

Original article

An examination of *Cryptosporidium parvum* infection in neonate BALB/c mice and rats

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Abstract

Introduction and objective: *Cryptosporidium parvum* is an intracellular, extracytoplasmic, zoonotic, coccidian parasite that infects the microvillus regions of the digestive tract, and respiratory tract and causes lethal disease mainly among individuals suffering from immune deficiency and children. Suitable animal models are needed to identify different aspects of *Cryptosporidium* infection and the means of treating and preventing this infection. This study was conducted to represent a simple and usable animal model for *C. parvum* so that it can be used in the drug-related studies.

Materials and methods: Forty neonatal BALB/c mice and forty neonatal rats as group I (case) were inoculated with 5×10^5 oocysts. At the same time the animals in group II (control) received only sterile PBS solution. The intensity of infection was determined through the microscopic observation of the homogenized intestines of all the samples at 6, 9, 12, and 16 days of post infection.

Results: In mice and rats, the intensity of infection in group I (case) reached its peak on the 9th day after infection, then it reduced gradually and on day 16 post infection, it showed its lowest point. Meanwhile the non-infected animals in group II (control) had no detectable oocysts.

Conclusion: As the intensity of infection in both models showed a similar pattern; and because of rat physiological similarity to humans and easier handling and manipulation during the procedure the rat model was considered experimentally preferable.

Significance and impact of the study: This study introduces an indigenous laboratory animal model which can be used in cryptosporidium-related investigations and *in vivo* oocysts reproduction and it has no precedence in Iran.

Keywords: *Cryptosporidium parvum*; BALB/c Mice; Animal model

Introduction

Cryptosporidium parvum is a world-widely distributed protozoan parasite which can infect both humans and animals. *Cryptosporidium* oocysts are very resistant to most common disinfectants, and they can survive for a month in moist and cold condition. Oocysts of *C. parvum* are infective when released in the feces and they can transmit from one host to another. *Cryptosporidium* has recently been considered as water borne pathogen which can be transmitted by potable water and is a great concern in water industry in the developed countries. Person to person transmission has been demonstrated. It causes cryptosporidiosis the symptoms of which are a mild to severe diarrhea, abdominal cramps, weakness, loss of appetite and weight.

Outbreaks of cryptosporidiosis among children in nursery houses have been recorded, some cases of waterborne outbreaks have been reported, and even this organism is now considered as a cause of traveller's diarrhea [1]. The illness may last for 2-3 weeks, it then resolves self-limiting. However, cryptosporidiosis can be relatively severe and chronic in persons with impaired immune systems such as persons with HIV/AIDS, cancer patients, and transplant patients, and it can involve respiratory and biliary tract or even lead to death [2].

Companion animals such as rodents and puppies may serve as reservoir hosts. Live stock such as calves are a source of human infection. Juvenile rodents during the first and the third weeks of age may suffer from the cryptosporidiosis and it can cause a self-limiting gastroenteritis, and a noticeable oocysts shedding in the feces [3,4]. So far, various animal models have been examined for studying different aspects of *Cryptosporidium* species *in vivo*. Each model has its own merits and shortcomings.

The current study aims at presenting a simple, economical, available, and applicable animal model for *C. parvum* by which the pharmaceutical or prophylactic controlling agents can be easily evaluated.

Materials and methods

Cryptosporidium parvum oocysts were collected from the feces of some naturally infected diarrheic calves of one to two months of age from a local dairy in Islamshahr, a suburban town of Tehran. After preparing fecal smears, the modified acid fast staining method was used to detect oocysts. Positive samples were washed through a 100 mesh sieve with Tween water (0.1% Tween 80 in distilled water). This fecal suspension was centrifuged at 3000g for 10mins in a centrifuge tube, then the supernatant fluid was discarded, and the pellet was diluted in Tween Water.

Cryptosporidium parvum oocysts from fecal suspension were extracted by using water-ether sedimentation procedure and it was then purified by discontinuous sucrose gradients. Then, the purified oocysts were preserved in 2.5% aqueous potassium dichromate solution and stored at 4°C. Application of these two stage techniques helped to achieve much more purified oocysts suspension [5,6].

Animals

Fifty suckling BALB/c mice (3-4-days-old) and fifty suckling rats (3-4-days-old) along with their mothers were the animals experimented on and they were purchased from Razi Institute located in Karaj, Iran. To be secured against any kind of potential side infections of the setting, all the infants and their mothers were separately placed in some plastic cages with wire mesh lids and wood shavings for bedding and they were kept at 21°C in the animal house of the Tarbiat Modares University over night.

Both BALB/c and rat infants were randomly divided into two groups (group I and group II, experimental and control groups, respectively). The experimental (case) group consisted of forty suckling animals and the control group consisted of ten suckling animals. The purified oocysts of *C. parvum* before the inoculation were washed three times with PBS and then the number of oocysts was measured with a haemocytometer. One hour before inoculation, the infants were isolated from their mothers to empty their stomach for easier inoculation.

By using a ball-point gavage, the neonatal BALB/c mice and rats in group I were orally inoculated with 50 μ l oocysts suspension containing 5×10^5 oocysts on the same day after infection. Simultaneously, mice and rats in group II (=10) received 50 μ l sterile PBS. On the 6th, 9th, 12th, and 16th days of post infection ten mice and ten rats of group I and two mice and two rats of group II were euthanized and killed [7,8].

Determination of infection intensity

In this step, small and large intestines of each killed animal were removed and separately put in 2ml PBS. In the next stage, the intestines were homogenized with an Ultra-Turrax homogenizer (three times for 10 second) and the total volume of the resulting suspension was recorded. Then, 0.8ml of an aqueous solution of malachite green (malachite green, 0.16g; sodium dodecyl sulphate 0.1g; distilled water, 100ml) was mixed with 0.2ml of homogenized suspension. The number of oocysts per 1ml of suspension was enumerated by using a Neubauer haemocytometer. The mean count of oocysts per animal \pm SE was used to show the intensity of infection [7, 8].

Results

All the BALB/c mice and rats in group I which were inoculated between 4-5 days of age were infected while the intestines of animals in group II showed no detectable oocysts. This phenomenon indicated that the occurrence of incidental infection is negative. It is pointed out that some of the samples in group I (case) show the symptoms of diarrhea and a relative reduction in appetite. Results showed that neonate BALB/c mice in six days of post infection produced $(12.06 \pm 0.58)10^5$ oocysts per mouse. The severity of infection reached its climax on the 9th post infection day with $(53.8 \pm 5.27)10^5$ oocysts per mouse and then the number of oocysts per mouse gradually decreased from $(27.13 \pm 5.90)10^5$ on the 12th post infection day to $(8.95 \pm 0.41)10^5$ on the 16th post infection day (Fig. 1).

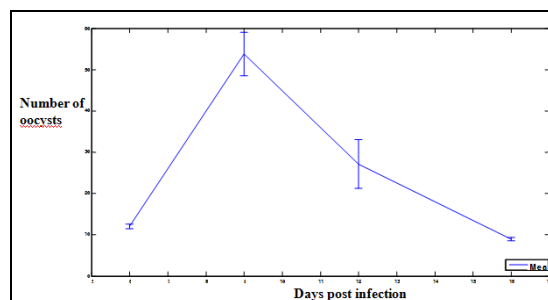


Fig. 1: Intensity of infection in BALB/c mice (Mean \pm SE) 10^5 , oocysts/mouse

A similar pattern of severity of infection was seen in neonate rats. On the 6th post infection day, the number of oocysts per rat was $(12.42 \pm 1.54)10^5$. The intensity of infection reached its peak with $(56.57 \pm 4.51)10^5$ oocysts per intestine on the 9th post infection day. Then a continuous reduction in oocysts production was seen and the number of oocysts per rat reduced from $(31.75 \pm 1.78)10^5$ on the 12th post infection day to $(10.22 \pm 0.48)10^5$ on the 16th post infection day (Fig. 2).

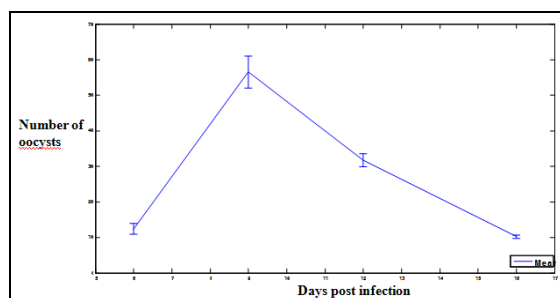


Fig. 2: Intensity of infection in Rats (Mean \pm SE) 10^5 , oocysts/rat

Discussion

Applying an animal model for the evaluation of anticryptosporidial agents has a long history and various sensitive rodent models have been developed for the last few decades [9]. Each animal model has its own advantages and disadvantages; however, most of the researchers have preferred to use the neonatal mouse or the immunosuppressed adult rat model [9,10]. Usually, neonatal rodents between one to six days of age are highly susceptible to *Cryptosporidium* infection. They remain clinically symptom free and shed oocysts for 10 to 14 days. Then, they recover and stop shedding of oocysts [11, 12]. In the light of these explanations, current study is trying to develop an appropriate simple and economical animal model which can be easily reached at and used in drug-related studies.

On the basis of the previous studies, the age of animals, the infectious dose, and the isolate of the organism and the technique by which the number of parasite is counted are the major factors which can affect the results of this kind of experiments. The following measures had to be adopted so that these affective factors can be fully controlled. At first, all the samples in the group I were age-matched at the time of oocysts inoculation. Secondly, the number of infants per dams was equally chosen. Consequently, the samples became

remarkably homogenized in terms of age and number [13].

The isolate of the parasite is the next factor which can affect the severity of infection in murine models and different results have been obtained from different isolates [14]. Bovine isolate of *C. parvum* has been more frequently used by previous researchers and it was also used in this study. Furthermore, the infectious dose of parasite to get an infectivity of 100% in murine models has been variously reported. Some authors reported that infant mouse could be inoculated with fewer than 100 oocysts [15]. Also some reports indicated that in murine models using 10^5 oocysts per mouse only 88.8% of mice were infected [16]. Therefore, in this paper a bovine isolate of parasite containing a high infective dose of 5×10^5 oocysts per animal was used so that we can ensure all the samples remain infective during the 6th to the 16th day of after infection.

The next factor is the technique by which the intensity of infection is determined. At present, there are three methods each of which has some degree of inherent variability. The most frequent one is histology which is based on a scoring system to determine the percentage of the infected intestinal cells [17]. The second method is counting the oocysts number in feces of rodents over a given period of time [18]. The last method which seems relatively simple and fast relies on counting oocysts following homogenization of intestines [19, 20].

It should point out that homogenization leads to loss of up to 41% of the oocysts; therefore it may not be a suitable method when the number of oocysts is not as high as those of this paper [21]. Therefore, the third method which is easier and less costly than the other two was used in this research. In addition, it is important to know when the intensity of infection reaches its climax,

so that we can correctly assess the efficacy of anticryptosporidial agents. Some authors reported the 7th post infection day as the peak of infection [21] and others have considered the 9th post infection day, the time at which the intensity of infection reaches its peak [8,22].

The intensity of infection in newborn BALB/c mouse and newborn rat showed a similar pattern. The intensity of infection peaked on the 9th post infection day in both experimentally infected animal models. Although both animal models in the present study are relatively economical, simple, easily reachable, and feasible, the neonate rat model seemed experimentally preferable because of its physiological similarity to humans [2,3], and easier manipulation; hence, it is a useful *in vivo* model to evaluate the efficacy of herbal, chemical, and immunological agents against *C. parvum* infection.

Conclusion

It is concluded that neonate rat can be considered an indigenous animal model which can be usefully applied to experimental studies on the *C. parvum* infection and even oocysts reproduction in laboratory.

Conflict of interest statement: All authors declare that they have no conflict of interest.

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