The genetic relationship for *Klebsiella pneumoniae* isolated from human urinary tract and beef

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Abstract

The present study aimed to describe the genetic relationships of zoonotic characterization of *Klebsiella pneumoniae* isolated from Human urinary tract and beef. The study includes (50) urine samples from human and (50) beef samples. The isolation and identification of Klebsiella pneumonia were done by using enrichment culture method and Vitek 2, then confirmed by PCR technique based on 16S ribosomal RNA gene which designed in this study using NCBI-GenBank (LT599801.1) and DNA sequencing was done on some positive isolates. The results show that *Klebsiella pneumoniae* was isolated from beef at 38 (76%) and from human at 32 (64%) by vitek2. The PCR technique was show highly sensitive and specific confirmative detection of *Klebsiella Pneumonia* isolates at Clarify DNA sequencing of a partial sequence of 16S ribosomal RNA gene was shown homology sequence identity highly with NCBI-Blast *Klebsiella pneumoniae* isolates. The phylogenetic analysis was show clear genetic similarity at (0.5 genetic change) between human and beef in *Klebsiella pneumoniae* isolates. The gene sequence deposited into NCBI-GenBank accession numbers (MF314450.1, MF314451.1, MF314452.1, MF314453.1). In conclusion, the study presents the first report in Iraq of genetic relationship among *K. pneumoniae* isolates from beef and humans. Therefore, it is essential to define the role of animals as an important source for the distribution of pathogen related to public health.

Keywords: *Klebsiella pneumonia*, 16s rRNA, Polymerase change reaction, phylogenetic tree
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العلاقة الوراثية لجرثومة الكلبيسيلا الرئوية والمعزولة من الجهاز البولي في الإنسان والأبقار

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الخلاصة

الدراسة الحالة تهدف العلاقة الوراثية للأهمية المشتركة للكليبسلا الرئوية التي تصيب الجهاز البولي للانسان ولحوم الأبقار. تضمنت الدراسة جمع ٥٠ عينة من إدرار الإنسان و٥٠ عينة من لحوم الأبقار تم تشخيصها والكشف عنها بواسطة استخدام الصفات الزرعية والاختبارات الكيموحيوية باستخدام فحص الفايتك٢و تقنية تفاعل سلسة البلمرة (بي سي ار). باستخدام الجين التشخيصي ١٦س رايبوسومال ار ان اي الذي صمم في هذه الدراسة باستخدام العترة العالمية التي أخذت من جين البنك العالمي يحمل رقم الانضمام LT599801.1. أظهرت النتائج أن نسبة الإصابة في الأبقار باستخدام فحص الفايتك ٣٨ (٧٦%) في الأبقار في حين نسبة الإصابة في الإنسان ٣٢ (٦٤%). تم استخدام التقنية الجزيئية للتحقق من النتائج وذلك عن طريق استخلاص الحامض النووي الرايبي DNA لبكتريا الكليبسلا الرئوية وقياس تركيزه ونقاوته بواسطة جهاز Nanodrop ليتسنى التحري عن قطعة الجين وذلك باستخدام بادئات primers لبكتريا الكليبسيلا الرئوية والعزلات تم إرسالها لغرض تتابع النيوكلوتيدات وتم الحصول على أرقام الانضمام لعزلتين من الإنسان وعزلتين من الأبقار (MF314450.1, MF314451.1, MF314452.1, MF314453.1) من بنك الجينات العالمي NCBI-Genbank. أظهرت الشجرة الوراثية تشابه بنسبة (٩٩ % -١٠٠%) مع العزلات العالمية لكليبسلا الرئوية المعزولة من الإنسان والأبقار. الخلاصة ,الدراسة الحالية

تسجل لأول مرة في العراق حيث سجلت العلاقة الوراثية للكليبسلا الرئوية بين الإنسان والأبقار لذلك من الضروري معرفة الدور الحقيقي لدور الأبقار كمصدر في نقل وانتشار الإصابة إلى الإنسان وخطرها على الصحة العامة.

Introduction

Klebsiella pneumoniae is a gram-negative bacterium that cause a liver abscess, bacteremia, pneumonia that is transmitted among hospital in Asia and the Middle East (1-3). In previous years, *K. pneumoniae* are known infectious pneumonia. Also, it causes Friedlander syndrome that associated with diabetic people (1,2).

Diabetic Mellitus summarize its pathogenesis can cause changing in mucosal immunity. Also causes serious of changes in normal flora also In the Europe and US, *K. pneumoniae* usually associated with antibiotic resistance that provided in the hospital. Also causes 6-17% of infection of the urinary tract, 3-20% all neonatal septicemia, 4-17% infections in intensive care units, 2-4% wound infections, 7-14% pneumonia, 6-17% infection of the urinary tract, 4-15% septicemia, 4-17% infections in intensive care units (4-9).

Klebsiella pneumoniae is opportunistic bacteria in animals and human and causes contamination in the meat (10). Also, *K. pneumoniae* causes diseases in horses and cows (11,12). In humans, *K. pneumoniae* can invade intestine and results in intestinal disease (13). *Klebsiella pneumoniae* causes many diseases such as meningitis, wound infections cystitis, liver abscess, bacteremia, pyelonephritis, osteomyelitis and septicemia, as well as pneumonia (13,14).

The aim of study is investigating the relationship of *K. pneumoniae* isolated from beef and infection of *K. pneumoniae* in human samples which isolated from urine by using polymerase chain reaction, sequencing and phylogenetic tree by using housekeeping gene 16s rRNA.

Material and method

Sample collection

Fifty urine samples were collected from human suffering from a urinary infection in Al-Diwanyah teaching hospital, and also fifty beef samples were collected from the different market in Al-Diwaniyah. All samples transported to microbiology laboratory in Veterinary Medicine College for bacterial isolation and identification. All samples were used for isolation of *k. pneumoniae* by culturing on MacConkey and blood agar plates and incubated for 24 hours at 37°C according to standard procedure (15). The isolates were activated by inoculated on CHROMagar Orientation and incubated at 37°C for overnight, Identification of isolates based on the morphology of colonies and by biochemical tests according to (16). The Vitek 2 biochemical reaction test was performed for identification *klebsiella pneumoniae* isolates.

DNA extraction of bacteria genome

Fresh bacterial genomic DNA of *K. pneumoniae* was extracted from 1ml nutrient broth samples in 1.5ml microcentrifuge tubes by using (Presto TM mini g DNA Bacteria Kit, Geneaid - China), the extract gDNA was checked by nanodrop spectrophotometer and store in deep free until usage.

Polymerase chain reaction (PCR)

PCR reaction which used for detection *K. pneumonia* by using housekeeping gene (16s rRNA). The housekeeping gene of *Klebsiella pneumoniae* -16s rRNA, gotten from NCBI GenBank *Klebsiella pneumoniae* strain K-18, F(GGAACTGAGACACGGTCCAG) and R (CCAGGTAAGGTTCTTCGCGT) from NCBI- GenBank with accession number (LT599801.1) complete genome with product sizes of 660 bp.

Preparation of PCR master mix is done according to (Bioneer. Korea). Master mix of the PCR consist of ($MgCl_2$ 1.5 mM, dNTPs 250 µM, KCl 30 mM, Taq DNA polymerase 1U, Tris-HCl (pH 9.0) 10 mM, stabilizer, and the stain). The reaction was done by adding DNA template (5 µl), (1.5 µl) forward and reverse primer (10 pmole) to mixture tube of PCR then complete the volume to reach (20 µl). Then mixed by vortex (Bioneer company. Korea). The reaction was done in PCR thermocycler apparatus (Mygene Bioneer company, Korea). The operation is done by setting up the initial denaturation for five minutes at (94°C). 30 cycle is a total cycle of denaturation at 94°C for 30 second, annealing for 30 second at 60°C, and extension for 30 seconds at 72°C. the last stage is extension stage for ten minutes at 72°C. All final products tested by electrophoresis apparatus 1.5% Agarose gel and stained with 3 µl ethidium bromide. The buffer which used in electrophoresis apparatus is Tris-borate- EDTA (TBE) (boric acid 5.5 g, Tris-base 10.8 g, EDTA (pH 8) 4 mL at (pH 8) (the mixture combined and shaken for complete dissolving). The DNA ladder that used in this study consist of 100-1500-base pair that made in the USA in Roche Company. Aliquots (10 µl) of PCR products were applied to the gel. A constant voltage for 1 hour was used for product separation. The Ethidium bromide used in the study as the stain of DNA then test under UV transilluminator light (UVItec, Paisley, UK).

DNA sequencing method

DNA sequencing method was performed for confirmative detection of *k pneumoniae* strain, two isolates were taken from human and two from cattle based on *16s rRNA* gene, the PCR product was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic, Canada). The purified 16s rRNA gene in PCR product samples was sent to Bioneer Company in Korea for performed the DNA sequencing using (AB DNA sequencing system) (17).

The genomic sequences were assembled and submitted in GenBank- NCBI then multiple sequence alignment was done by Basic local alignment Search Tool (BLAST) for phylogenetic tree construction and phylogenetic analysis by using the MEGA6 program.

Results

Isolation and identification of *Klebsiella pneumoniae*

The isolation of *K. pneumoniae* from human urine and beef was illustrated in table 1. Pink, mucoid, lactose fermented colonies were considered to be *Klebsiella* spp on MacConkey agar while on orientation medium, colonies are metallic blue colour, large, rounded (Figure 1). Further biochemical tests were done for confirmation of the isolates (Table 2).

Detection of *K. pneumoniae*

After culturing of the isolates, these isolates were positive produce for VATEK2 for biochemical tests from 50 isolates 38 (76%) positive for beef meet cattle while from 50 human urine isolates 32 (64%) positive in vitek2 (Table 3).

Molecular Identification of *K. pneumoniae*

The positive isolates of vitek2 biochemical tests are produced for confirmative endpoint PCR for detection *16s rRNA gene* (671bp) of *Klebsiella pneumonia* in Ethidium bromide-stained agarose gel using specific primers and the ladder in size (100-1500bp), 16 (50%) human urine isolates from different regions gave positive results (line 1-8) (Figure 2) (Table 4).

While cattle beef meat isolates gave 21 (55%) from different region of meat source line (8-15) (Figure 2) (Table 4). The comparative results by VITEK2 and PCR technique shown in (Figure 3). In addition to the confirmative diagnosis of the *Klebsiella,* the PCR products used in the sequencing for analysis of *16s rRNA gene* of a predominant strain of *Klebsiella pneumonia* in Iraq.

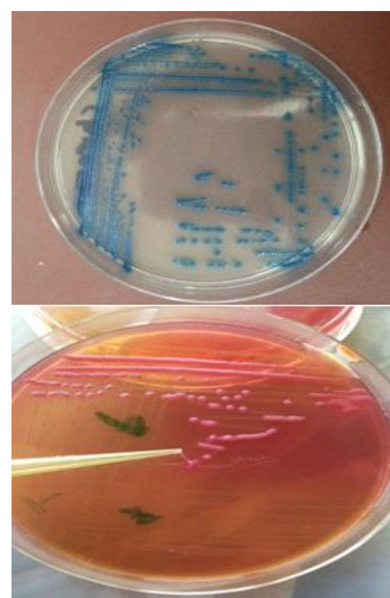

Figure 1: *Klebsiella pneumonia* on CHROM agar Orientation, produce metallic blue, rounded and large colonies, *Klebsiella pneumonia* on MacConkey agar characteristic by (rounded, mucoid, large and pink colonies).

Table 1: Cultural characters of *Klebsiella pneumoniae* on culture media

N	Culture media	Cultural characters for *Klebsiella pneumoniae*
1	MacConkey agar	rounded, mucoid, large and pink colonies
2	Eosin methylene blue	Purple, large, mucoid colonies
3	CHROM agar Orientation	metallic blue, mucoid, rounded and large colonies

Table 2: The result of the biochemical tests for *Klebsiella Pneumonia* which isolated from human and animal

N	Biochemical test	Result of Biochemical test
1	Triple sugar Irion Test	A/A- GAS -NO H_2S
2	Indole Test	Positive (red color Ring appear in the top of the test tube)
3	Citrate Utilization Test	Positive (The green colors converted to blue)

A=acid H2S = Hydrogen sulfide

Table 3: *K. pneumoniae* number and their isolation percentages of from cattle and human samples vitek2

Type of sample	*Klebsiella pneumoniae*		Total
	Positive	Negative	
Beef meat of cattle	38 (76%)	12 (24%)	50 (100%)
Urine samples from Human	32 (64%)	18 (36%)	50 (100%)

Table 4: *K. pneumoniae* number and their isolation percentages of from cattle and human samplesF by PCR technique

Type of sample	*Klebsiella pneumoniae*		Total
	Positive	Negative	
Beef meat of cattle	21(55%)	17 (45%)	38 (100%)
Urine samples from Human	16 (50%)	16 (50%)	32 (100%)

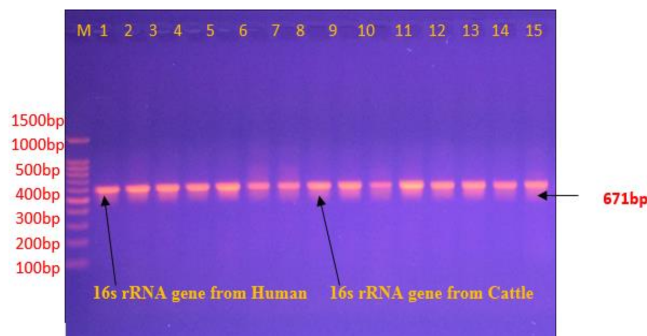

Figure 2: Agarose gel electrophoresis of *K. pneumoniae* stained with Ethidium bromide and product size 671 bp.

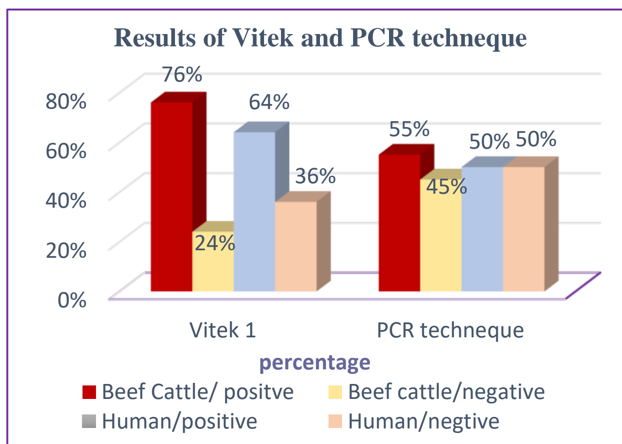

Figure 3: Comparative of *K. pneumoniae* infection by Vitek2 and PCR technique.

Sequencing and phylogenetic analysis

Two isolates from cattle, and two isolates from human sent for sequencing, then the four sequences submission in NCBI-GenBank database to get accession number codes (MF314450.1, MF314451.1, MF314452.1, and MF314453.1). Respectively, DNA sequencing method was performed for phylogenetic confirmative of *K. pneumoniae* detection, phylogenetic analysis, and zoonotic importance (Figure 4). The total percentage of substitution mutations rates between nucleotide gene sequences (Figure 5).

Discussion

K. pneumoniae is a cause of life-threatening diseases. However, the organisms have associated with disease occurrence (18). Particularly concerning are reports that *K. pneumoniae* producing bacteria can spread quickly through large geographic regions (19). The human clinical isolates reveal a strong relationship with beef isolates, but it was showed low percentage in some another study such as in beef meat, the prevalence of *K. pneumoniae* isolates was more than human 38 (76%), 32 (64%) of *K. pneumonia* by VITEK2. Also, it was 21 (55%), 16 (50%) in PCR results in both beef meat and human respectively. All meat that sold and distributed for use as food - animal production in Iraq. This confined with the result of united state (20,21). Thus, the distribution of *K. pneumoniae* population may reflect that food animal production vs hospital and general community use (22).

In contrast, genetic analysis demonstrates overlap between meat source and clinical *K. pneumoniae* population main strain of human and cattle isolates (MF314450.1), (MF314451.1), (MF314453.1) were related to the two strain, (KP091885.1) *Klebsiella pneumoniae* strain were isolated from *Mauremys mutica* from china and (KY471720.1) *K. pneumoniae* which isolated from water in India, while MF314452.1 that isolated from urine Human were more related to (MF429117.1) which isolated from buffalo milk from china this mean more relationship of strain of human with strains of cattle and the zoonotic relationship between them reveled the dangerous of *K. pneumoniae* that effect human with foodborne product (23).

The increase in infection of urinary tract lead of multidrug resistance with *K. pneumonia* and lead to be more virulence this cause may be mutation in the nucleotide that lead to be more resistance and need to scope on this point to know the reason of frequently infection of urinary tract system (24).

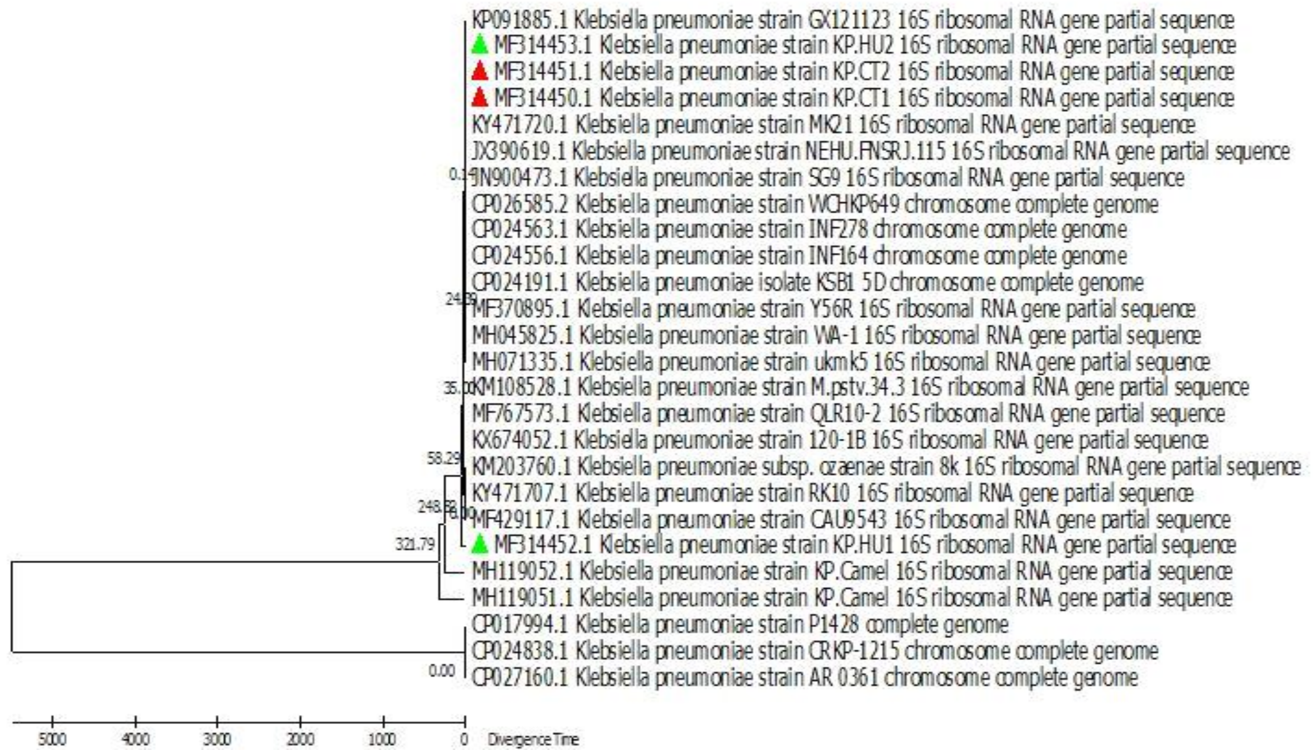

Figure 4: Phylogenetic tree of *K. pneumoniae* in human and cattle with world strains.

Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	*6.7*	*8.51*	**15.7**
T	*8.85*	-	**0.59**	*11.53*
C	*8.85*	**0.47**	-	*11.53*
G	**12.05**	*6.7*	*8.51*	-

NOTE:-- Each entry shows the probability of substitution (r) from one base (row) to another base (column)[1]. For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown in *italics*. The nucleotide frequencies are 24.86% (A), 18.83% (T/U), 23.91% (C), and 32.40% (G). The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 728 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [2].

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Figure 5: The total percentage of substitution mutations rates between nucleotide gene sequences.

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