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ORIGINAL ARTICLE

# Molecular identification of *Giardia duodenalis* isolates from domestic dogs and cats in Wroclaw, Poland

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## Abstract

**Introduction**. *Giardia duodenalis* (*G. intestinalis*) is a common protozoan causing gastrointestinal disorders in many species of mammals. The genus of *Giardia* has high molecular diversity. Dogs and cats, in addition to their typical infection with assemblages C, D and F, may be a reservoir of zoonotic assemblages (A and B).

**Objective.** The aim of this study was a genetic characteristic of *Giardia* isolates of dogs and cats from the area of Wroclaw (Poland).

**Materials and method.** A total of 128 and 33 faecal samples from dogs and cats, respectively, were analyzed by routine coprological methods. The animals were diagnosed on the presence of *G. duodenalis* antigens in faeces soluble with the use of SNAP *Giardia* (IDEXX Laboratories) immunosorbent assay. 27 DNA isolates of *Giardia* were subjected to molecular identification (PCR-RFLP).

**Results and conclusions.** The prevalence of *G. duodenalis* was 21.1% (27/128) in dogs and 15.1% (5/33) in cats. In dogs, C assemblage was present in 18 (81%) positive stool samples, D assemblage in 2 (9%) samples, B assemblage present in one (4.5%), and mixed assemblages (C and D) occurred in one (4.5%) sample. F assemblage was found in 4 (80%) cats' positive stool samples and A assemblage occurred in one case (20%). Confirmation of the presence of A and B zoonotic assemblages suggests that infected pets can be a threat to human health. This study describes for the first time the presence of mixed infections within host-specific C and D assemblages in dogs in Poland.

## Key words

*Giardia duodenalis,* assemblage, dogs, cats, nested-PCR, PCR-RFLP, zoonosis

## INTRODUCTION

*Giardia duodenalis* (*G. intestinalis*, *G. lamblia*) is a widespread protozoan parasitizing in humans and many species of mammals. Invasion is most commonly associated with the occurrence of gastrointestinal signs, though asymptomatic invasions have also been observed [1]. Studies using molecular techniques have shown that genetic diversity is very high within the *G. duodenalis* species. There are 7 basic assemblages, from A – G. The occurrence of A, B, C and D assemblages was confirmed in dogs, and A, B, D and F assemblages found in cats. Due to the occurrence of A and B assemblages in humans, and because of their relevance for zoonotic infections, there is a need for the monitoring of dogs and cats as companion animals that can be a direct source of human infection, as well as a source of environmental contamination [2, 3]. Asymptomatic and chronic course of the disease with frequent periodic expulsion of cysts is observed in older animals. Diagnosis of *Giardia* infection with the use of a faecal flotation or faecal smear is problematic due to the irregular shedding of cysts and their morphology, small size, and their similarity in appearance to many pseudoparasites such as yeast [4]. The use of immunoenzyme assay to detect *Giardia* coproantigens in faeces increases the probability of detecting invasion. Genotypic characterization of *G. duodenalis* is a very useful and essential tool used in epidemiological studies. PCR techniques for genotyping of *G. duodenalis* are based on polymorphic genes encoding 18S rRNA, glutamate dehydrogenase (gdh), triose phosphate isomerase (tpi), and ß-giardin [5, 6]. PCR amplification and RFLP/sequence analysis of all of these genes, with the exception of the 18S rRNA, can differentiate subgenotypes of assemblage A [7]. PCR methods for detection of gdh can provide information on *G. duodenalis* A and B sub-assemblages [8].

## OBJECTIVE

The aim of the study was the genetic characterization of isolates of *Giardia* in dogs and cats in the area of Wroclaw (Poland), which is important in determining the source of

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the invasion in humans and animals in a given area, as well as in assessment of the zoonotic potential of *G. duodenalis.*

## MATERIALS AND METHOD

**Samples collection and qualification.** Faecal samples of dogs and cats (age: 3 weeks – 10 years) from Wroclaw were collected in 2010–2012 and provided by their owners to the Division of Parasitology, Faculty of Veterinary Medicine (Wroclaw). The total number of stool samples was 161 (128 dogs and 33 cats), all of the animals had different gastric symptoms (e.g diarrhea, emaciation, loss of body weight). Faecal samples were stored at -20 °C**.** The animals were diagnosed on the presence of *G. duodenalis* antigens in faeces soluble with the use of SNAP *Giardia* (IDEXX Laboratories) immunosorbent assay. As a result of such qualifications, material for genetic research was obtained from 32 animals (27 dogs and 5 cats). Pets with positive test results ranged in age from 3 weeks – 2 years.

**DNA isolation.** Faecal samples (approximately 3–4 g) from infected animals were examined by the flotation method with the use of saturated NaCl solution. The upper part of the supernatant was harvested, rinsed in distilled $H_2O$, centrifuged at 1,000 rpm for 5 minutes. The resulting pellet was re-suspended in 300 µl of saline. This was a concentrated sample of *Giardia* cysts, which were the basis for DNA isolation using a Genomic Mini AX STOOL kit (DNA-Gdansk, Poland).

**Genotyping and nested PCR.** *Giardia* genotyping was performed based on the polymorphism of a gene fragment coding for β-giardin. Nested PCR technique for DNA amplification was applied. Reaction parameters and primers sequences were described in Lalle et al. (2005) [9]. In the primary PCR reaction forward G7: 5'AAGCCCGAC-GACCTCACCCGCAGTGC3' and reverse G759: 5'GAGGCCGCCCTGGATCTTCGAGACGAC3' primers were used. The reaction mixture consisted of 400 nM of each primer, 1 x reaction buffer for polymerase DNA Delta3, 500 µM of dNTP, 3 mM of $MgCl_2$, 1.25 U (0,05 U/µl) of polymerase DNA Delta3 (DNA-Gdansk, Poland) and 0.5 µl of DNA in a final volume of 25 µl. The amplification was carried out in a MJ Mini thermal cycler (Bio-Rad) using the following conditions: cycle of 96 °C for 5 min; 5 cycles of: initially denaturation for 30 sec at 95 °C, annealing for 30 sec at 55 °C, polymerization for 1 min at 72 °C. The PCR cycle was then carried out for 30 sec at 95 °C, 30 sec at 65 °C and 1 min at 72 °C, for a total of 30 cycles, followed by a final extension for 10 min at 72 °C. The secondary PCR reaction was amplified with forward 511: 5' GAACGAACGAGATCGAGGTCCG '3 and reverse 511: 5' CTCGACGAGCTTCGTGTT 3' primers. The amplification conditions were almost the same as in the first reaction with only 2 differences. The annealing temperature was changed, the first was 50 °C and the second was 55 °C and no final extension was carried out. The amplicons of the first PCR reaction (753 bp) was the matrix of secondary PCR reaction.

Amplicons of second PCR reaction (total volume = 100 µl) were subjected to electrophoresis in 2% agar gel. PCR products with a length of ~511 bp were isolated from the gel and purified using a set of DNA Extraction Kit (Fermentas). DNA concentration was determined by absorbance measurements at 260 nm wavelength. Purified products were digested with restriction enzyme BsuRI/HaeIII (Fermentas) for 2 hours at 37 °C. The required enzyme amount was calculated based on its activity on the lambda phage. Digestion products were electrophoresed in a 5% agarose gel for 2h at a constant 120V. Products were stained with ethidium bromide and photographed with a BioRad GelDoxXR device.

**Identification of genotypes of sequenced DNA.** Selected products of PCR reaction of approximately 511bp were subjected to DNA sequencing by GENOMED S.A. (Poland). The obtained results of sequencing were analyzed by comparing the sequence of individual genotypes of *G. duodenalis* with BLAST NCBI (http://blast.ncbi.nlm.nih.gov) database. The partial sequences of β- giardin gene obtained in this study were compared with sequences deposited in the GenBank database under Accession Nos.: Assemblage A- FJ560591.1; Assemblage B- AY072725.1; Assemblage C- AY545646; Assemblage D- AY545647.1; Assemblage F- AY647264. BioEdit and APE A Plasmid Editor software tools were used for verification of sequence compliance.

## RESULTS

In the study by nested PCR, *Giardia* DNA was detected in 27 of 32 tested stool samples (22 dogs and 5 cats). In 4 cats, the presence of assemblage F was revealed and in 1 cat it was assemblage A. Of the 22 dogs' stool samples, assemblage C was detected in 18 cases, assemblage D in 2 cases, assemblage B in 1 case.

Selected products of nested PCR reaction (~511bp) were sequenced and analyzed using the GenBank database. Analysis of the sequence results in cases where one assemblage was identified clearly confirmed the results obtained from RFLP. Figure 1 shows selected results of sequencing which, in turn, helped to identify the assemblages: A (Fig. 1A), B (Fig. 1B), C (Fig. 1C), D (Fig. 1D), F (Fig. 1E). There were no specific products for any of the previously identified assemblages in electrophoresis of 1 sample derived from a dog after digestion with HaeIII enzyme. The sense strand sequencing results of a β-gardin gene indicated the presence of assemblage C (Fig. 2A). However, sequencing of the antisense strand showed the presence of assemblage C or D, giving an appropriate sequence homology at the level of 0.988 and 0.949. In the analyzed antisense strand stool sample, the cut places were present at 194, 296, 311bp, while characteristic cut places for assemblage C were present at 194, 296, 311 and 461bp (Fig. 2B) and for assemblage D only in 194 and 311bp (Fig. 2C). Therefore, it is presumed that in this stool sample from dog we are dealing with a mixed infection of assemblages C and D, this observation was confirmed by the results of electrophoretic analysis of nested PCR product digested by BsuRI enzyme (data not shown).

In 5 dogs, despite the positive test of SNAP *Giardia* (IDEXX Laboratories), the PCR product was not detected. The nested PCR repeated one more time with the matrix volume increased to 2–4 µl in 25µl of reaction mixture and with the revised polymerase, did not produce satisfactory results. Re-isolation of DNA from thawed samples also did not allow DNA product to be obtained.

**Figure 1 A–E.** Multiple alignment of the *G. duodenalis* assemblage of β-giardin genes in nested PCR. Alignment based on maximum similarity of individual assemblages of *G. duodenalis* in the classification of Lalle et al. 2005. The underlined zone indicates a cut place of restrictive enzyme. The grey zone indicates differences in DNA sequence (no consensus at this position)

A) Assemblage A- Accession number **FJ560591.1**

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                 10         20         30         40         50
Assemblage A     GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTGAAGATGA
Cat No1          GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTGAAGATGA
                 60         70         80         90         100
Assemblage A     TCAAGGACGC CATCGCACAC CTCGACAGGC TCATCCAGAC GGAGTCGAGG
Cat No1          TCAAGGACGC CATCGCACAC CTCGACAGGC TCATCCAGAC GGAGTCGAGG
                 110        120        130        140        150
Assemblage A     AAGCGCCAGG CCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
Cat No1          AAGCGCCAGG CCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
                 160        170        180        190        200
Assemblage A     CGACAACATG TACCTAACGA TCAAGGAGGA GATCGACACC ATGGCTGCAA
Cat No1          CGACAACATG TACCTAACGA TCAAGGAGGA GATCGACACC ATGGCTGCAA
                 210        220        230        240        250
Assemblage A     ACTTCCGCAA GTCCCTTGCG GAGATGGGCG ACACACTCAA CAACGTTGAG
Cat No1          ACTTCCGCAA GTCCCTTGCG GAGATGGGCG ACACACTCAA CAACGTTGAG
                 260        270        280        290        300
Assemblage A     ACAAATCTCC AGAACCAGAT CGCCATCCAT AACGACGCCA TCGCGGCTCT
Cat No1          ACAAATCTCC AGAACCAGAT CGCCATCCAT AACGACGCCA TCGCGGCTCT
                 310        320        330        340        350
Assemblage A     CAGGAAGGAG GCCCTCAAGA GCCTGAACGA TCTCGAGACG GGCATTGCCA
Cat No1          CAGGAAGGAG GCCCTCAAGA GCTTGAACGA CCTCGAGACG GGCATTGCCA
                 360        370        380        390        400
Assemblage A     CGGAGAACGC AGAAAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
Cat No1          CGGAGAACGC AGAAAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
                 410        420        430        440        450
Assemblage A     GCAGAGGGCT TCGCCCGCAT CTCCGCCGCG ATCGAGAAGG AGACGATCGC
Cat No1          GCAGAGGGCT TCGCCCGCAT CTCCGCCGCG ATCGAGAAGG AGACGATCGC
                 460        470        480        490        500
Assemblage A     CCGCGAGAGG GCCGTTAGCG CTGCCACGAC AGAAGCGCTC ACAAACACGA
Cat No1          CCGCGAGAGG GCCGTTAGTG CTGCCACGAC AGAAGCGCTC ACAAACACGA
                 510
Assemblage A     AGCTCGTCGA G
Cat No1          AGCTCGTCGA G
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B) Assemblage B- Accession number **AY072725.1**

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                 10         20         30         40         50
Assemblage B     GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGT GTGAAGATGA
Dog No1          GAACGAGATC GAGGTCCGCC GCGTCGAGGA CGACACGCGT GTGAAGATGA
                 60         70         80         90         100
Assemblage B     TCAAGGACGC CATCGCGCAC CTTGACAGAC TCATCCAGAC AGAGTCGAGG
Dog No1          TCAAGGACGC CATCGCGCAC CTCGACAGAC TCATCCAGAC AGAGTCGAGG
                 110        120        130        140        150
Assemblage B     AAGCGCCAGG CCTCGTTCGA GGACATCCGC GAGGAAGTCA AGAAGTCTGC
Dog No1          AAGCGCCAGG CCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCTGC
                 160        170        180        190        200
Assemblage B     CGACAACATG TACCTGACGA TCAAGGAGGA GATCGACACT ATGGCCGCAA
Dog No1          CGACAACATG TACCTGACGA TCAAGGAGGA GATCGACACC ATGGCCGCAA
                 210        220        230        240        250
Assemblage B     ACTTCCGCAA GTCTCTTGCT GAGATGGGCG ACACGCTCAA CAACGTCGAG
Dog No1          ACTTCCGCAA GTCTCTTGCT GAGATGGGCG ACACGCTCAA CAACGTCGAG
                 260        270        280        290        300
Assemblage B     ACGAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCAGCCCT
Dog No1          ACGAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCAGCCCT
                 310        320        330        340        350
Assemblage B     TAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACA GGCATCGCCA
Dog No1          CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACA GGCATCGCCA
                 360        370        380        390        400
Assemblage B     CGGAGAACGC CGAGAGGAAG AAGATGTATG ACCAGCTCAA CGAGAAAGTC
Dog No1          CGGAGAACGC CGAGAGGAAG AAGATGTATG ACCAGCTCAA CGAGAAAGTC
                 410        420        430        440        450
Assemblage B     GCAGAGGGCT TCGCCCGCAT CTCCGCTGCC ATCGAGAAGG AGACGATCGC
Dog No1          GCAGAGGGCT TCGCCCGCAT CTCCGCTGCC ATCGAGAAGG AGACGATCGC
                 460        470        480        490        500
Assemblage B     CCGCGAGAGG GCCGTCAGCG CCGCCACGAC AGAGGCCCTC ACAAACACGA
Dog No1          CCGCGAGAGG GCCGTCAGCG CCGCCACGAC AGAGGCCCTC ACAAACACGA
                 510
Assemblage B     AGCTCGTCGA G
Dog No1          AGCTCGTCGA G
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C) Assemblage C- Accession number **AY545646**

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                 10         20         30         40         50
Assemblage C     GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTCAAGATGA
Dog No2          GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTCAAGATGA
                 60         70         80         90         100
Assemblage C     TCAAGGACGC CATCGCTCAC CTGGACAGGC TCATCCAGAC CGAGTCGAGG
Dog No2          TCAAGGACGC CATCGCTCAC CTGGACAGGC TCATCCAGAC CGAGTCGAGG
                 110        120        130        140        150
Assemblage C     AAGCGCCAGG GCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
Dog No2          AAGCGCCAGG GCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
                 160        170        180        190        200
Assemblage C     CGACAACATG TACCTGACGA TCAAGGAGGA AATCGACACC ATGGCCGCGA
Dog No2          CGACAACATG TACCTGACGA TCAAGGAGGA AATCGACACC ATGGCCGCGA
                 210        220        230        240        250
Assemblage C     ACTTCCGCAA GTCCCTCGCC GAGATGGGCG AGACCCTCAA CAACGTCGAG
Dog No2          ACTTCCGCAA GTCCCTCGCC GAGATGGGCG AGACCCTCAA CAACGTCGAG
                 260        270        280        290        300
Assemblage C     ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
Dog No2          ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
                 310        320        330        340        350
Assemblage C     CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACC GGCATCGCCA
Dog No2          CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACC GGCATCGCCA
                 360        370        380        390        400
Assemblage C     CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
Dog No2          CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
                 410        420        430        440        450
Assemblage C     GCAGAGGGAT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
Dog No2          GCAGAGGGAT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
                 460        470        480        490        500
Assemblage C     CCGCGAGAGG GCCGTCAGCG CAGCCACGAC CGAGGCGCTC ACAAACACGA
Dog No2          TCGCGAGAGG GCCGTCAGCG CAGCCACGAC CGAGGCGCTC ACAAACACGA
                 510
Assemblage C     AGCTCGTCGA G
Dog No2          AGCTCGTCGA G
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D) Assemblage D- Accession number **AY545647.1**

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                 10         20         30         40         50
Assemblage D     GAACGAGATC GAGGTCCGCC GCGTCGACGA TGACACGCGT GTCAAGATGA
Dog No3          GAACGAGATC GAGGTCCGCC GCGTCGACGA TGACACGCGT GTCAAGATGA
                 60         70         80         90         100
Assemblage D     TCAAGGATGC CATCGCACAC CTTGACAGGC TCATTCAGAC GGAGTCGAGG
Dog No3          TCAAGGATGC CATCGCACAC CTTGACAGGC TCATTCAGAC GGAGTCGAGG
                 110        120        130        140        150
Assemblage D     AAGCGCCAAA GCTCCTTCGA GGACATCCGC GAGGAGGTAA AGAAGTCCGC
Dog No3          AAGCGCCAGA GCTCCTTCGA GGACATCCGC GAGGAGGTAA AGAAGTCCGC
                 160        170        180        190        200
Assemblage D     TGACAACATG TATCTGACGA TCAAGGAGGA GATTGACACA ATGGCCGCAA
Dog No3          TGACAACATG TATCTGACGA TCAAGGAGGA GATTGACACA ATGGCCGCAA
                 210        220        230        240        250
Assemblage D     ACTTCCGCAA GTCCCTCGCA GAGATGGGCG AGACGCTCAA CAACGTCGAG
Dog No3          ACTTCCGCAA GTCCCTCGCA GAGATGGGCG AGACGCTCAA CAACGTCGAG
                 260        270        280        290        300
Assemblage D     ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCAGCTCT
Dog No3          ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCAGCTCT
                 310        320        330        340        350
Assemblage D     CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTTGAGACC GGCATCGCTA
Dog No3          CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTTGAGACC GGCATCGCTA
                 360        370        380        390        400
Assemblage D     CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
Dog No3          CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
                 410        420        430        440        450
Assemblage D     GCAGAGGGAT TCGCCCGTAT TTCCGCTGCC ATCGAGAAGG AGACGATCGC
Dog No3          GCAGAGGGAT TCGCCCGTAT TTCCGCTGCC ATCGAGAAGG AGACGATCGC
                 460        470        480        490        500
Assemblage D     CCGCGAGAGA GCCGTCAGCG CAGCCACAAC AGAGGCTCTC ACAAACACGA
Dog No3          CCGCGAGAGA GCCGTCAGCG CAGCCACAAC AGAGGCTCTC ACAAACACGA
                 510
Assemblage D     AGCTCGTCGA G
Dog No3          AGCTCGTCGA G
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E) Assemblage F- Accession number **AY647264**

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       10        20        30        40        50
Assemblage F    GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTGAAGATGA
Cat No2         GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTGAAGATGA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                      60        70        80        90       100
Assemblage F    TCAAGGACGC CATCGCGCAC CTCGACAGGC TCATCCAGAC GGAGTCGAGG
Cat No2         TCAAGGACGC CATCGCGCAC CTCGACAGGC TCATCCAGAC GGAGTCGAGG
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     110       120       130       140       150
Assemblage F    AAGCGCCAGG CCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
Cat No2         AAGCGCCAGG CCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     160       170       180       190       200
Assemblage F    CGACAACATG TACCTGACGA TCAAGGAGGA GATCGACACC ATGGCAGCCA
Cat No2         CGACAACATG TACCTGACGA TCAAGGAGGA GATCGACACC ATGGCAGCCA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     210       220       230       240       250
Assemblage F    ACTTCCGCAA GTCCCTTGCA GAGATGGGCG ACACACTCAA CAATGTTGAG
Cat No2         ACTTCCGCAA GTCCCTTGCA GAGATGGGCG ACACACTCAA CAATGTTGAG
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     260       270       280       290       300
Assemblage F    ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
Cat No2         ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     310       320       330       340       350
Assemblage F    CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACG GGCATCGCGA
Cat No2         CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACG GGCATCGCGA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     360       370       380       390       400
Assemblage F    CGGAGAACGC AGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
Cat No2         CGGAGAACGC AGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     410       420       430       440       450
Assemblage F    GCAGAGGGCT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
Cat No2         GCAGAGGGCT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     460       470       480       490       500
Assemblage F    CCGCGAGAGG GCCGTCAGCG CCGCCACGAC AGAGGCGCTC ACAAACACGA
Cat No2         CCGCGAGAGG GCCGTCAGCG CCGCCACGAC AGAGGCGCTC ACAAACACGA
                ....|....| .
                     510
Assemblage F    AGCTCGTCGA G
Cat No2         AGCTCGTCGA G
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B) The antisense strand comparison with assemblage C (lack of cut place in 461bp)- Accession number **AY545646**

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       10        20        30        40        50
Assemblage C    GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTCAAGATGA
Dog No4         GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTCAAGATGA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                      60        70        80        90       100
Assemblage C    TCAAGGACGC CATCGCTCAC CTGGACAGGC TCATCCAGAC CGAGTCGAGG
Dog No4         TCAAGGACGC CATCGCACAC CTGGACAGGC TCATCCAGAC CGAGTCGAGG
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     110       120       130       140       150
Assemblage C    AAGCGCCAGG GCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
Dog No4         AAGCGCCAGG GCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     160       170       180       190       200
Assemblage C    CGACAACATG TACCTGACGA TCAAGGAGGA AATCGACACC ATGGCCGCGA
Dog No4         CGACAACATG TACCTGACGA TCAAGGAGGA AATCGACACC ATGGCCGCGA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     210       220       230       240       250
Assemblage C    ACTTCCGCAA GTCCCTCGCC GAGATGGGCG AGACCCTCAA CAACGTCGAG
Dog No4         ACTTCCGCAA GTCCCTCGCC GAGATGGGCG AGACCCTCAA CAACGTCGAG
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     260       270       280       290       300
Assemblage C    ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
Dog No4         ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     310       320       330       340       350
Assemblage C    CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACC GGCATCGCCA
Dog No4         CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACC GGCATCGCCA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     360       370       380       390       400
Assemblage C    CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
Dog No4         CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     410       420       430       440       450
Assemblage C    GCAGAGGGAT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
Dog No4         GCAGAGGGAT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     460       470       480       490       500
Assemblage C    CCGCGAGAGG GCCGTCAGCG CAGCCACGAC CGAGGCGCTC ACAAACACGA
Dog No4         TCGCGAGAGA GCCGTCAGCG CAGCCACAAC AGAGGCTCTC ACAAACACGA
                ....|....| .
                     510
Assemblage C    AGCTCGTCGA G
Dog No4         AGCTCGTCGA G
```

A) The sense strand comparison with assemblage C- Accession number **AY545646**

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       10        20        30        40        50
Assemblage C    GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTCAAGATGA
Dog No4         GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTCAAGATGA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                      60        70        80        90       100
Assemblage C    TCAAGGACGC CATCGCTCAC CTGGACAGGC TCATCCAGAC CGAGTCGAGG
Dog No4         TCAAGGACGC CATCGCTCAC CTGGACAGGC TCATCCAGAC CGAGTCGAGG
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     110       120       130       140       150
Assemblage C    AAGCGCCAGG GCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
Dog No4         AAGCGCCAGG GCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     160       170       180       190       200
Assemblage C    CGACAACATG TACCTGACGA TCAAGGAGGA AATCGACACC ATGGCCGCGA
Dog No4         CGACAACATG TACCTGACGA TCAAGGAGGA AATCGACACC ATGGCCGCGA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     210       220       230       240       250
Assemblage C    ACTTCCGCAA GTCCCTCGCC GAGATGGGCG AGACCCTCAA CAACGTCGAG
Dog No4         ACTTCCGCAA GTCCCTCGCC GAGATGGGCG AGACCCTCAA CAACGTCGAG
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     260       270       280       290       300
Assemblage C    ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
Dog No4         ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     310       320       330       340       350
Assemblage C    CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACC GGCATCGCCA
Dog No4         CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACC GGCATCGCCA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     360       370       380       390       400
Assemblage C    CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
Dog No4         CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     410       420       430       440       450
Assemblage C    GCAGAGGGAT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
Dog No4         GCAGAGGGAT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     460       470       480       490       500
Assemblage C    TCGCGAGAGG GCCGTCAGCG CAGCCACGAC CGAGGCGCTC ACAAACACGA
Dog No4         TCGCGAGAGG GCCGTCAGCG CAGCCACGAC CGAGGCGCTC ACAAACACGA
                ....|....| .
                     510
Assemblage C    AGCTCGTCGA G
Dog No4         AGCTCGTCGA G
```

C) The antisense strand comparison with assemblage D (additional cut place in 296bp)- Accession number **AY545647.1**

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       10        20        30        40        50
Assemblage D    GAACGAGATC GAGGTCCGCC GCGTCGACGA TGACACGCGT GTCAAGATGA
Dog No4         GAACGAGATC GAGGTCCGCC GCGTCGACGA CGACACGCGC GTCAAGATGA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                      60        70        80        90       100
Assemblage D    TCAAGGATGC CATCGCACAC CTTGACAGGC TCATTCAGAC GGAGTCGAGG
Dog No4         TCAAGGACGC CATCGCACAC CTGGACAGGC TCATCCAGAC CGAGTCGAGG
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     110       120       130       140       150
Assemblage D    AAGCGCCAAA GCTCCTTCGA GGACATCCGC GAGGAGGTAA AGAAGTCCGC
Dog No4         AAGCGCCAGG GCTCGTTCGA GGACATCCGC GAGGAGGTCA AGAAGTCCGC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     160       170       180       190       200
Assemblage D    TGACAACATG TATCTGACGA TCAAGGAGGA GATTGACACA ATGGCCGCAA
Dog No4         CGACAACATG TACCTGACGA TCAAGGAGGA AATCGACACC ATGGCCGCGA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     210       220       230       240       250
Assemblage D    ACTTCCGCAA GTCCCTCGCA GAGATGGGCG AGACGCTCAA CAACGTCGAG
Dog No4         ACTTCCGCAA GTCCCTCGCC GAGATGGGCG AGACCCTCAA CAACGTCGAG
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     260       270       280       290       300
Assemblage D    ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCAGCTCT
Dog No4         ACAAACCTCC AGAACCAGAT CGCCATCCAC AACGACGCCA TCGCGGCCCT
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     310       320       330       340       350
Assemblage D    CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTTGAGACC GGCATCGCTA
Dog No4         CAGGAAGGAG GCCCTCAAGA GCCTGAACGA CCTCGAGACC GGCATCGCCA
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     360       370       380       390       400
Assemblage D    CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
Dog No4         CGGAGAACGC CGAGAGGAAG AAGATGTACG ACCAGCTCAA CGAGAAGGTC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     410       420       430       440       450
Assemblage D    GCAGAGGGAT TCGCCCGTAT TTCCGCTGCC ATCGAGAAGG AGACGATCGC
Dog No4         GCAGAGGGAT TCGCCCGCAT CTCCGCCGCC ATCGAGAAGG AGACGATCGC
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     460       470       480       490       500
Assemblage D    CCGCGAGAGA GCCGTCAGCG CAGCCACAAC AGAGGCTCTC ACAAACACGA
Dog No4         TCGCGAGAGA GCCGTCAGCG CAGCCACAAC AGAGGCTCTC ACAAACACGA
                ....|....| .
                     510
Assemblage D    AGCTCGTCGA G
Dog No4         AGCTCGTCGA G
```

**Figure 2 A–C.** Comparison of *G. duodenalis* sequencing product from a dog with the pattern of assemblages C and D. Alignment based on maximum similarity of individual assemblages of *G. duodenalis* in the classification of Lalle et al. 2005. The underlined zone indicates a cut place of restrictive enzyme. The grey zone indicates differences in DNA sequence (no consensus at this position)

## DISCUSSION

*Giardia duodenalis* occurs in dogs and cats all over the world and is one of the major parasites responsible for the symptoms of the gastrointestinal tract. Extensiveness of the invasion is varied and depends on the geographical location, different hygiene conditions in the region, as well as different diagnostic methods usage. Because the diagnosis of *Giardia* invasion based on classical methods (faecal flotation or faecal smear) is problematic, commercial immunoassays are therefore increasingly used to detect the presence of parasite protein secretion in animal faeces. Giardiasis is a very common disease and invasion prevalence ranges from 5–80%, depending on the age of the animals. The highest prevalence (46–50%) is observed in young dogs less than one year old, and in animals with diarrhea [10, 11]. In Poland in 2001–2006, the prevalence of *Giardia* invasion in dogs in Poznan was about 10%, in Warsaw it ranged from 9% to over 50%, in Lublin about 53%, in Pulawy – 10% and Gdansk over 16%. Significant differences in prevalence values resulted from the different number of animals in age groups and differences of their clinical condition [12]. In the presented study, the prevalence of *G. duodenalis* in dogs was 21.1% and in cats – 15.1%. The use of enzyme immunoassay test for detecting soluble *Giardia* antigens in faeces increases the probability of invasion detection. Studies conducted in Europe (especially in countries such as the UK, Spain, Netherlands, Italy, Germany, Belgium) using the SNAP *Giardia* Test (IDEXX Laboratories) showed the presence of *G. duodenalis* invasion in approximately 25% of dogs and 20% of cats with gastrointestinal signs [11]. In the USA, the prevalence of *G. duodenalis* invasion in animals with symptoms of the gastrointestinal tract was 15.6% in dogs and 10.8% in cats [13]. Similar results were obtained in Canada, where the prevalence was 13% in dogs and 4.1% in cats, and in Japan, where the prevalence of *Giardia* invasion in 1997–2007 in pet dogs remained unchanged at the level of about 15% [14]. In Brazil, the percentage of infected dogs was 16.9% [15]. Usually, for the molecular identification of *G. duodenalis*, PCR and its modifications (nested-PCR, semi-nested PCR, PCR-RFLP, real-time PCR) or *Giardia* DNA hybridization with a molecular probes (microarray technique and FISH technique) are used. Analysis of the genetic material is helpful in answering the question whether *Giardia* detected in a dog or cat can be a source of infection for humans. The most preferred is amplification of the β-Giardin encoding gene [9]. Giardins are specific structural proteins with a mass of 29–38 kDa, unique for this protozoan [16]. The integral part of each trofozoit sucker are α- and β-giardins, while on the surface of *G. duodenalis* cell membrane, a further 10 specific proteins are identified. Genetic research conducted in Poland revealed in affected dogs an occurrence of assemblages A-1, C and D in Warsaw, and only C and D assemblages in Poznan [17, 18]. Molecular analysis of isolates obtained from cats in Warsaw showed the presence of assemblages A, B and D [19]. Current studies in animals in Wroclaw confirmed the presence of specific assemblages C, D, mixed assemblages of C and D, and zoonotic assemblage B in stool samples derived from dogs. In cats, the presence of assemblage F was revealed, but the zoonotic assemblage A was also detected. The study of 55 dogs in Germany, with no apparent clinical signs of disease, revealed the presence of genotype A in 60% of tested animals, mixed infections with assemblages A and C in 27.3%, while the individual assemblages C and D were rarely recorded, respectively, in 9.1% and 3.6% of dogs.

In dogs in Spain, the most commonly circulating assemblages were B, D, followed by C, A, E and F [20]. The study not only confirmed the high prevalence of *G. duodenalis* among dogs with no clinical signs of disease, but also showed that zoonotic assemblage A is common in urban pet dogs, and even more common than assemblages typical for dogs. Although dogs owners' stools samples were not studied, the results indicate that a large proportion of urban dogs infected with zoonotic genotypes of *Giardia* are a reservoir for human invasion [21].

As in the presented study, the results of genotyping of *Giardia* isolates present in dogs in different regions of the world indicate that in these animals specific assemblages C and D usually predominate. Such results were obtained in dogs in Hungary, Brazil and Australia [22, 15, 23]. Similarly, non-zoonotic assemblages were identified more often in dogs in Italy (C and D), and assemblage A or mixed-induced invasion with zoonotic and non-zoonotic assemblages occurred only in individual animals [9]. Also in Australia, zoonotic assemblages A and B have been found only in individual animals, which indicated that dogs and cats are not a significant reservoir of invasive isolates of *Giardia* for humans [23]. In turn, other researchers from Germany, Japan and Thailand detected zoonotic assemblage A of *G. duodenalis* in dogs more often than assemblages C and D that are specific for canines [24, 25]. Higher prevalence of zoonotic *Giardia* assemblages were found in dogs that were kept singly than in dogs kept in groups [22]. This confirms earlier suggestions that individual dogs bred in house are infected by *Giardia* from the owners or householder, while the dogs kept in high density are infected with parasite assemblages specific for canids.

Although most of the invasion found in cats is caused by non-pathogenic to humans assemblages D and F, it has been shown that these animals are also a potential source of zoonotic *G. duodenalis* assemblages [5, 9, 22, 23]. Research carried out in Poland on cats from Warsaw confirmed the presence of assemblages A and B, next to assemblages D specific for cats [19]. Also, the results of the presented study, in which apart from assemblage F specific for cats, the zoonotic assemblage A was also found, indicating a potential threat to human health.

The issue that in 5 positive samples tested with the SNAP *Giardia* Test (IDEXX Laboratories) nested PCR failed can be explained by insufficient numbers of parasite's DNA for amplification [26].

## CONCLUSIONS

The results confirm the presence of assemblages C and D in dogs, and assemblage F in cats in the vicinity of Wroclaw. Stating the presence of assemblage A and B suggests that infected pets can pose a threat to human health. This study describes for the first time the presence of mixed infections within host-specific C and D genotypes in dogs in Poland.

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