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Morphological diagnosis and occurrence of *Blastocystis* spp. obtained from the stool samples of domestic bird species commercialized in municipal markets

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***Blastocystis hominis* is a gastrointestinal tract parasite whose pathogenic role, and zoonotic potential remains unclear. Several microorganisms similar to *B. hominis* have been reported in a variety of non-human hosts, and described as *Blastocystis* spp. The study aimed to verify the occurrence of *Blastocystis* spp. in three species of poultry of two municipal markets, by observing the microorganisms' forms in stool samples stained with Giemsa. A total of 214 birds, distributed into two markets, were studied. In each of the markets were observed, 35 ducks (*Anas platyrhynchos*), 35 Japanese quail (*Coturnix japonica*) and 35 chicks (*Gallus gallus*). In market A, 8 (22.9%) chicks, 15 (42.9%) quails and 13 (37.1%) and ducks were diagnosed with *Blastocystis* spp.; in market B, 15 (42.9%) chicks, 2 (5.56%) quails, and 21 (55.3%) ducks. A significant difference was observed only between quails from the two markets but no statistical difference was observed when all infected birds in the two markets were compared. In stool samples positive with *Blastocystis* spp. and stained with Giemsa were observed forms as vacuolar, granular, amoeboid, and cystic, and some types of reproduction, such as binary fission, plasmotomy and budding. Both markets had unhygienic conditions of animal facilities, favoring the infection among them.**

Key words: Poultry, pleomorphic organism, gastrointestinal parasite, giemsa, microscopy, unhygienic.

INTRODUCTION

The first description of the genus *Blastocystis* was given by Alexeieff (1911), and currently the correct diagnosis of the microorganism is still a challenge, especially due to its uncertain pathogenicity. In addition, *Blastocystis* is considered a pleomorphic organism that can be confused with many structures in fecal samples without staining. *Blastocystis* spp. obtained from the feces of humans and animals have been reported as morphologically similar, although some authors have described distinct differences between those isolates (Singh et al., 1996; Stenzel et al., 1994, 1997). However, to differentiate one isolate from another, morphology cannot be used as the single criterion. In the 1970s and 1980s, the studies of Charles Zierdt caught the attention of biologists and clinicians (Tan et al., 2002), and from there, many other studies were performed, mainly focusing on the morphology of *B. hominis* (Stenzel and Boreham, 1996). *Blastocystis* spp. is the most common microorganism in the gastrointestinal tract of humans in various parts of the world (Stenzel and Boreham, 1996). It is also found in a wide variety of animal hosts (Boreham and Stenzel, 1993) and is considered to be a pathogen with high

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zoonotic potential (Tan, 2004). New species of *Blastocystis* have been proposed for non-human hosts (Belova and Kostenko, 1990; Belova, 1992; Teow et al., 1991), but these species have not been accepted among researchers because most isolates of *Blastocystis* are morphologically indistinguishable by observation under optical and electron microscopy (Noël et al., 2005; Suresh and Smith, 2004). Nevertheless, until there is further confirmation using molecular data, *B. hominis* is defined as a parasite isolated from humans, and *Blastocystis* spp. is defined as a parasite obtained from other animals (Noël et al., 2005).

The diagnosis of *Blastocystis* is usually carried out by the examination of fecal samples under an optical microscope (Zierdt, 1991; Garcia and Bruckner, 1993), followed by other diagnostic methods, such as the molecular characterization of subtypes (Forsell et al., 2012). For diagnosis by light microscopy, fecal smears can be stained to observe the various forms of *Blastocystis* spp. with various dyes, such as the trichrome stain (Garcia and Bruckner, 1993), iron hematoxylin (Guimarães and Sogayar, 1993), Giemsa (Dawes et al., 1990), gram (Zierdt, 1991) and wright (Vannatta et al., 1985) stains. Because there is no indication of a method that is more efficient, and difficulties in identifying the various forms of the microorganism in fecal material, these dyes seem to be the procedure of choice for the diagnosis of *Blastocystis* spp. as they show some of its internal structures and morphological characteristics.

Many isolates of *Blastocystis* that have been obtained from diverse hosts have shown some similarities (Tanizaki et al., 2005). These authors observed that isolates from chickens, quails and geese were able to infect chickens, demonstrating the existence of cross infection between bird species. In addition, a non-infected chicken got infected with *Blastocystis* after being raised with infected ones, emphasizing that the transmission of *Blastocystis* may occur easily between the same species or between different species of birds. Tanizaki et al. (2005) observed that isolates obtained from chicken, quails and geese were able to infect chickens, and demonstrated the existence of cross infection among bird species. Besides, a non-infected chicken got infected after being raised with infected ones, indicating that transmission of *Blastocystis* may easily happen among birds from the same or different species, showing a lack of host specificity.

Abe et al. (2002) conducted a research with zoo animals and reported a great prevalence of *Blastocystis* infection in pheasant and ducks. In another study, the *Blastocystis* cystic forms were diagnosed to infect chickens (Stenzel et al., 1997). The experimental infection of chickens and mice using four subtypes isolated from humans confirmed that some subtypes could infect chickens and rats, indicating the zoonotic potential of *Blastocystis.* These results also suggested that these hosts may be adequate animal models for the study of this microorganism (Iguchi et al., 2007).

The aim of the present study was to verify the occurrence of *Blastocystis* spp. by morphological diagnosis in stool samples stained with Giemsa that were obtained from ducks (*A. platyrhynchos*), quail (*C. japonica*) and chickens (*Gallus gallus*), which were naturally infected and commercialized in markets in the state of Rio de Janeiro, Brazil.

MATERIALS AND METHODS

The current study was conducted with a total of 214 live domestic birds commercialized in two municipal markets, named A and B, in Rio de Janeiro City, Brazil. A total of 71 Japanese quails, 73 ducks and 70 chickens were acquired from these two markets. It was analyzed, with 105 birds (35 ducks, 35 quails and 35 chickens) from market A and 109 birds (38 ducks, 36 quails and 35 chickens) from market B. The animals were marketed at approximately one week of age. Both markets had similar characteristics and were approximately 110 km apart from each other. The animals of each market had different origins and suppliers. The birds were housed in metal cages that were placed on shelves and had a high population density, favoring the stress of the animals. Food and water were provided in the cages, but the containers were full of feces due to the high density of the birds.

In each market, the birds were randomly selected, immediately transported and housed in individual cages where they were provided with food and water. Individual fecal samples were analyzed daily. For each bird, 4 g of fecal samples were weighed and homogenized in 5 ml of sodium chloride 0.9%, and a small aliquot of this material was observed between a slide and a cover slip by microscopy to diagnose the presence of *Blastocystis* spp. Then, the positive fecal smears were fixed with methanol and stained with Giemsa to observe the detailed morphology of the microorganism. The forms of *Blastocystis* spp. observed by microscopy after staining were analyzed according to the shape and size of the microorganism, the number of nuclei, and the presence or absence of a vacuole. Fisher's exact test was used in the statistical analysis of the results, using program Epinfo's Statcalc (Dean et al., 2002).

RESULTS AND DISCUSSION

Occurrence of natural infection of *Blastocystis* spp. in poultry

In a total of 214 birds, 22.9% (8/35) of the chickens from market A presented *Blastocystis* spp. in the feces, while 42.9% (15/35) from market B were positive. Although market B had a higher number of positive chickens, no significant difference ($p = 0.74$) was observed between the two markets. With respect to the ducks, 37.14% (13/35) from market A presented *Blastocystis* spp. in the feces, while 55.26% (21/38) from market B were positive. As with the chickens, despite the higher number of positive animals found in market B, there was no significant difference ($p = 0.121$) between the two markets. With respect to the quails, 42.85% (15/35) from market A presented *Blastocystis* spp. in the feces, while only 5.56% (2/36) from market B were positive. In this

Table 1. Occurrence of *Blastocystis* in chickens (*G. gallus*), ducks (*A. platyrhynchos*) and Japanese quails (*C. japonica*) obtained from two municipal markets in Rio de Janeiro, Brazil.

Host	Market	Examined animals/Infected	Positive animals (%)	Positive animals (%) (N = 214)
Chicks	A	35/8	8/22.90	23/10.70
	B	35/15	15/42.90	
Ducks	A	35/13	13/37.14	34/15.90
	B	38/21	21/355.26	
Japanese quails	A	35/15	15/42.85	17/7.90
	B	36/2	2/5.56	
Total	A and B	214/74	74	74/34.60

case, a significant difference was observed between the two markets ($p < 0.001$) (Table 1).

The rate of *Blastocystis* spp. naturally infecting the chickens in the current study was similar to the one observed by Stenzel et al. (1997) who reported a natural infection rate of 31%. In the same study, they observed that infected chickens were held in cages that were stocked with two or three per cage, in contrast with the present study, where the birds were originally held in cages with a high population density. In another study, Lee and Stenzel (1999) observed the prevalence of *Blastocystis* in 227 domestic chickens from 5 commercial farms and found a high rate of infection that was approximately 95% in four of the five farms studied, which is a high percentage in comparison with the observed current study.

Regarding the ducks of the current study, the rates observed in the two municipal markets are very similar to that observed by Abe et al. (2002), in which the infection rate was 56% in ducks from the Zoo of Osaka City, in Japan. From the 214 birds investigated in markets A and B, 74 (34.6%) birds presented *Blastocystis* spp. in the feces, of which 15.9% (34/74) were ducks, 10.7% (23/74) were chickens, and 7.9% (17/74) were quails (Table 1). The results showed no significant difference between the infection of ducks and chickens or between chickens and quails; however, there was a difference between ducks and quails. These results suggest that ducks may be more susceptible to *Blastocystis* spp. infection than chickens and quails. Importantly, chickens and quails belong to the Order Galliformes, while ducks belong to the Order Anseriformes. Because they belong to different Orders, ducks may also have a different immune response from that of chickens and quails.

With respect to the birds positive with *Blastocystis* spp. infection, 36 (34.3%) were from market A, and 38 (34.9%) were from market B. These results indicate that there is no significant difference ($p = 0.92$) between the two municipal markets in the total number of birds with *Blastocystis* spp. infection. Thus, the markets likely did not have a great influence on the infection rates of these poultry because the hygiene and sanitary conditions of both markets, A and B, were very similar.

The observation of clinical signs, such as lethargy, appetite loss and fatigue, in poultry may suggest gastrointestinal infections, among them the *Blastocystis* spp. infection. One way to prevent such an infection would be the separation of the poultry after the observation of the first clinical signs, thus reducing the spread of the microorganism. The clinical signs mentioned above were previously reported by Stenzel and Boraham (1996) and Moe et al. (1997) in birds infected with *Blastocystis* spp. Therefore, avoiding the acquisition of symptomatic birds or separating the symptomatic from the asymptomatic birds would circumvent the dissemination of the infectious forms of this organism.

The consistency of the feces eliminated by the birds during the collection was another important aspect that was observed. Most of the fecal samples positive with *Blastocystis* spp. had an abnormal aspect; they were diarrheic and sometimes passed out blood colored with mucus feces. There was no correlation between the feces aspect and the occurrence of *Blastocystis* infection. In agreement, Tan (2004) observed that describing the clinical signs of *Blastocystis* infection as a real cause of disease is difficult. In contrast, several authors reported the occurrence of pasty feces and diarrhea in infections caused by *Blastocystis*. Quilez et al. (1995) observed pasty feces in infected pigs, Stenzel and Boreham (1996) described diarrhea in infected humans, and Moe et al. (1997) reported pasty feces in infected lab rats.

Lee and Stenzel (1999) investigated a property with high-quality hygiene and sanitary conditions, and none of the birds studied were positive for *Blastocystis*. They investigated the conditions of the (properly cleaned) floors, utensils and equipment, the removal of feces, and the cleanliness of the food and water containers. Their study indicated that good hygiene and sanitary conditions were possible inhibitors of environmental contamination

Table 2. Morphometric data of some of the forms of *Blastocystis* spp. observed in stool samples stained with Giemsa that were obtained from poultry sold at local markets.

Characteristic	Form			
	Vacuolar (n = 100)	Granular (n = 100)	Amoeboid (n = 17)	Cystic (n=200)
Maximum size (µm)	20.3±4.2	16.9±4.1	45.5	5.5
Minimum size (µm)	18.3±3.9	15.3±3.9	13.4	2.1
Number of nuclei	1 and 7	1 and 4	1 and 4	1 and 4
Index morphometrics	1.11±0.02	1.11±0.03	-	1.4±0.03

and of the fecal-oral transmission of *Blastocystis* infection among chickens. Therefore an adequate management of the birds could contribute to better health maintenance of the animals.

Diagnosis of *Blastocystis* spp. observed in stool samples stained with giemsa

Four forms of *Blastocystis* spp. were observed in stool samples stained with giemsa: vacuolar, granular, amoeboid and cystic. These forms have been reported by several authors, especially in studies related to *B. hominis*. With respect to the *Blastocystis* spp. isolated from animals, there are few descriptions of the morphological characteristics of the microorganism diagnosed in feces. The most common form observed was vacuolar, rounded and containing a central body resembling a large vacuole that occupies approximately 90% of the cell, with a thin layer of peripheral cytoplasm. In the cytoplasm, nuclei can be observed in a peripheral location, with up to seven nuclei per cell and an average of two nuclei in cells arranged at the opposite poles. Images of the form mentioned above from a fecal smear stained with Giemsa are shown in Figure 1; A and B.

The morphometric data presented in Table 2 are consistent with those described by Lee and Stenzel (1999) which were obtained from domestic chickens. The measurements of the vacuolar forms of *Blastocystis* spp. were quite varied, with a minimum measurement of 10.9 µm and a maximum of 32.1 µm.

The granular form of *Blastocystis* spp. was very similar to the vacuolar one, with multiple central granules in the vacuole. Dunn et al. (1989) proposed that these structures could be similar to myelin inclusions, small vesicles, crystalline granules and lipid droplets. As found in the vacuolar forms, more than one nucleus was observed in its restricted cytoplasm. Up to four nuclei, with an average of two per cell, were observed. Images of the granular form in stool smears stained with giemsa are shown in Figure 1C to F.

In the granular form, the *Blastocystis* spp. were slightly smaller than the vacuolar forms (Table 2) but similar to those reported by Stenzel and Boreham (1996). The diameter of the cells ranged from 9.0 to 28.3 µm. The granular form showed a different quantity of granules in their interior, which was noted because of the change in the intensity of staining when observed under microscopy. This observation may be due to the affinity of the dye used in this study. Similar to the vacuolar form, the

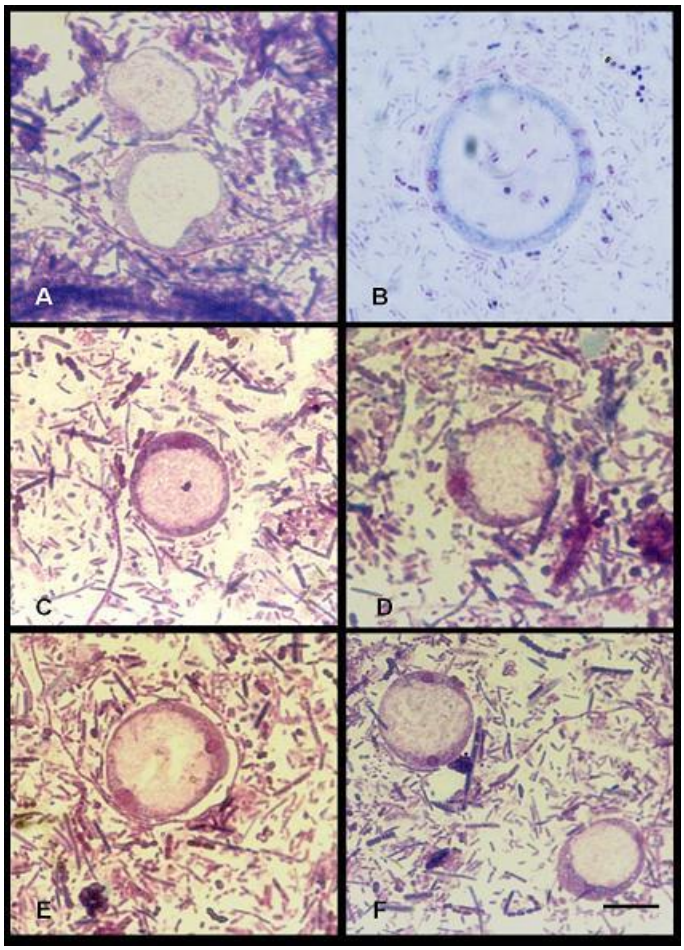

Figure 1. Forms of *Blastocystis* spp. obtained from the giemsa-stained fecal samples from poultry. Vacuolar form: A: central vacuole (→); B: seven nuclei in the cytoplasm. Granular form: C and D: some cytoplasmic inclusions, and one single nucleus; E and F: multiple nuclei (— = 10 µm).

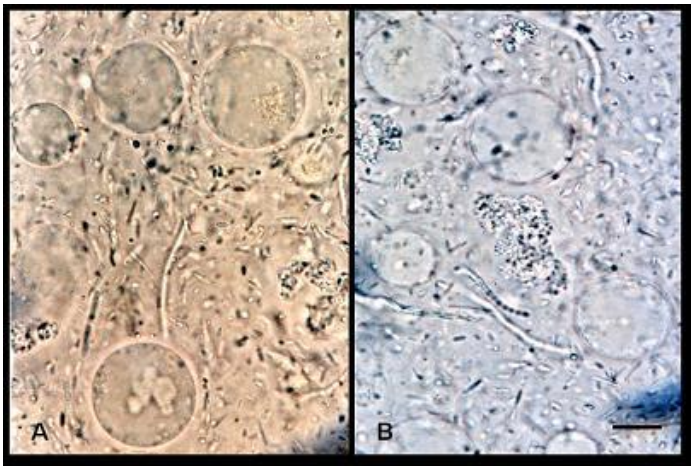

Figure 2. Forms of *Blastocystis* spp. obtained in the stool samples from poultry homogenized with sodium chloride 0.9%. A: bright field; B: phase contrast (— = 10 µm).

granular forms were also present in various samples. In addition, both were commonly observed in recently eliminated fecal samples and could be observed without staining (Figure 2; A and B).

In this study, the amoeboid form was found in small amounts in the stained fecal smears. In samples without staining, however, its observation was difficult. These forms presented irregular borders, often resembling extensions such as "pseudopods", and lacked a central vacuole (Figure 3; A and B). The measurements of the amoeboid forms of *Blastocystis* spp. are given in Table 2. The average size was 22 µm, and the measurements ranged from 13.4 to 45.5 µm. These values are similar to those found by Tan and Suresh (2006), who observed measurements ranging from 5 to 50 µm.

The morphological characteristics of the *Blastocytis* spp. ameboid forms are not in agreement with some reports. Dunn et al. (1989) reported cells of 2.6 to 7.8 µm in diameter that were irregularly shaped, had no central vacuole and had a structure similar to an extended pseudopod. In contrast, Tan and Zierdt (1973) reported oval amoeboid cells containing one or two long pseudopods and a large central vacuole. The cystic forms of the Blastocystis spp. observed in giemsa-stained fecal samples were in general, arranged in groups and rarely individually. The cysts, when grouped, were surrounded by a membrane or by a membrane trace. Stenzel et al. (1997), after observing the membrane of the cysts obtained from the feces of domestic chickens, using transmission electron microscopy, concluded that the membrane is composed of a fibrillar layer. In addition, Zaman et al. (1999) observed that the cysts might be surrounded by a fibrillar layer that could be intact or fragmented. Stenzel et al. (1997) also observed that each cyst might contain one to four nuclei. Nevertheless, in this study, the cysts were characterized as rounded or ovoid,

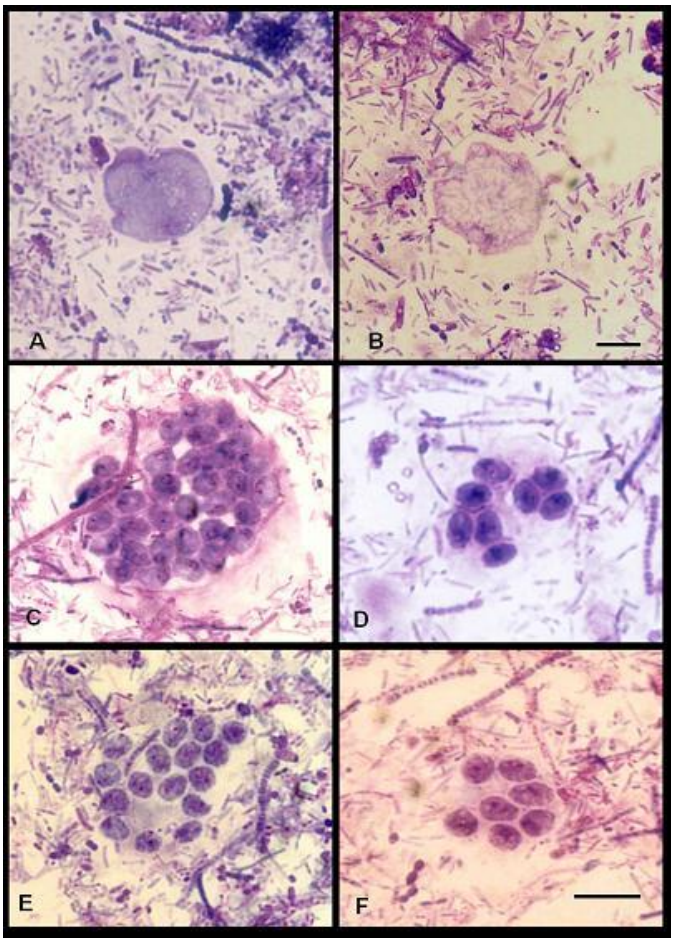

Figure 3. Forms of *Blastocystis* spp. obtained from the giemsa-stained fecal samples of poultry. Amoeboid form: A and B: projections from the cytoplasm similar to a pseudopod; cells with nuclei cyst: C: a clear fibrillar layer is observed covering the nucleated cysts; D, E and F: nucleated cysts with a trace fibrillar layer (— = 10 µm).

with one or two internal nuclei (Figure 3; C to F). The measurements of the cystic form of *Blastocystis* ranged from 2.1 to 5.5 µm (Table 2), agreeing with the measurements described by Stenzel and Boreham (1996). Zaman et al. (1999) found daughter cells within *Blastocystis* cysts, and some of them could be observed in the vacuoles. The rupture of these cysts resulted in the emergence of these daughter cells. In the present study, internal structures similar to those described by Zaman et al. (1999) were observed inside the cystic form, with one or two nuclei and a structure similar to a vacuole (Figure 4; A and B). This variation in the number of nuclei within the cysts was most likely related to the stage of maturity. Some of the cystic forms had daughter cells that were more clearly detailed when the cysts were disrupted (Figure 4C). In this study, three types of reproduction of the *Blastocystis* spp. could be observed: binary division, plasmotomy and budding. In addition, Zhang et al. (2007) described two more types of reproduction: endodyogeny

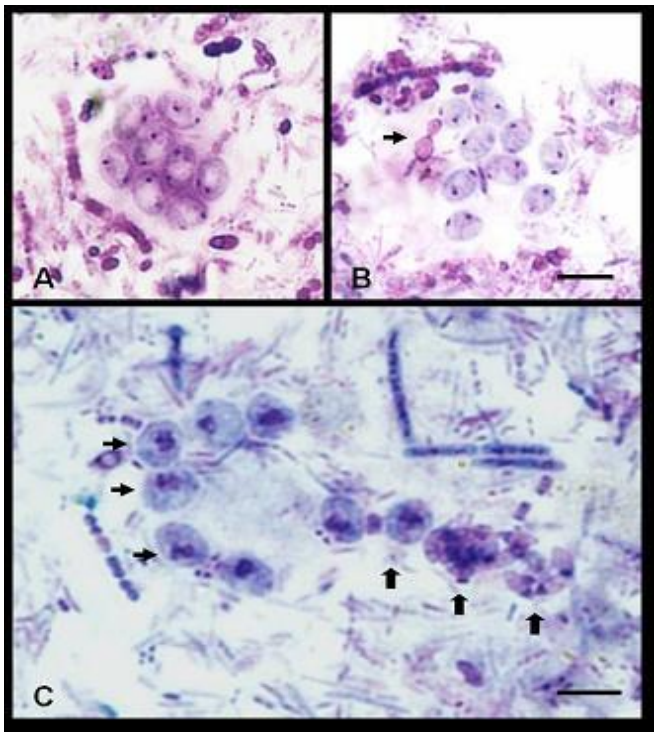

Figure 4. Cystic form of *Blastocystis* spp. observed in the giemsa-stained poultry fecal samples. A and B: presence of one or two nuclei inside and the vacuoles; B (→): budding yeast; C: daughter cells observed after the cyst break (— = 10 µm).

and schizogony. The most common reproductive form found in this study was binary division, which is characterized by the partition of the cytoplasm of the mother cell and results in two daughter cells with an equal size and shape (Figure 5; A to C). In binary division, the cytoplasm of the cell is elongated, and after the elongation of the cytoplasm, cell is compressed toward its center until it breaks, resulting in two daughter cells.

In this study, using fresh and stained fecal samples, plasmotomy was considered a rare observation. Zhang et al. (2007) were able to observe this type of reproduction only in *in vitro* culture. Plasmotomy is characterized by the extension of the cytoplasmic membrane and cell surface, by which the cell increases its size. The daughter cells are formed from the extension or expansion of the cytoplasmic surface of the mother cell (Figure 5D). The other type of reproduction found in this study using giemsa-stained fecal samples was budding, in which the mother cell forms a new daughter cell from its side. Sometimes this mother cell may give rise to two or even three daughter cells, which are always smaller than the original cell (Figure 5; E and F).

The different forms of *Blastocystis* spp. mentioned above were diagnosed in the three species of poultry used in the present study.

More studies in other host species should be conducted for further information on the morphology and identification of the various forms of *Blastocystis* spp. in stool samples to better elucidate its biological cycle and to establish the control and prophylaxis of the microorganism.

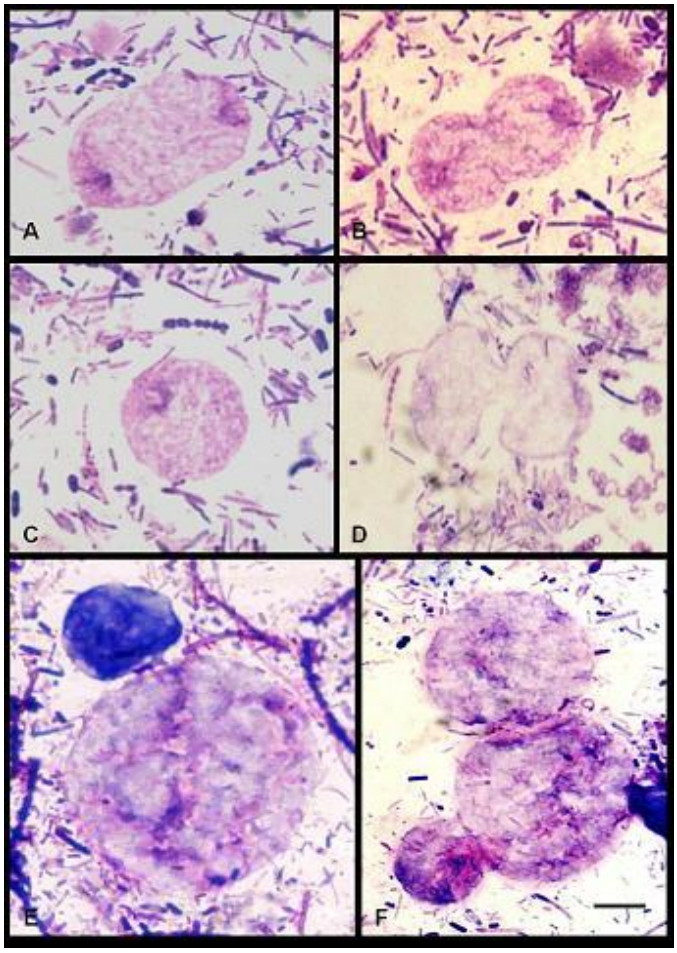

Figure 5. Types of reproduction of *Blastocystis* spp. obtained from the giemsa-stained fecal samples of poultry. Binary fission: A: beginning of the binary fission, with cell elongation and the nuclei in opposite poles; B: cell in cytoplasmic division; C: a cell possibly originating from binary fission; Plasmotomy: D: extension of the cytoplasmic membrane that will originate another cell; Budding: E: growth of one side of the cell, originating a new and smaller cell; F: several daughter cells of various sizes originating from a bigger mother cell (— = 10 µm).

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