Original Article

Microscopic, Molecular and Antigen Detection and Isolation of *Cryptosporidium parvumParasites in Diarrheal Disease of Calves* in Iran

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ABSTRACT

Cryptosporidiosis is a parasitic disease caused by the protozoan *Cryptosporidium* in vertebrates. In livestock, especially ruminants, infants develop diarrheal syndromes. The infection is common worldwide , including Iran, where it is reported in several species. Morphological diagnosis of *Cryptosporidium* species is associated with many limitations and has no taxonomic value on its own, so the use of molecular methods can overcome these limitations to some extent. The present aims at microscopic, molecular and antigen detection and isolation of *Cryptosporidium* parvum parasites. Firstly, 300 samples were collected from different parts of Iran. Subsequently oocysts from feces were purified by the method of Casemore *et al.* using the flotation technique and stained by the modified Ziehl-Neelsen method (Henriksen method) and identified by diagnostic keys. ELISA test was also performed on the samples with results ranging from 1 to 4 positive. The results of our study show that, of the 300 cases tested for Cryptosporidium, 48 cases (16%) and 54 (18%) were positive in ELISA and PCR, respectively. Microscopic evaluation also mainly confirmed the ELISA results. These cases were collected in summer, autumn, and winter, with, more than 50% of the positive cases found among the samples collected in autumn. In addition, 54 positive cases were found by PCR test, which is 6 cases more than ELISA results. Finally, the results of PCR detection and ELISA were subjected to chi-square analysis, where no significant difference was found between the collected data (p=0.0587).

Keywords: *Cryptosporidium*, ELISA, Molecular Identification, Microscopic *Examination*, *Parvum*

1. Introduction

Cryptosporidium parvum is an enteric protozoan parasite that causes gastrointestinal disease and diarrhea in a variety of animal species, including cattle, sheep, and also humans (1). The parasite has a monoxenic cycle, meaning that it completes its life cycle in a single host, reproducing alternately asexually and sexually (2). Cryptosporidiosis was first detected in Iranian cattle in 1971and 1984(1, 2, 3)and increasingly in a variety of hosts (3). Cryptosporidial infections are common in calves and can cause severe diarrhea, lethargy, anorexia, and dehydration, which can result in significant economic losses, especially because of reduced weight gain and the use of palliative and preventive treatments (4). Diarrhea, also known as calf diarrhea, is a diarrheal disease that primarily affects calves in the first few days of life (5). Claves that survive diarrhea have been shown to be more susceptible to other diseases, especially bovine respiratory disease, and often have growth retardation and poor performance in the first lactation (6). As a major cause of morbidity and mortality in dairy calves, neonatal calf diarrhea also raises serious concerns because of the overuse of antibiotics, which could lead to a potential increase in antibiotic resistance(7). Infected animals excrete oocysts of *C. parvum* in their feces, which transmits the disease Millions of these oocysts are transmitted during the first two weeks after the infection, and the presence of oocysts in feces indicates infection.(8, 9) Approximately half of dairy calves between 1 and 3 weeks of age actively excrete oocysts at any given time(10). The presence of oocysts in the feces is an indication of infection. Currently, 26 species are recognized as valid based on morphological, biological, and molecular data(11). There are 4 main species of Cryptosporidium in cattle, including Cryptosporidium parvum, Cryptosporidium Bovis, Cryptosporidium andersoni, and Cryptosporidium ryanae(12). In Iran, calf losses are 12-20% (average 16%), of which about 75% are due to diarrhea syndrome. Of these 75% deaths, about 16% are due to diarrhea syndrome, and the parasite is one of the most important protozoa causing diarrhea(13). The parasite is not specific and is considered one of the new disease common to humans and animals. This problem and the lack of effective treatments, on the other hand, double the importance of this disease (14). Although the cattle industry has made remarkable improvements in herd management, animal facilities and care, feeding and nutrition, and timely use of biopharmaceuticals, calf diarrhea remains problematic because of the multifactorial nature of the disease. Prevention and control of calf diarrhea should be based on a proper understanding of the complexities of the infection, such as multiple pathogens, coinfection, environmental variables, feeding, and management during the calving season before disease onset (15).

2. Materials and Methods

2.1. Collection and preparation of fecal samples

Initially, 300 fecal samples were collected from calves less than 75 days of age with diarrhea history from Tehran, Alborz, Qazvin, Kermanshah and Central provinces over a period of six months period. Rectal sampling was performed using disposable gloves. The characteristics and history of the herd and sampled calf ,including age, sex, clinical signs, etc. were recorded. Subsequently, the samples were stored at -20 °C. Purification of oocysts from feces was performed by flotation using saturated sugar or salt. Finally, following the method of Casemore et al. (16), a smear was prepared from direct fecal samples and concentrated oocyst samples and stained using the modified Ziehl-Neelsen staining method.

2.2 Live storage of oocysts

To store possible oocysts alive or in case of oocyst concentration and oocyst detection, the same volume of potassium dichromate 2.5% is added to the feces, and the oocyst suspension is stored in the refrigerator at 4 °C.

2.3 Diagnosis

Diagnosis of infection with this protozoan in cattle is based on isolation of Cryptosporidium oocysts and Ziehlneelsen staining of the feces. In the present study, the formal ether or sucrose suspension method was also used to concentrate possible oocysts in stool samples. Then, the prepared slide was stained by the modified Ziehl-Neelsen method. Finally, it was examined under a 100x light microscope according to the observation criteria of unicellular red oocysts with an approximate diameter of 3µm to 6µm.

2.4 ELISA

2.4.1 Preparation of samples for ELISA

First, a 10% suspension of the stool samples in phosphate buffer saline (PBS)was prepared. The samples were then homogenized before 200 µl of supernatant was collected

and centrifuged at 3000 rpm for 15 to 20 min at 4 °C. Finally, the supernatant was used for ELISA test.

Table 1: Cryptosporidium species, vertebrate hosts, type and location of disease and size of oocysts

Cryptosporidium species	Vertebrate hosts	Site of infection	Disease	Oocyst size
C. andersoni	cattle	gastric	chronic	7.4 x 5.5µm
C. baileyi	chickens	enteric, respiratory	acute	6.2 x 4.6µm
C. canis	dogs, humans	enteric	acute	5.0 x 4.7µm
C. fayeri	red kangaroo	enteric	?	4.9 x 4.3µm
C. felis	cats, humans	enteric	acute	5.0 x 4.5µm
C. galli	chickens	enteric	?	8.2 x 6.3µm
C. hominis	humans	enteric	acute-chronic	4.9 x 4.3µm
C. macropodum	eastern grey kangaroo	enteric	?	5.4 x 4.9µm
C. meleagridis	turkeys, parrots, humans	enteric	acute	5.2 x 4.6µm
C. molnari	fish	gastro-enteric	chronic	4.7 x 5.4µm
C. muris	mammals (mice, cats, humans)	gastro-enteric	chronic	7.4 x 5.6µm
C. nasorum	fish	gastro-enteric	chronic	4.3 x 3.2µm
C. parvum	mammals (humans, cattle, sheep, goats, horses, pigs, mice)	enteric	acute-chronic	5.0 x 4.5µm
C. ryanae	cattle	enteric	?	3.7 x 3.2µm
C. saurophilum	lizards	gastric	chronic	5.0 x 4.7µm
C. serpentis	snakes, lizards	gastric	chronic	6.2 x 5.3µm
C. suis	pigs	enteric	acute	4.6 x 4.2µm
C. wrairi	guinea pigs	enteric	chronic	5.4 x 4.6µm

2.4.2 Sandwich (capture antibody ELISA) method

A sandwich ELISA kit(BIO K 346 bio-X diagnostic ® company, Belgium) was used to identify *Cryptosporidium* oocysts according to the instructions provided with the kit. The stool samples were added in equal parts to the dilution buffer and stirred for 10 min, then the supernatant was removed and aliquoted into 100 µl volumes. Sample number 1 was then added to wells A1 and B1, and sample number 2 was added to wells C1 and D1. Positive controls were added to wells G1 and H1 before the plate was incubated at 21 ± 3 °C for 1 h. In the next step, the plate was washed with the wash solution and the contents of the microtiter plate were quickly emptied into a dish containing a neutralizing agent. Then, 300 µl of washing solution was added and the microplate was emptied again, and all the above steps were repeated two more times. After the plate was washed three times, 100 µl of the

conjugate solution was added to each well. The plates were incubated at a temperature of 21±3°C for 1 h. Then, 100 µl of the chromogen solution was added to each well and incubated again for 10 min at 21°C. The next step is to add 50 µl of stop solution to each well. Results were read immediately after addition of the stop solution using an ELISA reader. To interpret the absorbance rate results, the pure optical spectrum for each sample was calculated by subtracting the reading rate for each sample. In the same way, calculations were performed for the positive control samples. The test is valid if the positive control antigens give the same difference in light absorbance within 10 min that is greater than the value indicated on the insert.

2.5 Molecular identification

Molecular identification was performed by DNA extraction from oocysts of positive samples with specific primers branched from S-ribosomal RNA 18 of *C. parvum* by a semi-nested PCR method. The specificity of the *C. parvum* oocysts was confirmed by observation of the 845base pair fragment. The PCR mixture consisted of 5µL template DNA, 2.2 µL 10X PCR buffer, 2.4 µL MgCl, 2, 1.0 µL dNTPs, , 1.0 µL each of 10 pmol/L forward and reverse primers, 7.1 µL dH2O, and 0.3 µL Taq polymerase enzyme.

The sequences of the forward and reverse primers follow:

AWA72F: 5-AGTGCTTAAAGCAGGCAACTG-3

AWA1235R: 5- CGTTAACGGAATTAACCAGAC-3

The cycles used provided 40 cycles as follows: initial denaturation 96 C for 5 min, then (1) 95 °C for 30s; (2) annealing at 65 °C for 1 min; (3) extension at 72 °C for 3 min.

Gel Electrophoresis

A 2% agarose gel was prepared and then 12µl of sample was loaded with 10µlof the PCR product mixture and 2µl of loading dye along with 12µl of DNA ladder (100bp). The gel was run at a voltage of 90 volts for 40 min. The gel was then examined using a UV transilluminator. The specific DNA amplification product of each sample was determined by identifying 556-bp bands for C. parvum compared to the DNA ladder.

2.6 Direct Sanger sequencing of the samples

To confirm the specific bands belonging to *Cryptosporidium*, a sample of the PCR products that showed bands in electrophoresis was sent to NoorGenCo. Subsequently, 20µl of a positive PCR product with forward primer (*AWA72F*) was sent for direct Sanger sequencing.

2.7 Blast analysis (similarity search)

After reviewing the chromatogram of the sequenced sample, the obtained nucleotide sequence was aligned with the NCBI reference database (https://www.ncbi.nlm.nih.gov/) to confirm its similarity to the *Cryptosporidium* parasite.

2.8 Preparation and isolation of the antigen mixture

After the positive samples were confirmed and identified by the molecular method, they were first mixed and the oocysts were purified as much as possible by the flotation method. Then, the obtained suspension containing oocysts was processed to obtain sporozoite antigens using two methods: 1.melting and freezing in liquid nitrogen and at laboratory temperature, 2.treated sonication. The resulting suspensions are then be frozen in two forms (containing 10% glycerol) at a temperature of -70 °C and in formalin to be used for immunization and production of hyperimmune serum for farm animals in the next studies.

2.9 Statistical analysis

Finally, the obtained results were subjected to statistical analysis using SPSS 18 software. In addition, the relationship between the data on age, sex, season, geographical area, etc. and the incidence of cryptosporidial diarrhea was analyzed.

2.10 Staining according to the modified Ziehl-Neelsen method (Henriksen method)

The modified cold Ziehl-Neelsen method, first introduced by Henriksen and Pohlenz(17), is commonly used for staining Cryptosporidium oocysts.

1. Preparation of the fecal smear on the slide
2. Drying at room temperature
3. Fixation with 96% methanol for 2 to 6 min
4. Drying at room temperature
5. Short fixation with heat
6. Staining with thick carbolic fuchsin for 20 to 60 min
7. Complete washout and slight decolorization with 1% hydrochloric acid in 96% ethanol for 10 to 15 sec
8. Washing of the slide with water
9. Stain with malachite green 0.5% for 30 sec
10. Washing with water

2.10.1 Findings

For the initial diagnosis, smears are first examined with a 40x objective of a microscope and suspicious cases with a 100x objective with oil immersion; if even one oocyst is detected in the smear, the specimen is considered positive. For final diagnosis and confirmation of oocysts and observation of sporozoites, examine all positive smears with a 100x lens. To classify the intensity of contamination, the number of oocysts in 3 to 5 microscopic fields. Based on the average number in a microscopic field and with a 40x objective lens, the average contamination intensity (+), (++), (+++) was expressed as follows:

1. If 1 to 4 parasite oocysts are observed in each (+) field, the infection is said to be mild.
2. If 5 to 25 parasite oocysts are observed in each field (++), the infection is termed moderate.
3. If more than 25 parasite oocysts are observed in each field (+++), the infection is designated as severe.

Usually, the result is announced by observing the parasite in 3 to 5 microscopic fields and calculating its average. The presence of more than 25 oocysts in each visual field is often associated with clinical symptoms, and if more than 20 oocysts are counted in each microscopic field in a calf with diarrhea or the number of eggs is oocysts per gram of stool, the diarrhea may be considered cryptosporidial.

3. Results

3.1. Isolation and identification of *C. parvum*

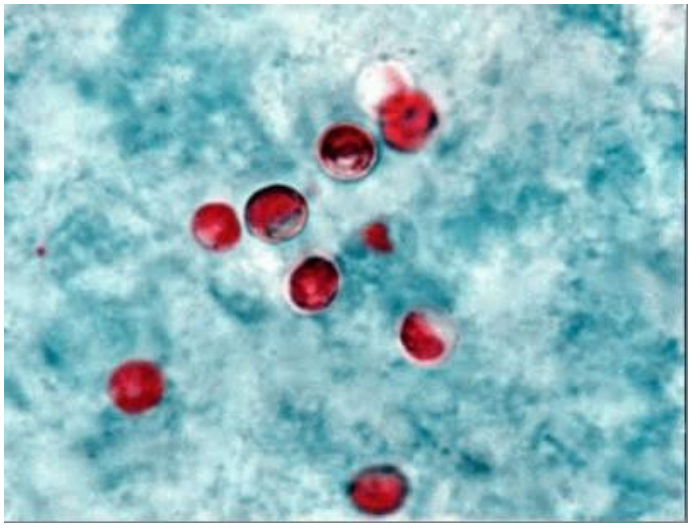

Figure 1. The positive sample identified in modified Ziehl-Neelsen staining

The oocysts of the parasite are very small (about 6 microns) and 22 microns in birds (Figures 1 and 2).In Ziehl-Neelsen staining, the oocysts are seen to be ovoid or spherical on a green ground containing numerous granules, which are sporozoites. 4 naked sporozoites, which are not in the sporocyst, are contained in the parasite oocyst.

3.2. ELISA Test Results

Out of 300 samples, 48 cases (16%) were positive for Cryptosporidium by sandwich ELISA method (capture antibody). These positive cases were observed in Varamin, Qazvin, and Kermanshah cities. Samples from the cities of Qom, Hamedan, Islamshahr, Robat Karim, and Shahriar were negative. Positive cases were observed in all three seasons (summer, fall, and winter), with most cases (about half of the positive material) occurring in the fall season. Thus, the disease is more common in the cold seasons. There were no sex differences between men and women (P> 0.05).

3.3. Molecular identification

3.3.1. PCR molecular test results

The size of the amplified fragment after PCR against the 18S rRNA gene of C. parvum was 556 base pairs (bps). In PCR evaluation, 54 positive samples (18%) were reported, and 6 samples (2%) differed from ELISA results, showing that the PCR method is more sensitive than ELISA in detecting positive cases of Cryptosporidium. The results show that the PCR method (16% vs. 18%)is more sensitive than the ELISA method.

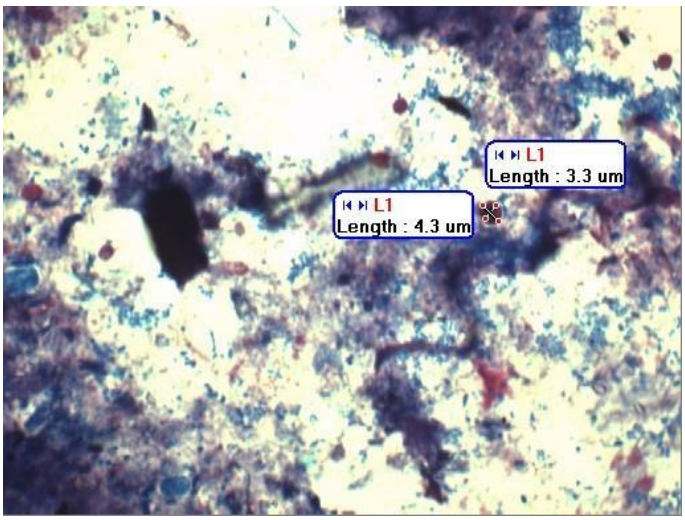

Figure 2. Samples measured with an optical microscope

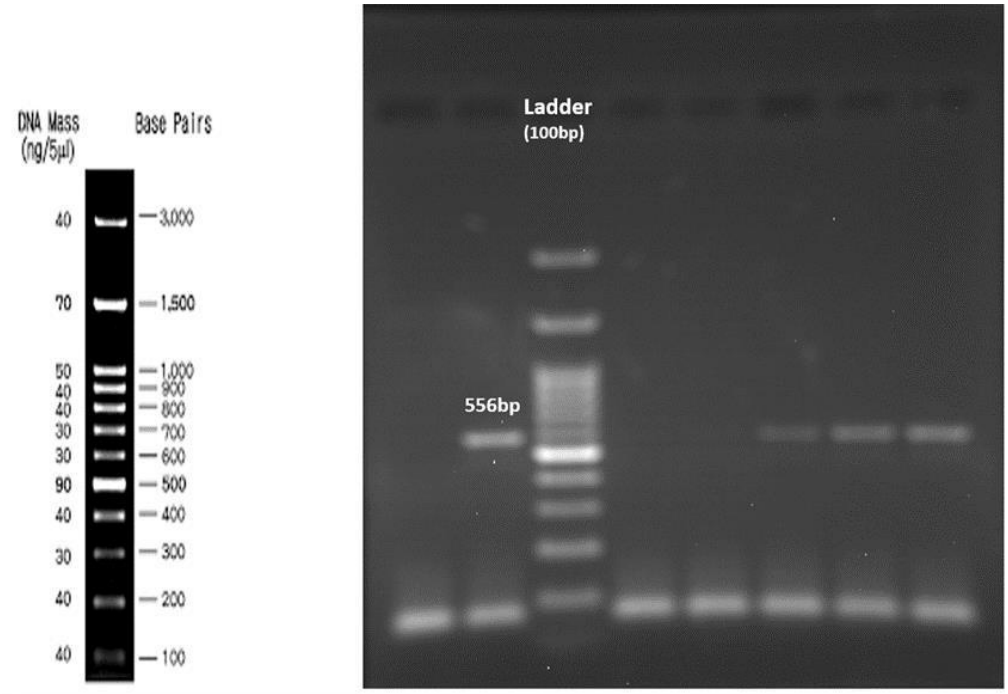

Figure 3. Electrophoresis results of some positive samples in PCR testalong with 100bp ladder.Gel electrophoresis shows 256-bp of the amplicon size targeting of 18S rRNA Cryptosporidium in positive samples. Lanes (from left to right): 1. Negative control of *C. parvum*; 2. Positive control of *C. parvum* ; 3. 100bp ladder; 4. Negative sample; 5.Negative sample; 6,7,8. Positive samples.

Moreover, th Blast results show 100%similarity of nucleotide sequence 550~ with the 18S rRNA gene of C. parvum. Thus, the band visualized was confirmed for *C. parvum*.

4. Discussion

The Cryptosporidium parasite is an enteric protozoan that is a major cause of diarrheal disease in both animals and humans(11). Cryptosporidiosis has been found in several species of domestic and wild mammals, birds, and rarely in reptiles and fish. In mammals, two species of Cryptosporidium, namely *C. parvum* and *Cryptosporidium andersoni*, of importance. Cryptosporidiosis has been reported from different parts of the world and it can be said to be a global disease (2). Diarrhea in calves is one of the major factors in deaths (18), productivity losses and loss of animal protein resources. Economic losses are caused by mortality, emaciation, treatment costs, and decrease in breast milk (19). In Iran, calf loss is 12-20% (average 16%), of which about 75% is due to diarrhea syndrome. One of the most important protozoa causing diarrhea is the parasite Cryptosporidium. It is not specific and is considered one of the new disease common to humans and animals. This problem and the lack of effective treatment make it all the more important to deal with this disease (14). In Iran, according to the studies conducted by Maleki et al. (20)in 2007 in Khorram Abad city, the level of infection in cows and calves with and without diarrhea was the same in four seasons. According to this study, in the population of animals without diarrhea, adult cows had the highest level of infection at 23.75%, while in the population of animals with diarrhea, calves had the highest level of contamination at 21.8%. Thus, the percentage of infections in the above animal populations in Khorramabad was 17.5%. MokhbarDezfuli and Meshki(21)reported 11% infections in the studied cow population without diarrhea in 2013. They also reported 20% of infections in calves under 3 months of age with diarrhea and 24% of infections in calves under 3 months of age without diarrhea. In 2013, Lotfollahzadeh et al. (22)reported 22.8% of contamination in calves under one month of age with diarrhea in Qaimshahr and Babol. In 2013, Rezazadeh et al. (23)reported 2.1% contamination in calves with diarrhea and 0% in apparently healthy calves in a dairy near Tehran. In 1999, Mohabali et al. (24) reported that the infection rate in apparently healthy calves in cattle farms in Islamshahr was 12.7% .

Table 2. The length and width of the oocysts observed in the studied stool samples

	Length (µm)	Width (µm)
1.	4.6	4.5
2.	4.6	3.8
3.	4.9	4.6
4.	3.4	3
5.	3.6	3.5
6.	5.0	4.4
7.	4.6	4.2
8.	4.7	4
9.	4.5	3.6
10.	3.8	3.6
11.	3.9	3.4
12.	3.9	3.0
13.	3.8	3.1
14.	4.0	3.2
15.	4.4	4.3
16.	3.9	3.8
17.	4.0	3.8
18.	4.3	3.6
19.	4.4	4.3
20.	4.2	3.7
21.	4.6	3.7
22.	4.9	4.2
23.	4.5	3.8
24.	4.5	3.9
25.	5.1	4.5
26.	4.4	4.1
27.	4.8	4.4
28.	4.4	4.3
29.	3.9	3.2
30.	4.9	3.2
31.	4.7	3.1
32.	4.0	3.2
33.	4.7	4.1
34.	4.1	3.5
35.	4.4	4.1
36.	4.5	3.9
37.	4.7	4.0
38.	3.9	3.5
39.	4.7	4.1
40.	3.3	3.1

In our previous study, Karimi et al. (13)conducted a research study in 2018 which showed that out of 150 samples of diarrheal feces collected randomly in Alborz province, 60 samples were positive and 40% had contamination. No parasites (oocysts) were detected in all 50 stool samples from calves without diarrhea. In this study, stool samples from calves without diarrhea showed no parasites (oocysts), and calves less than one month of age showed 40% contamination, which appeared to be *C. parvum* ,especially in the first two weeks. Moreover, our results (16 % by ELISA and 18% by PCR methods) in Tehran, Alborz, Qazvin and Central provinces by two methods of capture antibody ELISA and PCR showed lower prevalence compared to other reports (Jokar, et al., 2021, Safavi, et al., 2011, Radfar et al. 2006). This result is in agreement with some similar studies that showed that the prevalence of C. parvum ranged from 14.1% to 44.4% in different countries (Jokar, et al., 2021, Safavi, et al., 2011). Since infection in calves was relatively high, these animals could be a likely reservoir of infection for humans (as a zoonotic disease) in this area. In addition, further studies are needed to determine the prevalence of the disease in different provinces of Iran and the source of infection in order to control and prevent it.

Acknowledgment
Not Applicable

Authors' Contribution
K.,GH.R and R.,M.M planned and designed different phases of the study based on the general infectious disease megaproject in calf diarrhea. Microscopic investigations, isolation of the parasite and its identification, a part of the molecular method (PCR technique), as well as Persian writing of the diagnosis results were performed with the cooperation of K.,Gh.R and the Department of Parasitology (P.,H and A.G.,M). The serology test (ELISA) and some parts of molecular techniques were carried out by R.,M.M . Also, the English writing, editing, and submission of the article were done by their student D.B.,M .

Ethics
The paper reflects the authors' own research and analysis in a truthful and complete manner and the paper is not currently being considered for publication elsewhere. All of the experimental procedures involving animals were conducted in accordance with the institutional animal care guidelines of Razi Vaccine and Sera Research Institute, Karaj,Iran.

Conflict of Interest
The authors have no conflicts of interest to declare.

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