron microscope is an advanced device that can produce fascinating results. In this study the four selected biofilm positive isolates were confirmed to be biofilm producing strains. The level of magnification provided by this microscope could reveal the biofilm matrix that contains the bacterial cells as clamps of multiple layers. This is a confirmation for the positive results obtained from Congo red and crystal violet methods in spite of the incompatibility between the results obtained from these two methods.

Biofilm formation is proved to be one of the undeniable aspects as it contributes to the pathogenicity and cause some problems in the environment (Donlan 2002). In this study different methods used for the detection and evaluation of the biofilm were compared. More trustable methods need to be provided in the future in order to detect the biofilm formation more efficiently.

Conclusion

In conclusion, equine *Streptococcus equi zooepidemicus* strains forming biofilm could be a problem for effective therapy in the field.

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This article is a part of the first author's PhD thesis.

Conflict of Interest

The authors did not report any conflict of interest or financial support.

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Ethical Approval

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