Field application of a novel multiplex qPCR assay reveals the occurrence of the zoonotic hookworm *Ancylostoma braziliense* in Nigerian dogs

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Abstract

A number of gastrointestinal parasites have been reported to infect dogs in Nigeria, some of which have zoonotic potential. Of these, hookworms are the most prevalent, with both *Ancylostoma caninum* and *Uncinaria stenocephala* reported in the country. In this study, we subjected 203 hookworm microscopy-positive samples of the 885 individual faecal samples collected from dogs in Nigeria to a recently developed multiplex qPCR for the detection and characterisation of canine hookworm species. The qPCR demonstrated a diagnostic sensitivity of 98% (95% CI 95-99.4) allowing the detection of *A. caninum* and *A. braziliense* in 81.3% (165/203, 95% CI 75.3-86.1) and 51.2% (104/203, 95% CI 44.4-58) of the microscopy-positive faecal samples of dogs from

Nigeria, respectively and 35.4% (70/203, 95% CI 28.3-41.3) of mixed infections with both hookworm species.

The finding of *A. braziliense* is particularly worrisome given this is a well-known agent of persistent cutaneous larva migrans, commonly referred to as “creeping eruptions” in humans. Although this parasite has been diagnosed in locals and in people travelling in Nigeria suffering from dermatological illnesses, this represent the first molecular identification of *A. braziliense* in its canine reservoir in the country. These results update the occurrence and distribution of hookworm species affecting dogs in Nigeria highlighting the suitability of the newly developed multiplex qPCR assay as a high-throughput tool for the surveillance of zoonotic hookworms, globally.

Keywords

Hookworms; Zoonosis; *Ancylostoma braziliense*; dogs; qPCR; Nigeria.

1. Introduction

Dogs can harbour a plethora of intestinal parasites, some of which can also infect humans. A number of gastrointestinal parasites of veterinary or public health significance have been reported in Nigerian dogs including *Ancylostoma* spp., *Uncinaria stenocephala*, *Toxocara canis*, *Dipylidium caninum, Taenia* spp., and *Trichiuris vulpis.* (Anene et al., 1996; Sowemimo, 2009; Mustapha et al., 2016; Idika et al., 2017; Moro & Abah, 2019). Of these parasites, *Ancylostoma* spp. were the most common parasites detected in pet dogs, with

prevalence ranging from 14% to 70% (Anene et al., 1996; Sowemimo, 2009; Mustapha et al., 2016; Idika et al., 2017; Moro & Abah, 2019).

In addition to impacting canine health, the hookworms, *Ancylostoma caninum*, *Ancylostoma braziliense*, *Ancylostoma ceylanicum*, and *Uncinaria stenocephala* (Traub et al., 2004) are also zoonotic, therefore posing a risk to human health. Canine hookworms are well-known agents of cutaneous larva migrans in humans, with *A. braziliense* as the only species capable of causing "creeping eruptions", which clinically manifest as prolonged, highly pruritic serpiginous lesions in the skin of human patients that may persist for over 100 days (Dove, 1932; Beaver, 1956; Brenner et al., 2003). *Ancylostoma caninum* is a well-known agent of eosinophilic enteritis and aphthous ileitis in humans (Croese et al., 1994; Walker et al., 1995; Prociv & Croese, 1996). Recently, the finding of *A. caninum* eggs in the faeces of human subjects has led to speculation that this parasite might have the potential to complete its life cycle in humans (Ngcamphalala et al., 2019; Furtado et al., 2020). *Ancylostoma ceylanicum* on the other hand, is commonly reported to cause patent infections in humans throughout the Asia Pacific, sometimes with accompanying clinical signs of diarrhoea and anaemia (Stracke, Jex, & Traub, 2020; Traub, 2013).

To date, there is a paucity of data on the occurrence of canine hookworms in Africa, with the majority of the surveys performed using morphological identification of eggs to differentiate between the species of hookworms. For instance, based on faecal floatation and Kato-Katz techniques *A. caninum*, *A. braziliense* and *U. stenocephala* have been reported in dogs from Nigeria (Abraham & Gloria, 2009; Nwoha & Ekwuruike, 2010; Okoye et al. 2011 Ayinmode et al. 2016; Moro & Abah, 2019). However, these techniques are not reliable given marked similarities in egg morphology of hookworms species, making the use

of molecular tools fundamental to identify these parasites at species level (Lucio-Forster et al., 2012). Recently, PCR based assays allowed the identification of *A. caninum* and *A. braziliense* from dogs in South Africa (Lamb et al., 2012; Ngcamphalala et al., 2019) and Kenya (Mulinge et al., 2020) and, of all the four species of canine hookworms in Tanzania (Merino-Tejedor et al., 2018).

A recently published Taq-Man based multiplex qPCR targeting the internal transcribed spacer 1 (ITS-1) region of the rDNA of all four canine hookworms was recently developed for the specific detection and discrimination of canine hookworm species in faecal samples (Massetti et al., 2020). The Taq-Man qPCR was field-validated for *A. caninum*, *A. ceylanicum* and *U. stenocephala*, but not for *A. braziliense*. In this study we characterised the species of hookworm infecting dogs in Nigeria through a novel high-throughput qPCR and report the occurrence of *A. braziliense* in the country.

2. Methods

2.1 Parasite material

A cross-sectional national survey of gastrointestinal parasites of dogs in Nigeria was conducted between November 2016 to December 2017 across the Nigerian Federal Capital Territory (FCT), Abuja in 11 states of Nigeria. Faecal samples were collected from the rectum of 885 dogs sourced from breeding kennels, abattoirs, and veterinary clinics and transferred into previously labelled screw cap containers. The faecal samples were kept on ice and transported to the Helminthology Laboratory of the Parasitology Division of the National Veterinary Research Institute (NVRI) in Vom, Nigeria for parasitological

screening through microscopic examination. Faecal samples were refrigerated at 4 °C until processed. Aliquots of 203 faecal samples tested positive for hookworm eggs by microscopy were transferred to a separate screw cap container containing 2.5% w/v potassium dichromate solution for molecular analysis at the University of Melbourne, Melbourne Veterinary School.

2.2 Ethical statement

This study was approved by the Institutional Animal Ethics Committee (National Veterinary Research Institute, Vom, Nigeria), approval numbers AEC/03/21/15 and AEC/03/56/18. Oral consent was obtained from dog owners before faecal samples were collected.

2.3 Parasitological procedure

Fresh faecal samples were subjected to simple standing faecal floatation to screen for parasite eggs with saturated sodium chloride solution (S.G. 1.20) according to Soulsby (1982). Slides were analysed under an optical microscope (10x and 40x) for microscopic identification of parasite eggs based on morphological keys (Soulsby, 1982).

2.4 Molecular procedures

DNA was extracted from hookworm-positive faecal samples (150 mg each) using the ISOLATE Faecal DNA Kit (Bioline Sydney, Australia) according to the manufacturer's instructions. Final elution of DNA was made in 100 µl.

The multiplex qPCR for the characterization of the canine hookworms from faeces was performed according to Massetti et al. (2020).

Synthetic double stranded DNA fragments (gBlocks® Gene Fragments, IDT® Technologies, Skokie, Illinois, USA) containing individual sequence targets of each hookworm species and the genomic DNA of *U. stenocephala, A. braziliense, A. caninum*, and *A. ceylanicum* were used as positive controls. A four-channel Magnetic Induction Cycler (BioMolecular Systems, Sydney, Australia) was used for the amplification reaction, detection, and data analysis (micPCR software). The proportion of single-and mixed-species infections in dogs positive for hookworm eggs was calculated for each geographical region.

2.5 Sensitivity of the multiplex qPCR

Of the 218 samples positive for hookworm eggs on microscopy, 203 samples contained sufficient quantity of faeces to be subjected to qPCR. The diagnostic sensitivity of the qPCRs was assessed on 203 microscopy-positive samples using microscopy as a gold standard. These samples were shipped to the University of Melbourne for analysis. The diagnostic sensitivity was calculated as the number of hookworm samples found positive by qPCR (true positives) divided by the total number of microscopy positive samples (true positives + false negatives).

2.6 Data analysis

Analysis of the results was performed using Excel 2016 (Microsoft Corp., Redmond, WA) and the micPCR software (BioMolecular Systems, Sydney, Australia). Spatial analysis and

distribution maps were performed using QGIS (QGIS Development Team, 2019). The 95% confidence intervals (95% CI) were calculated using the Wald method.

3. Results

The frequency and distribution of dogs positive for *Ancylostoma* spp. at microscopic evaluation (A) and for *A. caninum* (B), *A. braziliense* (C) and mixed species infections (D) by qPCR in Nigeria is shown in Fig. 1. Data on the distribution of dogs positive for hookworms in each region are listed in Table 1.

Of the 885 animals, a total of 218 dogs (24.6%, 95% CI 21.8-27.6) were positive for hookworm eggs by microscopic evaluation. The multiplex qPCR for the species of canine hookworms successfully amplified and characterized 199 of the 203 microscopy positive samples subjected to qPCR, demonstrating a diagnostic sensitivity of 98% (95% CI 95-99.4). Of these, *A. caninum* was the most common hookworm, detected in 81.3% (165/203, 95% CI 75.3-86.1) of the microscopy-positive dogs, followed by *A. braziliense* which was recorded in 51.2% (104/203, 95% CI 44.4-58) of the microscopy-positive dogs. Single infections with *A. caninum* and *A. braziliense* were found in 46.7% (95/203, 95% CI 40.1-53.7) and 16.8% (34/203, 95% CI 12.2-22.5) of dogs respectively, and mixed infections with both hookworm species was recorded in 35.4% (70/203, 95% CI 28.3-41.3) of microscopy-positive dogs. *Uncinaria stenocephala* and *A. ceylanicum* were not detected in this study. Four of the 203 (2%; 95% CI 0.6-5.1) microscopy-positive samples did not yielded positive to hookworms on qPCR.

The reaction internal control (EHV) successfully amplified in all the 203 samples subjected to qPCR, excluding the presence of PCR inhibitors.

4. Discussion

In this study, we provide comprehensive information on the occurrence and species distribution of zoonotic hookworms infecting dogs in Nigeria. Further, we assessed the suitability of the newly developed high-throughput multiplex qPCR assays for the detection of *A. braziliense* for epidemiological investigations.

Based on classical parasitological methods, 24.6% of dogs from Nigeria were diagnosed with hookworm eggs in their faeces. Previous reports identified canine hookworms in 14-70% of dogs in Nigeria through microscopy-based methods, with *A. caninum* (14-70%) being the most common hookworm, followed by *A. braziliense* (12.8%) and *U. stenocephala* (0.4-2.5%; Anene et al., 1996; Abraham & Gloria, 2009, Edosomwan & Chinweuba, 2012; Idika et al., 2017; Moro & Abah, 2019; Mustapha et al., 2016; Sowemimo, 2009). However, the characterization of these species of canine hookworms relied solely on the morphological identification of the eggs without any further molecular evidence.

Recently a high-throughput qPCR developed to accurately detect and characterize species of canine hookworms (Massetti et al., 2020) was analytically and diagnostically validated for the simultaneous detection of *A. caninum, A. ceylanicum, U. stenocephala* and *A. braziliense*. This novel multiplex qPCR was further validated with field samples for all canine hookworm species but *A. braziliense* due to a lack of occurrence of this hookworm species in the previous geographical areas investigated. Nevertheless, the qPCR showed a high analytical sensitivity for *A. braziliense* being able to detect up to 0.00058 ng of genomic DNA. In the present study, we

183 detected *A. braziliense* in 51.2% of the microscopy-positive samples, demonstrating the
184 reliability of this assay to detect this hookworm species in field samples. The diagnostic
185 sensitivity of the assay compared to faecal floatation and microscopy was 98%, similar to that of
186 Massetti et al. 2020 (i.e. 97%).
187 Only microscopy-positive samples were available to be subjected to qPCR analysis, therefore the
188 diagnostic specificity of the assay could not be assessed. Furthermore, given that the prevalence
189 of canine hookworms was estimated solely on microscopy, it is likely that this study might have
190 underestimated the true prevalence of these parasites (Hii et al., 2018; Massetti et al., 2020). The
191 characterisation of the canine hookworm species by qPCR was only estimated for 203 of the
192 218 microscopy-positive samples received by University of Melbourne, therefore the true
193 proportion of hookworm species in dogs may be negligibly different. A small number of
194 samples were qPCR-negative and microscopy positive as a likely result of human error in
195 identifying hookworm eggs at microscopy or slightly lower sensitivity of the qPCR
196 compared to the microscopy. However, further studies are required to assess the analytical
197 sensitivity (limit of detections) of the qPCR against the intensity of hookworm eggs in
198 faeces. The reaction internal control (Equine Herpes Virus) successfully amplified with
199 consistent cycle threshold values in all the reactions, thereby excluding PCR inhibition as a
200 cause of false negative results.
201 Through the application of this novel multiplex qPCR assay we confirmed the presence of the
202 zoonotic hookworm *A. braziliense* in Nigerian dogs. This hookworm species was previously
203 reported from dogs in Nigeria in microscopy-based investigation (Abraham & Gloria, 2009;
204 Nwoha & Ekwuruike, 2010). However, morphological identification of the eggs alone is
205 insufficient to characterise hookworms at species level (Lucio-Forster et al., 2012). Therefore,

in this study, we confirm the occurrence of *A. braziliense* in its canine reservoir host in Nigeria using a highly specific molecular-based assay.

Although *A. caninum* was the most common hookworm infecting dogs in Nigeria (81.3% of the hookworm micrsocpy-positive samples), *A. braziliense* was detected in more than half (51.2%) of the microscopy positive samples. Several cases of human cutaneous larva migrants (CLM) have been described in Nigeria from locals or travellers who presented serpiginous and itchy skin lesions after returning from holidays (Ogunbanjo and Edungbola, 1989; Obunge and Onyejepu, 2008; Perez Lopez et al., 2017). Since infections are usually associated with people walking barefoot and lying or sitting in areas where soil is contaminated with dog faeces (Schantz, 2002), an increase in the dog population in regions endemic for zoonotic agents may also result in an increased risk of infection for humans (Chen et al., 2012; Colella et al., 2020). Therefore, the steady increase in the number of dogs in Nigeria - where these animals are also used for security, breeding and hunting purposes - may also represent an emerging zoonotic threat, exacerbated by poverty and poor hygienic conditions (Oboegbulem & Nwakonobi, 1989; Hambolu et al., 2014). Further, the risk for zoonotic infections may be favoured by the lack of awareness of Nigerian dog owners on the zoonotic risks posed by parasites (Ugbomoiko et al., 2008).

5. Conclusion

This study demonstrates that *A. braziliense* is endemic in Nigeria and poses a potential zoonotic threat to locals and travellers. The multiplex qPCR was demonstrated to be a valid and accurate tool for the surveillance of hookworms on a large scale and for the diagnosis and characterization of *A. braziliense* and *A. caninum* under field conditions.

Conflict of interest statement

The authors have no financial, personal or professional interests that could be construed to have influenced the here presented work.

Data availability statement

All data are available within this manuscript.

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Figure Legend

Figure 1. Distribution of dogs positive for hookworms by microscopy (A) and for *Ancylostoma caninum* (B) *Ancylostoma braziliense* (C) and mixed-species infections by qPCR in Nigeria.

Table 1. Distribution of *Ancylostoma* spp. positive dogs in each of the Nigerian states sampled.

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State	Sample size (%)	Hookworm microscopy positive (%; CI 95%)	*Ancylostoma braziliense** (%, CI 95%)	*Ancylostoma caninum** (%; CI 95%)	Mixed hookworm species infections (%, CI 95%)
Plateau	64 (7.2)	19 (29.7; 19.9-41.8)	2 (3.1; 0.23-11.3)	10 (15.6; 8.5-26.6)	4 (6.3; 2-15.4)
Benue	26 (2.9)	2 (7.7; 1-2.5)	1 (3.9; 0.01-20.4)	0	1 (3.9; 0-20.5)
Federal Capital Territory	146 (16.5)	18 (12.3; 7.8- 18.7)	1 (0.68; 0.014.2)	12 (8.22; 4.6-13.9)	4 (2.7; 0.8-7.1)
Kaduna	111 (12.5)	18 (12.3; 7.8- 18.7)	7 (6.3; 2.9-12.7)	6 (5.4; 2.2-11.5)	5 (4.5; 1.7-10.4)
Oyo	21 (2.4)	7 (33.3; 17-54.8)	0	5 (23.8; 10.2-45.5)	2 (9.5; 1.5-3)
Lagos	119 (13.4)	16 (13.4; 8.3-20.8)	0	9 (7.6; 3.9-13.9)	4 (3.4; 1-8.6)
Borno	103 (11.6)	8 (7.8; 3.8-14.8)	0	0	8 (7.8; 3.8-14.8)
Bauchi	65 (7.3)	55 (84.6; 73.8-91.6)	15 (23.1; 14.4-34.8)	6(9.2; 4-19)	26 (40; 29-52.1)
Kebbi	74 (8.4)	33 (44.6; 33.8-55.9)	8 (10.8-5.3-20.2)	5(6.8; 2.6-15.2)	16 (21.6; 13.7-32.4)
Akwa Ibom	60 (6.8)	31 (51.7; 39.3-63.8)	0	31 (51.7; 38.3- 63.8)	0
Abia	42 (4.7)	5 (11.9; 4.7-25.5)	0	5 (11.9; 4.7-25.5)	0
Rivers	54 (6.1)	6 (11.1; 4.8-22.6)	0	6 (11.1; 4.8-27.3)	0
Total	885	218	34	95	70

* Single species infection

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