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Molecular Diagnosis and Phylogenetic Tree of Histomonas Meleagridis in Iraq

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Abstract

THE CURRENT STUDY was aimed to diagnosis of Histomonas meleagridis by conventional polymerase chain reaction targeting the small sub unite ribosomal RNA gene 18S rRNA in cecal and liver samples . From September 2023 to the mid-January 2024, twenty five samples of naturally infected turkeys were collected from different areas of Hawija city in Kirkuk Province and Tikrit city and its suburbs in Saladin Province, Iraq. Cecal and liver samples were examined macroscopically and microscopically by direct smears using giemsa stain then, A conventional PCR test was used to confirm the diagnosis . PCR results demonstrated that 17 samples 68% from 25 turkeys samples was positive for parasite after gel electrophoresis and appearance of the DNA band with a size 209bp. The PCR product with the HISS primers was sent to Macrogen, Korea to know the genetic sequence of the target gene. The results demonstrated that only two out of 10 samples belonging to H. meleagridis . The results recorded in National Center for Biotechnology Information NCBI gene bank and for the first time in Iraq under accession No. PP669691 and PP669692 . we concluded that 18S rRNA gene proved to be highly effective in detecting the parasite, and genetic sequencing analysis identified the closest global isolates in France, Austria, and Poland.

Keywords: chitosan nanoparticles, biomedical, drug delivery, nanomaterials.

Introduction

Histomonas meleagridis is a protozoan parasite that live in anaerobic conditions [1]. It is the pathogen responsible for a disease known as histomoniasis, also referred to as enterohepatitis or blackhead, which is a fatal poultry disease that particularly affects turkeys [2]. This parasite was initially described as an Amoeba by Smith in 1895 and later renamed by Tyzzer in 1920 as *Histomonas meleagridis* [3].Morphologically the parasite can be exist in either flagellated form in ceca or amoeboid form in liver [4].

Turkeys can contract infections either through indirectly consuming *Heterakis gallinarum* eggs or directly from one bird to another through the cloaca without the presence of these nematodes [5]. Pathogenicity start when the parasite first invades the caecum, causing intense inflammation and tissue death. After damaging the intestinal tissue, the parasite can enter blood vessels and travel to the liver through the portal veins. Therefore, inflammation and damage can occur in the liver. In the last phase, the illness can turn systemic as the parasite moves to different organs of the body [6]. Turkeys are affected more severely, experiencing mortality rates as high as 100%. Common signs include lethargy, drooping wings and head, fluffed feathers, and yellow-colored feces [7]. The main methods for diagnosing histomonaisis were clinical signs, epidemiological data, and visible liver and caeca lesions, alongside with microscopic examination via direct smears and cultivation . In recent years, advances in molecular techniques like conventional PCR targeting the 18S rRNA gene have successfully been used for detecting *H. meleagridis* DNA in organ samples or feces [8].

Material and methods

Sample collection

From early of September 2023 to the mid-January 2024 25 samples of naturally infected turkeys were collected from different areas of Hawija city in Kirkuk Province and Tikrit city and its suburbs in Saladin Province, Iraq. The cecal and liver lesions were examined macro and microscopically at Parasitology Laboratory of the Faculty of Veterinary Medicine, University of Tikrit, then 20 mg of samples were taken and kept at -20 for DNA extraction and molecular diagnosis by conventional PCR.

Molecular examination

DNA isolation and PCR amplification

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DNA from liver and cecum samples was isolated according to AddPrep genomic DNA extraction kit from tissue mini Kit protocol (Add bio, Korea) . Amplification of *H. meleagridis* DNA was done using a species specific pair of primers HIS5F (5′-CCTTTAGATGCTCTGGGCTG-3′) and HIS5R (5′-CAGGGACGTATTCAACGTG- 3′) targeting 18 small sub unite ribosomal RNA18S rRNA genes [9].

Master Mix for all PCR was prepared using GeNetBio, Korea kit. The each reaction mixture consisted of 12.5 µl 2X Master Mix, 1 µl of each primer,8.5 µl PCR grade water and 2 µl DNA template, the additives were well mixed and distributed with a volume of 18 µL on small PCR tubes of 0.2 ml for the polymerase chain reaction procedure, then the DNA extracted from the samples with a size of 2 µL was added separately in the tube for each sample to make the total volume in each tube 20 µL. PCR was performed in Thermo cycler BioRad, USA as follows: primary denaturation at 94 °C for 10 min one cycle, followed by denaturation 35 cycles at 94 °C for 45 s , annealing at 55 °C for 45 s, extension at 72 °C for 60 s. Thereafter, the samples were maintained at 72 °C for 10 min for final extension step then cooled at 4°C. Amplification products 7 µl were analyzed by agarose gel 1.5 % electrophoresis after ethidium bromide staining and visualized under UV light using Gel Doc EZ Gel Documentation System, BioRad, USA.Fragment sizes were determined with reference to a 100 bp ladder GeneDirex H3, Korea (Addbio, Korea). The expected PCR product size was 209 bp. The PCR product with the HISS primers was sent to Macrogen, Korea to know the genetic sequence of the target gene in the study.

Phylogenetic analyses:

Phylogentic tree analysis of H. meleagridis using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings. Partial DNA sequences of partial fusion 18S small subunit ribosomal RNA gene were used as input data.

Results

Result of PCR technique

H.meleagridis DNA was detected by conventional PCR targeting 18S rRNA gene in liver and caecal samples, the result revealed that 17 samples 68% from 25 turkeys samples was positive for parasite after gel electrophoresis and appearance of the DNA packet with a size 209bp as shown in fig.(1) .

DNA sequence and Phylogenetic analyses

The PCR product with HISS primers was sent to Macrogen, Korea to know the genetic sequence of the target gene 18S rRNA for 10 samples. After that, the files of the genetic sequence were received in the form of a text file and then analyzed to find out the degree of genetic closeness Phylogenetic relationship. BLAST(Basic Local Alignment Search Tool) search algorithm was used to compare the obtained sequences with reference database obtained from NCBI genebank (http://www.ncbi.nlm.nih.gov/) as shown in figure (2) which indicates the identification of the query sample, *H. Meleagridis* , in alignment with NCBI GenBank . The results demonstrated that only two out of 10 samples belonging to *H. meleagridis* and given an accession number as shown in the table (1).

This study was conducted for the first time in Iraq and the isolates were recorded in NCBI genebank . The phylogenetic tree constructed from the analysis results is shown in the figure (3).

Discussion

H. Meleagridis is responsible for causing histomoniasis, commonly referred to as blackhead disease. The disease has emerged as a significant economic concern globally for poultry, particularly for turkeys and chickens, following the prohibition of effective feed additives and treatments in several nations [10].

Diagnosis of the parasite in poultry in labs has depended on microscopic examination, clinical signs, and cultivation. However, the similarity in symptoms to other bird diseases like coccidiosis, along with the morphological resemblance of the protozoan parasite to other protozoa such as Blastocystis sp. and *Tetratrichomonas gallinarum* pseudocysts under a microscope, as well as the presence of nonpathogenic microorganisms in poultry feces and cecum that can hinder the growth of *H. meleagridis*, have made culturing the parasite very difficult. These challenges have sparked interest in developing alternative diagnostic tools like PCR [11].

The current study was aimed to diagnosis of *H. meleagridis* by conventional PCR targeting the 18S rRNA gene in cecal and liver samples obtained from naturally infected turkeys , Cecal and liver samples were examined macroscopically and microscopically by direct smears using giemsa stain then, A conventional PCR test was used to confirm diagnosis. PCR results demonstrated that 17 samples 68% of turkeys samples were positive for the parasite after gel electrophoresis and appearance of the DNA band with a size 209bp .

Many studies used conventional PCR for detection of *H .meleagridis* DNA according to 18S rRNA gene in different samples for different reasons. The 18S rRNA gene is a useful molecular marker for researching systematic evolution, biogeography, and accurately identifying and classifying of *H. meleagridis*, with reliable results in multiple studies [12].In Iran a study investigated the frequency of *H. meleagridis* transmission in turkey groups and found

the parasite in fecal samples from both commercial and backyard turkey flocks using direct smears with giemsa stain and PCR test targeting 18S rRNAgene for diagnosis in a study conducted in Golestan, Mazandaran, Gilan, and Tehran provinces of Iran [13]. A study in India, aimed to determine the prevalence of histomoniasis in broiler chicken across various regions of Mizoram through microscopic analysis and c PCR targeting the 18S rRNA gene in samples from the ceca and liver [14]. Also, a study in Vietnam examined the frequency and genetic variations of *H. Meleagridis* strains in chickens in southern Vietnam through macroscopic diagnosis and PCR targeting the 18S rRNA gene in cecal and liver samples [15,16]. another study conducted in eastern China detected the parasite through clinical and histopathologic methods, and verified the diagnosis using conventional PCR targeting the 18S rRNA gene in cecal and liver chicken samples to investigate the prevalence and epidemic features of histomoniasis in chicken flocks [17,18]. In a study conducted in France, real time PCR was employed to detect the presence of *H.meleagridis* DNA in dust samples obtained from disease-free turkey flocks. Subsequent examination involved analyzing positive samples through conventional PCR and sequencing of the 18S rRNA gene [19,20]. A study conducted in Austria confirmed the efficiency of a vaccine by identifying the parasite in the caeca of turkeys through conventional PCR focused on the 18S rRNA gene [21,22].

Sequencing results of the current study in alignment with NCBI GenBank demonstrated that only two out of 10 samples belonging to *H. meleagridis* and given an accession number (PP669691 ,PP669692). The results identified 100% with AF293056 recorded in France which previously recorded in NCBI GenBank and documented the phylogenetic position of the trichomonad, *H. meleagridis* based on analysis of (Ss rRNAs) gene [23]. Also showed 99.49 % identical with EU647884 ,EU647885, EU647886, EU647887 recorded in France too based on SSU rRNAs gene sequencing which revealed that EU647886 and (EU647887) was belonging to genotype 1 and (EU647884 , EU647885 belong to genotype 2 according to NCBI gene bank sequencing [24] .The results also showed identity 99.49% with (AJ920323) recorded in Austria in a study conducted based on partial sequencing of the SS rRNA for detection the relatedness of *H.Meleagridis* to others protozoan parasites such as *Tetratrichomonas gallinarum* and a Blastocystis sp. [25]. Also 99.49% with (MZ956914) recorded in Poland by Bobrek ,K. and others according to NCBI gene bank (http://www.ncbi.nlm.nih.gov/) [26].

Conclusion

H. meleagridis was first detected in Iraq using conventional PCR targeting the 18S rRNA gene in turkey cecal and liver samples. The 18S rRNA gene proved to be highly effective in detecting the parasite, and genetic sequencing analysis identified the closest global isolates in France, Austria, and Poland.

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Conflict of interest

There are no conflicts of interest to be declared.

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Author contributions

Qudama abdullah obaid :Conceptualization, study design and sample collection.

Omaima Ibrahim Mahmood : Data analyses, Manuscript drafting, and Manuscript finalization:.

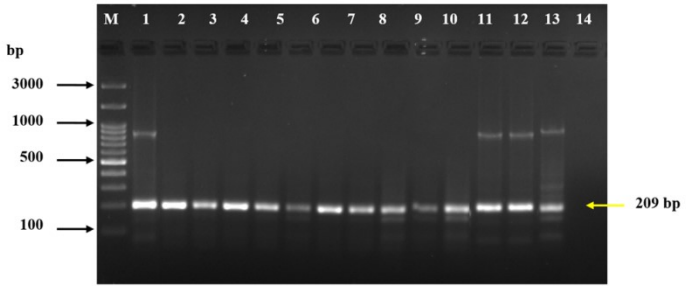

Fig. 1. Electrophoresis using agarose gel for PCR product for H. meleagridis. (M) represents the Marker indicator size 100 bp. Holes (1-14) represents positive samples and has a product volume of 209 bp, hole 15 represents negative control.

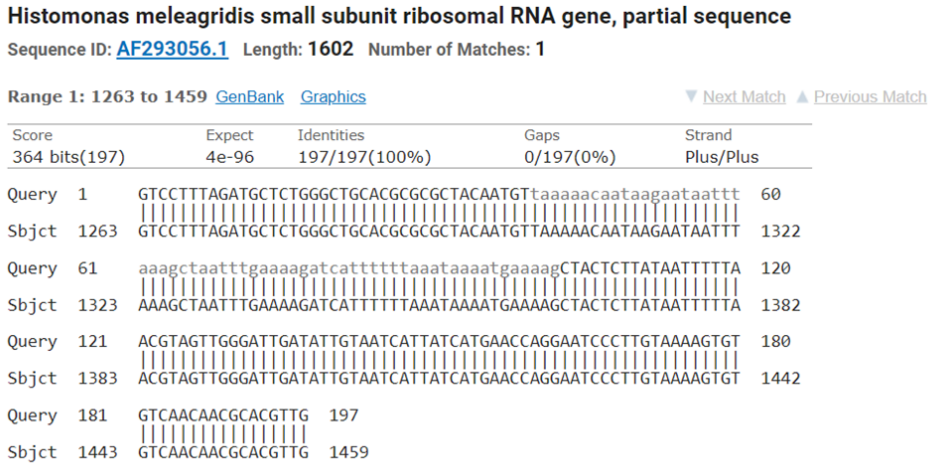

Fig. 2. Indicates the identification of the query sample, *H. meleagridis* , in alignment with NCBI GenBank.

TABLE 1. Percentage distribution of *H. meleagridis* based on (18S rRNA) gene according to blast in GenBank of NCBI.

Sample Accession Number	Identified	Query Cover %	Identic Number %	GenBank Accession Number	Country Identification
PP669691 PP669692	*Histomonas meleagridis*	100	100	AF293056	France
		100	99.49	EU647887	France
		100	99.49	EU647886	France
		100	99.49	EU647885	France
		100	99.49	EU647884	France
		100	99.49	AJ920323	AUSTRIA
		99	99.49	MZ956914	Poland

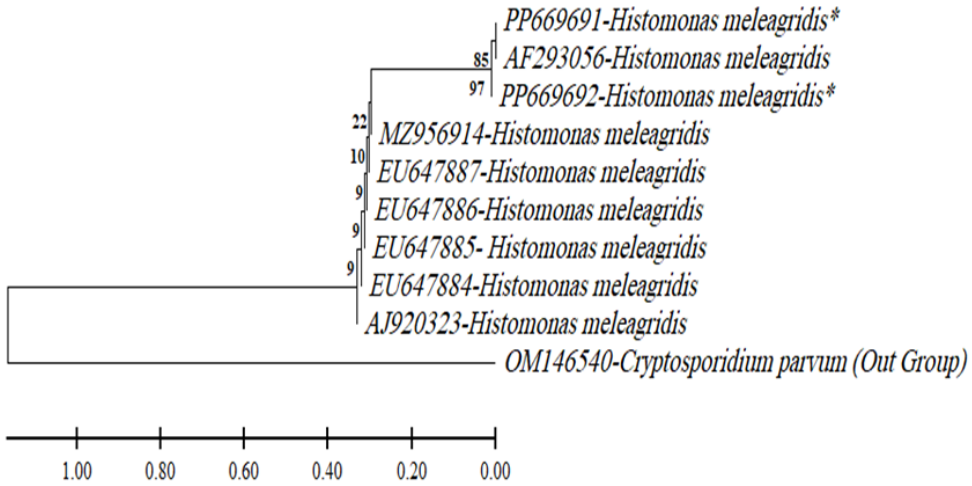

Fig. 3. Phylogenetic tree analysis of *H. meleagridis* using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings. Partial DNA sequences of partial fusion 18S rRNA gene were used as input data.

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التشخيص الجزيئي والشجرة الوراثية لطفيلي Histomonas meleagridis في العراق

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الملخص

منذ بداية شهر ايلول 2023 إلى منتصف كانون الثاني 2024 تم جمع 25 عينة من الديوك الرومية المصابة طبيعيا من مناطق مختلفة من مدينة الحويجة في محافظة كركوك ومدينة تكريت وضواحيها في محافظة صلاح الدين ، العراق. حيث هدفت الدراسة الحالية إلى تشخيص طفيلي H. meleagridis بواسطة تفاعل البلمرة المتسلسل التقليدي cPCR الذي يستهدف جين الحمض النووي الريبوزي الريبوسومي الصغير S rRNA 18في عينات الأعور والكبد . تم فحص هذه العينات عيانيا ومجهريا باستخدام صبغة كيمزا بطريقة المسحة المباشرة، ولتأكيد التشخيص تم استخدام تفاعل البلمرة المتسلسل التقليدي حيث أظهرت النتائج المتسلسل أن 17 عينة 68٪ من 25 عينة ديك رومي كانت إيجابية للطفيلي بعد الترحيل الكهربائي الهلامي وظهور شريط الحمض النووي بحجم . 209 bp بعدها إرسلت نتائج PCR مع بادئات HISS إلى شركة Macrogen في كوريا لمعرفة التسلسل الجيني للجين المستهدف حيث أظهرت النتائج أن اثنين فقط من أصل 10 عينات تعود إلى طفيلي H. meleagridis وتم تسجيل العزلات في بنك الجينات NCBI ولأول مرة في العراق تحت الرقم PP669691 و .PP669692 نستنتج من الدراسة الحالية ان الجين S rRNA 18 يظهر كفاءة عالية في تشخيص الطفيلي و ان تحليل التتابع الجيني قد حدد ان اقرب العزلات العالمية للطفيلي مسجلة في فرنسا ، النمسا وبولندا.

الكلمات الدالة : Histomonas meleagridis ، الديك الرومي، تفاعل البلمرة التقليدي، بادئ HISS ، التسلسل الجيني.