Molecular identification of Cryptosporidium andersoni *in healthy and in cattle with diarrhea of Mashhad, Northeast of Iran*

Alireza Sadrebazzaz

Razi Vaccine & Serum Research Institute, Agricultural Research, Education and Extension Organization, Mashhad, Islamic Republic of Iran

[*]Corresponding author at: Razi Vaccine & Serum Research Institute, Agricultural Research, Education and Extension Organization, Mashhad, Islamic Republic of Iran.
E-mail: a.sadr@rvsri.ac.ir.

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Summary
Cryptosporidium is an intracellular and extracytoplasmic protozoan that belongs to the phylum Apicomplexa. In this observational study, fecal samples were randomly collected from 800 dairy cattle, in 10 industrial dairy farms in Mashhad, Iran (from 2011 to 2015 years). The presence of *Cryptosporidium* oocysts was determined by modified cold Ziehl-Neelsen's staining. Results of microscopy observation showed that 23 samples (2.87%) were positive for *Cryptosporidium* oocyst. Twenty two samples were confirmed by PCR. The identification of *Cryptosporidium andersoni* was determined by restriction digestions of PCR products, using *SspI*, *VspI*, and *DdeI* enzymes. Differences between healthy and diarrheic groups as well as between age groups were not observed.

Introduction

Cryptosporidium is an intracellular protozoan parasite of a phylum of Apicomplexa. Ernest Edward Tyzzer first identified and reported the parasite, found frequently in the gut of the laboratory mice (Tyzzer 1907). This parasite causes acute and self-limiting gastroenteritis in humans. The parasite is detected in healthy individuals whereas persistent and fatal infection can be observed in immunocompromised individuals. It is estimated that millions of cases of disease occur annually in developing and developed countries (Putignani and Menichella 2010). Moreover, this parasite has been reported as one of the most common pathogens in human intestine (Mendonca *et al.* 2007, Paul *et al.* 2009).

The genus *Cryptosporidium* includes a variety of species found in many domestic animals and human (Dillingham *et al.* 2002, Fayer and Ungar 1986, Kvac and Vitovec 2003, Mendonca *et al.* 2007, O'Donoghue 1995). This protozoan often detected in calves, lambs, piglets, horses, puppies and kittens, chicken and turkeys, with diarrhea. This study aimed to establish *Cryptosporidium* prevalence and species identification in dairy cattle farms located in Mashhad, Northeast of Iran.

Materials and methods

Sampling

In this study, a cross-sectional study with single random sampling was done. A total of 800 stool samples were collected from healthy and diarrheal (from one month before the beginning of testing sampling) Holstein cattle in the city of Mashhad, within different age groups (less than 6 months, between 6-18 months and more than 18 months) from 2011 to 2015. Individuals with diarrhea among those showing diarrhea for a month before sampling.

Direct microscopic detection

The specimens were stained by modified Ziehl-Neelsen method and observed with a microscope (100X). Samples were then stored in a freezer at - 20 °C.

DNA extraction

Genomic DNA was extracted with two different procedures, a standard phenol-chloroform procedure [H.E. McKiernan, P.B. Danielson, in

Molecular Diagnostics (Third Edition), 2017] and a Nucleospin® Tissue Kit (MN), following manufacturer's instructions, from 23 positive samples by modified Ziehl-Neelsen method.

PCR-restriction fragment length polymorfism (RFLP)

Cryptosporidium oocysts were identified at species level using a nested PCR of 18S rRNA gene followed by RFLP (Xiao *et al.* 1999).

Primary PCR

DNA from positive samples was amplified using the protocol described by Xiao and colleagues (Xiao *et al.* 1999).

The PCR master mix was prepared by using the AccuPower® PCR PreMix kit (Bioneer, Korea). The PCR master mix reaction was prepared according to kit instructions in 40 µl total volume by adding 1 µl of purified genomic DNA and 1 µl of 10 pmol/µl of F1 and 1 µl of 10 pmol/µl of R1 and briefly mixed.

The following thermal profile was used: denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 45 seconds, extension at 72 °C for 1 minute, and then a final extension at 72 °C for 7 minutes. Then, PCR products were loaded on 1.5% agarose gel stained with SYBR safe, and electrophoresed. The bands were visualized by UV and photographed using the gel Doc system.

Secondary PCR

By using published primers (Xiao *et al.* 1999) the following thermal profile was used: initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of denaturation 94 °C for 45 seconds, annealing at 58 °C for 45 seconds, extension at 72 °C for 1 minute, and then a final extension at 72 °C for 7 minutes.

RFLP

A total volume of 10 µl of the secondary PCR product was digested for 2 h at 37 °C with 10 U of each enzyme, *SspI*, *DdeI* and *VspI* (Fermentas) in 32 µl following manufacturer's instruction. The digested products were separated on a 2% agarose gel electrophoresis using SYBR safe staining and photographed by UV transilluminator.

Statistical analysis

The chi-square test was used to evaluate significant differences ($P < 0.05$) by Statistix for windows, Trial version 9.0 (Analytical software, Tallahassee, FL 32317, USA).

Results

No differences have been observed between the two extraction methods in terms of final DNA concentration.

Results of microscopic tests

23/800 samples were positive for *Cryptosporidium* oocysts. Analysis according to health status and age is reported in Table I and II.

PCR-RFLP data

Out of 23 samples tested positive by ZN, 22 were correctly amplified by the adopted nested PCR. The PCR products were then digested with the *SspI* and demonstrated similar products (385 bp and 448 bp); with the *VspI* enzyme showing two bands of 731 bp and 102 bp, and with the *DdeI* enzyme showing three bands of 470 bp, 186 bp and 156 bp. All the 22 samples were identified positive for *Cryptosporidium andersoni* (2.75%).

Statystical analysis

No significant difference was found between age groups, and between healthy and diarrheal cows, using Chi-square test (overall $x^2 = 0.07$, p-value = 0.7847, d.f. = 1). It can be said that *Cryptosporidium andersoni* causes the same infection in all age groups (overall $x^2 = 1.27$, p-value = 0.5292, d.f. = 2) .

Table I. *Prevalence of* Cryptosporidium *spp. infection in cattle by age in Mashhad, Northeast of Iran.*

Age group (months)	N. of cattle	*Cryptosporidium* spp. positive	Prevalence (%)
≤ 6	220	6	2.72
6-18	200	8	4
≥ 18	380	9	2.36
Total	800	23	2.87

Table II. *Prevalence of* Cryptosporidium *spp. infection in healthy and diarrheic cattle in Mashhad, Northeast of Iran.*

Group	N. of cattle	*Cryptosporidium* spp. positive	Prevalence (%)
Healthy	500	15	3
Diarrheic	300	8	2.66
Total	800	23	2.87

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Nucleotide sequence accession number

One of the sequences used in this study has been deposited in the GenBank database under the accession no. MH395839.

Discussion

Molecular techniques for the detection and differentiation of *Cryptosporidium* oocysts, along with conventional methods such as condensation and staining of stool specimens have significantly increased our understanding of the parasite dispersion and epidemiology. Selection of molecular targets for species identification of the parasite should be performed appropriately, as the parasite genome plays an important role in the interpretation of the information obtained by the PCR method and real-time PCR (Burnet *et al.* 2013, Morgan *et al.* 1995). Molecular tools for *Cryptosporidium* identification at species level can provide valuable information about the detection of the protozoa in different hosts, and help to recognize the epidemiology of Cryptosporidiosis (Blears *et al.* 2000, Fayer and Xiao 2008, Fiuza *et al.* 2011, Ondrackova *et al.* 2009, Xiao *et al.* 2001).

According to the present study, from 800 samples from dairy farms in the city of Mashhad, 23 samples were positive for *Cryptosporidium* by direct microscopy. Of these 23 samples, using the PCR-RFLP procedure, as a very efficient, relatively simple and cost-effective method, 22 samples were positive for *Cryptosporidium andersoni*. These results confirmed the contamination rate of cows in the city of Mashhad and *Cryptosporidium andersoni* as dominant species.

It is worth noting that, due to the successful DNA extraction from 22 samples out of 23 positive samples, the manual DNA extraction method and the MN kit have a same results for DNA purity and concentration using a NanoDrop spectrophotometer and can be used as suggested methods in laboratories for veterinary and medical use.

The rate of *Cryptosporidium* infections in this study was consistent with the contamination rate found in China, 5.12%, in Sweden 1.8%, in Wales 6.9% and in Japan 1.5%, in India 12.85%, Brazil 5.88%, Portugal (4.5% in adult cattle), Western Czech Republic (4.1%) (Fiuza *et al.* 2011, Koyama *et al.* 2005, Kvac and Vitovec 2003, Mendonca *et al.* 2007, Ondrackova *et al.* 2009, Paul *et al.* 2009, Robinson *et al.* 2006, Silverlas *et al.* 2010, Wang *et al.* 2011).

The consistency of the results of the above-mentioned studies with our data suggests that *Cryptosporidium andersoni* has a relatively high rate in animal contamination in different countries.

In Iran, similar contamination rate was found in different cities, such as Isfahan, Tehran, Tabriz, Kerman and Ahvaz (Fotouhi Ardakani 2008, Nouri *et al.* 1995, Nouri 2003).

According to other studies and considering the role of *Cryptosporidium andersoni* in cattle industry prevention methods should be considered.

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