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ORIGINAL PAPER

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Infectivity of *Cryptosporidium parvum* genotype I in conventionally reared piglets and lambs

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Abstract Parasites of the genus *Cryptosporidium* are intracellular parasites that occur throughout the animal kingdom and have been reported in many species of mammals, including human. Most infections in humans are caused by two *C. parvum* genotypes, genotype I and genotype II; these are the human and the bovine (zoonotic) genotypes, respectively.

Successful experimental infection of *Cryptosporidium parvum* genotype I "human genotype" is described in four conventionally reared piglets and in a lamb. The inoculum was originally obtained from two diarrheic children, and the *Cryptosporidium* genotypes were determined by PCR and rDNA sequencing. The infective dose was between 10^6 and 2×10^6 oocysts. No clinical signs were observed in the infected animals, except in a piglet that showed watery diarrhea. The oocyst shedding period in positive animals ranged between 4 and 10 days. Histopathologic examination of the gastrointestinal tract of two positive piglets revealed shortening of the villi and denudation of the villous tips of the jejunum. In one piglet, the colon mucosa revealed numerous *Cryptosporidium* oocysts. The storage time of the inocula (≤ 3 weeks in PBS at 4°C) and the age of the animal (newborn) were important for the successful induction of infection.

Introduction

Human cryptosporidiosis is caused mainly by two genotypes of *Cryptosporidium parvum*. Genotype I "human genotype" has been detected almost exclusively in humans; the single non-human case that has been identified was that of a dugong (Morgan et al. 2000). Genotype II ("bovine genotype") has a wide range of animal hosts, including humans. It has been isolated particularly from calves, lambs and other newborn ruminants (Peng et al. 1997; Spano et al. 1998). Cryptosporidia are transmitted by the fecal-oral route or indirectly through food or drinking water that are contaminated with oocysts (Jokipii et al. 1983; MacKenzie 1994). *C. parvum* causes acute, self limiting diarrhea in immunocompetent individuals and in domestic animals. However, cryptosporidiosis has a chronic form and may have a fatal course in immunodeficient individuals (O'Donoghue 1995).

In the available literature, there is a paucity of information about the propagation of *C. parvum* genotype I oocysts. Despite the improvement of in vitro cultivation of *C. parvum* (Hijjawi et al. 2001), mass production of oocysts depends on animal inoculation (Petry et al. 1995). Recently, experimental infection of *C. parvum* genotype I in gnotobiotic piglets and in a lamb was reported (Widmer et al. 2000; Giles et al. 2001).

The aim of this study was to determine the infectivity of *C. parvum* genotype I in conventionally reared piglets and lambs.

Material and methods

Animals

Six large white breed piglets and six Swiss white alp lambs were used in this study. The piglets were obtained from Specific Pathogen Free (SPF) conventional farms and were colostrum-deprived by removing them from the sows immediately after birth. They were housed at 32°C in pairs in plastic containers with wire mesh top and with wood shavings for bedding. They were fed six times daily with commercial piglet milk replacement.

The lambs originated from the experimental sheep herd of the Institute of Parasitology at the University of Zurich. The lambs were kept with their dams throughout the study.

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In order to facilitate the collection of fecal material, piglets were kept on perforated aluminium flooring with an underlying tray during the period of oocyst shedding. In lambs, fecal collection bags held in place by a harness were used.

The experiment was approved by the animal experiment commission in accordance with the Swiss legislation on animal welfare.

The inocula

Two *C. parvum* genotype I isolates (Crypto-11 and Crypto-26) were obtained from diarrheic children treated at the Zurich University Hospital. The identification of the *C. parvum* genotype was based on PCR and direct sequencing of part of the 18S rDNA (Ward et al. 2002).

Fecal homogenates were kept at 4°C either in phosphate buffered saline (PBS) supplemented with penicillin G sodium 40 IU/ml, streptomycin 40 μg/ml and amphotericin-B 0.05 μg/ml (PSF) or in 2% (w/v) $K_2Cr_2O_7$ solution (Table 1). Oocyst number was determined semi-quantitatively by examining 10 μl aliquots of the fecal homogenate under the light microscope after staining with the modified Ziehl-Neelsen procedure (Casemore 1991). No bacteriological or virological examination of the fecal samples was attempted.

Before inoculation, the conserving medium was removed by washing the fecal material four times in sterile distilled water and centrifugation at 1000 *g* for 5 min.

Experimental procedures

The infective dose, which ranged between 10^6 and 2×10^6 oocysts suspended in 4 ml of sterile distilled water, was administered by stomach tube. The piglets were inoculated at one day of age except for one piglet (Table 1). The piglets were kept for 20 days post-infection and then euthanised with an overdose of barbiturates. Intestinal lavage was performed and the intestinal contents were collected. In two piglets (numbers 5 and 6), samples from the internal organs (liver, spleen, kidney and gall bladder) and samples from the intestinal tract (duodenum, jejunum, ileum, cecum, colon, and rectum) were fixed in 10% neutral formalin for histological examination. Histological sections of 4 μm thickness were prepared and stained routinely with H&E and with Giemsa stain.

Four lambs were inoculated few hours after birth and two were inoculated at the age of 6 weeks (Table 1). The lambs were discharged at the end of the study to join the experimental herd.

Clinical monitoring

The animals were examined clinically on a daily basis for changes in vital signs (body temperature, heart rate and respiratory rate) and for evidence of diarrhea. Body weight was recorded daily.

Fecal examination

All animals were examined for the presence of *Cryptosporidium* spp. oocysts in their feces prior to inoculation. Rectal swabs were taken from every animal daily until the end of the experiment and two smears thereof were examined microscopically.

To determine the *Cryptosporidium* genotype, a pool of fecal samples from every positive animal was subjected to PCR and direct sequencing of the 18S rDNA (Ward et al. 2002).

Results and discussion

In the present study, successful experimental infection with *Cryptosporidium parvum* genotype I was established in four conventionally reared piglets and in a lamb. Experimental *Cryptosporidium* infection has been reported in piglets (Moon et al. 1982; Arnault et al. 1994) and in lambs (Tzipori et al. 1982) using Cryptosporidia oocysts from diarrheic patients. However, interpretation of these studies was difficult because all the experiments were established without prior determination of the isolate's genotype. Recently, *C. parvum* genotype I was successfully propagated in gnotobiotic piglets (Widmer et al. 2000) and in a lamb (Giles et al. 2001).

The infective dose in this study of 10^6 –2×10^6 oocysts was similar to that of a previous report in gnotobiotic piglets (Widmer et al. 2000). Four out of six piglets started to shed oocysts 4–5 days post-infection (Table 1). No clinical signs were observed except in one piglet (no. 1) that showed watery diarrhea, and a change of fecal color from brown to yellow. The piglets were steadily gaining weight despite shedding oocysts (data not shown). The duration of shedding ranged between 4 and 10 days. In all the positive piglets, PCR and sequence analysis confirmed the presence of *C. parvum* genotype I as in the original inoculum. In two piglets (numbers 5 and 6), histological examination of the intestinal tract revealed shortening of the villi and denudation of the villous tips of the jejunum. The colon mucosa of piglet no. 5 showed numerous Cryptosporidium oocysts (Fig. 1). The ileum of piglet no. 6 showed atrophy of the lymphoid follicles. In piglet no. 6, no Cryptosporidia were detected in any histological section but there was shortening and denudation of the villous

Table 1 Experimental infection of piglets and lambs with *Cryptosporidium parvum* genotype I ("human genotype")

Animal	Age at time of inoculation	Inoculum (isolate)	Storage medium	Storage time of inoculum	Numberof oocysts in inoculum	Firstexcretion of oocyst PI / Duration of shedding
Piglet 1	Newborn	Crypto-11	PBS + PSF	2 days	10^6	day 4/9 days
Piglet 2	Newborn	Crypto-11	PBS + PSF	2 days	10^6	day 4/4 days
Piglet 3	Newborn	First passage from piglet 1	PBS + PSF	7 weeks	2×10^6	No shedding
Piglet 4	12days	Crypto-26	PBS + PSF	2 days	10^6	No shedding
Piglet 5	Newborn	Crypto-26	PBS + PSF	18 days	10^6	day 4/4 days
Piglet 6	Newborn	Crypto-26	2%$K_2Cr_2O_7$	18 days	10^6	day 5/6 days
Lamb 1	Newborn	Crypto-26	PBS + PSF	3 weeks	10^6	day 5/10 days
Lamb 2	6weeks	First passage from lamb 1	PBS + PSF	4 days	2×10^6	No shedding
Lamb 3	6weeks	First passage from lamb 1	PBS + PSF	4 days	2×10^6	No shedding
Lamb 4	Newborn	Crypto-26	PBS + PSF	10 weeks	10^6	No shedding
Lamb 5	Newborn	First passage from lamb 1	PBS + PSF	8 weeks	10^6	No shedding

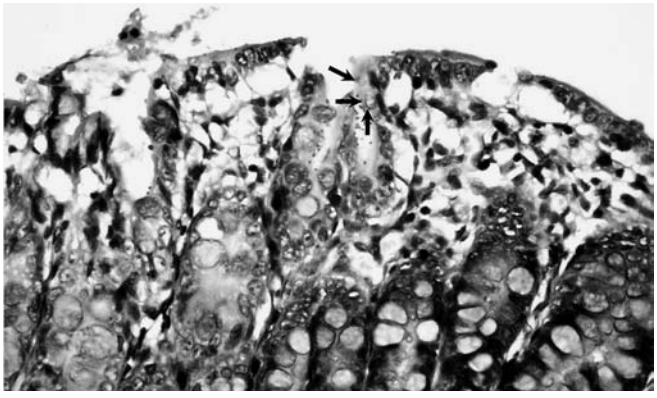

Fig. 1 Colon mucosa of infected piglet no.5 with numerous *Cryptosporidium* oocysts (*small arrows*) as basophilic rounded bodies that extend to the crypts. Gemisa × 500

tips along the small intestine (jejunum and ileum). These histological changes were consistent with the previous description of intestinal cryptosporidiosis in piglets (Moon and Bemrick 1981; Tzipori 1981; Moon et al. 1982; Argenzio et al. 1990; Vitovec and Koudela 1992). Two piglets (numbers 3 and 4) did not excrete the oocysts throughout the experiment. The failure to infect one piglet (no. 3) was possibly due to the prolonged storage of the oocysts used as inoculum. The half life of *C. parvum* genotype I was reported to be 1 week at room temperature (Widmer et al. 2000), in contrast to *C. parvum* genotype II, which showed a half life of 112 days at room temperature (Yang et al. 1996).

Failure to infect another piglet (no. 4) might be attributed to its age (12 days old) at the time of the inoculation. This assumption was supported by the fact that the same isolate was infectious in a newborn lamb (no. 1) and two newborn piglets (numbers 5 and 6).

Infection with *C. parvum* genotype I was also successful in a lamb that remained symptomless. Lambs numbers 2 and 3 were inoculated with oocysts collected from lamb no. 1. They were 6 weeks old at the time of the inoculation and remained symptomless, and no oocyst shedding was observed throughout the experiment. However, it should be noted that the detection of Cryptosporidia oocysts in formed stool with the Ziehl-Neelsen method requires 500,000 oocysts per g of stool for a 100% accurate detection of oocysts (Weber et al. 1991).

Due to logistic constraints, we could not obtain newborn lambs and piglets in the time required to investigate the viability of the oocysts. Therefore, it remains to be seen whether the failure to induce the infection in other lambs was attributable to the age of the inoculum.

Despite the fact that a small number of animals was used in this study, it seems that the storage time of the isolate might play a role in the infectivity of the oocysts. Isolates stored longer than 3 weeks in PBS at 4°C failed to induce infection in newborn animals. This point needs further investigations to explain waterborne outbreaks due to *C. parvum* genotype I oocysts. Inoculation with freshly collected oocysts was unsuccessful in 12-day-old piglets. It is plausible to assume that older animals require immunosuppression for the successful induction of the infection.

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