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Comparative Biochemical and Restriction Endonuclease Digestion Pattern Analysis of Indian and Portland-1 Strains of *Giardia lamblia*

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ABSTRACT

Biochemical and restriction enzyme analysis were carried out to compare two Indian and one Portland (USA) isolate of *Giardia lamblia*. Electrophoretic isoenzyme patterns of four enzymes namely, Acid phosphatase (ACP), Alkaline phosphatase (ALP), Isocitrate dehydrogenase (ICDH), and Phospho glucomutase (PGM) revealed three different zymodemes amongst three isolates in 5% polyacrylamide gel. Each isolate differs from the other in two or more enzymes out of four enzymes studied. Significant differences were observed within the two Indian isolates in ICDH and ALP enzymes. Differences were also noticed between the Indian and Portland isolates in the electrophoretic migration patterns in PGM and ICDH enzyme systems. Heterogeneity among *G. lamblia* isolates is, therefore, postulated within the same area and in different geographical locations based on the isoenzyme studies. ICDH and ALP enzymes were used for first time in the study and found useful in differentiation of *G. lamblia* isolates. The restriction enzyme digestion pattern of DNA from the isolates showed no marked differences, thereby suggesting the development of gene specific probe for such studies.

INTRODUCTION

Giardia lamblia, an intestinal protozoan parasite, is a well known causative agent of diarrhoea in humans and animals (Nash 1989). Despite the high prevalence of *G.lamblia* infection, very little is known about the host-parasite relationship, pathogenicity and immunology of the disease. The existence of zoonotic reservoir (Meloni *et al.* 1988) and confusion of the taxonomy of the genus *Giardia* (Bertram *et al.* 1983) made it impossible to ascertain whether the human *G.lamblia* species is homogenous or consists of multiple strains with antigenic variation and biological activity (Ey *et al.* 1993).

Antigenic variations in strains of *Giardia* isolated from humans and/or animals from different geographic origin have been reported (Smith *et al.* 1982; Aggarwal & Nash 1987; Stibbs *et al.* 1988 and Nash *et al.* 1988). Biological differences such as ability of *Giardia* to grow in culture (Meloni & Thompson, 1987), infectivity to gerbils and human (Faubert *et al.* 1983, Visvesvara *et al.* 1988) and the ability of a *Giardia* RNA virus to infect other *Giardia* isolates (Miller *et al.* 1988) were also observed. Phenotypic Differences among the *Giardia* isolates were demonstrated in isozyme studies by (Korman *et al.* 1986; Baveja *et al.* 1986; Meloni *et al.* 1989; Ey *et al.* 1992 and Andrews *et al.* 1993). Similarly, variation in *Giardia* isolates at the molecular level has been demonstrated by Thompson and Meloni (1993) and variation in the metabolism among the isolates has been suggested by Hall *et al.* (1992). Information are also available where restriction endonuclease digestion patterns of the genome (Nash *et al.* 1985; De Jonckheere *et al.* 1990 and Ey *et al.* 1992), southern blot hybridization with cloned DNA probes (Nash *et al.* 1985; De Jonckheere *et al.* 1989, 1990; and Ey *et al.* 1992), pulse field gel electrophoresis for biotyping (Sarafis *et al.* 1993) and heterogeneity of chromosome sizes (Upcroft *et al.* 1989 and De Jonckheere *et al.* 1990) were used in the differentiation of *Giardia* strains isolated from human and animals of same community and different geographic areas. Since strain differences contribute to the variation in pathogenicity, clinical manifestation and immune response, the present study was undertaken with the objective to compare biochemical, and restriction enzyme digestion patterns of three human isolates of *G. lamblia* from India (PD1 and PD2) and abroad (Portland-1) with a view to understand the existence of variations among the strains of *G. lamblia* from different geographic origin.

MATERIALS AND METHODS

Axenization and Cultivation of parasite:

Stools samples from patients admitted to the Infectious Diseases Hospital, Calcutta, India, with various gastrointestinal disorder and diarrhoea were routinely examined for the presence of diarrhoeagenic pathogens. Ten such patients, aged between 10-18 yrs. who had abdominal pain, flatulence, diarrhoea, fever and stool examination repeatedly showed the presence of *Giardia* cysts and trophozoites were selected for duodenal intubation. About 0.1 ml of the aspirated duodenal fluid containing about $2x10^5$ *Giardia* trophozoites were injected immediately into the culture tubes containing filter sterilized TPS-1 medium, supplemented with 10% fetal calf serum, 10% NCTC 135 (Sigma chemical Co. USA) and different combinations of antibiotics (Gordts *et al.* 1984). Attempts were made to axenize all the 10 isolates. However, only two strains (PD-1 and PD-2) survived. These two Indian isolates and Portland-1 (P-1, ATCC No. 30888) were routinely maintained in filter sterilized TYI-S-33 medium (Diamond *et al.* 1978) containing vitamin mixture and 10% heat-inactivated adult bovine serum. These isolates were subcultured twice a week.

Isoenzyme analysis:

Active trophozoites from late log phase of growth were harvested and centrifuged at 600 x g for 10 min. The pellet was washed three times with normal saline supplemented with

250 mM sucrose. Lysates were made by freezing at -70°C and then thawing at 4°C for several times, till no intact trophozoites could be seen under the microscope. The lysed material was centrifuged at 20,000 x g for 20 min. and the cell free supernatant was collected, stored at-70°C with equal volume of stabilizing reagent (250 mM sucrose, 1 mM DTT, 2 mM aminocapronic acid and bromophenol blue) until use. For electrophoresis, protein content of each lysate was estimated (Bradford, 1976) and run on 5% polyacrylamide gel (Siciliano & Shaw, 1976) in a horizontal electrophoresis apparatus (Pharmacia LKB Biotechnology, Sweden) for 5 hrs. at 300 v in cold condition. Four enzymes namely, Alkaline phosphatase (ALP-E.C. 3.1.3.1), Acid phosphatase (ACP-E.C. 3.1.3.2.), Isocitrate dehydrogenase (ICDH-E.C.1.1.1.42) and Phosphoglucomutase (PGM-E.C.2.7.5.1) were studied and their staining were performed according to the methods of Siciliano and Shaw (1976) and Harris & Hopkinson (1978). Complete axenic medium (Diamond *et al.* 1978) or bovine serum and standard enzymes were used as controls and run with the same experiment.

Preparation of Antigen:

Antigens were prepared according to method described earlier (Chaudhuri *et al.* 1992). Briefly, normal saline washed pellet of *G. lamblia* trophozoites was resuspended in 10 mM Tris-HCl pH 7.4 containing 1 mM Phenyl-methyl sulfonyl fluoride and 0.5% Triton X-100; sonicated with six 30 sec bursts in an ice bath. The sonicated materials were centrifuged at 15,000 x g for 30 min. to remove debris (Ortega *et al.* 1988). Protein contents of the supernatant was determined by the method of Bradford (1976).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

The protein constituents of the three isolates were compared in SDS-PAGE using discontinuous buffer system as described by Laemmli (1970). Briefly, a 100 μg of antigen per well was applied and electrophoresed in 10% separating gel on Protean II Vertical slab gel (BIO-RAD, USA) for 4 hrs. at 25mA constant current. The gel was fixed in methanol-acetic acid solution and stained with 0.2% coomassie brilliant blue. The molecular weights of *G. lamblia* trophozoite polypeptides were determined by comparing their electrophoretic mobilities with standard proteins (Sigma Chemical Co. USA).

DNA isolation:

DNA was isolated from late log phase of the **Giardia lamblia** trophozoites. The trophozoites were harvested by chilling the culture tubes in ice for 10 mins. and then centrifuged at 600 x g for 10 mins. After washing twice with cold PBS (pH 7.0), the harvested cells were resuspended in 1ml of PBS. Nuclei from the cells were obtained by cell lysis in 2ml of NP-40 (Sigma chemicals, USA) solution (1% NP-40 in PBS) followed by centrifugation at 500 x g. for 3 mins. Subsequently nuclei were lysed by 2ml of lysis buffer containing 20 mM Tris HCl pH-8 EDTA- 50 mM, 5% N-lauryl sarcosine (Sigma chemical Co., USA) and 500 μg/ml proteinase-K. The mixture was incubated overnight at 50°C. The lysate was mixed with CTAB/NaCl solution (Murray *et al.* 1980) and incubated at 65°C for 10 min. Subsequently an equal vol. of chloroform : isoamyl alcohol (24 : 1) was added, mixed gently and centrifuged for 5 min at room temperature. The aqueous phase was taken, extracted with equal volume of phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and DNA was ethanol precipitated.

DNA analysis:

The following five restriction endonucleases Alu-I, Bam-H1, Bgl-II, Hind-III and Pst-I(BRL, USA) were used to digest the DNA. Approximately 4 μg of the DNA/lane was digested with an excess of enzyme (normally 10 units of enzyme/μg of the DNA) under conditions suggested by the manufacturer. Digested DNA was electrophoresed in 0.7% agarose gel at 60 volts in a mini gel apparatus (Pharmacia, Sweden). Banding patterns were visible after staining with ethidium bromide under UV light.

RESULTS

Isoenzyme study:

Diagrammatic representation of four enzymes revealed three different zymodemes amongst the three isolates of *Giardia* (Fig. 1). Electrophoretic heterogeneity was distinctly observed in two enzymes viz. PGM and ICDH (Fig. 2a). Three bands were observed in PGM enzyme in both the local isolates i.e. PD-1 and PD-2 whereas only one band was seen in P-1 isolate. The electrophoretic mobilities of strain P-1 was found slower than PD-1 and PD-2 in ICDH enzyme analysis. Fig. 2b demonstrates the zymograph of other two enzymes, ALP and ACP. Isolate PD-1 showed two ALP bands whereas only one band could be observed with PD-2 and P-1 strains. However, similar banding patterns were observed among all the isolates in ACP enzyme. No enzymatic activity was observed in axenic medium or bovine serum.

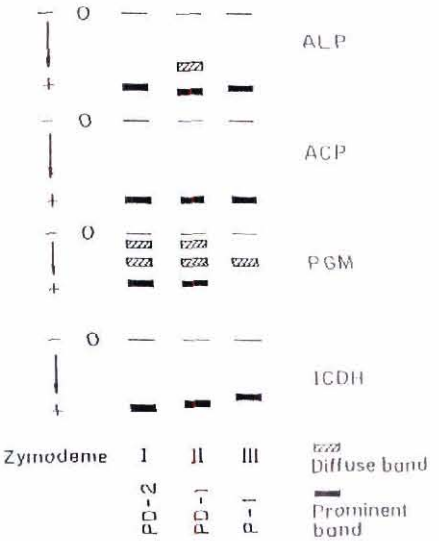

Figure 1. Diagrammatic representation of isoenzyme patterns of Alkaline phosphatase (ALP), Acid phosphatase (ACP), Isocitrate dehydrogenase (ICDH) and Phosphoglucomutase (PGM) showing the enzyme profiles of each isolates examined. Arrows indicated the direction of movement from the origin (O).

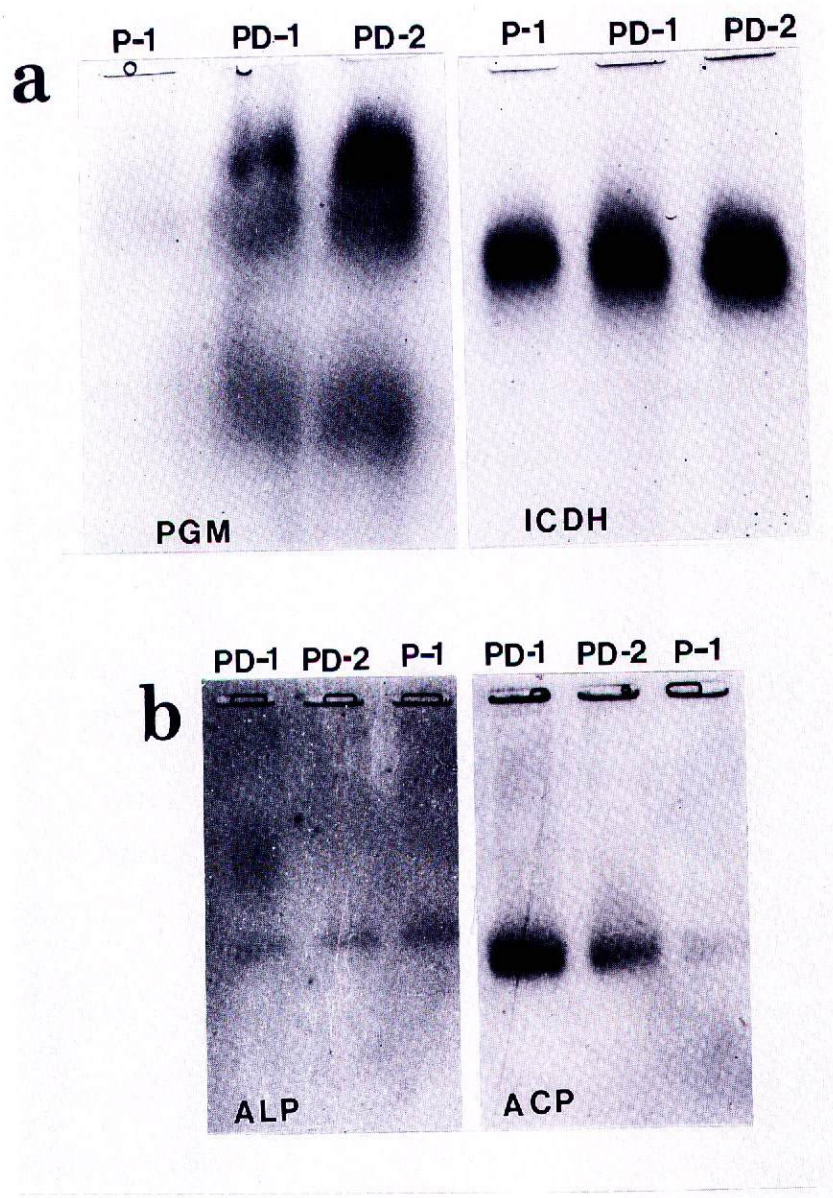

Figure 2. Photographs of polyacrylamide gels showing enzyme patterns of *Giardia lamblia* isolates: PGM & ICDH enzyme patterns Fig. 2a and ALP & ACP patterns Fig. 2b.

SDS-PAGE Analysis:

The SDS-PAGE protein profiles of three *G. lamblia* isolates showed 24 polypeptide bands with molecular mass ranging from 10 to 170 kDa (Fig. 3). The major polypeptides were observed in the 12 to 20 kDa, 28 to 38 kDa, 52 to 70 kDa and a single band at 94 kDa region. The bands above 118 kDa 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 kDa and 67 kDa regions could be detected which remain unaltered in repeated runs (Fig. 3).

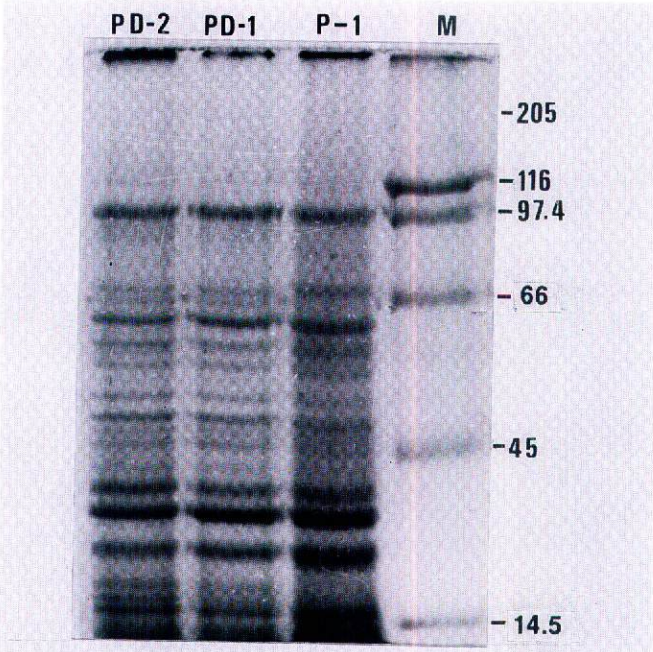

Figure 3. SDS-PAGE protein analysis of three *Giardia lamblia* isolates (P-1, PD-1 &PD-2) in a 10% gel stained with 0.2% coomassie brilliant blue. Marker proteins (M) and molecular mass(x10^3) are indicated.

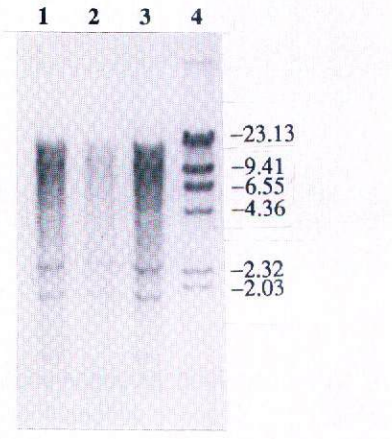

Figure 4. 0.7% agarose gel electrophoresis of BamH-1 restricted DNA from *Giardia*, Lane 1: P-1, Lane 2: PD-1, Lane 3: PD-2 isolates and Lane 4: \ Hind-111 marker (in kb).

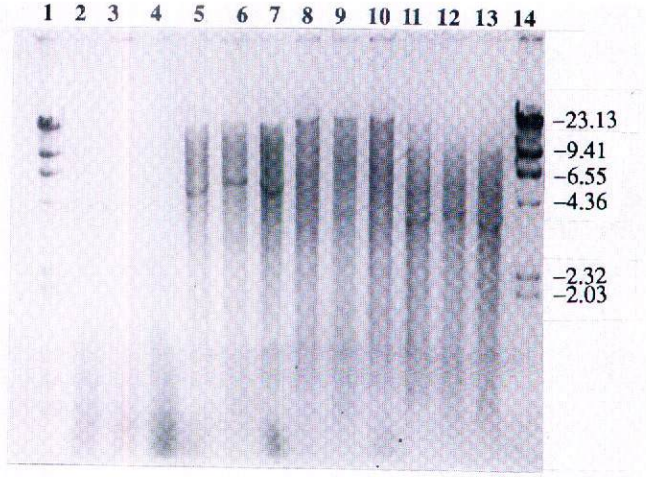

Figure 5. 0.7% agarose gel electrophoresis of DNA from 3 different isolates after treatment with restriction enzymes and staining with ethidium bromide. Lanes 1,14: \ Hind-III marker(in kb); Lanes 2,3, 4: Alu-I digestion profile of P-1, PD-1 and PD-2, respectively. Lanes 5,6,7: BgI-II digestion profile of P-1, PD-1 and PD-2, respectively. Lanes 8,9,10: Hind-III digestion profile of P-1, PD-I and PD-2, respectively. Lanes 11,12,13: Pst-1 digestion profile of P-1, PD-1 and PD-2, respectively.

Restriction enzyme analysis:

The restriction enzyme digestion patterns of the genomic DNA of different *Giardia* isolates with BamH-I and Alu-I, Bgl-II, Hind-III and Pst-I are shown in Figures 4 & 5, respectively. No difference in banding patterns amongst the isolates in any enzyme was observed.

DISCUSSION

The present investigation introduces the method for characterizing isolates of *G. lamblia*. Of the many existing methods isoenzyme analysis, surface antigen studies and genetic characterizations are the most recent approaches for strain differentiation in *Giardia* research. In this study two Indian human isolates (axenized in laboratory) and one reference, Portland-I (USA) strain, were compared by isoenzyme electrophoresis, SDS-PAGE analysis, and restriction digestion patterns of DNA. The isoenzyme profiles of the three isolates revealed three different zymodemes. Each isolate varies from the other in two or more enzymes out of four enzymes studied (Fig. 1). The significant differences were observed among the two Indian isolates PD-1 and PD-2 in ICDH and ALP enzymes (Fig 2a & 2b). Bertram *et al*. (1983) compared the isoenzymes of *Giardia* isolated from Portland, Bethesda and England. The later two isolates were observed indistinguishable and differed significantly from Portland-I strain. Similarly, Korman *et al*. (1986) observed differences among the four human *Giardia* isolates from Jerusalem and one from Bethesda using five isoenzymes. Out of four Jerusalem isolates,two were indistinguishable but rest differed from each other by one or more zymodemes. These observations and our result strongly suggest that heterogeneity exists among the strains of *G. lamblia* from different geographic localities as well as within the strains of same community. However, in contrast, a high degree of common sharing of zymodeme patterns have been reported among the genetically different Gardia isolates from human and animal (Moss *et al*. 1992). Efforts were made for the first time to use ICDH and ALP enzymes in the differentiation of Giardia isolates. The isoenzyme analysis of Portland-I (P-1) isolate in PGM enzyme analysis does not correlate with the observations of Baveja *et al*. (1986) but strongly resemble with the profiles obtained by Meloni *et al*. (1988), this probably suggest the existence of more than one P-1 strains in the scientific community (Miller *et al*. 1988). Although a considerable complexity in antigenic constituents of trophozoites of *G. lamblia* was observed (data not shown) among the three isolates but very meager or no major differences could be demonstrated in the protein profiles (Fig. 3). These observation are in accord with the data shown by Smith *et al*. (1982) and Einfeld *et al*. (1984) who also could not detect the differences in the antigenic components among isolates of geographically different Giardia by IEP and SDS-PAGE. This could be explained by the fact that the soluble proteins of different strains whether isolated from the same locality or from the different geographic locations are mostly conserved, in contrast to the studies conducted with surface antigens which showed marked differences (Nash *et al*. 1988; Nash, 1989; Nash and Mowatt, 1993 and Korman *et al*. 1986). The results of restriction enzyme analysis also suggests that *Giardia* genome is highly complex. Similarities in restriction endonuclease digestion pattern of these isolates indicated a general lack of DNA sequence

divergence although the geographic locality are different. This observation well corresponds with the results described by Nash *et al*. (1985). However, isolates of identical restriction digestion banding pattern do not necessarily be genetically identical. This was observed with the isolates having similar restriction digestion profile reacts differentially with the labelled DNA probes. Meloni *et al*. (1989) showed that the heterogeneity in zymodeme pattern was directly related to the variation of DNA banding pattern. For more precise strain differentiation work, future studies should be conducted on the preparation of gene specific probe. Amplification of rRNA gene using polymerase chain reaction have been found useful in classification of *G. lamblia*. The limitation of present investigation is the lesser number of isolates studied for comparison. However, our observations of isoenzyme studies strongly suggest that, heterogeneity exist among the *Giardia* isolates of the different geographic areas and also within the same locality. These differences could be associated with the variations in the clinical manifestation of giardiasis, differences in immune response in host and a varied degree of virulence among the parasite as shown by Meloni *et al*. (1988), Sargeaunt *et al*. (1984).

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