

# Detection of Experimental Cryptosporidiosis in Neonatal Mice and Rats by Nested-PCR

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## ABSTRACT

**Purpose:** The purpose of this study is to model Cryptosporidiosis in laboratory animals. The parasites were inoculated into animals and then multiplied. The process of proliferation was compared to control Cryptosporidiosis in humans.

**Materials and Methods:** Twenty-five laboratory mice (4-7 days of age) and twenty-five laboratory rats (5 days of age) were assigned to the category I while the category II (control group) consisted of twenty-five rats and twenty-five mice. The two categories were infected with  $5 \times 10^5$  Cryptosporidium parvum oocysts originated from a calf by using a 24-gauge & 20-gauge ball-point feeding needle. On 4-9 days of post inoculation the intestine, colon, and rectum were removed. Cryptosporidium infection was determined by detecting oocysts in intestinal homogenates by staining and PCR method. Simple extraction and purification method was used by ficoll gradient centrifugation. Also, twenty laboratory rats (4-6 weeks of age) were intramuscularly injected with dexamethasone (Sigma, Chemical Co. UK) two times per week, and the last injection was given with  $5 \times 10^5$  Cryptosporidium parvum oocyst on the same day as oral inoculation. The water was supplemented with tetracycline to avoid secondary infections.

**Results:** Two to four million purified oocysts with a maximum of 10 million were routinely obtained per mouse and rat. Also the day in which oocyst excretion is the highest was determined. The number of oocyst per neonatal mouse was  $(11 \pm 2) \times 10^5$  on 9-12 days of post infection while similarly it was  $(10 \pm 1) \times 10^5$  per neonatal rat.

**Conclusion:** The evaluation of the cryptosporidiosis in immunocompromised animal models can help us to understand and control the Cryptosporidium infections.

**Keywords:** cryptosporidium parvum; mice; rat; animal model

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## INTRODUCTION

Cryptosporidium was firstly isolated from gastric epithelial cells in 1895 by Clark. Cryptosporidiosis is a parasitic disease caused by protozoa of the genus Cryptosporidium that infects the microvillus regions of the digestive tract.<sup>(1,2)</sup> With the advent of acquired immunodeficiency syndrome (AIDS), the protozoa, as an important cause of morbidity and mortality in immunocompromised patients and diarrhea in children,

achieved international importance.<sup>(3)</sup> The first case of human cryptosporidiosis was in 1976 on a three-year-old child with severe gastroenteritis.<sup>(4)</sup> Children have different aspects of cryptosporidiosis, which can show the safety of the parasite is not stable. The infectious oocysts pass through the feces. These oocysts enter the environment to await ingestion by the next host. Cryptosporidium infection may be transferred to humans through food and water.<sup>(5,6)</sup>

*C. parvum* oocysts adhere to epithelium of the small intestine in humans and live on the margins of the microvilli of intestinal epithelium and cause clinical signs. Cryptosporidium is a cause of chronic diarrhea in human and ruminant, such as cattle, sheep, goats, deer and the parasite is completed in the digestive tract. Cryptosporidium oocyst stage is just outside the intestine.<sup>(7)</sup> Humoral immunity in mice against Cryptosporidium parvum produces small amounts of IgM and IgG. This reaction is coordinated by oocyst shedding, while Cellular immunity plays an important role in the defense and protection against Cryptosporidium parvum.<sup>(8,9)</sup>

In the immune competent population the infection is usually mild and self-limiting in hosts, and the symptoms include watery diarrhea, nausea, vomiting, and mild fever, but can be chronic and life-threatening in immunocompromised persons.<sup>(10)</sup> The prevalence of Cryptosporidium parvum infections in the general population has reportedly been 2.2~8.5%.<sup>(11)</sup>

In patients with complete immunity, many antimicrobial agents including nitazoxanide, paromomycin and spiramycin were tested.<sup>(12)</sup> Due to the effects of drug therapy in patients with immunosuppression, immunotherapy was recommended by using various combinations such as immune bovine colostrum, which may limit the clinical symptoms and lead to an improve in the patient's general condition.<sup>(13)</sup>

Owing to the lack of an invitro real system for amplification of Cryptosporidium, animal models have been introduced for cultivation. Most experimental studies on Cryptosporidium infection have been undertaken by using murine models because of their wide availability. Cryptosporidium infection models are needed to identify different aspects of Cryptosporidiosis and the means of treating and preventing this infection.

## MATERIALS AND METHODS

In the current study Cryptosporidium parvum (Iowa strain) oocysts were used. Diarrhea stool fresh samples from young calves were collected in the farm and oocysts were purified using sucrose flotation and stored at 4°C in 2.5% potassium dichromate.<sup>(14,15)</sup>

In this study twenty-five laboratory suckling BALB/c (4-7 days of age) and twenty-five laboratory suckling rats (5 days of age) were used and kept at 22-25°C, 60-80% relative humidity and a 12-hour dark-light cycle in the animal house. Also, twenty laboratory rats (4-6 weeks of age) were intraperitoneally injected two times in alternate days with 0.4-1 mg of immunosuppressive agent (dexamethasone).

The suckling BALB/c in category I were infected with *C. parvum* oocysts, and the suckling BALB/c in category II were considered as non-infected controls. The category I consisted of twenty-five BALB/c and twenty-five rats while the same formation was applied to the control category.

In the morning of the day 0, Cryptosporidium parvum oocysts were washed three times with PBS. Then, animals were inoculated with oocysts of *C. parvum*. Ninety minutes before inoculation, the sucklings were isolated from their mothers to void their stomach for easier inoculation. They were infected with 25 µl of the suspension of calf origin oocysts ( $5 \times 10^5$  oocysts) by using a 24-gauge & 20-gauge ball-point feeding needle. Category II received 25 µl sterile PBS.<sup>(16,17)</sup>

At the same time rats (male) with weights ranging from 250 to 300 gr were immunosuppressed by dexamethasone (0.4-1 mg/kg). Each vial, with a total volume of 2 ml, contained 8 mg of dexamethasone. Regular injections were performed two times per week intramuscularly: first week, in each time 0.1 ml containing 0.4 mg of dexamethasone. The second week, in each time 0.15 ml containing 0.6 mg of dexamethasone.

The third injection period was at the 7<sup>th</sup> week, in each time 0.2 ml containing 0.8 mg of dexamethasone. The same amounts were injected in the periods of 8<sup>th</sup> to 14<sup>th</sup> weeks. To protect animals from bacterial infection, tetracycline was added to drinking water and in each dexamethasone injection. Correspondingly, penicillin and streptomycin were injected intramuscularly.<sup>(18)</sup> The control group immune system was not weakened. The animals were kept in different cages in the same environment and diet.

After the 8<sup>th</sup> week, both case and control groups were infected to acquire Cryptosporidium parvum infection. Both BALB/c and rat infants were euthanized by chloroform asphyxiation at predetermined times. There were five groups in suckling mice and rats. Fecal samples were collected from all mice on 4-12 days of post-inoculation and diluted into PBS. Oocysts from feces were purified by discontinuous sucrose gradients and stained by a Ziehl-Neelsen.

Each groups contained five sucklings which were killed on the 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, 10<sup>th</sup> and 12<sup>th</sup> days of post infection. The control category consisted of ten suckling BALB/c & ten suckling rat. The same procedures were applied to the control category as well.

The small and large intestines of each neonatal mouse and rat were removed and individually placed in 2 ml PBS. The intestines (S&L) were homogenized for 25 seconds (two times) using a Tissue Tearer homogenizer

(Biospec Products, Bartlesville, OK).<sup>(16,17)</sup> The 15 µl of the homogenate was smeared onto a slide and stained using a Ziehl-Neelsen. After confirming the existence of parasites, the number of *Cryptosporidium parvum* oocysts per 1000λ of suspension was counted by Neubauer. Also during testing, fecal pellet was taken out from each infant cage and was examined daily for the presence of *Cryptosporidium* oocysts.<sup>(3)</sup>

Six of the rats in the case category and five of the rats in the control category died by the end of the 8<sup>th</sup> week. The remaining rats were euthanized by chloroform asphyxiation at the end of the 12<sup>th</sup> week.

The intestinal (S&L) contents of all the rats were emptied and placed into different falcon. The contents of the digestive system were smeared onto glass slides and stained using a Ziehl-Neelsen. After confirming the existence of parasites, the number of oocysts per 1000λ of suspension was counted by Neubauer.<sup>(3)</sup>

The genomic DNA was extracted from bowel samples by using QIAamp Stool Kit according to the manufacturer's instructions. Eventually precipitated DNA was eluted in 100 µl of buffer (Qiagen) and stored at -20°C until further use.<sup>(19)</sup> The DNA was also extracted by phenol-chloroform. The absorbance of the extracted DNA was measured by Eppendorf Biophotometer Plus. The OD (1.24) for extraction kit and the OD (1.01) for phenol-chloroform extraction method was resulted. Each PCR reaction (volume 15 µl) contained 7.5 µl master mix, 0.5 µl primer, 1 µl DNA, and 6 µl DW.

Distilled water samples were also used as a negative control for all sensitivity test reactions. To recognize the *Cryptosporidium*, a fragment of the GP60 gene (800 to 850 bp) was amplified by a nested PCR with the primer sets 5'-ATAGTCTCCGCTGTATTC-3' and 5'-GCAGAGGAACCAGCATC-3' in the primary PCR and 5' TCCGCTGTATTCTCAGCC-3' and 5'-GAGATATATCTTGGTGCG-3' in the secondary PCR (400 to 500 bp). Primers used in the GP60 gene were acquired from Invitrogen.

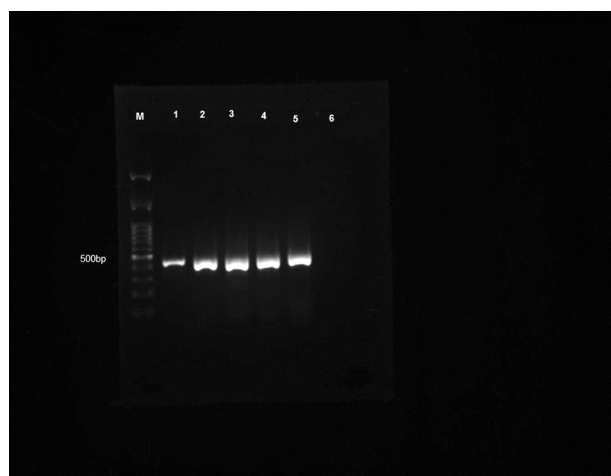
The PCR product was investigated by electrophoresis in 1.5% (w/v) agarose gel. The staining method, ethidium

bromide, was used to visualize the *Cryptosporidium* DNA with the used amount of 5 µg/ml.

## RESULTS

The Study of the bowl content of rats and mice showed that the *Cryptosporidium* oocyst was found in seventy-eight percent of the neonatal mice and sixty percent of neonatal rats. The neonatal mice showed a high percentage of infection. Furthermore, the immunosuppressed rats showed forty-five percent of infection with oocyst. In this study, both BALB/c and rat infants showed oocyst at the lowest ranges on days 10-12 PI, while the most values of oocyst were recorded on days 4-6 PI. Oocysts were indicated in 1/10 (10%) of the control group in mice and rats.

The number of *Cryptosporidium* oocyst per neonatal mouse was  $(11\pm 2)\times 10^5$  on 9-12 days of post infection while that of neonatal rats was  $(10\pm 1)\times 10^5$ . *Cryptosporidium* oocyst in immunosuppressed rats was  $(12-14)\times 10^5$  on 9-12 days of post infection.<sup>(20)</sup> The infected rodents display symptoms of diarrhea and Loss of appetite. The severity of infection attained its maximum with



**Figure 1.** Detection of *Cryptosporidium* by nested PCR of GP60 gene: M - DNA ladder 100bp, 1-Positive control, 2-*Cryptosporidium* from suckling BALB/c, 3-*Cryptosporidium* from suckling rat, 4&5-*Cryptosporidium* from immunosuppressed rat, 6-Negative control

**Table 1.** PCR program used in this study

Primer	Denaturing (temp(°C)/time(s))	Annealing (temp(°C)/time(s))	Extension	Cycles
PCR 1 F(5'-ATAGTCTCCGCTGTATTC-3') R(5'-GCAGAGGAACCAGCATC-3')	94/45	45/45	72/60	30
PCR2 F(5'-TCCGCTGTATTCTCAGCC-3') R(5'-GAGATATATCTTGGTGCG-3')	94/45	60/45	72/60	30

$(45\pm 1)\times 10^5$  oocysts per mouse intestine on the 4<sup>th</sup> post infection day. After the treatment by nitazoxanide, infected mice and rats displayed a significant decrease in symptoms and oocysts shedding.<sup>(21)</sup>

The results showed that DNA extraction was properly done and the amplification of the gp60 gene was satisfactory in all isolates. The Ziehl-Neelsen staining results was consistent with PCR.

## DISCUSSION

It has been shown that Balb/c neonates are the most susceptible to infection by *Cryptosporidium parvum* and different aspects of infectivity have been analyzed.

Applying an animal model for the evaluation of experimental cryptosporidiosis has a long history and various sensitive animal models have been developed in the last few decades.<sup>(22)</sup>

The neonatal mice are sensitive to experimental *Cryptosporidium parvum* infection in the initial week of life, but after some days will be resistant to the infection.<sup>(13)</sup> The animals during the experiment did not have clinical signs, therefore the cryptosporidiosis diagnosis was needed to quantify the intestinal parasitic load.

In immunosuppressed adult mice the shedding of *Cryptosporidium parvum* oocysts in stool makes a practical model of chronic cryptosporidiosis. A new neonate mice model was showed in a study for cryptosporidiosis as well as screening effective methods to limit the disease in gramivorous.<sup>(17)</sup>

In the most of surveys the neonate mouse and rat or the immunosuppressed adult rat model was preferred. In such studies no clinical symptoms and *Cryptosporidium parvum* oocysts shedding was reported for a 14-day period. Also after healing, the model members did not shed any *Cryptosporidium parvum* oocysts.

In this study, the severity of infection in neonatal mice was 78% while that in neonatal rats was 60%, however in other researches all the mice and rats were infected.<sup>(17)</sup> In the current study, the peak infection was on the 3<sup>rd</sup> and 4<sup>th</sup> days although in another study it was on the 9<sup>th</sup> day.<sup>(3)</sup>

The number of *Cryptosporidium parvum* oocyst in neonatal mice was  $(11\pm 2)\times 10^5$  on 9-12 days of post infection and the severity of infection, in this study, was attained its maximum with  $(45\pm 1)\times 10^5$  *Cryptosporidium parvum* oocysts in mice on the 4<sup>th</sup> post infection day. According to Martín-Gómez's study the number of *Cryptosporidium parvum* oocysts per intestine was  $15.8\pm 4.0$  on the 6<sup>th</sup> day of post infection. The peak of infection was on the 9<sup>th</sup> day (with  $32.9\pm 5.6$  oocysts per

mouse) and then decreased slowly until the 16<sup>th</sup> day of post infection.

In this report, immunosuppressed rats showed 55% *Cryptosporidium parvum* oocyst infection in their intestine while in another study 20% of immunosuppressed Balb/c and 80% of immunosuppressed C57BL/6 mice were infected by *Cryptosporidium* oocyst.<sup>(10)</sup> The C57BL/6 mice showed high percent of infection; it should be noted that 40% of them died at the end of the study whereas the infected Balb/c mice were alive at the end of the study. In our study, Balb/c neonates' sensitivity is the highest among the three groups.

## CONCLUSIONS

It is concluded that *Cryptosporidium parvum* oocyst is one of the infectious factors that may induce intestinal dysplasia. In the present study, the high infective power of *Cryptosporidium parvum* oocysts were detected in laboratory mice. The Balb/c neonates are a very convenient animal model which may facilitate the study of many aspects of experimental cryptosporidiosis.

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## CONFLICT OF INTEREST

None declared.

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