Diagnosis of *Klebssiella pneumonia* Isolated from Clinical Cases of Hospital -Acquired Infection in Al-Dewaniyah Teaching Hospital.

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Abstract:

Background: Hospitalized infections are major problem affecting millions of peoples each year . *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, the medically most important species of an important nosocomial pathogen, most frequently causing various clinical manifestation

Objective: To identify the role of *Klebsiella pneumoniae* isolates from different clinical cases in hospital –acquired infections .

Methods: During the period from March to September, 2018, a total of 110 clinical specimens were collected from patients with different nosocomial infections who were referred to Al-Diwaniya Teaching Hospital . Bacterial isolates were identified to the level of species using the traditional morphological and biochemical diagnostic tests. Multiplex PCR assay was performed to detection and genotyping *Klebsiella pneumoniae* based on capsular antigen (K) gene.

Results: Out of 110 of different specimens of patients with nosocomial infections, only 39 (35.4%) *Klebsiella pneumoniae* isolates were recovered. However, 47 specimens of sputum revealed 18 (16.36%) positive result; 37 urine specimens given 15 (13.36%) were positive for *Klebsiella pneumoniae* and 26 specimens of Burns and wounds gave 6 (5.45%) of *K. pneumoniae*, specimens. This study revealed that there was a predominance of K57, K1 and K2 serotypes in sputum, urine, burn and wound that isolated from patients with nosocomial infections.

Conclusion and Recommendation: The present study proved that K57 serotype was important virulence factor in the pathogenesis of *K. pneumoniae* in addition to predominant serotypes (K1 and K2) when compared with local serological studies.

Keywords: Hospital-acquired Infections; Klebseilla pneumonia; serotyping; clinical cases.

Introduction

Nosocomial infections, also called hospital-acquired infections are defined as infections acquired during hospital care which are not present or incubating at admission. Four types of infection account for more than 80% of all nosocomial infections: urinary tract infection (usually catheter-associated) accounting for about 35% of nosocomial infections but carry the lowest mortality, surgical-site infections are second in frequency (about 20%), bloodstream infection (usually associated with the use of an intravascular device), and pneumonia (usually ventilator-associated) are less common (about 15% each) but are associated with much higher mortality(1) .

The majority of *Klebsiella* infections are associated with hospitalization, as opportunistic pathogens, *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction(2) .

In recent years, diagnostic laboratories have been concerned with reducing the time required for diagnosis of *Klebsiella* infections. There has been a general move toward molecular methods of *Klebsiella* detection and typing, which were based less on phenotypic features and more on stable genotypic characteristics(3) .

The reported risks for nosocomial infection, many of which are linked, include an increased length of stay in the hospital particularly in intensive care unit, several outbreaks have been reported in hospitals where patients transfer is high which enhances the dissemination of such organisms(4) .Such infections may occur as an outbreak (epidemic) or may become established as a regular occurrence (endemic), endemic infections are most common, epidemic infections occur during outbreaks, an outbreak

is defined as an unusual or unexpected increase of cases of a known nosocomial infection or the emergence of cases of a new infection(4). The present study aimed to detect serotypes of *K. pneumoniae* using molecular methods.

Materials and Methods:

During the period from March to September ,2018, a total of 110 clinical specimens were collected from patients with different nosocomial infections who were referred to Al-Diwaniya Teaching Hospital according to MacFaddin(5) as follow: (26) burns and wounds; (37) urine; (47) sputum. All specimens were labeled and transported to the laboratory in portable container then streaked on general and selective media and incubated at 37˚C for 24 hours under aerobic condition. Bacterial isolates were identified to the level of species using the traditional morphological and biochemical diagnostic tests, according to the methods (*5)* then stored at maintenance medium until further tests.

Suspected *Klebsiella* spp. isolates were recoved from clinical andhospital specimens after culturing on MacConkey agar and incubated for overnight at 37C. This medium is specially made to distinguish lactose-fermenting (pink to red colonies) from non lactose fermenting bacteria (colorless or slightly beige). All lactose-fermenting isolates were subcultured and incubated for additional overnights. Suspected bacterial isolates which their cells are Gram negative and negative to Oxidase test which further identified by the following biochemical test according to Holt *et al.,(6)* and Baron and Finegold(7)

Multiplex PCR assay was performed to detection and genotyping *Klebsiella pneumoniae* based on capsular antigen (K) gene by using K1, K2, K5, K20, K54, and K57 gene primers and this assay was done according to method described by Fang *et al*.,(8) .

These Multiplex PCR Master mix reaction components were added into standard PCR tubes provided by kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Than mixed and centerige and placed in PCR Thermocycler. Experimental data were presented in terms of observed numbers and percentage frequencies was used (9).

Results and Discussion:

Klebsiella pneumoniae isolates culturing on test media, on MacConkey agar which appeared as colonies typically large, mucoid, with pink to red pigment, usually diffusing into the surrounding agar, indicating fermentation of lactose and acid reduction(10) .

For further identification, *K. pneumoniae* had the ability to grow on the CHROMagar Orientation with metallic blue colour(fig.1) , the major target of this medium is the detection of Gram negative pathogens (especially Urinary tract pathogens), *Klebsiella pneumoniae* appear as metallic blue colonies, also in some time appear as metallic blue with (+/- reddish halo)

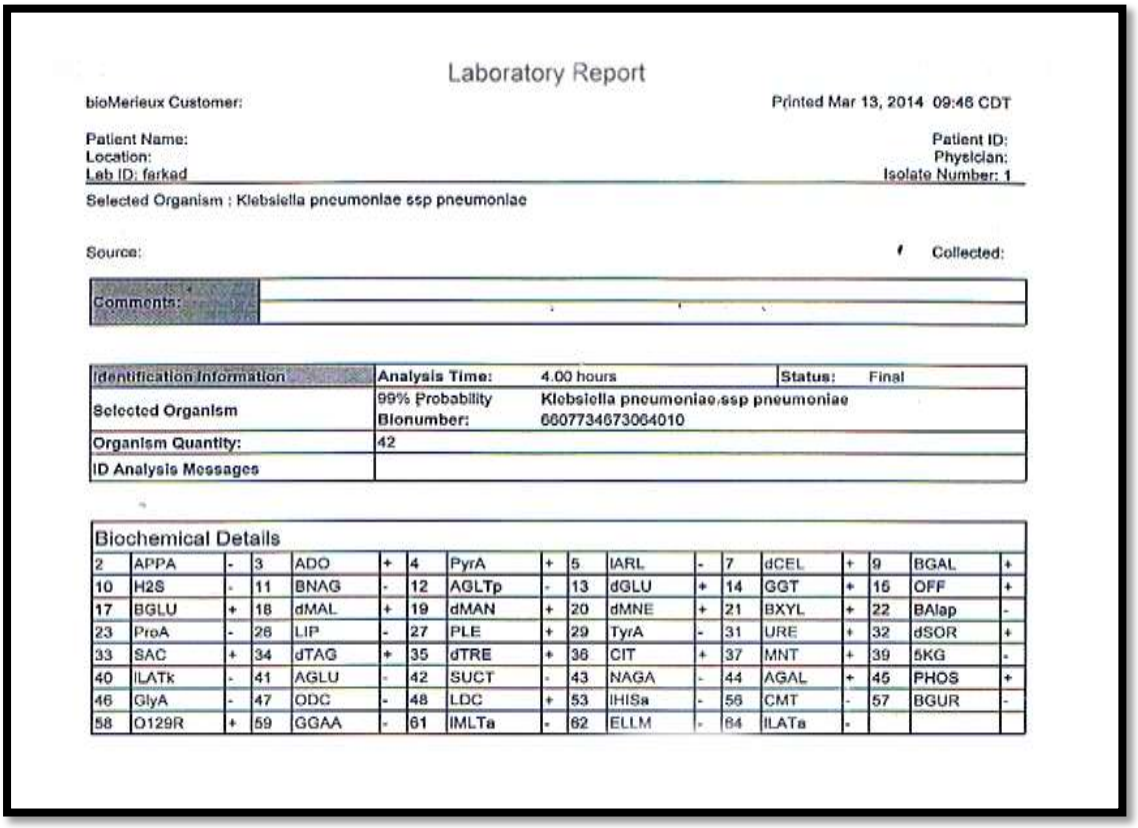
This media allows in most cases full differentiation of the pathogens, allows for reliable detection, enumeration and presumptive identification of urinary tract pathogens, easier recognition of mixed growth and provides higher detection rates.

Figure (1): Growth *K. pneumoniae* on CHROMagar Orientation, the colonies appearance are large mucoid colonies with metallic blue.

The microscopic examination by Gram's stain based on the morphological and staining, *Klebsiella* spp. exhibiting Gram-negative rods. All G-ve bacilli after stained negatively with indian ink (Negative stain) were examined under light microscope the result showed the presence of capsule that surrounded the cell as a hyaline layer as described by Brooks, *et al*.,(11) .

The confirmative diagnosis by VITEK-2 system of *K. pneumoniae* isolates was performed by using VITEK®2 GN kit manufactured by Biomerieux – France. The results showed that all isolates were identified by this technique, as *K. pneumoniae sub. pneumoniae* in ratio of 99% (fig.2).

Fig.2 **: VITEK2 result report for *K. pneumoniae* subsp. *pneumoniae* isolated from different clinical cases.**



Laboratory Report

bioMerieux Customer: Printed Mar 13, 2014 09:46 CDT

Patient Name: Patient ID:
Location: Physician:
Lab ID: farked Isolate Number: 1

Selected Organism : Klebsiella pneumoniae ssp pneumoniae

Source: Collected:

Comments:

Identification Information	Analysis Time:	4.00 hours	Status:	Final
Selected Organism	99% Probability Bionumber:	Klebsiella pneumoniae ssp pneumoniae 6607734673064010		
Organism Quantity:	42			
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	+	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	16	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	-	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Out of 110 of different specimens of patients with nosocomial infections, only 39 (35.4%) *Klebsiella pneumoniae* isolates were recovered. However, 47 specimens of sputum revealed 18 (16.36%) positive result; 37 urine specimens given 15 (13.36%) were positive for *Klebsiella pneumoniae* and 26 specimens of Burns and wounds gave 6 (5.45%) of *K. pneumoniae*, table (1), illustrate the distribution of *K. pneumoniae* among different clinical specimens.

(Table 1): Number and percentage of *Klebsiella pneumonia* occurrence in different clinical specimens.

Source of specimens	No. of Specimens	Positive results	Percentage %
Sputum	47	18	16.36%
Urine	37	15	13.36%
Burn & wound	26	6	5.45%
Total	110	39	35.4%

It was clear from table (1) that *Klebsiella pneumoniae* in present study were differed from previously studies (12) who reported that *Klebsiella pneumoniae* isolates percentage was (14%), in sputum sample were (16%), in urine samples (9.5%) and in Burns &wounds (15.5%) from patients and (13) who showed that *Klebsiella pneumoniae* in sputum were (11.6%), in urine (32.8%), in burn (39.5%) and in wound (16.2%).

Klebsiella pneumoniae is considered the micro flora of intestine; they pose important virulence factors as capsule helping in increasing the

opportunity to infect urinary system. The capsule protects the bacteria from harsh conditions and increases their resistance to immune system as phagocytosis process (2).

Increase rate occurrence of *K. pneumoniae* in hospitals reliant on many factors most patients had serious underlying disease, including diabetes mellitus, Urinary Tract Obstruction (UTO), chronic renal insufficiency or immunosuppression, the ability of this organism to spread rapidly often leads to nosocomial outbreaks, especially in neonatal units(14) the use of antibiotics rather than with factors connected with delivery of care in the hospital and the ability of this bacteria to survive under unsuitable environmental conditions, since it has a thick polysaccharide capsule, in addition to having different mechanisms of antibiotic resistance(15) .

However, the members of *Klebsiella* spp. causing infectious diseases were vary according to the geographic area, period of isolates collection and different bacterial diagnostic methods.

The results of amplification were performed on the DNA extracted from all the studied isolates were confirmed by electrophoresis analysis. By this analysis, the strands of DNA resulted from the successful binding between specific primers and isolates extracted DNA. These successful bindings appeared as single bands under the U.V light using ethidium bromide as a specific DNA stain. The electrophoresis was also used to estimate DNA molecular weight depending on DNA marker (2000 bp DNA ladder) and the result of this estimation revealed that the amplified DNA are K1 genotype at 1046 bp product size and K2 genotype at 1121bp product size (Fig. 3), K5 genotype at 999bp product size and K20 genotype at 1116 bp product size (Fig. 4), K54 genotype at 881 bp product size and K57 genotype at 1182 bp product size (Fig. 5).

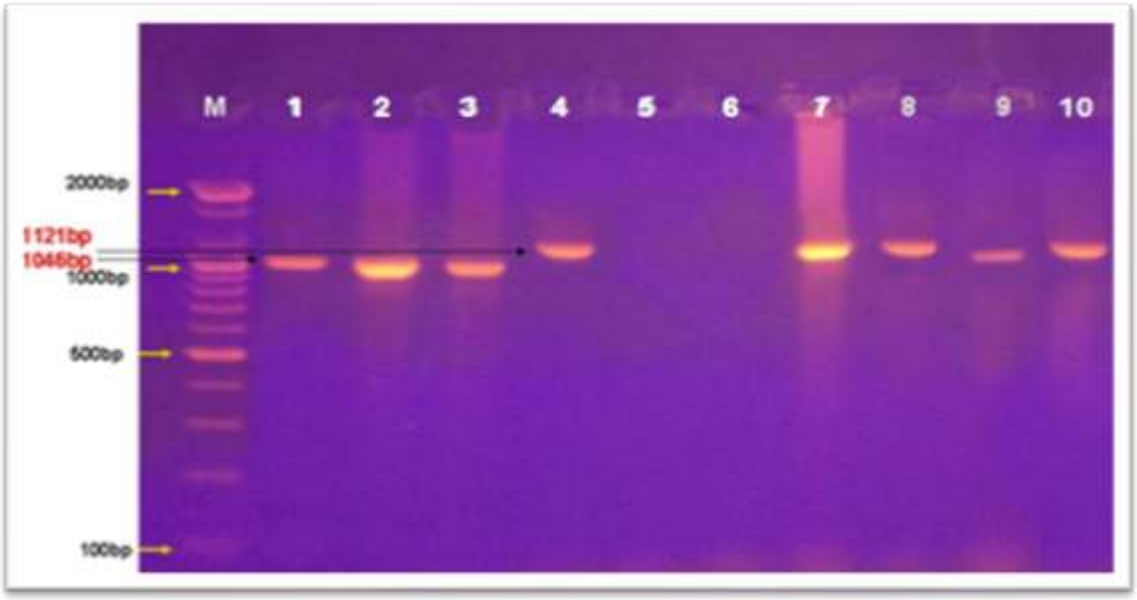


Figure (3): Agarose gel electrophoresis that explains the Multiplex PCR genotypes for *Klebsiella pneumoniae* type (K1 and K2). Lane M: Marker (2000bp), (1, 2, 3 and 9) are K1 genotypes at 1046 bp product size, and (4, 7, 8 and 10) are K2 genotypes at 1121 bp product size. Where, the isolate (5 and 6) are appeared non-typeable.

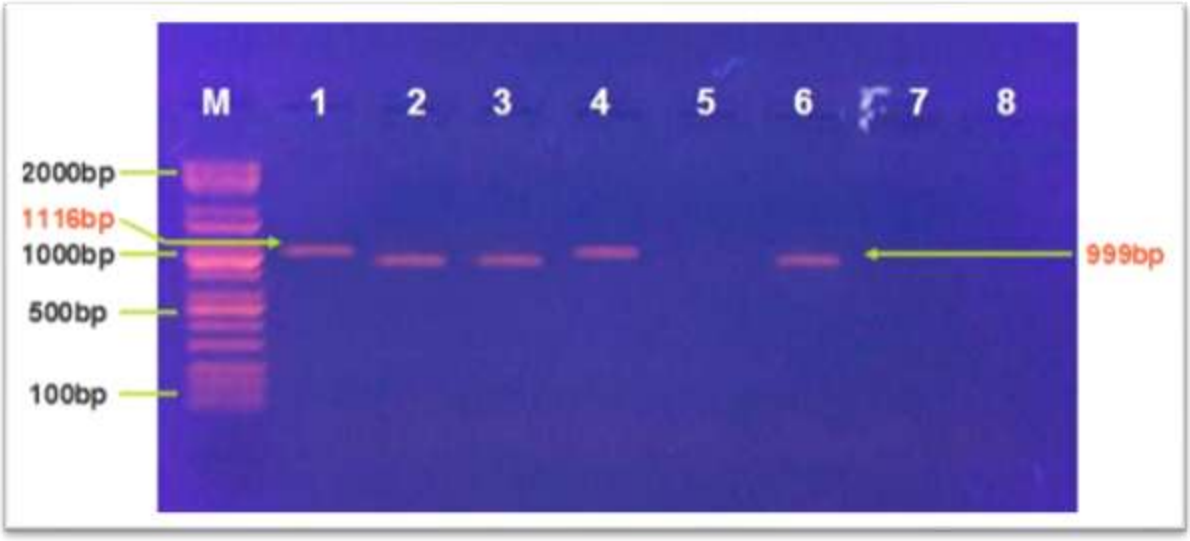


Figure (4): Agarose gel electrophoresis that explains the Multiplex PCR genotypes for *Klebsiella pneumoniae* type (K5 and K20). Lane M: Marker (2000bp) where 2, 3 and 6 are K5genotypes at 999bp product size, (1 and 4) are K20 genotypes at 1116 bp product size and (5, 7 and 8) are non-typeable.

Figure (5): Agarose gel electrophoresis that explains the Multiplex PCR genotypes for *Klebsiella pneumoniae* type (K54 and K57) Lane M: Marker (2000bp) where (2 and 7) are K54 genotypes at 881bp product size, and (1, 2, 5, 6, 8 and 9) are K57genotypes at 1182 bp product size. with isolate (10) are appeared non-typeable.

Polymerase Chain Reaction (PCR) is considered the best efficiency method for bacteria detection because it is faster than phenotypic detection method (16), and also detect the presence of poorly or non-expressed (silent) genes difficult to determine by phenotype; PCR may also be used to directly test patient specimens as an early predictor of infection (17).

In this study, found the PCR methods are highly sensitive and specific in comparison with routine techniques. The present study supports the ability of these specific primers sets to confirm the isolation of *Klebsiella Pneumoniae*.

In this study, 39 isolates from nosocomial infection belong to *Klebsiella Pneumoniae* under the PCR test revealed 28 specimens were success typing in to six types of serotypes of *K. pneumoniae* (K1, K2, K5, K20, K54 and K57) in different percentage, and 11 samples were non-typeable.

The method of multiplex PCR assay would offer an effective alternative to traditional typing methods for the identification and differentiation of the most clinically relevant *Klebsiella* types(18) .

The present results suggest that PCR analysis is a rapid and reliable method for identification of both capsular K1, K2, K5, K20, K54 and K57 serotypes of *K. pneumoniae*. However, the techniques most commonly used for identification of *K. pneumoniae* serotypes (Quellung and counter-current immunoelectrophoresis) are limited, because of costs of various antisera preparation. Thus, PCR assay may help to operate *K. pneumoniae* capsular type identification in routine diagnoses

The present study, depended on *cps* genotyping by PCR detection of K serotype-specific alleles at *wzx* loci because this loci don't have a conserved organization similar to the *Escherichia coli* group 1 capsule biosynthesis gene cluster, comprising 2 regions separated by a putative stem-loop transcriptional attenuator. The '3 region is serotype-specific and encodes enzymes for *wzy*-dependent biosynthesis system, including enzymes for producing sugar nucleotide precursors, gly consyltransferases, ana 2 integral inner membrane proteins (*wzy* and *wzx*)(19) .The polymerase *wzy* assembles undecaprenyl diphosphate- linked polymers using lipid-linked repeat units exported by the flippase *wzx(19)* .The *cps* gens clusters of serotypes K1, K2, K5, K20, K54 and K57 have different alleles at both *wzy* and *wzx* loci.
In this study the results of K1, K2, K5, K20, K54 and K57 serotyping were successfully detected in multiplex PCR (Table 2).

(Table 2): Distribution of capsular serotype in *K. pneumoniae* isolates from different cases (n=39):

PCR-serotype	Sputum	Urine	Burn and wound	Total percent
K1	4(10.25%)	2(5.12%)	1(2.5%)	7(17.94%)
K2	2(5.12%)	3(7.69%)	1(2.5%)	6(15.38%)
K5	2(5.12%)	1(2.5%)	0	3(7.69%)
K20	0	2(5.12%)	0	2(5.12%)
K54	1(2.5%)	1(2.5%)	0	2(5.12%)
K57	4(10.25%)	2(5.12%)	2(5.12)	8(20.51%)
Non-typable	5(12.82%)	4(10.25%)	2(5.12%)	11(28.20%)
Total percent	18/110 **(16.36%)**	15/110 **(13.36%)**	6/110 **(5.45%)**	39/110 **(35.4%)**

A total of 39 clinical isolates of *K. pneumoniae* were included in this study, showed 28 samples (71.79%) were type-able, among 7 positive K1 serotype isolates (four were sputum, two urine specimens and one in burn and wound). Also among 6 K2 serotype isolates (Two were sputum, Three urine and one was with burn and wound). Three positive of K5 serotype isolates (Two sputum and

one urine). Two positive result K20 serotype isolates were urine. Also two positive K54 serotype isolates appear with one sputum and one urine. Among 8 K57 serotype isolates four from sputum, two from urine and 2 were burn and wound. Whereas Among 11 (28.20%) of *K. pneumoniae* were non-typeable (Five sputum, four urine and two were burn and wound).

The present study revealed that there was a predominance of K57, K1 and K2 serotypes in sputum, urine, burn and wound isolates from patients with nosocomial infections, but other studies (20); showed that capsular serotypes are K1 and K2 only and they not referred to serotype K57, but in the present study found serotype K57 was predominant serotypes of *K. pneumoniae* isolated from different clinical cases of nosocomial infections.

In conclusion, *Klebsiella pneumoniae* represented one of the main causative agents of nosocomial infections especially the pulmonary infections. The serotypes K57, K1 and K2 were the more predominant rather than serotypes (K5, K20 and K54) of *K. pneumoniae* isolated from hospitalized infections. Based on the global previous literatures, the K57 serotypes is considered a minor serotypes of *K. pneumoniae*, in the present study, we found that the K57 comprise a major serotype with K1 and K2 of *K. pneumoniae.*

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