# Detection of Serotype Gene of *Klebsiella Pneumoniae* Isolated from Different Clinical Cases of Hospitalized Infections in Al-Diwaniya city

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**الخلاصة:**

الهدف من هذه الدراسة توصيف الانماط المصلية لبكتريا ***K. Pneumoniae*** المعزولة من حالات سريريه للعدوى المكتسبة في المستشفى (التهاب المسالك البولية؛التهاب الجهاز التنفسي والتهابات الحروق والجروح) بواسطة تقنية تفاعل إنزيم سلسلة البلمرة المتعدد.جمعت **110** عينة من الحالات المذكورة أعلاه من المرضى الراقدين في مستشفى الديوانية التعليمي للفترة من كانون الأول **2012** ولغاية اذار**2013**؛توزعت العينات بين (**47**) للالتهابات التنفسية؛(**37**) لالتهابات المسالك البولية و(**26**) لالتهابات الجروح والحروق.

أظهرت النتائج إن نسبة عزل بكتريا ***K. Pneumonia*** كانت( **35.4**%) ؛توزعت بين (**18.18**%) عزلات القشع؛(**10.90**%)عينات إدرار و(**6.36**%) عينات من الجروح والحروق. أظهرت النتائج نجاح طريقة الكشف للأنماط المصلية **K1,K2,K5,K20,K54 & K57** لبكتريا ***K. Pneumonia*** ؛حيث كانت **28** (**25.4**%) عينة من نوع معرف توزعت بين **7** عزلات ايجابية للنمط المصلي**K1** ؛ (حجم الدنا المضخم **1046** زوج قاعدي):**4** عينات قشع؛**2** عينات ادرار و **1** عينة من الحروق ؛ ومن بين **6** عزلات موجبة**K2** (حجم الدنا المضخم **1121** زوج قاعدي) :**3** عينات ادرارح**2** عينات قشع و**1**عينة من الجروح ؛**3** عينات للنمط المصلي**K5** (حجم الدنا المضخم **999** زوج قاعدي)؛وقد عزل النمط المصلي**K20** (حجم الدنا المضخم **1116** زوج قاعدي) من عينتين ادرار فقط؛عزل النمط المصلي**K54** (حجم الدنا المضخم **881** زوج قاعدي) في عينة قشع وعينة ادارواخيرا تم عزل **8** عزلات ايجابية للنمط المصلي **K57**(حجم الدنا المضخم **1182** زوج قاعدي):**4** عينات قشع؛**2** ادرار و **2** من الجروح والحروق. ،مع هذا من بين **11**(**10**%) من بكتريا ***K. Pneumonia*** غير معرفة بالأنماط المصلية في هذه الدراسة.كشفت الدراسة عن سيادة الانماط المصلية**K1,K2 & K57** في عينات القشع ؛الإدرار والجروح والحروق التي تم عزلها من المرضى الذين يعانون من حالات عدوى المستشفى.

**Abstract:**

This study aimed to identify serotype of *Klebsiella Pneumonia* isolated from different clinical cases of hospitalized infections( urinary tract infection, respiratory infection, burns & wounds) by using polymerase chain reaction(PCR).A total of 110 clinical specimens from in-patients with nosocomial infections(including 47 respiratory infection,37 UTI , burns & wound infections 26) in Al-Diwaniya Teaching Hospital during the period from December 2012 –March 2013 were collected. *K. Pneumonia* isolated from 35.4% of the specimens distributed as 18.18% sputum specimens,10.90% urine specimens & 6.36% burns & wounds specimens.

K1,K2,K5,K20,K54 & K57 serotypes of *K. Pneumonia* were successfully detected in multiplex PCR.The results revealed that 28 specimens(25.4%) were type able including 7 positive K1(Amplicon size 1046bp) serotype isolates ( 4 from sputum,2 from urine & 1 from burn), 6 positive of K2(Amplicon size1121) serotype isolates (3 from urine,2 from sputum & 1 from wound),3 positive for K5(Amplicon size 999bp) serotype isolates( 2 from sputum & 1 from

urine),2 positive for K20(Amplicon size 1116bp) serotype isolates from urine 2 positive of K54(Amplicon size 881bp) serotype isolates were one from sputum & the other from urine& finally 8 positive of K57(Amplicon size 1182bp) serotype isolates(4 from sputum,2 from urine & 2 from burns & wounds).
However, among 11 (10%) specimens, *K. Pneumonia* were non type able.
This study revealed that there were predominance of K1,K2 & K57 serotype isolates in sputum, urine burns & wounds of patients with nosocomial infections.

**Introduction:**

*Klebsiella Pneumonia*e is one of the most opportunistic pathogens commonly predominant in hospital environment , particularly in medical & surgical instruments like catheter & bed of the patients in burn & surgical intensive care units which are critical sources of *klebsiella* nosocomial infections(Fang et al.,2005.[1]

*Klebsiella Pneumonia*e are more commonly implicated in hospital acquired urinary tract & wound infections,particulary in immune compromised individuals & diabetics(Antoniadou,2006).[2]

Capsule is one of the important virulence factors of *Klebsiella Pneumonia*e,the capsular material forms thick bundles of fibrillous structures covering the bacteria surface in massive layers. A larger capsule composed of complex acidic polysaccharide; the capsular subunits consisting of 4-6 sugar attached with glucuronic acid & pyruvic acid molecules This protect bacterium from phagocytosis by polymorphnuclear granulocytes & prevents killing of bacteria by bactericidal serum factors(Carmen et al.,2001)[3].

There are more than 80 different capsular(K) serotypes & subtype with similar antigenicity but different polysaccharide backbones have been described(Sikarwar & Batra,2011).[4]

The degree of virulence conferred by a particular K antigen might be connected to the mannose of CPS.

Capsular types with low virulence, such as K7 or K21a antigen(Ofek et al.,1993)[5],contain repetitive of mannose-a-2/3-mannose or L-rhamnose-a-2/3-L-rhamnose. Thus, *Klebsiella* strains bearing capsule types devoid of these mannose or rhamnose sequences should be more closely associated with infectious diseases. Previous attempts to establish a correlation between individual *klebsiella* serotypes & the site of infection or clinical symptoms have produced a profusion of contradictory results,each study reports different capsular types as predominate(Crys et al.,1986)[6].

Aim of the study: to identify &detect capsular serotypes (K1,K2,K5,K20,K54 & K57) of *K. Pneumoniae* , using specific *caps* gene(WZC) which are required for biosynthesis of capsular polysaccharide depending on multiplex polymerase reaction(PCR) technique.

Method:

Specimens collection: During the period from December 2012 to March 2013, a total of 110 clinical specimens were collected from patients with different nosocomial infections who had been admitted to Al-Diwaniya Teaching Hospital .Detection of nosocomial infections according to CDC Definition of Nosocomial Infections, Garner JS,et al,1996[7].the collection of specimens according to MacFaddin,2000[8].

Identification of bacterial isolates: bacterial isolates were identified to the level of species using the traditional morphological & biochemical diagnostic tests, according to the methods of macfaddin,2000[8],then prepared for further tests.

Multiplex polymerase chain reaction(PCR):

Multiplex (PCR) assay was performed for detection & genotyping of *K.Pneumonia*
Based on capsular antigen(K) gene by using K1,K2,K5,K20,K54 & K57 gene primers. This assay was done according to the method described by Fang et al 2007[9]. Genomic DNA of *K.Pneumonia* isolates were extracted by using Genomic DNA kits.
Preparation of primers:
All primers were dissolved using TE buffer,1x(PH 8),which composed of 10 mM Tri- EDTA-Na2.At 1st <the primer stock tube prepared<then the working solution was prepared from primer manufacture(Bioneer/Solution Korea),The TE buffer was added to get 100 picomole/µmol concentration of primer stock solution. The working solution prepared from stock by dilution with Te buffer to get 10 picomole/µl.
Preparation of Multiplex PCR master mix

Multiplex PCR master mix reaction was prepared by using AccuPower PCR PreMix Kit and this master mix done according to company instructions as shown in Table1

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**Table (1): Preparation of Multiplex PCR master mix used in the study.**

| Multiplex master mix | | Volume |
|---|---|---|
| DNA template | | 5µL |
| K1 | Forward primer (10pmol) | 1.5µL |
| | Reverse primer (10pmol) | 1.5µL |
| K2 | Forward primer (10pmol) | 1.5µL |
| | Reverse primer (10pmol) | 1.5µL |
| PCR water | | 9µL |
| Total | | 20µL |

| Multiplex master mix | | volume |
|---|---|---|
| DNA template | | 5µL |
| K5 | Forward primer (10pmol) | 1.5µL |
| | Reverse primer (10pmol) | 1.5µL |
| K20 | Forward primer (10pmol) | 1.5µL |
| | Reverse primer (10pmol) | 1.5µL |
| PCR water | | 9µL |
| Total | | 20µL |
| Multiplex master mix | | volume |
| DNA template | | 5µL |
| K54 | Forward primer (10pmol) | 1.5µL |
| | Reverse primer (10pmol) | 1.5µL |
| K57 | Forward primer (10pmol) | 1.5µL |
| | Reverse primer (10pmol) | 1.5µL |
| PCR water | | 9µL |
| Total | | 20µL |

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.

**PCR Thermocycler Conditions:**

**Table 2: PCR Thermocycler Conditions used in the study.**

| Steps | Temperature C° | Time min. | No. of cycle |
|---|---|---|---|
| Initial denaturation | 96 | 3:00 | 1 |
| Denaturation | 96 | 0:30 | 30 |
| Annealing | 56 | 0:15 | |
| Extension | 74 | 1:00 | |
| Final extension | 72 | 10:00 | 1 |

## Statistical analysis:

Experimental data were presented in terms of observed numbers and percentage frequencies was used (Paulson, 2008)[10].

## Results & discussion:

Among 110 clinical specimens obtained from different nosocomial infections, *K. pneumonia* isolates were recovered from 39(35.4%)specimens.

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). **In this study the results of K1, K2, K5, K20, K54 and K57 serotyping were successfully detected in multiplex PCR (Table 3).**

**Table 3: 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%) |

For further identification, *K. pneumoniae* had the ability to grow on the CHROMagar Orientation with metallic blue colour (Figure 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).

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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 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%(Appendix 1).**

## Polymerase Chain Reaction (PCR):

## Genomic DNA extraction

**The DNA of all isolates were extracted and purificated using genome DNA purification kit. The results were detected by electrophoresis on 1% agarose gel and exposed to U.V light in which the DNA appeared as compact bands (Figure 2) which provide that technique and kits are successes in the extraction and purification of tested specimens.**

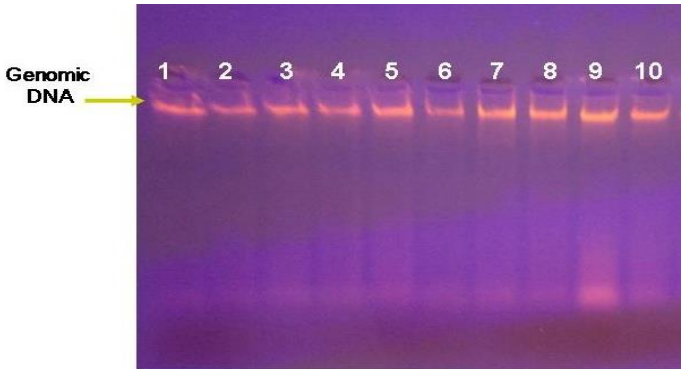


**Figure 2: Agarose gel electrophoresis that explains the genomic DNA extraction by using Genomic DNA mini kit Geneaid USA.**

## DNA Concentrations and Purity.

**Table 4 Shows concentration and purity of extracted DNA samples, where the concentration of DNA ranged from 15-100 (ng/µL) and their purities ranged from 1.45-1.85 these values are well sufficiently for amplification by PCR method.**

Table 4: Concentration and purity of extracted DNA

| Sample No. | Concentration (ng/µL) | Purity (260/280) |
|---|---|---|
| 1 to 10 | 20-65 | 1.4-1.78 |
| 11-20 | 15-100 | 1.60-1.89 |
| 21-30 | 40-70 | 1.45-1.85 |
| 31-39 | 20-50 | 1.6-1.91 |

## DNA Amplification using multiplex PCR:

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).

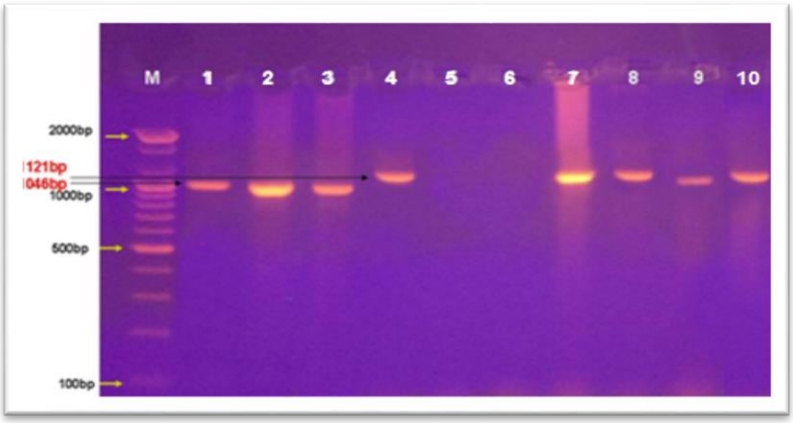


**Figure 3: Agarose gel electrophoresis that explains the Multiplex PCR genotypes for *Klebsiella pneumoniae* type (K1 and K2). Lane M: Marker (2000bp), (1, 2, 3 and 9) are K1 genotypes at 1046 bp product size, and (4, 7, 8 and 10) are K2 genotypes at 1121 bp product size. Where, the isolate (5 and 6)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

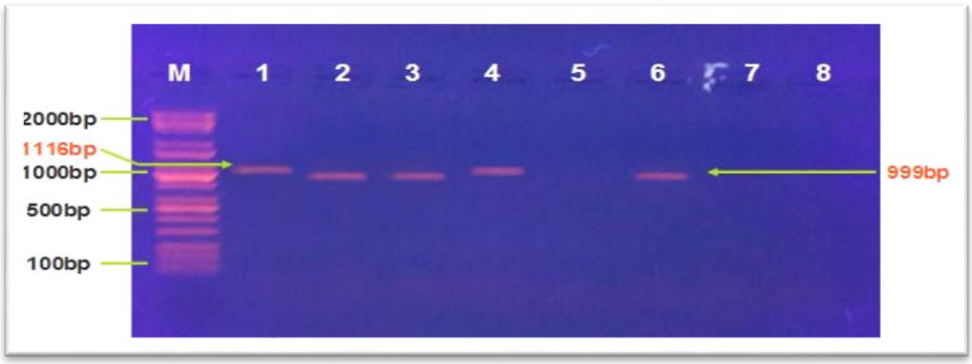


**Figure4: Agarose gel electrophoresis that explains the Multiplex PCR genotypes for *Klebsiella pneumoniae* type (K5 and K20). Lane M: Marker (2000bp) where 2, 3 and 6 are K5genotypes at 999bp product size, (1 and 4) are K20 genotypes at 1116 bp product size and (5, 7 and 8) are non-typeable.**

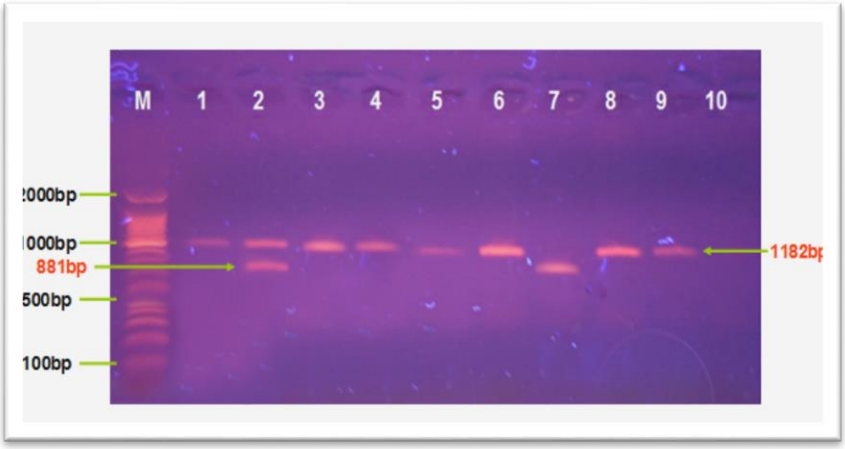


**Figure 5: Agarose gel electrophoresis that explains the Multiplex PCR genotypes for *Klebsiella pneumoniae* type (K54 and K57). Lane M: Marker (2000bp) where (2 and 7) are K54 genotypes at 881bp product size, and (1, 2, 5, 6, 8 and 9) are K57genotypes at 1182 bp product size. with isolate (10) are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

**Polymerase Chain Reaction (PCR) is considered the best efficiency method for bacteria**

detection because it is faster than phenotypic detection method (Chiangjong, 2006)[11], 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 (Diekema *et al*., 2004)[12].

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 successfully 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 (Alvarez *et al*., 2004)[13].

Multiplex PCR has got an important tool of modern molecular biology because it offers several important advantages over amplifications involving one DNA marker at a time or "singleplexes". First, the cost of analysis and labor to obtain a set of results from multiple markers can be reduced. Second, the amount of information obtained per unit time of investment improves. Third, the amount of template DNA required to obtain results is reduced (Chamberlain *et al*., 1988)[14].

Genotyping of *Klebsiella pneumoniae*:

In Iraq, this is the first report on the occurrence of molecular serotypes K1, K2, K5, K20, K54 and K57 the clinical specimens and shows the pathogenic potential of these isolates in different organs. Molecular Serotyping with PCR for serotypes had not been previously studied in Iraq. This may provide a new way to "genotype" an unknown capsular type strain but will require validation with several strains of each serotype to determine specificity and sensitivity. A multiplex PCR described which detects capsular types K1, K2, K5, K54 and K57, which are most associated with invasive disease or pathogenicity, these methods provide a rapid means of characterizing and typing isolates of this important agent of community-acquired or nosocomial infection. (Pan *et al*., 2008)[15].

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. Great advantage of molecular serotyping is that it does not undergo cross-reactions which sometimes render highly ambiguous results for conventional serotyping (Yu *et al*., 2009)[16]. Therefore, PCR genotyping seems to be a more sensitive and specific way for detecting these serotypes (Pan *et al*., 2008)[15]. Furthermore, molecular serotyping is capable to determine a potential serotype of capsule-deficient isolates (Gierczynski *et al*., 2007[17]; Turton *et al*., 2008)[18].

For the PCR assay, we selected *wzx* gene, encoding tyrosine-protein kinase and open reading frame 10 (orf-10) encoding putative inner membrane proteins for K1, K2, K5, K20, K54 and K57serotypes, respectively. There have been many reports linking *magA, rmpA* and *wcaG* with virulence, also *wzx* and orf-10 have been studied (Pan *et al*., 2008)[15].

*Wzx* protein was analyzed to evaluate K1, K2, K5, K20, K54 and K57 serotypes capacity to participate in the reversible phosphorylation of proteins on tyrosine. *Wzx* was found in other bacterial species which are all involved in the synthesis or export of exopolysaccharides. Since these are considered as important virulence factors, it could be suggested that reversible protein phosphorylation on tyrosine may be part of the cascade of reactions that determine the pathogenicity of bacteria (Vincent *et al*., 1999)[19]. The open reading frames (ORFs) of the complete cps regions suggested that this region was responsible for capsular polysaccharide synthesis (Pan *et al*.,

2008)[15]. 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*) (Whitfield, 2006)[20]. The polymerase *wzy* assembles undecaprenyl diphosphate-linked polymers using lipid-linked repeat units exported by the flippase *wzx* (Whitfield, 2006)[20]. The *cps* gens clusters of serotypes K1, K2, K5, K20, K54 and K57 have different alleles at both *wzy* and *wzx* loci.

## Conclusions:

1- *Klebsiella pneumoniae* represented one of the main causative agents of nosocomial infections especially the pulmonary infections.

2- The CHROMagar has advantages over traditional media due to full differentiation of the *K. pneumoniae* easier recognition of mixed growth.

3- The capsular (Caps) gene analysis by PCR allow determination of K serotype, while easier to perform and more discriminatory than classical serotyping.

4- The serotypes K57, K1 and K2 were the more predominant rather than serotypes (K5, K20 and K54) of *K. pneumoniae* isolated from hospitalized infections.

5- 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.*

## Recommendations:

1- It is necessary to give an attention for virulence of *K. pneumoniae* due to the variant of caps gene and then changing the strategies of chemotherapy against this bacteria (Antibiotic susceptibility).

2- The use of CHROMagar for reliable detection, enumeration and presumptive identification of nosocomial pathogens.

3- Conducting a numerous studies for other virulence genes that contribute in the molecular genotyping of *K. pneumoniae*.

4- More attention should be done for phenotypic and genotypic of K57 serotype of *K. pneumoniae* in clinical specimens.

5- The application of other types of PCR such as REFLP-PCR for determination the phylogeny of the serotypes of *K. pneumoniae*, in addition to the DNA sequencing of genotypes of *K. pneumoniae*.

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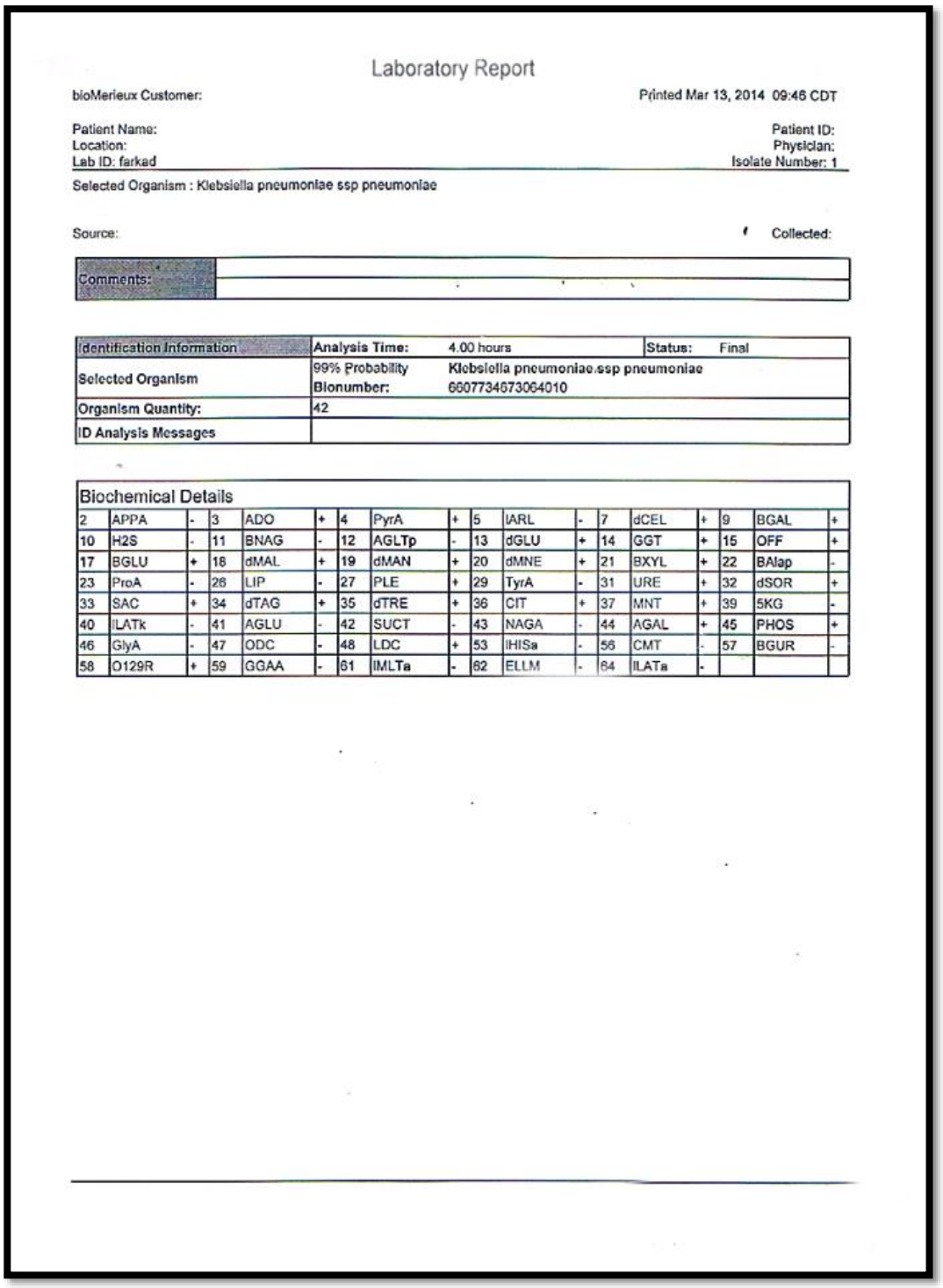
Appendix 1: VITEK2 result report for *K. pneumoniae* subsp. *pneumoniae* isolated from different clinical cases.



Laboratory Report

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

Patient Name: Patient ID:
Location: Physician:
Lab ID: farkad 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<br>Bionumber: | Klebsiella pneumoniae ssp pneumoniae<br>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 | + | 15 | 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 | - | | | |