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# MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *GIARDIA* ISOLATED FROM THE STRAW-NECKED IBIS (*THRESKIORNIS SPINICOLLIS*) IN WESTERN AUSTRALIA

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ABSTRACT: Following the first report of avian *Giardia* infection in Australia, isolates of the parasite recovered from naturally infected straw-necked ibis (*Theskiornis spinicollis*) were characterized using median body morphology, scanning electron microscopy, multilocus enzyme electrophoresis, random amplified polymorphic DNA (RAPD), and small subunit ribosomal RNA (SSU-rRNA) analyses. Results were compared with *Giardia* from other birds and mammals, and the extent of genetic diversity between a range of ibis isolates collected in Western Australia was determined. The ibis isolates of *Giardia* were genetically relatively homogeneous, which is in contrast to the extensive genetic heterogeneity often displayed by mammalian *Giardia* isolates. Morphologically, *Giardia* from ibis were similar to *Giardia ardeae* although they differed genetically and by the fact that the ibis isolates could not be established in in vitro culture. Sequence data of the DNA coding for the SSU-rRNA found a 96% homology between the ibis isolates from Western Australia and *G. ardeae,* suggesting that they represent distinct strains of the same species. In contrast, the ibis isolates were genetically and morphologically very different than *Giardia duodenalis* and *Giardia muris* from mammals.

*Giardia* is a flagellated protozoan in the phylum Sarcomastigophora, class Zoomastigophorea and order Diplomonadida (Levine et al., 1980; Cox, 1981). Species of *Giardia* inhabit the intestinal tracts of virtually all classes of vertebrates including humans (Kulda and Nohynkova, 1978; Thompson et al., 1990). Whereas it does not always cause disease, it is the most commonly recognized pathogenic intestinal parasite in developed countries and in developing areas where hygiene and nutritional standards are difficult to maintain, populations are particularly at risk (Warren, 1989; Meyer, 1990; Schantz, 1991).

Taxonomic speciation of *Giardia* has long been a contentious issue and remains unresolved (Lymbery and Tibayrenc, 1994). The traditional view, largely expounded by Hegner (1922a, 1922b, 1924) has been one of the existence of numerous distinct species based on strict host specificity. Most authorities now agree that the concept of rigid host specificity is untenable and favor the existence of a limited number of *Giardia* species that are not rigidly host specific (Thompson et al., 1990, 1993). This view is primarily based on the work of Filice (1952) who proposed that *Giardia* be classified into 3 species groups based on their morphological characteristics, e.g., *Giardia muris,* which occurs in rodents, birds, and reptiles, *Giardia agilis* in amphibians, and *Giardia duodenalis,* which is primarily found in mammals. The zoonotic potential of *G. duodenalis* from both domestic and wild animals is uncertain, although these hosts are considered important reservoirs due to their close association with humans and potential for environmental contamination with cysts (Davies and Hibler, 1979; Wenman et al., 1986; Kirkpatrick, 1987; Buret et al., 1990; Schantz, 1991; Thompson, 1996).

Apart from mammals, *Giardia* infection has been described in aviary birds (Jones and Carroll, 1977; Panigraphy et al., 1978, 1979, 1981, 1984; Hirai et al., 1980; Box, 1981; Yamashita et al., 1981; Scholtens et al., 1982). These reports have largely focussed on clinical rather than taxonomic aspects. An exception is the work of Erlandsen and Bemrick (1987) who used scanning electron microscopy to characterize *Giardia* trophozoites taken from the budgerigar (*Melopsittacus undulatus*) as a possible new species, *Giardia psittaci,* based on the absence of a ventrolateral flange.

There have also been numerous reports of *Giardia* from wild birds (Gonder, 1911; Noller, 1920; da Cunha and Muniz, 1922; Kotlan, 1922, 1923; Travis, 1939; Carini, 1943; Stabler, 1944; Ansari, 1952; Georgi et al., 1986; Erlandsen et al., 1990, 1991; Al-Sallami, 1991; Forshaw et al., 1992). Some indicate that further new species of *Giardia* may occur in wild birds. Based solely on comparative morphometric measurements, Travis (1939) described 4 new species from avian hosts; *Giardia sturnellae* (western meadowlark, *Sturnella neglecta*), *Giardia melsopizae* (swamp sparrow, *Melsopiza georgina*), *Giardia botauri* (American bittern, *Botaurus lentiginosus*), and *Giardia floridae* (little blue heron, *Florida caerulea*). Noller (1920) described a species of *Giardia* taken from the grey heron (*Ardea cinerea*) as *Giardia ardeae,* based on his observation of a single caudal flagellum. Using scanning electron microscopy (SEM) and orthogonal-field-inversion gel electrophoresis, Erlandsen et al. (1990) characterized *Giardia* similar to that described by Noller (1920), taken from a closely related species, the great blue heron (*Ardea herodias*), which they considered to be *G. ardeae.*

Forshaw et al. (1992) reported the first avian *Giardia* infection in Australia, occurring in the straw-necked ibis (*Threskiornis spinicollis*), but did not characterize the isolate. In the present study, SEM, multilocus enzyme electrophoresis (MLEE), random amplified polymorphic DNA (RAPD), and small subunit ribosomal RNA (SSU-rRNA) analyses were used to characterize *Giardia* isolates from the straw-necked ibis in comparison with isolates described from other birds and mammals and to evaluate the extent of genetic diversity between a range of ibis isolates collected in Western Australia.

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## MATERIALS AND METHODS

### Birds and *Giardia* samples

*Giardia* samples were obtained from straw-necked ibis in 2 breeding colonies, 1 approximately 150 km north and the other approximately 200 km south of Perth, Western Australia. Both colonies were of similar size and contained over 1,000 adult birds, and, although we have no direct evidence, it is likely that birds may cross-habitat colonies. Only moribund or freshly dead birds were selected for this study. Moribund birds appeared weak, hypothermic, and had a generalized unthrifty appearance. Selected birds did not display escape behavior when approached. The overall prevalence of *Giardia* in these colonies is approximately 70% and for this study *Giardia* was isolated from a total of 63 birds.

For comparative purposes, an isolate of *G. ardeae* (Erlandsen et al., 1990) and the reference mammalian isolate of *G. duodenalis* P1 (Meloni et al., 1988) were also included in the study. Isolates of *G. muris* were not available for inclusion in this study.

### Collection of trophozoites and in vitro cultivation

Trophozoites were obtained from killed (following injection with pentobartbitone sodium), moribund, or freshly dead birds by lightly scraping the mucosal surface of the proximal small intestine and resuspending the trophozoites in BI-S-33 (Meloni and Thompson, 1987) culture medium, containing 10% fetal bovine serum (FBS) and gentamicin 0.05 mg/ml, lincomycin 0.04 mg/ml, ampicillin 0.1 mg/ml. Suspensions containing *Giardia* trophozoites were used to inoculate culture tubes before incubating at 37 C. If mucosal debris was excessive, and to reduce the possibility of bacterial contamination in culture tubes, debris was removed from the bottom of culture flasks 1–2 hr after inoculation or the medium completely replaced.

Borosilicate culture (15 × 126 mm) tubes and 10-ml and 50-ml plastic flat-bottom flasks were used to culture trophozoites and were examined daily using an inverted microscope. The medium described by Erlandsen et al. (1990) used for the cultivation of *G. ardeae* was also used to culture trophozoites from ibis.

### Median body morphology

Trophozoite samples of *G. ardeae* and isolates from 2 ibis were washed twice by centrifugation in sterile phosphate-buffered saline (PBS; pH 7.2), resuspended in PBS, and small aliquots placed onto ethanol-cleaned microscope slides. The cells were left to adhere and the excess cell suspension was removed from the slide. Slides were air-dried overnight at room temperature and stained the following day.

Preliminary experiments had shown that excellent results could be obtained using the Giemsa stain as modified by S. S. Desser (pers. comm.). Briefly, slides were fixed for 1 min in absolute methanol (analytical grade) and washed in distilled water ($dH_2O$). Secondary fixation was in 10% buffered formalin for 10 min and slides were again washed in $dH_2O$. A commercial Giemsa preparation (Gurr Giemsa Staining Solution, British Drug House, BDH Poole, Dorset, England) was diluted 1:20 in PBS and the slides were stained for 45 min at room temperature. The slides received a final rinse in tap water to facilitate the color reaction. Slides were air-dried, mounted in DPX mountant (BDH), and the morphology of the median bodies of the trophozoites was examined.

### Scanning electron microscopy

Trophozoites for SEM were obtained from suspensions as described above and washed twice in sterile PBS before aliquots of concentrated trophozoites were placed onto 1-cm round coverslips coated with a thin layer of 1% poly-L-lysine. The specimens were then fixed in 2.5% glutaraldehyde, postfixed in Dalton's chromic acid, and dehydrated in a graded series of ethanol. They were then washed in absolute ethanol and critically point dried. The coverslips were mounted onto metal stubs and sputter coated with gold. Examination was in a Philips 501B scanning electron microscope with an attached Linof Superolex camera using FP4-120 film.

### MLEE

Trophozoite cultures from 1 adult and 4 chicks from the northern colony and from 6 chicks from the southern colony that had been amplified to numbers sufficient for enzyme electrophoresis after 2–5 days were collected by chilling flasks in an icebath for 15 min and washing twice in cold (4 C) PBS by centrifuging at 1,500 *g* for 3 min. Trophozoites were resuspended in cold PBS, recentrifuged in 1.5 ml microfuge tubes, and cell pellets immediately frozen and stored at −70 C.

Methods of processing trophozoite samples for enzyme electrophoresis have been described (Meloni et al., 1988, 1992). Thirteen enzyme systems used previously to characterize mammalian isolates of *Giardia* were used to characterize the isolates from ibis. The enzymes examined were acid phosphatase (AP) E.C. 3.1.3.2, esterase (EST) E.C. 3.1.1.1, fructose-1,6 diphosphatase (FDP) E.C. 3.1.3.11, glucose-6-phosphate dehydrogenase (G6PD) E.C. 1.1.1.49, glutamate dehydrogenase (GDH) E.C. 1.4.1.3, glutamate-oxaloacetate transaminase (GOT) E.C. 2.6.1.1, glucose phosphate isomerase (GPI) E.C. 5.3.1.9, hexokinase (HK) E.C. 2.7.1.1, malate dehydrogenase (MDH) E.C. 1.1.1.37, malic enzyme (ME) E.C. 1.1.1.40, nucleoside phosphorylase (NP) E.C. 2.4.2.1, phosphoglucomutase (PGM) E.C. 2.7.5.1 and 6-phosphogluconate dehydrogenase (6PGD) E.C. 1.1.1.44.

### RAPD analysis

Trophozoites cultured from 9 chicks from the southern colony were initially washed with warm culture medium 3 times to remove fecal debris and contaminating organisms and amplified to maximum numbers after 2–3 days. Trophozoites were collected and stored as described above.

For DNA extraction, trophozoites were resuspended in 200 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA), 400 μl lysis buffer (8% Triton X-100, 0.25 M sucrose, 50 mM EDTA, pH 8.0), and 50 μl of 10 mg/ml proteinase K. Tubes were mixed well and incubated at 50 C for 2–3 hr. DNA was then isolated from the lysate using the Prep-A-Gene DNA purification kit (Bio-Rad, Richmond, Virginia, U.S.A.).

A pair of primers (TR7—5′-CTGTTGTCGACGTTTATCCA-3′ and TR8—5′-GATCACCAGTGGAGGGTGTGTC-3′) designed by Riley et al. (1991) to characterize *Trichomonas vaginalis* were used. These primers have been used to detect variable DNA repeats in a variety of microorganisms, including *Giardia* (Riley et al., 1991; Morgan et al., 1993).

Polymerase chain reactions (PCRs) were set up as described by Riley et al. (1991), with some modifications. Approximately 80 ng of DNA were amplified in 67 mM Tris-HCl (pH 7.6), 16.6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 12.5 pmol primer, and 1 unit of *Tth* Plus. The PCR reaction was performed in a total volume of 25 μl under a layer of mineral oil. Amplifications were performed on a thermal cycler (Hybaid, Teddington, U.K.) for 2 cycles of 94 C for 5 min, 48 C for 5 min, and 72 C for 5 min, followed by 35 cycles of 94 C for 1 min, 50 C for 1 min, and 72 C for 2 min, and a final cycle of 72 C for 7 min. Amplification products were electrophoresed in ethidium bromide-stained 1.2% agarose gels.

### SSU-rRNA sequencing

Primers were designed from the *G. ardeae* sequence published by van Keulen et al. (1993) and were subsequently used to amplify the SSU-rRNA gene from an ibis isolate of *Giardia*. The 1.4 kb fragment was cloned into Invitrogens' T-vector and subcloned into pGEM 3Z (Promega) for sequencing with the Taq Dye-Deoxy® sequencing system from Perkin Elmer (Branchburg, New Jersey, USA). Several clones were subjected to sequencing on both the forward and reverse strands. The sequence data were analyzed and aligned to those sequences published by van Keulen et al. (1993) for rRNA of *G. duodenalis*, *G. muris*, and *G. ardeae* via a CLUSTAL global alignment (data not shown).

## RESULTS

### In vitro cultivation

Trophozoites isolated from 63 birds failed to establish in continuous culture in a medium used for growing mammalian isolates (Meloni and Thompson, 1987) and in a medium used

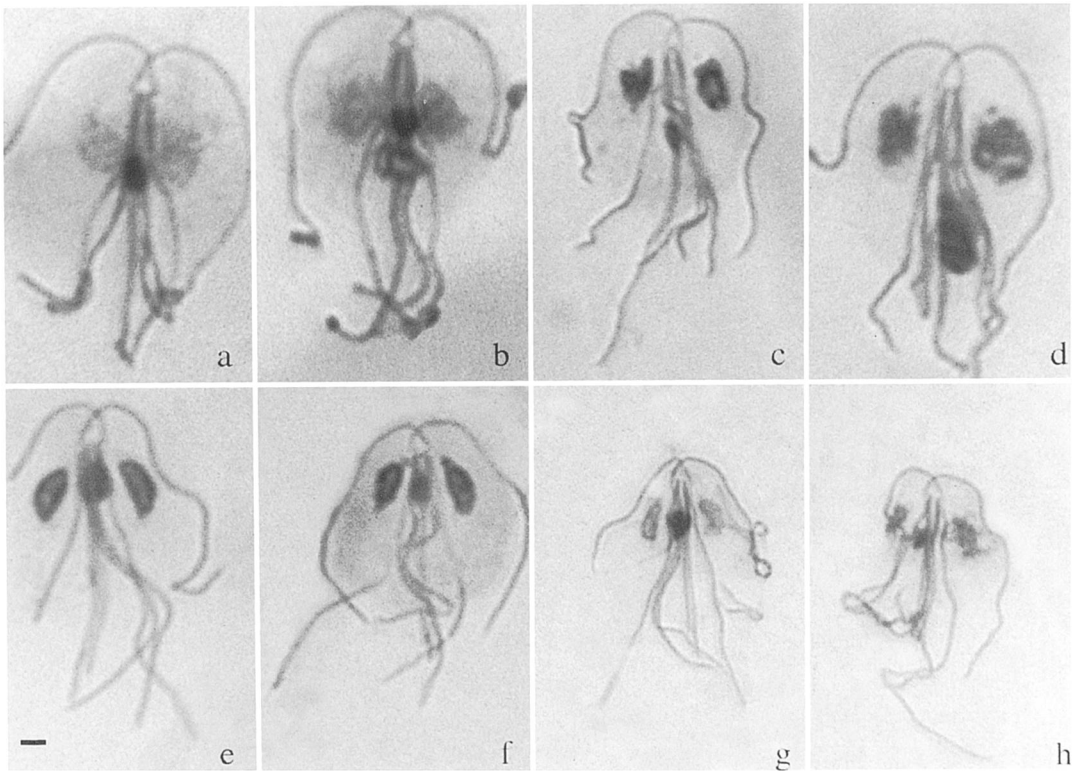


FIGURE 1. Giemsa-stained trophozoites of *Giardia ardeae* (a–d) and ibis isolates of *Giardia* (e–h); magnification (≈3,500×).

for growing the heron isolate, *G. ardeae* (Erlandsen et al., 1990), which we grew successfully in this study. Trophozoite numbers usually increased over the first 5 days of cultivation, forming partial monolayers before trophozoites slowly started to die. In most cultures, few viable trophozoites were left after 12–14 days and the longest surviving trophozoite culture was 22 days. Frequent changes of the medium, increasing the FBS concentration, and omitting bile from the medium did not improve trophozoite survival. It was also noted that during cultivation only a small proportion (10–20%) of trophozoites adhered to the culture vessel surface.

## Median bodies

The morphology of the median bodies observed in trophozoites obtained from *G. ardeae* and from ibis was different from any of the median body morphologies observed in our laboratories in a number of isolates belonging to the *G. duodenalis* morphological group (Fig. 1).

In trophozoites isolated from ibis, the median body could be described as a single rounded median body, invariably situated between the 2 nuclei of the trophozoite, rather than below them as seen in trophozoites of the *duodenalis* group. The median body in trophozoites from *G. ardeae* was also single but varied in shape and position in the trophozoite. Although it appeared identical to the median body observed in the ibis isolate, both in shape and position within the trophozoite, in some cells a teardrop-shaped median body (e.g., Fig 1d) or a small rounded median body situated well below the nuclei was seen.

Because the median bodies observed in the *duodenalis* group of isolates differed so much with respect to their shape, position, and orientation in the cell within even a single cloned cell line, it was not unusual for the median bodies of the 2 isolates from birds to differ as well. However, the variation between the 2 bird isolates on the basis of median body morphology was significantly less than that observed for the *duodenalis* isolates, and hence they may be considered to belong to the same morphological group/species.

## SEM

Using SEM, the morphology of trophozoites derived from all ibis isolates appeared to be identical and was characterized by a pyriform shape with the possession of a deep notch in the posterior border of the ventral adhesive disc (Fig. 2). The caudal flagella were markedly unequal in length. Viewed dorsally, the left caudal flagellum appeared rudimentary, with a length of approximately 1 μm. The right caudal flagellum was much longer, being approximately 7 μm in length. The overall length and width of the trophozoite (excluding flagella and flange) were approximately 8.5 μm and 6.5 μm, respectively.

## Characterization of isolates of *Giardia* using MLEE

Using the 13 enzyme systems described above, interpretable results were obtained for all enzymes except G6PD and MDH. *Giardia* trophozoites amplified by in vitro cultivation from 5 ibis (1 adult and 4 chicks) from the northern colony showed identical enzyme profiles. Similarly, 6 *Giardia* isolates obtained

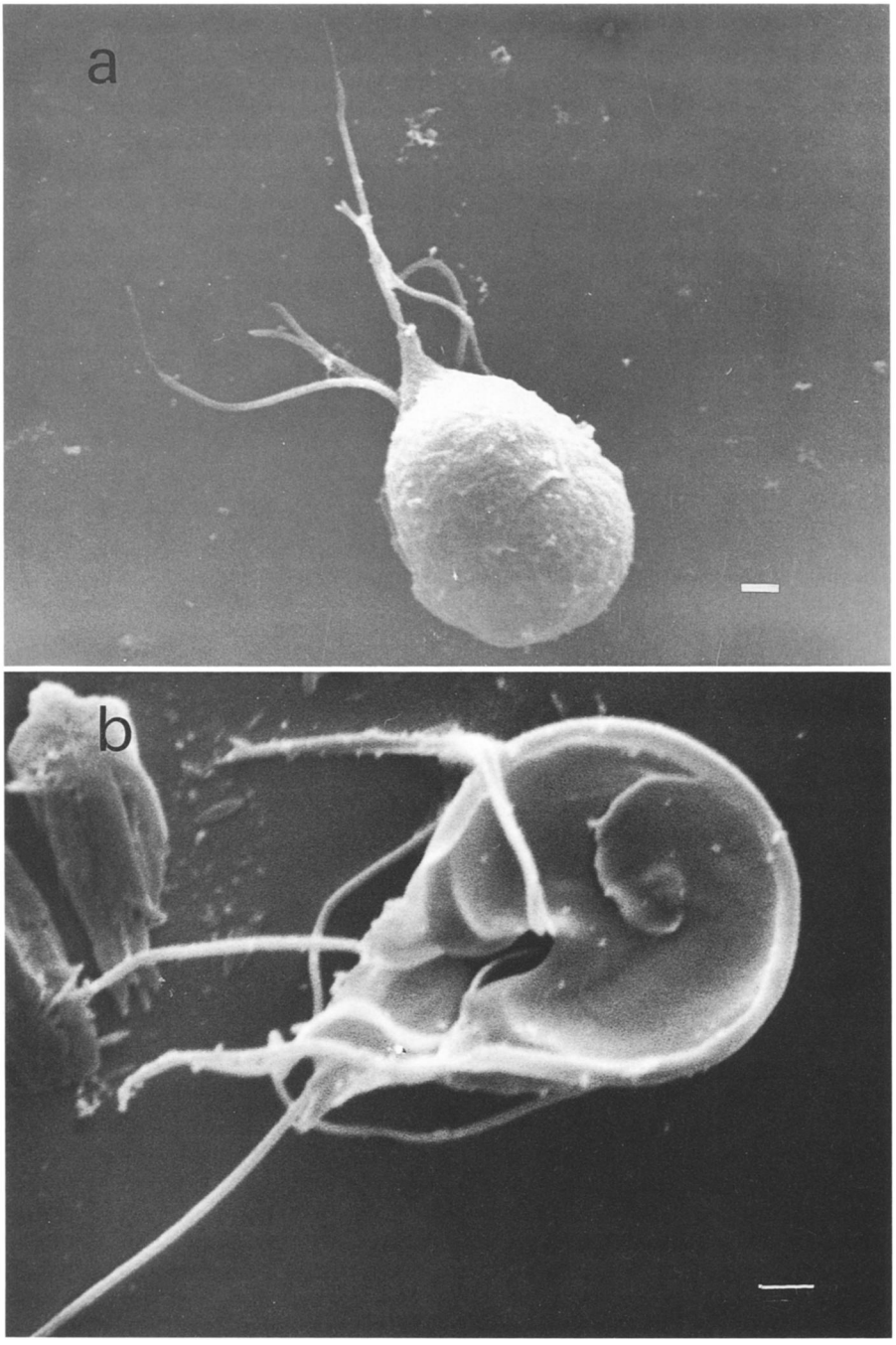


FIGURE 2. SEM of a *Giardia* trophozoite from ibis; (a) dorsal view (magnification, 4,250×), (b) ventral view (magnification, 7,000×).

from chicks from the southern colony and amplified in vitro produced identical enzyme profiles, which was the same profile observed for the ibis isolates from the northern colony. However, not all enzymes were detected in some samples, which was probably due to the low viability and the low numbers of trophozoites obtained from cultures used for enzyme detection.

The enzyme profile for *G. ardeae* was unique although the mobilities of 3 enzymes, AP, FDP, and GOT, were identical to *Giardia* isolates from ibis. The human-derived reference isolate of *G. duodenalis,* P1, did not share any bands with the heron or ibis isolates of *Giardia.* A diagrammatic representation of the enzyme profiles for all the isolates is shown in Figure 3, and a photograph of a gel stained for GDH is shown in Figure 4.

## RAPD analysis

The 9 ibis isolates of *Giardia* produced 2 distinct DNA profiles that were different to the unique profiles obtained for P1 and *G. ardeae* (Fig. 5). Seven ibis isolates produced 1 profile and 2 isolates produced a second profile.

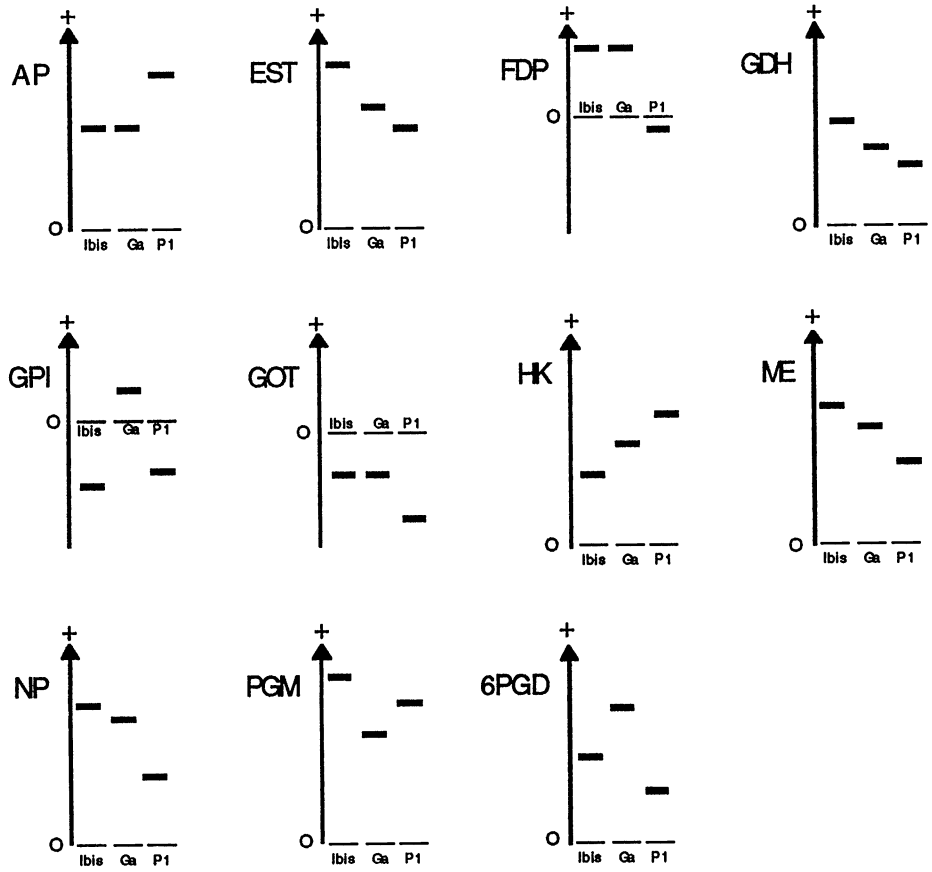


FIGURE 3. Diagrammatic representation of electrophoresis gels obtained for ibis isolates (ibis), *G. ardeae* (Ga), and P1 using 11 enzyme systems. See text for detail of enzymes. Thin dashed line at 0 indicates point of insertion of samples.

### SSU-rRNA sequencing

SSU-rRNA alignment displayed a 96% homology between the heron and the ibis isolate, whereas there was only a 76.5% and 74.25% homology with *G. muris* and *G. duodenalis*, respectively. The SSU-rRNA sequence obtained from the ibis isolate is available in the GenBank TM data base under the accession number U20351.

## DISCUSSION

Enzyme electrophoretic analysis produced similar profiles in 11 ibis isolates of *Giardia* and RAPD analysis of 9 isolates from the southern colony revealed 2 DNA profiles. Ibis isolates of *Giardia* with similar enzyme and DNA profiles were obtained from both colonies. Two isolates that produced a unique RAPD profile were obtained from the southern colony. The genetic

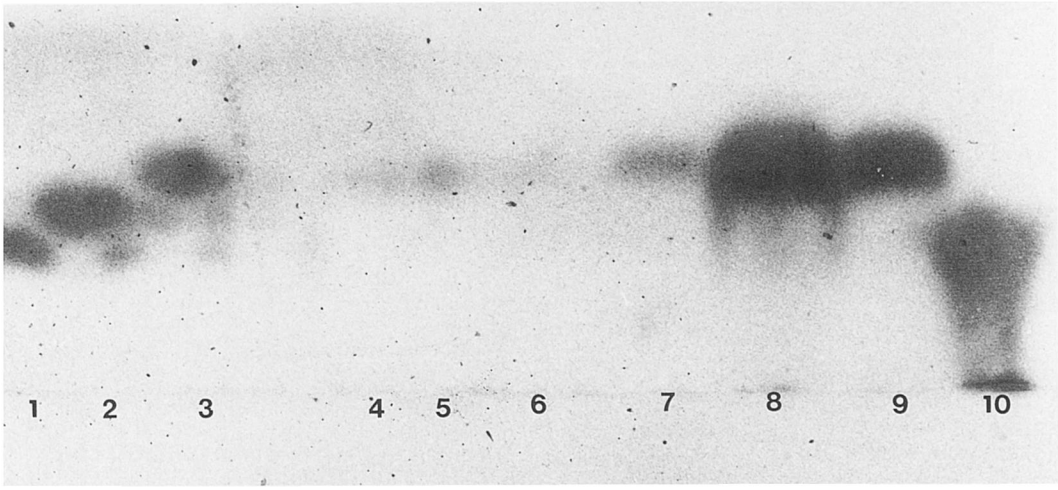


FIGURE 4. Starch gel showing glutamate dehydrogenase staining patterns for *Giardia* isolates. P1 lanes 1 and 10, *G. ardeae* lane 2, and ibis from lanes 3, 7, 8, and 9 (other ibis isolates in lanes 4–6 weakly staining).

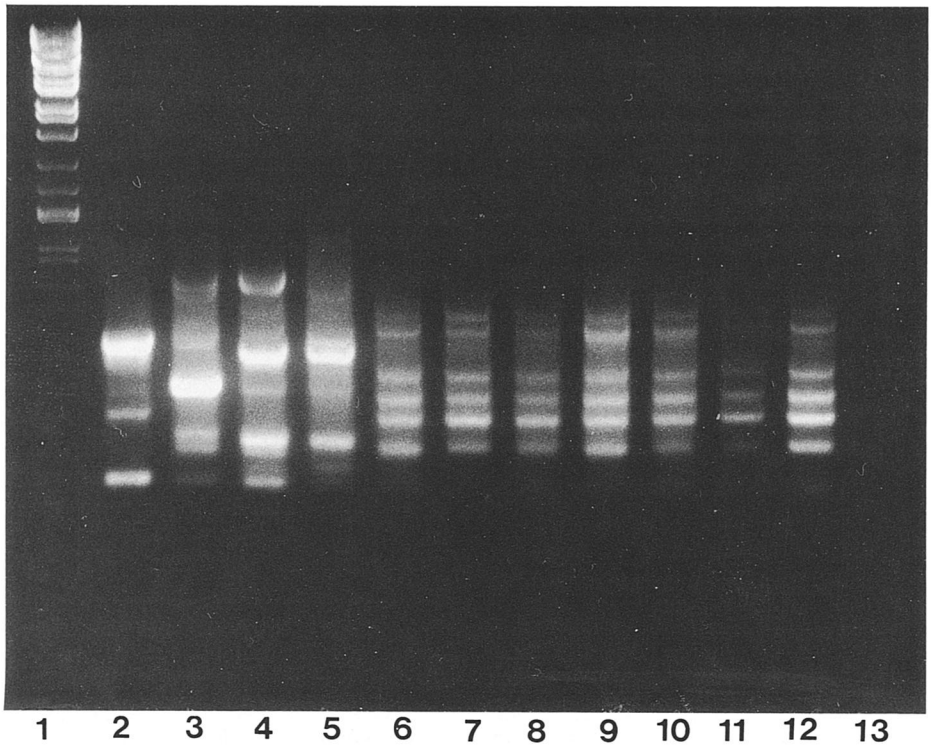


FIGURE 5. Ethidium bromide-stained gel showing profile obtained using Riley primers on ibis isolates lanes 4–11, *G. ardeae* lane 3 and P1 lane 2. Lane 1, Hi-Lo molecular weight markers (Bresatec, Australia); lane 13 negative control (no DNA).

similarity in the ibis isolates is surprising considering the geographical isolation of the 2 colonies and the extensive genetic heterogeneity often displayed by mammalian *Giardia* isolates even from localized geographical areas (Nash et al., 1985; Meloni et al., 1988, 1992, 1995; Proctor et al., 1989). However, the full extent of genetic heterogeneity may not have been revealed because enzyme electrophoresis and RAPD analysis were carried out on isolates amplified in vitro, thus imposing the possibility of selection.

When compared to other avian isolates of *Giardia,* the parasite from ibis bears little morphological resemblance to *G. psittaci* (Erlandsen and Bemrick, 1987). Genetically, the ibis isolates were very different from *G. ardeae,* but morphologically they were very similar, even to the pattern of possessing a rudimentary left caudal flagellum and a longer right caudal flagellum. The morphological similarity to *G. ardeae* is of interest considering the vast geographical distance separating the ranges of the straw-necked ibis and the great blue heron (the straw-necked ibis ranges Australia-wide and into Papua New Guinea; the great blue heron ranges over North America and into the Galapagos Islands). However, the colonial nesting of many herons with other heron species and also with the great white egret *Egretta alba* (see Hancock and Kushlan, 1984), which ranges from the Americas to Australia, may have led to the dispersal of avian isolates of *Giardia* that maybe are more closely related to each other than to mammalian species such as *G. muris* and *G. duodenalis.*

In contrast to the morphological similarities, however, is the failure of trophozoites from ibis to establish in vitro, even using the medium described by Erlandsen et al. (1990) that was used in this study to support the continuous growth of *G. ardeae.* Complementary studies compared sequence data of the DNA coding for the SSU-rRNA of the Western Australian ibis isolate with that of *G. ardeae* from herons in North America and found a 96% homology between the 2, which may suggest that they represent distinct strains of the same species.

The ibis isolates were genetically and morphologically very different from *G. duodenalis* on the basis of direct comparisons with the P1 isolate in this study, as well as with morphological descriptions in the literature (see Erlandsen and Bemrick, 1987; Erlandsen et al., 1990). Sequence data of the SSU-rRNA found only a 74.25% and 76.5% homology with that of *G. duodenalis* and *G. muris,* respectively, indicating strongly that *Giardia* from ibis represents a different species to that occurring in humans and other mammals. This suggests that the zoonotic significance of *Giardia* occurring in ibis in Western Australia is minimal.

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