HUMAN AND CAPRINE CRYPTOSPORIDIOSIS AMONG SMALLHOLD FARMS IN AURORA PROVINCE, PHILIPPINES

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ABSTRACT

This cross sectional study determined the prevalence of cryptosporidiosis among backyard animal raisers and goats in Baler, San Luis and Maria Aurora municipalities of Aurora province in the Philippines and determined risk factors associated with *Cryptosporidium* spp. infection among animal raisers and their goats. Systematic random sampling was used in selecting the animal raisers (n=678) and goats (n=168) from each barangay by municipality. The animal raisers were interviewed using a structured questionnaire containing all probable risk factors associated with cryptosporidiosis transmission. Animal raisers age 37-47 years had the highest cases of PCR positive reactors for *C. parvum* DNA. The general prevalence of human cryptosporidiosis was 21% regardless of age and sex of animal raisers.The prevalence of caprine cryptosporidiosis was 18% (30/168). All 30 goat fecal samples were positive reactors for the *C. parvum* DNA. Out of these 30 goats, 40% (12/30) were raised by infected goat raisers positive for *C. parvum*. After controlling all confounders, the potential risks significantly associated with human cryptosporidiosis were exposure to infected goats, herd size, garbage disposal and presence of scavengers. Interactions between length of experience in goat raising and hand washing and length of experience and herd size were also significantly associated. The only potential risk associated with caprine cryptosporidiosis was long standing diarrhea in the farm.

Keywords: Aurora province, cryptosporidiosis, goat, smallhold farm

INTRODUCTION

Goats are very popular among Filipinos because they require low initial capital investment, fit the small hold farm conditions and multiply fast. In addition, women and children can raise the animals, making it a sound option to augment the country's programs on livelihood. Among Muslims, goat farming is considered a "sunshine industry" and goat meat is a favorite Halal food. However, since goats are usually housed near human abodes and require low maintenance, health management factors could be taken for granted. Goats could serve as reservoirs of many zoonotic infections that can be transmitted to the animal raiser and one of

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these is *Cryptosporidium parvum.*

Small ruminants were reported to harbor *C. parvum* particularly in Spain where 40% of lambs coming from 90% of farms, aged 1–2 weeks old, were reported to be infected and in some cases mortality was high during spring. The same study reported high prevalence (70%) in goats with kids, 5-15 days old (Sari *et al.*, 2009). Published prevalence data for sheep was 10-78% and goats, 28-100%. In the Philippines, a variety of domestic livestock including cattle, swamp buffaloes, pigs, dogs, cats and chickens and also humans have been reported to shed *Cryptosporidium* oocysts, except in goats (Laxer *et al.,* 2003).

Hence, this study determined the prevalence of *C. parvum* among smallhold animal raisers and goats particularly in Baler, San Luis and Maria Aurora municipalities of Aurora province in the Philippines. Likewise, risk analysis on potential risk factors associated with *Cryptosporidium* transmission between goats and their owners was done.

METHODOLOGY

This study used a cross-sectional design with the aim of determining the prevalence of cryptosporidiosis among backyard animal raisers and goats in Baler, San Luis and Maria Aurora municipalities of Aurora province using fecal microscopy (Kinyoun Acid Fast) and molecular diagnostic methods (PCR and LAMP assay). Similarly, the study determined risk factors associated with *Cryptosporidium* spp. infection among animal raisers and their goats. Sample size of backyard animal raisers was based on the lowest expected prevalence of cryptosporidiosis reported in the Philippines and other Asian countries with a power of 80 and a confidence interval of 95%.

Selection of animal raisers, smallhold farms and goats

A livestock farmer was selected based on two criteria. The person must be exposed to direct handling of goats for the past year and should not have taken anti-protozoan medications, antibiotics, antacids for the past 2 weeks since these could distort protozoan morphology and serve as main reason for failure of identifying them under microscopy. Only one farmer was recruited to represent one farm. In case there were more than one who were eligible in the farm, the person who had the most number of direct contact with the animals was selected. Hence, a total of 678 farmers or farms were enrolled in the study.

From the 678 farms, those with 5 does and 20 growers were considered smallhold (Apelo,1998). The number of farms whose goat population fit the eligibility criteria was determined per municipality. Then the total number of farms per municipality was divided by the sample size per municipality to get the sampling interval k. The first farm in the municipality was selected at random from the first k farms and the next farm to be interviewed shall be every k^{th} farm within the sampling frame thereafter until the sample size per municipality was satisfied. Two animals were randomly sampled in farms with 4 heads of goats, 3 in farms with 5-10 heads and 5 in farms with more than 10. This method was repeated until the sample size of 168 goats was satisfied.

Stool collection

Specimen cups for human stool samples were distributed for collection. These were properly labeled with the name, age and sex of the farmer. All human stool samples were subjected to DNA extraction for PCR assay of *C. parvum*. Stool was collected directly from the rectum of goats and processed for microscopic examination of *Cryptosporidium* oocysts using Kinyoun Acid Fast stain and DNA extraction for Loop Mediated Isothermal Amplification Method (LAMP) assay.

Detection of *Cryptosporidium* oocysts using Kinyoun Acid Fast

A drop of fecal suspension from FECT specimen was placed on a glass slide and spread to form a thin smear (similar to a blood film). The slides were warmed at 60^{o}C to dry the smear completely. The fecal smears were flooded with Kinyoun's acid fast stain for 2 min. The smears were washed with 50% ethanol followed by tap water. This step was repeated three times until all the red stain was washed off. The smear was flooded with 1% sulfuric acid for 2 min or until no further color was seen. The slide was washed with tap water and flushed with Loeffler's alkaline methylene blue for 1 min. Finally, the slide was washed with tap water, air dried and examined under 40× and 100× oil immersion objective.

Genomic DNA extraction

DNA extraction was done from sediments of human and goat fecal samples. The pellets resulting from the sedimentation procedure were washed three times with PBS. The pellets from the last wash was resuspended in 200 ul TE buffer (10 mM Tris, ph 7.4 and 1 mM EDTA, pH 8.0).Two hundred µl lysis buffer (Tris, $Na_2EDTA.2H_20$,NaCl, SDS and sterile distilled water) were added followed by 8 µl of proteinase K (50 mg/ml). The resuspended pellets were incubated at 60℃ for one hour after which, an equal volume of phenol, chloroform, iso-amyl mixture (PCI) was added. The tube was mixed in a shaker for 30 min and centrifuged at 13,000 rpm for 4 min at 10℃. The acqueous phase was transferred into a new microcentrifuge tube (MCT) and an equal volume of PCI was again added. The tube was mixed by inverting for 5 min and centrifuged at 13,000 rpm for 4 min at 10℃. The aqueous phase was transferred to a new MCT. The DNA was precipitated with a thawed preparation of 3M frozen sodium acetate at 1:10 ratio followed by chilled absolute ethanol at 2:1 ratio. The tube was kept at -20℃ overnight. The following day, the tube was centrifuged at 12,000 rpm for 10 min at 4℃. The supernate was discarded and 1 µl of 70% ethanol was added into the tube. It was centrifuged at 12,000 rpm for 5 min at 4℃. The supernate was discarded and the tube was air dried by inverting on a paper towel to drip excess liquid. The resulting DNA was rehydrated with 30 µl of 10mM TRIS (pH 7.4)/ 1mM EDTA (TE buffer) and stored at -20℃ until conduct of PCR.

Detection of PCR product

The PCR was performed in a 10 µl reaction mix using Promega Biochemicals. The reaction contained 2 µl of 5X GoTaq buffer with dye, 0.2 µl of 10 mM dNTP, 1.2 µl of 25 mM MgCl, 0.5 µl of 10 uM forward primer, 0.5 µl 10 µM reverse primer, 0.1 µl of *Taq* polymerase, 4.5 µl of PCR water and 1.0 µl of DNA

isolate. The forward primer used was CPH2423 (5'-AAATGGTGAGCAATCCTCTG-3') while the reverse primer was CPH2764 (5'-CTTGCTGCTCTTACCAGTAC-3'). The protocol followed a PCR run of: 94℃ for 2 min, 35 cycles of 94℃ for 30 sec, 53℃ for 30 sec and 72℃ for 1 min and 30 sec, followed by an extension of 72℃ for 7 min and 4℃ for storage. Analysis of PCR products was carried out by subjecting 8 µl of amplified products to electrophoresis in 2% LO3 (TaKaRa Biochemicals) agarose gel. The DNA positive control isolate (HCNV4, human strain) was provided by Drs. Karanis and Kawazu of the NCRPD-OUAV, Obihiro, Hokkaido, Japan. The expected 361 bp amplified PCR band visualized with UV light after ethidium bromide staining (10 mg/ml) indicated positive result.

Detection of LAMP product

Four primers were designed based on the 60 kD glycoprotein gene (gp60) sequence for use in the LAMP assay and included two outer primers (F3 and B3), one forward inner primer (FIP) and one reverse inner primer (BIP) (Karanis *et al*.,2007).

LAMP primer designs:

F3	5'-TCG CAC CAG CAA ATA AGG C-'3
B3	5'-GCC GCA TTC TTC TTT TGG AG-'3
FIP	5'-ACC CTG GCT ACC AGA AGC TTC AGA ACT GGA GAC GCA GAA-'3
BIP	5'-GGC CAA ACT AGT GCT GCT TCC CGT TTC GGT AGT TGC GCC TT-'3

Each LAMP reaction mixture in a 0.2 ml tube contained a total volume of 25 µl final reaction. This mixture consisted of 1.0 ul of primer mix [20 pmol each FIP and BIP primers, 2.5 pmol of each F3 and B3 outer primers], 12.5 ul of LAMP buffer [1.0 M TrisHCl (pH 8.8) at 40 mM, 1.0 M KCl at 20 mM, 1.0 M $(NH_4)_2 SO_4$ at 20 mM, 1.0 M $MgSO_4$ at 16 mM, 10 mM dNTPs at 2.8 mM, 10% Tween 20 at 0.2% and 5 M Betaine at 1.6 M and distilled water adding up to 12.5 µl], 1.0 µl of 8 U Bst DNA polymerase large fragment (New England Biolabs), 2.0 µl genomic DNA (~100 ng) and 8.5 µl double distilled water. The LAMP reaction was carried out in a water bath at 60.5℃ for 60 mins and terminated at 90℃ for 3 min. A positive control (purified *C. parvum* DNA) and a negative control (distilled water) were included during each run. After the reaction, 5.0 µl of LAMP products were electrophoresed on 2% molecular-grade gel prepared in 0.5× tris-borate-EDTA buffer stained with 0.5 g ml-1 ethidium bromide. In addition, the amplification products were visualized under UV light following the addition of 1 µl of SYBR green I dye to the reaction tube.

Interview of animal raisers using questionnaires

Epidemiologic information was obtained from the identified animal raiser that fitted the inclusion and exclusion criteria from each farm through the use of a structured questionnaire. Risk variables in animal handlers focused on the following: educational attainment of animal raiser, quality of hand washing practices, high risk farm activities, characteristics of drinking water source, manner of excreta disposal, manner of garbage disposal practice, quality and usage of toilet in the farm, herd

size of farm. Risk variables in goats dealt with history of diarrhea for the past year, entry of new stock in the farm, access of farm animals to natural bodies of water, presence of animal scavengers (stray cats/rodents) in animal enclosure and fodder.

Data analysis

Crude analysis and logistic regression models using SAS were derived for risk variables related to animal raiser and human cryptosporidiosis, caprine cryptosporidiosis to human cryptosporidiosis and risk variables related to goats and caprine cryptosporidiosis. Since the data were collated from answers by the animal raisers to questionnaires and not from a controlled laboratory setting, the alpha level was set to 0.1 to determine statistical significance.

RESULTS AND DISCUSSION

Among the age brackets of animal raisers who were PCR positive for *C. parvum*, most belong to the 37-47 yrs age group while the lowest was in the 15-25 yrs old group (see Table 1). Most of them did not complain of diarrhea for the past 6 months at the time of sample collection. On the other hand, the general prevalence of the infection was 21% regardless of age and sex of animal raisers (Table 2).

Table 1. Prevalence of *Cryptosporidium parvum* infection among animal raisers by age using PCR.

Age of animal raisers	n	% Prevalence (95% C.I.)
<15 yrs	5	20.0 (0.5, 71.6)
15-25 yrs	38	15.8 (6.0, 31.3)
26-36 yrs	137	20.4 (14.0, 28.2)
37-47 yrs	203	23.2 (17.5, 29.6)
>47 yrs	295	21.0 (16.5. 26.1)

Table 2. Prevalence of *C. parvum* infection among animal handlers by sex using PCR.

Gender	Prevalence	95% CI
Male (n=410)	86 (21.00%)	17.1, 25.2
Female (n=268)	58 (21.60%)	16.9, 27.1

Table 3 shows that 18% (30/168) of goats was infected with *Cryptosporidium* spp. oocysts using microscopy after staining the fecal samples with Kinyoun acid

Table 3. Distribution of *Cryptosporidium parvum* infected goats and goat owners.

Goat owners	Kinyoun (+) goats	Kinyoun (-) goats	Total goats examined	Percentage
PCR (+)	12 (40%)	30	42	29% (12/42)
PCR (-)	18 (60%)	108	126	14% (18/126)
TOTAL	30* (100%)	138	168	18% (30/168)

Legend: PCR = polymerase chain reaction using specific *C. parvum* primers.
Kinyoun: acid fast stain specific for *Cryptosporidium* spp. oocysts.
*all 30 goats became positive in LAMP assay using specific *C. parvum* primers.

Table 4. Distribution of infected goats and goat owners.

Municipality	Infected owners	Infected goats
Baler	1	2
Maria Aurora	10	22
San Luis	1	6
TOTAL	12*	30

*goat owners with infected goats

fast. Out of 30 infected goats, 12 or 40% were raised by goat raisers positive for *C. parvum* by PCR. These 30 infected goat fecal samples were further subjected to LAMP assay and all were positive reactors. The municipality with the highest frequency of zoonotic strain based on the number of infected goats and infected animal raisers was Maria Aurora (see Table 4). This municipality has the most number of livestock and poultry, is highly rural and complaints of diarrhea for the past year among animals were reported by animal raisers upon the time of interview. Among goats with history of diarrhea, 39% were found to harbor *Cryptosporidium* and among those with no history of diarrhea, 16.4% were also positive for *Cryptosporidium* using Kinyoun Acid Fast stain and LAMP assay.

Within *C. parvum*, there are two distinct populations, one cycling only in humans and one cycling in both animals and man (Xiao *et al*., 1998). This is further corroborated using random amplified polymorphic DNA (RAPD) markers wherein two distinct groups of human *C. parvum* isolates were discovered, one containing most human isolates and the other containing some human isolates and all animal isolates indicating the possibility of zoonotic infection (Morgan *et al*., as cited by Xiao *et al*., 1998). Xiao examined the species and strain-specific types of *Cryptosporidium* spp. in clinical and environmental samples. Results indicated that anthroponotic organisms (genotype 1) accounted for the majority of the cases and person-to-person in non-outbreak cases. Meanwhile, the genotype 2 largely causes human infection through contamination of water or food or direct contact with

infected animals, especially in rural areas. Therefore, results of this study imply that the *C. parvum* diagnosed in both humans and goats could have been genotype 2, known to largely cause human infection through contamination of water or food or direct contact with infected animals.

Certain activities are associated with a greater chance of exposure to potentially zoonotic pathogens. Coming in contact with feces while working in a farm is inevitable and ingestion of infected feces through food and water or direct contact with fecal material through fecal-oral route or hand-to-mouth transmission is the main route of transmission for cryptosporidiosis.

Among the potential risks, the length of experience in goat raising (years) *per se* was not significantly associated with the transmission of caprine cryptosporidiosis to the animal raisers based on univariate analysis. After controlling all confounders, the following risks related to animal raisers were found to have significant associations with human cryptosporidiosis (Model 1, Table 5). Raisers with moderate to maximum number of herd size (5 does and 20 growers) were eight times more likely to get infected as compared to those with minimum number of herd size or less than 5 does and 20 growers (OR=8.082, p=0.0009). Poor manner of garbage disposal with the presence of scavengers predisposes the animal raisers to 2.24 times of acquiring cryptosporidiosis (OR=2.243, p=0.0042). Although the exact mode of transmission of *Cryptosporidium* oocysts is unknown, it would again be reasonable to assume that in these communities with human and caprine positive reactors, human-animal contact with subsequent hand-to-mouth transfer of oocysts is a potential source of infection (Laxer *et al.,* 2003).

Table 5. Final logistic regression model for human cryptosporidiosis.

MODEL 1 – Risk variables related to animal raiser and human cryptosporidiosis.
* p value of ≤0.1 is significant

Variables	Odds ratio	P value
Herd size (moderate to maximum)	8.082	0.0009*
Garbage disposal (poor manner with scavenger)	2.243	0.0042*
Hand washing (delayed with soap and water)	0.287	0.0734*
Experience (>4-10 yrs) * Hand wash (delayed with soap and water)	5.966	0.0927*
Experience (>10 yrs) * Herd size (moderate to maximum)	9.318	0.0189*

Hand washing with soap and water, although delayed, was observed to have a protective function to minimize if not to eliminate the risk factors for the transmission of the infection (OR: 0.287; p=0.0734).

The higher the animal density in the farm, the bigger is the number of susceptible animals that can serve as reservoir for sustaining zoonotic transmission. Protozoa infections that have a cyclozoonotic maintenance cycle require more than one vertebrate species but no invertebrates (Bomfin *et al*., 2005). According to Woolhouse *et al.* (2001), zoonotic protozoa that lack host specificity use animals as intermediate hosts to sustain the indirect route of transmission to man. Animal

density exceeding the available land area for waste disposal has become a major problem not only in the cost of disposing waste materials, but also in the pollution and/or contamination of water and land (Delafosse *et al.*, 2006).

Poor environmental sanitation attracts rodents bringing the rat population into closer contact with farmers and other rural residents. Cats which hunt rodents are more at risk of carrying protozoa oocysts. When they become acutely ill, they contaminate objects, animal fodder and feeding troughs in the farm with their feces seeded with oocysts. Warm blooded hosts, including man, get infected by accidentally ingesting objects contaminated with *Cryptosporidium* oocyts (Sari *et al*., 2009).

For fecal-oral and waterborne or water-washed transmission, the basic preventive strategies are to disrupt fecal-oral spread by hand washing and to improve water quality. Majority of the enteric protozoa require strict hand washing techniques as control and preventive measures (Tzipori, 1985). Hand washing is recognized as the single most effective method of controlling cross infection. Hands should be washed between each farm activity and before handling food. The wearing of gloves is not a substitute for hand washing (Molbak *et al.,* 1994).

On the other hand, interactions between length of experience in goat raising and two covariates resulted to increased risk of acquiring the infection among animal raisers. An individual with more than four to ten years raising experience who delays washing hands with soap and water was six (OR=5.966, p=0.0927) times more likely to be infected. The interaction of more than ten years of experience of raising a moderate to maximum herd size of ruminants makes the animal handler nine times more likely to contract the infection (OR=9.318, p=0.0189).

Similarly, after controlling the confounders, Table 6 showed that animal raisers of infected goats were almost 3 times (OR=2.701, p=0.0190) more likely to be infected compared to those raising healthy caprine.

Table 6. Final logistic regression model on the influence of caprine cryptosporidiosis to human cryptosporidiosis.

MODEL 2 – Caprine cryptosporidiosis and human cryptosporidiosis

Variables	Odds ratio	P value
Caprine cryptosporidiosis	2.701	0.0190*
New stock	1.434	0.3762
Water access	1.742	0.1593
Farm activity	0.844	0.6188

* p value of ≤0.1 is significant.

Table 7 shows that the only risk for goats to get infected with cryptosporidiosis is when they are exposed to diarrhea in the farm for the past year. The probability of goats getting infected with cryptosporidiosis is twice (OR=2.0, p=0.049) when exposed to diarrhea in the farm for the past year than those who came from farms that had no diarrhea.

Table 7. Final logistic regression model for caprine cryptosporidiosis.
MODEL 3 – Risk variables related to goat raising and caprine cryptosporidiosis

Variable	Odds ratio	P value
Diarrhea	2.0	0.049*
New stock	0.5	0.63
Access to water	1.0	0.089
Scavengers	2.7	0.632

***** p value of ≤0.05 is significant.

Diarrheic wastes from sick animals that are tethered in pasture or enclosed in pens allow accumulation of infectious excreta in one area. Moreover, whether or not the farmer adequately medicates his animals, *Cryptosporidium* infection requires a long period of chemotherapy which makes it more ubiquitous. Oocysts can then be directly transmitted by oral-fecal route to susceptible goats which are indiscriminate feeders. Sources of drinking water become contaminated when feces containing the oocysts are deposited or flushed into water. *C. parvum* is also waterborne and so other susceptible individuals and man can be infected through ingestion of contaminated water.

CONCLUSION

Kinyoun acid fast stain, PCR and LAMP assays have a sensitivity and specificity of above 90% for cryptosporidiosis. Using these tests, the following results were observed. In general, animal raisers aged 37-47 yrs had the highest prevalence of *C. parvum.* Majority of them were asymptomatic. On the other hand, the general prevalence regardless of age and sex was 21% (144/678) by PCR. The general prevalence among goats was 18% (30/168) by Kinyoun stain and all 30 infected goat fecal samples harbored *C. parvum* by LAMP assay. Twelve or 40% of these goats were raised by infected goat raisers. The detection of *C. parvum* in both humans and goats implies that the *C. parvum* strain present in smallhold farms is genotype 2 which is known to largely cause human infection through contamination of water or food or direct contact with infected animals. Maria Aurora municipality had the highest frequency of both animal raisers and goats infected with *C. parvum.*

After controlling all confounders, the potential risks significantly associated with human cryptosporidiosis were exposure to infected goats, herd size, garbage disposal and presence of scavengers. Interactions between length of experience in goat raising and hand washing and length of experience and herd size were also significantly associated. The only potential risk associated with caprine cryptosporidiosis was long standing diarrhea in the farm.

The study recommends that goat raisers should practice farm sanitation, personal hygiene, and provide proper attention and medication to diarrheic animals in order to prevent cryptosporidiosis among goats and eventually prevent the goat raisers from getting the infection from their animals as well.

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