First Molecular Study and Sequencing of *Cryptosporidium spp.* Isolated from Human, Cattle and Sheep in Wasit Province, Iraq

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Abstract

The current study aimed to investigate *Cryptosporidium* spp. that infect humans and some animal species as well as to compare among various isolates obtained. A total of 96 human stool samples and 96 animal fecal samples (48 for cattle and 48 for sheep) were collected and examined by using Nested PCR technique followed by DNA sequencing analysis through phylogenetic tree to know the parasite species. The whole infection rate in human was 36.4% (35/96), while in cattle was 22.9% (11/48) and in sheep was 14.5% (7/48) by nested polymerase chain reaction technique. The findings of DNA sequencing based on 18s rRNA gene (654 bp) showed the presence of three *Cryptosporidium* spp. which namely *C. hominis*, *C. parvum*, and *C. bovis*, all of them have identity 99 %, *Cryptosporidium parvum* was the most widespread species being detected in 60% (9/15), followed by *C. bovis* 33.33% (5/15), and *C. hominis* 6.66% (1/15). This is the first report revealed the molecular sequencing among human, cattle and sheep in Wasit province, Iraq and the role of livestock (cattle, sheep) in zoonotic transmission of the cryptosporidiosis.

Key Words: *Cryptosporidium spp*, Human, Cattle, Sheep, Sequences Analysis.

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Introduction

Cryptosporidiosis which is a significant diarrheal illness for both people and animals worldwide and caused by various species of the parasite [1]. There are at least 39 species and 70 genotype of *Cryptosporidium*, twenty one species & 4 genotypes have been documented in humans [2]. The most widespread species of *Cryptosporidium* spp in humans are *C. parvum* & *C. hominis*, while there are four species infecting cattle animals which include *C. bovis*, *C. parvum, C. andersoni and C ryanae.* [3,4]. The clinical signs in human is characterized via profuse watery diarrhea, nausea, abdominal cramps and some persons may also suffering from vomiting, low grade fever and weight loss [5]. In animals, the clinical signs are characterized via mild to heavy profuse watery diarrhea which is commonly yellow-pale brown and also experience lethargy, anorexia, weight loss, dehydration and in some cases can lead to death [6]. Human and livestock animals are potential reservoirs that collaborate to the contamination of food, water and the environment, therefore the infection transmitting to another hosts by the Fecal_oral route [7]. Molecular techniques were progressed to increase the diagnosis of specificity as well as sensitivity and to provide information for parasite on species and genotype level [8].

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The aimed of this study was to determine the molecular characters of *Cryptosporidium* spp. isolated from human and animals as well as to compare between various isolates obtained.

Materials and Methods

Molecular Analysis by Nested PCR

The Nested PCR technique was achieved to detect of *Cryptosporidium* spp. based on SSU Ribosomal RNA(18S rRNA) gene from (human, cattle, sheep) stool and fecal samples. This technique including DNA extraction stool and fecal samples using Presto™ stool DNA Extraction Kit (Geneaid, Taiwan) and this is was achieved according to the company instructions. The primers were used in this study to detect *Cryptosporidium* spp for human and animals, these primers were supplied by Scientific Researcher. Co. Ltd, Iraq as following: a forward primer was used (*Cryptosporidium* spp ; 5`-TGGCACCAGAATCAGCTGAA-3`) and a reverse primer was used (5`- GACAGGTTGAGTTGGAGCAGA -3`). The Master Mix of Nested PCR was prepared by GoTaq ® Green Master Mix and achieved according to company instructions. Finally master mix placed in PCR Thermocycler (Bio-Rad, USA), the first round thermocycler conditions was began with initial DNA denaturation (95°C for 5 min), followed by 35°C PCR cycles (95°C for 30sec, 60°C for 30 sec, 72°C for 2 min) then a final extension(72°C for 5 min). The products of PCR were analyzed by agarose gel electrophoresis and visualized using ethidium bromide.

DNA Sequencing Analysis

DNA sequencing method was achieved after some positive amplification of Nested PCR product (654 bp), then these samples were sent to Korea to do the DNA sequencing & analysis of phylogenetic tree using (MegaX.version) and analysis of SSU Ribosomal RNA(18S rRNA) based (Clustal.W alignment) as well as accounted of the evolutionary distances using (the Maximum Composite Likelihood Method)by phylogenetic treeUPGMA method. Finally identified isolates of *Cryptosporidium* species were presented into of NCBI-GenBank to obtain accession number.

Statistical Analysis

The Data were analyzed by using SPSS computer software for findings numbers and percentages (%) and the Chi-square test was used to compare between the percentages which (P<0.05) value was considered significant [9].

Results and Discussion

Molecular Diagnosis by Nested PCR

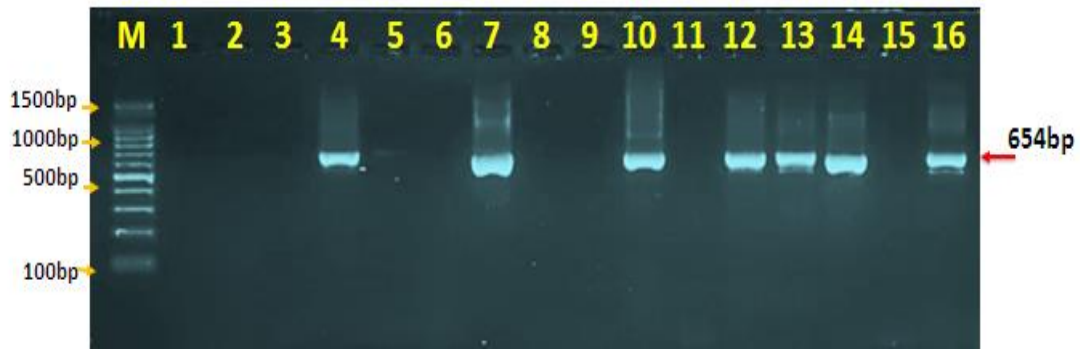
According to Nested-PCR investigation of human and animal DNA sample, the findings showed that the whole infection rate in human was 36.46% (35/96), while in cattle was 22.92% (11/48) and in sheep was 14.5 (7/48) at significant difference (p<0.05) Table (1).

Table 1. The infection rate of *Cryptosporidium* spp among human and animal by Nested PCR.

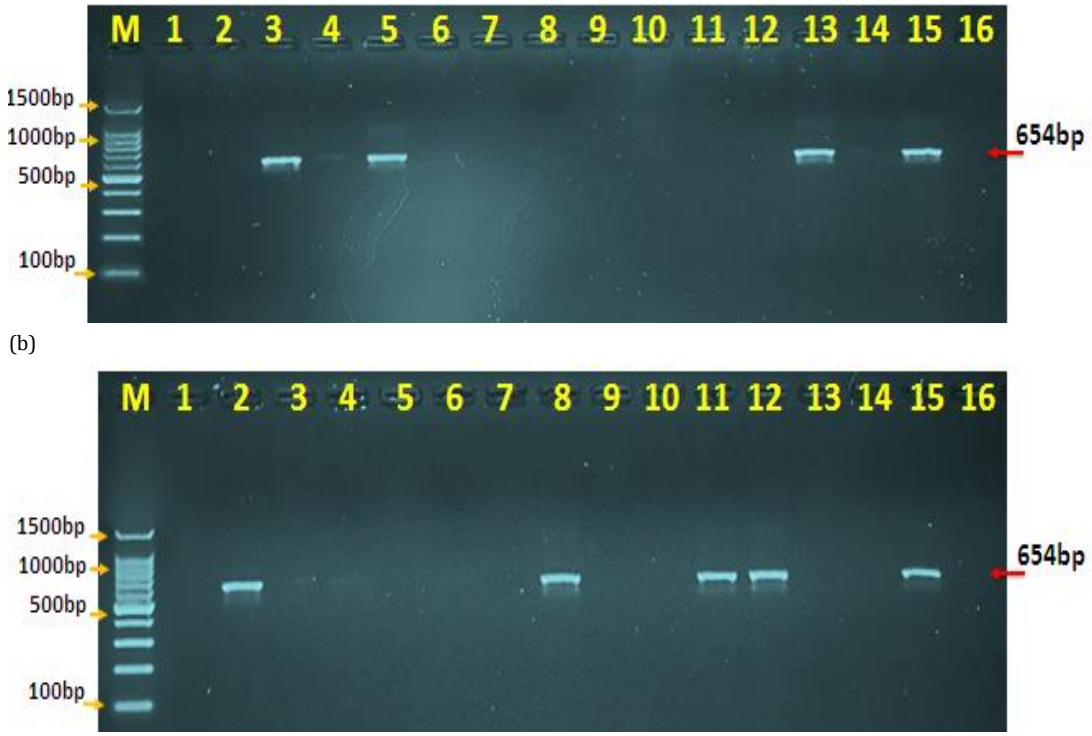
Type of species	No. of examined	Results of Nested PCR	
		+	%
Human	96	35	36.5
Cattle	48	11	22.9
Sheep	48	7	14.5
P value			0.0004

Significant difference at a level (p<0.05).

The genomic DNA obtained from 96 human stool samples and 96 animal fecal samples were undergo for molecular analysis using (18S rRNA gene) specific primers to determine the *Cryptosporidium* species. The fragment (654 bp) were amplified and analyzed by electrophoresis Fig (1).



(a)



(b)

(c)

Figure 1. Picture of agarose gel electrophoresis demonstrate the nested polymerase chain reaction product analysis of (SSU ribosomal RNA) gene in *Cryptosporidium* spp. from (a) human stool sample (b) cattle fecal sample and (c) sheep fecal sample, where M; marker: 1500-100 bp, Lane: 1-16 some positive samples at (654bp) product of Nested PCR

For human samples, the present results similar with a pervious results that revealed that the infection rate of cryptosporidiosis in Egypt 37.5% from diarrheic patients using Nested PCR [10]. The percentage is higher by [11] in Baghdad province recorded the total infection rate of *Cryptosporidium* spp in human was 47.33% by Nested PCR, while the lower percentage was by [12] in Bandar Abbas City/ Iran which record the prevalence of *Cryptosporidium* spp. by Nested PCR (12%) among diarrheic patients. For cattle samples, the current findings were in agreement with result by [13] in Guangdong, China showed that the whole infection rate was 24% from dairy calves using Nested PCR, the higher percentage of cryptosporidiosis was by [14] in Turkey they recorded (43.9%) from diarrheic calves, while the lower percentage was by [15] in northwest China registered the infection rate of *Cryptosporidium* spp in dairy cattle was 4.2%. For sheep samples, the current findings in sheep was nearly similar with result by [16] in Egypt revealed that the total rate of infection was 25.93% using nPCR, the higher percentage of cryptosporidiosis was by [17] in Al-Qadisiyah University/ Iraq recorded 40%, while the lower percentage was by [18] in Tehran, Iran recorded 1.69% using nested PCR/RFLP. These differences may be due to the animal age, season of samples collection or sample numbers among the various studies [19].

DNA Sequencing

The results of DNA sequencing of this study examined by the Basic Local Alignment Search Tool (BLAST analysis). Verification of sequences were proved by employing (18s RNA gene) of *Cryptosporidium* which involve *C. hominis, C. parvum, C. andersoni* and *C. bovis* gene sequences data that documented in genebank and the out groups for discovering the identity degrees and similarity score of *Cryptosporidium* spp 18s rRNA gene which effected on (human, cattle, sheep) and compared with current study isolates strains. The results of current local *Cryptosporidium* species that involves (5 samples of human, 5 samples of cattle and 5 samples of sheep) were revealed closed association with NCBI-BlAST *C. parvum* isolates, the whole percent identity score ranged (99.13-99.84%), one sample from human was revealed closed association with NCBI-Blast *Cryptosporidium hominis*, the percentage identity score (99.67%), 3 samples from cattle and 2 from sheep were revealed closed association with NCBI-BlAST *Cryptosporidium bovis*, the percentage identity score ranged (99.13-99.84%) at whole genetic alterations (0.04-0.01%).

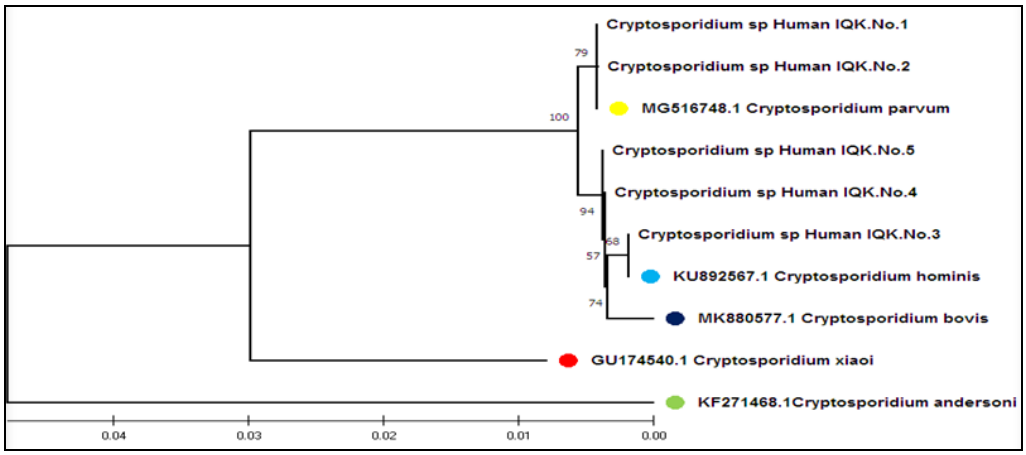


Figure 2. Analysis of phylogenetic tree depend on (SSU ribosomal RNA gene) partial sequence in "local *Cryptosporidium* Human isolates" that utilized in analysis of genetic species typing. Phylogenetic tree was built utilizing (UPGMA tree) in (MEGA6.0 version). The local *Cryptosporidium* spp. Human IQK isolates (No. 1, 2, 4 and 5) were revealed closed association with NCBI-BLAST *'Cryptosporidium parvum'* (MG516748.1), while the local *Cryptosporidium* spp. Human IQK isolate (No. 3) was revealed closed association with NCBI-BLAST *Cryptosporidium hominis* (KU892567.1) at whole genetic alterations (0.04-0.01%)

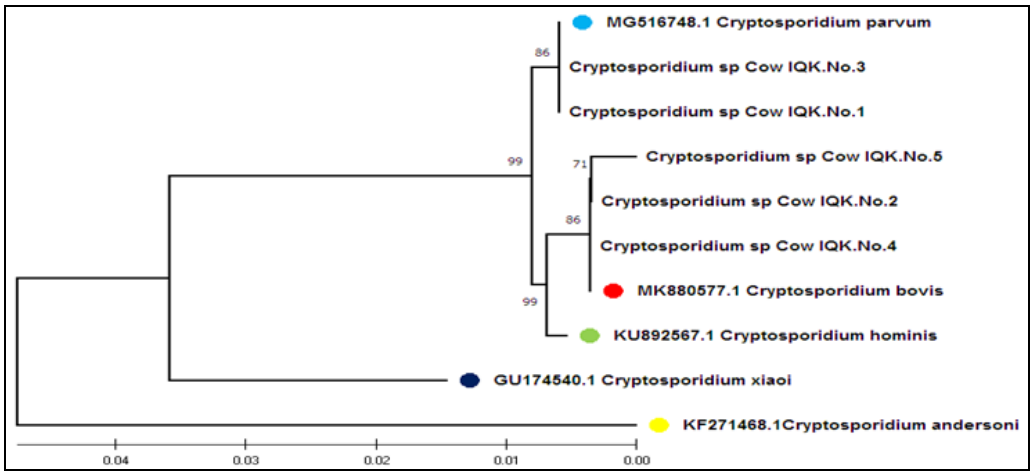


Figure 3. Analysis of phylogenetic tree depend on (SSU ribosomal RNA gene) partial sequence in "local *Cryptosporidium* Cow isolates" that utilized in analysis of genetic species typing. Phylogenetic tree was built utilizing (UPGMA tree) in (MEGA6.0 version). The local *Cryptosporidium* spp. Cattle IQK isolates (No.1 and No.3) were revealed closed association with to NCBI-BLAST *Cryptosporidium parvum* (MG516748.1), while the local *Cryptosporidium* spp. Cattle IQK isolates (No.2,4, and 5) were revealed closed association with NCBI-BLAST *Cryptosporidium bovis* (MK880577.1) at whole genetic alterations (0.04-0.01%)

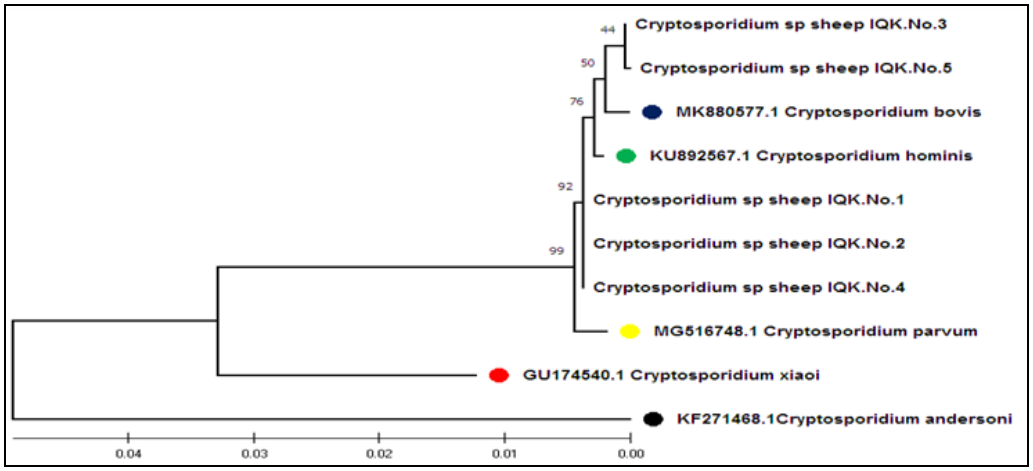


Figure 4. Analysis of phylogenetic tree depend on (SSU ribosomal RNA gene) partial sequence in "local *Cryptosporidium* Sheep isolates" that utilized for analysis of genetic species typing. Phylogenetic tree was built utilizing (UPGMA tree) in (MEGA6.0 version). "The local *Cryptosporidium* spp. Sheep IQK isolates (No.1, 2 and 4) were revealed closed association with to NCBI-BLAST *Cryptosporidium parvum* (MG516748.1), while the local *Cryptosporidium* spp. Sheep IQK isolates (No.3 and No.5) were revealed closed association with NCBI-BLAST *Cryptosporidium bovis* (MK880577.1) at whole genetic alterations (0.04-0.01%)

The current results of this study revealed the presence of three main *Cryptosporidium* spp. in human, cattle and sheep and the most widespread species was *C. parvum* being detected 60% (9/15), then *C. bovis* 33.33% (5/15) and *C. hominis* 6.66% (1/15). These isolates were considered to be adaptable for anthroponotic and zoonotic transmissions through direct or indirect contact.

For human isolates, our findings agreed with the findings that found by [20] in Yemen who registered 97% of human isolates were *C. parvum* and 3% were *C. hominis*, also agreed with results by [21] in AL-Diwaniyah province, Iraq record two species of human isolates which included *C.hominis* and *C. parvum*.

For cattle isolates, our findings agreed with the findings that recorded by [22] in Colombia record the presence of two species of *Cryptosporidium C.parvum* and *C.bovis*, while the present study disagreed with [23] in Thailand record the presence of *C. bovis* and *C. ryanae* but no *C. parvum* detected.

For sheep isolates, our findings agreed with the findings that recorded by [24] in United Kingdom who registered the presence of *C. bovis* and *C .parvum* in neonatal lambs of sheep, while the present results disagreed with [25] in China record the presence of *C. xiaoi* and *C. andersoni* but no *C. parvum detected.*

Conclusions

Cryptosporidium parvum was the most widespread species in human and animals and therefore the current study detect that human could obtain the infection through zoonotic and anthroponotic transmission. It is necessary to establish efficient strategies to control and reduce *Cryptosporidium* spp transmission among cattle, sheep and humans in Wasit province.

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