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Genotyping of *Giardia* in Dutch patients and animals: A phylogenetic analysis of human and animal isolates *

J.W.B. van der Giessen[a,], A. de Vries[a], M. Roos[a], Peter Wielinga[a], L.M. Kortbeek[b] and T.G. Mank[c]**

[a]National Institute for Public Health and the Environment (RIVM), Microbiological Laboratory for Health Protection, Antonie van Leeuwenhoeklaan 9, P.O. Box 1, Bilthoven, The Netherlands
[b]National Institute of Public Health and the Environment (RIVM), Laboratory for Infectious Diseases Surveillance and Screening, Antonie van Leeuwenhoeklaan 9, P.O. Box 1, Bilthoven, The Netherlands
[c]Laboratory of Public Health, Department of Parasitology, Boerhaavelaan 26, 2035 RC, Haarlem, The Netherlands

[*]Note: Nucleotide sequence data reported in this paper are available in the Genbank under the accession numbers: AY826191–AY826210; AY827496–AY827499; DQ100287, DQ100288.

** Corresponding author. Tel.: +31 30 2743926; fax: +31 30 2744434.

Abstract

Giardia duodenalis (syn. *Giardia lamblia*, *Giardia intestinalis*) is a protozoan organism that can infect the intestinal tract of many animal species including mammals. Genetic heterogeneity of *G. duodenalis* is well described but the zoonotic potential is still not clear. In this study, we analysed 100 *Giardia* DNA samples directly isolated from human stool specimens, to get more insight in the different *G. duodenalis* assemblages present in the Dutch human population. Results showed that these human isolates could be divided into two main Assemblages A and B within the *G. duodenalis* group on the basis of PCR assays specific for the Assemblages A and B and the DNA sequences of 18S ribosomal RNA and the glutamate dehydrogenase (*gdh*) genes. Genotyping results showed that *G. duodenalis* isolates originating from Dutch human patients belonged in 35% of the cases to Assemblage A (34/98) and in 65% of the cases to Assemblage B (64/98) whereas two human cases remained negative in all assays tested. In addition, we compared these human samples with animal samples from the Netherlands and human and animal samples from other countries. A phylogenetic analysis was carried out on the DNA sequences obtained from these *Giardia* and those available in GenBank. Using *gdh* DNA sequence analysis, human and animal Assemblage A and B *Giardia* isolates could be identified. However, phylogenetic analysis revealed different sub-clustering for human and animal isolates where host–species-specific assemblages (C, D, E, F and G) could be identified. The geographic origin of the human and animal samples was not a discriminating factor.

1. Introduction

The flagellated protozoan *Giardia* is an intestinal parasite that can infect many species in the animal kingdom including mammalian, avian and reptilian wildlife, domesticated animals and humans (Thompson, 2004 and Appelbee et al., 2005). Of the morphologically defined *Giardia* species, *Giardia muris*, *Giardia microti*, *Giardia agilis, Giardia psicatti* and *Giardia duodenalis* (syn. *Giardia lamblia, Giardia intestinalis*), only the latter is recovered from humans and a wide variety of other mammals. In humans, *G. duodenalis* can cause gastrointestinal infections ranging from mild to severe as well as chronic disease. In domestic animals, *G. duodenalis* is of considerable clinical importance and could have economic significance in cattle-based industries (Olson et al., 2004). Infection occurs by faecal oral route transmission, either by direct contact or by ingestion of contaminated food or water (Monis and Thompson,

2003). Despite morphological uniformity, considerable biotypic and genetic diversity exists within the *G. duodenalis* species (Monis et al., 1996 and Thompson et al., 2000). The species includes several 'assemblages' or genotypes, A–G, that can be discriminated on the basis of host selection and genomic mutations (Monis et al., 1999). Although several genes encoding proteins involved in meiosis are present in *Giardia* (Ramesh et al., 2005), direct evidence for sexual recombination has not yet been shown. Phylogenetic multilocus analysis using 18S rRNA, glutamate dehydrogenase (*Gdh*), elongation factor 1 alpha (EF-1α) and triose phosphate isomerase (*Tpi*) gene based molecular methods have been used on representatives of each major genetic group to study the relations among assemblages from different hosts (Monis et al., 1999, van Keulen et al., 2002 and Caccio et al., 2005). Zoonotic transmission of *G. duodenalis* is still under debate and despite increasing knowledge of the molecular identification of *Giardia* from different host species; the zoonotic potential of *G. duodenalis* is not clear (Monis and Thompson, 2003, Thompson, 2004 and Hunter and Thompson, 2005). One report in the literature has identified a clear animal to human transmission: waterborne transmission of *Giardia* from a beaver to humans was identified (Isaac-Renton et al., 1993). A survey on the transmission of *Giardia* from dogs to humans indicated that there was no transmission from dogs to humans but that the reverse, transmission from humans to dogs, could be possible (Hopkins et al., 1997). Dogs, however, might not only transmit human adapted *Giardia* genotypes as was described by a study in an endemic area where humans and dogs were living closely together (Traub et al., 2004) but may also be infected with host-adapted *Giardia* genotypes (Caccio et al., 2005). Studies designed to investigate zoonotic potential are still based either on limited numbers of isolates from a diverse source or limited molecular identification tools.

It was our aim to analyse the genetic diversity of *Giardia* isolates from human clinical cases in the Netherlands by different molecular typing methods and to compare the results of the different methods. We used the following methods: *gdh* PCR-restriction fragment length polymorphism (RFLP) assay and the two discriminative PCR assays. One PCR assay specifically detected Assemblage A and one was specific for Assemblage B genotypes of *G. duodenalis* as described by Homan et al. (1998). In addition, we performed DNA sequence analysis of two different genes, 18S rDNA and the *gdh* gene. As well as the human isolates, we sequenced these loci for several animal *Giardia* isolates. The different sequences were used to construct a database so we could compare our findings for human patient *Giardia* isolates with those from animals in the Netherlands and those published previously and available in GenBank. Using phylogenetic analysis, it was our aim to further elucidate the relationship of the different genotypes to each other, their hosts and the geographic origin to study the possibilities for zoonotic transmission.

2. Materials and methods

2.1. Origin of the samples

One hundred microscopically confirmed *Giardia*-positive faecal samples from humans with symptoms of diarrhoea were analysed. In addition, *Giardia*-positive faecal samples of two dogs three sheep/goat and one Dutch roe deer from the Netherlands were analysed using the same methods (Table 1A). The details of the DNA sequences acquired from Genbank and used in this study are shown in Table 1B and Table 1C.

Table 1A.

Giardia 18S rDNA and *gdh* DNA sequences submitted to Genbank for human and animal *Giardia* isolates determined in this study

Isolate	Host	Geographical origin	18S rRNA GenBank GI[a]	GDH GenBank GI	Assemblage[b]
NLH13	Human	The Netherlands	AY826201	AY826191	B
NHL20	Human	The Netherlands	AY826204	AY826194	A
NHL25	Human	The Netherlands	AY826203	AY826193	B
NHL28	Human	The Netherlands	AY826202	AY826192	B
NHL35	Human	The Netherlands	AY826207	AY826197	B
NHL37	Human	The Netherlands	AY826206	AY826196	A
NHL45	Human	The Netherlands	AY826205	AY826195	A
NLDE3	Dog	The Netherlands	AY827497	AY827498	D
NLD37	Dog	The Netherlands	AY827496	AY827499	D
NLG409	Goat	The Netherlands	AY826210	AY826198	E
NLR118	Roe deer	The Netherlands	DQ100287	DQ100288	A
NLS352	Sheep	The Netherlands	AY826208	AY826199	E
NLS387	Sheep	The Netherlands	AY826209	AY826200	E

[a] GenBank accession/GI number.
[b] Assemblage A corresponds to a positive A PCR, type 1 18S rRNA sequence and G1 glutamate dehydrogenase (GDH) restriction fragment length polymorphism (RFLP), Assemblage B to a positive B PCR, type 2 18S rRNA sequence and G2 GDH RFLP.

Table 1B.

DNA sequences from *Giardia* infecting humans, acquired from Genbank and used in this study

Isolate	Geographical origin	18S rRNA GenBank GI	GDH GenBank GI	Genotype[a]	Reference
BAH-12	Australia	AF113897	AF069059	B,III	Monis (1999), Thompson (2000)
Ad-2	Australia		L40510	A,II	Monis (1996)
Ad-28/Ad-19	Australia	AF113898	L40508	B,IV	Monis (1996, 1999)
BaH40c11	Australia	AF199446		A	Thompson (2000)
Ad-113	Australia		AY178736	A2	Ey (2002), Unpublished data
Bris-136	Australia		AY178737	A2	Ey (2002), Unpublished data
Ad-1	Australia		AY178735	A1	Ey (2002), Unpublished data
Ad-28	Australia		AY178738	B	Ey (2002), Unpublished data
Ad-45	Australia		AY178739	B	Ey (2002), Unpublished data
Ad-85	Australia		AY178755	B3	Ey (2002), Unpublished data
Ad-82	Australia		AY178754	B0 and B3	Ey (2002), Unpublished data
FCQ-21	Australia		AY178756	B3	Ey (2002), Unpublished data
Bris/91/Hepu1279	Australia	L29192			Upcroft (1994)
CA1	China	AJ293296		A	Yong (2000)
CA8	China	AJ293300		B	Yong (2000)
CA13	China	AJ293299		A	Yong (2000)
CA14	China	AJ293297		A	Yong (2000)
CA18	China	AJ293298		A	Yong (2000)
K2	South Korea	AJ293295		A	Yong (2000)
K–C1	South Korea	AJ293301		A	Yong (2000)
Portland 1	South Korea	M54878	M84604	1,A-I	Sogin (1989)
AMC-4	The Netherlands	U09491		B	van Keulen (1995)

[a] Genotype as listed in the references.

International Journal for Parasitology Volume 36, Issue 7 , June 2006, Pages 849-858

Table 1C.

DNA sequences from Genbank from various parasitic animal *Giardia* used in this study

Isolate	Host	Geographical origin	18S rRNA GenBank GI	GDH GenBank GI	Genotype[b]	Reference
Blue heron	Blue heron	USA	Z17210		*Giardia ardeae*	van Keulen (1993)
Ad-133	Calf	Australia		AY178740	E	Ey (2002), Unpublished data
Ad-23	Cat	Australia	AF113910	AF069057	Cat	Monis (1999)
Ad-131	Cat	Australia		AY178742	Cat	Ey (2002), Unpublished data
Ad-142	Cat	Australia		AY178743	Cat	Ey (2002), Unpublished data
Ad-154	Cat	Australia		AY178744	Cat	Ey (2002), Unpublished data
CH-105	Chinchilla	Czech Rep.		AY178751	B1	Ey (2002), Unpublished data
Ad-136	Dog	Australia	AF113899	U60982	C	Monis (1999)
Ad-148	Dog	Australia	AF113900	U60986	D	Monis (1998)
Ad-137	Dog	Australia		U60983	C	Monis (1998)
Ad-141	Dog	Australia		U60984	C	Monis (1998)
Ad-147	Dog	Australia		U60985	C	Monis (1998)
dog6	Dog	Australia	AF199443		Group 4	Thompson (2000)
dog19	Dog	Australia	AF199449		Group 3	Thompson (2000)
Vanc/89/UBC/059	Dog	Canada		AY178750	B	Ey (2002) Unpublished, Monis (1998)
CZ:D-47	Dog	Czech Rep.		AY178749	B	Monis (1999), Ey (2002), Unpublished data
GF-1	Ferret	Japan	AB159796	AB159795	A	Abe (2005)
BAG1	Goat	Australia	AF199448		Lifestock	Thompson (2000)
PRM 025	Ibis	Australia	U20351		*Giardia ardeae*	McRoberts (1996)
Ad-156	Marmoset	Australia		AY178752	B2	Ey (2002), Unpublished data
Ad-158	Marmoset	Australia		AY178753	B2	Ey (2002), Unpublished data
Ad-170	Mouse	Australia		AY178748		Ey (2002), Unpublished data
Peromyscus leucopus	(deer) Mouse	USA	AF473852		*Giardia. Microti*	van Keulen (2002)
Ondatra zibethicus	Muskrat	USA	AF006677		*G. microti*	van Keulen (1998)
Parakeet	Parakeet	USA	AF473853		*Giardia psittaci*	van Keulen (2002)
Microtus ochrogaster	Prairie vole	USA	AF006676		*G. microti*	van Keulen (1998)
Ad-157	Rat	Australia	AF113896	AF069058	Rat	Monis (1999)
Ad-155	Rat	Australia		AY178745	Rat	Ey (2002), Unpublished data
Ad-167	Rat	Australia		AY178746	Rat	Ey (2002), Unpublished data
Ad-171	Rat	Australia		AY178747	Rat	Ey (2002), Unpublished data
Rat2	Rat	Australia	AF199450		Rat	Thompson (2000)

[b] Genotype as listed in the references.

2.2. DNA isolation

Total DNA from *Giardia* cysts from fresh non-preserved stool samples was isolated as described earlier (Homan et al., 1998) with some modifications. Briefly, stools were broken up in distilled water and filtered through a 70 μm cell strainer (BD Falcon, Belgium), 2.5 ml stool suspensions were layered on 3 ml of 1.6 M sucrose and centrifuged at 750×*g* for 5 min. Cysts at the sucrose–water interphase were collected and washed with distilled water. The enriched cysts were resuspended in 1 ml distilled water and 100 μl of 10×buffer A, 100 μl of 10×buffer B and 10 μl anti-*Giardia* magnetic beads provided with the *Giardia*/*Cryptosporidium* purification kit (Dynal Biotech GmbH, Hamburg, Germany). After 1 h of gently mixing the suspension at room temperature the magnetic beads were washed with buffer A using a tube-holder with a magnetic strip (Dynal Biotech). For DNA isolation the beads were resuspended in 600 μl lysis buffer provided with the Puregene kit (Gentra systems, Minneapolis, Minnesota, USA). Cysts were lysed by five freeze/thaw cycles by exposing them to dry-ice ethanol followed by heating to 65 °C and the DNA was further purified according to the manufacturer's instructions.

2.3. Molecular analysis of samples

Genotyping of the *Giardia* samples was performed by two PCR assays specific for Assemblages A and B, which we call the A- and B-PCR, respectively, and by a PCR-RFLP analysis of the *gdh* described by Homan et al. (1998). For DNA sequence analysis of the 18S rDNA gene, the PCR described by van Keulen et al. (2002) was used, amplifying 298 bases of the 5′end of the 18S rDNA gene. PCR reactions of the *gdh* (human samples using primers gdh-1 and gdh-4; animal samples using primers gdh-1 and gdh-3) and the A (primers: P1F and P3R) and B (primers: B1F and B3R) loci were conducted in 50 μl reactions mixtures containing: 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2 mM MgCl 2, 5% dimethyl sulfoxide (Sigma), 0.1 mM of each dNTP, 1 U of AmpliTaq polymerase (Applied Biosystems, The Netherlands), 0.5 μM of each primer and 5 μl template DNA. The PCR was carried out with the following conditions: one cycle of 94 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, followed by one cycle of 10 min and 72 °C. Primers used in this study are listed in Table 2.

Table 2.

Primers used in genotyping and sequencing

Locus	Primers	Position	Sequence
Gdh	Forward gdh-1	362–382	5′-ATCTTCGAGAGGATGCTTGAG-3′
	Reverse gdh-4	1120–1140	5′-AGTACGCGACGCTGGGATACT-3′
	Reverse gdh-3	870–890	5′-TGTCCTTGCACATCTCCTCCA-3′
A	Forward P1F		5′-CTGCAGGGGCAAGGCGTAGAT-3′
	Reverse P3R		5′-CCACCGTGCCAGTCTTCTGGG-3′
B	Forward B1F		5′-CTGCAGTAACACTGGCAAG-3′
	Reverse B3R		5′-CTGCAGAGTCTCCGCAGCG-3′
18S rDNA	Forward 18S-1	3–21	5′-TCCGGTCGATCCTGCCGGA-3′
	Reverse 18S-A	285–301	5′-GCTCTCCGGAGTCGAAC-3′

PCR products were purified using a Qiaquick gel extraction kit (Qiagen, Westburg, The Netherlands), according to manufacturer's instructions. DNA sequencing reactions were performed in both directions using BigDye Terminator v3.1 (Applied Biosystems, The Netherlands). To perform sequencing of the 18S rDNA PCR product the forward primer 18S-1 and reverse primer 18S-A were used. For *gdh* sequencing, forward primer gdh1 and reverse primer gdh3 were used (Table 2). DNA sequences were stored and analysed using the software package BioNumerics of Applied Maths (Ghent, Belgium). Representative DNA sequences were submitted to GenBank (Table 1A). For phylogenetic analysis, DNA sequences from this study were used together with DNA sequences from the same loci obtained from GenBank (Table 1B and Table 1C). Phylogenetic DNA sequence analyses were performed by maximum parsimony tree calculation and bootstrap analysis. For the

combined or multilocus comparison of the 18S rDNA and the *gdh* sequences, one phylogenetic tree was generated using cluster analysis with the unweighted pair group method with arithmetic mean (UPGMA) as dendrogram type.

3. Results

3.1. Genotyping of human Giardia isolates

Giardia-positive human stool samples (100 isolates) were genotyped by the Assemblage A- and B-specific PCR, *gdh*-PCR and RFLP. In addition, an 18S rDNA and *gdh*-specific DNA sequence analysis was performed. Clearly two genotype groups could be recognised, similar to the two assemblages A and B described by Homan et al. (1998) and Monis et al. (1999) (Table 3). In total, 34 samples were identified as *Giardia* Assemblage A, 64 as Assemblage B and two samples remained negative. The *gdh* RFLP identified 33 human samples as G1 and 55 as G2 type; one sample both G1 and G2; 11 samples were negative with this method. Comparing the *gdh* RFLP results with the assemblage A- and B-specific PCR results showed that except for two samples all 33 G1 positive samples were positive in the A-PCR. One of the G1 isolates remained negative and one was positive in both the A- and B-specific PCR. The G2 samples only showed a positive result in the B-PCR. The 18S rDNA sequence analyses showed that 18S rDNA type 1 sequences were linked both to the Assemblage A-specific PCR samples and G1 positive samples. The 18S rDNA type 2 sequences were linked both to the Assemblage B-specific PCR samples and G2 positive human samples (Table 3). Of the 11 negative *gdh* RFLP samples one could be identified by the 18S rDNA sequence as type 1 *Giardia* isolate and eight as type 2 *Giardia* isolates. For one human sample, the *gdh* RFLP analysis revealed a combined G1 and G2 type, whereas in 18S rDNA sequence analysis this sample was identified as type 2 and thus was categorised in the Assemblage B.

Table 3.

Genotypic classification of 98 *Giardia* isolates originating from 100 clinical human patients

Patient isolate	Assemblage[a]	18S rRNA	*gdh*	A/B PCR[b]
1-29	A	Type 1	G1	A
30–31	A	Type 1	G1	A+B
32–33	A	Type 1	G1	Neg
34	A(*n*=34)	Type 1	Neg[c]	Neg
35–84	B	Type 2	G2	B
85	B	Type 2	G2	Neg
86	B	Type 2	G2+G1	Neg
87–94	B	Type 2	Neg	Neg
95–97	B	Neg	G2	B
98	B(*n*=64)	Neg	G2	Neg

[a] Assemblage A indicates a 18S type 1, G1, positive A PCR, Assemblage B type 2, G2, positive B PCR. Negative and inconclusive/mixed samples were not considered. Between brackets the total number (*n*) of each assemblage is given.
[b] An A indicates the sample was positive in the A PCR and negative in the B PCR, for B vice versa.
[c] Neg: no PCR product was obtained, for two patients' isolates this was the case for all three methods used.

For confirmation purposes, three of the Dutch human *Giardia* isolates (randomly chosen based on A and B PCR positivity) were sent to Dr R.C.A. Thompson at the WHO Centre in Australia, who confirmed our genotyping results.

Next to the 18S rDNA sequence comparison of *Giardia* isolates, which showed only two slightly different sequences linked to Assemblages A and B, diversity within the *gdh* DNA sequences analysed was much larger. We identified seven different *gdh* DNA sequences. Representatives of these seven different *gdh* sequences were submitted to GenBank and are

shown in Table 1A. For Assemblage A, we identified three different representative *gdh* sequences (NHL20, NHL37, NHL45) and for Assemblage B, four different representative sequences (NHL13, NHL25, NHL28, NHL35).

3.2. Genotyping of animal derived Giardia isolates by gdh and 18S rRNA sequences

In addition to the set of human samples, we tested several *Giardia*-positive animal samples from the Netherlands. Two dog samples (NLDE3, NLD37), one goat sample (NLG409), two sheep samples (NLS352, NLS387) and one roe deer sample (NRL118). Results of PCR and DNA sequence analyses showed that the roe deer *Giardia* isolate belonged to Assemblage A. The dog *Giardia* isolates belonged to Assemblage D, while the goat and sheep isolates belonged to Assemblage E (Table 1A). These results confirm the previously described host-specific assemblages for dogs and livestock (Thompson, 2004).

3.3. Phylogenetic analysis of 18S rRNA and gdh DNA sequences

From 18S rDNA sequences determined in this study, 229 nucleotides of representative sequences were aligned with a set of similar sequences from GenBank and were subjected to phylogenetic analysis using BioNumerics analysis software. Fig. 1 shows the phylogenetic tree resulting from 42 different *Giardia* 18S rDNA sequences. The tree is largely divided in seven clusters. The first cluster contains the human Assemblage A type isolated together with the cat (Assemblage F) and the livestock cluster (Assemblage E). The second cluster contains the human Assemblage B isolates and third and fourth are the dog clusters of either Assemblages C or D. The fifth contains the rat (Assemblage G) cluster, which was supported by a bootstrap analysis at 68%. The longest branches of the tree were the lineages giving rise to *G. psittaci* and *G. ardeae* (67% bootstrap support). Finally, *G. microti* isolates clustered together (84% bootstrap support). Besides the last three clusters, all other clusters were poorly supported by bootstrap analysis.

Similar to the 18S rDNA sequence comparison, we analysed a 399 bp fragment of the g*dh* gene of 47 different *Giardia* isolates. This resulted in a more specific division of clusters (Fig. 2) compared with the 18S rDNA analysis, indicated by higher bootstrap values for the different clusters. Again, human *gdh* DNA sequences belonging to either Assemblages A or B divided in two separate clusters, supporting the differentiation of the Assemblages A and B. The Dutch goat and sheep samples clustered together with the calf isolate (AY178740), Assemblage E and far distant from the Dutch dog samples (AY827498 and AY827499), which clustered together with the Australian dog (U60986), Assemblage D. The Dutch roe deer *gdh* sequence clustered together with the human isolates of Assemblage A, however, not in the same subcluster as the human Assemblage A isolates analysed. In addition, some animal *gdh* DNA sequences derived from GenBank clustered together with human Assemblage B. Subgenotyping of our human Assemblage B-derived *gdh* sequences showed that those with GenBank accession number AY826193 were located in the cluster of Australian human isolates of Assemblage B group III (AY178756/AF069059) (Monis et al., 1999), those with accession number AY826191 clustered in Assemblage B group IV containing the Australian human isolates (AY178738/AY178739, AY178754, L40508), the Canadian and Czech dog isolates (AY178750/AY178749) and the Czech chinchilla isolates (AY178751). Our human isolates with accession numbers AY826192, AY826197 belonging to Assemblage B clustered together in the IV-like group together with the marmosets AY178752, AY178753.

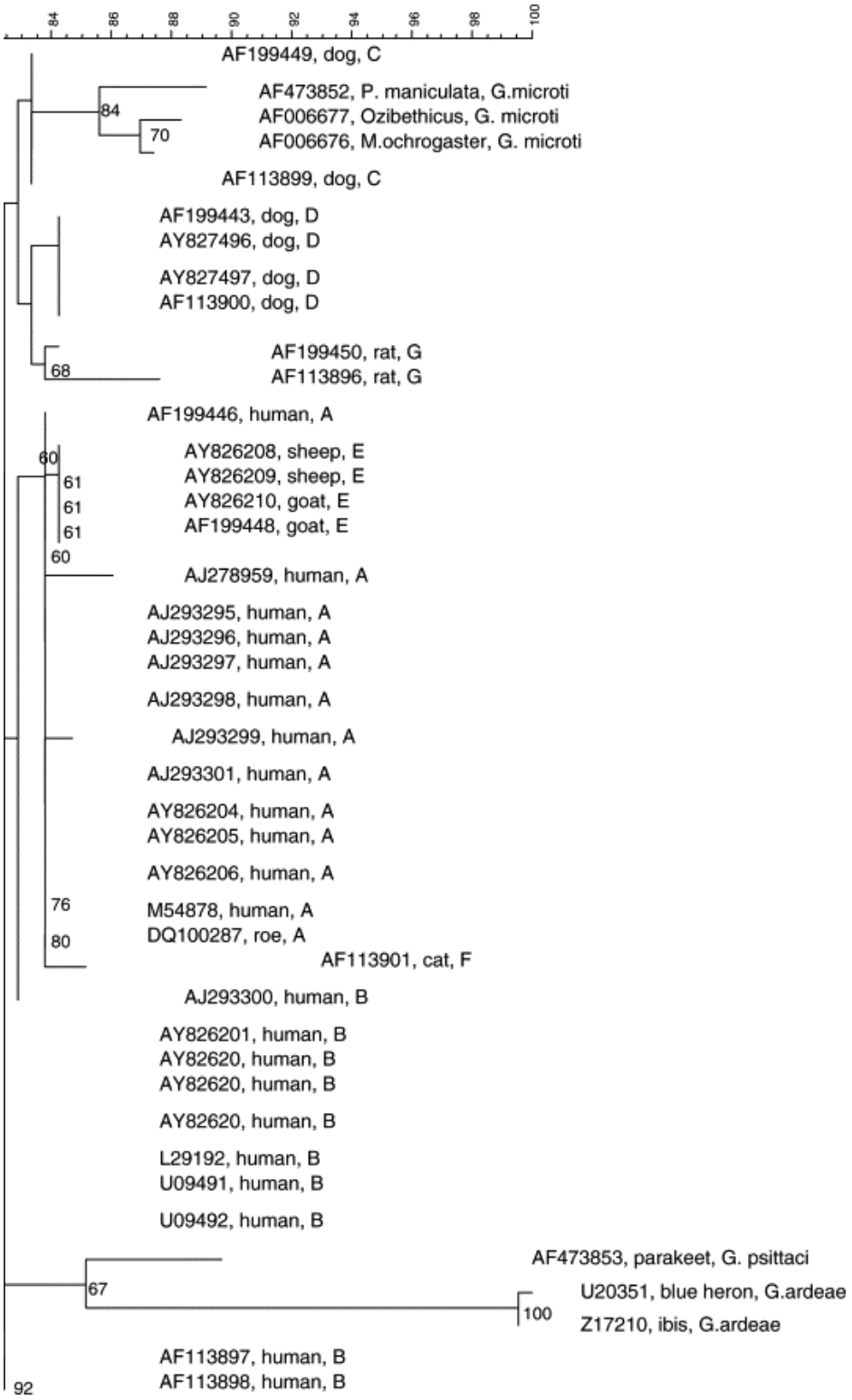

Fig. 1. Dendrogram of the phylogenetic analysis by maximum parsimony of the 18S rDNA *Giardia* sequences of the Dutch human clinical and animal isolates determined in this study (accession numbers starting with AY82 and DQ) compared with sequences taken from GenBank (Table 1A, Table 1B and Table 1C). Percentage bootstrap support (>60%) from 1000 replicate samples is indicated at the right of the supported node.

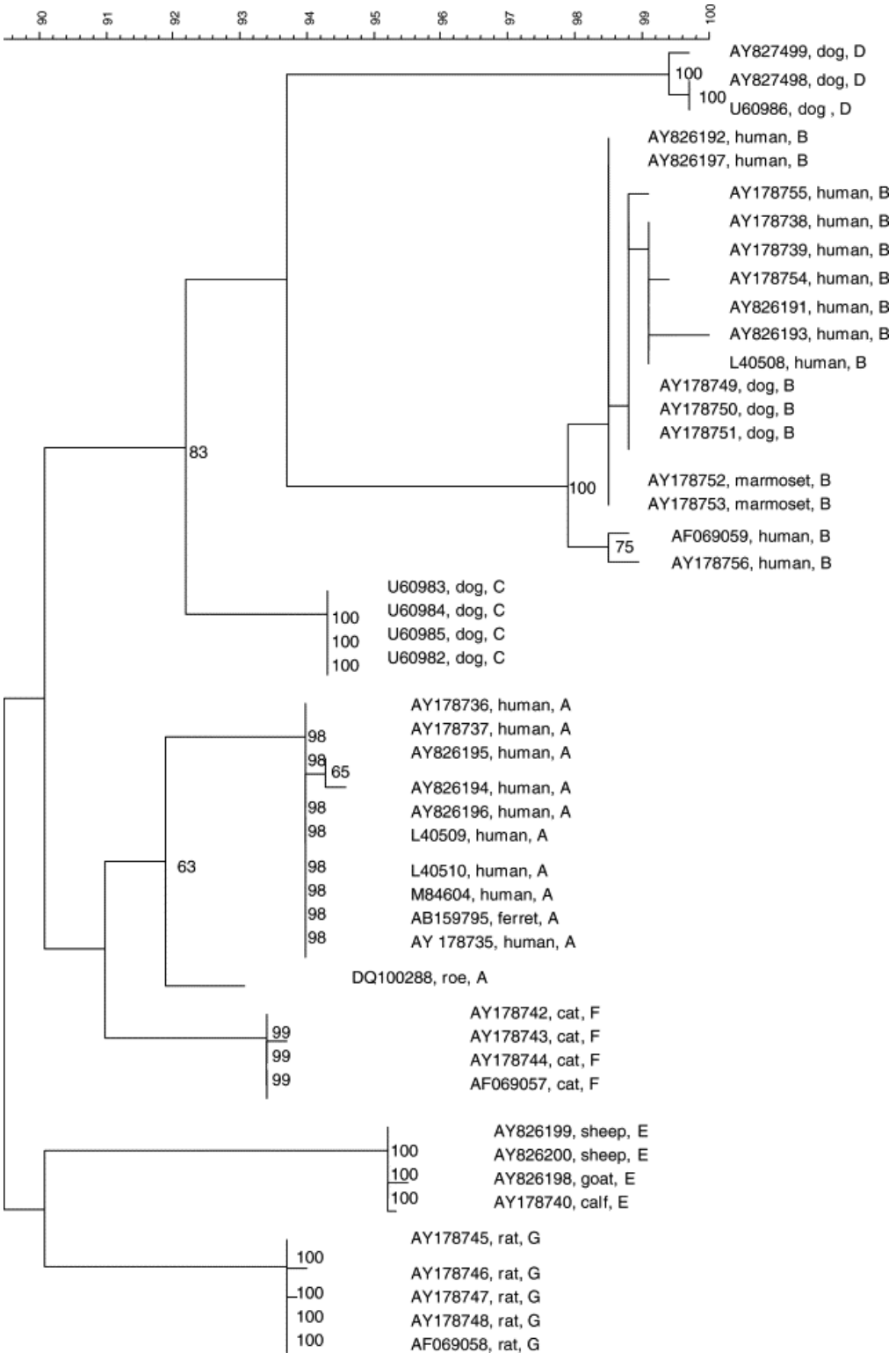

Fig. 2. Dendrogram of the phylogenetic analysis by maximum parsimony of the *gdh* sequences from *Giardia* of three different human clinical and the animal isolates determined in this study (accession numbers starting with AY82 and DQ) compared with sequences taken from GenBank (Table 1A, Table 1B and Table 1C). Percentage bootstrap support (>60%) from 1000 replicate samples is indicated at the right of the supported node.

Next, we performed a phylogenetic analysis of the combined 18S rDNA and *gdh* DNA sequences (Fig. 3), including only sequences from isolates that had both 18S rDNA and *gdh* GenBank sequence data (Table 1A, Table 1B and Table 1C). This resulted in a similar division of branches: two branches of human isolates typed as A and B assemblages clearly separated from each other, a branch containing goat and sheep isolates typed as Assemblage E, two dogs branches with the Assemblage D separate from the Assemblage C, a rat branch of the Assemblage G type and a cat branch of the Assemblage F type (Fig. 2). For the branch containing the human Assemblage B isolates, the subdivision in groups III, IV and IV-like was not as detailed compared with the *gdh*-based tree.

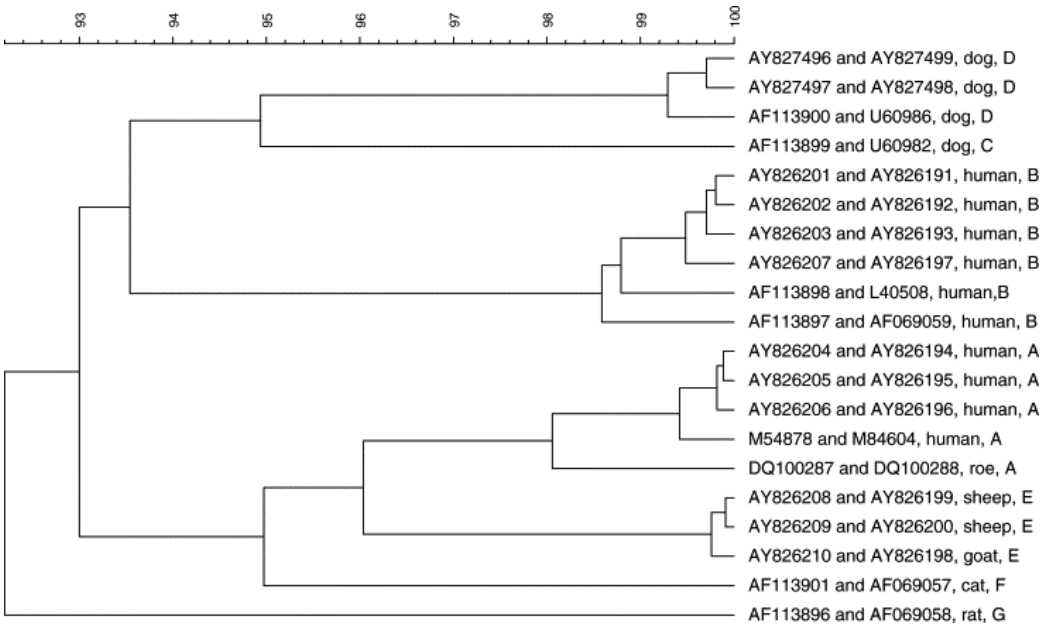

Fig. 3. Dendrogram of the multilocus phylogenetic analysis of the *Giardia* sequences for *gdh* and the 18S rDNA sequences from individual isolates as determined in this study and taken from GenBank Dendrogram is constructed using the unweighted pair group method with arithmetic mean. For the multilocus analysis, no bootstrap support could be calculated.

4. Discussion

In the current study, we genotyped 100 different *Giardia* DNA samples from human stool samples in the Netherlands. Genotypic analysis showed that the *Giardia* samples could be divided into two major groups or Assemblages, A (35%) and B (65%), similar to those described before (Monis et al., 1999 and Homan and Mank, 2001). Furthermore, we compared sequences from the 18S rDNA and *gdh* genes from *Giardia* isolates from one geographic region (The Netherlands) and a large set of DNA sequences available from the literature worldwide on these genes and performed a joint phylogenetic analysis for these 18S rDNA and *gdh* sequences. In accordance with other references, we only isolated Assemblages A and B from humans in the Netherlands. In these human samples, no other *Giardia* species or other *G. duodenalis* assemblages were identified, indicating that only these assemblages are able to infect humans in the Netherlands. Although only a few *Giardia* isolates originating from Dutch animals were analysed in this study, a large set of DNA sequences are available in GenBank from animals and humans and so these were included in this study. We confirm the findings of others that 18S rDNA sequences are useful to identify different *Giardia* species, however, they do not assist in the assemblage's classification for *G. duodenalis*. Genotyping by *gdh*-derived DNA sequences provides more detailed information within the different *G. duodenalis* assemblages. As more and more DNA sequences become available, the general picture that emerges is that each host species has its own host-specific *Giardia* assemblage or species but that some *Giardia* assemblages or species are found in more than one host. Although the typing results from different laboratories seem to agree, the

taxonomy is still poorly resolved. Using *gdh* sequences in phylogenetic analysis gives more detailed information than the Assemblages A–F; however, it should be noted that this detailed information for our Assemblage B based on less than 2% difference, might be too limited to make a significant differentiation between sub-clusters.

The question to which extent *Giardia* species are zoonotic is still unclear and under debate. An increasing number of *Giardia* animal-specific assemblages are being identified in phylogenetic comparisons: Assemblage C and D in dogs, F in cats, E in cattle, sheep and goats and G in rats. Thus far, in humans only *Giardia* species of the Assemblages A and B have been identified. The Assemblage B is found also in *Giardia* samples isolated from different animal species (Fig. 1 and Fig. 2; Table 1C). In the phylogenetic tree of *gdh* sequences the human and animals isolates of Assemblage B cluster together, with their closest neighbour the dog isolates from the Assemblage C, but far distant from the human samples of the Assemblage A. With one exception of the ferret Assemblage A, all human isolates typed as Assemblage A cluster together for the *gdh* sequences analysis, with the nearest neighbour being the cat (Assemblage F) (Fig. 2). The reason that we could find only minor differences in the human isolates of Assemblage A, whereas Monis et al. (1999) described two groups within this assemblage (AI and AII), might be due to the fact that we only analysed a 399 bp *gdh*-derived PCR fragment, whereas Monis and colleagues analysed a 1.17 kb fragment. In addition, it might be due to the fact that we only included human patients with clinical symptoms of diarrhoea in our study. For reasons of comparison, it might therefore, be interesting to study *Giardia* assemblages from asymptomatic patients. The majority of *G. duodenalis* in livestock belongs to Assemblage E and a minority to Assemblage A (Ey et al., 1997, Hunt et al., 2000, Becher et al., 2004 and Read et al., 2004; Appelbee et al, 2005; Giangaspero et al., 2005). However, in most studies the molecular identification was based on A- and B-specific PCR and not on analysing the *gdh*-derived DNA sequence product. Trout et al. (2004) described the presence of Assemblage A in cattle as determined by DNA sequence analysis of 18S rDNA (292 bp), TPI (500 bp) and beta-giardin (750 bp) derived PCR fragments. Assemblage A was detected on 15% of all farms, with varying levels ranging from 8 to 45%, concluding that calves might be a source of *G. duodenalis* infections in humans. Remarkably, we identified only one animal (roe deer) *gdh* DNA sequence and no other hoofed animal Assemblage A-specific *gdh* sequences are yet available in GenBank. The roe deer parasite *gdh* sequence, however, did not cluster with the human isolates typed as Assemblage A, indicating that hoofed and human *Giardia* Assemblage A might be different. Thus far, only one animal *gdh* DNA sequence typed as Assemblage A has been reported in GenBank, a ferret isolate from Japan (Table 1C). More *gdh* sequence data might be helpful in providing additional information to study the zoonotic potential of *G. duodenalis* Assemblage A and it would be interesting to analyse more of these *gdh*-derived Assemblage A DNA sequences of hoofed animals for the possibility of finding more Assemblage A in this group. Furthermore, other loci like non-coding sequences and microsatellites, which tend to have a higher mutation rate, may help in finding polymorphisms to make a more significant further subdivision of *Giardia* and elucidate the question of zoonotic potential for *Giardia.* This might also give us better tools to study outbreak investigations, transmission routes and eventually to settle the issue of zoonotic transmission.

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