5. Summary
The protozoan parasite *Entamoeba histolytica* infects an estimated 50 million individuals, causing and 40,000 to 100,000 deaths annually, placing it second only to malaria as a cause of death resulting from the parasitic protozoa. Amoebiasis is thus a major public health problem worldwide, particularly in developing countries such as India.

*E. histolytica* and *E. dispar* are two morphologically similar and genetically different species. Among these only *E. histolytica* is considered pathogenic and the disease it causes is called amebiasis or amebic dysentery. Since *E. dispar* is morphologically identical to *E. histolytica*, the two were previously considered to be the same species. However, genetic and biochemical data indicate that the non-pathogenic *E. dispar* and pathogenic *E. histolytica* are distinct species. Separation of *E. histolytica* and *E. dispar* as two different species has great impact on epidemiology and clinical management of the disease. Therefore, the epidemiological studies conducted so far considering *E. histolytica* and *E. dispar* as same species need to be reviewed. Individuals infected with *E. dispar* may not need any treatment.

In addition, we still do not understand why only small proportion of individuals infected with *E. histolytica* develop symptoms of disease and the others remain asymptomatic. In some cases *E. histolytica* become invasive and cross intestinal epithelium causing extraintestinal diseases such as liver abscess and brain abscess. It has been speculated that the varied organ tropism and clinical presentation of infection might be related to genetic diversity of *E. histolytica*. It has been established that some lab-maintained strains of *E. histolytica* differ in virulence. However, we need to develop accurate methods to differentiate strains of *E. histolytica* present in nature and correlate with their pathogenicity.

The work carried out in this thesis mainly deals with development of methods for *E. histolytica/E. dispar* detection and strain differentiation without cultivation. Various molecular markers were developed and validated with different strains of *E. histolytica* grown in axenic cultures. These markers were then used to screen samples from natural isolates without cultivation. The results obtained are summarized as follows:
Detection and differentiation of *E. histolytica*/*E. dispar* was performed by dot blot and PCR.

a. Detection of *E. histolytica*/*E. dispar* with DNA isolated from stool samples was performed by dot blot hybridization. HMe region of rDNA circle of EhR1 was taken as probe. Samples from symptomatic as well as asymptomatic individuals were tested. Our results indicate 16% of samples (total samples studied were 950 individuals) were positive for *E. histolytica* and *E. dispar*.

b. Species identification was performed by PCR amplification of EhSINE2 element in case of *E. histolytica*, and primers designed from ITS2 and 18S region of *E. dispar* rDNA circle. 59% of total *Entamoeba* positive samples in symptomatic people and 51% of total positive samples in asymptomatic people studied were *E. histolytica* positive and rest were *E. dispar* positive.

High prevalence of *E. histolytica* in symptomatic population was expected as *E. histolytica* is the pathogenic species.

In addition to species detection, strain identification of pathogenic species is necessary to understand the pathogenicity and virulence of the parasite. In order to study the genetic variation among the strains present in our population, following molecular markers were developed through this study.

a. HMg region of rDNA circle EhR1 was found to be absent in some *E. histolytica* strains. In these strains instead of two rRNA transcription units the rDNA circle has only one transcriptional unit. To understand the scenario in our population dot blot was performed using HMg as probe, with the DNA samples isolated from the stool samples. Our results indicate presence of only 3% HMg positivity among the samples (950) studied. This data indicates prevalence of single transcription units in most of *E. histolytica* strains. In addition to this, pus samples from amoebic liver abscess patients were also tested. Only 1 out of 30 samples tested came out to be HMg positive. This
result shows that both HMg positive and negative strains can cause liver abscess, however HMg negative strains are more prevalent.

b. Microsatellite tandem repeat primer sets R4/R9, R4/R10 and R5A/R6A developed by Zaki and Clark, 2001 were also tested for strain identification using axenic strains of *E. histolytica* as well as *E. histolytica* positive field samples. However, these primer sets could not differentiate between closely related strains HM-1:IMSS and HK-9. Polymorphism observed with *E. histolytica* positive samples indicated the presence of various strains in natural isolates. *E. histolytica* from liver abscess also shows polymorphism indicating more than one strain responsible for liver abscess. Primer set R4/R9 amplified a band size near 1.5 kb in all liver abscess samples which was not found in stool samples of symptomatic and asymptomatic people studied. This observation suggests presence of different strains in amoebic liver abscess which were not observed in intestinal isolates.

Primers based on tandem repeats can not differentiate the strains of *E. histolytica* that are closely related e.g. HM-1:IMSS and HK-9. Also, number of repeats are sometimes very small and therefore it is difficult to visualize variation in band pattern. Therefore, there is still a need for accurate and more reliable method for strain identification from natural isolates and clinical samples without cultivation.

In order to develop new methods for strain variation in *E. histolytica* we have exploited the presence of Non LTR retrotransposons (EhLINEs and EhSINEs) which are highly abundant in *Entamoeba* genome. We have developed methods called REP PCR and transposon display based on interspersed EhLINEs and EhSINEs in *Entamoeba* genome.

a. REP PCR is a widely studied genotyping method for strain identification in bacteria. REP PCR primers are designed from interspersed repetitive elements in opposite directions. The process allows amplification of many different sized fragments representing the DNA between the repetitive sequences in the genome. A unique REP-
PCR fingerprint profile is created containing multiple bands of varying sizes and intensities. The amplicons can be analyzed to generate a REP-PCR DNA “fingerprint” pattern which is specific to individual microbial clones or strains. These fingerprints can then be used to demonstrate the relationships between strains. We have desigened primers from EhLINEs and EhSINEs for REPPCR. Various combinations of the primers have been tested from which 8 combinations could differentiate *E. histolytica* strains tested (HM-1:IMSS, HK-9, NIH:200 and Rahman). This is the first report of REP PCR in *E. histolytica* for strain variation.

b. Transposon Display (TD) is also a PCR based method for strain variation. This procedure involves ligation mediated amplification of genomic fragment anchored to a transposon. Apart from genomic fingerprinting this technique is also useful in the study of transposon behaviour and transposition mechanism. TD is well studied in plants. In the present study we have exploited the unique properties of a Non autonomous, non-LTR retrotransposon EhSINE1 to develop a new class of molecular markers. TD technique is more accurate than AFLP since primers are designed from specific transposon. We have chosen EhSINE1 since it is absent in *E. dispar* and will only amplify fragments from *E. histolytica*. Due to its specificity, this technique can also be used in strain differentiation from mixed cultures or directly from clinical samples. We have tested TD with various primers designed from EhSINE1. One of the TD primers could successfully differentiate between all the strains tested including HM-1:IMSS and HK-9. The result suggests that TD is more specific and reliable technique than other molecular markers. We have developed TD method in a protozoan parasite which could be useful for strain variation and transposon based studies.
Future perspective would involve studies along the following lines-

- Elaborate epidemiological studies of *E. histolytica/E. dispar* in population based on molecular probes and markers designed.

- TD studies of *E. histolytica* strains directly from clinical isolates.

- Correlation with virulence patterns of strains based on transposon insertion in genome.

- Variation in transposon insertion in strains causing extraintestinal disease.
  
  Mechanism of transposition based on TD.

In summary we have developed advanced molecular tools which have potential to be used as markers for investigating the epidemiology of *E. histolytica* and the existence of genetically distinct invasive and noninvasive strains.