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Research paper

Molecular characterization of *Cryptosporidium* and *Giardia* in farmers and their ruminant livestock from the Coastal Savannah zone of Ghana

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ABSTRACT

Cryptosporidium and *Giardia* are major causes of diarrhoea in developing countries including Ghana, however, nothing is known about the species and subtypes of *Cryptosporidium* and *Giardia* in farmers and their ruminant livestock in this country. A total of 925 faecal samples from humans (n = 95), cattle (n = 328), sheep (n = 217) and goats (n = 285), were screened for *Cryptosporidium* and *Giardia* by quantitative PCR (qPCR) at the 18S rRNA and glutamate dehydrogenase (*gdh*) loci respectively. *Cryptosporidium* positives were typed by sequence analysis of 18S and 60 kDa glycoprotein (*gp60*) loci amplicons. *Giardia* positives were typed at the triose phosphate isomerase (*tpi*), beta-giardin (bg) and *gdh* loci. The prevalence of *Cryptosporidium* and *Giardia* by qPCR was 8.4% and 10.5% in humans, 26.5% and 8.5% in cattle, 34.1% and 12.9% in sheep, and 33.3% and 12.3% in goat faecal samples, respectively. *G. duodenalis* assemblages A and B were detected in humans and assemblage E was detected in livestock. *Cryptosporidium parvum* was the only species identified in humans; *C. andersoni*, *C. bovis*, *C. ryanae* and *C. ubiquitum* were identified in cattle; *C. xiaoi*, *C. ubiquitum* and *C. bovis* in sheep; and *C. xiaoi*, *C. baileyi* and *C. parvum* in goats. This is the first molecular study of *Cryptosporidium* and *Giardia* in livestock in Ghana. The identification of zoonotic species and the identification of *C. parvum* subtype IIcA5G3q in livestock, which has previously been identified in children in Ghana, suggests potential zoonotic transmission. Further studies on larger numbers of human and animal samples, and on younger livestock are required to better understand the epidemiology and transmission of *Cryptosporidium* and *Giardia* in Ghana.

1. Introduction

Cryptosporidium and *Giardia* are enteric protozoan parasites known to cause diarrhoea and other clinical symptoms in many mammals including humans (Xiao, 2010; Ryan and Cacciò, 2013). Both protozoans are included in the WHO “Neglected Diseases Initiative” (Savioli et al., 2006) and *Cryptosporidium* is considered the second greatest cause of diarrhoea and death in children in developing countries after rotavirus (Kotloff et al., 2013; Striepen, 2013). *Giardia duodenalis* is estimated to cause 280 million cases of gastroenteritis per annum worldwide (Lane and Lloyd, 2002), with high infection rates in developing countries including Africa and Asia (Feng and Xiao, 2011).

In livestock, *Cryptosporidium* and *Giardia* cause high morbidity and mortality, particularly in young animals, leading to significant economic losses (Olson et al., 2004; Noordeen et al., 2012). Infection in humans may be acquired through direct contact with infected persons (person-to-person transmission) or animals (zoonotic transmission), or through ingestion of contaminated food (foodborne transmission) (Xiao, 2010; Ryan and Cacciò, 2013). As both parasites shed environmentally robust oo/cysts in faeces, that are resistant to disinfectants used in water treatment, water is also a major mode of transmission (Baldursson and Karanis, 2011; Duhain et al., 2012).

Currently, 33 *Cryptosporidium* species have been recognized and of these > 17 have been identified in humans (Jezkova et al., 2016; Ryan et al., 2016). By far the most common species reported in humans worldwide are *C. parvum* and *C. hominis* (Xiao, 2010). *Giardia duodenalis* is the species which infects mammals and is composed of at least eight assemblages (A to H), with assemblage A in humans, livestock and other mammals; B in humans, primates and some other mammals; C and D in dogs and other canids; E mainly in ungulates including cattle, sheep and goats; F in cats; G in rats; and H in marine mammals (Ryan and Cacciò, 2013).

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Both *Cryptosporidium* and *Giardia* are recognized as important causes of diarrhoea in children and HIV/AIDs patients in Ghana (cf. Squire and Ryan, 2017). Little however, is known about the molecular epidemiology of these diseases in humans and livestock in Ghana, with genotyping studies to date confined to children (Anim-Baidoo et al., 2015; Eibach et al., 2015; Anim-Baidoo et al., 2016). Therefore, the aim of the present study was to determine the species and subtypes of *Cryptosporidium* and *Giardia* infecting farmers and their ruminant livestock in Ghana to better understand the transmission dynamics of these parasites in this country.

2. Materials & methods

2.1. Ethics statement

All participants enrolled in this study signed written and where appropriate oral informed consent prior to their participation, with the assistance of a translator. Approval and ethics clearance for this study was obtained from Murdoch University human (permit number: 2014/135) and animal ethics (permit number R2683/14) committees and the Ghana Council for Scientific and Industrial Research (CSIR) Institutional Review Board and Animal Care and Use Committee (permit number PRN 002/CSIR-IACUC/2014).

2.2. Study area and design

The study was carried out in Ghana in the Coastal Savannah agroecological zone. This is one of the six main recognized agroecological zones in Ghana, which lies along the coastal belt located in the southern part of the country (Oppong-Anane, 2006). Similar to most parts of the country, this region has a binomial rainfall pattern made up of major and minor rainy seasons. It covers about 20,000 km^2 of the total 238,539 km^2 land area of the country and encompasses much of the Accra, Keta and Ho Plains noted for livestock rearing (Oppong-Anane, 2006). Three of the country's ten administrative regions, the Central, Greater Accra and Volta lie fully or partially within the zone. Each region is subdivided into districts and two districts noted for livestock production were purposely selected from each region for the study with the help of Officers from relevant Regional and District Ministry of Food and Agriculture. Livestock in this study area, with the exception of a few cattle farms, were usually kept in or around households (within communities) and were predominantly local breeds (Sanga cattle and West African Dwarf goats and sheep). Sample collection for this cross-sectional survey was conducted from October 2014 to February 2015. Sample size calculations were conducted using EpiTools (http://epitools.ausvet.com.au), based on the estimated livestock population for the zone (SRID, 2011) and existing reports of a *Cryptosporidium* prevalence of 29% in cattle from Ghana (Squire et al., 2013), and a prevalence of 16% and 24% in sheep and goats respectively from Nigeria (Pam et al., 2013).

2.3. Study population, sample collection and analysis

The study population consisted of cattle, sheep and goats and the respective farmers from the Greater Accra (Shai Osudoku and Kpong Katamanso District), Central (Awutu Senya and Komenda Edina Eguafo Abirem Districts) and Volta (North Tongu and Central Tongu Districts) regions of the Coastal Savannah zone. A total of 925 faecal samples were collected from 95 farmers and 830 animals including cattle (n = 328), sheep (n = 217) and goats (n = 285). For human samples, coded clean stool containers with screw caps were given to the farmers with specific instructions on how to collect and submit their stool samples. For animals, stool samples were collected directly from the rectum of each animal into individually labelled airtight containers using sterile latex gloves to prevent cross contamination between samples. Faecal consistency scores (FCS) of animals were recorded using a scale of 1 (dry pellets) to 5 (liquid or fluid faeces) as previously described (Greeff and Karlsson, 1997) and body condition scores (BCS) using a scale of 1 (emaciated) to 5 (very fat) (Sutherland et al., 2010). Information on host, sex, age group, location and farm management system were also recorded. All samples were transported under appropriate conditions to the Animal Research Institute laboratory in Accra, Ghana and stored at 4 °C from 6 to 28 days prior to DNA extraction.

2.4. DNA isolation

Genomic DNA was extracted from 250 mg of each faecal sample using a PowerSoil DNA purification Kit (MolBio, Carlsbad, California) with some minor modifications as described by Yang et al. (2015), at the CSIR-Animal Research Institute laboratory in Accra, Ghana. Briefly, faecal samples for DNA extraction were subjected to four cycles of freeze and thaw in liquid nitrogen followed by boiling water to ensure efficient lysis of oocysts, before being processed using the manufacturer's protocol. DNA was shipped to Murdoch University, Australia for molecular analysis under Australian Quarantine and Inspection Service (AQIS) import permit IP14015324.

2.5. PCR amplification of Cryptosporidium

All DNA samples were screened at the 18S rRNA locus for *Cryptosporidium* using a quantitative PCR (qPCR) as previously described (Yang et al., 2014a). Each 15 µl PCR mixture contained, 1 × Go Taq PCR buffer (KAPA Biosystems), 5 mM $MgCl_2$, 1 mM dNTP's, 1.0 U Kapa DNA polymerase (MolBio, Carlsbad, CA), 0.2 µM each of forward and reverse primers (18SiF and 18SiR), 50 nM of the probe and 1 µl of sample DNA. The PCR cycling conditions consisted of a pre-melt at 95 °C for 10 min and then 45 cycles of 95 °C for 30 s (melt) and 60 °C for 1 min (annealing and extension) on a Rotor Gene Q (Qiagen). Samples that were positive by qPCR, were amplified at the 18S locus using a nested PCR as described by Silva et al. (2013) to identify the species. The PCR reaction volume of 25 µl contained 2.5 µl of 10 × Kapa PCR buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP's, 100 nM of each primer, 1 unit of Kapa Taq (Geneworks, Adelaide, SA) and 1 µl of DNA (about 50 ng). For samples that did not produce an amplification product, the PCR reaction was repeated multiple times by including 5% (DMSO), 2 µl of DNA as well as 1 µl of a 1:10 dilution of DNA. The PCR conditions at the 18S locus consisted of an initial cycle of 94 °C for 3 min, 40 cycles (94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min) and a final extension of 72 °C for 7 min for the primary amplification. Similar conditions were used for the secondary reaction except the annealing temperature was 55 °C and 45 instead of 40 cycles. Subtyping of *C. parvum* and *C. ubiquitum* was performed by sequence analysis of a fragment of the 60 kDa glycoprotein (*gp60*) gene (Zhou et al., 2003; Li et al., 2014). The nested PCR cycling conditions for the *gp60* gene were the same as for the 18S nested PCR described above. Approximately 20% of samples that were qPCR positive but negative by nested PCR, were randomly selected and spiked with 1 µl of *Cryptosporidium* positive control DNA and then re-amplified to test for inhibition.

2.6. PCR amplification of G. duodenalis

Samples were screened at the glutamate dehydrogenase (*gdh*) locus for *Giardia* using quantitative PCR (qPCR) as previously described (Yang et al., 2014c). Each 15 µl PCR mixture contained, 1 × Go Taq PCR buffer (KAPA Biosystems), 5 mM MgCl2, 1 mM dNTP's, 1.0 U Kapa DNA polymerase (MolBio, Carlsbad, CA), 0.2 µM each of forward and reverse primers (gdh F1 and gdh R1), 50 nM of the probe and 1 µl of sample DNA. The PCR cycling conditions consisted of a pre-melt at 95 °C for 3 min and then 45 cycles of 95 °C for 30 s, and a combined annealing and extension step of 60 °C for 45 s. All qPCR positive