Incidence And Risk Factors Of Cryptosporidium Spp. Infection In Water Buffaloes Confined In A Communal Management System In The Philippines

M Villanueva, C Domingo, N Abes, C Mingala

Citation

Abstract
The study determined the incidence of cryptosporidiosis (Cryptosporidium spp.) among water buffaloes in a confined management setting in the Philippines. Detection of oocysts in the feces of symptomatic and asymptomatic calves and their respective dams was performed using microscopy. The incidence proportion and density of the infection, the percentage distribution in calves according to sex and stool consistency, and the presence of association between several hypothesized risk factors such as birth weight, history of diarrhea and parasitologic state of the dam were described. Fecal samples from 38 Murrah buffalo calves were collected at 6 a.m. at days 4, 8 and 12 within the two-week observation period from day of birth. The fecal samples were processed using formalin-ether concentration technique, stained with Kinyoun acid fast and viewed under the microscope. Results showed that 10 out of 38 dams and one out of 38 buffalo calves were positive for Cryptosporidium spp. oocysts. The incidence proportion was 0.0263 for the 12-day observation period or three per 100 calves. The incidence rate or density was 2.2 per 1000 calf-days at risk with a point prevalence of 5%. Starting at day eight, 50% was diagnosed with Cryptosporidium spp. oocysts based on the stool consistency. However, test of association showed no sufficient evidence that cryptosporidiosis in neonatal calves was dependent on gender, birth weight and parasitologic state of the dam after delivery. Results of the test of association of these risk factors were undefined.

INTRODUCTION
Cryptosporidiosis is an emerging zoonotic disease caused by Cryptosporidium parvum (Hannahs, 1995). It affects a wide range of animals including newborn ruminants and people. In general, the development of cryptosporidiosis depends on the species, age, and immune status of the host. Younger animals and animals with less developed or compromised immune systems are generally more susceptible to severe infection than healthy adult animals. The disease is characterized clinically by profuse, watery, sometimes mucous, blood-stained diarrhea, dehydration, emaciation, anorexia, tenesmus and abdominal pain (Wiser, 2006). Disease is more severe and lethal when complicated with other enteropathogens such as E. coli, Salmonella, Rotavirus, Coronavirus infections, and in immunocompromised individuals, especially AIDS patients with low CD4-counts, but is now also recognized as widespread general pathogen of immunocompetent humans (Medema et al., 2006). There is no approved treatment for cryptosporidiosis. Supportive treatment like fluids and electrolytes, both orally and parenterally are necessary until the animal recovers (Aiello, 1998).

Philippine carabao and riverine buffalo, like cattle, harbor these zoonotic protozoa, Cryptosporidium spp. These serve as reservoir hosts, shedding infectious oocysts in feces and water causing infection in other animals and man. The protozoa are found in symptomatic and asymptomatic animals wherein calves are severely infected between one and three weeks of age causing diarrhea. The Cryptosporidium spp., having the smallest oocysts of all known enteric protozoa, may be difficult to detect under routine fecal examination. Also, water analysis is important knowing that cryptosporidiosis can be transmitted through water (Medema et al., 2006; Avery et al., 2003), which might infect the calves.

Cases of cryptosporidial infection among human (Anderson et al., 1982; Cross et al., 2003; Laxer et al., 2003; Capending and Saniel, 2003) as well as in water buffaloes (Fagiolo et al., 2007; Khan, 2000) has been documented on selected
urban and rural areas in the Philippines. Knowing that buffalo neonates are reservoir of Cryptosporidium spp. whether symptomatic or asymptomatic, they are sources of infective oocysts that could infect man. Moreover, these oocysts have the characteristics to be acid fast once stained with the carbol fuchsin stain, and then become resistant to decoloration with acid. In this case, Kinyoun acid fast stain can aid in the detection of these protozoa.

The results of cross-transmission experiments, using isolates from farm animals and human patients, suggested by Tzipori et al. (1980) that Cryptosporidium might be a single-species genus. If so, the domestic species may be a reservoir of infection for susceptible human individuals (Angus, 1983). The principal modes of contamination are fecal-oral spread among human beings and animals and water-borne transmission (Villacorta et al., 1991; Moon et al., 1995). Humans are the only significant source of C. hominis and humans and ruminants are the predominant sources of the cattle genotype of C. parvum. The cattle genotype of C. parvum has been found in other mammals, but infected humans, cattle and sheep shed oocysts in very high numbers, especially when infected in infancy, which probably contribute most to the environmental contamination (Medema et al., 2006). Case fatality rates in cryptosporidiosis are generally low unless complicated by other factors like concurrent infections, energy deficits from inadequate intake of colostrum and milk, chilling from adverse weather conditions (Aiello, 1998).

Generally, the present study determined the incidence of Cryptosporidium spp. infection among water buffaloes at a total confined management system. Specifically, the study aimed to demonstrate Cryptosporidium spp. oocysts using Kinyoun acid-fast stain among symptomatic and asymptomatic water buffaloes. Furthermore, to determine the incidence proportion and incidence rate of infected water buffaloes, described the percentage distribution of oocysts in the feces by sex and stool consistency; and describe the several hypothesized risk factors based on epidemiological measures of association.

MATERIALS AND METHODS

STUDY DESIGN

The study utilized a cohort study design where the neonatal calves were followed up starting zero to two weeks old. Thirty-eight (38) newly born calves were enrolled in the study. The dams who delivered them were also enrolled.

SAMPLE AND DATA COLLECTION

Stool collection of all newly born calves was done in the morning at days 4, 8 and 12 within the two-week observation period. This was based on the two to 10 days incubation period of Cryptosporidium spp. Approximately five grams of freshly expelled feces were collected right after defecation. The sample was collected from the middle part of the heap using a stick. However, if the animal has not defecated, stool samples were collected directly from the rectum with the use of a gloved hand. The same procedure was done to collect fresh fecal sample from the dam within 24 hours after parturition. All stool samples were kept in properly labeled canisters and stored in the refrigerator until processing and microscopic examination for Cryptosporidium spp. Stool consistency whether solid, mushy or watery was noted right after collection. Gender and age of calves was recorded. General characteristics of the calves, possible risk factors associated with the infection among buffalo neonates, and laboratory results on microscopy and oocyst density of Cryptosporidium spp. in stool samples were also recorded.

Five (5) samples of water contained in five different pails used for drinking by the calves were randomly collected throughout the duration of the study for water analysis using Kinyoun’s stain. Each water sample was collected using a sterile bottle. Water from the pail was agitated first before collecting 30 ml at the center. Thirty (30) milliliters of water from the source was also collected after the first stream of water has been released. Water used by the buffaloes for drinking was analyzed for the presence of Cryptosporidium oocysts.

SAMPLE PROCESSING AND EXAMINATION

Fecal-concentration procedure (Claveria et al. 2007). For semi-solid feces, 3 grams was mixed with seven milliliters of 10% formalin while for solid feces, two grams was mixed with eight milliliters of 10% formalin. The fecal-formalin mixture was stirred and the suspension filtered using three-fold surgical gauze. The contents were poured into a 15 ml centrifuge tube. Ten percent formalin was added to the filtrate to attain the 10 ml mark to which three milliliters of ether was added. The tube was capped with a rubber stopper and vigorously agitated (Plate 3) for 10 seconds and centrifuged at 2,500-2,800 RPM (400-500xg) for three minutes. The top three layers (ether, fatty debris and formalin) were discarded while the sediment was retained for the detection of Cryptosporidium oocysts.
Fecal sediment staining (Claveria et al., 2007). Preparations of Kinyoun acid fast stain was done prior to the actual staining of fecal samples. Briefly, 4 grams of basic fuchsina was dissolved in 20 ml 95% ethanol. Phenol crystals were melted in 56°C water bath. Eight ml of the melted phenol was combined in fuchsain and 100 ml of distilled water was added over the mixture. The stain was allowed to stand for 1-2 days after which it was filtered before storage.

For staining procedures, fecal smears were prepared in duplicates for each collection period from calves and from the dam. A drop of fecal suspension from the sediment of the concentration technique was placed on two separate glass slides and spread to form a thin smear. The slides were warmed at 60°C to dry the smear completely. The fecal smears were flooded with Kinyoun’s acid fast stain for two minutes. The smears were washed with 50% ethanol followed by tap water. This step was repeated three times until all the red stain is washed off. The smears were flooded with one percent sulfuric acid for two minutes or until no further color is seen. The slides were washed with tap water and flushed with Loeffler’s alkaline methylene blue for one minute. Finally, the slides were washed with tap water, air dried and examined.

Sample Examination. Per fecal smear, around 200 viewing fields were covered at 40X magnification. To confirm internal morphology, 100X oil immersion objective was used. Oocysts density was determined according to the oocysts scoring system of Dagnall Teaching Laboratory, Liverpool School of Tropical Medicine (1998), as follows:

- Rare (+) for < 5 oocysts per slide (all fields)
- Few to moderate (+++) for 1-10 oocysts per ocular field
- Numerous (++++) for 11 or more oocysts per ocular field

**ANALYSIS OF DATA**

Incidence proportion and incidence rate for infected cases were determined according to calf–days:

\[
\text{Incidence proportion of cases} = \frac{\text{No. of cases}}{\text{Total no. of calves examined}} \times 100
\]

for the 12 day observation period

\[
\text{Incidence density or rate of cases} = \frac{\text{No. of cases per calf-days}}{\text{Total no. of calves examined}}
\]

Point prevalence of Cryptosporidium spp. infection was calculated based on sex:

\[
= \frac{\text{No. of infected males} \times 100}{\text{Total no. males examined}}
\]

\[
= \frac{\text{No. of infected females} \times 100}{\text{Total no. females examined}}
\]

Percentage distribution of stool consistency by day of observation was computed:

\[
= \frac{\text{No. of stool consistency* by day 4, day 8 and day 12 x 100}}{38 \text{ stools examined}}
\]

*stool consistency = solid, mushy, watery

Crude analysis was done to have an idea of the strength and direction of the outcome to the independent variables. Odds ratios and P-values were computed for the association of birth weight of calf, diarrheic dam, and parasitological state of the dam after delivery in relation to cryptosporidiosis.

**RESULTS AND DISCUSSION**

**MICROSCOPIC EXAMINATION**

Only one female calf with watery stool was found to harbor Cryptosporidium oocysts using microscopy. Kinyoun acid fast stain demonstrated magenta red colored oocysts (Fig. 1) which was typical of Cryptosporidium oocysts. This stain is specific for Cryptosporidium spp. oocyst that has the characteristic to be acid fast once stained with carbol fuchsin stain and resistant to decoloration with acid. The oocysts measured an average of 5.25 µm under oil immersion (x100) which was within the expected size range of the Cryptosporidium spp. oocyst (Fig. 2).
INCIDENCE OF CRYPTOSPORIDIOSIS

There were 38 calves enrolled in the study. Each calf was followed up on day one until day 12. Stool collection was done every four days from day one of birth. Stool consistency and microscopic examination for the presence of Cryptosporidium spp. oocysts using Kinyoun acid fast stain was done.

Out of 38 calves, only one became infected on day 8. The incidence proportion is one over 38 or 0.0263 for the 12 day observation period. The average risk of developing cryptosporidiosis during a 12 day period is 2.6% or around 2.6 or three per 100 calves. The incidence rate or density is one per 452 calf-days at risk or 0.00221. The rapidity by which Cryptosporidium infection develop is 2.2 per 1000 calf-days at risk. Moreover, the waiting time for a calf to develop the infection right after birth is eight days. (Table 1)

DISTRIBUTION OF STOOL CONSISTENCY BASED ON THE TIME OF COLLECTION

Results showed that by the end of day 4, 26.31% (10/38) expelled solid formed stool, 63.15% (24/38) mushy stool and 10.5% (4/38), watery stool. At day 8, the percentage of solid stool was reduced to 18.42% while the mushy stool increased to 76.3%. On the other hand, the watery stool decreased to 5.3%. However, on this same day, one infected female calf was detected with oocyst density of (+) or < 5 oocysts per slide. The infected calf showed signs of watery diarrhea. The watery diarrhea appeared grayish in color with blood tinge and plenty of mucus, typical of catarrhal enteritis. At day 12, the solid stool was further reduced to eight percent, mushy stool and watery stool increased to 84.2% and 8%, respectively. The infected calf remained positive with the same stool consistency and oocyst density. On the other hand, no additional case was observed on this day. A watery stool consistency conform a symptom of cryptosporidiosis in infected buffalo calves. (Table 2)
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Figure 4
Stool consistency at the end of 4, 8 and 12 day observation period

<table>
<thead>
<tr>
<th>Initial Time (day)</th>
<th>Final Time (day)</th>
<th>Stool Consistency</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage distribution at Day 4</td>
<td>n = 38 calves</td>
<td>26.31%</td>
<td>63.15%</td>
</tr>
<tr>
<td>n = 38 calves</td>
<td>4</td>
<td>10</td>
<td>24</td>
</tr>
<tr>
<td>Percentage distribution at Day 8</td>
<td>n = 38 calves</td>
<td>18.42%</td>
<td>76.31%</td>
</tr>
<tr>
<td>n = 38 calves</td>
<td>8</td>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>Percentage distribution at Day 12</td>
<td>n = 38 calves</td>
<td>8%</td>
<td>84.25%</td>
</tr>
</tbody>
</table>

*1 infected female calf with oocyst density (%) or ≥ 5 oocysts per slide

DESCRIPTION OF HYPOTHESIZED RISK FACTORS BASED ON EPIDEMIOLOGICAL MEASURES OF ASSOCIATION

The point prevalence of Cryptosporidium spp. based on sex of calves since only one female calf was infected was 5% (1/20). Sex was evaluated for the presence of association with cryptosporidiosis using Chi-square Test of Association. The computed $X^2$ value was 0.9 with a p-value of 0.342. Therefore, there was no sufficient evidence that sex can influence the development of cryptosporidiosis at five to 10% level of significance. The small sample size of 38 calves apparently was the reason for having no association.

The infected calf in the study had a normal birth weight of 36 kg., which was the ideal weight for a normal buffalo calf. The mean ideal weight is 35.5 kg based on Chi-square Test of association. Birth weight had a computed $X^2$ value of 0.58 with a p-value of 0.445. Therefore, there was no sufficient evidence that birth weight had influence on the Cryptosporidium spp. transmission at five to 10% level of significance. Having a lower birth weight than the ideal birth weight is not a guarantee that the calf could become susceptible to cryptosporidiosis.

All 38 dams enrolled in the study did not have any history of diarrhea until the time of calving. The infected calf could not have contracted the infection from its mother. Since all calves had the same exposure, result of Chi square test for association was undefined.

Out of the 38 dams enrolled in the study, 10 were found to be infected with oocysts ranging from one to 14 per slide. However, no calf borne from these infected dams developed the infection. The only infected female calf which developed the infection at day 8 after birth came from a non-infected dam. Based on Chi-square Test of association, this risk factor had a computed $X^2$ value of 0.41 with a p-value of 0.52. No sufficient evidence that infected dams at the time of calving had an influence on transmission of the infection to their newly born calves.

There were other incidental findings that could probably explain the incidence of cryptosporidiosis at the farm. First, the 10 infected dams were asymptomatic because all 38 dams did not have history of diarrhea for the past 3 months before parturition. Therefore, cryptosporidiosis among adult animals is not clinically significant to warrant immediate medication. This could explain the lackadaisical attitude of the caretaker in adopting hygienic procedures during calving of dams. Second, two dams calved on the same day wherein one dam that calved earlier was found to be infected while the other dam that bore the calf which later became positive after eight days did not have cryptosporidiosis. Since only one caretaker handled these two dams it could be implied that the caretaker was responsible for mechanically transferring the oocysts to the calf through contaminated hands.

Analysis of water samples from the bucket of selected calf pens were performed by recovering the sediment after centrifugation and staining it with Kinyoun acid fast stain. No water sample was detected with oocysts.

CONCLUSION AND RECOMMENDATION

Test of association showed no sufficient evidence to conclude that sex, birth weight and parasitologic state of the dam after delivery had an influence over the development of cryptosporidiosis. All dams did not have diarrhea and all calves were exposed to other animals in the pen thus, results of the test of association of these risk factors were undefined. Analysis of water samples from the bucket of selected calf pens and water source using Kinyoun acid fast stain revealed the absence of oocysts.

It is recommended that the duration of cohort study should be lengthened in order to have an epidemiological significance in the incidence of cryptosporidiosis. It is also recommended that other possible risk factors contributing to the transmission of Cryptosporidium spp. infection in calves should be investigated such as health status of the calves, sanitation of the calf pen, and personal hygiene practices of the caretaker who assists in parturition and feeding of neonatal calves.
Furthermore, although the use of Kinyoun acid fast stain procedure has high specificity in detecting Cryptosporidium spp., its sensitivity should also be taken into account and confirm with other tests such as molecular based techniques to increase the reliability of the results. There is a high chance that the animals turned negative by microscopic exam if there is low level of parasitism.

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References

Author Information

Marvin A. Villanueva
College of Veterinary Science and Medicine, Central Luzon State University

Clarissa Yvonne J. Domingo
College of Veterinary Science and Medicine, Central Luzon State University

Nancy S. Abes
Philippine Carabao Center National Headquarters and Genepool

Claro N. Mingala
Philippine Carabao Center National Headquarters and Genepool