

Conventional, Molecular, and Phylogenetic-Based Study of *Pseudomonas Aeruginosa* Recovered from Cave Bats (Geoffroy's Bat: *Myotis Emarginatus*) of Northern Iraq

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Article History: | Received: 06.01.2025 | Accepted: 10.02.2025 | Published: 17.02.2025 |

Abstract: The cave bat; Geoffroy's bat (*Myotis emarginatus*), is an important component of the animal life in Northern Iraq, which lives in huge numbers in the mountain caves of that region. These numbers when they fly hunting for food may cause spreading of some pathogenic microbes. Thus, the current study looked at identifying the presence of *Pseudomonas aeruginosa* in the intestine content of these bats. In this case, 55 bats were captured, and 45 intestinal swab samples were taken. These samples were examined by using cultivation and biochemical methods. The colonies were subjected to a PCR and sequencing by recruiting the *16S rRNA* gene as a target. The sequencing data were processed for the identification of any evolution properties and displayed by presenting a phylogenetic tree. The cultivation and biochemical findings revealed the presence of the bacterium in 8/55 (14.6%) of the samples. These positive results were confirmed by using the PCR and found that 8/8 (100%) of the samples were positive for the pathogen. The sequencing and the phylogenetic analyses of three randomly selected PCR purified products revealed close similarity with global sequences isolated from human lung and animal food in the UK and Italy, respectively. The current results show important information about the possible pathogenicity of *Pseudomonas aeruginosa* present in the intestine of the examined bat, which may pose a real threat to the health of people who are in contact with these bats.

Keywords: Bat pathogens, *Enterobacteriaceae*, pneumonia.

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INTRODUCTION

Geoffroy's bat is a member of the *Myotis* genus (90 species) and the *Vespertilionidae* family [1]. Based on molecular analysis of mitochondrial genes, anatomical resemblances – the basis of the abovementioned classification – seldom correspond to the phylogenetic relations between the species concerned [2]. However, the opposite was found – the indicated species of different *Myotis* subgenera are often close relatives, sister taxa.

Geoffroy's bats are Mediterranean animals and partly endemic species habitually found in the areas. It can be numerous in one area while rare in the neighboring countries. Most of the geographical occurrence in Balkans is important for the species area, here particularly about Bulgaria. It is recognized in Syria,

Jordan, Saudi Arabia, Iran and Iraq [3]. *P. aeruginosa* is a multi-antibiotics resistant bacterium, an agent of opportunistic infection, ie it occurs in humans with weakened immunity caused by chronic obstructive pulmonary disease (COPD) or burns [4–6]. *P. aeruginosa* in biofilm form can survive in a hypoxic environment, or other extremely unfavorable conditions [7, 8]. Highest antimicrobial resistance in *P. aeruginosa* infections is another serious therapy challenge due to its rapid genetic variations and its capacity to develop antimicrobial resistance [9]. *P. aeruginosa* is a major pathogen in nosocomial infections, i.e., in infections with implanted medical devices (airways) where it grows especially on moist surfaces [10] *P. aeruginosa* exhibiting carbapenem-resistance from WHO (World Health Organization) is evaluated as a bacteria from the 'critically urgent' group – meaning an immediate need to develop a new drug, utilized in supporting therapy [11].

Citation: Jenan Nadhim Sadeq & Azhar Abdulsada (2025). Conventional, Molecular, and Phylogenetic-Based Study of *Pseudomonas Aeruginosa* Recovered from Cave Bats (Geoffroy's Bat: *Myotis Emarginatus*) of Northern Iraq. *SAR J Pathol Microbiol*, 6(1), 43-48.

Bacterial diseases resistant to antimicrobials are the cause of annual mortality of about 700 thousand people worldwide. Prevalence of *P aeruginosa* with mixed resistance in the European communities was 12.9 per cent. The percentage of infections in hospitals caused by *P aeruginosa* is still a major health problem as it seems to be susceptible to the medication used commonly for treatment [12, 13]. According to the 2016 EPINE questionnaire taken in the healthcare system in Spain, bacteria *Escherichia coli* and *P aeruginosa* are the major causatives of infections in the hospitalized patients. *P aeruginosa* accounts for 10.5 per cent of all bacterial diseases of medical provenience [14]. According to 2011–2012 report, Healthcare-Associated Illnesses in the European Union, *P aeruginosa* causes almost 9 per cent of infections in healthcare facilities, being the fourth most common bacterium in the hospitals in Europe [15]. In the United States, *P aeruginosa* causes 7.1 per cent of infections in healthcare facilities [16], and epidemiological investigation 2016 in Europe, showed *P aeruginosa* to be among diseases in the hospital's intensive care unit as pneumonia, urinary tract infections and infections of the blood [17, 18].

The cave bat is an important component of the animal life in Northern Iraq, which lives in huge numbers in the mountain caves of that region. These numbers when they fly hunting for food may cause spreading of some pathogenic microbes.

MATERIALS AND METHODS

Sampling

The current study looked at identifying the presence of *Pseudomonas aeruginosa* in the intestine content of these bats. In this case, 55 bats were captured, and 45 intestinal swab samples were taken. These bats were captured during January to March, 2019, using a nest placed in caves in Northern Iraqi mountains. These bats were overdose-chloroform-based euthanized at the Laboratory of Microbiology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq.

Cultivation and Tests

The samples were cultivated on nutrient broth under 24hrs-37°C incubation. Later, these growths were grown up on MacConkey plates. The resulted colonies were placed on nutrient plates under 24hrs-37°C

incubation. The conventional identification of the bacterium was done as detailed by [19].

Molecular Analyses

Extraction of DNA

After the conventional identification of *The bacterium*, the growth of the bacteria in the nutrient broth was exposed to the extraction of bacterial DNA by employing the Genomic DNA Mini Kit (Geneaid) and depending on the kit protocol steps. The DNA was calculated for its purity and amount by recruiting a NanDrop.

Polymerase Chain Reaction

The PCR methods utilized the primer sets; F: GGTGGTTCAGCAAGTTGGAT) and R: CGTAAGGGCCATGATGACTT that target a 633bp-fragment in the *16S rRNA* gene. The master mix was prepared according to the company protocol. The thermal cycling conditions were primary 94°C-30s-denaturation, 51°C for 30s of the annealing step, and elongation 72°C-30-step all for 30 repeats, and final 72°C-10min-elongation. The PCR products were examined by utilizing an agarose (1%) gel mixed with ethidium bromide. The resulted bands were visualized under a UB-transilluminating imaging machine.

16S rRNA gene Partial Sequencing Analysis

Three PCR (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobase. Canada)-purified products were sequenced at Bioneer Company (Korea) for confirmation and analysis of evolution. The obtained sequences were deposited in the GeneBank, and they were analyzed using the NCBI-website tools. The tree was constructed by employing the MEGA X.

RESULTS

The cultivation and biochemical findings revealed the presence of the bacterium in 8/55 (14.6%) of the samples. The colonies reflect pale appearance, which means that these isolates were from the non-lactose fermenting organisms. Moreover, the appearance of these colonies showed irregular ends as revealed on the MacConkey agar. In addition, the colony appearance on the nutrient agar revealed smooth, convex, and greenish-blue color (Figure 1). The biochemical properties of the isolates are displayed in Table 1.

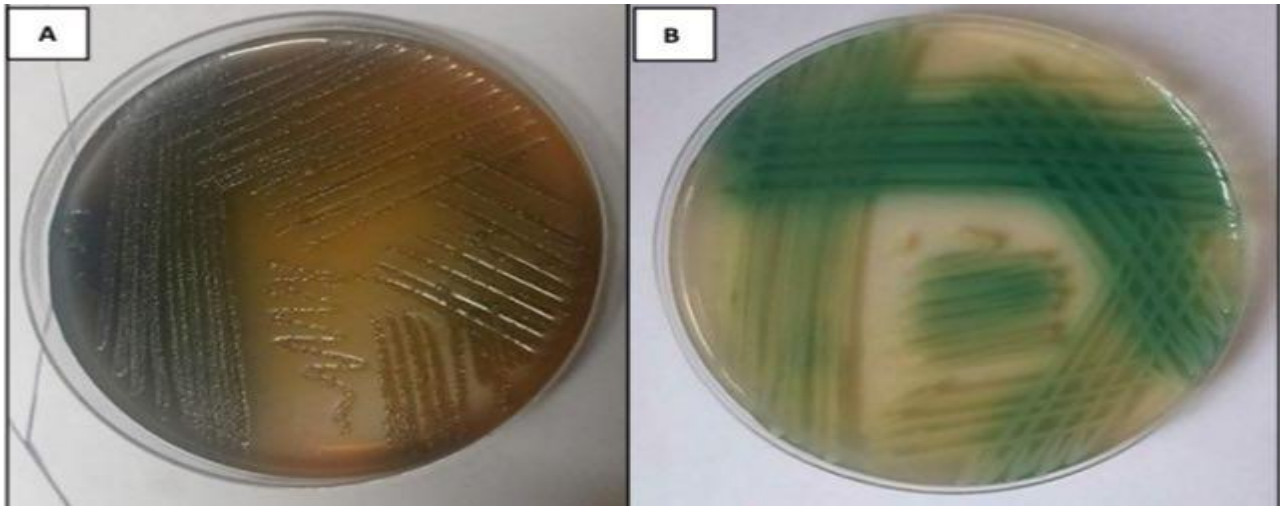


Figure 1: Colonies of *Pseudomonas aeruginosa* isolated from bat intestines grown on A. MacConkey agar and B. Nutrient agar

Table 1: Biochemical properties of *Pseudomonas aeruginosa* isolated from bat intestines

Subjected-to-test	Findings
Morphology	Rod-like appearance
Production of catalase enzyme	+ve
Ability of moving	+ve
Production of oxidase enzyme	+ve
Production Indole ring	-ve
Production of urease enzyme	-ve
Consumption of citrate	+ve
Production of methyl red reaction	-ve
Fermentation of triple iron sugar reaction	Alkali/Alkali (red/red/-H ₂ S)
Production of pigment	Pyoverdine

These positive results were confirmed by using the PCR and found that 8/8 (100%) of the samples were positive for the pathogen. The amplification of the DNA fragment was shown at 633bp piece of the *16S rRNA*

gene. The confirmation was for all the tested positive samples by the biochemical tests. Figure 2 shows the amplification of the fragment as revealed by the electrophoresis of the 1%-agarose gel.

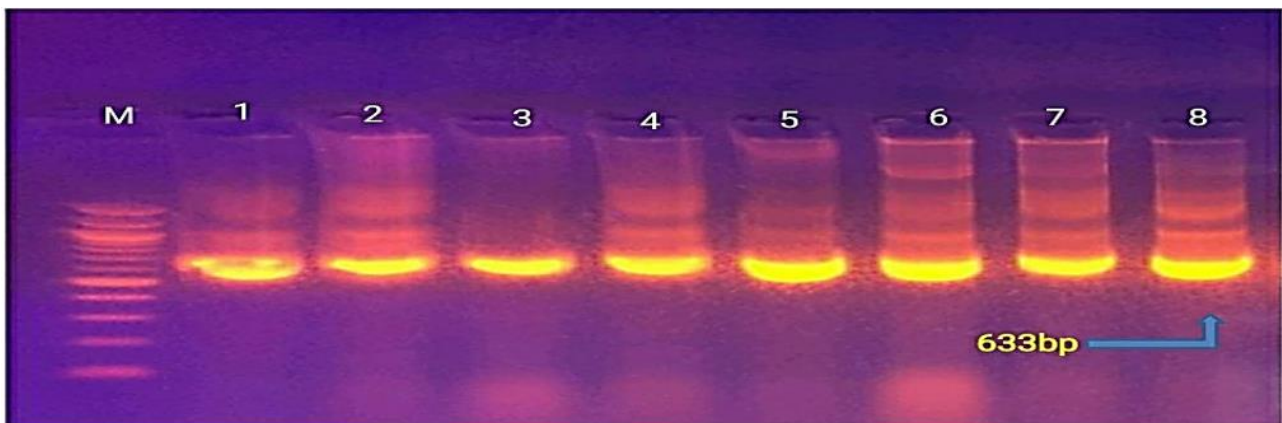


Figure 2: Image of the *16sRNA*-gene-PCR-based 1%-agarose gel electrophoresis of *Pseudomonas aeruginosa* isolated from intestine swabs from bats. M: 100-10000 ladder and 1-to-8: Positive PCR

The sequencing and the phylogenetic analyses of three randomly selected PCR purified products revealed close similarity with global sequences isolated

from human lung and animal food in the UK and Italy, respectively (Table 2 and Figure 3).

Table 2: Degree of identical similarity between current study intestinal isolates and global isolates of *Pseudomonas aeruginosa*

Accession number	Organ and origin country	identity
Current study isolates	MH118953.1	Bat intestine (Iraq)
	MH118952.1	Bat intestine (Iraq)
	MH118951.1	Bat intestine (Iraq)
Similar to the following global isolates		
CP006985.1	Human lung (UK)	99.36%
KY438756.1	Animal food (Italy)	95.77%

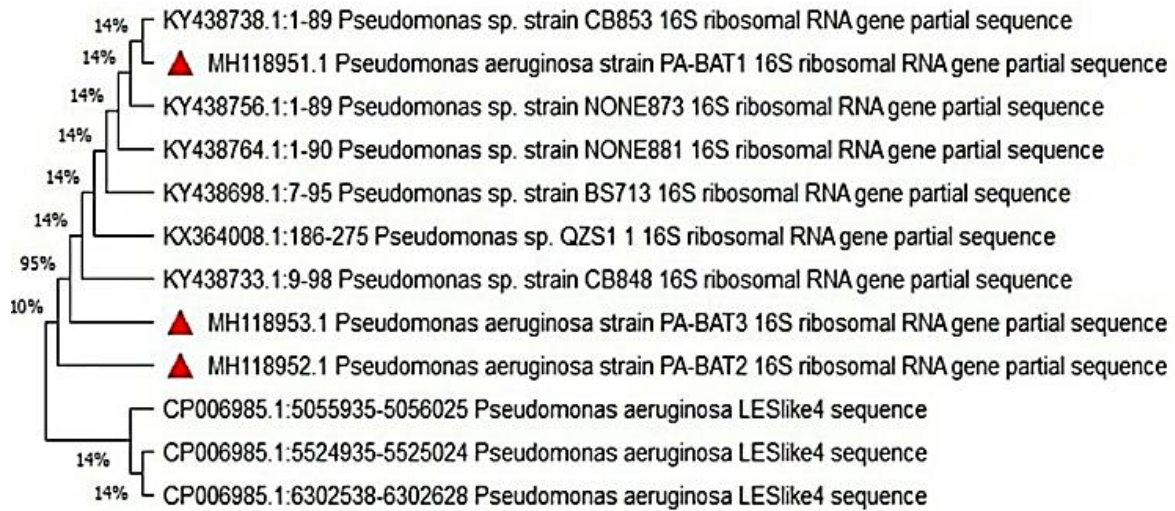


Figure 3: Tree of phylogeny related to the 16S rRNA gene belongs to *Pseudomonas aeruginosa* recovered from intestinal content of bats compared with global isolates

DISCUSSION

Bats are found in almost every place worldwide. At present, about 1300 species are recognized, which forms about 20% of all mammalian species on Earth. They are extremely diverse both in terms of ecology and together the evolutionary mechanism of feeding techniques is most varied among terrestrial vertebrates. Additionally, bats are extremely important for the environment as they perform environmental services such as spreading seeds and pollinating plants, as well as controlling pests. Moreover, they are rich in pathogens and zoonoses including those harmful to humans [20]. The bacterial composition of bats is still poorly understood due to lack of study. They have been studied most among species belonging to the Old World, of which the most attention is paid to microbiota of the gastrointestinal tract [21].

In our study, we analyzed 55 intestinal samples from the local bats. Out of all the samples tested, 8 (14.6%) indicated the presence of the bacterium in its intestine, which means almost 15 per cent of all of the studied bats. The fact that the bacterium was detected in the intestine of the Geoffroy’s bats suggests its possible role either as a commensal or pathogen. Commensal bacteria pose no health risks to animals and can even be useful for them, while pathogenic bacteria can cause infections and diseases [22]. To explain the role of the bacterium in the gut microbiota of Geoffroy’s bats,

further research is needed in order to determine its interaction with the host and the health implications. Factors that may indicate the causation behind the bacterium in being present in intestine of bats are their feeding habits. They usually consume food items such as insects, fruit and nectar but it is quite likely that the bacterium was acquired by this animal through the contaminated food or water. Additionally, bats in colonies can be quite dense, allowing the possible transmission of bacteria between each other [23].

Global antibiotic resistance by microorganisms, including the bacterium, has reached a peak of resistance, and poses a great challenge for humankind in the near future. In addition, many species of *P aeruginosa* strains carry resistance genes [24]. The presence of the bacterium in one of the upper layers of water used as drinking for animal is 10%. It is also known that bacteria of the bacterium can multiply in a variety of habitats or remain in a state of dormancy, when they remain alive though not cultured [25]. This fact can show the low level of the bacterium from freshwater. Antimicrobial use in agriculture and livestock control have spread the anti-bacterial resistance genes in the environment around them [26]. *Pseudomonas aeruginosa* produces two distinct toxins, namely exotoxin A (ETA) and exoenzyme S. Most of the bacterium strains synthesize the extremely toxic ETA, which suppresses the process of eukaryotic protein formation by acting on the elongation factor 2 responsible for the synthesis of

polypeptide chain elongation process. The wide distribution of the *toxA* gene (80%) in *P. aeruginosa* populations shows a high health risk [27].

CONCLUSION

The current results show important information about the possible pathogenicity of *Pseudomonas aeruginosa* present in the intestine of the examined bat, which may pose a real threat to the health of people who are in contact with these bats.

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