Conclusion
G. lamblia, a flagellated protozoan parasite of humans and other mammals is thought to be one of the most primitive extant eukaryotic organisms. It is notable for its lack of mitochondria, nucleoli and peroxisomes. The parasite inhabits the small intestine of humans and causes the enteric disease giardiasis (Nash, 1989; Wolfe, 1990; Adam, 1991). Millions of people worldwide are affected annually by the parasite, which recognizes no socioeconomic, political or geographical boundaries. The management of the disease is made difficult by the availability of only insensitive and nonspecific diagnostic tools. Also the currently available drugs for the disease are nonspecific and have numerous side effects. The present study addressed these problems and was aimed at developing a better diagnostic tool for giardiasis targeting the atypical rRNA gene of the parasite. We have also attempted to unravel the molecular mechanism of maturation of the rRNA from the pre-rRNA with a long-term goal of developing a specific drug targeting the maturation process.

A successful diagnostic tool should be highly sensitive, specific, reproducible, cost effective and also should not be time consuming. Conventionally Giardia was diagnosed by microscopic examination of stool samples for the presence of cysts or trophozoites (Goka et al., 1990) but only 50% to 70% sensitivity has been reported by this technique (Bruke, 1997). The most common reason for low sensitivity being the examination of stool sample by unskilled personnel. Another important reason was, Giardia cysts are sheded sporadically and despite symptoms some patients may not excrete cysts for several weeks (Hiatt et al., 1995). Though immunological method of detection of the parasite proved to be a better alternative yet there is always chance of getting false results due to cross reactivity with other microorganisms (Shetty et al., 1990). In this respect DNA based diagnosis is the best choice because it guarantees high specificity and at the same time sensitivity as it relies on the sequence of the genome. With the advent of PCR the field of molecular diagnosis underwent a major forward leap with lots of reports on PCR based detection of parasites appearing in major scientific journals. PCR suffice all the requirements of an ideal diagnostic tool. It is also very sensitive as it amplifies the target several thousand times. Other advantages like reproducibility, less time requirement and cost effectiveness has placed this diagnostic tool miles ahead of the other techniques.

While developing a PCR based diagnosis the most important requirement is meticulous choosing of the target for amplification. The target may be a gene, specific for the organism or a specific region within a conserved gene. As the initial number of the organism in the stool is very low, it will be helpful if the target is present in high copy number. The ribosomal RNA gene is a very good target for amplification by PCR (Waters and McCutchan, 1990). About 63-132 copies
of it is present in *Giardia lamblia* (Boothroyd et al., 1987; Sil et al., 1998). Also there are regions within the gene, which is species specific. Ribosomal RNA based diagnosis for giardiasis had been reported earlier where a 183bp region of the SSU rRNA of *Giardia* had been chosen as the amplification target (Weiss, 1993). But the inefficiency of choosing this target lies within the fact that the WEI primers that were used amplified DNAs of *Giardia muris* as also other parasites like *Entamoeba histolytica, Leishmania major* and *Trypanosoma brucei* (Rochelle et al., 1997).

Probably the region of the SSrRNA that was chosen as the target was not *Giardia lamblia* specific. We have chosen a 552bp-region target within the intergenic spacer region (IGS) of the rRNA gene of *Giardia lamblia*, which was found to be *Giardia* specific. The primers could not differentiate between two species of *Giardia* but was found highly specific in not amplifying the genomic DNAs of other enteric pathogens like *Entamoeba histolytica, Shigella dysenteriae* and *Escherechla coli*. The primers could detect even less than 10 *Giardia* trophozoites. In stool PCR these primers, AS1 and AS2 was found to be less sensitive due to the interfering substances present in stool. The simplest way of removing the inhibition was to dilute the fecal samples whereby the inhibitory substances gets diluted out. This had been successfully applied in case of cholera infection. But in our case the problem lay in the fact that the number of *Giardia* trophozoite that appeared in stool was too little and there was every possibility of diluting out the DNA. The sensitivity was restored by including a nested PCR step using another set of primers (SG3 and SG4) that amplified a 320bp region internal to the 552bp first PCR product. Inclusion of nested PCR increased the sensitivity of detection to as little as 10 parasites in 100μl stool. An added advantage of the nested PCR step was that it acted as a double check whereby if there was any false amplification in the first PCR the nested primers will not amplify to give the 320bp product.

The efficacy of the system was then evaluated with conventional microscopy, CIEP and ELISA in diagnosis of giardiasis from large number of stool samples collected from diarrhoea cases and control subjects. Comparative study of 145 clinical samples examined by microscopy, CIEP, ELISA and nested PCR showed the better efficiency of nested PCR detection system over other diagnostic method. A 100% correlation among nested PCR, microscopy and ELISA were found in both control subjects and suggestive giardiasis cases. In randomly chosen clinical samples (56 in number) nested PCR showed better diagnostic results where out of the 14 cases that were found to be positive by PCR, ELISA and CIEP, 9 were negative by only CIEP and 5 were negative by both CIEP and ELISA. None of these could be detected by microscopy. Further all the clinical samples that showed positive results by microscopy, CIEP and ELISA were also found to be positive by PCR. No PCR-negative cases were found to be positive by the other
detection system. Thus this new assay procedure appears to be a simple, rapid, accurate and sensitive method for the diagnosis of giardiasis. The PCR positive and ELISA negative results shows the greater sensitivity and specificity of PCR over ELISA.

Ribosomal RNA represents nearly 80% of the total RNA. It is a universal component of the ribosome. In all the three kingdoms namely eubacteria, archebacteria and eukaryotes the rRNAs namely LS rRNA, SSrRNA and 5.8SrRNA (5S in case of prokaryotes) are synthesized as a single long pre ribosomal RNA molecule which is acted upon by a series of endonucleolytic cleavages to generate the mature rRNA molecules. But the place and process of maturation of the rRNA molecules is different for each of the representative kingdoms. In eubacteria the pre-rRNA, synthesized by RNA polymerase, contains large inverted repeat sequences surrounding the 16S and 23S rRNA which forms helical structures causing the rRNA molecule to be looped out. It also contains the recognition feature for RNase III, which acts on it to generate the mature rRNAs. In archeabacteria though the rRNA operon is similar to eubacteria the enzymology of precursor processing is different. The recognition site for the excision endonuclease contains a well-defined structural motif and the processing activity requires RNA components similar to that of eukaryotes. In the more complex eukaryotes the pre-rRNA synthesis and processing occur in the nucleolus. The pre-rRNA is synthesized by RNA Polymerase I and the maturation of the rRNAs occur in a stepwise fashion involving different proteins like Fibrillarin and small nucleolar RNAs like U3, U8 etc. Thus in the three different kingdoms different strategies have been utilized to generate the mature rRNAs.

The evolutionary position and the atypical nature of giardial rRNA has encouraged us to study the biogenesis of the rRNA in molecular details with a hope to identify some novel pathway against which chemotherapeutic intervention could be designed.

A run on transcription assay has been developed using Giardia nuclei to follow the ribosomal RNA transcription in Giardia lamblia. The transcription machinery involved in synthesis of rRNA was found to be insensitive to α-amanitin at a concentration as high as 200μg/ml. This is similar to eukaryotes where the RNA polymerase I that synthesizes rRNA was also found to be insensitive to the drug.

Northern hybridization of nascent transcript generated by run on transcription using rDNA probe and primer extension analysis using giardial total RNA and end labeled oligonucleotide enabled us to identify the approximate initiation region of rRNA transcription. The transcription start site was found to be ~870bp upstream of the 5' end of mature SS rRNA. Primer extension allowed us to identify a processing site at ~420bp upstream of the SSrRNA.
This was further confirmed by primer extension with another primer upstream to the previous one that also gave ~50b product corresponding to the processing site at ~420bp.

Subcloning of the intergenic regions in vector pGEM4Z and successful in vitro RNA synthesis by run off transcription allowed us to follow the processing of rRNA further by developing an in vitro assay system using giardial nuclear extract. This allowed us to identify another processing site ~250bp upstream of the SSrRNA. The presence of this processing site was further confirmed by RNase protection assay. Thus at least three processing sites were found to be present in the 5' ETS-SSrRNA junction that is summarised in figure below.

Fig. 8.1. Schematic representation of the processing in 5'ETS of giardial pre-rRNA as inferred from the above studies. Transcription starts at ~870b upstream of 5' end of SS rRNA and is stepwise processed at ~420b, ~250b upstream and +1 of SS rRNA to generate the mature SS rRNA. Figure not to scale.
Conclusion

After identifying the processing sites we next wanted to study the factors involved in rRNA processing. Lack of any defined gene transfer system in *Giardia* had forced us to study with cloned genes. As mentioned above, rRNA processing in eukaryotes involves a protein Fibrillarin (Tollervey et al., 1993) and different snoRNAs (Balakin et al., 1996). The presence of Fibrillarin and snRNAs has been reported in *Giardia* (Narcisi et al., 1998; Niu et al., 1994). Three different giardial snRNAs namely RNA J, H and D have been cloned by RTPCR of total RNA isolated from *Giardia* cells using terminal primers designed from published sequences. The snRNAs could be synthesized *in vitro* and northern hybridization using antisense snRNAs confirmed their presence in nuclear extract of *Giardia*. Fibrillarin protein had also been cloned in pGEM4Z vector. Then the gene was subcloned in expression vector pET33b (+) after His.Tag sequence. The bacterial expression of the recombinant protein was standardized and the protein was purified using Ni-resin column chromatography. Polyclonal antibody was raised against the protein and western blot of nuclear extract with this antibody detected a band around 35Kd. Electrophoretic mobility shift assay with all the three snRNAs with recombinant fibrillarin protein demonstrated the positive RNA-protein interaction. The three snRNAs also interacted with a protein in nuclear extract in north-western analysis. Comparing the band position in western blot and north-western blot the protein in nuclear extract that bound to the different snRNAs was found to be Fibrillarin. The difference in migration between the bacterially expressed Fibrillarin protein and giardial Fibrillarin may be due to post-translational modification that has been reported to be present in *Giardia*. Recently direct and specific interaction of Fibrillarin was demonstrated with U16 box C/D snoRNA in Xenopus laevis oocyte nuclear extract as well as with U16 snoRNA alone (Fatica et al., 2000).

The different snRNAs and Fibrillarin were used in the *in vitro* processing reaction. We found that the three snRNAs alone can process the 0.87Kb primary RNA transcript corresponding to IGS- SsrRNA junction. The absence of any protein in the assay suggests the ribozyme activity of the snRNAs. Further studies with individual snRNAs may throw some light to characterize the ribozyme activity of the snRNAs. This will also help to elucidate the molecular function of the snRNAs in rRNA processing.