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To cite this version:
B Koudela, A Boková. The effect of cotrimoxazole on experimental Cryptosporidium parvum infection in kids. Veterinary Research, BioMed Central, 1997, 28 (4), pp.405-412. <hal-00902490>

HAL Id: hal-00902490
https://hal.archives-ouvertes.fr/hal-00902490
Submitted on 1 Jan 1997

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The effect of cotrimoxazole on experimental Cryptosporidium parvum infection in kids

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(Received 16 January 1997; accepted 13 March 1997)

Summary — The prophylactic and therapeutic effects of the folic acid inhibitor cotrimoxazole (trimethoprim in combination with sulfamethoxazole) was tested in goat kids experimentally infected with Cryptosporidium parvum oocysts. Of the twenty-four 6-day-old kids inoculated with $6 \times 10^6$ oocysts of $C$. parvum, ten kids were administered cotrimoxazole prophylactically at a dose of 20 mg/kg per day of trimethoprim/100 mg/kg per day of sulfamethoxazole for 14 consecutive days beginning 1 day before infection. Six kids were therapeutically treated at the same dose of cotrimoxazole for 9 consecutive days beginning 5 days post infection, and eight kids served as untreated controls. Experimental $C$. parvum infection caused a severe clinical disease with profuse watery diarrhea, oocysts shedding and intestinal lesions in all groups of kids. Total days and severity of diarrhea were similar for all groups of kids. However, the mean duration of oocysts shedding, mean number of cryptosporidia per ileal villus, and distribution of cryptosporidia in the intestine were increased in both groups of cotrimoxazole-treated kids. These findings indicate that cotrimoxazole failed to control cryptosporidiosis.

Cryptosporidium parvum / kid / experimental infection / therapy / cotrimoxazole

Résumé — Effets du cotrimoxazole sur l'infection expérimentale des chevreaux par Cryptosporidium parvum. Les effets prophylactiques et thérapeutiques d'un inhibiteur de l'acide folique, le cotrimoxazole (triméthoprim combiné au sulfaméthoxazole) ont été testés sur un troupeau de chevreaux infectés expérimentalement par les oocystes de Cryptosporidium parvum. Sur les 26 chevreaux, âgés de 6 jours et inoculés avec $6 \times 10^6$ oocystes de $C$. parvum, dix chevreaux ont reçu du cotrimoxazole dans un but prophylactique, à la dose de 20 mg/kg par jour de triméthoprim pour 100 mg/kg par jour de sulfaméthoxazole pendant 14 jours consécutifs à partir de la veille de l'infection ; six chevreaux ont été traités dans un but thérapeutique à la même dose de cotrimoxazole pendant 9 jours consécutifs à partir de 5 jours après infection et huit chevreaux non traités ont servi de témoins. L'infection expérimentale par $C$. parvum a provoqué une maladie clinique grave avec diarrhée profuse, une excrétion d'oocystes et des lésions intestinales dans tous les groupes de chevreaux. La gravité et la durée totale de la diarrhée ont été similaires pour tous les groupes de chevreaux. Cependant,

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la durée moyenne de l’excrétion des oocytes, le nombre moyen de cryptosporidies par villosité dans l’iléon, et la distribution des cryptosporidies dans l’intestin ont été augmentés dans les deux groupes de chevreaux traités par le cotrimoxazole. Ce travail indique que le cotrimoxazole ne permet pas de contrôler la cryptosporidiose.

Cryptosporidium parvum / chevreau / infection expérimentale / thérapie / cotrimoxazole

INTRODUCTION

Cryptosporidiosis is a disease caused by a coccidian parasite of the genus Cryptosporidium that inhabits the microvilli of the epithelial surface of the gastrointestinal and respiratory tracts of a wide variety of vertebrates including humans. In mammals, Cryptosporidium parvum primarily infects the intestine and clinical features of the infection include watery diarrhea, dehydration and weight loss. The infection is self-limiting, except in immunodeficient hosts, in which it may cause protracted and untreatable diarrhea (Fayer et al, 1990, O’Donoghue, 1995).

A variety of therapeutic and prophylactic pharmaceuticals have been tested for efficacy against C parvum in humans or animals but none of them produced satisfactory prophylactic or therapeutic benefits (Fayer et al, 1990; O’Donoghue, 1995).

Recently, Woods et al (1997) found that C parvum infectious ability was reduced by incubation with folic acid inhibitors in screening in vitro studies and supposed that this was due to the synergistic action of trimethoprim in combination with one of the sulfonamides. This study was conducted to test the prophylactic and therapeutic efficacy of cotrimoxazole (trimethoprim/sulfamethoxazole) against a single experimental C parvum infection in kids.

MATERIALS AND METHODS

Inoculum

Feces of a naturally infected scouring calf (12 days of age) were used as the source of cryptosporidial oocysts. Morphologically the oocysts corresponded to the species C parvum Tyzzer (1912). The fecal material was suspended in 2.5% potassium dichromate and, after removing large particles by passage through a series of graded sieves, the suspension was kept at room temperature for 1 week. Oocysts were concentrated by flotation in Sheather’s sugar solution and then washed with tap water to remove the flotation solution. To decontaminate the oocysts, the suspension was treated in bleach (SAVO, Bochemie, Bohumín, Czech Republic; 1.25% sodium hypochlorite) for 15 min. The purified oocysts were stored in 2.5% potassium dichromate at 4 °C for 27 days until used for kid inoculation. Before inoculation, the C parvum oocysts suspension was washed three times by centrifugation in sterile phosphate-buffered-saline (PBS, pH 7.2) and counted by a hemocytometer. The viability of the oocysts was verified by inducing experimental infection in 5-day-old suckling mice (Vitovec and Koudela, 1988).

Experimental design

Twenty-four 1-day-old kids (Saanen goat) of both sexes were obtained from a goat-cheese production unit of 80 spring kidding goats with no record of health problems in the newborn kids. The kids were separated from their dams immediately after birth. In the first 24 h, they were fed with goat colostrum and then transported to an isolator room and fed three times daily with commercial cow’s milk replacer (Sano, Germany). All 6-day-old kids were orally inoculated with a
syringe containing $6 \times 10^6$ C parvum oocysts in 10 mL of PBS. They were randomly separated into three groups. Each group of kids was reared in a separate pen in the same room and kids of all groups were kept in the same management conditions. Of the twenty-four 6-day-old kids inoculated with $6 \times 10^6$ oocysts of C parvum, ten kids (group P) were prophylactically administered with cotrimoxazole (Berlocid®, Berlin-Chemie, Germany) at a dose 20 mg/kg per day of trimethoprim/100 mg/kg per day of sulfamethoxazole in two divided doses for 14 consecutive days beginning 1 day before infection. Six kids (group T) were therapeutically treated at the same dose of cotrimoxazole for 9 consecutive days beginning 5 days post-infection, and eight kids (group C) remained untreated and served as an infected control.

**Assessment of infection**

All kids were monitored by observing twice daily for diarrhea and other clinical signs, and collecting a daily sample of feces from the rectum of each kid. The severity of diarrhea was scored as 0 for normal, 1 for loose and formless, 2 for semi-fluid, or 3 for watery. Each day, for each animal, 0.5 g of feces was examined microscopically for C parvum oocysts using Sheather’s sugar solution. The oocyst shedding was semi-quantitatively evaluated under a magnification of $\times 250$ after flotation, 0 for no oocysts, 1 for less than one oocyst per field, 2 for one to ten oocysts per field, and 3 for more than ten oocysts per field.

Beginning 3 days post-inoculation (3 DPI), two prophylactically treated kids (group P) and two control kids (group C), and beginning 7 DPI, two therapeutically treated kids were euthanized with an overdose of barbiturate (Thiopental®, Spofa, Czech Republic) every second or third day. At necropsy, the complete examination of all organs and tissue was conducted. Samples for histopathology were collected immediately after the animals were killed. The first specimen was taken from the ileum within 5 cm of the ileocecal valve (ostium ileocecal – OIC). More samples were removed 50 cm from OIC and then every 50 cm cranial from OIC so that the last jejunal specimen originated from duodenum. In the large intestine, specimens were collected from apex ceci, colon near ansa centralis, and rectum. Additional samples for histology were taken from abomasum, liver, kidneys, spleen, lung, pancreas, and regional mesenteric lymphonodes. The material was fixed in 10% neutral formalin and processed by routine histological methods. Histological sections were stained with hematoxylin-eosin, and with Wolbach’s modification of Giemsa staining. On the basis of the histological examination the distribution of cryptosporidia in the intestine and degree of infection were classified as follow:

- degree 0: no cryptosporidia found on the mucosal surface;
- degree 1: moderate infection, sporadic cryptosporidia distributed on the intestinal surface;
- degree 2: medium infection, regularly disseminated cryptosporidia on the intestinal surface;
- degree 3: massive infection, most of the epithelial surface covered by cryptosporidia.

The severity of the ileal infections, expressed as the mean number of cryptosporidia per villus, was determined by microscopic examination of the hematoxylin-eosin stained ileal sections and enumeration of the cryptosporidia, in various development stages, that were attached to the enterocytes of ten villi from the ileum within 5 cm from the ileocecal valve.

**Statistical analysis**

Each point in the graphs represents the degree and distribution of C parvum infec-
tion in the intestine. The severities of ileal infections were examined by analysis of variance (ANOVA) and comparisons of means (Newman-Keuls test). Statistical significance was considered to be $P < 0.05$.

**RESULTS**

Clinical signs and oocyst shedding

None of the kids had cryptosporidia in their feces prior to inoculation. All inoculated kids had a decreased appetite, and became depressed 3-4 days after inoculation. Their feces subsequently became watery with clumps of mucus, and color changed from brown to yellow. Kids in group C, infected but untreated, had diarrhea for 3-9 days (mean duration of diarrhea 4.8 days) and *C parvum* oocysts were shed for 4-11 days with a group average of 5.9 days. Prophylactically treated kids (group P) had diarrhea for 3-10 days (mean duration of diarrhea 5.6 days) and they shed oocysts for 5-13 days with a group average of 7.8 days. Therapeutically treated kids had diarrhea for 3-10 days (mean duration of diarrhea 4.8 days) and *C parvum* oocysts were detected for 4-13 days with a group average of 8.1 days. The severity of diarrhea and oocyst shedding, based on daily numerical scoring, were similar for all groups.

Histopathology

Pathological changes of cryptosporidiosis were similar or identical in all groups of animals. The most severe lesions were seen in the posterior jejunum and ileum from 3 to 7 DPI, and consisted of villus atrophy, villus blunting and crypt hyperplasia (fig 1). Many jejunal villi were fused together and

Fig 1. Histological section of the ileum from a prophylactically cotrimoxazole-treated kid inoculated with *C parvum*. 3 DPI. Villi are atrophic and fused together. Hematoxylin-eosin. Bar = 100 μm.
cross-bridged. The epithelium covering the atrophic villi was basophilic and usually low columnar to cuboidal. The crypt epithelium remained tall and columnar. In infected jejunal and ileal regions, the villus epithelium and lamina propria were infiltrated with numerous mononuclear cells and neutrophils. Cryptosporidia were located in the brush border of enterocytes (fig 2), primarily associated with villus enterocytes and were rarely observed in the crypts.

The distributions of cryptosporidia throughout the intestine of the untreated and cotrimoxazole treated kids are shown in figures 3-5. No cryptosporidia or associated pathological lesions were found in the large intestine of untreated kids (fig 3). In contrast, in kids treated with cotrimoxazole, small numbers of cryptosporidial developmental stages were found in the large intestine (figs 4 and 5). The differences between the distributions of cryptosporidia throughout the intestine were found in cotrimoxazole treated kids. On 9 DPI, more cryptosporidia were located on the intestinal surface in therapeutically treated kids than in
prophylactically treated kids. However, more cryptosporidia were found in prophylactically treated kids on 13 DPI. Data for the severity of ileal infections, expressed as the mean number of cryptosporidia per ileal villus are presented in table I. These data showed that the mean number of cryptosporidia per ileal villus was significantly higher in cotrimoxazole therapeutically treated kids on 9 DPI.

**DISCUSSION**

A major objective of many workers involved in Cryptosporidium research was to develop reproducible experimental animal models of the infection and clinical disease. Acute clinical infections have proven difficult to establish in small laboratory animals. As with other ruminants, neonatal kids are susceptible to *C parvum* infection and clinical infections have been established in experi-

**Table I.** Mean number of cryptosporidia per ileal villus in untreated and cotrimoxazole treated kids.

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>Untreated</th>
<th>Prophylactically treated</th>
<th>Therapeutically treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>57.2 ± 18.9</td>
<td>43.2 ± 29.7</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>31.7 ± 19.3</td>
<td>37.5 ± 11.9</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>32.6 ± 21.6</td>
<td>23.5 ± 9.2</td>
<td>38.8 ± 22.9</td>
</tr>
<tr>
<td>9</td>
<td>2.7 ± 2.05</td>
<td>5.3 ± 3.6</td>
<td>14.9 ± 7.1^a</td>
</tr>
<tr>
<td>13</td>
<td>ND</td>
<td>6.5 ± 3.6</td>
<td>10.7 ± 6.2</td>
</tr>
</tbody>
</table>

^a Differs significantly from the mean number of cryptosporidia per ileal villus in untreated kids (*P* < 0.05, ANOVA).
mentally infected kids (Tzipori et al, 1982; Current et al, 1983). Neonatal kids experimentally inoculated with *C. parvum* oocysts were also used to test anticryptosporidial activity of paromomycin (Mancassola et al, 1995). Recently, we have characterized kids as model for experimental cryptosporidiosis (Koudela and Vitovec, 1997).

Immunodeficient individuals who have cryptosporidiosis may develop life-threatening diarrhea (O'Donoghue, 1995). Treatment with cotrimoxazole has provided some clinical improvement in immunodeficient patients with cryptosporidiosis (Del Bono et al, 1987). In the present study, we used daily doses of cotrimoxazole which are recommended for therapy in toxoplasmosis and *Pneumocystis carinii* pneumonia (PCP) in immunodeficient patients.

Combinations of sulfonamides with folic acid inhibitors have long been used for the treatment of intestinal coccidiosis in animals (Long, 1993). However, studies on the efficacy of sulfonamides with folic acid inhibitors against cryptosporidiosis are few. Mirtschin and Ormerod (1990) reported that trimethoprim/sulfamethoxazole-based products used for snake cryptosporidiosis induced oocyst-negative stools. In another study, trimethoprim/sulfadiazine treatment also induced *Cryptosporidium serpentis* negative feces as determined by flotation, however, histological examination of the gastric mucosa revealed cryptosporidial developmental stages (Norton and Jacobson, 1989).

Studies of treatment of experimental cryptosporidiosis with sulfonamides presented mixed results. Sulfadimethoxine reduced cryptosporidial infection in immunosuppressed rats (Regh et al, 1988; Regh, 1991), and sulfonamide prophylaxis was obtained in another immunosuppressed rat model (Brasseur et al, 1991). In contrast, the results of another study have shown that sulfadimethoxine did not have anticryptosporidial activity in experimentally infected calves. Calves treated with 5 mg of sulfamethoxine for 21 consecutive days had diarrhea on average 2.3 days longer than untreated calves, and more *C. parvum* oocysts were shed per milliliter of feces by treated than by untreated calves (Fayer, 1992).

Our results show that treatment with cotrimoxazole did not significantly influence the clinical course of experimental cryptosporidiosis. Total days and severity of diarrhea were similar for all groups of kids. However, the mean duration of oocyst shedding, mean number of cryptosporidia per ileal villus, and distribution of cryptosporidia in the intestine were increased in both groups of cotrimoxazole-treated kids. These findings can be explained by the changes of intestinal microflora in cotrimoxazole-treated kids. It was noted that the intestinal microflora may stimulate nonspecific mechanisms contributing to resistance (Harp et al, 1992).

ACKNOWLEDGMENTS

We thank M Váňová and Š Kučerová for the animal care and V Schacherlová for her technical assistance in laboratory. This study was supported by the Grant Agency of the Czech Republic, grant no 508/93/2479.

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