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Original article

Molecular Study on *Cryptosporidium andersoni* Strains Isolated from Sheep Based on 18S rRNA Gene

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Abstract

Background: Cryptosporidiosis is one of the most important parasitic diseases infecting a broad variety of animals and humans. In the present study, Nested PCR-RFLP-based assay was applied for genotyping of sheep cryptosporidiosis. The target of amplification was the 18S rRNA gene used to identify *Cryptosporidium* species
Materials and Methods: In the first step, 1300 faecal samples were collected from sheep in Tehran province, then the samples were examined for the presence of *Cryptosporidium* using modified acid fast staining. In the second step, DNA was extracted from the positive samples. Next, 18S rRNA gene was amplified by Nested-PCR in order to differentiate between the species. The PCR product was digested by Ssp1 restriction enzyme.
Results: Twenty two positive sheep samples were detected by modified acid fast method. The results were confirmed by molecular techniques. The 845 bp fragment of 18S rRNA was digested by restriction enzymes. Twenty samples showed a similar band on 2.5% agarose gel whereas 2 samples demonstrated different pattern. The sequences of two patterns indicated two species of *C. andersoni* and *C. parvum.*
Conclusion: In spite of other studies results introducing *C. parvum* as the major agent of cryptosporidiosis in sheep, in our study, *C. andersoni* was found to be dominant.

Key words: Cryptosporidium, 18S rRNA, Nested-PCR, Sheep, IR Iran

1. Background

Cryptosporidium is considered as a important cause of diarrheal disease in humans, livestock, and other animals through the world, and as a major economic problem on the water industry (1). This protozoon has been reported in over 150 mammalian species, mostly as *C. Parvum* and *C. parvum* infections (2).
Direct characterization of cryptosporidium by PCR-based methods have a significant outcome not only on determining the variation of cryptosporidium species at the species level, but also on the molecular epidemiology of cryptosporidium infections.

(1). A number of genetic loci has proved to be useful particularly in studies on *Cryptosporidium* in terms of reproducibility between different laboratories, among which 18S rDNA, HSP70, actin, and COWP genes are of great importance (3-4).

The genotyping protocol proposed by Xiao et al. (1999) (5) targeting the small subunit of rRNA gene of *Cryptosporidium* is known to be PCR-RFLP based technique which can be used for detection of the most species and genotypes of *Cryptosporidium.* This protocol maintain a high level of sensitivity (5-6) and has been applied by some investigators in different parts of the world.

The *C. parvum* infection in sheep was first reported by Meutin et al. (1974) (7). Up to now, based on molecular study, nine *Cryptosporidium* species/genotypes have been identified in sheep. These include *C. hominis, C. parvum, C. suis, C. fayeri, C. andersoni*, the sheep genotype, *C.* cervine genotype, pig Genotype II, and a *C. bovis*-like genotype (8). Chalmers et al. (2002) described a novel isolate of *Cryptosporidium* from sheep, which had different *Cryptosporidium* oocyst wall protein (COWP) and PCR product sequences in comparison with *C. andersoni* and *C. parvum* (Genotype 2) (9).

In Iran, sheep breeding plays a significant role in tribal life, especially in mountainous grassland regions where raising sheep is a main source of people's income. While many studies were conducted on the prevalence rate of *Cryptosporidium* in sheep using mainly microscopic investigations, only few genetic data were reported for *Cryptosporidium* species in sheep in IR Iran.

2. Objectives

The aim of the current study was to identify *Cryptosporidium* species using 18S rRNA gene as the target of amplification.

3. Materials and Methods

3.1. Specimens

A total of 1300 faecal samples were collected from some sheep herds around Damavand city (Tehran Province) directly from the rectum of animals. After preparing a direct smear of each sample and diagnosing the positive samples, all positive samples were preserved in 2.5% potassium dichromate and stored at 4°C for use in PCR techniques.

3.2. Microscopic examination:

Microscopic diagnosis of *Cryptosporidium* was performed using modified Ziehl-Neelsen staining.

3.3. Oocyst Purification:

Oocysts were collected from the faeces using salt (NaCl) flotation method. Briefly, the oocysts were separated by flotation from faecal debris and centrifugation for 8 min at 1600×g. The floated materials containing the oocysts were washed with deionized water; the oocysts were resuspended in 1 mL deionized water and stored at 4°C prior to use.

The preservative was removed by resuspending the pellet in 10 mL of 55% sucrose, mixed it for 1 min, centrifuged at 1000×g for 15 min, overlaid with 1mL of distilled water and swelled gently, then the oocysts were collected from supernatant .

3.4. DNA extraction

QIAamp kit (QIAGEN Ltd., Crawley, West Sussex, United kingdom) was used to extract DNA from the washed faecal samples.

3.5. PCR amplification

A three-step nested PCR protocol was used to amplify the 18S rRNA gene from the genomic DNA for nucleotide sequencing. In the primary PCR amplification, a forward primer (Cr18PA: F-5`-TTCTAGAGCTAATACATGCG-3`) and a reverse primer (Cr18PB: R-5`-CCCATTTCCTTCGAAACAGGA-3`) were used, and in nested PCR amplification, a forward primer (Cr18NA: F-5`-GGAAGGGTTGTATTTATTAGATAAAG-3`) and a reverse primer (Cr18NB: R-5`- CTCATAAGGTGC TGAAGGAGTA-3`) were used for *C. andersoni* as well as a forward primer (Cr18NC: F-5`- TTTAGACGGTAGGGTATTG-3`) and a reverse primer (Cr18ND: R-5`- CTTCTTAGAGGGACTTTGC-3`) were used for *C. parvum*, which were designed from sequences of 18S rRNA gene from NCBI site.

The PCR mixture was consisted of PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl pH 8.3), 1µM of each forward and reverse primers, 0.2 mM of each dNTP, and 2.5 U taq DNA polymerase (Cinnagen Co). The fragment was amplified using 2 µL of DNA templete and 2 µL of forward and reverse primers in a total reaction volume of 30 µL. It was started with initial denaturation (94°C for 5 min), then thirty five PCR cycles (94°C for 40 s, 55°C for 40 s, 72°C for 60 s) and a final extension (72°C for 10 min). The PCR conditions for the secondary reaction were similar to those for the primary PCR reaction, except that the annealing temperature was 54°C. The PCR products were analyzed using agarose gel electrophoresis.

3.6. Sequencing

The PCR products were purified from gel by the PCR purification kit (Roche) according to the manufacturer´s instructions. Then it was sent to the Genfanavaran® Company (Iran) for sequencing. The comparison of the obtained sequences with the GenBank was performed using the Blast program.

3.7. RFLP assay

The PCR products were digested by restriction enzyme (Ssp1). Each 30µL restriction digest contained 20 µL of PCR product, 3 µL buffer, 2 µL of restriction enzyme, and 5 µL distilled water. Incubation times and temperatures were arranged 12 hours and 37ºC, respectively. The digests were ended by addition of 4 µL loading mixture (0.01% Bromo-phenol blue, 0.1 M ethylene diamine tetra acetic acid, 50% glycerol, pH=8). Digestion products were electrophorised on 2% agarose gel at 60 V (120 min). The gel was stained with ethidium bromide, and the fragments were visualized with UV transilluminator. Digestion reactions lacking restriction enzyme were used as negative controls. A 100bp ladder was run on each gel for the estimation of fragment sizes.

4. Results

Microscopic examination of 1,300 faecal samples showed the presence of *Cryptosporidium* oocysts in 22 (1.69%) samples.

The PCR yielded an amplicon with approximate length of 845bp in nested PCR for all samples (Fig. 1). Digestion by Ssp1, yielding the same electromorph pattern for 20 samples means that 90.90% of the samples were *C. andersoni*, and the rest (9.10%) were C. *parvum*. Figure 2 shows the RFLP pattern for the samples after digestion by Ssp1 restriction enzyme. Ssp1 enzyme cuts only two fragments of 448bp and 397bp at 448 sit produces for *C. andersoni* whereas cut 4 at 449, 461, 472, and 580 sit produces and produces 5 bands of 449bp, 12bp, 11bp, 108bp, and 265bp for *C. parvum*.

Phylogenetic tree of *C. andersoni* and C. *parvum* isolates based on partial small subunit Ribosomal RNA (18S rRNA) gene is shown in Fig. 3. Twenty (90.90%) isolates were found to be 100% aligned with *C. andersoni*, and only 2 (9.10%) isolates were *C. parvum*. The *C. parvum* isolates were submitted with accession numbers EU311201 and EU311202 while *C. andersoni* isolates were submitted with accession numbers EU311203 in GenBank.

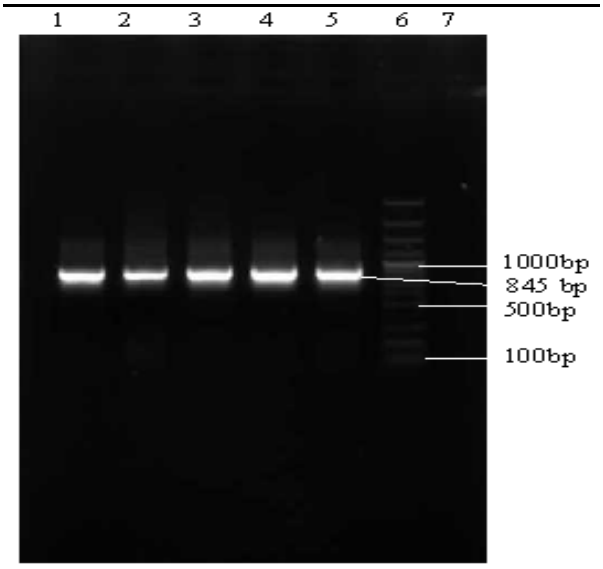

Figure 1. Nested PCR- Products of partial small subunit 18S rRNA gene of *Cryptosporidium* (845bp) isolated from sheep as visualized on 2% agarose gels (stained with ethidium bromide) in UV light : Lane 1 to 5 *Cryptosporidium* positive samples; Lane 6 DNA size marker (100 bp Ladder); Lane 7 negative control.

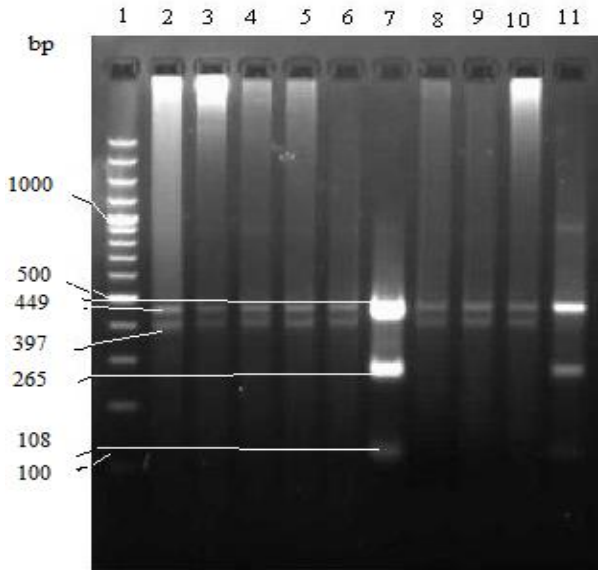

Figure 2. RFLP pattern of PCR product of partial small subunit ribosomal RNA (18S rRNA) gene of *Cryptosporidium* (845bp) with Ssp1 restriction enzyme as visualized on 2% agarose gels (stained with ethidium bromide) in UV light: Lane 1- DNA size marker (100 bp Ladder); Lane 2-6 and 8-10 Ssp1 enzyme digestion pattern (448bp, 397bp) represented *C. andersoni*; Lane 7 & 11 Ssp1 enzyme digestion pattern (449bp, 265bp & 108 bp) represented *C.parvum*.

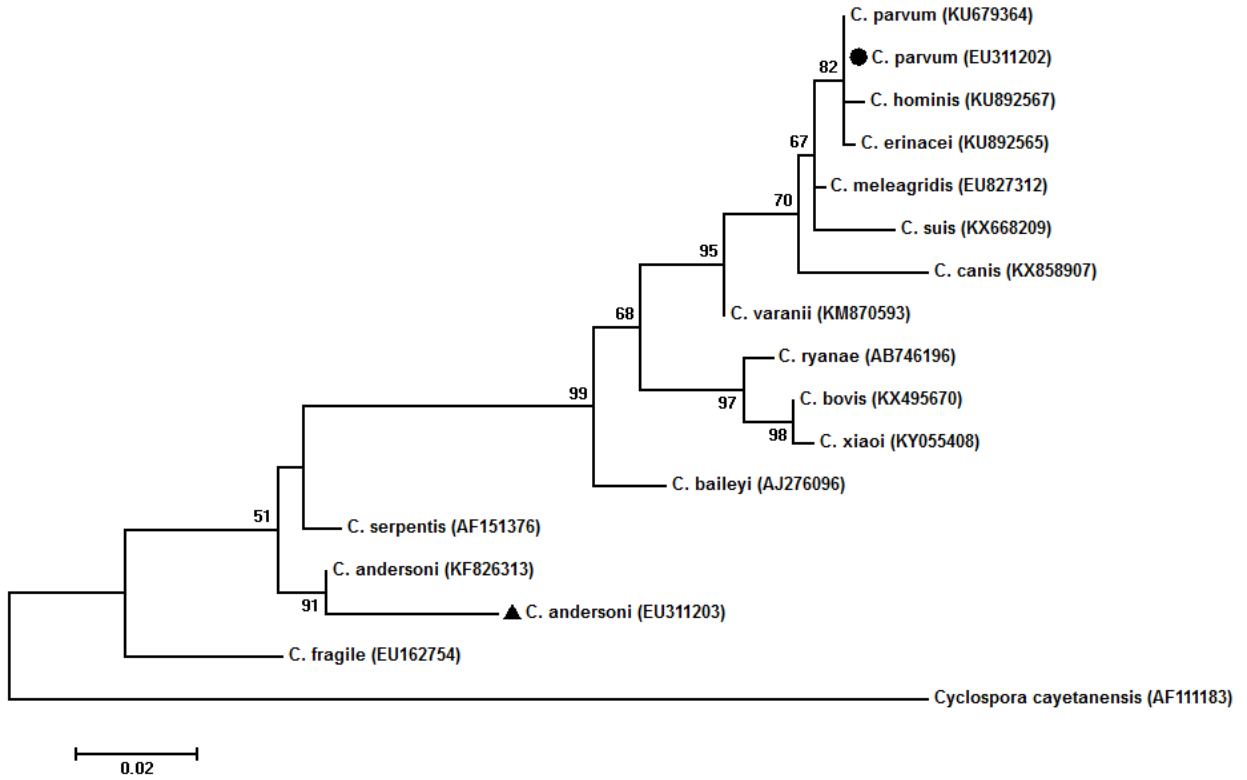

Figure 3. Phylogenetic tree of ●*Cryptosporidium parvum* (781 bp, GenBank: EU311202) and *Cryptosporidium andersoni* isolated from sheep (688 bp, GenBank: EU311203) based on partial small subunit ribosomal RNA (18S rRNA) gene sequence.

The evolutionary history was inferred using the neighbor-joining method, supported by 500 bootstrap replicates. The numbers above the branches indicate the percentage of bootstrap samplings. Samples isolated in the present study were compared with the isolates selected from GenBank.

5. Discussion

In the last decade, in order to provide a better understanding of the complicated biological and metabolic activities of *Cryptosporidium*, the complete genome sequence of *C. parvum* has been obtained and analysed (10). The 9.1Mb (megabase) genome of the parasite is distributed on eight chromosomes containing an estimated of 3807 genes. Information on host range and other phenotypic characters that has currently been acquired from different parts of the world supports the taxonomic validity of 14 species (1). In addition to these species, a large number of genetically distinct variants or genotypes have also been described, which were appeared to be host adapted and are thought to represent distinct species (1).

According to the results obtained from different parts of the world, *C. parvum* and a novel *C. parvum* genotype are responsible for infections in sheep and goats (9, 11-13). In a study conducted by Majewska et al. (2000), *C. parvum* was detected in 16 out of 159 sheep (10.1%) in the west-central region of Poland (14). The results of Mueller-Doblies et al.'s (2008) (15), Chalmers et al.'s (2002) (9), Elwin and Chalmers's (2008) (16) studies showed that *C. parvum* was significant in neonatal lambs diarrhea and prevalent in sheep flocks in the UK; however, other *Cryptosporidium* species (*C. bovis* and *C.* cervine) and genotypes were also present. In Yang et al.'s (2009) study at the 18S locus, 66 *Cryptosporidium* positive samples were detected, the majority of which were *C. bovis*, followed by *C. parvum* and the *C.* cervid genotype (17). In China, Wang et al.'s (2010) SSU rRNA-based PCR study identified two *Cryptosporidium* species and one genotype, including *C. xiaoi* (4/82), *C. andersoni* (4/82), and the *C. cervine* genotype (74/82), respectively while no *C. parvum* was detected (8).

In Spain, two species of *C. bovis*-like (18) and *C. parvum* (19) were reported; in Belgium, based on SSU rRNA and HSP70 genes, cervine genotype and *C. parvum* were reported (20); in addition, in Italy, *C. parvum* (21); in Tunisia, *C. bovis* (22); in the USA, (Maryland) *C. parvum*, cervine genotype, and *C. bovis*-like (23); in Australia, *C. bovis*, *C. fayeri*, *C. hominis*, *C. suis*, *C. andersoni*, cervine genotype, pig Genotype II, sheep genotype (24), as well as *C. parvum*, *C. bovis*, and cervine genotype (17) were reported.

The natural infection of *C. andersoni* in sheep has not been described previously. Although it is unique to the abomasum of cattle, but our results showed that 90.90% of the samples were *C. Andersoni*, and the rest (9.10%) were *C.parvum*.

The typical host of *C. andersoni* is cattle (*Bos taurus*), but *C. andersoni* can also infect the bobak marmot (*Marmota bobac*), Bactrian camel (*Camelus bactrianus*), and European wisent (*Bison bonasus*) (25). *C. andersoni* and *C. muris* (bovine isolate) are not found to be infective for laboratory outbreed, inbreed, immunocompetent and immunodeficient mice and rats. They are also non-infective for common and bank voles, rabbits, goats, and guinea pigs (26-29).

6. Conclusion

In spite of other studies results reporting *C. parvum* as the major agent of cryptosporidiosis in sheep, in our study, *C. andersoni* was found to be dominant.

Conflict of interest

All authors declare that they have no conflict of interest.

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Authors' Contribution

All authors contributed equally in this research.

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