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Infectivity of gastric and intestinal *Cryptosporidium* species in immunocompetent Mongolian gerbils (*Meriones unguiculatus*)

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ABSTRACT

We exposed juvenile (7-day old) and adult (8-week old) Mongolian gerbils (*Meriones unguiculatus*) to *Cryptosporidium andersoni* or *C. muris*, which infect the stomach, or to *C. parvum*, which infects the intestine. Both age groups could be successfully infected with each species in primary mono-infection with juvenile animals demonstrating higher peak oocysts per gram of feces and longer patent periods than adults. Concurrent exposure to mixed gastric and intestinal cryptosporidia resulted in successful infection with both species. The time course and infection intensities in the mixed infections were similar to those of primary mono-infection and for a given species. Similarly, sequential mixed infection of *C. andersoni* positive gerbils with *C. muris* 25 days after exposure to *C. andersoni* resulted in simultaneous infection with both gastric species. In contrast, following primary infection and clearance, animals re-exposed to the same *Cryptosporidium* species, failed to excrete oocysts in their feces and histological examination revealed no developmental stages in the stomach or intestine. In cross-infections, where the secondary exposure was with a different *Cryptosporidium* species than the initial cleared infection, successful infection was possible with a gastric species following *C. parvum*, or with *C. parvum* following a gastric species, but primary infection with one gastric species precluded secondary infection with the other gastric species. These results indicate cross-immunity between gastric *Cryptosporidium* species but not between intestinal and gastric species. Furthermore, our study demonstrates that Mongolian gerbils are susceptible to infection with many *Cryptosporidium* species and are a useful laboratory model for studies of mixed cryptosporidiosis.

1. Introduction

Parasites from the genus *Cryptosporidium* infect many vertebrates and both humans and animals can be infected with a variety of *Cryptosporidium* species/genotypes. For example, infections with at least 7 *Cryptosporidium* spp. have been detected in immunocompromised patients (Cama et al., 2003) and 3 species are specific for cattle or pigs (Morgan et al., 1999; Lindsay et al., 2000; Fretz et al., 2003; Ryan et al., 2003). While most described cases of cryptosporidiosis are infections with a single species, mixed *Cryptosporidium* infections have been described in humans (Fretz et al., 2003; Cama et al., 2006; Gatei et al., 2007) as well as animals (Tanriverdi et al., 2003; Kváč et al., 2008a).

It is difficult to detect mixed infections as identification of more than one species using oocyst morphology is

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almost impossible. Similarly, utilization of common molecular diagnostic tools such as polymerase chain reaction (PCR) or restriction fragment length polymorphism (RFLP) is limited as DNA from the predominant species/genotype overrides the assay, leading to poor sensitivity for detection of subpopulations (Tanriverdi et al., 2003). The aim of this study was to experimentally examine the dynamics of mixed infections, re-infections, and cross-infections with intestinal and gastric *Cryptosporidium* species using parasitologic, histologic, and molecular detection methods.

2. Materials and methods

2.1. Parasites

Cryptosporidium andersoni LI03, *C. muris* TS03, and *C. parvum* were used for experimental infection. Gastric cryptosporidia (*C. andersoni* and *C. muris*) were passaged in Southern multimammate mouse (*Mastomys coucha*) and molecular characterization of these isolates was performed as previously described (Kváč et al., 2008b) The *C. parvum* isolate was obtained from a naturally infected 1-month-old calf and had identical sequence to *C. parvum* GP60 genotype IIA A16G1R1 (GenBank accession number AM937009). Oocysts were purified from feces as previously described (Arrowood and Sterling, 1987; Kilani and Sekla, 1987) and stored at 4 °C in distilled water without antibiotics or antimycotics until use.

2.2. Experimental animals

Groups of 7-day or 8-week-old outbred Mongolian gerbils (*Meriones unguiculatus*) (AnLab, Czech Republic) were utilized for the experimental infections in this study. Groups of 7-day-old *M. coucha* (bred inhouse) were used as positive controls. Each group was housed separately in standard plastic cages and fed with sterile commercial rodent food and drinking water *ad libitum*. Groups of 7-day-old BALB/c mice (AnLab, Czech Republic) were used for verification of success of the sequential infection.

2.3. Infectivity assays

In all experiments, animals were inoculated with 1×10^6 oocysts (viability >95% by propidium iodide exclusion) in 200 μl distilled water via a gastric tube. For the primary mono-infection studies, groups of 7-day or 8-week-old gerbils (10/group) were infected with *C. parvum*, *C. andersoni* LI03 or *C. muris* TS03. In the re-infection studies, gerbils (10/group) that were 8 weeks old at the time of primary infection were re-exposed to the same dose of the same species (*C. parvum*, *C. andersoni* LI03 or *C. muris* TS03) 2 months after they had cleared their initial infection (no oocysts detected in feces for 25 days). In the mixed infection experiments, groups of 7-day or 8-week-old gerbils (10/group) were simultaneously inoculated with oocysts from 2 *Cryptosporidium* species. A sequential mixed infection study was performed in a group of 8-week-old gerbils (10/group) by exposing them to *C. andersoni* LI03 parasites at day 0, followed by *C. muris* TS03 parasites at day 25. *Cryptosporidium* oocysts isolated from these animals at day 60 were used for infection of 7-day-old BALB/c mice. Finally, cross-infection studies were performed by infecting groups of 8-week-old gerbils with one species of *Cryptosporidium*, allowing them to completely clear this infection (no detection of oocysts for 25 days) and exposing them to a second *Cryptosporidium* species 2 months later. Group of 5 animals were used as negative controls in each type of experiment. These animals were inoculated with 200 μl sterilized distilled water.

2.4. Assessment of infections

Fecal samples were collected daily starting from day 4 after exposure and examined for the presence of *Cryptosporidium* oocysts using the aniline-carbol-methyl violet staining method (Miláček and Vítovec, 1985). Infection intensity was determined as the number of oocysts per gram (OPG) as previously described (Kváč et al., 2007). In addition, DNA was extracted from feces (Sak et al., 2008). Molecular diagnosis of infections was based on sequencing a fragment of the 18S rRNA gene amplified by nested PCR (Jiang et al., 2005). In addition, sequence analysis of a fragment of the GP60 gene of *C. parvum* was performed according to the method of Alves et al. (2003). Three animals from each group were necropsied on day 7 (intestinal species) or day 25 (gastric species) after exposure and the stomach and intestinal tissue were processed for histology (Kváč et al., 2007). All animals were examined at necropsy 25 days after clearance of the final infection and examined using histology and PCR for presence of *Cryptosporidium* development stages in the tissues and feces.

2.5. Statistical analyses

For statistic analyses of group differences, Mann–Whitney *U*-test was used (Statistica, Release 5.1 Software (Statsoft, Tulsa, OK, USA, 1997)).

3. Results

3.1. Primary mono-infection

Both 7-day and 8-week-old gerbils were susceptible to infection with *C. parvum*, *C. andersoni* LI03 and *C. muris* TS03. The prepatent periods of *C. parvum* and *C. andersoni* LI03 infection were similar in juvenile and adult gerbils ($P > 0.01$) (Table 1, Fig. 1). Juvenile gerbils infected with *C. muris* TS03 started to shed oocysts significantly earlier than adult animals ($P < 0.01$). Statistic analyses revealed significant ($P < 0.01$) differences in patent periods between juvenile and adult gerbils infected with all *Cryptosporidium* species (Table 1). The juvenile gerbils infected with gastric *Cryptosporidium* species had not cleared the infection by 90 days of infection while adult gerbils cleared their *C. andersoni* LI03 infections as early as day 57 ($P < 0.01$). The juvenile animals also demonstrated higher maximum infection intensities and total quantity of shed oocysts than adult animals for all *Cryptosporidium*