

## Study on biofilm formation by Extended Spectrum $\beta$ -lactamase (ESBL) producing *Klebsiella* isolates

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### ABSTRACT

Infections caused by multidrug-resistant *Klebsiella* species that produce Extended-Spectrum  $\beta$ -lactamase (ESBL) enzymes have been reported with increasing frequency in intensive-care units and are associated with significant morbidity and mortality. Because of the high resistance to numerous antimicrobial agents, treatment can be problematic. Moreover formation of microbial biofilms has imposed a serious problem in treatment of microbial infections using conventional antibiotics. This has prompted researchers to identify alternatives such as plant products as antimicrobial agents. Researches on plant derived natural antimicrobials agents, has exclusively focused more on their effects against planktonic micro-organisms, however, the biofilm forms are more resistant to antimicrobial agents and therefore more difficult to control.

In this study we investigated the prevalence of (ESBLs) among *Klebsiella* isolates, the prevalence of biofilm formation among these isolates and the effect of five natural essential oils on inhibition of biofilm formation. Out of the 102 *Klebsiella* isolates, 52 isolates were (ESBL), and 44 isolates were strong and moderate biofilm producer. ESBL production was more common among strong and moderate biofilm producers; this probably is due to high rate of conjugation inside the biofilm, which facilitates transfer of drug resistant genes. Out of the five commercial essential oils investigated for their effect on inhibition of biofilm formation the Eucalyptus oil showed the maximum inhibition of biofilm formation followed by garlic oil, clove oil, ginger oil and black seed oil.

**Key Words:** *Klebsiella*, Extended Spectrum  $\beta$ -lactamase (ESBL), Biofilm, Essential oils.

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### INTRODUCTION

*Klebsiella* spp are opportunistic pathogens primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus. Nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, the medically most important species of the genus. To a much lesser degree, *K. oxytoca* has been isolated from human clinical specimens (Podschun and Ullmann, 1998).

$\beta$ -lactams antibiotics are widely used in the treatment of many bacterial infections and this has resulted in considerable selection pressure for emergence of resistance to the  $\beta$ -lactams (El-Daker *et al.*,2002). Although

several species of bacteria including *Klebsiella* are naturally susceptible to extended-spectrum cephalosporins these organisms acquire resistance to these antibiotics by several mechanisms, which include the production of ESBLs under the selection pressure of the extensive use of expanded-spectrum cephalosporins in clinical practice. The most abundant types of enzymes are represented by the genes SHV, TEM, CTX-M and OXA (Jacoby and Bush 2006). Infection with ESBL producing bacterial strains are encountered singly or in outbreaks, especially in critical care units (Shah *et al.*, 2004). Systemic infections with ESBL producing Enterobacteriaceae are associated

with severe adverse clinical outcomes (Canton *et al.*, 2002; Schwaber *et al.*, 2006).

Biofilms are currently defined as structured bacterial communities enclosed in a self-produced exopolysaccharide matrix and adherent to abiotic or biotic surface (Costerton *et al.*, 1995). Formation of biofilms by *K. pneumoniae* on urinary catheters, intra-venous catheters and prosthetic heart valves has been documented (Farber and Wolff, 1993; Galdiero *et al.*, 1987; Liu, 1993). Biofilm producing bacteria are responsible for many recurrent infections and are difficult to eradicate. They exhibit resistance to antibiotics by various mechanisms like restricted penetration of antibiotic into biofilms, decreased growth rate and increased expression of resistance genes (Kim, 2001). Most of the antibiotics used against *K. pneumoniae* have been rendered ineffective due to the biofilm formation (Stewart and Costerton, 2001). In order to limit the growth of bacterial biofilm, natural compounds are tested to replace or to be used in combination with the available chemotherapeutic agents (Mathur *et al.*, 2013). Many essential oils have been used for a long time to kill different infectious pathogens. Most of the essential oils from Rosewood, Cedarwood, Lime, Orange, Rosemary, Sage, etc, have been shown to have bactericidal effect on *Klebsiella pneumoniae* (Hammer *et al.*, 1999), but few of them, such as, *Cuminum cyminum* seed essential oil (Derakhshan *et al.*, 2010), have shown inhibition for biofilm formed by *Klebsiella pneumoniae*. The present study aimed to evaluate the susceptibility of *Klebsiella* isolates to different antibiotics, to investigate the prevalence of (ESBLs) among clinical isolates, the prevalence of biofilm formation and to study the effect of some natural essential oils on inhibition of biofilm formation.

## **MATERIAL and METHODS**

### **Bacterial isolates**

A total of two hundreds and fifteen clinical and thirty five environmental specimens were collected from different sources. Clinical isolates were obtained from urine, sputum, blood, pus, wounds swabs, catheters, diabetic foot and burns. Two environmental isolates are obtained from outside the hospital. These specimens were obtained from patients admitted to Zagazig and Mansoura University Hospitals during the period from April 2011 to November 2011. One hundred clinical isolates were identified as genus *Klebsiella*.

*Klebsiella* produce large, pink, mucoid colonies on MacConkey agar, were citrate positive and non-motile. *K. pneumoniae* is indole negative while *K. oxytoca* is indole positive. For long term maintenance of *Klebsiella* isolates, 400 µl of overnight culture were mixed with 800 µl of 50% glycerol and stored at -80 °C.

### **Antibiotic susceptibility tests**

Antibiotic sensitivity of the isolated *Klebsiella* strains was carried out by the disc diffusion assay according to Clinical and Laboratory Standard Institute (CLSI, 2013).

The antibiotic discs were obtained from Oxoid, UK. The tested antibiotics were Ofloxacin (OFX, 5µg), Gentamicin (CN, 10µg), Aztreonam (ATM, 30µg), Imipenem (IPM, 10µg), Sulphmethoxazole/Trimethoprim, (SXT, 25µg) Amikacin (AK, 30µg), Cefepime (FEP, 30 µg), Cefoperazone / Sulbactam (SCF, 105µg), Ceftriaxone (CRO, 30µg), Meropenem (MEM, 10µg), Piperacillin /Tazobactam (TPZ, 10µg) and Tetracycline (TE, 30µg).

### **Phenotypic detection of Extended Spectrum β-lactamases (ESBLs) by modified double disc synergy test.**

Isolates resistant to one of the following antibiotics (ceftriaxone, aztronam, cefipime, ceftazidime and cefotaxime) were subjected to the modified double disc synergy test (MDDST) according to Jarlier *et al.* (1988), using a disc of amoxicillin-clavulanate (20/10

$\mu\text{g}$ ) along with 3<sup>rd</sup> generation cephalosporins (cefotaxime and ceftazidime). Briefly a pure separate colony of each isolate were transferred into a tube containing 4-5 ml of Muller Hinton broth (MHB). The broth was incubated at 37°C, when the turbidity approximately equivalent to  $1 \times 10^8$  cells/ml, it was further diluted 1:200 in broth to obtain inoculum density ranged between  $10^5$ - $10^6$  cells/ml. A sterile cotton swab was dipped into the bacterial suspension. The surface of a dried Muller Hinton agar plate was streaked with the inoculated swab in three different directions. The inoculated plates were left to dry, then a disc of amoxicillin-clavulanate (20/10  $\mu\text{g}$ ) was placed in the centre of the plate. The discs of cefotaxime and ceftazidime were placed 15mm apart, centre to centre to that of the amoxicillin-clavulanate disc. Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate is considered as positive for ESBL production.

#### **Quantitative assessment of biofilm by spectrophotometric method**

All isolates were screened for their ability to form biofilm by micro-titer plate method as described by Stepanovic *et al.*, (2000) and Abdi-Ali *et al.*, (2005), with a modification in the duration of incubation which was extended to 48 hours. Overnight cultures of isolates from tryptic soya agar plates were inoculated in tryptone soya broth. The turbidity was adjusted to approximately 0.5 McFarland turbidity standard. Broth cultures were further diluted to 1:200 in broth to obtain inoculum density ranged between  $10^5$ - $10^6$  cells/ml. Aliquots of 100  $\mu\text{l}$  were distributed in 96-well polystyrene flat-bottomed micro-titer plates containing 100 $\mu\text{l}$  of tryptone soya broth with 0.25% glucose (TSB-glu) and incubated in stationary conditions for 48 hours at 37°C. Negative control wells are included. The content of each well was aspirated and washed three

The effect of different concentrations of oil on biofilm forming ability was tested in

times with sterile phosphate buffer saline (PBS), PH 7.3 to remove any non-adherent bacteria and then left to dry. 200  $\mu\text{l}$  of 99% methanol was added to each well for 15 minutes for fixation of adherent bacteria. Then, the wells were decanted, left to dry, and stained with 100  $\mu\text{l}$  of 2% Hucker crystal violet solution for 15 minutes. Excess stain was rinsed off gently by water. After the plates were air dried, the dye bound to the adherent cells was re-solubilized with 100  $\mu\text{l}$  of 33% (v/v) glacial acetic acid. The Optical Density (OD) was measured at 490 nm using spectrophotometer (UV-1800 Shimadzu, Japan). For each strain, the mean OD of three wells was calculated (ODt). The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control wells. The level of biofilm production was determined according to the following:

1. Non biofilm producer (0)  $\text{ODt} \leq \text{ODc}$
2. Weak biofilm producer (+) =  $\text{ODc} < \text{ODt} \leq 2 \times \text{ODc}$ ,
3. Moderate biofilm producer (++) =  $2 \times \text{ODc} < \text{ODt} \leq 4 \times \text{ODc}$
4. Strong biofilm producer (+++),  $4 \times \text{ODc} < \text{ODt}$

#### **Screening for anti-biofilm agents**

##### **Determination of minimum inhibitory concentration (MIC) of essential oils by agar dilution method**

The minimum inhibitory concentrations (MICs) of the oils under investigation were determined by agar dilution method. Oils from natural sources as Garlic, Clove, Black seed, Ginger and Eucalyptus oil were used. MIC of oils carried out according to CLSI (2011) with some modification by addition Tween-80, 0.5% (v/v) to enhance the solubility of oils. Plates with Tween-80 without any oils were used as control.

##### **Effect of essential oils on *Klebsiella* strong and moderate biofilm producers**

polystyrene flat-bottomed microtitre plates as described Hammer *et al.*, (2005) and Nostro *et*

al., (2007) with some modifications. As a treatment solution, the concentrations of oils were prepared in TSB with 0.25% glucose and 0.1% Tween 80 (TSBG) at the level of 0.25 MIC and 0.5 MIC. Then, cultures were grown overnight in TSBG and Broth cultures were further diluted to 1:200 in broth to obtain inoculum density ranged between  $10^5$  and  $10^6$  cells/ml. 100µl were dispensed into each well of micro-titer plate containing 100 µl of treatment solutions. For the negative controls, 100µl of TSBG were dispensed into each well consisting 100µl of treatment solutions. After incubation at 37°C for 48h, the contents of each well were aspirated and the wells were washed three times with sterile physiological saline solution PH=7.3. Plates were shaken vigorously to remove non-adherent bacteria. Adherent bacteria were fixed by adding 99% methanol to wells and leaving for 15 min at room temperature. The wells were then emptied and left to dry. Biofilm was stained by adding 150 µl of 2% crystal violet stain for 15 min. The plates were then rinsed with

water. After drying, stain was re-solubilised by adding 150 µl of 33% glacial acetic acid to each well and agitating gently, and then OD490 was measured by spectrophotometer using an ELISA reader. Each assay was performed in triplicate.

## RESULTS

### Strain identification

In the present study, a total of 102 isolates of *Klebsiella* species were isolated from 250 clinical and environmental samples as shown in **Table (1)**

### Differentiation between *K. pneumonia* and *K. oxytoca* using indole test

In the present study, 32 isolates (32 %) were *K. oxytoca* and 68 isolates (68 %) were *K. pneumonia*. The 2 environmental isolates were *K. pneumonia*.

### Determination of antimicrobial susceptibility pattern of *Klebsiella* isolates

According to (CLSI, 2013), the results of susceptibility are represented in **Table (2)**.

Table (1): Percentage of *Klebsiella* species isolated from different sources:

Specimens type	Number of specimens	Number of isolates	Percentage%
Urine	83	41	49.39%
Sputum	46	21	45.65%
Blood	16	9	56.25%
Pus swabs	25	11	44%
Catheters	35	16	45.71%
Diabetic foot	10	2	20%
Environment	35	2	5.74%
<b>Total</b>	<b>250</b>	<b>102</b>	<b>40.8 %</b>

Table (2): Susceptibility pattern of *Klebsiella* isolates

Antibiotic Disk	Klebsiella species (Number of isolates (N) =102)					
	Resistant		Intermediate		Sensitive	
	N	%	N	%	N	%
IMP	1	1%	6	5.88%	95	93.13%
MEM	1	1%	6	5.88%	95	93.13%
ATM	75	73.52%	0	0	27	26.47%
FEP	55	53.92%	0	0	47	46.07%
CN	33	32.35%	11	10.78%	58	56.86%
AK	15	14.7%	7	6.86%	80	78.43%
SXT	89	87.25%	1	1%	12	11.76%
TE	99	97.05%	0	0	2	1.96%
SCF	57	55.88%	5	4.90%	40	39.21%
CRO	87	85.29%	0	0	15	14.70%
TPZ	69	67.64%	8	7.84%	27	26.47%
OFX	57	55.88%	8	7.84	37	36.27%

### Phenotypic detection of extended spectrum $\beta$ -lactamase (ESBL) producers

Detection of ESBLs was done by the modified double disc synergy disc according to Jarlieret al. (1988). Among the 102 *Klebsiella* clinical and environmental isolate 50 isolates were ESBL producers (49.02%) and 52 isolates (50.98%) were non ESBL producers. As shown in figure (1) enhancement of zone of inhibition between either ceftazidime or cefotaxime and amoxicillin/clavulanic disc indicate positive test results.

### Quantitative assessment of biofilm producers

Assessment of biofilm was done according to Stepanovic *et al.*, (2000) and Abdi-Ali *et al.*, (2005). Out of 102 *Klebsiella* isolate, 19 isolates (18.6%) were strong biofilm producer, 25 isolates (24.5%) were moderate biofilm producers, 39 isolates (38.2%) were weak biofilm producer while 19 isolates (18.6%) were non biofilm producers, results are shown in figure 2.

### Relation between ESBLs and biofilm producing isolate

Out of the 19 strong biofilm forming isolates 12 isolates (63 %) were ESBL producers, 15 out of the 25 moderate biofilm isolates (60 %) were ESBL producers, 17 out of the 39 weak biofilm isolates (44 %) were ESBL

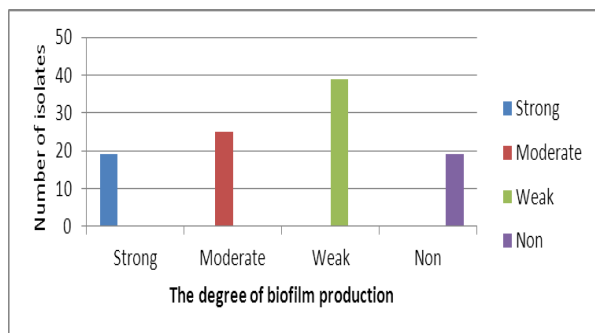


Figure (2): Classification of *Klebsiella* strains according to degree of biofilm production.

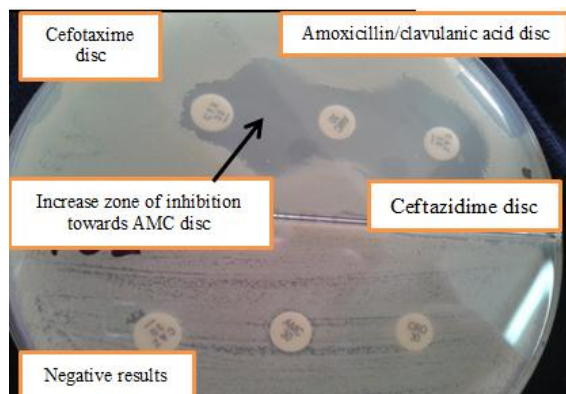


Figure (1): Modified Double Disk Synergy Test for detection of ESBL producers isolates showing positive and negative test.

producers, and only 5 out of the 19 non biofilm isolates (26 %) were ESBL producers, results are shown in Figure (3).

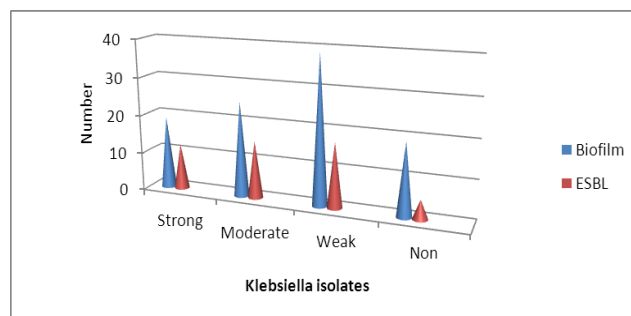


Figure (3): Relation between ESBLs and biofilm producer isolate.

### Detection of MIC of essential oils

MIC of essential oils was detected by agar dilution method for the strong and moderate biofilm producer *Klebsiella* isolates table (3).

Table (3): MIC of different essential oils against *Klebsiella* isolates.

Essential oils	Average MIC <sub>50</sub> (µg/ml) for 44 tested isolate
Black seed oil	40 µg/ml
Clove oil	40 µg/ml
Eucalyptus oil	30 µg/ml
Garlic oil	50 µg/ml
Ginger oil	40 µg/ml

### Effect of essential oils on inhibition of biofilm

The average percent of biofilm inhibition after addition of sub MIC (1/2 and 1/4 MIC) of essential oils were listed in table (4) and figure (4).

Table (4):The inhibition of biofilm by sub-MIC of different essential oils.

Essential oil	% of biofilm reduction using	
	1/2MIC	1/4MIC
Black seed oil	52.87%	43.42%
Clove oil	59.83%	47.65%
Eucalyptus oil	63.46%	51.51%
Garlic oil	59.90%	52.15%
Ginger oil	55.58%	40.56%

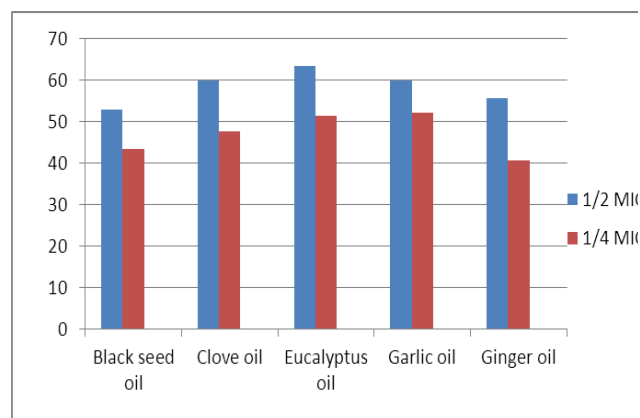


Figure (4): Effect of sub MIC of five essential oil on *Klebsiella* biofilm

### DISCUSSION

Over the past few decades, *K. pneumoniae* has been emerged as a significant cause of severe nosocomial infections due to the difficulty of its treatment and its ability to adhere and multiply on inanimate surfaces. *Klebsiella* infections were associated with increased mortality and prolonged hospitalization of survivors (Podschun and Ullmann, 1998).

In the present study out of 102 isolates 71 isolates were *K. pneumoniae* and 31 isolates were *K. oxytoca* in proportion 2:1. This result comes to accordance with

Bauernfeind *et al.* (1981) who estimated the same proportion.

In the present study resistance to Tetracyclin, (Sulphamethoxazole/Trimethoprim), Ceftriaxone, (Pipracillin/Tazobactam), Aztreonam, Ofloxacin, Cefoperazone/ sulbactam and Cefipime were (97.05%), (88.25%) (85.29) , (75.48%) , (73.52) , ( 63.72),( 60.78%) and (53.92%) respectively. These results were agreed with (Elsharkawy *et al.*, 2012). The high resistance rates could be attributed to the empirical usage of these antibiotics in treatment of nosocomial infection in hospitals (Koneman *et al.*, 1997).

On the other hand (6.86%) of *Klebsiella* isolates exhibited resistance to meropenem and imipenem, similar to the results obtained by (Elsharkawy *et al.*, 2012). The high resistance to these antibiotics which considered as “last-line agents” or “antibiotics of last resort” may be due to the emergence of multidrug resistant (MDR) pathogens (Queenan and Bush, 2007).

In this study *Klebsiella* isolates ESBL producers were (50.98%).These results come to accordance with (Amita and Rajesh ,2008) and ( El-Daker *et al.*, 2006) and less than that reported by (Paterson *et al.*, 2003) .The incidence of ESBLs producing *K. pneumoniae* is varying from country to another (Moubareck *et al.*, 2005).

In our study *Klebsiella* isolates were biofilm (strong, moderate) producers where *K. pneumoniae* produce copious amount of acidic polysaccharides capsules, which allowed the adherence of epithelial cells (Mathur *et al.*, 2013).

Strong and moderate biofilm producing isolates were ESBLs (61.3%) and multidrug resistant. Six isolates of biofilm producer were resistant to imipenem and meropenem. These data were higher than that reported by (Vasanthi *et al.*, 2014). The antimicrobial drug resistant profile of

biofilm producer of *Klebsiella* isolates are multiple drug resistant included ESBLs and carbapenemases (Revdwala, 2011). In this study the effect of five commercial essential oils (Black seed oil, Clove oil, Eucalyptus oil, Garlic oil and Ginger oil) on reduction of *Klebsiella* biofilm formation was detected. The results revealed that eucalyptus oil show maximum inhibition of biofilm followed by garlic oil, clove oil, ginger oil and black seed oil. These results are consistent with (Mathur *et al.*, 2013), who reported that Eucalyptus oil showed maximum inhibition of biofilm formation followed by garlic oil and clove oil. Plants are important source of potentially useful biomolecules for the development of new chemotherapeutic agents. Some reports are available on the antimicrobial effect of essential oil including Eucalyptus on various pathogens including *K. pneumonia* (Hammer *et al.*, 1999; Derakhshan *et al.*, 2010).

#### CONCLUSION

The prevalence of ESBL among *Klebsiella* biofilm producing isolates is due to increase rate of conjugation in biofilm which facilitate spread of drug resistant genes. Co-production of bacterial biofilm and ESBL reduces therapeutic options in treating infection. Eucalyptus oil show promising effect in inhibition of *Klebsiella* biofilm production.

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**دراسة عن تكوين البيوفيلم بواسطة جنس الكلبسيلا المنتج لأنزيم للبيتالاماميز ممتد الطيف  
إ.د. همت كمال عبد اللطيف، إ.د. رمضان حسن، د. أميره الجنائني، هبة عبد الصبور  
قسم الميكروبيولوجي والمناعة- كلية الصيدلة- جامعة الزقازيق**

يعتبر ميكروب الكلبسيلا مسبب هام لعدوى المستشفيات حيث ان لديه قدرة عالية على مقاومة العديد من المضادات الحيوية عن طريق انتاج بعض الأنزيمات المسببة للمقاومة وانتاج الغشاء الحيوي (البيوفيلم). في هذه الدراسة تم عزل مائه سلالة طبيعية من الكلبسيلا من مستشفيات جامعة المنصورة والزقازيق وعزلتان من خارج المستشفى وتم تعيين قدرة الميكروب علي انتاج البيوفيلم وانزيم البيتالاماميز ممتد الطيف ووجد ان السلالات المنتجة للبيوفيلم لها قدره كبيره علي انتاج انزيم البيتالاماميز. وبدراسة تأثير خمسة من الزيوت الاساسيه (زيت الكافور، زيت الثوم، زيت القرنفل، زيت الجنزبيل وزيت الحبه السوداء) علي تثبيط انتاج البيوفيلم في العزلات لوحظ ان زيت الكافور له كفاءه عاليه في منع تكوين البيوفيلم.