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Phylogenetic Tree Analysis of *Klebsiella pneumoniae* Isolated from Bat *Myotis emarginatus* based on *16srRNA* Prokaryote Sequence Alignment

AzharAbdulsada Neamah[1], Khilood Hamdan Fahad[2], JameelaRadi Esmaeel[3], JenanNadhim Sadeq[4]

[1]Department of Microbiology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.
E-mail: azhar.neamah@qu.edu.iq

[2]Department of Microbiology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.
E-mail: khilood.hamdan@qu.edu.iq

[3]Department of Microbiology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.
E-mail: jameela.esmaeel@qu.edu.iq

[4]Department of Microbiology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.
E-mail: jinan.sadeq@qu.edu.iq

ABSTRACT

Objective: Bats species are considered a reservoir of many pathogens which deemed important to human and animal health. However, there are studies was done on *Klebsiella pneumoniae (K. pneumoniae)* in these animals are very rare. The present study aimed to screen for the presence of *K. pneumonia* in bat species *Myotis emarginatus* and phylogenetic identification based on *16srRNA* prokaryote gene Sequence alignment. **Methods:***K. pneumoniae* selected from different organs of insectivory bat *Myotis emarginatus* (spleen, liver, kidney, lung, stomach, and intestines). *K. pneumoniae* recognized by cultures media and biochemical test than DNA extracted and submitted to PCR for amplification of *16srRNA* prokaryote gene and phylogenetic analysis for one isolate performed depend on *16srRNA* nucleotide sequences which searched in GenBank by using the BLAST. **Results:** Most bats harbor *K. pneumoniae* in the lung (20/30 (67%)) and the kidney (18/30 (60%)) whereas the less frequency of *K. pneumoniae* appeared in the spleen (5/30 (17%)).Nucleotide sequences of *16SrRNA* geneobtained from *K. pneumoniae* isolated from bat*Myotis emarginatus* were used to generate a phylogenetic tree based on the NCBI-GenBank database. GenBank number codes of these isolates are MK426995**. In Conclusion:***K. pneumoniae* isolated from different tissues of bat *Myotis emarginatus* and the phylogenetic analysis of *K. pneumoniae* in NCBI-GenBank depend on *16S rRNA gene*a good option to identification and detection comparable strain of *K. pneumoniae* worldwide

KEYWORDS

K. Pneumoniae, *16srRNA* Gene, Phylogenetic, Bat, *Myotis* Emarginatus.

Introduction

The bats are organism belongs to the mammals have more than (1300) species in the world (Adesiyun A.A. et, al. 2009) (Banskar S., *et, al*. 2016). The bats have a unique role and play the role as reservoirs for the pathogens and spread of bacteria and viruses for the great area (Abdullah M. and Jayaraj V. 2006). During the last ten years, the bats are considered reservoir hosts for many pathogens for the animals and humans such as COVID 19, SARs, Hendra, and Marburgvirus (Abdullah M. and Jayaraj V. 2006) (Calisher C.H., et, al. 2006) (Kupferschmidt K. (2013). Also, the bats have a great role in transferring the bacterial agent such as *Salmonella*, Bartonella, *Leptospira*, and *Yersinia* but the studies which isolated *K. pneumoniae* from bats is limited (Gloriana C. 2006).

K. pneumoniae has several types, some it is harmless and other are nosocomial pathogen which causes blood infection, lung infection and kidney infection, and causes rhinoscleroma, Friedländer's pneumonia and liver diseases (Blin C., *et, al*. 2017) (Paczosa M. and Mecsas J. 2018) (Brisse S., *et, al*. 2014). *K. pneumoniae* isolated from a different host and identified by numerous genetic methods from which PCR, RT–PCR, SSCP, DGGE, RFLP, whole-genome sequencing, and multilocus sequence typing (MLST). Recently, phylogenetic tree analysis of many bacteria is detected depend on *16S rRNA* prokaryotic gene sequences alignment (Seemann T. 2014) (Rodrigues C., *et, al*. 2018) (Naum M., *et, al.* 2008).

The *16S rRNA* gene consists of (1550) base pair with many polymorphisms that used in distinguishing it. The used primers are designed for complementary to the target area at a specific area at gene beginning or gene end while the variable area sequence used for the taxonomy (Clarridge J. *et, al*. 2001) (Cole J.R., *et, al*. 2009). Despite (500) and (1500) base pair is used wide common for comparing and sequencing. The sequence of *16S rRNA* is determined in

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many strains such as Genbank, which have more than (20) million of the deposited sequences (Gee J. E., *et, al*. 2003) (Dewhirst F. *et, al*. 2001) (Dixon B. 2001) (Drancourt M., *et, al*. 2000). The deposited sequences used for comparison of the sequencing. *16S rRNA* is a universal gene in bacterium therefore is used for the study of the relationships among the bacteria. The comparison among the bacteria depending on *16S rRNA* sequences is very beneficial for the detection of the bacteria, also, is used for classifying of the strains and determining the relationship tree among the bacteria ((Drancourt M., *et, al*. 2000) (Roth A., et, al. 2003). However, there are some occasional cases; *16S rRNA* sequence becomes useless due to similar sequences (Gee J. E., *et, al*. 2003) (Sacchi C. T., *et, al*. 2002). According to our knowledge, there is no previous study that determines the phylogenetic tree of *K. pneumoniae* which isolated from bat *Myotis emarginatus*, therefore the present study dealt with that.

Materials and Methods

Samples collection: thirty bats from insectivory species *Myotis emarginatus***(figure 1)** were caught with mist nets during the period from January to March of 2020 from the mountains in northern Iraq. Administration of the chloroform at a large dose was used in the bats. University of Al–Qadisiyah / Veterinary Medicine College/ In Anatomy Lab, The samples are including hepatic tissues, spleen; renal, intestines, stomach, and lung are taken. Taking the intestines lastly to prevent organs contamination, the samples kept in liquid nitrogen at (-4) °C (Daniel D.S., *et, al*. 2013).

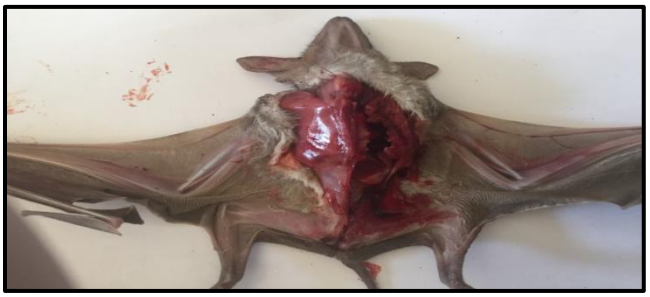

Figure 1. Anatomy of insectivory bat related to species *Myotis emarginatus* in lab

Isolation and Identification of *K. pneumoniae*

The sample was cultured on the MacConkey, then incubating for one day at (37) ˚C., then cultured on the Brilliant Green media and Xylose lysine Deoxycholate media for one day at (37) ˚C., then cultured on the Orientation - CHROM media and incubate. Identification of the isolates identifies depending on the general morphology, the color, and API20E (Ibrahim I. A., *et, al*. 2019) (Khazaal, M. 2017).

DNA Extraction

DNA was extracted from bacterial broth according to manufacturers' instructions of Genomic DNA Mini Kit (Geneaidfor bacteria). The extracted DNA was electrophoresed on agarose gel (0.5% agarose stained with 5**µ**L of ethidium bromide) to confirm that DNA present in each sample then microcentrifuge tubes that contain DNA stored at -20ºC in deep freeze even used in PCR (Khazaal, M. 2017).

Primer PreparationandPCR Reaction

Specific primers for *16S rRNA* gene (F-GGAACTGACACGGTCCAG, R-CCAGGTAAGGTTCTTCGCGT) are prepare based on the manufacturer's directions by adding the primers with water to produce stock solution at (100) pmol / µl (Klaif S.F., *et, al.* 2019). Diluting of the primer's stock with the water by using (**C1V1 = C2V2)** to producethefinal working (10) pmol / µl.

The master mix prepared based on (Bioneer Company, made in Korea). The master mix formed from (MgCl2, dNTPs, KCl, polymerase, Tris-HCl, stain, and stabilizer). The reaction was required DNA, and adding F and R primer to produce the mixture then reach volume to (20 µl) and mixing shaking or vortex.

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The reaction was done in Thermocycler (Biometra Company /made in Germany) as the table below:

Name of cycle	Number of cycle	Temperature	The time
The denaturation	30	94 C	30 seconds
The annealing	30	60 C	30 seconds
The extension	30	72 C	30 seconds
The extension stage	30	72 C	(10) minute

The final products examined by the electrophoresis by using Agarose gel (1.5) % with dyeing with ethidium bromide (3) µl. The used buffer in electrophoresis consists of Tris-borate- EDTA that consist of the boric acid, Tris-base, EDTA. The DNA ladder consists of (100) to (1500) bp (Roche, US). PCR products were used in the gel. The voltage was 110 V for (60) minutes. Ethidium bromide is a stain used for staining of DNA then watching under the ultraviolet light (Paisley company, made in the UK) at (660) bp band (Klaif S.F., *et, al*. 2019).

Identification of *E. coli* Using 16S rRNA

The DNA sequencing for one isolate was performed depended on *16S rRNA*, purifying of the PCR product from agarose by kit called (EZ-10 Spin Columni DNA) (Biobasic company, Made in Canada). The sequences were analyzed by GenBank-NCBI, after that submitted to alignment by BLAST for revealing the phylogenetic analysis. The phylogenetic tree was produced by compared with reference sequences by the neighbor-joining method software (MEGA) V. (6) (Ibrahim I. A., *et, al*. 2019) (Klaif S.F., *et, al*. 2019).

Statistical Analysis

Statistical analysis was done by using (SPSS) software V. (20) and Microsoft Excel (2010) at ($P<0.05$) ((Khazaal, M. 2017).

Results

Isolation and primary identification of *K. pneumoniae* done depend on colony's color, morphology on culture media, and biochemical tests **(table 1)**. Pink, mucoid, lactose fermented colonies were considered to be Klebsiella on MacConkey agar, and Brillant, the colonies appeared green to yellow to green and yellow color on XLDA. The orientation agar showed a metallic rounded large and blue colony. The final confirmation is done after making biochemical tests and API20E.

Table 1. Culture media and biochemical test for identification of *K.* pneumonia

Culture media	**Color and shape of colonies**
MacConkey agar	Rounded, mucoid, large and pink colonies
Brilliant Green Agar	Green yellow to green colonies without detected shape
XLDA	Yellow color large amorphous colonies
Orientation -CHROM agar	Metallic blue, mucoid, rounded and large colonies
Biochemical test	**Reaction result**
Citrate	Positive
Indole	Negative
Methyl Red	Negative
Motility	Negative
Triple Sugar Iron Agar	Acid/Acid
Urease	Positive

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Results in the **table (2),** showed that most bats harbor *K. pneumoniae* in the lung (20/30 (67%)) and the kidney (18/30 (60%)). *K. pneumoniae* also isolated from some bats intestinal tract (13/30 (43%)), liver (10/30 (33%)) and stomach (9/30 (30%)) whereas the less frequency of *K. pneumoniae* appeared in the spleen (5/30 (17%)). **Figure (2)** shows the disparities in the presence of *K. pneumoniae*, according to the bat organs

Table 2. Frequency of *K. pneumoniae* according to *Myotis emarginatus* organs

Bat organs	N. of infected bats/ total N. of bats	Frequency of *K. pneumoniae*
Stomach	**9/30**	**30%**
Intestinal	**13/30**	**43%**
Liver	**10/30**	**33%**
Spleen	**5/30**	**17%**
Lung	**20/30**	**67%**
Kidney	**18/30**	**60%**

***N =number**

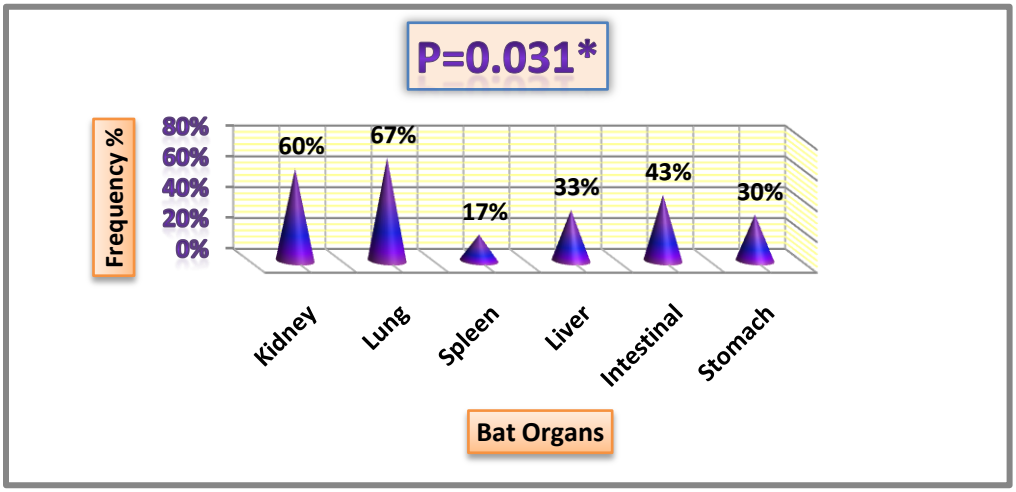

Figure 2. Frequency of *K. pneumoniae* according to *Myotis emarginatus* organ at (p<0.05)

16S rRNA gene is a sequence used in current research for molecular identification of *K. pneumonia.* According to conventional PCR reaction **(figure 3),** all *K. pneumoniae* isolates from different organs of *Myotis emarginatus* are carried *16S rRNA* gene when a 660 bp band visualized in agarose (1.5) % then dyeing by ethidium bromide then watched under ultraviolet light.

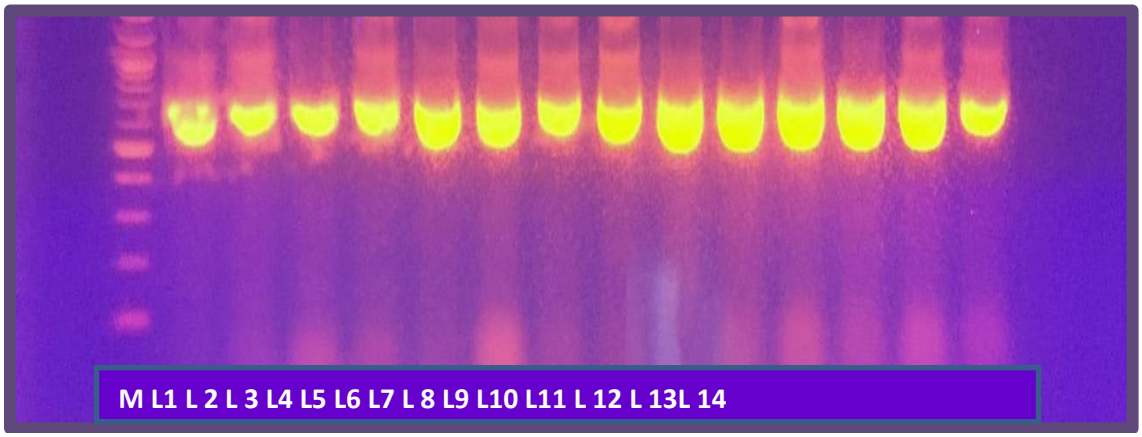

Figure 3. on the agarose that stianed by Ethidium bromide, PCR at (660) bp of *16S rRNA* gene. Lane (M) signify DNA molecular size marker (KAPA Universal Ladder) and other lines (1-14) represent *16S rRNA* gene

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Phylogenetic analysis of *K. pneumoniae*

Nucleotide sequences of *16SrRNA* were prepared from *K. pneumoniae* isolated from bat*Myotis emarginatus* were used to generate a phylogenetic tree based on the NCBI-GenBank database. GenBank number codes of these isolate is MK426995.1 as in **figure (4).** Six clusters (98, 85, 83, 100, 73, and 100) are detected by using the neighbor-joining method in MEGA 6.0. Accession number code (MK426995.1) of present strain located at cluster 85.

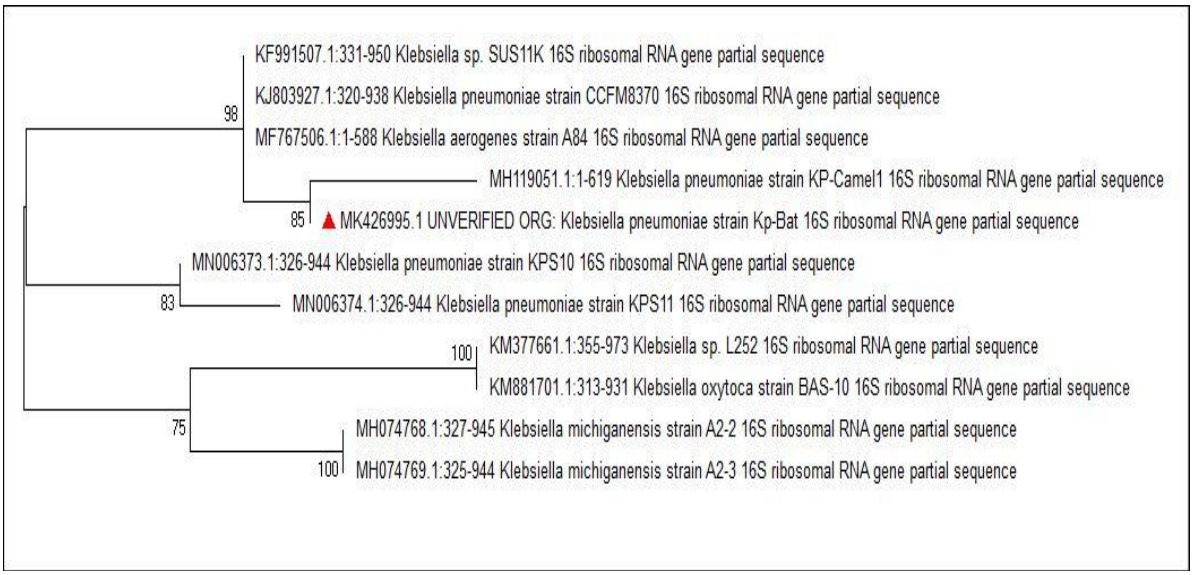

Figure 4. Phylogenetic tree of *K. pneumoniae* depend on *16SrRNA* gene detecting the phylogenetic aspect between present strain and worldwide strains

Discussion

The bats are most mammals that have a great role in the ecology system. The bats play an important role in insect pest control. New studies found that *Myotis emarginatus* is a reservoir of bacteria, viruses. On the other hand, many studies found that bacterial agents present in many animals spp (Hooda S., *et, al*. 2012) (Minamoto Y. *et, al*. 2012) (Suchodolski J.S., *et, al*. 2008) (Zhu L., *et, al*. 2011). The bacteria that carry by the Bats usually cause diseases after transmission (Banskar S., *et, al*. 2016). In the present study *K. pneumoniae* isolated from the different bat, *Myotis emarginatus* organs and this may be related to dietary habits (insects) and associated with *K. pneumoniae*. So Caves or ancient buildings where bats gather may be a source of this bacterium. Besides, most *K. pneumoniae*isolated from the lung and kidney of *Myotis emarginatus* and this may be due to its ability to adapt to these issues in terms of aeration and resistance to the immune system (Fodah R.A, *et, al*. 2014) (Struve C., *et, al*. 2008). Previously, Klebsiella are collected from the intestine of Cynopterusspp such as *C.brachyotis*, *C. sphinx*, and *C. tittaecheilus*(Graves S.R., *et, al*. 1988).Study of Cla´udio*et al*., (2018) on bats from an Atlantic Forest remnant in southeastern Brazil, found the most common bacteria within bat individuals were *Escherichia coli, Klebsiella oxytoca*and *Serratia marcescens (*Cla´udio V.C., *et, al*. 2018*).* However, we did not find any study isolated *K. pneumoniae*from different organs of bat *Myotis emarginatus* for more comparison and scrutiny.

The current study relied on *16SrRNA* geneto identify of *K. pneumoniae*and this gene obtained from all studied bacterial isolates. Bergey's Manual is most authoritative for the characterization of the bacteria. Using the *16S rRNA* as the template is done for determining the phylogenetic identification of the bacteria (Werner J., *et, al*. 2012) (Magray M.S., *et, al*. 2011). 16S rRNA was used to determine the phylogeny and taxonomy of the bacteria because it used as a housekeeping gene (Clarridge J.E. 2004).

The reasons are including (1) presence in all the bacteria, (2) 16S rRNA function is not altered, the changes of the sequence are key of evolution, (3) 16S rRNA is had high efficiency in the informatics (Clarridge J.E. 2004) (Brisse S.,*et, al.* 2009).

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Despite the 16S rRNA sequence is used in the determination of the bacteria, it has low phylogenetic at the species level not at the genera level. The limited new studies suggest that 16S rRNA could use in identification in most cases (Clarridge J.E. 2004) (Ewers C., *et, al*. 2014) (DeSantis T.Z., *et, al*. 2006). Determining of the genus and species of the bacteria is very difficult by using the 16S rRNA gene, the difficulties are included species sharing similar 16S rRNA, little sequences in the databases, and the recognition of novel taxa (Clarridge J.E. 2004) (Turenne C. *et, al*. 2001).

In the present study, the NCBI-GenBank database for Nucleotide sequences of *16SrRNA* geneobtained from *E. coli* isolates revealed a new stain with GenBank accession numbers; MK426995.1. The strain has high similarity with MH119051.1 and MN006373.1 previously recorded strains infect humans and animals. The relationship between these strains showed the risk of *K. pneumoniae* that affect the people by the bats (Ibrahim I. A., *et, al.* 2019).

Conclusion

The current study showed that the bat *Myotis emarginatus* is a good reservoir of *K. pneumoniae* that can be transferred to humans in different ways. Phylogenetic analysis of *K. pneumoniae* in GenBank depending on *16S rRNA gene*is a good option for identification and detection convergence or correlation among strains of *K. pneumoniae* from different sources.

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