

Detection of Potentially Zoonotic *Cryptosporidium* and *Giardia* among Livestock in Sariaya, Quezon, Philippines

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ABSTRACT

Livestock plays a great role in the economic development of smallholder farmers. However, the population of livestock has been affected by diseases such as cryptosporidiosis and giardiasis. Sariaya has a large production of livestock that is being distributed in Quezon Province. Thus, the current study aimed to identify the presence of *Cryptosporidium* sp. and *Giardia* sp. in livestock in selected farms of Sariaya, Quezon. Risk factors were also assessed in the present study. A total of 103 collected faecal samples from livestock were subjected to microscopic and molecular detection. Faecal samples were processed through Formalin-Ether Concentration Technique (FECT) and Polymerase Chain Reaction. Microscopy results revealed that 14 out of 103 (13.59%) samples were positive for *Giardia* with meant intensity of 13 cysts per gram (cpg) of faeces, while molecular detection confirmed that 13 out of 103 (12.62%) had amplified for the target gene of *Giardia*, *tpi*, with expected band size of 530 bp. Sequenced samples of *G. intestinalis* were characterised

as assemblages A, B and E. Moreover, pigs showed the highest prevalence (15.91%), while cattle had the highest mean intensity (14 cpg) for *Giardia*. *Cryptosporidium* were absent in all the samples. Statistical analysis of the risk factors such as diet, feeding floor, habit, presence of illness and faecal consistency of the animals revealed no significant association ($p > 0.05$) in the presence of *Giardia*. This study revealed the presence of zoonotic *Giardia* species in the

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area; however, future investigation in other possible risk factors such as the season and age is still recommended.

Keywords: *Cryptosporidium*, *Giardia*, livestock animals, zoonosis

INTRODUCTION

Livestock is one of the major sources of income in the Philippines, which contributes to the development of the country's agricultural and rural livelihood (PlantAsian Agro-forestry Management Systems Inc. [PlantAsian], 2017). The Department of Science and Technology (DOST) (2014) claimed that animal production in the Philippines has been increasing rapidly. In Sariaya, Quezon, where the lands are suited for grazing animals, the predominant livestock consists of carabaos, cattle and pigs. Carabaos are mostly used in land preparation and farm product transportation; cattle are raised for meat and dairy products; while pigs are used for home consumption and additional sources of income (Department of Agriculture-CALABARZON [DA- CALABARZON], 2017).

In 2016, a decline in livestock population was noted as a result of the lowering of inventory in backyards and diseases such as cholera and diarrhea, which raised death rate by 15.17% (Philippine Statistics Authority [PSA], 2016). As was observed, the increasing death rate could have been an effect of diseases such as parasitic infections, resulting in major economic problems. Livestock diseases and parasitic infections have direct (stunting growth,

infertility and death) and indirect (additional costs on vaccines, drugs, labour cost, and profit losses) effects on productivity and could thereby result in major economic implications to the producer (Lamy et al., 2012; Rajakaruna & Warnakulasooriya, 2011).

Giardia and *Cryptosporidium* are two of the neglected parasites that cause parasitic infections. *Giardia intestinalis* assemblages A and B and *Cryptosporidium parvum* subtype IIa, IIc and III are the recorded zoonotic strains of the two parasites (Feng & Xiao, 2011; Hunter & Thompson, 2005). Both parasites can be transmitted directly, from host to host, or indirectly, through ingestion of contaminated food or water (Lim et al., 2013). It has been found by Aloisio et al. (2006) that these parasites caused malabsorption, reduced feeding efficiency, and severe weight loss to the livestock which could lead to lower production and economic losses.

There are a number of studies that have reported the prevalence of *Giardia* and *Cryptosporidium* in some parts of Southeast Asia. In Thailand, 9.4% of 363 cows were found positive for *Cryptosporidium* (Jittapalapong et al., 2006). In Malaysia, *Cryptosporidium* and *Giardia* have been recorded to be present in different animals which may indicate that many Malaysian environments, particularly soil and water, are contaminated and may act as vehicles for the transmission of these parasites (Lim et al., 2008). In addition, cryptosporidiosis is frequently reported in both humans and animals from many countries such as Laos, Vietnam, Philippines, Myanmar,

Indonesia, Cambodia, Malaysia, and Thailand (Pumipuntu & Piratae, 2018). Meanwhile, the only reported case about *Cryptosporidium* relating to livestock animals from the Philippines was made by Laxer et al. (1988) in the rural areas of Palawan, where they obtained oocysts of *Cryptosporidium* from human, cattle, and carabao faeces. The *Cryptosporidium* was identified up to the genus level through microscopic examination, while *Giardia* showed negative result.

To date, there have been no updated published reports on the presence of *Cryptosporidium* and *Giardia* infection among livestock in the Philippines, particularly in Quezon. Moreover, there are also no recorded molecular characterisation and analysis of these zoonotic parasites from livestock animals in the Philippines. Hence, this study plays a significant role in the diagnosis of zoonotic parasites which may benefit the local agriculture and veterinary offices in the municipalities of Sariaya. Also, this study was designed to determine the prevalence of *Cryptosporidium* and *Giardia* in the livestock animals, specifically in cattle, pigs, and carabaos through molecular detection in selected farms of Sariaya, Quezon. This study also sought to correlate some risk factors that might be associated with the prevalence of these parasites.

MATERIALS AND METHODS

Study Design

This research was a cross-sectional study which determined the prevalence of *Cryptosporidium* and *Giardia* protozoan

parasites up to its species level. The barangays were purposively selected based on the livestock animal population number.

The final population sampling size was computed using the formula [1] adopted from Cochran (1977):

$$n = z^2 \frac{p(1-p)}{a^2} \quad [1]$$

where $z = 1.96$ (95%), $p = 7.2\%$ prevalence from a previous study of Wang et al. (2014) and $a = 0.05\%$. Using formula [1], the final sampling number of 103 livestock individuals was obtained and divided into 47 individual cattle, 44 individual pigs and 12 individual carabaos using formula [2].

$$n = \frac{\text{Population of special livestock}}{\text{Total population of livestock}} \times \text{Final sampling number} \quad [2]$$

Sampling Method

The animals were randomly selected regardless of their sex and age. The collection of faecal samples was done on the 14th of October 2018. The inner portion of the fresh faecal samples from each individual animal was collected to minimise contamination. Stool cups were filled with faeces to serve as the master sample. From each master sample, 2 g were preserved in 10% formalin for FECT, while the remaining were preserved in a cooler (2-8°C) and used for DNA extraction. The samples were immediately processed for

faecal analysis in the laboratory of Southern Luzon State University, Lucban, Quezon. Molecular characterisation was held in the Interactive Laboratory of the Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Nueva Ecija, Philippines.

Recovery and Identification of *Cryptosporidium* and *Giardia* through Microscopy

Kinyoun acid fast staining was used to recover *Cryptosporidium* oocysts while Lugol's Iodine staining was used for *Giardia* cyst (Abeywardena et al., 2014; Paller et al., 2013). All slides were observed under compound light microscope with 400× and 1000× magnification. The oocysts/cysts were manually and carefully counted for each slide. Morphological characteristics of the parasites were identified through comparison from the literatures and validated by a parasitologist from the University of the Philippines, Los Baños, Laguna.

Identification of *Cryptosporidium* and *Giardia* spp. using Polymerase Chain Reaction

Different protocols and gene markers were used for each parasite. For PCR reactions, positive and negative controls (distilled water) were included.

DNA Extraction. DNA was extracted from each sample using *NucleoSpin*® Soil Kit (Macherey-Nagel, USA) following the manufacturer's manual and stored at 4°C until further use. All the DNA samples were

subjected to Polymerase Chain Reaction (PCR) following specific protocol for *Giardia* and *Cryptosporidium*. The DNA was not quantified after extraction using spectrophotometric method. However, gel electrophoresis was performed to confirm the presence of the extracted DNA in the samples.

Nested PCR for the Detection of *Giardia* Species.

The triosephosphate isomerase (*tpi*) gene was the target gene for the nested PCR protocol in detecting *Giardia* (Sulaiman et al., 2003). For the primary reaction, an amplicon of 605 bp was amplified using primers AL3543 [forward: 5'-AAATATGCCTGCTCGTCG-3'] and AL3546 [reverse: 5'-CAAACCTTTTCCGCAAACC-3']. The primary reaction (20 µl) consisted of 10 µl of Premix (SolGent™ 2x h-Taq PCR Smart Mix), 1 µl for each primer, 5 µl of dH₂O and 3 µl of the DNA template. It was incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) having thermocycling condition of an initial hot start of 94°C for 5 min, followed by 35 cycles of 94°C for 45s denaturation, 50°C for 45s annealing, 72°C for 60s extension, and a final extension of 72°C for 10 min.

For the secondary reaction, an amplicon of 530 bp was amplified using primers AL354 [forward: 5'-CCCTTCATCGGTGGTAACTT-3'] and AL3545 [reverse: 5'-GTGGCCACCACICCCGTGCC-3']. The secondary reaction (20 µl) consisted of 10 µl of Premix (SolGent™ 2x h-Taq

PCR Smart Mix), 1 µl for each primer, 5 µl of dH₂O and 3 µl of the DNA template. The thermocycling conditions were as follows: initial hot start of 94°C for 5 min, followed by 40 cycles of 94°C for 45 seconds denaturation, 50°C for 45 seconds annealing, 72°C for 60s extension, and a final extension of 72°C for 10 min.

Nested Detection of *Cryptosporidium* Species.

The protocol of Nichols et al. (2003) was used to obtain the SSU rRNA gene, which was the target gene in detecting *Cryptosporidium*. For primary reaction, a 655 to 667 bp amplicon was amplified using a 26-mer primer N-DIAGF2 [forward: 5'-CAATTGGAGGGCAAGTCTGGTGC CAGC-3'] and N-DIAGR2 [reverse: 5'-CCTTCCTATGTCTGGACCTGGT GAGT-3']. The primary reaction (20 µl) consisted of 10 µl of Premix (X-Prime Taq Premix 2x), 1 µl for each primer, 5 µl of dH₂O and 3 µl of the DNA template. PCR thermocycling was performed as follows: 95°C for 5 mins, followed by 35 cycles of denaturing for 30 seconds at 94°C, annealing for 1 minute at 68°C and extension for 30 sec at 72°C, followed by a final extension at 72°C for 10 minutes.

For the secondary reaction, a 435 bp amplicon was amplified using primers CPB-DIAGF [5'-AAGCTCGTAGTTGGATTTCTG-3'] and CPB-DIAGR [5'-TAAGGTGCTGA AGGAGTAAGG-3'] (Johnson et al., 1995). The secondary reaction (20 µl) consisted 10 µl of Premix (X-Prime Taq Premix 2x), 1 µl for each primer, 3 µl of dH₂O and 5 µl of

the first PCR product. PCR thermocycling conditions were as follows: initial hot start of 80°C for 5 min, followed by 39 cycles of 98°C for 30s denaturation, 55°C for 30s annealing, 72°C for 60s extension and a final extension of 72°C for 10 min.

Sequencing and Analysis of Sequence Data

PCR products were visualized on 2% agarose gel-electrophoresis at 100V for 40 minutes for both *Cryptosporidium* and *Giardia* using Gel Documentation Systems (Uvitec Imaging Systems, United Kingdom). Positive bands were excised from the gel and purified using *NucleoSpin*® Gel and PCR Clean-up (Macherey-Nagel, USA), according to the manufacturer's instructions, and sent to 1st Base-Asia Gel, Ltd., Malaysia, for sequencing.

The sequence analysis of the DNA sequences obtained was assessed through BioEdit Sequence Alignment Editor (version 7.1.11) (Hall, 1999). Sequences from positive samples were aligned and ambiguous sites were removed prior to phylogenetic construction. The sequences were compared with known reference sequence using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

The phylogenetic construction and analyses were conducted using MEGA version 6 software, with bootstrap values establishing 1000 replicates. The Phylogenetic tree was constructed using neighbour-joining tree [NJ] (Tamura et al., 2013).

Survey of Risk Factors Associated with Prevalence of Parasites

A survey questionnaire, validated by a statistician, was distributed to the farm owners to retrieve information regarding the diet, feeding floor, habit, presence of illness and faecal consistency of their livestock animals.

Statistical Analysis. The prevalence rate and mean intensity of *Giardia* from faecal samples were obtained using formulas [3] and [4] by Ordoñez et al. (2018), respectively:

$$\text{Prevalence} = \frac{\text{No. of positive samples}}{\text{Total number of samples}} \times 100 \quad [3]$$

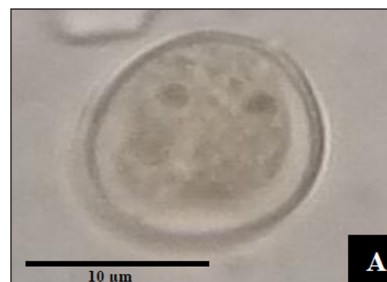
$$\text{Mean intensity} = \frac{\text{Individual count of parasites}}{\text{Total number of positive samples}} \times 10 \quad [4]$$

Chi-square test was used to determine the association between prevalence of *Giardia* and possible risk factors such as diet, feeding floor, habit, presence of illness and faecal consistency. Differences are considered statistically significant when $p < 0.05$. Statistical analysis was conducted using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

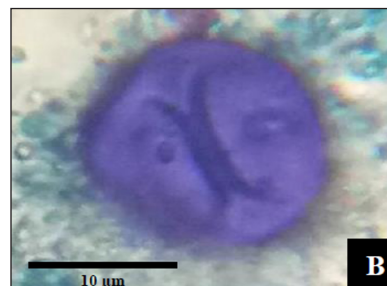
RESULTS AND DISCUSSION

Determination of Prevalence of *Cryptosporidium* and *Giardia*

In microscopy, *Giardia intestinalis* was detected in 14 out of 103 (13.59%) faecal samples from livestock animals, while no *Cryptosporidium* oocysts were observed in all the samples. The overall mean intensity of *Giardia* was 13 cpg wherein, cattle had the highest intensity (14 cpg) followed by pigs and carabao (10 cpg).



(a)



(b)

Figure 1. *Giardia intestinalis* recovered from faecal samples 1000× magnification. (a) Stained by Lugol's Iodine solution; (b) Stained by Methylene blue

The cysts of *Giardia* were observed to have oval shape, ranging between 7-11 µm in width and 12-16 µm in length. Most of them were with two visible nuclei and remnants of axonemes, although the axonemes were slightly disintegrated in some cysts (Figure 1).

Giardia can be found in environmental and faecal samples of livestock animals and has the potential to be zoonotic (Bawm et al., 2014). Sastry and Bhat (2014) described *Giardia* cysts to have 4 nuclei while Adam (1991) stated that it had 11–14 µm in length and 7–10 µm in width. Meanwhile, *Cryptosporidium* was found to be negative in all the faecal samples in the present study. Padilla and Ducusin (2015) studied the prevalence of endoparasites from smallholder farms in Sariaya, Quezon, and also did not recover *Cryptosporidium* in their samples. The absence of *Cryptosporidium* may have been caused by several factors such as ages of the livestock, shedding of oocysts/cysts in the faeces, long storage of samples before PCR methods and travel of DNA extracts (Abeywardena et al., 2014; Ghaffari & Kalantari, 2014; Olson et al., 2004; Qi et al., 2016).

All of the 103 faecal samples underwent PCR and only 13 out of 103 were amplified for the target gene of *Giardia*, *tpi*, with

expected band size of 530 bp. However, only 5 out of 13 positive samples were subjected to DNA sequencing since the other bands have low DNA intensity when subjected under blue light during excision. BLAST results of the obtained sequences for the parasites in the five faecal samples showed 98-99% identity (Table 1).

Phylogenetic analysis by Neighbour-Joining tree was performed on the five *Giardia* isolates (Figure 2). It showed that 3G-9P and 4G-19P clustered with assemblage B, while 1G-1K and 5G-19B clustered with assemblage A, and 7G-40B clustered with assemblage E.

The recovered *Giardia intestinalis* in the present study falls under assemblages A and B, which are zoonotic, while assemblage E is considered as livestock specific without zoonotic capability. The presence of zoonotic parasites indicates risks for humans especially farmers in the nearby area since they can contract infection caused by the livestock animals.

Table 1
The BLAST results for *Giardia intestinalis* recovered in positive faecal samples in molecular analysis

From this study	Closest match			
Faecal samples	Accession number from Genbank	Accession number from Genbank	Locality	Hosts
1G-1K Carabao	MK330179	KR075936.1	Mongolia, China	Sheep
		KY320581.1	Tehran, India	Human
3G-9P Pig	MK330180	KR902357.1	Shanghai, China	Water samples
		GU564279.1	Henan, China	Human
4G-19P Pig	MK330180	KR902357.1	Shanghai, China	Water samples
		GU564279.1	Henan, China	Human
5G-19B Cattle	MK330179	KR075936.1	Mongolia, China	Sheep
		KY320581.1	Tehran, India	Human
7G-40B Cattle	MK330181	KT922260.1	Central Ethiopia	Cattle
		EF654692.1	Georgia, USA	Cattle

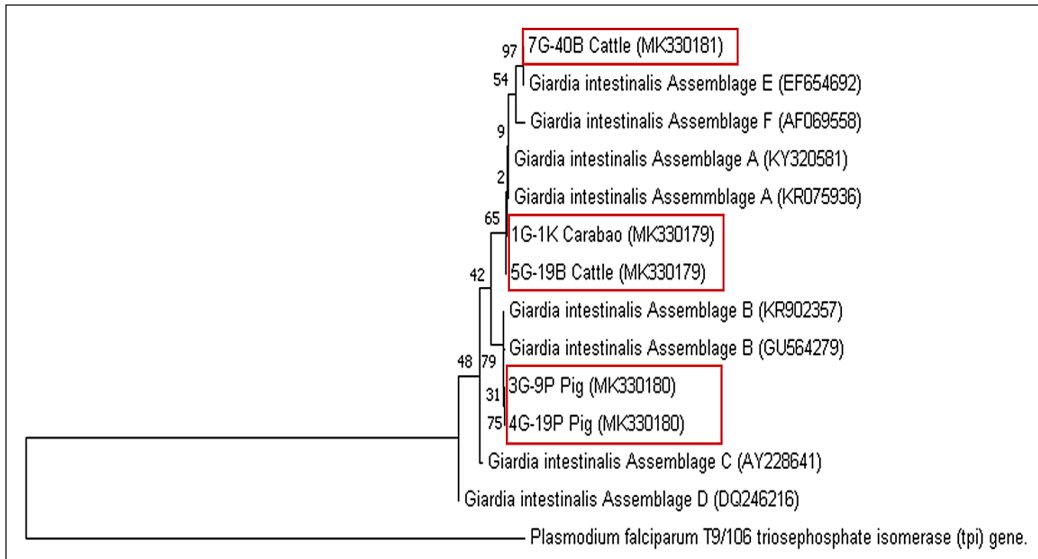


Figure 2. Phylogenetic relationship of sequences based on the TPI gene sequence by Neighbour-Joining Tree methods. Bootstrap: 1000 replicates

In the study of Abeywardena et al. (2013) in Australia, *Giardia* was detected under assemblage A among carabaos. In addition, the results in cattle are also the same with the study of Abeywardena et al. (2014) from Sri Lanka, where both assemblages A and E were found among the cattle. Although there are no reported cases of assemblage B among pigs in Asia, it is the most common assemblage among humans in Bangladesh (Ryan & Caccio, 2013). Additionally, in Asia, a significant number of infected cattle were found with assemblage A, but very few were infected with assemblage B. Meanwhile, Xiao and Fayer (2008) claimed that most pigs and ruminants from Western countries were infected with *Giardia* from assemblage E.

Possible Risk Factors among *Giardia*-positive Faecal Samples

The data showing the samples that were found positive with *Giardia* were used to see its association with the possible risk factors. However, the results of the current study showed that all the factors had no significant association with the prevalence of *Giardia* (Table 2). Several studies also revealed no association of the presence of *Giardia* with these factors such as feeding floor and diet (Kakandelwa, 2015), habit of livestock animals, whether it was freely roaming, tied or caged (Castro-Hermida et al., 2002), diarrhea and faecal consistency (Langkjaer et al., 2007; Maddox-Hyttel et al., 2006; Paz e Silva et al., 2012; Petersen et al., 2015; Toledo et al., 2016). Furthermore, Siwila (2017) stated that limited information was available on studies about *Giardia* infection since typically it came asymptotically.

Table 2
Association of risk factors with Giardia-positive faecal samples of livestock animals

Risk Factors	Livestock Animals			Total	*P value
	Pig n=44	Carabao n=12	Cattle n=47		
Diet					
Commercial Feeds	6 (40)	-	-	6 (40)	0.625
Grass	-	1 (12)	4 (40)	5 (52)	
Milk	1 (4)	-	1 (7)	2 (11)	
Feeding floor					
Open field	-	1 (12)	5 (47)	6 (59)	0.386
Cemented floor	7 (44)	-	-	7 (44)	
Habit					
Free roaming in the field	-	-	5 (47)	5 (47)	0.671
Tied	-	1 (12)	-	1 (12)	
Caged Indoor	7 (44)	-	-	7 (44)	
Presence of Illness (Diarrhea)					
Yes	4 (20)	-	2 (32)	6 (52)	0.682
No	3 (24)	1 (12)	3 (15)	7 (51)	
Faecal consistency					
Liquid	-	-	2 (8)	2(8)	0.661
Slightly Watery	2 (10)	1 (12)	2(31)	5 (53)	
Firm	5 (34)	-	1 (8)	6 (42)	

*p<0.05= significant

*The number of livestock species for each factor were enclosed in the bracket; the number outside the bracket is the positive samples from molecular detection

CONCLUSION

This study revealed the presence of *Giardia intestinalis* among livestock of Sariaya, Quezon. It is categorised as a zoonotic parasite of public health importance. Thus, it is recommended that water from their facilities and human faecal samples from livestock farms be tested for the presence of *Giardia* to better understand the possible source of this parasite. Although *Cryptosporidium* was not detected in the present study, it should still be considered in future researches. It is suggested to

assess other risk factors in livestock such as changes of season (dry/wet season) and age of the animals under longitudinal type of sampling method.

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