Journal of Applied Microbiology 2001, **90**, 836–842

Oligonucleotide probes for specific detection of *Giardia lamblia* cysts by fluorescent *in situ* hybridization

M.R. Dorsch and D.A. Veal

Centre for Fluorimetric Applications in Biotechnology, Department of Biological Sciences, Macquarie University, NSW, Australia

547/9/00: received 27 September 2000, revised 2 February 2001 and accepted 7 February 2001

M.R. DORSCH AND D.A. VEAL. 2001.

Aims: Our study focused on the design of oligonucleotide probes and a suitable hybridization protocol that would allow rapid and specific identification of potentially viable cysts of the waterborne parasite *Giardia lamblia*.

Methods and Results: Comparative analysis of ribosomal RNA (rRNA) sequences of *Giardia lamblia* and a number of closely and more distantly related species identified six regions that appear to be specific for the *G. lamblia* 16S rRNA. Fluorescently labelled probes targeting these regions were produced and employed in fluorescent *in situ* hybridization (FISH) experiments. Two of the six probes tested successfully.

Conclusions: Our study provides the first reported probes for specific FISH detection of *G. lamblia*. The method depends on sufficient amounts of intact rRNA in the target organism, which is unlikely to be present in nonviable cysts that have been exposed to the environment for a prolonged period.

Significance and Impact of the Study: Currently, detection of *G. lamblia* cysts is largely based on immunofluorescence assays (IFA) targeting cyst wall surface antigens. These assays lack specificity and will detect species others than *G. lamblia*. Further, IFA will detect nonviable cysts and cyst wall fragments that do not pose a public health risk. In contrast, FISH probes allow specific detection and are likely to only detect viable, infectious cysts.

INTRODUCTION

The protozoan parasite *Giardia lamblia* has been recognized as a common cause of diarrhoea worldwide (Adam 1991). Some controversy still surrounds the systematics of the species, which is also referred to as *Giardia duodenalis* or *Giardia intestinalis* (Lu *et al.* 1998). Other representatives of the genus *Giardia* described to date are *G. agilis* from amphibians and *G. muris* from rodents, birds and reptiles (Meyer 1994), *G. ardea* from herons (Erlandsen *et al.* 1990) and *G. microti* from muskrats and voles (van Keulen *et al.* 1998).

Giardia lamblia is the only species of the genus that is known to cause disease in humans. The infectious stage of the parasite is the cyst, which is transmitted between individuals by the faecal–oral route (Farthing 1994). Together with direct person-to-person, water and food may be significant vehicles of transmission. Little information is available about the relevance of contaminated food but infected persons handling freshly prepared food have been reported as a transmission source (Adam 1991). As a waterborne pathogen, the parasite is of concern for water utilities and is a target of routine monitoring of water samples. The methods used to detect cysts in water need to be very sensitive due to the low infectious dose (Akin and Jakubowski 1986). As it is not possible to culture *Giardia* from water samples, routine detection depends on direct detection of cysts in water concentrates. Most methods rely on immunofluorescence assay (IFA) using antibodies that target the cyst wall (Sauch 1985). Such IFA techniques alone provide results of limited value for determining public health significance of finding cysts in water samples as they do not provide information of whether the species present is infectious to humans.

Evaluating the public health risk by the application of viability dyes is considered inaccurate as it seems to lack

Correspondence to: Dr Matthias Dorsch, Department of Biological Sciences, Macquarie University, NSW 2109, Australia (e-mail: mdorsch@rna.bio.mq.edu.au).

reproducibility when conducted in different laboratories (Clancy *et al*. 1994) and, like IFA, does not enable speciation. *In vitro* excystation (Rice and Schaefer 1981) and subjecting cysts to animal infectivity models (Labatiuk *et al*. 1991) are considered too expensive, time consuming and impractical to be employed for routine testing of water supplies. Such animal or *in vitro* methods also may not correlate with infectivity in humans.

In contrast to the above methods, fluorescent *in situ* hybridization (FISH) employing species-specific oligonucleotide probes has the potential to overcome some of the drawbacks of IFA techniques. FISH can provide information about species identity and will not detect empty cysts or fragments of cyst walls. In the case of *Cryptosporidium parvum*, the percentage of oocysts found to give a positive FISH signal correlated with the viability of oocysts as measured via *in vitro* excystation (Vesey *et al*. 1995).

The potential of detection and speciation of *Giardia* spp. via oligonucleotide probes and FISH was discussed as early as 1994 (Erlandsen *et al*. 1994) but to the best of our knowledge no further progress has been reported.

In this study we describe the design of six oligonucleotide probes potentially specific for detection and identification of *G. lamblia*. Two probes successfully hybridized to their targets when applied for FISH. Hybridization protocols were optimized and the intensity of the FISH signal obtained from each specific probe and a combination of both probes was determined and compared to the signal intensity from hybridization with a FISH probe universal for all eukaryotic 18S rDNA/RNA. Initial specificity validations of the *G. lamblia* probes included hybridization with viable oocysts from *C. parvum* and viable cysts of *G. muris*.

MATERIALS AND METHODS

Probe design

Sequences of 16S rDNA of *G. lamblia* and a number of 16S and 18S rDNA sequences of phylogenetically closely and more distantly related organisms were obtained from GenBank and aligned using the program 'Clustal W' available through the Australian National Genomic Information Service (ANGIS). Sequences included in the alignment and accession numbers are: *Cryptosporidium baileyi* L19068, *Cryptosporidium parvum* L16997, *Cryptosporidium muris* L19069, *Cyclospora* sp. U40261, *Eimeria mitis* U40262, *Eimeria nieschulzi* U40263, *Eimeria tenella* U40264, *Giardia ardea* Z17210, *Giardia lamblia* M54878, *Giardia lamblia* U09492, *Giardia lamblia* U09491, *Giardia microti* AF006677, *Giardia microti* AF006676, *Giardia muris* X65063, *Giardia* sp. U20351, *Ichtyosporidium* L30110, *Nosema apis* X73894, *Nosema ceranae* U26533, and *Nosema furnucalis* U26532.

Comparative sequence analysis of the alignment generated as above was carried out manually and six oligonucleotide probes potentially specific for *G. lamblia* were designed. Designation of the probes, probe sequences and the position of the probes on their 16S rRNA target are: Giar-1 'GCGTCCCGGGTGAGCGGG', position 150–168; Giar-2 'GCCCGCGGGCGCCCGCCC', position 261–278; Giar-3 'TGGGCCCGCCTCGCTCGC', position 498–533; Giar-4 'CGGCGGGGGGCCAACTAC', position 638–655; Giar-5 'GCGGGTCCAACGGGCCTG', position 814–831; Giar-6 'CGGGGCTGCCGCGGCGCG', position 859–876. Probe sequences as according to international agreements are given in 5′-3′ direction. Probe/target positions are not in accord with an international numbering system of the 16S rDNA/RNA. The numbering is based on the sequence of *C. parvum* L16997. As the alignment includes gaps and insertions, respectively, individual position numbers may not be in accord with the total number of bases in a probe sequence. Signal intensity of hybridizations with the above probes was compared with the signal from a universal eukaryotic 18S rRNA targeted probe EUK, 'ACCAGACTTGCCCTCC' (Amann *et al*. 1995), position 502–516. A probe complementary to EUK, 'GGAGGGCAAGTCTGGT' designated Anti-EUK, was applied to determine the extent of fluorescent signal due to any nonspecific binding of probes.

Oligonucleotide probes

Genset, France manufactured all probes (5′-labelled with Texas Red^{Tm}, HPLC purified).

Cysts and oocysts

Viable cysts of *G. lamblia* and *G. muris* and oocysts of *C. parvum* were obtained from Waterborne Inc. (New Orleans, LA, US). Cysts and oocysts, respectively, were washed in phosphate buffered saline (PBS), pH 7·4, and fixed for 2 h in 4% (w/v) paraformaldehyde/PBS at 4°C. Cysts/oocysts were then washed in PBS, resuspended in PBS containing 0·05% sodium azide and stored at 4°C.

Fluorescent *in situ* hybridization

All incubation steps requiring higher than ambient temperature were carried out in a water bath. Suspension of fixed cysts/oocyst containing approximately 400 target organisms was transferred onto Millipore membrane filters (Isopore, pore size 0·8 μm, diameter 13 mm) employing a vacuum manifold ('Sort Stage', Macquarie Research Ltd, Sydney, Australia 2109) and washed with 500 μl cold PBS. Permeabilization and hybridization was then performed by transferring the membranes onto glass microfibre filters (Whatman

GF/C, diameter 25 mm) kept in screw-capped contact lens containers (Bausch & Lomb, inner diameter 25 mm). In these containers the amount of solution sufficient to soak the filter and enable contact with cysts and oocysts can be kept to a minimum (300 μl). Submerging the filters was avoided, as it will inevitably cause loss of target organisms.

To enable probes to penetrate the walls of cysts/oocysts, two permeabilization steps were applied prior to FISH. Membranes carrying *C. parvum* were incubated in 0·1 mol l^{-1} HCl at room temperature for 20 min and then transferred onto glass fibre filters soaked in 50% ethanol and 50% PBS prior to incubation at 80°C for 20 min. *Giardia lamblia* and *G. muris* were permeabilized with and without inclusion of the acid step and the FISH signal intensity was compared. Membranes were transferred onto filters soaked in standard hybridization buffer (0·9 mol l^{-1} NaCl, 20 mmol l^{-1} Tris/HCl, pH 7·2, 0·1% (w/v) SDS) containing oligonucleotide probe at a final concentration of 1–3 mmol l^{-1}. Prior to use, hybridization buffer was preheated at 48°C. In experiments requiring addition of 20% formamide, the hybridization buffer was made up containing 1·2-fold the amount of salts of the standard buffer. Formamide was added immediately before use. After membranes were placed on filters soaked in hybridization solution, containers were tightly closed and incubated 2 min at 80°C to denature any randomly forming secondary structure of the probe. During standard FISH, containers were then incubated 1–2 h at 48°C. Additionally, hybridizations with *G. lamblia* cysts were carried out at 60°C (probe Giar-4 and probe Giar-4 and Giar-6 in combination) and at 66°C (probe Giar-6). Increased temperatures were applied in order to subject Giar-4 and Giar-6 to the same hybridization stringency as probe EUK. Incubation at 48°C is 4°C below the melting point (T_m) of EUK. T_m of Giar-4 and Giar-6 are 64°C and 70°C, respectively. Subsequently, incubation of Giar-4 at 60°C and of Giar-4 at 66°C subjects both probes to the same stringency of probe EUK incubated at 48°C. T_m values were calculated by adding 2°C for each 'A' and 'T' residue and 4°C for each 'G' and 'C' residue within a probe sequence. After hybridization was completed, membranes were placed on a vacuum manifold. Hybridization was terminated and excess probe removed by washing membranes with 500 μl cold PBS.

Counter staining of cysts and oocysts

To facilitate detection of cysts/oocysts that appear FISH negative, fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (Mabs) directed towards surface antigens of cysts/oocysts were applied. *Giardia lamblia* and *G. muris* were labelled with Mab GIAR203. *Cryptosporidium parvum* was labelled with Mab CRY104 (both Mabs from Macquarie Research Ltd, Sydney).

After FISH was completed and excess probe was removed, membranes were washed with 500 μl cold TSPP (2 mmol l^{-1} tetra-sodium pyrophosphate, 0·5% (w/v) bovine serum albumin (BSA), 0·05% Tween 80, 0·05% (w/v) sodium azide, pH 8·0) prior to incubation with 100 μl TSPP containing either FITC conjugated GIAR203 or CRY104 at 10 μg ml^{-1} for 5 min at ambient temperature. Staining solution was removed with a vacuum manifold and the membranes washed with 500 μl cold TSPP. Following the final wash step, membranes were placed on microscope slides onto 5 μl 'AusFlow' mounting medium (2 ml glycerol, nonphotoreactive; 2·4 ml dist H_2O containing 100 mg ml^{-1} DABCO (1,4-diazobicyclo[2.2.2.]octene; Sigma); 4·8 ml 0·1 mol l^{-1} Tris-OH, pH 8·6; 0·5 ml formalin; 0·5 ml 5 mol l^{-1} NaCl). After placing a cover slip on top, membranes were examined at 40-fold magnification with an epifluorescence microscope (Zeiss Axioskop 2 equipped with an 'AttoArc' HBO 100 W adjustable power supply) using filter blocks for either FITC (excitation max. 490 nm, emission max. 525 nm) or Texas Red (excitation max. 590 nm, emission max. 615 nm).

Measuring FISH signal intensity

A 'National' TV camera (Model 1460 N/A; Matsushita Communication Industrial Co. Ltd, Japan) was mounted on the microscope and connected to a 'SPIRICON' laser beam analyser (Model LBA-100 A; Laser Electronics Pty. Ltd, Southport, Australia 4215). Signal intensity of individual cysts/oocysts was measured at 40-fold magnification with an output setting of the 'AttoArc' power supply of 100% and the laser beam analyser in 'frame averaging' mode. Each intensity value of individual objects represents an average of five images captured and the overall values given for different oligonucleotide probes are an average calculated from 50 single objects. Signal intensity measurements were conducted with *G. lamblia* cysts hybridized with EUK probe at 48°C, Giar-4 and Giar-6 separate and in combination at 48°C, Giar-4 at 60°C, Giar-6 at 66°C and Giar-4 and Giar-6 in combination at 60°C.

Determination of background from FISH

The probe Anti-EUK was applied in standard hybridization reactions in order to determine the signal caused by nonspecific binding of probes. An alternative method for background determination was conducted by destroying the probe target, the 18S rRNA, using RNase, prior to FISH. Cysts were permeabilized with 50% ethanol/50% PBS as described in the hybridization protocol. Cysts were then washed in PBS and resuspended in TE buffer, pH 7·5. RNase A (from bovine pancreas; Sigma), dissolved at 10 mg ml^{-1} in 10 mmol l^{-1} Tris-OH, 15 mmol l^{-1} NaCl,

pH 7·5, was added to a final concentration of 100 μg ml^{-1}. After incubating the cysts 30 min at 37°C, tubes were placed on ice and RNase was inactivated by adding Vanadyl Ribonucleoside Complex (VRC, Life Technologies) to a final concentration of 10 mmol l^{-1}. Cysts were washed once in PBS prior to FISH employing the *G. lamblia* specific probes.

For either of the above methods, background quantification was conducted via 'SPIRICON' and the results compared to the fluorescence from membranes subjected to simulated hybridizations without cysts.

RESULTS

Hybridization protocol

Preliminary experiments with the universal eukaryotic 18S rRNA targeted probe EUK showed satisfactory hybridization results with *G. lamblia, G. muris* and *C. parvum*, demonstrating that cysts/oocysts contained a sufficient amount of intact ribosomes and rRNA to enable detection via FISH. Extending the hybridization reaction at 48°C beyond 1 h did not increase the intensity of the fluorescent signal. Probe concentrations higher than 1 mmol l^{-1} only marginally improved signal strength but caused noticeably higher background. Subsequently, hybridization at 48°C for 1 h and a final probe concentration of 1 mmol l^{-1} were applied as standard conditions for all further experiments.

Permeabilization with 0·1 mol l^{-1} HCl at ambient temperature followed by incubation in 50% ethanol/50% PBS is routinely applied in our laboratory for FISH detection/identification of *C. parvum* and is a modification of recently published protocols (Vesey *et al*. 1998). Oocysts incubated in 0·1 mol l^{-1} HCl for 20 min prior to FISH showed significantly increased signal intensity compared to oocysts only permeabilized with 50% ethanol/50% PBS (data not shown). *Giardia lamblia* and *G. muris* did not require permeabilization with HCl as no signal increase was observed compared to cysts solely treated with ethanol/PBS.

FISH probes for detection of *Giardia lamblia* and specificity

Two of the six probes designed for specific detection/identification of *G. lamblia*, Giar-4 and Giar-6, tested successfully. No FISH signal above background was obtained from the four remaining probes. As an initial validation experiment regarding specificity, Giar-4 and Giar-6 were applied separately and in combination in FISH with *C. parvum* oocyst and *G. muris* cysts. No signal was observed from *C. parvum* but a weak signal from *G. muris* observed after FISH with Giar-4 indicated partial cross-reaction under the above hybridization conditions. The signal was eliminated, by increasing the stringency, through the addition 20% formamide to the hybridization buffer. All other parameters remained unchanged.

Intensity of FISH signals and background

Determination of the FISH signal intensity obtained from individual cysts revealed significant differences (Fig. 1). The weakest signal of 72·74 on an arbitrary scale from 0 to 255 was observed from FISH employing the probe EUK at 48°C. Hybridization at the same temperature resulted in much stronger signals from Giar-4 and Giar-6, averaging 124·88 and 160·98, respectively. A combination of both probes showed an average signal of 202·78. However, it was suspected that higher signal strength was at least partially due to the fact that hybridization at 48°C represents higher stringency for probe EUK than it does for Giar-4 and Giar-6, both probes showing a significantly higher T_m of 64°C and 70°C, respectively, compared to a T_m of 52°C for probe EUK. Adjusting the stringency by increased hybridization temperature of 60°C for Giar-4 and 66°C for Giar-6 decreased the signal strength. Average value was 123·48 for Giar-6 and 105·10 for Giar-6. Applying both probes in combination resulted in an average signal of 196·14. Determination of the background signal yielded similar results when either probe Anti-EUK was used or cysts were subjected to permeabilization and RNase A digest in order to destroy the targets of the FISH probes, the rRNA, prior to hybridization with Giar-4 and Giar-6. The average signal was 9 which equals 7·3–8·5% of the specific FISH reactions with Giar-4 and Giar-6 and 16·3% of the signal obtained from probe EUK. The same background was shown from the 'Isopore' polycarbonate membranes when subjected to simulated FISH without cysts.

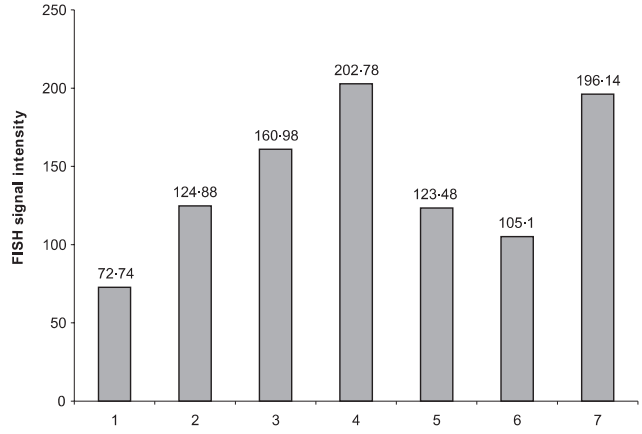

Fig. 1 Average FISH signal intensity obtained with probes/hybridization temperature. 1: EUK, 48 °C; 2: Giar-4, 48 °C; 3: Giar-6, 48 °C; 4: Giar-4 and Giar-6, 48 °C; 5: Giar-4, 60 °C; 6: Giar-6, 66 °C; 7: Giar-4 and Giar-6, 60 °C

DISCUSSION

Results of preliminary FISH experiments were largely in accordance with those obtained for *C. parvum*. The optimum probe concentration was determined as 1 mmol l^{-1}. Increase of the probe concentration caused only marginal increase of the specific signal but resulted in significantly higher nonspecific background fluorescence. Prolonged incubation time for the hybridization reaction past 1 h did not increase the signal. This observation is plausible as the hybridization reaction essentially is a saturation of target molecules that occurs over a certain period of time at given probe concentration and hybridization conditions. Comparing our current results of FISH targeting *G. lamblia* cysts with hybridizations involving bacterial species indicates that the number of FISH probe targets and thus ribosomes present in *Giardia* cysts is comparable to ribosome numbers in many actively growing bacteria. An *E. coli* cell from a culture in the exponential phase of growth contains 10^4–10^5 ribosomes (Malooe and Kjellgard 1966). Recent investigations in the protozoan parasite *C. parvum* concluded that the number of ribosomes per oocyst is substantially higher, averaging $3{\cdot}5 \times 10^5$ (Deere *et al*. 1998). However, no data is available to date regarding the ribosome content of *G. lamblia* cysts.

The lack of any detectable permeabilizing effect of 0·1 mol l^{-1} HCl on *G. lamblia* cysts was an unexpected result. The acid caused a significant degree of permeabilization in the oocyst wall of *C. parvum* as demonstrated by the increase in FISH signal compared to oocysts that were permeabilized at 80°C in 50% ethanol/50% PBS without prior acid incubation. A similar effect was anticipated when treating *G. lamblia* cysts with HCl as excystation of both *C. parvum* and *G. lamblia* is triggered by an acidic environment that causes the cyst/oocyst wall to rupture, but measuring fluorescent signal intensity clearly showed that maximum permeabilization of *G. lamblia* cysts was achieved through ethanol/PBS alone.

Only two of the six oligonucleotide probes designed for specific detection of viable *G. lamblia* cysts, Giar-4 and Giar-6, tested successfully in FISH experiments, leading to the conclusion that the target regions of the remaining probes are not accessible. The success of FISH probes targeting rRNA depends on identifying regions that do not form double strand-helix configuration and are not covered by ribosomal protein and are thus available for hybridization without additional denaturation of the rRNA or ribosomal proteins. Computerized secondary structure modelling can provide evidence for suitability of target regions but is not particularly reliable. More importantly, no program for secondary structure modelling is available that can take in account the interaction between rRNA nucleotides and the amino acids constituting the proteins of the ribosomal subunits. We attempted to manually create partial secondary structures using highly conserved regions of the eukaryotic 18S rRNA as starting points. The approach was abandoned as the corresponding rRNA in *G. lamblia* shows unusually low homology to all other 18S rRNA sequences available through the international databases. It has been reported that the small subunit rRNA from *G. lamblia* is highly unusual for eukaryotes (Sogin *et al*. 1989). The molecule consists of only 1453 nucleotides and therefore resembles the bacterial 16S rRNA rather than the eukaryotic counterpart, the 18S rRNA. Phylogenetic analysis suggests that it may show features of the common ancestor of bacteria and eukarya. In a published secondary structure (Sogin *et al*. 1989) the targets of probes Giar-1, Giar-2 and Giar-3 appear similar to the target region of the 'EUK' probe, as exposed loop regions that would be considered accessible for FISH probes. However, the above *G. lamblia* probes failed to give a signal and the conclusion is that these target regions are either covered by ribosomal protein or the 'true' secondary structure of the 16S rRNA in native ribosomes of *G. lamblia* is significantly different. No information could be obtained regarding the target regions of probe Giar-5 and the successfully tested probes Giar-4 and Giar-6. Their targets are located between positions 508–612 (numbering according to Sogin *et al*. 1989) of the *G. lamblia* 16S rRNA, and this sequence could obviously not be included in the published secondary structure model.

Both functional FISH probes Giar-4 and Giar-6 provided signals that can easily be visualized by epifluorescence microscopy. In contrast, the signal of EUK probe with less than 50% intensity of the specific probes appeared rather weak compared to FISH targeting *Cryptosporidium* spp. and yeasts (data not shown). Examination of the target region on the *G. lamblia* 16S rRNA revealed that the EUK probe is not universal for all eukaryotes as we assumed at the beginning of our study. EUK is designed for the 18S rRNA target 'GGAGGGCAAGUCUGGU'. The corresponding target region on the 16S rRNA of *G. lamblia* is 'GAGGGCAAGGUCUGGU', resulting in six mismatches that cause reduced binding and decreased signal intensity of the EUK probe.

Due to the fact that *G. lamblia* shows a very unusual 16S rRNA regarding sequence and secondary structure, it appears reasonable to assume that FISH probes Giar-4 and Giar-6 are *G. lamblia* specific and will not show cross-reactions with other protozoans under moderately stringent hybridization conditions. Table 1 shows a comparison of the target regions for Giar-4 and Giar-6 as extracted from all *Giardia* spp. 16S rRNA/DNA sequences available to date. A cross-reaction observed from probe Giar-4 with *G. muris* occurred under low stringency hybridization and was eliminated by increased stringency through the addition of 20% formamide to the hybridization buffer. The alignment

Table 1 Comparison of the target regions of probes 'Giar-4' and 'Giar-6' on the 16S rRNA of *Giardia* spp.*

Species/accession	Target 'Giar-4'	Target 'Giar-6'
G. lamblia M54878	GTAG TTGGCCCCCCGCCG	CGCGC CGCGGCA GCCCCG
G. lamblia U09492	GTAG TTGGCCCCCCGCCG	CGCGC CGCGGCA GCCCCG
G. lamblia U09491	GTAG TTGGCCCCCCGCCG	CGCGC CGCGGCA GCCCCG
G. microti AF006677	GTAG TTGGCCCCCCGCCG	**C**TCGC CGCGGCA GCCCCG
G. microti AF006676	GTAG TTGGCCCCCCGCCG	CGCGC CGCGGCA GCCCCG
G. ardea Z17210	G**C**AG**GC**G**T**CGC**GC**G**GC**GC**T**G	**TGGACCTACCGCCCGGGACGGCG**
G. sp. U20351	G**GC**G**C** TG**C**TG C**TGCAGTTA**	CGC C CG**G**GAC **GCG**CG
G. muris X65063	G**GAGTCGAGACGTC** CAG	Not applicable†

Nucleotide residues printed bold indicate mismatches to the *G. lamblia* target sequences of probes 'Giar-4' and 'Giar-6'; blank spaces represent nucleotide deletions.

*Analysis includes all 16S rDNA sequences of *Giardia* spp. available through GenBank in April 2000.

†Comparative sequence analysis and secondary structure modelling led to the conclusion that no corresponding target region exists on the 16S rRNA of *G. muris*.

of published sequences used to design the probes described in this study showed that the corresponding target of region Giar-4 on the *G. muris* 16S rRNA shows eight mismatches and one deletion compared to the target region on the *G. lamblia* 16S rRNA. A corresponding target region for Giar-6 does not exist on the *G. muris* 16S rRNA. The overall homology between the 16S rRNA of *G. lamblia* and *G. muris* is 75·7%, a value that seems surprisingly low between two species of the same genus. From database searches it would appear that the Giar-4 and Giar-6 probes might cross-react with a species designated *Giardia microti* (Koudela 1994; van Keulen *et al*. 1998), isolated from muskrats and voles. These sequences show overall homologies to the *G. lamblia* sequence of 95·2% (GenBank AF006677) and 96·8% (GenBank AF006676), respectively. Target regions for Giar-4 and Giar-6 are identical between *G. lamblia* and *G. microti* except for a single mismatch in the target of Giar-6 in sequence AF006677. *Giardia* spp. isolated from muskrats and voles were originally described as separate species *G. microti* and *G. ondatrae* (Kofoid and Christiansen 1915; Travis 1939). Describing these isolates as separate species was primarily based on morphological traits. It has been reported that cyst morphology of the species in question is different to that from *G. lamblia* and that, in contrast to other *Giardia* spp., the trophozoites of *G. microti* are fully differentiated (Januschka *et al*. 1988). Further, a monoclonal antibody that detects human *Giardia* cysts found in dogs and man (Riggs *et al*. 1983) failed to bind to *G. microti* cysts (Januschka *et al*. 1988). It may be argued that trophozoite morphology could depend on the host in which the cysts developed. Results obtained with a single monoclonal antibody appear insufficient to conclude antigenic differences in the cyst walls of *G. microti* and *G. lamblia*, which justify a separation into two distinct species. Heterogeneity in antigen recognition within an infected population has been observed (Olson *et al*. 2000) and has also been demonstrated *in vitro* through spontaneous appearance of variants in clones (Nash 1992). However, the relevance of variation is not clear and has been described either as significant (Nash 1992) or as minor (Stranden *et al*. 1990). It seems necessary to apply a variety of readily available antibodies that are currently being used for routine detection of *G. lamblia* to confirm the presence and relevance of different antigens on the cyst wall of *G. lamblia* and *G. microti*. No information is available as to whether or not *G. microti* can infect species others than muskrats and voles, including humans. Thus, the evidence of host specificity is lacking.

Given the unusual 16S rRNA sequence and secondary structure, resulting in an isolated phylogenetic position of *Giardia* spp., classification of species based on sequence homology or divergence, respectively, appears a difficult task.

In bacterial phylogeny 16S rDNA/RNA displaying such low degree of sequence homology between two species is generally sufficient for separation on genus level. Sequence homology of the 16S DNA of different species within phylogenetically tight genera may be as high as 98–99% (Martinez-Murcia *et al*. 1992). A comparison of 16S rDNA sequences of *Giardia* spp. described to date reveals a very different and unusual situation. *Giardia lamblia* and *G. muris* show an extremely low 16S rRNA homology of only 75·7% and the homology of *G. lamblia* and *G. ardea* (van Keulen *et al*. 1993) with 72% is even lower. It appears questionable that two *Giardia* spp., separated by host specificity, would share a comparatively very high sequence homology in the range of 96%. Further, it has to be taken in consideration that the amount of sequence data available for different *G. lamblia* isolates is very limited and currently does not allow valid conclusions about sequence diversity within the species *G. lamblia* or any other species of the genus.

ACKNOWLEDGEMENTS

Aspects of this work are covered by Australian Patent Provisional Application No. PQ1056 entitled 'Detection of Giardia'.

Images of *G. lamblia* subjected to FISH with the probes presented here can be viewed on our website http://www.bio.mq.edu.au/flowgird.

REFERENCES

Adam, R.D. (1991) The biology of *Giardia* spp. *Microbiological Reviews* **55**, 706–732.

Akin, A.W. and Jakubowski, W. (1986) Drinking water transmission of giardiasis in the United States. *Water Science and Technology* **18**, 219–226.

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**, 143–169.

Clancy, J.L., Gollnitz, W.D. and Tabib, Z. (1994) Commercial labs: How accurate are they? *Journal of the American Water Works Association* **86**, 89–97.

Deere, D., Vesey, G., Milner, M., Williams, K., Ashbolt, N. and Veal, D.A. (1998) Rapid method for fluorescent *in situ* ribosomal RNA labelling of *Cryptosporidium parvum*. *Journal of Applied Microbiology* **85**, 807–818.

Erlandsen, S.L., Bemrick, W.J., Wells, C.L., Feely, D.E., Knudson, L., Campbell, S.R., van Keulen, H. and Jarroll, E.L. (1990) Axenic culture and characterization of *Giardia ardeae* from the great blue heron (*Ardea herodias*). *Journal of Parasitology* **76**, 717–724.

Erlandsen, S.L., van Keulen, H., Brelje, T., Gurien, A., Jakubowski, W., Schaefer, F.W. III, Wallis, P., Feely, D. and Jarroll, E. (1994) Molecular approach to the speciation and detection of *Giardia:* Fluorochrome-rDNA probes for identification of *Giardia lamblia*, *Giardia muris*, and *Giardia ardeae* in laboratory and environmental samples by *in situ* hybridization. In *Giardia: from Molecules to Disease* ed. Thompson, R.C.A., Reynoldson, J.A. and Lymbery, A.J. pp. 64–65. Wallingford, Oxon, UK: CAB International.

Farthing, M.J.G. (1994) Giardiasis as a disease. In *Giardia: from Molecules to Disease* ed. Thompson, R.C.A., Reynoldsen, J.A. and Lymbery, A.J. pp. 15–37. Wallingford, Oxon, UK: CAB International.

Januschka, M.M., Erlandsen, S.L., Bemrick, W.J., Schupp, D.G. and Feely, D.E. (1988) A comparison of *Giardia microti* and *Spironucleus muris* cysts in the vole: An immunochemical light, and electron microscopic study. *Journal of Parasitology* **74**, 452–458.

Kofoid, C.A. and Christiansen, E.B. (1915) On *Giardia microti* sp. nov., from the meadow mouse. *University of California Publications in Zoology* **16**, 23–29.

Koudela, B. (1994) The ultrastructure of the intestinal microvillus border in the common vole (*Microtus arvalis*) naturally infected with *Giardia microti*. *Folia Parasitologica* **41**, 241–245.

Labatiuk, C.W., Schaefer, F.W. III, Finch, G.R. and Belosevic, M. (1991) Comparison of animal infectivity, excystation and fluorogenic dye as measures of *Giardia muris* inactivation by ozone. *Applied and Environmental Microbiology* **57**, 3187–3192.

Lu, S.Q., Baruch, A.C. and Adam, R.D. (1998) Molecular comparison of *Giardia lamblia* isolates. *International Journal of Parasitology* **28**, 1341–1345.

Malooe, O. and Kjellgard, N.O. (1966). *Control of Macromolecular Synthesis*. New York: W.A. Benjamin.

Martinez-Murcia, A.J., Benlloch, S. and Collins, M.D. (1992) Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: Lack of congruence with results of DNA–DNA hybridizations. *International Journal of Systematic Bacteriology* **42**, 412–421.

Meyer, E.A. (1994) *Giardia* as an organism. In *Giardia: from Molecules to Disease* ed. Thompson, R.C.A., Reynoldsen, J.A. and Lymbery, A.J. pp. 3–13. Wallingford, Oxon, UK: CAB International.

Nash, T.E. (1992) Surface antigen variability and variation in *Giardia lamblia*. *Parasitology Today* **8**, 229–234.

Olson, M.E., Ceri, H. and Morck, D.W. (2000) *Giardia* vaccination. *Parasitology Today* **16**, 213–217.

Rice, E.W. and Schaefer, F.W. III (1981) Improved *in vitro* excystation procedure for *Giardia lamblia* cysts. *Journal of Clinical Microbiology* **14**, 709–710.

Riggs, J.L., Dupuis, K.W., Nakamura, K. and Spath, D.P. (1983) Detection of *Giardia lamblia* by immunofluorescence. *Applied and Environmental Microbiology* **45**, 698–700.

Sauch, J.F. (1985) Use of immunofluorescence and phase-contrast microscopy for detection and identification of *Giardia* cysts in water samples. *Applied and Environmental Microbiology* **50**, 1434–1438.

Sogin, M.L., Gunderson, J.H., Elwood, H.J., Allonso, R.A. and Peattie, D.A. (1989) Phylogenetic meaning of the kingdom concept: An unusual ribosomal RNA from *Giardia lamblia*. *Science* **243**, 75–77.

Stranden, A.M., Eckert, J. and Köhler, P. (1990) Electrophoretic characterization of *Giardia* isolated from humans, cattle, sheep, and a dog in Switzerland. *Journal of Parasitology* **76**, 660–668.

Travis, B.V. (1939) Descriptions of 5 new species of flagellate protozoa of the genus *Giardia*. *Journal of Parasitology* **25**, 11–17.

van Keulen, H., Gutell, R.R., Gates, M.A., Campbell, S.R., Erlandsen, S.L., Jarroll, E.L., Kulda, J. and Meyer, E.A. (1993) Unique phylogenetic position of diplomonadia based on the complete small subunit ribosomal RNA sequence of *Giardia ardea*, *Giardia muris*, *Giardia duodenalis* and *Hexamita* sp. *FASEB Journal* **7**, 223–231.

van Keulen, H., Feely, D.E., Macechko, P.T., Jarroll, E.L. and Erlandsen, S.L. (1998) The sequence of *Giardia* small subunit rRNA shows that voles and muskrats are parasitized by a unique species *Giardia microti*. *Journal of Parasitology* **84**, 294–300.

Vesey, G., Ashbolt, N.J., Wallner, G., Dorsch, M., Williams, K.L. and Veal, D.A. (1995) Assessing *Cryptosporidium parvum* oocyst viability with fluorescent *in situ* hybridisation using ribosomal RNA probes and flow cytometry. *Protozoan Parasites and Water* ed. Betts, W.B., Casemore, D., Fricker, C., Smith, H. and Watkins, J. pp. 133–138. Royal Society for Chemistry.

Vesey, G., Ashbolt, N., Fricker, E.J., Deere, D., Williams, K.L., Veal, D.A. and Dorsch, M. (1998) The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labeling of viable *Cryptosporidium parvum* oocysts. *Journal of Applied Microbiology* **85**, 429–440.