Incidence and detection of *Blastocystishominis* among the infants in Dhaka

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA

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DOCTOR OF PHILOSOPHY in **Zoology (Parasitology)**

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DEDICATED

TO

MY RESPECTED PARENTS

AND

MY HUSBAND

DECLARATION

It is my greatest honour and privilege to declare that the dissertation on **"Incidence and detection of *Blastocystis hominis* among the infants in Dhaka"** is carried out by me under supervision and guidance of Professor **Dr. Hamida Khanum** (Chief Supervisor), Professor **Dr. Sharmin Musa** (Co-supervisor), Department of Zoology (Parasitology Branch) University of Dhaka and **Dr. Rashidul Haque** (Co-supervisor), Senior Scientist and Head, Parasitology Laboratory, icddr,b, for fulfillment of the degree of Doctor of Philosophy under the University of Dhaka.

I left no stone unturned to make the dissertation unique, informative and comprehensive one with the sincere co-operation and valuable guidance of my supervisors. In this regard, I would like to confirm that the works reported in this dissertation are original and had never been submitted for any other degree.

Sincerely,

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CERTIFICATE

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CONTENTS

	Page no
Abstract	i-ii
Abbreviations	iii-iv
Chapter 1: Introduction	1-32
1.1: TAXONOMY AND MORPHOLOGICAL FORMS OF BLASTOCYSTIS HOMINIS……	19-28
1.2: LIFE CYCLE AND ZOONOTIC IMPORTANCE OF BLASTOCYSTIS HOMINIS……	29-32
Chapter 2: Review of Literature	33-62
Chapter 3: Materials and Methods	63-87
Chapter 4: Results and observations	88-200
4.1: OBSERVATION BY MICROSCOPIC EXAMINATION ……	90
4.2: OBSERVATION BY IN VITRO CULTURE METHOD ……	103
4.3: COMPARISON BETWEEN MICROSCOPY AND IN VITRO CULTURE METHODS ……	116
4.4: SEASONAL PREVALENCE OF BLASTOCYSTIS HOMINIS ……	120
4.5: IMPACT OF CLIMATIC FACTORS ON MONTHLY INCIDENCE OF BLASTOCYSTIS HOMINIS ……	129
4.6: PREVALENCE OF DIFFERENT MORPHOLOGICAL FORMS OF BLASTOCYSTIS HOMINIS BY IN VITRO CULTURE ……	154
4.7: DIFFERENT COMBINATION OF FOUR MORPHOLOGICAL FORMS OF BLASTOCYSTIS HOMINIS ……	175
4.8: COMPARATIVE ANALYSIS OF PREVALENCE OF BLASTOCYSTIS HOMINIS BY MICROSCOPY, IN VITRO CULTURE AND PCR METHODS ……	183
4.9: SENSITIVITY AND SPECIFICITY OF MICROSCOPY AND IN VITRO CULTURE USING PCR AS STANDARD METHOD ……	187
4.10: INFECTION WITH PROTOZOAN AND HELMINTH PARASITES ……	190
Chapter 5: Discussion	201-210
Chapter 6: Summary	211-215
Chapter 7: Conclusion and Recommendation	216-217
Chapter 8: Bibliography	218-246
Appendix	i-vii

ABSTRACT

In the present study, three techniques: Direct microscopy, *in vitro* culture and PCR methods were applied to detect *Blastocystis hominis*. Multiple stool samples of 406 children were collected from Mirpur, Dhaka and 118 children from Mohammadpur slum area, Dhaka. The whole work was done in the Parasitology Laboratory, icddr,b, Mohakhali, Dhaka-1212. During the study period (from January 2012 to December 2013), in Mirpur area, out of 9322 stool samples, 8601 samples were observed on monthly basis called Monthly Stool (MS) samples and remaining 721 samples were Diarrheal Stool (DS) samples. In Mohammadpur area, out of 3218 stool samples, 2704 samples were MS samples and remaining 514 samples were DS samples. By microscopy, the prevalence of *B. hominis* in MS and DS samples were 3.72% and 5.83% in Mirpur area and 7.25% and 9.73% in Mohammadpur area respectively. According to culture examination, the prevalence of *B. hominis* in MS and DS samples were 34.25% and 27.32% in Mirpur area and 42.60% and 40.47% in Mohammadpur area respectively. It was observed that prevalence found higher by *in vitro* culture method than direct microscopy in all types of samples of both areas.

According to age groups of children, it was observed that prevalence was higher among 37-48 and 49-60 months age groups in MS and DS samples of both areas. Statistical analysis exhibited that there was no significant relationship between sexes in children for *B. hominis* infection. It was observed that female were more infected, by microscopy (4.16%) and by *in vitro* culture (35.07%) collected from Mirpur area. While, by *in vitro* culture method, female was more prevalent (42.79%) than male (41.59%) in overall samples from Mohammadpur area. By the analysis of variance it was revealed that there was a significant association between age groups of children for *B. hominis* infection.

Four morphological forms of *B. hominis*: cyst, vacuolar, amoeboid and granular, which were clearly distinguished by *in vitro* culture method. Prevalence of cyst, vacuolar, amoeboid and granular forms were 31.09%, 13.63%, 11.50% and 5.88% respectively in stool samples of Mirpur area, while comparatively higher prevalence

38.66%, 29.58%, 18.09% and 2.89% were observed respectively in stool samples of Mohammadpur area. To observe seasonal variation, by microscopy and *in vitro* culture method, in MS and DS samples of both areas, prevalence of *B. hominis* were higher both in summer and rainy seasons than winter. The seasonal variation (F-test), were significant (P=0.05* and p=0.000**) in DS samples of Mirpur and Mahammadpur areas. Monthly incidence of *B. hominis* in stool samples of study areas showed positive correlation with climatic factors (temperature, humidity and rainfall). It was observed that in DS samples of both areas, incidence were significantly correlated with temperature (p=0.004** and p=0.000**) and rainfall (p=0.004** and p=0.001**). The prevalence of *B. hominis* by direct microscopy was 4.50%, by *in vitro* culture method was 29.86% while by Real-time PCR method was 42.42% respectively in stool samples of Mirpur area, while these were 9.44%, 39.44% and 53.33% respectively in samples of Mohammadpur area. Comparisons among three methods, showed that the mean difference were highly significant (p=0.013** and p=0.000*) in stool samples of both areas. **The main goal of the present investigation was to prove the perfect technique for maximum detection of** *B. hominis.* As a result the **Real-time PCR is the gold standard as it showed highest sensitivity than direct microscopy and *in vitro* culture method.**

ABBREVIATIONS

A1-A8	:	Eight asymptomatic isolates
ANOVA	:	Analysis of Variance
AP-PCR	:	Arbitrarily primed Polymerase Chain Reaction
Bh	:	*Blastocystis hominis*
CDC	:	Centre for Disease Control and Prevention
dATP	:	Deoxyadenosine Triphosphate
dCTP	:	Deoxycytidine Triphosphate
dGTP	:	Deoxyguanosine Triphosphate
dTTP	:	Deoxythymidine Triphosphate
dNTPs	:	Deoxyribose Nucleoside Triphosphates
DNA	:	Deoxyribonucleic Acid
DS	:	Diarrheal Stool
dTTP	:	Deoxythymidine Triphosphate
ELISA	:	Enzyme-Linked Immunosorbent Assay
et al.	:	and others
FECT	:	Formal Ethyl Acetate Concentration Technique
FRET	:	Fluorescence Resonance Energy Transfer
GBD	:	Global Burden of Disease
GI	:	Gastro Intestinal
gm	:	gram
h	:	hours
HIV	:	Human Immune deficiency Virus
HPA	:	Helix Pomatia
HPJ technique	:	Hoffman, Pons and Janer technique
IBD	:	Inflammatory Bowel Disease
IBS-D	:	Irritable Bowel Syndrome-Diarrhea
icddr,b	:	International Centre for Diarrhoeal Disease Research, Bangladesh

IFA staining	:	Indirect Immunofluorescent Antibody staining
mg	:	milligram
MGG	:	May-Grünwald-Giemsa
ml	:	milli litre
mRNA	:	messenger Ribonucleic Acid
MS	:	Monthly Stool
^{o}C	:	Degree Centigrade
PCR	:	Polymerase Chain Reaction
Pfu DNA	:	*Pyrococcus furiosus* Deoxyribonucleic Acid
P^{H}	:	Negative Logarithm of Hydrogen ion Concentration
Q	:	Quencher
q-PCR	:	Quantitative Polymerase Chain Reaction
R	:	Reporter
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic Acid
S1-S8	:	Eight symptomatic isolates
SSUrRNA	:	Small Subunit ribosomal Ribonucleic Acid
ST	:	Sub Type
STS	:	Sequence-Tagged Site
Taq DNA polymerase	:	*Thermis aquaticus* Deoxyribonucleic Acid
TMP/SMX	:	Trimethoprim/sulfamethoxazole
WHO	:	World Health Organization

INTRODUCTION

Infestation in human with intestinal protozoan parasites is a worldwide problem. More than one quarter of world's population in developing countries is infected with one or more species of intestinal parasites (Khanum *et al*. 1998). The poorest people in the world suffer from this burden of infection (Crompton 1984). The protozoan parasites cause diarrhea, which is a major cause of childhood morbidity and mortality in developing countries (Islam *et al*. 1983).

Each year an estimated one billion cases of diarrhea results in 2.5 million deaths among children younger than 5 years in developing countries, most of them in Asia, Africa and Latin America. For example, in Bangladesh, 1 in 30 children die of diarrhea or dysentery by his or her fifth birthday (Haque *et al.* 2003). The median global incidence of diarrhea was 5 and 2.6 episodes per child per year in infants (6-11months) and for all children between 0-4 years respectively. Much higher rates are seen in children from low socioeconomic status (Kosek *et al.* 2003).

Bangladesh is one of the poor countries of the world with the highest density of population. About 70% of the population live in rural areas with a per capita income of only BDT 1,760/-. Poverty, illiteracy, ignorance, disease and disasters are the constant companion of them. With the increase of population, socio- economic conditions become poor and due to this population explosion, all the reversible socio-demographic condition goes in favor of disease occurrence, recurrence and complication. While knowledge regarding deaths are important for health sector planning, little is known from conventional sources about the causes of deaths in Bangladesh. About 20% of the child death is associated with diarrhea (Baqui *et al*. 1998).

Environmental pollution, sanitary condition and human behavior play an important role in the transmission of intestinal parasites (Anwar *et al*. 1993). Scientists are especially familiar with protozoan parasitic infections like Amoebiasis, Giardiasis and Cryptosporidiosis while Blastocystosis is not very familiar. *Blastocystis hominis* is an obligate single-celled anaerobic protozoan parasite found in the human large intestine, and is the most common eukaryotic organism reported in human fecal samples (Tan

2008). This parasite can be transmitted as cyst by the oro- faecal route, especially in areas with poor hygiene and sanitation (Stenzel *et al.* 1996).

Blastocystis is polymorphic, presenting such a variety of forms, even within a monoculture, that identification of specific stages is problematic (Tan 2008). The difficulty in identifying *Blastocystis* in fecal specimens has resulted in confusion and misinterpretation regarding its life cycle, host specificity, and pathogenicity. *B. hominis* is genetically and antigenically diverse, both within and among geographical regions, suggesting that several strains or species of this parasite exists (Licea *et al*. 2003).

Blastocystis comprises several species, living in the gastrointestinal tracts of species as diverse as humans, farm animals, birds, rodents, reptiles, amphibians, fish and cockroaches (Yoshikawa *et al.* 2007). *Blastocystis sp*. exhibits low host specificity, and many different species of *Blastocystis* can infect humans (Noël *et al.* 2005) and by current convention, any of these species would be identified as *Blastocystis hominis* if they were identified in a human. *Blastocystis hominis* is a parasite inhabit the human intestinal tract, probably the more common protistan found in human feces (Windsor *et al.* 2002).

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 tympanitis, and weight loss (Stenzel *et al.* 1996). Although its role in human diseases is controversial, this organism is considered in several studies as a human pathogen producing both intestinal and extra-intestinal disorders (Lakhanpal *et al.* 1991). In 1990, some author previously proposed *Blastocystis hominis* causes an intestinal infection known as human blastocystosis, for which the symptoms are not specific but include diarrhea, abdominal pain, cramps, nausea, fever, vomiting and flatulence, among other symptoms (Doyle *et al.* 1990).

However, the pathogenic mechanism of this protozoa remains controversial, because this parasite is very common in many healthy people without showing any symptoms and clinicians have diagnosed and treated patients with the infection (Tan *et al.* 2002). To solve the issue of the pathogenic mechanism of *Blastocystis hominis*, the fundamental knowledge, such as morphology, reproductive mode, life cycle, and mode of

transmission etc., must be well understood. Many works have focused on the above mentioned topics, and with the progression in depth and width, this parasite is becoming more and more fascinating on its biological aspects (Tan *et al.* 2000). The pathogenic potential of *Blastocystis* is controversial with numerous conflicting reports regarding its ability to cause disease (Vogelberg *et al.* 2010). *Blastocystis* has been found not only in individuals with gastrointestinal symptoms and skin rash, but also in apparently healthy and asymptomatic individuals.

In the clinical setting, a positive diagnosis of *B. hominis* infection depends on the confirmation of vacuolar, granular, or amoebic forms in diarrheal samples, or the presence of the cystic stage predominantly in formed feces as seen under light microscopy in samples prepared with wet mount smears, iodine staining, or permanent dyeing such as trichrome staining and iron hematoxylin staining (Garcia *et al.* 1997; MacPherson *et al*. 1994).

The wide range in prevalence of *Blastocystis* seen between countries can be attributed to several factors such as socioeconomic conditions, and also to the different diagnostic methods used for detection. The most common diagnostic technique used worldwide for identification of *Blastocystis* is the permanent stain. The use of xenic cultures, in which *Blastocystis* is grown *in vitro* with non-specific microorganisms, has been shown to be more sensitive in detecting *Blastocystis* but it is not commonly used in the diagnostic laboratory (Zaman *et al.* 1994; Leelayoova *et al.* 2002; Suresh *et al.* 2004).

Molecular diagnosis by polymerase chain reaction (PCR) using the small subunit (SSU) ribosomal RNA gene is becoming more widely used for detection of enteric parasites. Although this technique is more costly, it is known to be more sensitive than the direct smear and xenic culture (Stensvold *et al.* 2007). Because considerable diversity exists in the rDNA of *Blastocystis*, as the result of diversification of this species in a wide range of genotypes, the choice of primers is crucial from a diagnostic perspective. Some primers may amplify specific subtypes preferentially, which could result in some subtypes being missed in the analysis. Also, it is preferable that the PCR primers are compatible with DNA extracted directly from stool (Tan 2008).

HISTORY

Alexeieff *et al*. (1911) clearly define the genus *Blastocystis* as a distinct organism and proposed the name *Blastocystis enterocola*. Brumpt *et al*. (1912) proposed the name *Blastocystis hominid* for the organism isolated from the human fecal material. After a number of reports of *B. hominis* isolated from human fecal material, particularly in tropical countries, and its pathogenicity (Low 1916; Maplestone 1921; O'Connor 1919), very few data were published until the work of Zierdt *et al*. (1967) renewed interest in the organism.

The taxonomy of *Blastocystis spp*. remains controversial, and the history of the organism reflects the difficulty in defining its taxonomic position. The ultra-structural study by Zierdt *et al*. (1967) provided the first indisputable evidence that the organism was not a yeast or a fungus, as previously suggested by Alexeieff *et al.* (1911), Brumpt *et al.* (1912) and O'Connor (1919), or the cyst of another organism, such as a *Trichomonas spp.*, as proposed by Bensen (1909). It has been shown to be morphologically distinct from *Dientamoeba fragilis*. Further, ultra-structural studies have supported these conclusions (Boreham *et al.* 1993).

Morphologically, the *Blastocystis* cell shows protistan features: it contains one or more nuclei, smooth and rough endoplasmic reticulum, Golgi complex, and mitochondrion-like organelles (Boreham *et al.* 1993; Dunn *et al.* 1991). A number of morphologically distinct forms of the organism have been described by Boreham *et al.* (1993), Dunn *et al.* (1989), Stenzel *et al.* (1991), and Zierdt *et al.* (1967). Different forms of *Blastocystis spp.* other than the cyst form (Stenzel *et al*. 1991) are not surrounded by a cell wall but are enclosed by a bilaminar membrane.

Blastocystis spp. are strict anaerobes and are sensitive to oxygen and to changes in the tonicity of their environment (Zierdt 1967). Antibacterial agents such as ampicillin, streptomycin, and gentamicin do not appear to adversely affect the growth of *Blastocystis spp.* whereas, a number of antiprotozoan agents appear to inhibit their growth *in vitro* (Dunn *et al.* 1991).

Zierdt *et al.* (1978) classified *Blastocystis* in the Subphylum Sporozoa on the basis of proposed methods of division, in addition to morphological and cultural characteristics. A new class, Blastocystea, and a new order, Blastocystida, were suggested. More recently, Zierdt *et al.* (1988) has reclassified the organism to the Subphylum Sarcodina, order Amoebida, in a separate suborder, Blastocystina.

Molecular sequencing studies on a single axenic human isolate performed by small-subunit rRNA sequencing techniques, have shown that *B. hominis*is not monophyletic with the yeasts *Saccharomyces spp*., the fungi *Neurospora spp*., the amebae *Naegleria*, and *Dictyostelium spp*., or the flagellates *Trypanosoma* and *Euglena spp*. (Johnson *et al.* 1989). The apicomplexans *Sarcocystis* and *Toxoplasma spp*. were found to be monophyletic but were split from *B. hominis* by the ciliates *Oxytricha*, *Stylonichia*, *Tetrahymena*, *Paramecium*, and *Euplotes spp.* and the dinoflagellate *Prorocentrum sp.* These data suggest that *B. hominis* is not closely related to the yeasts, fungi, sarcodines, or sporozoans.

Four species of *Blastocystis* from nonhuman hosts have been proposed: *Blastocystis galli* (Belova *et al.* 1990) from chickens, *Blastocystis anatis* (Belova 1991) from domestic ducks, *Blastocystis anseri* (Belova 1992) from domestic geese, and *Blastocystis lapemi* (Teow *et al.* 1991) from a sea snake. The species isolated from birds were identified by morphological criteria, but the variation seen among individual organisms in isolates from humans (Dunn *et al*. 1989) suggests that these designations should be regarded cautiously until further confirmatory evidence is obtained. *B. lapemi* was differentiated from *B. hominis* by having different optimal culture requirements (*B. lapemi* grows better at 24ºC rather than at 37ºC) and a different electrophoretic karyotype from *B. hominis* (Teow *et al.* 1991).

Kukoschke *et al.* (1991) separated four isolates of *B. hominis* from human fecal samples into two distinct demes on the basis of immunological reactions and on polypeptide patterns obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein extracts. A more extensive study of 61 isolates identified four serologically distinct demes (Mu¨ller 1994). Analysis of 10 isolates by Boreham *et al*. (1992) also revealed at least two distinct demes. Proteins of the demes were immunologically distinct in immunoblots, and hybridization with random probes generated from the DNA of one

stock showed that the DNA content of the two demes also was different (Boreham *et al.* 1992). At least two zymodemes of *B. hominis* have been distinguished on the basis of isoenzyme patterns of nine isolates (Mansour *et al.* 1995). Electrophoretic karyotyping of seven isolates of *B. hominis* indicated that considerable genotypic heterogeneity occurred between isolates (Upcroft *et al.* 1989).

In a later study five isolates of *B. hominis* from Singapore showed only minor variations in karyotypic patterns. The number and size of chromosomes identified differed between the two studies (Upcroft *et al.* 1989). 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* is the only species of *Blastocystis* which is currently accepted to be present in human hosts (Stenzel *et al.* 1996).

SIGNS AND SYMPTOMS OF *Blastocystis hominis* INFECTION

Blastocystosis, as well as its diagnosis, is controversial because it has not been proven that associated symptoms come from *Blastocystis hominis* itself. It is argued that many people found to harbor *Blastocystis* might have other sources of their symptoms. Part of this debate spurs from the fact that it is found mostly in asymptomatic people, and only a minority experience symptoms. Those who do claim to experience symptoms related to blastocystosis claim the following:

- Watery diarrhea
- Abdominal pain / cramps
- Perianal pruritis (itch)
- Excessive flatulence

Heavy presence of *Blastocystis* replaces good bacteria and renders the intestinal lining helpless in combating the infection. Such cases will produce serious skin pathologies, allergies and sensitivities caused by the metabolic byproducts of these large parasite populations.

CLINICAL FEATURES

Name of the disease: Blastocystosis or travelers' diarrhea.
Causative agent: *Blastocystis hominis.*

Clinical manifestations

1. Intestinal

- Asymptomatic colonization
- Vague abdominal complaints: pain, bloating and flatulence
- Diarrhea-mild in immunocompetent, chronic in immunocompromised
- Inflammatory bowel disease: more frequently associated with ulcrative colitis
- Irritable bowel syndrome

2. Extra intestinal

- Urticaria-acute or chronic
- Palmoplantar pruritis
- Chronic angioedema
- Iron deficiency anemia

PATHOGENESITY

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 (Moe *et al.* 1997) infection of immunocompetent mice with fecal cysts of *B. hominis* caused weight loss and lethargy and histological examination of the cecum and colon showed intense inflammation with edematous lamina propria and mucosal sloughing. No organisms were seen invading the colonic wall and infection was self-limiting.

In spite of the above-mentioned experimental animal data, the pathogenic role of *B. hominis* in humans, if any, is far from settled. Arguments that are presented to support a pathogenic role of *B. hominis* in humans are mostly based on uncritical, anecdotal case reports or series and retrospective reviews (Sohail *et al.* 2005). 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. In a case control study from Taiwan, Chen *et al*. (2003) compared 99 immunocompetent adults infected with *B. hominis* with 193 controls, matched for age, gender and date of stool examination.

All subjects underwent upper GI (Gastro Intestinal) endoscopy and sigmoidoscopic examination. There was no statistically significant difference in gastrointestinal symptoms (nausea, abdominal discomfort, flatulence and diarrhea) between cases and controls and none of the subjects with a positive stool smear for *B. hominis* had the appearance of colitis or colonic ulcerations on sigmoidoscopy. Similar findings were reported in another study by Udkow *et al.* (1993). Some authors have suggested that the burden of organisms in stool correlates with symptoms in patients with *B. hominis* infection, but results of several studies have refuted this argument.

In another case-control study among expatriates and tourists in Nepal (Shlim *et al*. 1995), no correlation was found between higher concentrations [R10 *B. hominis* per high-power (400X) field] and severity of diarrhea. Also studies have not demonstrated that anti-protozoal treatment or eradication of *B. hominis* correlates with resolution of symptoms (Miller *et al.* 1988).

It has been suggested that a host's immune status may determine the presence and severity of symptoms. However, in a study of homosexual men with positive stool examination for *B. hominis*, (Church *et al.* 1992) there was no difference in symptoms or prevalence of infection between HIV positive and negative individuals. Several observational studies have suggested that an abnormal GI tract may predispose to colonization and overgrowth of *B. hominis.* Reported abnormalities in these reports include intestinal obstruction, GI malignancy and irritable bowel syndrome, suggesting that a positive stool smear with *B. hominis* may serve as a marker for organic or functional enteric diseases.

TREATMENT

The need to treat individuals infected with *Blastocystis* is equivocal, considering the controversial pathogenesis of the organism and the apparent self-limiting nature of symptoms (Albrecht 1995; Leder 2005). In instances where treatment is warranted, metronidazole has been the most frequently prescribed antibiotic (Cassano 2005; Moghaddam 2005; Nigro 2003).

Table 1. Treatment options and regimens for *Blastocystis* infections.

Drug	**Dose**	**Side effects**	**Reference(s)**
Metronidazole	Adult: 750 mg tid for 10 days Pediatric: 15 mg/kg/day for 10 days	Metallic taste, transient nausia	Gupta *et al.* 2006, Katsarou-Katsari *et al.* 2008. Zaki *et al.* 1991
TMP-SMP	Adult 320 mg TMP and 1,600 mg SMX daily in 2 equal doses for 7 days Pediatric: 6 mg/kg TMP and 1,600 mg/kg SMX daily in 2 equal doses for 7 days	Hives, lack or loss of appetite, nausea, skin rash, vomiting	Ok *et al.* 1999 Ok *et al.* 1999
Nitazoxanide	Adult: 500 mg bid for 3 days Padiatric: (1-3yr)100 mg bid for 3 days (4-11yr) 200 mg bid for 3 days	Yellowish urine, abdominal pain, headache, nausea	Rossignol, *et al.* 2005
Paromomycin	25 mg/kg tid for 10 days.	Nausea, abdominal cramps, diarrhea	Valsecchi, *et al.* 2004
Paromomycin-metronidazole combination	1000 mg paromomycin bid for 10 days and 750 mg metronidazole tid for 10 days	- - -	Waghorn, *et al.* 1991

Various drug regiments for metronidazole have been prescribed, ranging from 250 to 750 mg three times a day for 10 days (Valsecchi 2004) to 1.5 mg/day for 10 days (Cassano 2005), or used in combination with other drugs such as paromomycin (Pasqui 2004) or cotrimoxazole (TMP-SMX) (Andiran 2006). Nitazoxanide, a 5-nitrothiazole broad-spectrum antiparasitic agent, has been reported to be effective against *Blastocystis* (Rossignol 2005).

Paromomycin, a broad-spectrum antibiotic indicated for acute and chronic intestinal amoebiasis, was shown to successfully treat *Blastocystis* infections associated with cutaneous lesions, predominantly urticaria (Pasqui 2004; Valsecchi 2004).

In summary, a variety of drug treatment options are available for *Blastocystis* infections (Table 1), and metronidazole appears to be the most effective drug for *Blastocystis* chemotherapy despite some evidence for treatment failures (Moghaddam 2005). In such drugs treatment should be considered if diarrhea is persistent and no other pathogen apart from *Blastocystis* is identified in fecal specimens.

Future studies should investigate if there is an association between genotype and variations in drug sensitivity and should also focus on the mechanism of action of and resistance to metronidazole (Tan 2008).

BIOLOGY

Blastocystis is found to colonize the intestinal tract of a wide variety of hosts such as insects, reptiles, birds and mammals (Noël, 2005). The host specificity seems to correlate with the Sub Type (ST). Among the 17 ST identified, ST1-8 can colonize/infect human as well as non-human hosts, ST9 is found to occur only in humans and ST10-17 are exclusive to non human hosts (Stensvold 2013). *Blastocystis* is a strict anaerobe. Although, numerous intracellular organelles resembling mitochondria are seen, they are completely devoid of cytochrome enzymes (Zierdt 1986). These organelles have the property of both the mitochondria of aerobes and the hydrogenosomes of anaerobes and are involved in various metabolic pathways such as amino acid metabolism, iron-sulfur cluster biogenesis and tricarboxylic acid cycle (Stechmann 2008; Tsaousis 2012). It has

also been observed that the organism is capable of synthesizing various essential cellular phospholipids and accumulate them within storage vacuoles (Keenan 1994; Zierdt 1988).

The generation time of the organism observed *in vitro* in axenic media is 17-22 h; however, it varies on different media (Irikov 2009). In cultures on xenic media followed by axenization the generation time is shorter, ranging 7-12 h (Lanuza 1997). Generation time is also found to vary among different strains or ST (Zierdt 1981). The ability of the organism to undergo apoptosis under adverse conditions has been demonstrated (Nasirudeen 2001). This phenomenon has been observed when the cells are exposed to aerobic conditions and also in the presence of anti-parasitic agents such as metronidazole (Tan *et al.* 2001; Nasirudeen 2004). It has been postulated that apoptosis serves as a mechanism to increase the number of viable cells during stressed conditions (Dhurga 2012).

GENETIC DIVERSITY

Blastocystis spp. from humans and animals have been reported to be morphologically similar. This is probably an oversimplification, as there have been reports describing distinct morphological differences among *Blastocystis* isolates (Singh *et al.* 1996; Stenzel *et al*. 1994; Stenzel *et al.* 1997; Yoshikawa *et al.* 2007). However, it is nevertheless challenging to differentiate one isolate from another based on morphological criteria alone. Interestingly, extensive genetic variation has been observed among numerous isolates from both humans and animals.

A number of molecular techniques to study the genetic diversity of *Blastocystis spp*. have been described. The techniques commonly employed are PCR-restriction fragment length polymorphism (RFLP) (Abe *et al*. 2003; Gloning *et al.* 1997; Kaneda *et al*. 2001) PCR followed by dideoxy sequencing (Hoevers *et al.* 2005), and PCR with subtype-specific [sequence-tagged site (STS)] primers (Abe *et al*. 2003). A few studies employed the use of arbitrary primed PCR (Huang *et al.* 2006; Kuhls *et al*. 1994) or karyotyping (Clark 2000), by PCR-RFLP of the entire small-subunit rRNA (SSU rRNA) gene, revealed a remarkable amount of genetic variation that existed among 30 randomly selected human isolates.

These RFLP profiles (riboprints) could be grouped into seven distinct genotypes (ribodemes). It was previously observed that there was a 7% divergence between ribodemes 1 and 2, which is approximately four times the genetic distance between homologous genes of *Entamoeba histolytica* and *Entamoeba dispar* (Clark 1997).

In the most extensive phylogenetic study to date, (Noël *et al.* 2005) analyzed the SSUrRNA genes of 12 *Blastocystis* isolates from humans, rats, and reptiles together with 78 other *Blastocystis* sequences available in the GenBank database at the time of the study. They showed that *Blastocystis spp.* could be unambiguously placed within seven distinct clades, with six of the major groups comprising isolates from both humans and animals. Those authors concluded that numerous zoonotic isolates existed, with frequent animal-to-human and human-to-animal transmissions, and that animals represent a large potential reservoir for human infections. Thus, in the absence of genotype information and due to the extreme genetic diversity among *Blastocystis* isolates, caution is warranted when interpreting data or when extrapolating observations of morphology, drug sensitivity, and pathogenesis from one isolate to another. Since that time, a large number of case reports of *B. hominis* has been presented but very few experimental studies have been conducted. With the application of a number of new molecular biology-based techniques, tremendous advances have been made in our knowledge of the diagnosis, natural history, and epidemiology. As more is discovered about the molecular and cell biology of *B. hominis*, there is great potential for further understanding of the pathogenesis of blastocystosis.

EPIDEMIOLOGY AND PREVALENCE

Authors of early studies lamented the lack of epidemiological data on *Blastocystis spp.* (Boreham *et al.* 1993). However, recent years have shown a dramatic increase in prevalence studies, and these studies have shed light on the parasite's genotype distribution, mode of transmission, and pathogenesis. *Blastocystis* is an extremely ubiquitous parasite with a worldwide distribution. It is not uncommon for it to be the most frequently isolated parasite in epidemiological surveys. Prevalence varies widely from country to country and within various communities of the same country. In general, developing countries have higher prevalences of the parasite than developed countries,

and this has been linked to poor hygiene, exposure to animals, and consumption of contaminated food or water.

In some countries, the carriage rate can be rather variable, depending on the subpopulation studied. Prevalence ranges of 1.9 to 32.6%, 0.19 to 45.2%, and 1.04 to 18.3% in prevalence studies from China (Li *et al.* 2007) Thailand (Saksirisampant *et al.* 2006), and Turkey, respectively, have been reported. Such variations within the same country could reflect true differences between communities, especially if the same techniques were employed to identify the parasite. However, variations are also likely due to the use of different diagnostic approaches and the inherent difficulty in identifying stages other than the vacuolar form. Recent surveys incorporated genotype information by PCR of *Blastocystis* DNA from feces or from stool culture. Such studies are now shedding light on the distributions of genotypes among human populations (Table 2) and animal hosts and also provide information on transmission routes or sources.

A study by Yoshikawa *et al.* (2004) employed the use of PCR-based genotype classification to study the distribution of *Blastocystis* genotypes among isolates from Bangladesh, Germany, Japan, Pakistan, and Thailand. The most dominant subtype among four populations except Thailand was subtype 3 (41.7 to 92.3%), followed by either subtype 1 (7.7 to 25%) or subtype 6 (10 to 22.9%). Similar genotype distributions in Singapore (78% subtype 3 and 22% subtype 1), China (60.4% subtype 3 and 24.5% subtype 1), Greece (60% subtype 3 and 20% subtype 1), Germany (54% subtype 3 and 21% subtype 1), and Turkey (75.9% subtype 3) were also reported. In most studies, other genotypes were identified at lower frequencies (Table 2).

Collectively, some studies suggest that, subtype 3 is the subtype of human origin and that there is no correlation between *Blastocystis* geographic origin and genotype. It may be worthwhile to note that avian subtypes 6 and 7 may grow optimally at 40°C instead of 37°C, as is the case for the avian protozoan flagellate *Histomonas meleagridis* (Grabensteiner *et al.* 2006). Isolates belonging to subtype 7 have longer doubling times, about 50 h, when cultured at 37°C (Ho *et al.* 1993). In this case, these slow-growing subtypes may still be missed during in vitro culture expansion of stool samples, resulting in an underrepresentation of such subtypes in epidemiological surveys.

Table 2. Distribution of *Blastocystis* subtypes infecting humans in different geographic regions.

Country/region and type of isolates (no. of infected individuals studied)	Subtype distribution (%)[a]										No. of positive isolates/total no. of isolates (%)[b]
	1	2	3	4	5	6	7	8	9	Unknown/mixed	
Bangladesh (26)	7.7	—	92.3	—	—	—	—	—	—	—	NA
Guangxi, China (35)	37.1	—	40	—	—	—	5.7	—	—	17.2	NA
Yunnan, China (78)	20.5	1.3	70.5	1.3	—	—	—	—	—	6.5	NA
Denmark (28)	17.9	32.1	46.6	3.8	—	—	—	—	—	—	NA
Egypt (44)	18.2	—	54.5	—	—	18.2	9.1	—	—	—	NA
Germany (166)	21	1	66	7	—	—	—	—	—	5	NA
Germany (12)	25	16.7	41.7	16.7	—	—	—	—	—	—	12/67 (17.9)
Greece (45)	20	13.3	60	2.2	—	2.2	2.2	—	—	—	NA
Japan (55)	20	21.8	43.6	10.9	—	—	—	—	—	3.6	NA
Japan (50)	8	—	52	4	—	22	10	—	4	—	50/2,037 (2.45)
Pakistan (10)	20	—	70	—	—	10	—	—	—	—	NA
Singapore (9)	22.2	—	77.8	—	—	—	—	—	—	—	9/276 (3.3)
Thailand (153)	90.2	—	4.6	—	—	—	1.3	—	—	3.9	334/924 (36.1)[c]
Denmark (29)	3.4	20.7	51.7	24.1	—	—	—	—	—	—	NA

[a]—, subtype not detected. [b]NA, not available. [c]Three hundred thirty-four *Blastocystis*-positive samples were obtained by in-vitro culture of stool specimens. Out of these 334 isolates, only 153 were amenable to PCR amplification.

Although studies revealed that the majority of individuals are host to a particular *Blastocystis* subtype, mixed infections in a minority of individual have also frequently

been reported. Depending on the study, mixed subtypes have been seen among 1.1 to 14.3% of samples surveyed.

Most are coinfections with subtype 1 and subtype 3 while subtype 1/subtype2, subtype2/subtype 3, and subtype 3/subtype 5, combinations were infrequently reported. Intra-subtype 1 and subtype 2 variations in SSUrRNA sequence were also reported for single isolates. It may be difficult to ascertain the true distribution of mixed infections in a particular individual, as this depends on the method employed to determine the *Blastocystis* subtype (Dogruman-Al *et al.* 2008; Parkar *et al.* 2007). Genotyping of *Blastocystis* DNA obtained directly from stools may be more accurate for identifying mixed infections if PCR conditions are optimal. PCR employing subtype-specific STS primers are visually more discriminatory for mixed infections than is PCR-RFLP or sequence analysis of a single SSUrRNA amplicon.

In the former approach, the *Blastocystis* infection is commonly seen in children from various geographical settings, and accumulating epidemiological and case studies suggest that *Blastocystis* infection causes gastrointestinal disease in this cohort. Collectively, there is an increasing body of evidence suggesting that *Blastocystis* is pathogenic or is an opportunistic pathogen, with immunocompromised populations being more susceptible to infection and its associated symptoms (Chandramathi *et al.* 2012).

Blastocystis infections are common among certain occupations that involve exposure to animals, again reinforcing the zoonotic nature of the organism. These include food handlers and animal handlers such as zookeepers and abattoir workers. Longitudinal epidemiological studies add an important characteristic to point prevalence studies by permitting the characterization of temporal changes in affected patients and in disease characteristics, such as the frequency, complications, and outcomes of a disease. There are only a few such studies involving *Blastocystis spp.* (Tan 2008).

An earlier study involving young (10 to 28 months of age) Kenyan children over a 10-month period revealed a significant association between *Blastocystis* infection with unformed stools and diarrhea (Chunge *et al.* 1991) while a later study of Peace Corps volunteers in Guatemala over a 2-year period showed no correlation between *Blastocystis* infection and gastrointestinal symptoms. The discrepancy may be attributed

to the different age groups studied or to geographical differences in *Blastocystis* genotypes.

An increasing number of prevalence studies have implicated contaminated water as being a source of *Blastocystis* infections (Basualdo *et al.* 2007; Elshazly *et al.* 2007). This is not surprising since the transmissible form of the parasite is the water-resistant cyst. In a study involving a Thai army population, *Blastocystis* was found to be the most common (21.9%) intestinal parasite (Taamasri *et al.* 2000). This high prevalence among the soldier population was significantly linked to the consumption of unfiltered or nonboiled water. A recent study involving the use of STS primers on stool samples of 238 randomly selected individuals from a village in Yunnan province, China, revealed high infection rates (32.6%). It was observed that the consumption of raw water plants was associated with subtype 1 infections, while drinking unboiled water was associated with subtype 3 infections. This was the first study to investigate the association between subtypes and transmission routes, although more studies are needed before any firm associations can be made (Li *et al.* 2007).

Most authors seek statistical differences in subtype distribution between asymptomatic and symptomatic groups, while others consider the possibility that pathogenic subtypes can be present in approximately equal numbers in either group, possibly due to intrasubtype variations or the presence of pathological evidence within the symptomatic group (Özyurt *et al.* 2008; Yan *et al.* 2006). Due to these complications, Dogruman-Al *et al.* (2008) suggested that it is clearer to identify nonpathogenic subtypes since these subtypes should consistently be found in greater proportions within the asymptomatic group. Indeed, more studies with larger sample sizes are needed before this issue is resolved. Genotyping of isolates during outbreak situations may provide a valuable opportunity to identify pathogenic subtypes. The possibility of intrasubtype variation in pathogenesis should also be considered, as was suggested previously. Collectively, studies suggest that at least subtype 1 is associated with disease, while subtypes 2 and 3 may be nonpathogenic (Dogruman-Al *et al.* 2008).

JUSTIFICATION OF THE STUDY

The rate of mortality from diarrheal diseases in the world has decreased, mainly because of better therapy and interventions that promote sanitary conditions and that educate inhabitants to encourage them for taking part in primary health care activities. However, acute diarrheal diseases continue to be one of the major causes of morbidity and mortality in the developing world such as Bangladesh, where one in 30 children die before their fifth birthday (Haque *et al*. 2003).

An epidemiologic study of an infectious disease in a community is an initial step toward the introduction of the proper interventions for controlling the disease because the features and the patterns of isolation of etiologic agents of the disease vary from place to place depending on the local meteorology, geography, and socioeconomic elements (Haque *et al*. 2003).

"Prevention is better than cure." with this view, preventive measures were launched long ago to minimize the suffering of mankind. In our country, diarrheal disease is one of the major cause of mortality specially among infants. Lack of proper knowledge, poverty, and ignorance are the main contributing factors for this unwanted death. Though the pathogenecity of *B. hominis* is still controversial, it was suggested that, it can cause severe amebic dysentery and liver abscess while co-infecting with *E. histolytica*. Besides, *B. hominis* causes the disease Blastocystosis (Travelers' diarrhea). That means travelers are very susceptible to the infection with this disease. The author is very curious to be a part of the study as molecular detection PCR have to study on *B. hominis* for first time in Bangladesh. However, this study was based on the laboratory observation of the fecal samples of the chosen subjects, where children's age group was 1-72 months because they are more vulnerable than other age groups.

HYPOTHESIS

Molecular technique PCR and *in vitro* culture are more convenient method than direct microscopy to detect the presence of *B. hominis.*

OBJECTIVES OF THE STUDY

The present study was undertaken with some distinct aims as mentioned below:

1. To detect *Blastocystis hominis* by different techniques such as, microscopy, *in vitro* culture and PCR method.
2. To determine the prevalence of *B. hominis* among diarrhoeal and non diarrhoeal samples by microscopy and *in vitro* culture according to sex and age of children.
3. To compare the prevalence of *B. hominis* in non diarrhoeal (MS) and diarrhoeal stool (DS) samples.
4. Determine the seasonal variation in prevalence of *B. hominis* among the children during the study period.
5. Observed the monthly incidence of *B. hominis* among the children and correlate with climatic factors (temperature, humidity and rainfall).
6. Observe the prevalence of morphological forms of *B. hominis* in different samples of two study areas.
7. To study the epidemiological aspects of *B. hominis* by different methods in the infants of Mirpur and Mohammadpur slum areas.
8. To develop a molecular assay PCR for diagnosis of *B. hominis* for perfect identification in stool specimens and to compare the results of these three techniques.
9. To compare the sensitivity and specificity of these three diagnostic techniques.
10. Observe the infection rate of other protozoan and helminth parasites.

TAXONOMY OF *Blastocystis hominis*

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 (Arisue *et al.* 2002).

Superkingdom - Protista
Subkingdom - Protozoa
Phylum - Sarcomastigophora
Class - Blastocystea
Order – Blastocystida
Family - Blastocystidae
Genus - *Blastocystis*
Species - *Blastocystis hominis*

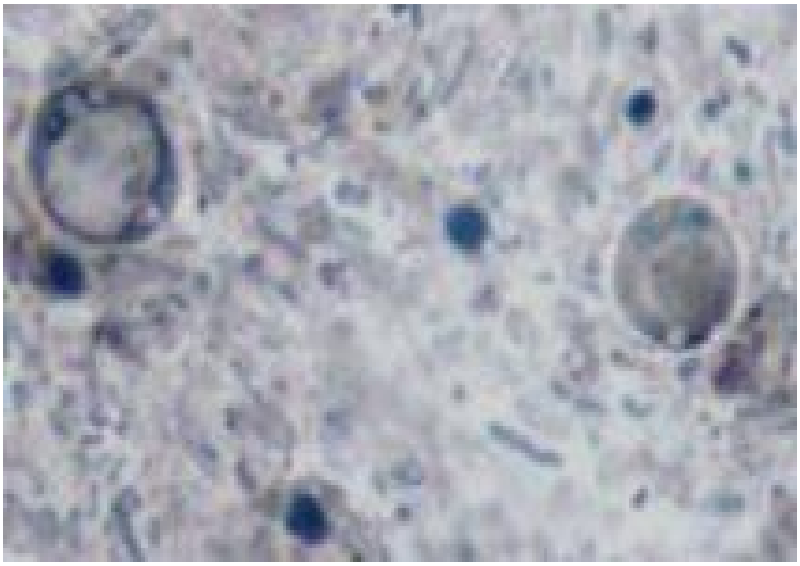

Fig. 1. *Blastocystis hominis* (Tan 2008).

MORPHOLOGY OF *Blastocystis 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 have made studies of its cell biology challenging, resulting in misinterpretations of data from time to time.

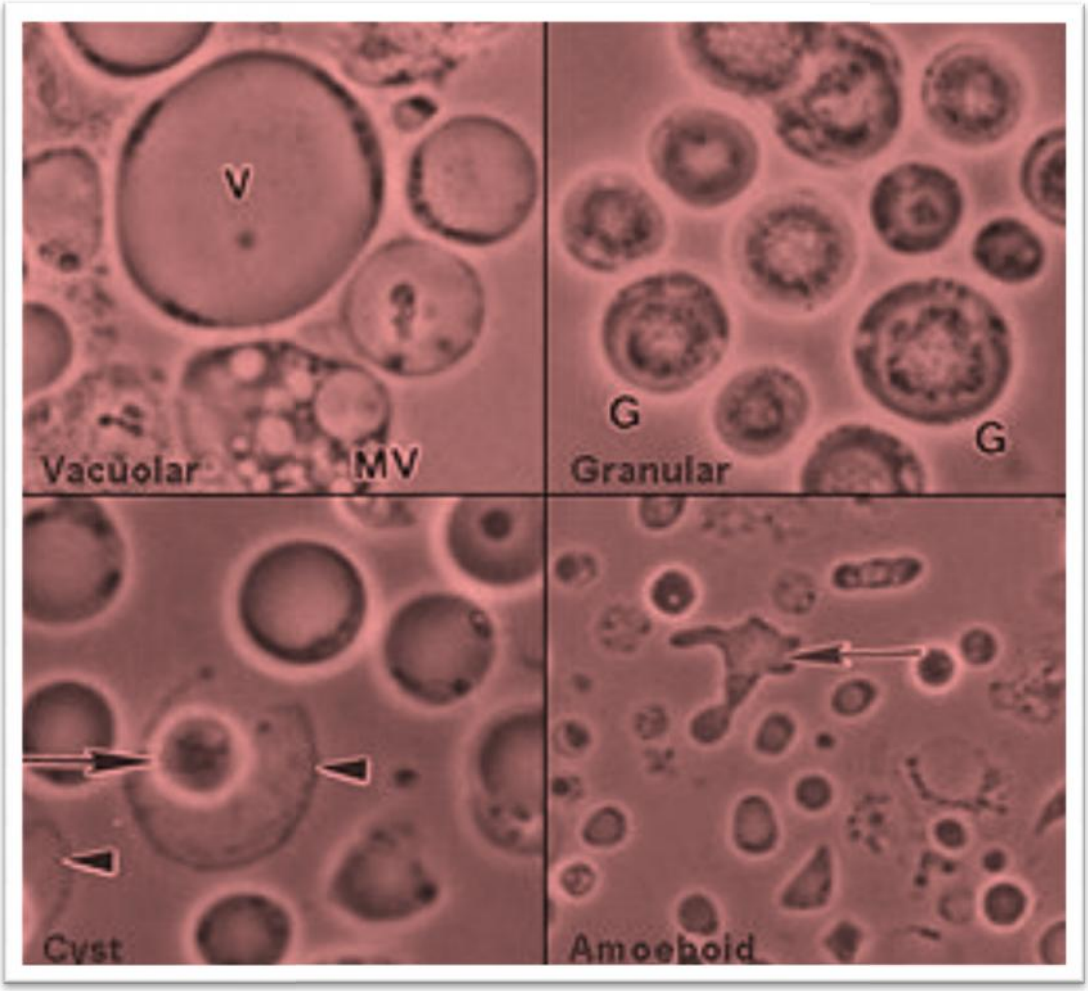

Fig. 2. Four morphological forms of *Blastocystis hominis* (Tan 2008).

The cyst form of *B. hominis:*

The cyst form 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, and the observation that cysts are infrequently seen in laboratory culture. The cysts are variable in shape but are mostly ovoid or 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. One report described the presence of large multinucleate cysts from the stools of *Macaca* monkeys, which were 15 μm in size, and this was suggested to be an indication of differences among *Blastocystis* species (Stenzel *et al.* 1997).

Blastocystis cysts were reportedly able to survive in water for up to 19 days at normal temperature but are fragile at extreme temperatures and in common disinfectants (Moe *et al.* 1996). A later study showed that cysts could survive up to 1 month at 25°C and 2 months at 4°C. The contrasting viabilities among the studies may be due to isolate variations (Yoshikawa *et al.* 2004). Vacuolar and granular forms, in contrast, are sensitive to temperature changes, osmotic shock, and exposure to air (Zierdt *et al.* 1991).

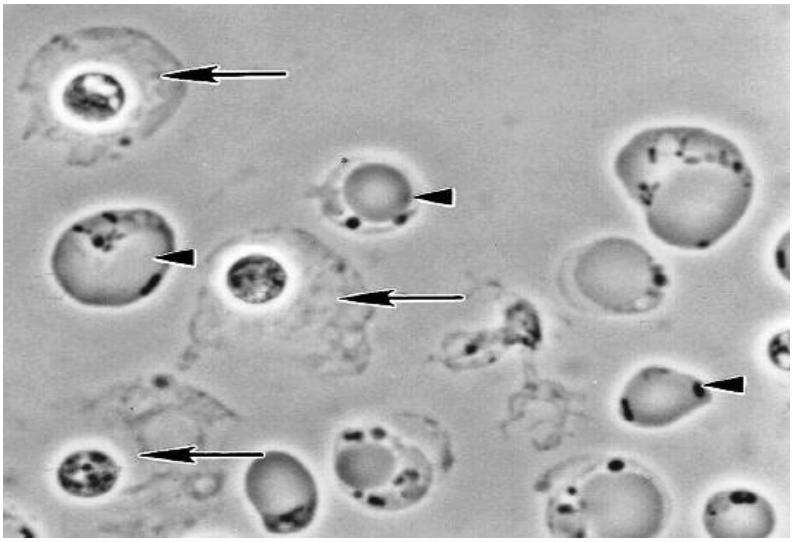

Fig. 3 . Cyst of *B. hominis* observed by phase-contrast microscopy (Tan 2008).

Experimental infectivity studies of BALB/c mice, Wistar rats and a variety of bird species with the cyst form indicate that this form is undoubtedly the transmissible form of the parasite. The formation of *in vitro*-derived cysts by the incubation of vacuolar forms in encystation medium was reported previously (Suresh *et al.* 1994; Villar *et al.* 1998). These “cysts” appear to be curiously similar to the classical granular forms, and the granules within the central vacuole were reported to be reproductive in nature (Suresh *et al.* 1994). It is likely that these are artifacts of culture induced by the encystation medium, as they bear no morphological similarity to the fecal cyst.

Interestingly, in vitro derived cysts are able to infect Wistar rats and were apparently resistant to osmotic lysis (Suresh *et al.* 1993; Villar *et al.* 1998).

By phase-contrast microscopy spherical cysts of *Blastocystis* subtype 4 from an *in vitro* axenic culture displaying a loose outer coat (arrows) among vacuolar forms (arrowheads) (Fig. 3). While, enrichment of subtype 4 cysts and loss of outer coat are apparent after overnight incubation in distilled water (Fig. 4).

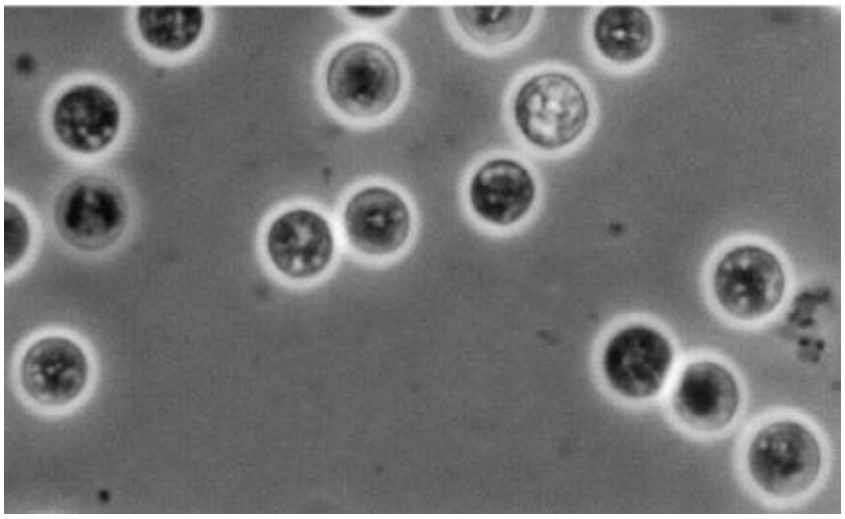

Fig. 4. Cyst of *B. hominis* observed by phase-contrast microscopy (Tan 2008).

The vacuolar form of *B. hominis:*

The central vacuole form, 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) (Stenzel *et al.* 1996). Extensive size variation can occur within and between isolates (Dunn *et al.* 1989; Rapeeporn *et al.* 2006).

Dunn *et al.* (1989) observed size variations of 4 to 63 µm among 10 human isolates; the mean diameters between stocks also varied significantly, with overlap between some isolates. A study of *Blastocystis* strains isolated from chickens revealed vacuolar forms ranging from 3 to 120 µm in diameter (Lee *et al.* 1999). The vacuolar form is characterized by a large central vacuole that occupies approximately 90% of the cell's

volume (Macpherson *et al.* 1994). This relegates the cytoplasm and organelles into a thin peripheral rim, which may sometimes be difficult to visualize under light microscopy.

Nuclei and mitochondrion-like organelles are usually located within thick cytoplasmic regions at opposite ends of the cell. Certain amphibian isolates possess thick cytoplasmic rims, which are easily discernible by conventional light microscopy (Singh *et al*. 1996). The central vacuole may appear empty or may contain fine to flocculant material. It was reported to contain carbohydrates, evidenced by positive staining with periodic acid-Schiff and Alcian blue staining (Yoshikawa *et al.* 1995a), or lipids, evidenced by Sudan black B and Nile blue staining (Yoshikawa *et al.* 1995b), suggesting a storage role for the organelle.

The vacuole has also been suggested to play a role in schizogony-like reproduction by providing an environment for the development of minute parasite progeny (Singh *et al*. 1995, Suresh *et al*. 1994). This is highly unlikely considering that these progeny appear strikingly similar to metabolic granules described previously and are therefore simply variants of the granular form (Singh *et al.* 1995).

Cytoplasmic contents, often containing organelles, may invaginate and deposit filament- or vesicle-like membrane-bound structures into the central vacuole (Dunn *et al.* 1989, Nasirudeen *et al.* 2004; Tan *et al.* 2001). The exact significance of this process is unclear, although it has been postulated to be a mechanism of apoptotic body deposition in *Blastocystis* cells undergoing programmed cell death (Tan *et al.* 2005). The cytoplasm contains organelles typically observed in eukaryotes. The features observable by transmission electron microscopy (TEM) include one or more nuclei, Golgi apparatus, endosome-like vacuoles, microtubules, and mitochondrion-like organelles.

The organism is often surrounded by a surface coat, sometimes referred to as the fibrillar layer or capsule, of various thicknesses (Dunn *et al*. 1989). The surface coat is often thicker in parasites freshly isolated from feces and gradually thins out during prolonged laboratory culture, and cells without surface coats have been observed *in-vitro* (Dunn *et al*. 1989). The reason for this thinning out is unknown, but may be due to the postulated role of the coat in trapping bacteria for nutritional purposes, which is not possible during axenic culture, or may be unnecessary if nutrients provided in laboratory culture are sufficient for growth (Zaman *et al.* 1999). Vacuolar form from *in vitro* axenic culture displaying extensive size variation (arrowheads) (Fig. 5).

The surface coat contains a variety of carbohydrates and has been postulated to play a role in trapping and degrading bacteria for nutrition protecting against osmotic shock or to provide a mechanical barrier for functionally important plasma membrane proteins from the immune system (Tan *et al.* 1997).

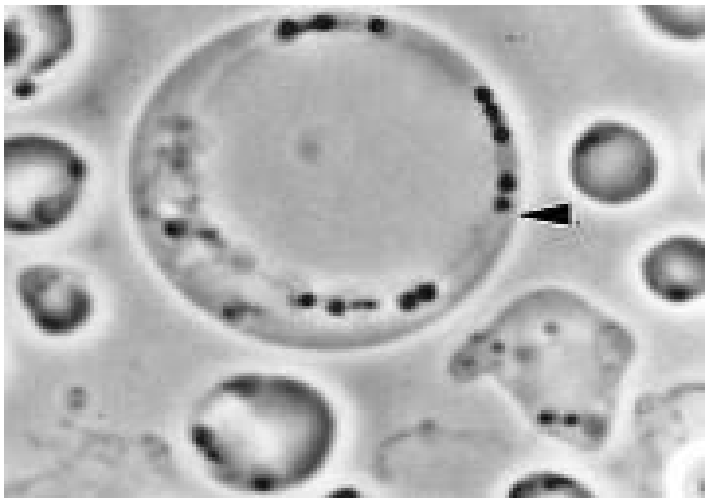

Fig. 5. Vacuolar form of *Blastocystis sp.* subtype 4 by phase-contrast microscopy (Tan 2008).

The granular form of *B. hominis:*

The granular form granular form has been suggested to arise from the vacuolar form and the transition induced by a variety of factors (Stenzel *et al.* 1996). These include increased serum concentrations in the culture medium, transfer of cells to a different culture medium, axenisation and addition of certain antibiotics (Stenzel *et al.* 1996). The granular forms shares many similarities with the vacuolar form except that numerous granules are found within the thin band of peripheral cytoplasm or, more commonly, within the central vacuole (Fig. 6). There is considerable morphological variation in the types of granules within the central vacuole. These may be myelin-like inclusions, small vesicles, crystalline granules and lipid droplets (Dunn *et al.* 1989). There have also been suggestions that the central vacuole functions in schizogony and endodyogeny with certain reproductive granules representing progeny of *Blastocystis* (Zierdt 1991, and Suresh *et al.* 1994). However, there has been some debate as to whether these are bona

fide progeny of *Blastocystis hominis* as, ultra structurally, they appear similar to granule types reported previously.

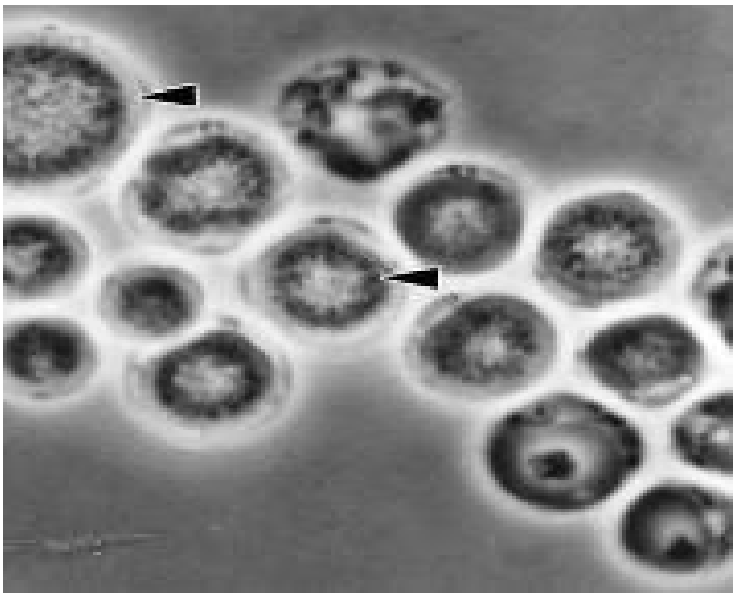

Fig. 6. Granular form with distinct granular inclusions with in the central vacuole (arrowhead) (Tan 2008).

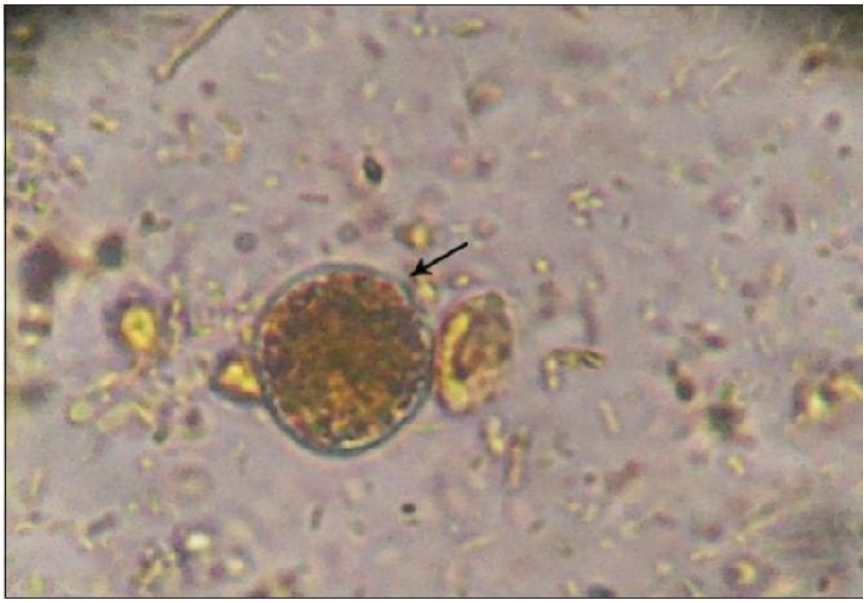

Fig. 7. Granular form of *Blastocystis spp*. in culture by ×1000 objective (Elghareeb *et al.* 2015).

One possibility for the confusion is that many of these observations were also made by conventional light microscopy, which can lead to inaccurate or biased interpretation. For example, it had been suggested (Boreham *et al.* 1993) that the less commonly seen multivacoular form may be misinterpret as a cell undergoing schizogony because the multiple vacuoles can resemble progeny. With the advent of improved live imaging systems, such as restoration microscopy, subcellular structures can be observed at very high resolving powers (< 0.2 µm). Using fluorescent markers and time-course assays, such systems should be helpful in confirming if certain granular structures reported earlier are indeed progeny of *B. hominis* (Tan *et al.* 2002).

The amoeboid form of *B. hominis.*

The amoeboid form (Fig. 8) of *Blastocystis* spp. is rarely reported, and there are contradicting descriptions of what constitutes this morphological type. An early report described numerous amoeba-like forms in the diarrheal fluid of a patient who died of aspiration pneumonia (Zierdt *et al*. 1976). These cells were irregularly convoluted, and some cells possessed one or two large pseudopods. In another study, amoeboid forms from in vitro culture were observed to be 10 to 15 µm, possessing features typical of vacuolar forms, with the exception of one or two pseudopods.

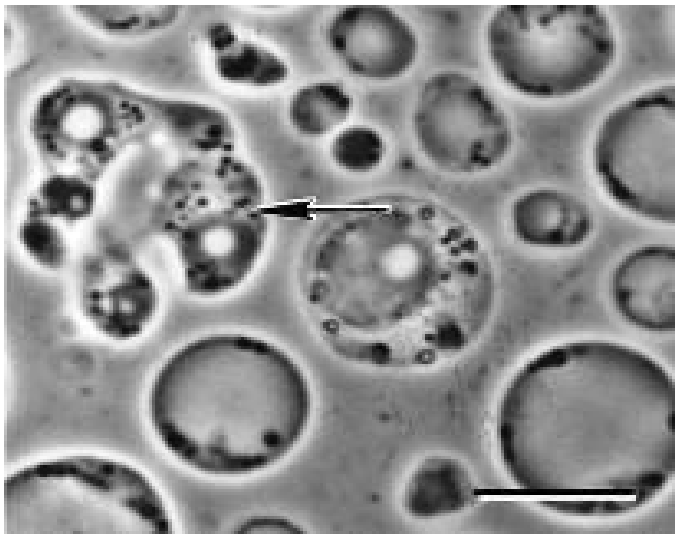

Fig. 8. Amoeboid forms occasionally seen in culture showing pseudopod-like cytoplasmic extensions (arrow). Bar, 10 µm (Tan 2008).

Tan *et al.* (2001) has reported the presence of numerous amoeboid forms from *Blastocystis* colonies grown in soft agar. Light microscopy and TEM showed cells with a central vacuole, a surface coat, and numerous golgi bodies and mitochondria within the cytoplasmic extensions of pseudopods (Tan *et al.* 2001). Dunn *et al*. (1991) previously described amoeboid cells ranging from 2.6 to 7.8 μm with extended pseudopodia and lysosome-like compartments containing ingested bacteria.

In contrast to the study of Tan (2008), these forms lacked a central vacuole, a golgi complex, a surface coat, and mitochondria. Considering the genetic diversity of the organism, it is plausible that the differing descriptions are due to genotypic variations among *Blastocystis* isolates. The presence of bacteria and bacterial remnants within the amoeboid form suggests a nutritional role for this form. The amoeboid form has been postulated to play a role in pathogenesis. However, the light and TEM micrographs in two of these reports were unconvincing for this form and appear more like irregular shaped central vacuole forms, a common artifact of TEM processing. Despite the observation of pseudopod-like cytoplasmic extensions, the amoeboid form appears to be non-motile. The identification of stage-specific molecular markers would be useful for studies of various developmental forms of the parasite and would obviate the problem of distinguishing the various forms by morphological criteria alone.

Other forms of *B. hominis*:

Other forms have also been described, and these forms include the avacuolar and multivacuolar forms. These forms reported from TEM studies of fresh stool samples were significantly smaller (5 to 8 μm) than culture forms and were suggested to be the form that occurs *in vivo*. (Fig. 9 and 10).

However, others observed typical vacuolar forms from fresh fecal samples. The central vacuole was absent in the avacuolar form, while the multivacuolar forms contained multiple small vacuoles. The small size and distinct multivacuolar or avacuolar morphology may be due to strain variations, or they are possibly cells in various stages of encystation or excystation, as similar morphologies were described in TEM studies of cells undergoing excystation (Chen *et al.* 1999).

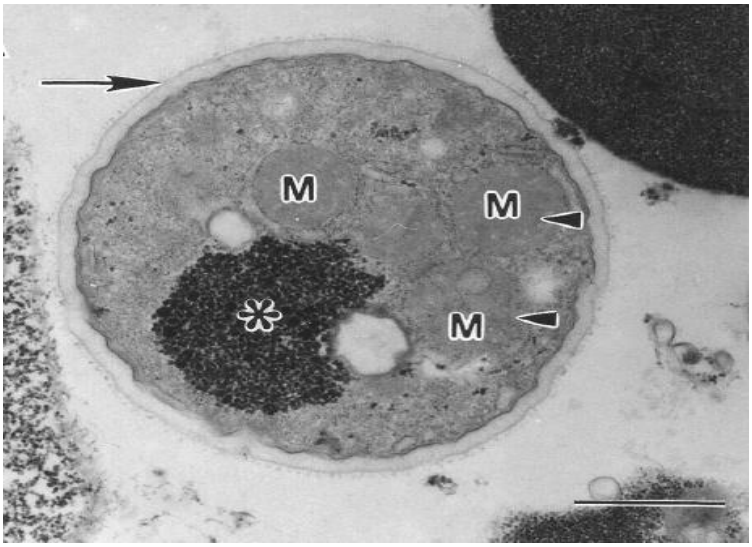

Fig. 9. Cyst of *Blastocystis* sp. subtype 4 by TEM. Mature cyst with a distinct double-layered cyst wall (arrow) and reduced glycogen mass (asterisk). Mitochondrion-like organelles (M) contain faint saccate cristae (arrowhead) (Tan 2008).

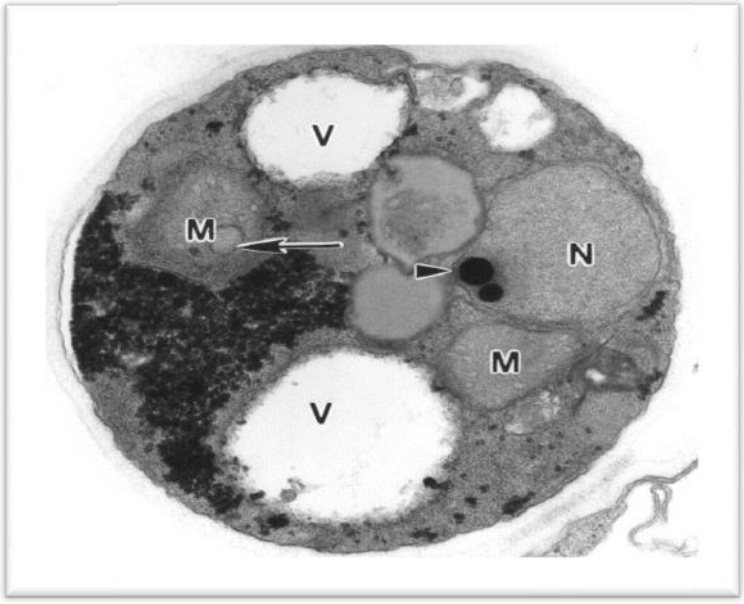

Fig. 10. Cyst of *Blastocystis* sp. subtype 4 by TEM. Mature cyst containing large vacuoles (V), a nucleus (N) with a dense chromatin mass (arrowhead), and mitochondrion-like organelles (M) with saccate and circular (arrow) cristae. Bar, 1 µm (Tan 2008).

LIFE CYCLE of *Blastocystis hominis*:

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. The suggestion that *Blastocystis* undergoes multiple fission has led to life cycles where schizogony is one of the modes of reproduction. This and other proposed modes such as plasmotomy (budding), endodyogeny, and sac-like pouches are more likely due to the pleomorphic nature of the organism and not true modes of reproduction (Tan 2008).

A life cycle comprising thick and thin-walled cysts from multiple fissions was proposed. The authors hypothesized that the thick-walled cysts are important for external transmission, while the thin-walled cysts were autoinfectious. There is little scientific evidence to support such a proposal, although schizogony-like reproduction in *Blastocystis* has been perpetuated in a number of authoritative sources. Until proven otherwise, the only accepted mode of reproduction is binary fission (Fig. 12).

The application of live-cell imaging technology should provide a better understanding of the modes of reproduction of *Blastocystis spp*.

A revised life cycle (Fig. 11) 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 excystation in the large intestines and develops into vacuolar forms. 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 (Fig. 10). Encystation 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 losing during cyst development.

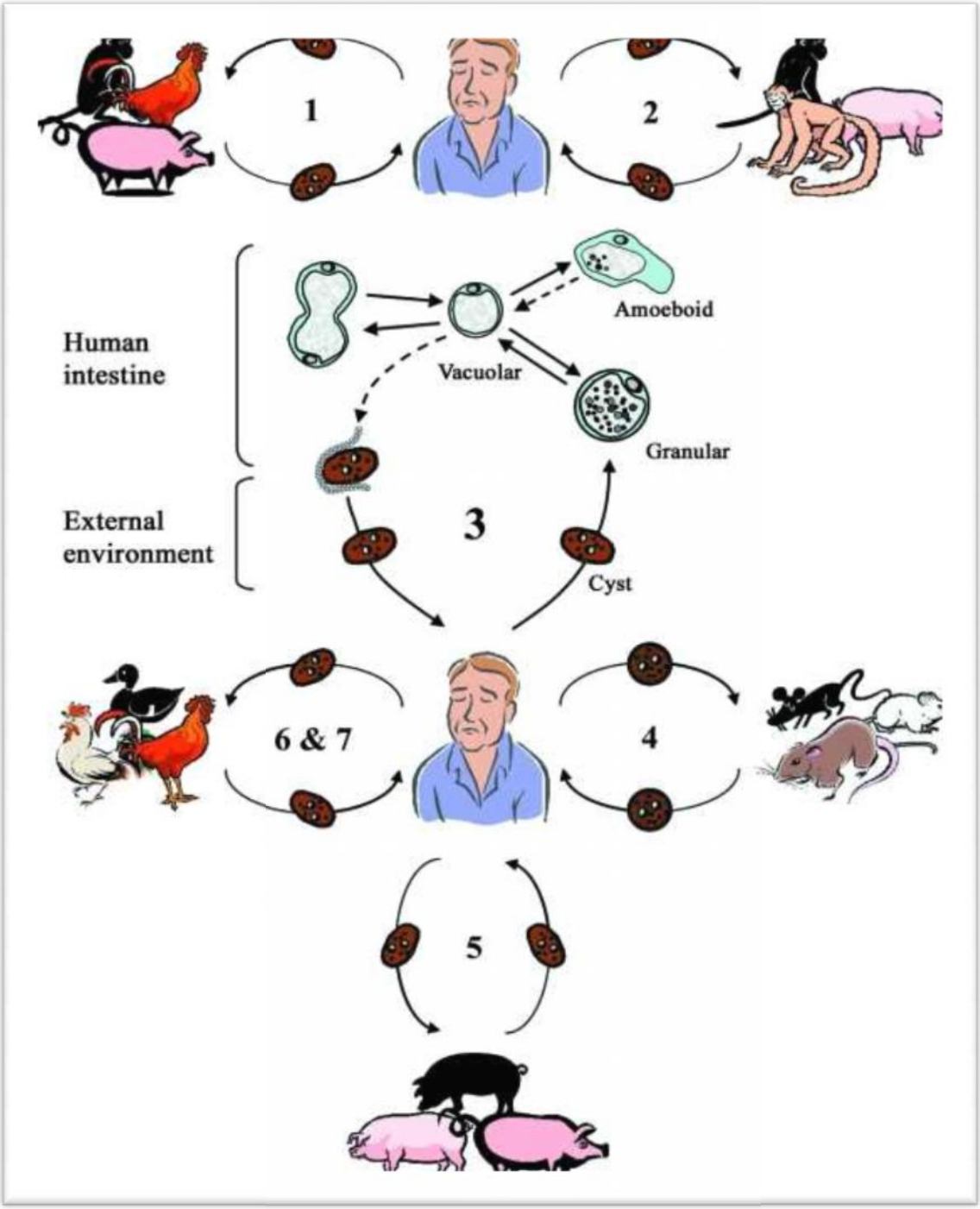

Fig. 11. **A proposed life cycle for *Blastocystis* cells taking into account recent studies suggesting the existence of zoonotic genotypes (subtypes 1 to 7) with various host specificities. Subtype 1 is cross-infective among mammalian and avian isolates; subtypes 2, 3, 4, and 5 comprise primate/pig, 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 (Adapted from Tan 2008).**

Apart from a few studies, the transitions from one of the classically described forms to another are not well understood. TEM (Transmission Electron Microscope) studies of the development of cysts to vacuolar forms were elegantly demonstrated with a human isolate and a rat isolate. In those reports, fecal cysts from both humans and rat develop similarly and dramatically into vacuolar forms within 24 h of inoculation into the growth medium (Chen *et al.* 1999; Moe *et al.* 1999).

The cells undergoing excystation apparently developed from cysts into granular forms before becoming vacuolar in morphology. Whether these granular forms are similar to those from patient samples and laboratory culture is not known. In a separate study, *Blastocystis* cysts enriched from a patient sample were cultured in Jones' medium and characterized by TEM at 24 h (Zaman 1999).

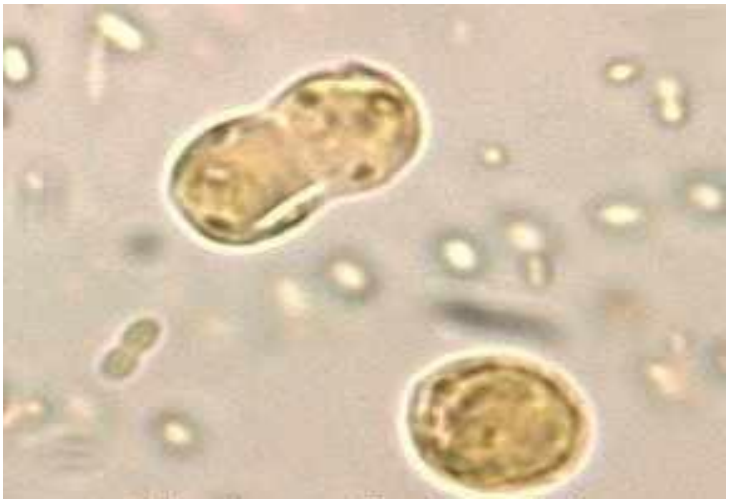

Fig. 12. Binary fission in *B. hominis*

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 (Tan *et al.* 1996).

ZOONOTIC IMPORTANCE OF *B. hominis*:

If, as Noël *et al.* (2005) deem likely based upon their own molecular work and a review of the literature, animal-to-human transmission is possible, then animals such as pigs and dogs could in fact be acting as a large reservoir capable of human infection. In animal, the prevalence of *Blastocystis* appeared to vary from animal to animal. It was also demonstrated that the prevalence of *Blastocystis* in dogs and in cattle differ from country to country (Tan 2004). Tan (2008) proposed that humans are potentially infected by seven or more species of *Blastocystis* and that certain animals represent as reservoirs for transmission to humans (Fig. 11).

Vassalos *et al.* (2008) diagnosed the disease has been in a wide range of animals in addition to being observed in humans, Extensive genetic heterogeneity has been demonstrated. *Blastocystis sp*. subtypes 1 to 9 were recently considered to be of zoonotic origin. While some suggested that *Blastocystis* might play a pathogenic role in intestinal disorders in humans. It might be speculated that in cases of zoonotic genotypes producing amoeboid forms, *Blastocystis sp*. infection might rather be considered a potential re-emerging zoonosis.

REVIEW OF LITERATURE

Prompt and accurate diagnosis of diarrhea is needed for implementation of appropriate treatment to reduce associated morbidity and mortality. Review of literature about diagnosis of *Blastocystis hominis* will enable us to know about many aspects that are relevant to the present study. There are number of literatures relevant to this study. Important of them are summarized below:

Morphology and life cycle of *Blastocystis hominis*

Abou *et al.* (2001) identified different morphological forms of *B. hominis* in human stool samples. Suspected of being the infective stage, cysts were studied in more detail as regards their morphology using both light and electron microscopy. By electron microscope, the cyst wall was evident surrounded by an additional fibrillar coat. The cytoplasm contained one nucleus, many mitochondria, glycogen deposits and a number of variable sized vacuoles. Histochemical studies detected carbohydrates in the cyst wall and fat globules in the cytoplasm. Oral inoculation of albino mice with these cysts led to inflammatory changes in the large and small intestine. The parasite was found at the mucosal epithelium but with no invasion. Different forms of the parasite were detected in the lumen of the intestine.

Qiao *et al.* (2006) studied on the biological characteristic of *B. hominis*: morphology, mode of reproduction and the relation to bacteria. Using the Iodine and Haematoxylin staining, the morphology of *B. hominis* from patients and RPMI 1640 medium were observed. The *B. hominis* positive mucous diarrheal specimens were cultured and identified any possible known pathogenic intestinal bacteria. *B. hominis* and *Colibacillus* were co-cultured to observe the interaction between them. Four modes of reproduction for *B. hominis* were confirmed: binary fission, endodyogeny, multiple fission and budding.

Tan *et al.* (2006a) described the predominance of amoeboid forms of *B. hominis* in isolates from symptomatic patients. Irregular and polymorphic amoeboid forms with multiple extended pseudopodia were observed in all isolates from symptomatic patients.

The amoeboid forms were initially noted on day 2 and the percentages increased from 2% to 28%, with peak percentages from day 3 to day 6. Transmission electron microscopy revealed two types of amoeboid forms; one containing a large central vacuole, and the other which revealed multiple small vacuoles. The amoeboid form could either be an indicator of pathogenicity of *B. hominis*, or the form likely to contribute to pathogenicity and be responsible for the symptoms seen in patients.

Vassalos *et al.* (2010) observed on the differences in clinical significance and morphologic features of *Blastocystis sp.* subtype 3. *Blastocystis* isolates were xenically cultured and subtyped. *Blastocystis sp*ecies subtype 3 was the predominant subtype. Diverse morphologic features, probably reflecting the progression from an asymptomatic to a symptomatic state, were observed in an asymptomatic subtype 3 carrier who later had symptoms. Searching for amoeboid forms might be helpful to presumptively screen symptomatic patients with subtype 3 or to follow up an asymptomatic subtype 3 carrier in case symptoms become evident before antiprotozoal treatment was attempted.

Different techniques for diagnosis of *Blastocystis hominis*

Ponce *et al.* (1991) described the importance of the diagnosis of *B. hominis* in the parasitological examination of feces. Out of 798 samples, 281 were collected after a purgative, and 517 by serial collection. By parasitological analysis, *B. hominis* appeared in 25.2% in the samples of the patients. *B. hominis* was associated with other parasites, appearing as the only parasite in only 29.4% of the cases. Both its statistical association with the patient's age and its independence from sex were determined. The search for this protozoa should be a parasitological routine analysis since it is the cause of frequent intestinal disorders.

Kukoschke *et al.* (1992) analysed the varying incidence of *B. hominis* in cultures from faeces of patients with diarrhoea and from healthy persons. Surprisingly, an increased detection rate was observed in samples from healthy persons after anaerobic cultivation. This increased frequency is obviously not dependent on the kind of serum used as a culture supplement and raises the question whether the protozoa morphologically

described as *B. hominis* represent a homogenous species. When rabbit and horse sera were used instead of human serum for cultivation, in both groups the share of positive cultures increased.

Aksoy *et al.* (2007) proposed a study by which fecal specimens were evaluated with native-lugol, formalin-ethyl-acetate sedimentation and with trichrome staining for protozoa and helminths and with cellophane tape for *Enterobius vermicularis*. Of the study group, 33.4% had one or more parasites. The most common parasite was *B. hominis* (14.6%) followed by *Enterobius vermicularis* (10.1%) and *Giardia intestinalis* (7.8%). When parasitic distribution was evaluated in association with demographic features, a significant relation was found between the income level and parasitic infection prevalence. Multiple parasitic infections were more prevalent in crowded families.

Akdemir *et al*. (2007) conducted a research that was based on microscopic examination of stool specimens. Different intestinal parasites were found in 7.7% of the stool specimens. At least one parasite species was found in 3.11% of women and 4.59% of men. The prevalence of helminths and protozoa were as follows; *Giardia intestinalis* 1.48%, *Entamoeba histolytica/dispar* 0.74%, *Isospora belli* 0.30%, *Iodamoeba butschlii* 1.33%, *B. hominis* 1.04%, *Endolimax nana* 0.44%, *Entamoeba coli* 1.04%, *Taenia saginata* 0.44%, *Trichostrongylus spp*. 0.15%, *Entamoeba coli + Blastocystis hominis* 0.15%, *Giardia intestinalis + Entamoeba coli* 0.15%, *Giardia intestinalis + Blastocystis hominis* 0.44%.

Dogruman *et al.* (2010) compared diagnostic methods for identifying *Blastocystis* in stool samples, and evaluated the frequency of detection of *Blastocystis* in patients with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). From that group of 30 positives, Lugol's stain, trichrome staining, and an immunofluorescence assay identified 11, 15, and 26 samples as positive respectively. Using culture as a standard, the sensitivity of Lugol's stain was 36.7%, trichrome staining was 50%, and the IFA stain was 86.7%. The specificity of Lugol's stain was 91%, trichrome staining was 100%, and the IFA stain was 97.3%. For comparison, trichrome staining alone, the method most frequently used in many countries, would have only identified *Blastocystis* infection in

29% (6/21) of the IBS patients. IFA staining may be a useful alternative to stool culture, especially if stool specimens have been chemically preserved.

Eymael *et al.* (2010) evaluated the effectiveness of different techniques for diagnosing *B.hominis*. These samples were subjected to the techniques of spontaneous sedimentation (HPJ), sedimentation in formalin-ether (Ritchie) and staining by means of Gram and May-Grünwald-Giemsa (MGG). The presence of *Blastocystis hominis* was observed in 40 samples, when staining techniques were used (MGG and Gram), while sedimentation techniques were less efficient (32 positive samples using the Ritchie technique and 20 positive samples using the HPJ technique). This result demonstrate that HPJ was less efficient than the other methods.

Yakoob *et al.* (2010) worked on irritable bowel syndrome-diarrhea (IBS-D) and healthy individual which were associated with genotypes of *B. hominis*. Stool microscopy, culture, and polymerase chain reaction for *B. hominis* genotyping were carried out. The dominant *B. hominis* genotypes were genotype 1 in 87 (65%) and type 3 in 49 (37%). In IBS-D, genotype 1 was present in 75 (86%; $P < 0.001$) compared to 12 (14%) in controls while type 3 was present in 23 (47%) compared to 26 (53%) in controls ($P < 0.001$), respectively. Majority of their patients had typeable *B. hominis* infection. The genotype of *B. hominis* in IBS-D was type 1 while in control genotype 3 was predominant.

Barua *et al.* (2015) worked on establishment of *B. hominis in vitro* culture using fecal samples from infants in slum area of Mirpur, Dhaka, Bangladesh. The aim of the study was to develop a sustainable technique to identify the pathogen. In culture, several morphological forms were observed. Through microscopy, various morphological forms were clearly observed. The higher percentage for all the morphological forms was observed in age group 25-36 months. By direct microscopy and culture from fresh samples, children from 37-48 months showed the highest percentage of infection ($P=0.000$ and $P=0.000$). The sensitivity of direct microscopy (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.

Marawan *et al.* (2015) worked on the distribution of *Blastocystis* subtypes in isolates from Qatar. *Blastocystis* subtypes were determined by sequencing of the small subunit

rRNA gene (SSU rDNA) PCR products. Phylogenetic analyses were done using Maximum Composite Likelihood method. 71.1% of samples were positive for *Blastocystis* infection based on PCR-detection methodology compared to only 6.9% by microscopy. Prevalence of *Blastocystis* did not differ between the sexes nor between age classes. Genetic analysis detected only three STs. ST3 was the most common (69.3%) and ST2 was the rarest (3.5%), while ST1 had a prevalence of 27.2%. ST2 showed a regional variation, being absent from the 64 Western Asian *Blastocystis*-positive subject. Both ST1 and ST3 showed significant differences in prevalence between the sexes.

Molecular diagnosis (PCR) of *Blastocystis hominis*:

Yoshikawa *et al.* (1996) conducted a study on DNA polymorphisms of different strains of *Blastocystis* isolated from humans, a chicken, and a reptile were examined by an arbitrary primer PCR method. Two strains of *B. hominis* isolated from humans in the USA and Japan yielded nearly identical PCR products. However, one strain of *B. hominis* (isolated from a human in Singapore) yielded quite different PCR products. *Blastocystis sp.* isolated from a chicken yielded PCR products similar to those of the former two strains, while *B. lapemi*, isolated from a reptile, shared no bands with any of the other isolates. These results indicate the possibility that the isolate from the chicken was a zoonotic strain, and that there was intraspecific variation of *B. hominis.*

Yoshikawa *et al.* (1998) worked on genomic polymorphisms among nine strains of *B. hominis* were examined by random amplified polymorphic DNA (RAPD) using four different arbitrary polymerase chain reaction (PCR) primers. Based on the RAPD patterns, nine strains were classified into three groups. Specificity of these diagnostic primers was tested against several common intestinal parasites and a yeast, and no amplification was confirmed. Since the current criteria indicates that *Blastocystis* organisms isolated from humans are designated as *B. hominis*, the authors propose to classify several subtypes among *B. hominis* groups based on the difference of genomic DNA using three diagnostic primers.

Yoshikawa *et al.* (2000) studied on genomic analysis of *B. hominis* strains isolated from two long-term health care facilities. 32 *B. hominis* isolates were collected from patients

and/or staff members of two long-term health care facilities (facilities A and B), and these organisms were subjected to genotype analysis based on diagnostic PCR primers and restriction fragment length polymorphism (RFLP) of small subunit rRNA gene (rDNA). Based on PCR amplification using diagnostic primers which were developed from randomly amplified polymorphic DNA analysis of known strains of *B. hominis*, the 32 isolates of *B. hominis* were classified into three different subtypes. These different subtypes were subjected to RFLP analysis, and the RFLP profiles were correlated with the results obtained by diagnostic PCR primers. This study presents the first molecular evidence of possible human-to-human *B. hominis* infection between and/or among two small communities.

Yoshikawa *et al.* (2003) compared specific PCR primers and phylogenetic tree analysis of restriction fragment length polymorphism using the small subunit ribosomal RNA gene in various *Blastocystis* populations obtained from humans and animals. A phylogenetic tree was constructed using 12 restriction enzymes and a sample pool of 22 isolates, including 2 reference strains and Proteromonas lacertae as an outgroup. The analysis showed that the 22 isolates could be separated into 7 clusters. Four of the 7 clusters were mixed groups that comprised isolates from both humans and nonhuman hosts. The other 3 clusters contained isolates from humans or nonhuman hosts only. PCR amplifications using previously described and newly defined specific primers mirrored the clusters obtained by the phylogenetic tree analysis. Their results show that primer PCR can be used as a powerful tool for the typing of *Blastocystis* populations.

Yoshikawa *et al.* (2004) were observed on PCR-based identification of zoonotic isolates of *Blastocystis* from mammals and birds. PCR-based genotype classification using known sequence-tagged site (STS) primers allows identification of zoonotic isolates of animal origin. To this end, 51 isolates from monkeys, cattle, pigs, chickens, quails and pheasants were subjected to genotype analysis using seven kinds of STS primers. These results were combined with previous studies on 41 isolates from animals and compared with the diversity of genotypes of 102 human *B. hominis* isolates, 67.4 % (62/92) of isolates from mammals and birds were identical to human *B. hominis* genotypes.

Stensvold *et al.* (2006) worked on detection of *B. hominis* in unpreserved stool specimens by using PCR. Primers were based on small subunit ribosomal DNA and able

to detect > or =32 parasites/200 mg stool artificially spiked with cultured *B. hominis*. In the evaluation of 43 clinical specimens, the PCR was tested against the formol ethyl acetate concentration technique (FECT) and a culture technique, proving 100% test specificity and a significantly higher sensitivity than the FECT. The PCR method is recommended for screening clinical specimens for *B. hominis* infection and for use in prevalence studies.

Tan *et al.* (2006.b) proposed a study on PCR fingerprinting of *Blastocystis* isolated from symptomatic and asymptomatic human hosts. Genomic DNA from 16 *B. hominis* isolates comprising of eight asymptomatic isolates (A1-A8) and eight symptomatic isolates (S1-S8) was amplified by arbitrarily primed polymerase chain reaction (AP-PCR) using 38 arbitrary 10-mer primers. The results of the phylogenetic analyses showed that all symptomatic isolates (S1-S8) formed a clade with >70% similarity among the isolates and which were clearly separate from asymptomatic isolates A1, A3, A4, A5, A6, and A7. The present study suggests that AP-PCR can be a valuable method for differentiating between isolates of *B. hominis* and their results support the hypothesis that their asymptomatic and symptomatic *B. hominis* isolates may represent two different strains/species with varying pathogenic potential.

Dogruman-Al *et al.* (2008) worked on possible link between subtype 2 and asymptomatic infections of *B. hominis*. The correlation between the genotype and the symptoms was evaluated. PCR subtyping indicated that subtype 3 was the most common genotype in both symptomatic and asymptomatic groups, and the second common genotype was subtypes 1 and 2 in symptomatic and asymptomatic groups, respectively. A significant correlation between subtype 2 and the asymptomatic groups was found among both in pediatric and adult patients ($chi^2cal = 4.38$, $df = 1$, $p = 0.044$).

Jones *et al.* (2009) using a real-time Light Cycler PCR assay, detected *Blastocystis* in nine patients from a metropolitan area of Corvallis, Oregon who presented with diarrhea, abdominal pain, fatigue, joint pain, skin rash and psychiatric co-morbidity. Phylogenetic analysis identified six infections with *Blastocystis sp*. subtype 3, and one with subtype 1, using the standard Stensvold nomenclature. Most patients with subtype 3 had previously tested negative with conventional parasitological diagnostics, had been symptomatic for over 4 years, and reported antibiotic failure.

Eroglu *et al.* (2009) identified *B. hominis* isolates from asymptomatic and symptomatic patients by PCR. Despite years of study, the pathogenic role of *B. hominisis* still controversial. Genotypic differences between the asymptomatic and symptomatic isolates should assist in determining the pathogenicity of *Blastocystis.* In this study, they genotyped 32 *Blastocystis* isolates obtained from 12 asymptomatic healthy individuals and 20 symptomatic patients pain by polymerase chain reaction using known seven kinds of sequence tagged site primers in the study. When they compared genotype of *Blastocystis* isolates between the symptomatic and asymptomatic patient group, they found that subtype 3 is the most dominant genotype in asymptomatic individual (9/12) and subtype1 determined all of symptomatic patients (20/20).

Parkar *et al.* (2010) studied on molecular characterization of *Blastocystis* which were isolate from zoo animals and their animal-keepers. To detect *Blastocystis* by PCR-based methods faeces were collected from various hosts, including non-human primates, Australian native fauna, elephants and giraffes, as well as their keepers from a Western Australian zoo. Overall, 42% and 63% of animals and zoo-keepers sampled from the Western Australian zoo were positive for *Blastocystis*, respectively. This was the first report of *Blastocystis* found in the elephant, giraffe, quokka, southern hairy nosed wombat and western grey kangaroo. These findings indicate that most zoo-keepers at the Perth Zoo were harbouring *Blastocystis*. Four of these zoo-keeper isolates were identical to the isolates from the southern hairy nosed wombat and five primate species.

Santín *et al.* (2011) developed a new PCR protocol to detect and subtype *Blastocystis spp.* from humans and animals. Infection of *Blastocystis spp.* has been reported as asymptomatic, acute symptomatic, and chronic symptomatic. The ~500 bp SSU rDNA gene fragment amplified by this PCR was highly sensitive compared with published primers and contains highly variable regions that allow phylogenetic analysis of *Blastocystis.* These primers were used to detect and subtype *Blastocystis* spp. Specimens from naturally infected humans, primates, cattle, pigs, and chickens. Based on these findings, application of this method can elucidate the complexity of this heterogeneous genus and its role in human and animal disease, as well as its zoonotic potential.

Poirier *et al.* (2011) presented a highly sensitive real-time quantitative PCR (qPCR) assay developed to detect *Blastocystis* parasites in stool samples. Direct-light microscopy

and xenic *in vitro* stool culture analysis showed only 29% and 52% sensitivity, respectively, compared to our qPCR assay. Of the 27 (14.5%) *Blastocystis*-positive patients, 8 (4%) experienced digestive symptoms. No correlation was found between symptomatic patients and immune status, parasite load, or parasite subtypes, although subtyping of all isolates revealed a high (63.0%) prevalence of ST4. Two unexpected avian subtypes were found, i.e., ST6 and ST7, which are frequently isolated in Asia but rarely present in Western countries. New diagnostic tools such as the qPCR are essential for evaluating the clinical relevance of *Blastocystis* subtypes and their role in acute or chronic digestive disorders.

Roberts *et al.* (2011) observed on comparison of microscopy, culture, and conventional polymerase chain reaction for detection of *Blastocystis sp.* in clinical stool samples. Ninety-eight (19%) samples were positive for *Blastocystis* in one or more of the diagnostic techniques. The PCR 2 method was the most sensitive at detecting *Blastocystis* with a sensitivity of 94%, and the least sensitive was microscopy of the permanent stain (48%). Subtype 3 was the most predominant subtype (present in 43% of samples assigned to this group). This study highlights the low sensitivity of microscopy when used as the sole diagnostic modality for detection of *Blastocystis sp.*

Santos *et al.* (2013) proposed a comparison of direct fecal smear microscopy, culture, and PCR for the detection of *Blastocystis sp*. in human stool samples. Culture method detected the highest number of *Blastocystis*-positive stool samples (n=36), followed by PCR of DNA extracted from culture (n=26), PCR of DNA extracted from stool (n=10), and direct fecal smear (n=9). Compared to culture, the sensitivity of the other detection methods were 66.7% for PCR from culture and 19.4% for both PCR from stool and direct fecal smear. Specificity of the methods was high, with PCR from culture and direct fecal smear having 97.3%, while PCR from stool at 95.9%. In this study, *in vitro* culture is the best method for detecting *Blastocystis sp.* in human stool samples.

Bart *et al.* (2013) diagnosed through advanced microscopy on two samples and sequence-confirmed PCR on a third sample from the same individual, were used for *Blastocystis* diagnosis and subtype analyses on routine clinical samples. With a combined gold standard of sequence-confirmed PCR and positive advanced microscopy, 107 out of 442 (24.2%) patients were diagnosed with *Blastocystis*. Infection. The

sensitivity of microscopy and sequence-confirmed PCR was 99.1% (106/107) and 96.3% (103/107), respectively. Among 103 typable samples, subtype 3 was most abundant (n = 43, 42%), followed by subtypes 1 and 2 (both n = 23, 22%), subtype 4 (n = 12, 12%), and single samples with subtypes 6 (1%) and subtype 7 (1%). The prevalence of *Blastocystis* infection was 38% in patients from the Department of Tropical Medicine and 18% in patients from other departments. A high prevalence of *Blastocystis* infection was found with both advanced microscopy and sequence-confirmed PCR in their patient population. Most cases were caused by subtypes ST1, ST2, ST3 and ST4.

Dagci *et al.* (2014) studied on the epidemiological and diagnostic features of *Blastocystis* infection in symptomatic patients in Izmir Province, Turkey. Totally, 617 stool samples of patients with gastrointestinal symptoms were examined. Diagnostic efficacies of microscopy, culture and Real-Time PCR were compared. PCR products were sequenced to identify the subtypes of *Blastocystis* isolates. Totally 94 (15.24%) samples were positive for *Blastocystis* after all methods. Among these, 83 of 94 (88.3%) samples were identified with all methods, while 11 were positive only with Real Time PCR. Diarrhea and abdominal pain were the leading symptoms in the patients. The only pathogenic agent identified in 80.9% patients was *Blastocystis.* Subtype 3 was the leading *Blastocystis* subtype (44.6%). Comparison of three diagnostic methods indicated Real Time PCR as the most sensitive and specific method.

Blastocystis hominis **as a pathogenic organism**

Sheehan *et al.* (1986) observed on the association of *B. hominis* with signs and symptoms of human disease. Purged stools from 389 patients were evaluated microscopically for the presence of *B. hominis*. A total of five or more *B. hominis* cells per 40X field were observed in 43 patients (11%), and *B. hominis* was the only intestinal parasite present in 23 (6%) of these patients. The proportion of eosinophils in the peripheral blood ranged from 4 to 12% in 11 (58%) of the symptomatic patients. Absolute eosinophil counts were greater than 250/microliter in 8 patients and greater than 400/microliter in 5 patients. This study supports the emerging concept of the role of *B. hominis* as an intestinal parasite causative of human disease.

Guirges *et al.* (1987) worked on the evidence for human pathogenicity of *B. hominis* and effectiveness of metronidazole therapy. Clinical symptoms and oral treatment with metronidazole were studied in 103 patients with pure infections by *B. hominis*. The results showed that excessive flatulence is the chief gastrointestinal symptom associated occasionally with diarrhoea and abdominal cramps. All the patients showed good responses with treatment of metronidazole and 74 patients whose stools were reexamined 1-2 months after the treatment demonstrated no signs of infections. It is concluded that *B. hominis* is a pathogenic intestinal parasite and the infection could be eradicated successfully by oral metronidazole.

Qadri *et al.* (1989) observed on the clinical significance of *B. hominis*. A total of 25.6% cases were observed to be in association with other enteric pathogens. Of these patients, 46.4% had symptoms, the most common being abdominal pain (87.9%), constipation (32.2%), diarrhea (23.4%), alternating diarrhea and constipation (14.5%), vomiting (12.5%), and fatigue (10.5%). Forty-three (18%) of the patients were treated with metronidazole (0.5 to 1.0 g/day) because of recurrent symptoms and the presence of large numbers of *B. hominis* cells in repeated stool specimens. After 7 to 10 days of treatment, all patients became asymptomatic with negative stools on follow-up examinations for *B. hominis*.

Tsang *et al.* (1989) observed on the terminal ileitis associated with *B. hominis* infection. They reported on the previously unobserved clinical presentation of terminal ileitis secondary to *B. hominis* in a 37-yr-old white male. When the patient was treated with metronidazole, the symptoms improved and the radiographic abnormalities resolved. They believed that this is the first well-documented instance of terminal ileitis secondary to *B. hominis*.

Bratt *et al.* (1990) investigated for intestinal symptoms of Blastocystosis in two children of one family. Two apparently healthy children from the same family were found to have moderate to heavy *B. hominis* in their stool samples whilst being investigated for intestinal symptoms: sporadic, painless, rectal bleeding in one and persistent diarrhoea in the other. After treatment with metronidazole, they had no further signs, and stool samples became negative. Eighteen months later, both were asymptomatic, and stool samples continued to be negative for the parasite.

Zaki *et al.* (1991) presented a clinical report of *B. hominis* infection in children. During a 9-month hospital-based survey, the intestinal parasite *B. hominis* was detected in high numbers in faecal specimens from 39 (2%) of 1960 children. Abdominal pain or discomfort with or without diarrhoea was present in 32 children categorized as acute, subacute or chronic cases. They included three with other enteropathogens. All symptoms of blastocystosis resolved with eradication of *B. hominis* or reduction to low numbers after metronidazole chemotherapy (28 cases) or with no treatment (four cases). This study would appear to support the role of the parasite as an enteropathogen in some children. A case control study is clearly needed to clarify the status of *B. hominis* as a pathogen.

Telalbasic *et al.* (1991) investigated on *B. hominis* to prove it may be a potential cause of intestinal disease. They presented 12 cases of prolonged or recurrent diarrhea associated with *B. hominis* found in a large number. No other intestinal parasites were recognized. All patients responded to metronidazole. This report confirms that *B. hominis* may be a cause of intestinal disease.

Fleta *et al.* (1993) reported the clinical features of *B. hominis* infection which is associated with abdominal pain in childhood. In 8 of the cases, no other enteropathogens (viruses, bacteria or other parasites) were found, whereas in 2 cases *Giardia lamblia* was also isolated. Nine out of ten of the patients presented with abdominal pain. In three of the cases it appeared as a pseudo-appendicular ailment which led to an appendectomy. Those children who were treated with metronidazole and those who were not treated with antibiotic recovered satisfactorily.

Nimri *et al.* (1994) worked on intestinal colonization of symptomatic and asymptomatic school children with *B. hominis*. Specimens were examined by using wet-mount preparation, formaline-ether concentration, and Sheather's flotation technique. Trichrome and acid-fast stains were done. *B. hominis* was observed in 203 (20.3%) of the specimens examined, and 175 specimens contained this organism in the absence of other pathogenic parasites. Older children had low *B. hominis* infections (6 to 7 years old, 50% infection rate; 8 to 9 years, 27.5%; 10 to 12 years, 9.5%). The most common complaints reported

by 75 children harboring the parasite were a mild recurrent diarrhea, abdominal pain, nausea, anorexia, and fatigue.

Jelinek *et al.* (1997) investigated on the role of *B. hominis* as a possible intestinal pathogen in travellers. *B. hominis* was detected in 14.7% patients with diarrhoea and in 5.7% controls. However, other organisms causing diarrhoea were detected in 26.1% symptomatic patients with *B. hominis*. Symptomatic patients 10.8% had *B. hominis* in the absence of other pathogens in their stool, significantly more than in the asymptomatic group (5.2%; P = 0.005). Irrespective of the development of symptoms, the organism was most frequently acquired during journeys to the Indian subcontinent.

Carbajal *et al.* (1997) evaluated the frequency of *B. hominis* parasitation and ascertained its role as an intestinal pathogen. The study included 2,039 patients, which were classified in three groups: asymptomatic (group A), with suspicion of parasitosis (group B) and with diarrhoea (group C). The frequency of parasitation was superior in adults ($p < 0.0001$), with a slight predominance in the female sex. The rate of asymptomatic carriers was 3.3%. In 21 patients *B. hominis* (group C) was observed in absence of other enteropathogens. Statistical significant association was found between *B. hominis*, in absence of other pathogens and the presence of clinical manifestations ($p < 0.0001$), the most common of which were diarrhoea and abdominal pain.

Moe *et al.* (1997) experiment on *B. hominis* infection in laboratory mice. Young (less than 8 weeks old) immunocompetent BALB/c mice became infected with *B. hominis* after inoculation of fecal cysts orally and of *in vitro* axenic-culture forms intracecally. This study confirmed that the fecal cyst was the form responsible for external transmission and that the mode of transmission was by the fecal-oral route. The infection was self-limiting and the infected BALB/c mice appeared normal except that some of them showed weight loss and lethargy. Both vacuolar and granular forms were found in the cecum, but only cyst forms were observed in the colon. Histological examination of the cecum and colon showed intense inflammatory-cell infiltration, edematous lamina propria, and mucosal sloughing. It is apparent that although *B. hominis* is not invasive, it is capable of causing pathogenesis in BALB/c mice.

Sadek *et al.* (1997) investigated on the intestinal parasites among 1700 male food-handlers in Qualyobia Governorate, with pathogenic parasite *B. hominis*. Clinical examination and stool samples collection in 3 alternative days were performed. The food handlers were divided into symptomatic (700) and symptomatic (1000). *B. hominis* was recovered from stools of 8.5% of symptomatic and 4% of asymptomatic. 2.4% symptomatic and 2% asymptomatic had *B. hominis* significant infection. Significant infection was higher among symptomatic than asymptomatic persons with detectable faecal leucocytes especially eosinophils.

Brites *et al*. (1997) stated that, the role of *B. hominis* as a human pathogen is controversial, although there is some evidence suggesting that it is the agent implicated in causing diarrhea in immunocompromised patients. They reported 6 cases of AIDS patients presenting with diarrhea with no agents identified in their stools except *B. hominis*. In all cases, treatment was followed by complete recovery from symptoms and clearance of *B. hominis* from the patients' stools. In 2 cases, relapse of diarrhea was followed by a positive stool examination for *B. hominis* oocysts, which again disappeared after treatment. These findings provide additional evidence for considering *B. hominis* a potential intestinal pathogen in AIDS patients.

According to Cirioni *et al.* (1999) the association between the presence of several intestinal parasites and gastrointestinal symptoms in diverse patient cohorts. The results showed a high prevalence of parasites in all the risk groups. Immunocompromised status, recent arrival from developing countries and the presence of behavioral aberrations were significantly related to presence of parasites. *B. hominis* showed a significant correlation with gastrointestinal symptoms only when detected in the group including subjects with a severe immunodepression.

Cheng *et al.* (2003) obesrved on hematological effects of *B. hominis* infection in male foreign workers in Taiwan. The prevalence of *B. hominis* was 14.1% in this study. 121 male Thai workers were examined hematologically and screened for stool parasites using the merthiolate-iodine-formaldehyde concentration method. Hematological values were compared in workers with and without a *B. hominis* infection. The workers infected with *B. hominis* had a lower leukocyte count (6.5+/-0.4 X 10(3)/microl) than those who were not (7.4+/-0.2 X 10(3)/microl). This was mainly caused by a reduced neutrophil count

(3.2+/-0.4 vs 4.2+/-0.2 X 10(3)/microl). Hemoglobin (13.9+/-0.3 vs 14.5+/-0.1 g/dl) and hematocrit (41.4+/-0.6 vs 42.9+/-0.2%) were also reduced in *B. hominis*-positive workers.

Barahona *et al.* (2003) proposed a prospective study on symptomatology and associated epidemiological factors of human blastocystosis. A statistical correlation was obtained ($p<0.05$) among symptomatic persons and presence of *B. hominis* (91,9%). The symptomatology associated with the *B. hominis* infection by order of statistical significance ($p<0.05$) was: Abdominal pain (OR=3) 1.47<OR<6.60, abdominal ballooning (OR=2.36) 1.06<OR<5.29, urticaria (OR=3.19) 0.81<OR<12.48. The only risk factor associated with the *B. hominis* infection was the consumption of unboiled water (OR=2.52) 1.01<OR<5.83. *B. hominis* is associated to symptomatic subjects with abdominal pain and ballooning and urticaria.

Baldo *et al*. (2004) performed a small scale survey to know the infection status of intestinal parasite in children. The study stool samples were examined by the formalin-ether concentration method. Among 172 children who gave detail information, the prevalence for *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm was 36.0%, 44.8%, and 7.0% respectively. 47.7% children were examined, found to be harboring parasitic protozoans such as *Entamoeba histolytica*, *Giardia lamblia*, and *B. hominis.* The most prevalent of these protozoans was *B. hominis* with an infection rate of 40.7%. The prevalence of these infections among children living in institutions was relatively high.

Leelayoova *et al.* (2004) observed on the evidence of waterborne transmission of *B. hominis*. Short-term *in vitro* cultivation was used to detect *B. hominis* in stool samples. In that population, *B. hominis* was the parasite most frequently found, and was identified in 36.9% stool specimens. A significant association between *B. hominis* infection and symptoms was identified that might emphasize the role of *B. hominis* as a human pathogen.

Yao *et al.* (2005) worked on experimental infection of mice with *B. hominis*. To seek a better pathway, *B. hominis* were used to infect normal mice through oral and rectum and also were used to infect immunocompromised mice through rectum. The infected

immunocompromised mice showed slow locomotion, lethargy and descended body weight. Some infected mice discharged mucus feces, a few of them died during the experiment. Parasites were found in the whole gastrointestinal tract. Severe edema, hyperemia and congestion were observed in the tissues of jejunum, ileum, cecum and colon. The epithelia of small intestine and colonic mucous membrane showed exfoliation and structural changes in glands. Mice were more susceptible to *B. hominis* infection through rectum than orally.

El-Shazly *et al.* (2005) evaluated, *B. hominis* in stool samples of symptomatic and asymptomatic individuals was as a possible cause of gastro-intestinal troubles. It was found in 10.1% individuals examined from six villages and one city in Talkha Center, Dakahlia Governorate. The highest infection rate was in Manshayt El-Badawy village (25.47%). Age group 10-20 years had higher infection (13.3%). The most common symptoms were diarrhoea (30.4%), abdominal pain (26.1%), flatulence (21.7%). vomiting (13.1%) and fatigue (8.7%). High concentrations of *B. hominis* were found in symptomatic patients than in asymptomatic ones with statistical significant difference (8.2 cells/100 x field versus 3.8 respectively).

Kaya *et al.* (2005) observed on clinical symptoms in cases caused by *Entamoeba coli* and *B. hominis*. The percentages of intestinal symptoms were found to be 67.2% and 79.4% for *E. coli* and *B. hominis*, respectively. As a result of these findings we concluded that intestinal symptoms may be seen frequently if *E. coli* and *B. hominis* are present. In conclusion, *E. coli* and *B. hominis* may be considered to be pathogens, especially when no other agents are present.

Su *et al.* (2007) used to Dot enzyme-linked immunosorbent assay for detection of serum antibody to *B. hominis* in humans. Serum and stool samples were collected from 322 undergraduate students in medical school. Using stool in vitro cultivation as golden standard, 178 cases were found *B. hominis* positive and 144 were negative. Dot-ELISA was used to examine the serum samples with a sensitivity of 92.1% (164/178) and specificity of 97.1% (141/144). This revealed that dot-ELISA can be used for antibody detection against *B. hominis*.

Ertug *et al.* (2007) examined on the effect of *B. hominis* on the growth status of children. *B. hominis* is a protozoan parasite commonly found in the human gastrointestinal tract. Healthy children with positive stool samples for *B. hominis* but negative for other parasites were selected as the case group (n=89). Two controls matched to each case by age and gender were selected by random sampling of children with negative stool samples for any parasite (n=178). The anthropometric measurements and body mass index were significantly lower in the case group than in the control group ($p<0.05$). According to this study there is a correlation between the presence of *B. hominis* and lower anthropometric indexes in children.

Ozçakir *et al.* (2007) worked on characteristics of *B. hominis* infection in a Turkish university hospital. Among the study group, 12.2% infection with *B. hominis* was found. It was mostly detected with *Dientamoeba fragilis*. Among the groups the incidence of *B. hominis* in allergic patients was higher than controls. Among the immunosuppressed patients, *B. hominis* was detected significantly higher in patients who had solid tumours. Concentration technique with trichrome stain was more sensitive than simple smear with Lugol solution for the detection of *B. hominis*.

Kaya *et al.* (2007) observed the pathogenicity of *B. hominis*, a clinical reevaluation. Metronidazole was administered for 2 weeks to the patients infected with *B. hominis*. After 2 weeks of treatment they were called for a follow-up stool examination. The frequency rate of intestinal symptoms was 88.4% in the *B. hominis* infestation. Abdominal pain was the most frequent symptom (76.9%), diarrhea and distention followed at a rate of 50.0% and 32.6%. Intestinal symptoms may be seen frequently together with the presence of *B. hominis.*

Tan (2008) studied on phenotypic and genotypic characterisation of *B. hominis* isolates. These discrepancies may be due to the varying pathogenic potential or virulence of the isolates studied. The present study represents the first to investigate both phenotypic and genotypic characteristics of *B. hominis* obtained from symptomatic and asymptomatic individuals. Symptomatic isolates had a significantly greater size range and lower growth rate in Jones' medium than asymptomatic isolates. The parasite cells of symptomatic isolates exhibited rougher surface topography and greater binding affinity to Canavalia ensiformis (ConA) and Helix pomatia (HPA). The present study also identifies further

phenotypic characteristics, which aided in differentiating the pathogenic forms from the non-pathogenic forms of *B. hominis*. *Blastocystis* subtype 3 was found to be correlated well with the disease.

Elwakil *et al.* (2010) observed on pathogenic potential of *B hominis* in laboratory mice. This study was carried out to clarify the pathogenecity of *B. hominis* infection and to study the proper number of parasites for mice infection. A total of 15 albino mice were orally inoculated with *B. hominis* and divided according to the inoculums, 10(2), 10(5), and 4 x 10(7) *B. hominis* forms/100 microl saline, into three groups consisting of five mice each, GI, GII, GIII, respectively. In addition with group IV (uninfected control) consisting of five mice. All mice were sacrificed 2 weeks post-infection. The results revealed that all mice of GIII and two mice of GII got the infection while all mice of GI showed a completely negative result. Histopathological examination of large intestine on highly infected group (GIII) showed that *B. hominis* infiltrated the lamina propria, the submucosa, and the muscle layers in the form of collection of vacuolar forms.

Zhou *et al.* (2010) worked on to study the interaction of *B. hominis* and immune system. Human *B. hominis* were isolated from diarrhea patients' feces and cultured *in vitro.* The *B. hominis* from living mice were collected and inoculated again to healthy mice. The *B. hominis* showed dose-dependent pathogenicity in the primary inoculation. No pathogenicity was observed in the secondary inoculation. The protozoan existed in the living mice abdominal cavity for more than 6 months and the cyst was the only form. These results showed that encystation enable the parasite to avoid the immune attack in competent host and simultaneously decrease the pathogenicity to host. Intraperitoneal inoculation to laboratory mice is a good method to maintain and propagate *B. hominis*.

Tai *et al.* (2011) proposed a study on six ulcerative colitis patients with refractory symptoms co-infective with *B. hominis* in China. *B. hominis* is an enteric parasite which has long been considered as an innocuous commensal living in the intestinal tract. Their research was to explore the role of *B. hominis* in refractory ulcerative colitis. Their department admitted 122 cases of ulcerative colitis patients. In these patients, there were 73 cases of patients who were responsive to sulfasalazinec, mesalazine in a standard dosage, according to the symptoms change. There was one patient who was detected to

have *B. hominis* infection through stool detection. There were 49 patients with relapse symptoms. In this group, there were six patients who were detected with *B. hominis* infection through stool detection. The six patients of refractory ulcerative colitis were treated with metronidazole for 10-14 days. They almost completely recovered 3 weeks later.

Tamalee *et al.* (2014) conducted a study to update on the pathogenic potential and treatment options for *Blastocystis sp.* They described the chronic nature of some infections and show the role of *Blastocystis* in immunocompromised patients and the relationship between irritable bowel syndrome and *Blastocystis* infection. There have been several studies that have suggested that pathogenicity may be subtype related. Metronidazole is the most widely accepted treatment for *Blastocystis* but several cases of treatment failure and resistance have been described. Other treatment options which have been suggested include paromomycin and trimethroprim-sulfamethoxazole.

Blastocystis hominis **as non-pathogenic organism**

Markell *et al.* (1986) worked to investigate the possibility that *B. hominis* may be associated with diarrhea and may respond to treatment with iodoquinol or metronidazole. Twenty-seven of the 32 persons were later found to have greater than or equal to 1 recognized pathogens-*Entamoeba histolytica, Giardia lamblia or Dientamoeba fragilis* and, after receiving appropriate therapy, became asymptomatic. The *B. hominis* infection, however, was unaffected by therapy. Five persons with only *B. hominis* infection were treated with iodoquinol without effect; these persons fulfilled the medical criteria for irritable bowel syndrome. They believed that when an apparently symptomatic *B. hominis* infection responds to therapy, the improvement probably represents elimination of some other undetected organism causing the infection.

Senay *et al.* (1990) observed the demographic profile of *B. hominis* carriers from Hamilton, Canada, the Regional Parasitology Laboratory records for 1988 were reviewed, and a prospective study on carriers was conducted to clarify the natural history of the infection and ascertain the role of *B. hominis* as an intestinal pathogen. Retrospective analysis revealed that 8% of stool samples harbored *B. hominis*. The

median age of the carriers was 37 years; 55% were female. Prospective analysis of 139 patients showed that most (76%) of 86 in whom *B. hominis* was the sole organism found. There was no correlation between the presence of *B. hominis* and symptoms.

A study on patients with exacerbated inflammatory bowel disease (IBD) to determine the effect of *B. hominis* on their disease was performed by Nagler *et al.* (1993). Bloody bowel movements were common with ulcerative colitis patients and watery diarrhea with Crohn's disease; other findings included abdominal pain, fever, nausea, and vomiting. All patients responded favorably to medical therapy. Three responded to treatment with corticosteroids alone, and one patient improved with bowel rest without medications. Five patients failed to improve on metronidazole; four of them responded to a subsequent course of corticosteroids, whereas the fifth patient became asymptomatic after erythromycin treatment for concomitant *Campylobacter jejuni*. Finally, three patients responded to treatment with metronidazole alone, which is known to eradicate *B. hominis* but may also have a beneficial effect on IBD. Their findings indicate that *B. hominis* is not a significant pathogen in IBD and treatment must be directed toward the underlying illness.

Shlim *et al.* (1995) conducted a case-control study among expatriates and tourists in Kathmandu, Nepal, in which they compared the prevalence of the organism among patients with diarrhea to that among a control group without diarrhea. *B. hominis* was detected in 56 (30%) of 189 patients with diarrhea, compared with 40 (36%) of 112 asymptomatic controls. Patients with diarrhea were significantly more likely to have > or = 10 *B. hominis* organisms per high-power (400x) field than were controls. Despite the high prevalence of the organism among travelers and expatriates in Nepal, the results of this study suggest that *B. hominis* does not cause diarrhea in this population.

Cirioni *et al.* (1999) observed on prevalence and clinical relevance of *B. hominis* in diverse patient cohorts. The results showed a high prevalence of parasites in all the risk groups. Immunocompromised status, recent arrival from developing countries and the presence of behavioural aberrations were significantly related to presence of parasites. *B. hominis* was the parasite most frequently detected in each studied group. *B. hominis* showed a significant correlation with gastrointestinal symptoms only when detected in

the group including subjects with a severe immunodepression. Immunodepression seems to be a factor of primary importance of the pathogenic role of *B. hominis*.

Chen *et al.* (2003) worked on clinical characteristics and endoscopic findings associated with *B. hominis* in healthy adults. The pathogenicity of *B. hominis* could not be demonstrated due to a lack of association with the development of gastrointestinal symptoms or pathologic findings on endoscopic examination. Multivariate analyses revealed that chronic hepatitis B infection was a predisposing condition to the acquisition of *B. hominis* (odd ratio = 2.848, 95% confidence interval = 1.299-6.242, P = 0.009), and concentration of urate was significantly lower in *B. hominis*-positive individuals (mean +/- SD = 361.64 +/- 87.44 versus 392.57 +/- 93.38 micromol/L; P = 0.009). Among the 64 individuals who underwent gastric biopsy, *Helicobacter pylori* was found more frequently in the individuals harboring *B. hominis*.

Leder *et al.* (2005) examined the association between *Blastocystis* and gastrointestinal symptoms in immunocompetent individuals. They monitored over 2800 healthy people for a period of 15 months, and took stool specimens during both asymptomatic periods and during periods of gastrointestinal symptoms. After exclusion of individuals who had simultaneous identification of other fecal pathogens, they compared the proportions of asymptomatic versus symptomatic individuals positive for *Blastocystis* and found no significant difference (P=0.5). Symptom status did not correlate with parasite abundance. The author found that some individuals were likely to have *Blastocystis* detected during both asymptomatic and symptomatic periods, possibly suggesting carriage of the organism. They found no correlation between clinical symptoms and the presence or absence of *Blastocystis* among this healthy cohort.

Kuo *et al.* (2008) determined the clinical significance of *Blastocystis hominis* in northern Taiwan. A total of 100 patients who had a positive *B. hominis* stool examination were retrospectively identified. Fifty nine adult patients had more than one underlying diseases, including malignancies. Twenty one patients presented with fever and 10 patients had gastrointestinal symptoms, including diarrhea and/or abdominal pain. Six patients had hyper eosinophilia that could not be attributed to other causes. Among 34 patients who had a further stool examination within one year, *B. hominis* was undetectable in 31 patients (91.2%), despite their having no specific antiprotozoal

treatment. The association of clinical symptoms and *B. hominis* could not be delineated from their study, even in immunocompromised patients. All of the patients improved without receiving any specific therapy.

Epidemiology of *Blastocystis hominis*:

An association between epidemiology and clinical features with *B. hominis* infection was observed by Kain *et al.* (1987). Of patients infected with *B. hominis*, 57.5% had recently travelled to the tropics or had consumed untreated water as compared to 12.2% of controls (p less than 0.001). Forty *B.hominis*-positive patients were assessed on more than one occasion. No significant differences appeared to exist in the clinical responses of those treated with Metronidazole (14/18; 77.8%) or with dietary management (6/6; 100%) as compared with those not receiving treatment (13/16:81.2%). Patients tended to become less symptomatic with time and in the absence of specific treatment, and therefore treatment with Metronidazole may not be warranted in light of the natural history of *B. hominis* infection.

Tirado *et al.* (1990) observed the frequency of infection *B. hominis* infection over a year of study. Since July 1987 to June 1988, out of a total of 2,009 stool examinations performed at a private laboratory, in Barquisimeto, Venezuela, we could identify *B. hominis* in 206 of them, using the methods of wet preparation with S.S.F., Iugol and Quensel. Clinical information was obtained in 73 patients. The group more affected was the one older than 12 years of age. They suggested the investigation and to report *B. hominis* in the feces test, because in presence of clinic manifestations and absence of other enteropathogenic can be the responsible.

O'Gorman *et al.* (1993) reviewed on the prevalence and characteristics of *B. hominis* infection in children. They sought to determine the prevalence of *Blastocystis* in stool and to characterize clinical features of infection with *Blastocystis* in children. Forty-six (3%) of 1,736 patients undergoing fecal microscopy at Children's Hospital of Pittsburgh between January 1, 1985, and December 31, 1988, harbored *Blastocystis*. Of these 75% had exposure to well water or had been in developing countries, 85% experienced gastrointestinal symptoms, such as abdominal pain, diarrhea, vomiting, and weight loss. *Blastocystis* was the only parasite found in 35 of those 39 symptomatic children.

Symptoms resolved within one month in 90% of patients receiving antiparasitic pharmacotherapy, but in only 58% ($P < .04$) of those receiving no therapy. They concluded that children infected with *Blastocystis* often experience gastrointestinal symptoms and that treatment increases the rate of symptomatic improvement. They speculated that *Blastocystis* is a human pathogen.

Logar *et al.* (1994) investigate on incidence of *B. hominis* in patients with diarrhoea. They studied the occurrence of the parasite *B. hominis* in 1066 stool specimens from patients with diarrhoea, and investigated the relationship between the presence of *B. hominis* in the faeces and the age of patients. The parasite was recovered from 3.7% samples, but as the sole species of micro-organism in the stool it was recovered from 1% samples. There was no statistically significant difference in the number of *B. hominis*-positive stools between the younger and the older patients ($P < 0.25$), yet in the latter, *B. hominis* was more frequently identified as the only species of micro-organism as compared with the younger group ($P < 0.005$). The presence of *B. hominis* in faecal samples of patients with diarrhoea harbouring no other intestinal pathogens suggests an aetiology that should receive more attention in Slovenia.

Devera *et al.* (1997) performed a prospective study to determine the prevalence of *B. hominis* infection in schoolchildren from Bolivar City. Results showed that *B. hominis* had a prevalence of 16.8%. They did not find a statistically significant association between sex ($P > 0.05$) or age and infection with *B. hominis* ($x2 = 1.94$ g.l = 4). In 52.0% school children they identified other parasites along with *B. hominis*, the most frequent was *Trichuris trichiura* as helminth and *Giardia lamblia* as protozoan. They observed *B. hominis* alone in 48.0% cases. There was a spectrum of clinical symptoms in 54.7% of all children evaluated. Diarrhea was the most frequently clinical manifestation observed.

Devera *et al.* (1998a), tried to evaluate the prevalence of *B. hominis* and its clinical relevance, 169 preschool children from the 'Los Coquitos' nursery school living in Bolivar City, Venezuela. Stool samples were obtained and examined by direct microscopic examination, and the Faust and Willis concentration techniques. Some 72 of the children had intestinal parasites, of whom 29.09% had *B. hominis*. Prevalence for the latter was 18.93 % +/- 5.93 %. No differences were observed by sex or age (X^2) =1.84 DF= 3; p > 0. 05). In the majority (53.13%) of the children, *B. hominis* was the only

parasite. *Giardia lamblia* was the parasite most frequently identified with *B. hominis* (39.13%). In 1994, in 12% of the cases more than five microorganisms per microscopic field were observed. Clinical manifestations were observed in 70.58% of the preschool children. Presence of parasites was not correlated with symptomatology, but only with severity. Proper clinical and parasitological response to treatment was observed in 80% and 90% of patients, respectively. The conclusion was that *B. hominis* is a relatively frequent intestinal parasite among the preschool children evaluated.

Giacometti *et al.* (1999) observed on irritable bowel syndrome in patients with *B. hominis* infection. The prevalence of *B. hominis* in stool specimens of individuals with gastrointestinal symptoms was evaluated to study a possible link between the protozoan and the irritable bowel syndrome. According to the Rome diagnostic criteria, 388 patients were evaluated. Altogether, 81 patients were classified as affected by irritable bowel syndrome. *B. hominis* was recovered from the stools of 38 subjects, 15 of whom belonged to the group with irritable bowel syndrome (P = 0.006). In addition, patients with irritable bowel syndrome were significantly more likely to have five or more *B. hominis* organisms per field (P = 0.031). The author found that there was a set of patients with irritable bowel syndrome in whom the presence of *B. hominis* may not be incidental.

Wang *et al.* (2002) studied on epidemiological survey of *B. hominis* in Huainan City, Anhui Province, China. *B. hominis* in fresh stools taken from 100 infants, 100 pupils, 100 middle school students and 403 patients with diarrhea was smeared and detected with method of iodine staining and hematoxylin staining. The cellular immune function of the patients with blastocystosis was detected with biotin-streptavidin (BSA). The positive rates of *B. hominis* in fresh stools taken from the infants, pupils, middle school students and the patients with diarrhea, were 1.0 % (1/100), 1.0 % (1/100), 0 % (0/100) and 5.96 % (24/403) respectively. Furthermore, the positive rates of *B. hominis* in the stool samples taken from the patients with mild diarrhea, intermediate diarrhea, severe diarrhea and obstinate diarrhea were 6.03 % (14/232), 2.25 % (2/89), 0 % (0/17) and 12.31 % (8/65) respectively. The prevalence of *B. hominis* as an enteric pathogen in human seems not to be associated with gender and living environment, and that *B. hominis* is more common in stool samples of the patients with diarrhea, especially with chronic diarrhea or obstinate diarrhea.

Giacometti *et al*. (2003) evaluated the prevalence of intestinal protozoans and helminths in stool samples of individuals with allergic cutaneous symptoms to study a possible link between parasites and allergy. Altogether, 218 patients who had chronic urticaria, atopic dermatitis, or pruritus of unknown origin were included in the study. Standard laboratory tests for the detection of allergic etiology were performed for all patients. The presence of intestinal parasites was investigated using microscopy, immunofluorescence, and immunoenzymatic assays. Overall, protozoans and helminths were recovered from the stools of 48 subjects (P = 0.004), 18 of whom were affected with intestinal symptoms (P = 0.023). The presence of *Giardia lamblia* in the stools was significantly associated with allergic cutaneous manifestations (P = 0.030). In addition, patients with allergy were significantly more likely to have > or = 5 *B. hominis* organisms per field (P = 0.046). There was a set of patients with allergic cutaneous diseases in whom the presence of intestinal parasites may not be incidental.

Graczyk *et al.* (2005) had worked to determine the prevalence of endoparasites and their association with diarrhea, a survey was conducted in the Southern Province of Zambia that used conventional and molecular techniques applied to stool and urine samples from school-age children (n = 93). Almost half of the stools (49.5%) were diarrhetic. The overall prevalence of *Endolimax nana*, *Schistosoma haematobium*, *Blastocystis hominis*, *Giardia lamblia*, *Cryptosporidium parvum*, *Encephalitozoon intestinalis*, and *Strongyloides stercoralis* was 64.3, 59.1, 53.8, 19.4, 8.6, 8.6, and 1.1%, respectively. Only the associations between infection with *B. hominis* and *E. nana* with diarrhea were statistically significant. Although *B. hominis* and *E. nana* are considered to be nonpathogenic organisms, this study demonstrated that they can be associated with diarrhea in children when they occur at high prevalence and intensity. This survey supports the recent evidence that *B. hominis* and *E. nana* infections are associated with deficient sanitation and low hygiene standards and can contribute to diarrhea in children in developing countries.

Luca *et al.* (2005) worked on epidemiologic and laboratory assessments of etiologic implications of *B. hominis* in gastrointestinal diseases. Authors presented the first laboratory and epidemiological results which reveal the circulation in a population of protozoan *B. hominis* and its implication in the determinations of some gastrointestinal troubles, with fever, diarrhea and constipation, intense intestinal meteorism, associated

with abdominal pain and cramps. Out of the 3106 investigated patients, 9.7% presented *B. hominis* as a unique etiologic agent, with an increased prevalence in adults (74.3%) and women (65.3%). *Blastocystis* infection with clinical manifestations or its asymptomatic form is included among emergent diseases.

A parasitological survey was carried out by Aguiar *et al.* (2007) among Terena Indians living in the Tereré settlement in the municipality of Sidrolândia, State of Mato Grosso do Sul, Brazil. Single samples of feces from 313 Indians were processed by means of the spontaneous sedimentation method. In the population studied, 73.5% were infected with at least one intestinal parasite or commensal. *B.* (40.9%), *Entamoeba coli* (33.2%) and *E. histolytica/E. dispar* (31.6%) were the most common. Bivariate analysis showed that females were generally more infected and presented higher rates of infection by *E. histolytica/E. dispar* and *Entamoeba coli.* Males were more infected by hookworms and *Strongyloides stercoralis* than females. The precarious sanitary conditions of the Tereré settlement are probably a contributory factor towards the high prevalence of intestinal protozoa.

Ozyurt *et al.* (2008) worked on molecular epidemiology of *Blastocystis* infections in Turkey. In this study 87 isolates from 69 symptomatic and 18 asymptomatic individuals were sequenced. Sequence data were phylogenetically analyzed and statistically tested against unmodifiable risk factors such as gender and age. *Blastocystis*-positive males were complaining mainly of gastroenteritis, whereas dyspepsia was the chief complaint among *Blastocystis*-positive females. *Blastocystis sp*. subtypes detected in the study included subtypes 1, 2, 3 and 4, subtype 3 being the most predominant (75.9%). No association was detected between *Blastocystis sp*. subtype and symptoms ($p>0.365$), or between infection intensity and symptoms ($p>0.441$). There was a tendency of subtype 2 isolates being more common among older study individuals, and subtype 2 isolates were significantly associated with higher parasite abundance ($p=0.017$). Compared to data from similar studies, the distribution of *Blastocystis sp*. isolates in Turkey was found to more or less reflect the one seen in other countries, and it was deduced that subtype 3 is generally by far the most common subtype infecting humans, followed by subtypes 1, 2 and 4.

Su *et al.* (2009) collected data on the prevalence of *B. hominis* infection in long-term care facilities in Taiwan. This study included 713 subjects (552 residents and 161 care workers) from ten long-term care facilities in Taiwan who completed stool microscopic examinations with Merthiolate-iodine-formalin stain technique. The prevalence rate of blastocystosis was the highest among foreign and domestic care workers followed by residents (12.2%, 4.6%, and 2.7%, respectively). Older age (p=0.04) and lower educational level (p = 0.008) were significantly associated with blastocystosis among care workers. Among residents, *B. hominis* infection was negatively associated with prolonged use of antibiotics within 3 months prior to examination (p = 0.05) and positively associated with tracheostomy in-place (p = 0.028). *B. hominis* infection was the most prevalent intestinal parasitic infection among both care workers and residents of long-term care facilities in Taiwan. Use of antibiotics was negatively associated with *B. hominis* infection among residents.

A case-control study was conducted to investigate the epidemiology and clinical features of *B. hominis* among Iranian patients with and without GI symptoms by Rostami *et al.* (2010). Six hundred and seventy patients with GI and Six hundred and seventy patients without GI symptoms were enrolled as cases and controls respectively during 2006-07. Standard microscopic examinations following in vitro culture were used to examine the stool samples for presence of trophozoites and cysts of *B. hominis.* Infection with *B. hominis* occurred most commonly in those with GI symptoms (5.67 %) compared with those patients without GI symptoms (3.43 %). The most common symptom in case group was abdominal pain (86.84 %). *B. hominis* was mostly found with *Giardia lamblia* in case group and with *Entamoeba coli* in control group. In addition, there was no significant relation between the presence of GI symptoms and the incidence of *B. hominis.* It has long been described as a non-pathogenic protozoan parasite until recently, when claims have been made that it could result in pathogenic conditions. Thus, to confirm the complication is needed to additional study especially on molecular pathogenesis of this organism.

Yakoob *et al.* (2010) suggested a possible role for *B. hominis* and *Dientamoeba fragilis* in the etiology of irritable bowel syndrome (IBS). They studied the prevalence of *B.*

hominis and *D. fragilis* in patients with IBS-diarrhea (IBS-D). Stool microscopy, culture, and polymerase chain reaction (PCR) for *B. hominis* and *D. fragilis* were done. *B. hominis* was positive by stool microscopy in 49% (83/171) of IBS compared to 24% (27/159) in control ($p < 0.001$). *B. hominis* culture was positive in 53% (90/171) in IBS compared to 16% (25/159) in control ($p < 0.001$). *B. hominis* PCR was positive in 44% (75/171) in IBS compared to 21% (33/159) in control ($p < 0.001$). *D. fragilis* microscopy was positive in 3.5% (6/171) in IBS-D compared to 0.6% (1/159) in control ($p = 0.123$). *D. fragilis* culture was positive in 4% (7/171) in IBS compared to 1.3% (2/159) in control ($p = 0.176$). *D. fragilis* PCR was positive in 4% (6/171) in IBS-D compared to 0% (0/159) in control ($p = 0.030$). *B. hominis* is common, while *D. fragilis* was less prevalent in our patients with IBS-D. *B. hominis* and *D. fragilis* culture had a better yield compared to stool microscopy and PCR.

Inceboz *et al*. (2011) was carried out an investigation of *B. hominis* prevalence in 17756 patients with gastrointestinal system complaints. One or more parasites were detected in 1510 (8.50%) of the patients. The distribution of the intestinal parasites was as follows: *B. hominis* 778 (4.38%), nonpathogenic amoebas 343 (1.93%), *G. intestinalis* 205 (1,15%), *E. vermicularis* 46 (0.25%), *E. histolytica/E. dispar* 34 (0.19%), and other rare parasites 104 (0.58%). The most frequently seen parasite was *B. hominis* in fecal samples of patients with gastrointestinal complaints in their study. Distribution of 778 patients with *B. hominis* due to parasite forms was determined as: vacuolar in 525 (67.49%), granular in 115 (14.78%), both vacuolar and granular in 138 (17.73%) cases. As *B. hominis* was the most frequently seen parasite in patients with gastrointestinal complaints, they suggested that the parasite should be considered as pathogenic and sufficient attention must be paid in routine stool examinations.

Treatment of diarrhea associated with *Blastocystis hominis*:

Ok *et al.* (1999) observed the effect of trimethoprim-sulfamethaxazole in *Blastocystis hominis* infection. Fifty-three symptomatic patients (38 children and 15 adults) with two consequent stool samples positive for abundant *B. hominis* (five or more organisms per x400 field) and negative for other parasitic and bacterial pathogens were treated with TMP-SMX for 7 days, children 6 mg/kg TMP, 30 mg/kg SMX, and adults 320 mg TMP,

1600 mg SMX, daily. Clinical symptoms disappeared in 39 (73.6%), decreased in 10 (18.9%), and no change was observed in one (1.9%) patient, whereas symptoms persisted in all three (5.7%) patients in whom *B. hominis* could not be eradicated. Mean number of stools per day was significantly decreased from 4.3 to 1.2 in the 33 children ($p < 0.001$), and decreased from 3.5 to 1.0 in the four adults ($p = 0.06$) with diarrhea. These results suggested that *B. hominis* may be pathogenic, especially when it is present in large numbers, and TMP-SMX is highly effective against this organism.

A study was done by Diaz *et al*. (2003), to evaluate the efficacy and the tolerance of nitazoxanide in children as a single broad-spectrum antiparasitic agent in the treatment of mixed parasite infections with both intestinal protozoa and helminths. Parasitic infections were confirmed by three stool examinations using direct smear, Ferreira concentration, and cold acid-fast Kinyoun staining methods. One hundred twenty-one (44%) children tested positive for protozoa such as *Giardia lamblia* (18%), *Entamoeba histolytica/E. dispar* (10%), *B. hominis* (7%), *Cryptosporidium parvum* (4%), and *Cyclospora cayetanensis* (3%), and helminths such as *Hymenolepis nana* (10%), *Trichuris trichiura* (6%), and *Ascaris lumbricoides* (6%). After a complete physical examination was performed, 121 patients received treatment with nitazoxanide. Overall, 84% of the protozoa and 95% of the helminths were completely eliminated from the patients. Nitazoxanide was very well tolerated, with no serious adverse effects reported.

Moghaddam *et al.* (2005) evaluated the effects of metronidazole and Trimethoprim/Sulfamethoxazole (TMP/SMX) on persons infected with *B. hominis*. All individuals were non-immunocompromised and subjects were monitored for 1 year after treatment. All stool samples were microscopically examined after staining with iodine and by culturing in an egg slant medium. Of the 104 infected individuals (52+/-16 years of age, M:F=60:44) with *B. hominis* infection, 28 were discharging large numbers of parasites before treatment. Of 28 severely infected individuals, 12 were treated with metronidazole/250-750 mg at a regimen of 3 x/day/10 days and 4 of the 12 were eradicated. Nine individuals were treated with TMP/SMX/1 tab at a regimen of 3 x/day/10 days and 2 of the 9 were eradicated. For severe *B. hominis* infections, it appears that metronidazole and TMP/SMX are effective in some individuals, but not all.

Ertuğ *et al.* (2009) observed on the effect of trimethoprim-sulfamethoxazole (TMP-SMX) in *B. hominis* infection. A total of 37 patients were selected, whose stool specimens were sent to the parasitology laboratory. Only five or more *B. hominis* were found during examination with direct wet mount using the 40x objective. The cases with blastocystosis were given TMP-SMX for 7 days. After the treatment, the cases were questioned as to symptoms once again, the stool specimen examinations were repeated with the same methods, and the results were evaluated. In 34 (91.89%) out of the 37 cases where *B. hominis* was found, various clinical symptoms such as stomach ache, flatulence, diarrhea, itching and fever were observed singly and/or together. After the treatment it was found that 36 (97.3%) out of 37 cases improved. This study supports the premise that TMP-SXT is effective in the treatment of *B. hominis*.

Dinleyici *et al.* (2011) worked on clinical efficacy of *Saccharomyces boulardii or* metronidazole in symptomatic children with *B. hominis* infection. This randomized single-blinded clinical trial included children presenting with gastrointestinal symptoms more than 2 weeks and confirmed *B. hominis* by stool examination. The primary end points were clinical evaluation and result of microscopic stool examination at day 15. Secondary end points were the same end points at day 30. Randomization was performed by alternating inclusion: group A, *S. boulardii* (250 mg twice a day, Reflor®) during 10 days; group B, metronidazole (30 mg/kg twice daily) for 10 days; group C, no treatment. There was no statistically significant difference between the three study groups for age, gender, and the presence of diarrhea and abdominal pain. At the end of the first month after inclusion, clinical cure rate was 94.4% in group A and 73.3% in group B ($p = 0.11$). Metronidazole or *S. boulardii* has potential beneficial effects in *B. hominis* infection (symptoms, presence of parasites). These findings challenge the actual guidelines.

MATERIALS AND METHODS

STUDY AREA

The present study was carried out in slum area of Mirpur and Mohammadpur, Dhaka. These areas were selected as the living conditions are unhygienic and the impoverished. Children are mostly affected by diarrheal disease than the others in these areas (Fig. 1 and Fig. 2). All the laboratory works were done in the Parasitology Laboratory of icddr,b, Mohakhali, Dhaka-1000, Bangladesh.

STUDY PERIOD

The study was conducted from 1st January, 2012 to 31th December, 2013 (2 years).

STUDY POPULATION

The study population was infants aged from 1 year to 6 years, enlisted during the study period.

SAMPLES FROM MIRPUR AREA (FIRST STUDY AREA)

In the present study, 9322 multiple stool samples were collected from 406 children of Mirpur slum area, Dhaka, to detect the presence of *Blastocystis hominis.* Among them, 8601 samples were observed as Monthly stool (MS) samples and 721 samples were observed as Diarrheal stool (DS) samples.

SAMPLES FROM MOHAMMADPUR AREA (SECOND STUDY AREA)

During the study period, total 3218 multiple stool samples were collected from 118 children of Mohammadpur area, Dhaka, to detect the presence of *Blastocystis hominis.* Out of 3218 samples, 2704 samples were observed as Monthly stool (MS) samples and remaining 514 samples were observed as Diarrheal stool (DS) samples.

AGE GROUP OF CHILDREN

For both areas, these infants were divided into the five (5) groups: 13-24 months; 25-36 months; 37- 48 months, 49-60 months and 61-72 months.

RESEARCH INSTRUMENTS

Laboratory facility of Parasitology Laboratory, icddr,b was utilized in this study.

SAMPLE COLLECTION TECHNIQUES

Fresh stool specimens were collected in a physically and chemically clean dry container. The following procedure was maintained while collecting the stool samples:

- The stool samples were collected in a clean, leak proof, transparent container.
- No antiseptic was used.
- Stool samples were not contaminated with urine.
- No de-worming medication was given before taking these samples.
- Identification number labels were put on the container (subject's number and date of collection).
- The sample containers were stored at the freezer at -80 degree C.
- In the laboratory, about180-220 mg of frozen stool samples (thawed at room temperature) was transferred to a 1.5 ml microcentrifuge tubes for DNA extraction.

PROCESSING OF STOOL SAMPLES

Examination of specimen was performed as soon as possible (within one hour of collection). The methods were used which are as follows:

1. Microscopy
2. Xenic culture
3. PCR (Polymerase Chain Reaction) method

Fig. 1. Map of Dhaka city showing Mirpur (First study area) area.

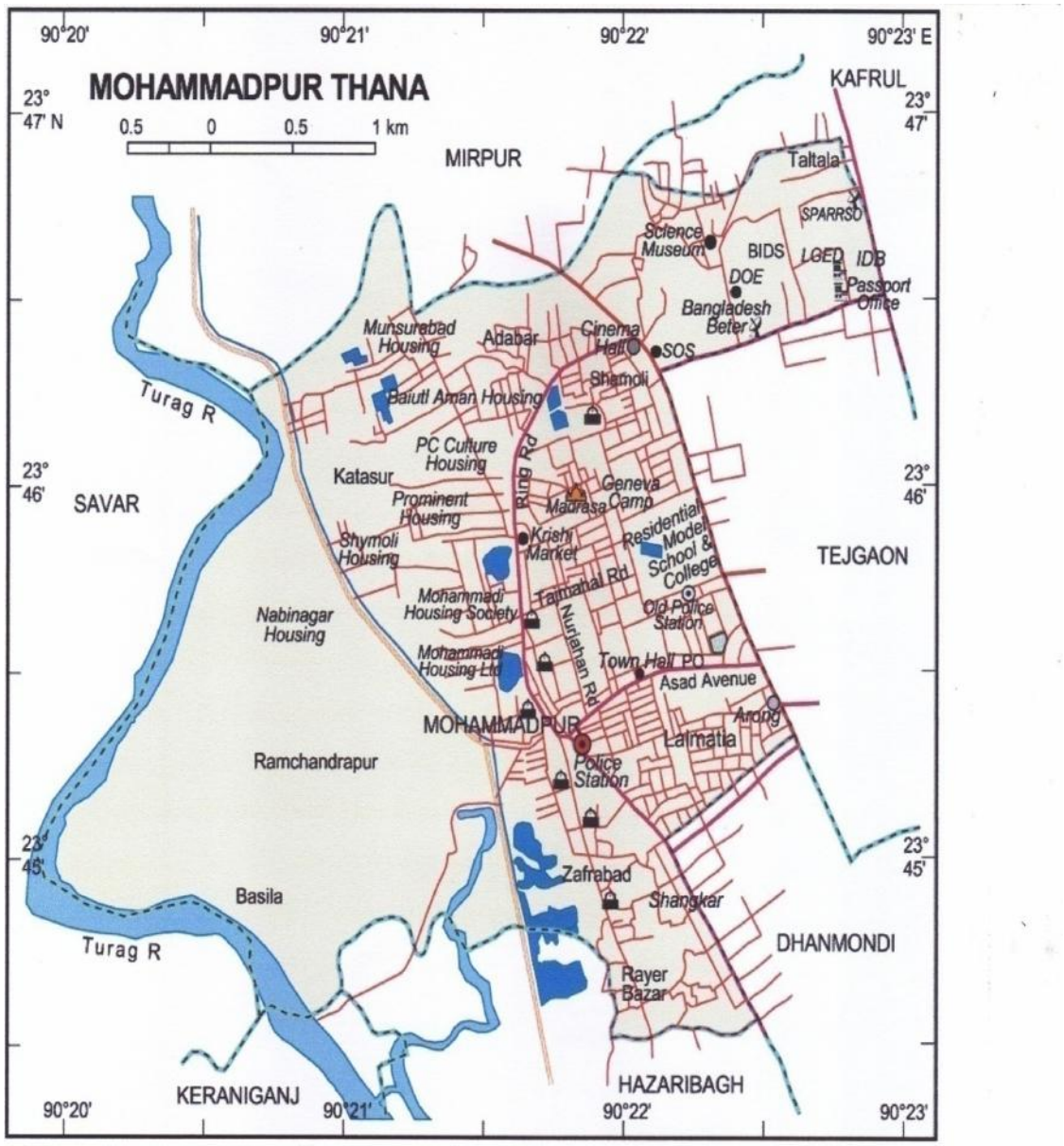

Fig. 2. Map of Dhaka city showing Mohammadpur (Second study area) area.

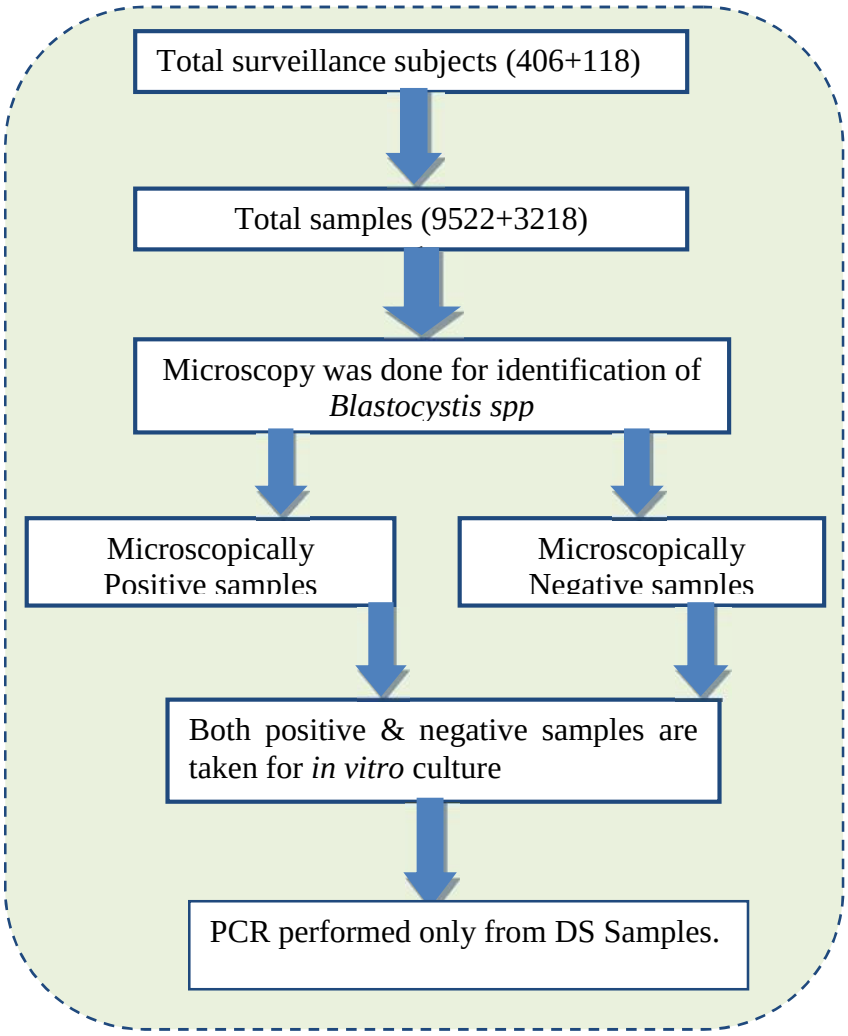

Fig. 3. Flow chart of sample selection.

1. MICROSCOPY

A. Materials for direct microscopy:

Following instruments were needed for direct microscopy:

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

B. Procedure of microscopic examination:

Microscopic examination was done by Olympus light microscope. In microscopic examination, liquid stool were taken on slide and examined whether it is positive or not. In case of solid stool, stool were diluted into normal saline and then taken on slide and examined whether it is positive or not. Microscopic examination must be performed on unpreserved specimens and must provide information on the age, consistency of stool and abnormality. The physical characteristics of the fresh stool specimen may aid to determine which type of parasite may be present. Fecal specimen are described as formed, semi formed, soft loose or watery.

2. XENIC CULTURE

A. Materials for bottle preparation:

- One Litre of distilled water
- 7g Nacl
- 14g Bacto-agar

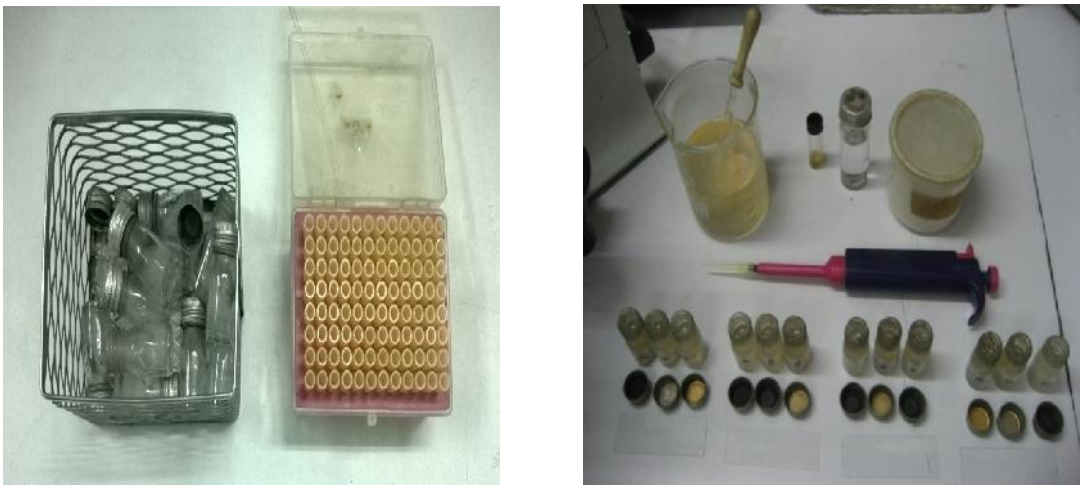

Fig. 4. Materials and media for Xenic culture of *Blastocystis hominis.*

B. Materials for medium preparation:

- Saline agar slopes
- Erythromycin
- DIFCO Bacto peptone
- Rice starch
- Pthalate solution
- Bovine serum
- Defined medium B
- BR Basal amoebic medium
- BRS complete amoebic medium

C. Procedure of Xenic culture:

1. 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.
2. Approximately 50mg of feces were placed, capped and incubated for 24 h at 37^{o} C.
3. Aspirated and discarded leaving only starch and feces
4. Overlay was replaced with sufficient BRS, diluted 1:4 with the phthalate solution to cover slope.
5. 0.06ml erythromycin solution, 0.06 ml Bacto-peptone solution and additional starch were added.
6. After the incubation of 24 hours, the drop of feces and starch was removed from the bottom of the slope.
7. It was examined under the optical microscope for the presence of the organism.

Fig. 5. Culture bottle for incubation of *B. hominis* at 37^{o} C for 48 h in incubator.

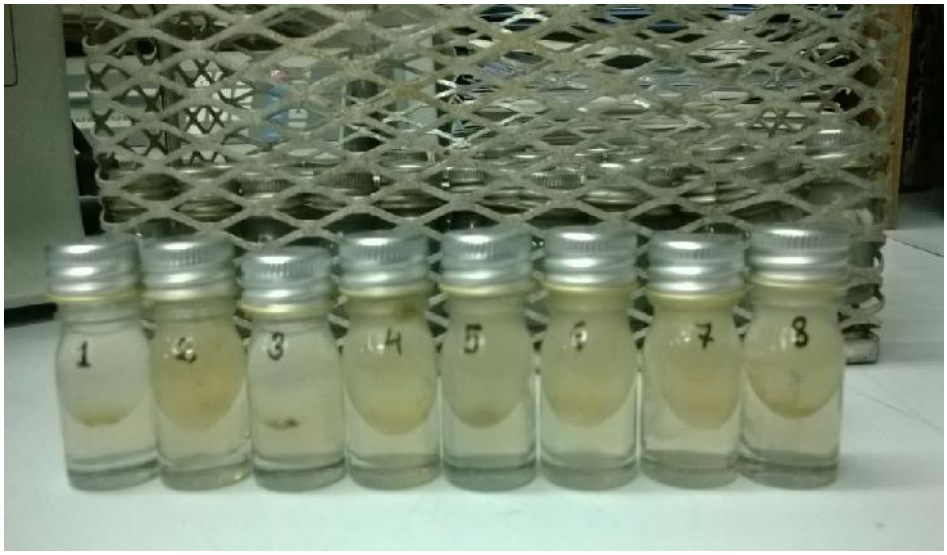

Fig. 6. Culture growth on agar slope.

D. Observation on different forms

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.

3. DNA extraction by QIAGEN method

The simple QIAamp spin procedure yields pure DNA ready for direct use in less than 1 hour. Purification requires no phenol-chloroform extraction or alcohol precipitation. DNA is eluted in low-salt buffer and is free of protein, nuclease, and other impurities or inhibitors. The purified DNA is ready for use in PCR and other enzymatic reaction, or can be stored at -20°C for later use. Stool samples typically contain many compounds that can degrade DNA and inhibit downstream enzymatic reactions. Inhibitex efficiently adsorbs these substances early in the purification process so that they can easily be removed by a quick centrifugation step. In addition, the kit contains buffer ASL, which is specially developed to remove inhibitory substances from stool samples.

A. Materials for the extraction of DNA by QIAGEN method

Materials that were provided by the QIAamp® DNA Stool Mini Kit as follows-

1. Buffer ASL,140 ml (stool lysis buffer)
2. Buffer AL, 33ml. This is a lysis buffer, which contains guanidine hydrochloride
3. Buffer AW1 (Concentrated), 19 ml. This is a wash buffer and contains guanidine hydrochloride. Before using for the first time, 25 ml ethanol (96-100%) was added to obtain 44 ml AW1
4. Buffer AW2 (Concentrated), 13 ml. This is a wash buffer. Before using for the first time, 30 ml, ethanol (96-100%) was added to obtain 43 ml AW2
5. InhibitEX™ tablets
6. Buffer AE, 12 ml. (elution buffer)
7. Proteinase K, 1.4 ml
8. QIAamp spin columns
9. Collection tubes (2 ml)

All the reagents were stored at room temperature between 15-25˚C. Other required materials which were used but not provided with QIAamp® DNA Stool Mino Kit-

1. Ethanol (96-100%)
2. 1.5 m and 2 ml. microcentifuge tubes
3. Micropipettes

4. Filter tips for micropipette
5. Microcentrifuge
6. Vortexes
7. Water bath
8. Deionized distilled water

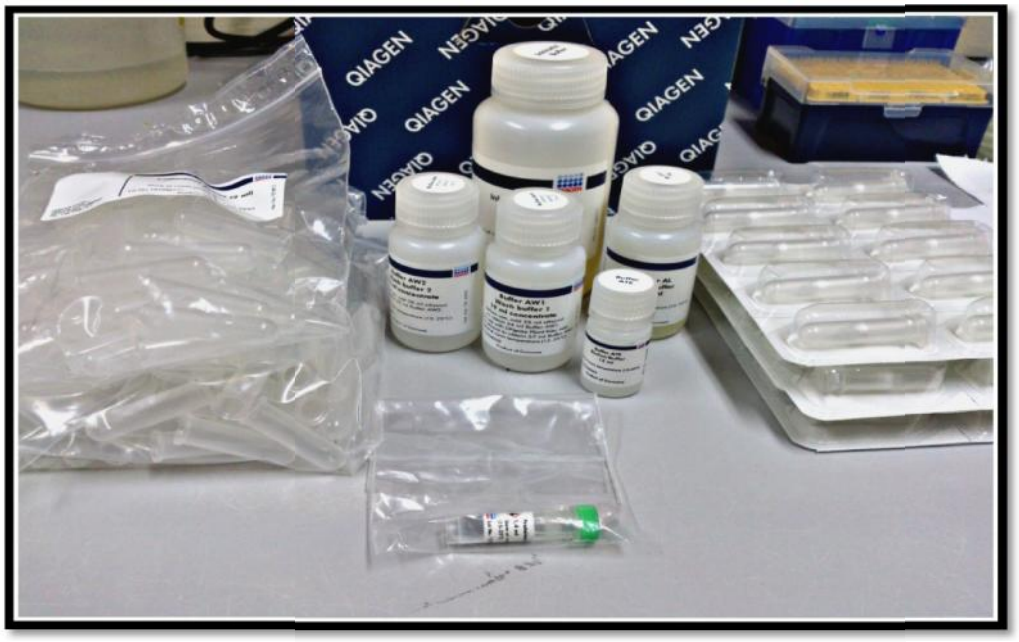

Fig. 7. QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany).

B. Procedure of the DNA extraction by QIAGEN method

1. About 180-220 mg of frozen stool samples (thawed at room temperature) or 200 µl of liquid stool samples was transferred to a 2 ml microcentrifuge tube and was placed on ice.
2. 1.4 ml Buffer ASL was added to each stool sample and was vortexed continuously until the stool sample was thoroughly homogenized. The Buffer ASL helps to lyse the stool sample.
3. The suspension was heated for 5 minutes at 70˚C in water bath. This heating increases total DNA yield 3 to 5 folds and helps to lyse other parasites and even those bacteria which are difficult to lyse (gram positive bacteria).

4. The sample was then vortexed for 15 seconds and centrifuged at full speed (14000 rpm) for 1 minute to pellet stool particles.
5. 1.2 ml of supernatant was pipette into a new 2 ml microcentrifuge tube and then the pellet was discarded.
6. One Inhibitex tablet was added to each sample and vortexed immediately and continuously until the tablet was completely suspended. The suspension was incubated for 1 minute at room temperature. DNA-damaging substances and PCR inhibitors present in the stool sample were adsorbed to Inhibitex.
7. The sample was centrifuged at full speed for 3 minutes to pellet inhibitors bound to InhibitEX.
8. All the supernatant was pipette into a new 1.5 ml microcentrifuge tube and the pellet was discarded. The sample was then centrifuged at 14000 rpm for 3 minutes.
9. 15 µl of proteinase K was pipetted into new 1.5 ml microcentrifuge tube. Proteinase K helps to digest the unnecessary proteins.
10. 200 µl of supernatant from step 8 was pipetted into the 1.5 ml microcentrifuge tube containing proteinase K.
11. 200 µl of Buffer AL was added and vortexed for 15 seconds. The sample and Buffer AL were thoroughly mixed to form a homogeneous solution.
12. Incubated at 70˚C for 10 minutes. A brief centrifugation was done to remove drops from the inside of the tube lid.
13. 200 µl of ethanol (96-100%) was added to the lysate, and mixed by vortexing. A brief centrifugation was used to remove drops from the inside of the tube lid.
14. The lid of new QIAamp spin column placed in 2 ml collection tube that was labeled. The complete lysate from step 13 was carefully applied to the QIAampspin column without moistening the rim. The cap was closed and centrifuged at full speed for 1 minute. The QIAamp spin column was placed in new 2 ml collection tube and the containing the filtrate was discarded.
15. The QIAamp spin column was carefully opened and 500 µl of buffer AW1 was added. Centrifuged at 14000 rpm for 1 minute. The QIAamp spin column was placed in a new 2 ml collection tube, and the collection tube containing the filtrate was discarded.

QIAGEN method

The DNA extraction methods and materials were provided by QIAamp® DNA Stool Mini Kit (QIAGEN).

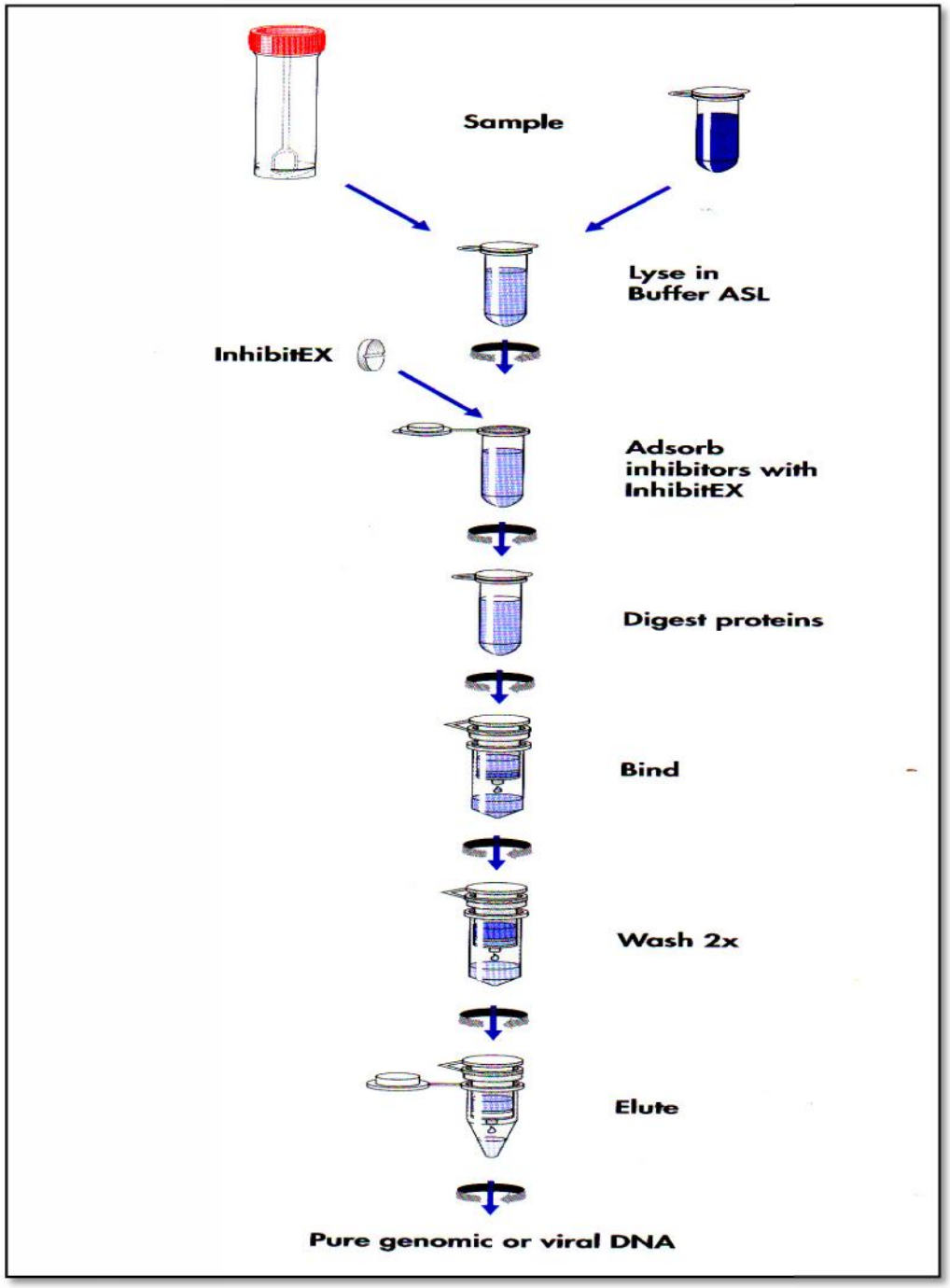

Fig. 8. The DNA extraction method of QIAGEN is shown in follow chart (www.qiagen.com).

16. The QIAamp spin column was carefully opened and 500 µl of Buffer AW2 was added. Centrifuged at full speed for 3 minute, the collection tube containing the filtrate was discarded.
17. The residual buffer AW2 in the elute may cause problems in downstream applications. To overcome this, the QIAamp spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. the collection tube containing the filtrate was discarded.
18. The QIAamp spin column was transferred into a new, labeled 1.5 ml microcentrifuge tube and 200 µl of Buffer AE was pipetted directly on to the QIAamp spin column membrane.
19. Incubated for 1min at room temperature, the centrifuge at full speed for 1 min to elute DNA.

3. POLYMERASE CHAIN REACTION (PCR)

PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

A. Principles of the PCR methods:

- Separation of the nucleic acid double strand (DNA).
- Annealing of short DNA-fragments (Primers) on their specific sequences.
- Elongation of these short fragments by Taq-Polymerase.
- Detection by specific probes.

B. Steps of PCR (Polymerase Chain Reaction) cycle:

PCR is closely patterned after the natural principle of DNA replication. It is a three-step process, referred to as a cycle, that is repeated a specified number of times. One PCR cycle consists of the following steps:

- Step 1: Denaturation by Heat
- Step 2: Annealing and
- Step 3: Extension

This process takes place in a thermal cycler, an instrument that automatically controls and alternates the temperatures for programmed periods of time for the appropriate number of PCR cycles (usually between 30 and 40 cycles).

- **Step 1: Denaturation by Heat**

During this initial step, heat (usually hotter than 90 degrees Celsius) separates double-stranded DNA into two single strands. This process is called "denaturation". Denaturation is possible because the hydrogen bonds linking the bases to one another are weak. The hydrogen bonds break at high temperatures, whereas the bonds between deoxyribose and phosphates, which are stronger covalent bonds, remain intact.

- **Step 2: Annealing Primer to Target Sequence**

The goal of PCR is not to replicate the entire strand of DNA but to replicate a target sequence of approximately 100-600 base pairs unique to the organism being studied. Targeting the sequence is achieved by using primers. Primers mark the ends of the target sequence. Two primers are included in the PCR, one for each of the complementary single DNA strands that was produced during denaturation. The beginning of the DNA target sequence of interest is marked by the primers that anneal (bind) to the complementary sequence. The reaction temperature lowered to 50-68◦ C depending on the length and base sequence of the primers, allowing hybridization of the primers to the single-stranded DNA template.

- **Step 3: Extension**

Once the primers anneal to the complementary DNA sequences, the temperature is raised to approximately 72 degrees Celsius and the enzyme Taq DNA polymerase is used to replicate the DNA strands. Taq DNA polymerase is a recombinant thermo stable DNA polymerase from the organism Thermus aquaticus and, unlike normal polymerase enzymes is active at high temperatures. Taq DNA polymerase, begins the synthesis process at the region marked by the primers. It synthesizes new double stranded DNA molecules, both identical to the original double stranded target DNA region, by facilitating the binding and joining of the complementary nucleotides that are free in solution (dNTPs).

Extension always begins at the 3′ end of the primer making a double strand out of each of the two single strands. Taq DNA polymerase synthesizes exclusively in the 5′ to 3′ direction. Therefore, free nucleotides in the solution are only added to the 3′ end of the primers constructing the complementary strand of the targeted DNA sequence.

End of the first PCR cycle:

At the end of the first PCR cycle, two new identical DNA strands produced to the original target. The DNA polymerase does not recognize the end of the sequence. The newly formed strands have a beginning, which is precisely defined by the 5′ end of the primer, but the 3′ end is not precisely defined. As the number of cycles increases, a strand with more defined length frequently serves as the template for the newly synthesized sequence. The DNA strand synthesized from such a template then has a precisely defined length that is limited at either end by the 5′ end of each of the two primers. These DNA strands are called an AMPLICON. After only a few cycles, DNA strands which correspond to the target sequence, are present in much larger numbers than the variable length sequences. In other words, the sequence flanked or defined by the two primers is the section that is amplified (Fig. 9).

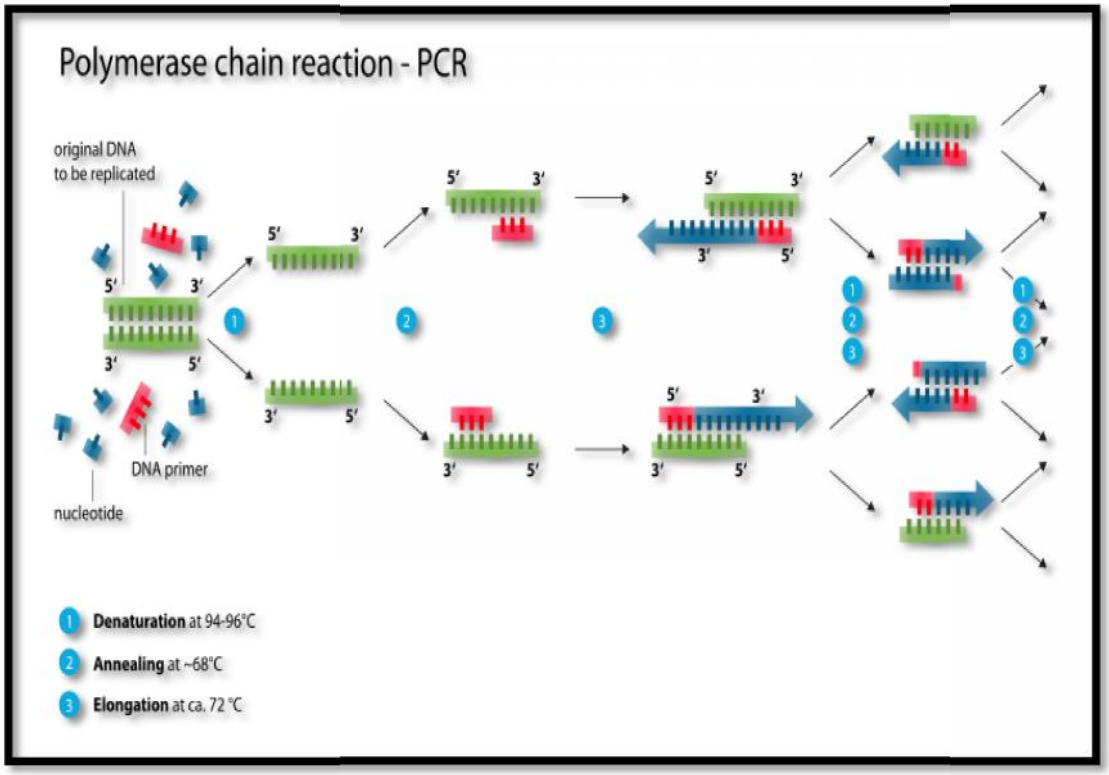

Fig. 9. Steps of Polymerase Chain Reaction (PCR) method.

C) Requirements for PCR:

1. **DNA template** - DNA segment to be amplified, the sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

2. **Primers**- short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer. These two primers are short segment of DNA (forward and reverse primers) about 20-25 bases long.

3. **DNA polymerase** - a type of enzyme that synthesizes new strands of DNA complementary to the target sequence. The first and most commonly used of these enzymes is *Taq* DNA polymerase (from *Thermis aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher

fidelity when copying DNA. Although these enzymes are subtly different, they both have two capabilities that make them suitable for PCR: 1) they can generate new strands of DNA using a DNA template and primers, and 2) they are heat resistant.

4. **Nucleotides (dNTPs or deoxynucleotide triphosphates)**- A nucleoside triphosphate (NTP) is a molecule containing a nucleoside bound to three phosphates (also known as a nucleotide). The dNTPs are the building blocks for DNA (they lose two of the phosphate groups in the process of incorporation). The nucleotide triphosphates containing deoxyribose are called dNTPs, and take the prefix deoxy- in their names and small d- in their abbreviations: deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP) and deoxyuridine triphosphate.

5. **Buffer solution** - a suitable chemical environment.
6. **Divalent cations**- Magnesium ions (Mg^{2+})
7. **Monovalent ions**- potassium ions (K^{+})
8. **PCR machine** – a thermal cycler.

D. Three-temperature cycle in PCR.

1. Temperature 90-98◦ C- separates two strands of target DNA.
2. Temperature 40-60◦ C- anneals two complementary primers to the ends of separated single strands of target DNA.
3. Temperature 72◦ C allows taq polymerase to use as target DNA and primers to synthesize new strands.

E. Mechanism of Real-time PCR

Real-time PCR was able to detect sequence-specific PCR products as they accumulate in "real-time" during the PCR amplification process. As the PCR product of interest

produced, real-time PCR can detect their accumulation and quantify the number of substrates present in the initial PCR mixture before amplification began (Fig. 10).

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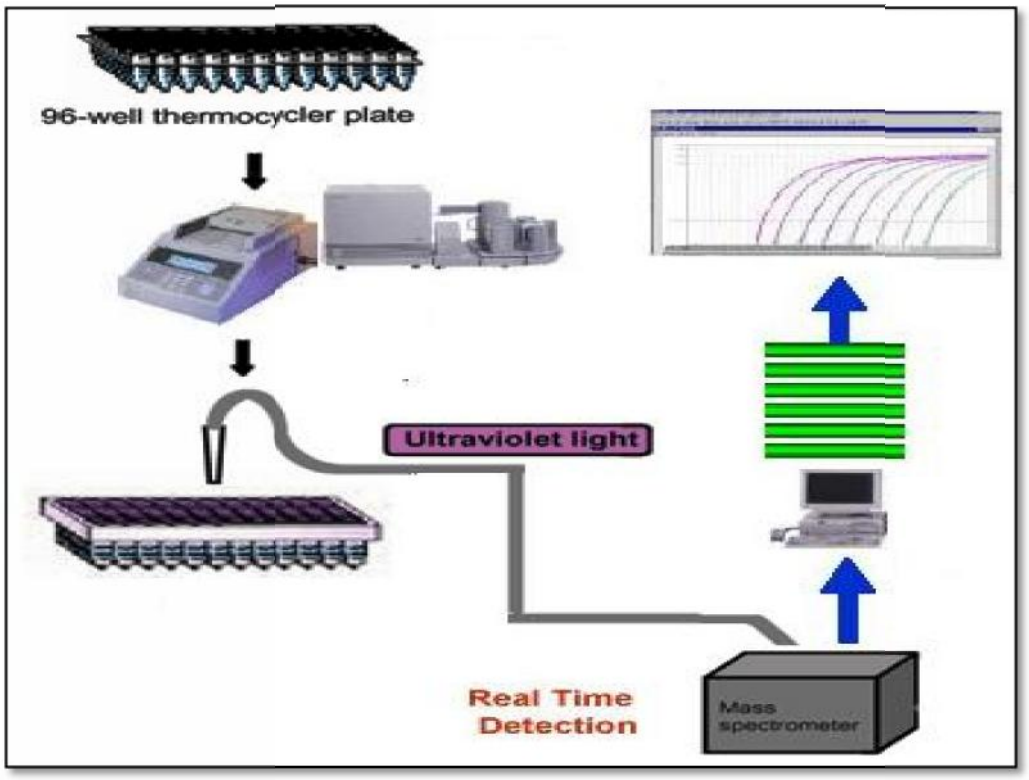


Fig. 10. Mechanism of real-time PCR.

F. Mechanism of TaqMan® probes

The TaqMan® probe consists of two types of fluorophores, which are the fluorescent parts of reporter proteins (Green Fluorescent Protein (GFP) has an often-used fluorophore). While the probe is attached or unattached to the template DNA and before the polymerase acts, the **quencher (Q)** fluorophore (usually a long-wavelength colored dye, such as red) reduces the fluorescence from the **reporter (R)** fluorophore (usually a short-wavelength colored dye, such as green). It does this by the use of Fluorescence (or Förster) Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. The reporter dye is found on the 5′ end of the

probe and the quencher at the 3′end [http://www.probes.com/handbook/boxes/0422.html 2003] (Fig. 11).

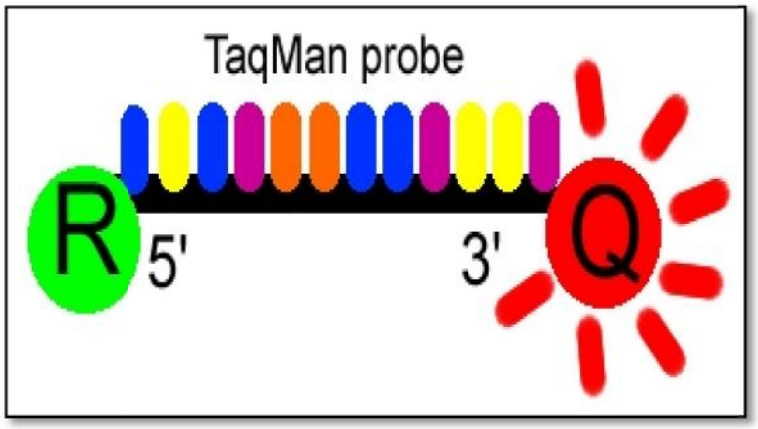


Fig. 11. The Taqman probe. The red circle represents the quenching «dye that disrupts the observable signal from the reporter dye (green circle) when it is within a short distance. (Image created by Dan Pierce. http://www.bio.davidson.edu/).

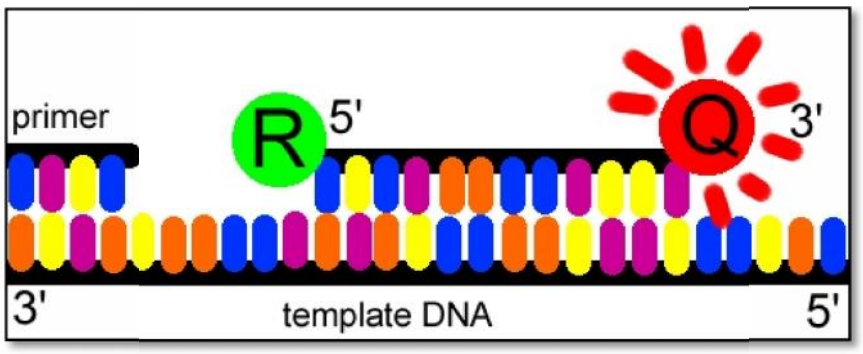


Fig. 12. The TaqMan® probe binds to the target DNA, and the primer binds as well. Because the primer is bound, *Taq* polymerase can now create a complementary strand. (Image created by Dan Pierce. http://www.bio.davidson.edu/)

Once the TaqMan® probe has bound to its specific piece of the template DNA after denaturation (high temperature) and the reaction cools, the primers anneal to the DNA. *Taq* polymerase then adds nucleotides and removes the Taqman® probe from the template DNA. This separates the quencher from the reporter, and allows the reporter to give off its emit its energy. This is then quantified using a computer. The more times the

denaturing and annealing takes place, the more opportunities there are for the Taqman® probe to bind and, in turn, the more emitted light is detected.

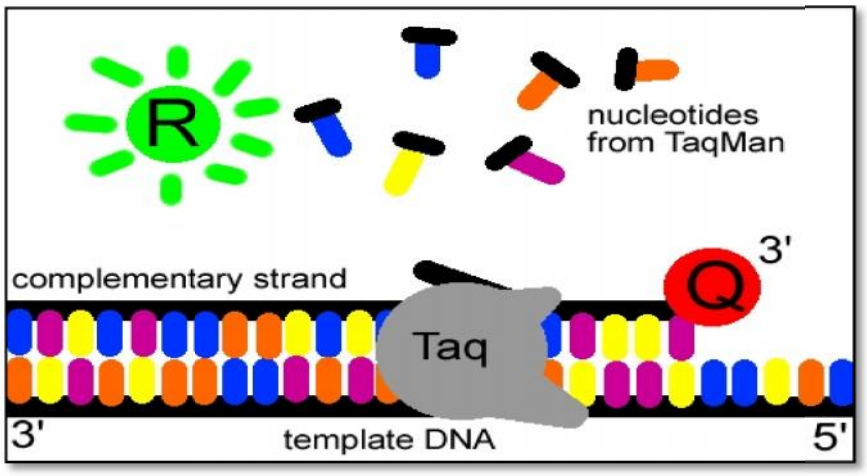


Fig. 13. The reporter dye is released from the extending double-stranded DNA created by the *Taq* polymerase. Away from the quenching dye, the light emitted from the reporter dye in an excited state can now be observed. (Image created by Dan Pierce. http://www.bio.davidson.edu/).

The oligonucleotide primers and TaqMan probe were designated to specifically amplify the target region of the rRNA gene of *Blastocystis hominis*. These primers amplified a 140-bp fragment inside the 16s small subunit of the rRNA gene of *Blastocystis hominis* strain HC06-28 (Gene Bank accession number: EF468654).

G. Description of kit and primers

1. Primers

Table 1. Oligonucleotide primers for real-time PCR assay for the simultaneous detection of *B. hominis.*

Name of organism	Primers	Position	Oligonucleotide sequence (5′–3′)
B. hominis	BlastoF1	326-347	GTATATGGGACTACCATGGCAG
	BlastoR1	446-465	TCACTACCTCCCTATGTCAG

Here, F for “Forward primer”; R for “Reverse primer”

2. Probes

Table 2. Oligonucleotide probes for real-time PCR assay for the simultaneous detection of *B. hominis.*

Name of organism	Position	Oligonucleotide sequence (5′–3′)
B. hominis	391-412	Taxas Red -CTGAGAGATGGCTACCACATCC-Dark Quencher

H. Reagents for Real-Time PCR

1. Qiagen IQ super mix
2. 96-well (200ul) thin walled PCR plates
3. i-Cycle Thermal Cycler
4. i-Cycle Optical Sealing Tape
5. Primers: a. 25 picomoles of each BlastoF1and BlastoR1
   b. 25 picomoles of probes
6. Deionized distilled water
7. Sealer

I. Protocol for Real time PCR

The Real-Time PCR was done in a total reaction volume of 25 µl.

QIAGEN PCR Master Mix contains

1. HotStarTaq DNA Polymerase.
2. QIAGEN Multiplex PCR Buffer Contains 6 mM $MgCl_2$, pH 8.7 (20°C).
3. dNTP Mix Contains dATP, dCTP, dGTP, dTTP; ultrapure quality.
4. Q-Solution 5x concentrated.
5. RNase-Free Water Ultrapure quality, PCR-grade.

Table 3. Master Mix composition.

Component	Concentration	Mix
Qiagen Master mix	-	12.5µl
$MgCl_2$	25.0mM	2 µl
B.hominis Primer F	20.0 µl	0.5 µl
B.hominis Primer R	20.0 µl	0.5 µl
B. hominis Texas red Probe	2.0 µl	0.5 µl
Total volume		16.0 µl

Here, F for "Forward primer"; R for "Reverse primer"

Table 4. Reaction Mix composition.

Mastermix	16.0 µl
dH_2O	6.0 µl
Sample DNA	3.0 µl
Total volume	25 µl

J. Procedure of Real time PCR

1. 22µl of freshly prepared master mix was added to the well of the experimental plate.
2. 3.0 µl of sample DNA was added to the well.
3. After adding mastermix to the well, experimental plate was sealed with i-Cycler Optical Sealing Tape and the plate was briefly spun to bring all the reagents to the bottom of the well.
4. The plate was placed into the i-Cycler and for the activation of Taq polymerase and initial denaturation at 95.C for 15 minutes was done.

5. Than a 40 cycles of PCR with denaturation at 95.C for 15 seconds, annealing and extension at 58◦ C for 1 minute was performed.
6. Following amplification, the fluorescence reading was taken after each extension step.

Table 5. Thermal cycling program used in Real-time PCR.

Cycle 1:	(1X)	Temperatu re	
	Step 1:	95.0ºC	for 15:00
Cycle 2:	(40X)		
	Step 1:	95.0ºC	for 00:15
	Step 2: Data collection and real-time analysis enabled	58.0ºC	for 01:00
Cycle 3:	(1X)		
	Step 1:	4.0ºC	infinity

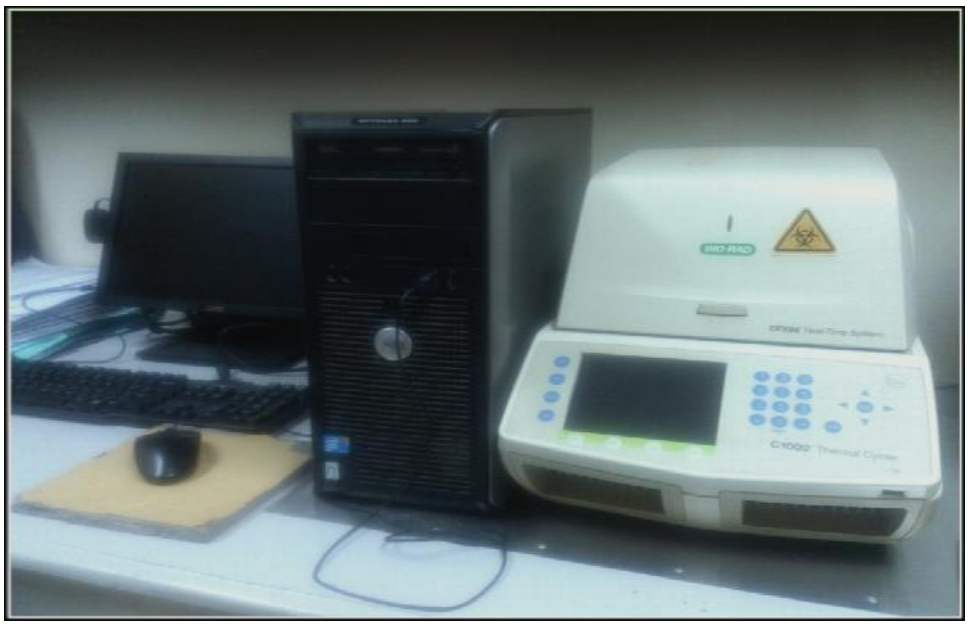


Fig. 14. The Real-time PCR machine (iCycler, BioRad) used in Parasitology Laboratory, icddr,b, Mohakhali, Dhaka.

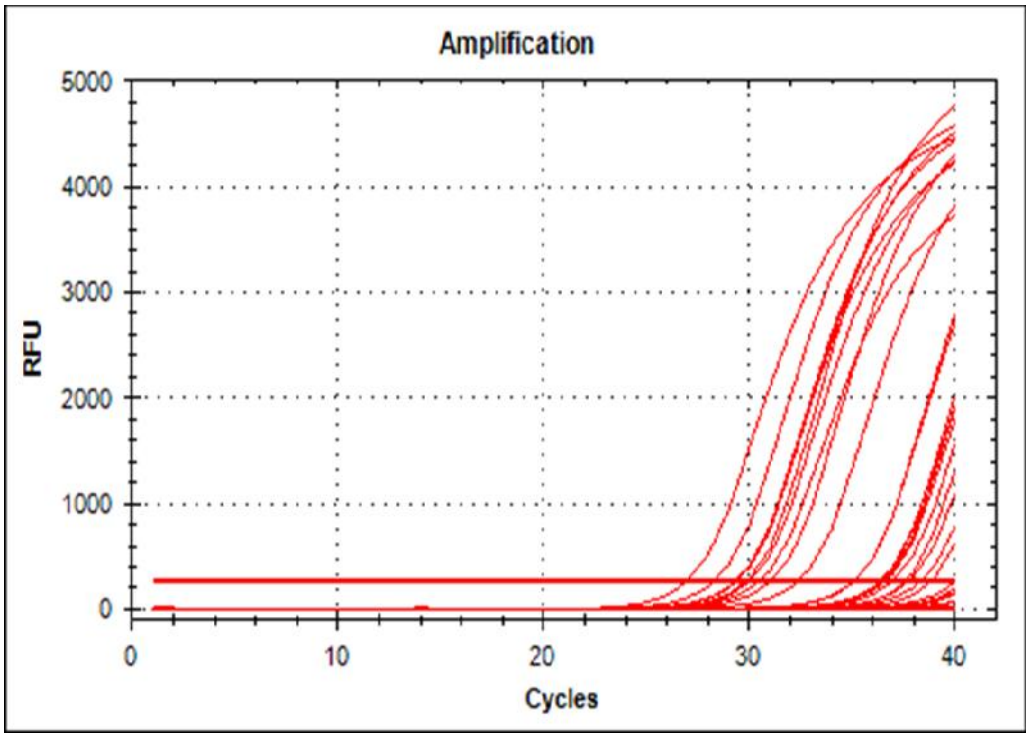


Fig. 15. Real-time PCR amplification cycle graph.

A sample was considered positive if the signal exceeded a preset threshold (Threshold cycle, C_T). For the detection of *Blastocystis hominis*, same Real-time procedure was used with corresponding primer and probe according to the parasite.

Sensitivity and specificity:

Sensitivity:

A measure of the ability of the screening test to identify positives expressed as the percentage of the positives correctly identified by the test out of the total number of positives actually tested (Lyng *et al.* 2015).

$$\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{Number of false negative}} \times 100$$

Specificity:

A measure of the ability of the screening test to identify negatives expressed as the percentage of the negatives correctly identified by the test out of the total number of negatives actually tested (Lyng *et al.* 2015).

$$\text{Specificit} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{Number of false positive}} \times 100$$

RESULTS AND OBSERVATIONS

Thepresent study was conducted in the Parasitology Laboratory, of the International Centre for Diarrheal Diseases Research, Bangladesh (icddr,b). The study period was from January, 2012 to December, 2013. During the study period, stool samples of the children were collected from slum area of Mirpur and Mohammadpur, Dhaka city, Bangladesh.

The present study was performed on 9322 fecal samples from 406 children in Mirpur Area and 3218fecal samples from 118 children in Mohammadpur area.Age range of these children's were 13-72 month's (1 to 6 years).The prevalence of *Blastocystis hominis* infection between the diarrheal and non-diarrheal children were investigated.

The children's who showed either *B. hominis* positive or negative stool, but didn`t show any symptoms of diarrhea called non-diarrheal children and samples collected from thesechildren are indicatedas MS (Monthly Stool) samples. The children who had either *B. hominis* positive or negative stool, but showed the symptoms of diarrhea called diarrheal childrenare indicatedas DS (Diarrhoeal Stool) samples.

Among 406 childrenfrom Mirpur area, 221 (54.43%) were male and 185 (47.54%) were female. Out of 406, 200 were diarrheal patients,111 (55.50%) were male and 89 (44.50%) female (Table 1).

While, among 118 childrenfrom Mohammadpur area,65 (55.08%) were male and 53 (44.92%) female and 37 were diarrheal patients, 22 (59.46%) were male and 15 (40.54%) female (Table 1).

Out of 9322 samples, 8601 were monthly stool (MS) samples and 721 were diarrheal stool (DS) samples while, out of 3218 samples,2704 were monthly stool (MS) samples and 514 were diarrheal stool(DS) samples (Table 2).

Table 1. Percentage of male and female children in the study areas (Mirpur and Mohammadpur slum area).

Study area	Total Children	Male		Female	
		Children	%	Children	%
Mirpur slum area	406	221	**54.43**	185	**45.57**
	200	111	**55.50**	89	**44.50**
Mohammadpur slum area	118	65	**55.08**	53	**44.92**
	37	22	**59.46**	15	**40.54**

Table 2.Samples collected from the study areas(Mirpur and Mohammadpur slum area).

Study Area	Total no of Children	Types of samplesexamined	No of stool samples examined
Mirpur slum area	406	MS	8601
	200	DS	721
Overall stool samples			**9322**
Mohammadpur slum area	118	MS	2704
	37	DS	514
Overall stool samples			**3218**

Prevalence of *B. hominis* in overall monthly stool (MS) samples from Mirpur area,according to sex and age groups:

During the study period (2012 and 2013), in MS samples from Mirpur area, the prevalence of *B. hominis*was found 3.72% by direct microscopy **(Table 3)**. In the age group of 13-24 months,the prevalence of *B. hominis*in male (2.59%) was higher than female (1.58%), while in theremaining age groupsof children,the prevalence of *B. hominis*was higher in female. The higherprevalence, 4.35% and 4.74% were observed in the age group of 37-48 and 49-60 months respectively**(Fig.1)**.By the analysis of variance prevalence of *B. hominis*among sexes of children was not significantly different (F=1.779 and p=0.182^{ns}), whileby age groups it was significantly different (F=1.377 and p=0.03*).

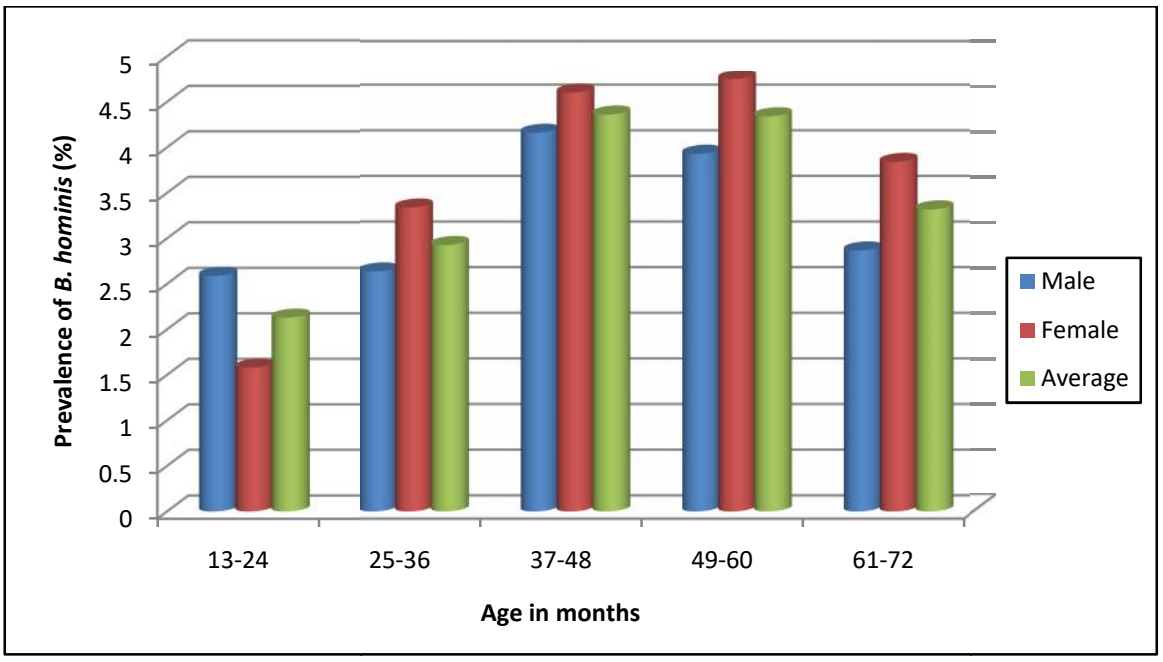


Fig. 1. Prevalence of *B. hominis* by direct microscopy in sex and age groups in overall MS samples from Mirpur area, during 2012-13.

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

Interpretation

The children of age group 37-48 months and 49-60 monthsweremore sensitive than other age groups.

Prevalence of *B. hominis* in MS samples from Mirpur area according to sex and age groupsduring 2012:

In 2012, out of 4481 MS samples, 3.53% found positive for *B. hominis* by direct microscopy**(Table 3)**.The prevalence of *B. hominis*, were higher in male children than female in the age group of 13-24 months and 37-48 months. While in the age group 25-36 months, prevalence was more in female. The highest prevalence (4.83%) was observed in the children of age group 49-60 months and the lowest prevalence (2.65%) was observed in age group 13-24 **(Fig. 2)**.By the analysis of variance, prevalence of *B. hominis*among the sexes of children was not significantly different (F=0.003 and p=0.954^{ns}), whileby age groups it was significantly different (F=1.496 and p=0.017*).

Prevalence of *B. hominis* in MS samples from Mirpur area according to sex and age groups during 2013:

In 2013, out of 4120MS samples, 3.93% found positive for *B. hominis* by direct microscopy **(Table 3)**.In all age group of children, the prevalence of *B. hominis*, was higher in female than male. The highest prevalence (4.40%) was observed in children of age group of 49-60 months and the lowest prevalence (3.31%) was observed in children of age group of 61-72 months**(Fig. 3)**. The prevalence of *B. hominis*among the sexes of children was not significantly different (F=3.469 and p=0.063^{ns}), whileby age groups it was highly significant(F=1.653 and p=0.003**) by the analysis of variance (ANOVA).

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

Table 3. Prevalence of *Blastocystis hominis*by direct microscopy inmale and female children from two areas.

Study period in year	Examine Sample Type	Total		Male		Female	
		No of samples	*B.h* positive (%)	No of samples	*B.h*positive (%)	No of samples	*B.h*positive (%)
Overall (Mirpur area)		**9322**	**362 (3.88%)**	**5137**	**188 (3.66%)**	**4185**	**174 (4.16%)**
2012	MS	4481	158 (3.53%)	2449	86 (3.51%)	2032	72 (3.54%)
2013	MS	4120	162 (3.93%)	2241	76 (3.40%)	1879	86 (4.58%)
Total	**Overall MS**	**8601**	**320 (3.72%)**	**4690**	**162 (3.24%)**	**3911**	**158 (4.04%)**
2012	DS	422	20 (4.74%)	263	12 (4.56%)	159	8 (5.03%)
2013	DS	299	22 (7.36%)	184	14 (7.61%)	115	8 (6.96%)
Total	**Overall DS**	**721**	**42 (5.83%)**	**447**	**26 (5.82%)**	**274**	**16 (5.84%)**
Overall (Mohammadpur area)		**3218**	**246 (7.64%)**	**1421**	**115 (8.09%)**	**1797**	**131 (7.29%)**
2012	MS	1169	107 (9.15%)	518	53 (10.23%)	651	54 (8.29%)
2013	MS	1535	89 (5.80%)	641	38 (5.93%)	894	51 (5.70%)
Total	**Overall MS**	**2704**	**196 (7.25%)**	**1159**	**91 (7.85%)**	**1545**	**105 (6.80%)**
2012	DS	237	22 (9.28%)	119	11 (10.08%)	118	11 (8.47%)
2013	DS	277	28 (10.11%)	143	13 (9.79%)	134	15 (10.45%)
Total	**Overall DS**	**514**	**50 (9.73%)**	**262**	**24 (9.92%)**	**252**	**26 (10.32%)**

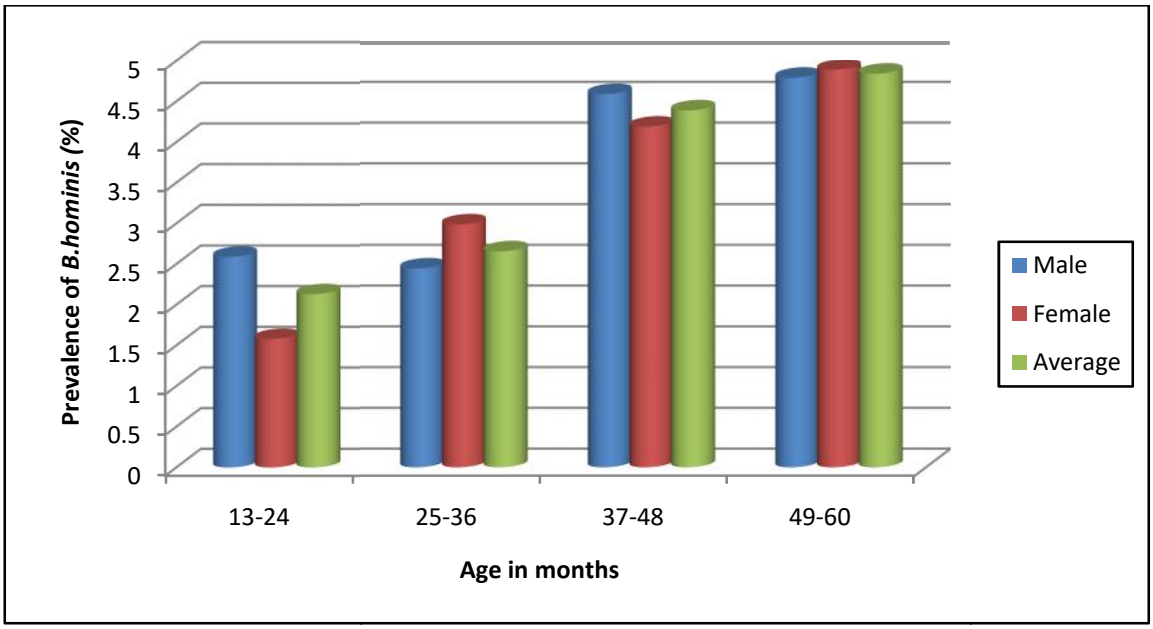


Fig.2. Prevalence of *B. hominis* by direct microscopy in sex and age groups in MS samples from Mirpur area (2012).

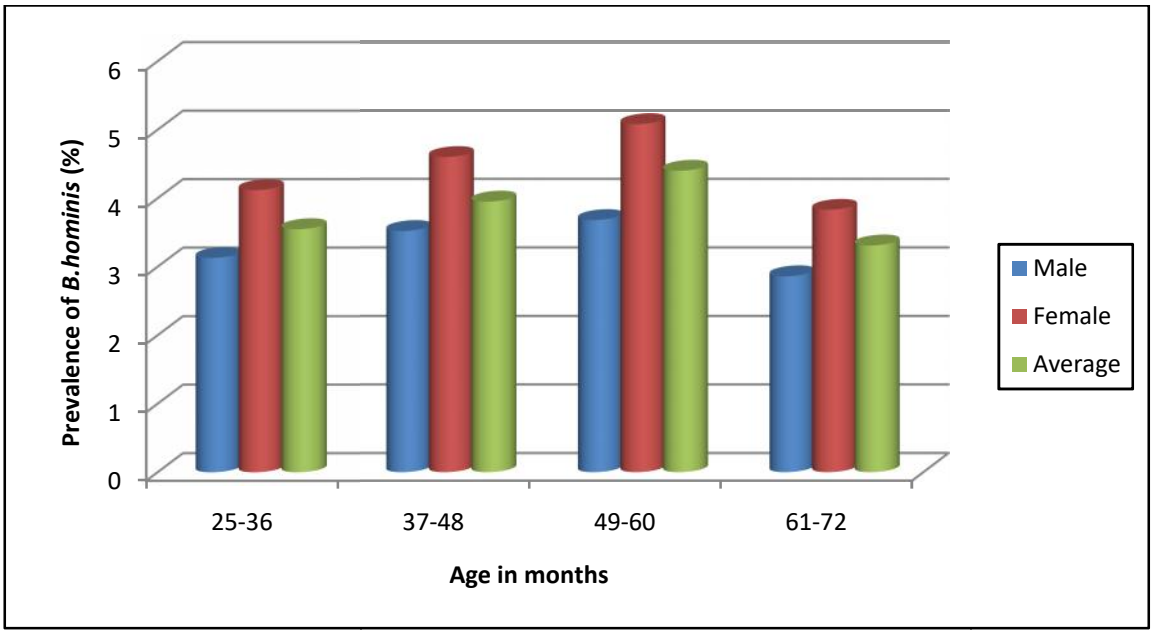


Fig.3. Prevalence of *B. hominis* by direct microscopy in sex and age groups in MS samples from Mirpur area (2013).

Prevalence of *B. hominis* in overall diarrheal stool (DS) samples from Mirpur area,according to sex and age groups during 2012-13:

During the study period (2012-2013),total 721 DSsamples were examined, and 5.83% were found positive for *B. hominis*by direct microscopy**(Table 3)**. The highest prevalence (15.49%) of *B. hominis*was observed in 49-60 months age group. In the age groupsof 25-36, 49-60 and 61-72 months, the prevalence of *B. hominis*were higher in male than that of female children**(Fig. 4)**.Statisticaltest (ANOVA), showed thatthe prevalence of *B. hominis*among the sexes of children was not significantly different (F=0.346 and p=0.557^{ns}) and it was also not significantly different in age groups (F=1.262 and p=0.103^{ns}).

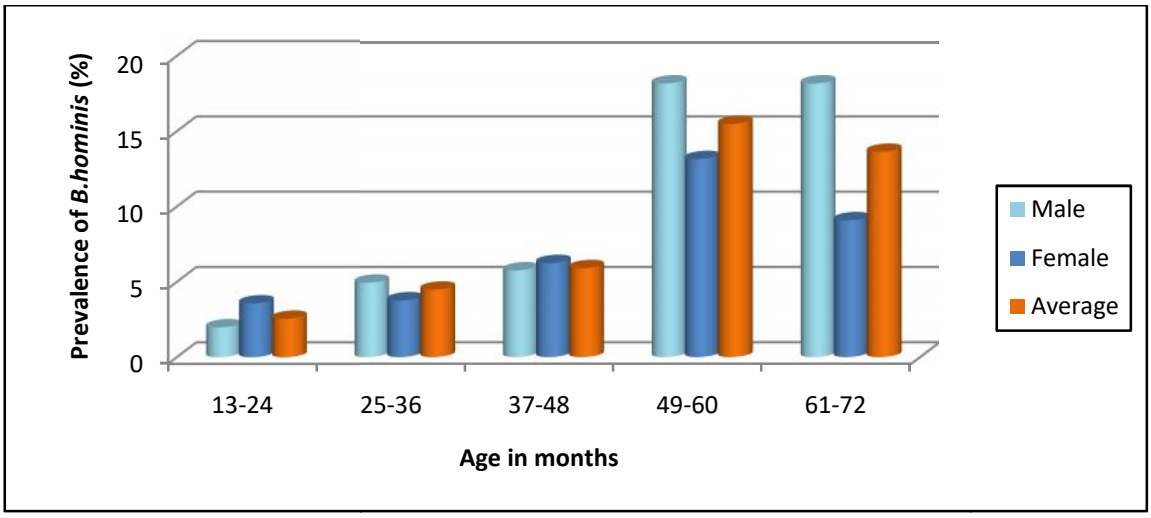


Fig. 4. Prevalence of *B. hominis* by direct microscopy in sex and age groups in overall DS samples from Mirpur area, during 2012-13.

Interpretation

The prevalence of *B. hominis* were higher among male, than that of female, as the male population are more exposed to external environment and cannot maintain proper personal hygiene when they are outside of their residence.

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

Prevalence of *B. hominis* in DS samples from Mirpur area according to sex and age groups during 2012:

In 2012, out of 422 DSsamples, by direct microscopy,4.74% found positive for *B. hominis***(Table 3)**. In children of age group 25-36 months, the prevalence of *B. hominis* was higher in male. While, in the remaining age groups of children the prevalence of *B. hominis* was higher in female children than male.InDS samples, the higher prevalence (8.97% and 9.52%) were observed in age group 37-48 and 49-60 months **(Fig. 5)**.By the analysis of variance, prevalence of *B. hominis*in sexes of childrenwas not significantly different (F=1.150 and $p=0.698^{ns}$) and also by age groups it was not significantly different (F=1.298 and $p=0.107^{ns}$).

Prevalence of *B. hominis* in DS samples from Mirpur area according to sex and age groups during 2013:

In 2013, out of 299 DSsamples, by direct microscopy,7.36% found positive for *B. hominis***(Table 3)**. In all age groups of children,the prevalence of *B. hominis*were higher in male children than female. The highest prevalence (18%) was observed in age group 49-60 months and the lowest prevalence (4%) was observed in 37-48 months**(Fig. 6)**. By the analysis of variance, prevalence of *B. hominis*among the sexes of children and also by age groups were not significantly different ($p=0.219^{ns}$and $p=0.152^{ns}$).

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

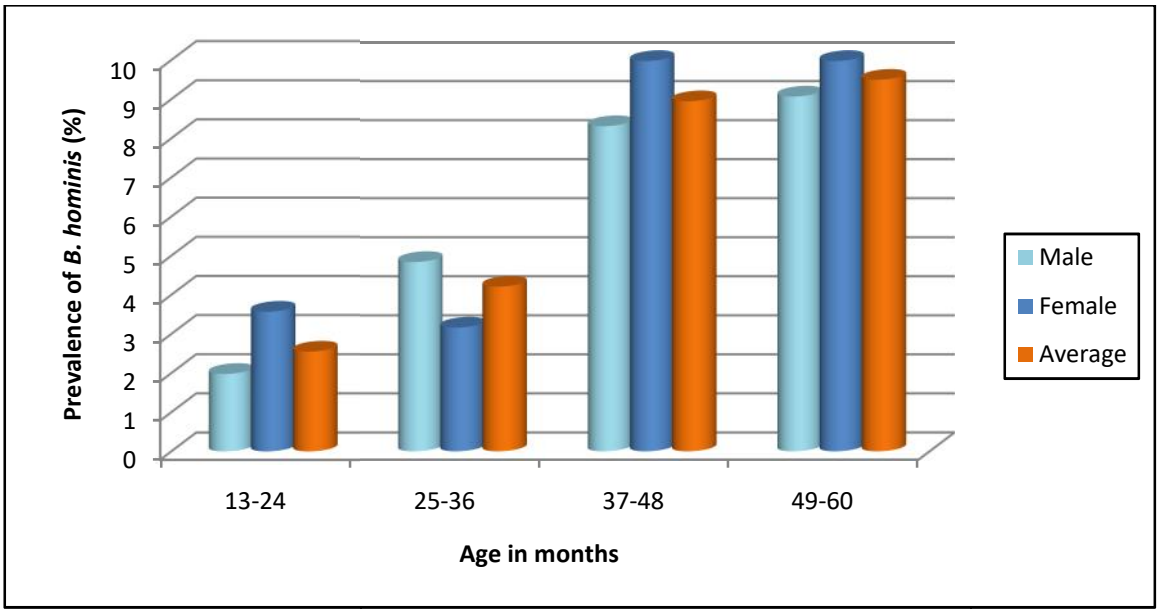


Fig.5. Prevalence of *B. hominis* by direct microscopy in sex and age groups in DS samples from Mirpur area (2012).

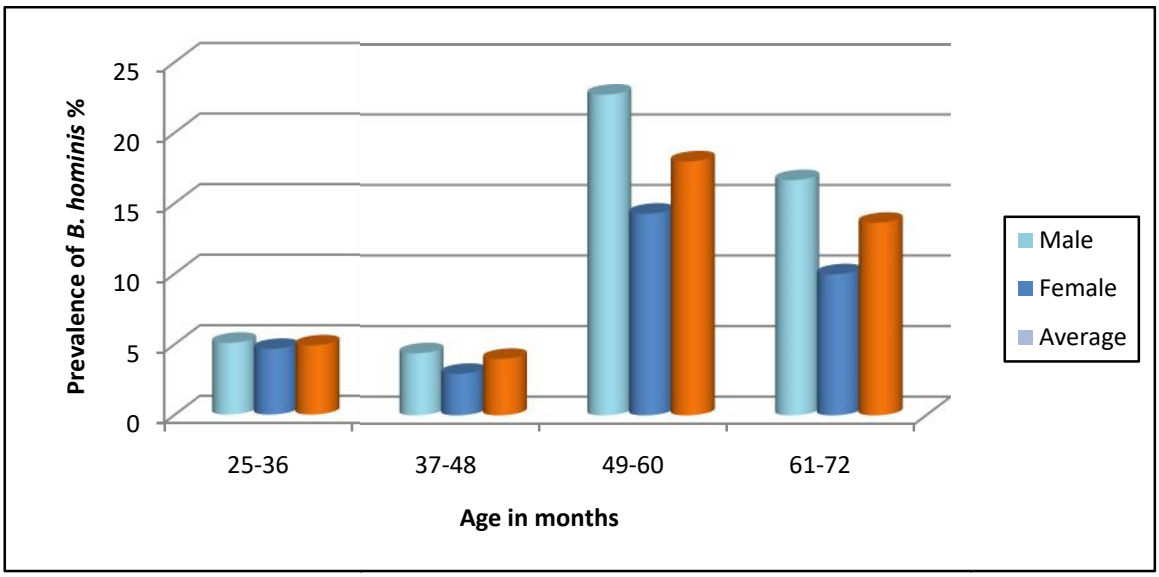


Fig.6. Prevalence of *B. hominis* by direct microscopy in sex and age groups in DS samples fromMirpur area(2013).

Prevalence of *B. hominis* in overall monthly (MS) stool samples according to sex and age groups during 2012-13 from Mohammadpur area:

During the study period (2012 and 2013), out of 2704 MS samples from Mohammadpur area,by direct microscopy,7.25% prevalence of *B. hominis*wasobserved **(Table 3)**.The highest prevalence (8.19%) was observed in age group 25 to 36 months and the lowest prevalence (5.86%) was observed in age group 13 to 24.Most of the age groupsof children showed that the higher prevalence of *B. hominis*in male than female group**(Fig. 7)**.The prevalence of *B. hominis*among the sexesand age groups of children werenot significantly different ($p=0.550^{ns}$and $p=0.063^{ns}$) by statisticaltest (ANOVA).

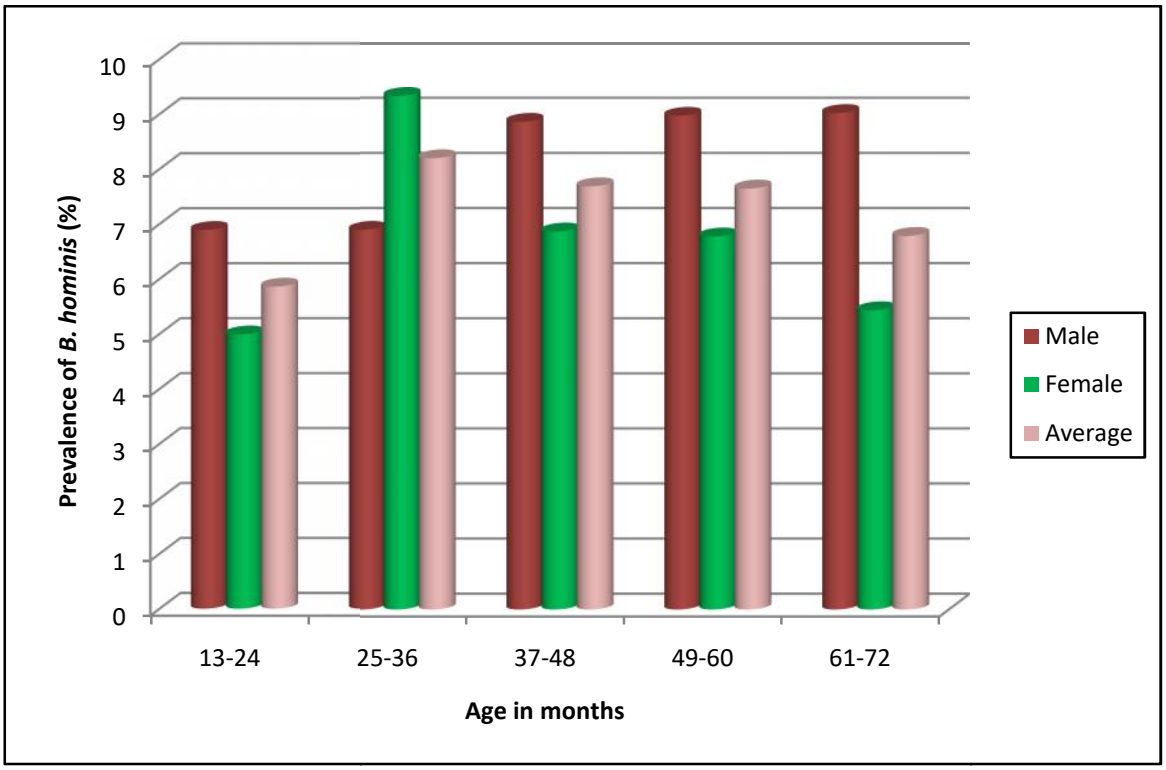


Fig. 7. Prevalence of *B. hominis* by direct microscopy in sex and age groups in overall MS samples from Mohammadpur area, during 2012-13.

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

Interpretation

From Mohammadpur area, in the children of age group 25-36 months were more sensitive than other age groups to the infection. The prevalence of *B. hominis* were higher among male, than that of female, as the male population cannot maintain proper personal hygiene when they are outside of their residence.

Prevalence of *B. hominis* in monthly stool samples according to sex and age groupsduring 2012:

In 2012, out of 1169 MS samplesfrom Mohammadpur area, by direct microscopy, 9.15% found positive for *B. hominis***(Table 3)**.The highest prevalence of *B. hominis*(10.81%) was observed in children of age group 49-60 months. In children of all age groups, the prevalence of *B. hominis* were higher in male than that of female **(Fig.8)**.While the prevalence of *B. hominis*among the sexesand age groups of children were not significantly different ($p=0.547^{ns}$ and $p=0.310^{ns}$).

Prevalence of *B. hominis* in monthly stool samples according to sex and age groups during 2013:

In 2013, out of 1535 MS samples from Mohammadpur area, by direct microscopy,5.80% found positive for *B. hominis***(Table 3)**. In children of age group 13-24, 37- 48 and 61-72 months, the prevalence of *B. hominis*in male were higher than female. The highest prevalence (7.14%) was found in the age group of 61-72 months**(Fig. 9)**.By the analysis of variance, the prevalence of *B. hominis*among the sexesand age groups of children was not significantly different ($p=0.867^{ns}$and $p=0.184^{ns}$).

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

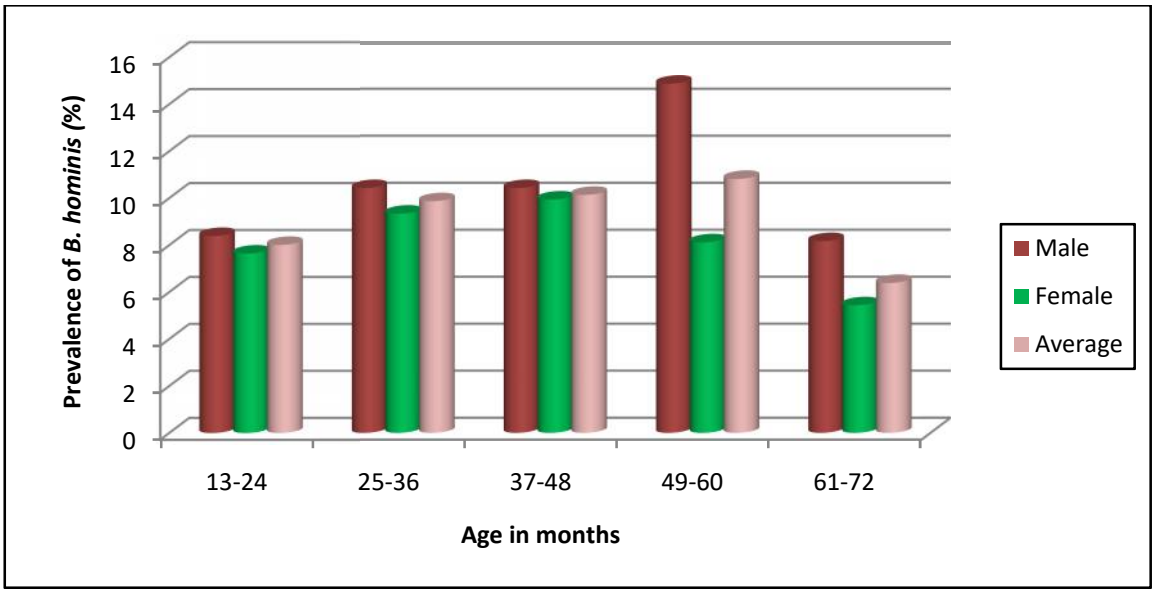


Fig. 8. Prevalence of *B. hominis* by direct microscopy in sex and age groups in MS samples fromMohammadpur area(2012).

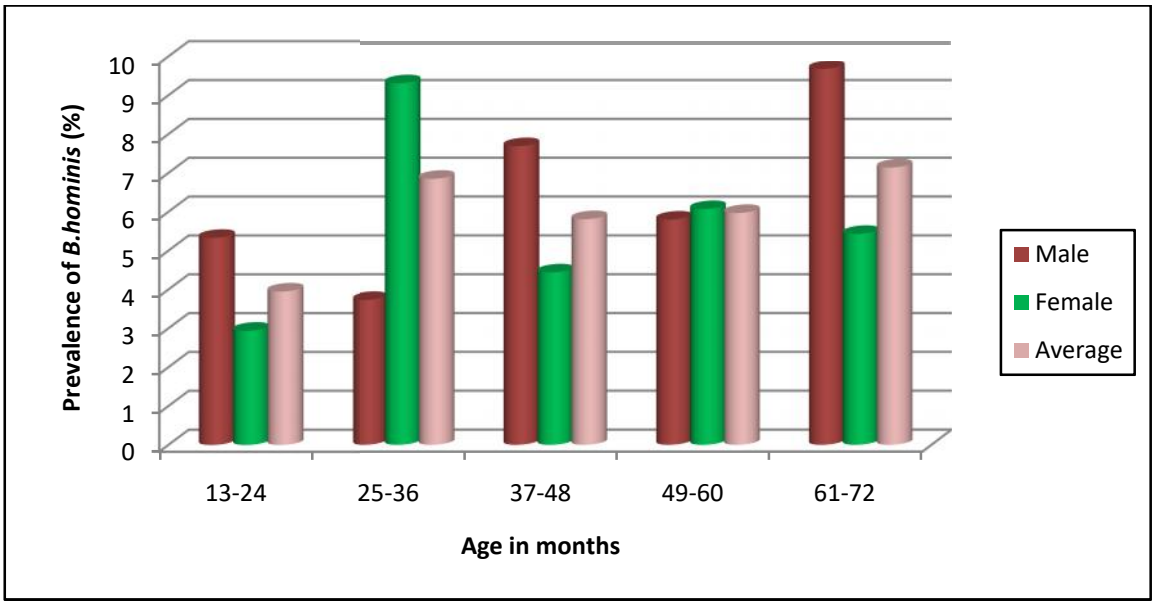


Fig. 9. Prevalence of *B. hominis* by direct microscopy in sex and age groups in MSsamples fromMohammadpur area(2013).

Prevalence of *B. hominis* in overall diarrheal stool (DS) samples according to sex and age groups from Mohammadpur area:

During the study period (2012 and 2013), in DS samplesfrom Mohammadpur area, prevalence of *B. hominis*was 9.73% by direct microscopy **(Table 3)**. The prevalence of *B. hominis* was higher in male group than in female group in the age group 37-48 months and 49-60 months. While the prevalence of *B. hominis*in male and female children was not significantly different (F=0.346 and p=0.557^{ns}). The highest prevalence (19.57%) was observed in the age group of 49-60 months and the lowest prevalence (3.31%) was in 25-36 months**(Fig. 10)**. By the analysis of variance, prevalence of *B. hominis*in age groupswas significantly different (F=1.462 and p=0.043*).

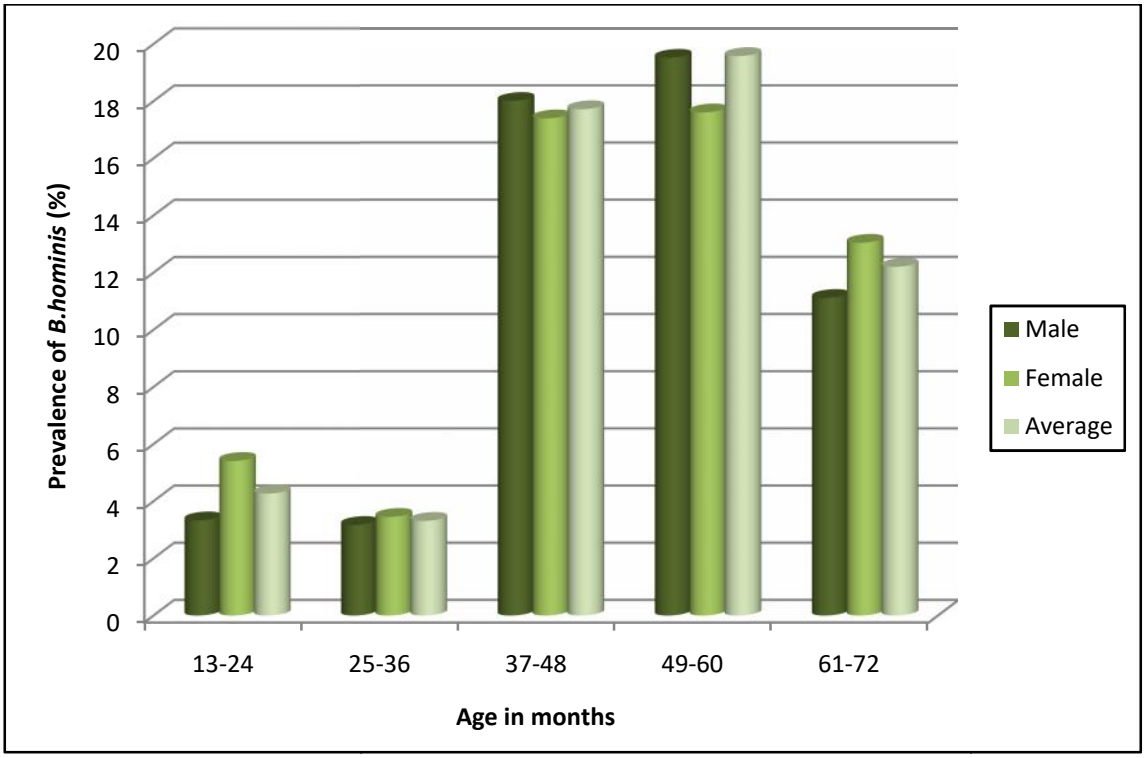


Fig. 10. Prevalence of *B. hominis* by direct microscopy in sex and age groups in overall DS samples from Mohammadpur area, during 2012-13.

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

Interpretation

The prevalence of *B. hominis* were higher among male, than that of female in the age group of 37-48 months and 49-60 months. The children of age group 49-60 months were more sensitive than other age groups to the infection.

Prevalence of *B. hominis* in diarrheal stool (DS) samples according to sex and age groups during 2012:

In 2012, out of 237DSsamples from Mohammadpur area, by direct microscopy, 9.28%found positive for *B. hominis***(Table 3)**. In children of age group 37-48 months, prevalence of *B. hominis* in male (21.05%) was higher than in female (14.29%). The highest prevalence (22.22%) was observed in age group 49-60 months and the lowest prevalence (2.74%) was observed in age group 13-24**(Fig. 11)**. By the analysis of variance prevalence of *B. hominis*by age groups of children was highly significant(**F=1.496 and p=0.017**)**

Prevalence of *B. hominis* in diarrheal stool (DS) samples according to sex and age groups during 2013:

In 2013, total 277 DS samples from Mohammadpur area were examined formicroscopy and 10.11%found positive for *B. hominis***(Table 3)**. In children of age group 13-24 months, 37-48 months and 61-72 months, the prevalence of *B. hominis* in male were higher than female group. Thehighest prevalence (17.86%) was observed in the age group 37-48 months and the lowest prevalence (3.39%) was observed in 25-36 months**(Fig. 12)**.And the differenceamong the prevalence of *B. hominis* in age groups was highly significant (**F=1.496 and p=0.017**)**.

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

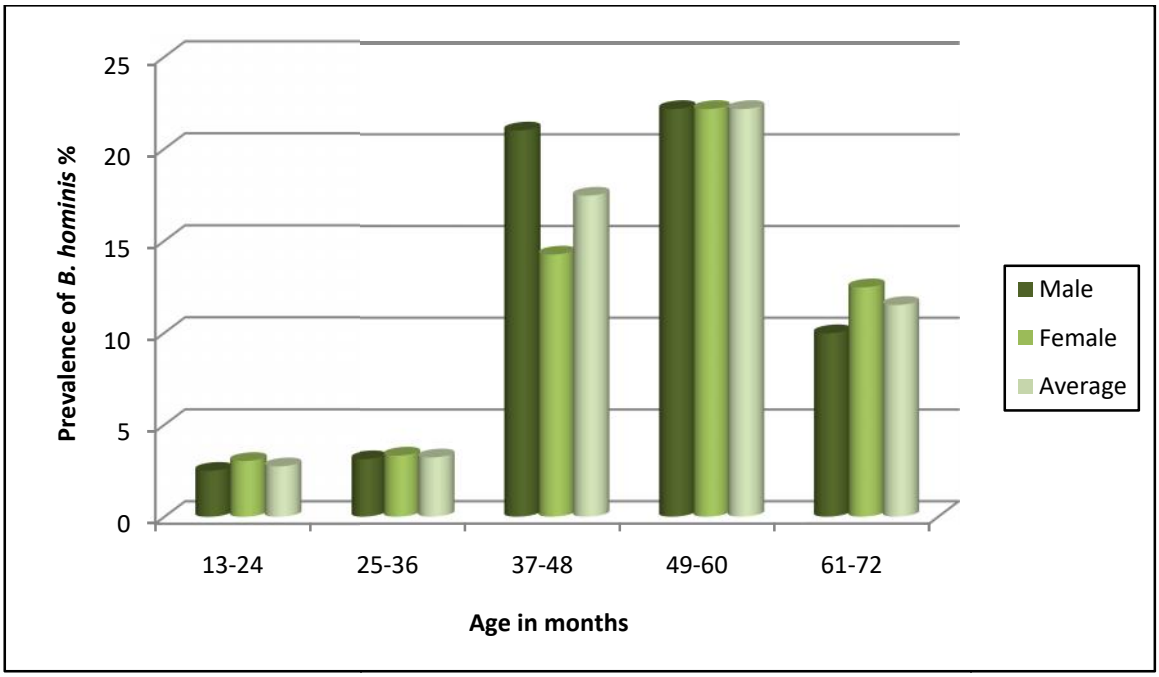


Fig.11. Prevalence of *B. hominis* by direct microscopy in sex and age groups in DS samples from Mohammadpur area (2012).

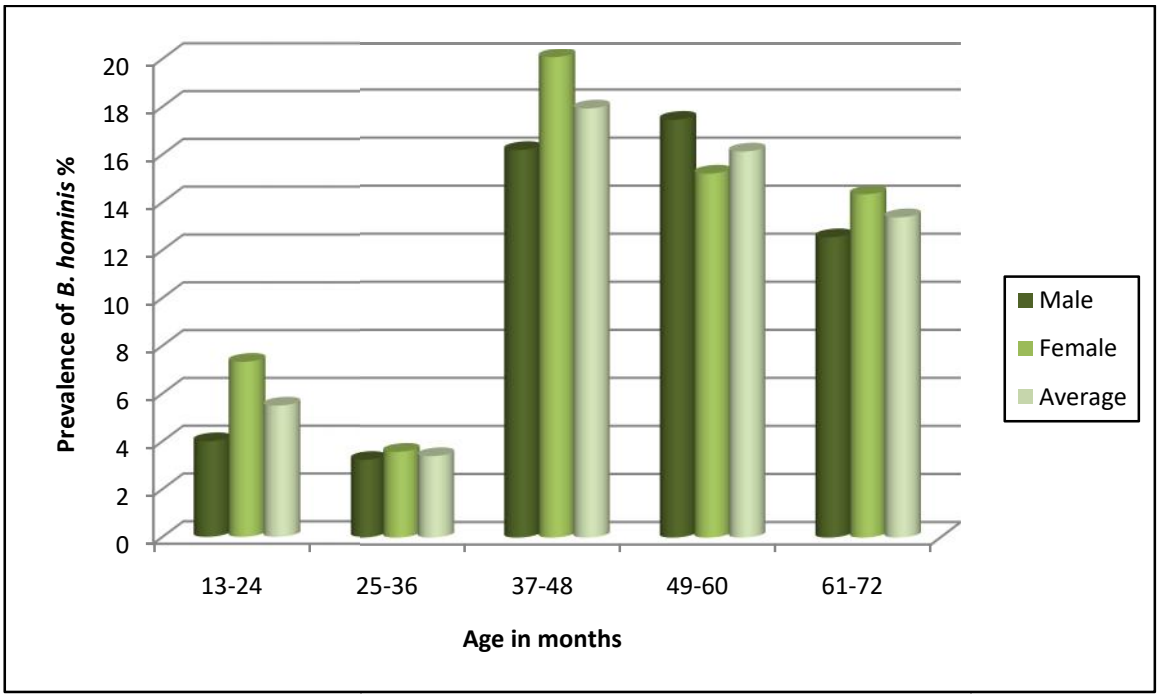


Fig. 12. Prevalence of *B. hominis* by direct microscopy in sex and age groups in DS samples from Mohammadpur area (2013).

Prevalence of *B. hominis* in overall monthly stool (MS) samples from Mirpur area according to sex and age groups during 2012-2013 from Mirpur area:

During the study period (2012-13), in MS samplesfrom Mirpur area, by *in vitro* culture, 34.25% prevalence of *B. hominis* was observed **(Table 4)**. The highest prevalence (43.92%) was observed in age group 61-72 months and the lowest prevalence (13.66%) in age group 13-24. By the analysis of variance prevalence of *B. hominis*by age groups of children was highly significant (F=5.186 and p=0.000**). In age group of 13-24 months and 61-72, the prevalence were higher in male than female **(Fig. 13)**. While, the prevalence of *B. hominis*among the sexes of children was significantly different (F=4.881 and p=0.027*).

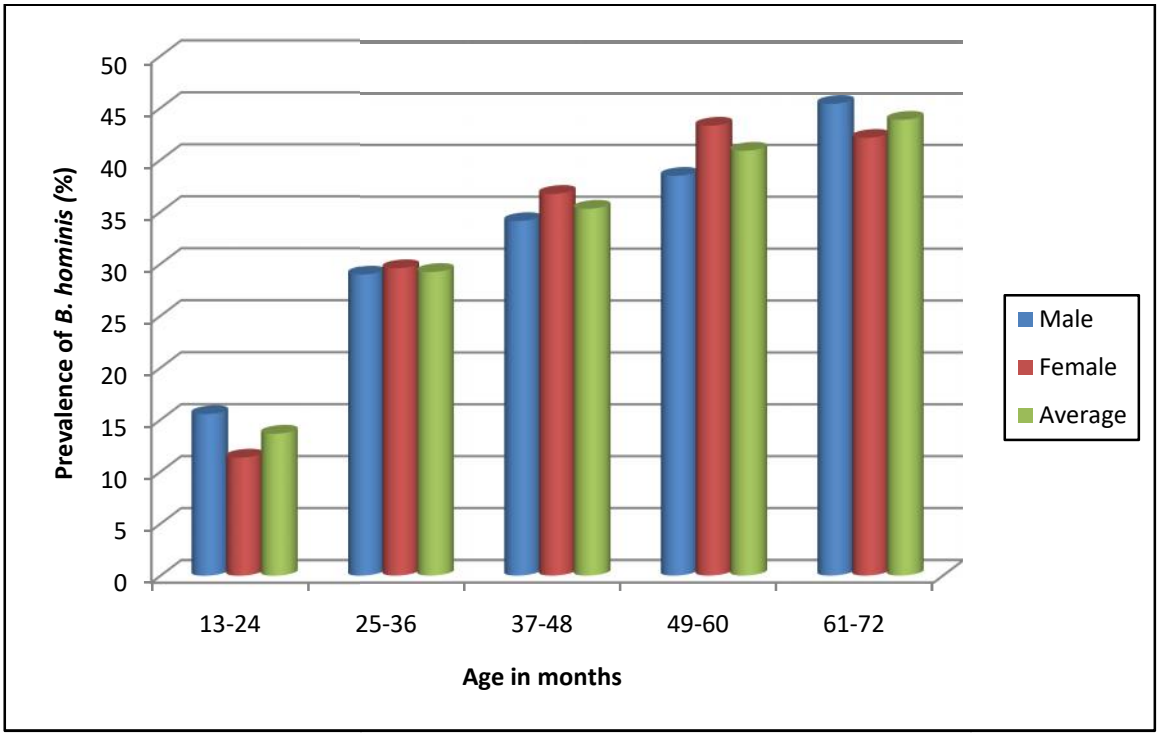


Fig. 13. Prevalence of *B. hominis* by *in vitro* culture in sex and age groups in overall MS samples from Mirpur area, during 2012-2013.

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

Interpretation

The prevalence of *B. hominis* in overall monthly stool samples from Mirpur area, between the age groups of children were significantly different. The children of age group 49-60 months and 61-72 months were more sensitive than other age groups to the infection.

Prevalence of *B. hominis* in monthly stool (MS) samples according to sex and age groups during2012:

In 2012, by *in vitro* culture, 31.58%prevalence wasfound for *B. hominis***(Table 4)**. In children of all age groups,except 13-24 months, the prevalence of *B. hominis* were higher in female.While the prevalence of *B. hominis*in male and femalewas not significantly different (F=2.892 and p=0.089^{ns}).The highest prevalence (42.95%) was observed in age group 49-60 months and the lowest (13.66%) was observed in age group 13-24 **(Fig. 14)**. By the analysis of variance, the difference among the prevalence of *B. hominis*by age groups was highly significant(F=5.451 and p=0.000**).

Prevalence of *B. hominis* in monthly stool (MS) samples according to sex and age groups during 2013:

In 2013, by *in vitro* culture, 37.16%samples found positive for *B. hominis***(Table 4)**. In age group of 25-36 months and 61-72 months, the prevalence of *B. hominis* were higher in male (32.10% and 45.45%)than that of female. The highest prevalence (43.92%) was observed in children of age group 49-60 months and the lowest prevalence (31.99%) was observed in children of age group 13-24 months**(Fig. 15)**.By the analysis of variance, the difference among the prevalence of *B. hominis*by age groups was highly significant(F=1.619 and p=0.005**)

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

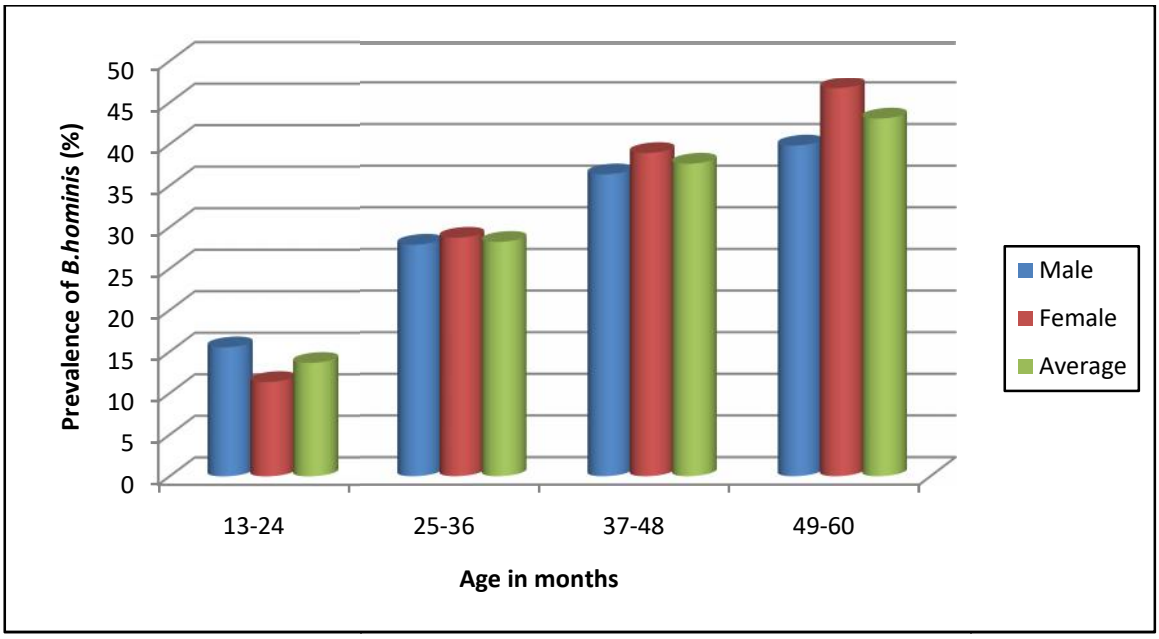


Fig.14.Prevalence of *B. hominis* by *in vitro* culture in sex and age groups in MS samples from Mirpur area (2012).

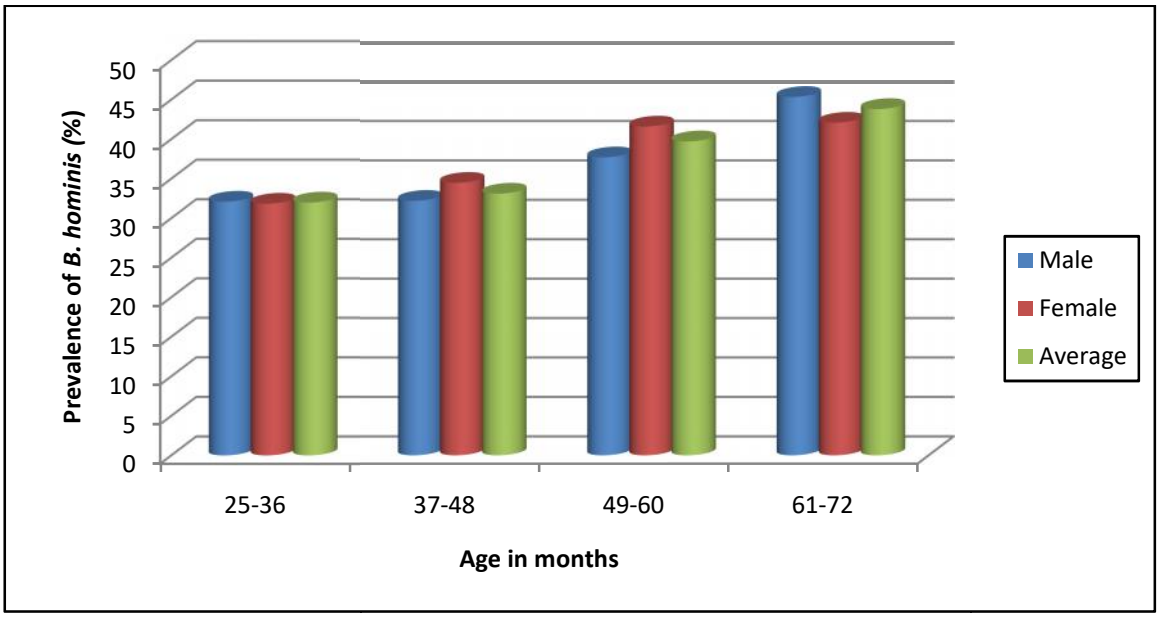


Fig.15.Prevalence of *B. hominis* by *in vitro* culture in sex and age groups in MS samples from Mirpur area (2013).

Prevalence of *B. hominis* in overall diarrheal stool (DS) samples according to sex and age group:

During the study period (2012 and 2013), in DS samples from Mirpur area,by *in vitro* culture, 27.32% was positive for *B. hominis***(Table 4)**.In the children of age group 13-24 months and 37-48 months, the prevalence of *B. hominis* were higher in female than that of male. While, in age group 61-72 months, 45.45% found in male and 36.36% in female. The highest prevalence (40.91%) was observed in age group 61-72 months and the lowest (13.38%) was observed in age group 13-24 months**(Fig. 16)**.The prevalence of *B. hominis*among the sexesand by age groups of children was not significantly different ($p=0.454^{ns}$ and $p=0.111^{ns}$)by statistical test (ANOVA).

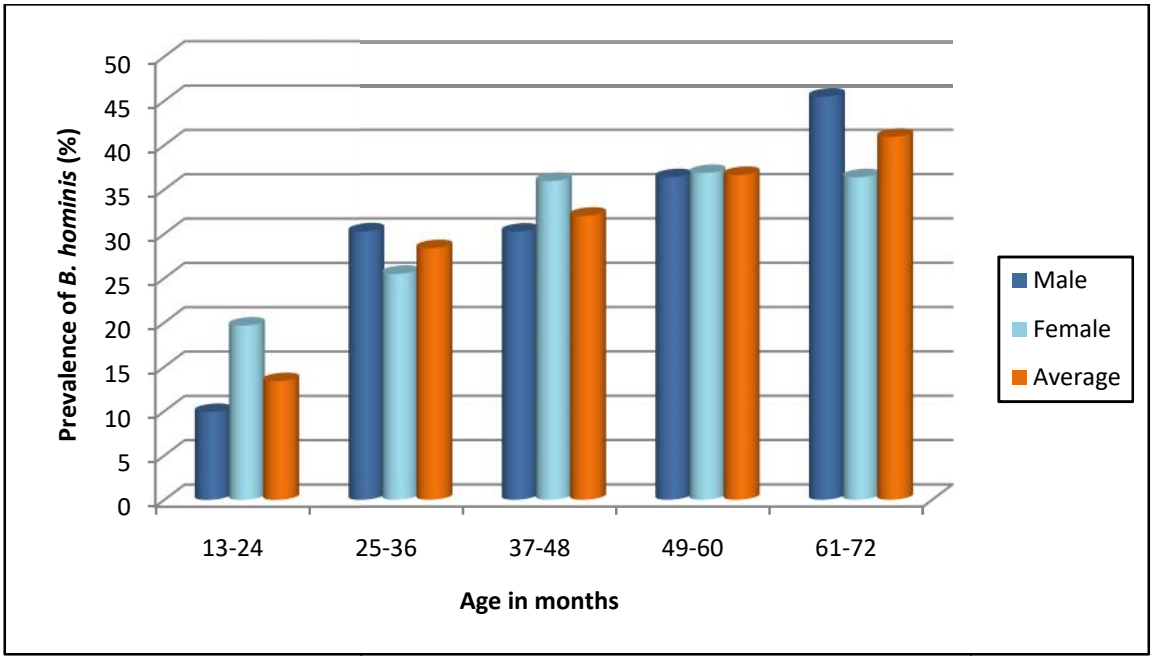


Fig. 16. Prevalence of *B. hominis* by *in vitro* culture in sexand age groups in overall DS samples from Mirpur area,during 2012-13

***significant, ** highly significant ($p\leq0.05$) and ^{ns} not significant ($p\geq0.05$) at 5% level**

Interpretation

The prevalence of *B. hominis* in overall diarrheal stool samples from Mirpur area, between the age groups of children were not significantly different. The children of age group 61-72 monthswasmore sensitive than other age groups to the infection.

Prevalence of *B. hominis* in diarrheal stool (DS) samples according to sex and age groups during 2012:

By *in vitro* culture, out of 422 DS samples, 29.62% found positive for *B. hominis***(Table 4)**.The prevalence of *B. hominis*in age group of 25-36 months was higher in male (39.81%) than female (28.57%). In the age group of 37-48 months, prevalence of *B. hominis*(50%)was higher in male than female (40%).The highest prevalence (46.15%) was observed in age group 37-48 months and the lowest (13.38%) was observed in age group 13-24 **(Fig. 17)**. The prevalence of *B. hominis*by age groups of children was highly significant(F=1.665 and p=0.007**).

Prevalence of *B. hominis* in diarrheal stool (DS) samples according to sex and age groups during 2013:

By *in vitro* culture, out of 299DS samples, 24.08% found positive for *B. hominis***(Table 4)**.In age group of 49-60 months was found more prevalent in male (40.91%)children than female (28.57%).The highest prevalence (40.91%) was observed in age group 61-72 months and the lowest (16.67%) in age group 25-36**(Fig.18)**. By the analysis of variance prevalence of *B. hominis*by age groups of children was not significantly different (F=1.047 and p=0.399^{ns}).

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

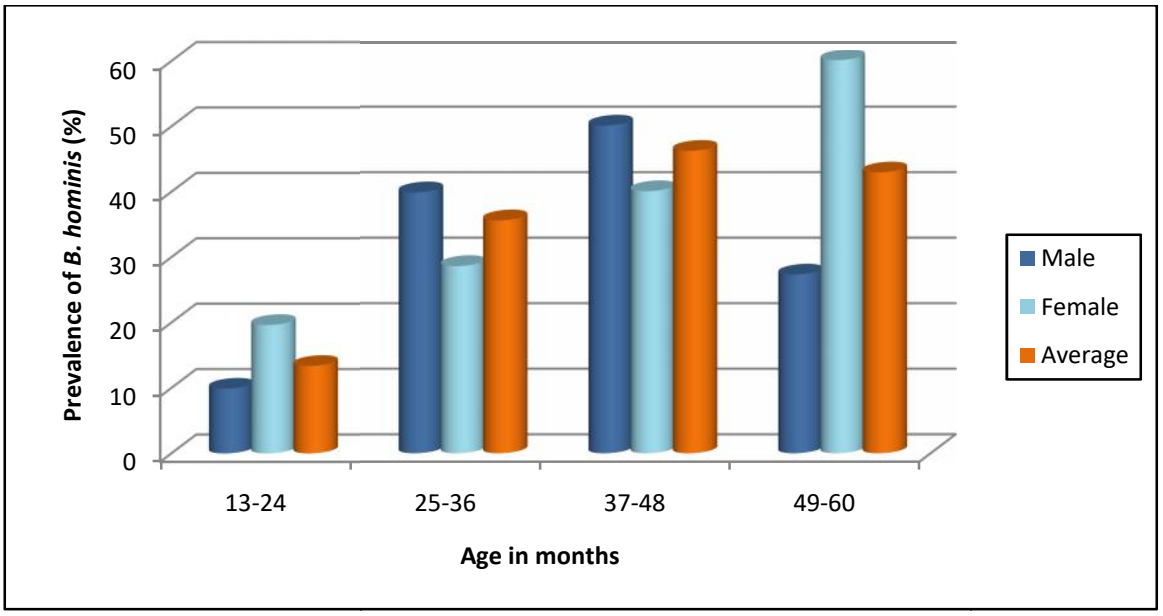


Fig.17. Prevalence of *B. hominis* by *in vitro* culture in sexand age groups in DS samples from Mirpur area (2012).

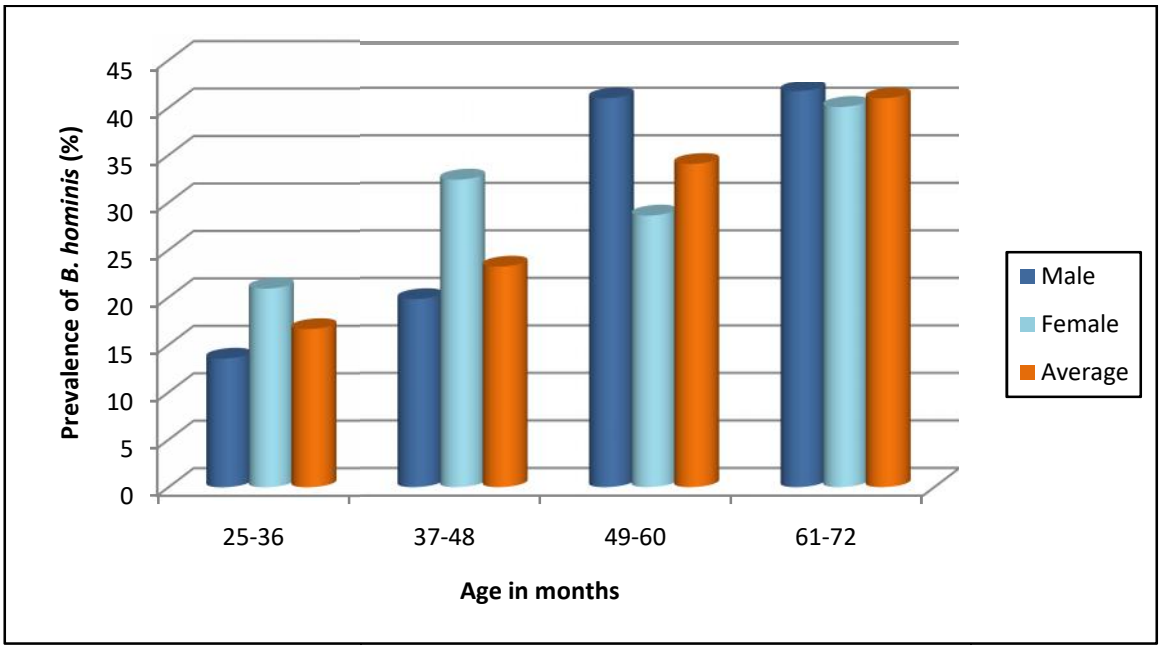


Fig.18. Prevalence of *B. hominis* by *in vitro* culture in sexand age groups in DS samples from Mirpur area (2013).

Table 4. Prevalence of *B. hominis*by *in vitro* culture in sex groups of children from two areas.

Study period in year	Sample Type	Total		Male		Female	
		No of samples	*B.h* positive (%)	No of samples	*B.h*positive (%)	No of samples	*B.h*positive (%)
Overall (Mirpur area)		**9322**	3143 **(33.72%)**	5136	1675 **(32.61%)**	4186	1468 **(35.07%)**
2012	MS	4481	1415 (31.58%)	2449	746 (30.46%)	2032	669 (32.92%)
2013	MS	4120	1531 (37.16%)	2241	811 (36.19%)	1879	720 (38.32%)
Total MS		8601	2946 **(34.25%)**	4690	1557 **(33.20%)**	3911	1389 **(35.52%)**
2012	DS	422	125 (29.62%)	263	78 (29.66%)	159	47 (29.56%)
2013	DS	299	72 (24.08%)	184	40 (21.74%)	115	32 (27.83%)
Total DS		721	197 **(27.32%)**	446	118 **(26.46%)**	275	79 **(28.73%)**
Overall (Mohammadpur area)		**3218**	**1360 (42.26%)**	**1421**	**591 (41.59%)**	1797	**769 (42.79%)**
2012	MS	1169	528 (45.17%)	518	243 (46.91%)	651	285 (43.78%)
2013	MS	1535	624 (40.65%)	641	248 (38.69%)	894	376 (42.06%)
Total MS		2704	1152 **(42.60%)**	1159	491 **(42.36%)**	1545	661 **(42.78%)**
2012	DS	237	103 (43.46%)	119	48 (40.34%)	118	55 (46.61%)
2013	DS	277	105 (37.91%)	143	52 (36.36%)	134	53 (39.55%)
Total DS		514	208 **(40.47%)**	262	100 **(38.17%)**	252	108 **(42.86%)**

Prevalence of *B. hominis* in overall monthly stool (MS) samples according to sex and age group from Mohammadpur area:

During the study period (2012 and 2013), by *in vitro* culture,out of 2704 MS samples from Mohammadpur area, 42.60% were found positive for*B. hominis***(Table 4)**.In children of age group 37-48 months and 61-72 months, the prevalence of *B. hominis* were higher in male children than female**(Fig. 19)**.The prevalence of *B. hominis*by age groups of children was not significantly different at 5 % level (F=0.651 and p=0.661^{ns}).

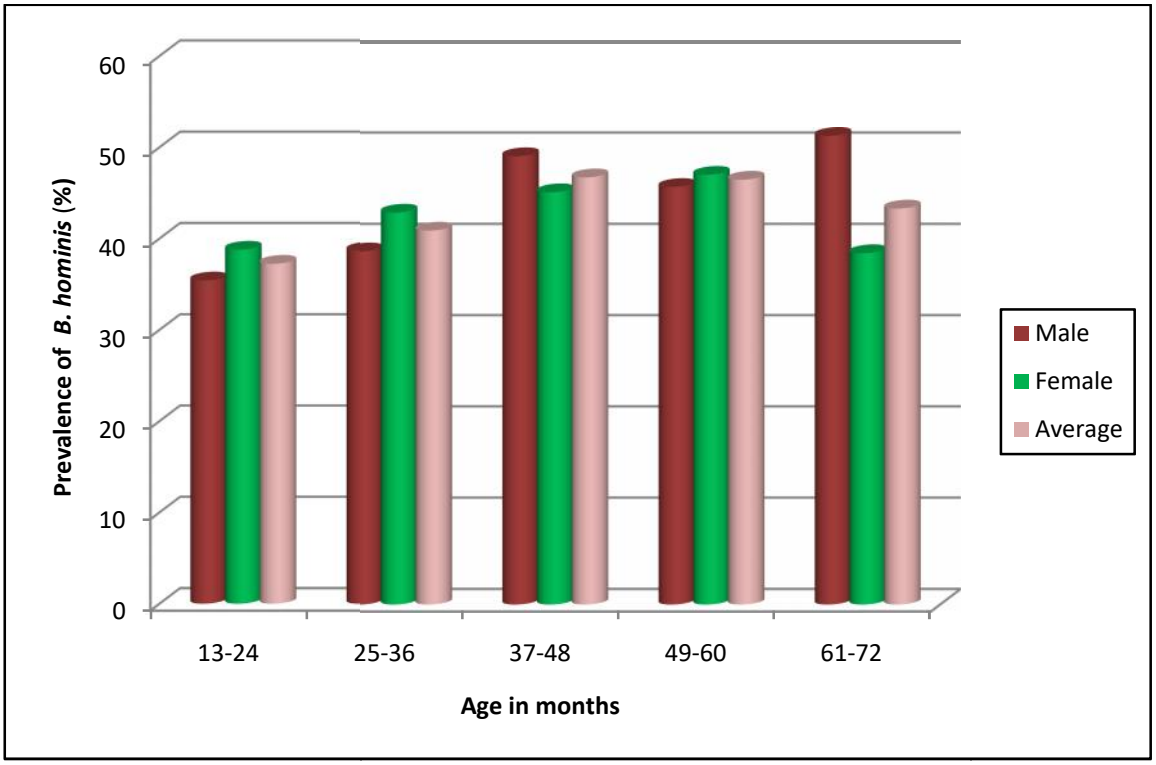


Fig. 19. Prevalence of *B. hominis* by *in vitro* culture according to sex and age groups in overall MS samples fromMohammadpur area during 2012-2013.

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

Interpretation

In the age group of children above 37 months age were more sensitive than below 36 months age. Infection rate increases, when age of children by month increases.

Prevalence of *B. hominis* in monthly stool (MS) samples according to sex and age groups during 2012:

Total 1169 monthly sampleswere examined, 45.17% were found positive for *B. hominis* by *in vitro* culture **(Table 4)**. The highest and almost similar prevalence (50.81% and 50.63%)were observed in the age group of 37-48 months and 49-60 months children, respectively**(Fig. 20)**.By the analysis of variance, prevalence of *B. hominis*among the sexesand age groups of children werenot significantly different ($p=0.343^{ns}$ and $p=0.200^{ns}$).

Prevalence of *B. hominis* in monthly stool (MS) samples according to sex and age groups during 2013:

Out of 1535 monthly stool samples, 40.65% were found positive for *B. hominis* by *in vitro* culture **(Table 4)**. Most of the age group of children showed similar prevalence of *B. hominis* in both sexes. Only in children of age group 61-72 months, the prevalence of *B. hominis* were higher in male than female **(Fig. 21)**.The difference among the prevalence of *B. hominis*in the sexesand by age groups of children were not significant at 5% level ($p=0.211^{ns}$ and$p=0.730^{ns}$) by ANOVA.

***significant, ** highly significant ($p \leq 0.05$) and ^{ns} not significant ($p \geq 0.05$) at 5% level**

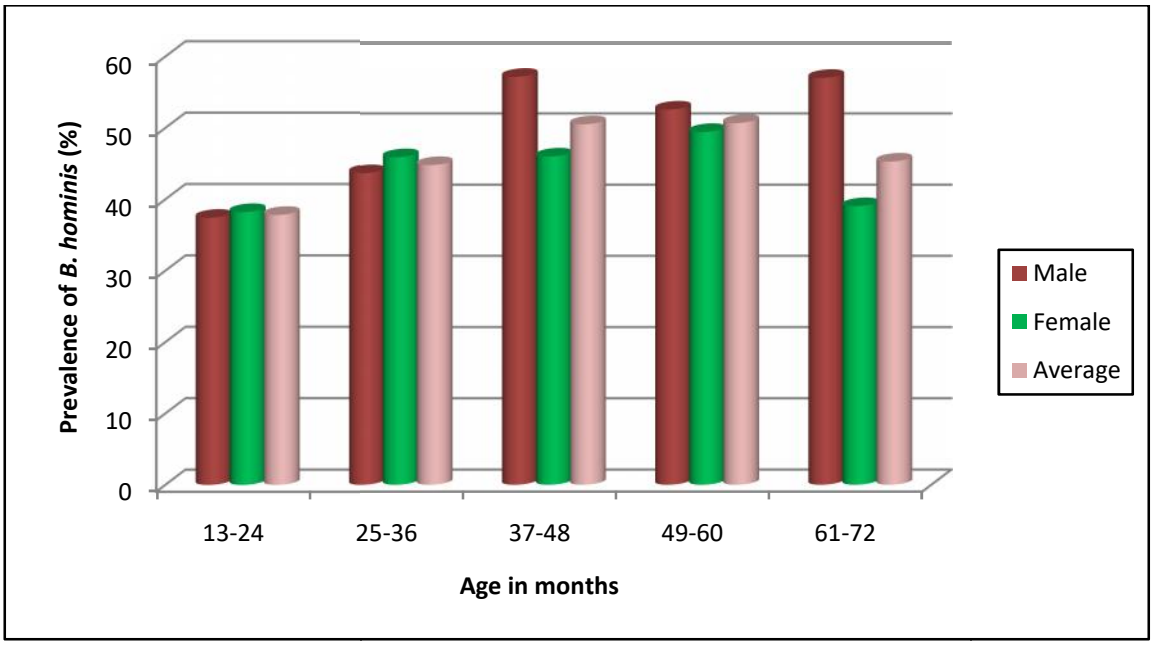


Fig.20. Prevalence of *B. hominis* by *in vitro* culture in sex and age groups in MS samples fromMohammadpur area (2012).

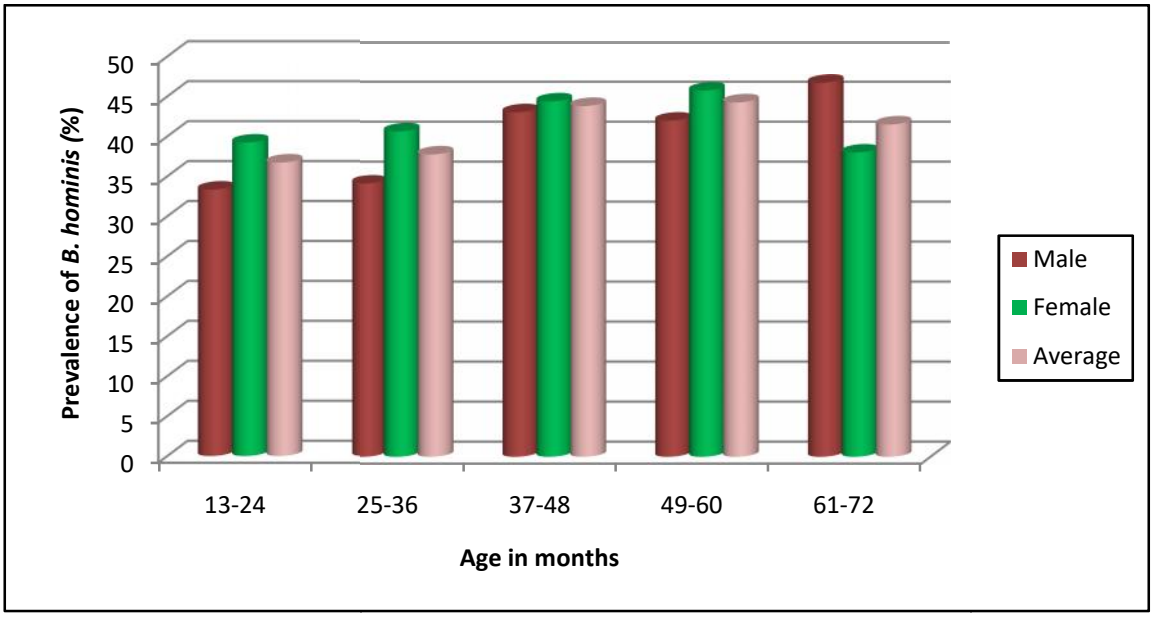


Fig.21. Prevalence of *B. hominis* by *in vitro* culture according to sex and age groups in MS samples fromMohammadpur area (2013).

Prevalence of *B. hominis* in overall diarrheal stool (DS) samples according to sex and age group

By*in vitro* culture method,out of 514 DS samples from Mohammadpur area,40.47% were found positive for *B. hominis*during 2012-2013 **(Table 4)**.The prevalence of *B. hominis*in female (41.38%) was higher in age group 25-36 months.While the prevalence of *B. hominis*among the sexesof children was not significantly different at 5% level (F=0.346 and $p=0.557^{ns}$).The higher prevalence observed in the age group of 37-48 months (60.42%) and 49-60 months (56.52%) children**(Fig. 22)**. The difference among the prevalence of *B. hominis*by age groups of children was not significantly different (F=1.262 and $p=0.103^{ns}$)by ANOVA.

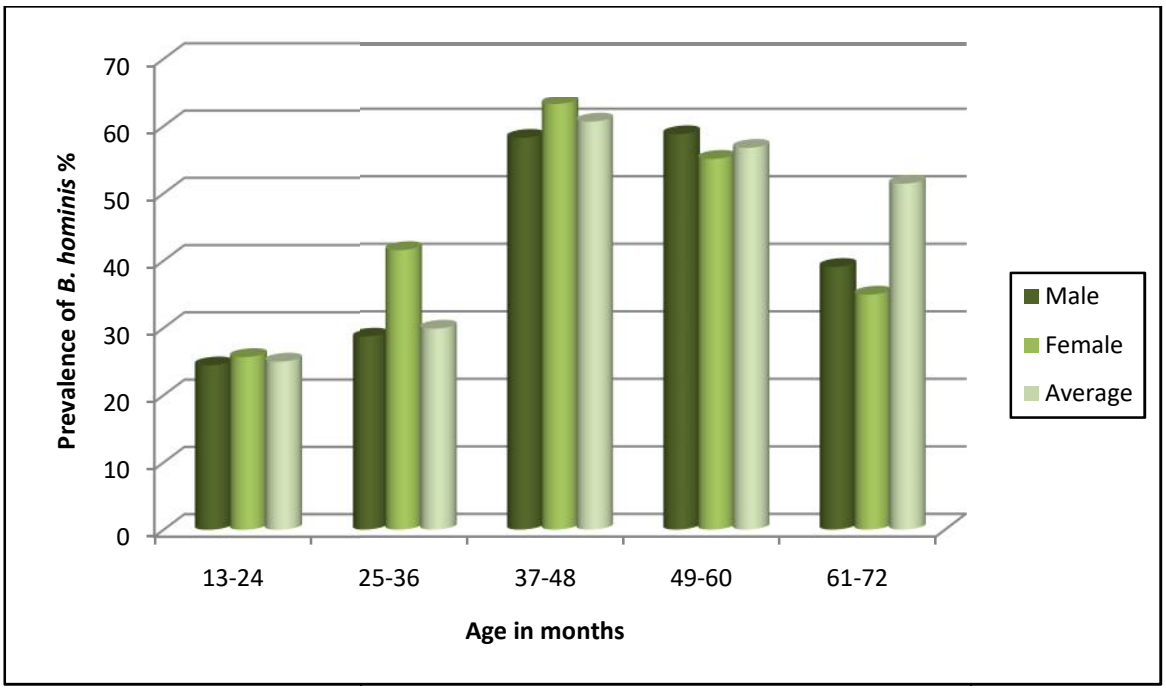


Fig. 22. Prevalence of *B. hominis* by *in vitro* culture according to sex and age groups in overall DS samples fromMohammadpur area during 2012-2013.

***significant, ** highly significant ($p \leq 0.05$) and ^{ns} not significant ($p \geq 0.05$) at 5% level**

Interpretation

In the age group of children above 37 months upto 72 months age were more sensitive than below 36 months age group to the infection for diarrhea. Because the age range 37-72 months age group were exposed to external environment. Infection rate increases, when age of children by month increases.

Prevalence of *B. hominis* in DS samples according to sex and age groups during 2012:

Out of 237 diarrheal examined stool samples, 103 found positive for *B. hominis* by *in vitro* culture (43.46%) **(Table 4)**. In children of age group 13-24 months, prevalence was higher in female (33.33%)than male (25%). While the prevalence of *B. hominis*among the sexesof children was not significantly different at 5% level (F=0.003 and p=0.954^{ns}).Comparatively the higher prevalence 70% and 53.85% were observed in children of age group 37-48 and 61-72 monthsrespectively**(Fig. 23)**. By the analysis of variance prevalence of *B. hominis*by age groups of children was significantly different at 5 % level (F=1.496 and p=0.017**).

Prevalence of *B. hominis* in DS samples according to sex and age groups during 2013:

Out of 277 diarrheal samples, 105 found positive for *B. hominis* by *in vitro* culture(37.91%) **(Table 4)**. In children of age group 13-24 monthsand 49-60 months prevalence of *B. hominis*were higher in male than female.The prevalence of *B. hominis*among the sexesof children was not significantly different at 5% level (F=0.003 and p=0.954^{ns}).Thehigher prevalence 53.57% and 51.79% were observed in children of age group 37-48 months and 49-60 monthsrespectively**(Fig. 24)**.The statistical test (ANOVA), showed that prevalence of *B. hominis*by age groups of children was significantly different at 5 % level (F=1.496 and p=0.017*).

***significant, ** highly significant (p≤0.05) and ^{ns} not significant (p≥0.05) at 5% level**

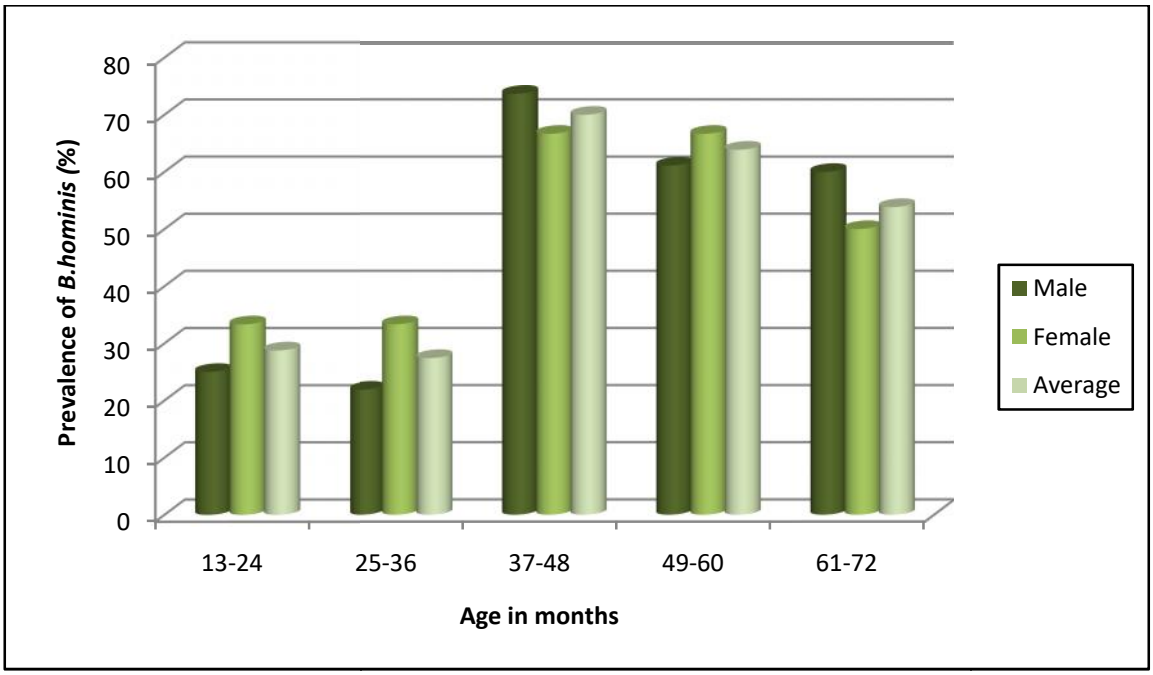


Fig. 23. Prevalence of *B. hominis* by *in vitro* culture according to sex and age groups in DS samples fromMohammadpur area (2012).

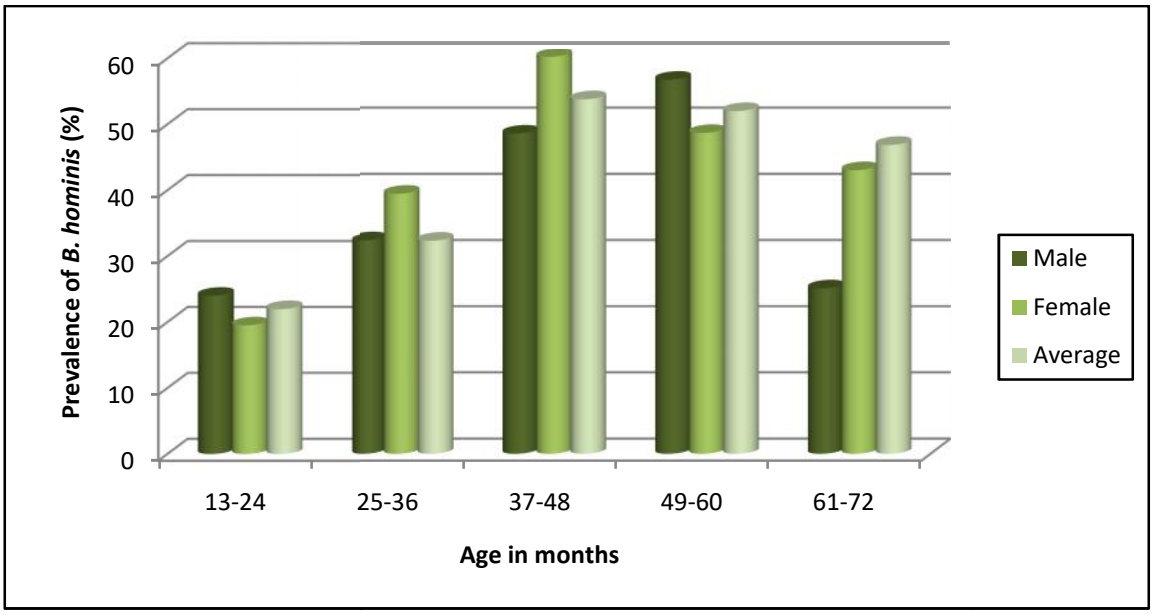


Fig. 24. Prevalence of *B. hominis* by *in vitro* culture according to sex and age groups in DS samples from Mohammadpur area (2013).

COMPARISON BETWEEN PREVALENCE OF *Blastocystis hominis*IN MS AND DS SAMPLES FROM MIRPUR AREA BY TWO METHODS:

During the study period (2012 and 2013), out of 8601overall monthly stool (MS) samplesfrom Mirpur area, the prevalence of *B. hominis* by direct microscopy was 3.72% while in culture it was 34.25%. In the same duration, out of 721 diarrheal stool (DS) samples, the prevalence of *B.hominis* by direct microscopy was 5.83% while in culture it was 27.32% (Fig. 25).

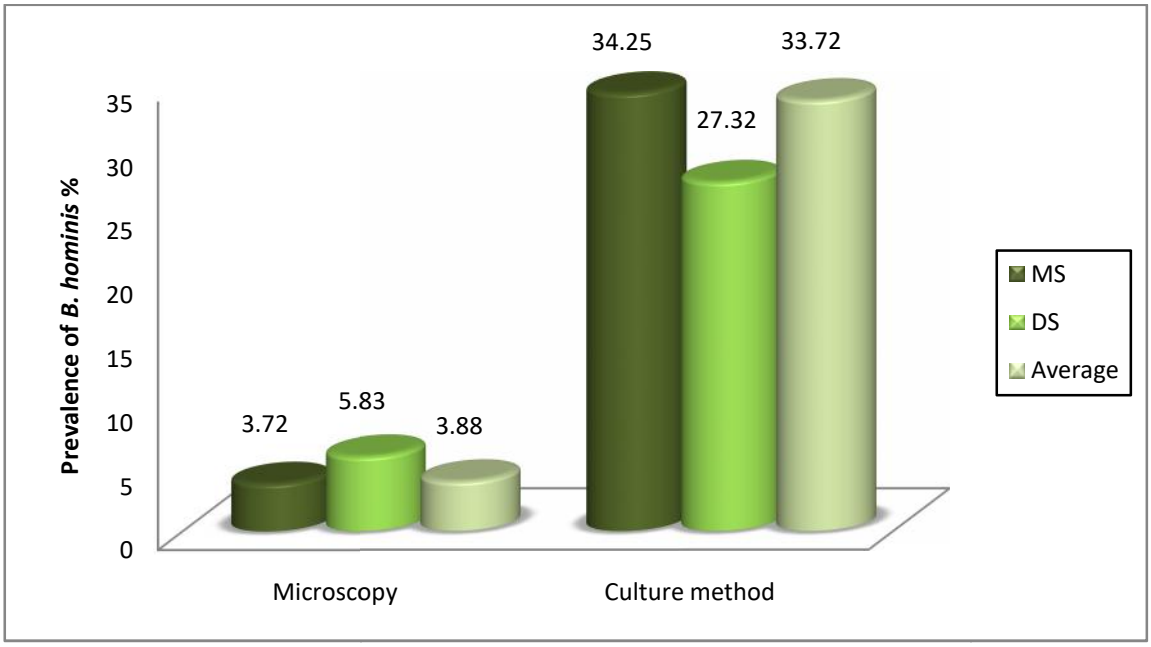


Fig. 25.The prevalence of *B. hominis* by microscopy and *in vitro* culture in MS and DS samplesfrom Mirpur area duringthe study period (2012 and 2013).

Interpretation

In culture method, prevalence of *B.hominis* found higher both in monthly and diarrheal stool samples from Mirpur area. So, it can be apprehend that *B.hominis* infection are not always show diarrheal symptoms.

In 2012, by direct microscopy, the prevalence of *B. hominis* was 3.53%, while by culture method it was 31.58% inMS samplesfrom Mirpur area. Out of 422DS samples, by direct microscopy the prevalence of *B.hominis* was 4.74%, while by*in vitro*culture method it was 29.62% (Fig. 26).

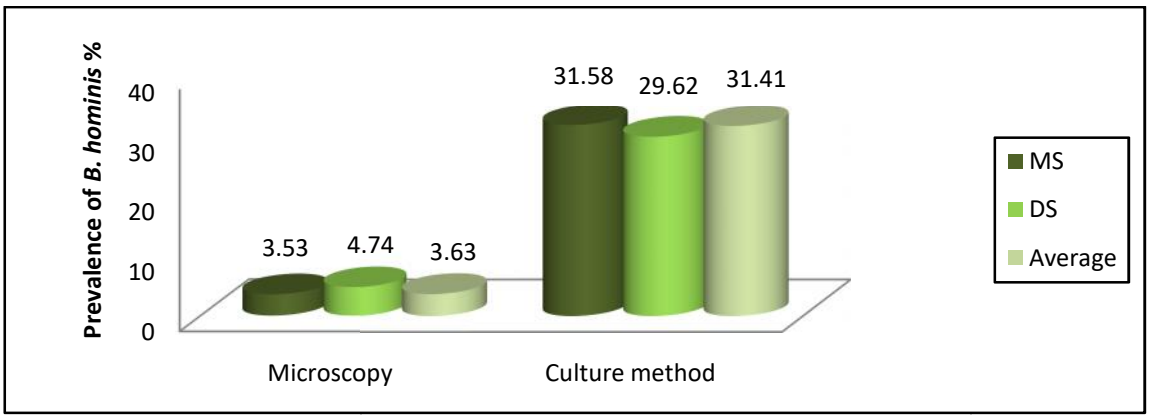


Fig.26.The prevalence of *B. hominis* by microscopy and *in vitro* culture in MS and DS samplesfrom Mirpur area during 2012.

In 2013, out of 4120MS samplesfrom Mirpur area, by direct microscopy the prevalence of *B. hominis* was 3.93% while by culture method it was 37.16%. In the same year, out of 299 diarrheal stool (DS) samples, by direct microscopy the prevalence of *B.hominis* was 7.36% while by *in vitro*culture method it was 24.08% (Fig. 27).

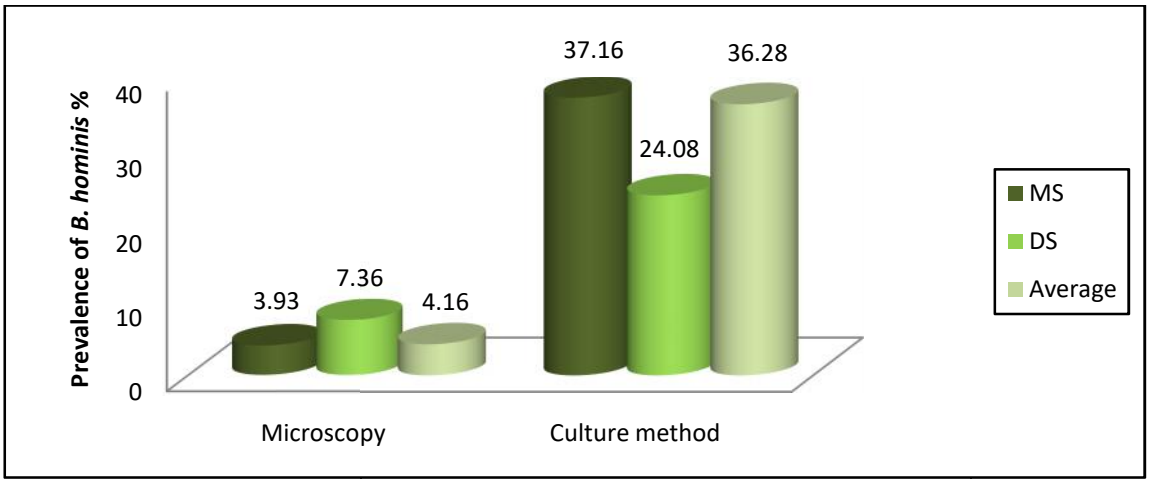


Fig. 27.The prevalence of *B. hominis* by microscopy and *in vitro* culture in MS and DS samplesfrom Mirpur area during 2013.

COMPARISON BETWEEN PREVALENCE OF *Blastocystis hominis*IN MS AND DS SAMPLES FROMMOHAMMADPUR AREA BY TWO METHODS:

During the study period (2012 and 2013), out of 2704monthly stool (MS) samplesfrom Mohammadpur area, by direct microscopy the prevalence of *B. hominis* was 7.25% while by culture it was 42.75%. In the same duration, out of 514 diarrheal stool (DS) samples, by direct microscopy the prevalence of *B.hominis* was 3.93% while by culture it was 40.47% (Fig. 28).

By microscopy, higher prevalence (9.73%) was observed in DS samples than MS samples. While by *in vitro* culture higher prevalence (42.75%) was observed in MS samples than DS samples (Fig. 28).

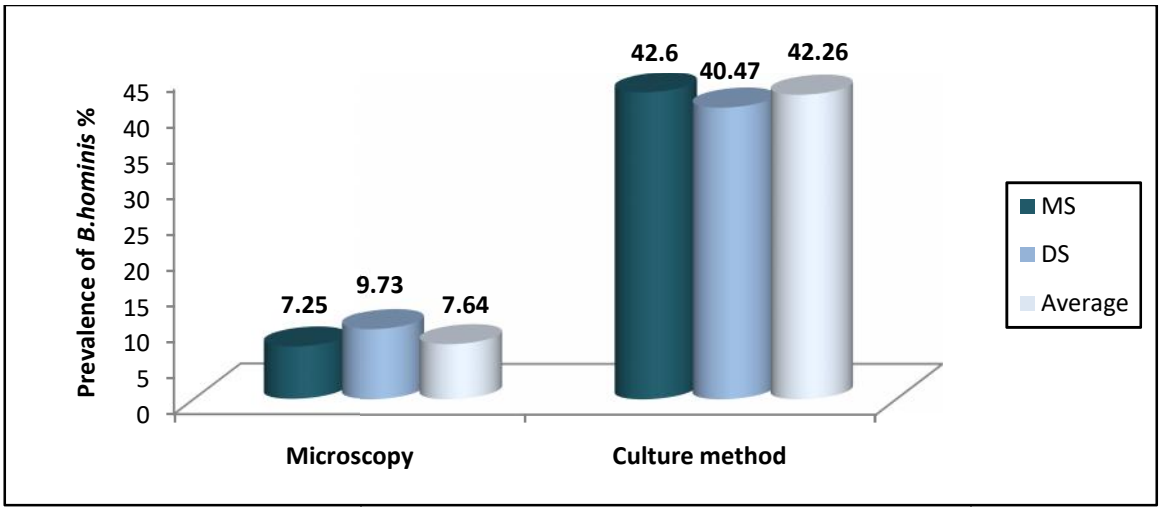


Fig.28.The prevalence of *B. hominis* by microscopy and *in vitro* culture in MS and DS samplesfrom Mohammadpur area duringthe study period (2012 and 2013).

Interpretation

Both in MS and DS samples, the prevalence of *B.hominis* found higher by *in vitro* culture than microscopy. So *in vitro* culture method is the more sensitive method than microscopy.

MS samples = Monthly Stool samples and DS samples = Diarrheal Stool samples

In 2012, out of 1169monthly stool (MS) samples of Mohammadpur area, by direct microscopy the prevalence of *B. hominis* was 9.15% while by culture method it was 45.51%. Out of 237 diarrheal stool (DS) samples, by direct microscopy the prevalence of *B.hominis* was 9.28% while in culture it was 41.77% (Fig. 29).

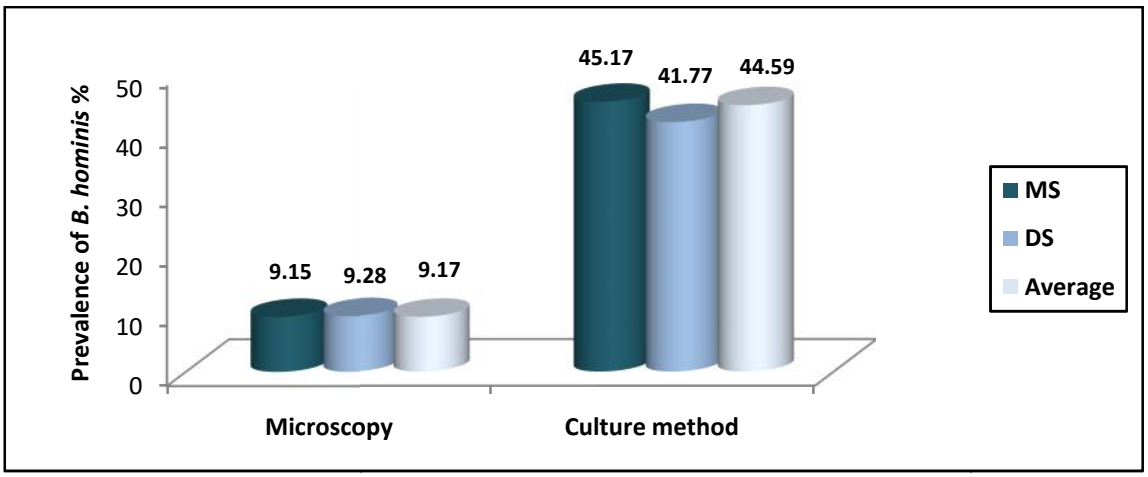


Fig. 29.The prevalence of *B. hominis* by microscopy and *in vitro* culture in MS and DS samplesfrom Mohammadpur area during 2012.

In 2013, by microscopy, higher prevalence (10.11%) were observed in diarrheal stool (DS) samples than monthly stool (MS) samples of children of Mohammadpur area. By *in vitro* culture higher prevalence (40.65%) were observed in monthly stool samples (MS) than diarrheal stool (DS) samples of children of Mohammadpur area (Fig.30).

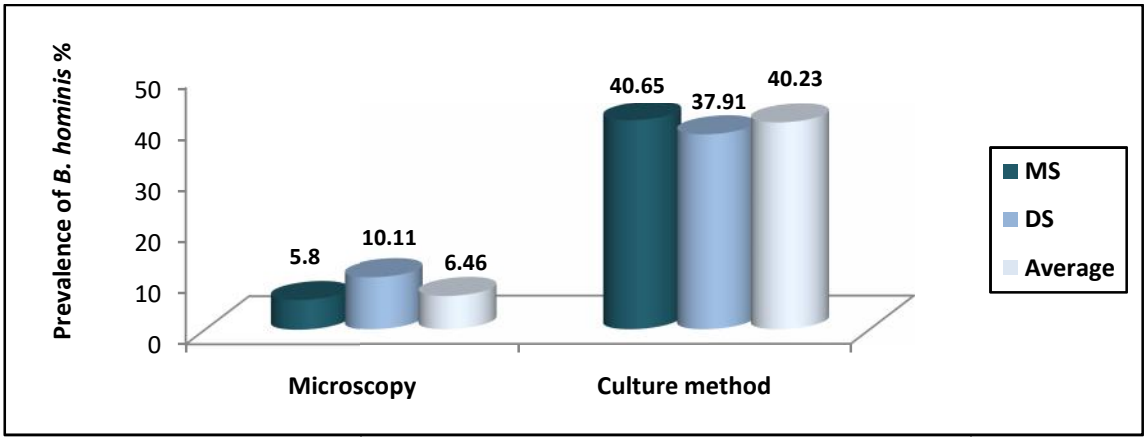


Fig.30.The prevalence of *B. hominis* by microscopy and *in vitro* culture in MS and DS samplesfrom Mohammadpur area during 2013.

Comparison of seasonal prevalence of *B. hominis* by two techniques in MS samples from Mirpur area.

By microscopy, the highest prevalence of *B. hominis* (5.03%) was in rainy season and the lowest was 2.98 % in winter (Fig.31) and the difference of prevalence of *B. hominis*among three seasons were significant (F=6.84 and p=0.016*) (Table 5). By *in vitro* culture method, the highest prevalence of *B. hominis* (35.73%) was in rainy season, while the lowest was 31.58 % in winter (Fig.31).

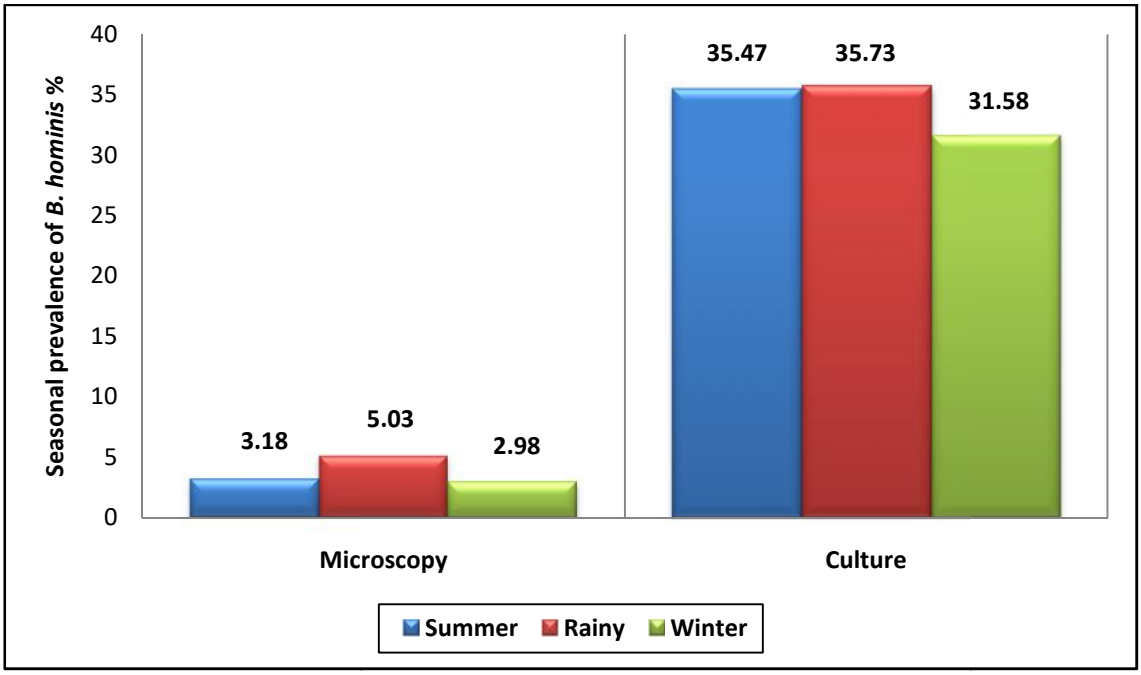


Fig. 31. Overall seasonal prevalence of *B. hominis* in MS samples by microscopy and *in vitro* culture.

Interpretation

Rainy season is more effective than other season to the infection in MS samples from Mirpur area.

Seasonal prevalence of *B. hominis*by microscopy and *in vitro* culture method in MSsamples from Mirpur Area.

By microscopy, in both years (2012 and 2013), the highest prevalence of *B. hominis*(5.03% and 5.04%) were found in rainy season. In 2012, the lowest prevalence (2.67%) was found in Summer'12, while in 2013,it was found in winter season (Fig. 32).

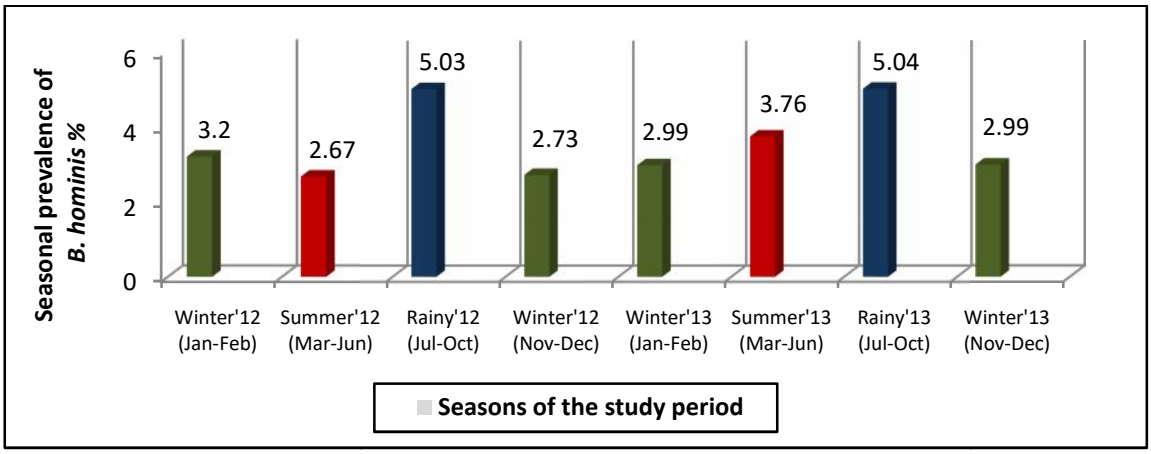


Fig. 32. Seasonal prevalence of *B. hominis* in MS samples by microscopy.

By *in vitro* culture method, in 2012, the highest prevalence of *B. hominis* was found 36.61% in Winter'12 (Nov-Dec) and in 2013 it was found 42.99% in Winter'13 (Nov-Dec). While in both years, the minimum prevalence of *B. hominis* (21.9% and 26.21%), observed in winter (Jan-Feb).In both years, prevalence(35.99% and 35.47%)almost similar in rainy season. (Fig. 33).

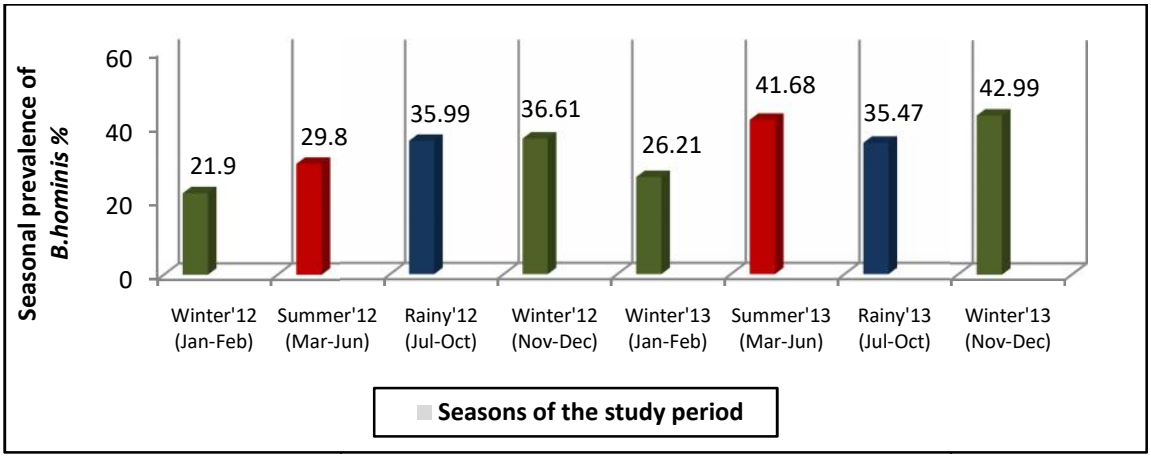


Fig. 33. Seasonal prevalence of *B. hominis* in MS samples by *in vitro* culture.

Comparison of seasonal prevalence of *B. hominis* by two techniques in DS samples from Mirpur area.

By microscopy,in rainy season the highest prevalence of *B. hominis* was 10.34 % and the lowest was 3.59 % in winter (Fig.34).The difference of prevalence of *B. hominis*among three seasons was not significant (F=1.994 and p=0.192^{ns}) (Table 5).

By *in vitro* culture method, in summer season the highest prevalence of *B. hominis* was 34.80 % and the lowest was 14.74 % in winter (Fig.34) and the difference of prevalence of *B. hominis*among three seasons was significant (F=3.465 and p=0.077*) at 7% level (Table 5).

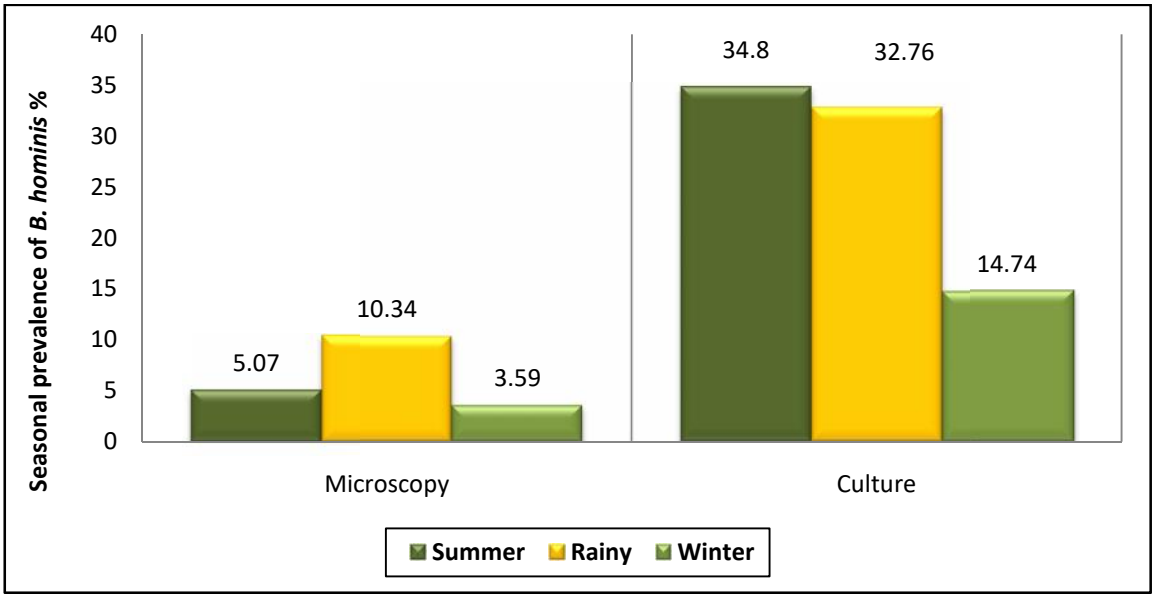


Fig.34. Overall seasonal prevalence of *B. hominis* in DS samples by microscopy and *in vitro* culture.

Interpretation

Summer and rainy seasonsare more effective than winter season to the infection in diarrheal stool (DS) samples from Mirpur area.

***Bh=Blastocystis hominis*, MS samples=Monthly Stool samples and DS samples=Diarrheal Stool samples**

Seasonal prevalence of *B. hominis* by microscopy and*in vitro* culture method in DS samples from Mirpur Area.

In 2012, by microscopy, the highest prevalence of *B. hominis* was found 8.05% in Rainy'12 and in 2013 it was found 9.82% in Summer'13. In both years, the lowest prevalence (3.31% and 4%) were found in winter (Fig. 35).

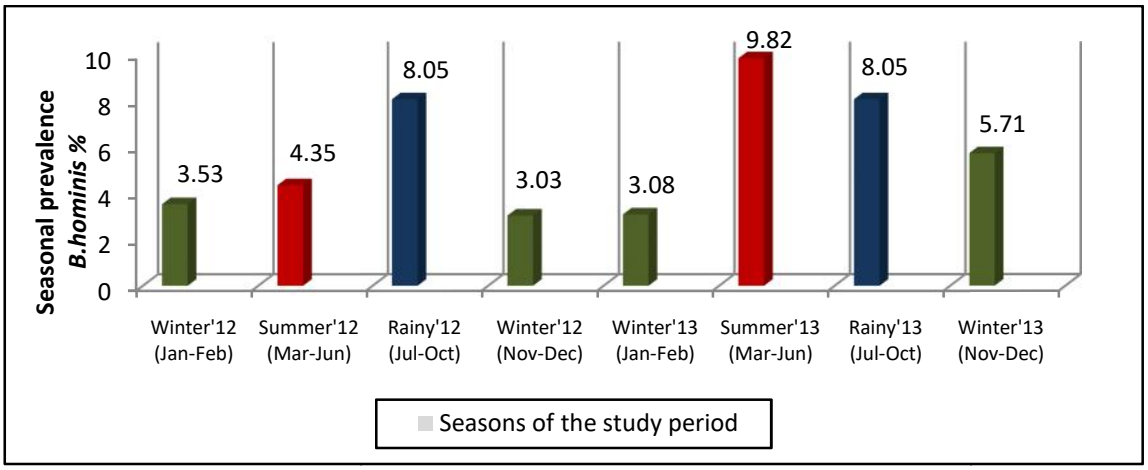


Fig. 35. Seasonal prevalence of *B. hominis* in DS samples by microscopy.

By *in vitro* culture, in 2012, the highest prevalence of *B. hominis* was found 37.93% in Rainy'12 and in 2013 it was found 33.04% in Summer'13. In both years, in winter from January to February, the lowest prevalence (16.47% and 4.62%) were found in winter (Fig. 36).

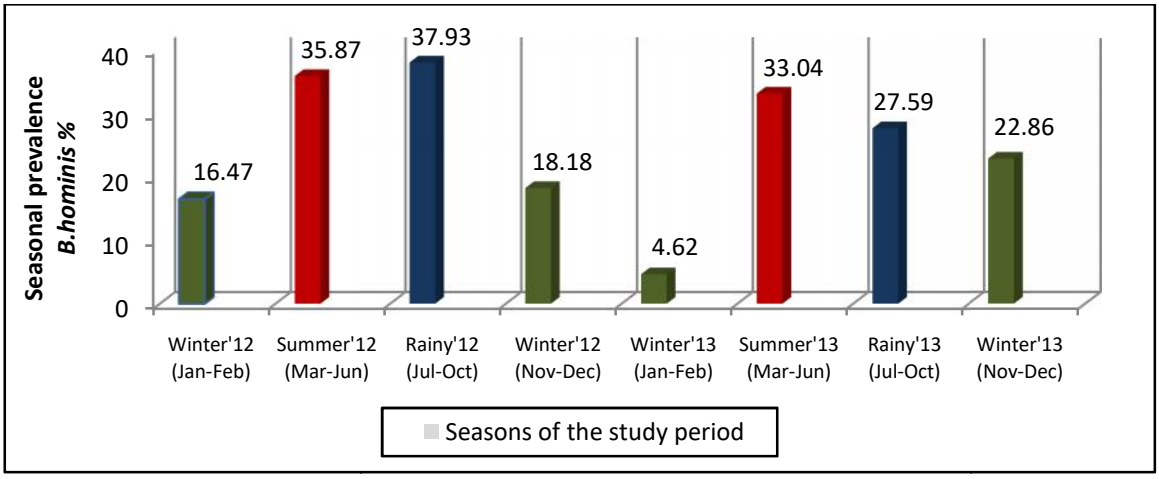


Fig. 36. Seasonal prevalence of *B. hominis* in DS samples by *in vitro* culture.

Comparison of seasonal prevalence of *B. hominis* by two techniques in MS samples from Mohammadpur area.

By microscopy, in summer season the highest prevalence of *B. hominis* was 8.78 % and the lowest was 5.40 % in winter. In rainy season 7.89% was observed (Fig. 37).The difference of prevalence of *B. hominis*among three seasons was highly significant (F=10.52 and p=0.004**) (Table 5).

By *in vitro* culture method, in rainy season the highest prevalence of *B. hominis* was 48.14 % and the lowest was 35.32 % in winter.In summer season 46.81% was observed (Fig. 37).The difference of prevalence of *B. hominis*among three seasons was highly significant (F=48.62 and p=0.000**) (Table 5).

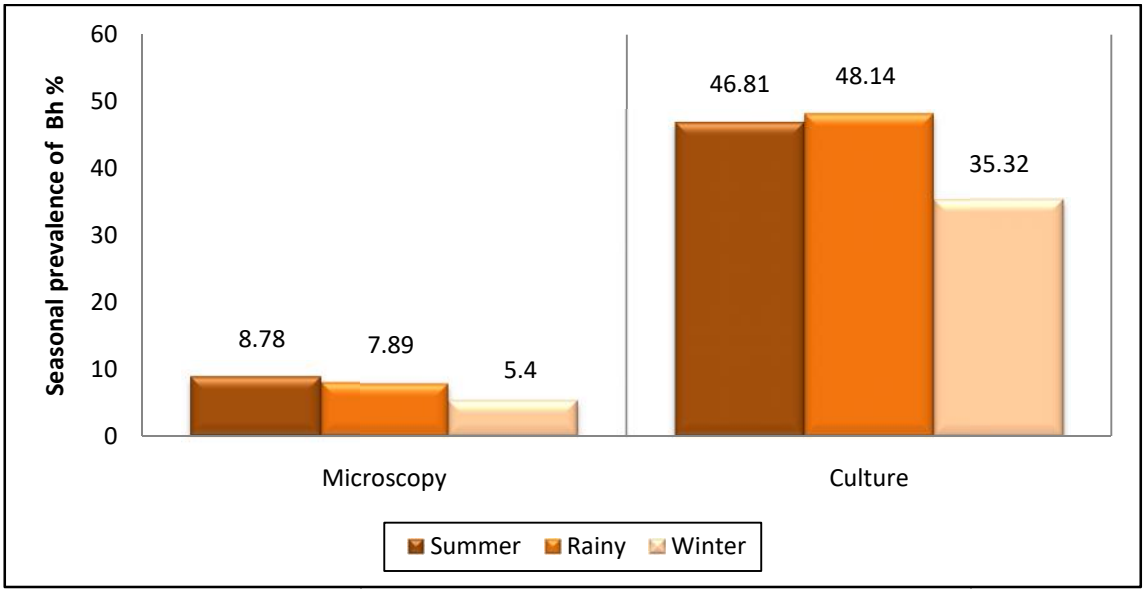


Fig.37. Overall seasonal prevalence of *B. hominis* in MS samples by microscopy and *in vitro* culture.

Interpretation

Summer and rainy seasonsare more effective than winter season to the infection in MS samples from Mohammadpur area.

***Bh=Blastocystis hominis*, MS samples=Monthly Stool samples and DS samples=Diarrheal Stool samples**

Seasonal prevalence of *B. hominis*by microscopy and by *in vitro* culturemethod in MS samples from Mohammadpur Area.

By microscopy, in 2012, the highest prevalence of*B. hominis* was found 11.86% in rainy'12 and in 2013 it was found 6.68% in summer'13. In both years, the lower prevalence were found in winter (Fig. 38).

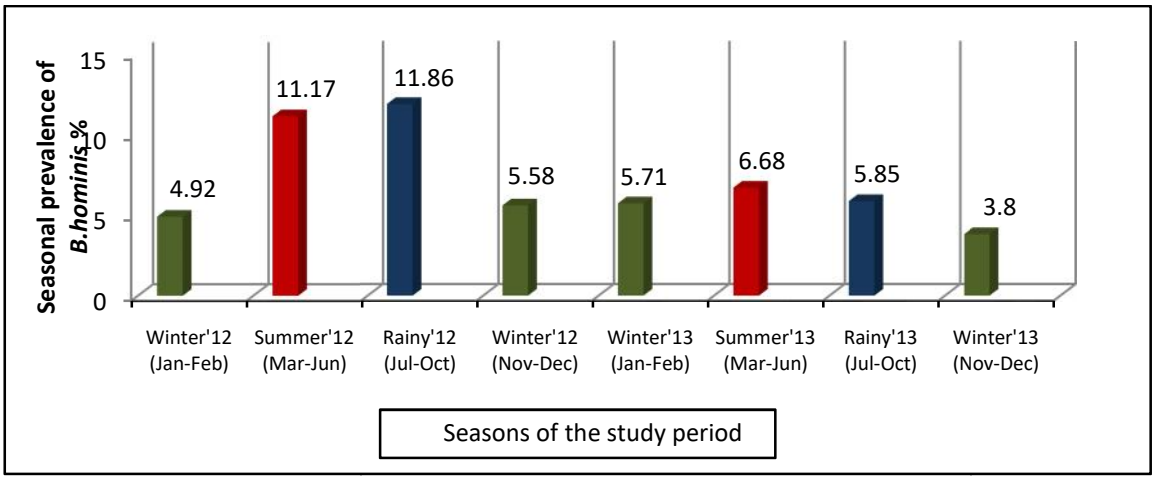


Fig.38. Seasonal prevalence of *B. hominis* in MS samples by microscopy.

By *in vitro* culture, in 2012, the highest prevalence of *B. hominis* was found 60.87% in rainy'12 and in 2013 it was found 44.07% in summer'13. In both years, the prevalence were found lower in winter (Fig. 39).

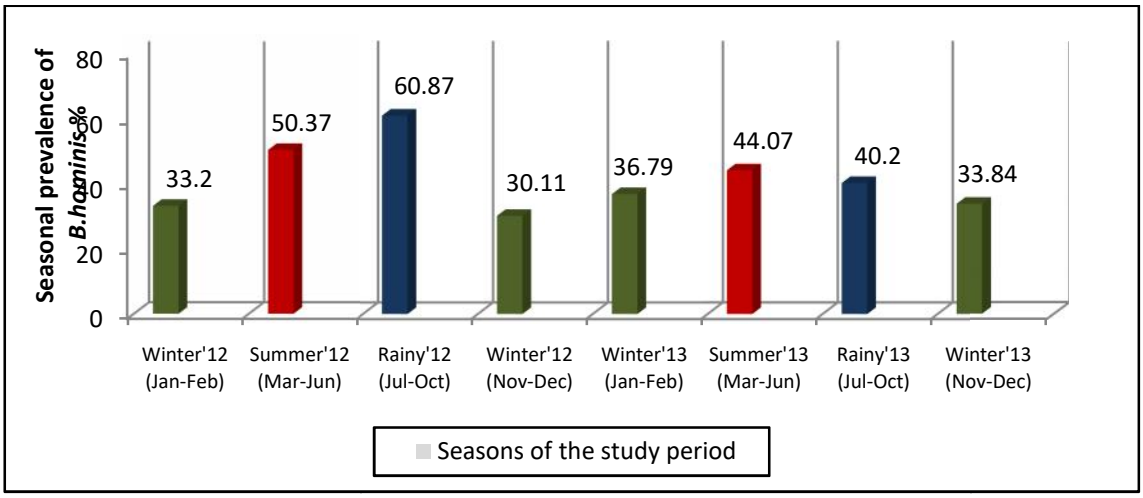


Fig.39. Seasonal prevalence of *B. hominis* in MS samples by *in vitro* culture.

Comparison of seasonal prevalence of *B. hominis* by two techniques in DS samples of Mohammadpur area.

By microscopy, in summer season the highest prevalence of *B. hominis* was 13.21 % and the lowest was 5.26 % in winter (Fig. 40). The difference of prevalence of *B. hominis*among three seasons was significant (F=5.84 and p=0.018*) (Table 5).

By *in vitro* culture,in summer season the highest prevalence of *B. hominis* was 54.09 % and the lowest was 21.53 % in winter (Fig. 40).The difference of prevalence of *B. hominis*among three seasons was highly significant (F=35.65 and p=0.000**) (Table 5).

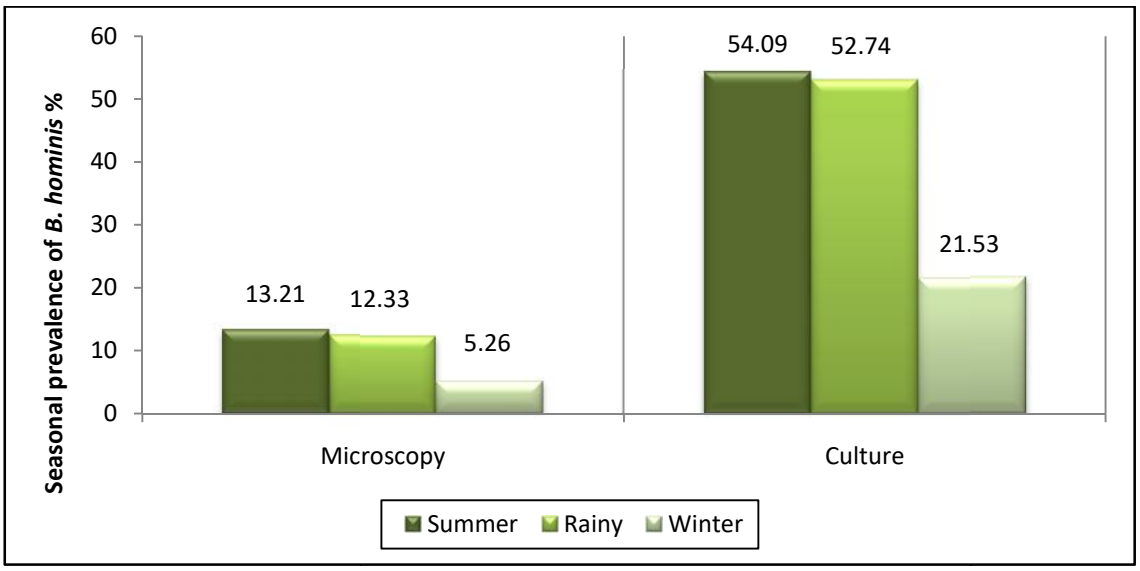


Fig. 40. Overall seasonal prevalence of *B. hominis* in DS samples by microscopy and *in vitro* culture.

Interpretation

Summer and rainy seasonsare more effective than winter season to the infection in DS samples from Mohammadpur area.

***Bh=Blastocystis hominis*, MS samples=Monthly Stool samples and DS samples=Diarrheal Stool samples**

Seasonal prevalence of *B. hominis*by microscopy and *in vitro* culture in DS samples from Mohammadpur Area.

By microscopy, in 2012, the highest prevalence of *B. hominis* was found 11.84% in summer'12 and in 2013 it was found 14.46% in summer'13. In both years, in winter the prevalence were found lower (Fig. 41).

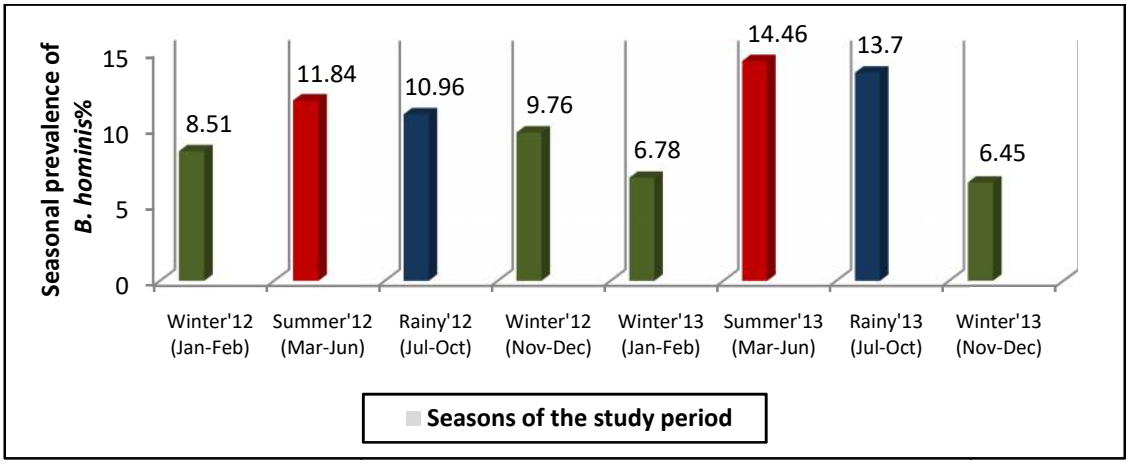


Fig. 41. Seasonal prevalence of *B. hominis* in DS samples by microscopy.

By *in vitro* culture, in 2012, the highest prevalence of *B. hominis* was found 55.26% in summer'12 and in 2013 it was found 54.79% in rainy'13. In both years, the lowest prevalence were found in winter (Fig. 42).

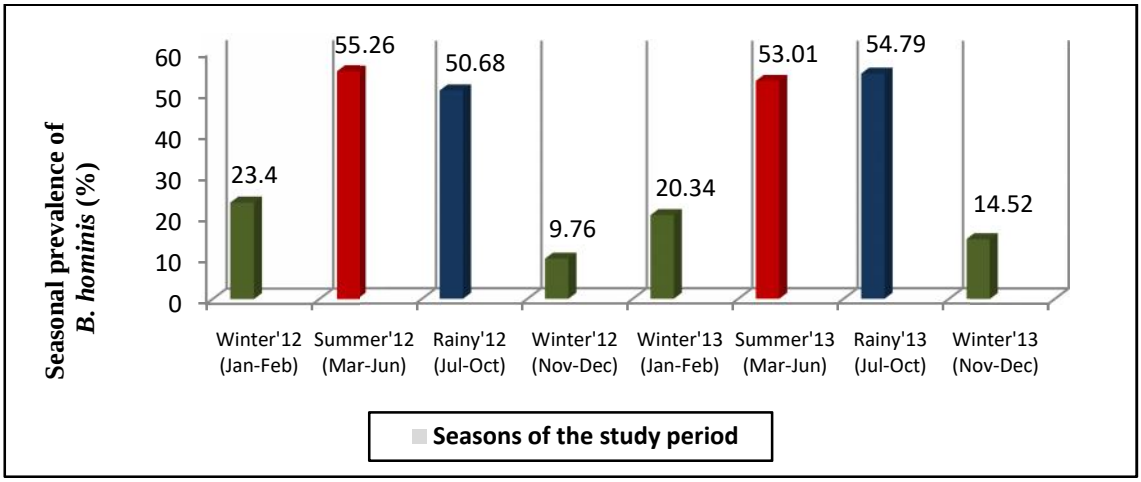


Fig. 42. Seasonal prevalence of *B. hominis* in DS samples by *in vitro* culture.

Table 5. Analysis of variance (F-test) for comparison of seasonal prevalence of *B. hominis* in overall monthly stool (MS) and diarrheal stool (DS) samplesof two areas.

Study area	Sample type	Source of variation	Prevalence by Microscopy (%)	Level of significance by F-test	Prevalence by *in vitro* culture (%)	Level of significance by F-test
Mirpur area	MS	Summer	3.25	0.016* (F=6.84)	**35.37**	0.643 ns (F=0.464)
		Rainy	**4.43**		**35.73**	
		Winter	2.32		31.58	
	DS	**Summer**	**6.42**	0.192 ns (F=1.99)	**35.47**	0.055* (F=3.47)
		Rainy	8.05		**31.61**	
		Winter	3.59		14.74	
Mohammadpur area	MS	**Summer**	**8.48**	0.004** (F=10.52)	**46.81**	0.000** (F=48.62)
		Rainy	8.20		**48.14**	
		Winter	5.97		35.32	
	DS	**Summer**	**13.21**	0.018* (F=5.84)	**54.09**	0.000** (F=35.65)
		Rainy	12.33		**52.74**	
		Winter	5.26		21.53	

Overall interpretation

By microscopy, either summer or rainy season showed higher prevalence than winter, while by culture method prevalence of *B. hominis* were higher both in summer and rainy season than winter. So infection of *B. hominis*is affected by seasonal variation.

MS samples = Monthly Stool samples and DS samples = Diarrheal Stool samples

IMPACT OF CLIMATIC FACTORS ON INTESTINAL PARASITES:

The distribution of intestinal parasitic infection depends on many factors. These include sociodemographic variables associated with poverty such as reduced access to adequate sanitation, pure water and health care hygiene as well as the prevailing climatic and environmental conditions (Mata 1982; WHO 1996; Montresor *et al.* 1998).The environmental factors including climate, season, temperature, humidity and rainfall play an important role in the development of parasites.

TEMPERATURE

The protozoan are an extremely diverse group of unicellular organisms occurring in almost all of the ecological niches known to humans, including the bottom of hot springs and the edges of ice flows (Melhorn 1988; Katz *et al.* 1989).Ambient air temperatures also have been linked to hospital admissions of Peruvian children with diarrhoeal disease (Checkley 2000). Some authors observed that hot tropical climate are the main factors associated with intestinal parasitic infections (Mehraj *et al.* 2008).

HUMIDITY

Humidity indicates the likelihood of precipitation, dew, or fog. Higher humidity reduces the effectiveness of sweating in cooling the body by reducing the rate of evaporation of moisture from the skin. This effect is calculated in a heat index table or humidex.Increased humidity can increases vector survival and decreased humidity can decreases transmission. Humidity can greatly influence transmission of vector-borne diseases, particularly for insect vectors. Saturation deficit (similar to relative humidity)has been found to be one of the most critical determinants in climate/diseasemodels, for example, dengue fever (Focks *et al.* 1995; Hales *et al*.2002)

RAINFALL

Seasonality of rainfall can exert a strong influence on animal condition and on host-parasite interactions. Rainfall seasonality directly influences resource availability and parasitism by altering the development, survival and transmission of parasite life stages in the environment (Fayer 1980; Banks *et al.* 1990; O'Connor *et al.* 2006).

Correlation of monthly incidence of *B. hominis*in MS samples with temperature (°C)by culture methodin Mirpur area:

By *in vitro* culture method, incidence of *B. hominis* was positively correlated with temperaturewhereas correlation is not significant ($p=0.328^{ns}$) in MS samples from Mirpur area(Fig. 43). This implies that incidence of *B. hominis* increases with the increase of temperature.

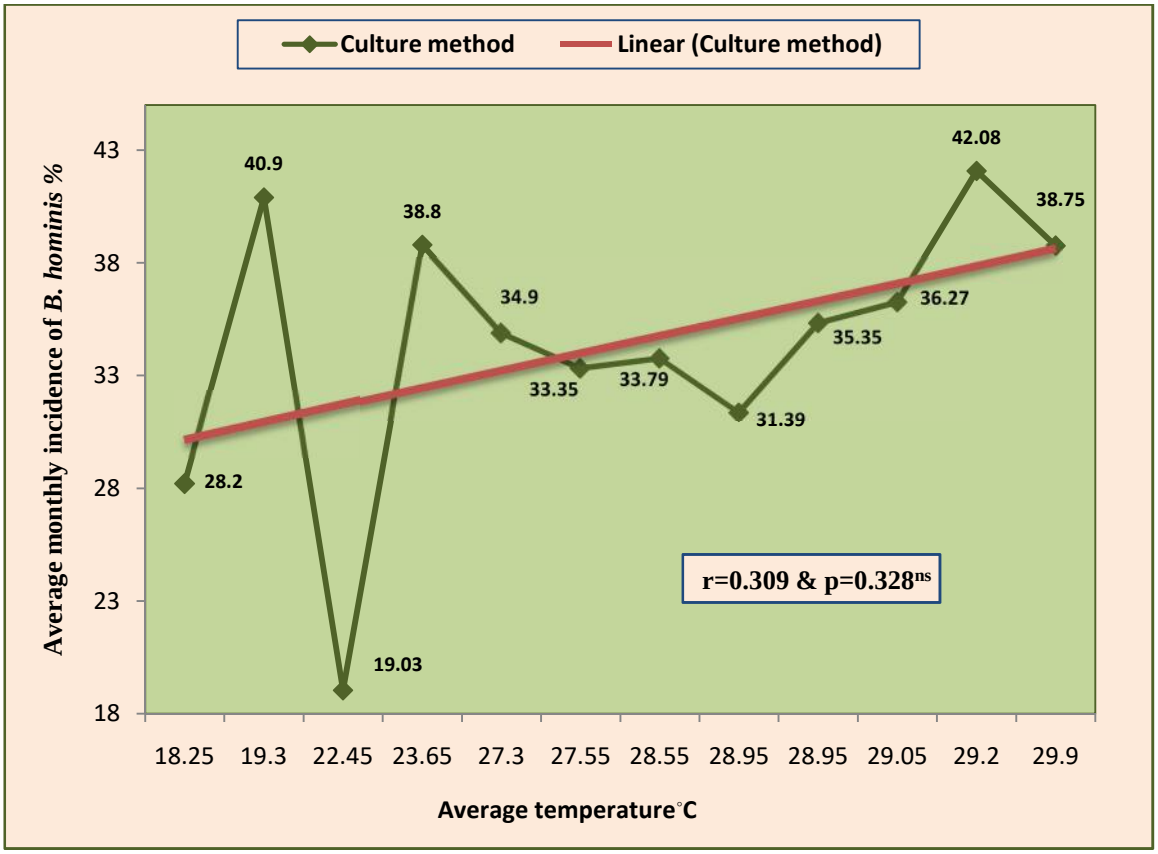


Fig. 43. Relationship of monthly incidence of *B. hominis*with temperature in overall MS samples by *in vitro* culture.

In 2012, lowest temperature 18.40°C was observed in the month of December, while lowest incidence (19.20%) was observed in January.In 2013, the highest temperature (30.10°C) was observed in month of June and the highest incidence was 46.15% in May. In both years 2012 and 2013, in MS samples, monthly incidence of *B. hominis* was positively correlated with temperaturewhereas, correlation was not significant ($p=0.418^{ns}$ & $p=0.596^{ns}$) with temperature(Table 6).

Table 6. Relationship of monthly incidence of *B. hominis* with temperature (°C)in MS samples in 2012-2013in Mirpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Temp◦ C	Year 2013	Total sample	*Bh* +ve	Incidence %	Temp ◦ C
Jan'12	401	77	**19.20**	18.90	Jan'13	379	141	37.20	**17.60**
Feb'12	380	94	24.74	22.10	Feb'13	323	43	**13.31**	22.80
Mar'12	365	97	26.58	27.10	Mar'13	324	140	43.21	27.50
Apr'12	408	127	31.13	28.10	Apr'13	354	129	36.44	29.00
May'12	413	109	26.39	**30.10**	May'13	351	162	**46.15**	28.00
Jun'12	351	128	36.47	29.70	Jun'13	329	135	41.03	**30.10**
Jul'12	411	166	**40.39**	29.10	Jul'13	377	165	43.77	29.30
Aug'12	327	87	26.61	29.20	Aug'13	307	111	36.16	28.70
Sep'12	361	130	36.01	29.00	Sep'13	346	120	34.68	28.90
Oct'12	332	132	39.76	27.90	Oct'13	360	97	26.94	27.20
Nov'12	388	135	34.79	23.50	Nov'13	320	137	42.81	23.80
Dec'12	344	133	38.66	**18.40**	Dec'13	350	151	43.14	20.20
Total	4481	1415	31.58		Total	4120	1531	37.16	
Correlations coefficient, r= 0.26 p = 0.42^{ns} Not significant at 5% level					Correlations coefficient, r = 0.17 & p = 0.60^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of monthly incidence of *B. hominis*in DS samples withtemperature (°C) by culture method in Mirpur area:

By *in vitro* culture method, incidence of *B. hominis* was positively correlated and correlation was highly significant (p=0.004**) with temperature in DS samples (Fig. 44), from Mirpur area. This implies that incidence of *B. hominis* increases with the increase of temperature.

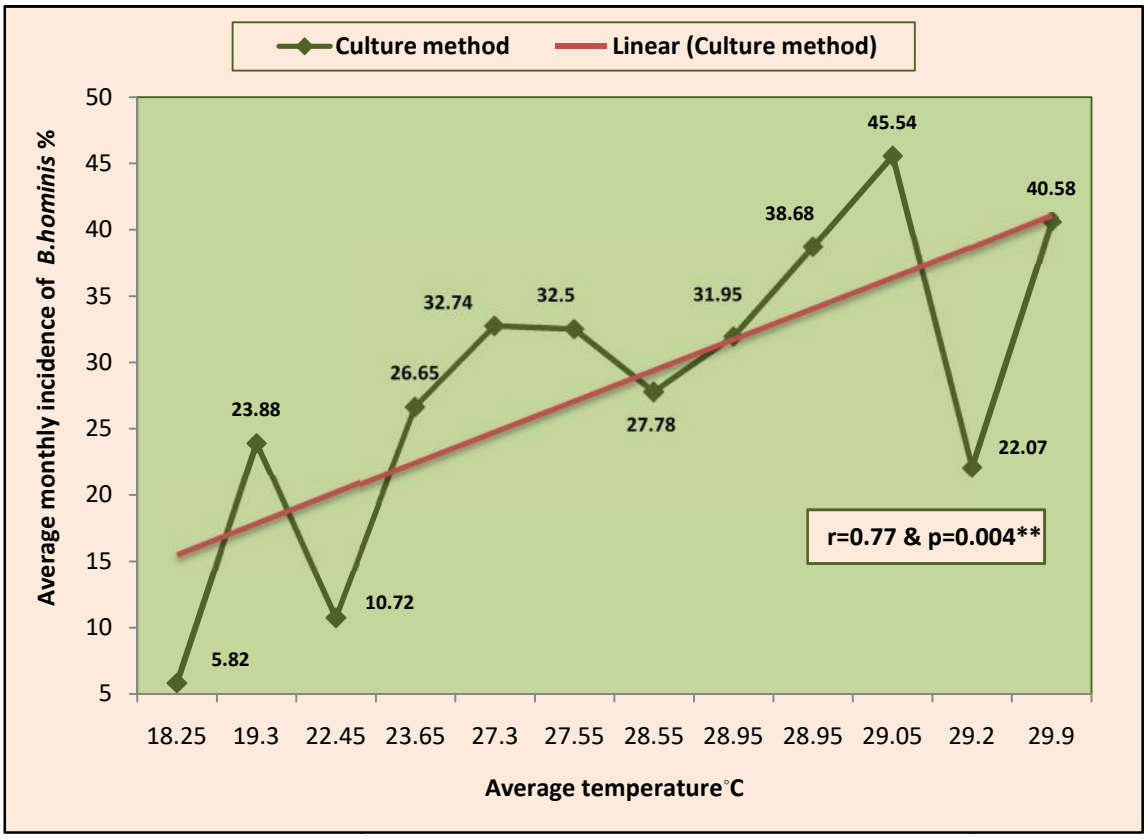


Fig.44. Relationship of monthly incidence of *B. hominis*with temperature overall DS samples by *in vitro* culture.

Interpretation

Climatic factor temperature is a growth factor for *B. hominis* infection in diarrheal patient in Mirpur area. Temperature as a growth factor, is more sensitive in diarrheal patient than non-diarrheal patient.

In 2012, the highest incidence (45.24%) was observed in the month of May and the highest temperature (30.10°C) was also observed in the same month (May'12). On the other hand, in

2013, the lowest incidence (3.57%) was observed in January and the lowest temperature (17.60°C) was observed in the same month (Jan'13)(Table 7).

Monthly incidence of *B. hominis* was positively correlated with temperature in DS samples, and correlation was highly significant (r=0.88 & p=0.000**) with temperature in 2012, while, correlation was not significant (r=0.53& p = 0.08^{ns}) in 2013 (Table 7).

Table 7. Relationship of monthly incidence of *B. hominis* with temperature (°C) in DS samples in 2012-2013 in Mirpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Temp◦ C	Year 2013	Total sample	*Bh* +ve	Incidence %	Temp ◦ C
Jan'12	43	5	**11.63**	18.90	Jan'13	28	1	**3.57**	**17.60**
Feb'12	42	9	21.43	22.10	Feb'13	37	2	5.41	22.80
Mar'12	48	16	33.33	27.10	Mar'13	28	9	32.14	27.50
Apr'12	63	17	26.98	28.10	Apr'13	35	10	28.57	29.00
May'12	42	19	**45.24**	**30.10**	May'13	24	9	37.5	28.00
Jun'12	31	14	45.16	29.70	Jun'13	25	9	36	**30.10**
Jul'12	26	9	34.62	29.10	Jul'13	21	4	19.05	29.30
Aug'12	18	7	38.89	29.20	Aug'13	16	4	25	28.70
Sep'12	18	7	38.89	29.00	Sep'13	26	10	**38.46**	28.90
Oct'12	25	10	40.00	27.90	Oct'13	24	6	25	27.20
Nov'12	38	6	15.79	23.50	Nov'13	16	4	25	23.80
Dec'12	28	6	21.43	**18.40**	Dec'13	19	4	21.05	20.20
Total	422	125	29.62		Total	299	72	24.08	
Correlations coefficient, r= 0.88 p = 0.000** Significant at 5% level					Correlations coefficient, r = 0.529 & p = 0.08^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of monthly incidence of *B. hominis*in MS samples withtemperature (°C)by culturemethod in Mohammadpur area:

By *in vitro* culture method, incidence of *B. hominis* was positively correlated (r=0.94) and correlation was highly significant (p=0.000**) with temperature in MS samples from Mirpur area (Fig. 45). This implies that incidence of *B. hominis* increases with the increase of temperature.

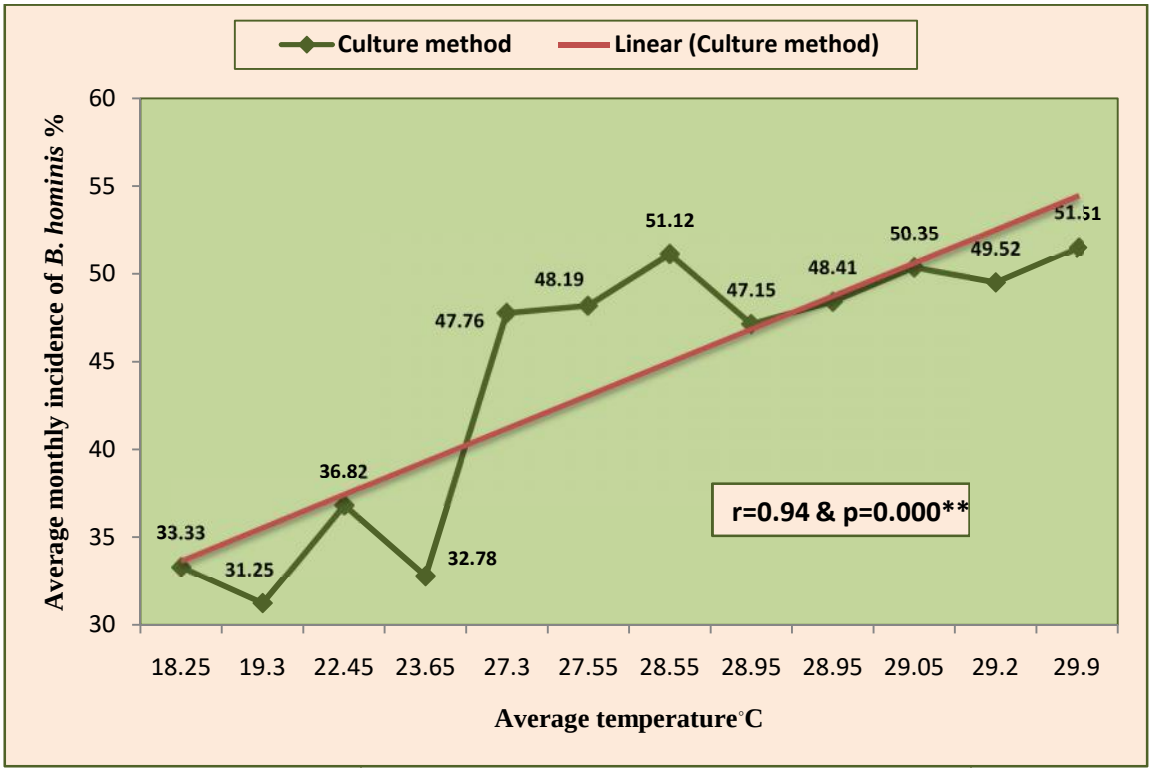


Fig. 45. Relationship of monthly incidence of *B. hominis*with temperature in overall MS samples by *in vitro* culture method.

Interpretation

Climatic factor temperature is a growth factor for *B. hominis* infection in non diarrheal patient in Mohammadpur area.

In 2012, lowest incidence (28.57%) was observed in the month of December when lowest temperature (18.40°C) was observed. Monthly incidence of *B. hominis* was positively correlated and highly significant (r=0.935 & p=0.000**) with temperature(Table 8).

In 2013, lowest incidence (32.17%) was observed in January and lowest temperature (17.60°C) was observed in the same month.Monthly incidence of *B. hominis* was positively correlated and significant (r=0.559 & p = 0.05*) with temperature(Table 8).

Table 8. Relationship of monthly incidence of *B. hominis* with temperature (°C) in MS samples in 2012-2013 in Mohammadpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Temp◦ C	Year 2013	Total sample	*Bh* +ve	Incidence %	Temp ◦ C
Jan'12	116	40	34.48	18.90	Jan'13	143	46	**32.17**	**17.60**
Feb'12	128	41	32.03	22.10	Feb'13	137	57	41.61	22.80
Mar'12	94	47	50	27.10	Mar'13	134	61	45.52	27.50
Apr'12	108	53	49.07	28.10	Apr'13	126	67	**53.17**	29.00
May'12	86	49	56.98	**30.10**	May'13	199	87	43.72	28.00
Jun'12	115	70	60.87	29.70	Jun'13	140	59	42.14	**30.10**
Jul'12	72	44	**61.11**	29.10	Jul'13	58	22	37.93	29.30
Aug'12	55	33	60	29.20	Aug'13	35	12	34.29	28.70
Sep'12	69	40	57.97	29.00	Sep'13	157	61	38.85	28.90
Oct'12	57	30	52.63	27.90	Oct'13	144	63	43.75	27.20
Nov'12	129	41	31.78	23.50	Nov'13	151	51	33.77	23.80
Dec'12	140	40	**28.57**	**18.40**	Dec'13	112	38	33.93	20.20
Total	1169	528	45.17		Total	1536	624	40.63	
Correlations coefficient, r = 0.94 p = 0.000** Highly significant at 5% level					Correlations coefficient, r = 0.56 & p = 0.05* Significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of monthly incidence of *B. hominis*in DS samples withtemperature (°C) by culture method in Mohammadpur area:

By *in vitro* culture method, in DS samples from Mohammadpur area,incidence of *B. hominis* was positively correlated (r=0.90) and correlation was highly significant (p=0.000**) with temperature (Fig.46). This implies that incidence of *B. hominis* increases with the increase of temperature.

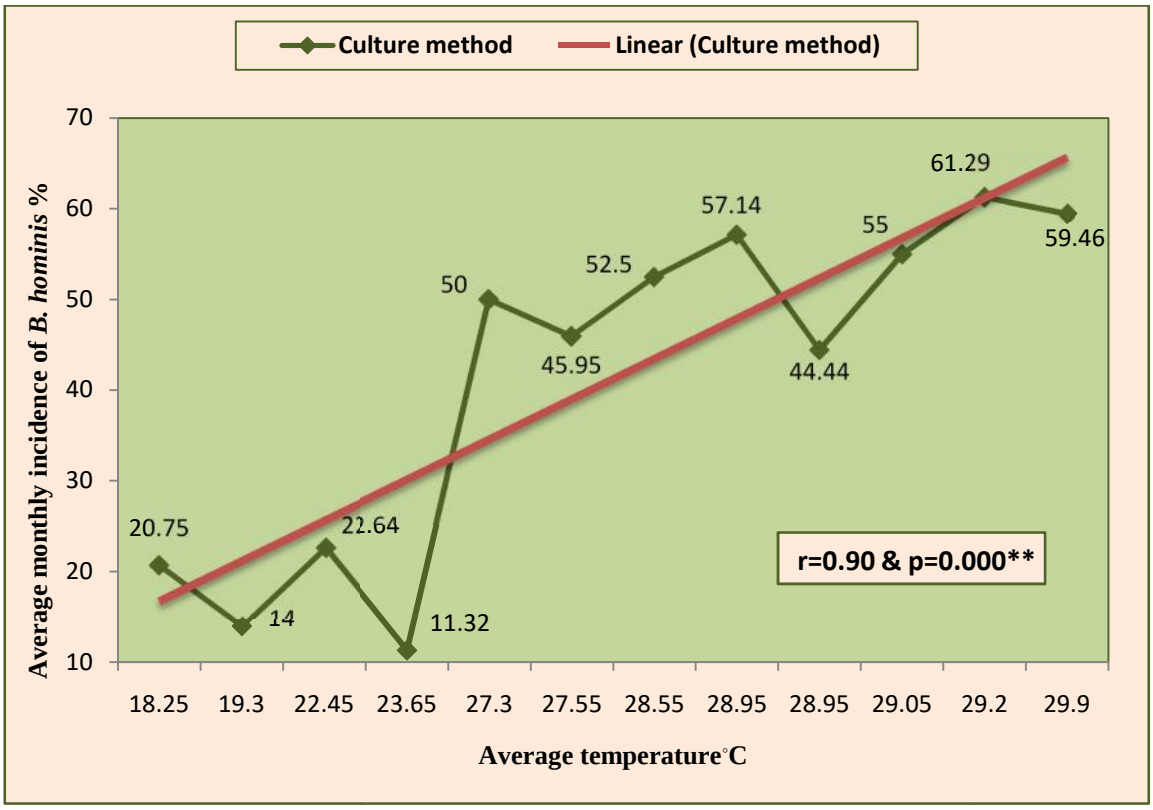


Fig.46. Relationship of monthly incidence of *B. hominis*with temperature in overall DS samples by *in vitro* culture.

Interpretation

Climatic factor temperature is a growth factor for *B. hominis* infection in diarrheal patient in Mohammadpur area.

In 2012, the highest incidence (57.89%) was observed in the month of March and temperature 27.10°C was observed in the same month which was lower than highest temperature (30.10°C) (Table 9).

In 2013, the highest incidence (66.67%) of *B. hominis*was observed in the month of July and temperature 29.30°C was observed in the same month which was almost nearer to highest temperature (30.10°C) (Table 13).Monthly incidence of *B. hominis* was positively correlated and highly significant (p=0.000**&p=0.000**) with temperaturein DS samples in both years 2012 and 2013 (Table 9).

Table 9. Relationship of monthly incidence of *B. hominis* with temperature (°C) in DS samples in 2012-2013 in Mohammadpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Temp° C	Year 2013	Total sample	*Bh* +ve	Incidence %	Temp ◦ C
Jan'12	25	6	24	18.90	Jan'13	28	5	17.86	**17.60**
Feb'12	22	5	22.73	22.10	Feb'13	31	7	22.58	22.80
Mar'12	19	11	**57.89**	27.10	Mar'13	23	10	43.48	27.50
Apr'12	23	12	52.17	28.10	Apr'13	17	9	52.94	29.00
May'12	18	10	55.56	**30.10**	May'13	22	12	54.55	28.00
Jun'12	16	9	56.25	29.70	Jun'13	21	13	61.9	**30.10**
Jul'12	13	7	53.85	29.10	Jul'13	18	12	**66.67**	29.30
Aug'12	26	14	53.85	29.20	Aug'13	16	10	62.5	28.70
Sep'12	14	6	42.86	29.00	Sep'13	22	10	45.45	28.90
Oct'12	20	9	45	27.90	Oct'13	17	8	47.06	27.20
Nov'12	21	2	**9.52**	23.50	Nov'13	32	4	**12.5**	23.80
Dec'12	20	2	10	**18.40**	Dec'13	30	5	16.67	20.20
Total	237	93	39.24		Total	277	105	37.91	
Correlations coefficient, r = 0.88 , p = 0.000** Significant at 5% level					Correlations coefficient, r = 0.89 & p = 0.000** Significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of monthly incidence of *B. hominis*in MS samples with humidity (%)by culture method in Mirpur area:

By *in vitro* culture method, in monthly stool samples from Mirpur area,incidence of *B. hominis* was positively correlated (r=0.35),while correlation was not significant ($p=0.26^{ns}$) with humidity at 0.01 level (Fig.47).

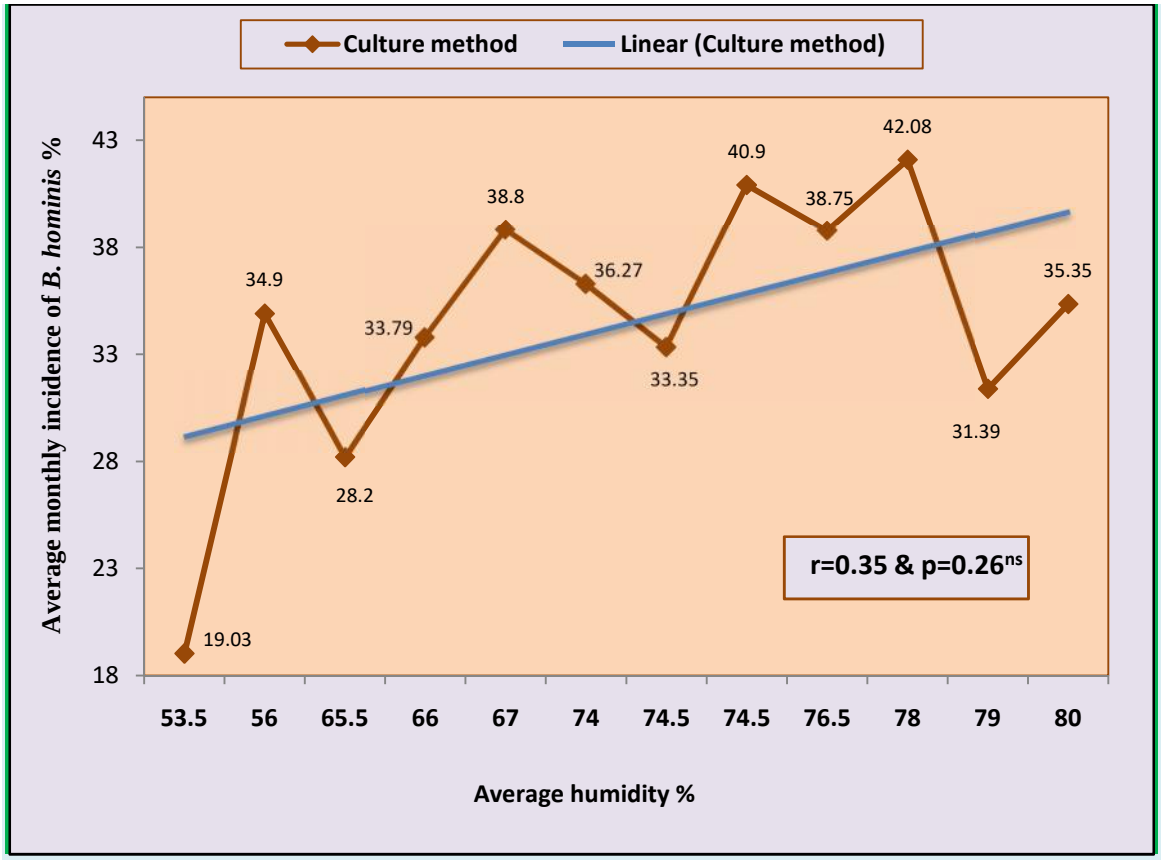


Fig.47. Relationship of monthly incidence of *B. hominis*with humidity in overall MS samples by *in vitro* culture.

In 2012, the highest humidity (79%) and the highest incidence (40.39%) were observed in the same month (Jul'12).In this year, monthly incidence of *B. hominis* was positively correlated and significant (r=0.61& p=0.04*) with humidityin MS samples(Table 10).

In 2013, the lowest humidity (79%) and the lowest incidence (40.39%) were observed in the same month (Jul'12).In this year, in MS samples, monthly incidence of *B. hominis* was positively correlated with humidity,whereas correlation was not significant (r=0.30& p= 0.35^{ns}) with humidity(Table 10).

Table 10. Relationship of monthly incidence of *B. hominis* with humidity (%) in MS samples in 2012-2013 in Mirpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Humidity %	Year 2013	Total sample	*Bh* +ve	Incidence %	Humidity %
Jan'12	401	77	**19.20**	66	Jan'13	379	141	37.20	65
Feb'12	380	94	24.74	**52**	Feb'13	323	43	**13.31**	**55**
Mar'12	365	97	26.58	57	Mar'13	324	140	43.21	**55**
Apr'12	408	127	31.13	69	Apr'13	354	129	36.44	63
May'12	413	109	26.39	70	May'13	351	162	**46.15**	78
Jun'12	351	128	36.47	77	Jun'13	329	135	41.03	76
Jul'12	411	166	**40.39**	**79**	Jul'13	377	165	43.77	77
Aug'12	327	87	26.61	78	Aug'13	307	111	36.16	80
Sep'12	361	130	36.01	**79**	Sep'13	346	120	34.68	**81**
Oct'12	332	132	39.76	71	Oct'13	360	97	26.94	78
Nov'12	388	135	34.79	68	Nov'13	320	137	42.81	66
Dec'12	344	133	38.66	77	Dec'13	350	151	43.14	72
Total	4481	1415	31.58		Total	4120	1531	37.16	
Correlations coefficient, r= 0.61 p = 0.04* Significant at 5% level					Correlations coefficient, r = 0.30 & p = 0.35^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of monthly incidence of *B. hominis*in DS samples with humidity (%) by culture method in Mirpur area:

By *in vitro* culture method, in DS samples from Mirpur area,incidence of *B. hominis* was positively correlated (r=0.51) while correlation was not significant ($p=0.09^{ns}$) with humidity (Fig.48). This implies that incidence of *B. hominis* increases with the increase of humidity.

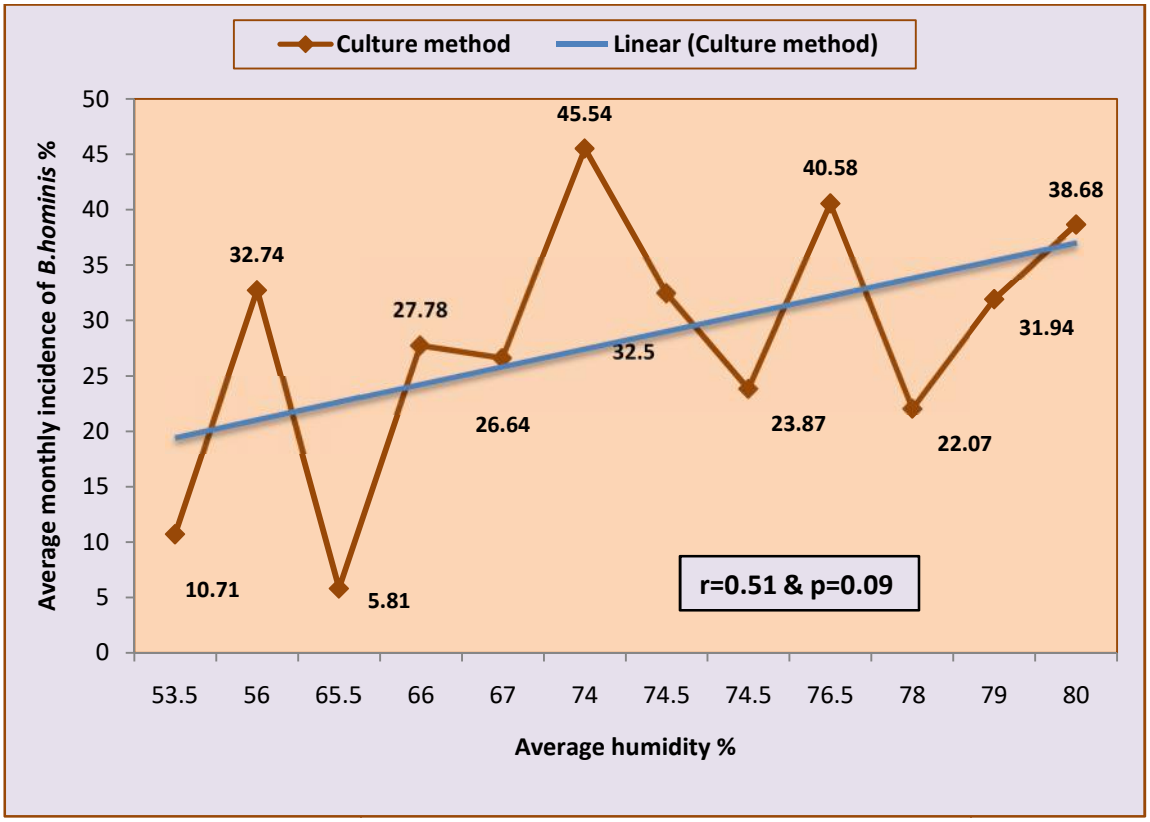


Fig.48. Relationship of monthly incidence of *B. hominis*with humidity overall DS samples by *in vitro* culture.

In 2012, the highest humidity 79% was observed in the month of July and September while the highest incidence (45.24%) was observed only in May. In 2013, the highest humidity (81%) and the highest incidence (38.46%) were observed in the same month (Sep'13) (Table 11).

Monthly incidence of *B. hominis* was positively correlated (r=0.42 & r=0.38) and not significant ($p=0.38^{ns}$&$p=0.22^{ns}$) with humidityin DS samples in both years 2012 and 2013 (Table 11).

Table 11. Relationship of monthly incidence of *B. hominis* with humidity (%) in DS samples in 2012-2013 in Mirpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Humidity %	Year 2013	Total sample	*Bh* +ve	Incidence %	Humidity %
Jan'12	43	5	**11.63**	66	Jan'13	28	1	**3.57**	65
Feb'12	42	9	21.43	**52**	Feb'13	37	2	5.41	**55**
Mar'12	48	16	33.33	57	Mar'13	28	9	32.14	**55**
Apr'12	63	17	26.98	69	Apr'13	35	10	28.57	63
May'12	42	19	**45.24**	70	May'13	24	9	37.5	78
Jun'12	31	14	45.16	77	Jun'13	25	9	36	76
Jul'12	26	9	34.62	**79**	Jul'13	21	4	19.05	77
Aug'12	18	7	38.89	78	Aug'13	16	4	25	80
Sep'12	18	7	38.89	**79**	Sep'13	26	10	**38.46**	**81**
Oct'12	25	10	40.00	71	Oct'13	24	6	25	78
Nov'12	38	6	15.79	68	Nov'13	16	4	25	66
Dec'12	28	6	21.43	77	Dec'13	19	4	21.05	72
Total	422	125	29.62		Total	299	72	24.08	
Correlations coefficient, r= 0.42 p = 0.38^{ns} Not significant at 5% level					Correlations coefficient, r = 0.38 & p = 0.22^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of monthly incidence of *B. hominis*in MS samples withhumidity (%)by culture methodin Mohammadpur area:

By *in vitro* culture method, incidence of *B. hominis* was positively correlated (r=0.35) and correlation was not significant ($p=0.26^{ns}$) with humidity at 0.01 level in monthly stool samples from Mohammadpur area (Fig.49). This implies that incidence of *B. hominis* increases with the increase of humidity.

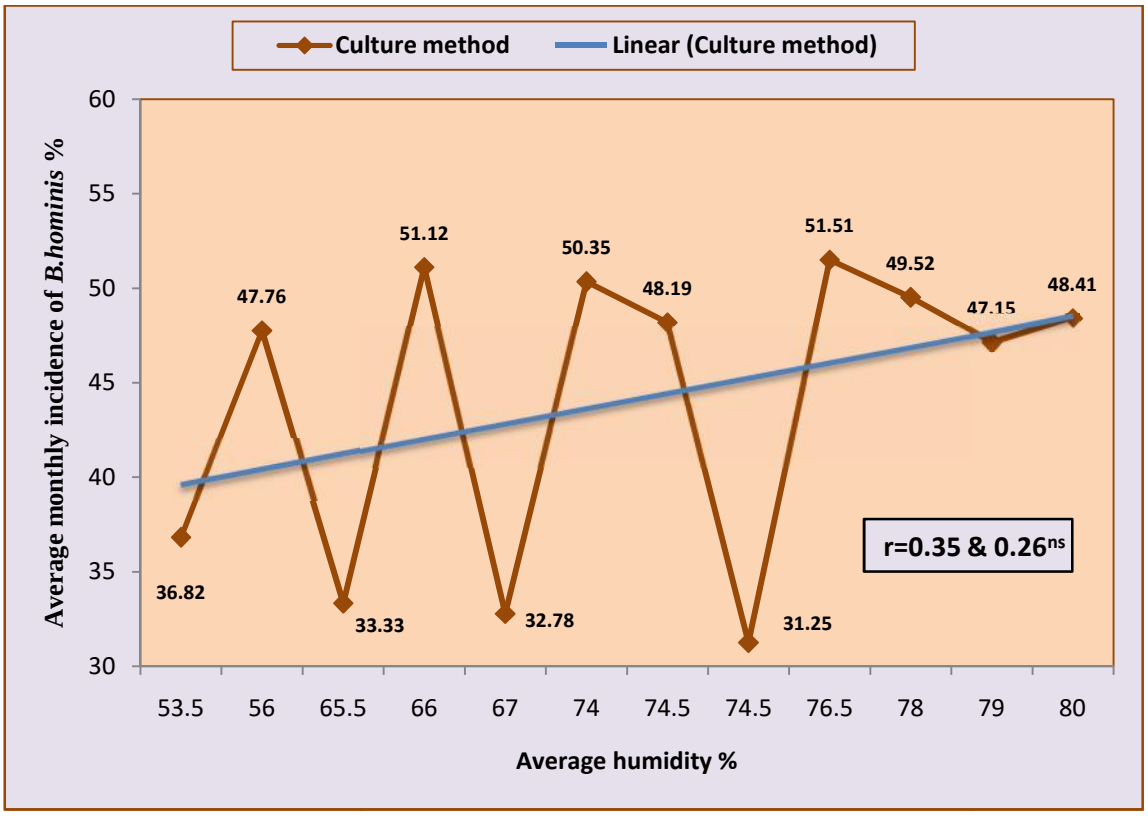


Fig.49.Relationship of monthly incidence of *B. hominis*with humidity in overall MS samples by *in vitro* culture.

Interpretation

Climatic factor humidity is a growth factor for *B. hominis* infection in non-diarrheal patient in Mohammadpur area.

In 2012, the highest humidity 79% was observed in the month of July and Sep'12 and the highest incidence (61.11%) was observed only in July. In 2013, the highest humidity 81% was observed in month of September, while the highest incidence (53.17%) was observed in April (Table 12).

Monthly incidence of *B. hominis* was positively correlated (r=0.50 & r=0.25) and not significant ($p=0.097^{ns}$&$p=0.432^{ns}$) with humidityin MS samples in both years 2012 and 2013 (Table 12).

Table 12. Relationship of monthly incidence of *B. hominis* and humidity (%) in MS samples in 2012-2013 in Mohammadpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Humidity %	Year 2013	Total sample	*Bh* +ve	Incidence %	Humidity %
Jan'12	116	40	34.48	66	Jan'13	143	46	**32.17**	65
Feb'12	128	41	32.03	**52**	Feb'13	137	57	41.61	**55**
Mar'12	94	47	50	57	Mar'13	134	61	45.52	**55**
Apr'12	108	53	49.07	69	Apr'13	126	67	**53.17**	63
May'12	86	49	56.98	70	May'13	199	87	43.72	78
Jun'12	115	70	60.87	77	Jun'13	140	59	42.14	76
Jul'12	72	44	**61.11**	**79**	Jul'13	58	22	37.93	77
Aug'12	55	33	60	78	Aug'13	35	12	34.29	80
Sep'12	69	40	57.97	**79**	Sep'13	157	61	38.85	**81**
Oct'12	57	30	52.63	71	Oct'13	144	63	43.75	78
Nov'12	129	41	31.78	68	Nov'13	151	51	33.77	66
Dec'12	140	40	**28.57**	77	Dec'13	112	38	33.93	72
Total	1169	528	45.17		Total	1536	624	40.63	
Correlations coefficient, r = 0.50 p = 0.097^{ns} Not significant at 5% level					Correlations coefficient, r = 25 & p = 43^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of monthly incidence of *B. hominis*in DS samples withhumidity (%)by culture method in Mohammadpur area:

By *in vitro* culture method, in DS samples from Mohammadpur area,incidence of *B. hominis* was positively correlated and not significant ($p=0.69^{ns}$) with humidity (Fig. 50). This implies that incidence of *B. hominis* increases with the increase of humidity.

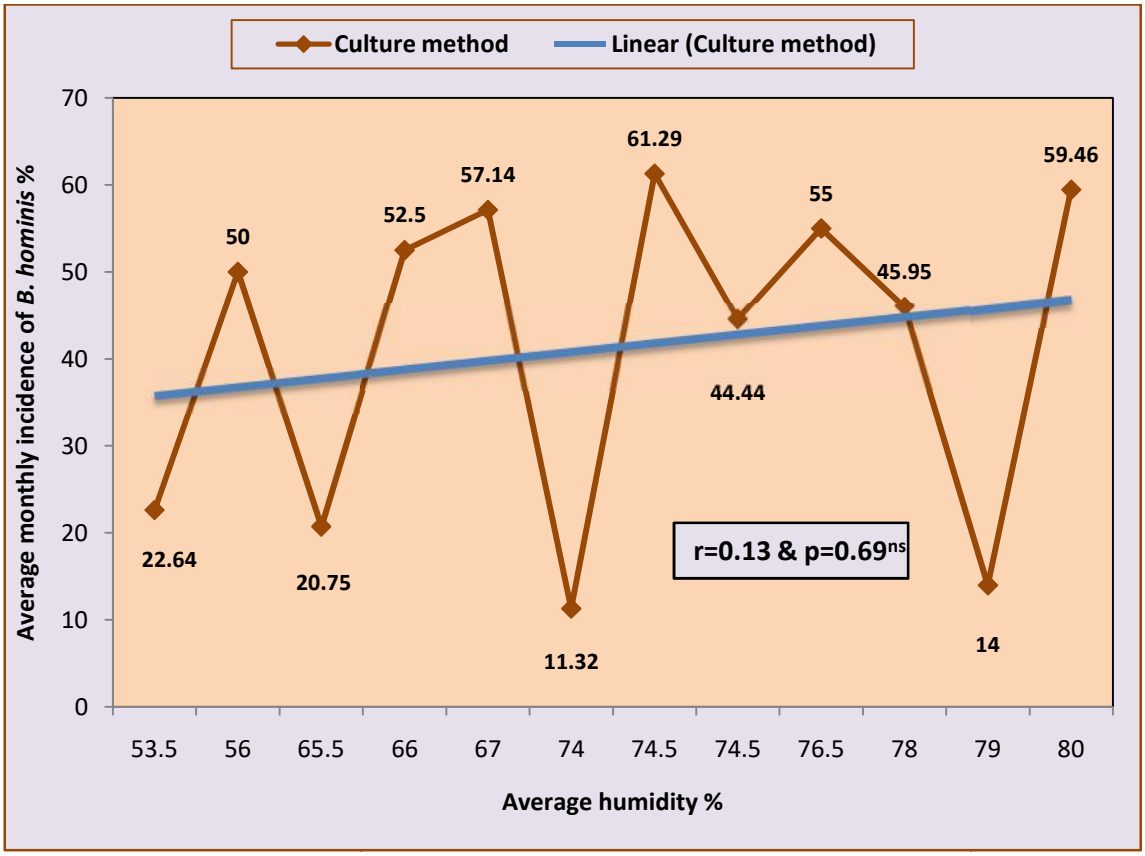


Fig.50. Relationship of monthly incidence of *B. hominis*with humidity in overall DS samples by *in vitro* culture.

Interpretation

Climatic factor humidity is a growth factor for *B. hominis* infection in diarrheal patient in Mohammadpur area.

In 2012, the highest incidence (57.89%) was observed in the month of March whereas,humidity 57% was observed in that month, which was not highest humidity (79%). In 2013, the highest humidity 81% was observed in month of September, while the highest incidence (66.67%) was observed in July (Table 13).

Monthly incidence of *B. hominis* was positively correlated (r=0.20 & r=0.52) and not significant ($p=0.53^{ns}$&$p=0.08^{ns}$) with humidityin DS samples in both years 2012 and 2013 (Table 13).

Table 13. Relationship of monthly incidence of *B. hominis* with humidity (%) in DS samples in 2012-2013 in Mohammadpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Humidity %	Year 2013	Total sample	*Bh* +ve	Incidence %	Humidity %
Jan'12	25	6	24	66	Jan'13	28	5	17.86	65
Feb'12	22	5	22.73	**52**	Feb'13	31	7	22.58	**55**
Mar'12	19	11	**57.89**	57	Mar'13	23	10	43.48	**55**
Apr'12	23	12	52.17	69	Apr'13	17	9	52.94	63
May'12	18	10	55.56	70	May'13	22	12	54.55	78
Jun'12	16	9	56.25	77	Jun'13	21	13	61.9	76
Jul'12	13	7	53.85	**79**	Jul'13	18	12	**66.67**	77
Aug'12	26	14	53.85	78	Aug'13	16	10	62.5	80
Sep'12	14	6	42.86	**79**	Sep'13	22	10	45.45	**81**
Oct'12	20	9	45	71	Oct'13	17	8	47.06	78
Nov'12	21	2	**9.52**	68	Nov'13	32	4	**12.5**	66
Dec'12	20	2	10	77	Dec'13	30	5	16.67	72
Total	237	93	39.24		Total	277	105	37.91	
Correlations coefficient, r = 0.20 , p = 0.53^{ns} Not significant at 5% level					Correlations coefficient, r = 0.52, p = 0.08^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of incidence of *B. hominis*in MS samples with rainfall (mm) by culturemethod in Mirpur area:

By *in vitro* culture method, incidence of *B. hominis* was positively correlated (r=0.39) and correlation was not significant ($p=0.22^{ns}$) with rainfall in MS samples from Mirpur area (Fig.51).

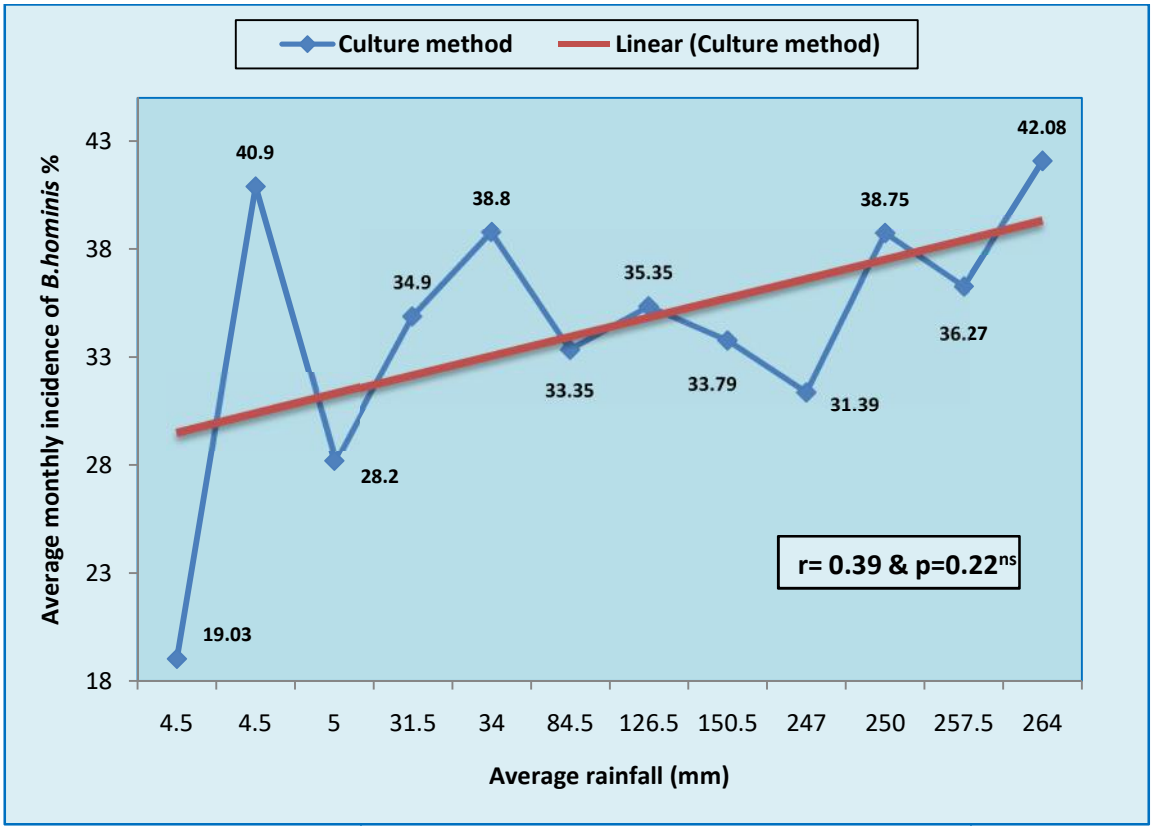


Fig.51. Relationship of monthly incidence of *B. hominis*with rainfall in overall MS samples by *in vitro*culture.

Interpretation

Climatic factor rainfall is a growth factor for *B. hominis* infection in non-diarrheal children in Mirpur area.

In 2012, the highest incidence (40.39%) was observed in the month of July and rainfall 226mm was higher, in the same month.In 2013, the highest rainfall 378 mm was observed in month of May'13 and the highest incidence (46.15%) was observed in the same month(May'13) (Table 14).

Monthly incidence of *B. hominis* was positively correlated (r=0.13 & r=0.33) and not significant ($p=0.69^{ns}$&$p=0.29^{ns}$) with rainfall (mm)in MS samples in both years 2012 and 2013 (Table 14).

Table 14. Relationship of monthly incidence of *B. hominis* with rainfall (mm) **in MS samples in 2012-2013in Mirpur area.**

Year 2012	Total sample	*Bh* +ve	Incidence %	Rainfall (mm)	Year 2013	Total sample	*Bh* +ve	Incidence %	Rainfall (mm)
Jan'12	401	77	**19.20**	10	Jan'13	379	141	37.20	**0**
Feb'12	380	94	24.74	**1**	Feb'13	323	43	**13.31**	8
Mar'12	365	97	26.58	37	Mar'13	324	140	43.21	26
Apr'12	408	127	31.13	269	Apr'13	354	129	36.44	32
May'12	413	109	26.39	137	May'13	351	162	**46.15**	**378**
Jun'12	351	128	36.47	175	Jun'13	329	135	41.03	325
Jul'12	411	166	**40.39**	226	Jul'13	377	165	43.77	302
Aug'12	327	87	26.61	**282**	Aug'13	307	111	36.16	212
Sep'12	361	130	36.01	81	Sep'13	346	120	34.68	172
Oct'12	332	132	39.76	38	Oct'13	360	97	26.94	131
Nov'12	388	135	34.79	68	Nov'13	320	137	42.81	**0**
Dec'12	344	133	38.66	5	Dec'13	350	151	43.14	4
Total	4481	1415	31.58		Total	4120	1531	37.16	
Correlations coefficient, r= 0.13 p = 0.69^{ns} Not significant at 5% level					Correlations coefficient, r = 0.33 & p = 0.29^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of incidence of *B. hominis*in DS samples with rainfall (mm) by culturemethod in Mirpur area:

By *in vitro* culture method, incidence of *B. hominis* was positively correlated (r=0.60) and correlation was highly significant (p=0.004**) with rainfall at 5% level in DS samples from Mirpur area (Fig.52). This implies that incidence of *B. hominis* increases with the increase of rainfall.

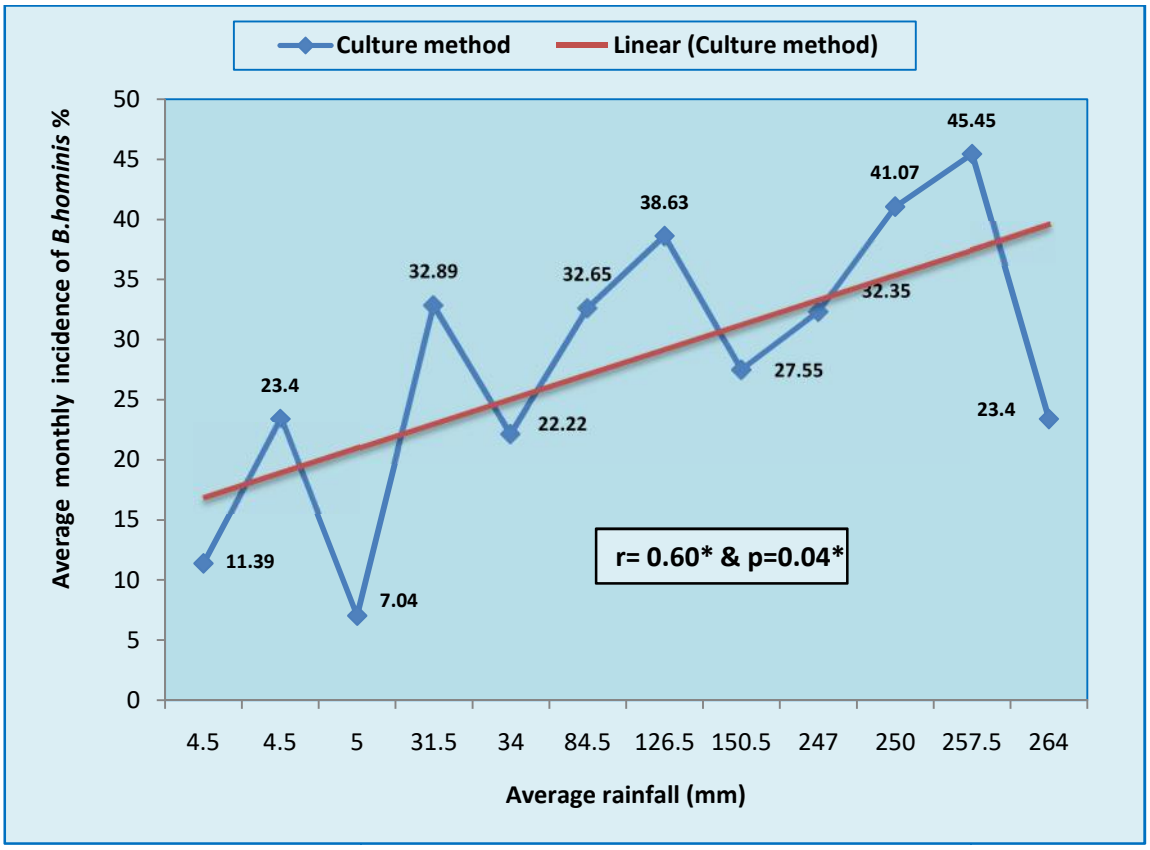


Fig.52. Relationship of monthly incidence of *B. hominis*with rainfall in overall DS samples by *in vitro* culture.

Interpretation

Climatic factor rainfall is a growth factor for *B. hominis* infection in diarrheal patient in Mirpur area.

In 2012, the highest rainfall (282 mm) was observed in month of August and incidence (38.89%) was observed in the same month. In 2013, lowest rainfall (0 mm) was observed in month of January and lowest incidence was (3.57%) in the same month (Jan'13) (Table 15).

Monthly incidence of *B. hominis* was positively correlated (r=0.46 & r=0.37) and not significant ($p=0.13^{ns}$&$p=0.24^{ns}$) with rainfall in DS samples in both years 2012 and 2013 (Table 15).

Table 15. Relationship of monthly incidence of *B. hominis* with rainfall in DS samples in 2012-2013in Mirpur area.

Year 2012	Total sample	*Bh* +ve	Incidence %	Rainfall (mm)	Year 2013	Total sample	*Bh* +ve	Incidence %	Rainfall (mm)
Jan'12	43	5	**11.63**	10	Jan'13	28	1	**3.57**	**0**
Feb'12	42	9	21.43	**1**	Feb'13	37	2	5.41	8
Mar'12	48	16	33.33	37	Mar'13	28	9	32.14	26
Apr'12	63	17	26.98	269	Apr'13	35	10	28.57	32
May'12	42	19	**45.24**	137	May'13	24	9	37.5	**378**
Jun'12	31	14	45.16	175	Jun'13	25	9	36	325
Jul'12	26	9	34.62	226	Jul'13	21	4	19.05	302
Aug'12	18	7	38.89	**282**	Aug'13	16	4	25	212
Sep'12	18	7	38.89	81	Sep'13	26	10	**38.46**	172
Oct'12	25	10	40.00	38	Oct'13	24	6	25	131
Nov'12	38	6	15.79	68	Nov'13	16	4	25	**0**
Dec'12	28	6	21.43	5	Dec'13	19	4	21.05	4
Total	422	125	29.62		Total	299	72	24.08	
Correlations coefficient, r= 0.46 p = 0.13^{ns} Not significant at 5% level					Correlations coefficient, r = 0.37 & p = 0.24^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of incidence of *B. hominis*in MS samples with rainfall (mm) by culture method in Mohammadpur area:

By *in vitro* culture method, incidence of *B. hominis* was positively correlated (r=0.77) and correlation was highly significant (p=0.003**) with rainfall in MS samples from Mohammadpur area (Fig. 53). This implies that incidence of *B. hominis* increases with the increase of rainfall.

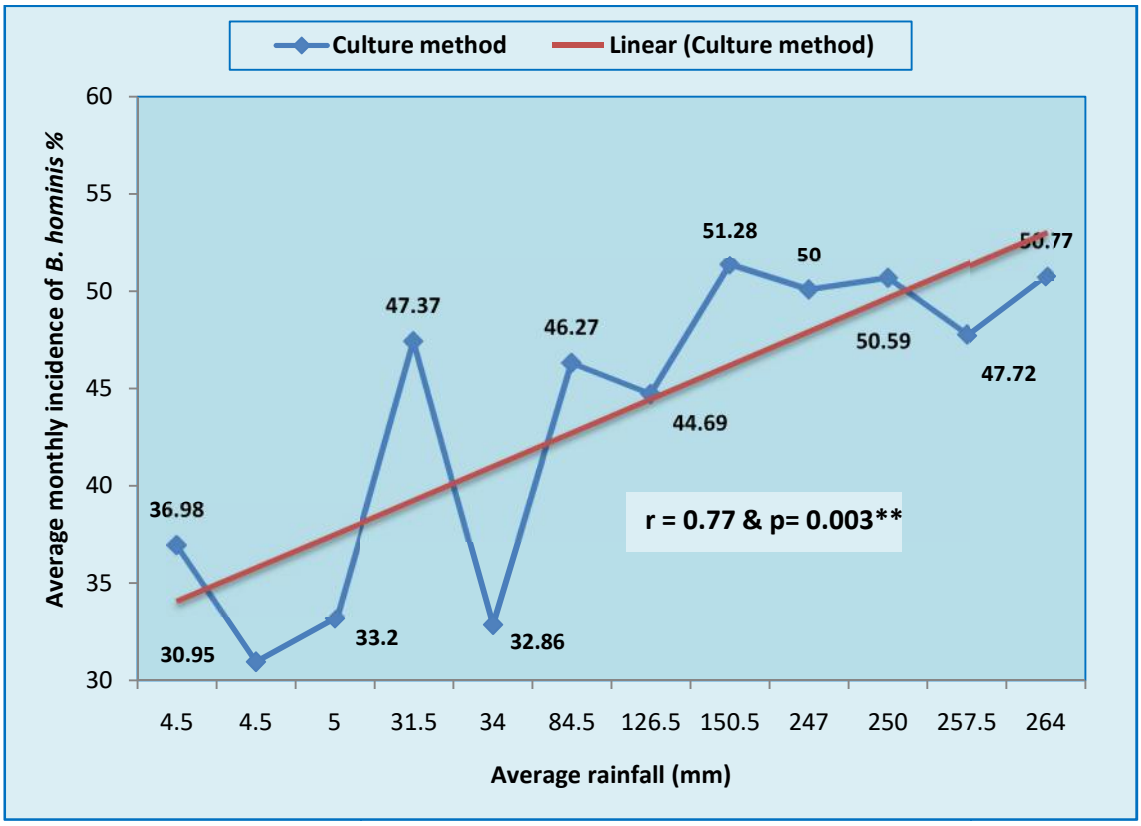


Fig.53. Relationship of monthly incidence of *B. hominis*with rainfall in overall MS samples by *in vitro* culture.

Interpretation

Climatic factor rainfall is a growth factor for *B. hominis* infection in non-diarrheal children in Mohammadpur area.

In 2012, the highest incidence (61.11%) was observed in the month of July and the rainfall 226 mm was observed in that month. In 2013, the highest incidence (53.17%) was observed in the month of April, while the rainfall 32 mm was observed in that month (Table 16).

Monthly incidence of *B. hominis* was positively correlated (r=0.68 & r=0.10) and only significant (p=0.02*) in 2012 and not significant (p=0.75^{ns}) in 2013, with rainfallin MS samples in (Table 16).

Table 16. Relationship of monthly incidence of *B. hominis* with rainfall (mm) **in MS samples in 2012-2013.**

Year 2012	Total sample	*Bh* +ve	Incidence %	Rainfall (mm)	Year 2013	Total sample	*Bh* +ve	Incidence %	Rainfall (mm)
Jan'12	116	40	34.48	10	Jan'13	143	46	**32.17**	**0**
Feb'12	128	41	32.03	**1**	Feb'13	137	57	41.61	8
Mar'12	94	47	50	37	Mar'13	134	61	45.52	26
Apr'12	108	53	49.07	269	Apr'13	126	67	**53.17**	32
May'12	86	49	56.98	137	May'13	199	87	43.72	**378**
Jun'12	115	70	60.87	175	Jun'13	140	59	42.14	325
Jul'12	72	44	**61.11**	226	Jul'13	58	22	37.93	302
Aug'12	55	33	60	**282**	Aug'13	35	12	34.29	212
Sep'12	69	40	57.97	81	Sep'13	157	61	38.85	172
Oct'12	57	30	52.63	38	Oct'13	144	63	43.75	131
Nov'12	129	41	31.78	68	Nov'13	151	51	33.77	**0**
Dec'12	140	40	**28.57**	5	Dec'13	112	38	33.93	4
Total	1169	528	45.17		Total	1536	624	40.63	
Correlations coefficient, r = 0.68 p = 0.02** Highly significant at 5% level					Correlations coefficient, r = 0.10 & p = 0.75^{ns} Not significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2-tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Correlation of incidence of *B. hominis* in DS samples with rainfall (mm) by culture method in Mohammadpur area:

By *in vitro* culture method, incidence of *B. hominis* was positively correlated (r=0.85) and correlation was highly significant (p=0.001**) with rainfall in DS samples from Mohammadpur area (Fig.54). This implies that incidence of *B. hominis* increases with the increase of rainfall.

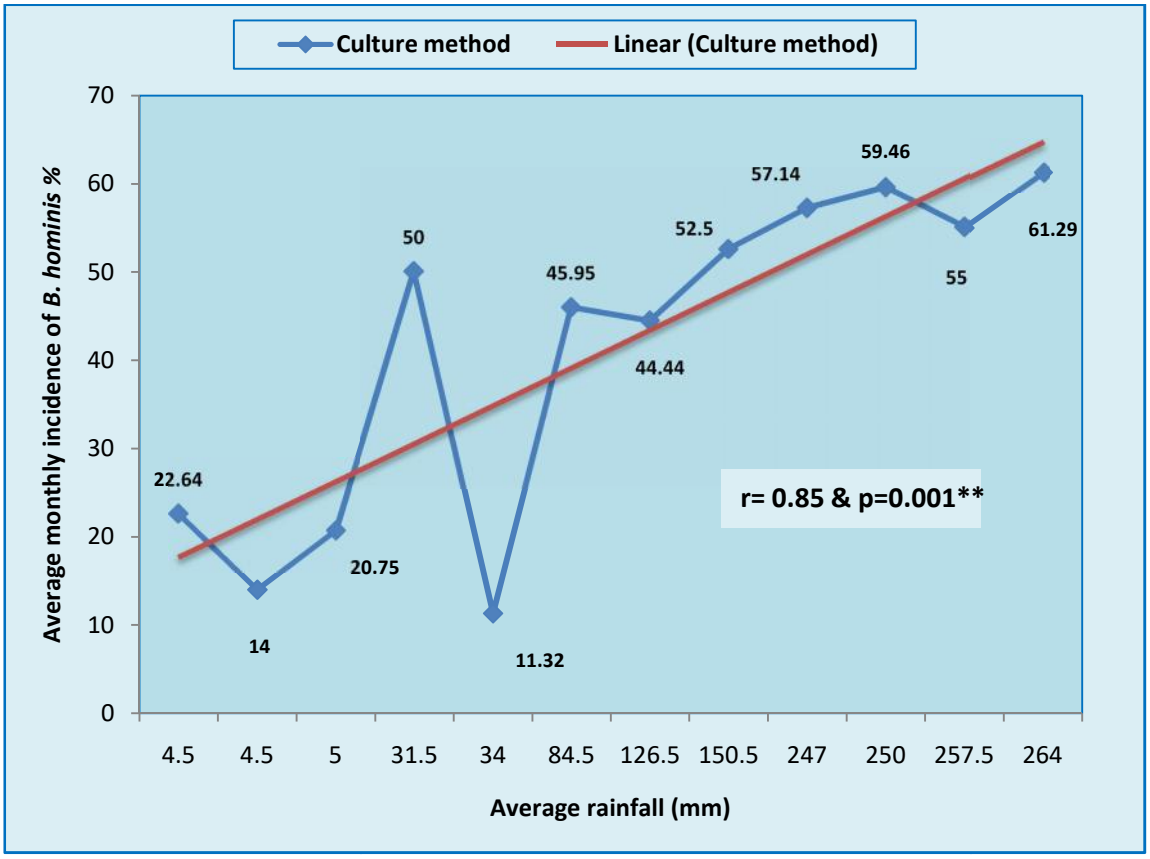


Fig.54. Relationship of monthly incidence of *B. hominis*with rainfall in overall DS samples by *in vitro* culture.

Interpretation

Climatic factor rainfall is a growth factor for *B. hominis* infection in diarrheal patient in Mohammadpur area.

In 2012, the highest incidence (57.89%) was observed in the month of March while very few rainfall (37 mm) was observed in that month. In 2013, lowest rainfall (0 mm) was observed in month of January and November and lowest incidence(12.50%) was observed only in November (Table 17).

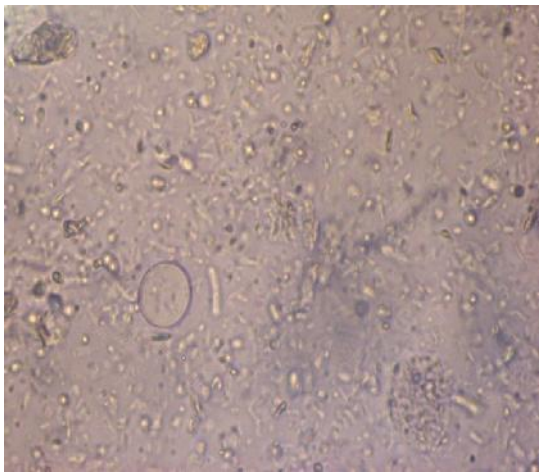
Monthly incidence of *B. hominis* was positively correlated (r=0.64 & r=0.80) and significant (p=0.03*&p=0.002**) with rainfall (mm)in DS samples in both years 2012 and 2013 (Table 17).

Table 17. Relationship of monthly incidence of *B. hominis* with rainfall (mm) **in DS samples in 2012-2013.**

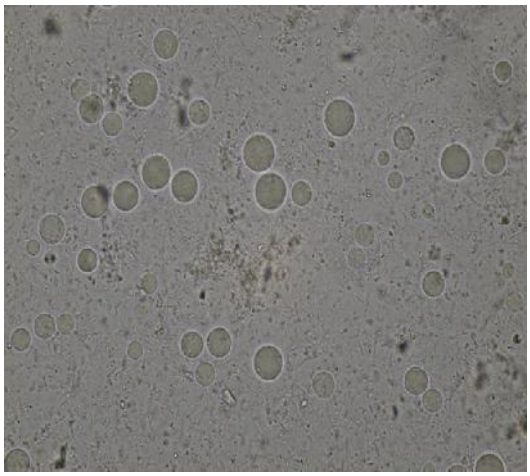
Year 2012	Total sample	*Bh* +ve	Incidence %	Rainfall (mm)	Year 2013	Total sample	*Bh* +ve	Incidence %	Rainfall (mm)
Jan'12	25	6	24	10	Jan'13	28	5	17.86	**0**
Feb'12	22	5	22.73	**1**	Feb'13	31	7	22.58	8
Mar'12	19	11	**57.89**	37	Mar'13	23	10	43.48	26
Apr'12	23	12	52.17	269	Apr'13	17	9	52.94	32
May'12	18	10	55.56	137	May'13	22	12	54.55	**378**
Jun'12	16	9	56.25	175	Jun'13	21	13	61.9	325
Jul'12	13	7	53.85	226	Jul'13	18	12	**66.67**	302
Aug'12	26	14	53.85	**282**	Aug'13	16	10	62.5	212
Sep'12	14	6	42.86	81	Sep'13	22	10	45.45	172
Oct'12	20	9	45	38	Oct'13	17	8	47.06	131
Nov'12	21	2	**9.52**	68	Nov'13	32	4	**12.5**	**0**
Dec'12	20	2	10	5	Dec'13	30	5	16.67	4
Total	237	93	39.24		Total	277	105	37.91	
Correlations coefficient, r = 0.64 p = 0.03* Significant at 5% level					Correlations coefficient, r = 0.77 p = 0.002** Highly significant at 5% level				

* Correlation is significant at the 0.05 level (2-tailed) and ** Correlation is highly significant at the 0.01 level (2- tailed) and ^{ns} Correlation is not significant at 0.05 level but positively correlated.

Plate 1.



A)



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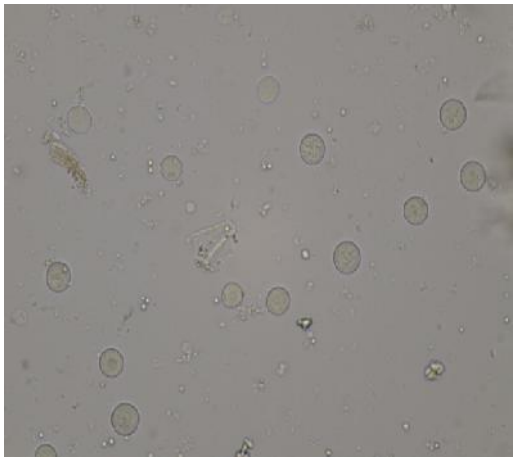
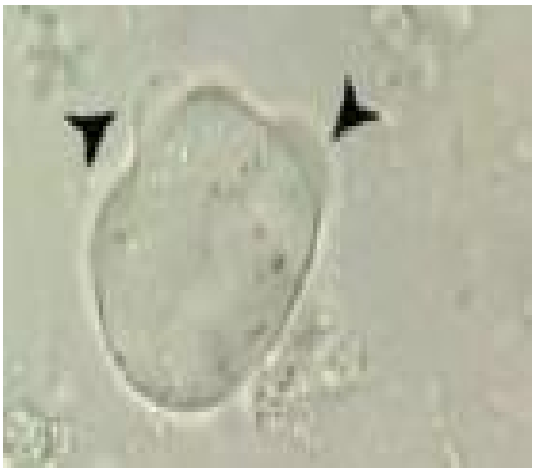


C)



D)

A) Cyst of *B. hominis* observed by direct microscopy (100 X magnification).B) Light microscopy of *B. hominis* showing vacuolar forms (magnification 40X).C) Light microscopy showing granular forms of *B. hominis* (40X magnification).D) Light microscopy showing single cell amoeboid form of *B. hominis* (100 X magnification).

OBSERVATION ON DIFFERENT FORMS OF *B. HOMINIS* IN MONTHLY STOOL (MS) SAMPLES IN DIFFERENT AGE GROUPS OF CHILDREN FROM MIRPUR AREA:

Out of 8601 monthly stool samples, in two years, 2946 samples were positive for *B. hominis*. As multiple forms were observed in the same sample, 92.83% for cyst, 40.97% for vacuolar, 35% for amoeboid and 17.89% for granular form of *B. hominis* (Fig. 54). The highest prevalence of cyst form of *B. hominis*96.86% observed in 61-72 months the age groups and the highest prevalence of vacuolar form 42.609%, amoeboid form 38.80% in the age group 49-60 months and granular form 21.88% within the age group 13-24 months (Table 18).The mean difference was highly significant (p=0.000**) among cyst, vocuolar, amoeboid and granular forms of *B. hominis* in overall MS samples from Mirpur area(Table 19).

Table 18. Prevalence of four morphological forms of *B. hominis*by*in vitro* culture in MS samples (n=8601) from Mirpur area during 2012-13.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	703	96	81 (84.38%)	38 (39.58%)	27 (28.10%)	**21 (21.88%)**
25-36	2126	622	560 (90.03%)	254 (40.84%)	211 (33.90%)	122 (19.61%)
37-48	2852	1008	933 (92.56%)	408 (40.48%)	328 (32.50%)	172 (17.06%)
49-60	2196	899	853 (94.88%)	**383 (42.60%)**	**349 (38.80%)**	171 (19.02%)
61-72	724	318	**308 (96.86%)**	124 (38.99%)	116 (36.50%)	40 (12.58%)
Total	8601	2946	2735 (92.83%)	1207 (40.97%)	1031 (35.00%)	527 (17.89%)

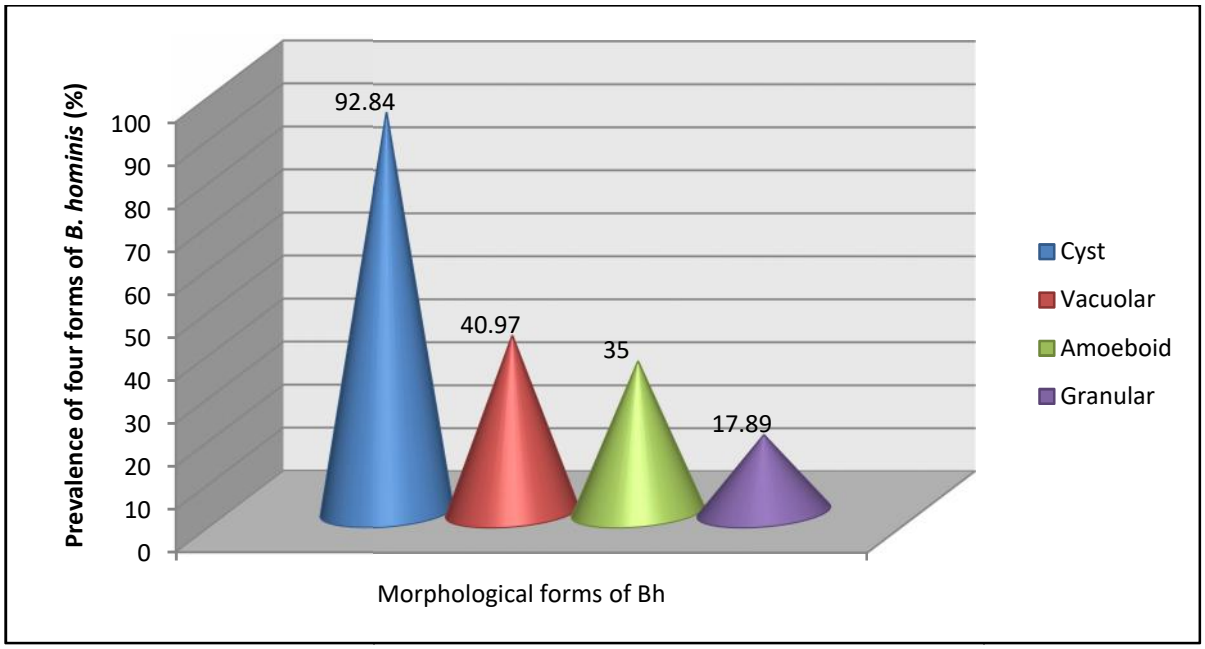


Fig. 54. Prevalence of four morphological forms of *B. hominis* in overall MS samples during 2012-13.

Table 19. F-test and multiple comparisons among **Cyst, Vocuolar, Amoeboid and Granular form of *B. hominis* in overall MS samples from Mirpur area.**

Source of variation	F-test	Level of significance	Multiple Comparisons by LSD[1]		Level of significance
Cyst, Vacuolar, Amoeboid and Granular	375.76	0.000 significant	Cyst	Vacuolar	0.000*
				Amoeboid	0.000*
				Granular	0.000*
			Vacuolar	Cyst	0.000*
				Amoeboid	0.000*
				Granular	0.000*
			Amoeboid	Cyst	0.000*
				Vacuolar	0.000*
				Granular	0.000*
			Granular	Cyst	0.000*
				Vacuolar	0.000*
				Amoeboid	0.000*

[1] LSD= Least Significant Difference and *The mean difference is significant at the 0.05 level

By*in vitro*culture method, various morphological forms were clearly identified. In 2012, out of 4481 examined samples, 1415 samples were positive for *B. hominis*. Multiple forms were observed: cyst (90.11%), vacuolar (42.83%), amoeboid (33.60%) and granular (20.78%) form of *B. hominis*(Fig. 55). The highest prevalence of most of the forms of *B. hominis*were observed within the age group of 49-60 months (Table 20).

Table 20. Prevalence of four morphological forms of *B. hominis*by*in vitro* culture inMS samples (n=4481) during 2012.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	703	96	81 (84.38%)	38 (39.58%)	27 (28.10%)	21 (21.88%)
25-36	1507	426	373 (87.56%)	178 (41.78%)	143 (33.60%)	96 (22.54%)
37-48	1484	555	505 (90.99%)	233 (41.98%)	177 (31.90%)	98 (17.66%)
49-60	787	338	316 (93.49%)	157 (46.45%)	128 (37.90%)	79 (23.37%)
Total	4481	1415	1275 (90.11%)	606 (42.83%)	475 (33.60%)	294 (20.78%)

In 2013, within 4120 examined monthly samples, 1531 samples were positive for *B. hominis*. Multiple forms were observed: cyst (95.36%), vacuolar (39.26%), amoeboid (36.30%) and granular (15.15%) forms of *B. hominis* (Fig. 55). The highest prevalence (96.86%) of cyst form of *B. hominis*observed within the 61-72 months age group (Table 21).

Table 21. Prevalence of four morphological forms of *B. hominis*by*in vitro* culture in MS samples (n=4120) during 2013.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
25-36	619	198	187 (94.44%)	76 (38.38.0%)	68 (34.34%)	26 (13.13%)
37-48	1368	454	428 (94.27%)	175 (38.55%)	151 (33.30%)	74 (16.30%)
49-60	1409	561	537 (95.72%)	**226 (40.29%)**	**221 (39.40%)**	**92 (16.40%)**
61-72	724	318	**308 (96.86%)**	124 (38.99%)	116 (36.50%)	40 (12.58%)
Total	4120	1531	1460 (95.36%)	601 (39.26%)	556 (36.30%)	232 (15.15%)

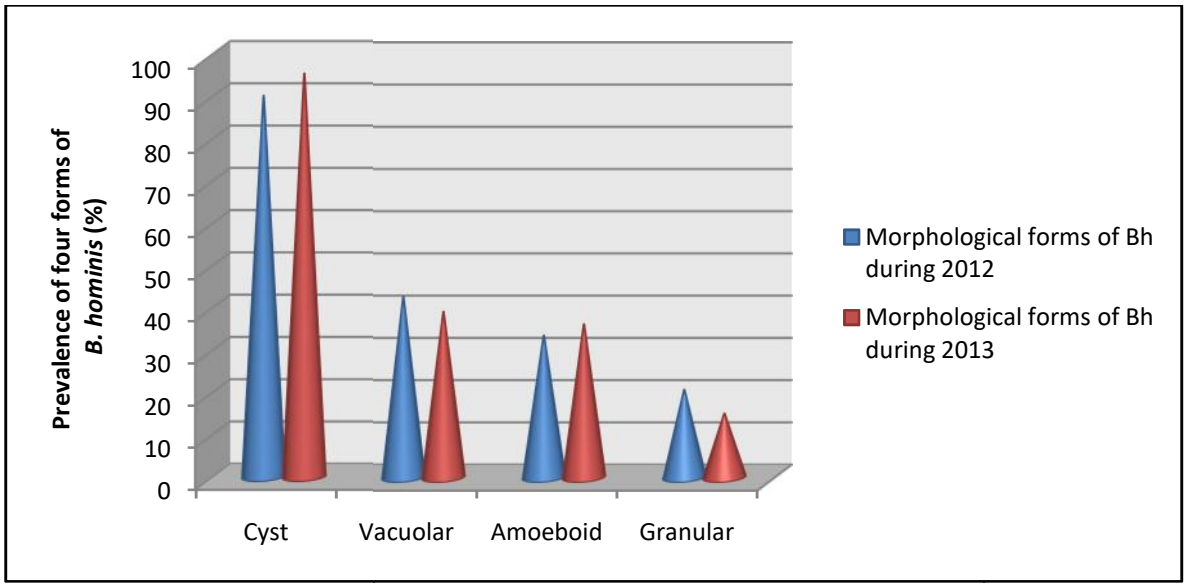


Fig. 55. Prevalence of four morphological forms of *B. hominis* in MS samples during 2012 and 2013.

OBSERVATION ON DIFFERENT FORMS OF *B. HOMINIS* IN DIARRHEAL STOOL (DS) SAMPLES IN DIFFERENT AGE GROUPS OF CHILDREN FROM MIRPUR AREA:

Out of 721 examined diarrheal samples of two years, 197 samples were positive for *B. hominis*. Multiple forms were observed: cyst (93.40%), vacuolar (35.53%), amoeboid (22.34%) and granular (10.15%) form of *B. hominis*(Fig.56). The highest prevalence (96.05%) of cyst form of *B. hominis*was observed within the age group of 25-36 months and the highest prevalence of other forms observed within the 13-24 months age group (Table 22).The mean difference was highly significant (p=0.000**) among cyst, vocuolar, amoeboid and granular forms of *B. hominis* in overall DS samples from Mirpur area(Table 23).

Table 22. Prevalence of fourmorphological forms of *B. hominis*by*in vitro* culture in DS samples (n=721) from Mirpur area during 2012-13.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	157	21	19 (90.48%)	**11 (52.38%)**	**6 (28.57%)**	**4 (19.05%)**
25-36	268	76	**73 (96.05%)**	23 (30.26%)	15 (19.74%)	8 (10.53%)
37-48	203	65	62 (95.39%)	26 (40%)	16 (24.62%)	6 (9.23%)
49-60	71	26	22 (84.62%)	9 (34.62%)	6 (23.08%)	2 (7.69%)
61-72	22	9	8 (88.89%)	1 (11.11%)	1 (11.11%)	0 (0.0%)
Total	721	197	**184 (93.40%)**	**70 (35.53%)**	**44 (22.34%)**	**20 (10.15%)**

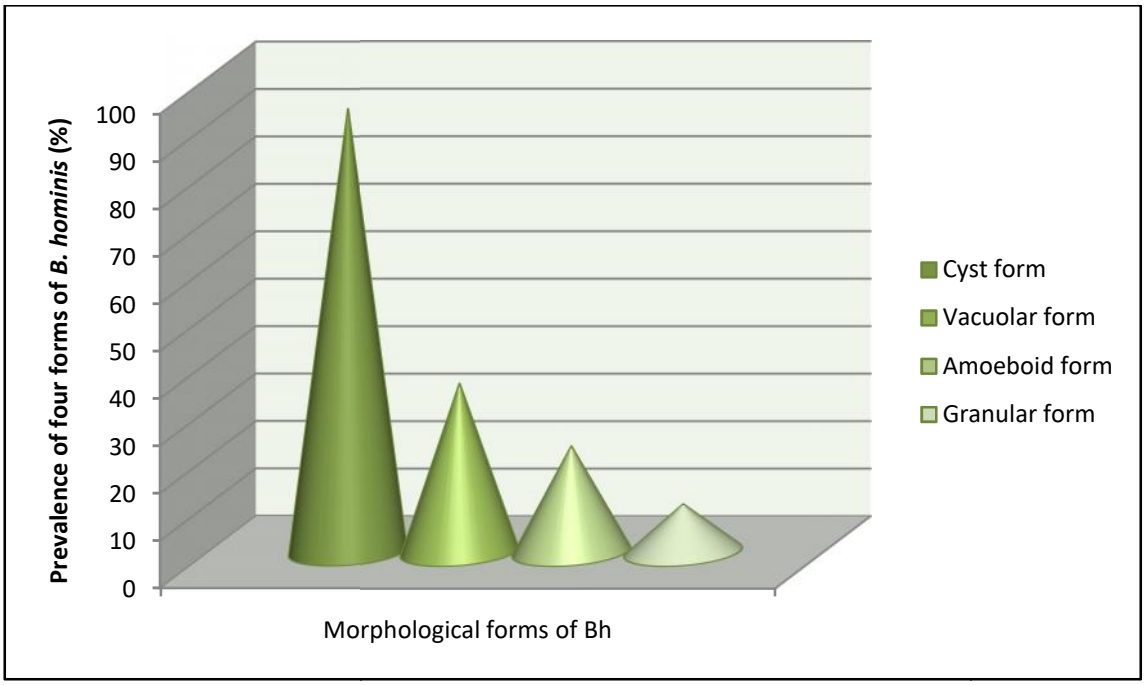


Fig. 56. Prevalence of four morphological forms of *B. hominis* in DS samples during 2012-13.

Table 23. F-test and multiple comparisons among **Cyst, Vocuolar, Amoeboid and Granular form of *B. hominis* in overall DS samples from Mirpur area.**

Source of variation	F-test	Level of significance	Multiple Comparisons by LSD[1]		Level of significance
Cyst, Vacuolar, Amoeboid and Granular	175.46	0.000** significant	Cyst	Vacuolar	0.000*
				Amoeboid	0.000*
				Granular	0.000*
			Vacuolar	Cyst	0.000*
				Amoeboid	0.005*
				Granular	0.000*
			Amoeboid	Cyst	0.000*
				Vacuolar	0.005*
				Granular	0.000*
			Granular	Cyst	0.000*
				Vacuolar	0.000*
				Amoeboid	0.000*

[1] LSD= Least Significant Difference and *The mean difference is significant at the 0.05 level

By*in vitro* culture method, various morphological forms were clearly identified. Out of 422 examined diarrheal stool samples, 125 samples were positive for *B. hominis*. As

multiple forms were observed in the same sample, 94.40% for cyst, 36% for vacuolar, 20.80% for amoeboid and 8% for granular form of *B. hominis*(Fig. 57). The highest prevalence of cyst form of *B. hominis* 96.61% observed within the age group 25-36 months and the highest prevalence of other forms observed within the 13-24 months age group (Table 24).

Table 24. Prevalence of fourmorphological forms of *B. hominis*by*in vitro* culture inDS samples (n=422)during 2012.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	157	21	19 (90.48%)	**11 (52.38%)**	**6 (28.57%)**	**3 (14.29%)**
25-36	166	59	**57 (96.61%)**	18 (30.51%)	12 (20.34%)	6 (10.17%)
37-48	78	37	35 (94.60%)	12 (32.43%)	6 (16.22%)	0 (0.0%)
49-60	21	8	7 (87.50%)	4 (50%)	2 (25%)	1 (12.50%)
Total	422	125	117 (94.40%)	45 (36.00%)	23 (20.80%)	10 (8%)

In 2013, out of299 examined MS samples, 72 samples were positive for *B. hominis*. Multiple forms were observed: cyst (91.67%), vacuolar (34.72%), amoeboid (25%) and granular (11.11%) form of *B.hominis*(Fig. 57).The highest prevalence (94.12%) of cyst form of *B. hominis*was observed within the age group 25-36 months and the highest prevalence of remaining three forms were found within the age group 37- 48 months (Table 25).

Table 25. Prevalence of fourmorphological forms of *B. hominis*by*in vitro* culture inDS samples (n=299) during 2013.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
25-36	102	17	**16 (94.12%)**	5 (29.41%)	3 (17.65%)	2 (11.76%)
37-48	125	29	27 (93.10%)	**14 (48.28%)**	**10 (34.48%)**	**5 (17.24%)**
49-60	50	17	15 (88.24%)	5 (29.41%)	4 (23.53%)	1 (5.88%)
61-72	22	9	8 (88.89%)	1 (11.11%)	1 (11.11%)	0 (0.0%)
Total	299	72	66 (91.67%)	25 (34.72%)	18 (25.00%)	8 (11.11%)

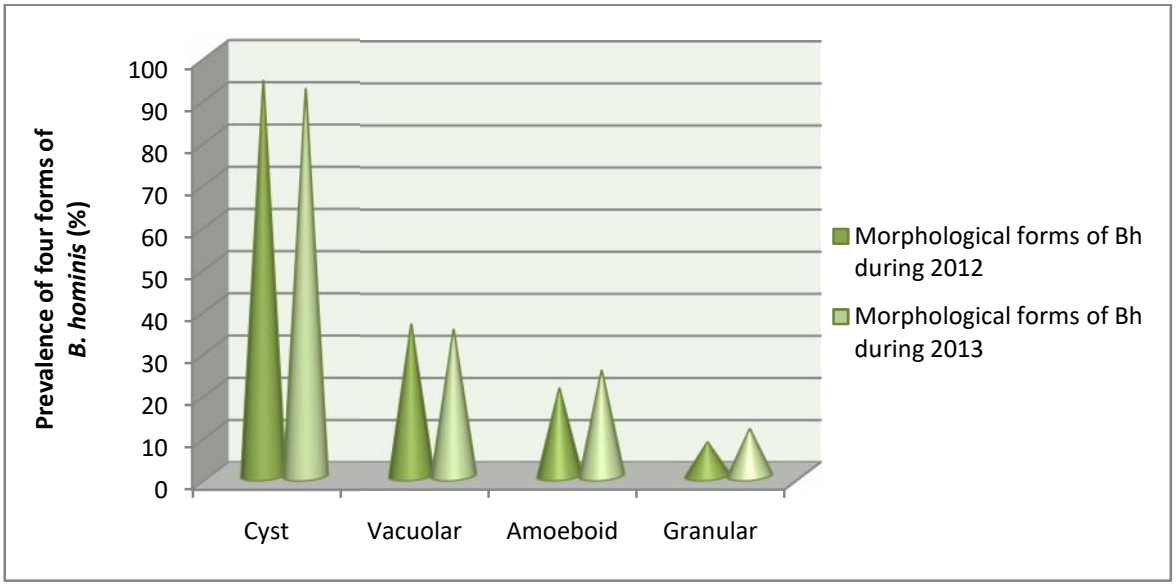


Fig. 57. Prevalence of four morphological forms of *B. hominis* in DS samples during 2012 and 2013.

Observation on morphological forms of *B. hominis* by *in vitro* culture found almost similar prevalence in MS and DS samples. Only cyst form found more prevalent (93.4%) in diarrheal stool samples than monthly stool samples (92.84%). Whereas other three

types of forms found more prevalent (40.97%, 35% and 17.89%) in MS than DS samples (35.53%, 22.34% and 10.15%) of Mirpur area (Fig.58)

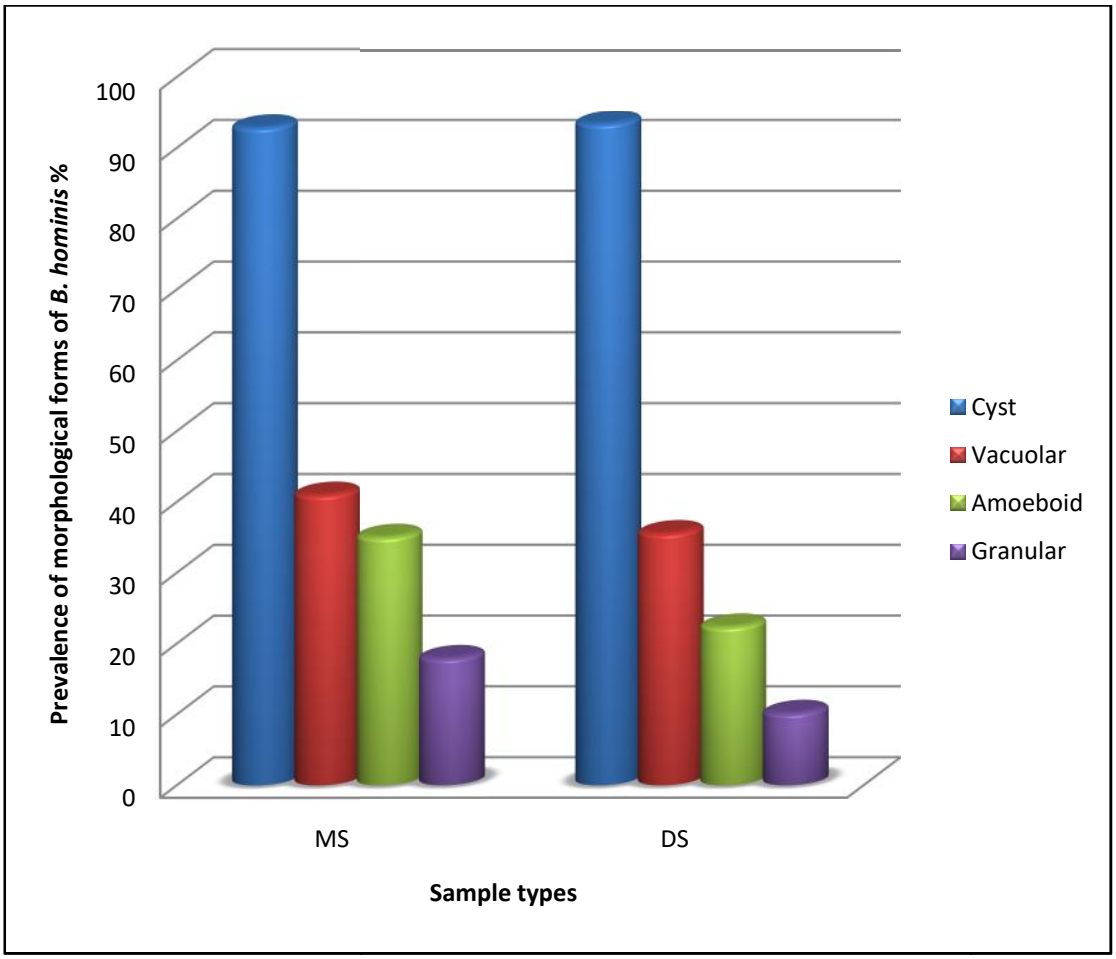


Fig.58. Comparative prevalence of fourmorphological forms of *B. hominis* betweenMS and DS samples from Mirpur area.

OBSERVATION ON DIFFERENT FORMS OF *B. HOMINIS* IN MONTHLY STOOL (MS) SAMPLES IN DIFFERENT AGE GROUPS OF CHILDREN FROM MOHAMMADPUR AREA:

Out of 2704 examined monthly samples of two years, 1152 samples were positive for *B. hominis*. Four major formsof *B. hominis* were observed: cyst (90.97%), vacuolar

(72.31%), amoeboid (42.90%) and granular (6.34%) form of *B. hominis* (Fig. 59). The highest prevalence of cyst form (100%), vacuolar form (78.63%), amoeboid form (50%) and granular form (8.47%)were observed within 13-24 months age group (Table 26).The mean difference was highly significant (p=0.000**) among cyst, vocuolar, amoeboid and granular forms of *B. hominis* in overall MS samples from Mohammadpur area(Table 27).

Table 26. Prevalence of four morphological forms of *B. hominis*by*in vitro* culture in MS samples (n=2704)from Mahammadpur areaduring 2012-13.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	666	248	**248 (100%)**	**195 (78.63%)**	**124 (50%)**	**21 (8.47%)**
25-36	659	270	255 (94.44%)	202 (74.81%)	127 (47%)	19 (7.04%)
37-48	547	256	213 (83.20%)	179 (69.92%)	98 (38.30%)	14 (5.47%)
49-60	537	250	215 (86%)	161 (64.40%)	94 (37.60%)	12 (4.8%)
61-72	295	128	117 (91.41%)	96 (75%)	51 (39.80%)	7 (5.47%)
Total	2704	1152	1048 (90.97%)	847 (72.31%)	504 (42.90%)	76 (6.34%)

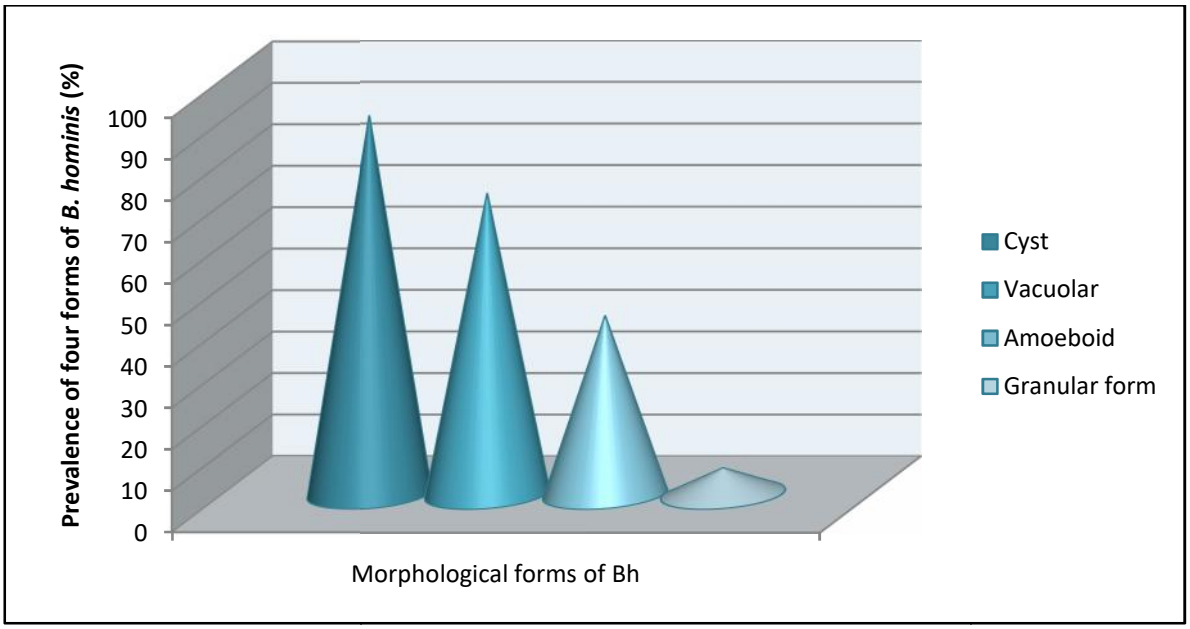


Fig. 59. Prevalence of four morphological forms of *B. hominis* in MS samples from Mohammadpur area during 2012-13.

Table 27. F-test and multiple comparisons among **Cyst, Vocuolar, Amoeboid and Granular forms of *B. hominis* in overall MS samples from Mohammadpur area.**

Source of variation	F-test	Level of significance	Multiple Comparisons by LSD[1]		Level of significance
Cyst,Vacuolar, Amoeboid and Granular	43.127	0.000** significant	Cyst	Vacuolar	0.000*
				Amoeboid	0.000*
				Granular	0.000*
			Vacuolar	Cyst	0.000*
				Amoeboid	0.000*
				Granular	0.000*
			Amoeboid	Cyst	0.000*
				Vacuolar	0.000*
				Granular	0.000*
			Granular	Cyst	0.000*
				Vacuolar	0.000*
				Amoeboid	0.000*

[1] LSD= Least Significant Difference and *The mean difference is significant at the 0.05 level

In 2012, within 1169 monthly stool (MS) samplesexamined, 528 samples were positive for *B. hominis*. Multiple forms were observed: cyst (92.42%), vacuolar (71.78%), amoeboid (45.3%) and granular (5.68%) form of *B. hominis*(Fig. 60). The highest prevalence (93.98%) of cyst form of *B. hominis*was observed within the 25-36 months age group (Table28).

Table28. Prevalence of fourmorphological forms of *B. hominis*by*in vitro* culture in MS samples (n=1169) during 2012.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	312	118	112 (94.92%)	**93 (78.81%)**	62 (52.5%)	9 (7.63%)
25-36	294	132	**125 (93.98%)**	101 (76.52%)	**71 (53.8%)**	**11 (8.33%)**
37-48	237	120	114 (95%)	78 (65%)	47 (39.2%)	3 (2.5%%)
49-60	185	94	80 (86.96%)	59 (62.77%)	34 (36.2%)	4 (4.26%)
61-72	141	64	57 (90.48%)	48 (75%)	25 (39.1%)	3 (4.69%)
Total	1169	528	488 (92.42%)	379 (71.78%)	239 (45.3%)	30 (5.68%)

In 2013, out of 4120 examined monthly samples, 1531 samples were positive for *B. hominis*. Various forms were observed: cyst (91.51%), vacuolar (72.76%), amoeboid (40.90%)and granular (6.89%)form of *B. hominis* (Fig.60). The highest prevalence of morphological forms of *B. hominis*observed within the age group 13- 24 months (Table 29).

Table 29. Prevalence of fourmorphological forms of *B. hominis*by*in vitro* culture in MS (n=1535)samples during 2013.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	354	130	**126 (96.92%)**	**102 (78.46%)**	**62 (47.70%)**	**12 (9.23%)**
25-36	365	138	130 (94.20%)	101 (73.19%)	56 (40.60%)	8 (5.80%)
37-48	310	136	120 (88.24%)	101 (74.26%)	51 (37.50%)	11 (8.09%)
49-60	352	156	135 (86.54%)	102 (65.38%)	60 (38.50%)	8 (5.13%)
61-72	154	64	60 (93.75%)	48 (75%)	26 (40.60%)	4 (6.25%)
Total	1535	624	571 (91.51%)	454 (72.76%)	255 (40.90%)	43 (6.89%)

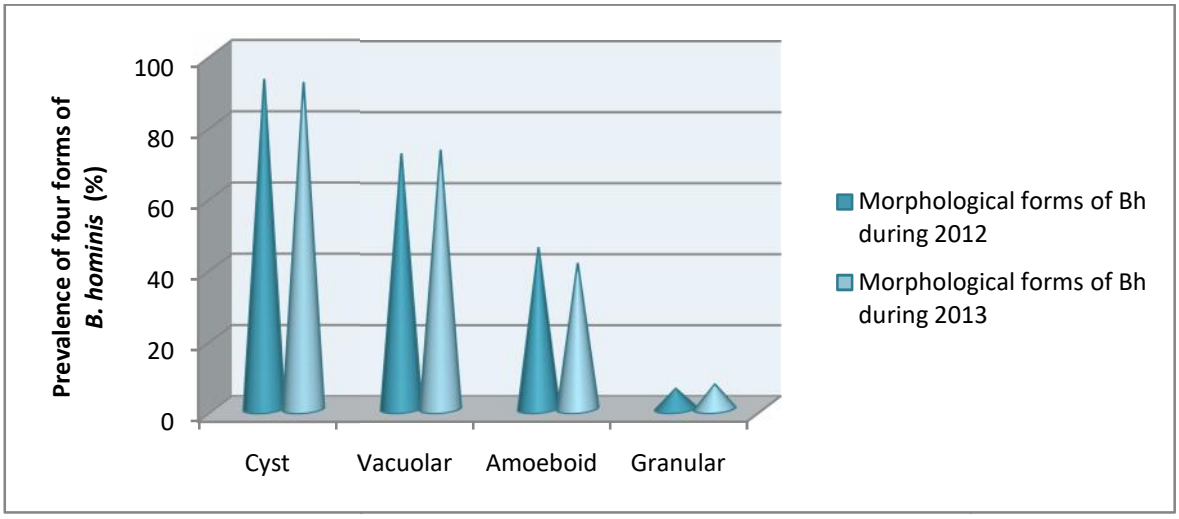


Fig. 60. Prevalence of four morphological forms of *B. hominis* in MS samples from Mahammadpur area during 2012 and 2013.

OBSERVATION ON DIFFERENT FORMS OF *B. HOMINIS* IN DIARRHEAL STOOL (DS) SAMPLES IN DIFFERENT AGE GROUPS OF CHILDREN FROM MOHAMMADPUR AREA:

By*in vitro* culture method,four morphological forms were clearly identified.Out of 514 examined diarrheal samples, 208 samples were positive for *B. hominis*. As multiple forms were observed in the same sample, 93.75% for cyst, 57.21% for vacuolar, 42.30% for amoeboid and 9.62% for granular form of *B. hominis*(Fig.61). The highest prevalence (95.24%) of cyst form was observed within the age group 61-72 monthsand the lowest prevalence (7.32%) of granular form observed within the age group 13- 24 months (Table 32).The mean difference was highly significant (p=0.000**) among cyst, vocuolar, amoeboid and granular forms of *B. hominis* in overall DS samples from Mohammadpur area(Table 33).

Table 32. Prevalence of fourmorphological forms of *B. hominis*by*in vitro* culture in DS samples (n=514) from Mohammadpur area during 2012-13.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	164	41	38 (92.68%)	25 (60.98%)	17 (41.50%)	3 (7.32%)
25-36	121	36	34 (94.44%)	**24 (66.67%)**	**18 (50%)**	4 (11.11%)
37-48	96	58	55 (94.83%)	32 (55.17%)	25 (43.10%)	**7 (12.07%)**
49-60	92	52	49 (94.23%)	29 (55.77%)	22 (42.30%)	4 (7.69%)
61-72	41	21	**20 (95.24%)**	9 (42.86%)	6 (28.60%)	2 (9.52%)
Total	514	208	195 (93.75%)	119 (57.21%)	88 (42.30%)	20 (9.62%)

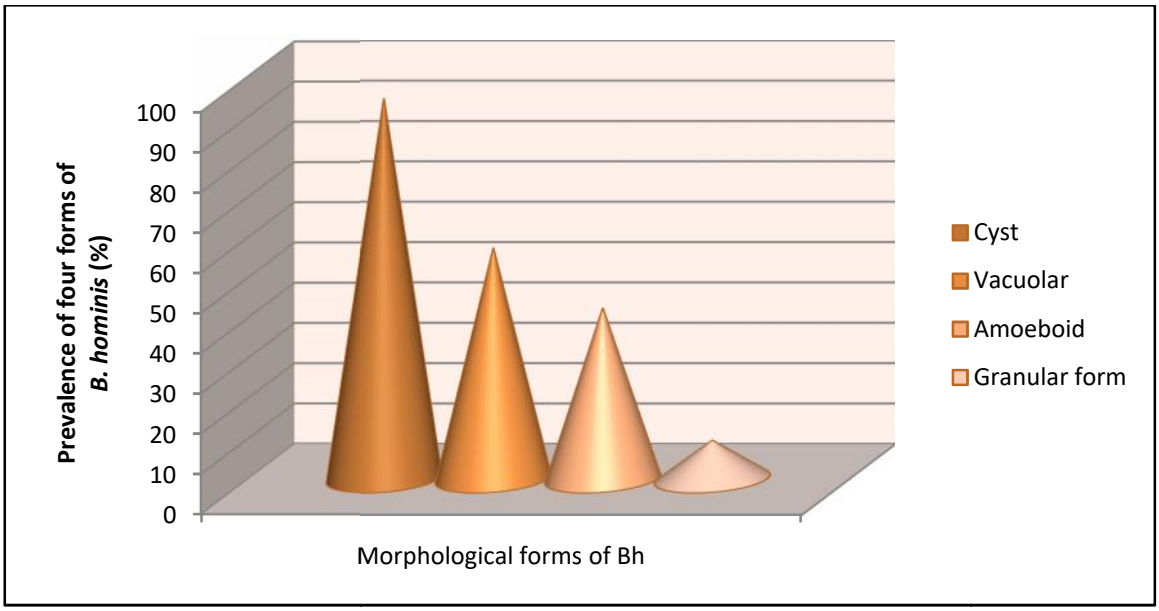


Fig.61.Prevalence of four morphological forms of *B. hominis* in DS samples during 2012-13.

Table 33. F-test and multiple comparisons among **Cyst, Vocuolar, Amoeboid and Granular forms of *B. hominis* in overall DS samples from Mohammadpur area.**

Source of variation	F-test	Level of significance	Multiple Comparisons by LSD		Level of significance
Cyst,Vacuolar, Amoeboid and Granular	180.55	0.000 significant	Cyst	Vacuolar	0.003*
				Amoeboid	0.000*
				Granular	0.000*
			Vacuolar	Cyst	0.003*
				Amoeboid	0.005*
				Granular	0.000*
			Amoeboid	Cyst	0.000*
				Vacuolar	0.005*
				Granular	0.000*
			Granular	Cyst	0.000*
				Vacuolar	0.000*
				Amoeboid	0.000*

*. The mean difference is significant at the 0.05 level
1. LSD= Least Significant Difference.

In 2012, DS samples from mohammadpur area, within 103 positivesamples, cyst (95.15%), vacuolar (61.62%), amoeboid (43.70%) and granular (9.71%) form of *B. hominis* were investigated (Fig. 62).The highest prevalence of cyst form (100%) was observed within the age group 49-60 months. The highest prevalence of other forms of *B. hominis*were observed within the age group 25-36 months (Table 30).

Table30. Prevalence of fourmorphological forms of *B. hominis*by*in vitro* culture in DS samples (n=237) during 2012.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	73	21	20 (95.24%)	13 (61.90%)	8 (38.10%)	2 (9.52%)
25-36	62	17	16 (94.12%)	**12 (70.59%)**	**10 (58.80%)**	**2 (11.76%)**
37-48	40	28	26 (92.86%)	17 (60.71%)	13 (46.40%)	3 (10.71%)
49-60	36	23	**23 (100%)**	14 (60.87%)	11 (47.80%)	2 (8.70%)
61-72	26	14	13 (92.86%)	5 (35.71%)	3 (21.40%)	1 (7.14%)
Total	237	103	98 (95.15%)	61 (61.62%)	45 (43.70%)	10 (9.71%)

Out of 277 examined diarrhealstool samples, 105 samples were positive for *B. hominis* and cyst (90.48%), vacuolar (55.24%), amoeboid (41%) and granular (9.52%) form of *B. hominis* were investigated (Fig. 62). The highest prevalence (93.33%) of cyst form, was observed within the age group 37-48 months, remaining were observed in other age groups (Table 31).

Table 31. Prevalence of fourmorphological forms of *B. hominis*by*in vitro* culture in DS samples (n=277) during 2013.

Age in months	Total no of samples examined	*B.hominis* positive	Samples with Cyst form	Samples with Vacuolar form	Samples with Amoeboid form	Samples with Granular form
13-24	91	20	18 (90%)	12 (60%)	9 (45%)	1 (5%)
25-36	59	19	17 (89.47%)	12 (63.16%)	8 (42.10%)	2 (10.53%)
37-48	56	30	28 (93.33%)	15 (50%)	12 (40%)	4 (13.33%)
49-60	56	29	26 (89.66%)	15 (51.72%)	11 (37.90%)	2 (6.90%)
61-72	15	7	6 (85.71%)	4 (57.14%)	3 (42.90%)	1 (14.29%)
Total	277	105	95 (90.48%)	58 (55.24%)	43 (41%)	10 (9.52%)

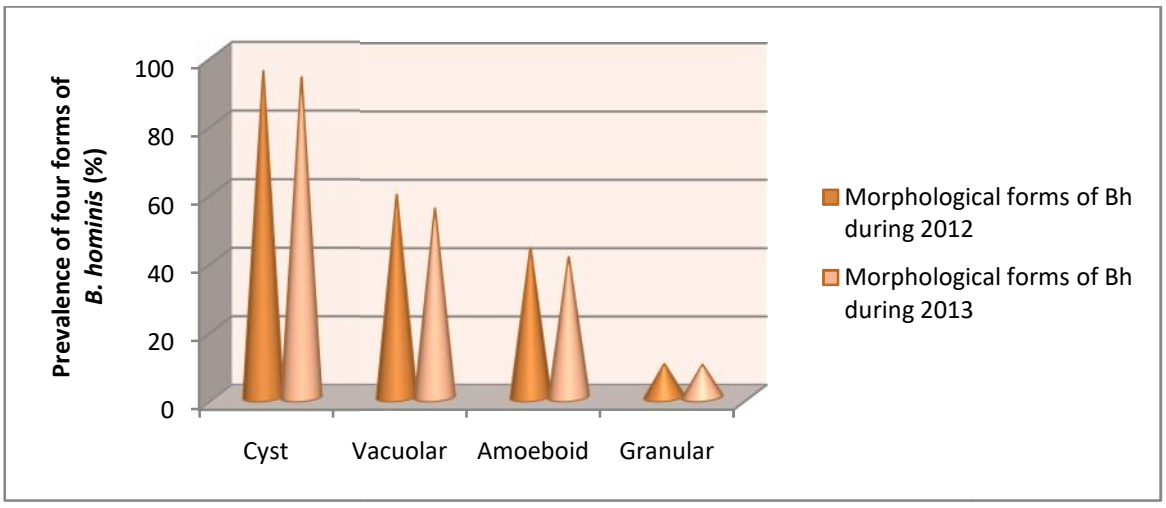


Fig. 62. Prevalence of four morphological forms of *B. hominis* in DS samples during 2012 and 2013.

By *in vitro* culture method,the prevalence of different morphological forms of *B. hominis* found almost similar prevalence in MS and DS samples from Mohammadpur area. In DS samples, cyst and granular formswere more prevalent (94.23% and 9.62%) than MS samples (90.97% and 6.34%). Whereas, remaining two vacuolar and amoeboid forms were more prevalent (72.31% and 42.9%) in MS than DS samples (57.21% and 42.30%) from Mohammadpur area (Fig.63).

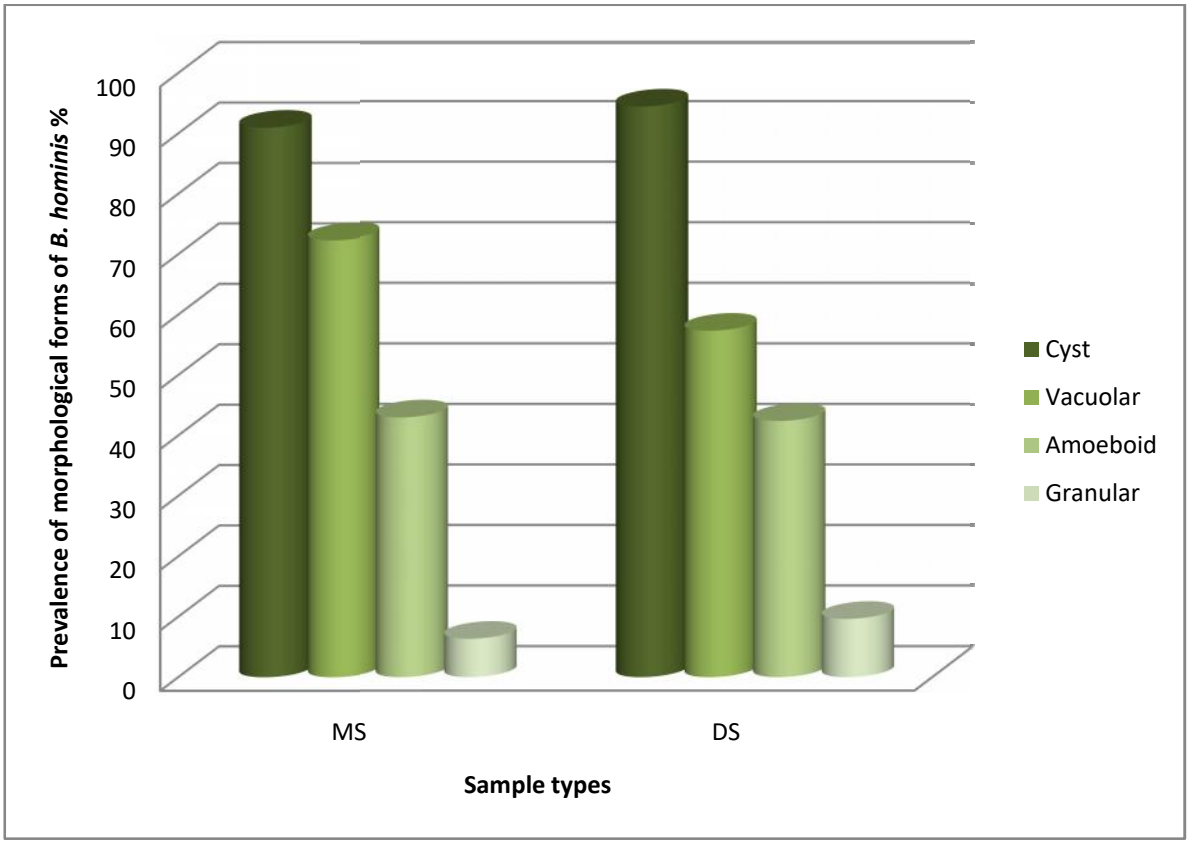


Fig. 63. Comparative prevalence of fourmorphological forms of *B. hominis* between MS and DS samples fromMohammadpur area.

Observation on overall prevalence of morphological forms of *B. hominis* in MS samples of both areas:

By *in vitro* culture method, a total of 8601 MS samples from Mirpur area, 31.80% cyst, 14.04% vacuolar, 11.99% amoeboid and 6.11% granular form were observed. While it was 38.78%, 30.81%, 18.30% and 2.70% in MS samples from Mohammadpur area (Table 34, 35).

Table 34.Overall prevalence of four morphological forms of *B. hominis* by*in vitro* culture in MS samples (n=8601)from Mirpur area.

Sample collection year	Total tested samples, n	Prevalence of Cyst form	Prevalence of Vacuolar form	Prevalence of Amoeboid form	Prevalence of granular form
2012	4481	1275 (28.45%)	606 (13.52%)	475 (10.60%)	294 (6.56%)
2013	4120	1460 (35.44%)	601 (14.59%)	556 (13.57%)	232 (5.63%)
Overall	**8601**	**2735 (31.80%)**	**1208 (14.04%)**	**1031 (11.99%)**	**526 (6.11%)**

Table 35.Overall prevalence of four morphological forms of *B. hominis* by*in vitro* culture in MS samples (n=2704)from Mohammadpur area.

Sample collection year	Total tested samples, n	Prevalence of Cyst form	Prevalence of Vacuolar form	Prevalence of Amoeboid form	Prevalence of granular form
2012	1169	477 (40.80%)	379 (32.42%)	239 (20.40%)	30 (2.57%)
2013	1535	571 (37.20%)	454 (29.58%)	255 (16.60%)	43 (2.80%)
Overall	**2704**	**1048 (38.78%)**	**833 (30.81%)**	**494 (18.30%)**	**73 (2.70%)**

Observation on overall prevalence of morphological forms of *B. hominis* in DS samples of both areas:

By *in vitro* culture method, a total of 721 DS samples from Mirpur area, 22.61% cyst, 8.74% vacuolar, 5.69% amoeboid and 3.05% granular form were observed. While it was 38.13%, 23.15%, 17.10% and 3.89% in DS samples from Mohammadpur area (Table 36, 37).

Table 36. Overall prevalence of four morphological forms of *B.hominis* by *in vitro* culture in DS samples (n=721) from Mirpur area.

Sample collection year	Total tested samples, n	Prevalence of Cyst form	Prevalence of Vacuolar form	Prevalence of Amoeboid form	Prevalence of granular form
2012	422	109 (25.83%)	40 (9.48%)	23 (5.45%)	12 (2.84%)
2013	299	54 (18.06%)	23 (7.69%)	18 (6.02%)	10 (3.34%)
Overall	**721**	**163 (22.61%)**	**63 (8.74%)**	**41 (5.69%)**	**22 (3.05%)**

Table 37.Overall prevalence of four morphological forms of *B.hominis* by*in vitro* culture in DS samples (n=514)from Mohammadpur area.

Sample collection year	Total tested samples, n	Prevalence of Cyst form	Prevalence of Vacuolar form	Prevalence of Amoeboid form	Prevalence of granular form
2012	237	98 (41.35%)	61 (25.74%)	45 (19%)	10 (4.22%)
2013	277	98 (35.38%)	58 (20.94%)	43 (15.5%)	10 (3.61%)
Overall	**514**	**196 (38.13%)**	**119 (23.15%)**	**88 (17.10%)**	**20 (3.89%)**

During the study period (2012-2013), from Mirpur area, 2946 samples were found positive for *B. hominis*,in **MS samples**. It was observed that cyst + vacuolar (C+V) combinationwas

highly prevalent (35.34%) and lowest (8.21%)was in cyst+vacuolar+amoeboid+granular (C+V+A+G) combination (Table 38).

Table 38. Prevalence of morphological forms of *B. hominis* in different combination by *in vitro* culture in MS samples (n=8601) from Mirpur area during 2012-13.

Combination of different morphological forms	Examined samples	Total *B h* positive samples	Number of combined forms of*Bh*	Prevalence (%)
Cyst + Vacuolar	8601	2946	1041	35.34
Cyst + Amoeboid			935	31.74
Cyst + Granular			493	16.73
Vacuolar +Amoeboid			636	21.59
Vacuolar + Granular			342	11.61
Amoeboid + Granular			331	11.24
Cyst+Vacuolar + Amoeboid			591	20.06
Cyst+Vacuolar + Granular			326	11.07
Cyst+ Amoeboid +Granular			317	10.76
Vacuolar+ Amoeboid + Granular			253	8.59
Cyst+ Vacuolar+ Amoeboid + Granular			242	8.21

In 2012, the combination of all morphological forms were observed 7.28%,in**MS samples** from Mirpur area. Highest prevalence (34.21%) was observed in cyst+vacuolar (C+V) combination. In case of combination of three forms like cyst+ vacuolar+ amoeboid (C+V+A) combination was18.37% (Fig.64).

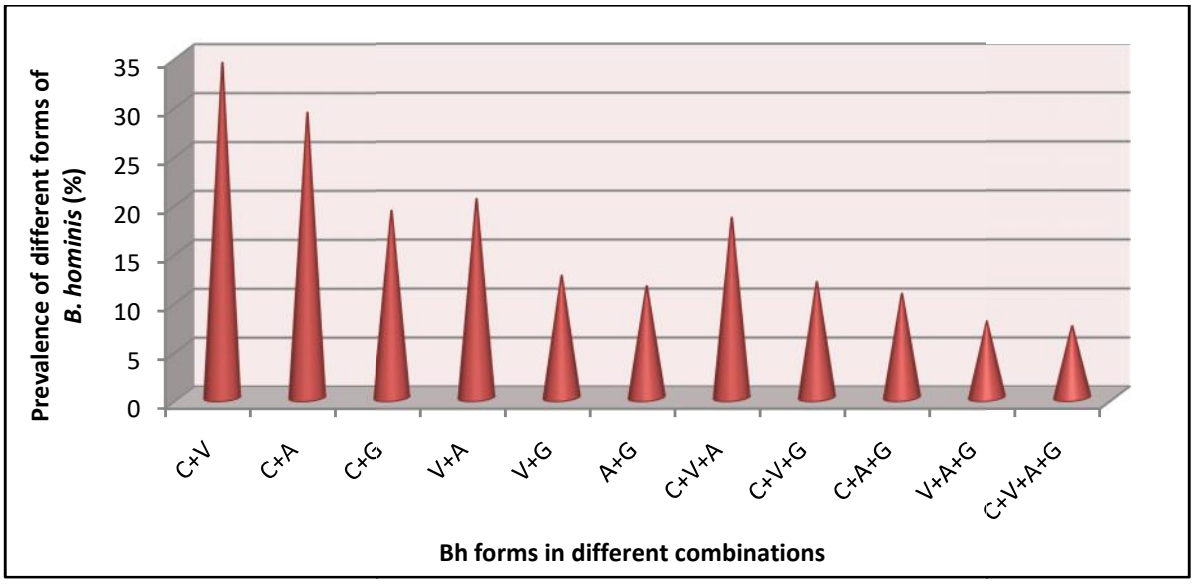


Fig.64. Prevalence of four forms of *B. hominis* in different combination in MS samples during 2012.

In 2013, the combination of all morphological forms (C+V+A+G) were observed 9.08%,inMSsamples from Mirpur area. Highest prevalence (36.38%) was observed in (C+V) combination. In case of combination ofthree forms (C+V+A) were 21.62% (Fig.65).

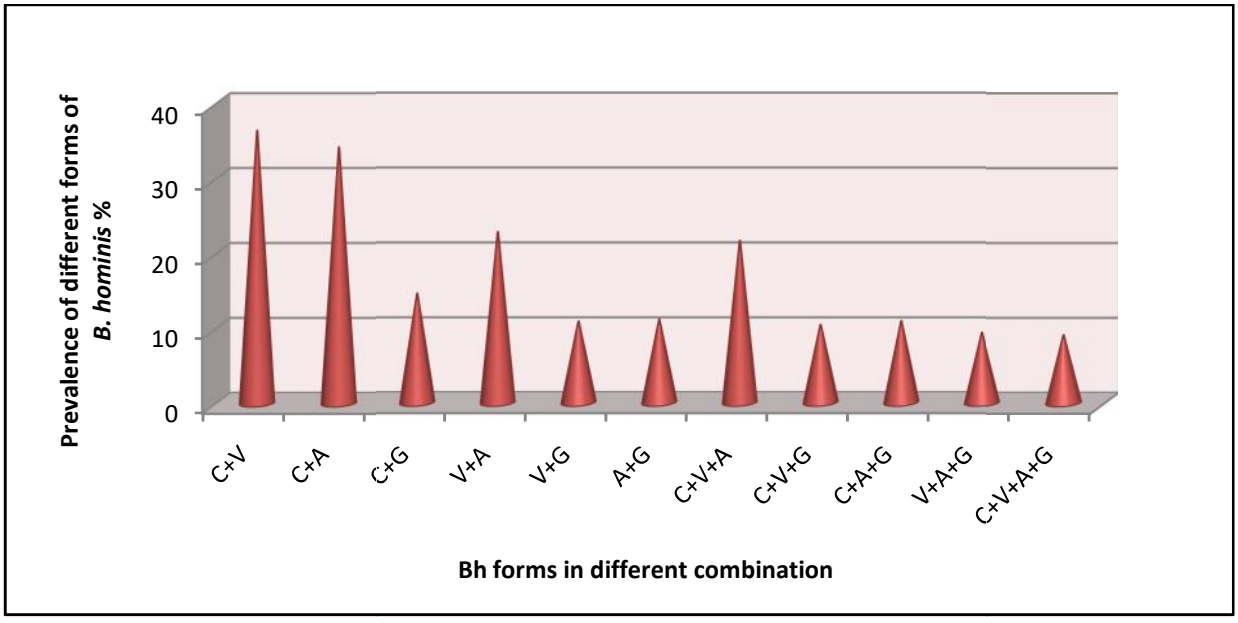


Fig.65. Prevalence of four forms of *B. hominis* in different combination in MS samples during 2013.

During the study period (2012-2013), in **DS samples** from Mirpur area, 191 samples for *B. hominis*were foundpositive. The highest prevalence (27.23%) was observed in cyst+vacuolar combination. All morphological forms were observed 8.21%, which was the lowest prevalence(Table 39).

Table 39. Prevalence of morphological forms of *B. hominis* in different combination by *in vitro* culture in DS samples (n=721) from Mirpur area during 2012-13.

Combination of different morphological forms	Examined samples	Total *B h* positive samples	Number of combined forms of*Bh*	Prevalence (%)
Cyst + Vacuolar	721	191	52	27.23
Cyst + Amoeboid			39	20.42
Cyst + Granular			21	10.99
Vacuolar +Amoeboid			29	15.18
Vacuolar + Granular			16	8.38
Amoeboid + Granular			14	7.33
Cyst+Vacuolar + Amoeboid			28	14.66
Cyst+Vacuolar + Granular			16	8.38
Cyst+ Amoeboid +Granular			15	7.85
Vacuolar+ Amoeboid + Granular			13	6.81
Cyst+ Vacuolar+ Amoeboid + Granular			13	6.81

In 2012, from Mirpur area, out of 422 **DSsamples**, the highest prevalence (27.91%) was observed in cyst+vacuolar (C+V) combination. All morphological forms were observed in 5.43% samples, which was the lowest prevalence (Fig. 66).In 2013, from Mirpur area, out of 299**DS samples**, the highest prevalence (38.71%) observed in cyst+vacuolar combination (C+V). The lowest prevalence (9.68%) was observed in the combination of all forms (C+V+A+G) only in one sample (Fig. 67).

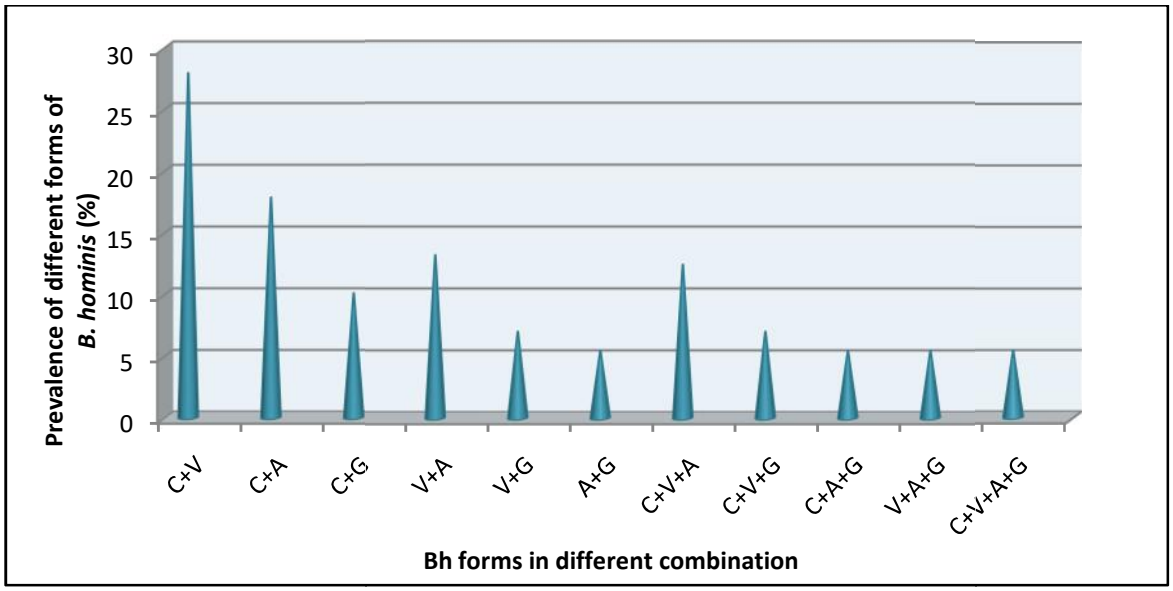


Fig.66. Prevalence of different forms of *B. hominis* in different combination in DS samples in 2012.

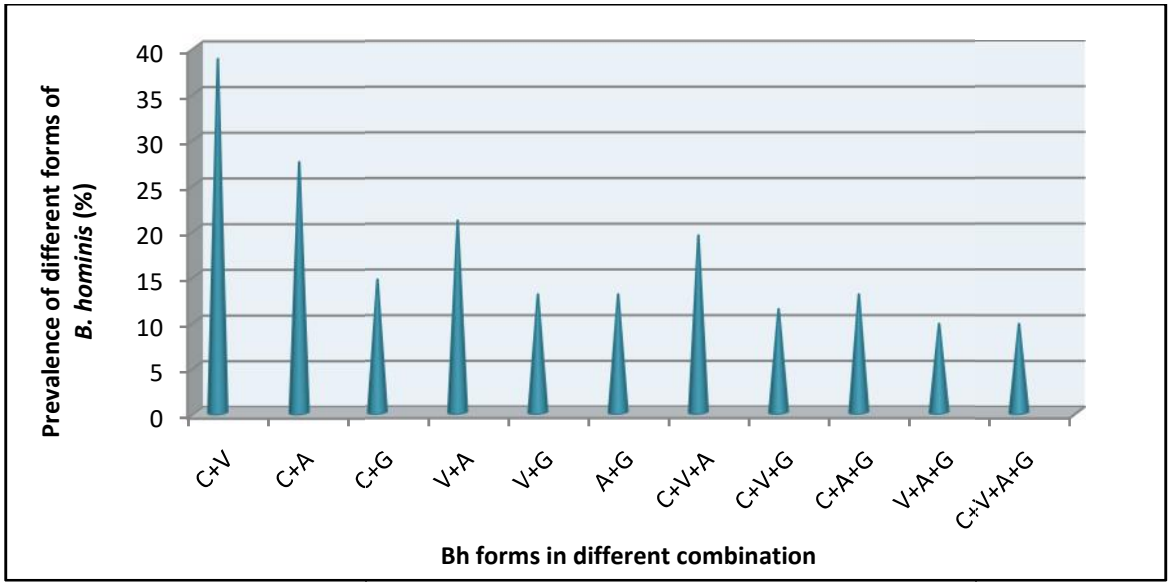


Fig.67. Prevalence of different forms of *B. hominis* in different combination in DSsamples in 2013.

Interpretation

From Mirpur area, cyst+vacuolar combination was more prevalent than other combination. Whereas, granular with other forms combination showed less prevalence.

During the study period (2012-2013), from Mohammadpur area, 1143 samples for *B. hominis*were found positive in **MS samples**. Highest prevalence (70.69%) wasobserved in cyst+vacuolar combination and lowest prevalence (4.20%)observed in cyst+vacuolar+ amoeboid+granular combination (Table 40).

Table 40. Prevalence of morphological forms of *B. hominis* in different combination by *in vitro* culture in MS samples (n=2704) from Mohammadpur area during 2012-13.

Combination of different morphological forms	Examined samples	Total *B h* positive samples	Number of combined forms of*Bh*	Prevalence (%
Cyst+Vacuolar	2704	1143	808	70.69
Cyst+Amoeboid			460	40.24
Cyst+Granular			75	6.56
Vacuolar+Amoeboid			342	29.92
Vacuolar+Granular			71	6.21
Amoeboid+Granular			57	4.99
Cyst+Vacuolar+Amoeboid			338	29.57
Cyst+Vacuolar +Granular			69	6.037
Cyst+ Amoeboid+Granular			50	4.37
Vacuolar+Amoeboid+Granular			52	4.55
Cyst+Vacuolar+Amoeboid+ Granular			48	4.20

In 2012, out of 1169**MS samples**, from Mohammadpur area, the highest prevalence (66.54%) observed in cyst and vacuolar combination.While, the prevalence 6.05% was observed in cyst and granular (C+G) combination, 5.29% in vacuolar and granular (V+G) and 3.40% in vacuolar and granular (A+G) combination. All morphological forms (C+V+A+G) were observed in 2.46% samples, which was the lowest prevalence (Fig.68).

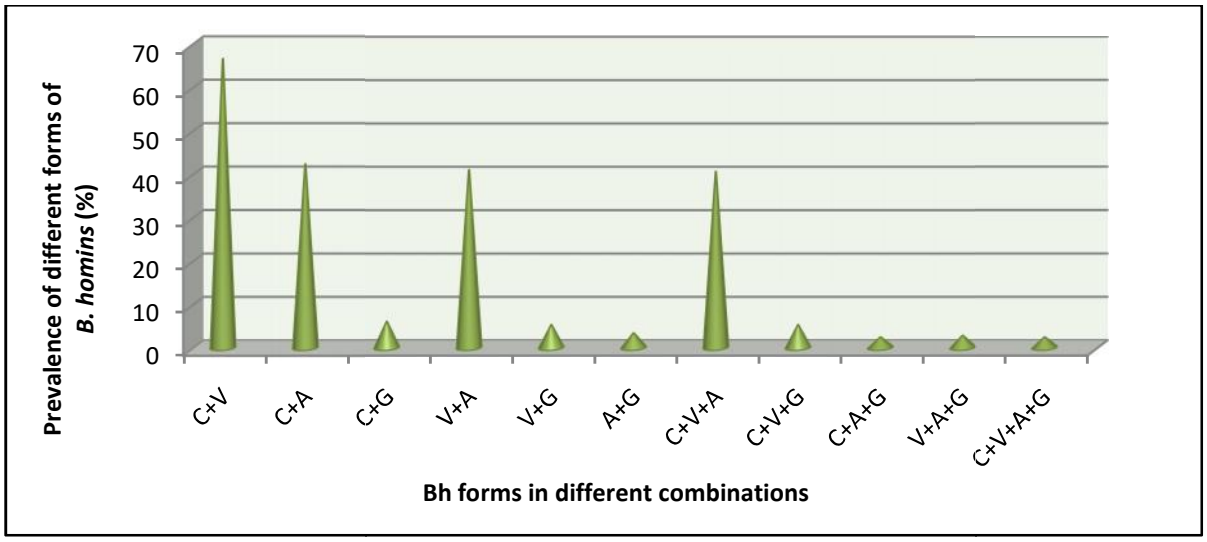


Fig.68.Prevalence of different forms of *B. hominis* in different combination in MSsamples from Mohammadpur area during 2012.

In 2013, out of 1535**MS samples**, from Mohammadpur area, the highest prevalence (73.08%) observed in (C+V) combination.While, in combination of three forms like (C+V+G) combination 6.57% was found, 5.93% was found in (C+A+G) and (V+A+G) combination(Fig.69).

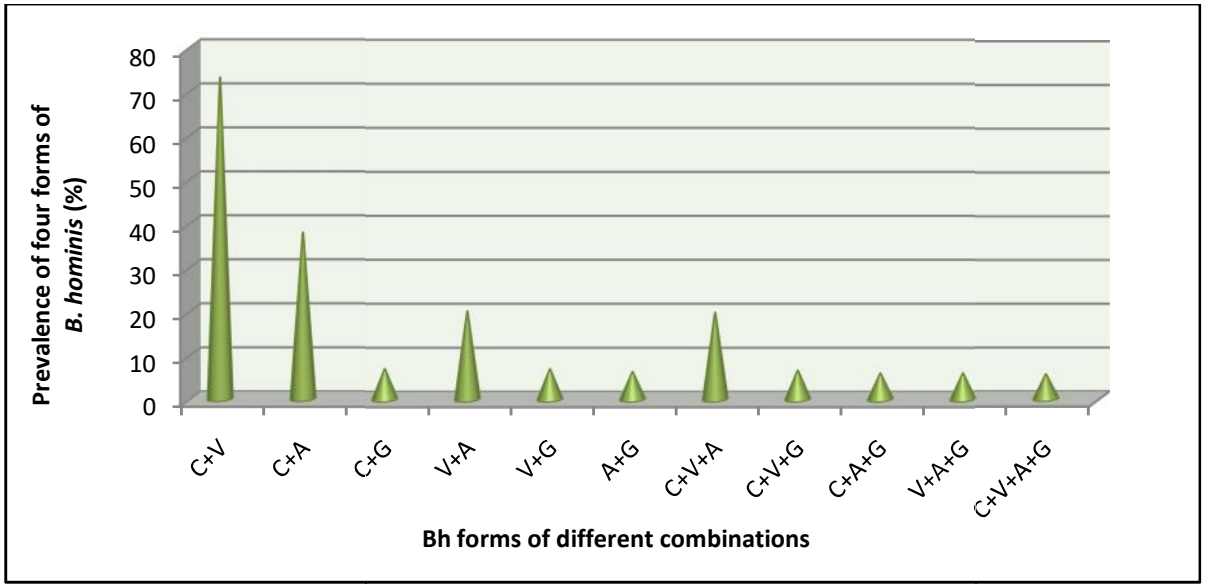


Fig. 69. Prevalence of different forms of *B. hominis* in different combinations in MS samples from Mohammadpur area in 2013.

During the study period (2012-2013), in **DS samples** from Mohammadpur area,208 samples for *B. hominis* were foundpositive. The highest prevalence (54.33%)was observed in cyst and vacuolar (C+V) combination. The lowest prevalence (5.29%) was observed in(A+G), (C+A+G),(V+A+G) and (V+A+ G) combination (Table 41).

Table 41. Prevalence of morphological forms of *B. hominis* in different combination by *in vitro*culture in DS samples (n=514) from Mohammadpur area during 2012-13.

Combination of different morphological forms	Examined samples	Total *Bh* positive samples	Number of combined forms of*Bh*	Prevalence (%)
Cyst + Vacuolar	514	208	113	54.33
Cyst + Amoeboid			63	30.29
Cyst + Granular			12	5.77
Vacuolar +Amoeboid			42	20.19
Vacuolar + Granular			12	5.77
Amoeboid + Granular			11	5.29
Cyst+Vacuolar + Amoeboid			40	19.23
Cyst+Vacuolar + Granular			12	5.77
Cyst+ Amoeboid +Granular			11	5.29
Vacuolar+ Amoeboid + Granular			11	5.29
Cyst+ Vacuolar+ Amoeboid + Granular			11	5.29

In 2012, out of 237 **DS samples**, from Mohammadpur area, the highest prevalence (56.31%) was observed in (C+V) combination. While, 6.80% was found in (C+V+G) combination, 5.83% in (C+A+G) and 4.85% in (V+A+G) combination. Lowest prevalence (3.88%) was observed in (C+V+A+G) combination (Fig. 70).In 2013, out of 277**DS samples**, from Mohammadpur area, the lowest prevalence (4.76%) was observed

in combination of four forms. 6.67% found in (V+G) and in(C+V+A+G) combination. While, the highest prevalence (52.38%) was observed in (C+V)combination(Fig. 71).

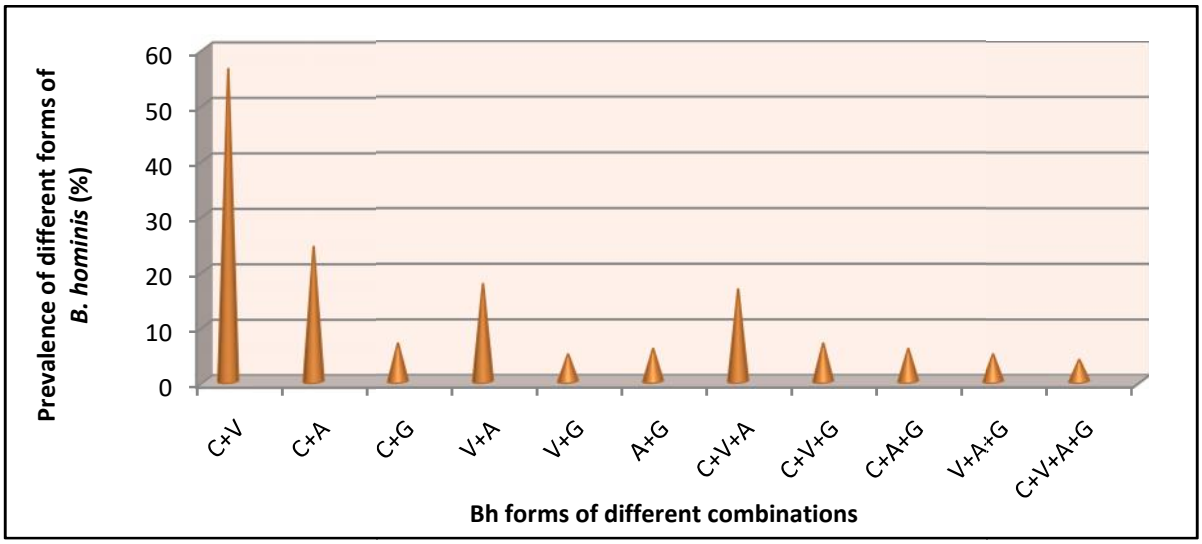


Fig. 70. Prevalence of different forms of *B. hominis* in different combination in DS samples in 2012.

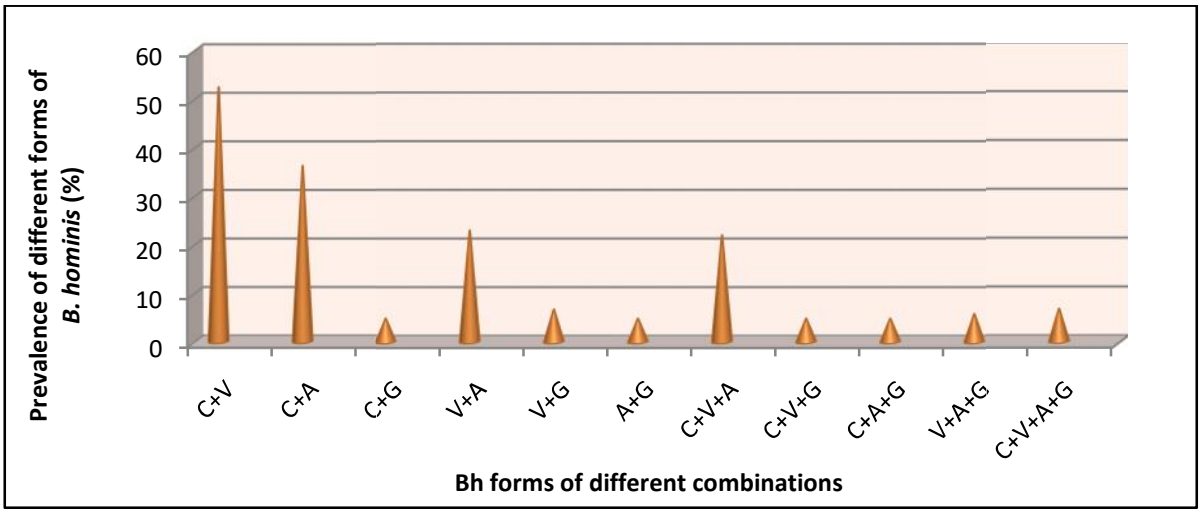


Fig.71. Prevalence of different forms of *B. hominis* in different combination in DSsamples in 2013.

Interpretation

From Mohammadpur area, cyst+vacuolar combination was more prevalent than other combination. Whereas, granular with other forms combination showed less prevalence.

In the present investigation, comparative results of *B. hominis* infestation was estimated by three methods. From Mirpur area, total 422 diarrheal stool (DS) samples were selected.By microscopy, 4.50% samples were positive for *B. hominis,*by *in vitro* culture, 29.86% positive and by PCR method, 42.42%. (Table 42).

Table 42. Comparison of prevalence of *B. hominis*by direct microscopy, *in vitro*culture and PCR method in DSsamples from Mirpur area.

Total examined samples	Prevalence of *B. hominis* by microscopy	Prevalence of *B. hominis* by *in vitro*culture method	Prevalence of *B. hominis* by PCR
422	19 (4.50%)	126 (29.86%)	179 (42.42%)

By the analysis of variance comparisons among three methods in DS samples of Mirpur area showed highly significant difference (p=0.013**) (Table 43).

Table 43. F-test and multiple comparisons among three methods in DS samples fromMirpur area.

Source of variation	**F-test**	**Level of significance**	**Multiple comparisons by LSD[1]**		**Level of significance**
Microscopy, culture and PCR methods	7.21	0.013**	Microscopy	Culture	0.053*
				PCR	**0.004****
			Culture	Microscopy	0.053*
				PCR	0.156
			PCR	Microscopy	**0.004****
				Culture	0.156

[1] LSD= Least Significant Difference.
*The mean difference is significant at the 0.05 level and **the mean difference is highly significant at the 0.05 level

From Mohammadpur area180 diarrheal samples were selected. By microscopy, 9.44% were positive for *B. hominis*,by *in vitro* culture, 39.44% were positive and by PCR method53.33% were positive for *B. hominis*. In this study population,PCR method showed highest prevalence (Table 44).

Table 44. Comparison of prevalence of *B. hominis*among direct microscopy, culture and PCR method in DS samples fromMohammadpur area.

Total examined samples	Prevalence of *B. hominis* by microscopy	Prevalence of *B. hominis* by *in vitro*culture method	Prevalence of *B. hominis* by PCR
180	17 (9.44%)	71 (39.44%)	96 (53.33%)

By the analysis of variance comparisons among three methods in DS samples of Mohammadpur area showed highly significant difference (p=0.000**). The difference between each of two methodswere highly significant (p=0.000**, p=0.000** and p=0.000**respectively)(Table 45).

Table 45.F-test and multiple comparisons among three methods in diarrhoeal stool samples from Mohammadpur area.

Source of variation	**F-test**	**Level of significance**	**Multiple comparisons by LSD[1]**		**Level of significance**
Microscopy, culture and PCR methods	107.87	0.000**	Microscopy	Culture	**0.000****
				PCR	**0.000****
			Culture	Microscopy	**0.000****
				PCR	**0.000****
			PCR	Microscopy	**0.000****
				Culture	**0.000****

[1] LSD= Least Significant Difference.
*The mean difference is significant at the 0.05 level and **the mean difference is highly significant at the 0.05 level

By multiple comparisons, three methods showed highly significant difference (F=47.32 and p=0.000**) among them (Table 46).

Table 46. F-test and multiple comparisons among three methods in overall DS samples of Mirpur and Mohammadpur area.

Method (number with *Bh*+ve=n)	Mean ± SD of proportion	F-test	Level of significance	Multiple comparisons by LSD[1]		Level of significance
Microscopy (n= 36)	7.697± 2.99	47.32	0.000**	Microscopy	Culture	**0.000****
					PCR	**0.000****
Culture (n= 197)	35.44±10.45			Culture	Microscopy	**0.000****
					PCR	**0.001****
PCR (n=275)	54.66± 16.33			PCR	Microscopy	**0.000****
					Culture	**0.001****

[1] LSD= Least Significant Difference.
*The mean difference is significant at the 0.05 level and **the mean difference is highly significant at the 0.05 level

Diarrheal stool samples ofchildren from Mohammapur area showed higher prevalence than Mirpur area by microscopy, *in vitro* culture and PCR method (Fig. 72).

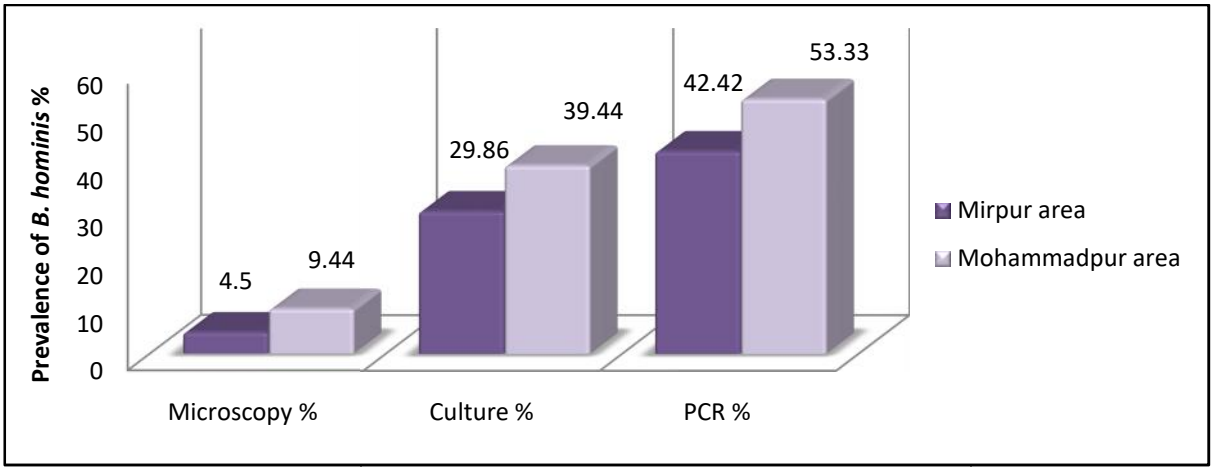


Fig.72. Comparison on three methods for detection of *B. hominis* in DSsamples of children collected fromMirpur and Mohammadpur area.

In the present investigation, proportion test between DS samples of Mirpurand Mohammadpurarea, only PCR method showed significant difference (p=0.032*). While, overall proportion test amongmicroscopy vs culture, microscopy vs PCRand culture vs PCRshowed highly significant difference (p=0.0000**, p=0.0000**and p=0.0005**) respectively (Table 47).

Table 47. Comparison of proportion by two areas and three methods.

Source of variation	**Total number of samples examined**	**Microscopy**		**Culture**		**PCR**	
		no of *Bh* +ve	%	no of *Bh* +ve	%	no of *Bh* +ve	%
Children of Mirpur area	422	19	4.5	126	29.86	179	42.42
Children of Mohammadpur area	180	17	9.44	71	39.44	96	53.33
Overall	602	36	5.98	197	32.72	275	45.68
Proportion test between DS samples of two area		$p = 0.52^{ns}$		$p = 0.095^{ns}$		p = 0.032*	
Overall proportion test among Microscopy, Culture and PCR		Microscopy vs Culture p = 0.0000**		Microscopy vs PCR p = 0.0000**		Culture vs PCR p = 0.0005**	

*The difference of proportion is significant **The difference of proportion is highly significant and

^{ns}The difference of proportion is not significant at the 0.05 level

Interpretation

In comparison with microscopy and culture, the Real-time PCR is the reliable and excellent diagnostic method for detecting *B. hominis* in human diarrheal stool samples.

ANALYSIS OF SENSITIVITY AND SPECIFICITY OFMICROSCOPY AND CULTURE METHOD IN DS SAMPLES OF MIRPUR AREA

Sensitivity and specificity of direct microscopy using PCR:

Total 422 selected diarrheal stool samples from Mirpur area were examined by two diagnostic methods: Microscopy and PCR. 12 samples were true positive (TP) both in microscopy and PCR. Among19 microscopic positive samples,7 were false positive (FP) in microscopic examination. Among 403 microscopic negative samples, 167 were false negative (FN). 236 samples were found true negative (TN) both in microscopy and PCR (Table 48).

Sensitivity and specificity of*in vitro* culture methodusing PCR:

From Mirpur area, total 422 diarrheal stool samples were also examined by two diagnostic methods: *in vitro*culture and PCR. By culture and PCR methods, 100 samples were true positive (TP). Whereas, true negative (TN) samples were 217 in both methods. Among 179 culture negative samples, 79 were false negative (FN) by PCR and 26 were false positive (FP) by culture(Table 49).

Table 48. Analysis of sensitivity and specificity of microscopy using PCR as gold standard in DS samples by cross tabulation.

		PCR as gold standard		Total
		Positive	Negative	
Microscopy	Positive	12 (TP)	7 (FP)	19
	Negative	167 (FN)	236 (TN)	403
Total		179 (TP+FN)	243 (TN+FP)	422

Sensitivity of microscopy:TP/(TP+FN)×100 = 6.70%

Specificity of microscopy: TN/(TN+FP)×100 = 97.12%

TP= True Positive, TN= True Negative, FP= False Positive and FN= False Negative

Table 49. Analysis of sensitivity and specificity of culture using PCR as gold standard in DS samples by cross tabulation.

		PCR as gold standard		Total
		Positive	Negative	
Culture	Positive	100 (TP)	26 (FP)	126
	Negative	79 (FN)	217 (TN)	296
Total		179 (TP+FN)	243 (TN+FP)	422

Sensitivity of culture method:TP/(TP+FN)×100 = 55.87%

Specificity of culture method: TN/(TN+FP)×100 = 89.30%

TP= True Positive, TN= True Negative, FP= False Positive and FN= False Negative

ANALYSIS OF SENSITIVITY AND SPECIFICITY OF MICROSCOPY AND CULTURE METHOD IN DS SAMPLES OF MOHAMMADPUR AREA

Sensitivity and specificity of direct microscopy using PCR:

Total 180 selected diarrheal stool samples from Mohammadpur area were examined by two diagnostic methods: microscopy and PCR. 13 samples were true positive (TP) both in microscopy and PCR and 4 were false positive (FP) by microscopy. Among 163 microscopic negative samples, 83 were false negative (FN) by PCR and 80 samples were found true negative (TN) by microscopy(Table 50).

Sensitivity and specificity of*in vitro* culture methodusing PCR:

From Mohammadpur area, total 180diarrheal stool samples were also examined by two diagnostic methods: culture and PCR. By culture and PCR methods, 66 samples were true positive (TP). Whereas, true negative (TN) samples were 79 in both methods. Among 109 culture negative samples, 30 were false negative (FN) by PCR (Table 51).

Table 50. Analysis of sensitivity and specificity of microscopy using PCR as gold standard in DS samples by cross tabulation.

		PCR as gold standard		Total
		Positive	Negative	
Microscopy	Positive	13 (TP)	4 (FP)	17
	Negative	83(FN)	80 (TN)	163
Total		96 (TP+FN)	84 (TN+FP)	180

Sensitivity of microscopy:TP/(TP+FN)×100 = 13.54%

Specificity of microscopy: TN/(TN+FP)×100 = 95.24 %

TP= True Positive, TN= True Negative, FP= False Positive and FN= False Negative

Table 51. Analysis of sensitivity and specificity of Culture using PCR as gold standard in DS samples from Mohammadpur area by cross tabulation.

		PCR as gold standard		Total
		Positive	Negative	
Culture	Positive	**66(TP)**	5 (FP)	71
	Negative	30 (FN)	**79(TN)**	109
Total		96(TP+FN)	(TN+FP)	180

Sensitivity of culture method:TP/(TP+FN)×100 = 68.75%

Specificity of culture method: TN/(TN+FP)×100 = 94.05 %

TP= True Positive, TN= True Negative, FP= False Positive and FN= False Negative

INFECTION WITH PROTOZOAN PARASITES

Different species of protozoans were found in monthly and diarrheal stool samples by microscopic observation along with *B. hominis.* Almost similar sp of protozoan parasites were found in the study samples of both areas.

The protozoan parasites observed by direct microscopy

1. *Blastocystis hominis*
2. *Giardia spp*
3. *Entamoeba histolytica*
4. *Endolimax nana*
5. *Entamoeba coli*
6. *Iodamoeba butschlii*

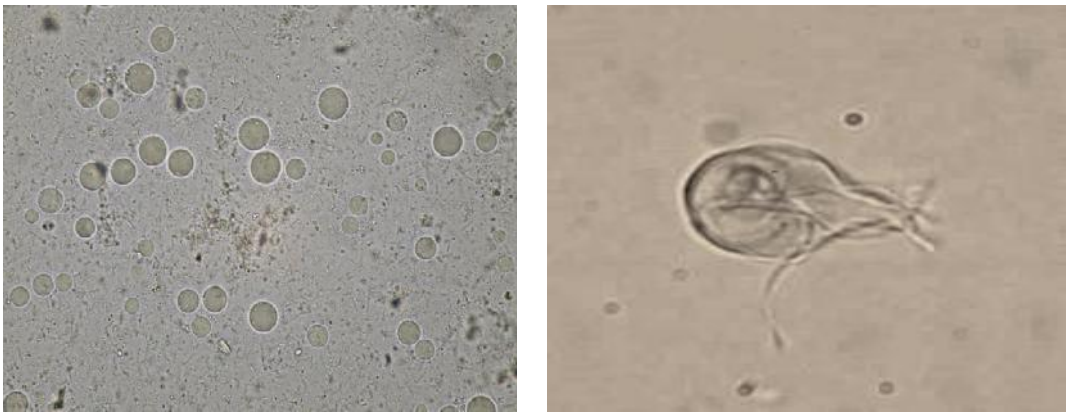
INFECTION WITH HELMINTH PARASITES

Different species of helminth parasites were found in monthly and diarrheal stool samples by microscopic observation along with *B. hominis.* Almost similar types of helminthparasites were found in the overall samples of both areas.

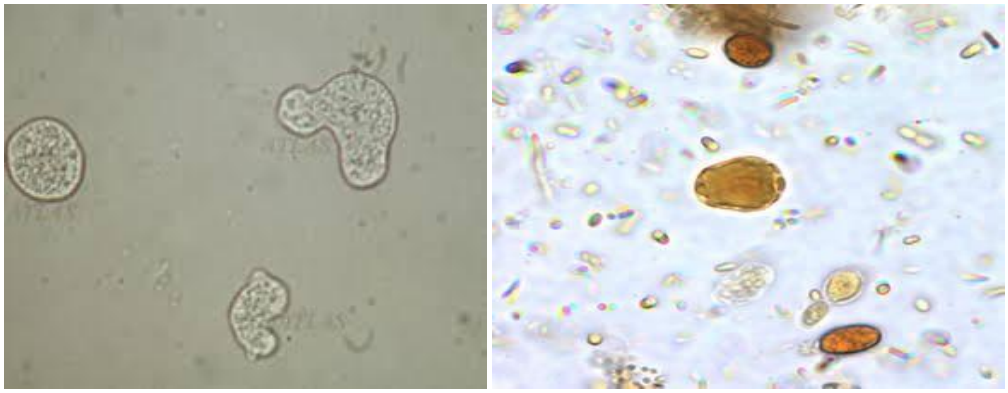
The helminth parasites observed by direct microscopy

1. *Ascaris lumbricoides*
2. *Trichuris trichiura*
3. *Hymenolepis nana*

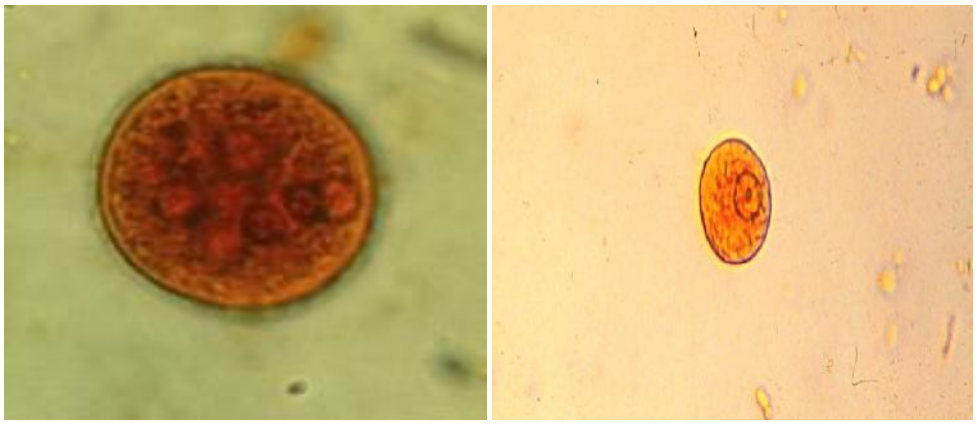
Plate 2.



A) Various forms of *Blastocystis hominis*.B) Trophozoite form of *Giardiasp.*



C) Trophozoite form of *Entamoeba histolytica*D) Cyst of*Endolimax nana*



E) Cyst of *Entamoeba coli* F) Cyst of *Iodamoeba butschlii*

Photographs of protozoan parasites A), B), C), D), E) and F).

PREVALENCE OF PROTOZOAN PARASITES IN MS SAMPLES FROM MIRPUR AREA:

A total of 8601 monthly stool samples examined, 8.64% samples were infected with *Giardia spp.*, 3.72% with *B. hominis*, 1.71% with *E. histolytica*, 0.47% with *E. nana*, 0.19% with *E. coli* and0.10% with *I. butschlii* (Table52).Among total infected MS samples from Mirpur area with protozoan parasites,the highest prevalence of *Giardia spp*(6.81% and 10.63%)were found in both years respectively (Fig.73).

Table52. Prevalence of protozoan parasites in MS samples from Mirpur area during 2012-13.

Total samples examined	Prevalence of *Giardia spp.*	Prevalence of *B. hominis*	Prevalence of*E. histolytica*	Prevalence of *E. nana*	Prevalence of *E. coli*	Prevalence of *I. butschlii*
8601	743 (8.64%)	320 (3.72%)	147 (1.71%)	40 (0.47%)	16 (0.19%)	9 (0.10%)

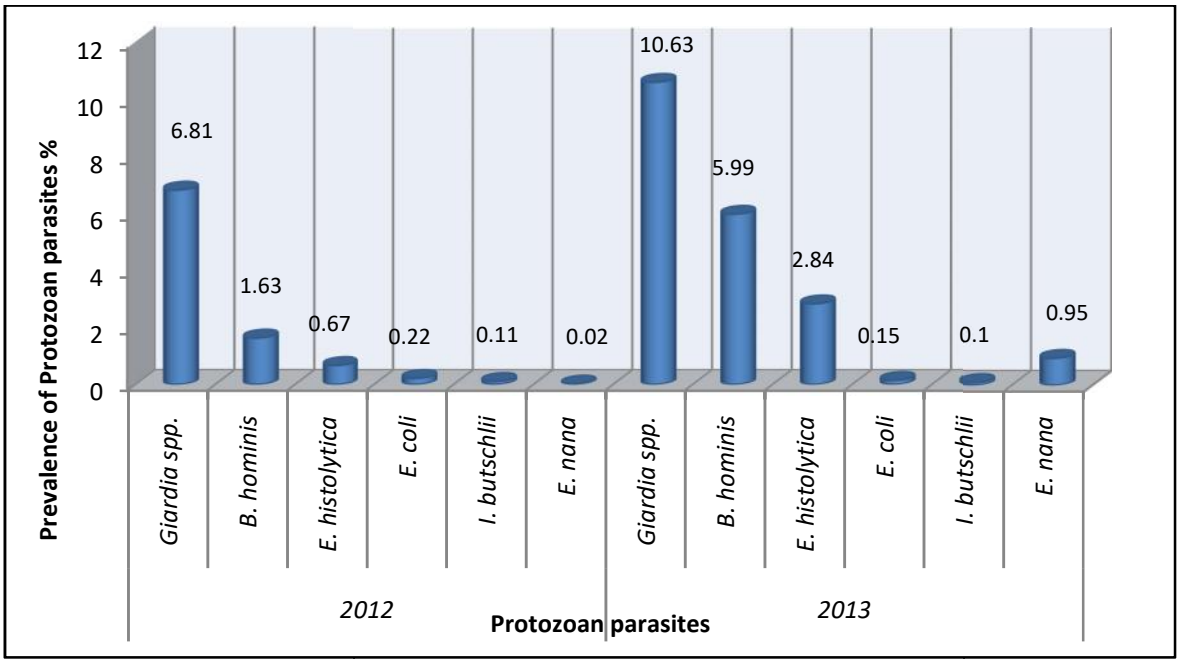


Fig.73.Prevalence of protozoan parasites in MS samples fromMirpur area in 2012 and 2013.

PREVALENCE OF PROTOZOAN PARASITES IN MS SAMPLES FROM MOHAMMADPUR AREA:

A total of 2704 monthly stool samples examined, 4.33% were infected with *Giardia spp.*, 7.25% with *B. hominis*, 3.48% with *E. histolytica*, 2.77% with *E. coli* and1.59% with *I. butschlii* (Table53).Among infected MS samples from Mohammadpur area,lowest prevalence (0.86% and 0.59%) were found for *Giardia spp*than other parasites in both years.(Fig. 74).

Table 53. Prevalence of protozoan parasites in MSsamples of Mohammadpur area during 2012-13.

Total samples examined	Prevalence of *Giardia spp.*	Prevalence of *B. hominis*	Prevalence of *E. histolytica*	Prevalence of *E. coli*	Prevalence of *I. butschlii*
2704	117 (4.33%)	196 (7.25%)	94 (3.48%)	75 (2.77%)	43 (1.59%)

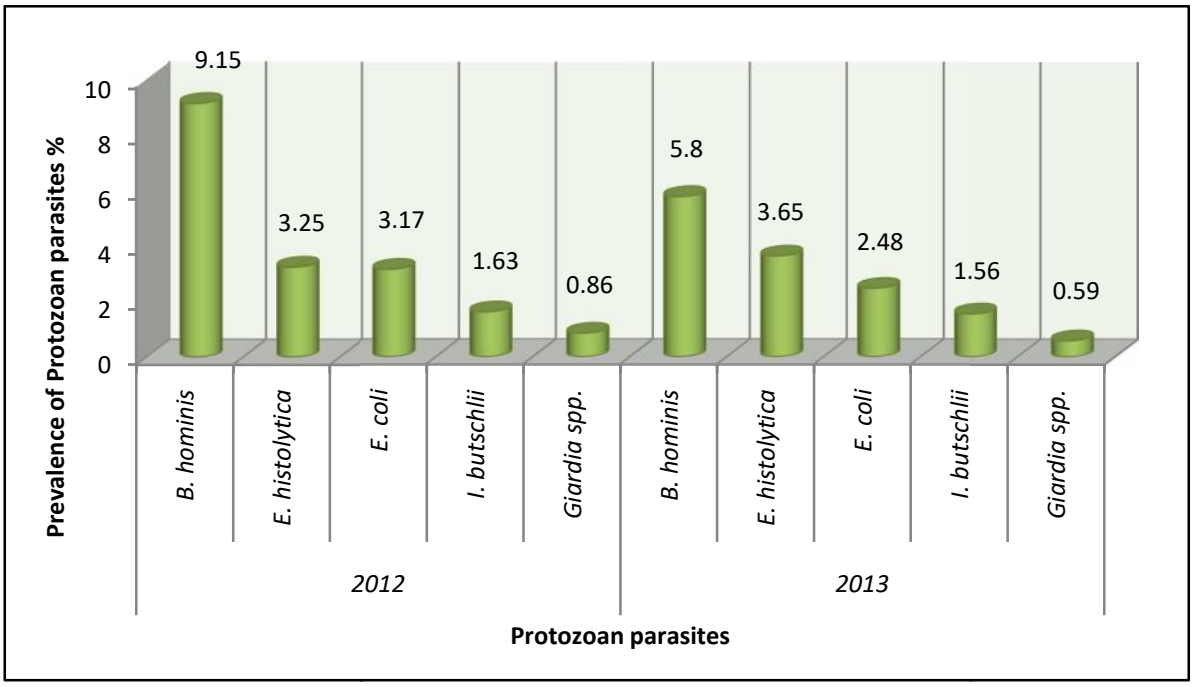


Fig.74. Prevalence of protozoan parasites in MS samples of Mohammadpur area in 2012 and 2013.

PREVALENCE OF PROTOZOAN PARASITES IN DS SAMPLES FROM MIRPUR AREA:

Out of 721 diarrheal stool samples, 5.96% samples were infected with *Giardia spp.*, 4.85% with *B. hominis*,0.55% with *E. histolytica*, 0.28% with *E. coli* and1(0.14%) with *I. butschlii* (Table54).Among infected Ds samples fromMirpur area with protozoan parasite,in 2012, 3.32% were found positive for *Giardia spp.* and 4.27% for *B. hominis*while, in 2012, 9.70% were found positive for *Giardia spp.* and 5.69% for *B. hominis* (Fig.75).

Table54. Prevalence of protozoan parasites in DSsamples from Mirpur area.

Total samples examined	Prevalence of *Giardia spp.*	Prevalence of *B. hominis*	Prevalence of *E. histolytica*	Prevalence of *E. coli*	Prevalence of *I. butschlii*
721	43 (5.96%)	35 (4.85%)	4 (0.55%)	2 (0.28%)	1 (0.14%)

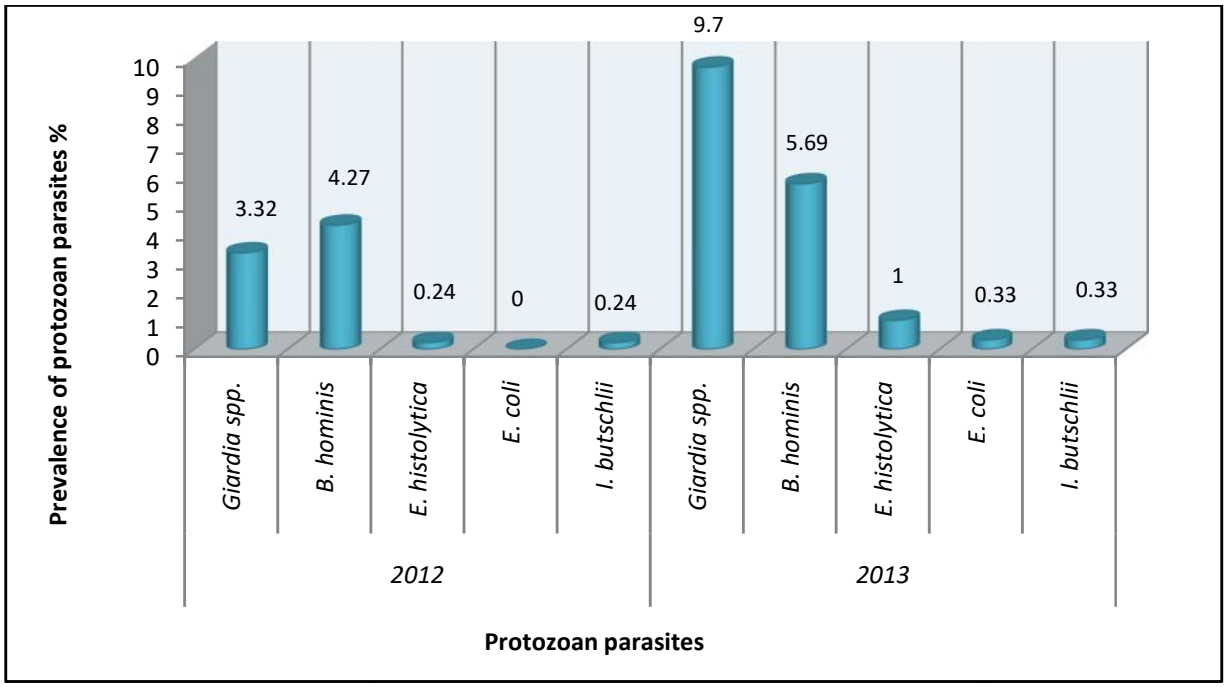


Fig.75. Prevalence of protozoan parasites in DS samples from Mirpur area in 2012 and 2013.

PREVALENCE OF PROTOZOAN PARASITES IN DIARRHEAL STOOL SAMPLES FROM MOHAMMADPUR AREA:

Out of 514,DS samples examined, 11.67% samples were infected with *Giardia spp.*, 9.73% with *B. hominis*, 1.56% with *E. histolytica*, 0.39% with *E. coli* and 0.39% with *I. butschlii* (Table55).Among infected DS samples from Mohammadpur area,*B. hominis* infection (45.15% and 32.13%) were more prevalent than other parasites in both years (Fig.76).

Table 55. Prevalence of protozoan parasites in DS samples from Mohammadpur area.

Total samples examined	Prevalence of *Giardia spp.*	Prevalence of *B. hominis*	Prevalence of *E. histolytica*	Prevalence of *E. coli*	Prevalence of *I. butschlii*
514	60 (11.67%)	50 (9.73%)	8 (1.56%)	2 (0.39%)	2 (0.39%)

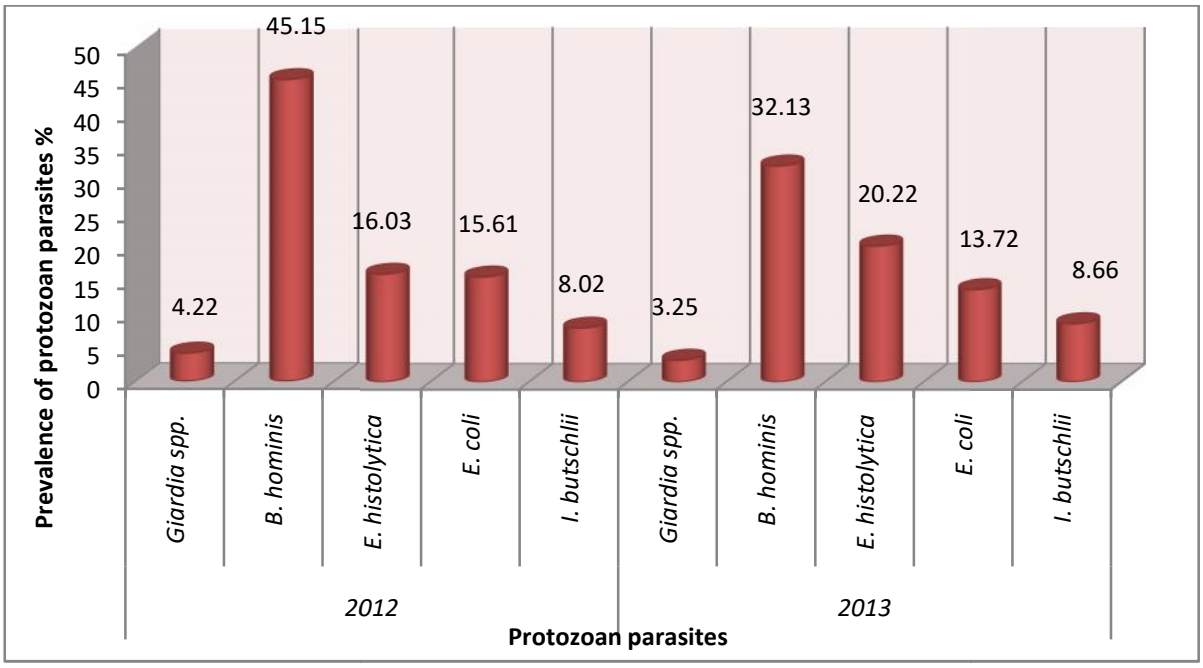
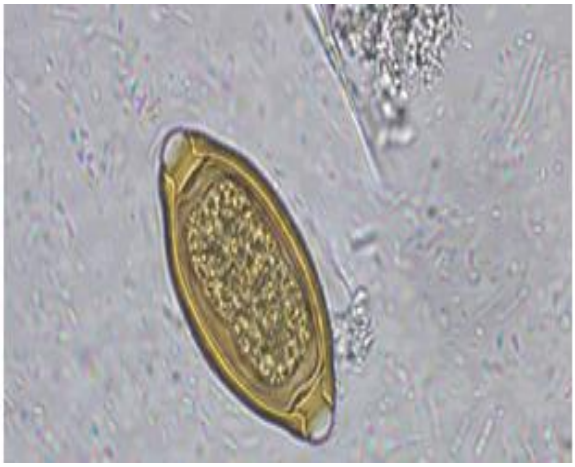


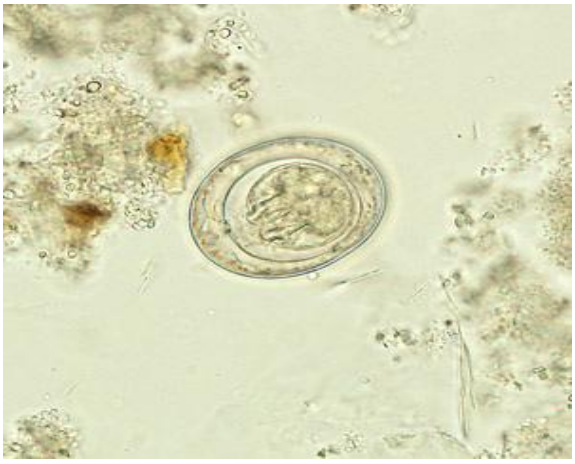
Fig.76. Prevalence of protozoan parasites in DS samples of Mohammadpur area.

Plate 3.



G)Egg of*Trichuris trichiura.*

H) Egg of *Ascaris lumbricoides*.



I)Egg of *Hymenolepis nana.*

Photographs of eggs of helminth parasites G), H) and I).

PREVALENCE OF HELMINTH PARASITES IN MS SAMPLES FROMMIRPUR AREA:

A total of 8601 monthly stool samples examined, 10.88% were infected with *A. lumbricoides*,5.35%with *T. trichiura*and0.30% with *H. nana* (Table56).Among infected MS samples fromMirpur area with helminth parasite,prevalence of*A. lumbricoides* (12.54%), *T. trichiura* (7.38%) and *H. nana* (0.51%) werehigherin 2013(Fig.77).

Table 56. Prevalence of helminth parasites in MSsamples from Mirpur area.

Total samples examined	Prevalence of *A. lumbricoides*	Prevalence of *T. trichiura*	Prevalence of *H. nana*
8601	936 (10.88%)	460 (5.35%)	26 (0.30%)

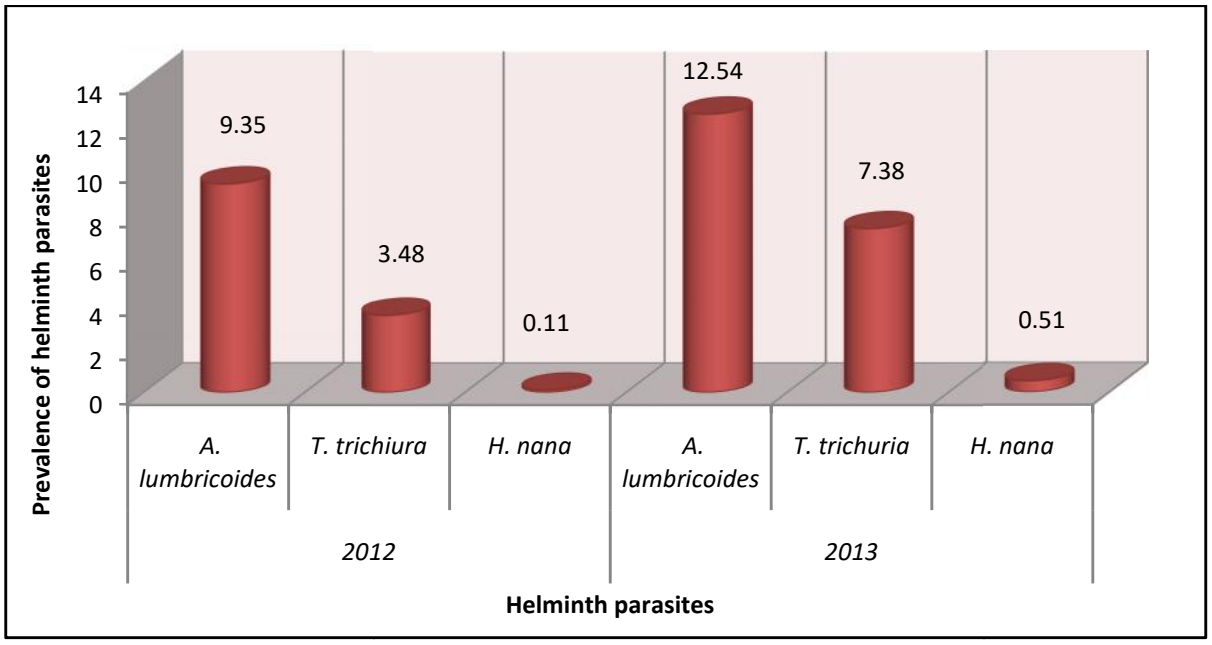


Fig.77. Prevalence of helminth parasites in MS samples fromMirpur area.

PREVALENCE OF HELMINTH PARASITES IN MONTHLY STOOL SAMPLES OF MOHAMMADPUR AREA.

Out of 2704 monthly stool samples examined, 12.24% were infected with *A. lumbricoides*,5.03% with *T. trichiura* and0.41% with *H. nana* (Table57).Among infected MS samples from Mohammadpur area with helminth parasite,in 2012, 17.37% were found positive for *A. lumbricoides*, 6.67% for *T. trichiura* and0.68% for *H. nana*, while these were 8.34%, 3.78% and 0.2% in 2013(Fig.78).

Table 57. Prevalence of helminth parasites in MS samples from Mohammadpur area.

Total samples examined	Prevalence of *A. lumbricoides*	Prevalence of *T. trichiura*	Prevalence of *H. nana*
2704	331 (12.24%)	136 (5.03%)	11 (0.41%)

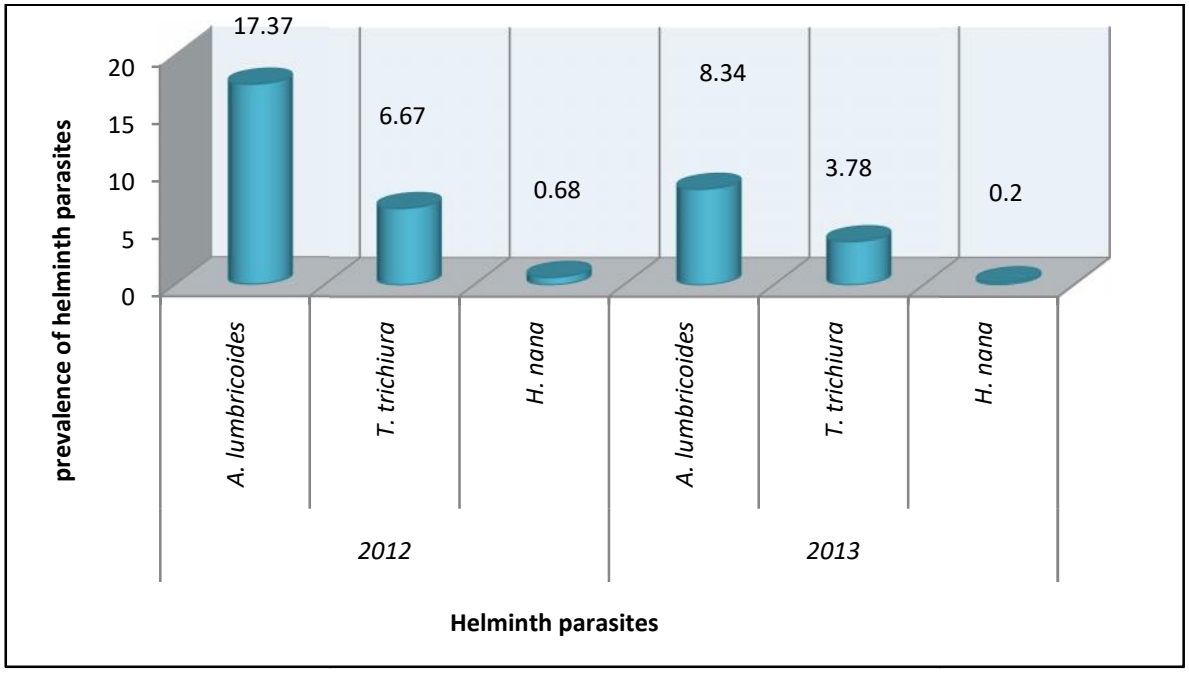


Fig.78. Prevalence of helminth parasites in MS samples fromMohammadpur area.

PREVALENCE OF HELMINTHS PARASITES IN DS SAMPLES FROMMIRPUR AREA:

Out of 721 diarrheal stool samples examined, 7.21% were infected with *A. lumbricoides*,3.05% with *T. trichiura*, and 0.28% with *H. nana* (Table58).Among infected DS samples fromMirpur area with helminth parasites,in 2012, prevalence of *A. lumbricoides* was 6.87% and in 2013, it was 7.69% (Fig. 79).

Table 58. Prevalence of protozoan parasites in DSsamples from Mirpur area.

Total samples examined	Prevalence of *A. lumbricoides*	Prevalence of *T. trichiura*	Prevalence of *H. nana*
721	52 (7.21%)	22 (3.05%)	2 (0.28%)

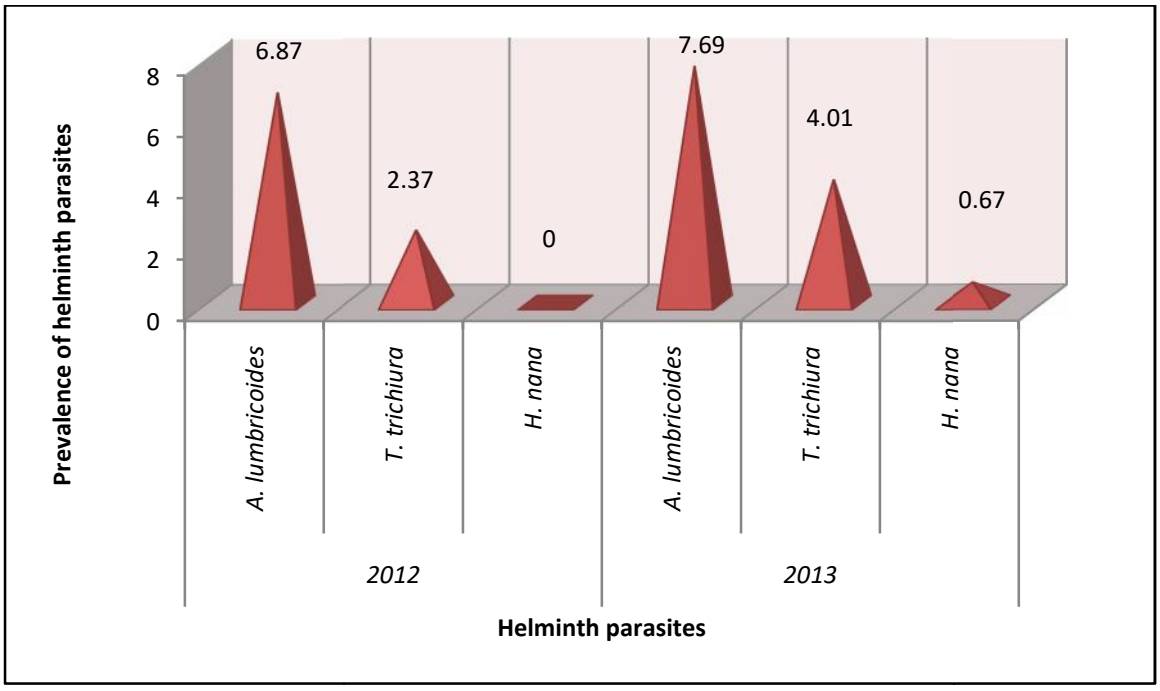


Fig.79. Prevalence of helminth parasites in DS samples fromMirpur area.

PREVALENCE OF HELMINTHS PARASITES IN DS SAMPLES FROM MOHAMMADPUR AREA:

A total of 514 diarrheal stool samples, 8.75% were infected with *A. lumbricoides*,3.31%with *T. trichiura*, and 0.19% with *H. nana* (Table59).Among infected DS samples from Mohammadpur area with helminth parasites, in 2012, prevalence of *A. lumbricoides* was 85.65% and *T. trichiura* was 32.91%. Whereas, in 2013, prevalence of *A. lumbricoides* was 46.21% and *T. trichiura* was 20.94% (Fig. 80).

Table 59. Prevalence of protozoan parasites in DS samples from Mohammadpur area.

Total samples examined	Prevalence of *A. lumbricoides*	Prevalence of *T. trichiura*	Prevalence of *H. nana*
514	45 (8.75%)	17 (3.31%)	1 (0.19%)

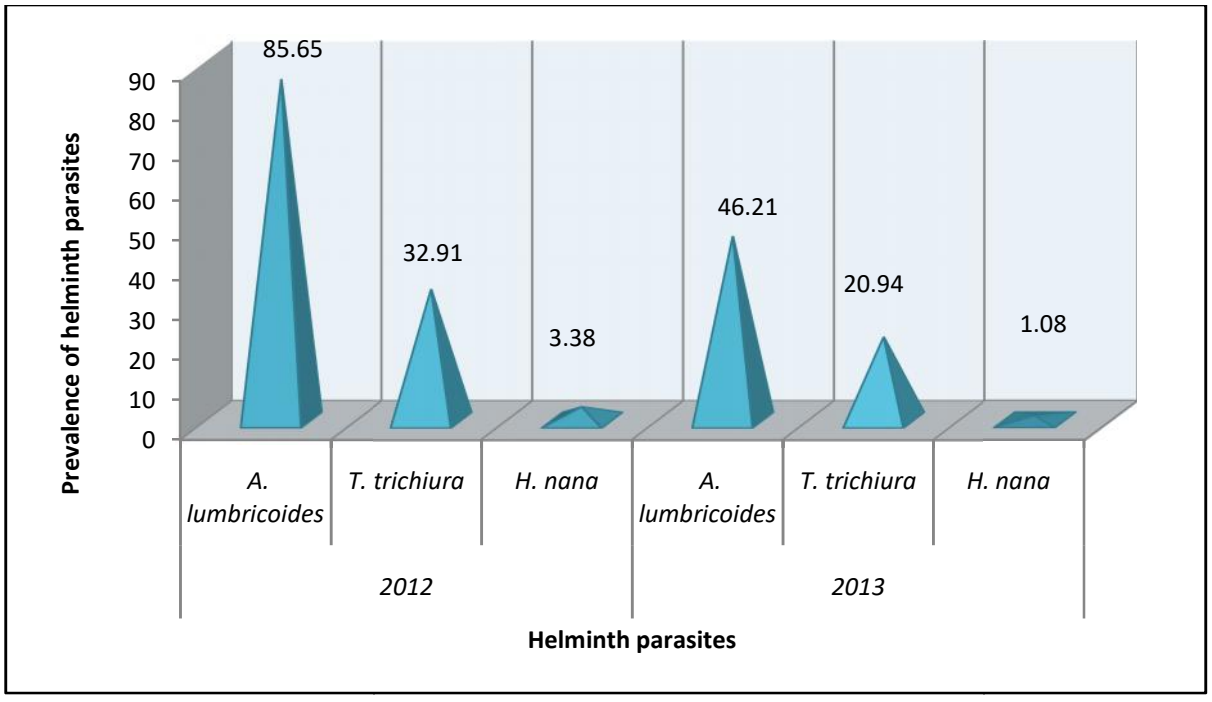


Fig.80.Prevalence of helminth parasites in DS samples from Mohammadpur area.

DISCUSSION

Diarrheal diseases continue to be the one of the leading causes of mortality and morbidity worldwide, especially in children, travelers and immune compromised patients. Diarrhea is defined by the World Health Organization as having 3 or more loose or liquid stools per day or as having more stools than is normal for that person. About 1.7 to 5 billion cases of diarrhea occur per year (WHO 2013; Abdelmalak *et al.* 2013; GBD 2013 Collaborators, *et al.* 2015). It is most common in developing countries, where young children get diarrhea on average three times a year (Abdelmalak *et al.* 2013). Total deaths from diarrhea are estimated at 1.26 million in 2013- down from 2.58 million in 1990 (GBD 2013, Collaborators, *et al.* 2014). In 2012, it was the second most common cause of deaths in children younger than five (11%) (Abdelmalak *et al.* 2013; GDB diarrhea 2013).

Frequent episodes of diarrhea are also a common cause of malnutrition (Abdelmalak *et al.* 2013). Other long term problems that can result include stunted growth and poor intellectual development (GDB, 2013). While knowledge of causes of deaths is important for health sector planning, little is known from conventional sources about the causes of deaths in Bangladesh. About 20% of the child death is associated with diarrhea (Baqui *et al*. 1998). Another study observed that in Bangladesh, 1 in 30 children die of diarrhea or dysentery by his or her fifth birthday (Haque *et al*. 2003). There are many etiologies for infectious diarrhea, which include viruses, bacteria and parasites. Diarrhea kills 2,195 children every day more than malaria, AIDS and measles combined. Diarrheal diseases account for 1 in 9 child deaths worldwide, making diarrhea the second leading cause of death among the children under the age of 5 (Liu *et al.* 2012).

Khanum (2005) reported that the prevalence of *E. histolytica* was 8.6% in asymptomatic stool samples. Dipstick test showed 10.32% infection in asymptomatic stool samples and 27.35% in symptomatic. The highest prevalence found in symptomatic stool samples. ELISA also detected higher prevalence 9.09% in symptomatic stool samples.

The pathogenic potential of *Blastocystis hominis* has not yet been established, although numerous case reports suggest that *B. hominis* may cause the development of various gastrointestinal symptoms and disorders (Stensvold *et al*. 2006). *B. hominis* infection is very common in many countries without any symptoms (Stenzel and Boreham, 1996). Besides causing mild to moderate diarrhea, bloating and flatulence, it is associated with inflammatory bowel disorder (IBD) where levels of specific IgG antibody were found to be significantly increased in patients with irritable bowel syndrome (IBS) compared with asymptomatic controls. This suggested a link between *B. hominis* and IBS, which is highly prevalent gastrointestinal motility disorder with a prevalence rate ranging from 3% to 20% (Amany *et al*. 2008). Despite years of study, the pathogenic role of *B. hominis* is still regarded by some as controversial as it is frequently found not only in individuals with enteric symptoms, but also in apparently healthy and asymptomatic subjects (Tan 2008).

To improve the health condition and for the implementation of control strategies against *B. hominis*, knowledge of both symptomatic patients and asymptomatic individuals is necessary. The present study was based on asymptomatic individuals who have *B. hominis* infection with symptom and without any symptoms. Diarrheal (symptomatic) and non diarrheal (asymptomatic) stool samples were collected from two slum area of Mirpur, and Mohammadpur area, Dhaka, Bangladesh. 9322 fecal samples were collected from 406 children of Mirpur area and 3218 fecal samples were collected from 118 children of Mohammadpur area. Duration of the collection period was from 1st January, 2012- 31st December, 2013. All the samples were from asymptomatic children and symptomatic children. As multiple samples from the same children were tested for the study, infection was defined when any sample was tested positive by the three diagnostic methods (microscopy, culture and Real-time PCR) for *B. hominis.*

Al-Fellani *et al*. (2007) reported that the prevalence of *B. hominis* was found to be 26.58% among outpatients at the Central Laboratory in Sebha. Of the 3,645 patients examined, 1,925 (52.81%) were male and 1,720 (47.18 %) were female. A total of 558 (28.98%) male and 411 (23.89%) female were harbouring *B. hominis* and male were

more infected (28.98%) than female (23.89%). The difference was significant ($p<0.05$). Khanum *et al.* (2013) reported that the prevalence of intestinal parasites was higher in female (30.56%) than in male (22.29%) children. Several studies have also reported that significantly higher prevalence observed in male than female patients (Nimri *et al.* 1993; Wang *et al.* 2002; Leder *et al.* 2005).

All these studies do not support the present results. The prevalence of *B. hominis* in male was 3.66% and in female 4.16% in Mirpur area in case of direct microscopy. So, the pathogen is more prevalent in female than male in Mirpur area. While, the prevalence of *B. hominis* in male was 8.09% and female was 7.29% in Mohammadpur area in case of direct microscopy. So, the pathogen is more prevalent in male than female in Mohammadpur area. And the difference among sex groups were not significant in asymptomatic and symptomatic children of both areas of microscopy.

In the present observation, by *in vitro* culture, the prevalence of *B. hominis* in male was 32.61% and female was 35.07% in Mirpur area. The prevalence of *B. hominis* in male was 41.59% and in female 41.59% in Mohammadpur area in case of *in vitro* culture. So, the pathogen was more prevalent in female than male in both area. The difference of infection among sex groups also were not significant among asymptonmatic and symptomatic children of both areas by *in vitro* culture.

A study was conducted in Spain, by Martin-Sanchez *et al.* (1992), in children of day care centres and primary schools where the incidence of *B. hominis* was greater in children older than 3 years in the day care centre and in the 10-14 year-old group in the primary schools. Khanum *et al.* (1999) worked on the intestinal protozoan parasites infection of age group 2-16 years, irrespective to sex, from lower income group employee in Dhaka city and noted 11.5% of double infection with *E. histolytica* and *Giardia lamblia*. In the present study, the prevalence of *B. hominis* was gradually increased from 37 to 48 months age group of children, in asymptomatic samples and symptomatic samples of both areas.

Al-Fellani *et al* (2007) reported another study on adult, most of them worked in agriculture, the highest positivity (10.28%) rate was found in 21-40 age group. The present study was on asymptomatic samples and symptomatic samples of children of 13-72 months age group. The highest positivity (19.57%) rate was found in the 49-60 months age group of diarrheal children of Mohammadpur area by microscopy, while the highest positivity (60.42%) rate was found in the 37-48 months age group of diarrheal children of Mohammadpur area by *in vitro* culture.

A study was designed by Yakoob *et al.* (2004) to examine stool specimens of irritable bowel syndrome (IBS) patients for *B. hominis*, a common intestinal parasite. In their study, they found 23% prevalence of *B. hominis* by direct microscopy and 32% prevalence by culture study. According to them, stool culture was more sensitive than microscopy. This study was quite similar with the present study because by direct microscopy, the prevalence of *B. hominis* was 3.88%, whereas by culture study the prevalence was 33.72% in Mirpur area, Moreover, by direct microscopy, the prevalence of *B. hominis* was 7.64%, whereas by culture study the prevalence was 42.26% in Mohammadpur area.

According to Al-Fellani *et al*. (2007) the seasonal variation of *B. hominis* among patients attending the Central Laboratory in Sebha was 17.80% in summer while 15.04% in winter in 2003 and 21.95% in summer while 19.09% in winter in 2004 and 27.39% in summer while 25.13% in winter in 2005. So, the results proved that the prevalence of *B. hominis* was more prevalent in summer than in winter in each year. The infection was significantly ($p<0.05$) more in summer than in winter over a three-year period. Khanum *et al.* (2013) studied the seasonal pattern showed that highest (30%) prevalence occurred in rainy season and lowest (17.19%) in winter season. These studies quite support the present study. In diarrheal samples of Mirpur area, 15.64% in summer while 6.16% in winter in 2012 and 13.04% in summer while 3.68% in winter in 2013. Moreover, in diarrheal samples of Mohammadpur area, 55.26% in summer while 27.27% in winter in 2012 and 53.01% in summer while 17.36% in winter in 2013. So, the results proved that the prevalence of *B. hominis* was more prevalent in summer than in winter in each year.

In both area, the infections were significantly ($p<0.05$ and $p<0.00$) more in summer than in winter over a two-year period.

The majority of the studies on seasonal patterns of infestation of helminthes have been in the temperature climate zone of the world, with very little information available on the tropical rainy climatic zones. There has been considerable speculation on the observed temperate zone seasonal patterns, with the most significant factors being thought to be water temperature variation (Aho *et al.* 1982; Camp *et al.* 1982).

Environmental conditions (e.g. temperature, humidity, wind and soil) and socio-economic factors are responsible for spreading and for developing infectious forms of *B. hominis* and *Giardia* spp., and for polluting the environment with these agents. The diversity of parasites endemic in any area is influenced by a variety of factors. These factors can be divided into social, cultural, economic, and environmental factors and the life cycle features of parasites, such as the nature of the environmental stage. Transmission and spread of these parasites is influenced by the availability of clean water, socio-economic conditions, education, personal and public hygiene practices, temperature, humidity and survival of the environmental stages of the parasites (Fan *et al.* 1998; Parekh *et al.* 1972; Kan *et al.* 1971; Sengbusch 1970; Gleason *et al.* 1970).

The occurrence of *B. hominis* infections in outpatients are probably related to weather conditions, with the suggestion that the hot, dry weather of the Sebha region favors the development and transmission of this organism. *B. hominis* infections might have a role in some pathological conditions, resulting in gastrointestinal symptoms (Al-Fellani *et al*. 2007).

Populations in different parts of the world face diverse parasitic challenges. For example, *Enterobius vermicularis* is more prevalent in temperate areas (Vermund *et al*. 2000) and *Ascaris lumbricoides* is more common in tropical regions (Stepek *et al.* 2006). The highest rates of *Ascaris* infection have been reported in China, Southeast Asia, coastal regions of West Africa, and Central Africa. *Trichuris* infestation is at its highest rate in Central Africa, southern India, and Southeast Asia. Hookworm infections are most common in sub-Saharan Africa, South China, and Southeast Asia. In industrialized

countries the prevalence of intestinal parasites such as *Giardia* ranges from 2% to 5%, whereas in developing countries it ranges from 20% to 40% (Ortega *et al.* 1997; Meyer *et al.* 1985; Ali *et al.* 2003).

The purpose of this study was to relate personal data, socio-cultural and environmental characteristics, and the presence of symptoms/signs with the frequency of *B. hominis* among study population of two areas, Mirpur and Mohammadpur. In the present study, monthly incidence of *B. hominis* related with climatic factors such as temperature, humidity and rainfall in overall samples of both area. Whereas, only significance observed with temperature (p=0.004, p=0.000 and p=0.000) and rainfall (p=0.040, p=0.003 and p=0.001) not with humidity.

On the other hand, Guignard *et al*. (2000) proposed *B. hominis* infects both humans and animals and also reported a higher prevalence (20-50%), especially in rural areas. Similarly in the present study, the infection with *B. hominis* in urban slum areas were 26%-42% in humans.

An investigation was done by Bergamo *et al*. (2013) to find out the various morphological forms of *B. hominis*. Most commonly found in their study was vacuolar rounded form, which is contradictory with the present findings. In the present study, most commonly found morphological form was cyst. Among the total samples of Mirpur area, cyst form was found 31.80% in monthly stool (MS) samples and 22.61% in diarrheal Stool (DS) samples. In case of Mohammadpur area, cyst form was found 38.78% in MS samples and 38.13% in DS samples.

Inceboz *et al.* (2011) investigated fecal samples by using the native-Lugol-trichorome and Kinyoun acid-fast method after sedimentation in fecal concentration tubes. Distribution of various morphological forms were observed, vacuolar form (67.49%), granular form (14.78%), and both vacuolar+granular forms (17.73%). It is quite similar with the present study where in MS samples, vacuolar form (40.97%), granular form (17.89%) and both vacuolar+granular forms (11.61%) and in DS samples, vacuolar form (35.53%), granular form (10.15%), both vacuolar+granular forms (8.38%) among the

total samples of Mirpur area. On the other hand, in MS samples of Mohammadpur area, vacuolar form (72.31%), granular form (6.34%), both vacuolar+granular forms (6.16%) and in DS samples, vacuolar form (57.21%), granular form (9.62%), both vacuolar+granular forms (5.77%) were found.

One of the key questions in *Blastocystis* biology is whether disease is genotype related. A few studies have been carried out to address this issue, although the results have been equivocal. A study by Kaneda *et al.* (2001) employed PCR-RFLP ribotyping on *Blastocystis spp.* isolated from asymptomatic individuals and patients with gastrointestinal symptoms. Their results suggested that ribodemes I, III, and VI (subtypes 1, 4, and 2, respectively) were associated with symptoms, with colonoscopic evidence of inflammation in patients harboring ribodemes III and VI. Ribodeme II (subtype 3), which was the most commonly isolated genotype, was not associated with symptoms (Kaneda *et al.* 2001)

In a similar study, genotyping was carried out with isolates of 28 patients with gastrointestinal disorders and 16 asymptomatic individuals. Subtype 1 was found exclusively in symptomatic patients, while subtypes 3 and 6 were found in both groups. Subtype 7 was found only in asymptomatic individuals. Those authors concluded that subtype 1 was the most virulent, while subtypes 3 and 6 consisted of pathogenic and nonpathogenic strains (Hussein *et al.* 2008).

A study of isolates from China revealed an association between subtype 1 and disease, while subtype 3 was isolated predominantly from asymptomatic individuals (Yan *et al.* 2006). Tan *et al.* (2008) employed arbitrary primed PCR on *Blastocystis* DNA and were able to distinguish among isolates obtained from eight symptomatic and eight asymptomatic isolates. In contrast, other studies indicated no association between disease and parasite genotype Böhm-Gloning *et al.* (1997) analyzed 158 isolates by PCR-RFLP and determined that the study population was infected by five subgroups (genotypes), none of which was significantly correlated with intestinal disease. A study involving isolates from asymptomatic and symptomatic individuals from Bangladesh revealed no association between genotypes and disease, although only 26 samples were analyzed (Yoshikawa *et al*. 2004).

In a recent case study, *Blastocystis* sp. subtype 8 was isolated from a Danish woman suffering from diarrhea, abdominal pain, bloating, and flatulence. No other infectious cause was evident, and her symptoms subsided after a course of trimethoprim-sulfamethoxazole (TMP-SMX) therapy (Stensvold *et al.* 2008). A recent study among *Blastocystis* isolates from a Turkish hospital revealed the presence of subtypes 1, 2, and 3 among adult and pediatric patients. Only subtype 2 showed a statistically different distribution between asymptomatic and symptomatic patients, with a greater proportion within the asymptomatic group. One reason for the discrepant conclusions on subtype association with disease is how the data were interpreted (Dogruman-Al *at el.* 2008).

Poirier *et al*. (2011) compared Real-time PCR assay with conventional diagnostic methods, including direct-light microscopy (DLM) and xenic *in vitro* culture (XIVC) of fresh stool samples. Only 8 *Blastocystis*-positive samples were detected by DLM and 14 by XIVC, whereas, by Real-time PCR assay detected 27 *Blastocystis*-positive samples, making it by far the most sensitive method. The Real-time PCR assay was used as the "gold standard" to calculate the sensitivity of both XIVC (52%) and DLM (29%). They also demonstrated that Real-time PCR assay was more sensitive than XIVC. The Real-time PCR assay showed an overall prevalence of 14.5%, among 186 patients. The specificity of their SYBR green-based real-time PCR assay was 95% (confirmed by sequencing of amplified products). Specificity would be enhanced by using probe-based Real-time PCR assays.

This result coincide with the present study. In DS samples of Mirpur area, by microscopy 4.50%, by *in vitro* culture 29.86% and by Real-time PCR 42.42% positive samples. Moreover, in DS samples of Mohammadpur area, by microscopy 9.44%, by *in vitro* culture 39.44% and by Real-time PCR 53.33% samples. Using the Real-time PCR the sensitivities of both microscopy and culture were observed 6.70% and 55.87% respectively in Mirpur area and 15.63% and 68.75% respectively in Mohammadpur area. When only one stool sample was tested per children 45.68% by the Real-time PCR assay.

Khanum *et al.* (2005) measured the sensitivity, specificity of microscopy and dipstick test compared with antigen detection test, ELISA for *E. histolytica.* The sensitivity of microscopy was 23% and specificity was 91%. Dipstick test showed 100% sensitivity and 92% specificity. Dipstick test was more sensitive and specific than microscopy when compared with ELISA for the diagnosis of amoebiasis. Amany *et al*. (2008) proposed a study to detect *B. hominis* by PCR, culture and microscopy were 44.6%, 41% and 30.1% samples respectively from patients with IBS. The sensitivity of microscopy and culture versus PCR were 62.2% and 89.2%, respectively. PCR-based detection directly from fecal specimens was shown to be more sensitive compared to *in vitro* culture for the detection of *B. hominis.* In the present study, *B. hominis* was detected in 45.68%, 32.72% and 5.98% patients with diarrheal, using PCR, culture and microscopy respectively. In stool samples of Mirpur area, the sensitivity of microscopy and culture versus PCR were 6.70% and 55.87% respectively, while these were 12.82% and 68.73% in stool samples of Mohammadpur area.

Global infections reported some of the most common intestinal parasites are *Ascaris spp*. (20%), hookworm (18%), *Trichuris trichiura* (10%), and *Entamoeba histolytica* (10%) (WHO, 1987). This finding do not match the present study. In the present study, no hook worm infection was observed. In MS samples, *Ascaris* (10.88%), *Trichuris trichiura* (5.35%), and *Entamoeba histolytica* (1.71%) were recorded, and in DS samples *Ascaris* (7.21%), *Trichuris trichiura* (3.05%), and *Entamoeba histolytica* (0.55%), were recorded in Mirpur area. In MS samples *Ascaris* (12.24%), *Trichuris trichiura* (5.03%), and *Entamoeba histolytica* (3.48%) and in DS samples *Ascaris* (20%), *Trichuris trichiura* (3.31%), and *Entamoeba histolytica* (1.56%) were recorded in Mohammadpur area.

The prevalence of *A. lumbricoides* was 10.88%, *E. histolytica* was 1.71%, *B. hominis* was 3.72% in MS samples and *A. lumbricoides* was 7.21%, *E. histolytica* was 0.55%, *B. hominis* was 5.83% in DS samples of Mirpur area. Moreover, prevalence of *A. lumbricoides* was 12.24%, *E. histolytica* was 3.48%, *B. hominis* was 3.72% in MS samples and *A. lumbricoides* was 8.75%, *E. histolytica* was 1.56%, *B. hominis* was 9.73% in DS samples of Mohammadpur area.

According to Clark *et al.* (2002), *B. hominis* is a parasite protist 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 *Dientamoeba fragilis*. This finding was exactly similar to the present study as the pathogen was observed in conjunction with several other diarrhea causing pathogen such as *E. histolytica* and *Giardia intestinalis*.

Cook *et al*. (2009) made an investigation in the Palajunoj Valley of Guatemala in which 5705 viable stool samples were screened for infection with *Ascaris hominis*. The overall prevalences of infection for specific parasites were- *A. lumbricoides* was 17.7%, *E. histolytica* was 16.1%, *B. hominis* was 2.8%. This finding was quite similar with the present study. The prevalence of *A. lumbricoides* was 10.88%, *E. histolytica* was 1.71%, *B. hominis* was 3.72% in MS samples and *A. lumbricoides* was 7.21%, *E. histolytica* was 0.55%, *B. hominis* was 5.83% in DS samples of Mirpur area. Moreover, prevalence of *A. lumbricoides* was 12.24%, *E. histolytica* was 3.48%, *B. hominis* was 3.72% in MS samples and *A. lumbricoides* was 8.75%, *E. histolytica* was 1.56%, *B. hominis* was 9.73% in DS samples of Mohammadpur area.

SUMMARY

During the study period from January, 2012 to December, 2013, a total of 9322 multiple stool samples from 406 infants of Mirpur slum area and 3218 samples from 118 infants of Mohammadpur slum area, Dhaka city, were collected and examined for the presence of *Blastocystis hominis*. Among them 8601 were monthly stool (MS) samples and remaining 721 were diarrheal stool (DS) samples in Mirpur area, while 2704 were MS samples and remaining 514 were DS samples in Mohammadpur area.

Among age groups and sexes of children:

By microscopy, in monthly stool (MS) samples of Mirpur area, the highest and almost similar prevalence (4.35% and 4.74%) were observed in age group 37-48 and 49-60 months respectively. In age group 37-48 months, 2.59% was found positive in male and 1.58% was positive in female. While by *in vitro* culture, in the same samples of Mirpur area, the highest prevalence (43.92%) was observed, in age group 61-72 months.

In case of diarrheal stool (DS) samples of Mirpur area, by microscopy, the highest prevalence (15.49%) were observed in age group 49-60 months. In the age groups of 25-36, 49-60 and 61-72 months, the prevalence of *B. hominis* were higher in male than that of female children. By *in vitro* culture method, in the same samples of Mirpur area, the highest prevalence (40.91%) was observed in age group 61-72 months, among them 45.45% found in male and 36.36% in female. In age group 13-24 months and 37-48 months, the prevalence of *B. hominis* were higher in female than that of male.

In monthly stool (MS) samples from Mohammadpur area, by direct microscopic examination, the highest prevalence (8.19%) were observed in age group 25-36 months. Most of the age groups of children showed that the higher prevalence of *B. hominis* in male than female group. While by *in vitro* culture, in the same samples of Mohammadpur area, the highest prevalence (46.80%) was observed, in age group 37-48 months.

By microscopy, in case of diarrheal stool (DS) samples of Mohammadpur area, the highest prevalence (19.57%) were observed in age group 49-60 months and the lowest prevalence (3.31%) was in 25-36 months. In the age groups of 37-48 and 49-60 months,

the prevalence of *B. hominis* were higher in male than that of female children. By *in vitro* culture method, in the same samples of Mohammadpur area, the highest prevalence (60.42%) was observed in age group 37-48 months. In the age group 25-36 months, the prevalence of *B. hominis* was much higher in female than that of male. While the data was interpreted statistically, it was found that there was no significant association between sexes and *B. hominis* infection among the children. However, the tested diagnostic methods explored that there was a significant association between various age groups and *B. hominis* infection among the children. The children aged over 37 months are at the most risk of the infection.

Microscopy and culture method in diarrheal and non-diarrheal samples:

By direct microscopy, from Mirpur area, out of 9322 multiple stool samples 3.88% were positive for *B. hominis.* Among them, prevalence of *B. hominis* was 3.72% in monthly stool (MS) samples and 5.83% in diarrheal stool (DS) samples. While from Mohammadpur area, 7.64% was found for *B. hominis*, among them, prevalence of *B. hominis* in MS and DS samples were 7.25% and 9.73% respectively. It was observed that, by microscopy prevalence of the organism was higher in diarrheal samples of both areas.

By *in-vitro* culture method, the prevalence of *B. hominis* was 33.72% in overall multiple samples from Mirpur area. Among them, prevalence of *B. hominis* was 34.25% in monthly stool (MS) samples and 27.32% in diarrheal stool (DS) samples. While, out of 3218 samples from Mohammadpur area, 1360 (42.26%) was found positive for *B. hominis.* Among them, prevalence of *B. hominis* in MS and DS samples were 42.60% and 40.47% respectively. It was observed that, by microscopy prevalence of this organism was higher in diarrheal samples of both area. It was also observed that, by *in-vitro* culture method prevalence of this organism was higher in monthly stool samples of both areas. So it reveals that *B. hominis* infection do not always shows diarrheal symptom.

Morphological forms of *B. hominis*:

Several morphological forms: cyst, vacuolar, amoeboid and granular were observed by *in vitro* culture method. The most commonly observed form was cyst. In overall MS samples from Mirpur area, the prevalence of cyst, vacuolar, amoeboid and granular of *B. hominis* were observed 31.80%, 14.04%, 11.90% and 6.11% respectively, while it was 22.61%, 8.74%,5.69% and 3.05% respectively in overall DS samples.

By culture method, from Mohammadpur area, in overall MS samples the prevalence of cyst, vacuolar, amoeboid and granular of *B. hominis* were observed 38.78%, 30.81%, 18.30% and 2.70% respectively, while it was 38.13%, 23.15%, 17.1% and 3.89% respectively in overall DS samples. One of the findings was that, the cyst form was the most prevalent form observed in infected individuals of both areas.

By *in vitro* culture, from Mirpur area, in MS samples it was observed that cyst + vacuolar (C+V) combination was highly prevalent (35.34%) and lowest prevalence (8.21%) was in cyst+vacuolar+amoeboid+granular (C+V+A+G) combination. In DS samples, the highest prevalence (27.23%) was observed in cyst+vacuolar combination. All morphological forms were observed 8.21%, which was the lowest prevalence.

During the study period (2012-2013), from Mohammadpur area, 1143 samples for *B. hominis* were found positive in MS samples. Highest prevalence (70.69%) was observed in cyst+vacuolar combination and lowest prevalence (4.20%) observed in cyst+vacuolar+ amoeboid+granular combination. In DS samples the highest prevalence (54.33%) was observed in cyst and vacuolar (C+V) combination. The lowest prevalence (5.29%) was observed in (A+G), (C+A+G), (V+A+G) and (V+A+G) combinations. The observation reveals that infection rate increased if cyst and vacuolar forms present in faecal samples.

Seasonal variation among *B. hominis* infection:

By microscopy, in MS and DS samples from Mirpur area, in rainy season the highest prevalence of *B. hominis* were found 5.03% and 8.05 % and the lowest was 2.98 % and 3.59 % in winter. By *in vitro* culture method, in MS samples, the highest prevalence (35.73%) was in rainy season, while it was 35.47% in summer for DS samples.

By microscopy, in MS and DS samples from Mohammadpur area in summer season the highest prevalence of *B. hominis* were 8.78 % and 13.21% respectively and the lowest were 5.40 % and 5.26 % in winter. By *in vitro* culture method, in rainy season the highest prevalence of *B. hominis* was 48.14% in MS samples, while it was 54.09 % observed in summer in DS samples.

By microscopy, either summer or rainy season showed higher prevalence than winter, while by *in vitro* culture method prevalence of *B. hominis* were higher both in summer and rainy season than winter. So infection of *B. hominis* is responsible for temperature and rainfall. The seasonal variation (F-test), were significant (P=0.05* and p=0.000**) in DS samples of Mirpur and Mahammadpur areas.

Impact of Climatic factors on monthly incidence of *B. hominis:*

In 2012, in MS samples of Mirpur area, the highest incidence (40.39%) and lowest incidence (19.20%) were observed in July and January. In 2013, the highest incidence was 46.15% in May. In 2012, in DS samples of Mirpur area, the highest incidence (45.24%) was observed in the month of May while in 2013 highest incidence (38.46%) was in September. On the other hand, in both years, the lowest incidence 11.63% and 3.57% were observed in January.

In 2012, in MS samples of Mohammadpur area, the highest incidence (61.11%) was found in July and lowest incidence (28.57%) was observed in the month of December. In 2013, the highest incidence was 53.17% in April, while lowest incidence (32.17%) was observed in January. In 2012, in DS samples of Mohammadpur area, the highest incidence (57.89%) was observed in the month of March while in 2013 it was 66.67% in July. On the other hand, in both years, the lowest incidence 9.52% and 12.5% were observed in November. Monthly incidence of *B. hominis* in all stool samples of study areas showed positive correlation with climatic factors (temperature, humidity and rainfall). It was observed that in DS samples of both areas, incidence were significantly correlated with temperature (p=0.004** and p=0.000**) and rainfall (p=0.004** and p=0.001**).

The prevalence of *B. hominis* by direct microscopy was 4.50%, by *in vitro* culture method was 29.86% while by Real-time PCR method was 42.42% respectively in stool

samples of Mirpur area, while these were 9.44%, 39.44% and 53.33% respectively in samples of Mohammadpur area. By comparisons among three methods, the mean difference were highly significant (p=0.013** and p=0.000*) in stool samples of both areas. **The main goal of the present investigation was to prove the sustainable technique for maximum detection of *B. hominis.* As a result the Real-time PCR is the gold standard as it showed highest sensitivity than direct microscopy and *in vitro* culture method.**

CONCLUSION

The present study was designed to identify a proper way of diagnosis of *Blastocystis hominis* infection. The aim of the study was to evaluate a molecular assay for detection of *B. hominis* that can be used in a routine diagnostic setting and in epidemiological studies. Finally to compare the results of three different techniques: Direct microscopy, *in vitro* culture and Real time PCR. Though the pathogenic mechanism of the parasite remains controversial, numerous studies confirmed the pathogenic potential of the parasite.

In Bangladesh, where acute diarrheal diseases continue to be the major cause of morbidity and mortality, the present study is an initial attempt to elucidate the pathogenic potential of the parasite. Apart from the individual hygiene practice, prospect of controlling a disease on a community basis are dependent upon the correct method of diagnosis.

The study included children of an impoverished slum area and the outcome of the study created a surge of interest as *B. hominis* was found in diarrheal children with significant difference in morphological forms. The most commonly observed morphological form was the cyst form in both direct microscopy and culture method. It indicated that the presence of cystic stage in samples is the easiest way to identify the infection. But in clinical setting, a positive diagnosis of *B. hominis* infection depends on the confirmation of vacuolar, granular or amoebic forms in diarrheal samples.

Compare to the Real-time PCR, sensitivity of culture and microscopy are 55.87% and 6.70% respectively in Mirpur area and 68.75% and 15.63% respectively in Mohammadpur area. Also by analyzing the specificity of culture and microscopy are 89.30% and 97.12% respectively in Mirpur area and 94.05% and 97.62% respectively in Mohammadpur area. The efficiency of culture method proves that this method can assist in making an accurate mode of diagnosis of *B. hominis* than microscopy. However, management decisions should not be based on utilizing only one method. So it is very important to apply three diagnostic methods simultaneously for the proper mode of diagnosis of *B. hominis*.

RECOMMENDATIONS AND LIMITATIONS

Recommendations:

- For the confirmed identification of the parasite, it is recommended to examine the samples with a microscope of high magnification.
- Microscopic examination must be performed on unpreserved specimens and should provide correct information on age, consistency of stool and presence of any abnormality.
- To prevent local transmission and endemic spread of *B. hominis*, obligatory routine health screening should be performed.
- As the pathogenesity of *B. hominis* and parasitic protists, increases with mixed infection, it is recommended to look for the presence of other parasites e.g. *E. histolytica* and *G. lamblia* etc.
- As the children of age group 37 to 60 months are the most susceptible group to the infection, proper care should be taken to reduce the mortality and morbidity of that specific risk group.
- Unlike other pathogenic protists of clinical importance, the stool antigen capture Enzyme linked immunosorbent assay (ELISA) kits are not very available for this parasite. But the addition of this method in cohesion with the utilized two may increase the percentage of correct diagnosis.
- Higher sensitivity and specificity recommends the molecular method PCR is the convenient method of detection.

Limitations:

- Cultivation of luminal protists has a limited role in diagnostic laboratory. For this reason, no diagnosis should rely on cultivation alone.
- One of the problem of cultivating *B. hominis* is that the organism often grows in the same or similar media with *E. histolytica, G. lamblia,* and *Balantidium coli* etc. As a result, it is difficult to identify and isolate the parasite for an inexperienced personnel.

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APPENDIX

Composition of Robinson's medium:

1. Saline agar slopes:
    a. 14gm of fine agar powder and 7gm of NaCl was dissolved in 1000ml of distilled water by heating.
    b. The solution was distributed in 2.5 ml portions to McCartney bottles.
    c. It was then sterilized by autoclaving(121▫C,15 min).
    d. Bottles were removed while agar is still molten.
    e. Bottles were positioned at an angle to form slope and allowed to cool.
2. Erythromycin: To prepare erythromycin, following steps were taken:
    a. 0.5 gm of pure erythromycin powder (Abott Laboratories) was dissolved in 20 ml of 70% ethanol in a sterile condition.
    b. The solution was allowed to stand at 4▫ C for 2 h or longer to sterilize the antibiotic.
    c. Add 30 ml of sterile distilled water.
3. DIFCO Bacto peptone: To prepare Bactopeptone, the following steps were taken:
    a. 20 gm powder was dissolved in distilled water.
    b. Then it was autoclaved for 15 minutes at 121▫C.
4. Rice starch: Rice starch was directly used without sterilization.
5. Phthalate solution:
    a. 204 gm Potassium phthalate was dissolved in 1800 ml distilled water by adding100ml of 40%NaOH until a pH of 6.3 is reached.
    b. Water was added to bring final volume to 2000 ml.
6. Serum: Bovine serum was used.
7. Defined medium B:
    a. Composition of stock solution:
        i. NaCl125gm
        ii. Citric acid monohydrate 50gm
        iii. $KH_2PO_4$12.5gm
        iv. $(NH4)_2SO_4$ 25gm
        v. $MgSO_4.7H_20$ 1.25gm

vi. Lactic acid(90%)100ml

vii. Distilled water 2500ml

b. Working solution:

i. 100ml of stock solution was added to 850 ml of distilled water.

ii. It was then titrated to pH 7.0 with 40% pure NaOH.

iii. 25ml portions was dispensed to flat sided 100ml screw capped bottles and autoclaved.

8. BR Basal amoebic medium: 25 ml of working solution was inoculated with *E. coli*and incubated at 37 ^{0}for 48h with bottle lying flat.

9. BRS complete amoebic medium:Anequal volume of serum was added to BR medium and continued to incubate for another 24-48h.

Agargaon, Dhaka-1207
Bangladesh

Monthly average Dry Bulb Temperature in degree Celsius (°C) of 2012

Station	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	A.Avg
Dhaka	18.9	22.1	27.1	28.1	30.1	29.7	29.1	29.2	29.0	27.9	23.5	18.4	(26.1)
Mymensingh	17.1	20.1	24.7	26.1	28.8	28.4	28.6	29.0	28.6	26.8	21.8	16.9	(24.7)
Sylhet	17.7	20.6	25.2	24.7	27.8	27.1	28.4	28.6	28.2	26.4	22.9	18.3	(24.7)
Rajshahi	16.7	19.8	25.2	28.6	31.4	30.6	29.0	29.5	28.9	26.4	21.3	16.6	(25.3)
Khulna	18.8	21.9	27.5	29.5	31.1	31.1	29.3	29.2	29.1	27.5	23.4	18.4	(26.4)
Barisal	17.8	21.1	26.6	28.2	29.8	29.6	28.5	28.6	28.2	26.8	22.9	17.5	(25.5)
Chittagong	20.4	22.8	26.2	27.2	29.1	28.2	27.5	28.1	28.3	27.3	24.2	19.0	(25.7)
Comilla	18.1	21.0	26.0	26.7	28.9	28.7	28.3	28.6	28.5	26.9	23.1	17.9	(25.2)

Monthly average Dry Bulb Temperature in degree Celsius (°C) of 2013

Station	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	A.Avg
Dhaka	17.6	22.8	27.5	29.0	28.0	30.1	29.3	28.7	28.9	27.2	23.8	20.2	(26.1)
Mymensingh	16.1	21.3	25.1	26.9	26.9	29.4	29.0	28.5	28.7	26.6	21.9	18.4	(24.9)
Sylhet	17.3	22.2	25.9	26.8	25.9	29.2	28.5	28.0	28.2	26.3	22.4	19.0	(25.0)
Rajshahi	15.5	20.2	25.5	28.9	28.7	29.9	29.8	29.0	29.2	26.5	21.8	18.1	(25.3)
Khulna	17.5	21.7	27.0	29.4	28.8	30.1	29.3	28.9	29.1	27.3	23.6	20.1	(26.1)
Barisal	16.6	21.1	26.3	28.4	27.7	29.4	28.5	28.4	28.3	26.9	22.8	19.3	(25.3)
Chittagong	18.4	22.9	25.9	28.2	27.2	28.6	28.3	27.9	28.2	26.9	24.2	20.8	(25.6)
Comilla	17.1	21.9	25.7	27.5	26.9	29.5	28.6	28.2	28.5	26.8	23.1	19.4	(25.3)

Bangladesh Meteorological Department
Climate Division
Agargaon, Dhaka-1207

Bangladesh

Monthly & Yearly Average Humidity in % during 2012-2013.

Station name	Year	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Annual
Dhaka	2012	66	52	57	69	70	77	79	78	79	71	68	77	70
Dhaka	2013	65	55	55	63	78	76	77	80	81	78	66	72	70
Mymensingh	2012	79	71	73	80	77	85	86	85	85	82	81	88	81
Mymensingh	2013	78	70	74	77	84	82	83	85	85	85	81	82	80
Sylhet	2012	76	60	63	77	77	88	84	84	84	81	77	82	77
Sylhet	2013	74	62	60	69	83	81	83	85	83	81	74	75	75
Rajshahi	2012	79	66	58	69	67	80	86	85	86	80	78	85	76
Rajshahi	2013	79	74	64	62	80	83	83	86	84	86	76	80	78
Khulna	2012	80	71	73	77	79	84	90	90	90	85	81	87	82
Khulna	2013	83	77	70	71	82	84	90	91	85	86	75	78	81
Barisal	2012	82	72	74	79	79	86	88	87	89	86	82	85	82
Barisal	2013	79	76	75	75	86	84	87	87	88	87	80	82	82
Comilla	2012	77	70	75	81	79	84	85	83	85	82	78	83	80
Comilla	2013	74	68	75	78	85	81	83	85	84	85	78	81	79
Chittagong	2012	68	62	76	80	80	86	88	86	85	84	77	80	79
Chittagong	2013	72	64	77	77	85	84	84	86	84	85	78	77	79

Bangladesh Meteorological Department
Climate Division
Agargaon, Dhaka-1207

Bangladesh

Monthly total rainfall 2012-2013

Station	Year	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec
Dhaka	2012	10	1	37	269	137	175	226	282	81	38	68	5
Dhaka	2013	0	8	26	32	378	325	302	212	172	131	0	4
Mymensingh	2012	18	0	1	202	85	241	409	238	221	45	19	0
Mymensingh	2013	0	18	21	69	308	267	318	343	132	263	0	0
Sylhet	2012	10	0	101	659	406	1185	700	738	261	502	48	0
Sylhet	2013	0	7	16	229	959	729	567	520	347	451	0	0
Rajshahi	2012	6	0	6	123	17	137	314	179	178	102	101	1
Rajshahi	2013	0	22	12	51	188	178	101	254	238	204	0	0
Khulna	2012	66	18	1	52	63	255	391	254	374	89	80	2
Khulna	2013	1	7	19	62	430	212	313	482	278	260	0	0
Barisal	2012	31	2	11	223	105	205	275	270	381	70	44	0
Barisal	2013	0	6	5	64	701	277	377	398	312	181	0	0
Comilla	2012	16	1	13	195	209	442	282	373	178	115	102	3
Comilla	2013	0	3	30	28	467	214	276	243	255	124	0	

Monthly non-diarrheal stool (MS) collection form (1)

Parasitology Laboratory, CCD,

icddr,b, Mohakhali, Dhaka 1212

1. Child ID:
2. Camp Code:
3. Serial MS number:
4. Stool collection date:
5. Was sample collected: Yes/No
6. Was sample in the original container: Yes/No

Microscopic examination status:

Protozoan parasite:

Entamoebahistolytica/dispar *:* Yes/No

Blastocystishominis *:* Yes/No

Giardia sp : Yes/No

Iodamoebabutschlii *:* Yes/No

Endolimax nana *:* Yes/No

Entamoeba coli *:* Yes/No

Helminth parasite:

Ascarislumbricoides :Yes/No

Trichuristrichiura *:* Yes/No

Hymenolepis nana *:*Yes/No

Diarrheal stool (DS) collection form (2)

Parasitology Laboratory, CCD,

icddr,b, Mohakhali, Dhaka 1212

1. Child ID:
2. Camp Code:
3. Serial MS number:
4. Stool collection date:
5. Was sample collected: Yes/No
6. Was sample in the original container: Yes/No

Microscopic examination status:

Protozoan parasite:

Entamoebahistolytica/dispar *:* Yes/No

Blastocystishominis *:* Yes/No

Giardia sp : Yes/No

Iodamoebabutschlii *:* Yes/No

Endolimax nana *:* Yes/No

Entamoeba coli *:* Yes/No

Helminth parasite:

Ascarislumbricoides :Yes/No

Trichuristrichiura *:* Yes/No

Hymenolepis nana *:*Yes/No