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Original article

Multilocus genotyping of *Giardia duodenalis* infecting rabbits in Ogun State, Nigeria

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ABSTRACT

Giardiasis is a cosmopolitan gastrointestinal protozoal parasite that infects humans and various animals worldwide. To assess the zoonotic transmission potential of *Giardia,* molecular characterization is required. We are unaware of any report on the genotypes of *Giardia* infecting rabbits in Nigeria. Molecular detection and genotyping of *Giardia duodenalis* were conducted in a herd of adult Chinchilla rabbits (*Oryctolagus cuniculus*) managed on the Teaching and Research farm of the Federal University of Agriculture, Abeokuta located in a southwestern state of Nigeria by analysis of the small-subunit ribosomal RNA (*ssu rRNA)*, glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi)* and beta-giardin (*bg)* genes. An overall prevalence of 72.3% (60/83) was recorded in the rabbits with no statistically significant ($p > .05$) influence of sex on the distribution of the infection in the herd. All the 19 isolates amplified at the four genetic loci were identified as *G. duodenalis* assemblage BIV by multiple alignment analysis of their consensus sequences. Novel nucleotide substitutions were identified in two isolates at the *ssu rRNA* locus. Phylogenetic analysis revealed that all *ssu rRNA* genotypes were closely related to *G. duodenalis* assemblage B of cattle and human origin. Findings of this study suggest that the rabbits harbour potentially zoonotic assemblage BIV that portends a high risk to students and staff of the University who are in regular contact with the animals.

1. Introduction

Giardia duodenalis is a cosmopolitan intestinal flagellate protozoan parasite that infects humans and a wide range of domestic and wild animal species (Feng and Xiao, 2011; Koehler et al., 2014; Squire and Ryan, 2017; Zhang et al., 2012). Infection is often characterized by acute or chronic diarrhoea whose severity depends on the age and health status of the host and concurrent infection with other pathogens (Geurden et al., 2010; Robertson et al., 2010).

Molecular techniques, especially the Polymerase Chain Reaction (PCR), in combination with Restriction Fragment Length Polymorphism (RFLP) or sequencing of the resulting PCR products, have confirmed the existence of eight assemblages (A-H) which show differences in host specificity (Castro-Hermida et al., 2007; Sprong et al., 2009; Xiao and Fayer, 2008). Assemblages A and B have the broadest host range, infecting humans and many other mammals. The other assemblages are reported to be host-specific and infect non-human mammals. Sporadic infection by assemblages C, D, E and F have however been reported in humans (Fantinatti et al., 2016; Foronda et al., 2008; Minetti et al., 2015; Zahedi et al., 2017).

Genotyping of *G. duodenalis* is most commonly achieved by amplification of target fragments of genes encoding the small subunit of ribosomal RNA (*ssu rRNA*), β-giardin (*bg*), triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*) (Caccio et al., 2002; Minetti et al., 2015; Sulaiman et al., 2003; Wegayehu et al., 2017; Zhang et al., 2016). The *tpi*, *gdh* and *bg* genes have high sequence variability and are useful for assemblage and sub-assemblage identification whereas the *ssu rRNA* gene is only sufficient for species and assemblage identification (Feng and Xiao, 2011; Koehler et al., 2014; Li et al., 2012; Wielinga and Thompson, 2007; Xiao and Fayer, 2008). Multilocus genotyping of *G. duodenalis* has been suggested to be more reliable in assemblage and sub-assemblage typing of isolates than single-locus genotyping especially for assessment of the true zoonotic potential of the infection (Feng and Xiao, 2011; Thompson and Ash, 2016). Multilocus genotyping is also useful for detecting and discriminating true mixed infections by different assemblages (or sub-assemblages) of the parasite.

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To the best of our knowledge, there are only six published reports on the *Giardia* genotypes infecting rabbits worldwide (Lebbad et al., 2010; Liu et al., 2014; Pantchev et al., 2014; Sulaiman et al., 2003; Qi et al., 2015; Zhang et al., 2012) with no previous genotyping study being reported in Africa.

In Nigeria, the prevalence of giardiasis ranges from 1.8% to 41.5% in humans with the infection being predominant in children < 15 years of age (Idowu and Rowland, 2006; Nwanguma and Alumanah, 2008; Obiukwu et al., 2008; Udeh et al., 2008; Biu et al., 2009; Inabo et al., 2011; Maikai et al., 2012; Efunshile et al., 2015). Additionally, contamination of water by *Giardia* cysts was reported by Obiukwu et al. (2008). However, only one genotyping study in humans has been reported in which only sub-assemblage AII was identified (Maikai et al., 2012). *Giardia* infection has been reported in cattle (Magaji et al., 2013), Kalahari Red goats (Akinkuotu et al., 2016), pigs (Agumah et al., 2015) and dogs (Abubakar et al., 2015) with prevalence ranging from 5.6% to 46.9%. In a recent study by Akinkuotu et al. (2017), *Giardia* coproantigens were detected in 100% of the rabbits in one herd located in a southwestern state. There are however no published report on the genotypes of *Giardia* species infecting animals in the country.

In this study, we determined the point prevalence and genotypes of *Giardia* species infecting recently acquired rabbits in the same herd using the *ssu rRNA*, *gdh*, *tpi*, and *bg* genes. This will provide novel information on the host-specificity and zoonotic potential of *Giardia* isolates from rabbits in Nigeria.

2. Materials and methods

2.1. Study location and animals

This study was performed in a herd of 25-weeks old Chinchilla rabbits (*Oryctolagus cuniculus*), consisting of 20 bucks and 63 does, intensively managed in the rabbit unit of the Teaching and Research farm of the Federal University of Agriculture, Abeokuta, Ogun state, southwest Nigeria. These rabbits were housed individually in cages placed in a pen constructed in 2016 and were bred for research purposes. Clinical signs of giardiasis including diarrhoea were not observed in any of the rabbits in the herd. The source of drinking water to the herd was a dug well while freshly cut forage was fed to the rabbits daily.

Other animal species reared on the farm include cattle, sheep, goats, pigs, poultry and fish. The proximities of these livestock units to the rabbit pen ranged between 23.50 and 597.20 m. Animal husbandry staff was regularly rotated among the various livestock units on the farm.

2.2. Sample collection and DNA extraction

Faeces were collected from all the rabbits in the herd. Freshly voided faeces found in individual cages were hand-picked, placed into separate labelled sterile universal sample bottles and stored at 4 °C with the DNA being extracted within 24 h.

Genomic DNA was extracted from the faeces using the AccuPrep™ stool genomic DNA extraction kit (Bioneer, Korea) according to the manufacturer's instruction. DNA was eluted in 100 µl of 10 mM Tris buffer and stored at -20 °C prior to use in PCR.

2.3. PCR amplification of G. duodenalis

Previously described nested PCR protocols were used to amplify target fragments of the *ssu rRNA* gene (Hopkins et al., 1997; Appelbee et al., 2003), the *gdh* gene (Caccio et al., 2008) and *tpi* gene (Sulaiman et al., 2003) while a semi-nested PCR protocol was used to amplify the *bg* gene (Mahbubani et al., 1992). Each PCR included a hot-start step of 94 °C for five minutes. The primers used, annealing temperatures and the expected sizes of PCR products for each genetic loci are summarized in Table 1.

All PCRs were conducted in a 50 µl reaction volume containing 1 X CoralLoad® PCR buffer containing 1.5 mM $MgCl_2$ (Qiagen), 1 X Q-solution (Qiagen), 200 mM of each dNTP, 0.5 µM of each primer, 2.5 units of HotStar® Taq polymerase (Qiagen) and 2 µl of the DNA sample. The secondary PCRs were similar to the primary PCR except that 2 µl of the primary PCR products was used as the DNA template and the annealing temperatures of the *ssu rRNA* and *bg* genes were changed (Table 1). For each round of PCR, a positive and negative control was included.

The secondary PCR products were separated by electrophoresis on a 1% agarose gel stained with SYBR™ Safe DNA gel stain (Invitrogen) and visualized using the VersaDoc™ 5000MP molecular imaging system (BioRad).

2.4. DNA sequencing

All *ssu rRNA* secondary PCR products and isolates detected at the *gdh*, *tpi* and *bg* genetic loci were sequenced in both directions with the secondary PCR primers. Sequencing was performed at the Macrogen USA Corp., MD, USA. All forward and reverse electropherograms were visually inspected for each of the isolates at each of the genetic loci. Thereafter, the consensus sequences were constructed for each isolate. Consensus sequences of each gene were aligned with reference GenBank™ sequences using the ClustalW multiple alignment function on the BioEdit version 6 program (Hall, 1999). Each consensus sequence was then compared with similar published sequences by Basic Local Alignment Search Tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov/blast).

Nucleotide sequence data for the *ssu rRNA* gene reported in this study are available at the GenBank™ under the accession numbers MG018738, MG018737 and MG018739 while those for the *gdh, tpi* and *bg* genes are available under the accession numbers MH475908, MH475909 and MH475910 respectively.

2.5. Phylogenetic analysis

A phylogenetic tree was constructed with three representative *ssu rRNA* sequences and four reference GenBank™ *ssu rRNA* isolates while another tree was constructed with one representative *gdh* sequence and 13 reference GenBank™ *gdh* isolates. These trees were constructed using maximum likelihood (ML) with the Tamura-Nei model of the Molecular Evolutionary Genetic Analysis (MEGA) 7.0 software (Kumar et al., 2016). *Giardia microti* (AF006676) and *G. ardeae* (AF069060) sequences were used as out-groups to root the *ssu rRNA* and *gdh* trees respectively. These sequences were subjected to bootstrap analysis with 1000 replicates (Felsenstein, 1985).

3. Results

3.1. Prevalence of Giardia duodenalis

Giardia duodenalis infection was detected in 72.3% (60/83) of rabbits in this study. A PCR-positive result was indicated by successful amplification at any of the genetic loci used. The prevalence rate in bucks, 75.0%, was not significantly higher ($\times^2 = 0.097$; $p = .756$) than the rate recorded in does, 71.4%. The highest and lowest diagnostic rates were recorded at the *ssu rRNA* and *bg* genetic loci respectively (Table 2).

3.2. Genotypes of Giardia duodenalis

The multilocus genotyping of the 19 positive isolates at the four genetic loci revealed the occurrence of *G. duodenalis* assemblage B in these rabbits. All the isolates were identified as sub-assemblage BIV at the *gdh*, *tpi* and *bg* genetic loci.

All 60 isolates detected at the *ssu rRNA* gene were identified as assemblage B. Fifty-eight of these isolates (GenBank™ accession number MG018738) were identical to one another and the *G. duodenalis*