**CORRELATION BETWEEN ANTIBIOTICS RESISTANCE AND BIOFILM FORMATION BY KLEBSEILLA PNEUMONIAE**

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**ABSTRACT**

Klebseilla pneumoniae is important gram negative opportunistic pathogenic bacteria associated with nosocomial acquired infections. Aim of the presented study was isolation and identification of Klebseilla pneumoniae and evaluate the prevalence of Klebseilla pneumoniae biofilm formed in microtiter plate by a spectrophometric assay as a risk and virulence factor. Also, study the resistance of this bacteria to many antibiotic which used in therapeutic, a fifty samples were collected from different patients from many Hospitals in Baghdad city during the period from February-August 2015. Klebsiella isolates (50 isolates) were as follows: sputum (14), urine (10), burn (9), wound (9) and blood (8). All isolate were identification by culturing on (blood, MacConkey) agar and analyzed according to morphological and biochemical tests, API 20 E system as well as vitek 2 system. Biochemical test performed were catalase, Oxidase, IMViC Test (including: indole test, methyl red, vogas prosquar and citrate utilization) as well as urease production, and fermentation of sugars (glucose, sucrose, lactose and manitol, additional to H2S production on Triple sugar iron), so the antimicrobial resistance testing was performed for 12 different antibiotics. Most of isolates were obtained from sputum samples by 28 % (14 isolates) followed urine 20 % (10 isolates) while both of burn and wound have 18 % (9) and finally blood infection 16 % (8 isolates). So (54%) of the isolates were produced strong biofilm, and (22%) were moderate produced biofilm, while (24%) were weak or non-produced biofilm. Bacterial strains were highly resistant to CEF (94.8,90.9)% respectively in K. pneumoniae form and non-form biofilm respectively, so more sensitive (0%) to GEN and IMP in form and non-form biofilm respectively, Also, K. pneumoniae positive biofilm showed (89.7, 7.6, 89.7, 94.8, 74.3, 46, 0, 0, 25.6, 56.44,
58.94, 58.94, 35.8, 64.1 and 76.9)\% respectively resistance to Ampicillin, Amikacin, Amoxicillin; Ceftriaxone, Cefotaxime; Ciprofloxacin; Gentamicin, Imipenem; nitrofurantoin; pipracillin; tobramycin; tetracycline; Norfloxacin; Nalidixic acid and trimethoprim/sulfamethoxazole respectively, compared to the (81.8, 0, 81.8, 90.9, 63.6, 27.2, 0, 27.2, 54.5, 18.1, 36.3, 18.1, 54.5 and 27.2) \% respectively resistance by non-form biofilm for the respective antibiotics. Conclusion: Our data indicate almost all isolates of Klebsiella pneumoniae form biofilm and these bacteria were more resistance to antibiotics comparative to isolate that non-form biofilm.

**KEYWORDS:** biofilm; Klebsiella pneumoniae, polystyrene microtiter plates, Antibiotics.

**INTRODUCTION**

Formation and development of biofilm (microbial city) by bacteria is important stage in the pathogenesis of Klebsiella, and it is relatively common phenomenon among many pathogenic bacteria which is development depending on many biological, chemical and physical factors [Bryers, 2008], biofilm composed of polysaccharides, proteins, DNA, surfactants, lipids, glycolipids, membrane vesicles and ions such as Ca\(^{2+}\) [Donlan and Costerton, 2002], the main molecule that responsible for intercellular adhesion is polysaccharide intercellular adhesion (PIA) which conceder as crucial virulence factors, also poly-N-acetylglucosamine (PNAG) [Mack, 1996].

Severity of K. pneumoniae infections causing by virulence factors as fimbriae type (1 and 3), LP, iron-scavening systems, urease and ability to produce many types of adherence factors, capsular polysaccharides, which conceder essential virulence of Klebsiella, these capsular polysaccharides help adhered bacteria to the epithelial cells as well as formed the biofilms on surfaces [Tarkkanen et al., 1992], so material of capsular formed fibrillous which covered the surface of bacteria, and it prevents killing bacteria by bactericidal serum factors so, protects it from phagocytosis [Amako, et al., 1988], also treC and sugE genes have been demonstrated to affect biofilm formation by modulating CPS production [Wu, et al., 2011].

Biofilms cause chronic infections because they causing increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of host immune system [Hoiby, et al., 2010], therefore strains which formed biofilm may cause problems in therapy [Wojtyczka, 2014], so increased resistant of Klebsiella pneumoniae to antibiotics caused complicated therapy of this infections, so high concentrations of antibiotics...
with Prolong treatment are normally needed to removing or reducing the biofilms (Holder, 1993).

K. pneumoniae can carried asymptomatically in throat, nose, skin and intestinal tract of healthy individuals but can also cause a range of infections in hospitalized patients [Brisse et al, 2006].

METHODS

1-Clinical specimens
Two hundred twenty five samples were collected from patient in many Hospitals of Baghdad city during the period from February to August 2015. The Klebsiella isolates (50 isolates) were as follows: sputum (14), urine (10), burn (9), wound (9) and blood (8).

2- Bacterial isolate
All samples collected from specimen (Blood, Burn, Wound, Urine and sputum) were analysed for K.Pneumoniae according to morphological on blood and maCconkey and identification by using api-20 and vitek2-Compact System.

3-Biochemical test
Biochemical test performed were catalase, Oxidase, IMVIC Test (including: indole test, methyl red, vogas prosquar and citrate utilization) as well as urease production, and fermentation of sugars. Sugar fermentation test performed were glucose, sucrose, lactose and manitol, additional to H2S production on Triple sugar iron.

4- Antimicrobial resistance
Antimicrobial resistance testing was performed for 12 different antibiotics by disk diffusion (Kirby bauer method), using the standard protocol for diffusion of antimicrobial agents on Mueller-Hinton agar as described in National Committee for Clinical Laboratory Standards NCCLS guidelines (CLSI, 2012). The antibiotics tested included: Ampicillin, Amikacin, Amoxicillin; Ceftriaxone, Cefotaxime; Ciprofloxacin; Gentamicin, Imipenem; nitrofurantoin; pipracillin; tobramycin; tetracycline; Norfloxacin; Nalidixicacidand Trimethoprim/sulfamethoxazole.

5- Biofilm assay on polystyrene microtiter plates
Ten ml of trypticase soy broth (TSB) with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. The TSB broth was incubated at 37oC for
24 hours, culture was diluted 1:100 with fresh medium and filled with 200µl of diluted cultures individually flat bottom tissue culture plates (96 wells), and incubated at 37°C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 200µl of phosphate buffer saline (pH 7.2) four times to remove free-floating bacteria. Biofilms which remained adherent to the walls fixed with 2% sodium acetate and stained by 0.1% crystal violet, then washed with deionized water to removed Excess stain, Eventually, the absorbance was read at a wavelength of 550 nm using Micro ELISA Reader. Experiments were performed in duplicate and the average of OD values of sterile medium were calculated [Mathur et al, 2006].

Statistical analysis
Analyzed data by using SPSS 17 version.

RESULTS AND DISCUSSION
K. pneumoniae have been known to coexist in wound, burn, cystic fibrosis, urinary tract infections and respiratory tract [Childers et al. 2013].

![Figure1: API 20 E system of Klebsiella pneumoniae code 5214773.](image)

The result in figure 1 showed API 20 E system used for identification of Klebsiella pneumoniae which has serial code number as 5214773, these results are quite consistent with the results of Mohammed (2012).
Table 1: Number and percentage of Klebsiella pneumoniae isolated from clinical source.

<table>
<thead>
<tr>
<th>Clinical source</th>
<th>Total sample</th>
<th>Klebsiella pneumonia isolate Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>50</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>Urine</td>
<td>50</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Burn</td>
<td>50</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Wound</td>
<td>50</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Blood</td>
<td>50</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

The results in table (1) showed that most of the isolates were obtained from sputum samples by 28% (14 isolates) followed by urine 20% (10 isolates) while both burn and wound have 18% (9) and finally blood infection 16% (8 isolates). This finding was also observed by Ansari et al. (2014) who have ensured that 6 (13.04%) were K. pneumoniae isolated from different clinical specimens as blood, pus and wounds.

The rate of Klebsiella infection increases urinary tract infection because the capsules of these bacteria are important virulent factors (Roberts, 2000). Similarly Hennequin and Forestier (2007) reported in a previous study the association of biofilm producing bacteria with colonization of urinary catheters [Hennequin and Forestier, 2007].

Table 2: Biochemical test for Klebsiella pneumoniae.

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Indole test</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>-</td>
</tr>
<tr>
<td>Vogas prosquar</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Urease production</td>
<td>+</td>
</tr>
<tr>
<td>Kligler iron agar (KIA)</td>
<td>Acidic/Acidic, with gas,No H2S</td>
</tr>
<tr>
<td>Lactose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 showed all isolate of Klebsiella pneumoniae have positive results for biochemical tests as Catalase, Vogas prosquar, Citrate utilization, Urease production and Lactose fermentation, while negative for Oxidase, Indole test, Methyl red as well as produced Acidic/Acidic, with gas, without H2S nor motility.

Mohammed showed in his study all isolate of Klebsiella pneumoniae have Oxidase negative and catalase positive, H$_2$S in not produced, reduce nitrates [Mohammed, W.F.2012].
Figure 2: Biofilm of Klebsiella pneumoniae by microtiter plate (Mtp)

In figure (2) appearance the levels of biofilm formed by K. pneumoniae isolates by Microtiter plate assays. Higher levels (strong) of biofilm formation were (No. = 27), moderate were (No. =11) and weak or non- biofilm form (No. =12).

Figure3. Distribution of Klebsella pneumoniae isolates according to biofilm Formation by microtiter plate (M.T.P).

The results observed in figure (3) that (54%) of the isolates were produced strong biofilm, so (22%) were moderate produced biofilm, while (24%) of the isolates were non or weak produced biofilm ,biofilm form that classification was based according to Mathur et al., 2006. This result was conformed to Ansari et al. (2014) who showed strongly positive of biofilm formed by microtitre assay in 2 (33.33%) Klebsiella spp., while the remaining isolates were either moderate adherent 1 (16.67%) or weak/non-biofilm producers 3 (50%). The importance of trec in gastrointestinal tract colonization suggests that biofilm formation contributes to the establishment and persistence of K. pneumoniae infection. While Yang and Zhang showed in his study 40% of K. pneumoniae isolated from wound swabs, sputum, urine, blood were reported to be able to form an in vitro biofilm [Yang and Zhang, 2008], so about 63% of K. pneumoniae isolates from urine samples form biofilm [Niveditha, etal, 2012].
Table 3 Results of Resistant test of Klebsiella pneumoniae (form and non-form biofilm) isolates against Antibiotics.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Number of isolates (50)</th>
<th>Resistant of Klebsiella pneumoniae (form biofilm) to antibiotics</th>
<th>Resistant of Klebsiella pneumoniae (non-form biofilm) to antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number (39)</td>
<td>%</td>
</tr>
<tr>
<td>AMP</td>
<td>35</td>
<td>89.7</td>
<td>9</td>
</tr>
<tr>
<td>AMK</td>
<td>3</td>
<td>7.6</td>
<td>0</td>
</tr>
<tr>
<td>AMO</td>
<td>35</td>
<td>89.7</td>
<td>9</td>
</tr>
<tr>
<td>CER</td>
<td>37</td>
<td>94.8</td>
<td>10</td>
</tr>
<tr>
<td>CEF</td>
<td>29</td>
<td>74.3</td>
<td>7</td>
</tr>
<tr>
<td>CIP</td>
<td>18</td>
<td>46</td>
<td>3</td>
</tr>
<tr>
<td>GEN</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IMP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NIT</td>
<td>0</td>
<td>25.62</td>
<td>3</td>
</tr>
<tr>
<td>PIP</td>
<td>22</td>
<td>56.44</td>
<td>6</td>
</tr>
<tr>
<td>TOB</td>
<td>23</td>
<td>58.94</td>
<td>2</td>
</tr>
<tr>
<td>TET</td>
<td>23</td>
<td>58.94</td>
<td>4</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>10</td>
<td>25.62</td>
<td>3</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>25</td>
<td>64.1</td>
<td>6</td>
</tr>
<tr>
<td>Trimethoprim/ sulfamethoxazol</td>
<td>30</td>
<td>76.9</td>
<td>3</td>
</tr>
</tbody>
</table>

AMP=Ampicillin, AMK=Amikacin, AMO=Amoxicillin, CER=Ceftriaxone, CEF=Cefotaxime, CIP=Ciprofloxacin, GEN=Gentamicin, IMP=Imipenem, NIT=nitrofurantoin, PIP=pipracillin, TOB=tobramycin, TET=tetracycline,

The antibiotic resistance pattern of fifty isolates from different clinical source were screened for their resistance to fifteen used antibiotics, table 3 showed the percentages of resistant this bacteria which form biofilm to antibiotics were as follow: 94.8% to CER, 89.7% to both AMP and AMO, So (76.9, 74.3) % respectively to Trimethoprim/Sulfamethoxazole and CEF respectively, while low resistance to AMK (7.6%) while no resistance (0%) compare to that non-form biofilm the percentage of resistance to antibiotics as 90.9% to CER and 81.8% to AMP, AMO but low resistance (18.1%) to TOB and norfloxacin, So 27.2% to CIP, NIT and Trimethoprim/Sulfamethoxazole but no resistance (0%) to AMK, GEN and IMP.

The present results showed klebseilla form biofilm for all antibiotics higher than non-form biofilm, Mohamed, 2012 showed in his study the Klebsiella pneumoniae are resistant to Ampicillin (AMP), Piperacillin/Tazobactam(PIT), Cefazolin(CZ) and Cefotaxime(CTX) while no resistance to Imipenem(IMP) and Amikacin(AK), In Taiwan Lee et al., (2004)

The results show the antibiotic resistance pattern of Klebsiella pneumoniae in samples from different clinical sources.
reported that Klebsiella pneumonia (10 isolates) resist to AMP (100%), CZ (50%), CTX (50%), CAZ (40%), GEN (50%), AK (20%) and IMP (0%), so Seyyed et al., (2011) noted that Klebsiella pneumoniae percent resisted to GEN, AMP, CTX, AK, CIP, and IMP respectively as (100, 100, 96, 70, 55, 10) % respectively.

In present study showed K. pneumoniae producers biofilm showed (89.7, 7.6, 89.7 , 94.8, 74.3, 46.0, 0, 25.6, 56.44, 58.94, 58.94, 35.8, 64.1 and 76.9)% respectively resistance to Ampicillin, Amikacin, Amoxicillin; Ceftriaxone, Cefotaxime; Ciprofloxacin; Gentamicin, Imipenem; nitrofurantoin; pipracillin; tobramycin; tetracycline; Norfloxacin; Nalidixic acid and trimethoprim/sulfamethoxazole respectively, compared to the (81.8, 0, 81.8, 90.9, 63.6, 27.2, 0, 0, 27.2, 54.5, 18.1, 36.3, 18.1, 54.5 and 27.2)% respectively resistance by biofilm non-producers for the respective antibiotics. These results are quite consistent with Subramanian et al, who found in his study K. pneumoniae producers biofilm showed 93.3%, 83.3%, 73.3% and 80% resistance to nalidixic acid, ampicillin, cefotaxime and co-trimoxazole, respectively, compared to the 70%, 60%, 35% and 60% resistance shown by biofilm non-producers for the respective antibiotics [Subramanian, etal., 2012].

While AL-Talib (2006) showed in his study Klebsiella pneumonia resisted as (100%) to both CAZ and IMP while (86%) to GEN and (29%) to CTX. These differences in resistance of some antibiotics may be occurred in some genera or species depending on the drug of choice that has been adopted in that hospitals and the time of using in addition to the nature of the strains. Mechanisms of resistence to antibiotics differ according to the antibiotics themselves (Landgren et al., 2005), as well as isolates which form-biofilm more resistant at least 10 times than their planktonic counterparts (Bellifa, S.et al., 2013).

One of the most commonly studied properties of biofilms is their increased resistance to the effects of antibiotics (Liaqat et al., 2009). Because of cells embedded in biofilms may include limited diffusion of antibiotics into the biofilm or decreased bacterial growth, also some antibiotics can react with matrix of biofilm, as well as, these biofilm functions as a diffusion barrier for some antimicrobial drug, and increasing resistance to antibiotics (Vuotto etal. 2014). On the other hand, cells in biofilms can adapt and form protected phenotypes (Monzon et al., 2001), as well as components of biofilm matrix form a mechanical shield and act to inhibit the effect of antibiotics(Anahit etal.,2015).

Antibiotic resistance is encoded by several genes, many of them can transfer between bacteria
[Jessica, 2015] therefore high resistance to antibiotics in biofilm-associated infections are very difficult to treat (Hall-Stoodley et al., 2004), cause more hazard of this pathogen in hospital settings.

**CONCLUSION**

Our data indicate the bacteria isolate form biofilm were more resistance to antibiotics from isolate that non-form biofilm.

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