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BACTERIOLOGICAL STUDY OF *KLEBSIELLA* SPECIES ISOLATED FROM DIFFERENT INFECTIONS AND DETECTION OF BIOFILM FORMATION BY THREE METHODS

Ansam Dawood Salman

Department of Biology, College of Science, University of Diyala, Iraq.
e-mail : ansamdawod83@gmail.com

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ABSTRACT : This study was conducted from the period from 1/10/2016 to 30/10/2017 in Baquba city in Iraq. Sixty nine samples were collected from different infections. The results of bacterial culture on media of MacConkey agar, blood agar, media of Eosin methylene blue, diagnostic phenotypic, biochemical tests, that 25 isolates are belonging to bacteria *Klebsiella* spp. The results of the investigation of some virulence factors of *Klebsiella*, that all isolates surrounded by capsule, and incapable of producing the hemolysine enzyme, while all isolates the ability to produce the urease enzyme and productivity isolates of Bacteriocin has amounted to 30%. The ELISA method was considered to be superior to tube method and congo red agar method for detection of biofilm. From the total of 25 clinical isolates, ELISA method detected 80%. All isolates showed pattern of multiple drug resistance towards 8 antibiotics, as were all isolates (100%) were resistance to antibiotic Ampicillin, while most of the isolates were sensitive to Amikacin and Ciprofloxacin and varied resistance ratio for the rest of antibiotics.

Key words : *Klebsiella*, biofilm, ELISA.

INTRODUCTION

Genus *Klebsiella* is due to intestinal family (*Enterobacteriaceae*), which is characterized as gram negative, bacilli and ranges in width from (0.3 – 1mm), either length ranges from (0.6 – 6mm) and characterized by these bacteria as an analyst for the blood and more kinds have ability to urea consumption and do not produce gas H_2S and it's colonies are pink, mucosa and smooth on the MacConkey agar media, and containing on the capsule and fermentation for the lactose, glucose and sucrose and non motile, facultative anaerobic (Sharmeen *et al*, 2012; Ahmed and Alaa, 2016).

Klebsiella is an important pathogen in both community and hospital setting and it is causative agent of many diseases, such as bacteremia, urinary tract infections, burns and wounds infections, sepsis, pneumonia and pyogenic liver abscesses (Korres *et al*, 2013; Rahamathulla *et al*, 2016).

Pathogenicity of *Klebsiella*is due to the presence of many virulence genes which encode virulence factors that cause much kind of diseases. Some of these virulence factors are: biofilm formation, capsule synthesis, adhesions, bacteriocin synthesis and lipopolysaccharides formation (Chung *et al*, 2015).

Klebsiella Spp. have been recognized as the most frequent cause of multidrug-resistant gram necative bacteria (MDR-GNB) outbreaks, particularly after the emergence of the extended-spectrum beta-lactamase (ESBL) enzymes. As a result, infections in hospitalized patients with this ESBL- producing *Klebsiella* Spp. (Tirza *et al*, 2015).

Aim of the study

1. Isolate and diagnose of *Klebsiella* Spp. from patients with different infections and to identify the epidemic of these bacteria in Baquba city.
2. Investigation of different virulence factors for *Klebsiella* Spp.
3. Study the pattern of antibiotic resistance prevailing between isolates.
4. Detection capability isolates the biofilm production by three ways.

MATERIALS AND METHODS

Antibiotics

The following antibiotics disks were used throughout the study as showing in Table 1.

Samples collection

Ninety six different clinical samples were collected

from patients in Baquba General Hospital and Al-Batool Hospital over period from 1/10/2016 to 30/10/2017. The samples were included (36 from urin, 22 from ear swabs, 32fromsputum and 6from stool). The samples were tested for all tests in microbiology unit in Al-Batool Hospital Laboratory.

Isolation and identification of *Klebsiella* spp.

The collected samples were inoculated on the blood agar and MacConkey agar, incubated at 37ºC for 24 hours. The isolates were examined for their shape, size, color, mucoid, lactose fermentation and haemolytic activity. Then transferred and streaked on EMB agar for detecting the ability of each isolate to differentiate the*Klebsiella* from *E. coli*. All plates were incubated at 37ºC for 24 hours then a single pure isolated colony was transferred to Nutrient agar medium for the preservation and to carry out other biochemical tests that confirmed the identification of isolates.

The isolates were identified according to the Bergey's Manual (Holt *et al*, 1994) and according to MacFaddin (2000) as the following: gram stain and biochemical tests which included (catalase test, oxidase test,Indole production test, Methyl Red test, Voges-Proskaure test, Simmons citrate test and urease test).

Detection of virulence factors

The *Klebsiella* spp. ability to produce some of virulence factorswas recognized and tests were applied on 25 isolates that identified. It included: Haemolysin production according to Atlas *et al* (1995), capsul production according to Atlas *et al* (1995), urease production according to Atlas *et al* (1995), bacteriocin production according to Atlas *et al* (1995) and biofilm formation.

Detection of biofilm

It was detected on the composition of the biofilm by *Klebsiella* spp. by three methods are:

1. Formation of biofilm test by ELISA

Biofilm was performed in wells microtitere plate as described by Wojnicz *et al* (2012). Briefly, *Klebsiella* spp. isolates were subcultured in brain heart infusion broth over night at 37^0C. 200 µl of bacterial cultures were transferred to beach well in triplicate then incubated 24h at 37^0 C and negative control contained media only. The media was removed and washed three times with phosphate buffer saline then 25 µl of crystal violate (1% w/v) was added to the wells for 15 min at room temperature. Crystal violate was then removed and washed three times with phosphate buffer saline. The crystal violate inside the cells was dissolved by absolute ethanol (100 µl) and the absorbance was measured by ELISA reader. After comparing the optical density (O.D.) of biofilm to the control and according to readings, the isolates was classified as follows: O.D. ≤ $O.D_c$ no biofilm producer, $O.D_c$< O.D ≤ 2x $O.D_c$ weak biofilm, 2x $O.D_c$<O.D ≤ 4 x $O.D_c$ moderate and 4 x $O.D_c$< O.D strong biofilm.

2. Tube method

Described by Christensen *et al* (1982) this is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/ none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times.

Congo red Agar method

Freeman *et al* (1989) have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium. CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red indicator 8 g/L. First Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121ºC for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55ºC. CRA plates were inoculated with test organisms and incubated at 37ºC for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.the experiment was performed in triplicate and repeated three times.

Antimicrobial susceptibility test

The sensitivity and resistance of *Klebsiella* spp. to antimicrobials agents was tested by the disc diffusion method on Muellar-Hinton agar using antibiotic discs according to Clinical and Laboratory Standards Institute (CLSI) guidelines (2013). Eight antibiotics were tested as shown in the Table 2.

Interpretation of inhibition zones was carried out based on the manufactures and CLSI guidelines.

Statistical analysis

A data analysis was computer aided. Statistical

Table 1 :

Antibiotics	Symbol	Concentration of disk (µg/ml)
Augmentin	AMC	10
Amikacin	Ak	30
Gentamycin	GEN	10
Ciprofloxacin	CIP	5
Tetracyclin	T	30
Ampicillin	AMP	30
Cefotaxime	CTX	30
Cefixime	Ce	5

Table 2 : Results of a screening test (tube, congored).

		Yes	No	Total
Results of a screening test (tube, congored).	**Yes**	a	b	a+b
	No	c	d	c+d
	Total	a+c	b+d	Total

a: True positive. b: False positive. c: False negative.
d: True negative.
The sensitivity of a screening test is (a / a+c).
The specificity of screening test is (d / b+d).

analysis was done using SPSS (Statistical Package of Social Science) version 18 computer software. Frequency distribution and percentage for selected variables were done first.

The validity of screening tests was examined by evaluate the sensitivity and specificity of tests (Niazi, 2004).

Whenever we examined the validity of a screening test, we must compare the results with a confirmatory or referral test.

RESULTS AND DISCUSSION

Isolation and identification

Ninety six samples were collected from patients, the samples comprised from (36 from urin, 22 from ear swabs, 32 from sputum and 6 from stool).

Fifty two isolates (26.04%) have the ability to grow on the MacConkey and blood agar which considered selective and differential media for genus *Klebsiella.* All 25 isolates had ability to ferment lactose and formed mucous colonies.

Microscopic examination was used to all 25 isolates after staining by gram stain and cells appeared as Gram-negative bacillirounded by capsule arranged in single or pairs. For further identification some of the biochemical tests were performed on 25 isolates, included: catalase test was all 10 isolated gave positive results. The 25 isolates gave the negative result to the oxidase test. Also all 25 isolates were positive to Indol production and Citrate utilization test. Additionally, urease test was applied for further identification because the *Klebsiella* often produce urease enzyme.

All 25 isolates were negative for methyl red test and positive for vogesproskauer test.

All these results were agreed with other results reported by other researchers (Ghusoon, 2015; Tirza *et al*, 2015 and Ahmed and Alaa, 2016).

Detection of virulence factors

In the present study detected of some virulence factors associated with pathogenecity of *Klebsiella.*

Hemolysin production

The results showed that all isolates of *Klebsiella* do not have to produce hemolysin on the blood agar.

These results were agreement with that reported by local researchers (Eman, 2013; Ahmed and Alaa, 2016).

The hemolysin important virulence factor for a wide range of gram negative bacteria and contributing to the occurrence of pathogenicity and there are several possibilities for the lack of the ability of these bacteria to produce hemolysin, including the effectiveness of hemolysin ruled by operon consists of four genes involved these genes together to express their production hemolysin, and it may exist between them a single gene is non active in the expression of this production (ÇáÒÚÇß, 1994).

Capsule

Microscopic examination showed the results of using a nigrocin stain to detect contain isolates under study on the capsule, that all isolates containing the capsule.

These results were agreement with that reported by other researchers (Eman, 2013; Claudia *et al*, 2014; Ahmed and Alaa, 2016).

Urease production

It has been investigating the ability of bacteria to produce urease and the results showed that all isolates (25) have the ability to produce urease. The importance of this enzyme from its ability to urea analysis to ammonia $NH_4$and carbonic acid H_2CO_3 and is an enzyme crystalline contain nickel, and represents a virulence factor of many pathogenic microorganisms and cause the formation of kidney stones, kidney inflammation basin, and causing a disorder brain due to ammonia (Ammoniaen Cephalopathy) and causing liver coma (Cartera *et al*, 2009).

Bacteriocin production

The present study results showed that 8 (32%) of the isolates of *Klesiella* spp. producing Bacteriocin (Table

Table 3 : The ability isolates of *Klebsiella* spp. to produce Bacteriocin

No. of isolate	Diameter of inhibition / mm
K1	-
K2	-
K3	-
K4	18mm
K5	-
K6	-
K7	25mm
K8	-
K9	8mm
K10	-

Table 4 : Results of different methods for detection of biofilm.

No. of isolates	Congo red method	Tube method	ELISA method
K1	+ weak	+ weak	+ strong
K2	+weak	+weak	+ strong
K3	-	-	-
K4	+ strong	+ strong	+ strong
K5	-	-	-
K6	+ strong	+ strong	+ strong
K7	+ strong	+ strong	+ strong
K8	-	+moderate	+moderate
K9	-	+ moderate	+ strong
K10	-	-	+moderate

3). These results are consistent with the results reached by Al- Zubaidi (2012) on the production of the bacterium *Klebsiella* Bacteriocin, amounting to production ratio (30.43%). And these results were comparable with other study reported by Eman (2013), where the percentage is (40.9%).

And that the results of this study do not mean that the rest of the isolates were unproductive for Bacteriocin has pointed out (Cursino *et al*, 2002) that modulating receptors for the occurrence of colicin result of mutations lead to the formation of resistant isolates.

Biofilm formation

Among 25 isolates, ELISA test, the standard method, detected 14 as strong and 6 as moderate biofilm producers. The biofilm production was (80%).By tube method, the number of strong biofilm producers were 10, moderate were 2 and weak or non-biofilm producers were8. Very different results were observed by the Congo red method, with which only six isolates showed black colonies with crystalline appearance (Table 4).

Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic into

Table 5 : Sensitivity and specificity of tube test and congo red test in detection of biofilm.

Detection Methods	Sensitivity	Specificity
Tube method	75 %	66%
Congo red method	50%	66%

Table 6 : Percentage sensitivity and resistance of different antibiotics for *Klebsiella* spp.

Antibiotics	(%) of Resistance	(%) of sensitivity
Augmentin	85.1%	14.9%
Amikacin	10%	90%
Tetracycline	70.2%	29.8%
Ciprofloxacin	23.4%	76.6%
Gentamycin	63.3%	36.7%
Ampicillin	100%	0%
Cefotraxime	83.4%	16.6%
Cefixime	49.2%	50.8%

biofilms, decreased growth rate and expression of resistance genes (Kim, 2001). There are various methods for biofilm detection.In this study we evaluated 25 isolates by three screening methods for their ability to form biofilms.

In the ELISA method, the number of isolates showing biofilm formation was 20 (80%), and non or weak biofilm producers were 5(20%). Regional data from Iraq also showed that out of 22 isolates tested, the number of biofilm producers identified by ELISA method was 100 % (Eman, 2013). Other researcher, Ghusoon (2015) was repotted 93% of isolates of produced biofilm.

Tube method detected 60% isolates as biofilm producers and 40% as non-biofilm producers. By this method, one isolate were found to be false positive and 2 were false negative. Tube method is 75% sensitive, 66% specific for biofilm detection (Table 4). This method correlated well with ELISA for identifying strong biofilm producers, but it was hard to differentiate between moderate, weak and non-biofilm producers due to the changeability in the results detected by different observers. In accordance with the preceding studies, tube method cannot be suggested as general screening test to identify biofilm producing isolates (Mathur *et al*, 2006; Afreenish Hassan *et al*, 2011).

With the congo red method, 6 were found to be biofilm producing bacteria and 19 as non-biofilm producers. The congo red method showed very little correlation with the other methods and parameters of sensitivity (50%), specificity (66%). By this method, three isolates were found to be false positive and 3 were false negative. Knobloch *et al* (2002) did not recommend the congo red method for biofilm detection in their study. Out of 128 isolates were congored detected only 3.8% as biofilm

producers as compared to ELISA which detected 57.1% as biofilm producing bacteria.

Susceptibility test

The sensitivity of 25 isolates was tested against 8 antibiotics. The susceptibility test was applied according to the Kirby-Baure Method (antibiotic disc diffusion method) (Table 6).

The results of the current study showed that isolates (85.1%) were resistance Augmentinantibiotic;this ratio was consistent with the ratio shown by Eman (2013), where it was 90.9%. The cause of the *Klebsiella* bacteria resistant to antibiotics B-lactam antibiotics is due to its ability to produce B-lactemase enzymes and the mechanisms of resistance to be several of which reduce the permeability of the counter inside the cell, the counter-analysis by B-lactemaseenzyme and reduce the affinity to Penicillin-Binding proteins (PBP) (Al- Charrakh *et al*, 2011).

Klebsiella was sensitivity to Amikacin (90%), this result consistent with others studies (Eman, 2013; Ahmed and Alaa, 2016) and this sensitive is due to narrow therapeutic index for this antibiotic (Barnhart, 2002).

The percentage of resistance to Gentamycin for isolates under study 63.3%, this result agreed with local studies by Eman (2013) and Ahmed and Alaa (2016) 63.6% and 61% reported.

The antibiotic tetracycline were resistant isolates under study, the proportion of 70.2% and this percentage is consistent with the findings of the researchers (Sarojamma and Ramakrishna, 2011) as was the proportion of resistance to this antibiotic 84%.

Klebsiella was sensitivity to Ciprofloxacin (76.6%), this result consistent with others studies (Eman, 2013; Ahmed and Alaa, 2016) that reported sensitivity to this antibitic 72.7% and 75.3%.

The result in Table 6 showed that all isolates (100%) were resistance to Ampicillin antibiotic and this result was agreed with other studies by Eman (2013), Ghusoon (2015) 100% resistance to this antibiotic.

The resistance of Cefotaxime was 83.4%, while *Klebsiella* resists Cefixime (49.2%), this result was consistent with other studies by Eman (2013), Ahmed and Alaa (2016).

CONCLUSION

1. The etiology of bacterium Klebsiella caused of urinary tract infections, respiratory infections and other acute in Baquba General Hospital and the Al-Batool hospital.
2. All isolates possess a number of virulence factors such as capsule, the ability to produce the enzyme urease and 100%, as well as the ability to produce Bactriocin by 30% and finally the ability to produce biofilm and 80%.
3. The best way to detect the production of biofilm of the three methods used in the study is the ELISA method.
4. Showed the multi- resistance to antibiotics was published between local isolares under study.
5. The Amikacin and Ceproflaxacin are the two most common antibiotics that have shown sensitive isolates.

Recommendations

1. To conduct an extensive study on the virulence factors that these bacteria possess genetically.
2. A study was conducted to isolate the *Klebsiella*from urinary catheters, intensive care units and dialysis machines to determine the prevalence and epidemiology of these bacteria on these devices.

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