Experimental Study on Mixed Infections of *Cryptosporidium muris* and *C. parvum* in Severe Combined Immunodeficient (SCID) and BALB/c Mice

Yuddhakarn Yananto[1,2], Punnarai Veeraseatakul[2]

[1]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro Hokkaido, Japan*
[2]*Regional Medical Sciences Center, Chiang Mai, Thailand*

Abstract

We sought to examine the possibility of mixed infections of *Cryptosporidium muris* and *Cryptosporidium parvum* in severe combined immunodeficient (SCID) and BALB/c mice. Oocysts of *C. muris* and *C. parvum* were injected per os into 6-week-old SCID and 4-week-old BALB/c mice. Thereafter, mouse feces were collected every 2-3 days, in 2.5% potassium dichromate. Following oocyst isolation by the sucrose flotation method, oocysts were detected using direct immunofluorescence assay. Mixed infection was demonstrated in both SCID and BALB/c mice. These results indicate that *C. muris* and *C. parvum* can infect a host simultaneously without oppressing the rival and that ongoing infection of one species cannot inhibit the establishment of subsequent infection of the other species.

Keywords: cryptosporidiosis, *Cryptosporidium muris*, *Cryptosporidium parvum*, SCID mouse, BALB/c mouse, mixed infection

Introduction

Cryptosporidiosis is a disease caused by coccidian parasites of the genus *Cryptosporidium*, inhabiting the microvilli of the epithelial surfaces of gastrointestinal and respiratory tracts of a wide variety of vertebrates, including humans. Various species of *Cryptosporidium* have been reported [1], which are well differentiated on the basis of oocyst morphology and site of infection [2]. *C. parvum* and *C. muris* are two species that infect a wide variety of mammalian hosts. Several studies have shown non-infectivity of *C. muris* oocysts obtained from cattle to neonatal BALB/c mice, adult BALB/c mice, SCID mice, common voles, common field mice, desert gerbils, guinea pigs, rats, rabbits or goats, and has transmissibility only to Mongolian gerbils [3,4]. Analysis of *Cryptosporidium* based on 18S small subunit suggested that *Cryptosporidium* possesses a specific host range [5]. Wade *et al* [6] had reported the recovery of *C. parvum* only from cattle younger than 6 months old, particularly from cattle 3-30 days old; while Olson *et al* [7] had recovered *C. muris* from cows older than 4 weeks.

Although *C. parvum* and *C. muris* inhabit separate organs and have been reported together in occurrence in farm and in wild animal populations [7,8], no simultaneous or concomitance of *C. muris* and *C. parvum* has been reported to date. In the present study, we sought to examine the possibility of mixed

Correspondence:
Yuddhakarn Yananto, Regional Medical Sciences Center, 191 M 5, Donkaew, Maerim, Chiang Mai 50180, Thailand
Tel: 66-53-211066; Fax: 66-53-219223
E-mail: yuddhakarn@thaimail.com

infections of *C. muris* and *C. parvum* in severe combined immunodeficient (SCID) and BALB/c mice.

Materials and methods

Oocysts and mice

The purified and viable oocysts of *C. parvum* (cattle origin) and *C. muris* (strain RN66) were supplied from Tokyo University and kept in 2.5% potassium dichromate ($K_2Cr_2O_7$) solution at 4˚C prior to the experiment. Nine female 6-week-old SCID mice, and 12 female 4-week-old BALB/c mice, were housed individually in a wire-floored cage placed on a tray containing 2.5% potassium dichromate solution.

Inoculation

SCID and BALB/c mice were divided into 3 groups. One group was inoculated orally with 10^5 oocysts of *C. muris*, another with 10^4 oocysts of *C. parvum*, and the remaining group with mixed 10^5 *C. muris* and 10^4 *C. parvum* oocysts. Mouse feces were collected starting the seventh day post-inoculation and at a 2-3 day interval, in 2.5% potassium dichromate.

Oocyst isolation

Oocysts were isolated using the cold sucrose flotation method. The fecal material was placed in a 50 ml centrifuge tube, to which 30 ml of sucrose solution (specific gravity 1.2) were added, shaken vigorously, laid-over with 5 ml of DW, and centrifuged at 4˚C at 411g for 30 minutes. Ten ml of the top layer of sucrose solution were transferred to new 50 ml centrifuge tubes plus 40 ml of DW. Following centrifugation at 1,643g for 15 minutes, 25 ml of supernatant were decanted and 25 ml of DW were added. After another centrifugation at 1,643g for 15 minutes, supernatant was decanted and 1,000 µl of DW were added. Finally, the suspension was mixed well and transferred to 1.5 ml tube.

Oocyst detection

Direct immunofluorescence assay was used. Five hundred ml of acetone in a 1.5 ml centrifuge tube were mixed with 10 µl of oocyst suspension. Oocysts were fixed for 3 minutes. Following centrifugation at 15,000 rpm for 2 minutes, the supernatant was discarded and 5 µl of fluorescein labeled anti-*Cryptosporidium/Giardia* (Cellabs) were added. The mixtures were incubated at 37˚C for 45 minutes then washed in 500 µl of PBS, 3x, and 490 µl of supernatant were discarded. All 10 µl of suspension were smeared on glass-slide and dried. The smears were mounted and covered with cover-glass. The oocysts whose sizes were 7.5-9.8 µm were identified as *C. muris* whereas 4.5-5.4 µm oocysts were identified as *C. parvum*.

Results

We have obtained data that demonstrate the establishment of mixed infection of *C. muris* and *C. parvum* in both SCID and BALB/c mice (Tables 1 and 2). One SCID mouse and one BALB/c mouse inoculated with *C. muris* did not shed oocysts, whereas all mice inoculated with *C. parvum* did. *C. muris* was detected in both mixed infection and the control (single species inoculation) as early as day 11 in BALB/c mice and only on day 14 in SCID mice. The maximum number of oocysts shed in BALB/c mice of *C. muris* was on days 16 and 18. In BALB/c mice, *C. muris* oocysts disappeared on day 23. *C. parvum* oocysts were detected as early as day 7 in both mouse models, suggesting a shorter prepatent period than *C. muris*. *C. parvum*, however, has a longer infection period than *C. muris*. The maximum number of oocysts shed in BALB/c mice of *C. parvum* was on days 9 and 11. We noted the same patterns of *C. parvum* and *C. muris* oocyst production in both SCID and BALB/c mice between mixed infection and single species infection.

Discussion

In this study, we used oocyst/day unit, not oocyst/gram, because fecal matter was soaked with $K_2Cr_2O_7$ solution. All fecal samples in mice were collected as the amounts were not as great compared with cattle fecal samples, which were difficult to collect because of the large amounts. The number of oocysts was independent of the amount of fecal sample and the host's behavior. Identification in this study was based on the

Table 1 Numbers* of oocysts shed by SCID mice.

Species (Mouse no.)	Day 7	Day 9	Day 11	Day 14	Day 16	Day 18	Day 21
Group I							
C. muris (1)	0	0	0	0	0	0	0
C. muris (2)	0	0	1	4	36	189	378
C. muris (3)	0	0	0	0	0	51	85
Group II							
C. parvum (1)	20	9	8	86	128	275	1,155
C. parvum (2)	6	13	50	11	22	48	67
C. parvum (3)	17	357	392	478	1,223	4,235	2,620
Group III							
C. m. + *C. p.* (1)	0 + 23	0 + 1	0 + 3	0 + 0	3 + 4	18 + 9	13 +12
C. m. + *C. p.* (2)	0 + 29	0 + 6	0 + 31	3 + 19	1 + 12	115 +32	235 +16
C. m. + *C. p.* (3)	0 + 1	0 + 4	0 + 12	0 + 0	4 + 77	3 +527	1 +433

*No. of oocysts x 100/day

Table 2 Numbers* of oocysts shed by BALB/c mice.

Species (Mouse no.)	Day 7	Day 9	Day 11	Day 14	Day 16	Day 18	Day 21	Day 23
Group I								
C. muris (1)	0	0	0	0	0	0	0	0
C. muris (2)	0	0	1	5	4	218	7	1
C. muris (3)	0	0	9	11	241	202	11	0
C. muris (4)	0	0	3	4	316	233	1	6
Group II								
C. parvum (1)	11	11	12	5	4	4	1	0
C. parvum (2)	5	4	6	4	1	2	1	1
C. parvum (3)	6	5	5	0	0	0	0	0
C. parvum (4)	11	19	5	1	0	0	0	0
Group III								
C. m. + *C. p.* (1)	0 +13	0 + 8	0 + 1	8 + 1	171 + 2	330 + 1	1 + 1	0 + 0
C. m. + *C. p.* (2)	0 + 4	0 + 5	0 + 2	6 + 3	102 + 3	55 + 1	1 + 0	0 + 0
C. m. + *C. p.* (3)	0 + 5	0 +24	2 + 4	1 + 3	92 + 2	28 + 1	3 + 1	0 + 2
C. m. + *C. p.* (4)	0 +17	0 +15	7 + 5	14 + 3	99 + 2	18 + 1	2 + 1	0 + 2

*No. of oocysts x 100/day

morphology characteristics of the oocysts ie the size of *C. parvum* oocysts is 5.0 x 4.5 μm and size of *C. muris* oocysts is 8.4 x 6.3 μm. It was easy to distinguish between these two species. Moreover, it was observed from our previous experiments (data not shown) that the more times that *C. parvum* oocysts were passaged to another mouse the shorter the prepatent period needed. It was also observed that the maximum number of oocysts shed by SCID mice was larger than BALB/c mice and that SCID mice could not get rid of *Cryptosporidium* until they died because their immune was very deficient. As the patterns of oocysts shedding in mixed infection and single infection (control) were similar in both species and both mouse types and the *C. muris*: *C. parvum* ratios in SCID mice were not on specific sides; it could be said that these 2 species did not express antagonism or synergism. The concomitance of them can take place maybe because they infect distinct organs but if they are of the same species or infect the same organ, they might not infect simultaneously or be antagonists, such as the interactions between the same species of schistosome where the infecting adult worms inhibit the establishment of a subsequent infection by larval forms [9]. The reasons why there may be no report of mixed infection of the *Cryptosporidium* species in nature may probably be their host specificity, preferred age of the hosts, low numbers and low susceptibility of non-experimental animals as they need immunocompetent hosts.

Why can hosts of *C. parvum* not inhibit subsequent infection by *C. muris*? Probably because their antigens, recognized by immune animals, are different. Répérant *et al* [10] have reported major antigens of *C. parvum* recognized by serum antibody from different infected animal species and man that were 15-17 and 23 kDa present on the sporozoites in the mouse and calf. However, there is no study that compares the antigen recognized by hosts between these two species. As it was shown by Tarazona *et al* [11] that younger mice have greater susceptibility to *C. parvum*, we would like to propose that *C. parvum* prefers a basic pH, whereas *C. muris* prefers an acidic pH. While *C. parvum* infects the bile duct and small intestine which is the place after the bile secretion site, as well as bile is a basic secretion, on the other hand, *C. muris* infects the stomach in which the conditions get more acidic with older age. Hijjawi *et al* [12] have described the complete development of *C. parvum in vitro* and have found that pH appeared to play an important role in successfully maintaining the growth of *C. parvum in vitro*. The optimum pH was 7.2-7.6. However, the *in vitro* complete life cycle of *C. muris* has not yet been reported.

Acknowledgements

We would like to thank Tokyo University for donation of *C. muris* oocysts and the National Research Center for Protozoan Diseases for the work place. This research was funded by the Japan International Cooperation Agency.

References

1. Fayer R, Morgan U, Upton SJ. Epidemiology of *Cryptosporidium*: transmission, detection and identification. *Int J Parasitol* 2000;30:1305-22.
2. O'Donoghue PJ. *Cryptosporidium* and cryptosporidiosis in man and animals. *Int J Parasitol* 1995;25:139-95.
3. Anderson BC. Experimental infection in mice of *Cryptosporidium muris* isolated from a camel. *J Protozool* 1991;38:16S-17S.
4. Koudela B, Modr'y D, Vi'tovec J. Infectivity of *Cryptosporidium muris* isolated from cattle. *Vet Parasitol* 1998;76:181-8.
5. Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montli RJ, *et al*. Phylogenetic analysis of *Cryptosporidium* parasites based on the small subunit sRNA gene locus. *Appl Environ Microbiol* 1999;65:1578-83.
6. Wade SE, Mohammed HO, Schaaf SL. Prevalence of *Giardia* sp., *Cryptosporidium parvum* and *Cryptosporidium muris* (*C. andersoni*) in 109 dairy herds in five counties of southeastern New York. *Vet Parasitol* 2000;93:1-11.
7. Olson ME, Guselle NJ, O'Handley RM, Swift ML, McAllister TA, Jelinski MD, *et al*. *Giardia* and *Cryptosporidium* in dairy calves in British

Columbia. *Can Vet J* 1997;38:703-6.

8. Chalmer RM, Sturdee AP, Bull SA, Miller A, Wright SE. The prevalence of *Cryptosporidium parvum* and *C. muris* in *Mus domesticus, Apodemus sylvaticus* and *Clethrionomys glaredus* in an agriculture system. *Parasitol Res* 1997;83:478-82.
9. Smithers SR, Terry RJ, Hockley DJ. Host antigens in schistosomiasis. *Proc R Soc Lond B Biol Sci* 1969;171:483-94.
10. Répérant JM, Naciri M, Iochmann S, Tilley M, Bout D. Major antigens of *Cryptosporidium parvum* recognised by serum antibody from different infected animal species and man. *Vet Parasitol* 1994;55:1-13.
11. Tarazona R, Blewett DA, Carmona MD. *Cryptosporidium parvum* infection in experimentally infected mice: infection dynamics and effect of immunosuppression. *Folia Parasitol* 1998;45:101-7.
12. Hijjawi NS, Meloni BP, Morgan UM, Thomson RCA. Complete development and long-term maintenance of *Cryptosporidium parvum* human and cattle genotypes in cell culture. *Int J Parasitol* 2001;31:1048-55.