COST EFFECTIVENESS IN THE DISCRIMINATION OF *LEISHMANIA* SPECIES CAUSING ANTHROPONOTIC LEISHMANIASES IN ASIA USING SELECTIVE ENZYMES

SM Shamsuzzaman[1,2] and Y Hashiguchi[1]

[1]Department of Parasitology, Kochi Medical School, Nankoku, Kochi 783-8505, Japan; [2]Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh

Abstract. In this study, an attempt was made to evaluate the usefulness of selective enzymes in the identification of *Leishmania* spp causing anthroponotic leishmaniasis in Asia, especially from a cost effectiveness point of view. For this purpose cellulose acetate electrophoresis was carried out to identify the *Leishmania* species of the Old World. After analyzing 11 enzymes 6PGDH was found to be the most polymorphic enzyme which could distinguish the WHO reference strains of the *Leishmania* species endemic in Asian countries like *L.(L.) donovani* (DD8), *L. (L.) infantum* (IPT-1), *L.(L.) major* (5ASKH), and *L.(L.) tropica* (K-27). Addition of another enzyme G6PDH improved the quality of diagnosis. Cost could be reduced manifold to discriminate the Asian *Leishmania* parasites by analyzing these two enzymes.

INTRODUCTION

In the Old World an estimated 150 million people in 40 countries are at risk of infection with cutaneous leishmaniasis (CL) and 180 million people of 39 countries are at risk of visceral leishmaniasis (VL) (Ashford and Bates, 1998). Amongst the 5 established *Leishmania* species of the Old World *L. (L.) donovani, L.(L.) infantum* and occasionally *L.(L.) tropica* cause VL; *L.(L.) major, L.(L.) tropica, L.(L.) aethiopica* and *L.(L.) donovani* (in the form of post kala-azar dermal leishmaniasis) cause CL. Of them *L.(L) donovani, L.(L.) infantum, L.(L.) major* and *L.(L.) tropica* are widely distributed in Asia, *L.(L.) infantum, L.(L.) major* and *L.(L.) tropica* in Europe and *L.(L.) donovani, L.(L.) major, L.(L.) tropica* and *L.(L.) aethiopica* in Africa. Despite their morphological similarity different *Leishmania* species vary in their pathogenicity and epidemiology but it is impossible to identify a particular *Leishmania* species precisely by their morphology. Different biochemical criteria, particularly isoenzyme analysis, have since been used to discriminate the *Leishmania* parasites (Kreutzer and Christensen, 1987; Andrews *et al*, 1988; Evans, 1987; Schnur *et al*, 1981). Analysis of multiple enzymes is not only expensive but also an exhaustive work. Moreover it is reported that above a certain level an increase in the number of enzymes does not increase the number of demonstrable zymodemes (Cibulskis, 1992) and certain enzymes with low levels of polymorphism need not be utilized for preliminary investigation in the New World (Cupolillo *et al*, 1995).

The present protocol was designed to evaluate the usefulness of selective enzymes in the discrimination of *Leishmania* spp causing anthroponotic leishmaniasis in Asia from cost effectiveness point of view. After analyzing 11 enzymes it was found that 6PGDH alone can distinguish most of the *Leishmania* parasites of the Old World especially the parasites prevalent in Asia.

MATERIALS AND METHODS

Five WHO *Leishmania* reference strains obtained from the London School of Hygiene and Tropical Medicine such as *L.(L.) major* (MHOM/SU/73/5 ASKH), *L.(L.) tropica* (MHOM/SU/74/K-27), *L.(L.) infantum* (MHOM/TN/80/IPT-1), *L.(L.) donovani* (MHOM/ET/67/HU3), *L.(L.) donovani* (MHOM/IN/80/DD8) and five known strains of *Leishmania donovani* isolated from Bangladeshi kala-azar patients such as *L.(L.) donovani* (MHOM/BD/91/PG 25), *L.(L.) donovani* (MHOM/BD/91 PG 31) *L.(L.) donovani* (MHOM/BD/91 PG 34) *L.(L.) donovani* (MHOM/BD/91/ PG 38) *L.(L.) donovani* (MHOM/BD/97/PG 192), were studied. Parasites were maintained in USMARU medium with PBSS (Evans, 1987). After doing mass cultivation in RPMI 1640 media with 5% healthy human urine without fetal calf serum (Shamsuzzaman *et al*, 1999), soluble enzymes of the parasites were extracted according to

Correspondence: Dr SM Shamsuzzaman, Department of Parasitology, Kochi Medical School, Nankoku City, Kochi 783-8505, Japan.
Tel:/Fax: +81-888-80-2415; Email: shamsuzz@kochims.ac.jp

the procedure of Evans (1989). Cellulose acetate electrophoresis was carried out following the methods of Evans (1989), Kreutzer and Christensen (1987) and Furuya *et al* (1998). A total of 11 enzymes were analyzed namely 6-phosphogluconate dehydrogenase (6PGDH), glucose-6-phosphate dehydrogenase (G6PDH), phosphoglucomutase (PGM), mannose phosphate isomerase (MPI), alanine amino transferase(ALAT), glucose phosphate isomerase (GPI), aspartate amino tranferase (ASAT), malate dehydrogenase(MDH), malic enzyme (ME), pyruvate kinase (PK) and nucleoside hydrolase (NH).

RESULTS

Of the 11 enzymes analyzed the electrophoretic mobility of the band of 6PGDH showed marked distinguishing features among the *Leishmania* species of the Old World specially those prevalent in Asia (Fig 1). The band could discriminate the *L. (L.) donovani* (DD8), *L. (L.) infantum* (IPT-1), *L.(L.) major* (5 ASKH) and *L.(L.) tropica* (K-27) strains. But it failed to separate the *L.(L.) infantum* strain from the *L.(L.) donovani* (HU3) reference strain which is prevalent in Africa. The enzyme G6PDH could distinguish the *L.(L.) donovani* (DD8), *L.(L.) tropica* (K-27), *L.(L.) major* (5-ASKH) reference strains (Fig 2). Again it failed to separate the *L.(L.) infantum* from *L. (L.) donovani* (HU3) reference strain. These two strains could be separated by minor difference in mobility of ASAT band. No single band could differentiate the 5 reference strains but addition of G6PDH with 6PGDH improved the quality of diagnosis of four *Leishmania* species prevalent in Asia. The enzyme profiles of all the five Bangladeshi isolates from kala-azar patients were consistent with *L.(L.) donovani* (DD8) reference strain. To do electrophoresis of 11 enzymes a total of 33 chemicals were used for 82 times and 13 of them were common for 2-8 enzymes. To carry out electrophoresis of 6PGDH only 8 chemicals were used which cost one tenth of that for 11 enzymes. By addition of only one more chemical one new enzyme G6PDH could be analyzed, *ie*, 9 chemicals were used for two enzymes, 6PGDH and G6PDH, and thus the cost could be reduced to one sixth of that for 11 enzymes.

DISCUSSION

For epidemiologic and therapeutic purposes *Leishmania* species identification is very important. But due to economic constraints and lack of laboratory facilities in most of the leishmaniases-endemic Asian countries very few studies have so far been carried out with limited number of isolates. VL in India and Bangladesh has been assumed to be due to *L. donovani* (Choudhury *et al*, 1991; Thakur, 1984). Very few reports regarding identification of *Leishmania* species supplemented this assumption (El-Masum and Evans, 1995; Chatterjee *et al*, 1995; Shamsuzzaman *et al*, 1999). VL caused by *L.(L.) infantum* and CL caused by *L.(L.) major* and *L.(L.) tropica* are endemic in the Middle East, Afghanistan, Pakistan, and the central Eurasia which includes China, Uzbekistan, Turkmenistan, Azerbaijan,

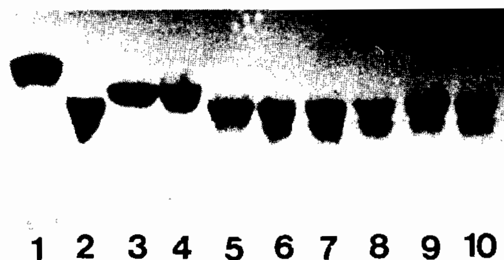

Fig 1–Photograph of 6PGDH enzyme profile of 5 reference strains in lanes 1-5 and 5 clinical isolates in lanes 6-10. Lane 1=*L.(L.) major* (5-ASKH), lane 2=*L.(L.) tropica* (K-27), lane 3=*L.(L.) infantum* (IPT-1), lane 4=*L.(L) donovani* (HU3), lane 5=*L.(L.) donovani* (DD8), lane 6=PG 25, lane 7=PG31, lane 8=PG 34, lane 9=PG 38, lane 10=PG 192. 6PGDH= 6-phosphogluconate dehydrogenase.

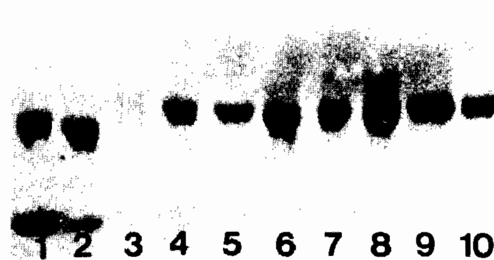

Fig 2–Photograph of G6PDH enzyme profile of 5 reference strains in lanes 1-5 and 5 clinical isolates in lanes 6-10. Lane 1=*L.(L.) major* (5-ASKH), lane 2=*L.(L.) tropica* (K-27), lane 3=*L.(L.) infantum* (IPT-1), lane 4=*L.(L.) donovani* (HU3), lane 5=*L.(L.) donovani* (DD8), lane 6=PG 25, lane 7=PG31, lane 8=PG 34, lane 9=PG 38, lane 10=PG 192. G6PDH= glucose 6-phosphate dehydrogenase.

Kyrgyzstan and Turkey (WHO, 1998; Matsumoto *et al*, 1999). CL caused by *L.(L.) major* and *L.(L.) tropica* is also prevalent in West of India (Peters *et al*, 1981). Recently *L.(L.) tropica* has been isolated from 6 veterans served in Operation Desert storm and 4 patients of India suffered from VL (Magill *et al*, 1993; Sacks *et al*, 1995). More over millions of people from Southeast Asia are working in the Middle East and there is a strong possibility of import as well as export of the disease among different countries of Asia.

In this study we examined 11 enzymes by cellulose acetate electrophoresis but few of them could distinguish different species of *Leishmania* parasite. Of the 11 enzymes analyzed 6PGDH was found to be the most appropriate enzyme to distinguish different strains of *Leishmania* of the Old World. But it could not distinguish the *L.(L.) infantum* from *L.(L.) donovani* (HU3) reference strain which is a causative agent of African VL. All the strains isolated from VL and post kala-azar dermal leishmaniasis (PKDL) from Bangladesh and all but 4 strains (identified as *L. tropica*) from Indian VL as well as PKDL cases were reportedly consistent with *L.(L.) donovani* (DD8) reference strain (El-Masum and Evans, 1995; Chatterjee *et al*, 1995, Shamsuzzaman's unpublished data). Therefore, all the *Leishmania* species prevalent in Asia could be distinguished by 6PGDH enzyme analysis. Addition of G6PDH enzyme analysis could improve the value of diagnosis. Cupolillo *et al* (1995) reported that 6PGDH could be used to separate strains of subgenus *Leishmania* from the subgenus *Viannia* in the New World. It was shown by Beach and Mebrahtu (1985) that GPI enzyme could distinguish the Kenyan strains *L.(L.) donovani*, *L.(L.) major* and *L.(L.) aethiopica*. It was observed in this study that to separate the *L.(L.) infantum* from African strain of *L.(L.) donovani* (HU3) electrophoresis of ASAT enzyme was necessary which showed only a minor difference in band mobility. But to visualize this band along with the bands of ALAT and FK an ultraviolet light system is necessary which may not be available in most of laboratories of leishmaniases-endemic countries of Asia. Furthermore, the concrete results obtained by analyzing these 2 enzymes could not be achieved even from the combined results of analysis of other 9 enzymes.

In conclusion, it can be said that rapid and cost effective identification of the *Leishmania* parasites isolated from CL and VL cases in Asia can be done by analyzing 6PGDH and G6PDH enzymes. So with the world's largest focus of VL in India and a large number of CL cases in Asia researchers and laboratory workers may proceed to identify the *Leishmania* species by analyzing these two enzymes. After preliminary identification if any new strain having different mobilities of bands are shown then it may be studied in detail by multiple enzymes analysis or may be sent to a reference laboratory having facilities for multiple enzyme analysis. This will be a cost-effective measure in terms of money as well as working hours especially in the countries endemic for the disease.

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