ORIGINAL ARTICLE

Establishment of *Blastocystis hominis* in-vitro Culture Using Fecal Samples from Infants in Slum Area of Mirpur, Dhaka, Bangladesh

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ABSTRACT

Introduction: *Blastocystis hominis (B. hominis*) is an obligate anaerobic protozoan found in the human large intestine, and is the most common eukaryotic organism reported in human fecal samples. **Method:** Multiple stool samples from 460 children (53.9% male and 46.07% female) were collected and examined for the presence of *Blastocystis hominis* in Parasitology Laboratory of International Centre for Diarrhoeal Diseases Research, Bangladesh during the period of 9th January to 28th December, 2011. Among them, 255 were diarrheal patients (56.47% male and 43.53% female). Direct microscopy was done for each of the samples and each sample was cultured in vitro for 48 hours and observed again for the presence of the pathogen. The aim of the study was to develop a sustainable technique to identify the pathogen. **Results:** In culture, several morphological forms were observed. Through microscopy, various morphological forms were clearly observed. Within 5679 tested samples, 795 samples (0.14%) were positive for *B. hominis*. As multiple forms were observed in the same sample, the most prevalent was cyst (0.125%) whereas least prevalent was granular (0.0072%). The highest percentage for all the morphological forms was observed in age group 25-36 months. In direct microscopy from fresh samples, children from 37-48 months showed the highest percentage (22.9%) of infection (p=0.000). In culture, the same age group showed the most infection rate (p=0.000). Among the different morphological forms observed in culture, the highest prevalence of cyst was in age group 37-48 months (p=0.000). The highest prevalence of vacuolar form(5.7%) was observed in the same age group (p=0.015). In contrast, the amoeboid forms were mostly observed in children of 25-36 months (p=0.002).The children aged in between 37 to 48 months are at the most risk of the infection. **Conclusion:** The sensitivity of direct microscopy was found only 38.46% in respect to in-vitro culture which strongly suggests that in-vitro culture is the gold standard for the diagnosis of this parasite.

Keyword: B. hominis, Diarrhoeal disease, Infant, In-vitro culture, Xenic culture

INTRODUCTION

Diarrhea is one of the deadliest diseases in children younger than 5 years in developing countries, most of them in Asia, Africa and Latin America. Each year, diarrheal disease kills around 760, 000 children under five. Although significant proportion of diarrheal disease can be prevented through safe drinking-water and adequate sanitation and hygiene, yet globally there are nearly 1.7 billion cases of diarrheal disease every year. It is also considered as a leading cause of malnutrition in children under five year old.[1]

Travelers and immune-compromised patients are also deeply affected by diarrhea caused by various pathogens. Scientific studies so far have mainly focused on *Entamoeba* sp, *Giardia* sp, *Clostridium* sp. etc. But there is another pathogen named *Blastocystis hominis*, which, despite being discovered a long time ago by Alexeieff[2] has recently caught eye of the scientists of different region fighting for diarrheal control worldwide. *Blastocystis hominis (B. hominis)* is an obligate anaerobic protozoan found in the human large intestine, and is the most common eukaryotic organism reported in human fecal samples.[3]

This parasite can cause blastocystosis (commonly known as traveler's diarrhea) with the symptoms of characteristic diarrhea accompanied by abdominal pain, dizziness, anorexia, nausea, vomiting, intestinal tympanites, and weight loss.[4]

The taxonomic classification of *Blastocystis* spp. has proven challenging and was only recently unambiguously placed within the stramenopiles despite the application of modern molecular phylogenetic approaches.[5]

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Super kingdom	- Protista
Sub kingdom	- Protozoa
Phylum	- Sarcomastigophora
Class	- Blastocystea
Order	- Blastocystida
Family	- Blastocystidae
Genus	- *Blastocystis*
Species	- *Blastocystishominis*

Recent data indicates that different groups of *Blastocystis* isolates can be distinguished in human hosts, and this has raised the possibility that more than one species of *Blastocystis* infect humans.

Despite the differences noted, there is insufficient evidence to designate new species of *Blastocystis* from humans without further biochemical and epidemiological data. Therefore, *B.hominis* (Figure 1) is the only species of *Blastocystis* which is currently accepted to be present in human hosts. There have been many attempts of culturing this parasite in axenic media in the past few decades i.e., Ho et al.[6] used Iscove's modified Dulbecco's medium for the axenic culture. But continuation of the culture in axenic media seems to have been problematic due to the inconstancy in the morphological forms in this pathogen. However, culturing the pathogen along with other pathogens seemed possible for a long time.

This article focuses mainly upon the in-vitro culture of *Blastocystis hominis* using BRS complete amoebic medium developed in parasitology laboratory of icddr,b (International Centre for Diarrhoeal Disease Research, Bangladesh) in xenic condition.

Morphology of *B. hominis*

Blastocystis is a polymorphic protozoan, and four major forms have been described in the literature. In reality, *Blastocystis* spp. can present with a bewildering array of forms within a single culture, and it may be difficult to assign a specific form to the cell in question. The extensive variation in *Blastocystis* forms has made studies of its cell biology challenging, resulting in misinterpretations of data from time to time. The several forms observed within in-vitro culture includes vacuolar, granular, amoeboid, cyst, avacuolar, multi-vacuolar forms. However, vacuolar, granular, amoeboid and cyst are the most observed forms in this study.

The Vacuolar Form

The central vacuole form (Figure 2), sometimes referred to as the central body form, is the most frequently observed form in laboratory culture and in stool samples. It is spherical and may display large size variations, ranging from 2 to 200 μm (average of 4 to 15 μm).[7] Extensive size variations can occur within and between isolates.[8]

The Granular Form

The granular form resembles the vacuolar form except that granules are present within the cytoplasm or, more commonly, within the central vacuole of the organism (Figure 3). These are more frequently observed in non-axenized, older, and antibiotic-treated cultures.

The Amoeboid Form

The amoeboid form (Figure 4) of *Blastocystis* spp. is rarely reported, and there are contradicting descriptions of what constitutes this morphological type.[9] These cells were irregularly convoluted, and some cells possessed one or two large pseudopods.

The Cyst Form

The cyst form (Figure 5) is the most recently described form of the parasite, and the late discovery is due to its small size (2 to 5 μm), which can result in confusion with fecal debris. The cysts are variable in shape but are mostly ovoid or

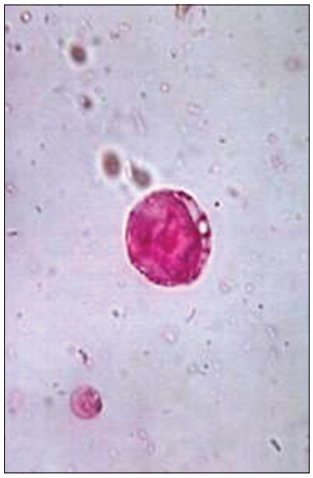

Figure 1: *Blastocystishominis*

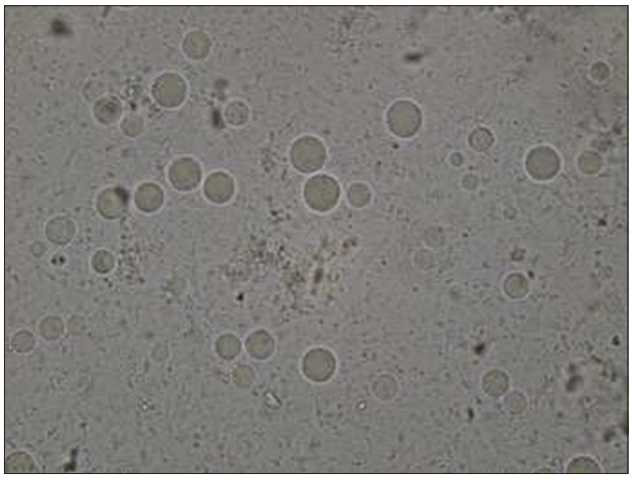

Figure 2: Light microscopy of *B. hominis* showing vacuolar forms (magnification 40X).

spherical. The cyst is protected by a multilayered cyst wall which may or may not be covered by a loose surface coat. The cytoplasm of the cyst may contain one to four nuclei, mitochondria, glycogen deposits, and small vacuoles.

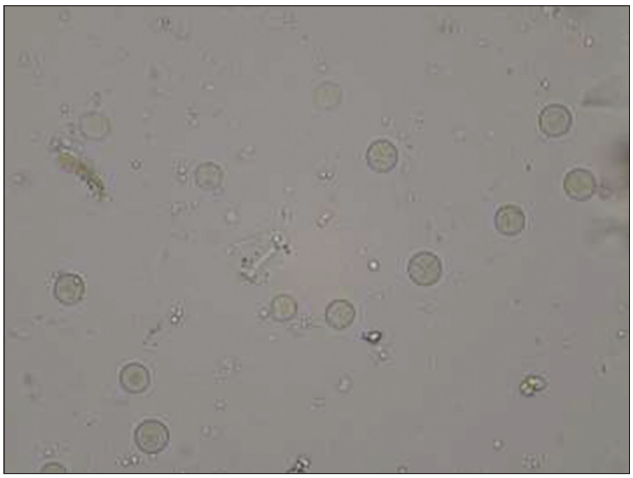

Figure 3: Light microscopy showing granular forms of *B. hominis* (40Xmagnification)

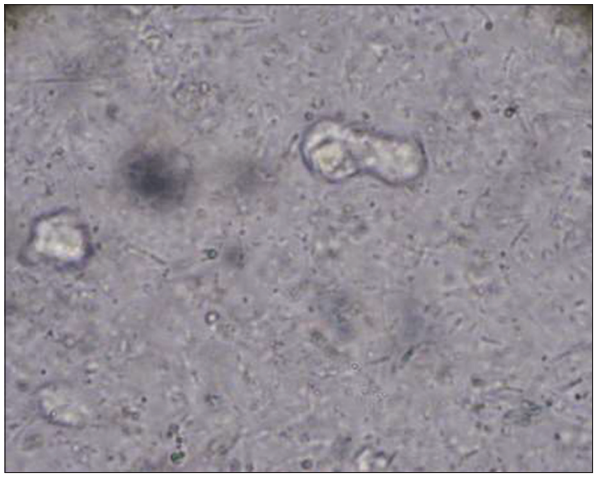

Figure 4: Light microscopy showing amoeboid forms of *B. hominis* (40X magnification)

Figure 5: Light microscopy showing cyst forms of *B. hominis*

Life Cycle

Numerous conflicting life cycles have been proposed by many authors and these discrepancies are due largely to the belief that *Blastocystis* exhibits multiple reproductive processes such as schizogony, plasmotomy (budding), endodyogeny.[10]

A revised life cycle (Figure 6) must take into account the large reservoir of *Blastocystis* spp. among various animal populations and that humans are potential hosts to numerous zoonotic genotypes (subtypes). Upon ingestion of cysts, the parasite undergoes ex-cystation in the large intestines and develops into vacuolar forms. En-cystation occurs during passage along the large intestines and is deposited in the feces. The fecal cysts may be covered by a fibrillar layer that is gradually lost during cyst development.

Humans and animals are infected by fecal cysts, which develop into vacuolar forms in the large intestines. In humans, vacuolar forms divide by binary fission and may develop into amoeboid or granular forms. Vacuolar forms undergo encystation in the host intestines, and intermediate cyst forms may be surrounded by a thick fibrillar layer that is subsequently lost during passage in the external environment. Information on the transition from the amoeboid to the vacuolar form and from the vacuolar to the cyst form is lacking. These hypothetical pathways are represented by dotted lines. Subtype 1 is cross-infective among mammalian and avian isolates; subtypes 2, 3, 4, and 5 comprise primate/pig, human, cattle/pig, and rodent isolates, respectively; and subtypes 6 and 7 include avian isolates. The proposed scheme suggests that humans are potentially infected by seven or more species of *Blastocystis* and that certain animals represent reservoirs for transmission to humans.

The micrographs revealed that cell division of vacuolar forms occurs while the parasite is still within the cyst wall and that both granular and vacuolar forms were observed in the same sample. Because only one time point was performed, it is difficult to conclude the order in which these forms developed. Certain culture conditions were reported to induce the development of the granular form from the vacuolar form. These conditions include old cultures, axenization, transfer to a different culture medium and increases in serum concentrations in the culture medium. Amoeboid forms probably arise from vacuolar forms. Some evidence for this is seen when vacuolar forms are cultured in agar, and after incubation, the resultant colonies contain numerous amoeboid forms.

Pathogenesity

The Center for Disease Control (CDC) states that the symptoms reported to be associated with blastocystosis

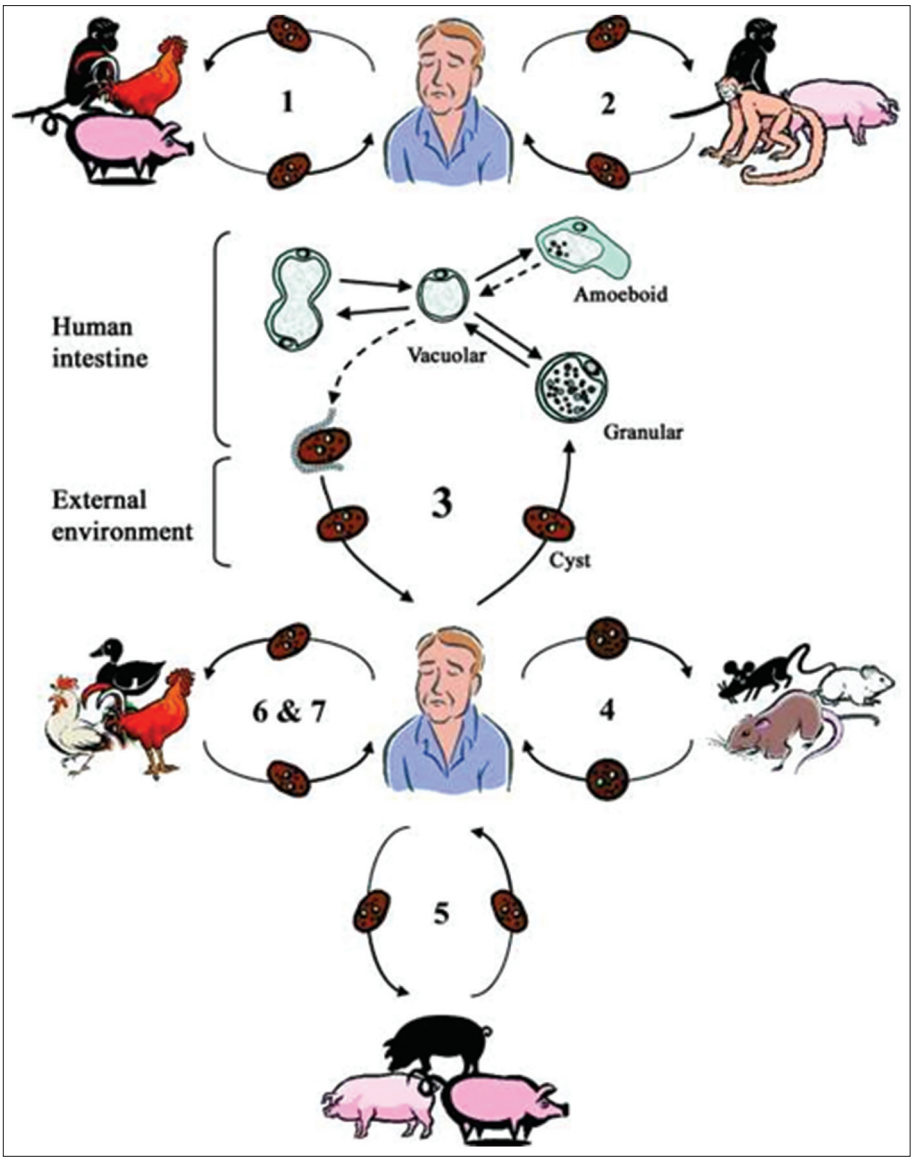

Figure 6: A proposed life cycle for *Blastocystis* cells taking into account recent studies suggesting the existence of zoonotic genotypes (subtypes 1 to 7) with various host specificities

infection (Figure 7) are diarrhea, watery or loose stools, anal itching, abdominal pain, weight loss, and excess gas. (CDC Fact Sheet) The pathogenic role of *B. hominis* in humans has been a subject of much controversy to date. Its pathogenic role in animals, however, has been demonstrated in some experimental studies. In a murine model.[11]

Most studies that include a control population have failed to show a significant difference in *B. hominis* prevalence or symptoms between symptomatic cases and asymptomatic controls. Other factors that further complicate the issue include the lack of standardized criteria for diagnosis, the self-limited nature of infection, the existence of an asymptomatic carrier state and the possibility that there may be both virulent and avirulent strains of the organism.

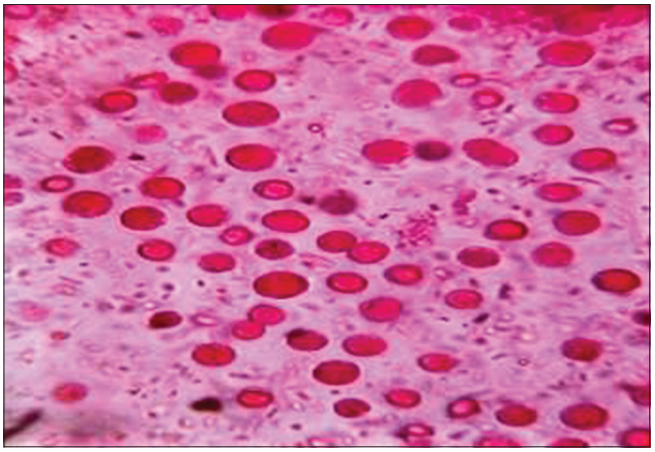

Figure 7: Heavy infection of *Blastocystishominis*

METHODS AND MATERIALS

A cross sectional and descriptive study was conducted among infants of 0 - 4 years of age group in Mirpur slum area (Figure 8) of Dhaka, Bangladesh from 9th January to 28th December,2011.Multiple fecal samples from 460 children enlisted in NIH birth cohort was selected for the study. Among them, 255 were diarrheal patients. They were divided into following age groups: 0-12 months; 13-24 months; 25-36 month; 37-48 months.

Materials for direct microscopy

Following instruments were needed for direct microscopy:

- Dry container
- Olympus light microscope
- Slide, cover slip
- Normal saline and pipette.

Microscopic examination

Microscopic examination was done by light microscope within one hour of collection. In microscopic examination, liquid stool were taken into slide and examined whether it is positive or not for *B.hominis*. In case of solid stool, stool were diluted into normal saline and taken into slide and examined. Microscopic examination was first performed on unpreserved specimens and included information on the age, consistency of stool and abnormality. Fecal specimen are described as formed, semi formed, soft loose or watery. The specimen containing *B. hominis* was later used for in-vitro culture.

Xenic culture: Materials for bottle preparation *(Figure 9)*

1. One Litre of distilled water
2. 7g Nacl
3. 14g Bacto-agar

Xenic culture: Materials for medium preparation *(Figure 10)*

1. Saline agar slopes
2. Erythromycin
3. DIFCO Bacto peptone
4. Rice starch
5. Pthalate solution
6. Bovine serum
7. Defined medium B
8. BR Basal amoebic medium
9. BRS complete amoebic medium

To a bottle containing a sterile agar slope, approximately 10 mg rice starch, 0.12 ml erythromycin solution and sufficient BR medium to cover slop were added. Approximately 50 mg of feces was placed, capped and incubated for 24 h at 37°C.After 24h,the media was aspirated and discarded leaving only starch and feces and was replaced with sufficient BRS, diluted 1:4 with the phthalate solution to cover slope. After that, 0.06 ml erythromycin solution, 0.06 ml Bacto-peptone solution and additional starch were added. After another incubation of 24 hours, the drop of feces and starch was removed from the bottom of the slope. Then it was examined under the optical microscope for the presence of the organism. A minute portion of cultured stool sample was taken with pipette and a drop of it was taken on a microscopic glass slide, a cover slip was taken gently to put over it to spread out the emulsion into a thin transparent layer and examined carefully under microscope in 10X and in 40X for confirmation of the presence of different forms.

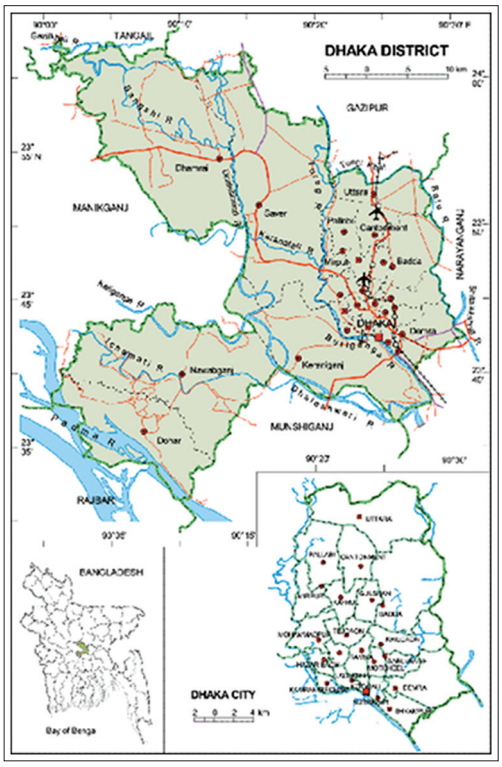

Figure 8: Map of Dhaka city showing Mirpur (the study area)

Figure 9: Bottle preparation for xenic culture of *B.hominis*

RESULTS

Baseline characteristics:

Basic epidemiological features of both symptomatic (diarrheal) and asymptomatic patients were collected:

Number of subjects	:	460
Range of age groups	:	0-48 months
Number of male children	:	248 (53.9%)
Number of female children	:	212 (46.07%)
Study period	:	January 9, 2011 to December 28, 2011

During this study period, multiple stool samples (5679) from 460 children were collected and examined for the presence of *Blastocystis hominis* (Table 1). Direct microscopy was done for each of the sample and then it was cultured in vitro for 48 hours and observed again for the presence of the pathogen (Table 2).

Among 5679 samples, 795 were found positive for *B.hominis*. Among them, 756 were found positive in asymptomatic samples and 39 was found positive in symptomatic samples (Table. 3).

Through microscopy, various morphological forms were clearly observed. As multiple forms were observed in the same sample, the most prevalent was cyst (n=713, 0.125%) whereas least prevalent was granular (n=41, 0.0072%) (Table 4, Figure 11).

In a total of 39 diarrheal samples positive for *B. hominis*, 0.53 % was found positive for both cyst and vacuolar forms, 0.93 % was positive for both cyst and amoeboid forms. 0.13% was found positive for both amoeboid and vacuolar forms. None was found positive for other criteria (Table 5).

The highest percentage for all the forms was observed in age group 25-36 months (Figure 12).

In direct microscopy, children from 37-48 months showed the highest percentage (22.9%) of infection (p=0.000). In culture, the same age group showed the most infection rate(p=0.000). Among the different morphological forms observed in culture, the highest prevalence of cyst was in age group 37-48 months (p=0.000). The highest prevalence of vacuolar form (5.7%) was observed in the same age group (p=0.015). In contrast, the amoeboid forms were mostly observed in children of 25-36 months.(p=0.002) (Table 6). So, the tested diagnostic methods explored that there is a

Table1: Baseline characteristics of the subjects

Total subjects	Test done	No. of samples
460	Microscopy from direct smear	5679
	Microscopy from in vitro culture	5679

Table 2: Number of samples collected during the study period

Total subject	Number of sample tested	Number of asymptomatic samples	Number of symptomatic samples
460	5679	5121	558

Figure 10: Materials and media for xenic culture of *B. hominis*

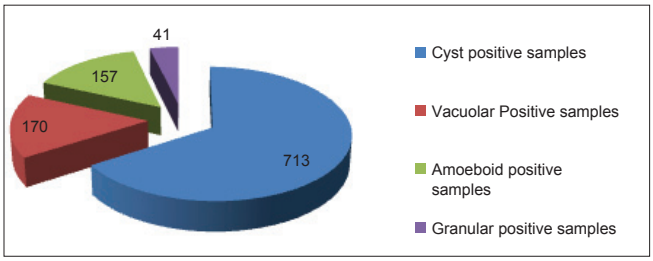

Figure 11: Number of morphological forms observed in culture

Table 3: Prevalence of *B. hominis* from in-vitro culturet

Total no of sample tested	No. of positive samples	Prevalence (%)	No. of positive in asymptomatic samples (*n*=5121)	%	No. of positive in symptomatic samples (*n*=558)	%
5679	795	13.99	756	14.76	39	6.98

Table 4: Morphological forms observed in culture

Total tested samples in culture	*B. hominis* positive samples in culture	Cyst positive samples	Vacuolar positive samples	Amoeboid positive samples	Granular positive samples
5679	795	713	170	157	41

Table 5: Percentage of morphological forms observed in culture in diarrheal samples

Morphological forms	Tested	Observed	%
Cyst & Vacuolar	39	4	0.53
Cyst & Amoeboid	39	7	0.93
Cyst & Granular	39	0	0.00
Vacuolar & Amoeboid	39	1	0.13
Vacuolar & Granular	39	0	0.00
Amoeboid & Granular	39	0	0.00
Cyst, Vacuolar, Amoeboid & Granular	39	0	0.00

Table 6: Relationship between age and *B. hominis* positive samples by direct microscopy and culture in diarrheal samples

	Age group (in months) (%)				P value	Chi-square value
	0-12	13-24	25-36	37-48		
Microscopy positive samples	2.5	3.6	7.7	22.9	0.000	24.469
Culture positive samples	0.8	3.6	13.5	22.9	0.000	28.812
Cyst	0.8	3.6	12.3	20.0	0.000	28.812
Vacuolar	0	0.4	1.9	5.7	0.015	10.514
Amoeboid	0	0.4	4.5	0.0	0.002	14.532

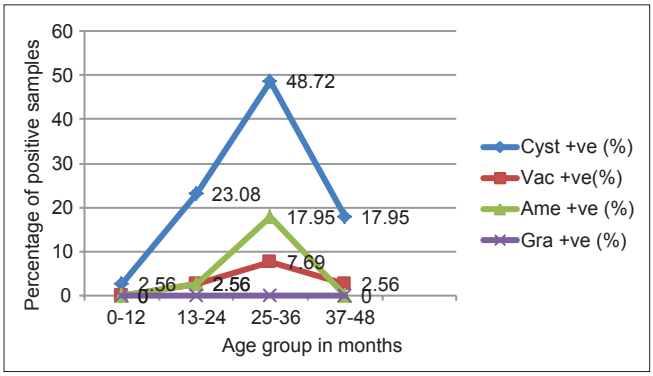

Figure 12: Different morphological forms according to age groups observed in culture in diarrheal samples.

significant association between various age groups and *B. hominis* infection among the children. The children aged in between 37 to 48 months are at the most risk of the infection.

Among 39 culture positive samples, 15 samples were found to be positive and 24 samples were found to be negative in direct microscopy. Beside this, among 519 culture negative samples, 17 samples were found to be positive and 502 samples were found to be negative in direct microscopy. Hence, the sensitivity of direct microscopy was found only 38.46% (Table 7) considering in-vitro culture as the gold standard.

DISCUSSION

Though the symptoms of *Blastocystis hominis* is controversial, it is already established that it causes blastocystosis or traveler's diarrhea, that means visitors from abroad are the first one's infected by this diarrheal pathogen.

Table 7: Cross-tabulation between direct microscopy and culture in diarrheal children

Diarrheal children	Culture		Total
	Positive	Negative	
Microscopy			
Positive	15	17	32
Negative	24	502	526
Total	39	519	558

According to a study,[12] *B. hominis* is a parasite protest of clinical importance. It is a very common infection in human and grows luxuriantly in all xenic media used of the isolation of *Entamoeba*sp. and *Dientamoe bafragilis*. This finding is exactly similar to the present study as the pathogen was observed in conjunction with several other diarrheas causing pathogen (*E. histolytica, Cryptosporidium* sp. and *Giardia intestinalis*). Zhang et al[13] supported the result by suggesting that the short-term in vitro culture method achieved the best performance with regard to sensitivity, and specificity of the five studied methods. They found that with the advantages of environmental safety, convenience in preparation and storage, facility in morphologic discrimination, and outstanding performance in terms of sensitivity and specificity, the in vitro culture method could be applied to identify *B. hominis* for both clinical diagnosis and field study purposes.

Yakoob et.al[14] suggested a possible role for *Blastocystis hominis* and *Dientamoeba fragilis* in the etiology of irritable bowel syndrome (IBS) by doing stool microscopy, culture, and polymerase chain reaction (PCR). *B. hominis* was positive by stool microscopy in 49% and by culture was

positive in 53% and PCR positive in 44%. *B. hominis* culture had a better yield compared to stool microscopy and PCR. These findings are also similar to the present study as the result clearly reflects that in vitro culture is the best method to detect the pathogen.

Termmathurapoj[15] investigated that when in vitro cultivation was used as the 'gold standard' for the detection of Blastocystis hominis in stool specimens, simple smear and trichrome staining showed sensitivities of 16.7% and 40.2% and specificities of 94% and 80.4%, respectively. Their data shows the usefulness of in vitro cultivation for the detection and molecular study of *B. hominis* in stool specimens. The prevalence of *B. hominis* using in vitro cultivation was 30.3% (95%CI, 23.4-35.2),which was six times higher and twice as high as those detected by simple smears and trichrome staining,respectively. When cultivation was used as the gold standard, simple smears and trichrome staining had sensitivities of 16.7% and 40.2%, respectively. The specificities of simple smears and trichrome staining were 94% and 80.4%, respectively.

The outcome of this investigation supported the present study, because using in vitro cultivation as the gold standard, the specificity and sensitivity was determined for direct microscopy.The sensitivity of direct microscopy was 38.46% and specificity was 96.72%.Thus from the present study, culture can be standardized as a gold standard. On the basis of the finding of present study, further molecular studies on large scale samples are recommended to draw conclusive inferences.

ACKNOWLEDGEMENTS

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