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Conventional and molecular identification of *Enterobacter cloacae* that carries SHV-related extended-spectrum-β-lactamase gene from bat intestinal contents

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Abstract

This study was conducted to identify SHV-extended-spectrum-β-lactamase (SHV-ESBL) gene-carrying *Enterobacter cloacae*, a crucial nosocomial bacterium, from bat using conventional and molecular techniques. Intestinal content samples from 50 *Myotis emarginatus* bats were cultivated and diagnosed using the VITEK 2 system. A *16S rRNA*- and *bla SHV* gene-based polymerase chain reaction (PCR) and partial gene sequencing techniques were performed to confirm the bacterium's identification and study its genetic evolution. The conventional and PCR methods revealed the presence of *E. cloacae* in 23 (46%) and 13 (26%), respectively, of bats. The *bla SHV* PCR uncovered that only ten out of 13 isolates were positive for the presence of the SHV-ESBL gene. Nucleotide-based similarity with world isolates of *E. cloacae* was detected based on the phylogenetic evaluation. This study confirms SHV-ESBL-gene-carrying *Enterobacter cloacae* in Northern Iraq's intestine of Myotis emarginatus bats.

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Introduction

Due to their widespread distribution, bats have been identified as a possible reservoir for several bacterial infections. They are thought to play a significant role in transmitting zoonotic diseases (1). Multiple encounters and possibilities for intraspecific disease transfer are facilitated in colonial species because of the near nesting location of humans (2). Familiar places for many species to nest in urban and human-associated regions include abandoned homes, structures, and trees (3). The possible function of animals as reservoirs of antibiotic-resistant bacteria has been gained from the frequent documentation of antibiotic-multi-resistant enterobacteria in wildlife animals in the past few years (4), even though different types of bacteria, such as *Escherichia coli*, *Salmonella* spp., and *Leptospira* spp. (5) have been identified from bats in different parts of the world; a minor is recognized about bat SHV-ESBL bacteria, such as *E. cloacae* in Iraq, which have emerged as nosocomial pathogens, especially in intensive care units (ICUs) (6). Among the *E. cloacae* complex, *E. cloacae* is the most well-known member (7). Some opportunistic illnesses, such as pneumonia, urinary tract infections, wound infections, and even hospital-acquired sepsis, may be caused by this organism in ICU patients (8,9). Due to widespread antibiotic resistance, the occurrence of *E. cloacae* continues to rise. The *bla* SHV gene produces SHV-ESBL enzymes responsible for high resistance to Beta-lactam agents (10). In the latter stage of the twentieth century, SHV enzymes began to be found in Enterobacteriaceae, responsible for healthcare-associated infections. Today, these isolates can be found in various epidemiological configurations, including those involving humans, animals, and the natural environment (11). There are now several different allelic variations of lactamases. These include ESBL, non-ESBL, and a few that have not yet been characterized. SHV

enzymes have expanded their hydrolyzing ability to retain monobactams and carbapenems due to amino acid modifications that have changed the arrangement surrounding the active site of the Beta-lactamases. SHV-ESBLs have become globally prevalent in several Enterobacteriaceae, underscoring their clinical relevance. These ESBLs are often carried by self-transmissible plasmids containing resistance genes to other drug classes (12,13).

This study was conducted to conventionally and molecularly identify the SHV-extended-spectrum-β-lactamase (SHV-ESBL) gene-carrying *Enterobacter cloacae*, a crucial nosocomial bacterium, from Bat in Iraq.

Materials and methods

Ethical approve

The study was approved and carried out at the College of Veterinary Medicine, University of Al-Qadisiyah with approval number (No. 576 in 2018) according to the international guidelines for the care and use of animals.

Samples

Fifty bats (*Myotis emarginatus*) were randomly collected using mist nets from a mountain in northern Iraq during January and May of 2018. Quickly, the bats were grabbed up and inserted into specific clothing bags. The University of Al-Qadisiyah- College of Veterinary Medicine received the live bats for a morphologically-based taxonomy classification project (14). Overdosing bats with chloroform put them in deep sleep and scarification. Aseptic intestinal material was recovered. Before further analysis, all samples were quickly submerged in liquid nitrogen and then stored at a temperature of -4ºC.

Isolation and identification of *Enterobacter cloacae*

The collected specimens were cultivated on blood and MacConkey agar media, then incubated at 37˚C for 24 hours; the colonies were identified based on their morphological characteristics and their VITEK®2.0 Compact 15-based results.

Extraction of DNA

To extract the DNA from intestinal contents, we followed the manufacturer's instructions for the Genomic DNA Kit (NOVOGEN). To determine the quantity and quality of the DNA Extracted, we used a NanoDrop-gel electrophoresis setting.

Primers

The *16S rRNA* gene was amplified by PCR using specific primers (F: GGGGGTAGAATTCCAGGTGT, R: TTCATGGAGTCGAGTTGCAG). Specific primers were used to identify whether or not *E. cloacae* isolates contained the *bla SHV* gene (F: AGCCGCTTGAGCAAATTAAA, R: AATGCGCTCTGCTTTGTTA). The PCR reactions were prepared by the methodology and the genes specified by the supplier of the master mix. The PCR settings for 35 cycles are as follows: initial denaturation at 94°C for 30s, annealing at 57°C for 30s for the first gene and 58C for the second gene, extension at 72°C for 60s, and terminal extension at 72°C for 7mins. To verify the existence of a 661bp *16S rRNA* gene and a 504bp *bla SHV* gene, PCR results were viewed on a 0.8% agarose gel pre-treated with ethidium bromide and utilizing a UV-illumination machine (15).

Gene sequencing and analysis

This study sequenced the *16S rRNA* gene in four different *E. cloacae* isolates after being separated off the PCR agarose gel by (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobase. Canada). The sequencing of the *16S rRNA* gene was established by sending the PCR products to the Bioneer Company/Korea. The complete sequences were submitted to the GenBank-NCBI database, where an online query tool was used to do the alignment. Next, we used the BLAST (NCBI) database to evaluate the matched sequences depending on the proportion of shared homology to classify the bacterium. The neighbor-joining method was used to construct a phylogenetic tree in MEGA (X) (16-18).

Results

23 (46%) bacterial isolates *of E. cloacae* were recovered from 50 bat intestinal tract cultures. The bacterial microorganism was a Gram-negative rod with peritrichous flagella that was facultatively anaerobic. Colonies grew on blood agar as large, smooth, and flat with no beta hemolysis. On MacConkey agar, *E. cloacae* had distinct pink to red-colored mucoid colonies. Table 1 shows different biochemical tests performed in this study to identify *E. cloacae* isolates.

Table 1: *Enterobacter cloacae* based VITEK 2 system

Test	Findings
Indole	-
Methyl red	-
Vogas Proskaure	+
Simmon citrate	+
Catalase	+
Urease	V
CO_2	+
H_2S	-
Oxidase	-
Gelatin liquefication	-
Triple sugar iron	A/A
Sucrose	+
Mannitol	+
Motility	+

(+) Positive, (-) Negative, A/A (Acid/Acid), V (variable).

PCR results

According to conventional PCR assay, 13 (26%) isolates showed positive results for the *16S rRNA* gene at 661bp (Figure 1). According to conventional PCR assay, 10 of 13 isolates showed positive results for the *bla SHV* gene at 504bp (Figure 2). The results of different methods used for detection of *E. cloacae* from bats (Table 2).

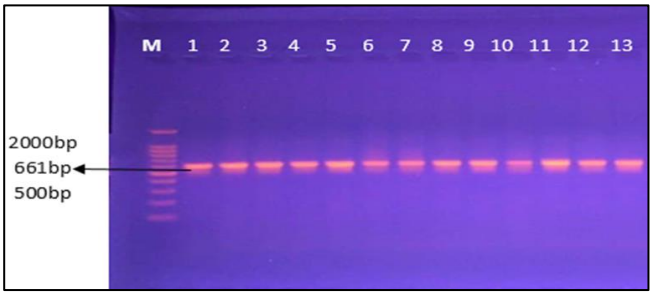

Figure 1: The PCR products for *16s rRNA* gene from *Enterobacter cloacae* in bat intestinal contents.

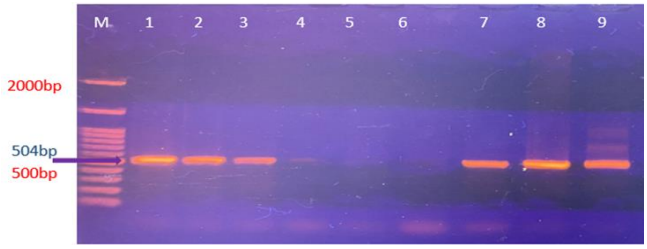

Figure 2: The PCR products for the *bla*$_{SHV}$ gene from *Enterobacter cloacae* in bat intestinal contents.

Phylogenetic analysis

Four positive PCR isolates were sent to Korea for sequencing and constructed in the Gene Bank NCBI, with accession numbers OK605772.1, OK605773.1, OK605878.1, and OK605879.1. The *E. cloacae* isolates from the present work were aligned with global NCBI-based isolates. The phylogenetic tree of E. cloacae shows 99 % identity with CP039318.1 from the United Kingdom/homo sapiens and 99 % identity with CP046116.1 from China/Human (Table 3 and Figure 3).

Table 2: comparison results obtained from the other techniques used to identify *E. cloacae*

Diagnosis methods	Positive samples No. (%)	Negative samples No. (%)	Total
Conventional culture	23 (46%)	27 (54%)	50 (100%)
PCR-based 16s rRNA gene sequence	13 (26%)	37(74%)	50(100%)
PCR-based *bla* $_{SHV}$ gene sequence	10(76.9%)	3(23.1%)	13(100%)

Table 3: Accession number of our study with world global strain

Accession No.	Country	Source	Identity
OK605772.1	Iraq	Bat	This study
OK605773.1	Iraq	Bat	This study
OK605878.1	Iraq	Bat	This study
OK605879.1	Iraq	Bat	This study
CP039318.1	UK	Human	99%
CP046116.1	China	Human	99%

Discussion

Bats are efficient vectors and potential reservoir hosts for numerous infectious agents due to their flexible eating pattern, flying ability, and inherent features. And people and bats both frequently visit each other environments. This theory predicts an elevation in the occurrence of vector-borne and zoonotic illnesses (19,20). It has previously been shown that bat guano contains harmful enteric bacteria and other pathogenic organisms often associated with human and animal disorders (21-23). This investigation confirmed previous findings that *E. cloacae* may be found in bat poop. We successfully culture 23 isolates, and 13 of them were identified as *E. cloacae* using a polymerase chain reaction (PCR) based on the 16S rRNA gene sequence. No positive result was obtained for other isolates, suggesting they were misclassified as *E. cloacae* or another member of the *E. cloacae complex*. This proves the accuracy of the molecular assay in detecting these bacteria.

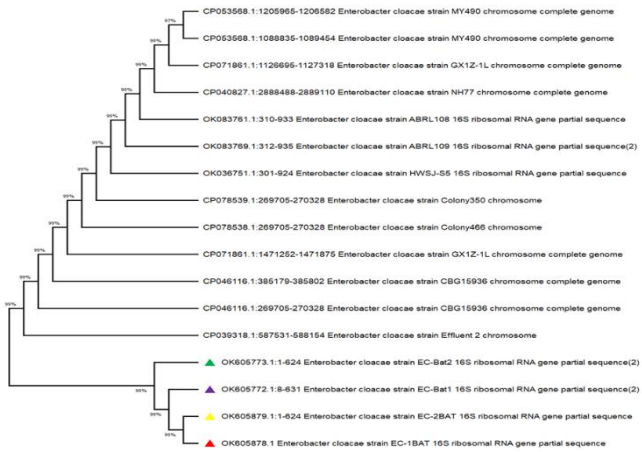

Figure 4: Phylogenetic relationship of *E. cloacae* isolates with world isolates.

Analysis of 16S rRNA gene sequences was the most accurate approach for identifying bacterial isolates (24,25). Progress has been made in many areas of microbiology due primarily to 16S rRNA sequencing, which will remain the standard for determining bacteria. Accordingly, on-cultivable microbes can now be classified, which is a huge step forward in the field (26). It also aids in clarifying the relationship between previously discovered bacterial species and those already recognized. Therefore, difficult to identify microorganisms need to have their 16S rRNA sequenced (27). This agrees with a previously published study that found 16S rRNA sequencing superior to other approaches depending on cellular fatty acid patterns or carbon sources for identifying 72 rare aerobic Gram-negative bacilli (28). The bacterial community makeup in bat guano has been studied extensively recently. Several investigations have found drug-resistant harmful bacteria in the guano of various bat populations, including ESBL and carbapenemase-producing Enterobacteriaceae (29).

Antimicrobial resistance has grown in both pathogenic and endogenous bacteria and has been observed in domesticated and wildlife animals (30,31). Thus, bats may contribute to disseminating drug-resistant bacteria and transferring resistant pathogens to people. *Yersinia*, *Campylobacter*, and *Vibrio* are only a few other taxa discovered in bats. However, their impact on the host species is largely unknown (32). While environmental antibiotic resistance is still poorly understood, the spread of ESBLs in Enterobacteriaceae from people and animals has been investigated in depth (33-35). Results showed that the *bla SHV* gene was present in 76.9% (10/13) of isolates in this investigation. The significant threats to public health and support for a one health research strategy are highlighted by the widespread occurrence of SHV ESBL genes, suggesting transmission in humans, animals, and the environment.

Bats may pick up antibiotic-resistant *E. cloacae* via the foo d, drink, and environment they come into contact with. This may represent the widespread utilization of antimicrobials in humans and animal agriculture (36-39). Mbehang Nguema *et al.* described the existence of antibiotic-resistant pathogens in bats due to food contamination by other mammalian species that already hold this form of resistance via consuming the same food and water sources once in the same living habitat. Most bats get their hydration from areas of open water, such as lakes and slow-moving streams. Especially in urban areas, these water supplies are often contaminated (29). Multiple studies show sewage drainage is a significant source for the dispersal of ESBLs in the ecosystem (40,41).

Sequencing and phylogenetic tree analysis revealed that the E. cloacae isolated from bats with accession numbers OK605772.1, OK605773.1, OK605878.1, and OK605879.1 were closely related to the Humans in China and the United Kingdom with accession numbers CP039318.1 and CP046116.1, implying that bacteria can infect humans and cause disease in domestic animals. Bats are a significant transmitter and reservoir of dangerous microorganisms like *E. cloacae*, which transmit evolved resistance because of the connection between human and bat variants and the zoonotic interaction among them. These bacterial infections are transmitted from bats to people via bat-contaminated food and water, threatening human health. This can be a very problematic issue for public health.

Conclusion

Our results highlight the necessity of tracking the prevalence of antimicrobial resistance in the animal world, and further research is required to determine the source of the reported resistance and determine the impact that bats play in the spread of antibiotic-resistant genotypes with significant public health implications.

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Conflict of interest

There is no conflict of interest for the publication of this manuscript.

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التحديد التقليدي والجزيئي لبكتيريا الامعائية المذرقية التي تحمل جين مقاومة الطيف الواسع البيتا لاكتميز والمرتبط بالشف في أمعاء الخفاش

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الخلاصة

أجريت هذه الدراسة لتشخيص بكتيريا الامعائية المذرقية والحاملة لجين مقاومة الطيف الواسع البيتا لاكتميز والمرتبط بالشف من الخفاش باستخدام الطرق التقليدية والجزيئية. تمت زراعة عينات المحتوى المعوي المأخوذة من ٥٠ خفاش تابع للمايوتيس ايمارجينيتس والتي تم تصنيفها في هذه الدراسة بعد التقاطها في شمال العراق. تم إجراء تفاعل انزيم البلمرة المتسلسل مستهدفا جين ١٦ س الحمض النووي الريبوزي الريبوسومي وجين الشف بيتا لاكتام وتقنيات فحص التسلسل النيوكليوتيدي الجيني الجزئي لتأكيد وجود البكتيريا ودراسة تطورها الجيني. أظهرت الطرق التقليدية وطريقة تفاعل البلمرة المتسلسل الى وجود بكتريا الامعائية المذراقية في ٢٣ (٤٦٪) و ١٣ (٢٦٪) على التوالي من عينات الخفافيش. وضح فحص تفاعل البلمرة المتسلسل لجين البيتا لاكتام شف إلى أن ١٠ عزلات فقط من أصل ١٣ كانت إيجابية لوجود الجين. تم الكشف عن التشابه القائم على تسلسل النيوكليوتيدات مع العزلات العالمية من الامعائية المذراقية بناءً على تقييم النشوء والتطور. تؤكد هذه الدراسة وجود بكتيريا الامعائية المذراقية والحاملة لجين البيتا لاكتاميز واسعة الطيف نوع شف في أمعاء خفافيش المايوتيس ايمارجينيتس في شمال العراق.