APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3234–3237 Vol. 62, No. 9
0099-2240/96/$04.00+0


# Viability and Infectivity of *Cryptosporidium parvum* Oocysts Are Retained upon Intestinal Passage through a Refractory Avian Host

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Received 18 April 1996/Accepted 1 July 1996

**Six *Cryptosporidium*-free Peking ducks (*Anas platyrhynchos*) were each orally inoculated with $2.0 \times 10^6$ *Cryptosporidium parvum* oocysts infectious to neonatal BALB/c mice. Histological examination of the stomachs jejunums, ilea, ceca, cloacae, larynges, tracheae, and lungs of the ducks euthanized on day 7 postinoculation (p.i.) revealed no life-cycle stages of *C. parvum*. However, inoculum-derived oocysts extracted from duck feces established severe infection in eight neonatal BALB/c mice (inoculum dose, $2.5 \times 10^5$ per mouse). On the basis of acid-fast stained direct wet smears, 73% of the oocysts in duck feces were intact (27% were oocyst shells), and their morphological features conformed to those of viable and infectious oocysts of the original inoculum. The fluorescence scores of the inoculated oocysts, obtained by use of the MER*IFLUOR* test, were identical to those obtained for the feces-recovered oocysts (the majority were 3+ to 4+). The dynamics of oocyst shedding showed that the birds released a significantly higher number of intact oocysts than the oocyst shells ($P < 0.01$). The number of intact oocysts shed (87%) during the first 2 days p.i. was significantly higher than the number shed during the remaining 5 days p.i. ($P < 0.01$) and significantly decreased from day 1 to day 2 p.i. ($P < 0.01$). The number of oocyst shells shed during 7 days p.i. did not vary significantly ($P > 0.05$). The retention of infectivity of *C. parvum* oocysts after intestinal passage through an aquatic bird has serious epidemiological and epizootiological implications. Waterfowl may serve as mechanical vectors for the waterborne oocysts and may enhance contamination of surface waters with *C. parvum*. As the concentration of *Cryptosporidium* oocysts in source waters is attributable to watershed management practices, the watershed protection program should consider waterfowl as a potential factor enhancing contamination of the source water with *C. parvum*.**

*Cryptosporidium parvum*-associated cryptosporidiosis is a zoonosis which contributes significantly to the mortality of immunocompromised or immunosuppressed humans (17). The transmissible stage, the oocyst, is the most important contaminant in drinking water in the United States (36). Oocysts represent a public health threat (3), having caused numerous outbreaks in recreational and finished drinking waters (27), including 403,000 cases in an outbreak in Milwaukee, Wis. (28). Cryptosporidia are also a major cause of gastrointestinal illness in nonoutbreak settings (31). The oocysts, prevalent in source waters, are resistant to conventional water disinfection procedures applied to other waterborne pathogens and remain viable in the environment over long periods, particularly when in association with fecal material (27).

Although previous epidemics of diarrheal disease were associated with contamination of source waters and suboptimal water treatment processes (27), the recent outbreak in municipal water in Clark County (Nevada) occurred despite state-of-the-art water treatment (16). Such outbreaks resulted in the U.S. Environmental Protection Agency issuing procedures for water monitoring and watershed management to protect source waters from contamination with the oocysts (36). The most significant deficiency of the current watershed protection program is that environmental factors that contributes to contamination, or has the potential to do so, are not clearly recognized (27).

*C. parvum* infects a variety of domestic and free-ranging mammals, particularly ruminants (9, 13), which may disseminate oocysts within a watershed area. *Cryptosporidium* infections have been reported to occur in wildlife (7, 11, 29), and the large game animals have been suggested as a cause of oocyst contamination in pristine waters (22). Although *C. parvum* is noninfectious to lower aquatic and semiaquatic vertebrates, those poikilothermic animals can disseminate ingested oocysts as well (20).

Cross-transmission of *C. parvum* to birds has yielded variable results which are difficult to interpret (32). One study demonstrated experimental establishment of a weak tracheal infection in 1- and 7-day-old chickens (26). Even if *C. parvum* is not infectious to birds, the question of whether wild aquatic birds, with unlimited access to most surface waters, can disseminate *C. parvum* oocysts acquired by ingestion remains unanswered. Pastures, grazing lands, and animal manure are recognized as significant sources of viable *C. parvum* oocysts

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(4, 23, 27). For example, cider from applies collected from pastures caused a massive outbreak of cryptosporidiosis (30). During spring and fall migrations, thousands of waterfowl use fields, pastures, ponds, and cattle grazing lands for feeding and resting (14). Yet waterfowl have not received epidemiological or epizootiological attention as a vector for waterborne *Cryptosporidium* oocysts.

The purpose of the present study was to determine if *C. parvum* could establish intestinal or respiratory tract infections in Peking ducks (*Anas platyrhynchos*) or if experimentally infected birds would excrete infectious *C. parvum* oocysts that simply passed through the digestive system. Peking ducks were selected because their wild strain (Mallard duck, *A. platyrhynchos*) is abundant in the wild and because they represent a convenient laboratory model for other Anatidae, which include most waterfowl species.

## MATERIALS AND METHODS

*C. parvum* AUCP-1 oocysts obtained from the feces of experimentally infected Holstein calves (10) were purified by cesium chloride (CsCl) gradient centrifugation (24) and stored for 2 weeks at 4°C in potassium dichromate ($K_2Cr_2O_7$). To remove $K_2Cr_2O_7$, the oocysts were washed five times with phosphate-buffered saline (PBS) (pH 7.4) by centrifugation (750 × *g*, 5 min) at 4°C. The oocysts were numerically evaluated with a hemacytometer (10). The infectivity of the oocysts was tested by gastric intubation of six neonatal BALB/c mice (12).

Thirteen 2-day-old Peking ducks (*A. platyrhynchos*) obtained from a commercial hatchery (The Ridgway Hatcheries, Inc., LaRue, Ohio) were housed indoors in a room with a tap water swimming pool and pelleted food (PURINA; Purina Mills, Inc., St. Louis, Mo.) available ad libitum. At 2 months of age, the ducks were divided into groups of seven and six birds and housed in separate rooms without swimming pools but with food and water available ad libitum. Each of the six ducks in one group was intubated directly in the stomach with 5.0 ml of PBS containing $2.0 \times 10^6$ *C. parvum* oocysts by using the ARGYLE Premature Infant Feeding Set (Sherwood Medical, A Brunswick Company, St. Louis, Mo.). The remaining seven ducks served as uninoculated controls.

Duck feces were collected every 2 weeks after the birds' arrival and examined by acid-fast stained direct wet smears (AFS DWS) for *Cryptosporidium* oocysts. The feces were collected daily from 3 days prior to inoculation until 7 days postinoculation (p.i.). Because ducks in each group were housed together, collective fecal specimens contained stools of all animals in the group. The feces collected daily were examined for *Cryptosporidium* oocysts by both AFS DWS and the MER*IFLUOR Cryptosporidium*/*Giardia* test kit (Meridian Diagnostic, Inc., Cincinnati, Ohio) for direct immunofluorescence according to the manufacturer's instructions. Fecal specimens obtained after inoculation were weighed and mixed to homogeneity with 10% (wt/vol) PBS (pH 7.4). Ten AFS DWS were prepared (2). Each was examined for 10 min by light microscopy, and the numbers of oocyst shells and intact oocysts were counted. The ratio of oocyst shells to intact oocysts (expressed as a percentage) was computed on the basis of the mean number obtained from 10 counts and adjusted for the 1/10 dilution. Of each fecal specimen collected 1 and 2 days p.i., 100 g was subjected to Sheather's sugar coverslip flotation (SSCF) (2). The SSCF-recovered material was examined by AFS as described above and by immunofluorescence microscopy using the MER*IFLUOR* test. Examination of slides and scoring of fluorescence followed the previous protocol (15). Fecal specimens positive for oocysts by AFS DWS and the MER*IFLUOR* test were used for oocyst purification conducted at 4°C (18) by CsCl gradient centrifugation. The number of oocysts was assessed both by using a hemacytometer (10) and by using the MER*IFLUOR* test (15).

A total of $2.5 \times 10^6$ *C. parvum* oocysts purified from duck feces 1 and 2 days p.i. were divided evenly into 10 vials, and eight 5-day-old suckling BALB/c mice were individually inoculated by gastric intubation with $2.5 \times 10^5$ oocysts in 50 μl of PBS. To further determine if CsCl processing altered oocyst viability, eight 5-day-old suckling BALB/c mice were individually inoculated in the same manner with the same number of *C. parvum* oocysts removed from $K_2Cr_2O_7$ storage and processed by CsCl gradient centrifugation. All 16 mice were euthanized (12) on day 7 p.i., and their intestinal sections were processed and histologically examined for development of *Cryptosporidium* stages (12). The intensity of infection was scored as previously described (8).

All ducks were euthanized in a saturated $CO_2$ atmosphere on day 7 p.i. and necropsied. For each duck, the entire digestive tract, larynx, trachea, and one lung were removed and fixed separately in 10% neutral buffered formalin (2). The respiratory tract was collected because it has been reported that *C. parvum* can establish respiratory infections in chickens (26). Segments of the stomach, jejunum, ileum, cecum, cloaca, larynx, trachea, and lung were embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin (12). Histologic sections were examined for developmental stages of *C. parvum* by light microscopy.

Statistical analysis was performed with Statistix 4.1 (Analytical Software, St. Paul, Minn.). The variables were examined by the runs test to determine if their distribution conformed to a normal distribution. Nonparametric tests were used (Kruskal-Wallis analysis of variance and rank sum test) to assess the significance of differences between variables. The *G* heterogeneity test (35) was used to assess differences between fractions. The mean values ($\bar{x}$) were associated with standard deviations. Statistical significance was considered to be a $P$ value of $<0.05$.

## RESULTS

Fecal specimens collected from all 13 ducks prior to inoculation with *C. parvum* oocysts and all feces from the 7 noninoculated ducks were negative for *Cryptosporidium* oocysts.

The weight range of seven fecal specimens from the inoculated ducks was 540 to 920 g ($\bar{x} = 714 \pm 177$ g), and the range was 500 to 1,150 g ($\bar{x} = 728 \pm 206$ g) for the controls. Although the total mean weight was higher for the control group, which included one more bird, the difference between groups in mean weight of produced feces was not significant (rank sum test; $t = 1.35$, $P > 0.05$).

When examined by light microscopy, *C. parvum* oocysts used for inoculation displayed, in AFS, a nonuniform, bright red coloration, contained characteristic black granules, and had densely packed cytoplasm. In contrast, oocyst shells, which constituted approximately 20% of the oocysts in the original inoculum were pale, were uniformly stained (pink to light red) and did not contain black granules. The oocysts shed by the six inoculated ducks revealed two AFS variations. The features of intact oocysts and oocyst shells conformed to the morphological features observed for the original-inoculum oocysts. The oocyst shells constituted 27% of all duck-released oocysts.

*Cryptosporidium* oocysts were detected by AFS DWS in feces from all seven inoculated ducks. The dynamics of shedding of intact oocysts and oocyst shells are presented in Fig. 1. Enumeration of intact oocysts and oocyst shells in AFS DWS yielded the following observations. (i) Over time, ducks shed a significantly higher number of intact oocysts than oocyst shells (rank sum test; $t = 4.85$, $P < 0.01$). (ii) The number of intact oocysts shed during the first 2 days p.i. was significantly higher than that for the remaining 5 days p.i. (rank sum test; $t = 4.09$, $P < 0.01$); however, the number of intact oocysts significantly decreased from day 1 to day 2 p.i. (rank sum test; $t = 2.87$, $P < 0.01$). (iii) The number of oocyst shells shed during 7 days p.i. did not vary significantly (Kruskal-Wallis analysis of variance; $F = 1.33$, $P > 0.05$). Overall, the oocyst shells constituted 27% of all duck-released oocysts and 16% for the first 2 days p.i., when the oocyst output was most intense. The ratios of oocyst shells to intact oocysts (×100%) were 15, 25, 91, 75, 49, 38, and 142% for the 7 consecutive p.i. days, respectively.

AFS examination of the material recovered by the SSCF from fecal specimens obtained on days 1 and 2 p.i. revealed both intact oocysts and oocyst shells at ratios of 17 and 26%, respectively. The ratios did not differ significantly from those of 15 and 25% from fecal DWS from days 1 and 2 p.i., respectively (*G* heterogeneity test; $G = 4.6$, $P > 0.05$). However, when the SSCF-recovered material was examined by the MER*IFLUOR* test, no difference in immunofluorescence between oocyst shells and intact oocysts was observed. The fluorescence scores for the *C. parvum* oocysts from the original inoculum were identical (the majority were 3+ to 4+) to those of oocysts recovered from duck feces by SSCF.

The total number of oocysts recovered from duck feces was $2.85 \times 10^6$ as determined with a hemacytometer and $2.90 \times 10^6$ as determined by the MER*IFLUOR* test. This represented 23.8 and 24.5%, respectively, of the total of $1.2 \times 10^7$ oocysts administered as the inoculum. The majority of oocysts (approximately 87% of the total recovered) was released by birds during the first 2 days p.i. Oocyst concentration significantly

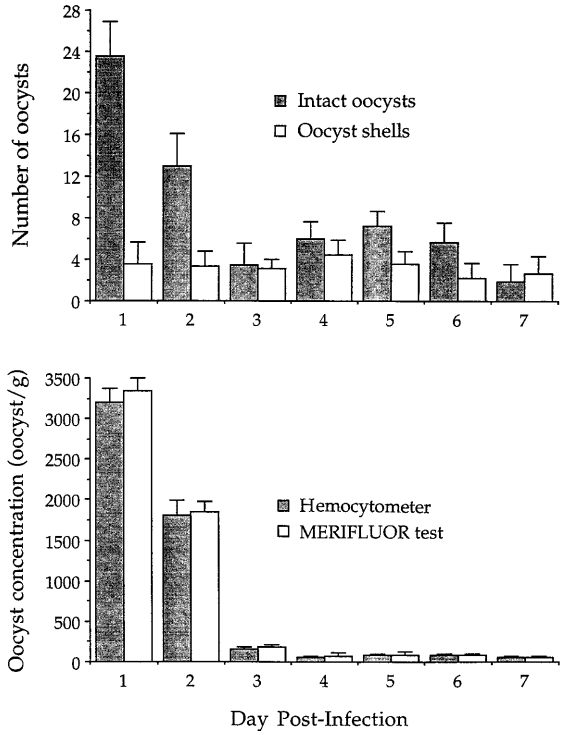


FIG. 1. Temporal distribution of the mean numbers (plus standard deviations) of *C. parvum* oocysts shed in the feces of six Peking ducks (*A. platyrhynchos*), each gastrically intubated with $2.0 \times 10^6$ of *C. parvum* (AUCP-1 strain) oocysts. The number of oocysts was determined by 10-min examination of 10 AFS DWS, and fecal oocyst concentration was determined by extraction of oocysts from feces, purification by cesium chloride gradient centrifugation, and enumeration with a hemacytometer and by immunodetection using the MER*IFLUOR Cryptosporidium/Giardia* test kit for direct immunofluorescence.

decreased from day 1 to day 2 p.i. (rank sum test; $t = 2.71$, $P < 0.01$). When the oocysts recovered from feces and purified by CsCl gradient centrifugation were assessed by AFS it appeared that oocyst shells constituted less than 10%.

The ducks inoculated with *C. parvum* oocysts did not exhibit clinical signs of infection. Histological examination of the stomachs, jejunums, ilea, ceca, cloacae, larynges, tracheae, and lungs of all six ducks inoculated with *C. parvum* oocysts and euthanized on day 7 p.i. revealed no development of cryptosporidia in any part of the digestive or respiratory system.

The ilea of all eight neonatal BALB/c mice inoculated with *C. parvum* oocysts extracted from duck feces contained large numbers of life-cycle stages of cryptosporidia; more than 67% of the epithelial cells harbored developmental stages. The eight control mice challenged with the *C. parvum* oocysts recovered from $K_2Cr_2O_7$ storage and purified by CsCl gradient centrifugation had similarly high numbers of intracellular cryptosporidia in the ilea.

## DISCUSSION

Because developmental stages of cryptosporidia were not detected in histological sections of Peking ducks inoculated with infectious *C. parvum* oocysts, we conclude that the pathogen was unable to establish gastrointestinal or respiratory infection. However, the viability and infectivity of *C. parvum* oocysts were retained after passage through the intestines of the refractory avian hosts.

The fact that *C. parvum* oocysts, which are pathogenic to humans and other mammals, remained viable and infective after intestinal passage through an aquatic bird has serious epidemiological and epizootiological implications; waterfowl may serve as a mechanical vector for this waterborne pathogen and disseminate infectious oocysts in an aquatic environment. Much of the waterfowl daily activity involves grazing on land and in shallow waters with defecation into the water (5). It has been demonstrated that grazing frequency is a factor predisposing waterfowl to contact with pathogens that can be transmitted via water (5). Grazing of aquatic birds around water supplies with agricultural drainage causes waterfowl toxicosis (34).

Results of the present study may suggest useful modifications to the management of watershed treatment protection plans. The small size of oocysts (3.5 to 5.5 μm in diameter), their low sedimentation velocity (0.5 μm/s), and water movement may prevent oocyst sedimentation in water reservoirs (27). If oocysts shed by aquatic birds are detected at water treatment facilities (WTF) by the immunofluorescence antibody (IFA) method (1, 21), which can be used in conjunction with existing and proposed regulations, even noninfectious waterborne oocysts (i.e., oocyst shells) will produce a positive reaction. The majority (approximately 75%) of waterborne *Cryptosporidium* oocysts recovered at WTF were suggested to be nonviable, as they did not contain sporozoites (25). Although oocyst shells are not of epidemiological importance, their presence will still alert WTF diagnostic laboratories by yielding positive IFA assay results. Considering the potential for movements and migrations of waterfowl, the timing of oocyst detection at WTF water intake may result in failure to correlate the presence of oocysts with the presence of birds.

Both dynamics observed in the present study (oocyst concentration in the duck feces and oocyst output as determined by AFS DWS) were consistent; the majority of the inoculated oocysts (approximately 87%) were shed during the first 48 h p.i. However, the oocyst shells, undistinguishable by IFA from the viable and infectious oocysts, were detected in similar quantities during all 7 days p.i. The present experiment utilized a single-dose inoculum, but if waterfowl graze in contaminated fields or pastures, ingestion of *C. parvum* oocysts can be continuous or intermittent. Consequently, the pattern of shedding of ingested oocysts may vary from the pattern observed in the experiment, in that the birds may constantly shed the oocysts.

Except for a single case of *Cryptosporidium baileyi* infection in an AIDS patient (6), human cryptosporidiosis is exclusively due to *C. parvum*. The seven other valid species of *Cryptosporidium* (13) are medically unimportant. However, the oocysts of these non-human-pathogenic species of *Cryptosporidium* have produced positive IFA reactions with commercially available test kits (19). The oocysts recovered from waterfowl-contaminated waters will produce IFA reactions similar to those obtained with *C. parvum* even if other species of *Cryptosporidium* are present, leaving the question of species identification unanswered.

The striking observation of the present study is that the majority of viable oocysts of *C. parvum* (approximately 73% of oocysts detected) did not excyst in the intestines of homothermous vertebrates, even if the host species is refractory to the pathogen. Cross-transmission of *C. parvum* to birds has yielded variable results (32, 33); however, it was demonstrated that in 1- and 7-day-old chickens a weak, short-lasting tracheal (but not intestinal) infection can be established (26). In that experiment (26), oocysts of tracheal origin were found in the chicken feces. This provides evidence that *C. parvum* oocysts did not excyst in the digestive tract, and this observation is confirmed with regard to ducks by the results of the present experiment.

The rate and dynamics of in vivo excystation of *C. parvum* oocysts were not measured in the present study. The oocyst shells, which may represent excysted oocysts, constituted approximately 27% of the oocysts; however, considering host-related factors (e.g., peristalsis) and the efficiency of oocyst detection, it is undetermined if the observed dynamics of oocyst shell shedding reflect the dynamic of in vivo excystation.

On the basis of our results, we conclude that birds, particularly migratory waterfowl, abundantly residing in the watershed area should be considered a potential factor enhancing contamination of the water with *Cryptosporidium* oocysts. As oocyst concentration in the source waters is attributable to watershed management practices (22), modifications of watershed control activities regarding waterfowl may enhance prevention or reduce contamination and may therefore decrease the number of cases in a nonoutbreak setting (31) due to recreational contact with surface water. Although a flock of Canada geese visiting a protected water supply may help meet the wildlife conservation goal of the watershed protection program and of state and federal environmental laws, their presence also may have epidemiological implications.

## ACKNOWLEDGMENTS

We thank B. Haynie, L. Campbell, and E. Kovacs for technical assistance and G. Gurri-Glass for facilitating this study.

The study was sponsored in part by grant 3 H-680-951-2118 from the Maryland Zoological Society and the AKC Fund of New York.

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