นิพนธ์ต้นฉบับ

Isoenzyme characterization of *Opisthorchis viverrini* from man+

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Cellulose acetate electrophoresis and enzyme activity staining have proven to be remarkably successful techniques in studying the taxonomy of microorganisms. Three enzymes, namely glucose phosphate isomerase (GPI; EC 5.3.1.9), phosphoglucomutase (PGM; 2.7.5.1) and glucose-6-phosphate dehydrogenase (G-6-PD;EC 1.1.1.49), all of which are involved in glucose metabolism, were studied in the homogenate ground from a single live adult worm of ***Opisthorchis viverrini (O. viverrini)****. Only the live adult worms were collected either from intrahepatic bile ducts of autopsy cases or from the common bile duct of patients who had undergone surgical operations. A total of 109 worms from human sources were analysed. Eight different patterns of GPI (GPI 1-8); five PGM patterns (PGM 1-5) and three patterns for G-6-PD (G-6-PD 1-3) were identified. As a result, the 109 worms were classified into 47 zymodemes based on the isoenzyme polymorphic patterns of the three enzymes.*

Key words : *Isoenzymes,* ***Opisthorchis viverrini.***

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ธาดา สืบหลินวงศ์, อติรัฐ ปิยัมบุตร, สุชาติ ปริยานนท์, สดศรี ไทยทอง. ลักษณะรูปแบบ ไอโซเอนไซม์ของ โอพิสโทรคิส ไวเวอร์รินิ จากคน. จุฬาลงกรณ์เวชสาร 2536 มิถุนายน; 37(6) : 375-385

เทคนิคการแยกโปรตีนด้วยกระแสไฟฟ้าแบบ เซลลูโลส อะซีเตท อิเล็กโตรฟอรีซิส และย้อมแถบไอโซไซม์ที่แยกได้โดยอาศัยประสิทธิภาพการทำงานของเอ็นไซม์นั้น มีประโยชน์ต่อการศึกษาด้านอนุกรมวิธานของจุลชีพและปรสิต การศึกษานี้ได้ศึกษาเอ็นไซม์ 3 ตัว ซึ่งเกี่ยวข้องกับวิถีเมตาบอลิสมของกลูโคส ได้แก่: กลูโคสฟอสเฟตไอโซเมอเรส (GPI; EC 5.3.1.9) ฟอสโฟกลูโคมิวเตส (PGM; EC 2.7.5.1) และกลูโคส -6 - ฟอสเฟต ดีไฮโดรจิเนส (G-6-PD, EC.1.1.1.49) ในโฮโมจิเนท ที่ได้จากการบดพยาธิใบไม้ตับตัวเต็มวัยเป็น ๆ 1 ตัว พยาธิใบไม้ตับที่นำมาศึกษานี้ ได้เก็บจากตับของศพที่ถูกผ่าตัดเพื่อชันสูตรและจากท่อน้ำดีรวมของผู้ป่วยที่เข้ารับการผ่าตัดรวมจำนวนพยาธิทั้งหมด 109 ตัว ผลการศึกษาพบรูปแบบของ GPI ได้ 8 รูปแบบ คือ GPI 1-8, PGM 5 รูปแบบ (PGM 1-5) และ G-6-PD 3 รูปแบบ (G-6-PD 1-3) จากผลการศึกษาที่ได้ทำให้สามารถจำแนกพยาธิตับใบไม้ 109 ตัวออกได้ 47 ชนิดย่อยตามความแตกต่างในรูปแบบไอโซเอนไซม์ของเอ็นไซม์ทั้ง 3 ชนิด

Northern and Northeastern Thailand are endemic areas for opisthorchiasis, a disease caused by *Opisthorchis viverrini,* which presents with a wide variety of clinical features ranging from symptomless to cholangiocar cinoma.[1-3] Although the disease has been studied extensively, there remains some gap with regard to the biological character of the worm.

Isoenzyme pattern analysis has been widely used in both taxonomy and the genetic study of parasites[4-6] and vectors[7] in order to subclassify into types and subtypes. These types or subtypes of the living organisms have then been related to other biological properties, namely drug resistance, geographical distribution[8,9] or as genetic markers in the cloning of the parasite.[10]

The purpose of this study was to use the isozyme patterns for the classification into various types of *Opisthorchis viverrini* collected from human subjects. The enzymes studied were glucose phosphate isomerase phosphoglucomutase and glucose-6-phosphate dehydrogenase. The distribution frequency and isozyme pattern of *Opisthorchis viverrini* were then analysed.

Materials and Methods

Opisthorchis viverrini

One hundred and nine adult live worms were collected from the bile ducts of three autopsied livers and three patients who had undergone cholecystostomy operations at Khon Kaen University Hospital. All worms were washed three times with sterile NSS which contained 200 U penicillin/ml and 200 ug streptomycin/ml; they were resuspended in Basal Medium Eagle (BEM. pH 7.2, Gibbco)with antibiotics added during transportation to the laboratory.[11]

Preparation of liver fluke homogenate

Individual live adult worms were homogenized manually with 10 µl 1% Triton X-100 in 0.2 M Tris/citrate buffer (pH 7.4) in a welled slide placed on ice. The crude homogenate was then applied to prewetted cellulose acetate strips. The isozyme patterns of the three enzymes, namely glucose phosphate isomerase (GPI; EC.5.3.1.9),glucose-6-phosphate dehydrogenase (G-6-PD;EC.1.1.1.49) and phosphoglucomutase (PGM; EC.2.7.5.1), were studied.

Electrophoretic separation of enzymes

Cellulose acetate electrophoresis technique was used for the separation of isozyme throughout the study. The cellulose acetate strip (Titan III Iso-Vis, Helena Laboratories) was presoaked in the electrophoresis buffer and placed on the sample applicator. Eight parasite homogenates were applied simultaneously at the cathodal end, using a Gelman Sample Applicator; then the strip was put into a Gelman electrophoretic chamber for running. The electrophoretic buffer system and time of the run are summarized in Table 1.

Table 1. Cellulose acetate electrophoresis systems used for the study of GPI, PGM and G6PD in *Opisthorchis viverrini*

Enzyme	Buffer	Voltage, Time of run
1. Glucose phosphate isomerase(GPI)	0.1M Tris EDTA pH8,3	250 volts,40 mins
2. Phosphoglucomutase (PGM)	0.1M Tris,0.1M Maleic acid,0.01M Na2EDTA,0.01M NaOH,pH 7.1	150 volts,30 mins
3. Glucose-6-phosphate dehydrogenase (G-6PD)	Same as PGM	150 volts,30 mins

Enzyme activity staining

To visualize the isozyme patterns of any enzyme, it is necessary to stain that enzyme via its reaction activity, since the different bands separated by electrophoresis can catalyse the same reaction. By using methyl thiazolyl tetrazolium (MTT) as electron acceptor, every positive reaction will give a violet-bluish band of insoluble formazan when MTT is reduced in the staining reaction. This can be linked to a specific enzyme activity when the specific substrate is added. The different bands visualized in a specific enzyme activity staining are the isozymes of that particular enzyme.

The staining mixes for the three enzymes were modified, according to the method of Harris and Hopkinson,[12] for both the volume and the concentration of substrates used. The reaction mixture for staining the GPI isozyme pattern consisted of 25.0 mg fructose-6-phosphate, 5.0 mg NADP, 5.0 mg MTT, 2 mg PMS in 2 ml of Tris HCl (pH 8.0). For the staining of the PGM isozyme pattern, the solution contained 5.0 mg PMS, 10.0 mg $MgCl_2$ and 10 µl of glucose-6-phosphate dehydrogenase in 2.0 ml of 0.01 M Tris HCl (pH 8.0) whereas the G-6-PD activity stain needed 10.0 mg glucose-6-phosphate, 5.0 mg NADP, 5.0 mg MTT, 5.0 mg PMS and 10.0 mg $MgCl_2$ in 2.0 ml of 0.01 M Tris HCl (pH 8.0).

After electrophoretic separation, each strip was placed on a glass plate with the cellulose acetate surface facing upwards; the strips were placed into a 37 °C incubator. The freshly prepared dye mixtures were poured onto each strip and sandwiched between another prewetted strip which was carefully layered over the former to avoid the trapping of air bubbles. A one-kilogram weight was then put on top of the cellulose acetate sandwich. The staining time varied from 3 to 6 minutes. The strips were then pulled apart and washed with tap water to remove any excess dye. Photographic pictures were taken immediately to avoid darkening of the background. The number of isozyme bands. their position, intensity of stain and distance from the origin were recorded and compared.

Results

Isozyme patterns of the three enzymes studied

There were eight GPI isozymes found in the homogenate of *Opisthorchis viverrini* which had been collected from human sources. Grouping the isozyme patterns was based on the number of bands and the distance the bands moved away from the site of sample application. The isozyme bands occurred in eight patterns varying from 3 to 7 bands (Fig 1). The darker stained band signified higher enzyme activity than the lighter ones.

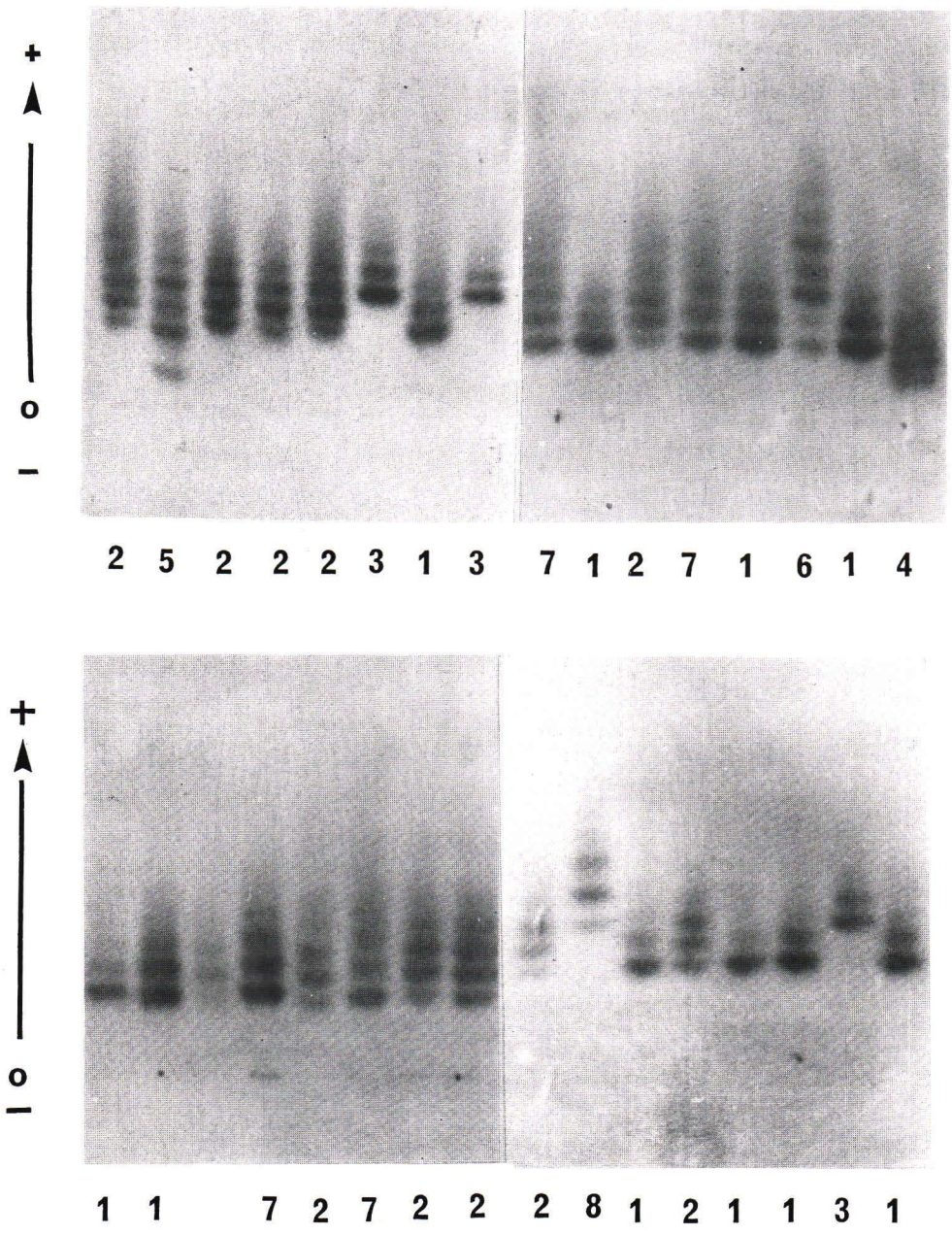


Figure 1. The eight isozyme types of glucose phosphate isomerase (GPI 1-8) found in adult worms of *Opisthorchis viverrine*.

The number of different banding patterns demonstrated by both PGM and G-6-PD were two bands. But owing to the distance differences, the isozyme patterns of PGM and G-6-PD from the 109 adult worms were grouped into 5 and 3 patterns, as shown in Figures 2a and 2b, respectively. Diagrammatic representations of the isozyme patterns of all three enzymes studied are shown in Figure 3.

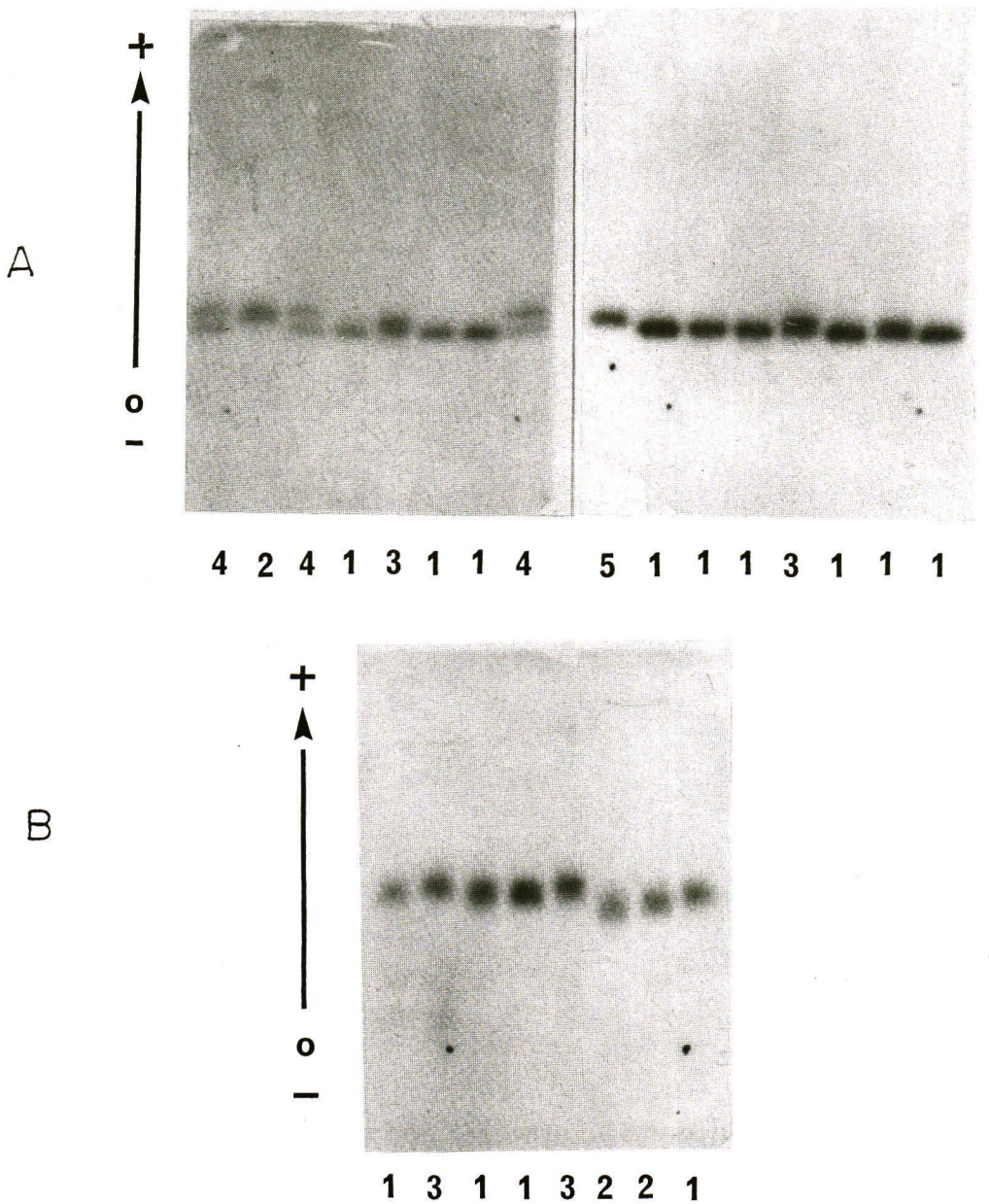
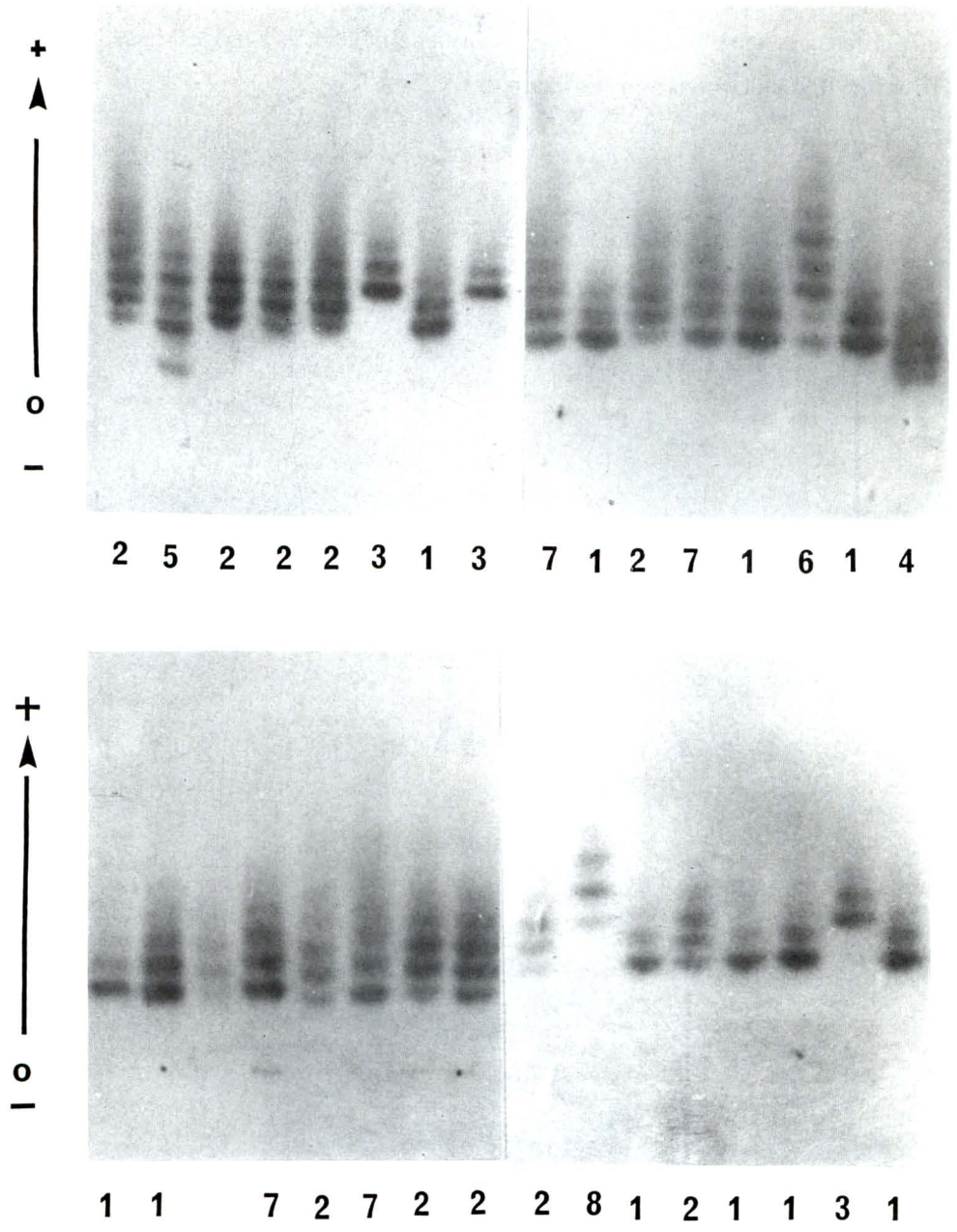


Figure 2. A : Phosphoglucomutase isozyme pattern. (PGM 1-5)
B : Glucose-6-Phosphate dehydrogenase isozyme patterns. (G6PD 1-3)



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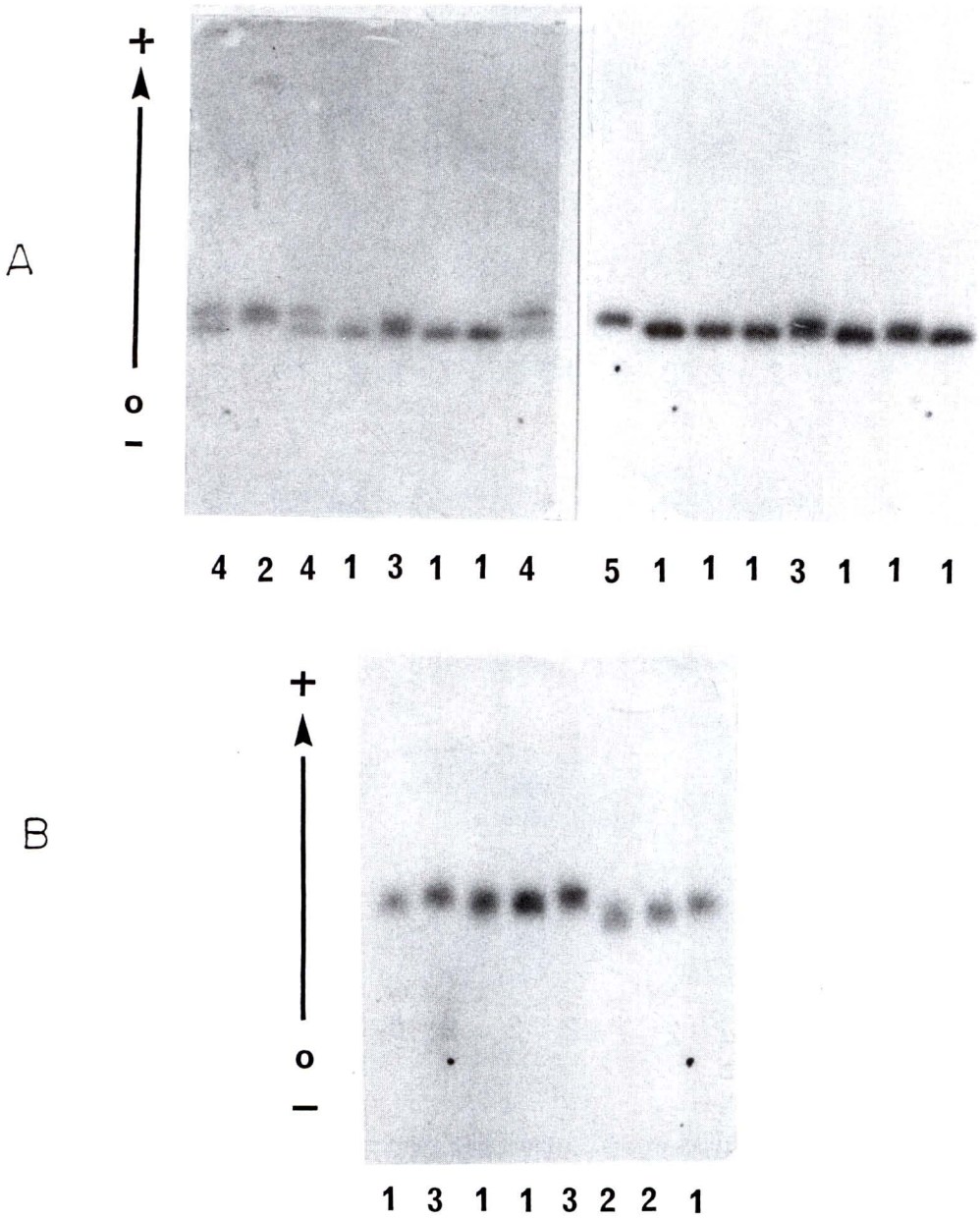

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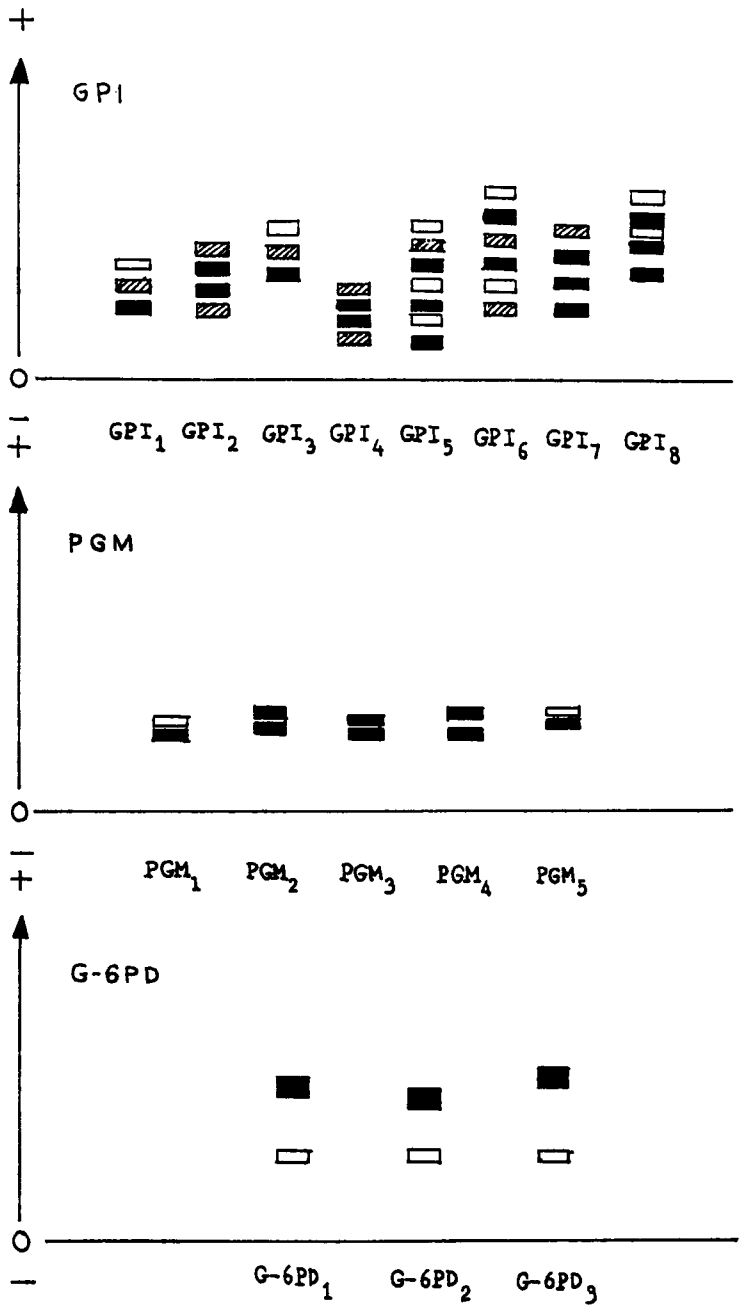

Figure 3. Diagrammatic representation of the eletrophoretic patterns of Glucose phosphate isomerase (GPI), Phosphoglucomutase (PGM) and Glucose 6 phosphate dehydrogenase (G6PD) found in *Opisthorchis viverrini*

▬ Dark band ▨ Lighter band ▭ Faint band

Isozyme analysis in *Opisthorchis viverrini*

The isozyme profiles of the 109 *Opisthorchis viverrini* adult worms were grouped into 47 zymodemes or types using three enzymes as shown in Table 2. Zymodemes 1 and 2 were the types that occurred most frequently in all the isolates. Six common zymodemes, with the occurrence varying from 3.57% to 14.29%, out of the 109 adult worms are diagrammatically shown in Figure 4. There were 25 zymodemes found in only one parasite per type. The number of bands and different banding patterns demonstrated by each enzyme system which ranged from two (G-6-PD and PGM) to seven bands(GPI),are shown diagrammatically in Figure 3.

Table 2. The 109 adult worms of *Opisthorchis viverrini* were classified into 47 zymodemes based on profile of GPI,PGM and G6PD.

Zymodemes (Types)	Isoenzyme patterms			%
	GPI	PGM	G6PD	
1	1	1	1	13.1
2	1	1	2	14.29
3	1	1	3	3.57
4	1	2	1	0.6
5	1	2	2	1.79
6	1	2	3	1.2
7	1	3	1	1.2
8	1	3	2	1.79
9	1	3	3	1.79
10	1	4	1	2.98
11	1	4	2	2.38
12	1	4	3	0.6
13	1	5	1	0.6
14	1	5	2	0.6
15	2	1	1	10.71
16	2	1	2	6.55
17	2	1	3	1.2
18	2	2	1	0.6
19	2	2	2	0.6
21	2	3	2	2.38
22	2	3	3	1.79
23	2	4	1	2.38
24	2	4	2	1.79
25	2	4	3	0.6
26	2	5	1	0.6
27	3	1	1	2.98
28	3	1	3	0.6
29	3	3	2	0.6
30	3	3	3	0.6
31	3	4	2	0.6
32	4	1	1	0.6
33	4	1	2	0.6
34	4	1	3	0.6
35	4	3	1	0.6
36	4	4	2	1.2
37	4	5	1	0.6
38	5	1	2	1.2
39	5	1	3	0.6
40	5	5	2	0.6
41	6	1	1	1.2
42	6	4	2	0.6
43	7	1	3	0.6
44	7	3	1	0.6
45	7	4	1	0.6
46	7	4	3	0.6
47	8	1	3	0.6

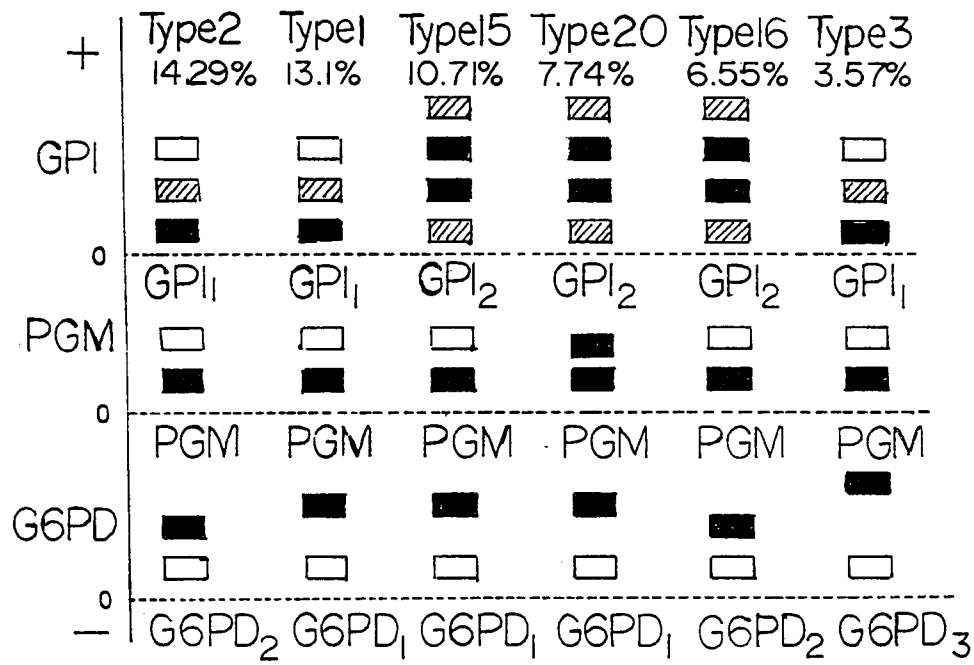

Figure 4. Diagrammatic representation of the enzyme profile of the 6 common zymodemes (types) of *Opisthorchis viverrini*.

Discussion

Our preliminary study on glucose phosphate isomerase isozyme pattern in *Opisthorchis viverrini* was done in -20 °C frozen worms collected from autopsied patients. The activity stain gave several intense bands with variable patterns which were named GPI 1-8. Agatsuma and Suzuki[13] had studied the GPI pattern in the *Fasciola sp.*, which is also an intrahepatic and biliary tract parasite. They found only one GPI pattern, with six bands, all of which moved anodally. Apart from GPI, we had screened from the -20 °C frozen specimens five more enzymes, namely glutamate dehydrogenase (GDH; EC 1.4.1.3), malate dehydrogenase (MDH; EC.1.1.1.47), malic enzyme (ME; EC.1.1.1.40), adenine deaminase (ADA; EC.3.5.4.4) and lactate dehydrogenase (LDH; EC 1.1.1.27); we obtained totally negative activity. A literature search revealed that no study on the isoenzyme characterization in *Opisthorchis viverrini* had been done even up to the time of the preparation of this manuscript. It took quite a while before we noticed that the only enzyme activity that could be detected in the -20 °C frozen parasite was GPI. This encouraged us to examine the carbohydrate metabolic pathway before deciding to embark on a study of PGM and G-6-PD, which are closely linked to GPI, as shown in Figure 5.

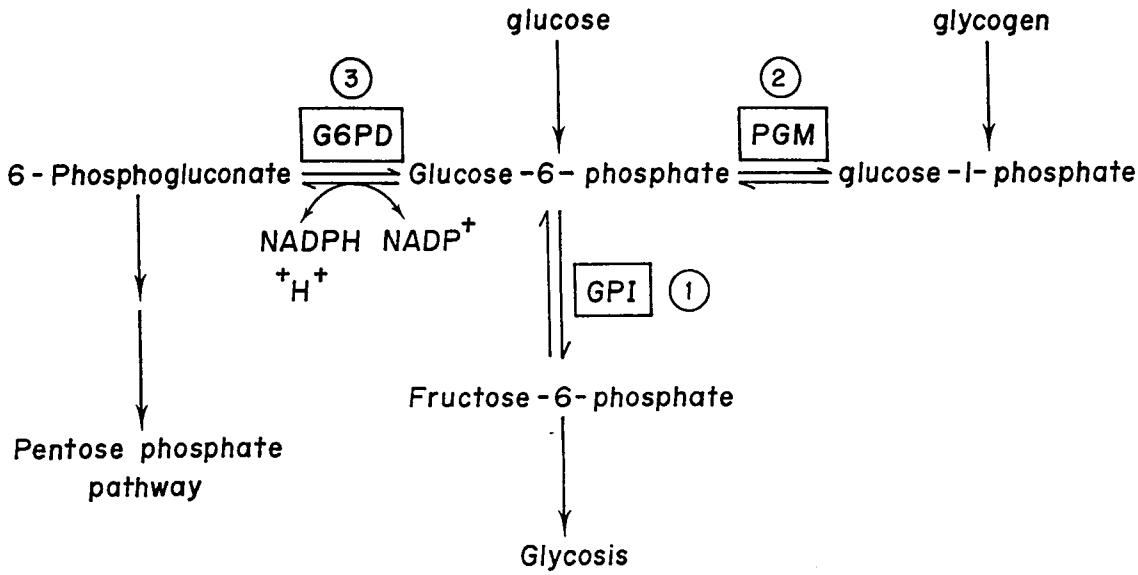

Figure 5. The enzymes studied, all participating in intermediary metabolism of carbohydrate in *Opisthorchis viverrini*.

The abundance, high activity and multi-isozyme patterns of GPI found in the -20° C frozen *Opisthorchis viverrini* adult worms from our preliminary study indicated that this enzyme might play a vitally important role in the life of the parasite. We proposed that the parasite(a) needs multi-isozyme patterns in order to better adapt to living in a host biliary tract and (b) depends for its main source of energy on carbohydrate via the anaerobic glycolytic pathway. It was difficult to compare the differences in the GPI patterns between specimens of live *Opisthorchis viverrini* and the frozen ones, since the resolution of bands after GPI staining was not good in the frozen specimens.

In this study, the common GPI patterns found in adult worms collected from human sources were GPI1 and GPI2 (Figure 2) with the occurence being 45.83% and 37.5%, respectively. It is possible that, in nature, GPI1 and GPI2 are equally distributed, but some host factors may contribute to the survival selection of one isozyme being carried by one parasite over another.

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