Multidrug resistance in amoebiasis patients

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Background & objectives: Amoebiasis, caused by Entamoeba sp. a protozoan parasite, is a major public health problem in tropical and subtropical countries. The symptomatic patients are treated by specific chemotherapy. However, there are reports of treatment failure in some cases suggesting the possibility of drug resistance. The present study was therefore planned to assess the presence and expression of mRNA of multidrug resistance (MDR) gene in clinical isolates of Entamoeba histolytica and E. dispar.

Methods: Forty five clinical isolates of Entamoeba sp. [E. histolytica (15) and E. dispar (30)] were maintained in polyxenic followed by monoxenic medium. DNA and total RNA were extracted from clinical isolates of Entamoeba sp. and from sensitive strain of E. histolytica (HM1: IMSS) and subjected to polymerase chain reaction (PCR) and multiplex reverse transcription (RT)-PCR techniques.

Results: The 344 bp segment of E. histolytica DNA was seen by PCR using primers specific to EhPgp1 in all clinical isolates and sensitive strain of E. histolytica. Over expression of EhPgp1 was observed only in resistant mutant of E. histolytica; however, transcription of EhPgp1 was not seen in any clinical isolates and sensitive strain of E. histolytica.

Interpretation & conclusion: The findings of the present study indicate that, so far, drug resistance in clinical isolates of E. histolytica does not seem to be a major problem in this country. However, susceptibility of clinical isolates of E. histolytica against various antiamoebic drugs needs to be investigated for better management.

Key words E. dispar - EhPgp1 - Entamoeba histolytica - multidrug resistance - multiplex RT-PCR

Entamoeba histolytica, an enteric protozoan parasite causing amoebiasis, is one of the major causes of morbidity and mortality and an important public health problem in developing countries. It affects 500 million people annually with 50 million clinical cases of dysentery or amoebic liver abscess (ALA), and 70,000 to 100,000 deaths every year¹. The disease in symptomatic patients is primarily treated by antiamoebic drugs². The occurrence of Entamoeba into two defined species i.e., pathogenic, E. histolytica and
non-pathogenic, *E. dispar* and the limited number of drugs available to treat amoebiasis require a new approach to treat infected individuals. In asymptomatic individuals, inappropriate usage of drugs or overdosing could lead to drug resistance. Till date, drug resistance in *E. histolytica* is uncommon. However, differences in drug susceptibility between different isolates have been reported. Reports on treatment failure also indicate that drug resistance may become clinically important in the near future. Moreover, drug resistant clones have been generated in the laboratory, suggesting that *E. histolytica* may naturally develop drug resistance.

The various mechanisms of antimicrobial resistance in bacteria are well known, one of which may be the efflux of antimicrobials. Little is known about drug efflux from eukaryotic cells which has been best studied in mammalian tumour cells. The product of multi drug resistance (MDR) loci such as the P-glycoprotein (Pgp) is involved in drug efflux from the cells. Tumour cells become resistant to chemotherapeutic agents via the mechanism of efflux where the drug is pumped out from the cell. This efflux is energy dependent and is inhibited by calcium ion channel blockers.

So far, six Pgp like genes (*EhPgp1-EhPgp6*) have been cloned and sequenced, but copy number has not been described in the laboratory mutants. Of these six genes, four were clearly expressed in drug-resistant line (*EhPgp1, EhPgp2, EhPgp5, EhPgp6*), while two were pseudogenes (*EhPgp3, EhPgp4*). The *Ehpgp1* and *EhPgp5* genes are overexpressed in the emetine resistant clone and their expression is regulated at transcriptional level. The *EhPgp5* gene is transcribed only in the presence of a high drug concentration and after the *EhPgp1* gene is overexpressed. Although the mechanisms of MDR in *Entamoeba* mutants have not been studied in detail, the MDR gene encoding for the P-glycoprotein (170 kDa protein) was isolated from drug resistant cancer cells. The cDNA sequence of the MDR gene shows that it is a protein with two homologous halves, each containing a hydrophobic and hydrophilic segment.

The overexpression of surface P-glycoprotein (Pgp) also produces the MDR phenotype in protozoan parasites, including *Plasmodium*, *Trichomonas*, *Giardia*, *Leishmania*, and *Entamoeba*.

At present, the models available to study the mechanism of drug resistance in *E. histolytica* are the emetine resistant mutant clones derived from virulent strain HM1: IMSS, selected after mutagenesis with the alkylating agent, ethyl methanesulphonate (EMS) and laboratory induced metronidazole resistant cell lines of *E. histolytica*, maintained indefinitely in medium with drug. Studies on the mechanisms of multi drug resistance in clinical isolates of *E. histolytica* and *E. dispar* are lacking. Therefore, we investigated the presence and expression of MDR gene in *EhPgp1* clinical isolates of *E. histolytica* and *E. dispar*, and in sensitive and resistant mutants of *E. histolytica* by molecular techniques.

**Material & Methods**

**Source of Entamoeba clinical isolates:** A total of 4700 stool samples were collected from randomly selected patients and are subjects without history of amoebiasis attending the Out Patient Department of Nehru Hospital attached to the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, during January 2002 to October 2004 and from subjects who were residing at low socio-economic area of Sector-25, Chandigarh and all the samples were examined for the presence of *Entamoeba* sp. by routine microscopy as reported earlier. Forty five stool samples found positive for cysts/trophozoites of *Entamoeba* sp. on microscopic examination were cultured in polyxenic modified Boeck and Drbohlav (NIH) medium, followed by culture in Robinsons medium. These isolates were identified, either as *E. histolytica* (15) or *E. dispar* (30) by hexokinase isoenzyme analysis and by Techlab ELISA.

**Standard strain of *E. histolytica***: Standard strain of *E. histolytica* (HM1: IMSS), being maintained in the Department of Parasitology, PGIMER, Chandigarh, in
TYI-S-33 medium was used in this study. In our earlier study, this strain was found sensitive to metronidazole, chloroquine, emetine and tinidazole and was labeled as sensitive strain for the present study.

**Preparation of nucleic acid:**

DNA isolation–DNA was isolated from all clinical isolates of *E. histolytica/E. dispar* and sensitive strain of *E. histolytica* using the standard phenol: chloroform: isoamyl alcohol method described by Sambrook et al.

RNA isolation–Total RNA was extracted from sensitive strain and all clinical isolates of *E. histolytica/E. dispar* using commercial RNAwiz solution (Ambion, USA). RNA of emetine resistant mutant of *E. histolytica* was obtained as gift (Dr Perez Gullimero Ishiwara, Mexico).

Polymerase chain reaction (PCR)–The PCR assay was performed in a DNA thermal cycler (Model Eppendorf mastercycler gradient, Germany) using sense primer specific to *EhPgp1* 5’-GAATAGGTCAGGTGAGC-3’ and an anti-sense primer 5’-TCATGTGGCATTTCCATTTTA-3’ to the amino acid Ala-Lys-Met-Ala-Asn-Ala, which is conserved in ATP binding sites of identified *Entamoeba* P-glycoproteins. The PCR reaction was performed in 50 µl reaction mixture containing 10 x PCR buffer, 1.25U Taq polymerase (MBI, Fermentas, USA), 0.5 µM of each primer, 200 µM dNTPs (MBI, Fermentas, USA) and 2 µl DNA. The reactions were run in thermalcycler (Eppendorf master cycler gradient, Germany). The optimum PCR conditions were initial denaturation of 5 min a 94°C for one cycle and 28 cycles of each denaturation at 94°C for 1 min, annealing for 2 min at 55°C and elongation of one min at 72°C, final elongation of 3 min at 72°C for one cycle. The PCR product was analysed in 2 per cent agarose gel (Sigma-Aldrich, USA), after electrophoresis and staining with ethidium bromide, gels were photographed on UVI-gel documentation system, UK.

Multiplex reverse transcription-polymerase chain reaction (RT-PCR)–To check the expression of P-glycoprotein mRNA in drug sensitive and resistant mutants of *E. histolytica* and in clinical isolates, semiquantitative multiplex RT-PCR was carried out. Total RNA was extracted and reverse transcription into cDNA was performed with the Revertaid™ first strand cDNA synthesis kit (MBI, Fermentas, USA), according to manufacturer’s instructions. The PCR was run by using the sense and antisense primers Eh1 and Eh2 mentioned earlier, 18s rRNA primer (internal control) and 18s competimers. Briefly, one-tenth of the cDNA mixture of each sample was amplified in single tube including 40 µm of both forward and reverse primers of Eh1/Eh2 and 5 µm of 18s rRNA and 18s competimers, 2U XT-Taq polymerase (Bangalore Genei, India) and 10mM dNTPs (MBI, Fermentas, USA) in a total volume of 50 µl reaction mixture by 30 cycles with the condition of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 50 seconds PCR products were analyzed by electrophoresis in 2 per cent agarose gel (Sigma-Aldrich, USA) made in 0.5 x TBE-buffer and visualized in UV-transilluminator by ethidium bromide staining. To quantify the intensity of ethidium bromide signals, the gels were photographed and images were analysed by densitometry using NIH IMAGE analysis software. Results were expressed as the ratio of MDR area/18s rRNA area and designated as MDR expression rate.

**Results**

To identify a portion of the MDR gene (*EhPgp1*) in *E. histolytica* which is similar to the mammalian MDR gene, PCR was carried out. It was observed that sensitive strain and all clinical isolates of *E. histolytica/E. dispar* showed amplified product of 344bp, identified on an ethidium bromide stained agarose gel (Fig. 1). There was no amplification in control reaction in which target DNA was omitted.
Multiplex RT-PCR was done to see the mRNA expression of \textit{EhPgp1} gene in all clinical isolates of \textit{E. histolytica/E. dispar} and both drug sensitive and resistant mutants of \textit{E. histolytica}. The results of densitometry showed 2-2.5 fold increase of \textit{MDR} gene transcription in resistant mutant as compared to the internal control 18s rRNA gene, whereas no expression of mRNA was seen in any of the clinical isolates and also in the sensitive strain of \textit{E. histolytica} (HM1:IMSS) (Fig. 2).

**Discussion**

In the present study, the expression of \textit{MDR} gene in clinical isolates of \textit{E. histolytica} and \textit{E. dispar} was investigated. Resistance is based on the overexpression of P-glycoprotein (\textit{MDR} gene product) which results in drug efflux from resistant tumour cells. We identified 344 bp segment by PCR using primers to conserved region of the P-glycoprotein. Similar findings have also been reported earlier using human \textit{MDR} gene as primers (Eh1 & Eh2)\textsuperscript{35}. In the present study, expression of mRNA transcription was determined by multiplex semiquantitative RT-PCR. Semiquantitative RT-PCR allows the relative quantification of mRNA levels. We used the 18s rRNA gene as an internal control, which showed less variance in expression across a variety of treatment conditions than \textit{\beta}-actin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) to avoid the biasness of variations from sample to sample. However, 18s rRNA being abundant, amplifies rapidly during RT-PCR, quickly exhausting the reaction reagents making the detection of expression very difficult. To overcome this problem, we also used the 18s comeptimers (modified at their 3' ends to block extension by DNA polymerase) which reduced the overall PCR amplification efficiency of 18s cDNA without the primers becoming limited and produced the expression without any loss of relative quantitation. The result showed overexpression in resistant mutant but not in the sensitive and clinical isolates of \textit{Entamoeba}. Study reported from Mexico also indicated that the expression of mRNA was increased three to four times when resistant clones were grown under emetine pressure compared to the same parasite grown without emetine\textsuperscript{15}.

Till date, drug resistance in amoebiasis has not been described as a major problem. But, with increased drug usage, it may well emerge as an important health problem and if mechanism of multi drug resistance exists in \textit{E. histolytica}, resistance to multiple drugs in clinical isolates may arise simultaneously similar to that observed in...
mammalian tumour cells. To the best of our knowledge this is probably the first report on the MDR gene in clinical isolates of *E. histolytica* and *E. dispar*.

Singh *et al.* have reported that the field isolates of *Leishmania donovani* have novel mechanism of drug resistance which was different from the mechanisms of resistance postulated for laboratory mutant of *Leishmania* species. Similarly, it may be possible that drug resistance mechanisms in clinical isolates of *E. histolytica* and *E. dispar* might be different from those for laboratory mutants.

In conclusion, the present study indicates thereby that more than one mechanism might be operating in the development of resistance in clinical isolates of *E. histolytica*. Most of the studies carried out so far have only tried to analyze the mechanism of resistance in emetine resistant clones. Further studies to evaluate MDR phenotype in clinical isolates of *E. histolytica* are desired. Further, data on resistance to other antiamoebic agents including metronidazole are also lacking and therefore the antiamoebic susceptibility of pathogenic clinical isolates needs to be investigated to help in developing strategies to increase the efficacy and the life span of few currently available antiamoebic drugs.

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