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MOLECULAR AND PHYLOGENETIC ANALYSIS OF CRYPTOSPORIDIUM SPP. ISOLATED FROM CHILDREN, LAMBS AND GOAT KIDS.

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Abstract

To offer details on the infection rate of cryptosporidiosis in farmed small ruminants and the possibility of goat and lamb offspring acting as zoonotic reservoirs for the cryptosporidiosis virus in youngsters. 200 fecal samples from 100 children,65 lambs and 35 goat kids,were collected between September 2022 and May 2023 from various regions within the Diyala governorate. To check for Cryptosporidium spp. infection, traditional methods (Sheather's flotation technique and modified ziehl-neelsen staining microscopic examination) were employed. The total *Cryptosporidium*-positive isolates by conventional methods were (53/65) 81.5% in diarrheic cases of lambs, (15/35) 42.9% in goat kids and (21/100) 21% in children. Furthermore, forty *Cryptosporidium*-positive isolates were investigated utilizing subtyping and genotyping methods. Through sequencing of a selection of 20 isolates and restriction studies of PCR products from small-subunit 18s rRNA genes from microscopy-positive isolates, *Cryptosporidium parvum* and *C. scrofarum* were identified. The first application of phylogenetic analysis is to help resolve the controversy surrounding the taxonomy of the genus Cryptosporidium. Ten children, five lambs, and five children's genomes were used to create a phylogenetic tree that describes the 20 sequenced isolates of Cryptosporidium species. These results imply that a significant source of the zoonotic *C. parvum* and *C. scrofarum* for humans is goat offspring, specifically lambs. It was discovered that the disease mainly afflicted sheep and goats by comparing newly isolated strains of Cryptosporidium scrofarum from Iraq with previously documented cases.

Key words: Molecular, Phylogenetic Analysis, Cryptosporidiosis, Lambs, Goat Kids, Children.

Introduction

Cryptosporidiosis is a newly discovered zoonotic illness caused by an intracellular protozoal parasite that causes intestinal and extraintestinal diseases in both humans and animals. It has only been recognized recently that the illness is a major protozoal cause of diarrhea in humans, despite being well recognized in veterinary medicine (Diaz, 2010). Cryptosporidiosis, a dangerous zoonotic infection caused by a protozoan parasite that has detrimental effects on both

human and animal health, is recognized for affecting a wide spectrum of animal species as well as humans. The cause of cryptosporidiosis is an internal apicomplexan protozoan parasite from one of the species of Cryptosporidium. In humans and other vertebrates, including domestic animals, a total of 23 species of Cryptosporidium have been identified. These species range in pathogenicity from asymptomatic to highly debilitating gastrointestinal sickness (Ryan and Hijjawi 2015).

According to Leitch and He (2012), this parasite completes both its sexual and asexual life cycles in a single host, which is often a herbivorous mammal. Usually, people get Cryptosporidium hominis by drinking or eating tainted water that has been infected with the parasite's oocysts. According to Feng *et al*. (2018), the parasite causes severe diarrhea and destroys the small intestine after invading the digestive tract.

The sporulated oocyte, or infected stage, which infects humans and contaminates the environment, can originate from a wide variety of domestic and wild animals. These animals often have cryptosporidiosis (Robertson *et al*. 2014). Molecular techniques are used to verify. It is essential to discover Cryptosporidium spp. because their identification cannot be made by morphological or biological characteristics alone, nor by employing adequate culture techniques. To identify isolates of Cryptosporidium species, only molecular methods are employed, such as DNA sequencing of PCR products and PCR-based genotyping (Robinson *et al.,* 2006; Feng *et al*., 2007).

Materials and method

Molecular Diagnosis

A total of 200 fecal samples was collected from 100 children, 65 lambs and 35 goat kids from different regions in Diyala governorate, were collected from September 2022 to May 2023 and traditional techniques (Sheather's,flotation technique,modified ziehl-neelsen,staining microscopic examination) were used to check for Cryptosporidium spp. infection. For conventional PCR screening, 40 positive stool samples from children (20 samples), lambs (10 samples), and goat kids (10 samples) were molecularly analyzed. The small subunit ribosomal RNA gene based on Cryptosporidium spp. was detected using the ConventionalPCR technique in fecal samples.

This method was applied, according to (Yu et al.,2009) and Ruecker *et al*.,2013). This work used the NCBI-Genbank *Cryptosporidium sp*. to create the 18S ribosomal rRNA gene. Conventional PCR was used to detect Cryptosporidium spp. Sequence of the small subunit ribosomal gene and primer forward F-AGTGACAAGAAATAACAATACAGG and revers R-CCTGCTTTAAGCACTCTAATTTTC), then placed in PCR thermocycler. Primary PCR master mix was prepared according to to company instructions kit(**AccuPowerTM)** the PCR master mix components (Taq DNA polymerase, ,dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye) depending on standard **AccuPower® PCR PreMix Kit** which needed to PCR reaction.

Every PCR tube was placed in an Exispin vortex centrifuge and spun at 10,000 rpm for a minute. after which it was put in a MJ-Mini,BioRad/USA PCR,thermocycler. A hot start at 94˚C for five minutes was followed by thirty-five cycles of denaturation at 94˚C for thirty seconds, annealing at 56˚C for thirty seconds, and extending at 72˚C for five minutes before being held at 4˚C indefinitely (Ruecker *et al.,* 2013).

Results

Conventional and molecular identification of *Cryptosporidium* sp.

From September 2022 to May 2023, 200 fecal samples were taken from 100 children, 65 lambs and 35 goat kids from various regions in the Diyala governorate. The samples were tested for Cryptosporidium spp. infection using standard methods (Sheather's flotation,,technique, modified,,ziehl-neelsen staining microscopic examination). The total *Cryptosporidium*-positive isolates by conventional methods were (53/65) 81.5% in diarrheic cases of lambs, (15/35) 42.9% in goat kids and (21/100) 21% in children. Furthermore, a forty *Cryptosporidium*-positive isolates were examined by using genotyping,,and subtypeing techniques. Restrictions analysis of PCR results from small-subunit 18s rRNA genes from microscopy-positive isolates and sequencing of a sample of 20 isolates were used to identify *Cryptosporidium parvum* and *C. scrofarum*.

Detection of *Cryptosporidium parvum* using Conventional PCR

Forty positive stool samples from children (20 samples), lambs (10 samples), and goat kids (10 samples) were molecularly examined in the conventional examination, and the results revealed that the molecular examination matched the microscopic inspection. 80% of children, lambs, and kids, respectively, have Cryptosporidium DNA. (Table 1)

Table (1) Number of DNA extraction from Positive conventional methods examination of children ,lambs and goat kids.

Species	No. of Positive conventional methods examination	No. of positive conventional PCR	Infection rate %
Children	20	16	80%
Lambs	10	8	80%
Goat kids	10	8	80%
Total	40	32	80%

Genomic DNA was isolated from fecal samples and subjected to conventional PCR using small subunit ribosomal RNA,gene specific primers in order to identify the species of Cryptosporidium.

All 32 samples will use band at (290bp) for conventional PCR. Confirmation of the presence of *Cryptosporidium parvum* and *Cryptosporidium scrofarum* using conventional PCR product size on agarose gel (Figure 1)

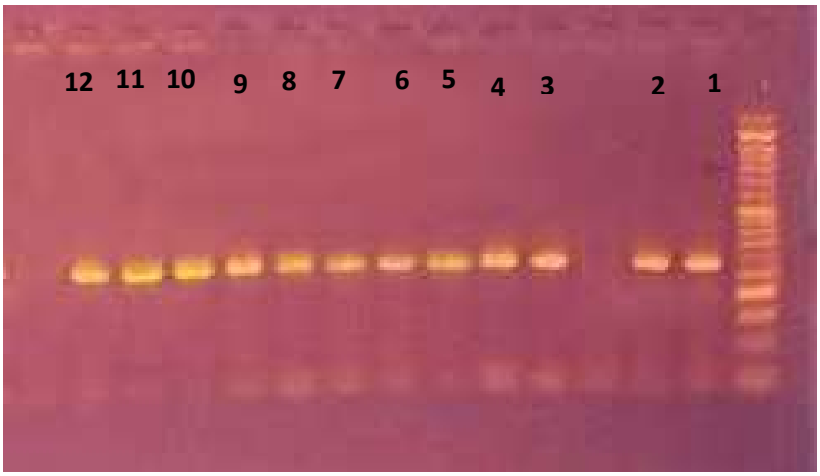

Figure (1) : The PCR product analysis of the small subunit ribosomal RNA gene in Cryptosporidium sp. from fecal samples was displayed in an agarose gel electrophoresis image. Whereas lanes revealed some positive Cryptosporidium sp. at the M:

Sequences analysis

Using the NCBI BLAST tool to analyze the sequences of conventional PCR products, the findings showed that children, lambs, and kids in the Iraqi region of Diyala were infected with *Cryptosporidium parvum*. To examine the genetic links between various Cryptosporidium species in children, lambs, and young animals, a phylogenetic tree was constructed using the Unweight Pair,.Group method with Arithmetic,Mean (UPGMA tree) in the MEGA.X version utilizing all 20 local Cryptosporidium isolate PCR data. (Table 2).

To create the association between Cryptosporidium spp., sequences from children, lambs, and youngsters obtained from Gen,Bank at total genetic alterations (0.010-0.060%) were aligned with the NCBI BLAST *Cryptosporidium sp*p.. The first use of phylogenetic aanalysis is to assist in settling the taxonomy of the genus Cryptosporidium debate.

In contrast, the iidentity of *C. scrofarum* was 100% confirmeddunder accession numbers (MH178036.1) in China and (MG516763.1) in Australia. With 100% homology observed with their respectivesspecies sequences reported on Gen Bank under accession numbers (DQ833278.1)

in Ireland and (KT948751.1) in Brazil, the sequencing analysis verified the identification of Cryptosporidium parvum (Figure 2).

Table (2) The percentage identity of the NCBI-BLAST Homology Sequence among nearby Cryptosporidium species. Isolates of children, lambs, and goat young, as well as Cryptosporidium species submitted to NCBI-BLAST. isolates

Local Children, Lambs, and goat kids *Cryptosporidium*,sspp. No.	**Accession, No. of Gen –Bank**	**NCBI,BLAST Homology,ssequence identity**		
		NCBI BLAST *Cryptosporidium* sp.	**Gen –Bank accession No.**	**Identity (%)**
1Children	OQ996400	***Cryptosporidium parvum***	**KT948751.1**	98%
2Children	OQ996401	***Cryptosporidium parvum***	**KT948751.1**	98 %
3 Children	OQ996402	***Cryptosporidium scrofarum***	**MG516763.1**	100%
4 Children	OQ996403	***Cryptosporidium parvum***	**KU382244.1**	100%
5 lambs	OQ996404	***Cryptosporidium parvum***	**MG516763.1**	100%
6 lambs	OQ996405	***Cryptosporidium parvum***	**KT948751.1**	98%
7 lambs	OQ996406	***Cryptosporidium parvum***	**MG516763.1**	98%
8 lambs	MZ377137.1	***Cryptosporidium parvum***	**DQ833278.1**	98%
9 kids	OQ996407	***Cryptosporidium parvum***	**DQ833278.1**	98%
10kids	OQ996408	***Cryptosporidium scrofarum***	**MG516763.1**	100%
11 kids	OQ996409	***Cryptosporidium scrofarum***	**MG516763.1**	100%
12 Kids	OQ996410	***Cryptosporidium parvum***	**DQ833278.1**	98%

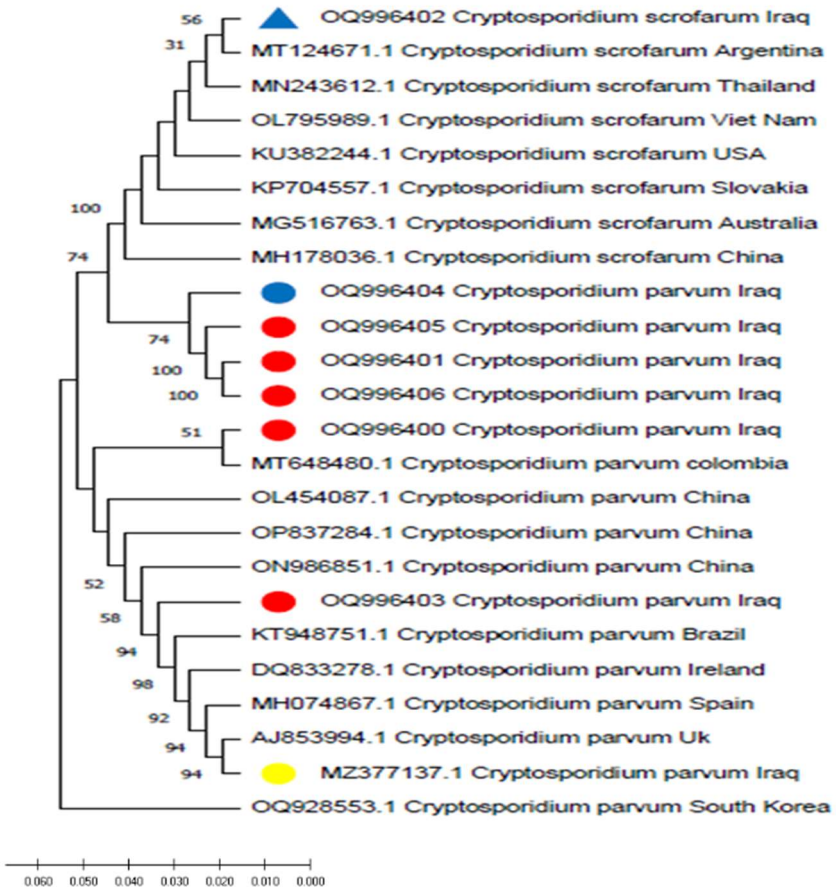

Figure (2): phylogenetic tree analysis using partial sequences of the small subunit ribosomal RNA gene in nearby Cryptosporidium species. The Unweighted Pair Group technique with Arithmetic Mean (UPGMA tree) in (MEGA.X version) was used to create the phylogenetic tree. Children (No.1, No.2, and No. 4) from the local Cryptosporidium isolate IQ were shown to be closedly linked to NCBI-BLAST Cryptosporidium parvum (KT948751.1), whereas children (No. 3) were shown to be closedly related to NCBI-BLAST Cryptosporidium scrofarum (MG516763.1). Lambs (No.5, No.6, and No.7) were shown closed linked to NCBI-BLAST Cryptosporidium pavum (KT948751.1) and No.8 was shown closed related to NCBI-BLAST Cryptosporidium pavum (DQ833278.1), according to the local Cryptosporidium isolate IQ. The IQ of the nearby Cryptosporidium isolate.The goat offspring, identified as Nos. 9 and 12, were found to be closed in relation to NCBI-BLAST Cryptosporidium parvum (DQ833278.1). However, at total genomic alterations (0.010-0.060%), (Nos. 10 and 11) were shown to be closed connected to NCBI-BLAST Cryptosporidium scrofarum (MG516763.1).

Discussion

A protozoan parasite called Cryptosporidium infects numerous mammal species, including humans, through the gastrointestinal route. There are several genetically different forms in the genus, the most prevalent zoonotic species being *C. parvum* (cattle genotype, type 2). Cryptosporidium infection, an endemic enteric protozoal sickness, continues to be the second most prevalent cause of mild to severe diarrhea in children under two years old and a substantial global cause of mortality. It usually occurs during waterborne outbreaks in immunocompromised hosts (small ruminants and children).

Molecular Identification: Detection of *Cryptosporidium parvum* using Conventional PCR

Only 32 species out of 40 that tested positive using the conventional method tested positive for cryptosporidium (80%) in children, lambs, and kids, respectively. This finding can be explained by the possibility that specimens stored for an extended period of time before testing could result in false negative results because parasite DNA is labile in faecal medium. These agreement with (*Paulos*, *et al.,* 2016).

Using gene-specific primers,and small subunit ribosomal RNA, genomic DNA from fecal samples was submitted to conventional PCR molecular analysis to determine the species of Cryptosporidium, these results were recorded in NCBI from many Iraqi researcher with many differences in genotypes and will employed in many different band depending in primers used in these studies. The study is the first molecular reports of cryptosporidium isolate from human, lamb, kids in Diyala province. Since *C. parvum* is not host-specific, it is more common in other animals and is the second most common species in humans, after C. hominis (Chen, 2018; Certad, 2018) which may explain a high infection rate of *C. parvum* compared to the other Cryptosporidium species.

Sequences analysis

The identification of *Cryptosporidium parvum* 100% homology was observed with their respective species sequences reported on Gen Bank on accession numbers (DQ833278.1) in Ireland and (KT948751.1) in Brazil which confirmed the sequence analysis, also we reported the first time in Iraq a new species of cryptosporidium was *C. scrofarum,* it is a Cryptosporidium pig genotype II with identity 100 % on accession number (MH178036.1) in china (MG516763.1) in Australia . these a new species may be infected human and animal from contaminated of water and vegetable's with adult wild pigs feces in many Iraq provinces .

Phylogenic analysis

The percentage of homology sequence identity (%) between isolates of Cryptosporidium spp. from goats, lambs, and children in the area of study and those submitted by NCBI-BLAST. In order to establish a correlation, Cryptosporidium spp. sequences of children, lambs, and goat

kids obtained from GenBank at total genetic alterations (0.010-0.060%) were aligned with the NCBI BLAST *Cryptosporidium* spp.sequences.,Phylogenetic analysis is first applied to help settle the taxonomy dispute for the genus Cryptosporidium. Twenty Cryptosporidium species isolates that were sequenced from the genomic DNA of ten children, five lambs, and five kids of goats were described in a phylogenetic tree along with the corresponding reference sequences that were obtained from GenBank. The primary Cryptosporidium species, *C. scrofarum*, was revealed by the DNA sequencing investigation. The genotypes of *C. parvum, C. suis, C. hominis, C. bovis*, and the new sheep genotype (Morgan, *et al*. 1998) have been examined in sheep feces in a different study (Alkhaled and Hamad 2017) identified *C. parvum, C. hominis, and C. suis.* Five species of Cryptosporidium were identified in the goat sample; these included *C. parvum, C. xiaio, C. hominis, C. andersoni, C. ubiquitum,* and *C. xiaio.* These results were reported in another study by (Wang *et al*., 2014), and included *Cryptosporidium,,ubiquitum* (24 from 44) in Henan and Chongqing, *Cryptosporidium,,andersoni* (16 from 44), and *Cryptosporidium,,xiaio* (4 from 44) in Henan. To date, several Cryptosporidium species and genotypes have been identified in goats, including *C. parvum, C. xiaio, C.,hominis*, a goat genotype, and a new Cryptosporidium genotype (Rose, *et al*., 1997).

The primary species of Cryptosporidium, *C. parvum*, was identified from goats in Egypt, Zambia, Sri Lanka, Italy, Spain, Belgium, the Czech Republic, the Netherlands, France, and Zambia (Rose *et al*., 1997). & *C. ubiquitum* and *C. andersonire* offer the first studied species of Cryptosporidium in goats; both species were previously created in sheep in Henan, China (Smith, 2008).

Conclusions

These results imply that the zoonotic *C. parvum* and *C. scrofarum* that affect humans are primarily reservoir in lambs and goat kids. Consequently, to reduce human and domestic animal cryptosporidiosis, safety precautions and programs for health education are required.

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