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Occurrence and Molecular Identification of Zoonotic Cryptosporidium Species in Fish in Mosul City, Iraq

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Abstract

Cryptosporidium is a protozoan parasite infecting human and a wide range of animals causing severe diarrhea. A total of 200 fresh fish samples belonging to five species (*Arabibarbus grypus*, *Cyprinus carpio*, *Mesopotamichthys sharpeyi*, *Chondrostoma regium and Luciobarbus xanthopterus*) were collected from local markets for the period from August 2023 - February 2024. Total infection rate of Cryptosporidium spp. was (53/200) (26.5%). Highest infection rate recorded in October (34.28%), and the lowest was in February (14.28%). Scraping smears from stomach and intestines stained with Ziehl Neelsen (mZN), oocysts of Cryptosporidium appeared spheroidal or ovoidal with diameter ranged from 4.6 - 5.5 X 3.8 - 4.7µm. Nested PCR and sequence analysis of four samples using 18SrRNA gene showed positive for both species under accession numbers PP593584 for *C. parvum* and three isolates PP593585, PP593586 and PP593587 for *C. hominis* were 100% identical to the same species in other studies in Iraq and other countries according to blast in GenBank of NCBI. Nested PCR is considered a good tool for species identification and conformation of microscopical results. This is the first study in Iraq proved that fish became a new source for transmission of both species to human and livestock.

Keywords: *C. parvum*, *C. hominis*, fish, nested PCR, occurrence, phylogenetic analysis.

Introduction

Fish production increased remarkably with annual fish consumption of about 18.1 kg per person [1]. Fish are important for human food and therefore considered important for the economy of various countries in the world and are considered a key source of proteins [2]. Fish like other animals could be infected with various diseased including parasitic infection such as protozoa which affect the quality of fish products which negatively affecting economic industry led to reduction in growth reaches up to 10% and with hatcheries losses (20%) [3]. Cryptosporidium is an intracellular protozoan worldwide spread belonging to the Apicomplexa, Cryptosporidae infect microvillus of gastrointestinal epithelium of human, birds, reptiles and fish [4]. Infections with Cryptosporidium causes large economic losses and zoonotic implications [5]. Cryptosporidiosis is foodborne and waterborne disease where water is considered the major course for Cryptosporidium transmission [6]. Until now, more than 25 novel Cryptosporidium genotypes have been identified in fish and more common species are *C. molnari* [7], *C. huwi* [8] and *C. scophthalmi* [9, 10], while *C. parvum* and *C. hominis* infect human [4]. In addition to other species identified in other animals like *C. parvum*, *C. scrofarum*, *C. hominis* and *C. xiaoi*, which have also been identified in fish. Other 15 Cryptosporidium fish genotypes, and one Cryptosporidium genotype in rat, have been identified [4, 11, 12]. Both species *C. parvum* and *C. hominis* are known as zoonotic pathogens of public health importance even in the developing countries with good sanitary infrastructures, but frequent with food and water-borne parasitic infections [13]. Species of Cryptosporidium are found either in the intestine or stomach of fish causing pathological effects and increase the mortality mainly in juvenile fish [9].

The aims of the present study are to perform microscopical investigation of Cryptosporidium spp and molecular analysis, sequencing and phylogenetic analysis of 18S rRNA for Cryptosporidium species.

Material and Methods

Sample collection

A total of 200 fresh fish samples belonging to five species (*Arabibarbus grypus*, *Cyprinus carpio*, *Mesopotamichthys sharpeyi*, *Chondrostoma regium* and *Luciobarbus xanthopterus*) were collected from local markets for the period from August 2023 - February 2024.

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Microscopic identification

After dissecting of fish, stomach and intestines were isolated and put in a petri dish and divided into two parts, part one was examined by direct smear by scraping stomach and intestine mucosa then kept in methanol and stained with mZN according to [14] and examined microscopically for identification of Cryptosporidium oocysts under light microscope 100X, measurements were done with and eyepiece micrometre. While the second part was minced and kept in -20C° for DNA extraction and molecular analysis using nested PCR [11, 15].

DNA extraction

Genomic DNA was extracted from fish intestine samples according to the instructions for the extraction kit (AddPrep genomic DNA extraction kit from tissue mini-Kit Addbio, Korea).

Nested Polymerase Chain Reaction (PCR)

The region of 18S rRNA was amplified by using primer as [12] (Table 1) the PCR mixture was prepared using Addbio (Korea) kit; by measuring the sizes needed for the reaction elements for each specimen as shown in (Table 2), each reaction used a pair of particular primers representing a specific gene to be identified. The extracts were well mixed and distributed in small PCR tubes size 0.2 ml for the PCR and then the DNA extracted from the samples with a volume of 2 µl added discretely in the tube of each sample so that total volumes in each tube became 20 µl.

Amplification was performed using Bio-Rad thermocycler (Bio-Rad, USA) as follows: one cycle at 95°C /10 minutes, 35 cycles at 95°C / 45 seconds, 67°C / 45 seconds, and 72°C /45 seconds. Final extension, one cycle at 72°C/ 7 minutes was set. Finally, the reactions were cooled at 4°C until proceeding to the gel electrophoresis. The amplified products were verified in 1.5 % agarose gel prepared with 1x Tris-Borate-EDTA buffer and stained with a red safe DNA staining solution (GeNetBio, South Korea). Results were visualized using UV transilluminator and digital camera (Bio-Rad, USA). In all electrophoresis performed, DNA molecular weight marker 100bp (AddBio Inc., South Korea) was presented. The presence of specific amplified DNA fragment with 784 and 588 bp indicating a positive result for *Cryptosporidium*.

DNA sequencing

The PCR products were sent to Macrogen, Korea to find out the genetic sequence of the target gene18S rRNA. Genetic sequence was analyzed to find out the degree of genetic affinity and phylogenetic relationship.

Statistical analysis

The percentages of data in this study were calculated and confirmed significant differences using the chi-square test, and in the event of significant differences between the groups, the Bonferroni correction test was used to stabilize the positions of these differences, all tests were conducted using the program (IBM spss v27, UK) at a significant value $p<0.05$ [16].

Results

The morphological identification of intestine scraping samples by stomach and intestine scraping revealed the presence of *Cryptosporidium spp*. in (53/200) with total infection rate (26.5%). The results in (Table 3) show infection rate with Cryptosporidium according to the months of the study. There are no statistically significant differences between the infection rates during the months of August to February at $p \leq 0.05$. The infection rates ranged between (34.28%) and (14.28%), where the highest percentage of infection was in October (34.28%), and the lowest in February (14.28%).

Through the morphological identification by scraping smear from stomach and intestine stained with mZN stain; the results revealed that oocysts of Cryptosporidium appeared spheroidal or ovoidal with diameter ranged from 4.6 - 5.5 X 3.8 - 4.7µm. as in (Fig.).

The results of molecular analysis for *Cryptosporidium spp.* identification. Using the external initiator JerExtF and JerExtR. The amplified product was 784 bp.as shown in (Fig.2). While the internal initiator JerExtF and JerExtR, the amplified product was 588 bp.as shown in (Fig.3).

The PCR sequencing to four scraped intestine and stomach samples of infected fish showed positive PCR of Cryptosporidium identified were *C. parvum* and *C. hominis* and the isolates are registered in the NCBI under accession numbers PP593584 of *C. parvum* and three isolates PP593585, PP593586 and PP593587 for *C. hominis* according to phylogenetic analysis (Fig.4).

The results also showed the species of *C. parvum* and *C. hominis* in Mosul -Iraq were 100% identical to the same species in other studies in Iraq and other countries according to blast in GenBank of NCBI as in (Table 4).

Discussion

Microscopical examination of both stomach and intestine scraping revealed the presence of *Cryptosporidium* oocysts. Species of *Cryptosporidium* are considered homogeneous morphologically and the molecular analysis is critical for species differentiation. In this study showed oocyst of *Cryptosporidium* as red spherical or ovoidal in shape by mZN and mean size ranged from 4.6 - 5.5 X 3.8 - 4.7µm. This morphological identification microscopically is not enough at the species level because oocysts sizes has no significant differences among species for example; the species infecting fish *C.molnari* is 4.7X4.5 µm is similar in size with the zoonotic species *C.parvum* infecting

cattle, sheep, goats and human 5X4.5 µm and *C.hominis* infecting human and sheep5.5X4.5 µm [17] and [18] also mentioned that all species of *Cryptosporidium* ranged from 5-6 µm except for *C.muris* which is larger 7 µm. A total of 200 samples of five species of fish were examined of which 53 were positively infected with *Cryptosporidium* spp. with the infection rate (26.5%) which is high due to the fact that all fish collected were in same conditions which is suitable for the spread of this parasite [19] our results match with [20] who recorded (28.97%) in *Liza abu* fish in Mosul, but disagree with another study in Thi-Qar (12.02%) [21] and in Al-Diwaniya (6.18%) [22]. Occurrence of *Cryptosporidium* species was observed in this study showing that higher infection rates were observed during hotter months while the lowest rates were observed in colder months. This agrees with the results of [15] and [23] who recorded higher infection rate (87.5%) in July but the lowest (44.4%) in January. This may be attributed to different factors such as influence of physiology of the host, immunity, feeding and *Cryptosporidium* is characterized by the shedding of high numbers of oocysts that remain for long periods in water, in addition their ability to cause infection immediately after shedding with feces, resistance of these of oocysts to most of the sterilizers used in water sterilization due to the thickness of oocysts wall, and their vitality in water for 66 days [15, 17]. The molecular tool analysis by nested PCR is an ideal tool for species identification of *Cryptosporidium* by amplification of 18SrRNA gene [24, 25]. Molecular results in our study by nested PCR detected both *Cryptosporidium* species; *C. parvum* and *C. hominis* in five fish species and the 18SrRNA sequence and phylogenetic tree of the two isolated species in this study were identical 100% with reference sequences in many countries collected from GenBank database. These findings in the current study of are positive for *C. hominis* and *C. parvum* species which are considered zoonotic parasites and important from a public health infecting specially fishermen who are at risk of cryptosporidiosis and patients suffer from immunosuppression are also at risk of explosion to *Cryptosporidium* infection, either by consumption of undercooked fish or by direct contact during handling and preparation of fish [26, 27].

Conclusion

High infection rates during summer months with *Cryptosporidium* was recorded. Because of the morphological similarities among species, Nested PCR is considered a good tool for species identification and conformation of microscopical results. Due to water contamination with oocysts of *Cryptosporidium*, fish became a new source for transmission of parasite to human in addition to livestock. Fish are considered good sign for water contamination with parasites.

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Declaration of Conflict of Interest

Researchers declare that they have no conflicts of interest regarding the publication of this research.

Ethical of approval

Samples were collected and transferred after obtaining approval from the Veterinary Medicine/ Mosul University Ethics Committee, Iraq. UM.VET.2023.091 in 22-3-2023.

TABLE 1. PCR mixture

	Parasite	Primer	Primer sequences (5'-3')	bp	Reference
1.	*Cryptosporidium*	JerExtF	GACATATCWTTYAAGTTTCTGACC	784	Certad *et al.*, 2015
2.	External Primers	JerExtR	CTGAAGGAGTAAGGAACAACC		
3.	*Cryptosporidium*	JerIntF	CCTATCAGCTTTMGACGGTAGG	588	
4.	Internal Primers	JerIntR	TCTAAGAATTTCACCTCTGACTG		

TABLE 2. PCR mixture

#	Contents	Total size (µl)
1.	2X Master Mix	12.5
2.	Forward primer 10 pmol/µl	1
3.	Reverse primer 10 pmol/µl	1
4.	PCR grade water	8.5
5.	DNA template	2
6.	Total volume	25

TABLE 3. Monthly occurrence of Cryptosporidium spp. in fish

Month	No. of fish Exam.	No. of fish Inf.	Percentage of Inf.
August	37	10	%27.02 [a]
September	31	9	%29.03 [a]
October	35	12	%34.28 [a]
November	27	8	%29.62 [a]
December	33	8	%24.24 [a]
January	23	4	%17.39 [a]
February	14	2	%14.28 [a]
Total	**200**	**53**	**%26.5**

Different letters show significant differences between months.

TABLE 4. Sequence identity between local Cryptosporidium parvum isolate IN-C1-M24 (PP593584) and Cryptosporidium hominis isolate IN-C2-M24 (PP593585- PP593587), and others have recorded in the GenBank.

No	***Cryptosporidium parvum* isolate IN-C1-M24 and *Cryptosporidium hominis* isolate IN-C2-M24**	Gene name	GenBank accession number	Country	Sequence identity
1	*Cryptosporidium parvum* isolate RAYS341	Small subunit ribosomal RNA	PP038021.1	China	100
2	*Cryptosporidium parvum* isolate 278	Small subunit ribosomal RNA	KM065509.1	Spain	100
3	*Cryptosporidium parvum* isolate SCAU27715	Small subunit ribosomal RNA	ON023860.1	China	100
4	*Cryptosporidium parvum* isolate ZY51	Small subunit ribosomal RNA	MW769926.1	China	100
5	*Cryptosporidium parvum* isolate LZ98	Small subunit ribosomal RNA	MW769897.1	China	100
6	*Cryptosporidium hominis* isolate Black-capped	Small subunit ribosomal RNA	MT648439.1	China	100
7	*Cryptosporidium hominis* isolate GPH309	18S ribosomal RNA	MK982462.1	Bangladesh	100
8	*Cryptosporidium hominis* isolate ET91	Small subunit ribosomal RNA gene	MK990042.1	China	100
9	*Cryptosporidium parvum* isolate CryTeh-5	Small subunit ribosomal RNA	MH215514.1	Iran	100
10	*Cryptosporidium parvum*	Small subunit ribosomal RNA	MF326949.1	India	100
11	*Cryptosporidium hominis* isolate Har270	18S ribosomal RNA	MG516758.1	Australia	100
12	*Cryptosporidium hominis* isolate SCA631	18S ribosomal RNA	MG516757.1	Australia	100
13	*Cryptosporidium hominis* isolate Et14N1	18S ribosomal RNA	KX856002.1	Ethiopia	100
14	*Cryptosporidium hominis* isolate ED2	Small subunit ribosomal RNA	KY483987.1	Spain	100
15	*Cryptosporidium parvum* isolate CP3	Small subunit ribosomal RNA	PP327379.1	Iran	100
16	*Cryptosporidium hominis* isolate YPW2	18S ribosomal RNA	KJ019854.1	China	100
17	*Cryptosporidium parvum* LF1	18S ribosomal RNA	LC794437.1	Iraq	100
18	*Cryptosporidium parvum* isolate CSP	18S ribosomal RNA	KU882704.1	Iraq	100

No	***Cryptosporidium parvum* isolate IN-C1-M24 and *Cryptosporidium hominis* isolate IN-C2-M24**	Gene name	GenBank accession number	Country	Sequence identity
19	*Cryptosporidium parvum* isolate 122	Small subunit ribosomal RNA	OQ676143.1	Brazil	100
20	Cryptosporidium *sp.* isolate SSU-rRNAIS10	Small subunit ribosomal RNA	KX056091.1	India	100
21	*Cryptosporidium hominis* isolate DF001	Small subunit ribosomal RNA	OP999667.1	United Kingdom	100
22	Cryptosporidium *sp.* isolate LE8C18	18S ribosomal RNA	KU311859.1	France	100
23	*Cryptosporidium hominis* isolate UKH5	18S ribosomal RNA	KM012041.1	United Kingdom	100
24	*Cryptosporidium hominis* isolate PE3	18S ribosomal RNA	KP098564.1	Brazil	100
25	*Cryptosporidium parvum* isolate: CCAhv1	18S ribosomal RNA	AB986573.1	Iran	100
26	*Cryptosporidium hominis* isolate 06-012	Small subunit ribosomal RNA	MT648496.1	Colombia	100
27	*Cryptosporidium hominis* isolate 30000	Small subunit ribosomal RNA	KC734570.1	China	100
28	*Cryptosporidium hominis* isolate BRW_apr'11	18S ribosomal RNA	KF944361.1	Belgium	100
29	*Cryptosporidium hominis* isolate 43C	Small subunit ribosomal RNA	MN955482.1	Spain	100
30	*Cryptosporidium hominis* voucher IQ10	Small subunit ribosomal RNA	MW947224.1	Iraq	100
31	*Cryptosporidium hominis*	Small subunit ribosomal RNA	JN571735.1	USA	100
32	*Cryptosporidium hominis* isolate 31372	18S ribosomal RNA	JX644911.1	China	100
33	*Cryptosporidium hominis* strain W22638	Small subunit ribosomal RNA	JQ413431.1	United Kingdom	100
34	*Cryptosporidium hominis* isolate B6212B-1st	Small subunit ribosomal RNA	JQ313971.1	Canada	100

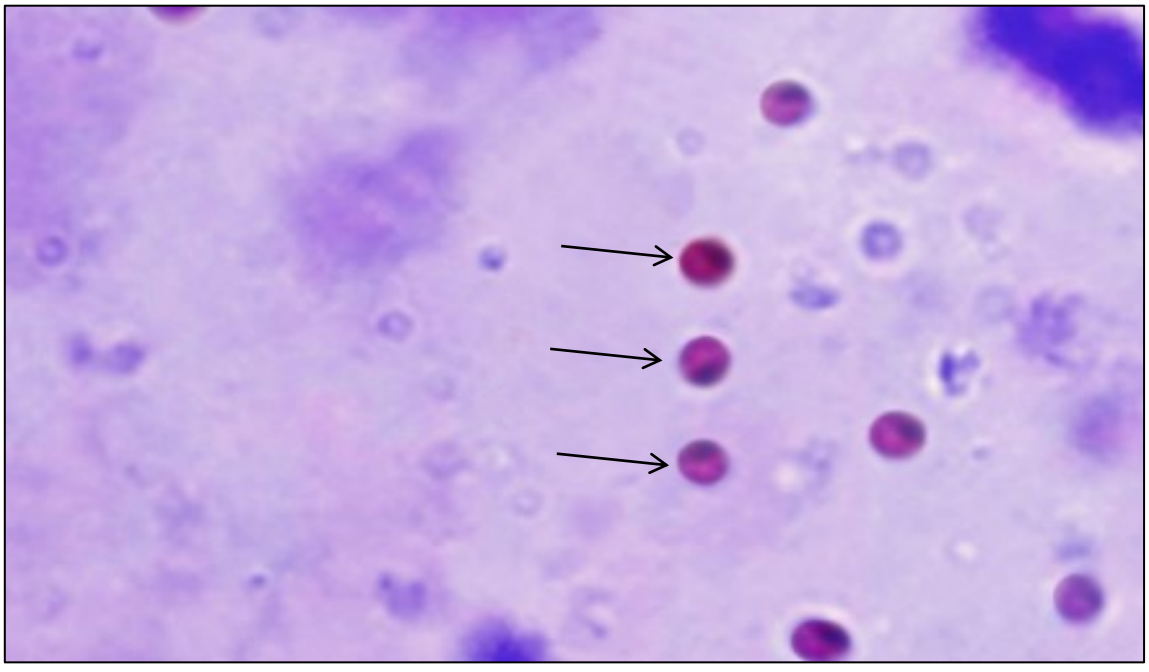

Fig. 1. Oocyst of *Cryptosporidium spp*. in stomach and intestine stained with mZN stain 100X.

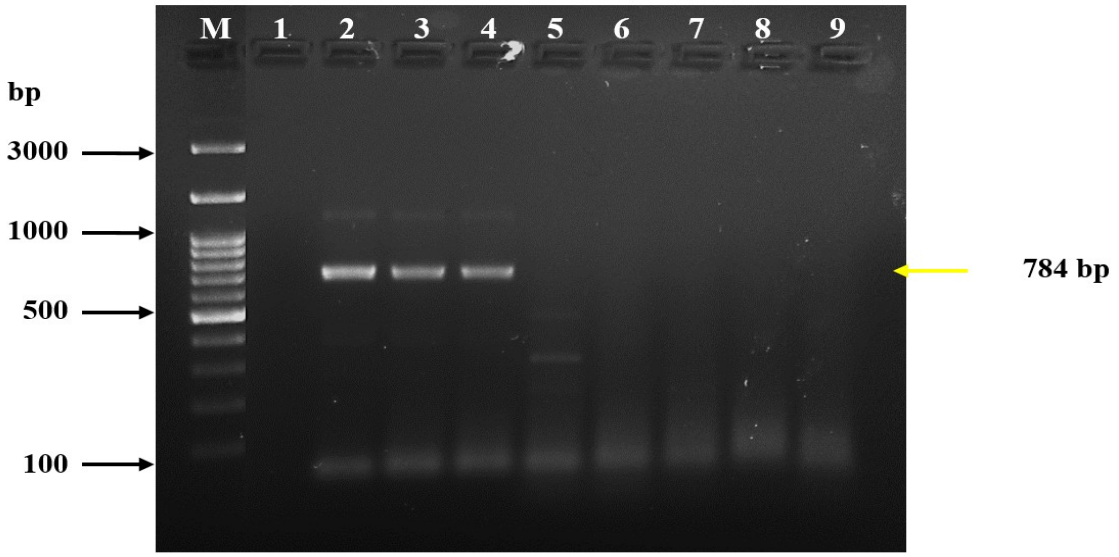

Fig. 2. Electrophoresis of agarose gel of Nested PCR products for the detection of Cryptosporidium spp. Using the external initiator JerExtF and JerExtR. Path M: The Marker indicator represents a volume of 100 bp. Track 2, 3, 4 represents positive samples and a product volume of 784 bp, Path 1, 5-9 represents negative samples.

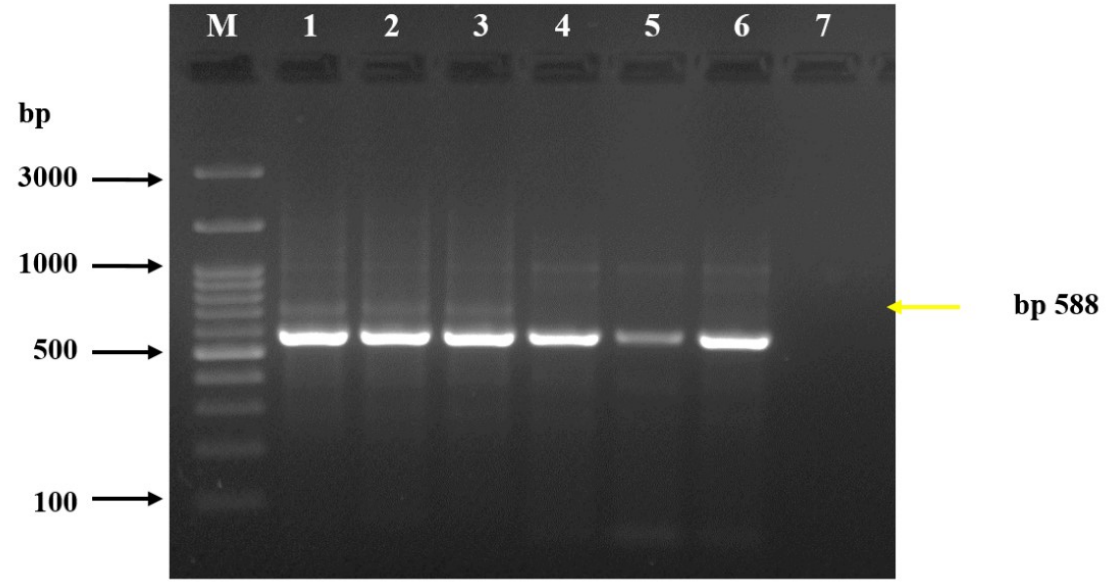

Fig. 3. Electrophoresis of agarose gel of Nested PCR products for the detection of Cryptosporidium spp. Using the internal initiator JerExtF and JerExtR. Path M: The Marker indicator represents a volume of 100 bp. Track 1, 2, 3, 4, 6 represents positive samples and a product volume of 588 bp, Path 5,7 represents negative samples.

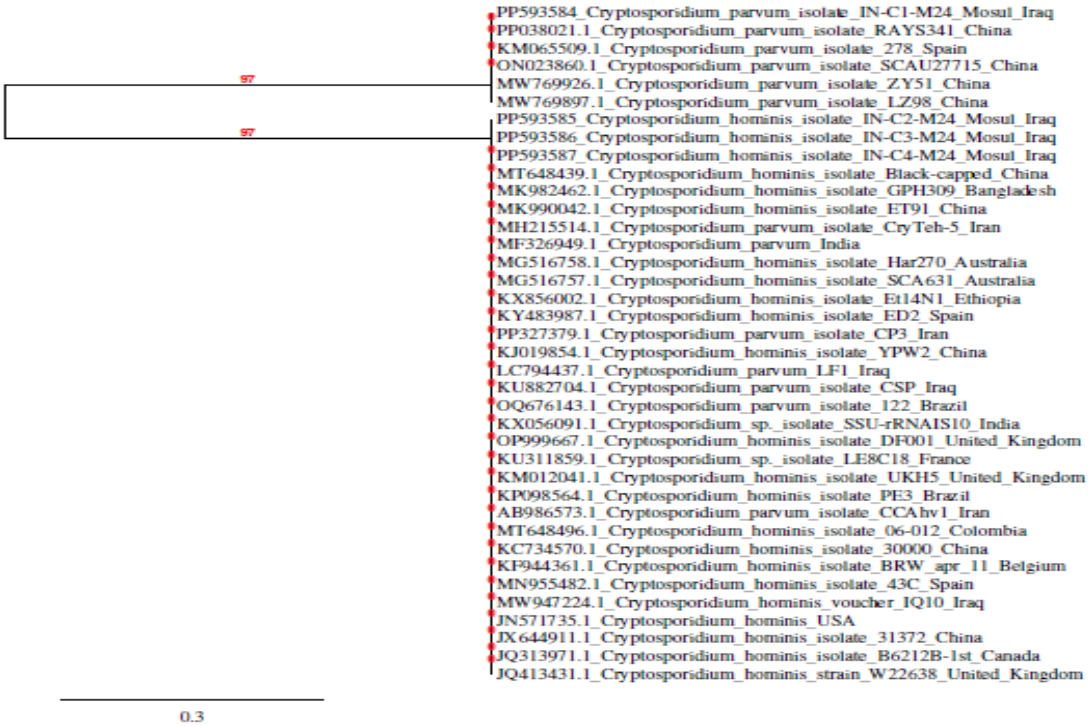

Fig. 4. Neighbor-Joining (NJ) phylogenetic tree analysis according to the 18SrRNA gene sequencing from Cryptosporidium spp. Mosul isolates from fish.

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حدوثية وتحديد جيني للأنواع المشتركة لطفيلي الابواغ الخبيئة في الاسماك في مدينة الموصل – العراق

اسراء عبد الواحد الطائي ونادية سلطان الحيالي
فرع الاحياء المجهرية - كلية الطب البيطري - جامعة الموصل – الموصل - العراق.

الملخص

طفيلي الابواغ الخبيئة هو من الاوالي الطفيلية حيوانية المنشأ يصيب مجموعة واسعة من مضائف الفقريات مما يسبب الإسهال الشديد. تم جمع 200 عينة من الأسماك الحية تعود لخمسة أنواع (الشبوط، الكارب الاعتيادي، البني، البلعوط الملكي (الزولي) والكطان) من الأسواق المحلية للفترة من آب 2023 - شباط 2024. بلغت نسبة الخمج الكلية لجنس الابواغ الخبيئة (200/53) (26.5٪). أعلى معدل إصابة كان في تشرين الاول (34.28٪)، وأدنى معدل كان في شباط (14.28٪). اظهرت قشطات من المعدة والأمعاء المصبوغة بصبغة زيل نيلسن (mZN) اكياس بيض الطفيلي بأشكال كروية أو بيضاوية وبحجم يتراوح من 4.6 - 5.5 × 3.8 - 4.7 ميكروميتر. أظهر تفاعل البلمرة المتسلسل وتحليل تتابع التسلسل الجيني لأربع عزلات باستخدام الجين SrRNA18 كانت موجبة لكلا النوعين وبالارقام التسلسلية PP593584 للنوع *C. parvum* وثلاث ارقام PP593585 وPP593586 وPP593587 للنوع *C. hominis* كانت متطابقة بنسبة 100٪ لنتائج دراسات أخرى في العراق وبلدان أخرى والمسجلة في بنك الجينات GenBank of NCBI. يعد تفاعل البلمرة المتسلسل تقنية جيدة لتحديد الأنواع وتأكيد النتائج المجهرية. تعد هذه الدراسة الأولى في العراق التي أثبتت أن الأسماك أصبحت مصدرا جديدا لانتقال لكلا النوعين إلى الإنسان فضلا عن حيوانات المزرعة.