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

Sylvia Afriyie Squire[a,b], Rongchang Yang[a], Ian Robertson[a,d], Irene Ayi[c] , Una Ryan[a]

[a]School of Veterinary and Life Sciences, Murdoch University, 90 South Street, Murdoch, Western Australia, Australia

[b]Council for Scientific and Industrial Research-Animal Research Institute, P. O. Box AH 20, Achimota, Accra, Ghana

[c]Department of Parasitology, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, P. O. Box LG 581, Legon, Accra, Ghana

[d]China-Australia Joint Research and Training Center for Veterinary Epidemiology, Huazhong Agricultural University, Wuhan, China

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 (b*g*) 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.

Keywords: Cryptosporidium; Giardia; Ghana; Livestock; Farmers; Genotyping

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).

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 samples were further analysed by nested PCR of the *gdh*, beta-giardin (*bg*), and triose-phosphate isomerase (*tpi*) genes using primers and protocols previously described (Cacciò et al., 2002; Sulaiman et al., 2003; Read et al., 2004; Lalle et al., 2005; Cacciò et al., 2008; Geurden et al., 2008; Levecke et al., 2009; Hijjawi et al., 2016). Similar PCR reaction volumes as stated for *Cryptosporidium* at the 18S locus was used for both *gdh* and beta-giardin gene amplification with different PCR conditions as follow; For the *gdh* gene, PCR conditions for both primary and secondary reactions was performed with an initial cycle of 94 °C for 3 min, 48 cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min) and a final extension of 72 °C for 7 min. The PCR condition for the beta-giardin gene, consisted of an initial cycle (94 °C for 2 min, 65 °C for 1 min and 72 °C for 2 min),

40 cycles (94 °C for 30 s, 65 °C for 1 min and 72 °C for1 min) and a final extension of 72 °C for 7 min for the primary reaction. The PCR conditions were similar for the secondary reaction except for a lower annealing temperature of 55 °C. For the tpi gene, the primary PCR reactions had an initial cycle of 94 °C for 5 min, 45 cycles (94 °C for 45 s, 50 °C for 45 s and 72 °C for1 min) and an extension of 72 °C for 10 min. The secondary reactions were performed using assemblage specific primers with annealing temperatures; 64 °C for assemblage A, 62 °C for assemblage B and 67 °C for assemblage E. As with *Cryptosporidium*, a subset (~ 20%) of samples that were qPCR positive but negative by nested PCR were randomly selected and spiked with a 1 µl of *Giardia* positive control DNA and then re-amplified to test for inhibition.

2.7. Sequencing

The amplified DNA fragments from the secondary PCR products were separated by gel electrophoresis and purified using an in-house filter tip method and used for sequencing in both directions without any further purification as previously described (Yang et al., 2013). Sequence searches were conducted using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences were analysed and aligned with reference sequences from GenBank.

2.8. Statistical analysis

Prevalence was estimated as a percentage of positive samples with 95% confidence intervals calculated using the exact binomial method (Ross, 2003). Odds ratios were calculated using Woolf's Method (Kahn and Sempos, 1989). Chi-square and Fisher's Exact tests for independence were used to determine the associations between prevalence and various categorical data (host, sex, age group, location, farm management system, FCS and BCS) using SPSS 21 for windows (Statistical Package for the Social Sciences) (SPSS Inc. Chicago, USA). P-values < 0.05 were considered statistically significant. The numbers of *Cryptosporidium* and *Giardia* oocysts per gram of faeces in positive samples were also determined by qPCR as previously described (Yang et al., 2014b; Yang et al., 2014c).

3. Results

3.1. Prevalence of Cryptosporidium and Giardia in livestock farmers

The prevalence of *Cryptosporidium* and *G. duodenalis* in the farmers was 8.4% (8/95, 95% CI 3.7–15.9) and 10.5% (10/95, 95% CI 5.2–18.5) by qPCR at the 18S rRNA and *gdh* loci respectively (Table 1 and Supplementary Table 1). A total of 17.9% (n = 17, 95% CI 10.8–27.1) out of the 95 human samples were positive for at least one protozoan; 7.4% (7/95) for *Cryptosporidium* only, 9.5% (9/95) for *Giardia* only and 1.1% (1/95) had mixed infections. There was no significant difference between the prevalence of *Cryptosporidium* and *Giardia* among the different age groups or sexes. Infection did not cause poor faecal consistency, as *Cryptosporidium* prevalence was significantly higher (24.1%, $p = 0.008$) in farmers with formed faecal samples. Unlike the Greater Accra and Volta regions, none of the farmers from the Central region tested positive for *Cryptosporidium* (Table 1). Oo/cyst numbers for human qPCR positive samples ranged from 393 to 1.4×10^5 oocysts/g of faeces (median = 586 oocysts/g) and 52 to 2.8×10^6 cysts/g (median = 244 cysts/g) for *Cryptosporidium* and *Giardia*, respectively.

3.2. Prevalence of Cryptosporidium and Giardia in livestock

Table 2 provides a summary of the prevalence of *Cryptosporidium* and *Giardia* in livestock as categorized by livestock species, district, management type, faecal consistency score (FCS) and body condition score (BCS). Overall, 35.4% (294/830, 95% CI 32.2–38.8) of the livestock tested positive for at least one protozoan by qPCR at the 18S and *gdh* loci; 24.5% (203/830) for *Cryptosporidium* only, 4.6% (38/830) for *Giardia* only and 6.4% (53/830) for mixed infections. The prevalence of *Cryptosporidium* and *G. duodenalis* in livestock was 30.8% (256/830, 95% CI 27.7–34.1) and 11.0% (91/830, 95% CI 8.9–13.3) respectively. The prevalence of *Cryptosporidium* and *Giardia* were both associated with the location of livestock (district) and FCS ($p < 0.05$), but not sex, BCS or farm management systems.

The prevalence of *Cryptosporidium* species was higher in sheep, 34.1% (95% CI 27.8–40.8), and goats 33.3% (95% CI 27.9–39.1), compared to cattle, 26.5% (95% CI 21.8–31.7), but this was not

statistically significant (p = 0.092). Similarly, *Giardia* prevalence was higher in sheep, 12.9% (95% CI 8.7–18.1) and goats, 12.3% (95% CI 8.7–16.7), than in cattle, 8.5% (95% CI 5.7–12.1) but again was not statistically significant (p = 0.190). However, among the different livestock species, both goats and sheep were slightly at more risk of infection with *Cryptosporidium* and *Giardia* compared to cattle (p = 0.024) (Table 2).

Cryptosporidium and *Giardia* positives were found in all six districts, however, the prevalence of *Cryptosporidium* was highest in the North Tongu district (Volta Region) (53.0%, 95% CI 43.7–60.3) and Shai Osudoku (50.0%, 95% CI 41.8–58.2) and Kpong Katamanso districts (Greater Accra Region) (26.6%, 95% CI 19.1–35.1) (Table 2). In addition, livestock from the Shai Osudoku and North Tongu districts were over five times more likely to be infected with *Cryptosporidium* and *Giardia* (OR 10.25, 95% CI 5.54–18.91 and OR 6.36, 95% CI 3.48–11.63, respectively) than those from the Awutu Senya district. Similarly, the prevalence of *Giardia* was highest in livestock from the North Tongu district (29.1%, 95% CI 21.9–37.1) (Table 2).

Cryptosporidium prevalences seemed to increase with increasing FCS with the highest prevalence (69.6%, 95% CI 47.1–86.6) in livestock with a FCS of 5 (fluid/liquid faeces) (p < 0.001). Animals with fluid/liquid faeces also had a higher prevalence of *Giardia* (39.1%, 95% CI 19.7–61.5) and were more than four times at risk of being infected with both *Cryptosporidium* and *Giardia*, than livestock with a FCS of 1 (firm balls/hard pellets) (OR 4.54, 95% CI 1.75–11.77) (Table 2). *Cryptosporidium* oocyst numbers in livestock ranged from 42 to 2.9×10^7 oocysts/g of faeces (median: 1.3×10^4 oocysts/g) and *Giardia* cyst numbers ranged from 47 to 4.6×10^7 cysts/g of faeces (median: 690 cysts/g) as determined by qPCR.

3.3. Prevalence of Cryptosporidium and Giardia in different age groups of livestock

In general, the prevalence of *Cryptosporidium* and *Giardia* in particular seemed to reduce with increasing age, but this was not significant for *Cryptosporidium* (p = 0.104 and p = 0.007, respectively) (Table 3). The prevalence of *Giardia* was higher in younger livestock (0–12 months old) (14.5%, 95% CI 11.1–18.3), compared to older animals (> 24 months old) (7.0%, 95% CI 4.3–10.7)

(Table 3). However, there was no association ($p > 0.05$) between *Giardia* prevalence and different age groups of cattle and goats. In sheep, *Giardia* prevalence was higher ($p = 0.027$) in lambs 0–12 months old (19.1%, 95% CI 12.0–27.9), than in 13–24 month old sheep (5.3%, 95% CI 1.1–14.6). There was no significant difference in *Cryptosporidium* prevalences among the various age groups of cattle, sheep and goats (Table 3).

3.4. Distribution of Cryptosporidium spp. and Giardia assemblages in farmers, livestock and different locations

Of the 264 *Cryptosporidium* qPCR positives, only 79 samples were successfully genotyped at the 18S locus, although a spike analysis of ~ 20% of negative samples indicated no evidence of inhibition. Seven *Cryptosporidium* species were identified; *C. xiaoi*(44), *C. bovis* (19), *C. ryanae* (7), *C. ubiquitum* (4), *C. andersoni*(2), *C. baileyi* (1) and C. *parvum* (2) (Table 4). *Cryptosporidium parvum* was identified in one positive farmer, four species (*C. andersoni*, *C. bovis*, *C. ryanae* and *C. ubiquitum*) were identified in cattle, three (*C. bovis*, *C. ubiquitum* and *C. xiaoi*) in sheep and three (*C. baileyi*, *C. parvum* and *C. xiaoi*) in goats (Table 4). The 18S nucleotide sequences for the *Cryptosporidium* species identified have been submitted to GenBank under accession numbers KY711393 - KY711403. With the exception of *C. xiaoi* (KY711402) identified in sheep which showed 99.8% similarity with *C. xiaoi* (KY055408) from the GenBank with a single nucleotide polymorphism, all the 18S nucleotide sequences (KY711393-KY71143) showed 100% similarity to corresponding species nucleotide references acquire from the GenBank (*C. andersoni* - KX259131, *C. baileyi* - KY352489, *C. bovis* - MF074602, *C. parvum* - MF353928, *C. ryanae* - KX668207 *C. xiaoi* - KY055408 and *C. ubiquitum* - KT027455).

All *C. ubiquitum* and *C. andersoni* positives were from livestock in the Volta region. The remaining *Cryptosporidium* species, with the exception of *C. parvum* and *C. baileyi* that were identified in goats from the Greater Accra Region, were spread throughout all three regions within the study area.

Subtyping of *C. parvum* and *C. ubiquitum* at the *gp60* locus identified *C. parvum* subtype IIcA5G3q from a goat which was 100% identical to subtype IIcA5G3q (GenBank accession numbers KM539028, KM539034, KM539035 and KM539041), previously isolated from children in Ghana. *Cryptosporidium ubiquitum* subtype XIIa was identified from one sheep and one of the cattle and both sequences (KY711404 - KY711406) were 100% identical to *C. ubiquitum* subtype XIIa (JX412915) from GenBank. Unfortunately, subtyping of the single *C. parvum* from the positive farmer and the remaining two *C. ubiquitum* positive samples (one from cattle and one from sheep) at the *gp60* locus was not successful.

For *Giardia*, of the 101 qPCR positives, 34 were successfully typed; 5 from farmers and 29 from livestock using a combination of three loci (*gdh*, *bg* and *tpi*) (Table 4). Again, spike analysis indicated no evidence of inhibition. All 29 positives from livestock were typed as assemblage E (*tpi* only = 4; *gdh* and *bg* = 2; *bg* and *tpi* = 8; *gdh*, *bg* and *tpi* = 15). Three of the positives from farmers were typed as assemblage A (*tpi* only-1, *gdh* and *tpi* = 1, *gdh*, *bg*and *tpi* = 1), all of which were 100% identical to subtype AII (EF507673) and the remaining two (isolates 262 and 658) were assemblage B (*gdh* and *tpi* = 1 and *gdh*, *bg* and *tpi* = 1). At the *gdh* locus, they exhibited 99% similarity to both subtype BIV (EF507665) (5 and 4 single nucleotide polymorphisms, SNP's, respectively) and subtype BIII (KX228242) (9 and 5 SNPs). *Giardia* nucleotide sequences have been submitted to GenBank under accession numbers KY711407 - KY711417. Detailed data on qPCR Ct values, genotyping data etc. are provided in Supplementary Table 1.

4. Discussion

This is the first molecular study of *Cryptosporidium* and *Giardia* species in livestock and respective farmers in Ghana. The prevalence of *Cryptosporidium* and *Giardia* in the 95 farmers screened (8.4% and 10.5% respectively), was within the range of previous prevalence reports for *Cryptosporidium* (1.0% to ~ 38%) and *Giardia* (4.5% to ~ 9%) in both children and adults in Ghana (cf. Squire and Ryan, 2017). Similarly, the prevalences of *Cryptosporidium* in cattle (26.5%),

sheep (34.1%) and goats (33.3%), were within the range of prevalences reported previously in livestock in Africa and globally (< 5–70%) (Robertson, 2009; Squire et al., 2013; cf. Squire and Ryan, 2017). The prevalence of *Giardia* in cattle (8.5%), sheep (12.9%) and goats (12.3%) was also within the range of previous studies (Robertson, 2009; Geurden et al., 2010; cf. Squire and Ryan, 2017).

Both protozoans were more prevalent in livestock with fluid/liquid faeces (FCS of 5) and this difference was significant. *Cryptosporidium* and *Giardia* are known to cause morbidity and other clinical symptoms including diarrhoea in livestock, particularly young animals (Olson et al., 2004; Noordeen et al., 2012). This suggests that *Cryptosporidium* and *Giardia* may have a clinical impact on infected livestock by causing diarrhoea, however confounding factors including infection with other pathogens and diet need to be examined. Neither protozoan appeared to be associated with reduced BCS in livestock, farm management systems and sex, however, further studies using a larger number of samples is required to determine the clinical impact of *Cryptosporidium* and *Giardia* in livestock in Ghana.

In contrast to previous studies (e.g. Zhang et al., 2015; Li et al., 2016), there was no significant difference in the prevalence of *Cryptosporidium* in different age groups of animals but overall, *Giardia* prevalence was significantly higher ($p = 0.007$) in younger animals (0–12 months old) and was also higher in sheep 0–12 months old, but not in cattle and goats.

In the present study, only ~ 30–34% of the *Cryptosporidium* and *Giardia* qPCR positives were successfully typed. Why this is so is unclear, as spike analysis indicated no evidence of inhibition but could be due to the fact that (1) qPCR is much more sensitive than conventional PCR (Hadfield et al., 2011), (2) the size of the amplicons generated, as the qPCR amplicon sizes for *Cryptosporidium* and *Giardia* were 298 bp and 261 bp respectively, whereas the secondary amplicon size for the 18S nested PCR for *Cryptosporidium* was 611 bp and ranged from 332 to 743 bp for *Giardia*. It is well known that shorter amplicons amplify much more efficiently than longer amplicons (Shagin et al., 1999). It is also possible that some of the qPCR positives were due to non-specific amplification, however both qPCR assays have been extensively validated (Yang et al., 2014a, 2014c). Although samples were stored at 4 °C, analysed within 28 days of collection and spike

analysis showed no evidence of inhibition, the presence of inhibitors and degradation of DNA during storage cannot be ruled out.

In the current study, C. *parvum* was identified from one farmer, but unfortunately could not be subtyped. Previous genotyping studies in children from the Greater Accra Region identified *C. parvum*only (Anim-Baidoo et al., 2015), while both *C. parvum* (42.1%) and *C. hominis* (58.0%) were identified in *Cryptosporidium*-positive children from the Ashanti Region (outside the present study area) (Eibach et al., 2015). In the latter study, *C. parvum* (IIc, IIe) and *C. hominis* (Ia, Ib, Id and Ie) *gp60* subtype families were identified, with subtypes IIcA5G3q, IbA13G3 and IaA21R3, being the most frequent (Eibach et al., 2015).

This is the first molecular study of *Cryptosporidium* and *Giardia* species in livestock in Ghana and the single *C. parvum* from a goat was typed as IIcA5G3q. The IIc subtype family was previously thought to be anthropologically transmitted only, but subtype IIcA5G3j has recently been detected in hedgehogs (*Erinaceus europaeus*) in Germany and the UK (Dyachenko et al., 2010; Sangster et al., 2016). Subtype IIcA5G3 was also reported in hedgehogs in Holland, but as sequences were not submitted to GenBank, it is not possible to obtain more information (Krawczyk et al., 2015). The present study is the first report of the IIc subtype in livestock and the fact that identical subtypes (IIcA5G3q) were detected in livestock and in children from Ghana (KM539028, KM539034, KM539035 and KM539041) (Eibach et al., 2015), suggests potential transmission between goats and humans in Ghana. Further studies however are required to confirm this.

Cryptosporidium bovis was the most commonly detected species in cattle (62.1%) followed by *C. ryanae* (24.1%), *C. andersoni* and *C. ubiquitum* (6.9% each). This is similar to results of previous studies in cattle from Nigeria (Maikai et al., 2011), with the exception of *C. ubiquitum*, which was not reported in cattle in that study. *Cryptosporidium parvum* was not detected in cattle in the present study, which may have been due to the ages of the cattle sampled, as the majority of cattle in the 0–12 month category were approximately 12 months old (data not shown). Previous studies have shown that the prevalence of *C. parvum* is much higher in pre-weaned calves (0–2 months old) (Santin et al.,

2008). Further studies on faecal samples from cattle of all ages, in a wider range of locations is required to determine the role that cattle may play in the zoonotic transmission of *Cryptosporidium* in Ghana and West Africa.

In the present study, *C. xiaoi* accounted for 88.9% and 90.9% of infections in sheep and goats, respectively. *Cryptosporidium bovis* (3.7%) and *C. ubiquitum* (7.4%) were also detected in sheep and *C. baileyi* (4.5%) and *C. parvum* (4.5%) were detected in goats. In other African countries including Egypt and Tanzania, *C. xiaoi* was the only species detected in sheep and goats, while *C. parvum* was identified in sheep and goats form Zambia, *C. suis* in sheep from Zambia, *C. bovis* in lambs from Tunisia and *C. ubiquitum* in goats from Algeria (cf. Squire and Ryan, 2017). This is the first report of *C. baileyi* (which commonly infects birds), in an 18 month old goat from the Kpong Katamanso district. The goat was emaciated (BCS of 1) and recovering from a severe diarrhoea outbreak on the farm, that had previously resulted in the loss of a number of goats. Further investigation is however needed to ascertain if this was mechanical transmission from ingesting bird faeces or an actual infection.

Of the species identified in sheep and goats in this study, *C. parvum* and *C. ubiquitum* are the most important in terms of potential zoonotic transmission, as *C. parvum* is the second most common species infecting humans (Xiao, 2010) and *C. ubiquitum*is considered an emerging human pathogen (Li et al., 2014). Subtyping of *C. ubiquitum* identified XIIa in one of the cattle and one sheep, which is a common subtype found in ruminants and humans worldwide and is therefore potentially zoonotic (Li et al., 2014).

Cryptosporidium xiaoi is not considered a major zoonotic species as it has only been reported once in two HIV-positive individuals in Ethiopia (Adamu et al., 2013). Similarly, *C. bovis* is predominantly a parasite of livestock and has only been reported in humans on a few occasions (Khan et al., 2010; Ng et al., 2012). *Cryptosporidium baileyi* is not considered to be of public health significance, as it has not been identified by molecular analysis in humans (Zahedi et al., 2015).

Giardia duodenalis assemblages A and B were detected in the farmers in the present study, which are the main assemblages in humans globally (Ryan and Cacciò, 2013). A previous study identified *G. duodenalis* assemblage B (subtype BIII) in samples from children in Ghana (Anim-Baidoo et al., 2016). In the present study, all 29 typed *Giardia* isolates from livestock belonged to assemblage E, which is commonly reported in livestock in other parts of Africa (cf. Squire and Ryan, 2017). The only other molecular study of animals in Ghana, identified assemblage B in wild Ursine colobus monkeys (Teichroeb et al., 2009). Assemblages A and/or B have been identified in cattle from Egypt, Tanzania and Uganda and goats from Côte d'Ivoire and Tanzania (cf. Squire and Ryan, 2017). Assemblage E and F have been identified in humans in Africa (cf. Squire and Ryan, 2017) and in a recent study in Egypt, assemblage E was detected at a prevalence of 62.5% in human samples (Abdel-Moein and Saeed, 2016), therefore assemblage E should be considered potentially zoonotic.

In conclusion, ruminant livestock in Ghana are hosts to various species of *Cryptosporidium* and to *G. duodenalis* assemblage E, all of which, with the exception of *C. ryanae* and *C. baileyi* are potentially zoonotic and have been previously identified in humans in Africa. This is the first report of *C. parvum* subtype IIcA5G3q in livestock and suggests potential zoonotic transmission in Ghana. Further studies however, 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.

Author contributions

Sylvia Afriyie Squire performed most part of the experiment under the supervision of Rongchang Yang and Una Ryan. Sample collection was done by Sylvia A. Squire under the supervision of Irene Ayi and Una Ryan, data analysis under the supervision of Ian Robertson. All authors particularly, Sylvia A. Squire and Una Ryan contributed to the design of the experiment and writing of the manuscript of this manuscript.

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Potential conflicts of interest

None of the authors have any conflict of interest to report.

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Table 1. Prevalence of *Cryptosporidium* and *Giardia* in faecal samples from livestock farmers by qPCR at the 18S and *gdh* loci respectively.

Category	**Number examined (%)**	***Cryptosporidium***			***Giardia***			**Any protozoan (*Cryptosporidium*and/or *Giardia)***		
		Number positive (%)	**95% CI**	**p-Value**	**Total number positive (%)**	**95% CI**	**p-Value**	**Number positive (%)**	**OR (95% CI)**	**p-Value**
Age group (years)										
≤ 30	34 (35.8)	5 (14.7)	5.0–31.1		5 (14.7)	5.0–31.1		9 (26.5)	2.16 (0.5–9.1)	
31–50	40 (42.1)	2 (5.0)	0.6–16.9	0.258	3 (7.5)	1.6–20.4	0.594	5 (12.5)	0.86 (0.2–4.0)	0.262
> 50	21 (22.1)	1 (4.8)	0.1–23.8		2 (9.5)	1.2–30.4		3 (14.3)	1.00	
Sex										
Female	24 (25.3)	2 (8.3)	1.0–27.0		2 (8.3)	1.0–27.0		4 (16.7)	1.00	
Male	71 (74.7)	6 (8.5)	3.2–17.5	0.675	8 (11.3)	5.0–21.0	0.513	13 (18.3)	1.12 (0.3–2.8)	0.563
Location (region)										
Central	30 (31.6)	0	0–11.6		2 (6.7)	0.8–22.1		2 (6.7)	1.00	
Greater Accra	36 (37.9)	6 (16.7)	6.4–32.8	0.049	4 (11.1)	3.1–26.1	0.665	9 (25.0)	4.67 (0.9–23.6)	0.138
Volta	29 (30.5)	2 (6.9)	0.8–22.8		4 (13.8)	3.9–31.7		6 (20.7)	3.65 (0.7–19.9)	
Faecal consistency										
Watery	1 (1.1)	0	0–97.5		0	0–97.5		0		
Semi-formed	58 (61.1)	1 (1.7)	0–9.2	0.01	6 (10.3)	3.9–21.2	0.936	7 (12.1)	1.00	
Formed	36 (37.9)	7 (19.4)	8.2–36.0		4 (11.1)	3.9–31.7		10 (27.8)	2.80 (0.96–8.21)	0.139
Total	95	8 (8.4)	3.7–15.9		10 (10.5)	5.2–18.5		17 (17.9)		

Table 2. Prevalence of *Cryptosporidium* and *Giardia* in livestock by qPCR (at the 18S and *gdh* loci respectively), as categorized by livestock species, district, management type, faecal consistency score (FCS) and body condition score (BCS).

Category	**Number of samples examined (%)**	***Cryptosporidium* species**			***Giardia duodenalis***			**Any protozoan (*Cryptosporidium* and/or *Giardia*)**		
		Number positive (%)	**95% CI**	**p-Value**	**Number positive (%)**	**95% CI**	**p-Value**	**Number positive (%)**	**OR (95% CI)**	**p-Value**
Livestock										
Cattle	328 (39.5)	87 (26.5)	21.8–31.7		28 (8.5)	5.7–12.1		98 (29.9)	1.00	
Sheep	217 (26.1)	74 (34.1)	27.8–30.8	0.092	28 (12.9)	8.7–18.1	0.19	87 (40.1)	1.57 (1.10–2.25)	0.024
Goat	285 (34.3)	95 (33.3)	27.9–39.1		35 (12.3)	8.7–16.7		109 (38.3)	1.45 (1.04–2.03)	
Sex										
Female	626 (75.4)	195 (31.2)	27.5–34.9		70 (11.2)	8.8–13.9		221 (35.3)	1.00	
Male	204 (24.6)	61 (29.9)	23.7–36.7	0.737	21 (10.3)	6.5–15.3	0.724	73 (35.8)	1.02 (0.73–1.42)	0.901
Location: district (region)										
Awutu Senya (Central)	117 (14.1)	16 (13.7)	8.0–21.3		2 (1.7)	0.2–6.0		17 (14.5)	1.00	
Komenda Edina Eguafo Abirem (Central)	158 (18.9)	31 (19.6)	13.7–26.7		15 (9.5)	5.4–15.2		40 (25.3)	1.99 (1.07–3.74)	
Kpong Katamanso (Greater Accra)	128 (15.4)	34 (26.6)	19.1–35.1	< 0.001	15 (11.7)	6.7–18.6	< 0.001	40 (31.3)	2.67 (1.42–5.05)	< 0.001
Shai Osudoku (Greater Accra)	154 (18.6)	77 (50.0)	41.8–58.2		11 (7.1)	3.6–12.4		80 (52.0)	6.36 (3.48–11.63)	
North Tongu (Volta)	148 (17.8)	77 (52.0)	43.7–60.3		43 (29.1)	21.9–37.1		94 (63.5)	10.24 (5.54–18.91)	

Central Tongu (Volta)	125 (15.2)	21 (16.8)	10.7–24.5		5 (4.0)	1.3–9.1		23 (18.4)	1.33 (0.67–2.63)	
Farm management system										
Extensive	50 (6.0)	10 (20.0)	10.0–33.7		2 (4.0)	0.5–13.7		11 (22.0)	1.00	
Semi-Intensive	742 (89.4)	233 (31.4)	28.1–34.9	0.216	87 (11.7)	9.5–14.3	0.123	270 (36.4)	2.03 (1.02–4.04)	0.119
Intensive	38 (4.6)	13 (34.2)	19.6–51.4		2 (5.3)	0.6–17.7		13 (34.2)	1.84 (0.72–4.75)	
Faecal consistency score										
1 (Firm balls/hard pellets)	385 (46.4)	131 (34.0)	29.3–39.0		41 (10.7)	7.8–14.2		148 (38.4)	1.00	
2 (Soft pellets)	225 (27.1)	54 (24.0)	18.6–30.1		23 (10.2)	6.6–14.9		64 (28.4)	0.64 (0.45–0.91)	
3 (Firm faecal mass)	163 (19.5)	42 (25.8)	19.2–33.2	< 0.001	15 (9.2)	5.2–14.7	0.001	51 (31.3)	0.73 (0.49–1.08)	< 0.001
4 (Soft faecal mass)	35 (4.2)	13 (37.1)	21.5–55.1		3 (8.6)	1.8–23.1		14 (40.0)	1.07 (0.53–2.16)	
5 (Fluid/liquid faeces)	23(2.8)	16 (69.6)	47.1–86.8		9 (39.1)	19.7–61.5		17 (73.9)	4.54 (1.75–11.77)	
Body condition score										
1 (Emaciated)	6 (0.7)	1 (16.7)	0.4–64.1		0 (0)	0–45.9		1 (16.7)	1.00	
2 (Thin)	55 (6.6)	26 (47.3)	33.7–61.2		5 (9.1)	3.0–20.0		26 (47.3)	4.48 (0.49–40.92)	
3 (Average)	384 (46.3)	111 (28.9)	24.4–33.7	0.077	42 (10.9)	8.0–14.5	0.899	130 (33.9)	2.56 (0.30–22.13)	0.313
4 (Heavy)	328 (39.5)	99 (30.2)	25.3–35.5		37 (11.3)	8.1–15.2		116 (35.4)	2.74 (0.32–23.70)	
5 (Very fat)	57 (6.9)	19 (33.3)	21.4–47.1		7 (12.3)	5.1–23.7		21 (36.8)	2.92 (0.32–26.68)	
Total	830	256 (30.8)	27.7–34.1		91 (11.0)	8.9–13.3		294 (35.4)		

Table 3. Prevalence of *Cryptosporidium* and *Giardia* in different age groups of livestock by qPCR at the 18S and *gdh* loci respectively.

Animals and ages (months)	**Number of samples examined (%)**	***Cryptosporidium* spp.**			***Giardia duodenalis***			**Any protozoan (*Cryptosporidium* and/or *Giardia duodenalis*)**		
		Number positive (%)	**95% CI**	**p-Value**	**Number positive (%)**	**95% CI**	**p-Value**	**Number positive (%)**	**OR (95% CI)**	**p-Value**
Cattle	N = 328									
0–12	140 (42.6)	38 (27.1)	20.0–35.3		17 (12.1)	7.2–18.7		44 (31.4)	1.09 (0.66–1.78)	
13–24	33 (10.1)	7 (21.2)	9.0–38.9	0.767	2 (6.1)	0.7–20.2	0.131	8 (24.2)	0.76 (0.32–1.81)	0.718
> 24	155 (47.3)	42 (27.1)	20.3–34.8		9 (5.8)	2.7–10.7		46 (29.7)	1.00	
Sheep	N = 217									
0–12	105 (48.4)	40 (38.1)	28.2–48.1		20 (19.1)	12.0–27.9		48 (45.7)	2.36 (1.17–4.77)	
13–24	55 (25.3)	20 (36.4)	23.8–50.4	0.204	5 (9.1)	3.0–20.0	0.027	24 (43.6)	2.17 (0.98–4.80)	0.046
> 24	57 (26.3)	14 (24.6)	14.1–37.8		3 (5.3)	1.1–14.6		15 (26.3)	1.00	
Goats	N = 285									
0–12	149 (52.2)	49 (32.4)	25.4–41.0		20 (13.4)	8.4–20.0		57 (38.3)	1.30 (0.69–2.47)	
13–24	77 (27.0)	31 (40.3)	29.2–52.1	0.189	8 (10.4)	4.6–19.4	0.8	33 (42.9)	1.58 (0.78–3.21)	0.448
> 24	59 (20.7)	15 (25.4)	15.0–38.4		7 (11.9)	4.9–22.9		19 (32.2)	1.00	
Overall age group	N = 830									
0–12	394 (47.5)	127 (32.2)	27.6–37.1		57 (14.5)	11.1–18.3		149 (37.8)	1.45 (1.04–2.02)	
13–24	165 (19.9)	58 (35.2)	27.9–43.0	0.104	15 (9.1)	5.2–14.6	0.007	65 (39.4)	1.55 (1.03–2.33)	0.044
> 24	271(32.7)	71 (26.2)	21.1–31.9		19 (7.0)	4.3–10.7		80 (29.5)	1.00	

Table 4. Distribution of *Cryptosporidium* species and *G. duodenalis* assemblages in farmers, livestock and different locations in Coastal Savannah zone in Ghana.

Category	***Cryptosporidium* species (n = 79)**								***G. duodenalis* assemblages (n = 34)**			
	***C. andersoni* (%)**	***C. baileyi* (%)**	***C. bovis* (%)**	***C. parvum* (%)**	***C. ryanae* (%)**	***C. ubiquitum* (%)**	***C. xiaoi* (%)**	**Total**	**A (%)**	**B (%)**	**E (%)**	**Total**
Host												
Farmers	0	0	0	1 (100.0)	0	0	0	1	3 (60.0)	2 (40.0)	0	5
Cattle	2 (6.9)	0	18 (62.1)	0	7 (24.1)	2 (6.9)	0	29	0	0	14 (100.0)	14
Sheep	0	0	1 (3.7)	0	0	2 (7.4)	24 (88.9)	27	0	0	12 (100.0)	12
Goat	0	1 (4.5)	0	1 (4.5)	0	0	20 (90.9)	22	0	0	3 (100.0)	3
Location: district (region)												
Awutu Senya (Central)	0	0	2 (40.0)	0	2 (40.0)	0	1 (20.0)	5	0	0	1 (100.0)	1
Komenda Edina Eguafo Abirem (Central)	0	0	2 (18.2)	0	0	0	9 (81.8)	11	0	1 (11.1)	8 (88.9)	9
Kpong Katamanso (Greater Accra)	0	1 (9.1)	2 (18.2)	1 (9.1)	0	0	7 (63.6)	11	1 (14.3)	0	6 (85.7)	7
Shai Osudoku (Greater Accra)	0	0	7 (35.0)	1 (5.0)	1 (5.0)	0	11 (55.0)	20	0	0	3 (100.0)	3
North Tongu (VR)	2 (7.4)	0	4 (14.8)	0	3 (11.1)	2 (7.4)	16 (59.3)	27	2 (18.2)	1 (9.1)	8 (72.7)	11
Central Tongu (Volta)	0	0	2 (40.0)	0	1 (20.0)	2 (20.0)	0	5	0	0	3 (100.0)	3
Total	2 (2.5)	1 (1.3)	19 (24.1)	2 (2.5)	7 (8.9)	4 (5.1)	44 (55.7)	79	3 (8.8)	2 (5.9)	29 (85.3)	34