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# Occurrence of oocysts of *Cryptosporidium* sp. in *Larus* spp. gulls

H. V. SMITH[1], J. BROWN[2], J. C. COULSON[3], G. P. MORRIS[2] AND R. W. A. GIRDWOOD[1]

[1] *Scottish Parasite Diagnostic Laboratory, Stobhill General Hospital, Glasgow G21 3UW, UK*

[2] *Division of Environmental Health, University of Strathclyde, Glasgow G4 1AX, UK*

[3] *Department of Biological Sciences, University of Durham, Durham DH1 3LE, UK*

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## SUMMARY

Between November 1990 and February 1991 101 gull faecal samples, collected in central Scotland, and 50 cloacal lavages, from gulls captured at two refuse tips near Durham, England were examined for the presence of *Cryptosporidium* sp. oocysts. Five of 101 (*c* 5%) of faecal samples and 11 of 50 (22%) of cloacal lavages contained oocysts, of which 64% and 83%, respectively were considered viable when examined with propidium iodide and 4′-6-diamidino-2-phenylindole. Since there is insufficient evidence to ascribe these oocysts to a recognized species they are therefore referred to as *Cryptosporidium* sp. oocysts. There were significant differences in the occurrence of oocysts between gulls captured at the different refuse tips ($P \leqslant 0{\cdot}01$), but no significant difference between the distribution of oocysts in two species of gull, *Larus argentatus* (Herring Gull) and *L. ridibundus* (Black-head Gull). The differences may be explained by different food sources and feeding habits. The contribution of gulls to environmental contamination with *Cryptosporidium* sp. oocysts is probably generally small, but may be more significant when large numbers roost on surface waters.

## INTRODUCTION

*Cryptosporidium parvum* and *C. muris* which infect mammals, and *C. baileyi* and *C. meleagridis* which infect birds are accepted as distinct species of the genus *Cryptosporidium*, although other species may exist [1]. On the basis of oocyst morphology, *C. parvum* is considered to be the species responsible for gastro-intestinal illness in humans and other mammalian species [1]. In the last decade it has become evident that *C. parvum* is a significant human pathogen causing severe, protracted diarrhoea in the immunocompromised patient and acute, self-limiting enteritis, in the immunocompetent patient. The transmissive stage is the thick walled, environmentally robust, oocyst. Human infection may be acquired from animals, and a variety of mammals can be infected with human derived

oocysts. Transmission can occur via any route by which material contaminated with viable oocysts excreted by infected individuals can reach the mouth.

Water is emerging as an important vehicle for transmission and since 1984, six documented community-wide outbreaks of cryptosporidiosis have been linked with contaminated potable supplies, and in two instances, agricultural contamination was thought to be the cause [2].

Roosting gulls, defaecating into surface waters, have been associated with the decline in water quality throughout this century and have been implicated in the waterborne transmission of disease to humans [3]. Most gull populations in Britain and elsewhere in Europe have increased during this century, probably because of reduced persecution. Because of the relatively mild winters, an appreciable proportion of the gulls which breed in northern Europe overwinter here. Most of these gulls feed on refuse produced in urban areas and they are now more frequent inland and use reservoirs as safe overnight roosts [4]. Their increased numbers have enhanced the likelihood of their feeding at sewage treatment works and outfalls, on discarded offal at abattoirs, on pasture land beside grazing farm stock and on refuse tipped at landfill sites; all of which can be contaminated with *C. parvum* oocysts. Should gulls ingest and excrete viable oocysts, they would contribute to the dissemination of oocysts and the contamination of the environment, and those which utilize reservoirs as safe overnight roosts have the potential to contaminate water supplies.

Carriage of other enteropathogens, such as salmonella, by gulls is recognized, and numerous serotypes have been identified in their droppings [5]. Girdwood and colleagues [6], demonstrated that whilst there was a 7·8% carriage rate of salmonella spp. in 5888 *Larus* spp. gulls examined over a 2 year period, the small number of organisms excreted and the short duration of excretion suggested that gulls were a good indicator of environmental contamination.

Because oocysts are not totally removed by conventional water treatment processes, and because normal chlorination regimes used by the water industry are not oocysticidal [2, 7], the water industry is currently faced with the requirement of assessing the potential for oocyst contamination of their catchment areas [2]. Therefore, it is important to recognize all possible sources of contamination in order to attempt to reduce the numbers of viable oocysts which enter water treatment works and to implement practicable control measures. The aim of this investigation was to determine whether gulls are involved in the dissemination of *Cryptosporidium* sp. oocysts and the potential significance of such dissemination should it occur.

## MATERIALS AND METHODS

### *Collection sites and sampling*

One hundred and one freshly deposited gull faecal droppings were collected between November 1990 and February 1991 from various locations in the central belt of Scotland (Table 1), where gulls had been observed to stand, feed or roost. Individual samples were collected with separate wooden applicator sticks into sterile containers and stored at 4 °C until analysed.

Cloacal lavage samples were obtained from 50 gulls which were trapped at two refuse tips, A or B, where different categories of waste were collected. Site A was

licensed for the disposal of sewage sludge; veterinary, pail closet and industrial wastes and domestic refuse whereas site B was licensed for the disposal of domestic refuse; building construction and inert industrial waste. Approximately 1 ml of distilled water contained in a disposable plastic pipette (Pastette, Alpha Laboratories, UK) was introduced into the cloaca, and as much as possible of the lavage sucked back into the pipette and transferred into a clean labelled specimen tube.

Both refuse tips are within 10 miles of Durham, England, and are used for an ongoing survey into the ecology of gulls by the Department of Biological Sciences, University of Durham [8, 9]. Twenty-eight samples were obtained from site A on 18 January 1991, of which 17 were from Black-headed Gulls and 11 from Herring Gulls. A further 22 samples were obtained from Herring Gulls from site B on 25 January 1991. Samples were sent to the Scottish Parasite Diagnostic Laboratory by first class post and were processed without concentration.

*Processing of samples*

Faecal samples were concentrated as follows: individual samples (*c.* 1 g) were made up to 5 ml with reverse osmosis (RO) water in separate sterile 12 ml conical centrifuge tubes, and emulsified thoroughly. Two ml of diethyl ether (Analar) were overlaid onto the aqueous slurry, and the tube was capped and shaken for 5 min in order to defat the sample. Tubes were centrifuged at 900 g for 5 min and the fatty plug together with the fluid above and below it were removed by aspiration. The pellet was resuspended in RO water and centrifuged at 900 g for 5 min. This washing procedure was repeated twice in order to remove all traces of diethyl ether. Oocysts were concentrated further by clarification on cold sucrose. Defatted faecal suspensions were made up to 10 ml with RO water and underlaid with 10 ml of sucrose solution (sp. gr. 1.18). Care was taken not to mix the two layers, and the sample was centrifuged at 900 g for 5 min. The interface, which should contain oocysts, was removed, made up to 50 ml in RO water and centrifuged at 900 g for 10 min. The supernatant was aspirated to waste, and the pellet washed a further two times in RO water to remove traces of the sucrose solution. The pellet was finally resuspended in a minimum volume of RO water: this was between 250 and 1000 $\mu$l depending on the consistency of the original sample. All faecal droppings were clarified before being subjected to the fluorogenic viability assay (see below). The recovery efficiency of this method for oocysts seeded into negative gull faeces was 32–40% ($n = 10$).

*Detection of oocysts by direct immunofluorescence*

Between 20 and 30 $\mu$l suspensions of concentrated faeces and unconcentrated lavages were examined on four well multispot slides (C. A. Hendley, (Essex) Ltd, UK). Slides were air dried, fixed in absolute methanol for 3 min and air dried. Twenty-five $\mu$l of a FITC-labelled *Cryptosporidium* spp. oocyst-specific monoclonal antibody (MAB) (Northumbria Biologicals Ltd, UK), optimally diluted, were applied onto each well to cover the concentrate and incubated in a humidified chamber in the dark at 37 °C for 30 min. Slides were immersed in three changes of phosphate buffered saline (PBS; 0·1 M, pH 7·2), each of 5 min duration, and

following the last immersion, the excess PBS was drained. Specimens were mounted in PBS:glycerol (40:60; v/v) and covered with a glass cover slip. Samples were viewed under the ×20, ×40 and ×100 objectives of an Olympus BH2 microscope equipped with Nomarski differential interference contrast (DIC) and epifluorescence systems. Known positive and negative *C. parvum* oocyst controls were included in each analysis. All fluorescent objects were noted, and spheres measuring 4–6 μm in diameter, with or without a surface fold, were recorded as putative *Cryptosporidium* sp. oocysts. Where possible, these fluorescent spheres were subjected to DIC microscopy in order to attempt to reclassify putative oocysts as definitive oocysts, if recognizable oocyst contents were observed (see below).

*Identification of oocysts*

Samples identified as containing putative oocysts by direct immunofluorescence, following air-drying and methanol fixation, were also tested by direct immunofluorescence in suspension, by incubating the sample with optimally diluted FITC-labelled MAB for 30 min in the dark. Oocysts were pelleted by centrifugation, and the excess MAB aspirated. Oocysts were rinsed in three changes of Hank's Balanced Salts Solution (HBSS), mounted in HBSS, viewed under epifluorescence optics to locate putative oocysts and under DIC optics to identify any internal morphology.

The following criteria were adopted: viable *C. parvum* oocysts are smooth, thick-walled spherical or slightly ovoid bodies measuring 4·5 × 5·0 μm (range 4–6 μm), containing, four elongated, naked banana-shaped sporozoites (each with one nucleus) and a cytoplasmic residual body which can be seen under DIC microscopy.

*Assessment of oocyst viability*

Oocyst viability was assessed following their incubation with a mixture of two fluorescent dyes, propidium iodide (PI) and 4′-6-diamidino-2-phenylindole (DAPI), according to the method of Campbell and colleagues [10]. Briefly, 100 μl aliquots of either clarified faecal concentrate or cloacal lavage, suspended in HBSS, were incubated with a working solution of both DAPI (2 mg/ml in absolute methanol) and PI (1 mg/ml in PBS) for a minimum of 2 h at 37 °C and the viability of each oocyst detected was determined by epifluorescence microscopy (see below). The inclusion/exclusion of these fluorogenic vital dyes have been shown to be a good indicator of oocyst viability when compared to maximized excystation *in vitro* (correlation coefficient = 0·997). Following incubation with the fluorogenic vital dyes, samples were incubated with 100 μl of FITC-labelled *Cryptosporidium* spp. oocyst-specific monoclonal antibody, optimally diluted, in the dark at 37 °C for 30 min. Samples were the pelleted by centrifugation, the excess MAB aspirated, and the pellet resuspended in HBSS. This washing procedure was repeated three times to remove residual, unbound MAB. The inclusion of the MAB step, following incubation with the fluorogenic vital dyes, aided the identification of oocysts in samples containing a high bacterial load. The demonstration of the staining of four sky-blue nuclei by DAPI,

together with the exclusion of PI is indicative of a viable oocyst by epifluorescence microscopy. The microscope was equipped with green, blue and UV filter blocks for epifluorescence microscopy. FITC fluoresces apple green with the blue filter block, PI fluoresces bright red with the green filter block and DAPI fluoresces sky-blue with the UV filter block. Morphometric assessments were made on 20 oocysts in suspension at $\times 1000$ magnification by DIC microscopy.

*Statistics*

Fisher's Exact Probability Test was used to compare the proportion of positive to negative samples between refuse tips A and B and between the species of gull at tip A.

## RESULTS

*Measurement and microscopic appearance of oocysts*

When stained in suspension with the MAB, all oocysts detected in both the faecal droppings and the cloacal lavages were spherical, appeared intact and measured between 4·5 and 6·0 $\mu$m on a calibrated eyepiece micrometer under the $\times 40$ objective (1·5–2 small divisions on the eyepiece graticule). Measurement of 20 viable oocysts from various samples by DIC under $\times 100$ oil immersion indicated a mean and range of $4{\cdot}7 \times 5{\cdot}2$ ($4{\cdot}4–5{\cdot}2 \times 4{\cdot}7–5{\cdot}8$) $\mu$m. In addition, the FITC-labelled MAB bound to the outer surfaces of all oocysts detected. Viable oocysts contained four banana-shaped naked sporozoites and a residual body by DIC microscopy.

*Gull faecal droppings*

Of the 101 faecal droppings examined five (4·95%) contained oocysts. Three of the positive samples were collected in the west of the central belt of Scotland whereas two were collected in the east of the central belt (Table 1). The number of oocysts recorded, adjusted to 1 ml of concentrate, ranged from 100 to 200 (mean 144) oocysts. Viability studies based on the inclusion/exclusion of the two fluorogenic vital dyes indicated that nine of 14 (64·3%) oocysts observed were viable (Table 2). Insufficient samples were collected from the various locations to make any statistical comparisons.

*Cloacal lavage specimens*

Of 50 cloacal lavages analysed, 11 (22%) contained oocysts. Significant differences were observed in the percentage of positive specimens from each site ($P \leqslant 0{\cdot}01$). At tip A, ten of 28 (35%) samples were positive, whereas at tip B one of 22 (4·5%) lavages was positive for oocysts. 23·5% of lavages from Black-headed Gulls and 54% of lavages from Herring Gulls captured at tip A contained oocysts (no significant difference), and the number of oocysts recorded, adjusted to 1 ml of lavage fluid, ranged from 50 to 150 (mean 77). Lavages from Black-headed Gulls contained 50–100 (mean 75) oocysts per ml of fluid, whereas lavages from Herring Gulls contained 50–150 (mean 78) oocysts per ml of fluid. Viability studies revealed that 19 of 23 (83%) oocysts detected in lavages were viable (Table 3).

Table 1. *Site distribution and location of* Cryptosporidium *sp. oocyst-positive faecal droppings*

| Location of sampling site | No. of samples collected | No. of samples positive for oocysts |
|---|---|---|
| Loch Goil, Argyll | 8 | 1 |
| Craigmaddie Reservoir, Nr. Glasgow | 6 | 0 |
| Dalmarnock Sewage Treatment Works, Glasgow | 7 | 0 |
| Edinburgh Abattoir | 6 | 2 |
| Hogganfield Loch, Glasgow | 4 | 0 |
| Pond Hotel, Glasgow* | 14 | 1 |
| Scottish Zoological Park, Edinburgh | 27 | 0 |
| Shieldhall Sewage Treatment Works*, Glasgow | 9 | 1 |
| Dock Wasteland, Shieldhall*, Glasgow | 12 | 0 |
| Helensburgh | 2 | 0 |
| Clydebank Walkway | 4 | 0 |
| Portobello Promenade | 2 | 0 |
| Total | 101 | 5 |

* Sites which were visited on two separate occasions.

Table 2. *Viability of* Cryptosporidium *sp. oocysts in faecal droppings*

| Sample location | Viable | Non-viable |
|---|---|---|
| Edinburgh Abattoir | 2/2 | 0/2 |
| Edinburgh Abattoir | 2/2 | 0/2 |
| Pond Hotel, Glasgow | 1/4 | 3/4 |
| Shieldhall Sewage Works | 2/3 | 1/3 |
| Loch Goil | 2/3 | 1/3 |

Table 3. *Viability of oocysts in cloacal lavage samples*

| Sample no. | Viable | Non-viable |
|---|---|---|
| 136 | 2/3 | 1/3 |
| 137 | 1/1 | 0/1 |
| 140 | 7/8 | 1/8 |
| 657 | 3/3 | 0/3 |
| 660 | NR* | 0/1 |
| 661 | 2/2 | 0/2 |
| 662 | 1/1 | 0/1 |
| 663 | 1/2 | 1/2 |
| 665 | 1/1 | 0/1 |
| 719 | 1/1 | 0/1 |

* Not readable with DAPI.

## DISCUSSION

We suggest that the objects detected are oocysts of the genus *Cryptosporidium* because they conform to the following criteria: (a) smooth, thick-walled spherical or slightly ovoid bodies measuring 4–6 $\mu$m, containing four banana-shaped inclusions, without sporocysts, when viewed in suspension under Nomarski optics. (b) the DNA selective fluorogenic dye, DAPI, highlighted four nuclei which

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appeared to be within these banana-shaped inclusions. Further evidence comes from the fact that the anti-*Cryptosporidium* MAB bound onto surface-exposed epitopes of intact organisms. Whilst this MAB appears to be genus specific, it is reported to bind surface-exposed epitopes of oocysts of *C. baileyi*, *C. muris* and *C. parvum* [7]. This MAB does not cross-react with a variety of enteric organisms [11], although surface fluorescence of similar sized non-cryptosporidial organisms has been observed in water samples (Smith, unpublished). No data were available on the reactivity of the MAB used in that report with *C. meleagridis* oocysts. Because of the documented size differences between oocysts of *C. parvum* ($4{\cdot}5 \times 5{\cdot}0\ \mu$m, [12, 13]) and oocysts of either *C. baileyi* ($4{\cdot}6 \times 6{\cdot}2\ \mu$m, [14]; $5{\cdot}0 \times 6{\cdot}8\ \mu$m, [15]) or *C. muris* ($5{\cdot}6 \times 7{\cdot}4\ \mu$m, [13]) it seems reasonable, on the basis of oocyst morphometry, to exclude the possibility that the oocysts detected in this study were either *C. baileyi* or *C. muris*. It was not possible to exclude the possibility that the oocysts detected were those of *C. meleagridis* ($4{\cdot}0 \times 4{\cdot}5\ \mu$m, [15]; $4{\cdot}6 \times 5{\cdot}2\ \mu$m, [16]) on the basis of oocyst size and shape. Little is known about the occurrence of *C. meleagridis* in the UK, although surveys of occurrence are underway (M. Taylor, personal commun.).

We have insufficient evidence to ascribe these oocysts to a recognized species, and until cross-infection studies are performed, and/or methods are available to identify other species-specific characteristics within the genus *Cryptosporidium*, the *C. parvum*-like oocysts identified in this study will be referred to as *Cryptosporidium* sp. oocysts.

During cryptosporidial infections large numbers of oocysts are excreted. The mean total output of experimentally infected calves was $2{\cdot}5 \times 10^{10}$ oocysts [17], and up to $1{\cdot}4 \times 10^{4}$ oocysts per litre occur in raw sewage, with up to 79% being retained in the sludge [18]. Seasonal increases in the occurrence of cryptosporidiosis have been documented in the spring and late autumn in human beings and domestic animals in the UK [2], being coincident, in domestic animals, with lambing and calving. This study was undertaken between November and February when it was anticipated that contamination of the environment with *C. parvum* oocysts was likely to be high.

Between 4·5 and 35% of samples analysed contained *Cryptosporidium* sp. oocysts (100–200 per ml of faecal concentrate, 50–150 per ml of lavage fluid), with over 60% of oocysts analysed being viable. We consider these numbers to be low and suggest that they probably reflect carriage as opposed to infection.

One explanation why lavages from gulls netted at refuse tip A contained significantly more *Cryptosporidium* sp. oocysts than those netted at refuse tip B, could be because these gulls fed on *C. parvum* oocyst-contaminated waste at that tip. However, other oocyst-rich sources may also be available to these gulls. For example, gulls captured at site A also feed at coastal sewage outfalls, whereas gulls captured at site B roost and feed only inland. Sewage is only available to birds captured at site B at sewage treatment works where Black-headed Gulls, but not Herring Gulls, are known to feed.

The contribution from gulls to the contamination of the terrestrial environment is probably minimal when compared to the output of oocysts from infected domestic animals kept at pasture. However, their contribution to the contamination of surface waters may be more significant. Up to $10^{5}$ gulls have been

counted flying on to a single reservoir at dusk [4], and over a quarter of a million birds may roost on the London reservoirs [19, 20]. Sage [21] observed that, during December and January, 73% of gulls which roosted on reservoirs were Black-headed Gulls and 11% were Herring Gulls; these species have been shown to excrete *Cryptosporidium* sp. oocysts in this study.

Irrespective of the likely species of the oocysts detected, contamination of the aquatic environment would occur following defaecation into reservoirs at night roosts. Because their exposed epitopes are recognized by one of the MABs used for the detection of oocysts in water samples, and their size range is similar to that of *C. parvum*, such oocysts would be identified as *C. parvum* oocysts according to the consensus standard method for the analysis of water for the presence of *Cryptosporidium* spp. oocysts [22].

Coulson and colleagues, [23] implicated Herring Gulls as vectors of *Salmonella montevideo* in outbreaks affecting cattle and sheep, and stated that they might transmit this pathogen both locally and over considerable distances. Such a possibility also exists for oocysts of *Cryptosporidium* sp., and their dispersal between the different regions of Britain, and between Britain and Europe may be due to: (a) the high mobility of gulls [24], (b) their rapid and synchronous return to breeding areas [25, 26] and the ability of oocysts to remain in the digestive tract, in a viable state, for a period of a day or so.

In addition, we suggest that gulls may be a good indicator of *Cryptosporidium* sp. oocyst environmental contamination for reasons similar to those suggested by Girdwood and colleagues for *Salmonella* spp. [6].

Attempts to infect chickens with *C. parvum* oocysts have yielded variable results. Chickens inoculated with oocysts *via* the intra-tracheal route exhibited light infections in the trachea, larynx and primary bronchi, and produced small numbers of oocysts in faeces, whereas chickens inoculated orally with oocysts had no detectable developmental stages in mucosal smears, tissue sections or faeces [27].

The ability of *Cryptosporidium* sp. oocysts isolated from gulls to infect mammals, and conversely the ability of *C. parvum* to infect gulls is worthy of investigation to elucidate further the zoo-epidemiology of cryptosporidiosis.

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