CHAPTER-3
MATERIALS AND METHODS
Chapter- 3

MATERIALS AND METHODS

3.1 Materials:

3.1.1 Study design, period and study area:

A comparative cross-sectional study was conducted to determine the prevalence of amoebiasis and co-infection of major diarrhoeogenic Protozoan parasite among amoebiasis patients from January, 2011 to January, 2014. The study was carried out in selected North Eastern states of India (Assam, Manipur, Meghalaya and Tripura) at the levels of community and hospitals. The study was based on a single stool sample per person.

In parallel, in a two and half year follow up study, sequential stool samples from HIV infected persons attending Antiretroviral Therapy (ART) Centre of Silchar Medical College and Hospital, Silchar, Assam, India were examined for the presence of stool *E. histolytica* between July, 2011 and January, 2014. Those patients who were negative for parasitic infection were asked to provide stool samples for follow-up testing using the same method in order to assess the incidence of new acquisition. The interval between the stool samples collection were 6 months.

3.1.2 Subject consent and ethical considerations:

The study protocol was reviewed and approved by the Institutional Ethical Committee (IEC) Gurucharan College, Silchar, Assam before the commencement of the study and Silchar Medical College and Hospital, Silchar. The participants were informed that the procedure used did not pose any potential risk and their identities and personal particulars will be kept strictly confidential. After explaining the importance, purpose and procedure of the study briefly consent was obtained from study participants either in written form (signed) or verbally followed by their thumb prints (for those who are illiterate) and for children aged 1 to 9 years consent was systematically sought from the family heads or guardians. Any study participant who was positive for intestinal parasite was referred to physicians for treatment.
3.1.3 Sampling and data collection:

Stool samples were collected randomly from 1260 (274 HIV+ and 986 HIV-) inhabitants of the selected North Eastern states of India (Assam, Manipur, Meghalaya and Tripura) without discriminating age, sex and symptom through apposite survey work in different Hospitals, Community Health Center, Primary Health Center, Pathological Laboratory and Community level (Figure 3.1). Fresh stool samples were collected in wide mouth screw-capped containers, pre-labelled with identification code and were distributed to each participant for the collection of around 5g fecal samples. The samples were collected within 2-3 h of defecation, delivered to the laboratory and processed through a systematic approach (Figure 3.2).

To study incidence of amoebiasis in HIV+ patients stool samples were collected sequentially from 310 HIV seropositive subjects of different areas of Barak Valley, Southern Assam, attending the Antiretroviral Therapy (ART) Centre of Silchar Medical College and Hospital, Silchar, Assam, India. The subjects were confirmed as HIV positive in Integrated Counseling and Testing Centre (ICTC), SMCH, Silchar by three successive serological rapid tests using Combs Aids, Pareekshak® HIV ½ Triline card test (Bhat Bio-Tech, India) and SD Bioline anti HIV ½ test kits(Standard Diagnostics Inc., India), as per protocol provided by the supplier. The CD4+ T cell count was performed using CyFlow® Counter (Partec, Germany) to monitor disease status at their enrolment.

The outcome variable was stool parasite status of the study subjects, whether positive or negative for *E. histolytica*, which was determined from a stool sample. Data on the selected independent variables were collected by interviewing all the subjects using pre-structured questionnaire (appendix) which consists of three sections:

1. General socio-demographic data: age, gender, residence, education, marital status, income and occupation etc.
2. Environmental factors: toilet facility, water supply, animal contact, contacts with animal feces etc.
3. Clinical information: anti-amoebic treatment taken previously, previous history of infection symptomatic or asymptomatic, CD4 T cell count (HIV+ subjects) etc.
Figure 3.1: Map showing the location of sample collection site. 1- Silchar, 2- Karimganj, 3- Hailakandi, 4- Dibrugarh, 5- Jorhat, 6- Nagaon, 7- Goalpara, 8- Darmanagar, 9- Kailashahar, 10- Kamalpur, 11- Agartala, 12- Udaipur, 13- Khliehriat, 14- Shillong, 15- Tura, 16- Jiribam, 17- Imphal.
Materials and Methods

Figure 3.2: Systematic approach attempted to derive the prevalence rate of the parasite *E. histolytica* and co-infection rate of major diarrhoeagenic protozoan parasite in amoebiasis patients. (*Eh* = *E. histolytica*, *Cp* = *C. parvum*, *Gi* = *G. duodenalis*, *Eb* = *E. bieneusi*, *Cc* = *C. cayetanensis*, *Tt* = *T. trichiura*).
3.1.4 Chemicals:

It mainly includes those required for microscopy, xenic culture, DNA isolation, radio-labeled probe preparation, dot blot hybridization, PCR amplification, DNA sequencing and other associated techniques such as gel electrophoresis and gel purification.

3.1.4.1 Sources of chemicals:

DH5α strain of E. coli was obtained from Bethesda Research Labs (United States of America) and was used for recombinant DNA work. Vector pBluescript II KS+ was obtained from Stratagene (United States of America). Sequence version V3.1 DNA sequencing kit was obtained from Applied Biosystem, USA; random-priming oligo labeling kit was obtained from NEB. All other molecular biological reagents including PCR enzymes, dNTPs, BSA, EtBr, agarose, were purchased from Fermentas (Vilnius, Lithuania) and Sigma-Aldrich (United States of America). Oligo nucleotide primers were synthesized from Sigma-Aldrich (United States of America.). 32P-α dATP was obtained from Bhaba Atomic Research center (BARC, India). Stool DNA isolation kit was obtained from Qiagen (Valencia, CA), while culture DNA isolation kit and Gel extraction kit were obtained from Real Genomics (Taiwan). Adult Bovine Serum was purchased from PAA Laboratories (Austria) and E. coli and amoeba culture components were obtained from Amersham Biosciences, and DIFCO (United States of America).

All solution concentrations expressed in percentage are in (w/v) basis unless specified otherwise. All solutions and reagents were prepared in double distilled water. All sterilizations were performed at a pressure of 15 lbs per square inch for 20 min. at a temperature of 121°C.

3.1.4.2 Reagent and solution preparation:

✓ Lugols iodine solution:

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine crystal</td>
<td>5 gm</td>
</tr>
<tr>
<td>Potassium iodine</td>
<td>10 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Potassium iodide was dissolved in distilled water and iodine crystals were slowly added. The solution was then filtered and kept in an amber colour stopped bottle. The solution was diluted five times with distilled water before using. The stain deteriorates very quickly; hence it was prepared for every two weeks.

**Schaudinn’s fixative:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated aqueous mercuric chloride</td>
<td>100 ml</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>50 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Glacial acetic acid was added just before (30 min- 1 hr) use.

**Heidenhains’s iron haematoxylin:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric ammonium sulphate</td>
<td>2 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Solution I</td>
<td></td>
</tr>
<tr>
<td>Haematoxylin crystal</td>
<td>1 gm</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>20 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>200 ml</td>
</tr>
<tr>
<td>Solution II</td>
<td></td>
</tr>
</tbody>
</table>

Equal volume of solution I and solution II were mixed before use and was used for next seven days.

**Robinson medium:**

Defined medium "R" for growing *Escherichia coli* is prepared by mixing 125 gm sodium chloride, 50 gm citric acid monohydrate, 12.5 gm potassium dihydrogen phosphate, 25 gm ammonium sulphate, 1.25 gm magnesium sulphate heptahydrate and 100 ml lactic acid in 2.5 liters water. For use, 100 ml of the medium is diluted 10 times by adding 900 ml distilled water to 100 ml medium, adjusted to pH 7 by adding 40% NaOH (approximately 7.5 ml) and autoclaved at 15 lbs pressure (121°C) for 15 minutes.
Materials and Methods

To prepared basal amoebic medium "BR" in a flat sided screw capped bottle 100ml of working solution (1X R) is inoculated with 100µl of E. coli strain B (kept in 1% bactopeptone) and incubated at 37⁰C for 48 hours with bottle lying flat. To complete the medium for amoebic growth "BRS" equal volume of adult bovine serum and BR are mixed, incubated 24-48 hours at 37⁰C and stored at room temperature for use up to one month.

✓ Difcobactopeptone (20%):

20gm is dissolved in 100ml of distilled water and then sterilized by autoclaving at 15 lb/in² pressure at 121⁰C for 15 min.

✓ Rice starch:

The powder is used untouched, as supplied by Sigma-Aldrich Corporation (St. Louis, Missouri, United States).

✓ LB Agar:

LB agar was prepared by adding 1.5 % (w/v) of Bacto-Agar to LB medium and sterilized by autoclaving. Ampicillin 100µg/ml to a final concentration was added after cooling the LB agar to around 55⁰C and culture plates were made under aseptic conditions.

✓ Saline agar slopes:

15gm fine agar powder and 7 gm NaCl are dissolved in 1000ml of distilled water and distributed 2.5ml to screw capped McCartney bottles and sloped after autoclaving.

✓ Luria Broth:

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 gm</td>
</tr>
<tr>
<td>Bacto-Yeast extract</td>
<td>5 gm</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>10 gm</td>
</tr>
</tbody>
</table>

The components were dissolved in double distilled water and pH adjusted to 7.0 using 2N NaOH. The medium was sterilized by autoclaving.
✓ **Erythromycin:**

0.5gm of pure erythromycin powder was dissolved in 20ml of 70% ethanol in sterile container. The solution was then allowed to stand at 4°C for 2 hour or longer to sterilize the antibiotic, and 30ml of sterile distilled water was added to it.

✓ **Phthalate solution (10X):**

204gm of phthalate is dissolved in 1800ml of distilled water and then pH is adjusted to 6.3 by adding 40% NaOH (approximately 100 ml). Final volume is made up to 2000 ml with distilled water and then sterilized by autoclaving at 15 lb/in² pressure at 121°C for 15 min. The solution is diluted 1 in 10 in sterile water for use as 0.05 M phthalate.

✓ **0.5M Ethylenediamine tetra-acetic acid (EDTA) solution, pH 8.0:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium EDTA</td>
<td>18.61gm</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>80 ml</td>
</tr>
</tbody>
</table>

18.61 gm of disodium EDTA is added to 80 ml of distilled water. After stirring vigorously the pH is adjust to 8.0 by adding NaOH pellets (approximately 2.0 gm). The disodium salt of EDTA will not get dissolve until the pH of the solution is adjusted to ~8.0 by the addition of NaOH. Once pH becomes stable at 8.0, final volume is make-up to 100 ml with nuclease free water and the solution is autoclaving at 15 lb/in² pressure at 121°C for 15 min. The solution is stored at room temperature.

✓ **1M Tris-HCl, pH 8.0:**

12.11 gm of Tris base is dissolved in 80 ml of distilled water. pH is adjust to 8.0 by adding concentrated HCl (approximately 4.2 ml). The solution is then allowed to cool to room temperature before making final adjustments to the pH. Final volume is make-up to 100 ml with distilled water. Then the solution is sterilized by autoclaving at 15 lb/in² pressure at 121°C for 15 min. It is stored at room temperature.

✓ **Ethidium Bromide (10mg/ml):**

10mg of EtBr is dissolved in 1ml of Nuclease free water. It is mixed properly and wrapped the container with aluminum foils and stored at 4°C.
Materials and Methods

 ✓ **TBE (Tris-borate-EDTA) Buffer (10X):**

108 gm of Tris-base, 55 gm boric acid and 40 ml of 0.5M EDTA (pH 8.0) are mixed and is adjusted to 1 L. To make 1X TBE, 50 ml of stock is diluted with 950 ml of distilled water.

 ✓ **10% (w/v) Sodium Dodecyl Sulphate (SDS):**

10 gm of SDS is dissolved in 80 ml of distilled water. It is heated to 68°C and stirred with a magnetic stirrer to assist dissolution and adjusted pH to 7.8 with sodium hydroxide (NaOH) pellets. The final volume is adjusted to 100 ml with distilled water. Sterilization is not necessary. It is then stored at room temperature.

 ✓ **20X SSC:**

175.3 g of NaCl and 88.2 g of sodium citrate are added to 800 ml of distilled water. After stirring vigorously the pH is adjusted to 7.0 by adding dilute HCl (4N). Final volume is make-up to 1000 ml with distilled water and the solution is autoclaving at 15 lb/in² pressure at 121°C for 15 min. The solution is stored at room temperature.

 ✓ **Heat Inactivation of Serum:**

Adult Bovine Serum was stored at -20°C. Before use the serum was thawed at room temperature and incubated in a water bath at 37°C for 30 min with intermittent shaking for heat inactivation. The serum was then kept at 55°C for 45min with intermittent shaking. The inactivated serum was stored at 4°C for use.

 ✓ **Phenol:Chloroform:Isoamyl Alcohol (25:24:1), pH 7.8-8.0:**

Tris-saturated phenol (pH 7.8-8.0), chloroform and isoamyl alcohol is mixed in the ratio of 25:24:1. The mixture is stored under 100 mM Tris-HCl (pH 8.0) (some volume of 100 mM Tris-HCl (pH 8.0) is added to the mixture; the Tris solution will be layered above while the mixture will be layered below the Tris solution) in a light tight dark bottle at 4°C for period of up to 1 month. This is because phenol is readily oxidized in the presence of air and light, and oxidized phenol damage DNA by breaking down of phosphodiester bonds or cause cross-linking of RNA and DNA.
Materials and Methods

✓ Chloroform: Isoamyl Alcohol (24:1):

Chloroform and isoamyl alcohol is mixed in the above ratio and store at room temperature.

3.1.5 Stock primer: Primers supplied in lyophilized form were resuspended in the required volume of sterilized millipore water to obtain a final concentration of 100pmol/µl and was stored refrigerated at -20ºC as stock. The solution was divided into small aliquots by making the concentration at 20pmol/µl...

3.2 Methods:

3.2.1 Microscopic analysis:

3.2.1.1 Direct Microscopy (Wet mount):

Iodine wet mounts of fresh unpreserved stool samples were examined microscopically for demonstrating *E. histolytica/E. dispar/E. moshkovskii* complex and other protozoan parasite oocysts, cysts and trophozoites. Briefly, iodine wet mounts were prepared by adding approximately 2 mg of stool to a drop of Lugol's iodine (5% iodine and 10% potassium iodide, diluted 1:5 with distilled water) on a glass microscope slide and placing a cover slip on the stool suspension. The mounts were then examined microscopically under compound light microscope (Olympus CX-31, Japan) initially by using 10X eyepieces and a low-power (10x) objective and then using an oil-immersion objective of 100X and magnification. The wet mount was read in approximately 5 min to view at least 90-100 fields per slide.

3.2.1.2 Modified Ziehl–Neelsen staining:

Presence of oocysts in fecal sample was detected using modified Ziehl-Neelsen staining technique as previously described with some modifications (Potters and Van Esbroeck, 2010). Briefly, it involves staining of a methanol fixed thin smear of fecal material with Kinyoun’s carbol fuchsin stain for 30 minutes. Subsequently, the slide is rinsed in tap water and placed in an acid-alcohol solution to remove the stain, while acid-fast structures will resist to the acid-alcohol's destaining action. After rinsing again, the slide is placed for 10 minutes in a counter-stain methylene blue, providing contrast between background material and acid-fast structures. The slide is rinsed once more in
tap water and after that slide has been air-dried and examined under microscope at 10X eyepieces and an oil-immersion objective of 100X magnification (Olympus CX-31, Japan).

3.2.1.3 Heidenhain’s iron haematoxylin staining technique:

On clean a slide, thin smear of faecal material was then immersed in Schaudinn's fixative solution for about at least three minutes. Schaudinn's solution fixed thin smear was then passed through 30% and 50% alcohol, two minutes each. The smear was then immersed in 70% iodine alcohol for 3-5 minutes and then again rinsed in 50% and then 30% alcohol two minutes each and finally rinsed the slide in distilled water (two changes). The slide was kept in 2% aqueous iron alum for 30 minutes. After washing with distilled water staining of thin smear was done using 0.5% aqueous haematoxylin stain for 30 minutes. After single washing with distilled water, differentiation was done by rinsing the slide in 1% aqueous iron for 2 minutes and then in 4% aqueous iron alum for 1-3 minutes. After this step slide was observed under microscope for proper staining. If the staining was perfect the slide was rinsed in tap water and the dehydrated by immersing the slide sequentially in 30%, 50%, 70%, 95% alcohol for about 2-3 minutes each and finally two changes in absolute alcohol for 2-3 minutes each. After clearing in xylol: alcohol (1:1) for 3-5 minutes, slide was once immersed in xylene for another 3-5 minutes and finally mounted using DPX.

3.2.2 Cultivation of amoeba:

3.2.2.1 Isolation of amoebae from stool sample:

Cultivation of Entamoeba under xenic condition was performed using biphasic (solid and liquid) Robinson’s medium (Robinson, 1968). Briefly, to a McCartney bottle containing sterile agar slope, completed medium for amoebic growth "BRS" diluted with phthalate solution (1:4) was added to cover the slope followed by the addition of 2 drops (0.06 ml) of 20% bactopeptone and 10mg rice starch. It was then inoculated with approximately 50mg of fresh untreated faeces by a loop and incubated at 37°C. After 48 hours a drops of culture sediment from the bottom of slope was taken in a slide and examined with the aid of an optical microscope for the presence of amoebae. During subculture 0.1ml of faecal-starch layer was taken to a culture bottle with fresh agar
Materials and Methods

slope containing BRS and phthalate (1:4), 2 drops (0.06 ml) of 0.5% erythromycin, 1-2 drops (0.03-0.06 ml) penstrep, 2 drops (0.06 ml) of 20% bactopeptone and 10mg rice starch and incubated at 37\(^0\)C for 24 hours.

Growth of *Blastocystis sp.* in biphasic Robinson’s medium is very common and it interferes with growth of *Entamoeba*, hence it was always necessary to remove *Blastocystis* from the culture. Briefly, after 1-2 subculture starch layer along with BRS from the two culture tubes was taken into a 50ml falcon. 10ml of tape water was then added to the content and kept at room temperature for 10-15 minutes. Following, centrifuged at 13000 rpm for 5 minutes at 4\(^0\)C. The supernatant was then decanted by inverting the tube and fresh BRS was added to the tube and finally taken the content to a new culture tube. 2 drops (0.06 ml) of 0.5% erythromycin, 2 drops (0.06 ml) of 20% bactopeptone, 1-2 drop (0.03-0.06 ml) penstrep and 10mg rice starch were added to the culture tube and incubated at 37\(^0\)C for 24 hours.

3.2.2.2 Harvesting of cell and DNA isolation:

The cells were harvested at 600g for 5 minute at 4\(^0\)C. The cell pellet was then washed with 20 ml of PBS twice and finally stored in 70% ethanol at -20\(^0\)C. The DNA was isolated from the cells harvested from at least 5 tubes using HiYield\textsuperscript{TM} Genomic DNA mini kit (Real Genomics, Taiwan) following manufacture’s instruction with some modifications. Briefly, cells were pelleted through at 13000 rpm for 4 mins and kept under laminar air flow for 3-4 mins to remove all traces of ethanol and then resuspended in 150 µl of RBC lysis buffer (provided with kit). After adding 200 µl of GB buffer to the sample, it was vortexed for 5-10 secs and then incubated at 70\(^0\)C for 10-15 mins untill the lysate was clear. 200 µl of ethanol was then added to sample lysate and after vortexing the lysate applied to a GB column provided with the kit. Centrifuged at 13000rpm for 2 mins at room temperature. After discarding the flow-through the column was washed first with 400 µl of W1 buffer and then with 600 µl of wash buffer. The tube was then centrifuged at maximum speed for 3 mins to remove all traces of ethanol present in the wash buffer. 30-50 µl of elution buffer was then added at the centre of DF column matrix and incubated at room temperature for 3 mins and finally eluted the purified DNA through centrifugation at full speed for 2 mins.
3.2.2 Screening of samples by dot blot:

3.2.3.1 Restriction enzyme double digestion:

Restriction enzymes digestions were in generally carried out in small volume usually 20 µl (50 µl in case of genomic DNA digests). Approximately 200ng of DNA was digested in a reaction mixture containing 2X enzyme Tango™ buffer as per manufacturer’s instructions (Thermo scientific, Wattham, USA) and 5-10 units of enzymes (EcoRI and HindIII) at the recommended temperature (37°C) in water bath for 4-16 hours. After incubation the reaction mixture was loaded with 1X gel loading dye into agarose gel and run for appropriate times.

3.2.3.2 Purification of insert DNA from agarose gel:

The agarose slice containing the relevant DNA fragment was cut out from gel and trimmed to remove any extra agarose. DNA from gel bend was isolated using HiYield™ Gel/PCR DNA Mini kit (Real Genomics, Taiwan) following manufacturer’s protocol. Briefly, approximately 300 mg of the gel slice containing the desired DNA fragment was mixed with 500 µl of DF buffer in 1.5 ml microcentrifuge tube followed by vortexing. After proper vortexing the tube was incubated at 58°C for 10-15 mins until the gel slice completely dissolved. After cooling the dissolved sample mixture at room temperature, it was passed through a new DF column. Centrifuged at 13000rpm for 30 sec at room temperature. After discarding the flow through the column containing the desired DNA fragment was washed twice with 600 µl of wash buffer. The tube was then centrifuged at maximum speed for 3 mins to remove all traces of ethanol. 20-30 µl of elution buffer was then added at the centre of DF column matrix and incubated at room temperature for 3 mins and finally eluted the DNA through centrifugation at full speed for 2 mins.

3.2.3.3 Ligation of vector and insert:

The vector and respective insert DNA was mixed with 1X T4 DNA ligase buffer and 1µl of T4 DNA ligase (NEB) in a total volume of 10µl, with a total DNA concentration of 100 to 200 ng and was incubated at 16°C for 16 hours and subsequently transformed into competent DH5α cells. Vector and insert DNA having almost similar length or insert DNA is smaller in size than vector, a molar ratio of 1:3 (vector: insert) was taken.
whereas a molar ration of 1:1 was used in case of insert DNA larger in size than the vector DNA.

3.2.3.4 Preparation of competent cells:
Competent *E. coli* cells (DH5α) were prepared following the method as previously described by Hanahan et al. (1991). A single colony of *E. coli* strain (DH5α) was inoculated in 10 ml of LB medium and was allowed to grow overnight at 37°C at 200 rpm in a 50ml conical flask. 1% of overnight culture was added to 50 ml LB in 500 ml conical flask. The cells were grown with continuous shaking at 37°C at 200 rpm to an optical density at 600 nm of 0.38-0.42. The cells were immediately chilled on ice for 2 hours with vigorous shaking and thereafter harvested at 5000 rpm for 10 min at 4°C. The pellet was resuspended in a 10 ml of pre-chilled 0.1 M CaCl₂. After an incubation of 1 hour on ice with intermittent shaking, cells were pelleted through centrifugation at 5000 rpm for 10 min at 4°C. The cell pellet was finally re-suspended in 2 ml of 0.1 M CaCl₂ and kept on ice for another 15 min. For storing purposes 50% glycerol was then added to a final concentration of 15% (v/v) and after proper mixing was stored at –80°C as 100μl aliquots.

3.2.3.5 Transformation of competent cells:
Competent cells were thawed on ice and to 100 μl cells, plasmid DNA of 7-10ng (~10 μl) was added. The cells were incubated on ice for 15 min and subsequently given a heat shock at 42°C for 60 seconds and again incubated on ice for another 4-5 min. 900 μl LB (without antibiotic) was added to the cells and the tube was incubated at 37°C for 1 hour at 220 rpm. The transformation efficiency was checked by plating 200 μl cells obtained after centrifugation in presence of IPTG, X-Gal and ampicillin following the protocol as previously described (Sambrook and Russell, 2001). A part of transformed cells was stored in 15% glycerol (v/v) in 100 μl aliquot at -80°C for future use.

3.2.3.6 Midi prep of plasmid DNA:
This is adapted from the alkaline lysis method as previously published (Brinboin and Dolly, 1979). The bacterial cells were grown overnight in 50 ml LB containing antibiotic at 225 rpm at 37°C. The cells were collected by centrifugation at 6000 rpm at
Materials and Methods

4°C for 15 min. The cell pellet was resuspended in 1.5 ml of lysis buffer (25 mM Tris-HCl, pH 7.5, 10 mM EDTA pH 8.0, 15% sucrose and 2 mg/ml lysozyme) and kept on ice for 10 min. To the lysed cells, 3 ml of denaturing solution (freshly prepared solution containing 0.2 N NaOH and 1% SDS) was added and mixed smoothly by inverting the tube and incubated on ice for 10 min. After adding 1.8 ml of 3 M sodium acetate (pH 4.6) the tube was incubated on ice for another 20 min and then centrifuged at 12000 rpm for 30 min at 4°C. The supernatant was then treated with 10 μl RNase A (10 g/ml) at 37°C for 45 min to remove RNA. The supernatant was further purified by extracting twice with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with equal volume of chloroform: isoamyl alcohol (24:1, v/v). The aqueous layer was transferred to a tube and the plasmid DNA was precipitated by addition of 2.5 volume of pre-chilled ethanol and collected by centrifugation at 12000 rpm for 10 min at 4°C. The DNA was further purified by re-suspending in 0.4 ml of autoclaved nuclease free water and 0.12 ml of 4M NaCl and 0.5 ml of 13% PEG 8000. The tube was incubated for 1 h on ice and centrifuged at 12000 rpm for 15 min. The pellet was then once washed with 200 μl of 70% ethanol, dried at 37°C and resuspended in 50 μl of buffer TE.

3.2.3.7 Preparation of radiolabeled DNA by random priming method:

Radiolabeling was done using random priming kit (New England Biolabs, Massachusetts, United States) following manufacture’s instruction with minor modifications. Briefly, about 50 ng of DNA was denatured by heating for 10 min at 95°C and immediately chilled on ice for 5 min. In addition to the denatured DNA the random priming reaction mixture containing 1 μl each of 0.5 mM dTTP, dGTP, dCTP, 2.5 μl hexanucleotide labeling mixture containing random primers and reaction buffer at 10X concentration (2 M HEPES pH 6.6, 2 mM Tris-Cl pH 7.0, 0.1 mM EDTA and 4 mg/ml BSA), 3 μl of α32P dATP and 1 μl of Klenow enzyme. The reaction was made up to a final volume of 25 μl by adding nuclease free water and incubated for 3 hour at 37°C. The reaction was stopped by the addition of 2 μl of 0.5M EDTA (pH 8.0). The unincorporated dNTPs were removed by ethanol precipitation using 25 μg of salmon sperm DNA as carrier and 2.5M ammonium acetate. The labeled DNA was washed with 70% ethanol and dried and resuspended in 200 μl of T10E1.
3.2.3.8 Enrichment of cysts for dot blot:

Cysts were enriched following formalin ether concentration method as described previously with slight modifications (Knight et al., 1976). A portion of each fresh stool sample was taken and processed. Briefly, 1-2 gm of stool was homogenized in a 15 ml conical centrifuge tube containing 10 ml of autoclaved distilled water. The resulting suspension was strained through doubled cheesecloth in a 50 ml falcon tube. This suspension was centrifuged at 2000 rpm for 5 min and pellet was re-dissolved in 10 ml of 10% formaldehyde. 3 ml of diethyl ether was added to the tube and this mixture was vortexed and incubated at RT for 30 min. The mixture was then subjected to centrifugation at 2000 rpm for 5 min, supernatant was removed and pellet was washed with double distilled water. The pellet containing concentrated cysts was re-dissolved in 400 µl of TE buffer.

3.2.3.9 DNA dot blots:

Cysts in T10E1 buffer was subjected to five freeze-thaw cycle and thereafter to sonication in order to obtain crude DNA for dot-blot hybridization experiment. The crude cyst DNA was denatured by addition of NaOH to final concentration of 0.25 N in a total volume of 300 µl. The DNA was kept at room temperature for 30 minutes and then transferred on to the ice. The GS positive nylon membrane of required size was cut and saturated in 0.4 M Tris-Cl, pH 7.5 for 15 min and the DNA were spotted on to the membrane with the help of mini-fold apparatus from Whatman, Germany. The blots were air dried and UV cross linked before hybridization.

3.2.3.10 Hybridization:

DNA blot was first pre-saturated in 5 ml of pre-hybridization solution (1% SDS and 1 M NaCl, 0.3-0.4 ml per square cm of membrane) at 65°C in hybridization bottle. After 3 h, heat denatured radio labeled probe (25 µl) and 50 µl denatured salmon sperm DNA (100 µg/ml) were added to the pre-hybridization solution and hybridization was carried out for 16 h in a hybridisation oven. The membranes were washed sequentially to remove non-specifically bound probe using the steps: twice with 2X SSC at RT for 5 min, twice with 1X SSC containing 1% SDS at 65°C for 20 min and finally twice with 0.1 X SSC at RT for 15 min each.
3.2.3.11 Autoradiography:

After hybridization and washing, the blots were wrapped in common saran wrap. Autoradiography was then carried out by exposing the hybridized blot against imaging plate (IP) and scanning in a Phosphor imager.

3.2.4 Species identification by PCR:

3.2.4.1 Isolation of genomic DNA with QIAamp™ kit:

An aliquot of 200 mg stool sample was used for isolation genomic DNA using QIAamp DNA stool kit (Qiagen, Valencia, CA) as per protocol provided by the supplier with some modifications. Briefly, following five freezing-thawing cycles, samples were incubated at 95°C for 10 minutes in buffer ASL (Lysis buffer, Qiagen). Sample vortexed for 15 sec and centrifuged at 12000rpm/1 min to pellet stool particles. One inhibitex tablet was added to 1.2 ml of supernatant and vortexed immediately for 1 min at room temperature to allow inhibitors to absorb to the inhibitex matrix. Centrifuged at 12000rpm/3 min to pellet inhibitors bound to the inhibitex and supernatant transferred to a new microfuge tube. 15 µl of proteinase K and 200 µl of buffer AL (provided with kit) were added and incubated at 70°C for 10 mins. 200 µl of ethanol was then added and the lysate applied to the QIAamp spin column provided with the kit. Centrifuged at 12000rpm/ 1min at room temperature, washed with buffer AW1 and AW2 and finally eluted out with 50 µl of elution buffer.

3.2.4.2 Selection, designing and in-silico analysis of primer:

Primers for PCR chosen were highly specific for diagnosis of the respective parasite. The published primer sequences used in this study for detection of *E. histolytica*, *E. dispar*, *E. moshkovskii*, *C. parvum*, *T. trichiura* and *G. duodenalis* were blasted in the genome database of all organisms in the website (http://www.ncbi.nlm.nih.gov/blast/) and were found to be specific.

The generic oligonucleotide primers targeting the intergenic spacer region of *Entamoeba* spp. were designed with the aid of online tool Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers for the detection of *Entamoeba* genus were picked up from the end of 18S and the start of the 28S rRNA gene of *E. histolytica* rDNA circle. The primer pairs specific for the detection of two
emerging protozoan parasites *C. cayetanensis* and *E. bieneusi* were designed using the Primer-Blast targeting the small subunit ribosomal RNA gene found in the National Center for Biotechnology Information GenBank database. Sequences were aligned with CLUSTAL W version 1.8.1 and primers were designed from respective conserved region to anneal at these unique signature sequence locations.

The primers were checked for sequence specificity by comparison to the GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov) using nucleotide blast. Final selections of the designed primers were made after passing them through online analysis tool: OligoCalc (http://www.basic.northwestern.edu/biotools/OligoCalc.html), PCR Products (http://www.bioinformatics.org/sms2/ pcr_products.html) and PCR Primer Stats (http://www.bioinformatics.org/sms2/pcr_primer_stats.html). List primers used in this study along their signature sequence locations and annealing temperature were mentioned in Table 3.1.

### 3.2.4.3 Reaction condition for PCR:

Forward and reverse oligonucleotide primers targeting the signature sequence of the infecting parasite were used for PCR. All the PCR amplifications were performed in a final volume of 20µl with approximately 100ng of template DNA, 1 µM of each primer, 1X PCR buffer with 2.5 mM MgCl₂, 1X BSA, 0.2 mM dNTPs, and 1U of Taq DNA Polymerase (Thermo scientific, Wattham, USA) in the MJ Mini™ thermal cycler (Bio-Rad Laboratories, Hercules, CA).

In a nested PCR protocol, amplifications were performed as mentioned above in the primary PCR step, while in the secondary PCR step 2-3 µl of PCR products from primary PCR amplification was used as DNA template along with other components of the PCR mixture as mentioned above in a final volume of 20 µl.

PCR cycling conditions comprised initial denaturation at 94°C for 4-5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at X°C (X=depends on Tₘ) for 30 sec-1 min and extension at 72°C for 1 min. The final extension step at 72°C was done for an additional 7 min. The annealing temperatures for respective PCR assay were mentioned in the Table 3.1
### Table 3.1: List of primers and probe used in this study

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Primer name and sequence</th>
<th>Location in genome</th>
<th>Amplicon/Probe size (bp)</th>
<th>Annealing temperature</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. histolytica</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EhF: 5’ AAGCATTTTCTAGATCTGAG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eh R: 5’ AAGAGGTCTAACCAGAAATTAG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSU rRNA</td>
<td>439</td>
<td>48°C</td>
<td>Khairnar and Parija, 2005</td>
</tr>
<tr>
<td>2</td>
<td><em>E. dispar</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EntaF: 5’ ATGCACGAGAGCGAAAGCAT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EdR: 5’ CACCACCTTATCCCTACC 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITS-2/18S rRNA</td>
<td>752</td>
<td>58°C</td>
<td>Hamzah et al., 2006</td>
</tr>
<tr>
<td>3</td>
<td><em>E. moshkovskii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EntaF: 5’ ATGCACGAGAGCGAAAGCAT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EmR: 5’ TGACCAGGACAGACACAT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>small-subunit rRNA</td>
<td>580</td>
<td>55°C</td>
<td>Hamzah et al., 2006</td>
</tr>
<tr>
<td>4</td>
<td><em>E. bieneusi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPLEBF: 5’ GAAACTTGTCACACTCTTAC 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPLEBR: 5’ TCCTGCCATTCTACGATAATC 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>small-subunit rRNA</td>
<td>596</td>
<td>55°C</td>
<td>In this study</td>
</tr>
<tr>
<td>5</td>
<td><em>G. duodenalis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGL639-658: 5’ AAGTGCGTCACCGGACGGCT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGR789-809: 5’ TTAGTGCTTTGACCACTCGA 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>giardin</td>
<td>171</td>
<td>60°C</td>
<td>Mahbubani et al., 1992</td>
</tr>
<tr>
<td>6</td>
<td><em>C. cayetanensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPLCCF: 5’ CCCTCGAATCGCTTTTCTC 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPLCCR: 5’ AAACACAGCGGAAATGCCA 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITS-1</td>
<td>302</td>
<td>55°C</td>
<td>In this study</td>
</tr>
<tr>
<td>7</td>
<td><em>Entamoeba spp.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18S/28SF: 5’ CTCTTACCGATTGATAAAAGAGG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18S/28SR: 5’ AAATCATTGTTATTTTTCTCCTC 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITS+5.8S+28S</td>
<td>609</td>
<td>53°C</td>
<td>In this study</td>
</tr>
<tr>
<td>8</td>
<td><em>Cryptosporidium parvum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B1: 5’ CCGAGTGGTTATCCCAAAGTTCAGG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B2: 5’ TCCTAAAGAGGAAAGACTCCAAGG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B3: 5’ GCGAAGATGGACTTTTGTGATTTG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B4: 5’ ATCGATTAGTATACGGAATAACTCAT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>small-subunit rRNA</td>
<td>400</td>
<td>45°C</td>
<td>Balatbat et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>194</td>
<td>47°C</td>
<td></td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th></th>
<th><strong>T. trichiura</strong></th>
<th><strong>HMe probe</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>HTT18SF0: 5’ TCTTGATTCAGTGGTAGG 3’  &lt;br&gt;HTT18SR0: 5’ CTTACTGGGAATTCTCGTC 3’  &lt;br&gt;HTT18SF: 5’ AGCGCTCCGGGAGGACCT 3’  &lt;br&gt;HTT18SR: 5’ CTGTCCAGTCACGAGAAC 3’</td>
<td>SSU rRNA</td>
</tr>
<tr>
<td>10</td>
<td>HMe probe</td>
<td>HMe region of EhR1 (EcoRI+ HindIII fragment)</td>
</tr>
</tbody>
</table>
3.2.4.4 Agarose gel electrophoresis: The agarose concentrations (0.6-1.5%) used in the electrophoretic separation of DNA were chosen on the basis of the size of the DNA to be resolved. Agarose was melted in 0.5X TBE by heating and was cooled to about 50°C before adding 0.5µg/ml of EtBr. The molten agarose was poured into a gel tray with comb and allowed to solidify. DNA samples were then loaded with 1X Gel loading buffer and electrophoresed in 0.5X TBE at an appropriate electric filed 4 V/cm for an appropriate time period for optimum separation. The DNA was visualized using an UV transilluminator.

3.2.5 DNA sequencing:

3.2.5.1 Purification of PCR product: The single uniform diagnostic band was excised and purified using HiYield™ Gel/PCR DNA Mini kit (Real Genomics, Taiwan) according to the instructions given by the manufacturer with minor modifications as mentioned in the previous section. Eluted DNA was further checked by agarose gel electrophoresis.

3.2.5.2 Sequencing of diagnostic amplicons:

Sequencing was performed for further wet lab validation of the signature diagnostic fragments of the respective infecting parasites under study. Dideoxy chain-termination reaction was carried out in a 10 µl reaction consisting of approximately 50ng of purified PCR product, 5 pmole of primer, 4 µl of Big dye terminator V3.1 (Applied Biosystem, USA) and 3 µl of double distilled water. Chain termination reaction was performed under the following conditions: 20 cycles of 96°C for 10sec, 55°C for 5 sec and 60°C for 4 min. Products were purified by ethanol precipitation followed by washing with 70% ethanol. Purified samples were dissolved in 10 µl of Hi-Di formamide and run in an ABI 3500 Genetic analyzer (Applied Biosystems Inc., CA, USA) as per manufacturer’s instructions.

3.2.5.3 Sequence annotation and similarity search:

The generated sequence chromatograms were viewed using Sequence scanner™ v1.0 (Applied Biosystems Inc., CA, USA). After editing overlaying peaks if any along DNA sequences were then checked by alignment of the bidirectional sequences with the help of BioEdit Sequence Alignment Editor to find out the region of homology (overlap).
The region of homology (overlap) was selected as the final sequence which was then subjected to similarity search using the online program nucleotide blast (blastn) in National Centre for Biotechnological Information (NCBI, http://www.ncbi.nlm.nih.gov) database for further wet lab evaluation of the respective diagnostic amplicons of the parasite species under study. The following reference sequences were used in this study: (i) *E. histolytica* (KC763012); (ii) *E. dispar* (AB282661); (iii) *E. moshkovskii* (AB520687).

### 3.2.6 Data analysis:

Statistical analysis was carried out using the statistical software SPSS version 16.0 (SPSS, Chicago, IL, USA). Mean, range and percentage were used to describe different characteristics of study subjects as appropriate.

Continuous variables were described using medians with interquartile ranges (IQR) and categorical variables were described using numbers and percentages. Descriptive statistics were used to give a clear picture of background variables like age, sex and other variables in well-structured questionnaire.

A Pearson’s Chi-square test was used to test the associations between each variable in univariate statistical model, with prevalence of parasite as the dependent variable, while the independent variables were environmental factors, socio-demographic factors and clinical status. Evaluation of determinants of parasitic infection and potential covariates that were independently associated with outcome was performed by univariate logistic regression analysis for statistically significant factor showing level of significance P<0.05.

The frequency distribution of both dependent and independent variables were worked out and the association between the independent and dependent variables were measured and tested using OR and 95% CI.

Sensitivity, specificity, PVP (positive predictive value), and NPV (negative predictive value) values were calculated with the following formula: sensitivity: $100\times\frac{a}{a + c}$; specificity: $100\times\frac{d}{b + d}$; PVP: $100\times\frac{a}{a + b}$; and NPV: $100\times\frac{d}{c + d}$, where 'a' represents real positive samples, 'b' are false positive samples, 'c' are false negative samples and 'd' are real negative samples.