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SHORT PAPER

Cryptosporidium parvum: oocyst excretion and viability patterns in experimentally infected lambs

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SUMMARY

Cryptosporidium parvum infections of domestic animals can have a considerable economic impact and as oocysts are voided in the faeces of infected hosts, environmental contamination with agricultural waste has also become a matter of concern. Since only viable oocysts are potentially infectious, the numbers of oocysts excreted during infection can have important implications for both veterinary and public health. During the course of infection in experimentally infected lambs, oocyst viability was assessed by a fluorogenic vital dyes assay and by a maximized *in vitro* excystation assay. The excreted oocyst populations contained a higher proportion of viable oocysts 5–11 days post infection (d.p.i.) than later in the infection. Oocyst viability declined consistently 11–15 d.p.i. and coincided with periods when peaks in serum and intestinal anti-*Cryptosporidium* antibodies have been reported to occur. Infected lambs excreted a mean of 4·8 (standard error [S.E.] $\pm$ 0·4) $\times 10^9$ oocysts per g of faeces, of which half were non-viable and therefore of no significance for disease transmission. This study demonstrates that the numbers of viable oocysts excreted by infected lambs is smaller than previously suspected.

Cryptosporidium parvum is a protozoan parasite capable of causing moderate to severe but self-limiting enteric disease in immunocompetent hosts. However, in the malnourished or immunocompromised individuals cryptosporidiosis can cause severe dehydration, loss of weight or even death. The low host specificity of *C parvum* and the excretion of large numbers of oocysts over the course of infection [1] suggest that various animal species can be potential reservoirs for human infection. Transmission to human beings following the handling of livestock, especially lambs, during educational farm visits has been documented [2].

Experimental infectivity trials with *C. parvum* in various animal species [3–5], including adult human volunteers [6]), indicate that ingestion of low numbers of viable oocysts can initiate infection in susceptible hosts. Thus, it is recognized that environmental contamination with small numbers of infectious oocysts can pose a significant threat to livestock and to public health. However, at present, it is unclear what proportion of excreted oocysts are viable, and hence potentially infectious. In this study the viability of oocysts excreted during the course of infection was determined. Such information is of importance when assessing the likely impact of excreted oocysts on veterinary and public health.

A cervine-ovine isolate of *C. parvum* (Moredun Research Institute, Edinburgh, UK), maintained by passage through lambs, was used. Oocysts were isolated and purified from lamb faeces [1], and stored in Hanks' balanced salt solution (HBSS), containing antibiotics, at 4 °C until used.

Two pre-infection faecal samples, taken when each lamb was 2 and 4 days old, were examined to ensure the lambs did not have naturally-acquired *Cryptosporidium* infections. Each of 11 5-day-old Finnish

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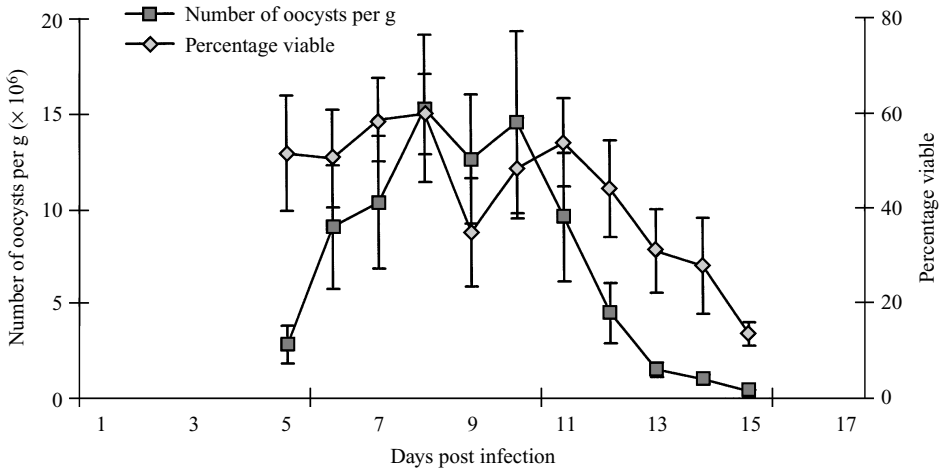

Fig. 1. *Cryptosporidium parvum* oocyst excretion and viability patterns during the course of infection. All figure values are arithmetic mean ± standard error ($n = 11$).

Landrace × Dorset, Dorset and Suffolk × Greyface lambs was inoculated orally, by syringe, with 10^6 oocysts. The infectious doses (suspended in PBS, [150 mM pH 7·2]) were prepared from stock suspensions of oocysts (viability 85–90%) following enumeration with an improved Neubauer haemocytometer. Neonatal lambs were maintained on three daily feeds of ultra-high-temperature treated (u.h.t.) cow's milk. The total daily faecal output from each individually-penned lamb was collected in faecal collection bags, which were attached to lambs with harnesses. For each lamb, the bags were changed daily, the faeces weighed and a sub-sample (in the range 1–5 g) was removed and purified by a water–ether concentration procedure [7]. The concentrated samples were examined for *C. parvum* oocysts, using an Olympus BH2 fluorescence microscope (×40 objective, ×12·5 eyepieces) equipped with Nomarski DIC optics, and oocyst enumeration was performed using an improved Neubauer haemocytometer. Oocyst viability was determined, within 48 h of oocyst excretion, primarily by the fluorogenic vital dyes (4′,6-diamidino-2-phenyl indole and propidium iodide) assay [8], but viability was also assessed by the maximized *in vitro* excystation assay [9] on randomly chosen samples.

The mean, standard error (S.E.) and median values for oocyst excretion, oocyst viability patterns and the correlation coefficient (r) between the vital dyes assay and maximized *in vitro* excystation assay were calculated.

The pre-patent period of infection was between 4 and 5 days. Mean oocyst output increased from 2·8 (S.E. ± 1·0) × 10^6 (median 1·4 × 10^6) per g of faeces (4–5 days post infection (d.p.i.)) to 1·5 (S.E. ± 0·4) × 10^7 (median 1·1 × 10^7) on 8 d.p.i., and mean oocyst output began to decline 10 d.p.i. (Fig. 1).

The mean viability of excreted oocysts varied between 50% and 69% (median 58–80%) from 5–8 d.p.i. (the first 4 days of oocyst excretion) (Fig. 1). Although a decline in the mean oocyst viability to 35% (S.E. ± 11·5) occurred on 9 d.p.i., it was not statistically significant ($P > 0·05$) and was followed by an increase in oocyst viability to 54% (S.E. ± 9·4) (median 60%) on 11 d.p.i. By 15 d.p.i. the mean percentage viability had declined to 13% (S.E. ± 2·4) (median 18%). The mean period for which oocysts were excreted in the absence of diarrhoea was 3·2 days. During this period a mean total of 5·2 × 10^7 oocysts were excreted of which 1·4 × 10^7 (27%) were viable.

Similar excretion profiles were observed for both mean of total oocyst output and mean of total viable oocyst output and peaks in these numbers (total output: 1·3 × 10^9, total viable output: 0·76 × 10^9) occurred at 8 d.p.i. (Fig. 2). During the course of oocyst excretion (9–11 days), the mean of the total oocyst output was 4·8 (S.E. ± 0·4) × 10^9 of which half [2·4 (S.E. ± 0·2) × 10^9] were viable.

The clinical signs in the experimentally-infected lambs were similar to those described for ovine cryptosporidiosis [1] and the oocyst excretion patterns were similar to those recorded in experimentally infected gnotobiotic lambs [16]. Whereas in the investigation by Hill [1, 11], Scottish blackface lambs excreted 10^7–10^9 oocysts 4–10 d.p.i., in this study, and that of Ortega-Mora and Wright [12], this range was observed 5–14 d.p.i. The duration of high oocyst

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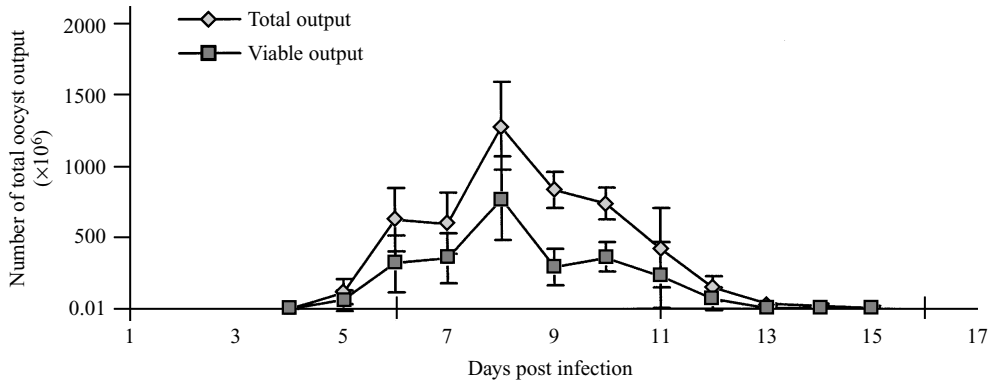

Fig. 2. Comparison of total oocyst excretion with total viable oocyst excretion per day. All figure values are arithmetic mean ± standard error ($n = 11$).

excretion was longer in our investigation when compared to that of Hill [1, 11], who used the same isolate of *C. parvum*. This may be due to lambs of different breeds exhibiting differing susceptibilities to infection. Susceptibility differences to *C. parvum* have previously been suggested for calves of different breeds by Peeters and colleagues [13].

Comparison between maximized *in vitro* excystation and the fluorogenic vital dyes assay [8] revealed a correlation coefficient (r) of 0·90 from 45 samples (40 %) taken throughout the oocyst excretion period indicating that the fluorogenic vital dyes assay is a satisfactory alternative for the maximized *in vitro* excystation assay. As *in vitro* excystation correlates well with animal infectivity [14, 15], we assume that oocysts which are viable by the fluorogenic vital dyes assay are potentially infective to susceptible hosts.

Whilst large numbers of viable oocysts were excreted 5–11 d.p.i. (during the first week of oocyst excretion), nevertheless a considerable number of non-viable oocysts were also voided. It is possible that non-specific defence mechanisms [such as the *in vivo* production of nitric oxide, as demonstrated by Leitch and He (16)], may have been responsible for this. The decline in the percentage viability of recovered oocysts was more consistent between 12 and 15 d.p.i. and may have been a consequence of immunity developing in these lambs. Between 7 and 16 d.p.i., rising titres of serum and intestinal anti-*Cryptosporidium* antibodies have been reported and correspond with declining oocyst numbers in experimentally infected lambs [1, 17]. A consistent decline in the proportion of viable oocysts that were excreted also occurred within this period of infection in our study.

Although humoral and/or cellular immune responses have been demonstrated in lambs, calves and mice, following infections with *C. parvum* oocysts [1, 18, 19], their effect(s) on oocyst viability are at present unknown and warrant investigation, as does the role of non-specific defence mechanisms.

The fact that up to half of the oocysts excreted during infection were dead is of interest. A possible reason for this could be that protective immune mechanisms generated during infection can render the zygote non-viable, but are incapable of preventing either the production of the oocyst wall or the release of dead oocysts into the intestinal lumen.

Ortega-Mora and colleagues [17] demonstrated that *C. parvum* oocyst excretion profiles for naturally and experimentally infected lambs were similar, hence similar viability patterns may also occur between experimentally and naturally acquired infections. This would result in the release of up to $2{\cdot}5 \times 10^9$ viable oocysts per infected animal. However, the epidemiological impact of viable oocysts is dependent upon their survival following a variety of environmental pressures. For example a 55 % reduction in viability occurred when oocysts were stored in bovine faeces for 176 days while exposure to freezing, desiccation or physical abrasion either reduced oocyst viability or infectivity under experimental conditions [20–22]. These reports, coupled with ours suggest that more oocysts than previously suspected, which contaminate the environment, may be non-viable. Such oocysts have no significance with respect to disease transmission.

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