COMPARATIVE BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON HUMAN ISOLATES OF *GIARDIA LAMBLIA*
5. COMPARATIVE BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON HUMAN ISOLATES OF GIARDIA LAMBLIA

5.1 Materials & Methods

5.1.1 Source of parasite and cultivation

Stool samples from patients admitted to the Infectious Diseases Hospital, Calcutta, India, with various gastrointestinal disorders and diarrhoea were routinely examined for the presence of intestinal parasites. Ten such patients, aged between 10 and 18 years with complaints of abdominal pain, flatulence, diarrhoea, fever and with positive stool samples (on three separate occasions) for Giardia cysts and trophozoites were selected for duodenal intubation.

1) Axenization of Giardia isolates

The isolation of G. lamblia trophozoites from the duodenal fluid and their subsequent cultivation was done according to the methods of Meyer (1976) and Gordts et al (1984). Briefly, 0.1 ml of the aspirated duodenal fluid containing $1 \times 10^5$ active Giardia trophozoites were introduced immediately into 15 mm x 125 mm culture tube (Borosil) containing prewarmed (32°C) filter sterilized TPS-1 medium (Diamond, 1968). The medium was supplemented with 20% fetal calf serum (instead of horse serum originally used by Diamond). Subsequently, 10% NCTC 135 solution, supplemented with L-glutamine (GIBCO Laboratories Cat. No. 320-1350) and a combination of antibiotics (penicillin, streptomycin, gentamycin and cyclofloxacin) at final concentration of 100 IU/ml, 100 mg/ml, 50 IU/ml and 50 mg/ml, respectively was added to the medium. The tubes were kept at 45°C angle at 37°C and the live trophozoites were examined under inverted microscope (Leitz, Labovert, West Germany) after 4-6 hours of incubation at a magnification of X100. The cultures were incubated for 10
days and screened every day, with fresh addition of medium twice in between. As soon as the attached trophozoites on the glass surface increased in numbers, the broth was aspirated out from the tube and refilled with the fresh medium containing 5-fluorocytocine (an antifungal) at a concentration of 0.02 mg/ml. This process was done quite a few times to eliminate the intestinal fungal contamination. Once the cultures were established the concentration of the antibiotics were gradually reduced and finally omitted. These axenized strains were finally adapted to TY1-S-33 medium (Diamond et al., 1978) containing 10% heat inactivated bovine serum and vitamin mixture. These isolates and the P-1 reference strain were routinely subcultured twice-a-week (vide Chapter 3, section 3.1.1).

5.1.2 Biochemical and Immunological analysis of strains

1) Isoenzyme studies

The isoenzyme analysis of G. lamblia isolates were carried out according to the methods of Siciliano and Shaw (1976), Harries and Hopkins (1978) and Bertram et al. (1983).

a) Preparation of lysates

Active trophozoite of Giardia in the late log phase were harvested after 72 hrs of incubation at 37°C. Culture tubes were chilled for 30 min at 4°C to release the viable trophozoites adhering to the glass surface and then were centrifuged at 600 g for 10 mins. The cell pellet was washed twice with cold normal saline and once with normal saline supplemented with 250 mM sucrose. Lysates of the cells were made by freezing at -70°C and then thawing at 4°C several times till no intact trophozoites were seen. The cell free extracts were prepared by centrifugation of the lysed cells at 20,000 x g for 20 min. The protein concentration of the lysates was estimated by the method of Bradford (1976) and stored at
### TABLE

**Enzymes assayed and buffer systems used in electrophoretic analysis of G.lamblia**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Abbreviation</th>
<th>E.C. number</th>
<th>Buffer system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>ALP</td>
<td>E.C.3.1.3.1</td>
<td>Tris-Citrate</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>ACP</td>
<td>E.C.3.1.32</td>
<td>Tris-Citrate</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>G$_6$PDH</td>
<td>E.C.1.1.1.49</td>
<td>Tris-Versene Borate</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>ICDH</td>
<td>E.C.1.1.1.42</td>
<td>Tris-Versene Borate</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>ME</td>
<td>E.C.1.1.1.40</td>
<td>Tris-Versene Borate</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>PGM</td>
<td>E.C.2.7.5.1</td>
<td>Tris-Versene Borate</td>
</tr>
</tbody>
</table>
-70°C with equal volume of stabilizing reagent (250 mM sucrose, 1 mM DTT, 1 mM amino caproic acid and bromophenol blue) until used.

b) Electrophoresis

The electrophoretic isoenzyme analysis was carried out using 5% polyacrylamide gel electrophoresis (PAGE). Gels were run in a horizontal LKB Multiphor II electrophoresis apparatus (Pharmacia LKB United, Upsala, Sweden) for 5 hrs at 300V in cold condition. The electrode buffers were 0.13 M Tris-0.043 M citrate, pH 7.0 and 0.5 M Tris-0.016 M Versene - 0.65 M Borate, pH 8.0. Gels were prepared either in 0.009 M Tris - 0.003 M Citrate, pH 7.0 or 0.05 M Tris pH 6.0, 0.016 M Versene - 0.065 M Borate, pH 8.0 which ever was applicable. The lysates (100 μg - 200 μg of protein) of bovine serum and complete medium (Diamond et al, 1978) were applied to the respective wells to serve and controls.

c) Detection of enzyme activity in gels

After electrophoresis, the gels were sliced and subjected to different staining solutions for visualization of different enzymes viz., Alkaline phosphatase, Acid phosphatase, Glucose-7-phosphate dehydrogenase, Isocitrate dehydrogenase, Malic enzyme and Phosphoglucomutase (Harris & Hopkinson, 1978; Sicilians & Shaw, 1976).

- **Alkaline phosphatase (ALP-E.C. 3.1.3.1)**

  The electrophoresed gel was incubated at 37°C with a staining solution which contained α-Naphthyl phosphate Na salt : 25 mg, MgSO₄, 7H₂O : 60 mg, Fast Blue B salt : 25 mg, in 50 ml of 0.06 M Borate buffer pH 9.7. Red bands appeared as zones of enzyme activity on the gel. Reaction was stopped by washing the stain gel with a mixture of methanol, water and acetic acid (5:5:1) and preserved in glycerin water solution.
• **Acid phosphatase (ACP-E.C. 3.1.3.2.)**

The staining solution containing sodium \(\alpha\)-naphthyl acid phosphate 50 mg, Black K salt mg in water 50 ml was poured over the gel and incubated at 37°C for one hour. Red bands appeared as zones of enzyme activity. The excess stain was poured off, rinsed with water and fixed in glycerin water.

• **Glucose-6-phosphate dehydrogenase (G\(_6\)PDH - E.C. 1.1.1.49)**

The gel was incubated in the dark at 37°C with a solution consisting of 200 mg of Glucose-6-phosphate (disodium salt), 15 mg NADP, 15 mg NBT, 1 mg PMS, 50 mg MgCl\(_2\) in 10 ml 0.2 M Tris-HCl pH 8.0 and 40 ml of water. Zones of enzyme activity appeared as dark blue bands. The gel was rinsed with water and fixed in 10 ml of alcohol gel wash solution.

• **Isocitrate dehydrogenase (ICDH-E.C.1.1.1.42)**

The enzyme activity in the gel was detected by incubating the electrophoresed gel in a solution containing 8 ml of 0.1 M sodium isocitrate pH 7.0, NADP - 15 mg, NBT - 15 mg, PMS - 1 mg MgCl\(_2\), 50 mg and 31 ml of 0.2 M Tris-HCl pH 8.0. The enzyme activity was detected as dark blue bands. The gel was rinsed in water and stored in alcohol gel wash for preservation.

• **Malic enzyme (ME - E.C.1.1.1.40)**

The staining mixture consisted of 5 ml of 1 M Na-L malate pH 7.0, 15 mg NADP, 15 mg NBT, 1 mg PMS, 50 mg MgCl\(_2\), 10 ml of 0.2 M Tris-HCl, pH 8.0 and 35 ml of water. The gel was incubated with staining solution in the dark at 37°C. The zones of enzyme activity appeared as dark blue bands. The gel was rinsed and preserved in alcohol wash.

• **Phosphoglucomutase (PGM- E.C.2.7.5.1)**

The enzymatically active bands were visualised by incubating the electrophoresed gel at 37°C in dark with the staining solution consisting...
of 300 mg Glucose-1-phosphatase (disodium salt), 15 mg NADP, 20 mg MTT, 1 mg PMS, 80 units Glucose-6-phosphate dehydrogenase, 50 mg MgCl₂, 10 ml of 0.2 M Tris-HCl, pH 7.0 and 40 ml of water. The gel was rinsed with water as soon as the dark blue bands appeared and preserved in glycerine water.

11) **Preparation of antigens for SDS-PAGE and Immunoblotting**

Antigens were prepared from trophozoites of the three isolates (P-1, PD-1 and PD-2) of *G. lamblia* as described in Chapter 3, Section 3.1.2 (1). Briefly, the pellet of washed *G. lamblia* trophozoites was resuspended in 10 mM Tris-HCl buffer [pH 7.4] containing 1 mM Phenylmethylsulphonyl fluoride (Sigma Chemical, U.S.A.) and 0.5% Triton X-100, sonicated with six 30 sec bursts in an ice bath. The sonicated materials were centrifuged to remove the debris (Ortega Pierres et al., 1988). The supernatants were pooled separately and protein content was estimated by a modified Lowry's method (Markwell et al., 1978).

111) **SDS-PAGE**

The protein constituents of the three isolates were compared in SDS-PAGE using discontinuous buffer system as described by Laemmli (1970) [vide Chapter 3, section 3.1.2. (vii)]. Briefly, 100 μg of antigen per well was applied and electrophoresed in 10% separating gel on Protean II vertical slab gel unit (BIORAD, Richmonds, USA) for 4 hrs at 35 mA constant. The gel was fixed in methanol acetic acid solution and stained with 0.2% Coomassie brilliant blue. The molecular weights of *G. lamblia* trophozoite proteins were determined by comparing the electrophoretic mobilities of unknown protein with the standard proteins (Sigma Chemical Co., USA) which was electrophoresed in the same gel.
iv) **Immunoblotting**

Immunoblotting was employed to determine the immunoreactive polypeptides of the three isolates and their molecular size. Details of the method has been elaborated in Chapter 3, section 3.1.2. (viii).

v) **Sera**

Sera was collected from five male patients aged between 10-15 years admitted to the Infectious Diseases Hospital, Calcutta, India, with complaints of diarrhoea, abdominal cramps, flatulence and fever. Stool examination and/or duodenal aspirates of these patients showed the presence of active *Giardia* trophozoites and cysts. No other causative agents for diarrhoea were detected from these stools. All the patients responded to metronidazole therapy and symptoms resolved within 15 days.

Control sera were obtained from 5 healthy individuals (5-16 yrs old). No cysts or trophozoites of *Giardia* were detected in their faeces and none had a history of giardiasis for the past 6 months.

5.2 **Results**

5.2.1 **Isoenzyme analysis**

Diagrammatic representation of the six enzymes revealed three different zymodemes among the three (Fig.1) isolates of *Giardia*. A distinct electrophoretic heterogeneity was observed in the two enzymes namely PGM and ICDH. PD-1 and PD-2 appeared to be quite different from P-1 since the PGM profile showed three bands in PD-1 and PD-2 whereas only one band appeared in P-1 isolate. The migration band of enzyme ICDH in P-1 isolate was found to be slower than PD?1 and PD-2 (Fig.5.2a) Significant heterogeneity was also observed in the Zymogram Q of the other two enzymes
Figure 5.1

Diagrammatic representation of isoenzyme patterns showing the enzyme profiles of each *G. lamblia* isolate examined. Arrows indicate the direction of movement from the origin (O). For abbreviations see materials and methods.
Figure 5.2

Photographs of polyacrylamide gels showing isoenzymes
a) PGM and ICDH; b) ALP and ME of *G.lamblia* isolates.
Fig. 5.2

(a) PGM

(b) ALP

ICDH

ME
Photographs of polyacrylamide gels showing ACP and G₆PDH enzyme patterns of G.lamblia isolates.
Fig. 5.3
namely ALP and ME. Two ALP bands could be seen in PD-1 whereas only one band appeared in PD-2 and P-1. The band of ME was found to migrate fast, moderate and slow in PD-2, PD-1 and P-1 isolates, respectively (Fig 5.2b). No difference in the mobility of the two enzymes namely ACP and G6PDH was discernible in the three isolates (Fig 5.3).

No enzymatic activity was observed in the axenic medium or in bovine serum, a major constituent of the axenic medium, which served as controls.

5.2.2 SDS-PAGE analysis

The SDS-PAGE protein profiles of soluble antigen of the three *G. lamblia* isolates are shown in Fig 5.4. SDS-PAGE showed about 24 protein bands in all the three isolates with mol. wt. ranging from 10,000 to 170,000 dalton. The major protein bands were observed in the mol. wt. region of 12,000 to 20,000; 28,000 to 38,000 dalton; between 52,000 to 70,000 dalton and one band at 94,000 dalton. The bands above 118,000 dalton mol. wt. although present in the original gel but were too faint to compare. No major differences were observed in the protein profiles of the three isolates; however, minor differences at 20,000 and 67,000 dalton mol. wt. regions could be detected which remained unaltered in repeated runs (Fig 5.4).

5.2.3 Immunoblotting

Antigenic analysis by immunoblotting against pooled sera from acute giardiasis patients demonstrated that all the three *Giardia* isolates shared the major protein antigens (Fig 5.5). Prominent conserved antigens of 118,000; 94,000 and 55,000 dalton mol. wt. were detected. However, a few less prominent immunoreactive bands at 32,000 dalton and some in
Figure 5.4

SDS-PAGE protein analysis of three G.lamblia isolates P-1 (A), PD-1 (B) and PD-2 (C) in a 10% gel stained with 0.2% Coomassie brilliant blue. Marker proteins (M) and molecular weights (kDa) are indicated.
Antigens from three *G. lamblia* isolates P-1 (A), PD-1 (B) and PD-2 (C) immunoblotted with pooled acute giardiasis patients sera. Proteins were separated in 10% polyacrylamide gels and transferred to nitrocellulose. Marker proteins (M) and molecular weights (kDa) are indicated.
Fig. 5.5
between 45,000 to 55,000 dalton mol wt. were also recognized in the patients sera (Fig. 4). No major differences were observed except the higher intensity of antigen-antibody reaction in Indian isolates when compared to the Portland isolate. The immunoblot analysis of three isolates against pooled control sera did not exhibit any immunoreactive polypeptide.

5.3 Discussion

Smith et al (1982) compared antigenic constituents of four G.lamblia isolates from widely separated localities but failed to distinguish them by IEP and PAGE. Einfeld and Stibbs (1984) identified and characterized a major surface antigen of G.lamblia and found that it was almost common to all the four isolates examined. However, it was almost common to all the four isolates examined. However differences in surface antigens among isolates have been shown by agglutination assay (Korman et al, 1986) and by indirect fluorescent antibody test (Ungar and Nash, 1987). In the present study for the first time, an attempt was made to differentiate three human isolates of G.lamblia, two from India and one from Portland, using a combination of electrophoretic isoenzyme patterns, SDS-PAGE analysis and immunoblot techniques. The isoenzyme profiles of the three isolates revealed three different zymodemes and each isolate differed from the other in two or more enzymes out of six enzymes studied. Significant differences were observed among PD-1 and PD-2 (the two Indian isolates) in ICDH, ALP and ME enzymes. This suggest that different pathogenic forms of G.lamblia are present in the same community. The genetic interpretation of multiple banded pattern of PGM and ALP among the two Indian isolates raise the possibility of the existence of genetically
different clones or diploid nature of the isolates with isoenzymes specified by different alleles at the same locus. Since cloning of Giardia was not done, these questions are yet to be answered. However, Meloni et al (1988) have used clonal culture of Giardia and found that the original multiple banded profiles in PGM were retained in the clones, thus ruling out the probability of genetically different clones in one isolate. The double banded pattern of ALP enzyme observed in PD-1 isolate could explain it as a heterozygous organism in which the enzyme might be coded by different alleles at the same locus. Bartram et al (1983) compared isoenzymes of Giardia isolated from Portland, Bethesda and England. The later two isolates were indistinguishable and differed significantly from Portland-1. Similarly, Korman et al (1986) observed differences among the four human Giardia isolates from Jerusalem and one from Bethesda using five isoenzymes. The two Jerusalem isolates were indistinguishable but the rest differed from each other by one or more zymodemes. These observations along with those presented here, strongly suggest that heterogeneity exists among the strains of G. lamblia from different geographic localities as well as within the strains of the same area.

This is probably the first report on ICDH and ALP isoenzymes studied in Giardia and considerable differences were observed in these enzyme profiles of the three isolates. The isoenzyme profile of PGM in Portland-1 (P-1) isolate in this study does not correlate with the results of Baveja et al (1986) but strongly resemble the profiles obtained by Meloni et al (1988). This difference in PGM profile of the same isolates in different studies may be a reflection of the existence of more than one P-1 strains in the scientific community (Miller et al, 1988). No major differences were observed in any of the isolates in immunoblot
analysis except for the higher intensity of the antigen-antibody reaction in Indian isolates than the Portland-1 isolate, when blotted against the pooled giardiasis patients sera. The probable explanation could be the long in vitro continuous cultivation of P-1 isolate in synthetic medium, may have altered or reduced the antigenic activity as was noticed with axenic *E.histolytica* (Philips, 1973). Antigenic differences among isolates have been well demonstrated by Korman *et al* (1986) with the antisera directed against the surface antigens of live trophozoites. However, in this study, soluble antigens and sera from patients were used, which could be the reason for the failure in establishing antigenic differences among the three isolates. Despite the dissimilarities among the isolates of *G.lamblia* some isolates of *Giardia* share certain common antigens (Korman *et al*, 1986; Ungar and Nash, 1987). Capon *et al* (1989) have demonstrated the similarities among the *Giardia* antigens, which correlated well with the findings of electrophoretic protein profile studies and immunoblot assay.

The present observations on isoenzyme studies certainly suggest that, heterogeneity exists among *Giardia* isolates of different geographic areas and also within the same locality. Although the observations with protein profile and immunoblot analysis did not show any marked differences, the isoenzyme studies definitely suggest a remarkable variation. This could be associated with variations in the clinical manifestation of giardiasis patients, differences in immune response in host and a varied degree of virulence among the parasites (Meloni *et al*, 1988; Sargeaunt *et al*, 1984). Correlation of genetic variation to pathogenicity and susceptibility to drugs of *G.lamblia* should be the future line of research in this regard.