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 Cryptosporidium andersoni was amplified by Nested-PCR in order to differentiate between the species. This protocol maintains a broad variety 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.

2. The genotyping protocol proposed by Xiao et al. (1999) 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 maintains a high level of sensitivity and has been applied by some investigators in different parts of the world.

3. Cryptosporidium andersoni was found to be dominant.

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

1. Background

Cryptosporidium is considered as a major cause of diarrheal disease in humans, livestock, and other animals throughout the world, and as a major economic problem on the water industry. This protozoan has been reported in over 150 mammalian species, mostly as C. Parvum and C. parvum infections.

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 cryptosporidiosis infections.

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The C. parvum infection in sheep was first reported by Meuten 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 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 1600g. The floated materials containing the oocysts were washed with deionized water; the oocysts were resuspended in 1mL deionized water and stored at 4°C prior to use.

The preservative was removed by resuspending the pellet in 10mL of 55% sucrose, mixed it for 1 min, centrifuged at 1000g 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 fecal samples.

3.5. PCR amplification

A three-step nested PCR protocol was used to amplify the 18S rRNA gene from the genomic DNA. In the primary PCR amplification, a forward primer (Cr18PA: F-5’-TTC TAGGCTAGTACGGC-3’) and a reverse primer (Cr18PB: R-5’-CCATTTCTCTGCACAGGA-3’) were used, and in nested PCR amplification, a forward primer (Cr18NA: F-5’-GGAGGTTGTATTTATAGTAAAG-3’) and a reverse primer (Cr18NC: F-5’-TTTAGACGTTAGGATTTG-3’) and a reverse primer (Cr18ND: R-5’-CTCTTTAGAGGACTTGTG-3’) were used for C. parvum as well as a forward primer (Cr18NC: F-5’-TTTAGACGTTAGGATTTG-3’) and a reverse primer (Cr18ND: R-5’-CTCTTTAGAGGACTTGTG-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), 1 µM 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 template 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 30 µL 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 37°C, 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 electrophoresed 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 fecal 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 electrophoretic 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 at 449, 461, 472, and 580 sit produces and produces 5 bands of 449bp, 12bp, 11bp, 108bp, and 260bp 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.
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. xiaoii (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 outbred, inbred, 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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