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Study of biofilm formation by ESBL producing and non-producing clinical isolates of *Klebsiella pneumoniae*

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ABSTRACT

This study was conducted to explore the biofilm forming capabilities of extended spectrum beta-lactamase (ESBL) producing and non-producing *Klebsiella pneumoniae* strains by three different methods and to compare the methods as well. Two ESBL producers and two non-producers strains were subjected to biofilm detection methods. These isolates were previously confirmed by standard methods and API (Analytical Profile Index) scoring. Their ESBL production capabilities were confirmed by double discs synergy test as recommended by the Clinical and Laboratory Standards Institute. Biofilm formation was detected by Congo Red Agar method (CRA), Test Tube method (TTM) and Microtiter Plate Assay method (MTP). Both the ESBL producing *Klebsiella pneumoniae* were characterized as strong biofilm formers. Between the two ESBL non-producers, one was assessed as weak and the other as moderate biofilm former by quantitative TTM and MTP. The MTP and quantitative TTM were considered superior to qualitative TTM and CRA method. Our findings highlighted the relatively higher biofilm forming ability by the ESBL positive *Klebsiella pneumoniae* that may additionally contribute to their resistance against extended spectrum antibiotics. We can conclude that *K. pneumoniae* strains, isolated from blood, to form biofilm have a significant association with ESBL production.

Introduction

The incident that free-living microorganisms are capable of continuing their life attached to a surface was first described through a phenomenon known as 'bottle effect' [1]. It then took more than 3 decades to accept that for microorganisms the biofilm mode of life is a rule not an exception [2-3]. Biofilms can be seen as consortia of microorganisms that are attached to a biotic or abiotic surface and its formation is a multistage process in which microbial cells adhere to the surface while an extracellular matrix is produced subsequently results in a firmer attachment [4-5].

Biofilm is a complex polymer matrix composed of cells and matrix material that can accumulate on medical devices and immune-compromised or dead tissues [6]. Biofilm formation is often considered the underlying reason of treatment failure with an antimicrobial agent and an estimated 65–80% of all infections are thought to be biofilm-related [3, 7-8]. The involvement of *Klebsiella pneumoniae* in biofilm-related infections is well known and this bacterium is one of the commonest species known to produce extended spectrum beta lactamase (ESBL) among the Enterobacteriaceae [9-10]. ESBL producing gram-negative bacteria are considered as the causative agents of acute and chronic infections and are responsible for high mortality and morbidity rate as well as high treatment cost [11-12].

In biofilm, penetration rate of antimicrobial agents is low and the transfer of genetic material such as conjugative plasmid is relatively higher than under planktonic conditions [3, 6]. Since ESBL producing capability is usually plasmid mediated, these phenomena make it possible to hypothesize that ESBL producing *Klebsiella pneumoniae* are more likely to be biofilm formers.

There are various methods to detect biofilm formation. These include the Microtiter Plate Assay (MTP) method [13], Test Tube method (TTM) [14], Congo Red Agar method (CRA) [15], bioluminescent assay [16], piezoelectric sensors [17] and fluorescent microscopic examination [18]. We designed the current research to investigate the biofilm forming abilities of ESBL producing and non-producing *K. pneumoniae* strains by three different methods (CRA, TTM and MTP). This study also compared all three methods for the detection of biofilms.

Materials and methods

Bacterial strains

Four *Klebsiella pneumoniae* were subjected to biofilm detection, which were isolated from the blood cultures performed at Chittagong Maa Shishu O General Hospital. These isolates were confirmed as *Klebsiella pneumoniae* using standard methods and API scoring (Analytical Profile Index, 20E strip, BioMerieux) and their ESBL production was confirmed by double discs synergy test as recommended by the Clinical and Laboratory Standards Institute and described in a thesis work [19]. Among the 4 *Klebsiella pneumoniae* taken for the current study, 2 were ESBL producers (designated as 'ESBL1+' and 'ESBL 2+') and 2 were ESBL non-producers (designated as 'ESBL 1-' and 'ESBL2-').

Inoculum preparation

The *K. pneumoniae* isolates were sub-cultured into Luria Bertani (LB) broth (Difco, USA) individually and incubated at 37°C for 20 hours. After incubation, the broths were centrifuged and the pellet cells were dissolved in normal saline solution until the turbidity of the solution matched with McFarland turbidity

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standard number 0.5 to give counts of approximately 10^8 cells/ml.

Congo Red Agar method (CRA)

Freeman *et al.* [15] have described a simple qualitative method to detect biofilm formation by using Congo Red Agar (CRA) medium. This CRA medium contains 3.7% (w/v) brain heart infusion (BHI) base (Oxoid, UK), 5% (w/v) sucrose, 1.5% agar (Oxoid, UK) and 0.8 % (w/v) Congo red. Congo red stain was prepared as concentrated aqueous solution and autoclaved (121°C for 15 minutes), separately from other medium constituents and was then added to the medium when it had cooled to 55°C. The CRA plates were inoculated with test organisms and incubated aerobically for 24 hours at 37°C. Positive result is indicated by black colonies with a dry crystalline consistency. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicates an intermediate result [20].

Test Tube method (TTM)

A qualitative test for biofilm formation was done by the modified method of Christensen *et al.* [14]. Test tube containing 9 ml of LB broth or nutrient broth (NB) were inoculated with 1 ml fresh inoculum and then incubated at 37°C for 24, 48 and 72 hours separately. After incubation, the tubes were decanted and washed with phosphate buffer saline (PBS, pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%). Then excess stains were removed and tubes were washed with deionized water. The tubes were dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formed was scored as 0-absent, 1-weak, 2-moderate or 3-strong.

Beside visible observation, a quantitative assessment biofilm formation was also performed by a modified method [21]. After incubation the broth was discarded and the test tubes were washed with deionized water and stained the with 1% (w/v) crystal violet solution. Then the tubes were kept in room temperature for 30 minutes. After that, excess stain was removed by deionized water and air-dried. Then 5ml 95% ethanol was added to the test tubes and waited for 30 minutes. Ethanol solubilized the remaining crystal violet attached to cells. Finally, the optical density (OD) of the retained dye was measured by spectrophotometer (UV-VIS RS spectrophotometer, LaboMed. Inc) at 600 nm. Negative control was maintained without inoculating the media with bacteria. The experiment was carried out in triplicate, repeated three times and mean values were expressed. The interpretation of biofilm production was done according to the criteria of Stepanovic *et al.* [22] (Table 1).

Microtiter Plate Assay method (MTP)

This method was described by Christensen *et al* [13] and was considered as standard quantitative test for biofilm formation. Freshly prepared 20 µl of inoculum and 180 µl of NB/LB/BHI broth were dispensed in the wells of sterile 96 well flat-bottomed microtiter plates and kept for incubation at 37^0C for 48 hours. Only broth served as control to check the sterility and non-specific binding of media. After incubation, content of each well was gently removed by tapping the plates. The wells were washed three times with 0.2 ml of PBS. Subsequently, adhered cells were fixed for 30 min at 80^0C. Adhered cells were stained by addition of 220 µl of 1% (w/v) crystal violet for 1 min. The stain was removed by exhaustive washing with deionized water. The plates were then allowed to dry. In order to quantify adhered cells, 220 µl of decolorizing solution (95% ethanol: acetone = 8:2) was added to each well and waited for 15 min. The optical density (OD) was measured at 590 nm by using ELISA reader (ELx 800, Absorbance Microplate Reader, BioTek Instruments Inc.). The interpretation of biofilm formation was done according to the criteria of Stepanovic *et al.* [22] (Table 1).

Table 1. Interpretation of biofilm formation

Average OD Value	Biofilm formers
OD_C> OD	Non
OD_C< OD < 2 x OD_C	Weak
2 x OD_C< OD < 4 x OD_C	Moderate
4 x OD_C< OD	Strong

Results:

Congo red agar (CRA) showed typical colonies

We found black colonies with a dry crystalline consistency in CRA plates for ESBL producers, which was an indication of the strong biofilm formers. Black colonies were appeared without dry crystalline appearance in the plates of ESBL non-producing *Klebsiella pneumoniae*, which meant they were intermediate biofilm formers. No pink colonies (weak or non-formers) were appeared in the CRA media (Figure 1).

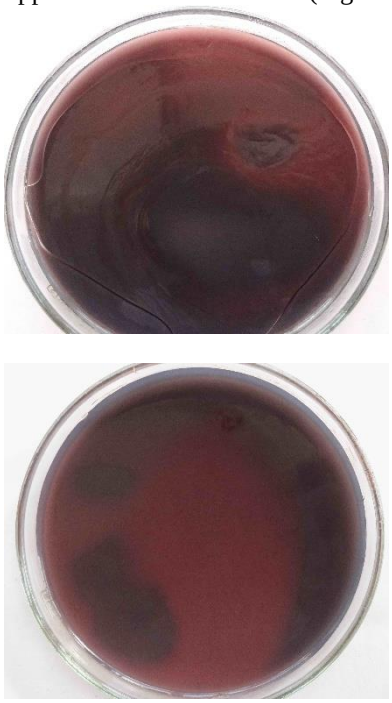

Figure 1. CRA plate showing characteristics growth of 'ESBL1+' (Top) and 'ESBL1-' (Bottom)

Test tube assay method (TTM)

In TTM qualitative assessment, ESBL producers were seemed to be strong biofilm formers by visible observation whereas ESBL non-producers were moderate. The TTM quantitative assessment was seemed to be more precise than visible observations. In LB broth, biofilm forming capacities of the ESBL producers were found to be higher than the ESBL non-producers. We tested biofilm formation in three different incubation periods. Incubation period was found to be an important factor in biofilm formation because increased in incubation period resulted in increased OD in both NB and LB broth media. After 72 hours of incubation, the OD values reached top. 'ESBL1+' was the strongest biofilm formers among the test organisms and both the ESBL producers were strong biofilm formers by this method. 'ESBL1-' was the weakest while 'ESBL2-' was relatively better biofilm former and

counted as moderate biofilm former. On day 3, a six to seven times increase in the OD of ESBL producing *Klebsiella pneumoniae* in comparison to negative control was seen in the result (Figure 2).

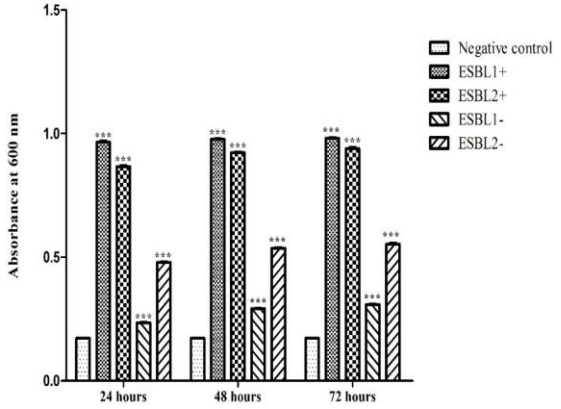

Figure 2: Biofilm formation by ESBL producing and non-producing *Klebsiella pneumoniae* while grown in LB broth. The data is representative of three independent experiments. Here, *** = P< 0.001, P = significance level.

A little increase in biofilm formations by the test organisms was observed when we used NB in comparison to LB broth and incubation period showed the same positive impact. The ESBL non-producers showed significantly less biofilm formation than the ESBL producers (Figure 3).

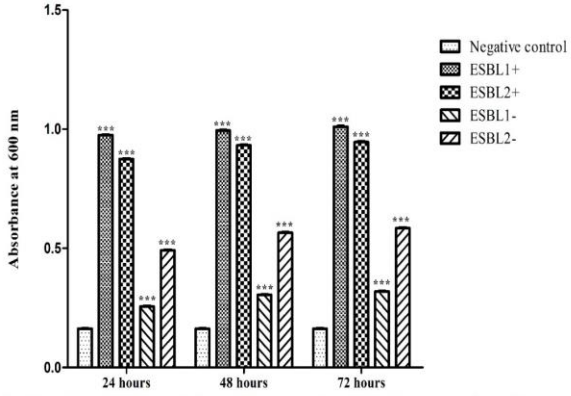

Figure 3: Biofilm formation by ESBL producing and non-producing *Klebsiella pneumoniae* while grown in Nutrient broth. The data is representative of three independent experiments. Here, *** = P< 0.001, P = significance level.

Microtiter plate assay method (MTP)

This standard test was carried out in three different media namely LB broth, NB and BHI broth and the impact of these media on biofilm formation was explored. Neither of the investigated bacteria were assessed as non-biofilm producer disregarding the media used. The highest OD values were obtained in BHI medium. In all media, both the ESBL producing *Klebsiella pneumoniae* were quantified as strong biofilm formers. Between the two ESBL non-producers one was classified as weak (ESBL1-) and the other as moderate biofilm former (Table 2).

Table 2. Mean optical density (OD) measured in three repeated tests in microtiter plates in nutrient broth (NB), LB and BHI broth

Isolate	OD in LB	OD in NB	OD in BHI
ESBL1+	0.726***	0.743***	0.813***
ESBL2+	0.694***	0.708***	0.737***
ESBL1-	0.242*	0.271*	0.298*
ESBL2-	0.487**	0.525**	0.561**
Control	0.168	0.151	0.171

*- weak biofilm former; **- moderate biofilm former; ***- strong biofilm former

Discussions

Klebsiella pneumoniae of being strong, moderate, weak biofilm formers or non-formers have been reported in many studies [6, 23-24]. While working on 54 clinical isolates of *K. pneumoniae*, Sanchez *et al.* [25] determined 76% were positive for biofilm formation. In our study ESBL producing and non-producing *K. pneumoniae* were assessed as strong and weak or moderate biofilm formers respectively by quantitative TTM and MTP methods. The MTP method was considered as the gold standard for this study and compared with the observations from TTM and CRA methods.

By CRA method, we could not characterize any strain as weak biofilm former though one ESBL non-producer isolate was assessed as weak biofilm former by TTM and MTP methods. There are opinions in both sides whether the CRA method is reliable for detecting biofilm formation or not. Some researchers reported to find a fine consistency between CRA and other biofilm detecting methods and found it reliable [26-27], but Nagaveni *et al.* [28] concluded that CRA method is not remarkable compare to TTM and MTP. Hassan*et al.* [29] found *Klebsiella pneumoniae* isolates as strong biofilm formers but while comparing among the methods, CRA showed very little correlation with test tube and microtiter plate methods with a very low sensitivity (11%), specificity (92%) and accuracy (41%). Based on our observations we also cannot recommend the CRA method as a suitable method for categorizing the biofilm formers as strong, moderate or weak but as screening method to detect biofilm formation. We found the qualitative assessment by TTM is convenient in detecting biofilm formers in general but by naked eye observations, it had been difficult to grade the biofilm formers due to the changeability in the results detected by different observers. On the other hand, quantitative assessment by TTM correlated well with MTP method for identifying strong, moderate and weak biofilm formers. In accordance with the previous studies [14, 30] qualitative TTM cannot be suggested as general screening test to identify biofilm producing isolates though we can strongly recommend quantitative TTM is as good standard test as MTP method.

Yang D*et al.* [31] investigated the biofilm and ESBL producing capability of 150 *K. pneumoniae* clinical isolates and discovered that 83.6% of biofilm positive strains had the ability to produce ESBL. Our ESBL producing *K. pneumoniae* were strong biofilm former and ESBL negatives were weak or intermediate biofilm formers. We can conclude that *K. pneumoniae* strains from blood to form biofilm have a significant association with ESBL production. Scientists tried to explain the reason for the higher rate in biofilm positive among ESBL producers strains which may be due to the nature of biofilm where sharing of genetic material is high [32] and induction of ESBL by low antibiotic concentration which is a function of low penetration into biofilm [33].

Conclusions

We found the ESBL producing *Klebsiella pneumoniae* as strong and the ESBL non-producers as weak or moderate biofilm formers. To our knowledge, this study is the first to evaluate and compare biofilm formation by ESBL producers and non-producers. Our findings highlight the relatively higher biofilm forming ability by the ESBL producing *Klebsiella pneumoniae* that may additionally contribute to their resistance against extended spectrum antibiotics. As biofilm facilitates the transfer of plasmid rapidly, the ESBL non-producers, which were found to be moderate or weak biofilm formers, may

acquire this ESBL producing ability and may emerge as multidrug resistant strains.

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