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Biofilm Formation and Extended Spectrum Beta Lactamase (ESBL) Production of Bacterial Strains Isolated From Sewage Water

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Research Article

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Abstract

The production extended spectrum beta lactamase (ESBL) and metallo-beta lactamase of strains grown biofilm that were isolated from Kilis State Hospital sewage water were investigated in this study. Congo red agar, tube adherence, tissue culture plate tests and SEM analysis were performed for the detection of biofilm production. The extended-spectrum beta lactamase and metallo-beta lactamase expression of biofilm producing bacteria were tested by using Kirby-Bauer disc diffusion method according to CLSI guidelines. 5 clinical isolates were revealed to be biofilm producers. From of these isolates, *Enterococcus* spp. (C2) and *E. coli* strains showed strong biofilm producing. One of *Enterococcus* isolates, SEM micrograph of C1 has viewed the weak adherence on glass surface. Only one from 5 isolates, *Enterococcus* spp. (C2) the strain was determined to synthesis ESBL enzyme. None of the strains was observed to produce metallo-beta lactamase. ESBL and biofilm production provide an important pathogenic character allowing protection against antibiotic treatments to *Enterococcus* spp. (C2).

Keywords: Biofilm, ESBL, metallo-beta lactamase, SEM.

Atık Sudan İzole Edilen Bakteri Suşlarının Biyofilm Oluşumu ve Genişletilmiş Spektrum Beta Laktamaz (ESBL) Üretimi

Özet

Bu çalışmada Kilis Devlet Hastanesi kanalizasyon suyundan izole edilen biyofilm oluşturan suşların genişlemiş spektrumlu beta laktamaz ve metallo-beta laktamaz üretimi incelenmiştir. Biyofilm üretiminin belirlenmesi için kongo kırmızısı agar, tüp adherens, doku kültürü plak testleri ve SEM analizi uygulanmıştır. Biyofilm üreten suşların genişlemiş spektrumlu beta laktamaz ve metallo-beta laktamaz ekspresyonu CLSI yönetmeliğine göre Kirby-Bauer disk difüzyon metodu kullanılarak test edilmiştir. 5 klinik izolatın biyofilm üreticisi olduğu belirlenmiştir. Bu izolatlardan *Enterococcus* spp. (C2) ve *E. coli* suşları güçlü biyofilm üretmiştir. *Enterococcus* izolatlarından C1'in SEM mikrografında cam yüzeye zayıf tutunma gözlenmiştir. 5 izolattan yalnızca biri, *Enterococcus* spp.'nin (C2) GSBL enzimi sentezlediği ortaya konmuştur. Suşlardan hiçbirinin metallo-beta laktamaz üretmediği belirlenmiştir. GSBL ve biyofilm üretimi *Enterococcus* spp. (C2) suşuna antibiyotik tedavilerine karşı koruyarak önemli bir patojenik karakter sağlamaktadır.

Anahtar Kelimeler: Biyofilm, GSBL, metallo-beta laktamaz, SEM.

INTRODUCTION

Antoni van Leeuwenhoek, a Dutch scientist, observed microbial material scraped from surfaces of his teeth by using a simple microscope, about 300 years ago. This microscopic object, not visible to the naked eye, is called *animalcules (tiny living animals)* by him. This discovery is the first description of the microbial biofilm (Dufour, 2012; Jamal et al., 2015).

Bacterial cells demonstrate two growth forms in nature; planktonic and biofilm known as the ubiquitous and predominant life form (Maric et al., 2007; Sala et al., 2012; Jamal et al., 2015). Biofilm can grow many different areas such as surfaces of aqueous environments, plant, and animal tissues, implanted medical materials, wastewater channels, and industrial places etc. (Dufour, 2012; Jamal et al., 2015). This structure attached to surfaces is a microbial community consisted of different microbial species or genera sharing the same ecological niche (Sala et al., 2012; Bogino, 2013).

Biofilm is a porous matrix that encased to surface within extracellular polymeric substances produced by bacteria inside this community (Bogino et al., 2013; Jamal et al., 2015). The bacterial dense in the biofilm matrix is regulated via the chemical signaling pathway (quorum sensing) that is defined as cellular communication between bacteria (Sharma et al., 2016; Temel and Erac, 2018). The extracellular polymeric substance (EPS) is included the several cellular components such as proteins, DNA, polysaccharides, RNA, and water. Biofilm formation is exhibited the basic steps: adherence to a surface, micro-colony formation (the sessile form), production of polymeric matrix, biofilm maturation and breakaway (Jamal et al., 2015).

Biofilm phenotype enhances the survival efficiency of the microbial community by protecting bacteria against antibacterial agents, phagocytic animal cells, adverse environmental conditions and providing the high-osmolarity conditions, oxygen limitations and high cell density (Maric, 2007; Dufour, 2012; Adamus-Bialek, 2015).

Biofilm-associated antimicrobial resistance is disseminated by genetic material exchange included conjugation, transduction, and transformation with varying from organism to organism (Sala et al., 2012; Dias et al., 2018). This matrix causes various clinic infections that are especially associated with the use of intravascular and urethral catheters of orthopedic devices, contact lenses, prosthetic heart valves, vocal cord prosthesis (Maric, 2007). The biofilm formation and extended-spectrum beta-lactamases production synergistically give rise to develop multi-drug resistant strains such as *Enterobacteriaceae* species by prolonging the treatment periods of these infections (Dumaru et al., 2019).

Biofilm formation and ESBL production in bacteria synergistically contribute to the development and dissemination of multi-drug resistant strains. In this regard, it is important to the knowledge of biofilm formation and antibiotic resistance profile of bacterial strains in antibiotic therapy of the patients. So, we aimed to discuss ESBL-synthesizing in biofilm producer clinic strains isolated from sewage water of Kilis state hospital in this study.

MATERIALS and METHODS

Biofilm producer bacteria isolation from hospital sewage

Isolation of biofilm-producing bacteria from Kilis hospital sewage was performed. Sample collected in an autoclaved dark bottle was brought to the microbiology lab. in a short time and kept at 4ºC.

Congo red agar was described as screening biofilm formation by bacterial strains. This medium consisted of brain heart infusion broth (BHI) supplemented with 5% sucrose and congo red stain (0.8 g/L). The serial dilutions were prepared by using 1 mL of a sewage water sample. 100 µL from five and eight-fold dilutions were spread on congo red agar. And plates were incubated for 24 h at 37°C (Mathur et al., 2006; Ivana et al., 2015; Lima et al., 2017). After incubation, the colors of colonies on plates were observed. The dark red or blackish colonies with dry or crystalline consistency and red colonies with a smooth and darkened appearance in the center were evaluated as biofilm producers; biofilm non-producers, respectively (Lima et al., 2017). Dark red or blackish colonies were selected and strains were identified by applying morphological (Gram staining) and standard microbiological procedures (indole, methyl red, voges proskauer, citrate, and mug agar test systems). The isolates were identified by comparing with standard description reported in Bergey's Manual of Determinative Bacteriology.

Determination of biofilm formation

Characterization of biofilm morphotype: Colony morphology of biofilms was tested on Luria Bertani (LB) agar without salt and supplemented with Congo red (40 µg/mL) as an indicator stain. Biofilm producer strains were inoculated into Luria Bertani (LB) broth without NaCl. Test tubes were incubated for 24 h at 37°C. After overnight growing, 10µL, 20µL and 50µL of cultures were

transferred by dripping onto the agar surface. The plates were incubated for 168 h at 37°C. Following the incubation period, colony morphology of biofilms was stated as rdar (red, dry and rough), bdar (brown, dry and rough), pdar (pink, dry and rough) and saw (smooth and wet) (Akyıldız, 2015; Ivana et al., 2015).The experiment was separately performed in triplicate.

Tube adherence method (TM): Biofilm production at the liquid medium was analyzed by the tube adherence method. A loopful from an overnight culture of test organisms was transformed into the tubes including 10 mL of Trypticase soy broth (TSB) with 1% glucose and incubated for 24-168 hours at 37°C. And then, tubes were gently poured and washed with phosphate buffer saline pH 7.4. The tubes dried in the air and at room temperature were stained with 0.1% crystal violet. The excess of stain in tubes was removed by running water. The intensity of color adhered to the wall and bottom of the tubes dried in inverted position was observed for biofilm formation. According to the depth of color, biofilm formation was detected as weak, moderate, and strong (Christensen et al., 1982; Mohamed et al., 2016).

Tissue culture plate test (TCP): This method was spectrophotometrically investigated biofilm formation. The bacterial suspensions were acquired by growing in TSB with 1% glucose for 24 h at 37°C. The wells of sterile flat-bottomed 96-well polystyrene tissue culture plates were filled with 200 µL of these suspensions. 150 µL of the non-inoculated sterile medium was added to the wells and plates were then incubated 168 h at 37°C. After incubation, the content of wells was removed and the adherent biofilm layers in the inoculated wells were stained with 150 µL of with crystal violet (1%) for 30 min. The stains in the wells were poured and wells were washed by distilled water three times. After washing, the biofilm matrix was fixed by waiting with 200 µL of ethanol-acetone (70:30 w/w) mixture for 30 min. Optical density (OD) of stained adherent biofilm was measured by using microplate reader at 595 nm. For each bacteria, test was performed in triplicate. According to the absorbance difference between the average values of optic density of the inoculated and control wells (ODc), biofilm formation was categorized: weak biofilm: ODc<OD≤2xODc Moderate biofilm: 2xODc<OD≤4xODc and Strong biofilm: 4xODc<OD (Hassan et al., 2011; Ozdemir and Arslan, 2018).

ESBL production of strains

Production ESBL of biofilm producer strains was tested by double disc synergy assay. The turbidity of the overnight bacterial culture was adjusted to 0.5 McFarland standard reference range. Following the inoculation on Mueller Hinton Agar (MHA), the plates were incubated at 37ºC for 24 h. 30 µg of Cefotaxime (CTX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), and Cefoxitin (FOX) discs were placed 20 mm apart from each other and amoxicillin-clavulanic acid disc on the centre of plates. After incubation at 37ºC for 18-24 h. Enhancement of inhibition zones around test discs towards to amoxicillin-clavulanic acid disc and being wider (by ≥ 5mm) from inhibition zones of test discs on MHA plates without amoxicillin-clavulanic acid disc were indicated to ESBL production.

10 µg of imipenem (IMP) and meropenem (MER) standard discs were used for metallo beta lactamase (MBLs). 10 µL of 0.5 M EDTA was embrued on these standart discs. The plates were incubated at 37ºC for 18-24 h. The being broader (by ≥ 7mm) of inhibition zone diameters around the combined discs (IMP+EDTA; MER+EDTA) than discs without EDTA was a positive result.

Scanning electron microscopy analysis of biofilm

Authocleved glass shards with a smooth surface were added to test tubes including TSB. A loopful from an overnight culture of test organisms was inoculated to test tubes. After 168 h incubation, the biofilm formation on dried glass surfaces in the air was observed by using scanning electron microscopy without fixation protocol. The surface of the sample covering-stubs was coated by a gold particle (Quorum Q150R Sputter Coater). It was viewed by using scanning electron microscopy (FEI Quanta FEG 650) at 10 kV accelerating voltage.

RESULTS and DISCUSSION

Biofilm formation was analyzed by the Congo red agar test. In a total, 5 bacterial strains produced biofilm were isolated from Kilis State Hospital sewage and entitled C1, C2, C5, C6 and C7. Figure 1 is shown the strains with colonies dark red or blackish colonies on CRA. Bacteriological profiles of the isolates associated with biofilm production were determined for C1 and C2 as *Enterococcus* spp.; C5 as *Enterobacter* spp.; C6 as *Escherichia* spp.*; C7* as *E. coli.*

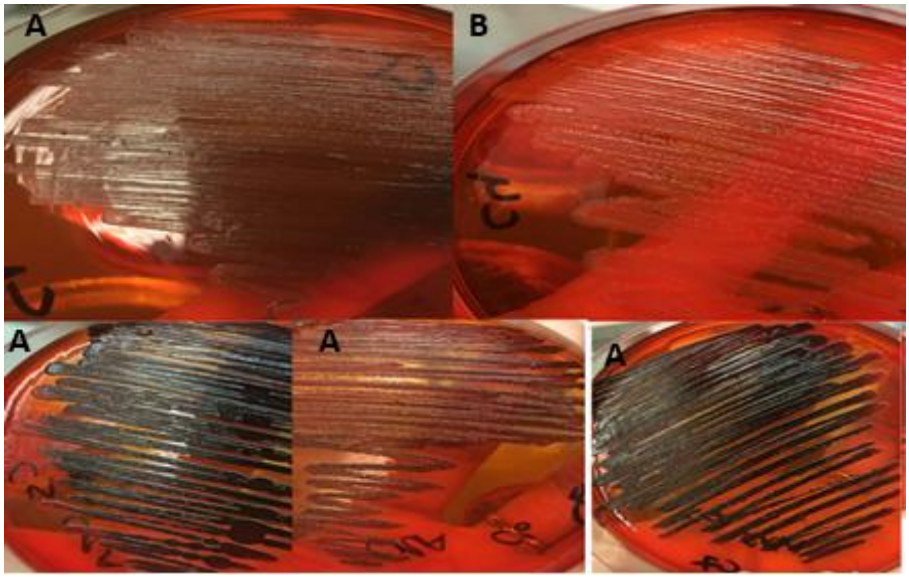

Figure 1. Colony colors of biofilm formation on CRA plates indicating biofilm producers of black and brown colonies; non-biofilm producers of red colonies

The presence of black colonies correlated with biofilm formation on CRA has been reported by other authors. The biofilm production of *S. epidermidis*, *P. aeruginosa*, *E. coli*, *Candida* spp. *and Staphylococcus* spp. isolates showing colonies with black colors on CRA plates were determined (Mathur et al., 2006; Darwish and Asfour, 2013; Kaiser et al., 2013; Saxena et al., 2014; Nachammai et al., 2016; Lima et al., 2017; Kırmusaoglu, 2017). The CRA method used to identify biofilm formation in different strains has advantages such as speed, reproducibility, and preservability. For this reason, the CRA method is the first analysis chosen to demonstrate of the biofilm formation ability. CRA plates are also performed to visualize the individual biofilm morphology.

The morphotype of biofilm colonies onto CRA plates was classified based on the colony color and morphology analysis. The morphology of all biofilm colonies is demonstrated in Figure 2.

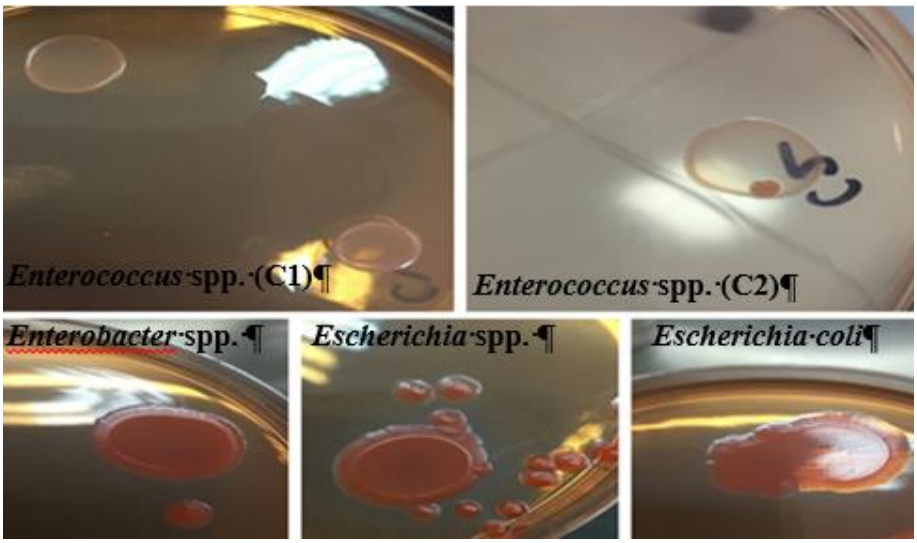

Figure 2. Screening of biofilm morphotype on CRA plates

Enterococcus spp. strains and the other ones were showed bdar type morphology categorized with brown, dry, and rough colonies. In biofilm positive *S. aureus* strains, biofilm characterization with dry and smooth black; dry and smooth red morphology was noted by Darwish and Asfour (2013). The similarity to our result, bdar, rdar, and pdar biofilm morphologies for *Salmonella* spp strains were observed (Karaca, 2011). In another study, the biofilm formation *Salmonella* spp. strains having bdar

and rdar type morphology was exhibited by Akyıldız (2015). It is declared that the isolates having rdar, bdar and pdar biofilm morphotype produced cellulose and curli fimbriae, curli fimbriae, and cellulose as extracellular matrix components, respectively (Karaca, 2011; Akyıldız, 2015) Therefore, the biofilm producer strains isolated in our study can be expressed to be curli fimbriae producing. This fimbriae type finds on the extracellular surface of the many bacteria such as *Escherichia*, *Enterobacter* and *Salmonella* spp. belong to *Enterobacteriaceae*. (Barnhart and Chapman, 2006).

Concerning biofilm production on CRA plates, the biofilm formation ability of the strains was tested by tube adherence and tissue culture plate methods. The analyzed isolates were categorized as non-adherence, weak, moderate and strong producer based upon crystal violet binding of biofilm adhered to surfaces.

By the standard tube adherence analyses, biofilm characterizations after 24-168 h incubation period are depicted in Figure 3.

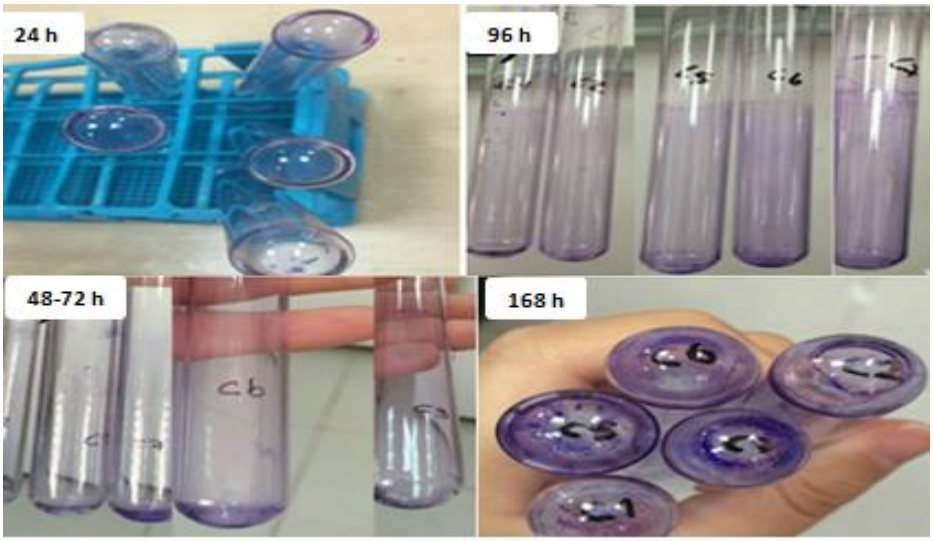

Figure 3. Categorization of biofilm production by using tube adherence method (TM)

In the first 24 h, none of the analyzed strains was not formed biofilm. Following 48-72 h, while *Enterobacter* spp., *Escherichia* spp., and *E. coli* produced moderate biofilm, biofilm of *Enterococcus* spp. strains were weak. *E. coli* was the strongest biofilm producer between strains within 96 h by adhering more cells. *Enterobacter* spp., one of *Enterococcus* spp. (C2) and *Escherichia* spp. formed a moderate biofilm. Biofilm characterization of *Enterococcus* spp. strain (C1) showed similarity for 96 and168 h: weak adherence. *Enterococcus* spp. (C2) and *E. coli* were determined as strong biofilm producers after 168 h incubation period. This observation is stated to need a long incubation period for adhering more cells to create a biofilm.

Table 1. Comparison of biofilm adherence of clinic strains by standard tissue culture plate method (TCP)

Strains	**Biofilm formation** **Mean OD values**	**Adherence**
Enterococcus spp. (C1)	0.177	Weak
Enterococcus spp. (C2)	0.828	Strong
Enterobacter spp.	0.372	Moderate
Escherichia spp.	0.466	Moderate
Escherichia coli	0.674	Strong

Similar results to tube adherence method were recorded in tissue culture plate analyses in this study. Quantification of biofilm production is expressed in Table 1/Figure 4.

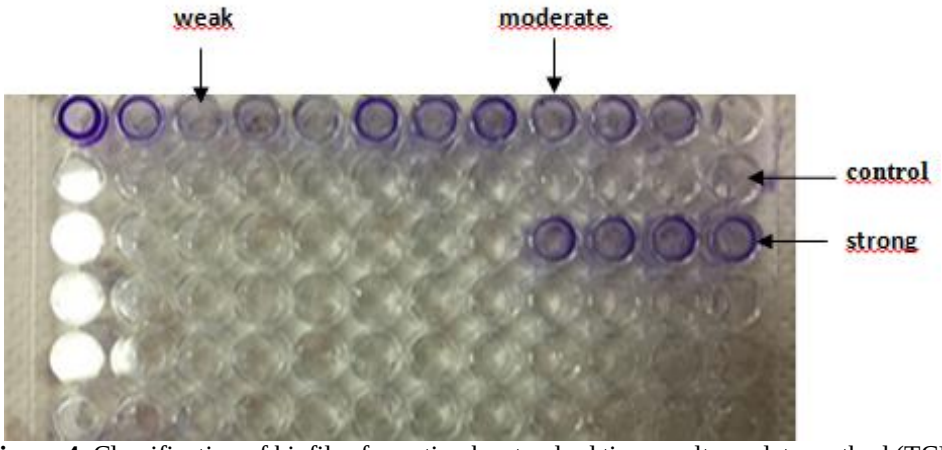

Figure 4. Classification of biofilm formation by standard tissue culture plate method (TCP)

The strains of strong biofilm producers were *Enterococcus* spp. (C2) and *E. coli* with 0.828 and 0.674 optic density, respectively. *Enterococcus* spp. strain (C1) was considered to be weak biofilm producers according to TPC method. This is indicated the low expression of genes responsible for the synthesis of surface materials that provide adhesion and the synthesis of EPS in weak biofilm producer strains. *Enterobacter* spp. and *Escherichia* spp. were classified as moderate producers.

Similar data was recorded in a previous study showing strong/high biofilm production of %27 out of 121 *Staphylococcus* sp. isolates (Kırmusaoglu, 2017). Similarly, Kristich et al. (2004) indicated a significant biofilm accumulation for *Enterococcus faecalis*. In the other study, 22, 60, and 70 from 152 *Staphylococcus* spp. isolates were considered to be strong, moderate, and non or weak biofilm producers in TCP method (Mathur et al., 2006). Asati and Chaudhary (2018) determined that 160 strains including *Klebsiella* spp., *Escherichia coli, Citrobacter* spp., *Proteus* spp. *Enterobacter* spp. showed biofilm formation by using TCP and modified TCP method.

The results obtained in this research are similar to the other studies which determined none or weak biofilm formation by *E. coli* after 24 hours of incubation (Adamus-Białek, 2015). As similar to our results regarding incubation time, Dias et al. (2018) detected that *K. pneumoniae* produced weak biofilms after the first 24 h incubation. This formation capability increased at 48 h.

Hassan et al. (2011) identified biofilm producer *S. epidermidis, E. faecalis, E. coli, K. pneumoniae* and *S. aureus* clinic strains by using three standard methods (CRA, TM and TCP) as similar to our data's.

Our results related to biofilm produced *Enterococcus* spp. were similar to others which indicated strong and weak adherent by *E. faecalis* strains isolated from urinary tracts (Zheng et al., 2018).

After 168 h incubation on glass, SEM micrograph of biofilm formation is viewed in Figure 5.

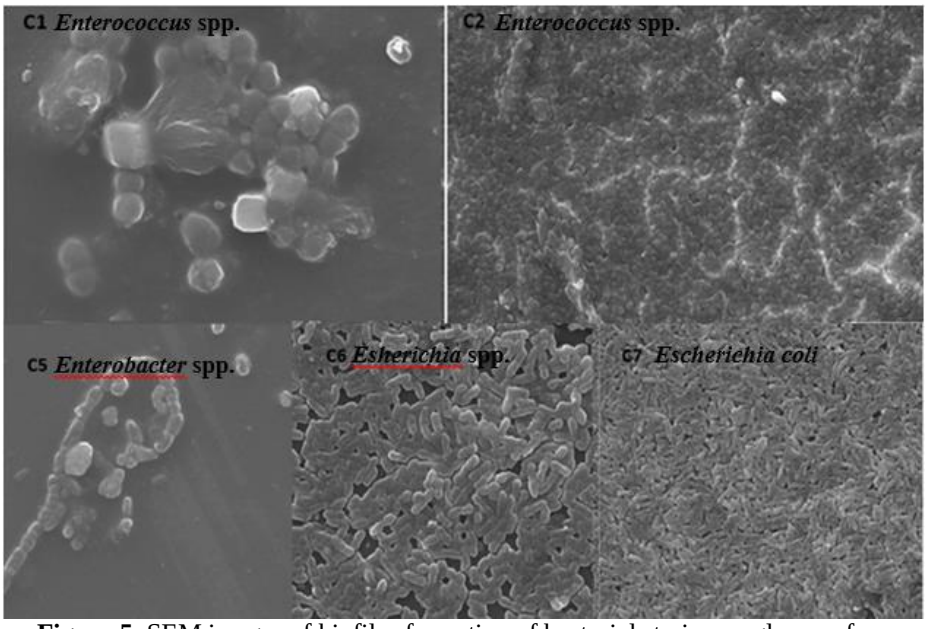

Figure 5. SEM images of biofilm formation of bacterial strains on glass surface

Regarding biofilm formation, *Enterococcus* spp. (C2) and *E. coli* was the stronger biofilm producer strains. This characterization is indicated the high amount of adhered cells on the glass surface for theirs. According to these results, the SEM analysis showed a good correlation with the TCP and TM assay for biofilm-forming characterization. In opposition to the adhered cells, *Enterococcus* spp. strain (C1) and *Enterobacter* spp. were observed to form a slimy biofilm as individualized cells and not encase on the glass surface. SEM micrographs revealed that *Enterobacter* spp. produced weak biofilm on the glass surface by contrast with TM and TPC. For SEM analysis, the variations concerning biofilm formation between the different strains of the same genus in addition to different genus were observed. This can be explained by the different responses of bacterial strains to the external parameters such as population density, incubation time and surface produced biofilm.

Variations regarding ESBL and metallo-beta lactamase production between the biofilm formation strains were detected. *Enterococcus* spp. (C1), *Enterobacter* spp. and *Escherichia* spp. showed a low and moderate ability to biofilm formation were exhibited to not produce ESBL and metallo-beta lactamase. Even if the density of the biofilm was high, the ESBL and MBL expressions were not observed. The association between antimicrobial drug susceptibility pattern and biofilm formation was only noted for *Enterococcus* spp. (C2) strain. Despite of ESBL producer, it was found to not synthesize MBL. The antibiotic resistance of *Enterococcus* spp. (C2) was observed to increase depending on the association between ESBL and biofilm production by comparison with other isolates.

CONCLUSION

With regard to *Enterococcus* spp., biofilm formation by ESBL producing strains has been rarely demonstrated by the other authors. Generally, biofilm formation researches have been worked on *Enterobacteriaceae* strains causing multidrug-resistant infections. Because of this, our studies regarding ESBL expression of clinic *Enterococcus* spp. isolates produced biofilm preferred research. However, the drawback of our results is the lack of determination of biofilm, ESBL, and MBL production by using molecular applications.

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