Lethal *Giardia* from a wild-caught sulphur-crested cockatoo (*Cacatua galerita*) established *in vitro* chronically infects mice

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SUMMARY

An axenic culture of *Giardia* was established from a sample of infected intestine obtained following autopsy of a sulphur-crested cockatoo (*Cacatua galerita*). The cockatoo recently captured in the wild and with good muscle tone died along with several other cage mates, apparently of an overwhelming, acute infection of *Giardia*. Trophozoites which established in the traditional, axenic *Giardia* medium (TYI-S-33 with supplementary bile) were morphologically identical to *G. duodenalis*. When outbred Quackenbush Swiss neonatal mice were infected with trophozoites a chronic infection was established and parasites were still present at 38 days post-inoculation. Weight gain by infected mice was reduced by 20%, thus mimicking failure-to-thrive syndrome in children, and maximum parasite load was more than 3-fold higher in comparison with other *G. duodenalis* strains. Analysis of the electrophoretic karyotype, rDNA and hybridization studies together with Giemsa- and trichrome-stained samples, and scanning electron microscopy indicated that the bird-derived *Giardia* belonged to the *duodenalis* group. This is the first report of infection of mammals with *Giardia* isolated from a bird. These data may have potentially serious implications for contamination of watersheds and establishment of zoonotic infections.

Key words: avian *Giardia*, *Giardia duodenalis*, cross-species transmission, sulphur-crested cockatoo (*Cacatua galerita*), zoonosis.

INTRODUCTION

The protozoan parasite, *Giardia duodenalis*, infects the small intestine of humans and other animals, often causing chronic diarrhoea and in children, failure to thrive. It has been claimed to be a pathogen associated with 10000 deaths per year (Warren, 1989). There are several species of *Giardia* including *G. duodenalis* (synonymous with *G. lamblia* and *G. intestinalis*), which infect humans and mammals, *G. muris*, confined to rodents (Feeley, Erlandsen & Chase, 1984), *G. ardeae*, found typically in blue herons (Erlandsen *et al.* 1990) and *G. psittacae*, a parasite of the *Psittacidae* particularly the budgerigar (Erlandsen & Bemrick, 1987). All of these species are morphologically distinct and attempts to cross-infect host species outside of the normally wide host-range, with other than transient infections, have been unsuccessful.

Giardia from birds has been the subject of several studies (Erlandsen, Bemrick & Jakubowski, 1991, and references therein; Box, 1981). In Australia, flocks of sulphur-crested cockatoos (*Cacatua galerita*), belonging to the *Psittacidae* family, and other related birds range over vast areas of the country. Trapping licences allow small numbers of these birds to be caught and sold as pets and exhibits all over the country. *Giardia* has not previously been implicated as a pathogen in these or other birds in Australia and the only previous report of *Giardia* in birds in Australia was that in a Straw Necked Ibis (*Threskiornis spinicollis*) in Western Australia (Forshaw *et al.* 1992; McRoberts *et al.* 1994). We have established *Giardia* isolated from a sulphur-crested cockatoo in axenic culture and have chronically infected mice with trophozoites. This work may have serious implications for water management especially in regions where the water is likely to be cold enough to preserve viable *Giardia* cysts.

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MATERIALS AND METHODS

Parasite culture

A wholesaler of wild-caught sulphur-crested cockatoos (*Cacatua galerita*), concerned about the health of a recent arrival of birds from Victoria, consulted his veterinarian. One bird was moribund on arrival at the surgery and on autopsy *Giardia* was diagnosed as the only apparent cause of death

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(Gallagher, Gartrell & Upcroft, 1995). Excellent muscle tone indicated an acute infection. Following this diagnosis, the remaining birds were treated with ronidazole at recommended doses (4 g/l) in their drinking water but later died.

Within approximately 1 h of autopsy a section of the intestine was placed in ice-cold TYI-S-33 medium containing bile, antibiotics and 20% foetal calf serum in a glass tube (Upcroft *et al*. 1995). The tube was vortexed, the large tissue masses were removed and parasites incubated at 37 °C with frequent changes of medium. Once the culture, BRIS/95/HEPU/2041, was established (within approximately 4 weeks) and cryopreserved (Phillips, Boreham & Shepherd, 1984), foetal calf serum was reduced to 10%, antibiotics removed and glass tubes replaced with plastic.

Details of other parasite cultures used in this study, BRIS/83/HEPU/106 and BRIS/91/HEPU/1279, are documented in Capon *et al*. (1989) and Upcroft *et al*. (1995), respectively. All cultures were maintained in TYI-S-33 medium supplemented with bile (Boreham, Phillips & Shepherd, 1986).

Parasite staining and SEM

Within the first week of culture a sample was fixed in PVA fixative (Meridian Diagnostics, Cincinnati) and trichrome stained by Dr Robyn Boreham of Sullivan and Nicolaides and Partners, pathologists, Brisbane. Once the culture was established, methanol-fixed parasites were stained with Giemsa. Scanning electron microscopy (SEM) was performed on trophozoite-infected mouse intestinal sections. The sections were fixed in 3% glutaraldehyde (Fluka) in 0·1 M sodium cacodylate, pH 7·2, and SEM performed by the Electron Microscopy Facility at Queensland University of Technology.

Infection of mice

Litters of outbred Quackenbush Swiss mice younger than 5 days were infected intragastrically with 10^5 *Giardia* trophozoites growing in log phase (Boreham *et al*. 1986). At 3–4 day intervals and finally at 38 days post-inoculation (p.i.), 2 or 3 pups were killed, the small intestine removed, opened longitudinally and placed in cold phosphate-buffered saline (PBS) for at least 10 min. Parasite counts were performed on the whole intestine. All inoculated and control pups were weighed prior to inoculation and at regular intervals.

Electrophoretic karyotype

Whole chromosomes prepared in agarose blocklets (Upcroft, Boreham & Upcroft, 1989) were separated by contour-clamped homogeneous electric field electrophoresis (CHEF) in a CHEF-DRII BioRad apparatus (Upcroft, Chen & Upcroft, 1996). Separated chromosomes were transferred (Southern, 1975) to nylon membrane (Amersham, Hybond-N) and hybridized (Church & Gilbert, 1984) with ^{32}P α-dCTP (110 TBq/mmol, Dupont, Wilmington, DE) labelled and randomly primed (Feinberg & Vogelstein, 1984) chromosome-specific probes derived from individual chromosome libraries (Upcroft, Healey & Upcroft, 1993; Chen, Upcroft & Upcroft, 1994).

RESULTS

Cultured avian Giardia

The culture established from the sulphur-crested cockatoo, BRIS/95/HEPU/2041, was indistinguishable from *G. duodenalis* in culture. By trichrome and Giemsa staining the characteristic median body of *G. duodenalis* was evident (Meyer, 1994) (Fig. 1A and B). BRIS/95/HEPU/2041 grew slower than the stock BRIS/83/HEPU/106, and at about the same rate as BRIS/91/HEPU/1279.

SEM data supported the observation that BRIS/95/HEPU/2041 trophozoites are *G. duodenalis*. The parasites did not have the roughened or pitted surface of *G. psittaci* nor the folded ventral flange observed in *G. ardeae* (Fig. 1C). By Giemsa staining 2 caudal flagella were evident (Fig. 1A).

Infection of mice

At 7 days p.i., parasites were observed in all litters, but not in control litters inoculated with PBS. In 3 separate experiments, peak parasite numbers in litters infected with trophozoites of BRIS/83/HEPU/106 and BRIS/91/HEPU/1279 occurred at 10–11 days p.i. while the parasite load in mice infected with avian-derived *Giardia* peaked at 14–17 days p.i. (Fig. 2). The mean total parasite load in animals infected with BRIS/95/HEPU/2041 in the 3 experiments was 3·23, 2·97 and 3·98 times greater than that with BRIS/83/HEPU/106 at the peak of the infection (Fig. 2 and 2 other experiments not shown). Compared with BRIS/91/HEPU/1279, parasite load was 4·5 and 4·39 times greater in mice infected with BRIS/95/HEPU/2041 (data for 2 experiments not shown). At 20 days p.i. there were no detectable parasites in mice infected with BRIS/83/HEPU/106 (Fig. 2) and the numbers in mice infected with BRIS/91/HEPU/1279 had dropped significantly. However, in mice infected with BRIS/95/HEPU/2041 parasite numbers at 20 days p.i. were still higher than at the peak of the infection with the 2 human *Giardia* isolates tested. At 27 days p.i. mice infected with BRIS/95/HEPU/2041 were still carrying greater than 10^6 parasites, and after 38 days $1{\cdot}5 \times 10^4$ parasites/mouse were still present.

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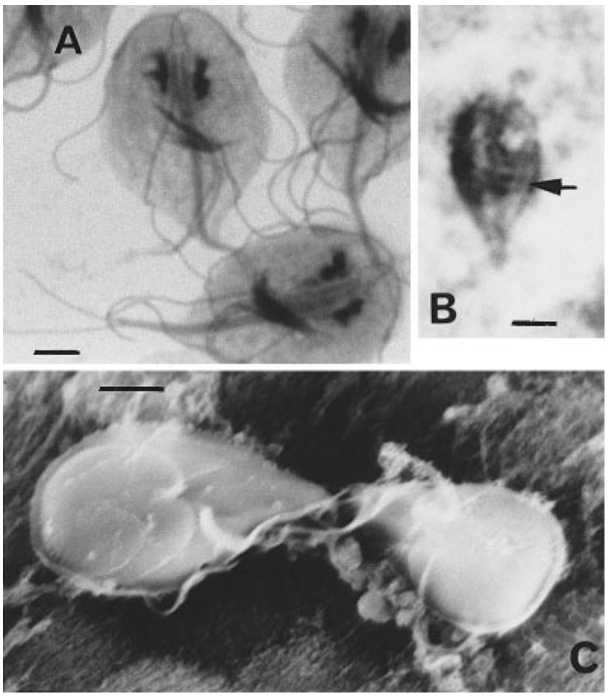

Fig. 1. Fixed trophozoites of *Giardia* strain BRIS/95/HEPU/2041. Giemsa stain of trophozoites cultured *in vitro* (A). Trichrome stain of the original sample taken from the dead bird (B). The arrow indicates the clawed hammer-shaped median body. Scanning electron micrograph of trophozoites associated with mouse intestinal epithelial cells (C). The scale bar in each panel represents 2μm.

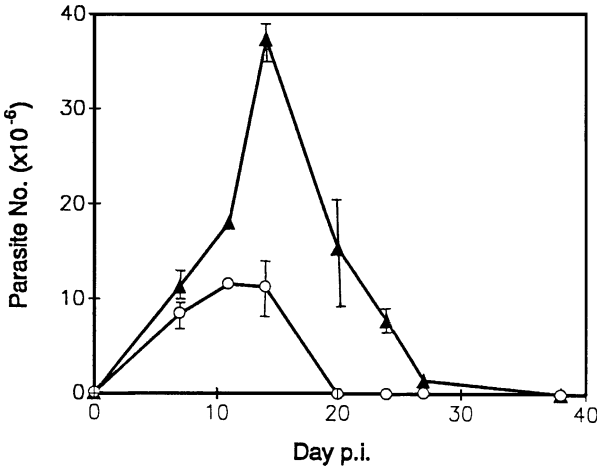

Fig. 2. Trophozoite counts in the small intestine of mice following inoculation with *Giardia* isolated from a bird and with a human isolate of *G. duodenalis*. Three-day-old mouse pups were infected with 10^5 trophozoites of strain BRIS/95/HEPU/2041 (isolated from a bird) (▲) and BRIS/83/HEPU/106 (○) (isolated from a human). Separate litters were used for different isolates. Counts were performed on 1–3 pups, depending on the litter size, for each time-point. Error bars indicate standard deviations between pups in the same experiment. At the same time as this experiment was conducted a third litter was infected with trophozoites of BRIS/91/HEPU/1279. The results were similar to those obtained following infection with BRIS/83/HEPU/106, with fewer parasites overall.

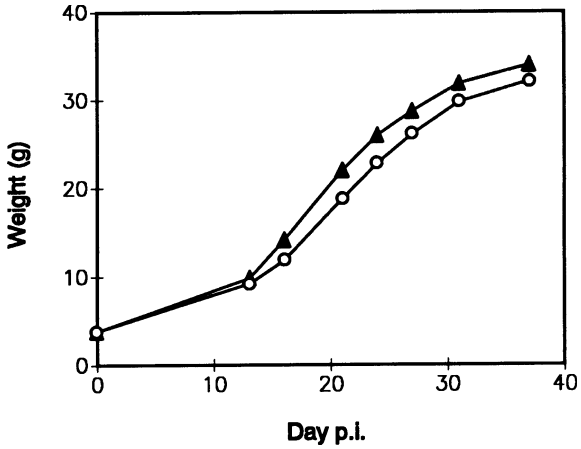

Fig. 3. Percentage weight deficit between mice infected with *Giardia* strain BRIS/95/HEPU/2041 and uninfected mice. Half of the litter was infected (○) and the other half was maintained as controls (▲) to prevent variation between litters. The number of mice for each time-point was 6 i.e. a total of 12 pups in the litter. As determined by the Two-Sample *t*-test the results were significant to a 99% confidence limit. Other litters were infected separately with BRIS/83/HEPU/106 and BRIS/91/HEPU/1279 but the weights of these mice were slightly higher than their uninfected littermates.

The long-term infection of mice with BRIS/95/HEPU/2041 correlated with slower weight gain in pups infected with this stock (Fig. 3). At the peak of the infection with BRIS/95/HEPU/2041 there was a 16% weight deficit. The weight gain in mice infected with BRIS/83/HEPU/106 and BRIS/91/HEPU/1279 was the same as control uninfected mice (data not shown). After 38 days p.i. the weight of BRIS/95/HEPU/2041 infected mice was still lower than control mice.

Genomic comparison between avian and human derived Giardia

The electrophoretic karyotype of the strain BRIS/95/HEPU/2041 was very similar to that of WB-1B (Fig. 4A). Both strains carried brightly stained chromosome bands 3/4. BRIS/95/HEPU/2041 had an additional, faint-staining band between the latter and chromosome band 5. (A similar band is also seen in BRIS/83/HEPU/106 and BRIS/89/HEPU/1065 (Upcroft & Upcroft, 1994; Upcroft *et al.* 1995).) Both karyotypes had readily detectable chromosome bands 6, 7 and 8. However, BRIS/95/HEPU/2041 also had extra faint bands migrating more slowly than the 2 Mb chromosome 5 and another migrating more slowly than chromosome 6 but ahead of chromosome 7 (Fig. 4A). When transfers of these electrophoretically separated chromosomes were hybridized with chromosome-specific probes it was revealed that BRIS/95/HEPU/2041 contained duplications of both chromosomes 3 (or 4) and chromosome 6 (Fig. 4B and D). Hybridization patterns of the same

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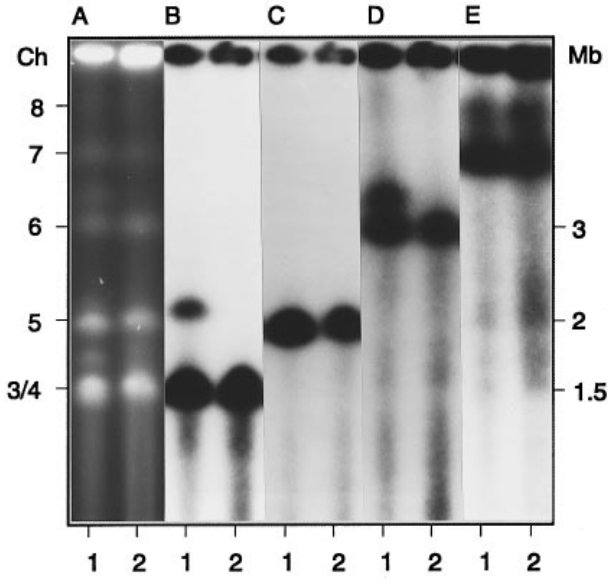

Fig. 4. Chromosomes of *Giardia* strain BRIS/95/HEPU/2041 compared with WB-1B and hybridized with chromosome-specific probes. Electrophoretically separated chromosomes were stained with ethidium bromide (A) and transferred to nylon membrane. Chromosomes were hybridized with a chromosome 3/4 specific marker G6/1 (Upcroft *et al.* 1993) (B); a chromosome 5 specific marker (Chen *et al.* 1994) (C); a chromosome 6 specific marker (unpublished) (D) and a chromosome 7/8 marker (E) (unpublished). Chromosome sizes are given in Mb and chromosome designation (Ch) is indicated. Lane 1, BRIS/95/HEPU/2041; 2, WB-1B.

filter shown in Fig. 4 with probes specific for chromosomes 5, 7 and 8 were the same for both BRIS/95/HEPU/2041 and WB-1B (Fig. 4C and E).

Other characteristics that BRIS/95/HEPU/2041 shared with BRIS/83/HEPU/106 and WB-1B were identical rDNA repeat units as determined by *Pst*I cleavage of genomic DNA and hybridization with rDNA probes as previously described (Upcroft, Healey & Upcroft, 1994; Upcroft *et al.* 1995) (data not shown), identical β-tubulin genes (unpublished observations) and similar protein profiles as determined by polyacrylamide gel electrophoresis and Coomassie staining as previously described (Capon *et al.* 1989) (data not shown).

DISCUSSION

This work describes the first axenically grown *Giardia* strain of the *duodenalis* group isolated from a bird and which infects mammals. This isolate established a chronic infection of *G. duodenalis* in an outbred mouse model which resulted in weight loss and mimicked failure-to-thrive syndrome in children chronically infected with *G. duodenalis*. After 38 days p.i. fully developed Quackenbush Swiss mice carried significant numbers of trophozoites.

In 1981, Box reported *Giardia* in budgerigars which was lethal to some young birds but she was unable to infect DBA-2 mice or canaries with cysts obtained from the budgerigars. It was suggested that the budgerigar *Giardia* was of the *duodenalis* type (Box, 1981) but Erlandsen & Bemrick (1987) showed by electron microscopy that this *Giardia*, now known as *G. psittacae*, was morphologically different from *G. duodenalis*. Furthermore, Erlandsen and his colleagues (1991) reached the conclusion that avian *Giardia* species could not be transmitted to chicks, ducklings or mammalian hosts.

Previously the only *Giardia* cultured axenically from a bird was isolated from the great Blue Heron (Erlandsen *et al.* 1990). These parasites have only 1 caudal flagellum, variable median body ranging from small round-oval to an elongate rod shape whose axis lies parallel with the axonemes of the caudal flagella, tear dropped-shaped nuclei and very different electrophoretic karyotype (Erlandsen *et al.* 1990). The report of *Giardia* from an ibis, *T. spinicollis*, in Western Australia, claims that morphologically the parasites more closely resembled *G. ardeae* than *G. psittacae* (McRoberts *et al.* 1994). The parasites we have established in culture are morphologically identical to *G. duodenalis* on the basis of trichrome and Giemsa staining and SEM data. It might be expected that *Giardia* isolated from a cockatoo is likely to be the same as that which infects the not too distantly related budgerigar, *G. psittacae*, especially considering that budgerigars originated in Australia. However, *G. psittacae* has not been identified in Australia and the parasites we established in axenic culture were of the *duodenalis* group. Furthermore, SEM data reveal a smooth dorsal surface on the trophozoites of strain BRIS/95/HEPU/2041, unlike the pitted surface of trophozoites of *G. psittacae* and there is clearly a ventral flange encircling the antero-lateral border of the adhesive disc unlike its appearance in *G. psittacae* (Erlandsen & Bemrick, 1987).

In our experience CBA, BALB/c and C57 neonatal mice, which are all inbred strains, established infections following inoculation of trophozoites from human isolated cultures. The mice carried maximum parasite loads of 10^7 or fewer trophozoites (unpublished data) and cleared their infection by 23 days. This is consistent with earlier reports by Hill *et al.* (1983) of a maximum parasite load less than 10^7 parasites per suckling mouse around 10 days p.i. Quackenbush Swiss mice (*Mus musculus*) which showed the same course of infection as the inbred mice with all of our earlier tested strains (BALB/c, CBA, C57) were chosen for these studies because of their robustness and their large litter size, up to 20 pups/litter.

Most studies of *G. duodenalis* infections in mice indicate that the mouse can clear the parasite by 3–4 weeks p.i. However, Waight Sharma & Mayrhofer (1988) isolated 2 *G. duodenalis* strains from a non-

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laboratory colony of mice and another from a laboratory colony of rats: both of these isolates established chronic but asymptomatic infections (longer than 10 weeks) in C3H/HeJ adult mice, while infections in BALB/c mice were not detectable after 6 weeks, as measured by cyst excretion. This is similar to the situation in mice infected with *G. muris* where C3H/He mice appear to be more susceptible to chronic infections (they are unable to eliminate the parasite) than DBA/2 or BALB/c (Underdown *et al*. 1981). Mice chronically infected with *G. muris*, the natural mouse parasite, are not generally symptomatic (Underdown *et al*. 1981; Hill *et al*. 1983). However, Roberts-Thomson *et al*. (1976) reported an approximate 10% weight deficit between outbred Swiss albino young females heavily infected with *G. muris* and uninfected controls. This is comparable to the 16% weight deficit we have observed at the peak of infection with *G. duodenalis* strain BRIS/95/HEPU/2041.

It has proven difficult to infect adult mice with isolates of human *G. duodenalis* and Byrd, Conrad & Nash (1994) have claimed consistent infection in 4 week C3H/HeJ mice with only 1 out of 9 strains tested. Several 7 week adult Quackenbush mice that we inoculated with trophozoites of BRIS/95/HEPU/2041 had detectable parasites following inoculation but none was detected in mice inoculated with BRIS/83/HEPU/106 or BRIS/91/HEPU/1279 (data not shown). One mother which ate her infected litter, 2 days p.i., carried 3×10^7 trophozoites 5 days later. We did not, however, detect cross-infection of *Giardia* between uninfected and infected litter mates during the time-course of our experiments.

Chromosome duplications are frequently associated with drug-resistance in *Giardia* (Upcroft *et al*. 1992; Chen *et al*. 1994; Upcroft *et al*. (1996) but are also seen in a few isolates which have apparently not been stressed (Upcroft, Chen & Upcroft, 1996). The only obvious difference that we observed between the strain BRIS/95/HEPU/2041 isolated form a bird and the human strain WB-1B is the duplication of two chromosomes, chromosome 3 (or 4) and chromosome 6. Chromosome 3 and 6 rearrangements were also seen in a metronidazole-resistant and albendazole-resistant line (Chen, Upcroft & Upcroft, 1995; Upcroft *et al*. 1996) and chromosome 5 rearrangements were involved in metronidazole and furazolidone-resistant lines (Chen *et al*. 1994).

WB-1B belongs to a deme of *G. duodenalis* which is characterized by several criteria including a very similar karyotype to that shown here for both WB-1B and BRIS/95/HEPU/2041. Strains of *Giardia* belonging to a second deme have very different karyotypes (Upcroft *et al*. 1995; Upcroft, Chen & Upcroft, 1996) and again the karyotype of *G. ardeae* is distinctly different from all human isolates (Campbell *et al*. 1990). By this criterion alone the parasites isolated from the sulphur-crested cockatoo clearly belong to one of the *duodenalis* groups.

There were no obvious differences in polyacrylamide protein profiles between trophozoites of BRIS/95/HEPU/2041 and BRIS/83/HEPU/106 that might account for the pathogenic phenotype stably rendering BRIS/95/HEPU/2041 trophozoites more pathogenic than all others we have examined in mice. The pathogenicity of the continuously cultured strain has not changed since its establishment in January 1995 and the completion of the work reported here (September 1995). In addition to the karyotype data, rDNA and hybridization studies supported our conclusion that the parasite isolated from the sulphur-crested cockatoo belongs to the *duodenalis* group. Furthermore, these parasites can cross-infect mammals. Work that was carried out by Erlandsen and his colleagues (1991) led to the conclusion that birds as carriers of zoonotic *Giardia* were not a threat to the environment. However, we must now consider the possibility that birds can contaminate waterways with *Giardia* cysts that are potentially a danger to humans, livestock and native animals.

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