Journal of Kerman University of Medical Sciences

JKMU

Journal of Kerman University of Medical Sciences, 2021; 28 (2): 179-186

Isoenzyme Characterization of *Trichomonas vaginalis* Isolated from HIV Patients in Fars and Kerman, Southeast Iran

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Received: 26 July, 2020 Accepted: 21 February, 2021

ARTICLE INFO

Article type:
Original Article

Keywords:
Trichomonas vaginalis
HIV
Isoenzyme Electrophoresis
Zymodeme

Abstract

Background: *Trichomonas vaginalis* is an anaerobic flagellated protozoan which is responsible for human urogenital infections. Several zymodemes of *T. vaginalis* have been reported from various parts of the worlds on the basis of isoenzyme patterns. This study was conducted to characterize the isolated organisms of *T. vaginalis* from HIV patients using isoenzyme electrophoresis in Fars and Kerman provinces, southeast Iran.

Methods: Eighteen mass cultivated isolates of *T. vaginalis* in the modified TYI-S-33 medium were analyzed using isoenzyme electrophoresis. Polyacrylamide gel electrophoresis (PAGE) of five different enzyme systems were used to characterize *T. vaginalis* isolates: (i) Glucose-6-phosphate dehydrogenase (G6PD), (ii) Glucose phosphate isomerase (GPI), (iii) Malate dehydrogenase (MDH), (iv) Malic enzyme (ME), and (v) Phosphoglucomutase (PGM).

Results: MDH, GPI, PGM, and ME enzyme systems showed a homogeneity and detected an identical enzyme pattern in all isolates. Meanwhile, G6PD revealed two different enzyme patterns. The isoenzyme electrophoretic profiles divided 18 *T. vaginalis* isolates into two zymodemes. Zymodeme 1 contained Shiraz isolates and zymodeme 2 contained Kerman isolates.

Conclusion: The polymorphism of Iranian human isolates of *T. vaginalis* could be assessed by biochemical study using appropriate enzyme systems. Isoenzyme analysis is a promising method for the characterization of *T. vaginalis*. New molecular studies with increased number of enzyme loci and genetic markers are suggested to classify more zymodemes of *Trichomonas* in Iran.

Citation: Vafafar A, Ziaali N, Tavakoli R, Rayani M, Hatam G.R. Isoenzyme Characterization of Trichomonas vaginalis Isolated from HIV Patients in Fars and Kerman, Southeast Iran. *Journal of Kerman University of Medical Sciences*, 2021; 28 (2):179-186.

Introduction

Trichomonas vaginalis, an anaerobic flagellated protozoan, is the most common sexually transmitted infection (STI) with an estimated 250 million new cases all over the world every year (1,2). The rate of *T. vaginalis* infection is relatively high among the Iranian population. Several studies have reported the

prevalence rate of 0.5% to about 39% in different age groups of women (3,4). Trichomoniasis is related to serious health consequences such as adverse pregnancy outcomes and increased risk of cervical cancer. Women who are infected can be asymptomatic or have different symptoms, consisting a yellowish-green frothy discharge purities, dysuria, and the strawberry cervix (3). Recent advances in genetic characterization of *T. vaginalis* isolates have shown that the extensive clinical variability in trichomoniasis and its disease sequelae are matched by a significant genetic diversity in the organism itself, suggesting a relationship between the genetic identity of isolates and their clinical manifestations (5). Different studies have shown that *T. vaginalis* has been associated with human immunodeficiency virus (HIV), which increases the number of high-risk members (6).

Isoenzyme analysis is an efficient and powerful biochemical tool with numerous applications for studying the population genetics, taxonomic purposes, and epidemiology of parasites (7). Isoenzyme analysis is commonly used to make recommendations on the separation or combination of species, subspecies, and varieties (8). Genetic variation detected by enzyme electrophoresis can be used to evaluate genetic relationships within and between species, therefore, it is suitable for evaluating the taxonomy and providing information about epidemiological factors such as transmission patterns and population structure for a given organism achieved by the generic interpretation of the observed electrophoretic banding patterns (9). Electrophoretic analyses of isoenzyme have been proven to be valuable tools for studying the genetic relationships between taxons of protozoa such as *Leishmania*, *Trypanosoma*, *Toxoplasma*, *Entamoeba*, and *Giardia* (10-15). These studies divided the isolates of parasites into various zymodemes on the basis of isoenzyme patterns and associated them with the geographical distribution. The aim of this study was to characterize the *Trichomonas vaginalis* isolates from HIV patients with characteristic symptoms of trichomoniasis in Fars and Kerman provinces, southeast Iran, using isoenzyme profile.

Materials and Methods

Sample collection

In this cross-sectional descriptive study, HIV patients were selected using convenience sampling. Specimens of vaginal fluid and urine samples were collected from 380 female and male HIV-positive patients referring to health centers and hospitals located in Shiraz and Kerman cities in the south of Iran. These patients were convicted prisoners from central prison in Kerman as well as drug-addicted and HIV-positive in the same age group who were referred to the obstetrics and gynecology clinics in Shiraz. Written ethical consent was obtained from all the patients participated in this study. Vaginal discharge of the posterior fornix was collected by two sterile cotton swabs and the collected samples were immediately transferred into sterile tubes containing normal saline. For urine specimen, the midstream urine sample was also collected in a sterile tube. All samples were transferred immediately to our laboratory for microscopic examination and subsequent examination. All samples were examined by a light microscope for diagnosis based on the morphological characteristics and jerky movements in wet mount preparations.

T. vaginalis cultivation

All collected samples were inoculated into Dorset medium and incubated at 35˚C for up to 5 days. The axenic culture was

initiated in the modified TYI-S-33 culture medium (16) supplemented with 10% heat inactivated adult bovine serum, mix vitamin (No.18), streptomycin (100 µg/ml; Sigma), and penicillin (100 U/ml; Sigma) at 35.5˚C for 48 h. Trophozoites were passaged at every 72- and 96-hour interval, twice weekly based on the method developed by Clark and Diamond (17).

Preparation of lysates

Samples of logarithmic stage, including 500 ml medium containing 1×10^7 parasites/ml, were prepared. The logarithmic phases of the organisms were obtained 3 days after cultivation (18). The culture tubes were centrifuged at 2,000×g for 20 min at 4˚C, the supernatant was discarded, and the pelleted organisms were washed three times by resuspension and recentrifugation in cold PBS. An equal volume of hypotonic aqueous solution of enzyme stabilizers (1 mM amino-n-caproic acid, 1 mMdithiotheritol, and 1 mM EDTA, Sigma) was then added and mixed thoroughly. Freeze/thaw cycles were performed five times (19). The extract was centrifuged at 18,000×g for 30 min at 4˚C, and the supernatants were stored at −70˚C (20).

Enzyme electrophoresis

Eighteen mass cultivated of *T. vaginalis* isolates in the modified TYI-S-33 medium were analyzed using isoenzyme electrophoresis. Each strain was tested for five enzyme systems, namely, a) Glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.444); b) Glucose phosphate isomerase (GPI, E.C. 5.3.1.9); c) Malate dehydrogenase (MDH, E.C. 1.1.1.37); d) Malic enzyme (ME, E.C. 1.1.1.40); e) Phospho glucomutase (PGM, E.C. 2.7.5.1) on polyacrylamide gel electrophoresis (PAGE). The electrophoretic band developing conditions were as described earlier (8,19). Analysis was performed by discontinuous polyacrylamide gel electrophoresis using 3% stacking gel and 7.5% separating gel. The stacking buffer was composed of Tris-HCl (pH 6.7), resolving buffer of Tris-HCl (pH 8.9), and the tank buffer of Tris-HCl (pH 8.3), which ran under a constant current of 2 mA/well.

After electrophoresis, the prepared specific reagent for each enzyme system was poured onto the gel so that covers all the gel, and then, incubated at 37˚C for 30, 25, 30, 40, and 35 min for G6PD, GPI, MDH, ME, and PGM, respectively. Finally, the gel was removed from the incubator and examined. The relative migration distance or relative factor (RF) graphically was determined for the all bands in the samples. The migration distance was measured from the top of the resolving gel to each band and to the dye front using a ruler. RF value was calculated using the following equation:

RF = Migration distance of the protein band/Migration distance of the dye front

RF was defined as the mobility of a protein divided by the mobility of the ion front.

Results

Successful mass cultivation of *T. vaginalis* trophozoites was achieved with the modified TYI-S-33 medium. Eighteen isolates of the parasites were analyzed by isoenzyme electrophoresis on polyacrylamide gel electrophoresis (PAGE). Isoenzyme patterns obtained from *T. vaginalis* isolates gave reproducible results for five enzymes: G6PD, GPI, MDH, ME, and PGM. In most cases, the enzyme patterns appeared within 10 minutes after specific staining and rapidly diffused on further incubation. The enzyme patterns were classified into two groups: Similar electrophoretic patterns in MDH, GPI,

PGM, and ME enzyme systems and different patterns in the G6PD enzyme system.

Same zymodeme was detected in the MDH enzyme system in Kerman and Shiraz isolates. This zymodeme showed an identical 5-bands mobility pattern. Also, one zymodeme was detected in the GPI enzyme system and showed an identical triplex banding pattern. In addition, one identical zymodeme was detected in the PGM enzyme system. An identical single band mobility was obtained from all isolates. No difference in the mobility was detected with the PGM enzyme. Beside, one zymodeme was detected in the ME enzyme system that showed an identical 5-bands mobility pattern. Finally, two zymodeme were detected in the G6PD enzyme system. The first one showed an identical 5-bands mobility pattern and the second showed an identical quaternary band mobility pattern (Table 1) (Figure 1). MDH, GPI, PGM, and ME enzyme systems showed the same enzyme pattern, however, G6PD enzyme system showed 2 different enzyme patterns. Moreover, isoenzyme analysis revealed a total of 2 zymodemes among the 18 isolates of *T. vaginalis*. Zymodeme 1 contained Shiraz isolates and zymodeme 2 contained Kerman isolates (Figure 2).

Table 1. Summary of isoenzyme patterns of *T. vaginalis* isolates using 5 enzyme systems

City	MDH					GPI			PGM	ME					G6PD				
	RF1	RF2	RF3	RF4	RF5	RF1	RF2	RF3	RF	RF1	RF2	RF3	RF4	RF5	RF1	RF2	RF3	RF4	RF5
Shiraz	.23	.26	.32	.42	.67	.6	.72	.89	.6	.13	.25	.32	.37	.67	.26	.3	.4	.48	.66
Kerman	.23	.26	.32	.42	.67	.6	.72	.89	.6	.13	.25	.32	.37	.67	---	.3	.4	.48	.66

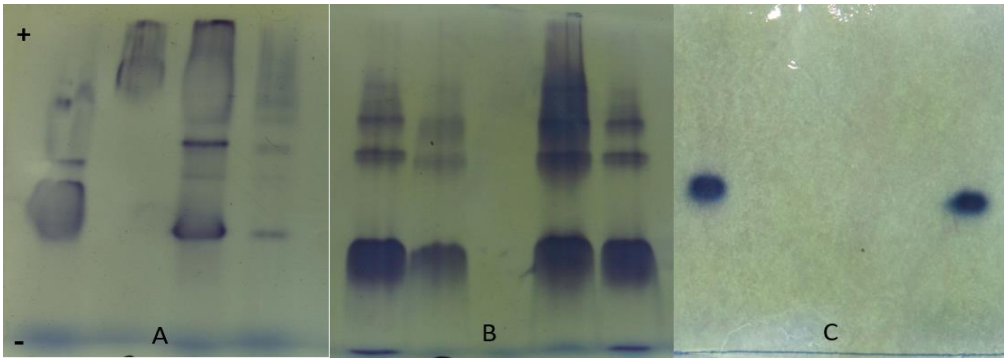

Figure 1. Photographs of the enzyme profiles for different stages of *T. vaginalis*. A: G6PD pattern, B: MDH pattern, C: PGM pattern.

A: Photograph pattern (photograph of a gel plate after the assays for G6PD) showed an identical 5-band mobility pattern with five RF (0.26, 0.3, 0.4, 0.48, and 0.66) and an identical 4-band mobility pattern with four RF (0.3, 0.4, 0.48, and 0.66).

B: Photograph pattern (photograph of a gel plate after the assays for MDH) showed an identical 5-band mobility pattern with five RF (0.23, 0.26, 0.32, 0.42, and 0.67).

C: Photograph pattern (photograph of a gel plate after the assays for PGM) showed an identical single band mobility pattern with RF of 0.6 for all isolates.

RF= Migration distance of the protein band/Migration distance of the dye front.

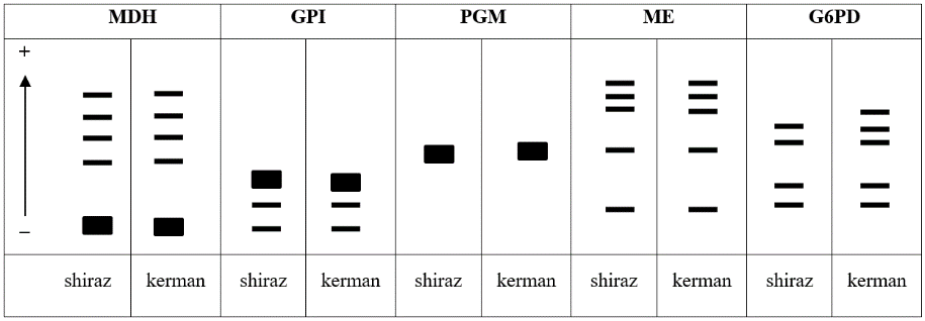

Figure 2. Diagrammatic representation of the enzyme from *T. vaginalis* isolates using five enzyme systems.

Diagrammatic representation of banding pattern for five enzyme systems from *T. vaginalis* isolates in Shiraz and Kerman.

Discussion

Isoenzyme analysis is available for identification and studying phenotypic characteristics of the parasites isolates. Electrophoretic analyses have been used to study the levels of genetic differentiation among strains and clones of Trichomonads (5,21-29). In the present study, 18 human isolates of *T. vaginalis* from two important provinces in the south of Iran (Shiraz and Kerman) were identified by biochemical method (Isoenzyme electrophoresis). Isoenzyme analysis by vertical acrylamide gel electrophoresis was a promising method for the characterization of *T. vaginalis* using five enzyme systems. Also, the mass cultivation of Iranian human isolates of *T. vaginalis* from Shiraz and Kerman provinces was well-established in the modified TYI-S-33 medium.

In this study, MDH enzyme pattern showed one identical profile with five bands. Soliman et al. (1982) found three different patterns, including 1 with three bands and 2 with double bands (22). Meanwhile, Proctor et al. (1988) discovered two isoenzyme patterns, one with four bands and the other with five bands (26) for this system. GPI enzyme pattern showed one identical profile with three bands of each extract of *T. vaginalis*. Soliman et al. (1982) observed two distinct patterns, one with three bands and the other with two bands (22). PGM enzyme pattern revealed one identical thick profile for all isolates. Soliman et al. (1982) found a homogeneous PGM isoenzyme pattern similar to that observed in the present study (22). ME enzyme pattern demonstrated one identical profile with five bands. A three band electrophoretic pattern was identified by Soliman et al. (1982) (22). Meanwhile, Proctor et al. (1988) showed four isoenzyme patterns, including 2 different three bands and 2 different four bands (26). G6PD enzyme pattern revealed two different profiles including pattern I constituted one identical pattern with five bands in Shiraz isolates and pattern II constituted one identical pattern with four bands in Kerman isolates. On the other hand, Soliman et al. (1982) and Proctor et al. (1988) reported that their results were not satisfactory for G6PD enzyme (22, 26).

Isoenzyme electrophoresis have been proven to be a useful tool for studying protozoan genetics and systematics. Soliman et al. (1982) compared 32 strains of *T. vaginalis* isolated from patients for eight enzymes (GPI, PGM, ME, HK, MDH, G6PD, ALD, LDH). A high degree of intraspecific variation among strains of *T. vaginalis* was found in isoenzyme patterns for MDH, HK, LDH, and GPI. PGM showed differences in only one strain, while two other enzyme patterns (ME and

ALD) were the same for all the strains of *T. vaginalis* tested (22). Gradus and Matthews (1985) used polyacrylamide gel electrophoresis (PAGE) to compare the proteins and isoenzymes of esterase, superoxide dismutase, and acid phosphatase insoluble, whole-cell extracts of four strains of *T. vaginalis*. Intraspecific, interspecific, and intergeneric differences were found in protein and isoenzyme profiles (24). Nadler et al. (1988) used isoenzyme electrophoresis to study the levels of genetic differentiation among strains and clones of trichomonads. Phenetic clustering of the biochemical data suggested that levels of genetic divergence among the species studied were extensive (25). Proctor et al. (1988) categorized 63 isolates of *T. vaginalis* into 15 groups using IE with four enzyme systems (ME, MDH, HK, and LDH) (26). Vohra et al. (1991) characterized *T. vaginalis* isolates from symptomatic and asymptomatic patients with IE and concluded that the isolates could not be classified based on their isoenzymic patterns alone (27). Azab et al. (1992) compared three *T. vaginalis* isolates from Egypt for their isoenzyme electrophoretic patterns on cellulose acetate. The three isolates shared the same isoenzyme banding patterns using MDH, GPI, HK, and LDH. Besides, two of these isolates were similar in their banding patterns of G6PD, PGM and different from those of the third isolate (28). Yuan and Gao (2003) studied the isoenzymes patterns on the seven isolates of *T. vaginalis* using five isoenzyme systems including MDH, LDH, G6PD, PGI, and PGM. The results showed some diversities in the patterns between the isolates and it seems reasonable to assume that there are at least three different biological types of *T. vaginalis* in China (29). Overall, in the current study the MDH, GPI, PGM, and ME enzyme systems showed a homogeneity, with one identical enzyme pattern detected in all isolates. The G6PD system, on the other hand, revealed two different enzyme patterns. Thus, the G6PD enzyme system was found to be more efficient in characterizing *T. vaginalis* in this study. Finally, 2 zymodemes were observed among 18 examined isolates in this study.

Conclusion

In the present study, isoenzyme analysis was a promising method for the characterization of *T. vaginalis* isolates. The MDH, GPI, PGM, ME, and G6PD were identified as differentiating enzyme systems for characterization of *Trichomonas*. The lower enzyme polymorphism in the present project compared to some studies in other parts of the world, indicates the need to study more enzyme systems in future studies.

Acknowledgments

This is a joint project between Shiraz University of Medical Sciences and Kerman University of Medical Sciences in Iran. It is a part of the MSc thesis of Arghavan Vafafar. The authors appreciate the Vice-Chancellor of Research, Shiraz and Kerman University of Medical Sciences, for the collaboration and financial support of this research.

Ethical approval

This research was approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethical code: IR.SUMS.REC.1390.S5885).

Conflict of Interests

The authors declare that they have no conflict of interests.

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