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New Primers for Fast Detection of *Giardia duodenalis* Assemblages A and B Using Real-time PCR

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Abstract. *Giardia duodenalis* is one of the six *Giardia* species and it is the most common, cosmopolitan flagellate that infects humans and many species of animals. This species exhibits considerable genetic diversity; to date, eight assemblages (A–H) have been defined. These assemblages differ in host specificity: assemblages A and B have been found in both humans and in many animal species. Mixed infections with *Giardia* (A and B) assemblages have been reported in humans and in animals. Many molecular techniques are effective and rapid for the detection of *G. duodenalis* and also for the determination of genetic variability of isolates in clinical and environmental samples. In this context, the aim of this study was to design new assemblage-specific primers for rapid detection and identification of *G. duodenalis* assemblages A and B and both of these assemblages simultaneously using quantitative real-time polymerase chain reaction (qPCR). Fragments of glutamate dehydrogenase and triose phosphate isomerase were used as targets in the design of primers. In conclusion, the use of *G. duodenalis* assemblage-specific primers designed in this study allows quick identification of human infectious *G. duodenalis* assemblages A and B as well as mixed AB assemblages in a sample without further sequencing of the amplification products, which reduces the cost of study and the waiting time for the results.

Key words: *Giardia duodenalis*, real-time PCR, specific primers, genotyping, zoonoses

INTRODUCTION

Giardia duodenalis (syns. *G. intestinalis*, *G. lamblia*) is one of the six *Giardia* species and it is the most common, cosmopolitan flagellate that infects humans and numerous animal species. This species exhibits considerable genetic diversity; to date, eight assemblages (A–H) have been defined (Ryan and Cacciò 2013). These assemblages differ in host specificity: assemblages A and B have been found in both humans and in many animal species, particularly in mammals and birds, whereas the remaining assemblages (C–H) are more host-adapted (Sprong *et al*. 2009, Heyworth 2016). *G. duodenalis* assemblages A (except sub-assemblage AIII) and B are responsible for approximately

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99% of human giardiasis cases worldwide (Sprong *et al*. 2009). Mixed infections with the *Giardia* (A and B) assemblages have been reported in humans and in animals (Amar *et al*. 2002, Feng and Xiao 2011, Pallant *et al*. 2015). Considerable genetic variability among *G. duodenalis* isolates obtained from different hosts and from a variety of geographic regions largely complicates the determination of the role of animals as a source of human infection (Sprong *et al*. 2009). Therefore, in view of public health and control of zoonotic giardiasis, rapid identification of *Giardia* assemblages occurring in pets is very important. Since not all species or assemblages or even sub-assemblages are able to infect humans, it is necessary to use fast and reliable molecular techniques to identify the genotype and/or the sub-genotype of *G. duodenalis* in faecal specimens from humans, animals and environmental samples. Nowadays, many molecular techniques are effective and rapid for the detection of *G. duodenalis* as well as for the determination of genetic variability of isolates in clinical and environmental samples (Guy *et al*. 2003, Zhang *et al*. 2012, Gotfred-Rasmussen *et al*. 2016). Real-time PCR is a rapid, very sensitive, quantitative technique for detecting many pathogens including *G. duodenalis* (Verweij *et al*. 2003, McGlade *et al*. 2003, Guy *et al*. 2004, Schuurman *et al*. 2007, Almeida *et al*. 2010, Feng and Xiao 2011, Prasetbun *et al*. 2012, Zhang *et al*. 2013, Alonso *et al*. 2014, Gizzi *et al*. 2014).

Dogs can be infected with *Giardia* canid-specific genotypes C and D and/or with zoonotic genotypes belonging to assemblages A and B. Moreover, mixed infection with *Giardia* A–D genotypes in dogs has been documented (Xiao *et al*., 2008). Cats may be infected by *G. duodenalis* cat-specific assemblage F and may harbour other assemblages such as A, B and D (Xiao *et al*. 2008).

In this context, the aims of this study were (i) to design new assemblage-specific primers for rapid detection and identification of *G. duodenalis* A and B assemblages, including both of them simultaneously, using quantitative real-time polymerase chain reaction PCR (qPCR), (ii) to apply qPCR and the new assemblage-specific primers for the detection of DNAs of the two major *G. duodenalis* assemblages A and B in human and pet stool specimens.

MATERIALS AND METHODS

Primer designation

Five new specific primer pairs for a fragment of references genes (*gdh* – glutamate dehydrogenase and *tpi* – triose phosphate isomerase) were designed in oligo 6.71 software. Assemblage-specific primers were designed for the detection of *G. duodenalis* zoonotic assemblages A and/or B in faecal samples. The sequences and their GenBank accession numbers are as follows for *gdh*: (EF685702 – A); (AB618784 – B); (EF507621 – C); (EF507614 – D); (EF507644 – E); (EF507592 – F); (AY178748 – G); (GU176101 – H) and for *tpi* (EF688042 – A); (KM926543 – B); (KP258397 – C); (DQ246216 – D); (EU272164 – E); (KM977655 – F); (JX571040 – G). Sequences of these primers are shown in Table I.

Faecal samples and *G. duodenalis* DNA

Thirteen DNA templates extracted from different *G. duodenalis* isolates belonging to various assemblages were used to determine the specificity of new primers (Table II). Methods of DNA isolation and assemblages establishment have been described previously (Solarczyk and Majewska 2010, Solarczyk *et al*. 2010, Solarczyk *et al*. 2012, Solarczyk *et al*. 2014, Stojecki *et al*. 2015, Solarczyk *et al*. 2016). Moreover, a total of 131 DNA probes isolated from faecal samples obtained from various hosts were also included in the study. Stool specimens were derived from five humans and 126 pets, including 79 dogs and 47 cats. All samples were collected in Poland from two localities – Poznan (n = 25) and Warsaw (n = 106). Most of the specimens (n = 106) originating from pets were obtained from a shelter and the rest of the samples (n = 20) were collected from privately owned dogs and cat. All stool samples were examined by microscopy for the detection of *Giardia.* Samples were preserved in 2.5% potassium dichromate solution and stored at 4°C until laboratory analysis.

DNA isolation, and real-time PCR analysis

Total genomic DNA was directly extracted from each faecal specimen using the QIAamp Fast DNA Stool Mini Kit (Hilden Germany) according to the kit instructions.

Fast detection of different genotypes of *G. duodenalis* (assemblages A and B) were measured by qPCR using the Light Cycler real-time PCR detection system from Roche Diagnostic GmbH (Mannheim, Germany), SYBR Green I as detection dye, and target DNA. For amplification, 1 µl of total (10 µl) DNA solution was added to 5 µl of LightCycler 480 DNA SYBR Green I Master (Roche) as well as assemblage-specific primers. All reactions were performed in triplicate. The qPCR cycling conditions for *gdh* gene primers were 10 min at 95°C and 45 cycles of 95°C for 8 s, 66°C for 8 s and 72°C for 8 s. The following cycling conditions were used for *tpi* gene primers: 10 min at 95°C, 45 cycles of 95°C for 8 s, 56°C for 8 s and 72°C for 8 s. qPCR data were collected and analyzed using the Excel program (Microsoft Office). Amplified DNA together with 100 bp DNA Ladder (Novazym, Poland) were electrophoresed on a 2% agarose gel in TAE buffer (2 M Tris, 0.05 M EDTA, 5.7% glacial acetic acid) for 90 min at 50 V, stained with a solution of 0.5 mg per ml of ethidium bromide, and visualized under UV light.

Table I. Primer sequences for A and B assemblages of *G. duodenalis*.

Assemblages	Gene/primer name	Sequences	Size of product	Annealing temp.	Cycles
AB	*gdh* GuniAB-F GuniAB-R	F: 5′GCTGTCTACTTCCTGGAGG3′ R: 5′ATGAGGTGGGCCAGCTTCT3′	206 bp	66	45
A	*gdh* GspecA-F GuniAB-R	F: 5′CGTCCTTCTTTCTGGCTCC3′ R: 5′ATGAGGTGGGCCAGCTTCT3′	147 bp	66	45
B	*gdh* GspecB-F GuniAB-R	F: 5′TCCTCTCTGGCTCTGGCAA3′ R: 5′ATGAGGTGGGCCAGCTTCT3′	142 bp	66	45
A	*tpi* Giar.A-F Giar.A-R	F: 5′CGTCGTCATTGCCCCTTCC3′ R: 5′GCTTTGCTCGTCGGTCTCC3′	217 bp	56	45
B	*tpi* Giar.B-F Giar.B-R	F: 5′CGTTGTTGTTGCTCCCTCC3′ R: ′TTCGCCTTCTTAGCACTCTG3′	231 bp	56	45

Table II. Different genotypes of *G. duodenalis* used in the study.

Number	Origin/host	Assemblage/sub-genotype	Accession number	Reference
1	Human	A	KT731989	Stojecki *et al*. 2015
2	Sheep	A	KT732004	Stojecki *et al*. 2015
3 4	Cattle	A	KT731980; KT731978	Stojecki *et al*. 2015
5	Red deer	AIII	EU621373	Solarczyk *et al*. 2012
6	Human	B	FJ009207	Solarczyk *et al*. 2010
7	Pig	B	KT731994	Stojecki *et al*. 2015
8	Thomson's gazelle	B	EU626199	Solarczyk *et al*. 2014
9	Raccoon dog	D	HQ538709	Solarczyk *et al*. 2016
10	Dog	D	FJ009206	Solarczyk and Majewska 2010
11 12	Sheep	E	KT732003; KT732001	Stojecki *et al*. 2015
13	Pig	E	KT731992	Stojecki *et al*. 2015

RESULTS AND DISCUSSION

The specificity of the primers was verified by performing qPCR using genomic DNA of *G. duodenalis* isolates belonging to the assemblages A, B, D and E. A summary of the qPCR results is presented in Table III. Correct amplicons were obtained only when primer pairs designed to detect the DNA of *G. duodenalis* from genotypes A or B were used. The GuniAB-F/GuniAB-R, GspecB-F/GuniAB-R and GiarB-F/GiarB-R primers were successfully used to amplify the DNA from parasite isolates belonging to assemblage B. On the other hand, the GuniAB-F/GuniAB-R, GspecA-F/GuniAB-R and GiarA-F/GiarA-R primers only amplified the DNA of *G. duodenalis* assemblage A, also including the DNA of a *Giardia* isolate from red deer belonging to sub-assemblage AIII. Since *G. duodenalis* isolates belonging to sub-assemblage AIII have never been identified in humans so far (Sprong *et al*. 2009), it is necessary to find significant sequence differences of the examined target genes that allow parasite isolates to be distinguished at the sub-assemblage level.

Table III. Specificity of primers for detection of *G. duodenalis* using qPCR.

DNA source	qPCR result				
Specific primers/(assemblages)	*Gdh* GspecA-F GuniAB-R (A)	*Gdh* GspecB-F GuniAB-R (B)	*Gdh* GuniAB-F GuniAB-R (AB)	*Tpi* Giar.A-F Giar.A-R (A)	*Tpi* Giar.B-F Giar.B-R (B)
Negative control (sterile water)	–	–	–	–	–
			Assemblage A		
Human (KT731989)	+	–	+	+	–
Sheep (KT732004)	+	–	+	+	–
Cattle (KT731980)	+	–	+	+	–
Cattle (KT731978)	+	–	+	+	–
Red deer (EU621373)	+	–	+	+	–
			Assemblage B		
Human (FJ009207)	–	+	+	–	+
Pig (KT731994)	–	+	+	–	+
Thomson's gazelle (EU626199)	–	+	+	–	+
			Assemblage D		
Raccoon dog (HQ538709)	–	–	–	–	–
Dog (FJ009206)	–	–	–	–	–
			Assemblage E		
Sheep (KT732003)	–	–	–	–	–
Sheep (KT732001)	–	–	–	–	–
Pig (KT731992)	–	–	–	–	–

+ detected, – not detected

Table IV. *G. duodenalis* assemblages in tested stool samples.

No.	Source	Microscopy analysis	qPCR				
				GDH		TPI	
			AB	A	B	A	B
1	Human	–	+	+	–	+	–
2	Privately owned dog	+	+	–	+	–	+
3	Privately owned dog	–	+	–	+	–	+
4	Privately owned dog	–	+	–	+	–	+
5	Dog kept in shelter	–	+	–	+	–	+
6	Dog kept in shelter	–	+	–	+	–	+
7	Privately owned cat	–	+	–	+	–	+
8	Privately owned cat	+	+	–	+	–	+

+ detected, – not detected

Furthermore, the GuniAB-F/GuniAB-R primers also amplified the DNA of *G. duodenalis* assemblages A and B and both of them simultaneously.

Nevertheless, the designed primers proved to be specific because they did not cross-amplify *Giardia* DNA belonging to assemblages D and E. This indicates the

proper choice of the primer sequence fragments of two molecular markers (*tpi* and *gdh*), since the primer pairs used in this study did not match the genomic homologous sequence of isolates belonging to other *G. duodenalis* groups of genotypes. The high genetic heterogeneity among *G. duodenalis* isolates obtained from different hosts and geographic regions complicates the determination of the role of animals as a source of human infection (Caccio and Ryan, 2008; Sprong *et al*. 2009). The vast majority of human giardiasis cases are caused by *G. duodenalis* isolates belonging to assemblages A and B, which infect animals as well (Monis *et al*. 2009, Jerlstrom-Hultqvist *et al*. 2010, Feng and Xiao 2011, Vanni *et al*. 2012, Xu *et al*. 2012). Therefore, assemblage-specific primers would be very useful for rapid identification of human-infectious genotypes of *G. duodenalis* in animals and environmental samples.

This study provides useful results concerning detection of zoonotic *G. duodenalis* isolates in faecal samples from dogs, cats and human. Of the 131 stool specimens examined by microscopy only two were positive for *Giardia.* In the *Giardia*-positive faecal samples a few cysts per slide were detected. While using qPCR and the new assemblage-specific primers in eight out of 131 tested specimens *Giardia* DNA was found (Table IV). All positive probes in real-time PCR were confirmed in two molecular markers, TPI and GDH. No mixed infections were found in analyzed stool samples since using assemblage-specific primers in qPCR *G. duodenalis* assemblage A was identified in human while assemblage B was identified in five dogs and two cats (Table IV). However, usage of primers (GuniAB-F/Guni AB-R) that allow for simultaneously detection of both A and B assemblages of *G. duodenalis* can be useful for fast screening of human infectious *Giardia* in animal and environmental samples.

The qPCR technique has been developed to provide information on the *Giardia* species/assemblages. The results of our study demonstrated that newly designed assemblage-specific primers for qPCR were able to detect *G. duodenalis* DNA in the stool samples and the primers enabled recognition of zoonotic assemblage B in pets and assemblage A in humans. Moreover, results of the preliminary studies indicate that these new assemblage-specific primers are also very useful for the detection of zoonotic *G. duodenalis* assemblages in water samples (Skrzypczak 2016).

In conclusion, the use of *G. duodenalis* assemblage-specific primers designed in this study allows quick identification of human-infectious *G. duodenalis* assemblages A and B as well as mixed AB assemblages in the sample without further sequencing of the amplification products, which reduces the cost of testing and the waiting time for the results. Therefore, we are of the opinion that a real-time assemblage-specific PCR assay is a useful tool for the screening of animals' faecal specimens and environmental samples in order to detect *G. duodenalis* assemblages A and B and mixed A+B.

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