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Microscopic and molecular investigation of *Cryptosporidium* spp. in sheep in Nineveh governorate, Iraq

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Abstract

The research aimed to investigate *Cryptosporidium* spp. that infect sheep (different ages) by gathering 200 fecal samples from sheep in various regions of Nineveh province during the period from August 2024 to the end of February 2025.All samples were Examined by Microscopic methods and PCR technique followed by DNA sequencing tests and phylogenetic tree analysis to identify the species of parasite, which were then entered into the NCBI-GenBank database. This study was conducted for the first time in Nineveh province for identify *Cryptosporidium* species in sheep through PCR analysis were recorded in NCBI-GenBank database under accession numbers. The microscopic examination by using Modified Ziehl-Neelsen stain (MZN), showed that the total infection rate was 52 % (104/200), with the severity of infection with the *Cryptosporidium* spp. in sheep was 62.5%, while the percentage of mild infection in sheep was 37.5% of the total (104) examined samples. Three species of Cryptosporidium were identified in the results of DNA sequencing based the 18s rRNA gene. Which namely *C. parvum*, *C. hominis*, and *C. ubiquitum*, six strains were registered which received the registration number GenBank accession number. Furthermore, through analysing the genetic evolutionary tree of the isolated strains, confirmed the existence of a convergence between the isolated local strains and the rest of the strains recorded in the gene bank and isolated in other countries of the world.

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Introduction

Cryptosporidium is an intestinal protozoan parasite, accountable for cryptosporidiosis. Cryptosporidium species can spread a broad variety of hosts vertebrate, enclosing persons. An infection with Cryptosporidium is linked to serious, perhaps fatal illnesses in immunocompromised patients and among the primary reasons of diarrhea and death in kids less than five years (1,2). Because they can infect a variety of hosts (3). Some species, like *C. parvum*, characterized by lacking specificity of host. Because of their tremendous genetic variety, the later species actually exhibit significant adaptive skills (4). Different species of Cryptosporidium identified in sheep by molecular examination of fecal samples, there are *C. parvum*, *C. xiaoi*, *C. ubiquitum*, *C. fayeri*, *C. hominis*, and *C. andersoni* (5). Because animals can serve as reservoir and rigin of zoonotic cryptosporidiosis, the disease has become an issue for both public health and veterinary (6,7). Transmission of the Cryptosporidiosis via the consumption of oocysts from the infected Humans by through contaminated of food, water and pasture, Cryptosporidium infections indifferent animals and peoples, having significant importance for both public health and veterinary medicine. *C. parvum* is mostly responsibility for producing infections and dissemination via the pollution of drinking water on its surface (rivers, lakes, springs and streams) or oocysts - contaminated water for swimming which are the main infection sources among

animals and individuals (8-11). Most diarrheal infections have the same cause in both peoples and animals in Cryptosporidium infection. It is held responsible for 60% of documented outbreaks of water-borne and presents a serious concern to drinking water in developed country populations (12). The significance of cryptosporidiosis in farm animals, particularly sheep, is that it causes diarrhea solely from this parasite and can also coexist with other infections that impact the animals' health and productivity. Diarrhea is the main clinical indicator of infection in sheep, although other symptoms include dehydration, cramps, abdominal pain, sadness, listlessness, unthriftiness, and weight loss. As a result, morbidity and mortality rates are high worldwide (13,14). Sheep are considered to be a principal source of infection with Cryptosporidium, either directly or by environmental contamination, and they may transmit the zoonotic Cryptosporidium spp. that causes clinical symptoms in humans (15,16). Different techniques were used in an investigate of Cryptosporidium. Morphological identification with a 100× lens yields a direct microscopic method. It employs a variety of staining methods, including auramine phenol and cold or hot modified Ziehl-Neelsen, to identify oocysts that contains four sporozoites (17). Although the microscopical technique is an excellent instrument as well as very cost-effective, it needs information and skilled diagnostic staff to reduce false-positive outcomes and therefore, it may take a lot of time. In addition, the microscopical examination unable to detected parasites species, by using the traditional microscopic technique (modified Ziehl-Neelsen staining), Al-Zubaidi (18) found the infection rate with Cryptosporidium 37.77%. However, using the PCR analysis, the infection rate is recorded 48. 88% of cases with highest sensitivity in sheep in Baghdad city.

The variations in infection rats were attributable to a number of components, including systems of management and techniques of rearing, unaltered factors of risk, including aged, conditions in the environment, and animal breed, and altered variables of risk, such as technique of sampling and Methods for detection. Thus, purpose of this scientific research to evaluation the detection of *Cryptosporidium* spp in sheep by examination of fecal samples which are collected from various regions of Nineveh Province, Iraq. Utilizing microscopic examination and molecular techniques, using 18S rRNA sequences, it also seeks to record the phylogenetic diversity of Cryptosporidium across species.

Materials and methods

Ethical approval

The study was done in acceptance with the rules of ethics established by Institutional Animal Care and Use Committee at Mosul University, College of Veterinary Medicine under at number of approved animals: 250 at 9/7/2024.

Study design

Current study was done at sheep with and without diarrhea in different regions in Nineveh Province -Iraq from the beginning of August 2024 to the end of February 2025 of both genders, with various ages.

Collection of fecal samples

Two hundred Samples of feces were taken from sheep in various regions in Nineveh Province -Iraq. Each sample was placed in a cleaned plastics container, numbered on the label and the day of samples collection, then transported in cooling box, to the parasitology laboratory for examination.

Microscopic examination

Direct method and Modified Ziehl-Neelsen stain was done (19-21). And the oocysts which were measured by using an ocular micrometer. The severity of the infection was determined by examining fecal smears by different methods. The oocysts were counted by light microscopy under 100X magnification. If 5 or more oocysts were found in a single microscopic field, the infection was considered heavy. If 1-4 oocysts were found in a single microscopic field, the infection was considered mild. The examination was carried out on more than 40 observations in different microscopic fields (22). All fecal samples were positive by microscopical examination have been kept at -20ºC for later DNA extraction.

Molecular examination and processing

Sixty-five positive fecal samples were severing infection by microscopic examination were used for molecular detection of Cryptospordium using PCR technique. The conventional PCR process was accomplished for investigate species of Cryptosporidium, according to18s ribosomal rRNA gene in sheep fecal samples by multiple steps.

DNA extraction from fecal samples

The fecal samples underwent extraction of nucleic acid by using DNA extraction kit provided with company Geneaid from Bioneer-Corporation, Korea (Accu-Prep®stool-DNA Extraction kit). It was carried out as follows: 200 µl the parasite precipitate transferred in to an Eppendorf tube 1.5 ml, 200 µl were included in Eppendorf tube containing the Lysozyme enzyme at a concentrating of 0.8 mg/200 ml, and using Vortex the mixing was carried out. Then tube was incubation at 37°C for about Half an hour, during period of incubation the tube was inverted each three minutes. Then add 20 µl Proteinase K and mix by Vortex. At 60°C incubate the mixture was incubation for ten minutes. Add 200 µl GB buffer solution and mixing by using Vortex then incubate at 70°C. Add 200 µl absolute ethanol then mix by hand, and transport the mix to the GD tube fixed in collecting tube then discard for 30seconds at 16000g and throw the filter fluid, then add 600 µl of the solution for washing and discard at identical speed and time as mention

above and discard the precipitate, then centrifuge again for 3 minutes in order to eliminate all remnants of the solution for washing. Transfer GD column to a clean tube 1.5 ml, add 100 µl of dissolution fluid, quit for three minutes, then discard for 30 seconds at 16000 g, then Store the DNA until you need it.

Agarose gel preparation and DNA electrophoresis

In order to Prepare of 1% agarose gel. By dissolved 0.5 gram of agarose grind in 50 ml of X1 TBE (Tris/Borate/EDTA, is a buffer solution) and safe red dye 3 microliters were adding. This carried out with continuous stirring over a heat source up until it boils and let cooling at temperature between 50 and 60 degrees Celsius, after that pour the gel fluid into the container of the transfer apparatus after getting the comb installed to create the gel's outer wells at the gel's edges, Considering that the pouring needs to be completed gently to Prevent bubbles from forming, and if they are present, they are eliminated by using a pipette, then the gel is remains for solidifies. Then the container is positioned in electrophoresis containing a suitable quantity of X1 TBE solution, then the comb is raised carefully. The samples of immigration were preparation by mixing 5 µl of the DNA sample with 3 µl of the solution for loading. After that migration apparatus was worked for 2 - 1.5 hours via moving by A current of electricity with a differential voltage of 5volts per centimeter. Then gel was scanned under UV light using a Gel Records instrument to be prepared to view the DNA bands and also results of PCR reaction.

PCR technique

Detection of amplified region by addition 4 µl template DNA and 1 µl of every gene -specific primer adding to materials of the Master mix (Table 1) utilizing PCR amplification and electrophoresis (23,24). Later all reaction tubes were placed through the process thermocycler to carry out the multiplication reaction utilizing the specific reactions program (Table 2).

Table 1: Primers for *Cryptosporidium* spp. using 18s rRNA

primer	Sequence
Cry-F	TGGCACCAGAATCAGCTGAA
Cry-R	GACAGGTTGAGTTGGAGCAGA

Table 2: PCR program

Phase	ºC	Period	Cycle (n)
Initial denaturation	95	6 min.	1
denaturation	95	1.30 min.	
Annealing	56	1.30 min.	35
Extension	72	2 min.	
Final extension	72	5 min.	1

DNA sequence analysis

Following some positive amplification of conventional PCR results (320 bp), the DNA sequencing procedure was accomplished, later these samples were sent to Korea to carry out DNA sequencing and evaluation of phylogenetic tree utilizing (MegaX version) as well as accounted of the distances in evolution. Then determined isolates of Cryptosporidium species were transferred in to the NCBI-GenBank to recorded the accession no.

Results

Microscopic results

In This study results showed the rate of Cryptosporidium infection according to the microscopical examination was 52% (104/200) based on its Modified Ziehl-Neelsen stain, the size 4-6 µm of the oocysts (Figure 1). This investigation revealed the severity infection rate with Cryptosporidium spp. in sheep was 62.5%, while the percentage of infection with mild infection in sheep was 37.5% of the total 104 samples. The incidence of severe infection varied, reaching more than 5 oocysts per microscopic field, while in mild infection the number of oocysts reached 1-4 oocysts per microscopic field.

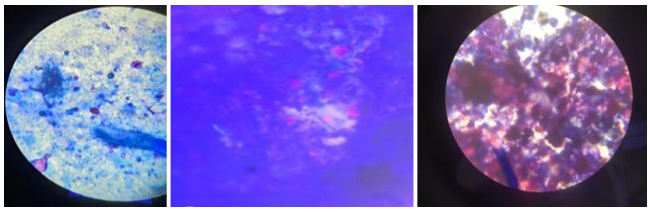

Figure 1: Oocysts of Cryptosporidium, MZN stain, 100X.

Results of molecular examination

Results of molecular examination revealed that all the samples (65 samples) which were sever infection by microscopical examination and All samples showed positive by PCR reaction. The results of DNA sequencing were done to confirmed results of conventional PCR for detection of *Cryptosporidium* spp. according 18S-ribosmal RNA gene. Where, the nucleotides sequence was examined through NCBI (BLAST analysis data base) that in line with the nucleotide inquiry with 18s ribosomal RNA, gene of Cryptosporidium species that include *C. parvum*, *C. hominis*, *C. ubiquitum* (Figures 2-4). Six strains were registered under the name RFMN-2024, which received the registration number Gen Bank accession number (Table 3).

Table 3: Local gene sequences of Cryptosporidium in 12 positive reaction products of PCR technology

Name of isolate	Nucleotides size	GenBank number	Isolation repetitions (n)
Cryptosporidium ubiquitum strain RFMN1-2024	393 bp	PQ836187.1	1
Cryptosporidium parvum strain RFMN2-2024	350 bp	PQ836188.1	3
Cryptosporidium parvum strain RFMN3-2024	386 bp	PQ836189.1	3
Cryptosporidium hominis strain RFMN4-2024	380 bp	PQ836190.1	2
Cryptosporidium hominis strain RFMN5-2024	467 bp	PQ836191.1	2
Cryptosporidium ubiquitum strain RFMN6-2024	378 bp	PQ836192.1	1

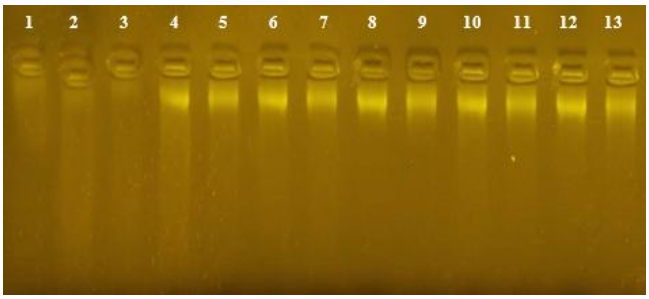

Figure 2: DNA extraction and agarose gel electrophoresis.

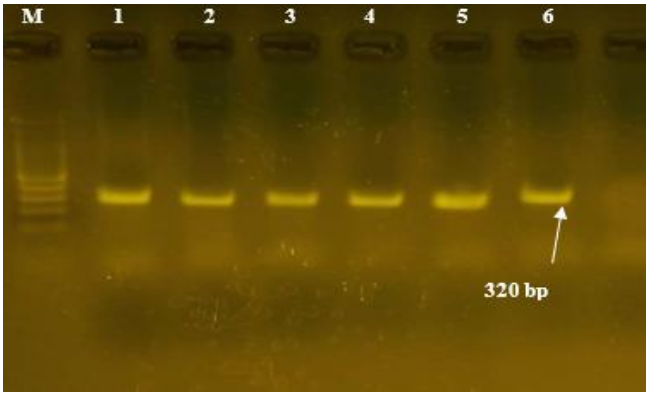

Figure 3: Cryptosporidium parasite diagnostic wells (1-6) with molecular weight 320 pb.

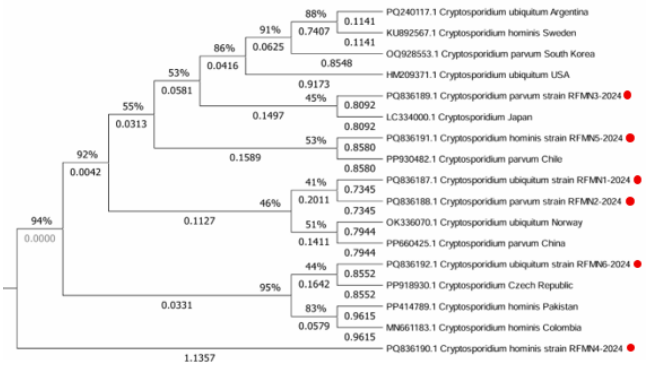

Figure 4: The Phylogenetic tree plotted from the 18SrRNA gene of the *Cryptosporidium* spp. isolates (local Cryptosporidium species referred as red circular).

Discussion

Results of present research recorded total rate of infection with *Cryptosporidium* spp. was 52% according the microscopical examination of sheep fecal samples which were obtained from various regions of Ninevah Governorate. Results are closely with previous studies in different cities in the world. The rate of infection was 3.8% in Turkey (25), and in Nigeria recorded rate 22.7% in sheep (26). In China recorded rates 1.8-6.8% in sheep farms (27). In Bangladesh recorded rate 11.3% (28), but in Iraq recorded high rate of infection with *Cryptosporidium* spp. oocysts 64.37% (56/87) (29) and rate 35.12% (22) were found in lambs in Mosul city. Out of one hundred examined sheep fecal samples, 51 (51%) were positive results to *Cryptosporidium* spp. in Al-Qadisiyah province (30). The cause of these variations in rats of infection belong to many components; such as amount of fecal sample, conditions for breeding, diagnostic procedure may which has significantly affected the infection rate. Widespread of Cryptosporidium in most regions of the world and in various hosts is due to the fact that oocysts of Cryptosporidium are passed on through directly contact with fecal infected persons (Transmission from person to person) or by fecal animals (zoonotic spread), as well as by the consuming of food contaminated (transmission of foodborne disease) and water contaminated (water-borne infection) (31,32).

Current study found the severity of infection with the *Cryptosporidium* spp. in sheep was 62.5%, however the rate of mild infection in sheep was 37.5%. This result was agreement with Hassan (22) who pointed out the percentage of lambs infected with severe infection was 36.11% while mild infection was 63.88% in lambs in Mosul city. The variation in infection rates may be due to several factors, including the number of animals examined, the examination method, the age of the animals, nutritional quality, animal immunity and the breeding system (30).

Results of Molecular examination revealed that all the samples (65 samples) which are sever infection based on microscopical examination, the results showed all samples were positive by PCR reaction based on 18S-ribosmal RNA gene (33). The findings indicate the detected of Cryptosporidium by PCR and identifying the species according sequence analysis, recorded 3 species of Cryptosporidium can be infected sheep *C. parvum*, *C,*

hominis, *C. ubiquitum*. However, previous studies in Iraq (34) were identified 4 species *C. parvum*, *C. hominis*, *C. ubiquitum* and *C. andersoni* according to sequence examination of the heat shock protein 70 (Hsp70) in sheep of Babylon province, Iraq. Hussein and Khadim (23) isolated three species of Cryptosporidium there are *C. hominis*, *C. parvum*, and *C. bovis* from sheep in Wasit Province, Iraq.

Most scientific studies showed the high incidence of infection with *C. parvum* in contrast to other species of parasite could be explained that *C. parvum* is not specific to a host, and it is the most common species in other animals and the second common species in humans, behind *C. hominis* (35). By Gene sequencing recorded six new strains in Gen Bank and the reference squads to examine the levels of complete identity and overall score for similarity of the 18s ribosomal RNA gene of cryptosporidium spp. This was compared to other isolates and typically affected sheep. Local species Cryptosporidium that had been recorded in the GenBank database in named *Cryptosporidium ubiquitum* strain RFMN1-2024 in accession no. PQ836187.1were displayed as closed in relation to the reference. *C. parvum* isolates in named *Cryptosporidium parvum* strain RFMN2-2024 in accession no. PQ836188.1 the total percent identity score 41%. The strain under named *Cryptosporidium parvum* strain RFMN3-2024 in accession no. PQ836189.1 were showed closed related to strain in Japan at total percent identity score 45%. The strain *Cryptosporidium hominis* strain RFMN5-2024 in accession no. PQ836191.1 were showed closed related to strain in Chile at total percent identity score 53%. The study utilized the 18S rRNA gene for genetic investigation and phylogenetic evaluation the species of *Cryptosporidium* spp in Iraq (23,33). In Eastern region of Saudi Arabia (36,37). In Egypt (38). In Jordan (39). Future studies on Cryptosporidiosis in sheep should be focused on species which is a zoonotic aspect, aligning with previous studies (40).

Conclusion

This study demonstration the Cryptosporidium parasite in fecal samples of sheep by usig microscopical examination and conventional PCR in Mosul city, Iraq. The Microscopical examination of fecal samples for detection of Cryptosporidium should be accompanied by PCR and Gene sequences to get a precise diagnosis of infection with species of *Cryptosporidium* spp. based on fecal samples examination.

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Conflict of interest

Authors of this manuscript affirm that They don't have any conflicts of interest.

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الكشف المجهري والجزيئي لطفيلي الأبواغ الخبيئة في الأغنام في محافظة نينوى، العراق

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[١]طبيبة بيطرية، قطاع خاص، [٢]فرع الأحياء المجهرية، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

صُممت هذه الدراسة للكشف عن تواجد من طفيلي الأبواغ الخبيئة الذي يصيب الأغنام (مختلف الأعمار). جُمعت ٢٠٠ عينة براز من الأغنام من مناطق متفرقة في محافظة نينوى خلال الفترة من شهر أب ٢٠٢٤ إلى نهاية شباط ٢٠٢٥. فُحصت جميع العينات بالطرق المجهرية وتقنية تفاعل البلمرة المتسلسل متبوعة بتحليل تسلسل الحمض النووي من خلال الشجرة الوراثية لمعرفة أنواع طفيلي الأبواغ الخبيئة، وسُجلت الأنواع المحددة في قاعدة بيانات بنك الجينات. وتعتبر هذه الدراسة الأولى في محافظة نينوى في تشخيص أنواع طفيلي الأبواغ الخبيئة في الأغنام بواسطة تفاعل البلمرة المتسلسل وسجلت الأنواع في قاعدة البيا نات في بنك الجينات وبأرقام محددة. أظهرت نتائج الفحص المجهري باستخدام صبغة زيل نلسن المحورة، أن نسبة الإصابة الكلية بلغت ٥٢% (١٠٤/٢٠٠)، وبلغت شدة الإصابة بطفيلي الأبواغ الخبيئة في الأغنام نسبة ٦٢,٥%، بينما بلغت نسبة الإصابة المتوسطة في الأغنام ٣٧,٥% من إجمالي العينات المفحوصة (١٠٤). أظهرت نتائج تسلسل الحمض النووي باستخدام جين ١٨ وجود ثلاثة أنواع من الأبواغ الخبيئة، وهي *C. parvum* و *C. hominis* و *C. ubiquitum*. وقد سُجلت ست عزلات وحملت رقم التسجيل في بنك الجينات. كما أكد تحليل الشجرة التطورية الوراثية للسلالات المعزولة وجود تقارب بين السلالات المحلية للطفيلي المعزولة مع بقية السلالات المسجلة في بنك الجينات والمعزولة في دول أخرى حول العالم.