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Original Article

Isolation and Molecular Identification of Gram-negative Bacteria from The Leech *Limnatis paluda* in Iraq

Dur Kasim Hazim * [1], Luay Abdul-Qader Ali [1]

[1] *Department of Biology, College of Education, University of Salahaddin, Erbil 44001, Kurdistan Reign, Iraq.*

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ABSTRACT

Limnatis paluda is a sanguivorous, endoparasitic leech that has the ability to parasitize the mucus membranes of human and domestic animals. Leech has been used for many years to treat a wide range of diseases. However, Leeches' gut is known to harbor variable bacterial species which can cause moderate to severe infections. Thus, we aimed in this study to isolate and study Gram-negative bacteria from *L. paluda*. From September 2024 to July 2025, 53 leech samples were collected from Karokh mountain's water springs in Erbil province/ Iraq. Morphological identification was done and followed by a molecular study using COI gene. Subsequently, bacteria were isolated from three parts of the leech's body and Gram staining, biochemical tests and molecular analysis using 16S rRNA gene were used to identify the isolated bacteria. The results revealed the isolation of 21 Gram- negative bacterial isolate that comprised of 6 species: *Aeromonas hydrophila, Aeromonas veronii, Morganella morganii, Serratia sarumanii, Serratia ureilytica* and *Yokenella regensburgei.* It is worth mentioning that depending on NCBI, *Serratia sarumanii and Serratia ureilytica* were recorded for the first time in Iraq during this study. Moreover, *Yokenella regensburgei* was molecularly studied and identified using 16S rRNA for the first time in Iraq. Moreover, the results revealed that *Aeromonas veronii* was the predominant bacteria in the *L. paluda*. Furthermore, the highest percentage of Gram- negative bacteria was found in crushed buccal cavity swab 9(42.86%). Biofilm assay using microtiter plate method showed that 14(66.67%) were biofilm producers. Antibiotic susceptibility test was performed using 12 antimicrobial agents. The results showed that Amikacin, Ciprofloxacin, Imipenem and Netilmicin were the most effective antibiotics against all the isolates. However, all the isolates were 100% resistant to Ampicillin, Cefoxitin, Ceftazidime and Ertapenem.

*Keywords: Leech, **Limnatis** paluda, Gram-negative bacteria, COI gene, 16S rRNA gene.*

1 Introduction

Limnatis paluda (Tennent, 1859) is a species of jawed leeches, which has been classified under the order Arhynchobdellae (leeches lacking proboscis) that is further subdivided into the suborders Pharyngobdellae (worm-leeches) and Gnathobdellae (jawed leeches). Gnathobdellae members possess jaws lined with hundreds of small, sharp teeth. They are well-known sanguivorous that are able to consume huge amounts of blood [1,2]. Among the genus *Limnatis*, only three species were identified as *L. nilotica* (Savigny, 1822), *L. paluda* (Tennant, 1859) and L. *bacescui* (Manoleli, 1972) [3]. Moreover, *L. paluda* is known as an endo-parasitic leech species, due to its ability to infest the mucus membranes and cause a considerable bleeding of different body organs such as larynx, nasopharynx, pharynx, trachea, esophagus, bronchial tubes, and the female reproductive organs in both human and domestic mammals such as cattle, deer, camels, dogs, and horses [4,5]. Since ancient times and in various countries such as China, Egypt, Europe and India, leech have been used in many traditional therapeutic practices [6,7] known as leech-therapy which has been utilized in the treatment of various chronic and acute diseases, by utilizing the bioactive compounds that present in the leech's saliva [8], including Hirudin, Hirustatin, Eglin, Calin, Saratin, Apyrase, Hyaluronidase and Destabilase [9,10]. The function of these compounds is variable, which could be analgesic, anti-coagulant, anti-inflammatory, regulation of thrombin activity and inhibition of platelet aggregation [11]. Leech therapy has been used in post-reconstructive and plastic surgeries, also in the treatment of cardiovascular diseases, postphlebitic syndrome, deep vein thrombosis as well as chronic and acute otitis, tinnitus, relieving osteoarthritis pain and diabetes mellitus complications [12,13]. However, the clinical use of these organisms in hirudotherapy has been found to be associated with significant infectious complications caused by the commensal microbiota that colonize the leech's gut [14,15]. These bacteria, include mainly *Aeromonas* spp., *Morganella* spp. and

* Corresponding author
E-mail address dur.hazim@su.edu.krd (Instructor).
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Pseudomonas spp. that are known for their potential of causing several infections to the human being [16]. Notably, these bacteria have been found to be able to transmit to the human body via several mechanisms, such as passing these microorganisms at the bite site through the feeding process when the leech becomes in direct contact with the wound and secretes saliva; another route could be through regurgitating the digested blood while the leech is removed [17].

The current research was conducted for the purpose of morphological and molecular study of the leech *Limnatis paluda*. In addition, isolating and identifying Gram-negative bacteria from *L. paluda* in Erbil province/ Iraq, using conventional methods and confirmed by molecular identification using 16S rRNA gene. Furthermore, investigating the resistance patterns of Gram-negative bacterial isolates via antibiotic susceptibility test.

2 Materials and Methods

2. 1 Collection of leech samples

During the period from September 2024 to July 2025, 53 leech samples were collected from five water springs in Karokh mountain, located about 134 km from Erbil city. The samples were collected from under the rocks using tweezers and were placed directly in clean plastic jars containing spring water, which were then covered with a piece of porous cloth to maintain adequate aeration and prevent the escape of leeches. In the laboratory, the jars that contained the samples were kept in a good temperature (about 25 – 30 °C) and the water in the jars was changed daily with de-chlorinated water. After that, the collected leeches were primarily identified depending on their morphological characteristics after fixing them in 70% ethanol according to Mann, (2013) [18] and Utevsky et al. (2022) [4].

2. 2 Isolation of bacteria from the collected leech

In this study, bacteria were isolated from the following body parts of the leech: the body surface, buccal cavity, and the crushed buccal cavity. At first, the leech body was rinsed with sterilized double-distilled water and body surface samples were collected, where a sterile cotton swab was utilized to rub the ventral and dorsal body sides gently; while buccal samples were collected from the buccal cavity by using sterile, disposable loop. After that, for crushed buccal cavity sampling, the leech was submerged in 5% - 8% ethanol solution in order to be anesthetized then rinsed with sterile double-distilled water and the anterior part of leech (which contains the buccal cavity) was cut by using a sterile scalpel, homogenized in sterile normal saline. All swabs were immediately streaked on two sets of culture media for each sample; each set includes blood agar, nutrient agar, MacConkey agar and mannitol salt agar. Then one set was incubated aerobically at 37°C for 24 hours and the other set was incubated anaerobically at 37 °C for 48 hours. Following incubation, all the bacteria were sub-cultured on blood agar in order to obtain pure colonies to be used for further tests. Subsequently, all bacterial isolates underwent Gram staining which played a significant role in determining the upcoming biochemical tests. Moreover, cultural, morphological and some biochemical tests, including oxidase, catalase, urease, DNase, lipase and protease tests were performed as primary identification[19,20], then confirmed by molecular methods using PCR, sequencing of 16S rRNA gene as well as submitting to NCBI for acquiring the accession numbers.

2. 3 Biofilm assay

In order to detect the biofilm production from the isolated bacteria, the microtiter plate method was used as described by Manandhar et al. (2018) [21] with minor modifications. Briefly, 200µl of Brain- Heart Infusion broth supplemented with 5% sucrose was added to the wells of a 96- well flat bottom microtiter plate. Following this, 50µl of bacterial suspension was added to the wells and incubated at 37°C for 24 hours. Following incubation, the wells' contents were discarded and washed with phosphate-buffered saline (PBS) three times then stained with 1% crystal violate [22]. Optical density (OD) has been measured by using ELISA reader at OD 630nm.

2. 4 Antibiotic susceptibility test

Antibiotic susceptibility test was conducted on the bacterial isolates using the Disc Diffusion method (Kirby-Bauer method) as recommended by Clinical and Laboratory Standards Institution (CLSI) [23] in order to evaluate their resistance patterns. All bacterial isolates were tested against the following 12 antimicrobial agents: Amikacin (30 µg), Ampicillin (10µg), Cefoxitin (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg), Ertapenem (10 µg), Imipenem (10 µg), Netilmicin (30µg), Nitrofurantoin (300µg), Piperacillin (100µg), and Ticarcillin-clavulanate (75/10 µg).

2. 5 Molecular study

2. 5. 1 Molecular identification of leech

2. 5. 1.1 DNA extraction of leech

To extract the genomic DNA, the posterior sucker of the leech was cut into tiny pieces, mashed by using mortar and pestle and then 20 mg of its tissue was placed into Eppendorf tube for further steps. The genomic DNA extraction process was performed by using (Add bio, Korea) DNA extraction kit where the manufacturer's instructions were followed with a few modifications, where the procedure included: cell lysis, DNA binding, washing twice and elution.

2. 5. 1. 2 Primers

In the current study, the universal primers that have been used were provided by (Macrogen, Korea) as lyophilized form as shown in Table 1. Lyophilized primers were suspended in nuclease free water at a specific volume that was provided by the manufacturer to get 100 µM stock solution. After that, 10 µM was prepared as a working solution for the PCR reaction.

Table 1: Forward and reverse primers used in the sequencing of COI gene.

Primer code	Sequence 5'-3'	Amplicon size(bp)	Reference
Forward LCO1490	5'-GGTCAACAAATCATTAAAGATATTGG-3'	720bp	[24]
HCO2198 Reverse	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'		

2. 5. 1. 3 Leech Identification via COI gene amplification

Polymerase chain reaction (PCR) was used to amplify partial Cytochrome C Oxidase subunit one (COI) gene by using Bioresearch PTC-200 Gradient thermal cycler, where a total volume of 50 µl comprised of 25 µl Master mix, 1 µl of each primer forward (LCO1490) and reverse (HCO2198), 3 µl of the DNA template and finally the volume was adjusted to 50 µl by adding nuclease free water. The PCR protocol started with a primary denaturation step at 95°C for 5 min followed by forty cycles of amplification including denaturation at 95°C for 35 sec, annealing of primer at 59°C for 35 sec and then extension at 72°C for one minute. Subsequently, the final extension step at 72°C for 10 min.

2. 5. 1. 4 PCR products analysis utilizing Gel Electrophoresis

The PCR products were subjected to electrophoresis technique (Biobase, China) with DNA ladder (1000 bp), where 1% agarose gel was used to run the DNA fragments and visualized under UV trans-illuminator (Biobase, China) [25].

2. 5. 1. 5 Evaluation of DNA purity and concentration

DNA concentration and purity of the leech samples were measured by using Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, USA). The genomic DNA concentration varied between 5-25 ng/ µl, while the purity ratios were between 1.7 and 2.0.

2. 5. 1. 6 DNA sequencing, alignment and phylogenetic analysis

PCR products were subjected to (Macrogen, Korea) in order to be sequenced by Sanger sequencing method. The resulting sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) to achieve species- level identification, while a phylogenetic tree for the collected leeches was constructed by utilizing MEGA-11 to illustrate the evolutionary relation between the collected leeches.

2. 5. 2. Molecular identification of the isolated Gram-negative bacteria

2. 5. 2. 1 DNA extraction of the bacterial isolates

Firstly, all the tested bacterial isolates were inoculated in 5 ml Brain-Heart Infusion broth and incubated for 24 hours at 37°C; then DNA extraction process was accomplished using (Add bio, Korea) genomic extraction kit, where the provided leaflet instructions were applied with minor modifications.

2. 5. 2. 2 Primers used for PCR

In the current study, the universal primers that have been used were provided by (Macrogen, Korea) as lyophilized form as shown in Table 2. Lyophilized primers were suspended in nuclease free water at a specific volume that was provided by the manufacturer to get 100 µM stock solution. After that, 10 µM was prepared as a working solution for the PCR reaction.

Table 2: Forward and reverse primers used in the sequencing of 16S rRNA gene.

Target gene	Primer	Sequence 5'-3'	Amplicon size(bp)	References
16S rRNA	27F	5'- AGT TTG ATC MTG GCT CAG -3'	797bp	[26,27]
	805R	5'- GGA CTA CHA GGG TAT CTA AT-3'		

2. 5. 2. 3 Bacterial isolates identification through 16S rRNA gene

Polymerase chain reaction was used to amplify 16S rRNA gene by using Bioresearch PTC-200 Gradient thermal cycler, where a total volume of 25µl comprised of 12.5µl Master mix, 1 µl of each primer forward and reverse, 3 µl of the DNA template and finally the volume was adjusted to 25µl by adding nuclease free water. The PCR protocol started with a primary denaturation step at 94°C for 5 min followed by thirty-five cycles of amplification including denaturation at 94°C for 30 sec, annealing of primer at 55°C for 60 sec and then extension at 72°C for one minute. Subsequently, the final extension step at 72°C for 10 min [26, 27].

2. 5. 2. 4 Analysis of PCR products using Gel Electrophoresis

The PCR products were subjected to electrophoresis technique (Biobase, China) with DNA ladder (1000 bp), where 1% agarose gel was used to run the DNA fragments and visualized under UV trans-illuminator (Biobase, China) [25].

2. 5. 2. 5 Evaluation of DNA purity and concentration

DNA concentration and purity of bacteria were measured by using Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, USA). The genomic DNA concentration varied from 197 to 603 ng/ µl, while the purity ratios were between 1.7and 2.0 [28].

2. 5. 2. 6 DNA sequencing, alignment and phylogenetic analysis

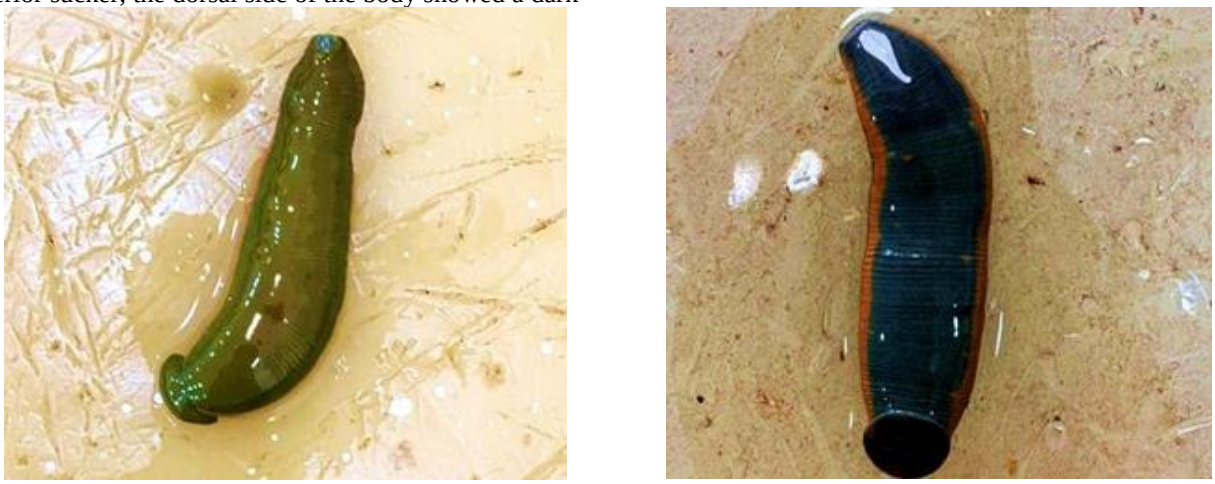
PCR products were subjected to Macrogen, Korea in order to be sequenced by Sanger sequencing method. The resulting sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) to achieve species- level identification, while a phylogenetic tree for the isolated bacteria was constructed by

utilizing MEGA-11 to illustrate the evolutionary relation between the isolated bacteria.

3 Results and Discussion

3. 1 Identification of the Collected Leech

The morphological examination of all leeches that were collected in this study indicated that they belong to the species *Limnatis paluda*, where our study showed that they have an extremely large posterior sucker, the dorsal side of the body showed a dark green color without any spots which allow to distinguish *L. paluda* from other species of the same genus; While the ventral side was almost black in color; In addition to the presence of two laterally orange lines along both body sides as illustrated in Figure 1. These findings were similar to the findings of Adawi et al. (2025) [29] and Farzali et al. (2025) [30] who reported *L. paluda* in Palestine and Azerbaijan respectively. However, Nakano et al. (2015) [31] mentioned that *L. paluda* has a reddish-brown dorsal color and this difference may be due to adaptation of the leech with its surrounding environment.



A B

Figure 1: (A) Dorsal view of *L. paluda*; (B): Ventral view of *L. paluda*.

However, to confirm the morphological identification of samples molecular analysis was accomplished by amplification of COI gene of *L. paluda*, where the primers that have used effectively amplified the target gene, giving PCR products of the expected size (720bp) as shown in Figure 2.

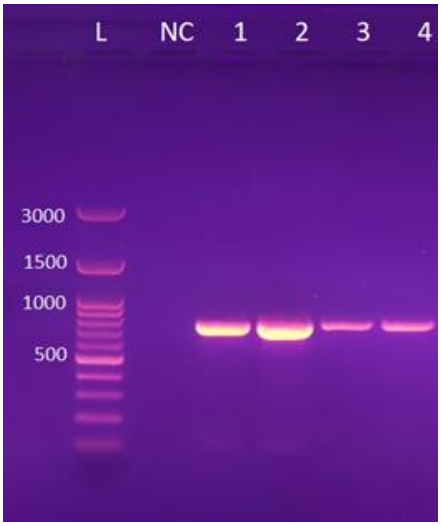


Figure 2: Agarose gel electrophoresis of PCR amplified partial COI gene of *L. paluda*. where L: ladder (3000-100), lanes from 1 to 4 displaying the amplified PCR products of about 720bp; while lane NC: negative control.

Subsequently, sequence similarity analysis was done by subjecting the 4 obtained sequences to BLAST within Genbank (http://blast.ncbi.nlm.nih.gov/). Following, submission for the 4 obtained sequences was done to Genbank as well as a specific accession number for each sequence was acquired as clarified in Table 3. The NCBI results confirmed the leech species of this study was *L. paluda*.

Table 3: The accession numbers of *Limnatis paluda* of this study.

The obtained leech	Source	Country	Accession number
Limnatis paluda	Karokh mountain water springs	Iraq/Erbil province	PX447715
			PX447716
			PX447718
			PX447719

For the phylogenetic study, the phylogenetic analysis program (MEGA 11) was used to conduct a Neighbor-Joining (NJ) phylogenetic tree depending on COI gene. Our sequences with the following accession numbers (PX447715, PX447716, PX447718, PX447719) were clustered tightly with *L. paluda* clade and supported by a bootstrap value of 100 which greatly confirm the species-level identification of our sequences as clear in Figure 3.

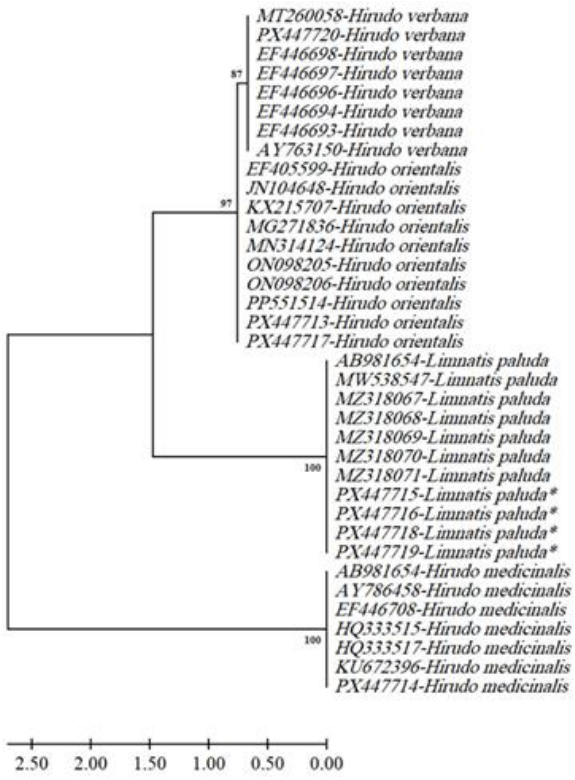


Figure 3: Phylogenetic tree for partial COI gene using Neighbor joining analysis within Mega 11 program.

3. 2 Identification of Gram-negative Bacterial Isolates

In this study, 21 bacterial isolates were obtained from 53 *L. paluda* (39.62%) and Gram staining procedure indicated that these 21 isolates were Gram-negative bacteria. Following, cultural and morphological characteristics of the colonies were studied and gave the first expectation of the bacterial types. Further, a series of biochemical and virulence enzymes were tested in order to identify the bacterial species as shown in Table 4. Catalase is known for its significant defending role in bacteria verses oxidative stress through splitting H_2O_2 into H_2O and O_2 [32,33]. The results of this study showed that all the 21(100%) isolates were notable for their ability to produce catalase enzyme. These findings matched the findings of Assouma et al. (2023) [34] and Ahmed et al. (2024) [35]. Moreover, the results had shown that out of 21 Gram-negative bacterial isolates 11(52.38%) were oxidase producers while 10(47.62%) were non oxidase producers, these results were nearly similar to the results of Hassan et al. (2017) [36]. Urease represents an important virulence enzyme in bacteria due to its role in establishment and persistence of bacteria in the hosts' tissue [37]. In this study, only 8(38.1%) showed positive results for urease, which had slightly matched with the results of Alam and Imran, (2018) [38]. In this study, it was found that among 21 bacterial isolate underwent DNase test, 13(62%) were DNase producers, which is significant due to crucial role of DNase enzyme in bacteria in degrading neutrophil extracellular traps (NET) which allow the bacteria to evade the host's immunity and spreading to other sites within the host [39]. Similarly to DNase, this study indicated that 13 (62%) isolates showed positive results for protease, which were similar to the study of Dienye et al. (2025) [40]. These results are important as protease plays a significant role in invasion by destroying host's extracellular proteins and immunity evasion by inactivating immunoglobulins [41]. Moreover, 12 (57.14%) of the bacterial isolates were lipase producers, which considered a crucial virulence enzyme; Since bacteria are using the lipase enzyme to degrade the lipids that found in the host's tissue in order to be used as nutrition source and enhancing colonization and pathogenicity [42].

Table 4: The results of biochemical tests of the bacterial isolates.

Gram-negative isolates	No.	Catalase No. (%)	Oxidase No. (%)	Urease No. (%)	DNase No. (%)	Protease No. (%)	Lipase No. (%)
Aeromonas hydrophila	2	2(100)	2(100)	0(0)	2(100)	2(100)	2(100)
Aeromonas veronii	9	9(100)	9(100)	0(0)	9(100)	9(100)	9(100)
Morganella morganii	7	7(100)	0(0)	7(100)	0(0)	0(0)	0(0)
Serratia sarumanii	1	1(100)	0(0)	0	1(100)	1(100)	0(0)
Serratia ureilytica	1	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)
Yokenella regensburgei	1	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)
Total	21	21(100)	11(52.38)	8(38.1)	13(62)	13(62)	12(57.14)

Furthermore, molecular analysis was performed to confirm the biochemical identification. The 16S ribosomal RNA gene sequencing is considered as the most significant identification technique for bacterial identification at the genus and species level due to its presences in various bacterial species [43,44]. During the current study, 16S rRNA gene was targeted and amplified by PCR, where the primers that have used effectively amplified the target gene, giving PCR products of the expected size (797bp) as shown in Figure 4.

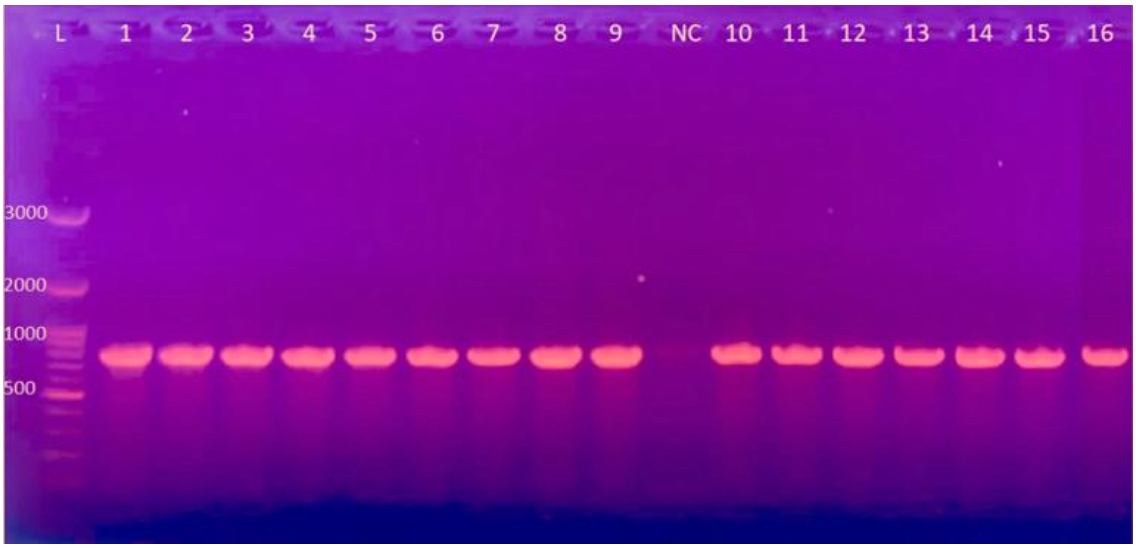


Figure 4: Agarose gel electrophoresis of PCR amplified 16S rRNA gene of the bacterial isolates; where L: ladder (3000-100), NC: negative control and the lanes from 1 to 16 displaying the amplified PCR products of around 797bp for isolated bacteria.

Subsequently, sequence similarity analysis was done by subjecting the obtained sequences to BLAST within Genbank (http://blast.ncbi.nlm.nih.gov/). Following, all obtained sequences were submitted to Genbank as well as a specific accession number for each sequence was acquired as shown in Table 5. During this study, 4 genera and 6 species of Gram-negative bacteria were identified as the following: *Aeromonas hydrophila, Aeromonas veronii, Morganella morganii, Serratia sarumanii, Serratia ureilytica* and *Yokenella regensburgei.* It is worthy to mention that depending on NCBI results the species of *Serratia sarumanii* and *Serratia ureilytica* were recorded for the first time in Iraq in this study. Moreover, *Yokenella regensburgei* was molecularly studied and identified using 16S rRNA for the first time in Iraq.

Table 5: The accession numbers of isolated Gram-negative bacteria of this study.

Isolated bacteria	Accession number
Aeromonas hydrophila	PX901910
Aeromonas veronii	PX901919
Aeromonas veronii	PX901920
Aeromonas veronii	PX901921
Aeromonas veronii	PX901923
Aeromonas veronii	PX901925
Aeromonas veronii	PX901927
Aeromonas veronii	PX901928
Morganella morganii	PX901903
Morganella morganii	PX901904
Morganella morganii	PX901905
Morganella morganii	PX901909
Morganella morganii	PX901899
Serratia sarumanii	PX901914
Serratia ureilytica	PX901916
Yokenella regensburgei	PX901906

For the purpose of demonstrating the molecular and phylogenetic evolutionary analyses. The phylogenetic analysis program MEGA 11 was utilized to conduct a Neighbor-Joining (NJ) phylogenetic tree depending on 16S rRNA gene as shown in Figure 5.

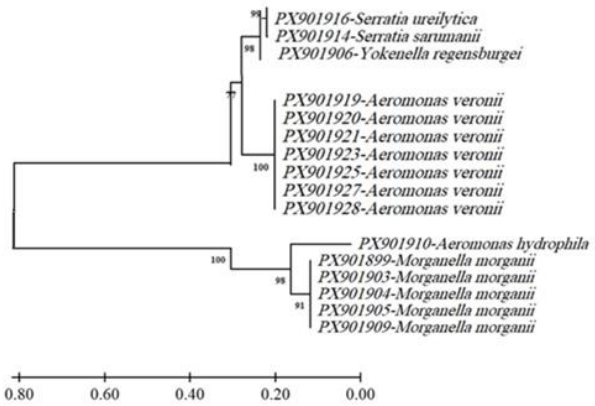


Figure 5: Phylogenetic tree of isolated Gram-negative bacteria using 16S rRNA gene.

3. 3 Prevalence of Gram-negative bacteria in *L. paluda*

In the current study, bacteria were isolated from the body surface, buccal cavity and crushed buccal cavity of the leech. The results revealed that out of 21 bacterial isolates, the most bacterial abundance was obtained from crushed buccal cavity swab with a proportion of 9(42.86%), followed by surface swab 8(38.1%) and the lowest abundance in buccal cavity swab 4(19%) as shown in Table 6. Additionally, it was found that *A. veronii* was the most prevalent species among the isolates with a proportion of 9 (42.86%). These findings interestingly aligned with many studies like Litwinowicz and Blaszkowska, (2014) [17] and Wagner and Ulrich, (2024) [45] that indicated *A. veronii* as the predominant bacterium in the leech gut. Moreover, *M. morganii* also presented high abundance rate 7 (33.33%); however, only few studies have isolated this bacterium from leech and some others have indicated it in low rates in leech Karasartova et al. (2025) [15]. Furthermore, other isolated species represented the following rates: *A. hydrophila* 2 (9.52%); *S. ureilytica, S. sarumanii* and *Y. regensburgei* species each with 1 (4.76%) isolate. Indeed, several factors such as leech's environment, source of the consumed blood and the fasting time of the leech are all affecting the abundance and variety of bacterial species among leech [15]. To the best of the authors' knowledge, it is worth mentioning that *Y. regensburgei* has isolated from leech gut for the first time, since no studies in the literature has indicated its isolation from leech before, moreover, all other isolated bacteria were reported for the first time from leech in Iraq.

Table 6: Prevalence of Gram-negative bacteria among *L. paluda* leech.

Gram-negative isolates	Gram-negative isolates No. (%)	Surface swab No. (%)	Buccal cavity swab No. (%)	Crushed buccal cavity swab No. (%)
Aeromonas hydrophila	2(9.52)	1(50)	-	1(50)
Aeromonas veronii	9(42.86)	4(44.44)	2(22.22)	3(33.33)
Morganella morganii	7(33.33)	2(28.57)	2(28.57)	3 (42.86)
Serratia sarumanii	1(4.76)	1(100)	-	-
Serratia ureilytica	1(4.76)	-	-	1(100)
Yokenella regensburgei	1(4.76)	-	-	1(100)
Total	21(100)	8(38.1)	4(19)	9(42.86)

3. 4 Biofilm Assay

The development of biofilm is considered an adaptive strategy for survival and growth that allows the bacteria to tolerate harsh environments in their host's body [46]. In this study, as shown in Table 7, the isolated bacteria showed various potentials of biofilm formation. Indeed, the results demonstrated that out of 21 bacterial isolates, 14(66.67%) showed the ability to form biofilm including weak and moderate biofilm with percentage 10(47.62%), 4(19.05%) respectively, while no strong biofilm producer were found and these results were similar to the results of Dumaru et al. (2019) [47]. Moreover, the isolated *A. hydrophila* and *A. veronii* were biofilm producers with a percentage of 100% and 55.56% respectively. These results agree with the findings of Igere et al. (2021) [48] who indicated that *Aeromonas* spp. are biofilm producers and it is further considered important in their pathogenicity [49]. Furthermore, *S. ureilytica* and *Y. regensburgei* were both 100% positive to biofilm assay, while 71.42% of *M. morganii* isolates were also found to be biofilm producers. However, *S. sarumanii* isolate was found to be negative to biofilm formation.

Table 7: Biofilm formation among Gram-negative isolates of this study.

Isolated bacteria	No. of isolates	Strong No. (%)	Moderate No. (%)	Weak No. (%)	Non No. (%)	Total of biofilm producers No. (%)
Aeromonas hydrophila	2	0(0)	0(0)	2(100)	0(0)	2(100)
Aeromonas veronii	9	0(0)	2(22.22)	3(33.33)	4(44.44)	5(55.56)
Morganella morganii	7	0(0)	2(28.57)	3(42.86)	2(28.57)	5(71.42)
Serratia sarumanii	1	0(0)	0(0)	0(0)	1(100)	0(0)
Serratia ureilytica	1	0(0)	0(0)	1(100)	0(0)	1(100)
Yokenella regensburgei	1	0(0)	0(0)	1(100)	0(0)	1(100)
Total	21	0(0)	4(19.05)	10(47.62)	7(33.33)	14(66.67)

3. 5 Antibiotic Susceptibility Test

In this study, antibiotic sensitivity for the 21 bacterial isolates was tested against twelve antibiotics by using Kirby-Bauer method. The results demonstrated variable resistance patterns as shown in Table 8. The infections that are caused by antimicrobial-resistant bacteria comprise a serious threat to human and animal health [50]. In the recent years, the antimicrobial resistance has increased due to the overuse of antibiotics in both human and aquaculture treatment [51]. This could be seen clearly in the results of this study, where all bacterial isolates showed a notable resistance with a percentage 100% against Ampicillin, Cefoxitin, Ceftazidime and Ertapenem. These results are nearly align with the results of Drk et al. (2023) [52] and Öztürk et al. (2025) [53] for *A. veronii and A. hydrophila*; Rahmati Holasoo et al. (2022) [54] for *M. morganii*; Ferreira et al. (2020) [55] and Awoderu and Omololu, (2021) [56] for *S. sarumanii and S.ureilytica*; Zhou et al. (2020) [57] for *Y. regensburgei*. Moreover, most of the bacterial isolates were resistant to Piperacillin and Ticarcillin-clavulanate with percentage of 95.24%.

On the other hand, Amikacin, Ciprofloxacin, Imipenem and Netilmicin showed the highest effective percentage 100% against all isolated bacteria. These findings matched with Shuang et al., (2020) [58] and Montalvo et al. (2025) [59] for *Aeromonas* spp., Zhu et al. (2025) [60] for *Serratia* spp., Aziz, (2015) [61] for *Y. regensburgei*. Furthermore, other antibiotics showed variable resistance percentages against the tested bacteria. For example, all bacterial isolates were susceptible to Nitrofurantoin except *A. veronii and A. hydrophila.* Similarly, all isolates showed susceptibility to Ceftriaxone a part of *S. sarumanii*.

Table 8: Resistance percentages among Gram-negative bacteria isolated from *L. paluda* leech.

Name of Antibiotic	Symbol	Bacterial isolates No. (%)						
		*A. hydrophila** n=2 No. (%)	*A. veronii** n=9 No. (%)	*M. morganii* n=7 No. (%)	*S. sarumanii* n=1 No. (%)	*S. ureilytica* n=1 No. (%)	*Y. regensburgei* n=1 No. (%)	Total 21 (%)
Amikacin	AK	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Ampicillin	AMP	2(100)	9(100)	7(100)	1(100)	1(100)	1(100)	21(100)
Cefoxitin	CX	2(100)	9(100)	7(100)	1(100)	1(100)	1(100)	21(100)
Ceftazidime	CAZ	2(100)	9(100)	7(100)	1(100)	1(100)	1(100)	21(100)
Ceftriaxone	CTR	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	1 (4.76)
Ciprofloxacin	CIP	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Ertapenem	ETP	2(100)	9(100)	7(100)	1(100)	1(100)	1(100)	21(100)
Imipenem	IPM	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Netilmicin	NET	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Nitrofurantoin	NIT	2(100)	9(100)	0(0)	0(0)	0(0)	0(0)	11(52.38)
Piperacillin	PI	2(100)	8(88.89)	7(100)	1(100)	1(100)	1(100)	20(95.24)
Ticarcillin-clavulanate	TCC	2(100)	8(88.89)	7(100)	1(100)	1(100)	1(100)	20(95.24)

*CLSI-M45[62] was used to interpret the results of *A. veronii* and *A. hydrophila* except for Netilmicin, Nitrofurantoin and Ticarcillin-clavulanate the breakpoints of Enterobacterales were used.

4 Conclusion

The current study indicated the presence of several Gram-negative bacterial species within the leech *L. paluda.* These bacterial isolates showed resistance to many antibiotics suggesting that they were multi-drug resistant bacteria which can be responsible for serious infections when being used for hirudotherapy. These outcomes highlight the importance and need of studying the microbiota of the other leech species that found in Iraq since hirudotherapy is popular there, and understanding the resistance patterns of the bacteria within these leech species in order to find solutions that help to get benefit from hirudotherapy and avoid its associated infections.

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Conflict of interests

The authors declare they have no conflict of interest.

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