1. INTRODUCTION
In history, during the “Hadean aeon” (around 4,600 million years ago) the mother earth came into existence in the form of a planetary material followed by “Archaeozoic aeon” (from 3,800 million years to 2,500 million years ago) when the earth's crust was formed (Wilde et al., 2001). The atoms of hydrogen, oxygen, nitrogen and carbon were elementary to constitute life this hypothesis popularly known as Oparin and Haldaen hypothesis (Oparin, 1924) was eventually proved by the classic experiment conducted by Miller-Urey in 1953 that simulated hypothetical conditions present on the early Earth and tested for the occurrence of chemical evolution (Miller, 1953; Miller and Urey, 1959), this landmark experiment deciphered invaluable clues for the understanding of origin of life and showed that “life” might have originated from “non-life”. During the “Proterozoic aeon” (from 2,500 to 544 million years ago) one of the first forms of 'life' to develop was the lowly amoeba (Stanley and Steven, 1999). Through the Proterozoic aeon, the amoeba evolved into different species, one of them Entamoeba histolytica a protozoan parasite that causes amoebiasis. During the “Phanerozoic aeon” which constituted; “Miocene period” (from 23 to 5.3 million years) when the first hominoids appeared to the “Holocene period” (from 10,000 radiocarbon years to the present) the modern civilization (Felix et al., 2005), man evolved and explored many places on earth during this he drank water contaminated with amoeba and sometimes developed amoebiasis, which includes both intestinal and occasionally extra-intestinal amoebiasis (Parija, 2006).

*E. histolytica* is a single celled eukaryote. It has two stages that are cyst and trophozoite. The cysts measure 10–16 μm in diameter and typically contain four nuclei (WHO, 1997; Parija, 2006). The cysts are resistant to acidification,
chlorination, desiccation, and capable of surviving in a moist environment for several weeks. The trophozoites usually measures 20 μm to 40 μm with a single nucleus (WHO, 1997; Parija, 2006). Transmission of *E. histolytica* occurs through ingestion of cysts in faecally contaminated food or water, use of human excrement as fertilizer, and person to person contact (Parija, 2006).

*E. histolytica* remains in the large intestine where in most infected humans the symptoms of intestinal amoebiasis are intermittent and mild (various gastrointestinal upsets, including colitis and diarrhea) (Kasper, 2005; Parija, 2006). In more severe cases the gastrointestinal tract hemorrhages can result in dysentery (Haque et al., 2003).

In some cases the trophozoite of *E. histolytica* can be transmitted extra intestinally to other organs like liver, lungs, brain, spleen and skin etc. The trophozoite enters the circulatory system and infects other organs, most often the liver (hepatic amoebiasis), or it may penetrate the gastrointestinal tract resulting in acute peritonitis; such cases are often fatal (Haque et al., 2003).

Amoebiasis can occur in any age group and there is no sex or racial difference in the occurrence of the disease. Most of the infected people who remain asymptomatic are called as carriers (Parija, 2006). The human carrier can discharge up to $1.5 \times 10^7$ cysts per day (Archana, 2002).

Amoebiasis is responsible for 50 million cases and 100,000 deaths annually (WHO, 1997; Walsh, 1986), placing it second only to malaria in terms of mortality.
caused by protozoan parasites occurring worldwide (WHO, 1997). It is an important health risk to travelers from other geographical areas to endemic areas (WHO, 1997). This infection spreads worldwide but is most common in tropics where dense population and poor sanitation exist and lead to health hazards (Haque et al., 2003). In developing countries such as Mexico, India and Bangladesh the diarrheal diseases continue to be major causes of morbidity and mortality in children (Caballero-Salcedo et al., 1994; Haque et al., 1997; Martinez-Palomo and Martinez-Baez, 1983; Radvin, 1995).

In India about 15–20% of Indians are affected by the parasite (Archana, 2002) and in Bangladesh 1 in 30 children from an urban slum in Dhaka die of diarrhea or dysentery by his or her fifth year of age (Huston et al., 2000; Haque et al., 2002).

*E. histolytica*, the pathogenic amoeba and the causative agent of invasive amoebiasis, is indistinguishable in its cyst and trophozoite stages from those of *Entamoeba moshkovskii*, considered to be primarily a free-living amoeba (Clark and Diamond, 1991a), and *Entamoeba dispar*, a noninvasive amoeba, except in cases of invasive disease when *E. histolytica* trophozoite may contain ingested red blood cells (Clark and Diamond, 1997); such a finding is rarely seen (Gonzalez-Ruiz et al., 1994). This leads to confusion in the definite identification and differentiation of *E. histolytica* from *E. histolytica* like amoebae such as *E. moshkovskii* and *E. dispar* in the diagnosis of intestinal amoebiasis (Parija, 2006).

The acceptance of *E. dispar* as a distinct but closely related protozoan species and reporting of free living *E. moshkovskii* in humans have a profound impact on the
true epidemiology of amoebiasis. *E. dispar* and *E. moshkovskii* have been reported to be found in most asymptomatic amoebic infections, although *E. histolytica* asymptomatic colonization is not uncommon (Jackson, 2000; Gathiram and Jackson, 1987; Tachibana et al., 2000; Ravdin et al., 1990).

In most of the laboratories of the world especially in developing countries like India, intestinal amoebiasis is diagnosed by demonstration of either cysts or trophozoites or both in the stool by light microscopy (Archana, 2002). There is ambiguity regarding the earlier reports about the true prevalence of amoebiasis in such populations as interpretation is very difficult because older data did not differentiate between morphologically identical nonpathogenic species *E. dispar* and *E. moshkovskii* from that of pathogenic *E. histolytica*. In addition, these subjects might have been treated unnecessarily with anti-amoebic drugs, as the infection with *E. dispar* and *E. moshkovskii* doesn’t warrant anti-amoebic chemotherapy (Parija, 2006).

The definite detection of pathogenic *E. histolytica*, the causative agent of amoebiasis, is an important aspect of the clinical microbiology laboratory. A rapid and cost effective laboratory test for the differential detection of *E. histolytica* from *E. dispar* and *E. moshkovskii* infections is required for a clinical microbiology laboratory.

The development of valuable diagnostic tool for use in clinical laboratories and large-scale epidemiological studies has been made a priority (WHO, 1997) and is the subject of intense research (Abd-Alla et al., 2000; Barker, 1994; Garcia, 1990;
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Pillai et al., 1999; Ravdin, 1995). Of late, the diagnosis of intestinal amoebiasis has been done by applying microscopy, culture/zymode assimilation, antigen detection and molecular biology based techniques.

The sensitivity of light microscopy to the best is limited to only 60% (Krogstad et al., 1978; Haque et al., 1995). Isoenzyme analysis of cultured amoeba allows the differentiation of *E. histolytica* from *E. dispar* (Huston et al., 1999). The isoenzyme analysis takes long time usually between one to several weeks to report the results, and also requires special laboratory facilities, making it impractical for use in the routine diagnosis of intestinal amoebiasis. Moreover, the factors like delays in the stool specimen processing and initiation of anti-amoebic treatment prior to sample collection can lead to *E. histolytica* - negative culture results even in those patient stool samples showing cyst/trophozoite by light microscopy.

Presently, amoebic coproantigen detection and polymerase chain reaction (PCR) on stool samples are being reported as specific and sensitive methods to detect *E. histolytica* in stool specimens (Haque et al., 2000; Furrows et al., 2004; Gonin and Trudel, 2003; Sharma et al., 2003). The sensitivity and specificity of monoclonal antibody based enzyme linked immuno-sorbent assay (ELISA) for the detection of *E. histolytica* specific antigen in the stool specimen of intestinal amoebiasis patients has been reported to be 85% and 90% respectively (Haque et al., 1998a). The ELISA for antigen detection and nested PCR targeting 16S-like ribosomal RNA (rRNA) gene of *E. histolytica* had shown comparable sensitivities as 85% and 87% respectively, when performed directly on fresh stool specimens (Haque et
al., 1998a). The main limitation of ELISA test is that it can specifically detect *E. histolytica* and *E. dispar* but not *E. moshkovskii*.

Till now *E. moshkovskii* in humans has been reported from North America, Italy, South Africa, Bangladesh and India (Clark and Diamond, 1991a; Parija and Khairnar, 2005). We have reported *E. moshkovskii* from our laboratory at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry. This was the first such report of *E. moshkovskii* from India (Parija and Khairnar, 2005). The presence of morphologically similar forms like *E. dispar* and *E. moshkovskii* has made the differentiation of the three species by PCR necessary, especially in those countries and in India where all the three species, *E. histolytica*, *E. dispar* and recently *E. moshkovskii* have been reported to be prevalent. The differential diagnosis of pathogenic *E. histolytica* from non-pathogenic *E. dispar* and free living *E. moshkovskii* will enable the clinicians to avoid unnecessary treatment with anti-amoebic drugs as colonization with *E. dispar* and *E. moshkovskii* does not warrant anti-amoebic therapy. In addition, the differential diagnosis will also enable us to estimate the relative proportions of each of these species in patients attending JIPMER hospital, Puducherry, India.

Mortality from amoebiasis is mainly due to extra-intestinal pathology, in which amoebic liver abscess (ALA) is the most common. It is difficult to differentially diagnose ALA from pyogenic liver abscess (PLA) as the treatment for ALA is different from PLA. If left untreated, ALA can rupture into neighboring tissue and spread to the brain and other organs via hematological route. If untreated PLA is usually fatal (Smoger et al., 1998). It is also important to differentiate ALA from
other space occupying lesions of liver such as hydatid cyst and liver hepatoma. ALA is diagnosed clinically on the basis of tenderness and pain in the right upper quadrant, fever, weight loss, hepatomegaly and rarely jaundice. Unfortunately these features are often nonspecific (Kasper et al., 2005).

Imaging techniques like ultrasound, computed tomography, and magnetic resonance are highly sensitive in detecting liver abscess arising from any cause but cannot distinguish ALA from PLA. Although the initial site of infection is the colon, less than one third of patients with an amoebic abscess have active diarrhea (Kasper et al., 2005). Majority of the patients suffering from ALA do not have coexistent amoebic colitis. Therefore, stool microscopy or antigen detection in stool samples is not very helpful for diagnosis of ALA, in fact less than only 10% of patients have identifiable *E. histolytica* in stool specimens (Katzenstein et al., 1982).

Laboratory diagnosis of ALA is usually done by serological tests. The drawback with antibody detection is that antibody levels in individuals living in endemic areas can remain positive for years even after eradication of infection with *E. histolytica* (Gandhi et al., 1987; Jackson et al., 1984; Yang and Kennedy, 1979). In addition up to 10% of the patients with acute ALA may have negative serology findings (Kasper et al., 2005). The demonstration of *E. histolytica* trophozoite in liver abscess pus aspirates by microscopy confirms the diagnosis of ALA, but in best of the laboratories it has a sensitivity of only 15% (Parija, 1993). For this reason diagnosis of ALA by culture of parasite is also unsatisfactory (Zaman et al., 2000).
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A monoclonal antibody-based TechLab *E. histolytica* II ELISA has been reported to be 78% and 40.7% sensitive for the detection of *E. histolytica* antigen in the serum and liver pus of ALA patients respectively (Haque et al., 2000).

Study conducted in various laboratories including ours show that PCR has proven itself to be a sensitive, specific and rapid method for detecting *Entamoeba* DNA in stool samples and for differentiating the morphologically similar *E. histolytica* from *E. dispar* and *E. moshkovskii* (Clark and Diamond, 1997; Katzwinkel-Wladarsch et al., 1994; Aguirre et al., 1995; Novati et al., 1996; Walderich et al., 1997; Haque et al., 1998b; Nunez et al., 2001; Ali et al., 2003; Parija and Khairnar, 2005). However, there are only few reports in which PCR has been used for the detection of *Entamoeba* DNA in liver abscess pus specimens (Tachibana et al., 1992; Zengzhu et al., 1999; Zaman et al., 2000; Haque et al., 2000). PCR has been reported to have a varied sensitivity ranging from 33% to 100% for detecting *E. histolytica* DNA in liver abscess pus (Haque et al., 2000; Khan et al., 2006; Zengzhu et al., 1999; Zaman et al., 2000; Parija and Khairnar, 2007). In this study we have applied PCR for detection of *E. histolytica* DNA in liver pus abscess aspirates for diagnosis of ALA.

Blood or liver abscess pus specimen collection involves invasive procedures, henceforth in the recent past much impetus has been shown towards the use of urine and saliva as a non-invasive alternative for the diagnosis of various infectious diseases (Parija, 1998; Crotchetelt et al., 1997; Peyer et al., 2001; Fuentes et al., 1996; Aceti et al., 1999; Parkash et al., 2004; Crowcroft et al., 1998; Chaita et al., 1995; Abd-Alla et al., 2000; LaDuka et al., 1999; Lucht et al., 1998; Pozo and
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Tenorio, 1999; Crepin et al., 1998; Jiang et al., 1998). There is only one report on the detection of *E. histolytica* Gal/GalNAc lectin antigen in the saliva of ALA patients (Abd-Alla et al., 2000). To the best of our knowledge till now there is no report available on detection of *Entamoeba* DNA in the urine and saliva. Therefore, these observations prompted us to make an attempt to search for *Entamoeba* DNA getting released in urine and saliva specimen of ALA patients by applying PCR and to evaluate the possible utility of urine and saliva specimen as a clinical specimen for the diagnosis of ALA.

Since the first description of amoebiasis more than a century ago (Lösch, 1975), we still do not know aptly why the symptoms of amoebiasis appear in only minority of those infected with *E. histolytica*. There are few speculations in this regard such as the range of virulence levels among the *E. histolytica* strains and variability in the host immunity against amoebic invasion. While the variability of human immunity against amoebic infection is still not well understood, the existence of genetic variation in *E. histolytica* has recently been revealed (Bhattacharya et al., 1992; Clark and Diamond, 1993; Ghosh et al., 2000; Sehgal et al., 1993; Zaki and Clark, 2001; Li et al., 1992). However, whether the intra-species genetic variation also exists in an *E. histolytica*, *E. dispar* and *E. moshkovskii* population in a restricted geographic area like Puducherry remains unknown. The 16S rRNA gene is mostly preferred for establishing genetic variation because of their conservative nature and universal distribution. Henceforth, an attempt was made to investigate for the existence of genetic variation in *E. histolytica*, *E. dispar* and *E. moshkovskii* isolated from patients coming from in and around Puducherry, India.