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An Assay for Detection of Uropathogenic Escherichia coli Biofilm using Calcofluor

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Abstract: *The biofilm is a community of microorganisms found adhered to a surface, growing in an exopolysaccharide matrix whose composition varies between bacterial species. It has been reported that chronic infections are closely related to the formation of biofilm; it represents a defense mechanism in which bacterial cells survive the bacterial stress, to host immune response and even resist some antimicrobial agents. The calcofluor white is a fluorophore that binds to glycosidic bonds β (1-3) and β (1-4), generally used for identifying various species of fungi. This paper shows an assay to the detection of uropathogenic E. coli biofilm using the calcofluor staining.*

Keywords: *Biofilm, Detection, Bacteria, Calcofluor white, Exopolisacharide.*

1. INTRODUCTION

The biofilm is a community of microorganisms that are attached to a living or inert surface, having a extracellular matrix synthesized in the biofilm. Also it can also be found in a gas-liquid interface (Costerton, 1999; Costerton *et al.*, 1995; Characklis and Marshall, 1990; Donland, 2002; Donland and Costerton, 2002; Flores-Encarnación *et al.*, 2014; Hall-Stoodley *et al*., 2008; Nazar, 2007). The biofilm consists of an extracellular matrix composed of proteins, DNA, exopolysaccharides, bacteria and water (98% volume approximately) (Nazar, 2007; Sack *et al.*, 2014). The water, nutrients and waste pass through small channels formed between the bacterial extracellular matrix (Flores-Encarnación *et al*., 2016; Kalbbach *et al*., 1997; Nazar *et al*. 2007; Zhang *et al.*, 2012). The history of Microbiology has focused on studying microorganisms in planktonic state (those free-floating cells that are dispersed in the environment where they grow) (Costerton, 1999; Costerton *et al.*, 1995). However, it has been observed that there are metabolic differences between cells in biofilm state and planktonic state (Donland, 2002; Nazar, 2007). The biofilm represents an old strategy prokaryotic survival which offers advantages such as protection against environmental fluctuations (temperature and pH changes, nutrient concentrating and facilitating the removal of waste (Donland and Costerton, 2002; Post, 2001).

Currently, bacterial infectious diseases have gained particular interest because of their capacity to produce chronic infections, which in many cases respond poorly to treatment with antibiotics (Castrillón *et al*., 2010; Donland and Costerton, 2002; Foxman, 1990). It has been reported that the biofilm allows bacteria to be more resistant to action of antibiotics and even they are able to evade the host immune response (Bjarnsholt, 2013; Nazar, 2007; Stewart and Costerton, 2001). It has been reported that one of the factors that has contributed to the development of chronic infections and repeat offenders is the biofilm formation. So it has been observed the formation of bacterial biofilm in otitis, endocarditis, periodontitis, osteomyelitis, cistitis, orthopedic devices, among others (Barbara and Rabih, 2004; Castrillón *et al*., 2010; Christensen *et al*., 1985; Foxman, 1990; Kanamaru *et al*., 2006; Post, 2001). The bacteria commonly found in biofilm and associated with infectious processes are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, *Ureaplasma urealyticum*, some fungi species belonging to the genus *Candida* sp. and *Aspergillus* sp. (Barbara and Rabih, 2004; Castrillón *et al*., 2010; Donland and Costerton, 2002; Foxman, 1990; Foxman *et al*., 2000; Kanamaru *et al*., 2006).

One of the most common techniques for the detection of biofilm is the plaque assay using violet crystal described by Christensen *et al*., (1985) and O'Toole and Kolter (1998). However, not all clinical diagnostic laboratories have this technology. The calcofluor white is a fluorophore which binds to bonds β (1-3) and β (1-4) of exopolysaccharides (Ramos *et al*., 2006). It has been widely used for identifying fungi and yeast (García *et al*., 2001; Ramos *et al*., 2006). The present study aimed to assay of a rapid method for detection of bacteria forming biofilm using calcofluor white staining.

2. MATERIAL AND METHODS

2.1. Bacterial Strain

A strain of uropathogenic *Escherichia coli* from a clinical isolate was used. As reference *E. coli* K12 strain was used. In both cases the strains were stored into cryovials at -40°C until analysis.

2.2. Culture Conditions and Calcofluor Assay

The LB broth (Luria Bertani: tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) was used for bacterial culture. The exopolysaccharides of biofilm were stained with calcofluor white according to methodology described by Ramos *et al.*, (2006). For that, a total of 20 µL of uropathogenic *E. coli* and *E. coli* K12 overnight incubated starter cultures were extended on 0.02% calcofluor white/LB agar plates. Plates were incubated at 37°C during 24-48 hours into a chamber to keep moisture. A similar procedure was performed using test tubes containing 0.02% calcofluor/LB broth. The test tubes were incubated at 37°C during 24-48 hours. The biofilm of gas-liquid interface was evidenced by the addition of 0.1% crystal violet on the surface of the culture broth. Also it was tested the calcofluor staining in a microplate assay. For that, 10 µL of an uropathogenic *E. coli* overnight incubated starter culture was transferred to a pre-sterilized 96-well polystyrene microtiter plate containing 200 µL of 0.02% calcofluor/LB broth for well. The microtiter plate was incubated for 24-48 hours at 37ºC. Then, the plates and probe tubes were exposed to UV light and the fluorescence emitted by exopolysaccharides of cells forming biofilm was observed. Assays on all samples were repeated in duplicate.

2.3. Calcofluor white Staining

For calcofluor white staining the bacterial sample was obtained from uropathogenic *E. coli* strain growing on LB agar plate. The sample was placed on a glass slide and then the sample was stained with 0.02% calcofluor white. The glass slide was incubated at room temperature for 20 min in the dark and it was then exposed to UV light. The light emission confirmed the presence of exopolysaccharides in the samples. Subsequently, the samples were observed at 40x in a fluorescence microscope using an exciter filter 460 nm and 500 nm barrier filter. Assays on all samples were repeated in duplicate.

3. RESULTS

The uropathogenic *E. coli* and *E. coli* K12 strains were grown on 0.02% calcofluor white/LB agar plates. Plates were incubated at 37°C during 24-48 hours. As shown in Fig. 1A and Fig. 1B, 0.02% calcofluor not inhibited the growth of uropathogenic *E. coli* and *E. coli* K-12 strains on LB agar plates. The cells de *E. coli* absorbed the calcofluor dye and good growth was recorded. When culture plates were exposed to UV light, cells forming biofilm were identified by fluorescence emission related to presence of exopolysaccharides (Fig. 1C and Fig. 1D). Fluorescence was more intense along the seed line of the bacteria. Furthermore fluorescence on the surface of tested culture medium was observed, which suggested that exopolysaccharides spread in the culture medium. As shown in Fig. 1, the biofilms from uropathogenic *E. coli* and *E. coli* K-12 were recorded. The fluorescence emitted by the control plate (without inoculum) was negligible, so it was found that LB agar components not interfered in the detection of exopolysaccharides by this technique (data not shown). Also the calcofluor staining was carried out in test tubes containing 0.02% calcofluor/LB broth. The results obtained are shown in Fig. 2. Fig, 2A shows the test tubes containing *E. coli* in active growing. The biofilm formation of *E. coli* was observed in the gas-liquid interface by adding violet crystal. However, *E. coli* biofilm was weak and moved to the bottom of the culture medium (Fig. 2A). Fig. 2B and Fig. 2C show the probe tubes exposed to UV light. The results showed that the fluorescence emission was located at the bottom of the test tubes containinig *E. coli* in active growing and in the bottom walls of the test tube, while the gas-liquid interface showed no significant fluorescence emission. The fluorescence emitted by the culture medium used as control (without inoculum) was

negligible (Fig. 2B, Fig, 2C, left). The microplate assay for calcofluor staining was done for both strains. Fig. 2D shows the results obtained when the plate was exposed to UV light. In Fig. 2D is shown the fluorescence emitted by *E. coli* cells located in a well of polystyrene microtiter plate. The fluorescence emission was located at the walls and bottom of well containinig *E. coli* in active growing. The fluorescence emitted by the culture medium used as control (without inoculum) was negligible (Fig. 2D, top). On the other hand, the calcofluor direct staining was carried out from samples of uropathogenic *E. coli* strain growing on LB agar plates. The sample was placed on a glass slide, it was stained with calcofluor and then exposed to UV light. The Fig. 3A shows the fluorescence obtained from the samples of *E. coli*. As it can be seen, the calcofluor technique quickly detected the fluorescence of cells forming biofilm, confirming the presence of exopolysaccharides in the samples. When the samples were observed at 40x in a fluorescence microscope, the cell clusters were observed emitting fluorescence (Fig. 3B).

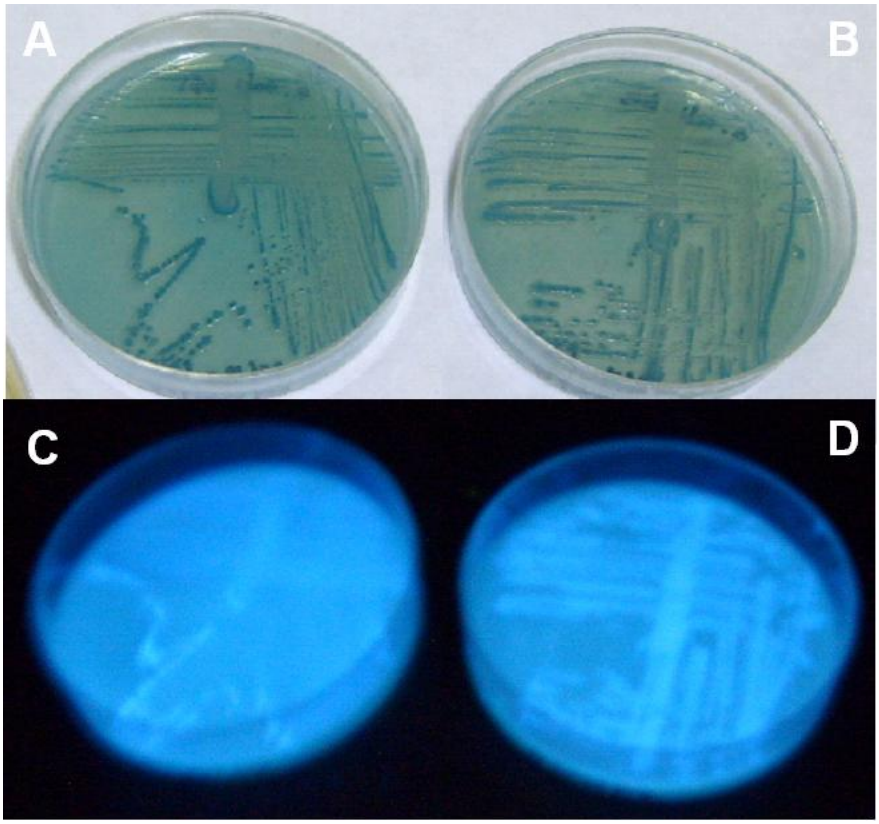

Fig1. *The uropathogenic E. coli (A, C) and E. coli K12 (B, D) strains growning on calcofluor/LB agar plates. The plates were not exposed to UV light (A and B). The plates were exposed to UV light (C and D).*

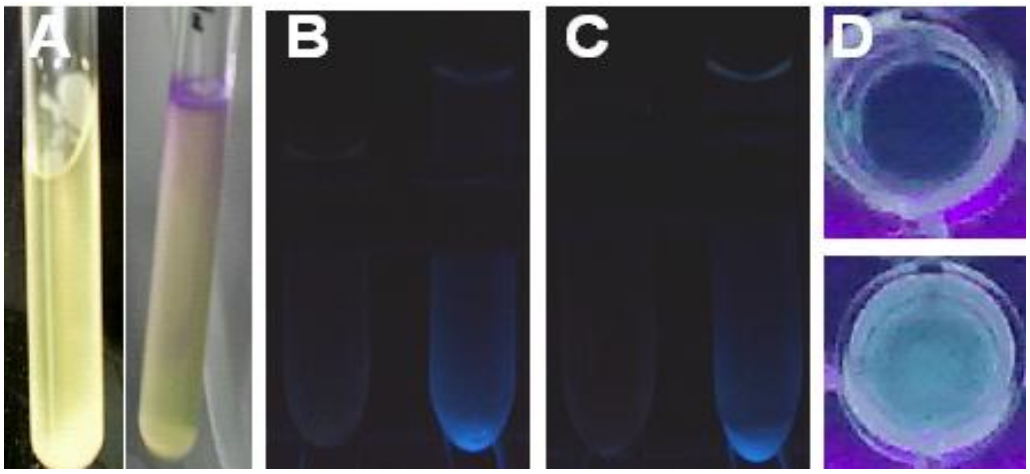

Fig2. *The calcofluor staining of uropathogenic E. coli and E. coli K12 forming biofilm in test tubes. Biofilm of E. coli observed in the gas-liquid interface (A). Fluorescence emission of uropathogenic E. coli and E. coli K-12 (Right: B and C); Fluorescence emission of only culture medium (Left). D. Microplate assay for calcofluor staining; only culture medium (Top) and uropathogenic E. coli forming biofilm in well (Botton).*

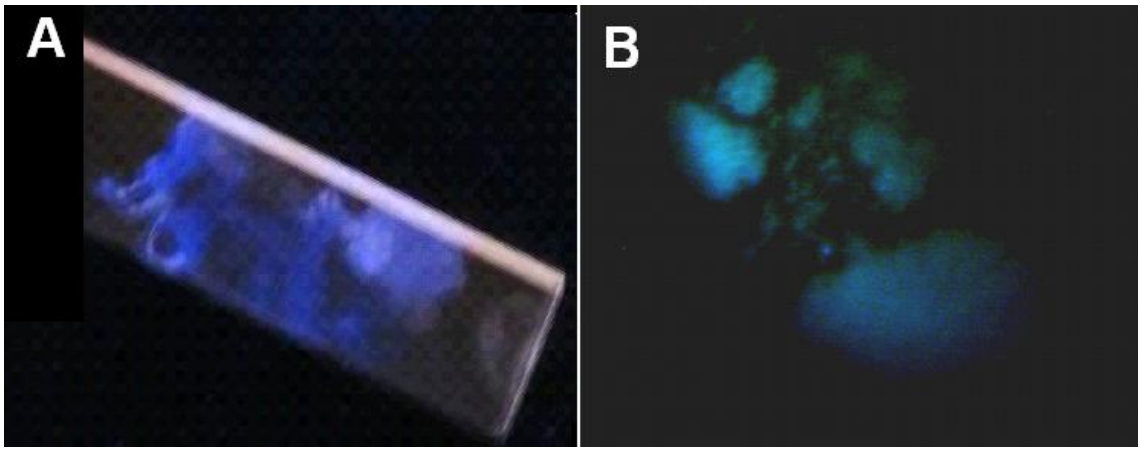

Fig3. *The calcofluor direct staining. Uropathogenic E. coli placed on a glass slide and exposed to UV light (A). Cell clusters observed in fluorescence microscope (B).*

4. DISCUSSION

Bacteria can live in planktonic (free living) or biofilm state. In biofilm, bacteria are attached to a surface, where proliferate themselves, aggregate and secrete exo-polysaccharides and other substances (Naher *et al.*, 2014). Bacteria inside the biofilm show properties that are different from their free-living state (Costerton, 1999). The biofilm structure confers the bacteria more resistant against environmental stresses, antibiotics and host immune (Donland and Costerton, 2002; Marin, 2009; Serra *et al*., 2013). On the other hand, it has been reported that bacterial biofilm is measured through a technique using violet crystal; that staining has been reported by many authors. The violet crystal staining provides a biofilm qualitative assay because it evidences the adherence to the walls of the containers used in the assay. The violet crystal stains bacterial organic matter attached to the test surfaces, while calcofluor staining has confirmed that it is indeed biofilm. The calcofluor white is a fluorescent dye that binds in the glycosidic linkages β-(1-3) and β-(1-4); it is generally used for to observe the exopolysaccharides in biofilm (Ramos *et al*., 2006). The violet crystal provides a good measure of biofilm mass. However, it does not give a measure of biofilm viability (Welch *et al*., 2012). In this paper, the production of bacterial exopolysaccharides was indicative of the viability of bacteria forming biofilm. The exopolysaccharides were detected using calcofluor white in cells of uropathogenic *E. coli*. As shown in Fig. 1, the uropathogenic *E. coli* and *E. coli* K-12 strains growth on calcofluor/LB agar plates and the growth not inhibited by calcofluor. The assay showed the viability of the bacteria when the plates were exposed to UV light. By the fluorescence emission the polysaccharides from bacterial strains were observed. This is consistent with studies by other authors in different biological models (Brandl *et al.*, 2011; Eriksson de Rezende *et al.*, 2003; Mathur *et al*., 2006). It has been reported that calcofluor white binds to cellulose (for example: *E. coli* exopolysaccharide) (Brandl *et al*., 2011; Serra *et al*., 2013). Therefore fluorescence colonies on calcofluor agar denoted the bacterial cellulose production as some authors have described (Monteiro *et al*., 2009; Zogaj *et al*., 2001). In this work, biofilm of *E. coli* in the gas-liquid interface and botton from test tubes was observed as it described in other bacteria (Spiers *et al*., 2003) (Fig. 2). The fluorescence emission was related with cellulose production for uropathogenic *E. coli*. As shown in Fig. 2, the uropathogenic *E. coli* and *E. coli* K-12 strains formed biofilm in test tubes, however the fluorescence intensity produced by calcofluor was poor. The results showed that *E. coli* biofilm was weak, it moved to the bottom of test tubes and it had poor adherence to glass. In microplate assay, fluorescence intensity emitted by *E. coli* cells in polystyrene wells was higher than fluorescence emitted by *E. coli* cells in glass (Fig. 2D). This difference is according with studies reported by other authors (Donland, 2002; Moreira *et al*., 2015; Ryu and Beuchat, 2005; Van Houdt and Michiels, 2010). Apparently the best result was obtained using polystyrene as support material to do this test. It has been reported that polystyrene is used by bacteria to form biofilm easily (Chaves-Simões *et al*., 2010). Finally, the uropathogenic *E. coli* strain extended on a glass slide was stained with calcofluor and then exposed to UV light. The results shown that calcofluor quickly detected to cells forming biofilm by presence of exopolysaccharides. To fluorescence microscope the cells of uropathogenic *E. coli* were organized in clusters (Fig. 3), which is consistent with studies by other authors. Rowe *et al*., (2010) reported that uropathogenic *E. coli* forms large biofilm aggregates when it was grown in iron-restricted tissue culture media.

5. CONCLUSION

The calcofluor agar showed significant detection of exopolysaccharide present in biofilms of uropathogenic *E. coli*. The use of this technique is suggested for rapid detection of bacteria forming biofilm. Also the fluorescence microscopy facilitates identification of biofilm-forming strains, having a corroboration of the presence of bacterial polysaccharides in clusters.

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