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Phylogenetic Analysis of Proteus SPP from Cave-Dwelling Bats That are Risk to Human in Iraq

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Abstract
The present study aimed to describe the genetic relationships of zoonotic characterization of proteus isolated from intestine of Bat and close relation with proteus that caused disease to the human. The study includes (40) Bat insect ivory species Myotisemarginated. The isolation and identification of Proteus Spp were done by using enrichment culture method, and then confirmed by PCR technique based on 16S ribosomal RNA gene which designed in this study using NCBI-GeneBank (CP034105.1) and DNA sequencing was done on some positive isolates. The results show that proteusspp was isolated from intestine of Bat 20(50%) by culture method. The PCR technique was show highly sensitive and specific confirmative detection of Proteus sppisolates result occur about 10(25%) at Clarify DNA sequencing of a partial sequence of 16S ribosomal RNA gene was shown homology sequence identity highly with NCBI-Blast Proteus isolates. The phylogenetic analysis was show clear genetic similarity at (0.5 genetic change) between proteusmiribilus and proteus vulgaris isolates. The gene sequence deposited into NCBI-GenBank accession numbers (MH119074.1, MH119075.1). In conclusion, the study presents focus on main pathogen that infects Bat in Iraq of genetic relationship among Proteus sppisolates Bat. Therefore, it is essential to define the role of Bat an important source for the distribution of pathogen related to public health
Keyword: Phylogenetic analysis, Proteus Spp, cave-dwelling

Introduction

Bats are a common source of pollution of a many microorganisms and wellsprings. As possible reservoir hosts and vectors of zoonotic microorganisms, the incidence of irresistible illnesses and their impact on human well-being has increased interest in bats. A many bacteria, including Proteus spp, Salmonella spp., Pasturella spp., Leptospira sp., Escherichia coli, Similarly, Bartonella spp. Wild bats in various nations have been isolated from wild Bats [1]. In urban areas, bats are common and come into close contact with both domestic and human animals, contaminating houses with guano and urine. In addition, humans sometimes invade bat habitats. That means that in urban areas with guano and urine, many zoonotic and bacterial pathogens can spread. The prevalence of pathogenic bacteria in bats and their possible danger to humans remain poorly investigated[2,13].Bacteria from the genus Proteus can also be differentiated on the basis of their O-antigen variability, although serotyping is not included in the routine diagnosticsof these rods. So far, there have been established 80 O-antigenic serogroups in the genus, some of them divided into subgroups [3-4]and many new O serotypes are still being discovered. The chemical structure of the sugar part of the lipopolysaccharide may play an important role in the adaptation of Proteus spp. bacteria to environmental conditions and enhancing their pathogenicity, as some O serotypes are more prevalent and more frequently isolated from clinical sources than the others [5].P. mirabilis and P. vulgaris were found in fecal samples of western lowland gorillas (Gorilla gorilla), collected at two locations in south-central Cameroon, proving to inhabit the intestines of these great wild apes [6].Like in humans, the presence of Proteus spp. in animal intestines may pose a threat of autoinfection and cross-

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infection. The example of such autoinfection was described by[7]who in the Netherlands isolated P. mirabilis strains from feces and urine of dogs suffering from recurrent urinary tract infections,although Proteus spp. bacteria are commonly known as opportunistic pathogens, there can be found interesting examples of positive relations between the microorganisms and the host animal. P. mirabilis and P. vulgaris strains isolated from the intestine of the Indian flying fox (Pteropusgiganteus) were recognized as the members of symbiotic physiological microflora of this big fruit bat [8].Although this is unusual for the two species, the isolates were able to produce cellulolytic and xylanolytic enzymes just as three other isolated species, Citrobacterfreundii, Serratialiquefactions, and Klebsiellaoxytoca, and contrary to the other six gut isolates unable to digest cellulose. Fruits and leaves,which are the animal's main food, are built of up to 50 % of cellulose, hemicelluloses (xylem), lignocelluloses, and pectin.However, it is important to remember that the bacteria belonging to the genus Proteus are opportunistic human and animal pathogens. It would be useful and interesting to compare the features (especially virulence factors, serotypes, metabolic apparatus, and the antibiotic resistance) expressed by Proteus spp.Identification of bacterial pathogens from bat caves which are a risk to human, animal, and environment health in Iraq is needed as early detection of the presence of bacterial pathogens on the bat.

Material and Methods

Samples collection: forty bats from insectivore species Myotisemarginated (figure 1) were caught with mist nets during period from January to March of 2019 from a mountain in northern Iraq. An overdose of chloroform was used in euthanizing the bats. In Anatomy Lab/College of Veterinary Medicine/University of Al -Qadisiyah, the tissue samples of spleen, liver, kidney, lung, stomach and intestines were aseptically taken. Intestines were removed last to avoid contamination of organs with intestinal content. All samples were immediately preserved in liquid nitrogen and stored at -4°C until further analyses.

Figure (1): A dead insectivore bat related to species Myotisemarginated

Isolation and Identification of proteus in Bacteriology Lab: taken of 40 intestine swabs from Bat cultured on blood agar with three replications for each specimen and incubated overnight at 37˚C in bacteriological incubator under aerobic conditions. The identification of proteus was done depending on morphological features and the rose-pink color of the colonies on MacConkey agar plats and nutrient agar with incubated for 24 hours at 37˚C, then the result confirmed Gram stain and by Analytical Profile Index 20 (API 20). After identification of proteus bacterial cell cultured in Nutrient broth for DNA extraction which use in molecular study

DNA Extraction: DNA was extracted from bacterial broth according to manufacturers' instructions of Genomic DNA Mini Kit (Geneaid). The extracted DNA was electrophoresed on agarose gel (0.5% agarose stained with 5µL of ethidium bromide) to confirmation that DNA

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present in each sample then micro-centrifuge tubes that contain DNA stored at -20ºC in deep freeze even used in PCR.

Primer Preparation and PCR Reaction: Specific primers for 16S rRNA gene (F-GGAACTGACACGGTCCAG, R-CCAGGTAAGGTTCTTCGCGT) were prepared according to manufacturer's instruction by dissolving the lyophilized primers with deionized distal water to form stock solution with concentration of 100 pmol / µl. Primer's stock solution diluted with deionized water, using the equation C1V1 = C2V2 to get final working solution (10 pmol / µl) for each primers. The PCR reaction mixtures were preformed according to manufactures procedures of the master mix and the genes that targeted in the methods. The PCR tube vortexed until the lyophilized pellet dissolved and all mixtures are mixed, then PCR tube was entered into Professional TR/O Thermocycler for thermocycling condition. Optimized PCR conditions for 30 cycles are as follow; initial denaturation at 94 ◦C for 30 second, annealing at 60 ◦C for 30 second (optimized temperature), elongation at 72 ◦C for 30 second and final elongation at 72 ◦C for 10 min. PCR products were visualized on a 1% agarose gel stained with ethidium bromide under UV light to confirm the presence of a 671 bp band.

Identification of *proteus* using 16S rRNA gene sequence analysis: The DNA sequencing for two isolates was performed based on 16S rRNA gene, the PCR product was purified from agarose gel by using (EZ EZ-10 Spin Columni DNA Gel Extraction Kit, Biobase. Canada). Then purified PCR products were sent to Bioneer Company/ Korea for testing and confirm DNA sequence of the 16S rRNA gene. The final genomic sequences were submitted in GenBank-NCBI website then alignment was made by search Tool (BLAST) for showing phylogenetic analysis. Phylogenetic tree was generated using the neighbor-joining method in Molecular Evolutionary Genetics Analysis (MEGA) version X software.

Results

Isolation and identification of proteusspp

The isolation of proteusspp from bat showed that exhibit the swarming growth on blood agar. Culture characteristic of P.mirabilis in mac Conk agar appear that pink color colonies with swarming inhibited because to presence of bile salts,charecristic in nutrient agar ,small colony, glistening and growth irregular due to swarming,while bacteria proteus. Vulgaris appear are pale or colorless du in mac Conk agar, the same characteristic to p.mirabilis in nutrient agar further biochemical test were done for confirmation of the isolate,table (1)

Table (1):Biochemical tests were done for confirmation of the isolates

N	Biochemical test	Result of Biochemical Test for P.mirabilis	Result of Biochemical test For P.vulgaris
1	Test (MR – VP)	Positive (red to pink color signifies	Negative
2	Indole	Negative (Indole reagent retained it yellow color indicating	Positive cred color ring appears in the top the test table
3	Triple sugar Iron (TsT)	R/y + H2 s	R/y + H2 S
4	Citrate utilization	Positive (blue precipitate observe)	Positive

Key = MR = methyl red, VP =vogesproscaur R= Red y = yeuow H2 s = hydrogen sulfate TSI = Triple Iron sugar

Molecular Identification of Proteus Spp

The positive isolates of biochemical tests are produced for confirmative endpoint PCR for detection 16s rRNA gene (671bp) of Proteus Spp in Ethidium bromide-stained agarose gel using specific primers and the ladder in size (100-10000bp), 20 (50%)cultured cell isolates from

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different regions gave positive results (line 1-4),figure (2), table (2).In addition to the confirmative diagnosis of the Proteus Spp,the PCR products used in the sequencing for analysis of 16s rRNA gene of a predominant strain of Proteus SppinBatin Iraq.

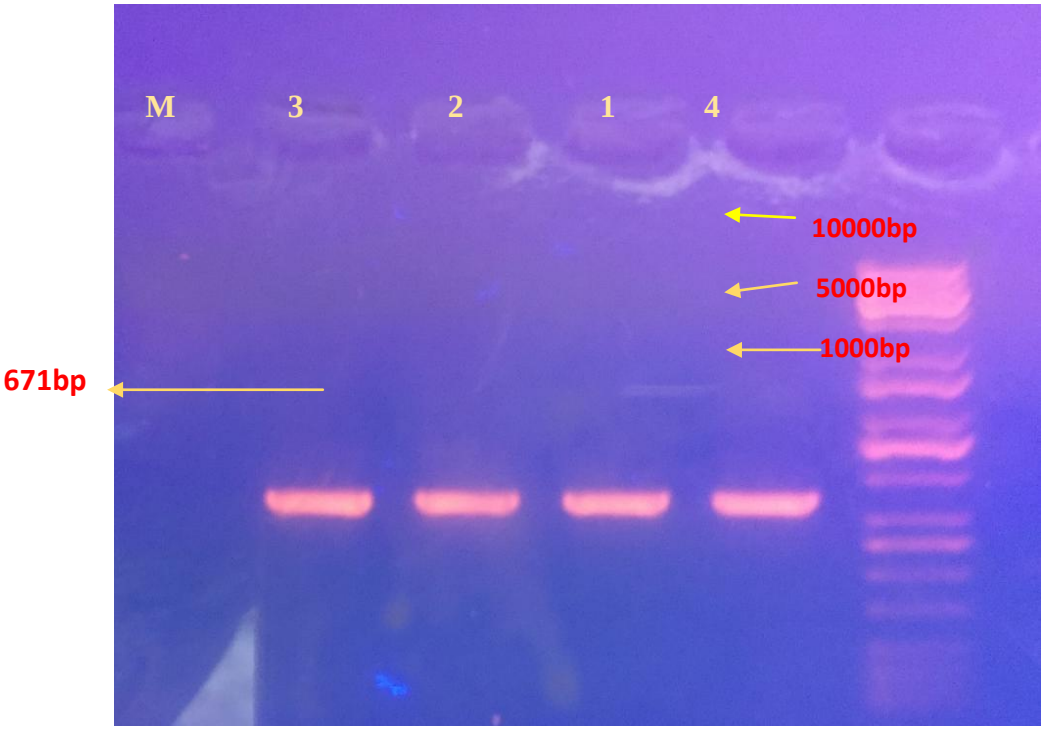

Figure (2): Electrophoreses image of 1% agarose gel with the PCR products of 16sRNA gene from intestine samples of Bat. M lane is the ladder (100-10000), Lanes (1 to 4) are positive samples at 671bp.

Table (2): Proteus Spp number and their isolation percentages offrom Bat by cultured cell and PCR technique .

Type of sample	*Proteus spp*		Total
	Positive	Negative	
Intestine swab sample cultured cell	20(50%)	20(50%)	40(100%)
Intestine swab sample PCR sample	10(25)	30(75%)	40(100%)

Sequencing and phylogenetic tree construction of 16S rRNA gene

From 10(25%) PCR positive results two send to Korea for sequencing and record in GenebankNCBI, on proteusmirabilis with accession number MH119074.1 while the second Proteus vulgaris with accession number MH119075.1 the two isolate of proteus were aligned with global reference strains for proteusmiribilus and proteus recorded in the GenBank. Phylogenetic tree of ProteusMiribilusshowed that local strains ofproteusMiribilus were close 99%, in their identity to isolates from of both human and animal isolatesfigure (3), table (3) while phylogenetic tree of Proteus vulgaris showed that local strain of Proteus vulgaris isolate from Bat were identity with Human and animal isolate by 99% of India and USA isolates, figure (3) Table (4).

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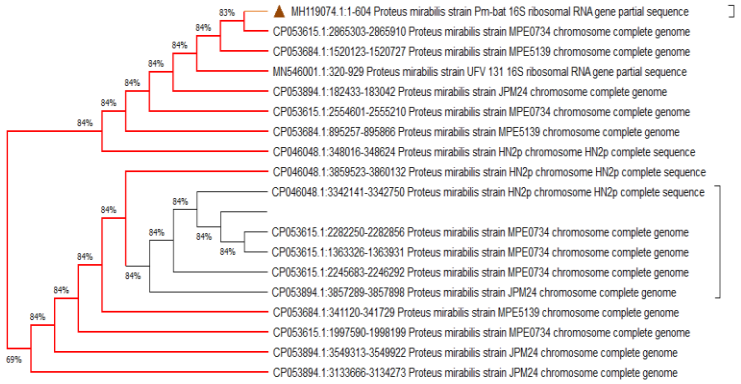

Figure (3) Phylogenetic tree of Proteus MirbilusinBat with world strains

Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method (9). The optimal tree with the sum of branch length = 4.21254519 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (10) and are in the units of the number of base substitutions per site. This analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 724 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (11)

Table (3): The current study proteusmiribilus isolates and their nucleotide-based similar global isolates

Accession No.	Country	Source	Identity
MH119074.1:1	Iraq	Bat	This study
CP053615.1	China	Malayan pangolin	99%
CP046048.1	china	swine	99%
KJ937078.1	India	Soil	99%
CP053684.1	china	human	99%

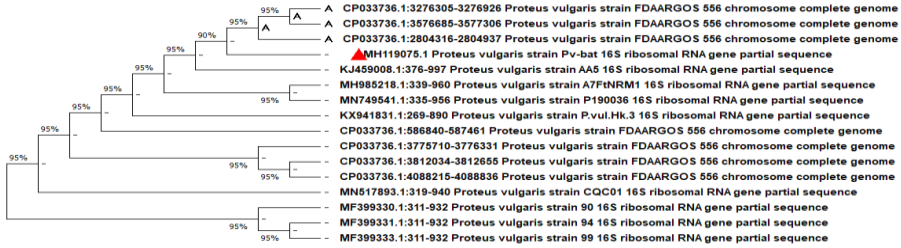

Figure (4): Phylogenetic tree of Proteus Vulgaris in Bat with world strains

Inferred Ancestral Sequences

Maximum Likelihood method [9] and Tamura-Nei model [10]. The tree shows a set of possible nucleotides (states) at each ancestral node ased on their inferred likelihood at site 1. The set of states at each node is ordered from most likely to least likely, excluding states with probabilities below 5%. The initial tree was inferred using the method. The rates among sites were treated as being uniform among sites (Uniform rates option). This analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 655 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [11], figure (4), the current study *proteus Vulgaris* isolates and their nucleotide-based similarglobal isolate.

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Accession number MH119075.1	country Iraq	source Bat	Identity This study
KJ459008.1	India	marine	99%
KX941831.1	India	river water	99%
CP033736.1	USA	Human	99%

Discussion

Insectivore species Myotisemarginated Bats are important reservoirs for many zoonotic pathogens. However, little research of bacterial pathogen in Bat in Iraq.Nowadays, molecular techniques provide proper tools to investigate the microorganisms in human and animals. The differences in the biochemical features do not exclude the strain from a genus as long as the similarities on the genome level are big enough. It is important to employ sequencing and phylogenetic tree in the identification of Proteus spp.Bacteria proteus spp. are known to be human opportunistic pathogen, from all clinical sources proteusspp [12] bacteria present in soil or water habitats as indicated of fecal pollution,posing a threat of poisoning when the contaminated water or seafood is consumed.Besides thus fact,cave-dwelling bats can transmit bacterial pathogen to human population and environment by drooping their feces in water, fruit and other ways [13].Sequencing and phylogenetic tree analysis show that proteusmiribilus with accession number MH119074.1 in insectivore species Myotisemarginated Bat was close related to the Human and many animal in china and USA suggest that genetic overlapping that bacteria can infect human and cause disease to the domestic animal ,while sequencing and phylogenetic tree of Proteus Vulgaris with Accession number MH119076.1 in Bat was close related to the Human with accession number CP033736.1 and many animal KJ459008.1this mean more relationship of strain of human with strains of cattle Bat and the zoonoticrelationship between them reveled the dangerous of proteusmiribilus and proteus vulgaris that effect human with food borne product [14] Study of wildlife showed that Proteus vulgaris was isolated from Varanasisalvation Lombok Island, Indonesia [15]. These bacteria are known to be human opportunistic pathogens, isolated from urine, wounds, and other clinical sources [16], thus, there is the risk of such fruit being consumedhealth of humans in riskimportant, as well as constant monitoring of these populations, mainly chiropteran, due to the risk of disseminating bacteria, which could contaminate the environment, changing common factors and unbalancing the host/parasite status

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