Int.J.Curr.Microbiol.App.Sci (2017) *6*(8): 203-210

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 8 (2017) pp. 203-210**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.608.028

Isolation and Identification of *Klebsiella pneumoniae* using API-20E analytical system and conventional PCR assay

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Keywords

Klebsiella, API-20E, *wbbZ* gene

Article Info

Accepted:
04 June 2017
Available Online:
10 August 2017

A B S T R A C T

Klebsiella spp., particularly *Klebsiella pneumoniae*, are important causes of nosocomial infections due to the presence of capsular polysaccharide which is a major surface-located virulence properties associated with the pathogenesis of *Klebsiella pneumoniae*. The capsule is an elaborate polysaccharide matrix that encases the entire cell surface and provides resistance against many host defense mechanisms. D-galacton II has an important role in synthesising the lipopolysaccharide of *Klebsiella pneumoniae* and many other gram negative bacteria. This study included the isolation of *Klebsiella pneumoniae* bacteria from different sources in the environment and identification by using API-20E system and a conventional Polymerase chain reaction assay which detects *Klebsiella pneumoniae wbbZ* gene that involved in expression of D-galacton. The results of our study confirmed the positive identification of the bacteria using API-20E analytical system and also the presence of *wbbZ* gene in two of *Klebsiella pneumoniae* tissue invasive strains using conventional PCR assay.

Introduction

Klebsiella is one of the most important members of *Klebsiella* genus in Enterobacteriacae family, which is responsible for pneumonia (Puspanadan *et al.*, 1998). Besides it is found to cause infections in the urinary and lower biliary tract (Lopes, *et al.*, 2005; Ryan, 2004).

Klebsiella species were found to be the most frequently isolated gram negative bacteria in cases of primary bacteremia (Cross A., 1983). It is the second pathogen, next to E. coli that causes urinary tract infection. It normally affects persons with low immune system such as hospital patients, diabetes patients and people with chronic lung disease. Many a times, alcoholics also suffer from *K. pneumoniae* infections.

Thus, the infections are either hospital-acquired or community-acquired (Sikarwar and Batra, 2011).

Characteristically, *Klebsiella* spp. produce large mucoid colonies because of the synthesis of large amounts of capsular

polysaccharide (CPS). There are 72 recognized K antigen serotypes of *Klebsiella* spp., which are based on the structures of the CPS (Kenne and Lindberg, 1983).

The genus *Klebsiella*

General characteristics

The genus *Klebsiella* is non-motile, non-sporulating, lactose-fermenting, oxidase negative, and Gram-negative with a prominent polysaccharide capsule of considerable thickness which gives the colonies their glistening and mucoid appearance on agar plates. *Klebsiella* is rod shape 0.3-1 µm in diameter and 0.6-6 µ min length arranged singly, in pairs or in short chains.

Klebsiella is facultative bacteria and the colonies appear large, mucoid, and red with diffusing red pigment on MacConkey agar indicating fermentation of glucose and acid production. *Klebsiella* are normal inhabitant of the intestinal tract of human and animal, soil, water and botanical environment (Ørskov 1984; Podschun and Ullmann 1998; Brisse *et al.*, 2006).

Classification

The bacterial genus *Klebsiella* was designated in honor of the German microbiologist (Edwin Klebs by Trevisan in 1885), who also described the *Klebsiella pneumoniae* species in 1887.

Historically, the classification of *Klebsiella* species, like that of many other bacteria, was based on their pathogenic features or origin. Taxonomic keys were proposed that included characteristics such as substrate utilization and enzymatic activities.

A great confusion has prevailed in *Klebsiella* nomenclature (Kanki *et al.*, 2002) with new bacteria being synonymous with species already proposed (Grimont *et al.*, 2000; Hedegaard *et al.*, 1999; Podschun and Ullman, 1998) or with the reclassification of some species even within other genera (Drancourt *et al.*, 2001).

Based on DNA-DNA hybridization data *K. ozaenae* and *K. rhinoscleromatis*, taxonomically, are regarded as subspecies of *K. pneumoniae* (Podschun and Ullmann, 1998; Drancourt *et al.*, 2001).

Pathogenesis and clinical importance

K. pneumoniae is the most medically important species of the group. *K oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens (Podschun and Ullmann, 1998).

K. pneumoniae is also a potential community-acquired pathogen (Ko *et al.*, 2002).

In humans, *Klebsiella* species may colonize the skin, pharynx, or gastrointestinal tract.

They may also colonize sterile wounds, urine and may be regarded as normal flora in many parts of the colon, intestinal and biliary tract (Podschun and Ullmann, 1998; Brisse *et al.*, 2006).

Materials and Methods

Collection and isolation of bacteria

At the period from September 2016 to March 2017, 40 samples from different sources were collected as follows:

Culturing of isolates

The samples which transported to the laboratory were inoculated into nutrient broth at 37^0C for 5 hr. (enrichment step) to increase bacterial level.

Loopful from the incubated broth was streaked onto each surface of MacConkey agar then incubated at 37^0C for 24 hr.

Identification of some selected pathogenic bacterial isolates using API-20E analytical system (confirmation test)

This system was used for approving the diagnosis of bacterial isolates and study the rest of the biochemical tests for K. pneumonia.

The test conducted as (Atlas *et al.,* 1995) according to the French manufactured company (BioMarieux).

Procedure out line

Oxidase test performed prior to inoculation.

Preparation of strip

A 5ml of sterilized distal water were added to the holes of the tray in order to provide humidity conditions then the strip was put in the tray.

Preparation of bacterial suspension

Three to four bacterial colonies isolated from MacConkey agar culture were suspended in a test tube filled with 5ml of phosphate buffer saline.

Inoculation of strip

The upper and lower part of caplets were filled with bacterial suspension by a sterile Pasteur pipette of the following caplets (CIT, VP, GEL) as for the rest of caplets only the lower part were filled with the suspension to concave line.

The lower part filled with sterile liquid paraffin in order to provide anaerobic conditions to the following caplets (H_2S, URE, ADH, LDC, ODC). The band was covered and incubated for 24 hour at 37ºC.

Reading of strip

After incubation period the following reagents were added:

A drop of Kovac's reagent to IND caplet and read immediately.

A drop of Ferric chloride 10% to TDA caplet and read immediately.

A drop of VP1 to VP caplet then a drop of VP2 were added and read after 10 minutes.

On the result page, the tests were separated into 7 sets and a number (1, 2 and 4) is specified for each test. Every positive result has given its own number while the negative result is zero.

Adding the numbers correspondingly to results within each group, a 7-digit profile numbers were attained and then compared with numbers that found in the analytical profile index for documentation of tested bacterium (Fig. 1).

Molecular identification using PCR technique

Extraction of DNA

Samples tubes were mixed thoroughly, and then DNA was extracted from each sample by using Wizard Genomic DNA purification kit (Promega, USA) with modification according to the following protocol:

A loop full of activated colony culture grown for 18-20 hours was added to a 1.5ml micro centrifuge tube containing 200µl of TE buffer and then mix by vortexing. The samples were centrifuged at 13,000 rpm for 2 minutes and then the supernatant was removed. For cell

lysis, 600 µl of Nuclei Lysis Solution was added to the cell pellet and gently pipet to mix. Mixture was incubated for 15 min at 70°C. For RNA removal, 4 µl of RNase solution was added to the cell lysate and inverted 2-5 times to mix. It was incubated at 37ºC for 15 min, and then it was cooled to room temperature.

To start DNA purification, 300 µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vortexed at high speed for 20secs. Then it was incubated on ice for 5 mins, centrifuged at 13000 rpm for 10 min. Diluting DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl of isopropanol to start DNA concentrating step.

The tube was then gently mixed by inversion until the thread-like strands of DNA form a visible mass, centrifuged at 13000 rpm for 2mins. The supernatant was carefully poured off and the tube was drained on clean absorbent paper. Five hundred microliter of 70% ethanol was added and the tube was gently inverted several times to wash the DNA pellet, centrifuged at 13000 rpm for 2mins and ethanol then was aspirated carefully. The tube was drained on clean absorbent paper and the pellet was allowed to air-dry at room temperature for 10-15mins.

One hundred microliter of DNA Rehydration Solution was added to the tube and rehydrated the DNA by incubating at 56ºC for 1hrs, solution was mixed by gently tapping the tube. The resultant DNA was stored at 2ºC.

Primers selection

All primers (Table 2) were supplied in lyophilized forms. Dissolved in nuclease-free water to give a final concentration of 100 picomol/µl as recommended by provider and stored in deep freezer as stock solution until used in PCR amplification. Work solution was prepared by added 90 µl of nuclease free water to 10 µl of stock solution of primer to get 10 picomol/ µl concentration.

Oligonucleotide primers

The oligonucleotide primer sequences were used for amplification of the highly conserved region of target gene from bacterial isolates as previously described by Hsieh *et al.,* 2014. The sequence of the forward primer KP0663+228 –F was 5' AGGATTGTA TTCTGAAGGTC 3' and for the reverse primerKP0663 – R was 5' TCAACTTGC CGTAATAAAGC 3' will produce expected product of 567 bp. Primers were obtained commercially from Sigma, USA.

Determination of DNA concentration and purity

Quantus florometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 99 µl of diluted Quanty Flour Dye was mixed. After incubation at room temperature for 5min, DNA concentration values were determined.

Results and Discussion

Isolation

From a total 40 samples which represents 9 urine samples from healthy and sick persons in hospitals, 16 swabs from operation room of a hospital, 3 river water, 8 salad vegetables, 2 milk samples from a cow and 2 swabs from sheep nose *Klebsiella* spp. were isolated only 3 urine samples and 2 swabs from sheep nose were positive to be *Klebsiella pneumoniae* on MacConkey agar, further more only 2 (1 urine sample and 1 sheep nose swab) were subjected to API-20E analytical test and PCR assay and proved to be positive *Klebsiella pneumoniae* (Tables 1–6; Fig. 2).

Table.1 Total number of samples

Samples	Total No.
Human Urine samples	9
Swabs from operation room of Baqubah's hospital	16
Water from Diyala's river	3
Salad vegetables	8
Cow's milk	2
Sheep nose	2
Total	40

Table.2 The primers and their sequences used in conventional PCR technique

Primer	Primers sequences (5`-3`)	Target gene	Size	Reference
KP0663+228 –F	AGGATTGTATTCTGAAGGTC	*wbbZ*	567bp	Hsieh *et al.*, 2014
KP0663 – R	TCAACTTGCCGTAATAAAGC	*wbbZ*	567bp	Hsieh *et al.*, 2014

Table.3 Reaction volume and components of PCR

Components	Conc.	Volume(µl)\Reaction
GoTaq PCR master mix	2X	10 µl
Forward Primer	10µM	1µl
Revers Primer	10µM	1 µl
DNA	10-50ng	6 µl
Neuclease-free water	-	2 µl
Total per reaction	20 µl	

Table.4 Thermal cycle programming

Steps	C°	min:sec	Cycles
Initial Denaturation	95 C°	5 min	1
Denaturation	95 C°	30 sec	35
Annealing	53 C°	30 sec	
Extension	72 C°	30 sec	
Final Extension	72 C°	7 min	1
Hold	4 C°	-	1

Table.5 Total number of samples and the isolates from them

Type of samples	Total No.	No. of isolates					
		Klebsiella	***E. coli***	***Proteus***	***Salmonella***	***Fungi***	***Pseudomonas***
Urine samples from human	9	3	4	2	-	-	-
Swabs from operation room of hospital	16	-	-	4	3	5	4
Water from river	3	-	3	-	-	-	-
Salad vegetables	8	-	5	3	-	-	-
Cow's milk	2	-	-	-	-	2	-
Sheep nose	2	2	-	-	-	-	-
Total	40	5	12	9	3	7	4

Table.6 API-20E

Test	Code	Negative result	Positive result
β-galactosidase	ONPG	Colorless	Yellow
Arginie Dihydrolysis	ADH	Yellow	Red-orange
Lysine Decarboxylase	LDC	Yellow	Red-orange
Ornithine Decarboxylase	ODC	Yellow	Red-orange
Citrate Utilization	CIT	Yellow	Green-blue
Hydrogen Sulfide	H2S	Colorless	Black sediment
Urease production	URE	Yellow	Red-orange
Tryptophan Deaminase	TDA	Yellow	Dark brown
Indole production	IND	Yellow ring	Red ring
Acetone production	VP	Colorless	Pink-red
Gel Hydrolysis	GEL	No pigments	Black pigments
Glucose	GLU	Blue	Yellow
Manitol	MAN	Blue	Yellow
Insonitol	INO	Blue	Yellow
Sorbitol	SOR	Blue	Yellow
Rhaminose	RHA	Blue	Yellow
Sucrose	SAC	Blue	Yellow
Melibiose	MEL	Blue	Yellow
Amayloid	AMY	Blue	Yellow
Arabinose	ARA	Blue	Yellow

Fig.1 API-20E test showing the 7-digit profile number according to positive and negative results that confirms the diagnosis of *Klebsiella pneumoniae* 97% in the API-20E analytical profile index book

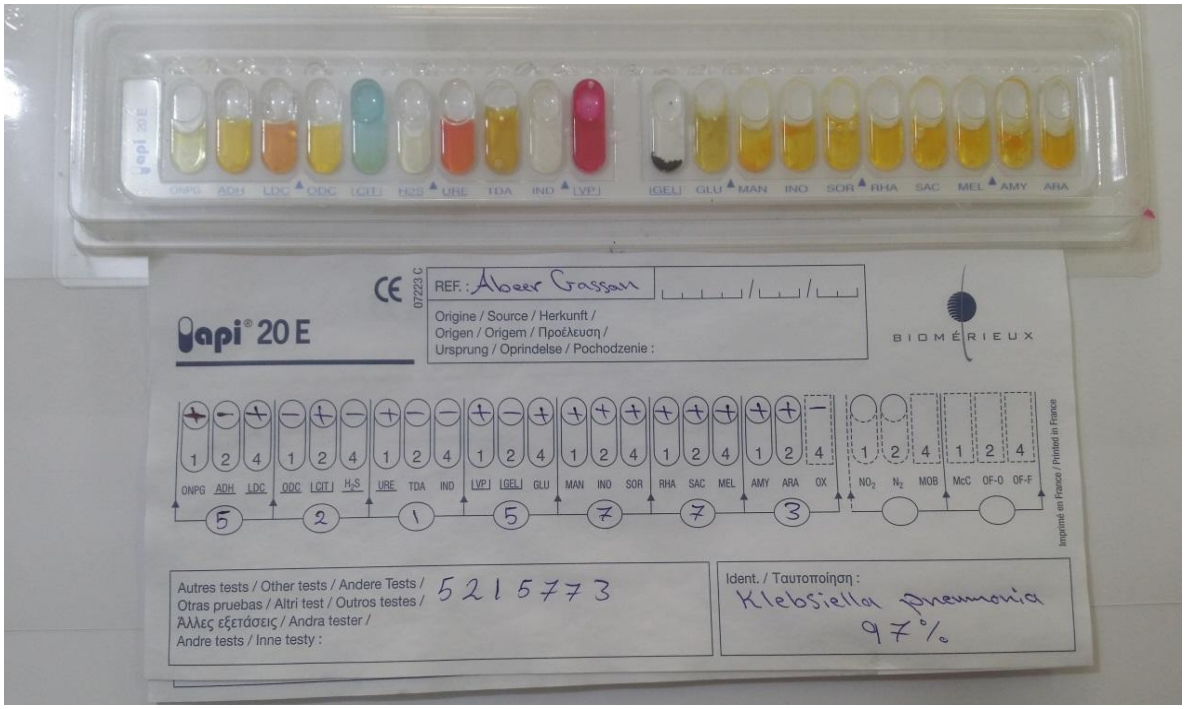
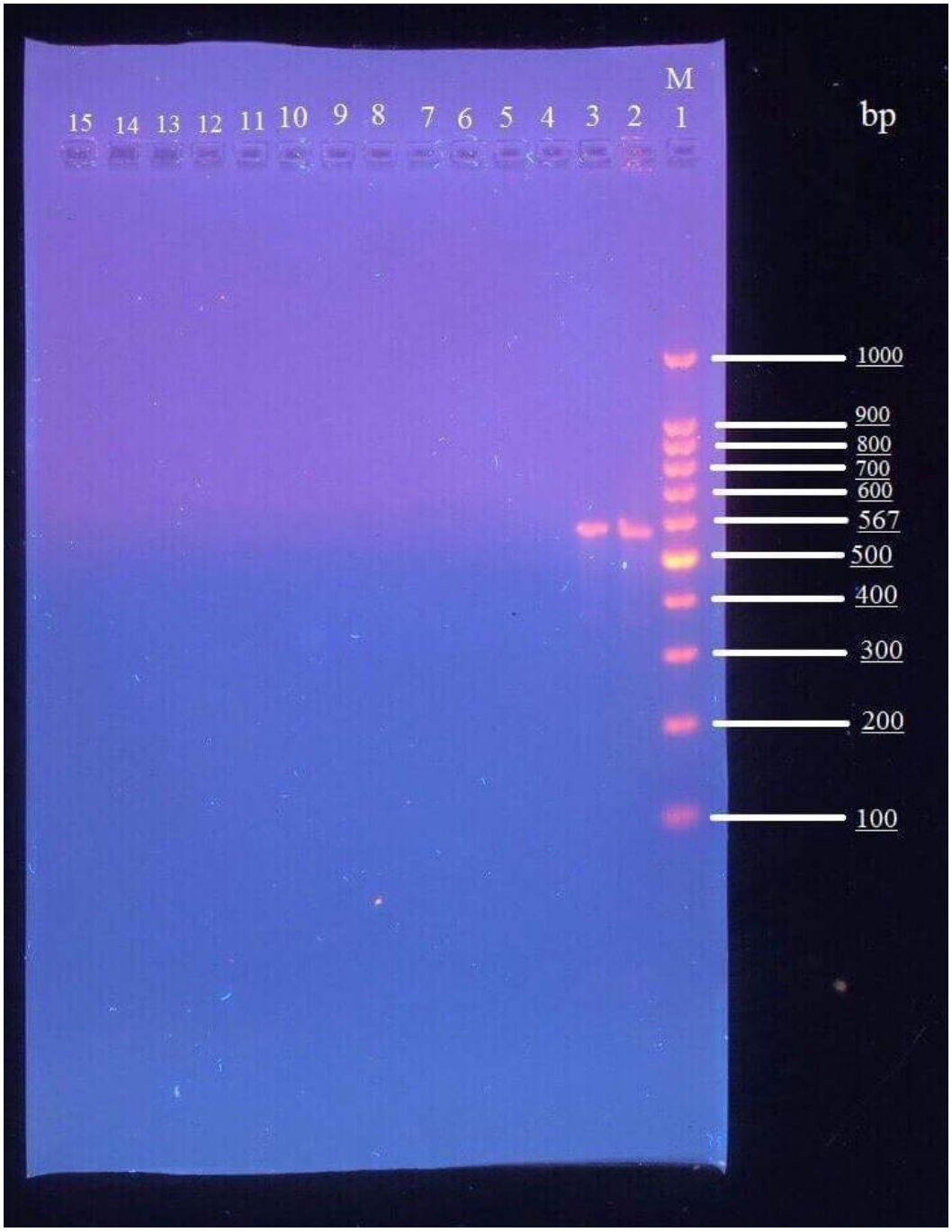


Fig.2 Agarose gel electrophoresis PCR product of a 567*wbbZ* gene in tissue invasive *Klebsiella pneumoniae*. Lane 1 is M, represents the 100-bp ladder DNA marker; Lane 2 is a urine sample from a patient while Lane 3 is a swab from sheep nose



Identification of isolates

On MacConkey agar a large dome shaped highly mucoviscous colonies of lactose fermenting bacteria were grown confirmed by string test reaching 6 mm in length. Then smears were prepared from the isolated colonies on a glass slide and stained by Gram stain. Short Gram- negative rods of bacteria appears in stained smear.

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How to cite this article:

Abeer Ghassan Munther Al-Agha, Nazar Jabar Muslih Al-Khafaji and Amer Khazal Salih Al-Azawi. 2017. Isolation and Identification of *Klebsiella* pneumoniae using API-20E analytical system and conventional PCR assay. *Int.J.Curr.Microbiol.App.Sci.* 6(8): 203-210.
doi: https://doi.org/10.20546/ijcmas.2017.608.028