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First Molecular Detection of *Giardia duodenalis* Assemblage B in a Free-Living European Wildcat (*Felis s. silvestris*) from Luxembourg

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Abstract. *Giardia duodenalis* is one of the most widespread intestinal parasites of humans and other vertebrates. In terms of public health, identification of *Giardia* assemblages in wildlife is important because only some assemblages of *G. duodenalis* can infect humans. Here, we use loop-mediated isothermal amplification (LAMP) and genotyping of analysis of the *β-giardin* gene to screen the zoonotic assemblages of *G. duodenalis* recovered from faeces of free-living European wildcats (*Felis s. silvestris*) from Luxembourg. *Giardia* DNA was detected in one animal (10%) and assigned to assemblage B by both methods. This is the first detection and genotyping of *G. duodenalis* in a European wild felid in general, and of assemblage B in particular. Free-living wildcats may act as reservoirs of *G. duodenalis* infectious for humans and other wildlife and domestic animals. Using a combination of LAMP- and genotyping-based methods allowed effective, sensitive, and rapid detection of a zoonotic *G. duodenalis* assemblage B in wildlife.

Key words: *Giardia,* molecular diagnosis*,* LAMP, PCR, epidemiology, zoonosis.

INTRODUCTION

The unicellular flagellate *Giardia duodenalis* (syns. *G. intestinalis, G. lamblia*) is one of the most widespread intestinal parasites of humans and many other vertebrate species (Cacciò *et al*. 2018). In terms of public health, identification of *Giardia* assemblages in wildlife is very important because only some assemblages or even sub-assemblages of *G. duodenalis* are capable of infecting humans (Feng and Xiao 2011). *Giardia* is one of the most common enteric protozoa with great environmental contamination abilities because is transmitted by excreted feaces of infected host. Transmission of the parasite may be favoured by wild species that spread the *Giardia* in the nature. Deposited cysts may be directly or indirectly via contaminated water ingested by other hosts belonging to wild and domestic animals or humans. However, some aspects of *Giardia* spillover between groups of a potential host

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species remains still unclear. Free ranging protozoan-wildlife hosts including wild cats, may play a role with zoonotic transmission and public health threat. In spite of *G. duodenalis* cosmopolitan distribution it is difficult to make inferences in the role of animals as a source of human infection. Felidae may be infected by different *G. duodenalis* assemblages belonging to both zoonotic assemblages A (sub-assemblages AI, AII) and B or host-adapted assemblages D (dog specific), F (cat specific) and also sub-assemblages AIII associated with wild ruminants. Most of the previous data concerning *G. duodenalis* identification in felids derived from research on domestic or stray cats (*Felis catus*) (Cacciò *et al*. 2008, Cacciò *et al*. 2010; de Lucio *et al*., 2017; Gil *et al*., 2017; Kváč *et al*. 2017; Li *et al*. 2017; Lebbad *et al*. 2010; Suzuki *et al*. 2011; Read *et al*. 2004; Pallant *et al*. 2015; Palmer *et al*. 2008; Jaros *et al*. 2011; McDowall *et al*. 2011; Sotiriadou *et al*. 2013; Souza *et al*. 2007). In addition to reports from individual captive wildcats (Beck *et al*. 2011, Li *et al*. 2017, Liu *et al*. 2017), only one study has used molecular-based techniques and sequence analysis to identify *G. duodenalis* assemblages in feacal material from free-living wild felids (Oates *et al*. 2012). Given the lack of molecular genotyping of *Giardia* isolates from European wildcats (*Felis s. silvestris*), the aims of the present study were (*i*) to use loop-mediated isothermal amplification (LAMP) for rapid detection of *G. duodenalis* zoonotic assemblages, (*ii*) and to genotype *Giardia* isolates recovered from wild wildcats by polymerase chain reaction (PCR).

MATERIAL AND METHODS

Sampling

Between 2008 and 2013, ten road-killed wildcats were collected in Luxembourg and stored at -20°C. We collected carcasses from: Mamer (49°38'N/6°1'E); Garnich (49°37'N/5°57'E); Rombach (49°50'N/5°45'E); Bous (49°33'N/6°20'E); Hoscheid (49°57'N/6°5'E); Angelsberg (49°45'N/6°10'E); Koedange (49°44'N/6°13'E); Rippweiler (49°45'N/5°57'E); Kopstal (49°40'N/6°4'E); Buderscheid (49°56'N/5°56'E). During dissection, we collected tissue samples for genetic analysis and 30-50 g fecal samples from the large intestine. The fecal samples were placed in a plastic container containing 2.5 % potassium dichromate solution and stored at 4°C until further analysis. The age of the wildcats was determined by the Ansorge (1995) method using incremental growth lines in the enamel of a lower-jaw canine. After demineralization with 5 % nitric acid (HNO_3), the teeth were cross-sectioned (width, 5 µm) with a rotary microtome (RM 2050, Leica Biosystems Nussloch GmbH, Germany) and stained with hematoxylineosin. The growth lines were counted under a B1-220A light microscope (Motic, Wetzlar, Germany) at ×40-100 magnification. Following Piechocki and Stiefel (1988), animals were either classified as subadults (≤ 24 months; one growth line) or adults (≥ 25 months; two or more growth lines). The dataset consisted of 5 adults and 5 subadults (comprising 6 males and 4 females). The genetic investigations of the wildcats were carried out at the Senckenberg Research Institute (Gelnhausen, Germany) and showed that all animals are pure wildcats (Steyer et al. 2016).

Microscopy

Each faecal specimen was examined as a wet mount. Each sample was concentrated using the 0.85 M sucrose gradient centrifugation technique, with the final sediment being examined using a light microscope (Axioskop, Zeiss, Germany). In order to demonstrate *Giardia* stages, the entire coverslip area of wet mounts was microscopically screened under high power (total magnification of x600).

DNA extraction

From each sample, total genomic DNA was directly extracted using the QIAamp® DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, except for an overnight incubation with Proteinase *K*. DNA was eluted in 50 µl of elution buffer and stored at -20 °C until LAMP and PCR assays.

LAMP, PCR, sequencing and phylogeny

The *Giardia* LAMP assay was designed based on a set of primers specific to the *EF1-α* gene of *G. duodenalis* described by Plutzer and Karanis (2009). Six complementary primers of 40 pmol each were used: F3, forward outer primer; B3, backward outer primer; FIP-forward inner primer; BIP-backward inner primer; LoopF (LF), forward loop primer; LoopB (LB), backward loop primer. LAMP reactions were conducted in 13 µl of reaction mixtures: 7.5 µl of 1x Isothermal Master-mix Fluorescence Dye (OptiGene Ltd., UK), 3.5 µl of Primers Mix, 1 µl of ddH_2O and 1 µl of DNA template. The reaction mixtures were incubated at 63ºC for 60 min with fluorescence recording/detection every 45 sec and subsequently, to melt the reaction products, heated to 95ºC for 15 sec, with fluorescence recorded after every temperature change of 0.5ºC. The LAMP products were detected with the Line Gene-K Fluorescent Quantitative Detection System (Hangzou Bioer Technology Co. Ltd.). LAMP results were directly analyzed by viewing of the amplification melting curves and the threshold cycle.

We amplified a 753-base pair fragment of the *β-giardin* gene (*bg*) using 0.6 µM of the G7 forward primer and the G759 reverse primer, as previously described (Cacciò et al. 2002). PCRs were performed in a total volume of 20 µl including 10 µl of AmpliTaq Gold Fast PCR Master Mix UP, 1 µl of 0.6 µM of primer final concentration, 5 µl of DNA template and 3 µl of ddH_2O. Amplified DNA and 100 bp DNA Ladder (Novazym, Poland) were separated on a 2% agarose gel in TAE buffer (2 M Tris, 0.05M EDTA, 5.7% glacial acetic acid) for 90 min at 50 V, stained with ethidium bromide (0.5 µg/ml), and visualized under UV light. All PCR and LAMP experiments included *G. duodenalis* positive controls (total genomic DNA extracted from axenically cultured trophozoites of the human isolate HP-124) and negative controls (water template) to exclude contamination of the PCR components. The PCR prod-

uct was sequenced in both directions with the same set of primers. Sequencing was performed with BigDye Terminator v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems, USA). Sanger sequencing trace files were checked and edited using MEGA software v.7.0 (Kumar et al. 2016). Contigs were manually aligned and assembled in GeneDoc v. 2.7.000 (Nicholas and Nicholas, 1997). Sequences were clustered using the neighbor-joining algorithm in program MEGA version 7.0, based on the Kimura 2-parameter substitution model. Bootstrap values were calculated by performing 1,000 pseudo-replicates. The nucleotide sequence of the obtained *bg* gene fragment was deposited in GenBank® (NCBI) under accession number KX685669.

RESULTS AND DISCUSSION

One (10%) of the ten examined wildcats, a sub-adult female from Rippweiler (collection dates: 09.01.2012), was *Giardia*-positive. While no microscopy sample was positive, *Giardia* DNA was detected in the faecal sample both by the LAMP and PCR methods. The LAMP amplicon melting curve indicated a product melting temperature (T_m) of 60.5ºC (±0.5ºC). The sequence of the *bg* marker was identical to *G. duodenalis* sequences obtained from humans isolates. In the neighbor-joining algorithm, the wildcat *Giardia* isolate clustered with *Giardia* reference sequences isolated from humans and representing assemblage B (Fig. 1).

In this study, we report the first detection of the potentially zoonotic *G. duodenalis* assemblage B in wildlife in Luxembourg. The *Giardia* infection rate in wildcats from Luxembourg was low. Nevertheless, we report the first record of *G. duodenalis* in a free-living felid species and the first detection of *Giardia* in the wild member of the genus *Felidae* in Europe. A study on free-living felids which were conducted on 11 samples from bobcats (*Lynx rufus*) and mountain lions (*Puma concolor*), reported one *Giardia* positive mountain lion from the USA. The genotype of the *G. duodenalis* isolate from mountain lion clustered with assemblage E (Oates *et al*. 2012).

A few species of wild felids kept in zoos were found to be *G. duodenalis* positive. Most of the *G. duodenalis* isolates from chinese leopard (*Panthera pardus japonensis*), serval (*Laptailurus serval*) and lynx (*Lynx lynx*) were genotyped as assemblage A (sub-assemblage AI), whereas a singular *Giardia* isolates from a cheetah (*Acinonyx jubatus*) and one from a snow leopard (*Panthera uncia)* were characterized as assemblage D and C, respectively (Beck *et al*. 2011). As stated, isolates of *G. duodenalis* assemblage F were also identified in one chinese leopard (*Panthera pardus fontanierii*) [= *P. p. japonensis*], two siberian tigers (*Panthera tigris altaica*) and mix assemblages D/F in a leopard (*Panthera* sp.) in Chinese zoo (Li *et al*. 2017, Liu *et al*. 2017).

LAMP was effective and rapid for the screening of *G. duodenalis* in wildcat feacal samples. The specificity of the LAMP assay was high, because it used six specifically designed primers recognizing six loci on the elongation factor 1-alfa DNA target. In the present study, the LAMP reactions required lower amounts of DNA template than PCR. According to the literature, eight assemblages of *G. duodenalis* have been established. Only assemblages A and B have been found in a wide range of hosts, including various species of wild animals and humans (Cacciò *et al*. 2018). The LAMP technique was based on strongly conserved *EF1-α* markers which can be used only to identify two assemblage A and B (Plutzer and Karanis 2009). To overcome the limitation, the positive sample was subsequently assessed by PCR using the variable *β-giardin* marker to exclude presence of assemblage F, which is host-specific for cats. Sequencing data at the *bg* molecular marker confirmed *G. duodenalis* assemblage B in the wildcat feacal sample. In conclusion, using LAMP and PCR based methods seems to be effective, sensitive, and rapid for the detection of zoonotic *G. duodenalis* assemblages in animals.

It is known that A and B assemblages of *G. duodenlis* are responsible for infections in humans globally. Moreover, both *G. duodenalis* assemblages may be present in the environment. In the other studies that were carried out in Luxemburg, *Giardia* cysts were mainly detected in environmental samples (Helmi *et al*. 2011; Burnet *et al*. 2014, Burnet *et al*. 2015). Unfortunately, in these long termed studies there was lack of molecular data about *G. duodenalis* genotypes probably due to the negative PCR inhibitors effects in a surface water samples. Despite the evidence of *Giardia* presence both in drinking and recreational water reservoirs without molecular designation and in wildlife that might contaminate water sources, implications for public health become still unresolved in the Luxembourg. According to the National Health Laboratory of Luxembourg (LNS) there were reported no more than 20 human infections per year by gastrointestinal protozoa, including *Giardia* and the addressed cases were no linked with water activities or consumptions (Helmi *et al*. 2011). Our data indicated, that wild animals spread *Giardia* in nature and may contaminate water bodies but there is low risk of humans infection with *G. duodenalis* by drinking or

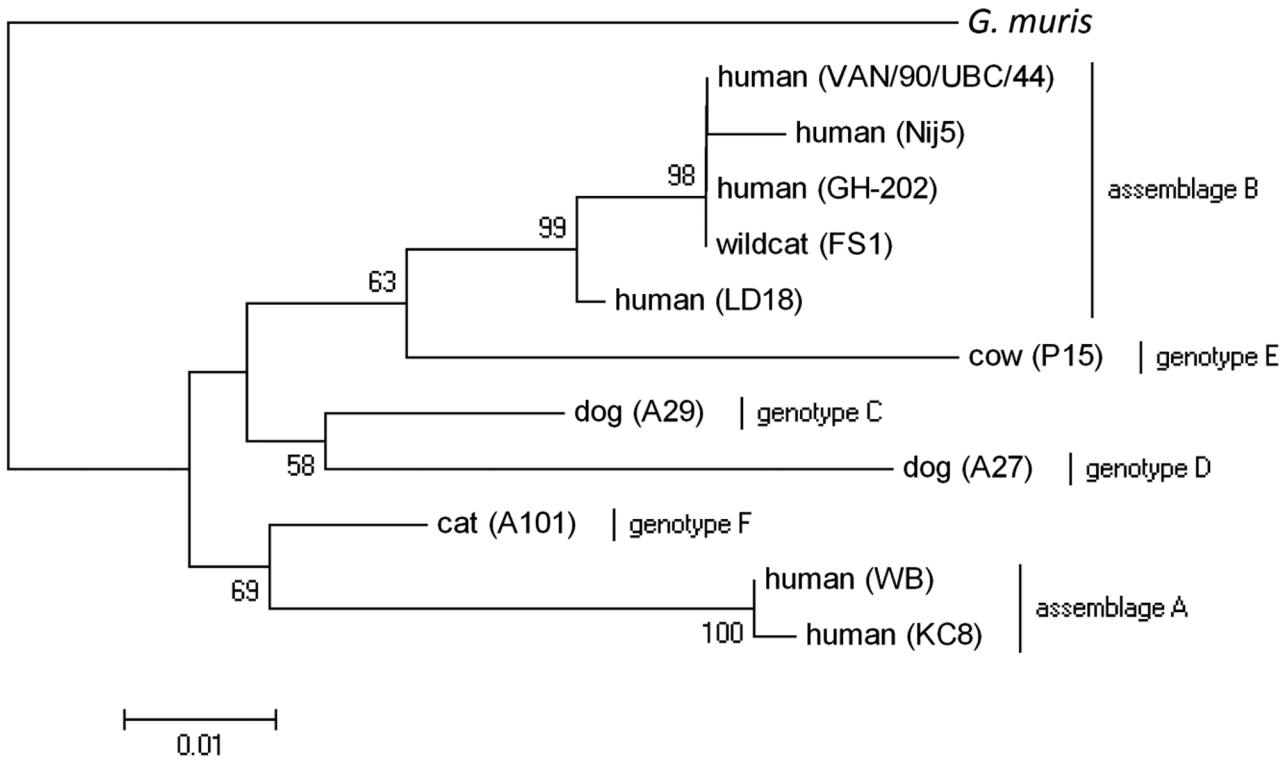

Fig. 1. Neighbor-joining distance analysis of the *β-giardin* nucleotide sequences. FS1 – sequence of partial *β-giardin* gene of the isolate from wildcat (KX685669). Reference human isolates: WB and KC8, (X85958 and AY072723); LD18, Nij5, VAN/90/UBC/44, GH-202 (AY072727, AY072725, KP687755, AB618785); A101 and – cat isolate, (AY647264 and EU769206); P15 – reference cow isolate, (AY072729); A29 and A27 – reference dog isolates (AY545646 and AY545648). *G. muris* (AY258618) represents an outgroup.

other water activities. Wildcats shed zoonotic *Giardia* assemblage B and may thus be a potential source of human infection. However, environmental contamination with wildcat fecal samples that propagate the *Giardia* dispersive stages is probably low. Based on the low population densities and the solitary life style of wild felids, the role of wildcats in contaminating soil and surface and ground waters with the protozoa parasite is probably limited. To ultimately protect water quality and reduce indirect waterborne transmission of zoonotic *G. duodenalis* assemblages, it is necessary to focus on the role that free-ranging domestic cats play in spreading these potentially pathogenic protozoa. Nevertheless, it should be underlined that *Giardia* cross-infection among wildcats, other wildlife and domestic animals could be possible. Therefore, further studies are necessary to explain the impact of direct *Giardia* infection on other wild protected and/or farm animals and zoonotic transmission to human should also be elucidated.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

All animals had been road-killed and were collected with the authorisation of the Luxembourg Ministry of Sustainable Development and Infrastructures (Ref.: 70646 GW/sc).

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