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# Genotyping of *Klebsiella* spp. isolated from different clinical sources

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**Abstract**

A total of 172 clinical were obtained over 6 months. *Klebsiella* spp. was detected in 58 (33.7%) samples with a high percentage 29 (50%) in urine in female and low percentage 1(1.7%) in pus and burn swabs in male, and the vaginal swab was 1(1.7%). The female to male ratio was 3.1:1. PCR detection showed that 51(87.93%) out of 58 produce 108 bp. product with *rpo*B specific primer that represented *K. pneumonia*. Whereas 7(12.07%) showed PCR product with 343 bp by *K. oxytoca* specific primer (*peh* X), furthermore, the sequences of two selected isolates showed that the species related to *K. oxytoca* strain CAV1335, and to *K. oxytoca* strain CAV1374. Five selected isolates were re-tested by the *gyr* A primer, all were showed specific band product with 441bp. Sequencing blast analysis for these isolates showed that one was related to *K. pneumoniae* subsp. pneumoniae strain RJF999, two isolates related to *K. pneumoniae* strain 17265 *Gyr*A, one related to *K. oxytoca* strain 7102 *Gyr*A and one related to *K. pneumonia* isolate 103 G*yr*A gene. Phylogenetic tree analysis showed the relation of 3 *K. pneumoniae* isolates to USA and UK strains and one with the Asian strains, and 2 *K. oxytoca* isolates have a relation within the Iranian strains and one has a genetic variation.

**Keywords**: *K.oxytoca*, PCR, sequencing, phylogeny.

# التنميط الجيني لبكتريا *Klebsiella* spp المعزولة من مصادر سريرية مختلفة

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**الخلاصة**

جمعت 172 عينة سريرية على مدى اكثر من ستة أشهر، وتم الكشف عن الكليبسيلا في 58 (33.7%) عينة وكانت اعلى نسبة 29 (50%) في عينات الادرار في النساء، واقل نسبة 1 (1.7%) من مسحات القيح والجروح في الذكور وبنفس النسبة في مسحات المهبل. وأشارت النتائج إلى أن نسبة عزلات الكليبسيلا في الإناث إلى الذكور هي 1:3.1. اظهر فحص انزيم البلمرة المتسلسل أن نسبة 51 (87.98%) من اصل 58 باستخدام بادئ *rpo* B قطعه بحجم 108 والتي تشير الى *pneumoniae K.* بينما اظهرت 7(12.07%) قطعة بحجم 343 بأستخدام بادئ *pehx* الخاص ببكتيريا *K.oxytoca*. وكان تطابق تسلسل القواعد النتروجينية لعزلتين منتخبتين منها الى انها ترجع الى strain *K. oxytoca* CAV1374و CAV1335 *K. oxytoca* strain تم اختبار خمس عزلات منتخبة باستخدام بادئ gyra واظهرت قطعة بحجم 441 باستخدام طريقة انزيم البلمرة المتسلسل للقواعد النتروجينة الى ان واحدة تعود الى *K. pneumoniae* subsp. Pneumonia strain RJF999 واثنين الى *K. pneumonia* strain

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*K.pneumonia* isolate 103 وواحدة الى *K.oxytoca* strain 7102 GyrA وواحدة 17265 GyrA
. اظهر فحص شجرة العائلة الى ان ثلاث عزلات من *K.pneumonia* قريبة بصفاتها من العزلات الامريكية والبريطانية وواحدة قريبة من صفات العزلات الاسيوية بينما كانت عزلتين *K.oxytoca* قريبة بصفاتها من العزلات الايرانية وعزلة واحدة مغايرة وذات صفات جينية بعيدة.

## Introduction

The *Klebsiella* belongs *Enterobacteriaceae* family, has three important species associated with illness in humans: *K. pneumoniae, K. oxytoca,* and *K. granulomatis*. Klebsiellae has become important pathogens in nosocomial infections in recent years [1,2]. *Klebsiella* has a polysaccharide capsule, which play an important role in virulence. Capsule protects *Klebsiella* from phagocytosis and and inhibiting the activation of complement components, especially C3b [3,4]. Clinically and epidemiologically important species are *K. pneumoniae* and *K. oxytoca*. A large number of molecular typing methods for their epidemiology of outbreaks have been used: plasmid pattern, restriction endonuclease analysis, ribotyping, random amplified polymorphic DNA, multilocus enzyme analyses and pulsed-field gel electrophoresis.

These were soon followed by polymerase chain reaction technique. A number of conventional PCR assays have been developed targeting the four most common nosocomial pathogens ( *A. baumannii*, *E. coli*, *K. Pneumonia* and *P. Aeruginosa*) , a Real-time PCR that can target all five clinically important species was developed that are highly specific that allow researchers to rapidly identify these pathogens directly from culture in less than 90 minutes with a high degree of accuracy and sensitivity [5-7].

Aims of the work is to determine the predominant species of *Klebsiella* spp. that distributed in Baghdad and Kut hospitals, and to study the relation between the sequenced isolates to the reference strains using the phylogenic tree.

## Materials and Methods

### Samples collection

One hundred and seventy two clinical specimens (burns, wounds and vaginal swabs, urine, sputum, stool and blood), were collected during the period from October 2014 until April 2015, from four hospitals (Al-Yarmouk hospital, Child Center hospital, Al-Swera hospital and Al-Karrama Teaching hospital),.

### Bacterial isolation and Identification

The clinical specimens were taken carefully from the site of infection. Primary isolation were done according to the general culture characteristic and lactose fermentation [8], further identification was achieved by microscobical examination using anthony's capsule stain [9] and the API20E Biochemical Test Strip [10].

### DNA Extraction and primers preparation

All bacterial genomic DNA was extracted by Promega DNA extraction and purification kit (USA) according to the manufacture instructions. Extracted genomic DNA was used as template in PCR reactions [11]. DNA purity was determined by measuring the UV absorbance at 260 and 280 nm with using the nanodrop [12]. All primers were used in this study [13, 14], synthesized as a lyophilized product of diverse picomols concentrations, and were dissolved in sterile deionized distilled water give a final concentration of 100 picomols\ml of suspension and then diluted to 10 picomols\ml according to the manufacture instructions. Three specific primers Table-1 were used to identify *Klebsiella* as a genus by targeting *gyr*A and as *K. pneumonia* and *K. oxytoca* by targeting *rpo*B and *peh*X respectively.

**Table 1-** Primers used in this study for the genus and species detection.

| Primers | Sequence 5-3 | Target gene | PCR-size (bp) |
|---|---|---|---|
| KP- F<br>KP - R | CAA CGG TGT GGT TAC TGA CG<br>TCT ACG AAG TGG CCG TTT TC | *rpo* B | 108 |
| KO- F<br>KO- R | GAT ACG GAG TAT GCC TTT ACG GTG<br>TAG CCT TTA TCA AGC GGA TAC TGG | *Peh* X | 343 |
| Kleb- F<br>Kleb- R | CGC GTA CTA TAC GCC ATG AAC GTAACC GTT<br>GAT CAC TTC GGT CAG G | *Gyr* A | 441 |

***Gyr*A for *Klebsiella* as a genus; *rpo*B for *K. pneumonia* and *peh*X for *K. oxytoca.***

**PCR reaction preparation**

PCR was performed in a DNA thermal cycler using aliquot as reported in Table-2, according to the program condition shows in Table-3. Positive and negative controls were amplified with each run Table-4. After gel electrophoresis, the ethidium bromide stained PCR products were visualized under UV light. The size of the bands was compared to the molecular weight marker [12].

**Table 2-** PCR reaction preparation for detection *Klebsiella* spp

| Reaction component | Volume µl | Final concentration |
|---|---|---|
| Promega Green PCR Master mix (2X) | 12.5 | 1X |
| upstream primer 10µM | 1 | 0.1–1.0µM |
| downstream primer 10µM | 1 | 0.1–1.0µM |
| DNA template | 3 | <250ng |
| Nuclease free water | 25 | N.A. |
| Total volume | 25 | |

**Table 3-** The program of PCR technique used to detect *Klebsiella* spp.

| Steps | Temperature | Time | No. of cycle |
|---|---|---|---|
| Initial denaturation | 95 °C | 5min. | 1 |
| Denaturation | 95 °C | 30 sec | 35 |
| Annealing | 55 °C | 30 sec | |
| Extention | 72 °C | 30 sec. | |
| Final extention | 72 °C | 7 min. | 1 |
| Holding | 4 °C | | |

**Table 4-** Positive and negative controls

| Control | Bacteria | Source |
|---|---|---|
| Positive control | *K. pneumomia* | Central Health Laboratory |
| Negative control for PCR | D.W. without DNA | |

**DNA Sequencing**

Five isolates were retested with genus specific primers targeting the *gyr*A gene Table-1, under the same reaction conditions as for PCR with the species specific primers, in addition to two PCR products of *K. oxytoca*. The PCR products were sequenced by promega company (USA) and the data obtained were matched with the online database using BLAST [15, 16].

**Result and Discussion**

A total number of 172 clinical samples were collected over six months. The samples were selected randomly, from four hospitals (Al-Yarmouk hospital, Child Center hospital, Al-Swera hospital and Al-Karrama Teaching hospital). Out of 172 samples, the urine samples were 108 (62.8%), blood samples were 20 (11.6%), wound swabs were 15 (8.7%), stool samples were 12(7.0%), vaginal swabs were 9 (5.2%), sputum samples were 4(2.4%), burns swabs were 3(1.7%) and the pus swab was 1(0.6%), and the collected samples in female were 120 (69.8%) much more than in male 52 (30.2%), as shown in Table-5. This study revealed that urine samples were represented in high percentage followed by blood then wound swabs, this due to that the urinary tract infection is a very common reason for consultation and antibiotic prescription in current practice while the pus sample is very low. The female urine samples are much more than the male urine samples, this because that the women suffering from UTIs more than men due to that the urethra is more shorter, wider and adjacent to the anus in female [17, 18].

**Table 5-**Sex distribution frequency among 172 samples

| Sample source | percentage % | Female | Male |
|---|---|---|---|
| Urine | 108 (62.8) | 88 | 20 |
| Wound swabs | 15 (8.7) | 5 | 10 |
| Stool | 12 (7.0) | 5 | 7 |
| Sputum | 4 (2.4) | 3 | 1 |
| Blood | 20 (11.6) | 9 | 11 |
| Burns swabs | 3 (1.7) | 1 | 2 |
| Pus swabs | 1 (0.6) | 0 | 1 |
| Vaginal swabs | 9 (5.2) | 9 | 0 |
| Total | 172 (100) | 120 | 52 |

*Klebsiella* spp. were detected in 58 (33.72%) from the total 172 samples by colonies morphology, capsule staining and the biochemical tests. As shown in Figure-1, the *Klebsiella* isolates in female were more than in male with a highest percentage in urine samples which represented 29 (50%), followed by wound swabs samples 6(10.34%) then by stool 4 (6.8%), while in male urine samples were 6 (10.34%) then followed by wound and stool 3 (5.17%) for each. The sputum, blood and burn samples presented 2(3.44%) for each from female only. While pus and burn swabs represented 1(1.72%) for each in male only, the same percent for vaginal swab. In a study by Ravichitra [19] found that the highest number of *K. pneumonia* was isolated from pus samples (69.1%) followed by sputum then urine. Whereas Romanus [20], showed that the urine was with high percentage, followed by sputum then pus. Battikhi and Battikhi [21] also reported that the urine and vaginal *K. pneumonia* isolates in female were more than in the male. Female to male ratio was 3.1:1, as shown in Figure-2, the female percentage was 75.86%, while the percentage of male was 24%. Almost, the same ratio was presented by Anil and Chandrika [22].

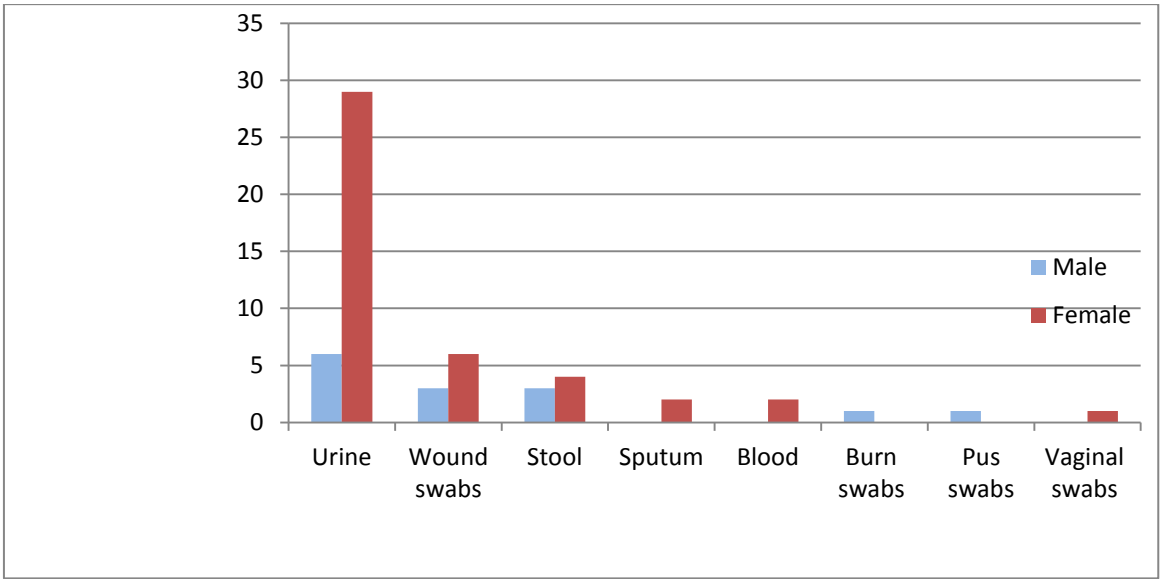


**Figure 1-** The distribution of 58 *Klebsiella* isolates according to gender and samples source.

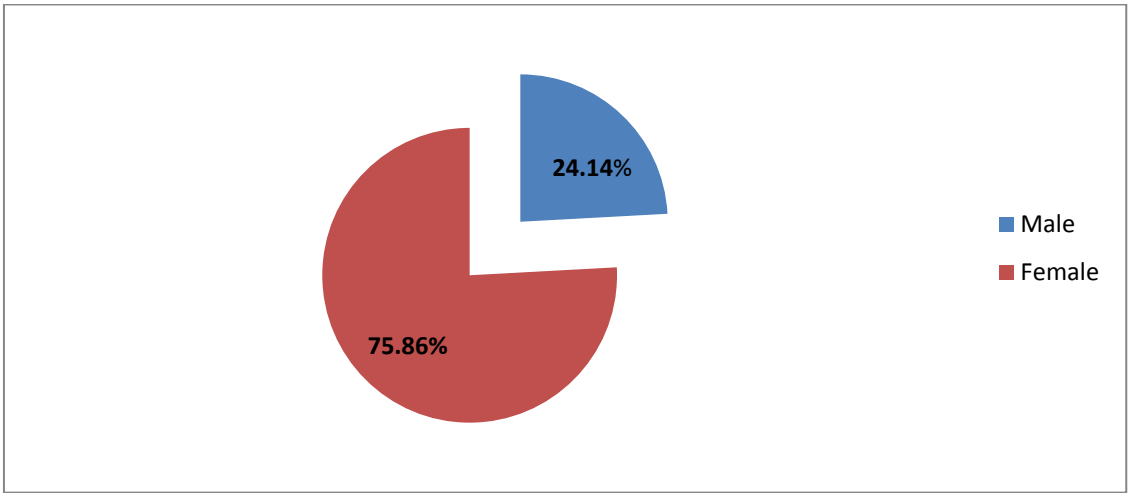


**Figure 2-** Sex distribution of 58 *K. pneumonia* isolates.

**Conventional Methods for Identification of *K. pneumonia*:**

Fifty eight isolates (58) were identified as *Klebsiella* spp. depending on the cultural characteristics, microscopic examination, capsule stain and API 20E biochemical test. Bacterial isolates were identified on their appearance on the MacConkey agar and EMB agar. Those exhibiting mucoid pink color colonies on the MacConkey and good growth of brown, dark-centered, mucoid colonies in EMB agar plate were processed for biochemical testing [23]. All isolated bacteria were encapsulated 58(100%), this in agreement with a study by Ruaa and Abbas [24], small straight rod-

shaped arranged single or in pairs and the capsule appears as a faint blue or white halo around a purple cell under light microscope. The crystal violet stains both the bacterial cell and the capsule, then the capsule stain only eliminated by copper sulfate which serves as both decolorizer and counter stain [9]. Colonial morphology and microscopical examination with the API 20E system test should be considered before reaching a final identification steps. Misidentification made either by the API 20E system or by the diagnostic laboratory or by both. The API 20E strip identified 100% of the isolates. Identifications by the API 20E system and the diagnostic laboratories were in agreement in 87.9% of the isolates, disagreement on 12% of the isolates, a same result reported by Evryll and Michael [25]. Differences in identification occurred primarily in distinguishing between *Klebsiella*, *E. coli.* and *Enterobacter*, and these disagreements were most often due to incorrect identifications by the diagnostic laboratory rather than by the API20E system. So the API 20E system more reliable in detection especially when confirmed by molecular method. The percentage of *K. pneumonia* about 54 (93.1%), and the remaining isolates were *K. oxytoca* with a percentage 4 (6.9%). The prevalence of *K. oxytoca* was less common than *K. pneumoniae*, this result in disagreement with a study by Asal, [26], who's found that the prevalence of *K. oxytoca* from patients with suspected urinary tract infections in Kirkuk hospital recorded the percentage of (25.92%).

**Molecular identification of *Kelbsiella* spp.**

**DNA Extraction**

The genomic DNA was extracted from 58 *Klebsiella* spp. isolates by using promega kit. This kit used to extract genomic DNA from gram negative and Gram positive bacteria to yield intact DNA with a good quality and high purity to use in PCR and sequence techniques. The purity of DNA samples were range from (1.6 – 2), and the concentration range (90-600) ng/ul.

**PCR technique**

*K. pneumoniae* and *K. oxytoca* are the two members of the genus *Klebsiella* responsible for most human infections. *K. pneumoniae* is the most common reason of nosocomial infections. Members of the genus *Klebsiella* are opportunistic pathogens, there identification are difficult and misclassified in laboratories [27], so correct identification is necessary for taxonomic and molecular description. Fifty one (87.93%) out of 58 showed PCR product with 108 bp by *K. pneumonia* specific primer (*rpoB*), that represented *K. pneumonia* Figure-3. Whereas 7 (12.07%) showed no product which re-tested by *K. oxytoca* specific primer (*Peh* X), and all showed a product with 343 bp that represented *K. oxytoca*, Figure-4, furthermore, the sequences of two selected isolates showed that the species related to *K. oxytoca* strain CAV1335 Figure-5A, and to *K. oxytoca* strain CAV1374. Figure-5B. This disagreed with a study by Hadi [28] in Kufa University, who's found that only *K. pneumoniae* subsp. *Pneumonia* were identified among all *Klebsiella* isolates from UTLs, this may explained by using a variety of sources that obtained from different locations in this study. Ekrem *et al*., [29], showed that the *K. oxytoca* percent was 1(1.43) among 70 bacterial isolates in patients with UTIs from Rzgary Teaching Hospital in Erbil. *K. pneumoniae* are the most important cause of nosocomial *Klebsiella* infections and the most medically important species of the genus. *K. oxytoca* is too much lesser degree, this result in agreement with a study by Podschun and Ullmann [30], they showed that *Klebsiella* spp. in the U.S and in Europe cause 8% of all nosocomial bacterial infections.

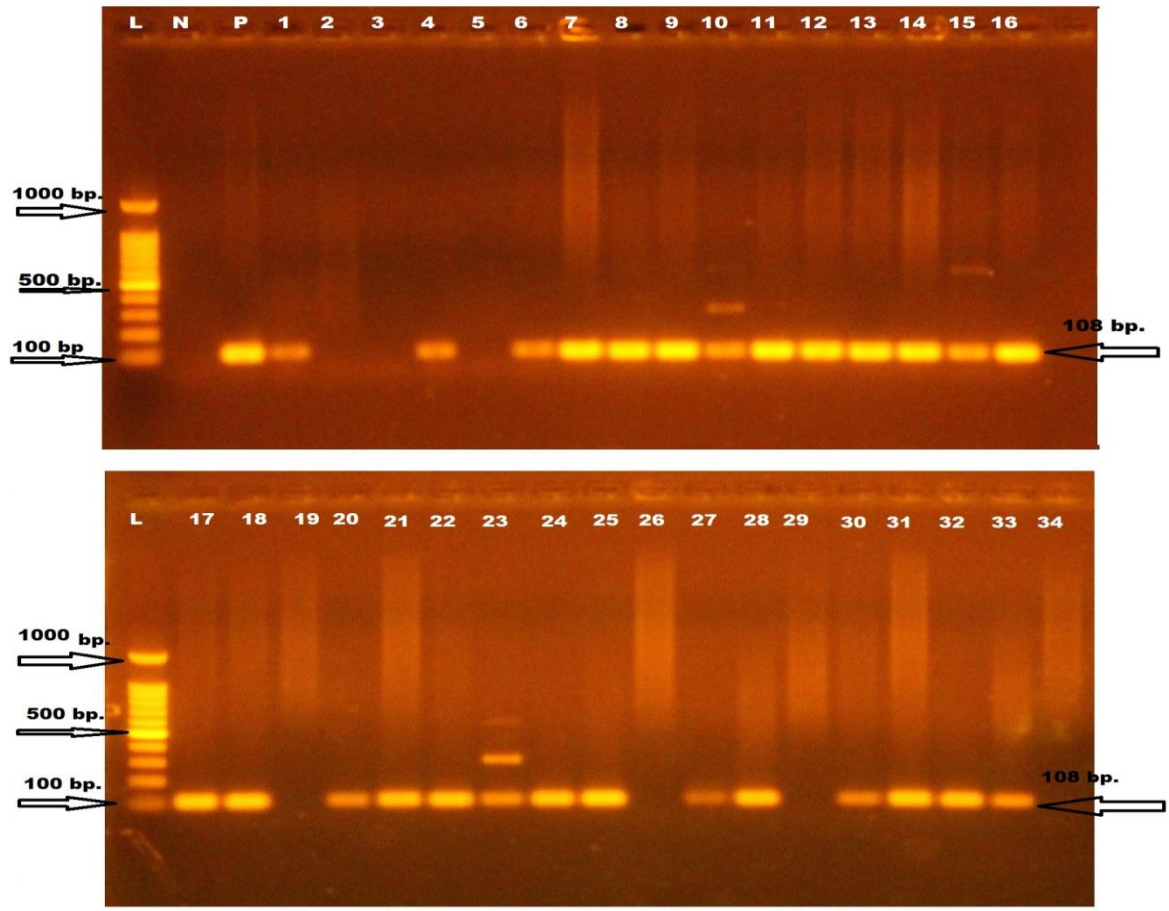


**Figure 3-** Agarose gel electrophoresis of PCR product of the gene specific for *K. pneumonia* (*rpo*B) in 1.5% agarose gel, 1.5 hr., 5v/cm, 1x tris-borate buffer and visualized under U.V light after staining with ethidium bromide. Lane L: 100bp DNA ladder. Lane N: negative control (PCR product without the DNA template). Lane p: positive control. Lanes 1-34: Clinical isolates. The amplified products and their sizes are indicated in the right.

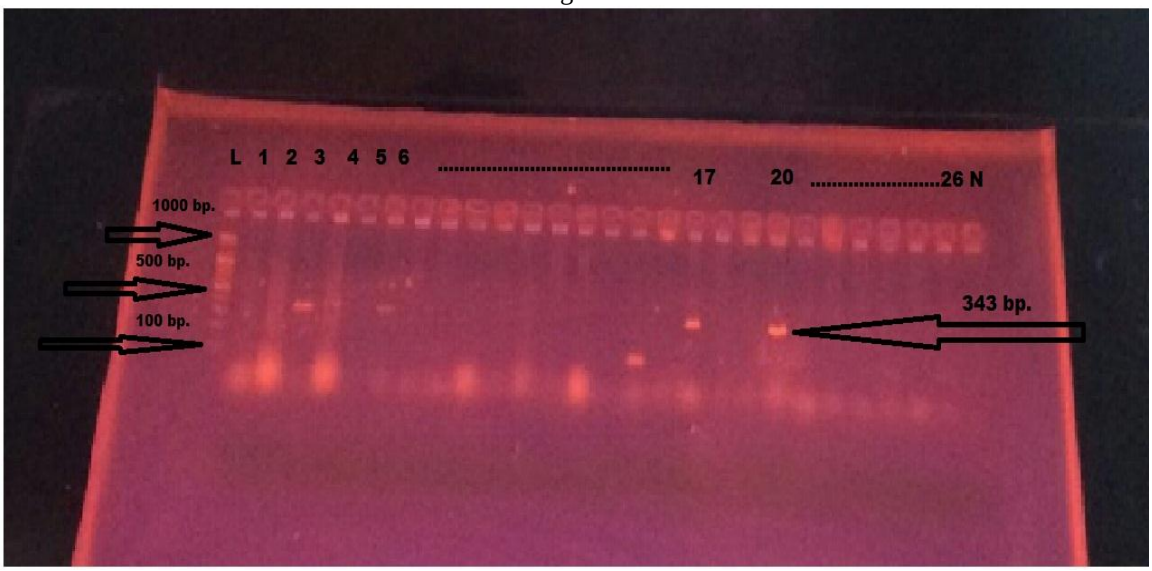


**Figure 4-** Agarose gel electrophoresis of PCR product of the gene specific for *K. oxytoca* (*peh*X) in 1.5% agarose gel, 1.5 hr., 5v/cm, 1x tris-borate buffer and visualized under U.V light after staining with ethidium bromide. Lane L: 100bp. DNA ladder. Lanes 1-26: clinical isolates. The amplified products and their sizes are indicated in the right. Lane N: Negative control (PCR product without the DNA template).

*K. oxytoca* strain CAV1335, complete genome/Sequence ID: gb|CP011618.1|Length: 6229565 Number of Matches: 1 / Range 1: 2031903 to 2032205GenBankGraphics

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
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SAM-dependent methyltransferaseexo-poly-alpha-D-galacturonosidase

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2032146

Query 71 GCTCTTGAATATGCCGCCGGAAATCAGCACCTTGCAGCCCTGAGGATAGTGCGAGACCGT  130
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Sbjct 2032145 GCTCTTGAATATGCCGCCGGAAATCAGCACCTTGCAGCCCTGAGGATAGTGCGAGACCGT
2032086

Query 131 ACAGTTATCGATCGCCTGCTGCAGCGCCTGGGTATTCAGCGTAGTGCCATCACCCTTTGC  190
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Sbjct 2032085 ACAGTTATCGATCGCCTGCTGCAGCGCCTGGGTATTCAGCGTGGTGCCATCACCCTTTGC
2032026

Query 191 ACCAAAGGTTCTGGCTTCGATGACGTGCGGCGTTTTGCGGGTTTGCGCTTTAACCACCGC  250
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2032025  ACCAAAGGTTCTGGCTTCGATGACGTGCGGCGTTTTGCGGGTTTGCGCTTTAACCACCGC
2031966

Query 251 GCTGTCCGGAGATTCCTGGCCATCGGCGTAGACCGCGCGCACCGTAAAGGCATACTCCGT  310
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Sbjct 2031965  GCTGTCCGGAGATTCCTGGCCATCGGCGTAGACCGCGCGCACCGTAAAGGCATATTCCGT
2031906

Query 311      ATC 313
                 |||
Sbjct 2031905  ATC  2031903
```

**Figure 5A-** Sequences producing significant alignments of PCR product (343bp) using *K. oxytoca* specific primer (*peh*X). Sample A.

*K. oxytoca* strain CAV1374, complete genome/Sequence ID: gb|CP011636.1|Length: 6257473Number of Matches: 1

Range 1: 5017083 to 5017393GenBankGraphics

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| **534 bits(289)** | **3e-148** | **304/311(98%)** | **2/311(0%)** | **Plus/Plus** |

**Features: cupinexo-poly-alpha-D-galacturonosidase**

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           |||| |||||||  |||||||||||| ||||||||||||||||||||||||||||||||
Sbjct 5017083  TACGCCAATGGCAAAGAGTCGCCGGATAGCGCGGTGATTAAAGCGCGGACCAGCAAAACG
5017142

Query 65  CCGCACATCATCGAAGCCAGAACCTTTGGCGCGAAGGGTGACGGCACAACGCTGAATACC  124
          |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct 5017143  CCGCACATCATCGAAGCCAGAACCTTTGGTGCGAAGGGTGACGGCACAACGCTGAATACC
5017202

Query 125 CAGGCGCTGCAGCAGGCGATCGATAGCTGTACGGTCTCGTACTATCCTCAGGGCTGCAAG  184
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```

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Sbjct5017203    CAGGCGCTGCAGCAGGCGATCGATAGCTGTACGGTCTCGTACTATCCTCAGGGCTGCAAG
5017262

Query 185 GTGGTGATTTCCGACGGCGAATTCAAAACCGGCGCGCTGTTTCTGCACAGCGATATGACC  244
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5017322

Query 245 CTGGAGATTGCCGCTGGCGCCACCCTGCTGGGTTCGGACGATCCGGCCCAGTATCCGCTT  304
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Sbjct 5017323  CTGGAGATTGCCGCTGGCGCCACCCTGCTGGGTTCGGACGATCCGGCCCAGTATCCGCTG
5017382

Query 305      GATAAAGGCTA 315
                || ||||||||
Sbjct 5017383  GAAAAAGGCTA  5017393
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**Figure 5B-** Sequences producing significant alignments of PCR product (343bp) using *K. oxytoca* specific primer (*peh*X). Sample B.

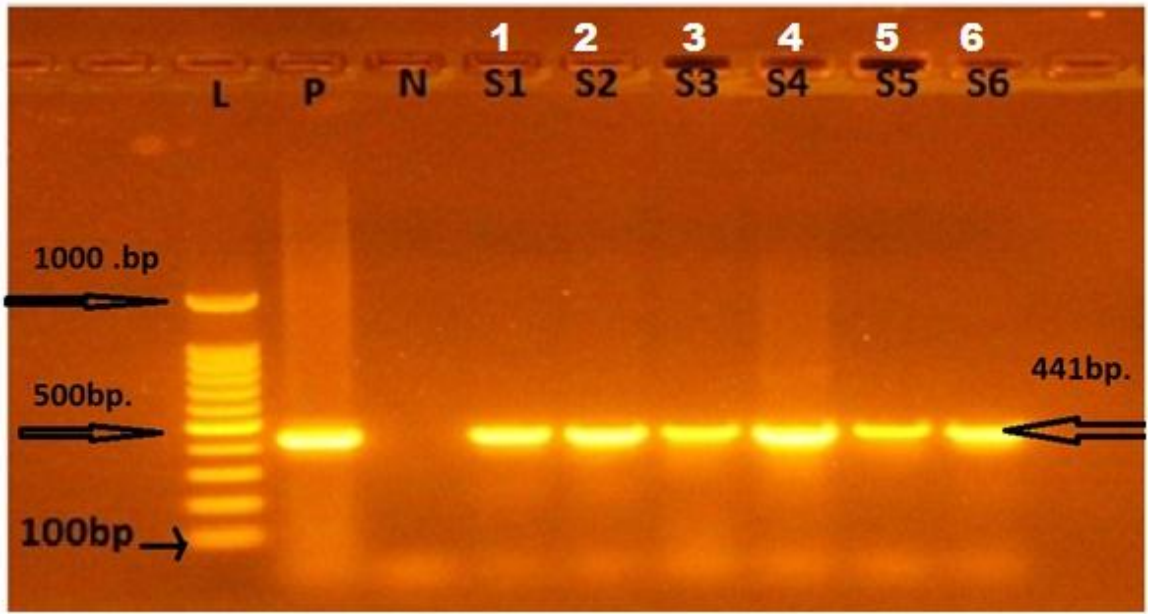


**Figure 6-** Agarose gel electrophoresis of PCR product of the gene specific for *Klebsiella* spp. (*gyr*A) in 1.5% agarose gel, 1.5 hr., 5v/cm, 1x tris-borate buffer and visualized under U.V light after staining with ethidium bromide. PCR. Lane L: 100bp-DNA ladder, Lanes 1-6: Clinical isolates. Lane N: Negative control. Lane p: posative control.

Five isolates were re-tested by the *Gyr* A primer which responsible for the genus detection, all were showed specific band product with 441 bp Figure-6. Blast sequence analysis for these isolates showed that one was related to *K. pneumoniae* subsp. pneumoniae strain RJF999, two isolates related to *K. pneumoniae* strain 17265 G*yrA*-like gene, one related to *K. oxytoca* strain 7102 *Gyr*A-like gene and one related to *K. pneumoniae* isolate 103 G*yrA* gene Figure-7 A,B,C,D,E.

*K. pneumoniae* subsp. pneumoniae strain RJF999, complete genome/Sequence ID: gb|CP014010.1|Length: 5461919Number of Matches: 1/Range 1: 3844331 to 3844727GenBankGraphics

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 697 bits(377) | 0.0 | 391/397(98%) | 3/397(0%) | Plus/Minus |

Features:tRNA uridine(34) 5-carboxymethylaminomethyl synthesis enz...DNA gyrase subunit A

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3844668

Query 73  GCGACTCCGCGGTATACGACACCATCGTGCGTATGGCGCAGCCGTTCTCGCTGCGTTACA  132
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Query 133 TGCTGGTGGACGGCCAGGGTAACTTTGGTTCCATCGACGGCGACTCCGCCGCGGCGATGC  192
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Sbjct 3844607  TGCTGGTGGACGGCCAGGGTAACTTTGGTTCCATCGACGGCGACTCCGCCGCGGCGATGC
3844548

Query 193 GTTATACCGAAATTCGTCTGGCGAAAATCGCTCATGAGCTGATGGCCGATCTTGAAAAAG  252
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Sbjct 3844547  GTTATACCGAAATTCGTCTGGCGAAAATCGCTCATGAGCTGATGGCCGATCTTGAAAAAG
3844488

Query 253 AGACGGTCGATTTCGTCGACAACTATGACGGTACGGAGCGTATTCCTGACGTCATGCCGA  312
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3844428

Query 313 CCAAAATTCCTAACCTGCTGGTGAACGGCGCCTCCGGGATCGCCGTAGGGATGGCCACCA  372
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 3844427  CCAAAATTCCTAACCTGCTGGTGAACGGCGCCTCCGGGATCGCCGTAGGGATGGCCACCA
3844368

Query 373     ACATACCGCCGCATAACCTGACCGAAGTGATCAACGG  409
                |||||||||||||||||||| |||||||| |||||
Sbjct 3844367 ACATACCGCCGCATAACCTGACGGAAGTGATTAACGG  3844331
```

**Figure 7A-** Sequences producing significant alignments of PCR product (441bp) using *Klebsiella* spp. specific primer (*gyr*A). Sample 1.

*K. oxytoca* strain 7102 GyrA-like (*gyr*A) gene, partial sequence/Sequence ID: gb|HM452909.1|Length: 411Number of Matches: 1/Range 1: 2 to 394GenBankGraphics

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|

```
9 bits(373)          0.0          387/393(98%)          4/393(1%)          Plus/Plus
Query  9   GATGG-ACAAGCCTAT---AAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCA  64
           |||||  ||||||||||   ||||||||||||||||||||||||||||||||||||||||
Sbjct  2   GATGGTACAAGCCTATAAAAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCA  61
Query 65   CCCTCATGGTGATACTGCCGTGTATGACACCATTGTACGTATGGCGCAGCCATTCTCCCT  124
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 62   CCCTCATGGTGATACTGCCGTGTATGACACCATTGTACGTATGGCGCAGCCATTCTCCCT  121
Query 125  GCGCTATATGCTGGTAGATGGCCAGGGTAACTTTGGTTCGGTCGACGGCGACTCCGCCGC  184
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 122  GCGCTATATGCTGGTAGATGGCCAGGGTAACTTTGGTTCGGTCGACGGCGACTCCGCCGC  181
Query 185  AGCGATGCGTTATACGGAAATCCGTATGTCGAAGATCGCCCATGAGCTGATGGCCGACCT  244
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 182  AGCGATGCGTTATACGGAAATCCGTATGTCGAAGATCGCCCATGAGCTGATGGCCGACCT  241
Query 245  CGAAAAAGAGACGGTGGATTTCGTCGATAACTATGACGGCACGGAAAAGATCCCTGACGT  304
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 242  CGAAAAAGAGACGGTGGATTTCGTCGATAACTATGACGGCACGGAAAAGATCCCTGACGT  301
Query 305  TATGCCGACTAAAATCCCGAACCTGTTAGTCAACGGTTCGTCAGGTATTGCGGTAGGTAT  364
           |||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 302  TATGCCGACCAAAATCCCGAACCTGTTAGTCAACGGTTCGTCAGGTATTGCGGTAGGTAT  361
Query 365  GGCGACGAATATTCCGCCGCACAACCTGACCGA  397
           ||||||  |||||||||||||||||||||||||
Sbjct 362  GGCGACCAATATTCCGCCGCACAACCTGACCGA  394
```

**Figure 7B-** Sequences producing significant alignments of PCR product (441bp) using *Klebsiella* spp. specific primer (*gyr*A). Sample 2.

*K. pneumoniae* strain 17265 GyrA-like (*gyr*A) gene, partial sequence
Sequence ID: gb|HM452905.1|Length: 412Number of Matches: 1
Range 1: 3 to 402GenBankGraphics

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 715 bits(387) | 0.0 | 397/401(99%) | 3/401(0%) | Plus/Plus |

```
Query 9    GACTGGCACAAGCCTAT--AAAATCAGCCCGTGTCGTTGGTGACGTAATCGGTAAATACC  66
           ||||||  ||||||||||  |||||||||||||||||||||||||||||||||||||||
Sbjct 3    GACTGGTACAAGCCTATAAAAAATCAGCCCGTGTCGTTGGTGACGTAATCGGTAAATACC  62
Query 67   ACCCGCACGGCGACTCCGCGGTATACGACACCATCGTGCGTATGGCGCAGCCGTTCTCGC 126
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 63   ACCCGCACGGCGACTCCGCGGTATACGACACCATCGTGCGTATGGCGCAGCCGTTCTCGC 122
Query 127  TGCGTTACATGCTGGTGGACGGCCAGGGTAACTTTGGTTCCATCGACGGCGACTCCGCCG 186
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 123  TGCGTTACATGCTGGTGGACGGCCAGGGTAACTTTGGTTCCATCGACGGCGACTCCGCCG 182
Query 187  CGGCGATGCGTTATACCGAAATTCGTCTGGCGAAAATCGCTCATGAGCTGATGGCCGATC 246
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 183  CGGCGATGCGTTATACCGAAATTCGTCTGGCGAAAATCGCTCATGAGCTGATGGCCGATC 242
Query 247  TTGAAAAAGAGACGGTCGATTTCGTCGACAACTATGACGGTACGGAGCGTATTCCGGACG 306
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 243  TTGAAAAAGAGACGGTCGATTTCGTCGACAACTATGACGGTACGGAGCGTATTCCGGACG 302
Query 307  TCATGCCGACCAAAATTCCTAACCTGCTGGTGAACGGCGCCTCCGGGATCGCCGTAGGGA 366
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 303  TCATGCCGACCAAAATTCCTAACCTGCTGGTGAACGGCGCCTCCGGGATCGCCGTAGGGA 362
Query 367  TGGCCACCAACATACCGCCGCATAACCTGACCGAATGATTC  407
           |||||||||||||||||||||||||||||||||| ||||
Sbjct 363  TGGCCACCAACATACCGCCGCATAACCTGACCGAAT-ATTC  402
```

**Figure 7C-** Sequences producing significant alignments of PCR product (441bp) using *Klebsiella* spp. specific primer (*gyr*A). Sample 3.

*K. pneumoniae* strain 17265 GyrA-like (*gyr*A) gene, partial sequence
Sequence ID: gb|HM452905.1|Length: 412Number of Matches: 1
Range 1: 6 to 409GenBankGraphics

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|

715 bits(387) 0.0 400/405(99%) 5/405(1%) Plus/Plus

```
Query 11   TGG-ACAAGCCTAT--AAAATCAGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCACC  67
           ||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 6    TGGTACAAGCCTATAAAAAATCAGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCACC  65
Query 68   CGCACGGCGACTCCGCGGTATACGACACCATCGTGCGTATGGCGCAGCCGTTCTCGCTGC 127
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 66   CGCACGGCGACTCCGCGGTATACGACACCATCGTGCGTATGGCGCAGCCGTTCTCGCTGC 125
Query 128  GTTACATGCTGGTGGACGGCCAGGGTAACTTTGGTTCCATCGACGGCGACTCCGCCGCGG 187
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 126  GTTACATGCTGGTGGACGGCCAGGGTAACTTTGGTTCCATCGACGGCGACTCCGCCGCGG 185
Query 188  CGATGCGTTATACCGAAATTCGTCTGGCGAAAATCGCTCATGAGCTGATGGCCGATCTTG 247
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 186  CGATGCGTTATACCGAAATTCGTCTGGCGAAAATCGCTCATGAGCTGATGGCCGATCTTG 245
Query 248  AAAAAGAGACGGTCGATTTCGTCGACAACTATGACGGTACGGAGCGTATTCCGGACGTCA 307
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 246  AAAAAGAGACGGTCGATTTCGTCGACAACTATGACGGTACGGAGCGTATTCCGGACGTCA 305
Query 308  TGCCGACCAAAATTCCTAACCTGCTGGTGAACGGCGCCTCCGGGATCGCCGTAGGGATGG 367
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 306  TGCCGACCAAAATTCCTAACCTGCTGGTGAACGGCGCCTCCGGGATCGCCGTAGGGATGG 365
Query 368  CCACCAACATACCGCCGCATAACCTGACCGAATGATTC-CAACGG  411
           |||||||||||||||||||||||||||||||| |||| ||||||
Sbjct 366  CCACCAACATACCGCCGCATAACCTGACCGAAT-ATTCTCAACGG  409
```

**Figure 7D-** Sequences producing significant alignments of PCR product (441bp) using *Klebsiella* spp. specific primer (*gyr*A). Sample 4.

*Klebsiella pneumoniae* isolate 103 gyrase subunit A (*gyr*A) gene, partial cds
Sequence ID: gb|EU430282.1|Length: 447Number of Matches: 1

| **Score** | **Expect** | **Identities** | **Gaps** | **Strand** |
|---|---|---|---|---|
| 688 bits(372) | 0.0 | 399/411(97%) | 6/411(1%) | Plus/Plus |

```
Query 8    ATGACTGG-AC-AAGCCTAT--AAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAAT  63
           |||||||| || |||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct 25   ATGACTGGAACAAAGCCTATAAAAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAAT  84
Query 64   ACCATCCCCATGGTGACTTGGCGGTTTATAACACGATCGTCCGTATGGCGCAGCCATTCT 123
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 85   ACCATCCCCATGGTGACTTGGCGGTTTATAACACGATCGTCCGTATGGCGCAGCCATTCT 144
Query 124  CGCTGCGTTACATGCTGGTAGACGGTCAGGGTAACTTCGGTTCCATCGACGGCGACTCTG 183
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 145  CGCTGCGTTACATGCTGGTAGACGGTCAGGGTAACTTCGGTTCCATCGACGGCGACTCTG 204
Query 184  CGGCGGCAATGCGTTATACGGAAATCCGTCTGGCGAAAATTGCCCATGAACTGATGGCTG 243
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct 205  CGGCGGCAATGCGTTATACGGAAATCCGTCTGGCGAAAATTGCCCATGAACTGATGGCCG 264
Query 244  ATCTCGAAAAAGAGACGGTCGATTTCGTTGATAACTATGACGGTACGGAAAAAATTCCGG 303
           |||||||||||||||||||||||||||||||||||||||| |||||||||||||| |
Sbjct 265  ATCTCGAAAAAGAGACGGTCGATTTCGTTGATAACTATGACGGCACGGAAAAAATTCCCG 324
Query 304  ACGTCATGCCAACCAAAATTCCTAACCTGCTGGTGAACGGTTCTTCCGGTATCGCTGTAG 363
           |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct 325  ACGTCATGCCAACCAAAATTCCTAACCTGCTGGTGAACGGTTCTTCCGGTATCGCCGTAG 384
Query 364  GTATGGCAACCAACATCCCGCCGCACAACCTGACCGAA-TGATCCAACGGT  413
           |||||||||||||||||||||||||||||||| ||| | ||| ||||||
Sbjct 385  GTATGGCAACCAACATCCCGCCGCACAACCTGACGGAAGTTATC-AACGGT  434
```

**Figure 7E-** Sequences producing significant alignments of PCR product (441bp) using *Klebsiella* spp. specific primer (*gyr*A). Sample 5 .

**Phylogenetic relationship through the partial sequence**

To clarify the phylogenetic relationships of the four *Klebsiella* isolates, the 441 bp sequences of the *Gyr*A regarding *K. pneumonia* were subjected to phylogenetic tree analysis using ClustalW2 Figure-8A. The Iraqi isolates found in to 2 clusters (I and II). The phylogeographic grouping of isolates number 1, 2 and 4 in cluster I was strongly associated with geographic origin of the strains in Europe which aligned with separated cluster which contain strains from (USA and UK). Isolate number 3 showed more genetic variation than cluster I, which related more to the Asian strains. The morphological and *gry*A sequencing and phylogeny of *Klebsiella* spp. were also explained by Jessica *et al*. [31], they showed that *K. pneumoniae* strain MRSN2404 was isolated from the chronic wound of a soldier who had been wounded in Iraq in 2006. Using *gyr*A gene sequencing by comparing them with the six global strains give a good scale of area to show the exact position of the local isolates in the phylogenic tree.

Regarding *K. oxytoca* isolates, the 343 bp. sequences of the *peh*X and the 441 bp. sequences of the *gyr*A were subjected to phylogenetic tree analysis using ClustalW2, the isolates were separated into two groups, the first group Figure-8B contain isolates number 2 and 3 which showed relation to the Iranian strains, concerning this to the mixing among the travelers. On the other hand, the isolate number 1 Figure-8C has a genetic variation and far from reference strains in GenBank and located in between the Asian and European isolates with a separation time 0.002 ,which mean that the gene of this isolate can be changed very fast. .

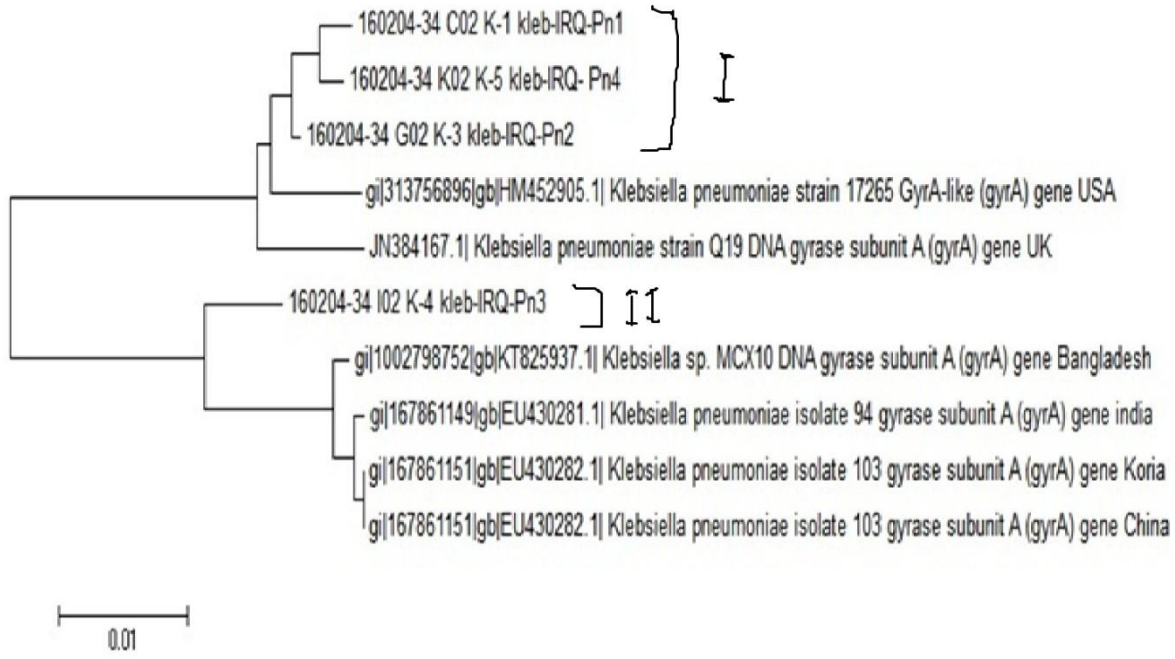


**Figure 8A-** Neighbor – joining tree analysis of the 441 bp. of *GyrA* of four *K. pneumonia* and six reference strains reported from different parts of the world available in public database GenBank (https://www. ncbi.nlm.nih.gov/genbank).

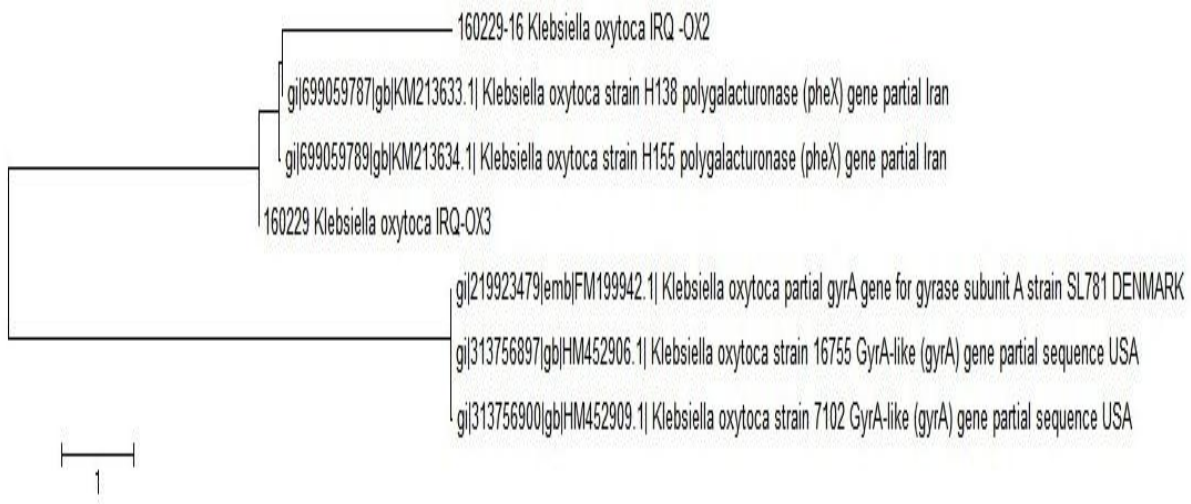


**Figure 8B-** Neighbor – joining tree analysis of 343 bp. of the *peh*X gene of 2 *K. oxytoca* isolates and 5 reference strains reported from different parts of the world available in public database GenBank (https://www. ncbi.nlm.nih.gov/genbank).

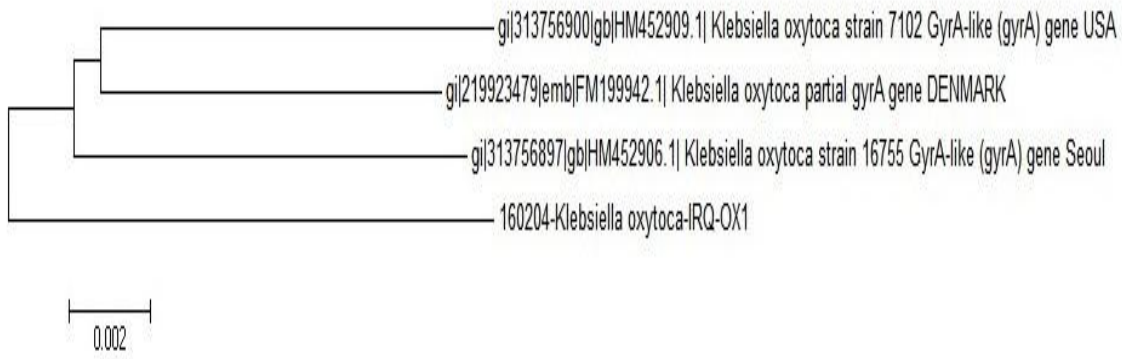


**Figure 8C-** Neighbor – joining tree analysis of 441 bp. of the *gyr*A gene of one *K. oxytoca* isolate and 3 reference strains reported from different parts of the world available in public database GenBank (https://www. ncbi.nlm.nih.gov/genbank).

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