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Original papers

Molecular identification of *Buxtonella sulcata* from associated-diarrhea in water buffaloes (*Bubalus bubalis*) in the Philippines

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ABSTRACT. Sixty suspected protozoan oocysts were demonstrated from 260 fecal samples collected from water buffaloes aged one month to seven years old with clinical signs of diarrhea in four provinces in the Philippines after conventional methods of isolation, sporulation, morphological characteristics and Kinyoun Acid Fast Staining techniques. The recovered protozoan oocysts were subjected to molecular analysis. Amplification of DNA extracted from recovered *Eimeria* oocysts using universal primers for the ITS-1 region of 18S rRNA revealed PCR products with 348 bp size demonstrated by samples collected from Benguet, La Union and Nueva Ecija provinces in the Philippines while DNA extracted from oocysts of suspected *Cryptosporidium* spp. samples that applied primers for the SSU of 18S rRNA registered PCR products but no genes were amplified from diarrheic water buffaloes from these provinces. Alignment of the DNA sequences of the suspected *Eimeria* and *Cryptosporidium* species revealed sequences for three isolates of *Buxtonella sulcata* with product lengths that varied from 235 to 252 bp. This is an initial observation on the involvement of *B. sulcata* in diarrhea condition of water buffaloes in the Philippines. Phylogenetic analysis of the three local isolates of *B. sulcata* revealed no similarity with other protozoan constructed according to Neighbor-Joining method.

Key words: water buffaloes, diarrhea, *Buxtonella*, *Eimeria*, *Cryptosporidium*

Introduction

The population of water buffaloes in Asia comprising the riverine and swamp types is more than 95.8% of the total world population [1]. India has a water buffalo population of 97.9 million head representing 56.5% of the world population and China has the second largest buffalo population in Asia with 97.9%. Similarly, there were 3.2 million swamp type buffaloes in the Philippines as of 2003; nearly 3 million swamp buffalo were in Vietnam; 772,764 buffaloes in Bangladesh; 889,246 water buffaloes in Nepal and 750,000 head in Sri Lanka [1]; and 1.24 million in Thailand [2].

Water buffaloes often manifest long-standing diarrhea. Several causes of diarrhea in animals include bacterial, viral, intestinal parasites, certain chemicals, poor diet, overfeeding on milk or lush grass, poisonous plants, other toxins, food allergies and stress [3]. This condition is usually accompanied by bloody diarrhea due to GIT hemorrhage, poor growth in young animals and often associated with coccidiosis. The disease results in death especially in young animals and cause considerable economic losses in farm animal's worldwide.

Diarrhea continues to be single cause of death in neonatal and young calves caused by protozoan-

related diarrhea such as *Cryptosporidium* spp., *Eimeria* spp. and *Giardia* species. On the other hand, *Buxtonella sulcata* has been discussed as a parasitic cause of diarrhea in calves but it is not fully documented [4,5]. The involvement of *B. sulcata* in diarrhea of young calves and neonates is reported to be related to the presence of suitable conditions in the intestinal lumen that promote multiplication of the parasite [6].

All domestic animals are prone to infections resulting to diarrhea and every case of diarrhea requires immediate diagnosis and strategies for effective treatment and management. At present, morphological observation of oocysts is the most used practical method of diagnosis for diarrheic-related bovine coccidiosis. The morphological characterisation method is not confirmatory since several species have confusing features, compounded by the presence of intra-species morphological variation. Furthermore, morphological observations combined with fecal examination are very labor-intensive and require skilful technique [7]. The oocysts of the coccidia are robust structures, frequently isolated from the feces or urine of their hosts which provide resistance to mechanical damage and allow the parasites to survive and remain infective for other animals in the herd. The diagnosis of coccidiosis, species description and systematics are all dependent upon the characterization of the oocysts [8].

The utilization of molecular techniques has allowed accurate diagnosis of protozoan parasites and recognition of coccidian infections in water buffaloes [9]. Sequencing provides a way of confirming the identity of the protozoan parasite, bacterial and viral agent of diarrhea where phenotypic characters demonstrate conflicting information [10]. The study was undertaken to detect the protozoan oocysts in water buffaloes through conventional methods and to identify the recovered protozoan oocysts through PCR and confirmed by DNA sequencing.

Materials and Methods

Collection of fecal samples from water buffalo. Fecal samples of 260 water buffaloes aged one month to seven years with clinical signs of diarrhea were collected in the provinces of Nueva Ecija, La Union, Mountain Province and Benguet for the recovery of 60 suspected protozoan parasites (30 for *Eimeria* and 30 for *Cryptosporidium*). Thirty grams of fresh fecal samples (liquid and semi-solid in consistency) were collected per rectum from each water buffalo. Each sample was placed either in clean plastic bottles, sterile plastic cellophane and or sterile collection gloves, held in ice chest and brought to the laboratory for microscopic evaluation and sporulation.

Pre-screening evaluation of fecal samples for the presence of protozoan parasites. The parasitological technique for the demonstration of *Eimeria* oocysts was done following the procedures as described by Hansen and Perry [11]. Fecal samples positive with suspected *Eimeria* oocysts were properly labelled for processing. The procedures were repeatedly done individually to fecal samples collected until 30 positive fecal samples with *Eimeria* spp. were determined.

For the recovery of suspected *Cryptosporidium* oocysts, Kinyoun Acid Fast Staining (KAFS) was utilized following the procedures adapted from Domingo [12]. The demonstration of magenta red color of oocyts was found typical feature that signifies presence of *Cryptosporidium* oocysts. Individual fecal samples showing magenta red color of oocysts were properly labelled and stored at 4°C until processing and microscopic examination for the presence of *Cryptosporidium* sp. The procedures were repeatedly done individually until 30 fecal samples demonstrated magenta red color oocysts.

Microscopic evaluation of coccidian oocysts. The sixty fecal samples positive with suspected coccidian parasites were individually processed and analysed by sugar flotation technique. The recovered *Eimeria* and *Cryptosporidium* oocysts from 60 positive samples were collected as described by Dibner et al. [13]. The sediments containing the suspected *Eimeria* oocysts were washed two times with distilled water and the sediments were added with a 2.5% potassium dichromate and transferred to a petri dish for sporulation. The individual sediments containing the suspected *Cryptosporidium* oocysts were placed in individual 10 ml plastic tubes and stored at –20°C ready for DNA extraction.

DNA extraction. DNA extractions for the 60 positive samples (30 *Eimeria* spp. and 30 *Cryptosporidium* spp.) were processed individually using QIAamp Fast DNA Stool Mini Kit (Cat. no. 51604) as per manufacturer's protocol. The elute DNA was preserved at –20°C until further use.

Primers for PCR. Universal primers for the amplification of the target ITS-1 region of the 18S

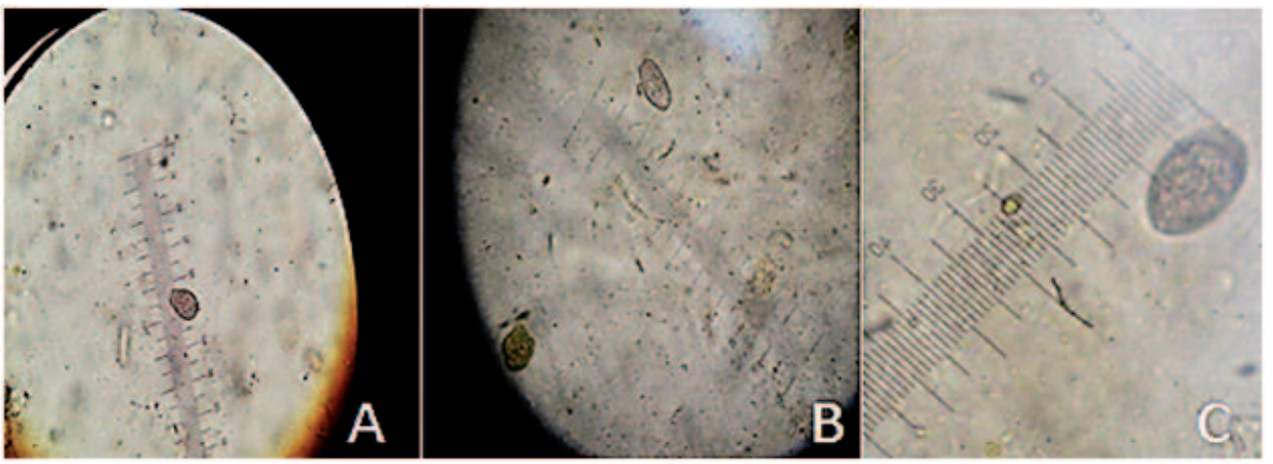

Fig. 1. *Eimeria* oocysts using Electric Binocular Microscope (Ken-a-vision®) (40×) with arrow. **A.** 27×20.25 µm, pyriform, light yellowish-brown, 2-layered oocyst wall, micropyle present and polar cap absent; **B.** 31.5×22.5 µm, ellipsoidal, light yellowish-brown, 2-layered oocyst wall, inconspicuous micropyle, and polar cap absent; **C.** 31.5×27 µm, ovoidal, yellowish brown, 2-layered oocyst wall, micropyle present and absent of polar cap.

rRNA of *Eimeria* sp. using primer sets, Forward: GCAAAAGTCGTAACACGGTTTCCG and Reverse: CTGCAATTCACAATGCGTATCG. Amplification of the ITS-1 region of 18S rRNA gene of *Eimeria* was in a thermal cycler (9800 Fast Thermal Cycler, USA) that need 20-µl reaction mixtures containing 10 µl Premix Taq (Ex Taq Version, Takara Bio Inc., Shiga, Japan), 1 µl of the 10 µM primer set and 1 µl of genomic DNA template. Reaction cycles consisted of an initial denaturing step at 94°C for 30 s followed by 35 cycles at 94°C for 10 s, 55°C for 30 s, 72°C for 30 s with final extension at 72°C for 2 min [7].

Cryptosporidium sp. was amplified using Primers targeting the SSU-18S rRNA gene set for, Forward: CTCGTAGTTGGATTTCTGTT and Reverse: TAAGCACTCTAATTTTCTCA. The full-length SSU of 18S rRNA gene was amplified in a thermocycler (9800 Fast Thermal Cycler, USA) programmed to function at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, followed by 72°C for 10 min [14].

Agarose Gel Electrophoresis. After amplification, 10µl of PCR products were separately resolved in a 1.5% agarose gel with the 100 kb bp DNA ladder (New England Biolabs, MA, USA) and 4 µl loading dye. An electric current of 110 volts were applied during the electrophoresis for 30 min as described by Ho et al. [15]. The PCR products were visualized in a trans-illuminator (Gel Doc, USA) and the amplicon sizes of the DNA products were noted separately for *Eimeria* and *Cryptosporidium* species.

Sequencing of suspected ITS-1 region in *Eimeria* and SSU genes in *Cryptosporidium*. The genetic sequence of the ITS-1 region of *Eimeria* and SSU gene of *Cryptosporidium* were aligned using MEGA 5 software and compared with the sequences of other ITS-1 region and SSU genes in the

Table 1. Morphological characteristics of isolated coccidian oocysts in water buffalo

Characteristics	*Eimeria* sp.	*Cryptosporidium* sp.
Size (mean)	31.95×22.5 µm	5.0–5.5 µm
Shape	Ovoid somewhat flattened, somewhat flattened at the small end, ellipsoidal, pyriform	Spherical to oval
Color	Light brown, colorless pale yellow	
Oocysts wall	2 layered which either appeared smooth or rough	
Polar cap	Present or absent	
Reaction to Kinyoun Stain		Magenta red
Clinical sign	diarrhea	diarrhea

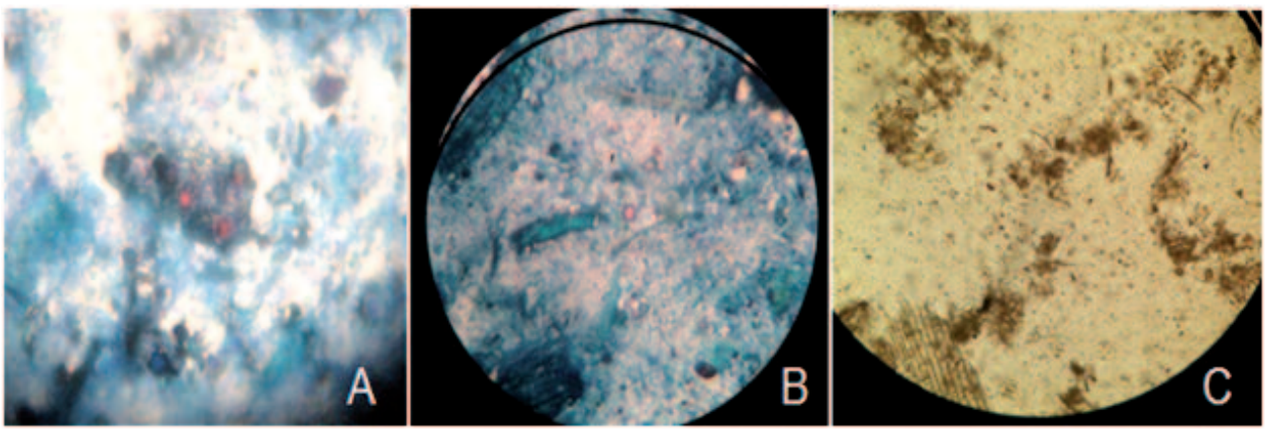

Fig. 2. *Crystosporidium* oocysts in magenta red in color using Electric Binocular Microscope, (Ken-a-vision®) (arrow) (40×). **A.** spherical, absent of micropyle and polar cap; **B.** spherical, absent of micropyle and polar cap; **C.** unstained oocysts, spherical with arrow (10×).

GenBank using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (NCBI).

Phylogenetic analysis. The aligned DNA sequences of the identified local isolates were used as reference for the construction of the phylogenetic tree using the MEGA 6 software. The sequences of the identified local isolates were used for sequence alignment and phylogenetic analysis with reported protozoan parasite strains deposited in GenBank.

Results

Morphological characteristics

Following conventional procedures, 60 fecal samples (30 positive samples for *Eimeria* and 30 positive samples for *Cryptosporidium* oocysts) were detected with protozoan parasites from 260 fecal samples collected from water buffaloes. Presumptive *Eimeria* oocysts were recovered based on their morphological features and measurements as shown in Fig. 1 while *Cryptosporidium* oocysts appeared magenta red based on KAFS and unstained oocysts as shown in Fig. 2. A summary of the morphological characteristics of coccidian oocysts in the feces of water buffalo is shown in Table 1.

The observed morphological characteristics and microscopic measurements of the isolates of *Eimeria* and *Cryptosporidium* oocysts were in conformity with the descriptions of Florião et al. [16] and Levine [17], respectively. KAFS demonstrated a magenta red color of the *Cryptosporidium* oocysts which conformed with the findings of Villanueva et al. [18] (Fig. 2).

Sporulation of oocysts

Microscopic evaluation of *Eimeria* oocysts were subjected to sporulation and exhibited four sporocysts and within each sporocysts are two ellipsoidal shape sporozoites slightly pointed ends. The observed *Cryptosporidia* oocysts showed no sporocysts but with emerging four sporozoites.

These observations conformed to the report of Pyziel and Demiaszkiewicz [19] who observed sporulated oocysts of *Eimeria* and *Cryptosporidium* with immature, not fully formed sporocysts. There was difficulty in the identification of other structures in the sporulated oocysts through conventional microscopic evaluation. Long et al. [20] described the tediousness of performing microscopic evaluation of the morphological features of sporulated oocysts as protozoans which may share similarity in structural characteristics.

Molecular characteristics of isolated coccidian parasites in water buffalo

Conventional methods of protozoan oocysts identification established a positive recovery of both *Eimeria* and *Cryptosporidium* oocysts from diarrheic feces of water buffalo while PCR products showed three positive and 27 negative for *Eimeria* spp. and 0 recoveries of PCR products for *Cryptosporidium* oocysts. The low and zero recoveries of PCR products of *Eimeria* and *Cryptospostridium* spp., respectively, may be attributed to inefficient DNA extraction and high amounts of impurities that prevented inhibition of the enzymatic reactions. These observation concurred with the findings of other authors such as obtaining high-quality genetic material from a given

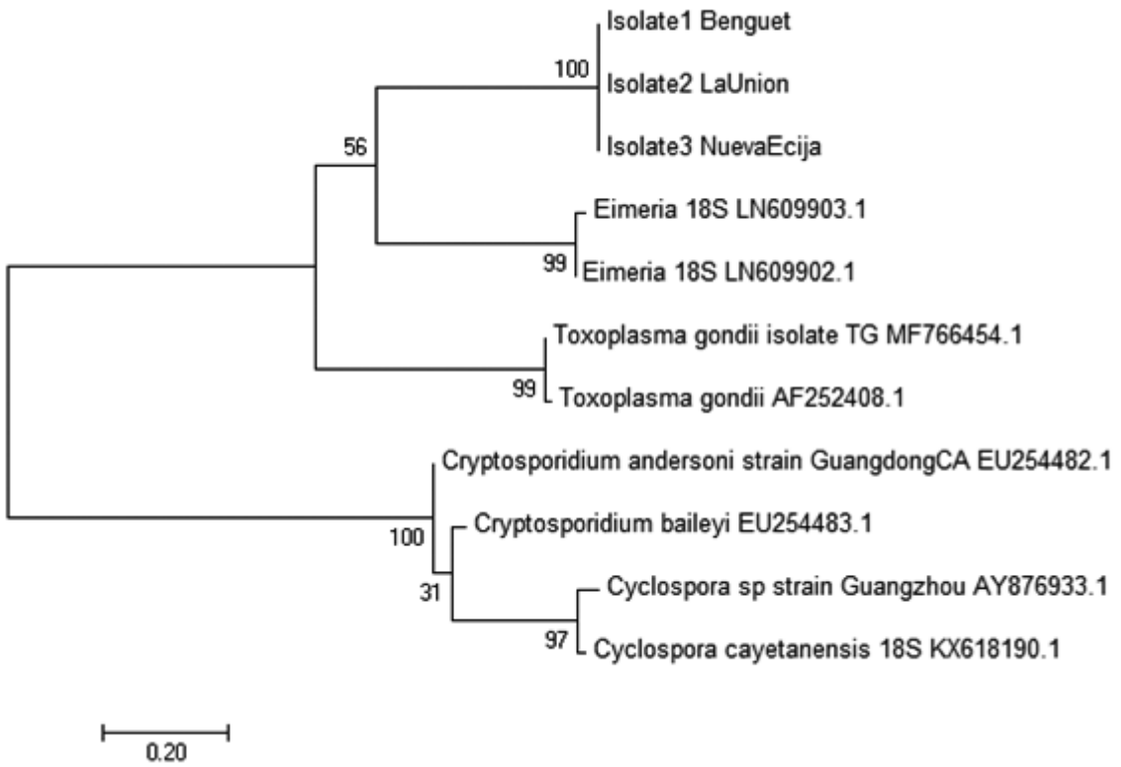

Fig. 3. Phylogenetic relationship of three local isolates of *Buxtonella sulcata* and other protozoa registered in the GenBank constructed according to the Neighbor-Joining method. Local isolate 1 (Benguet), Local isolate 2 (La Union) and Local isolate 3 (Nueva Ecija).

extraction method is essential for successful amplification [21]. Samples must contain minimal amount of impurities that prevent inhibition of the enzymatic reactions or interference with the gel migration patterns [22,23]. Therefore, the higher the purity of the extracted samples, the better results of the PCR reactions [24].

Amplification of PCR products of *Eimeria* species

Amplification of collected DNA extracts from recovered sporocysts of 30 *Eimeria* positive samples that applied the universal primers for the ITS-1 region of 18S rRNA of genus *Eimeria* revealed PCR products with 348 bp molecular weight as demonstrated by *Eimeria* species obtained from water buffaloes with diarrhea.

Amplification of PCR products of *Cryptosporidium* species

Amplification of the DNA extracts from recovered oocysts from 30 *Cryptosporidium*-positive samples that utilized the primers for the SSU of 18S rRNA of *Cryptosporidium* exhibited PCR products but no genes were amplified. The PCR product was sent for DNA sequencing to identify what was amplified and after aligning the gene sequence using BLAST, the protozoa identified was *B. sulcata.* Result of gene aligning implied that PCR primers for *Cryptosporidium* were not specific at the genus level.

DNA sequences of coccidian parasites

Results of DNA sequencing were processed and confirmed by NCBI- BLAST. *Eimeria* was not demonstrated, instead a 97% to 99% degree of similarity and to *B. sulcata* was revealed with a product length of 235 bps and 252 bps in the sequencing of *Eimeria*. Similarly, *Balantidium* and *Neobalantidium* isolates with degree of similarity of 85% to 88% and 85% to 87% were identified, respectively.

After DNA sequencing, all PCR products showed high homology to *B. sulcata.* The nucleotide sequences of the identified isolates of *B. sulcata* were used as a reference in determining their genetic relationship with other protozoan parasites registered in the GenBank.

Data in Fig. 3 demonstrated that the three isolates of *B. sulcata* (local isolate 1, Benguet; local isolate 2, La Union; and local isolate 3, Nueva Ecija) belonged to the first clade. Based on the data, the three isolates were phylogenetically related with bootstrap values of 100 percent. Data also showed that the three local isolates had low rate of phylogenetic relationship with *Eimeria* species (56%) and were not related with other protozoa like *Toxoplasma gondii*, *Cryptosporidium* species and *Cyclospora* species which presented high bootstrap values only with protozoans of the same genus, independent from the three local isolates of *B. sulcata.*

Discussion

In the identification of potential protozoan parasites incipient with diarrhea in water buffaloes, conventional methods of screening parasitism through flotation, microscopic evaluation and measurement of structures with diagnostic importance were considered essential steps in parasitological studies. As these may have some limitations in the capacity to demonstrate cellular structures distinct and diagnostic for specific parasites, the study showed the importance of relying on advance techniques that evaluated DNA components to confirm a diagnosis. In this study, *Eimeria* and *Cryptosporidium* species were successfully demonstrated based on conventional method.

DNA extracts of morphologically-evaluated samples of *Eimeria* sporocysts were successfully amplified by the ITS-1 region of 18S rRNA as demonstrated by the molecular weight of the targeted amplified product size of 348 bp, while the amplification of the SSU of the 18S rRNA of DNA products of *Cryptosporidium* oocysts were not successful to demonstrate the expected products size of 122 base pairs. Other researchers have successfully explored the importance of the ITS-1 region of rRNA gene in their studies for *Eimeria* in cattle [7,25,26]. Studies that evaluated *Cryptosporidium* in snake and lizards and cattle were efficiently detected by the application of the SSU 18S rRNA [14,27]. However, in this study, Agarose gel electrophoresis revealed that the amplicon sizes of DNA products were not demonstrated for *Cryptosporidium* as the expected product size of 122 bp was not established.

Despite the application of universal primers for *Eimeria* and specific primers for *Cryptosporidium*, result of DNA sequencing resulted to the incidental identification of a protozoan ciliate *B. sulcata*. This finding raised the contention that the target genes used in nucleic acid amplification was not specific for *Eimeria* and *Cryptosporidium*. Hence, further study using other target genes must be done.

Result of DNA sequences established the occurrences of *B. sulcata* in water buffaloes raised in Benguet, Nueva Ecija and La Union. *B. sulcata* is a parasitic protozoa which inhabits the colon of ruminants [28] and as a commensal protozoa of the alimentary tract of ruminants and participative in the digestion of plant aliment [29]. The parasite is under Kingdom *Protozoa*; Phylum *Ciliophora*; Class *Kinetofragminophora*; Order *Trichostromatida*; Family *Pyenotrichidae*; Genus *Buxtonella* [30]. The life cycle was not described [31]. The presence of *B. sulcata* was recorded in England, Poland, Denmark, Serbia, Pakistan, Japan, Turkey, Nepal, Iraq, Thailand, North America, Korea, Costa Rica and Egypt [5,29,32]. At present, no record of *B. sulcata* infection among buffaloes has been reported in the Philippines.

Recent studies showed that *B. sulcata* may be a potential causative agent of periodical recurrent diarrhea of unknown etiology in cattle [33]. In a study conducted by Tung et al. [34], mixed infections of *Cryptosporidium* spp. (41.6%), *Eimeria* spp. (11.9%) and *B. sulcata* (8.4%) were demonstrated in 310 fecal samples of yellow cattle raised in Taiwan and its offshore islands Penghu and Kinmen using coprological techniques. Buxtonellosis has not yet been considered of veterinary importance in ruminants; however, previous investigations reported that *B. sulcata* might be a causative agent of diarrhea in ruminants [5,28,30]. High incidence of *B. sulcata* infection in ruminants may be responsible for the incidence and intensification of diarrhea symptoms [30]. Urman and Kelly [35] and Skotarczak [36] suggested that *B. sulcata* can lead to pH changes of large intestinal content of cattle and multiplication of the parasite causes a cytotoxic effect in the large intestine which is manifested as lesions of the intestinal mucosa followed by secondary bacterial infections.

PCR detection of intestinal protozoa was reportedly restrained by poor DNA recoveries or by inhibitors present in the feces [37]. Amplification of *Eimeria* and *Cryptosporidium*-positive samples subjected to DNA extraction protocol was cited to be speculative and low sensitivity had been

attributed to the inhibition of the reaction by impurities present in the stool samples with target DNA and in the extraction procedure such as inefficient nucleic acid isolation or purification [37].

Phylogenetic relationships of the identified 3 local isolates of *B. sulcata* belonged to the first clade which were closely related with each other with bootstrap values of 100% and are not related to other protozoa registered in the GenBank. Relatively, little descriptions regarding the molecular phylogenetic data of *B. sulcata* had been reported [38].

Other studies that elucidated other conventional methods of oocysts isolation and Fecal Ether Concentration Technique are recommended for processing *Cryptosporidium* oocysts. It is recommended that specific primers be utilized for the DNA amplification for *Eimeria* and *Cryptosporidium* and synthesized positive control for *Eimeria* and *Cryptosporidium* be used for confirmation. Further, follow-up studies related to the presence or absence of *B. sulcata* in water buffaloes in the Philippines is recommended.

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