Original Article

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Unique *Trichomonas vaginalis* gene sequences identified in multinational regions of Northwest China

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Summary ***Trichomonas vaginalis* (*T. vaginalis*) is a flagellated protozoan parasite that infects humans worldwide. This study determined the sequence of the 18S ribosomal RNA gene of *T. vaginalis* infecting both females and males in Xinjiang, China. Samples from 73 females and 28 males were collected and confirmed for infection with *T. vaginalis*, a total of 110 sequences were identified when the *T. vaginalis* 18S ribosomal RNA gene was sequenced. These sequences were used to prepare a phylogenetic network. The rooted network comprised three large clades and several independent branches. Most of the Xinjiang sequences were in one group. Preliminary results suggest that Xinjiang *T. vaginalis* isolates might be genetically unique, as indicated by the sequence of their 18S ribosomal RNA gene. Low migration rate of local people in this province may contribute to a genetic conservativeness of *T. vaginalis*. The unique genetic feature of our isolates may suggest a different clinical presentation of trichomoniasis, including metronidazole susceptibility, *T. vaginalis* virus or *Mycoplasma* co-infection characteristics. The transmission and evolution of Xinjiang *T. vaginalis* is of interest and should be studied further. More attention should be given to *T. vaginalis* infection in both females and males in Xinjiang.**

Keywords: *Trichomonas vaginalis*, genetic diversity, 18S ribosomal RNA gene, multinational regions, Northwest China

1. Introduction

Trichomonas vaginalis (*T. vaginalis*) is a flagellated protozoan parasite that inhabits the vagina of females and the urethra, prostate gland, and epididymis of males (*1*). The WHO estimates that trichomoniasis affects more than 276 million people each year, with a worldwide prevalence of 22% for women and 2.2% for men (*2*). Trichomoniasis is more symptomatic in females than males. Symptoms in females can be debilitating and severe, such that 89% of the diagnosed cases are in women. *T. vaginalis* has emerged as a major pathogen of non-gonococcal urethritis and may contribute to male infertility (*3-5*).

The traditional method for detecting trichomoniasis involves examination of samples using direct light microscopy. More recent studies have used culturing, serological diagnosis, and other molecular methods to detect trichomoniasis. For example, the 18S ribosomal RNA gene is conserved in primary structure, making it a good target for detecting *T. vaginalis* by PCR. In addition, PCR-based gene sequencing has been used to examine T. vaginalis genotypes (*6-9*). Conrad *et al*. used 27 polymorphic markers, including 21 microsatellite and 6 single-copy genes, to study genetic diversity in *T. vaginalis* and identified two population types (*6*). Previous studies had also been done on the relationship between its genotype and the clinical presentation of trichomoniasis, including drug susceptibility and

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microbial symbiosis (*6-9*).

Most studies of *T. vaginalis* focus on women, with samples collected from vaginal swabs. Genetic information from *Trichomonas* in male patients remains largely unknown. In this study, we extracted DNA from vaginal swabs of women and semen samples of men, respectively, infected with *T. vaginalis* in Xinjiang Province, China. The 18S ribosomal gene of *T. vaginalis* was amplified and analyzed to evaluate the molecular epidemiology of *T. vaginalis* in this multi-ethnic region. Preliminary results suggest that a unique 18S ribosomal RNA gene of *T. vaginalis* was detected in these samples.

2. Materials and Methods

2.1. *Sample collection*

Seventy-three women and 28 men of childbearing age (range, 20-39 years) who attended the First Affiliated Hospital of Xinjiang Medical University and produced leukorrhea or semen infected with *T. vaginalis* were selected for the present study. The participants included several ethnic groups, including 63 Han, 20 Uyghur, 6 Mongolian, 6 Kazakh, 3 Hui, 1 Khalkhas, and 2 foreigners (from Russia, 22 and 38 years old). Detailed participant information is presented in Table 1. The institutional ethics committees of the First Teaching Hospital of Xinjiang Medical University approved the protocol (Ethics approval number: 20150402-06).

2.2. *Sample evaluation by microscopy and genomic DNA extraction*

Leukorrhea from women or semen from men was collected after 3-5 days of sexual abstinence. Leukorrhea was examined from a direct smear, and semen analysis was conducted strictly according to the WHO guidelines (*10*). All samples were examined using a microscope to verify the presence of a *T. vaginalis* infection. Samples were then sent immediately to the protozoan laboratory. Total genomic DNA from all fresh samples was extracted using the DNeasy Blood and Tissue Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. Extracts were stored at -20°C before being tested.

2.3. *PCR amplification and sequencing*

A set of primers was designed to target the conserved regions of the 18S ribosomal genes of *T. vaginalis*. The sequences were selected from regions of the 18S

Table 1. Ethnicity and age of patients included in this study

Sample	Ethnicity	Age	Sample	Ethnicity	Age	Sample	Ethnicity	Age
XJ-F3	Uyghur	28	XJ-F62	Han	27	XJ-F130	Han	39
XJ-F4	Han	36	XJ-F64	Han	39	XJ-F132	Uyghur	29
XJ-F8	Han	23	XJ-F65	Han	28	XJ-F133	Uyghur	23
XJ-F9	Han	27	XJ-F68	Han	37	XJ-F134	Han	33
XJ-F11	Han	27	XJ-F73	Han	38	XJ-F135	Uyghur	38
XJ-F13	Han	24	XJ-F74	Han	34	XJ-M1	Han	28
XJ-F15	Uyghur	29	XJ-F77	Uyghur	36	XJ-M2	Han	29
XJ-F16	Mongolian	38	XJ-F78	Kazakh	24	XJ-M3	Han	27
XJ-F17	Kazakh	27	XJ-F79	Han	25	XJ-M4	Han	39
XJ-F18	Han	39	XJ-F81	Kazakh	27	XJ-M5	Han	39
XJ-F19	Han	24	XJ-F83	Uyghur	25	XJ-M6	Han	25
XJ-F21	Han	32	XJ-F85	Han	24	XJ-M7	Uyghur	39
XJ-F22	Han	32	XJ-F86	Han	37	XJ-M8	Han	39
XJ-F23	Uyghur	26	XJ-F88	Han	26	XJ-M9	Uyghur	28
XJ-F25	Han	32	XJ-F90	Hui	30	XJ-M11	Uyghur	35
XJ-F27	Uyghur	26	XJ-F93	Kazakh	31	XJ-M12	Han	24
XJ-F28	Han	21	XJ-F94	Han	26	XJ-M13	Other ethnic	22
XJ-F32	Mongolian	36	XJ-F98	Khalkhas	26	XJ-M14	Hui	26
XJ-F33	Han	29	XJ-F101	Mongolian	25	XJ-M15	Han	38
XJ-F34	Han	34	XJ-F102	Uyghur	38	XJ-M16	Han	38
XJ-F36	Han	25	XJ-F104	Uyghur	37	XJ-M17	Hui	22
XJ-F37	Uyghur	32	XJ-F106	Han	39	XJ-M18	Han	31
XJ-F38	Kazakh	24	XJ-F107	Uyghur	39	XJ-M19	Other ethnic	38
XJ-F42	Mongolian	31	XJ-F109	Han	38	XJ-M21	Han	39
XJ-F45	Han	20	XJ-F110	Uyghur	31	XJ-M22	Han	30
XJ-F47	Han	25	XJ-F111	Uyghur	23	XJ-M23	Han	31
XJ-F50	Han	25	XJ-F113	Uyghur	39	XJ-M24	Han	26
XJ-F51	Han	20	XJ-F117	Mongolian	27	XJ-M25	Han	28
XJ-F52	Han	39	XJ-F119	Han	38	XJ-M26	Han	34
XJ-F57	Han	32	XJ-F120	Mongolian	27	XJ-M27	Han	38
XJ-F58	Kazakh	30	XJ-F123	Han	32	XJ-M28	Han	36
XJ-F59	Han	24	XJ-F127	Han	25	XJ-M29	Han	31
XJ-F60	Uyghur	26	XJ-F128	Han	35	XJ-M30	Han	39
XJ-F61	Han	23	XJ-F129	Han	27			

ribosomal gene that differed from those of *T. tenex*, *Tritrichomonas foetus*, *Candida albicans*, and other common pathogens found in the human urogenital system. The primer sequences were as follows: *T. vaginalis* S, 5′-ATC AGA GGC ACG CCA TTC-3′; *T. vaginalis* AS, 5′-CGC CCT TGA TCG ACA GAA-3′. PCR was performed using the thermal cycler Gene Amp PCR system (BIO-RAD).

Standard PCR was conducted using a total volume of 50 μL. The master mix contained 5 μL 10× *ExTaq* buffer (Mg^{2+} plus), 4 μL of the four deoxynucleoside triphosphates (2.5 μM each), 2.5 μL of each primer (20 μM each), 0.25 μL *ExTaq* DNA polymerase (5 U/μL, Takara), 2 μL DNA, and 33.75 μL double distilled sterile water. The amplification procedure included 3 min of denaturation at 94°C followed by 35 cycles of 15 s denaturation at 94°C, 30 s annealing at 60°C, and 30 s extension at 72°C. A final extension step at 72°C for 7 min was also included in each cycle.

PCR products were separated by horizontal gel electrophoresis at 100 volts on a 2% agarose gel in Tris-acetate-EDTA buffer and visualized using an ultraviolet transilluminator after ethidium bromide staining. The size of the amplified product (582 bp) was determined by comparison to a commercial 100-bp DNA ladder (Takara). The AxyPrep DNA Gel Extraction kit (Corning, Inc., Corning, NY, USA) was used to extract the desired band, and the product was sequenced. For the double peak signal products, the pMD 19T vector (Takara), *E.coli* JM109 (Takara), and the AxyPrep Plasmid Miniprep kit (Corning) were used to clone the products. At least five clones of each sample were sent for sequencing.

2.4. *Gene analysis*

Gene sequences were analyzed to ensure that the sequences were from *T. vaginalis*. Multiple sequence comparisons were made using Clustal W in software BioEdit 7.0.5.3 (*11*). Genetic distances were calculated using the Maximum Composite Likelihood settings in the software MEGA 5 (*12*) and were then sent to SplitsTree 4.13.1 (*13*) to create a phylogenetic network that accounts for reticulation events such as hybridization, horizontal gene transfer, and recombination.

A factorial correspondence analysis (FCA) representing the proximity between each individual genotype in a 2D plot was performed based on the 18S ribosomal gene frequencies using GenAlEx 6.5. The haplotype diversity and nucleotide diversity of these gene sequences between different groups were calculated using DnaSPv5 software.

3. Results

After extracting DNA from fresh samples, partial *T. vaginalis* 18S ribosomal gene sequences were amplified and sequenced. PCR analysis of 73 samples from females identified 75 *T. vaginalis* 18S ribosomal RNA sequences (labeled XJ-F1 through XJ-F75). In the samples from 28 males, 35 *T. vaginalis* 18S ribosomal RNA sequences were identified (labeled XJ-M1 through XJ-M35)To construct a rooted phylogenetic network, the 110 sequences identified in this study and another 46 reference sequences reported in Genbank were included.

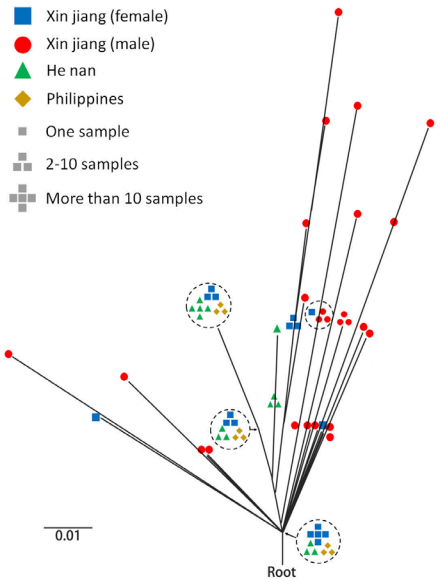

Figure 1. Network based on the 18S ribosomal RNA gene sequence of *T. vaginalis*. Network presenting the genetic relationships between Chinese and Philippine isolates as determined using SplitsTree 4. Colors indicate Xinjiang females (blue), Xinjiang males (red), Chinese Henan (green), and Philippine (yellow) sequences. Single symbol, one sample included; 3-symbol stack, 2-10 samples included; 5 symbol stack, > 10 samples included.

In the distance-based network generated from the 18S ribosomal RNA gene, *T. vaginalis* separated into three main clusters (Figure 1). The majority of Philippine sequences were located close to the root, while the majority of the Xinjiang sequences were located far from the root. The network revealed high diversity in 18S ribosomal RNA gene sequences in isolates from Xinjiang males' isolates.

Sequences of *T. vaginalis* clearly separated into three groups using visual assessment of the FCA plot (Figure 2). The majority of Xinjiang sequences differed from the majority of Henan and Philippine sequences. Group sequence compositions are as follows: Group 1, 59 from Xinjiang females, 32 from Xinjiang males of this study, with 16 previously reported sequences (4 Philippine, 12 Henan); Group 2,12 Xinjiang females, 3 Xinjiang males, 8 Henan, and 3 Philippine; Group 3, 4 Xinjiang females, 12 Henan, and 7 Philippine. No significant differences between groups were observed with respect to Xinjiang sample ethnicity or age in this study (Figure 3).

Quantifiable data revealed both high haplotype diversity (0.957) and nucleotide diversity (0.00738) of 18S ribosomal RNA gene sequences from Xinjiang

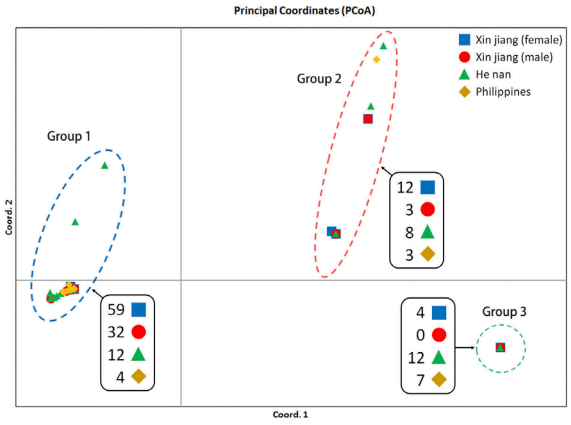

Figure 2. Principal coordinate analysis of *T. vaginalis* populations. The first and second coord extracted 38.75% and 11.76% of the total genetic variance, respectively. Colors indicate Xinjiang females (blue), Xinjiang males (red), Chinese Henan (green), and Philippine (yellow) sequences. The number of different populations included in each group is presented in the black textbox.

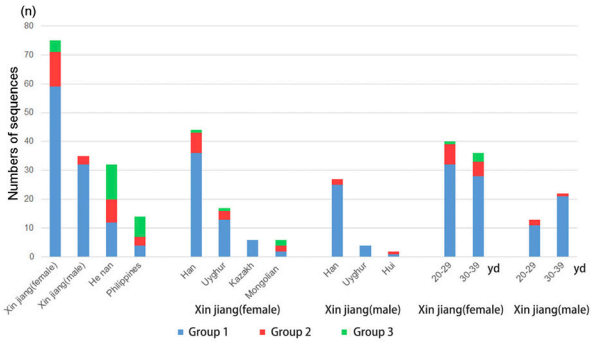

Figure 3. Group distribution of Xinjiang samples with respect to ethnicity and age. Y-axis, number of sequences; X-axis, sequence diversity of Xinjiang samples with respect to ethnicity and age. Sequences were divided into 3 groups according to principal coordinates analysis (as in Figure 2); Xinjiang females (blue), Xinjiang males (red), Chinese Henan (green), and Philippine (yellow) sequences

males. Mongolian samples of Xinjiang females also indicated high haplotype diversity (0.867) and nucleotide diversity (0.00289). No significant diversity was observed between Xinjiang samples with respect to ages (Table 2).

4. Discussion

Approximately 1% of male patients in the First Affiliated Hospital of Xinjiang Medical University were positive for *T. vaginalis* upon examination of urine samples by microscopy. However, the detection rate of *T. vaginalis* in semen samples was about 4%. PCR has been used to study the related molecular epidemiology of *T. vaginalis* infection (*14-16*). In this study, all the samples were confirmed for the existence of trophozoites of *T. vaginalis* in semen by microscopy and unique sequences of the 18S ribosomal RNA gene of *T. vaginalis* existed in Xinjiang male patients.

Table 2. Diversity of 18S ribosomal RNA gene sequence in *T. vaginalis*

Items	Haplotype diversity ± SD	Nucleotide diversity ± SD
China Xinjiang (females)	0.737 ± 0.036	0.00374 ± 0.00034
China Xinjiang (males)	0.957 ± 0.026	0.00738 ± 0.00083
China Henan	0.81 ± 0.031	0.00325 ± 0.00026
Philippines	0.703 ± 0.095	0.0023 ± 0.00042
China Xinjiang (females)		
Han	0.397 ± 0.09	0.0015 ± 0.00039
Uyghur	0.404 ± 0.13	0.00103 ± 0.00038
Kazakh	0	0
Mongolian	0.867 ± 0.129	0.00289 ± 0.00053
China Xinjiang (male)		
Han	0.961 ± 0.028	0.00711 ± 0.00095
Uyghur	1 ± 0.177	0.01055 ± 0.00261
Hui	1 ± 0.5	0.00842 ± 0.00421
China Xinjiang (females)		
20 - 29 years old	0.39 ± 0.092	0.00122 ± 0.00034
30 ± 39 years old	0.442 ± 0.1	0.00168 ± 0.00042
China Xinjiang (males)		
20 ± 29 years old	0.987 ± 0.035	0.00864 ± 0.00104
30 ± 39 years old	0.931 ± 0.046	0.00632 ± 0.00106

Several *T. vaginalis* sequences have been reported to date, and most of the 18S gene sequences have been reported in Chinese and Philippine studies (*17-19*). Previous studies indicate a relationship between *T. vaginalis* genotype and the clinical presentation of trichomoniasis (*7,20,21*). In this study, a distance-based network was constructed with three large clusters and many branches. The data suggest that Xinjiang *T. vaginalis* isolates might be genetically unique, although the results are preliminary. Thus the transmission and evolution of Xinjiang *T. vaginalis* is of interest and should be studied further.

A previous study showed that ethnicity is associated with *T. vaginalis* infection (*22*). In our study, Mongolian Xinjiang females were highly diverse with respect to haplotype and nucleotide sequences, comprising 3 groups. Samples from individuals other than Han were too few to make a reliable conclusion. The relationship between patient age and *T. vaginalis* 18S ribosomal RNA gene sequence was also investigated, but no significant clustering of samples was indicated based on age. The clusters indicated in this study must be confirmed using a larger patient cohort. Awareness of the molecular epidemiology of *T. vaginalis* in both females and males may be beneficial for disease diagnosis and treatment.

Previous study determined high genetic diversity within the *T. vaginalis* parasite isolated from world-wide regions in North America, Africa, Europe, Asia and Australia, and it was also found that the genotype characteristics remained stable in parasites. These studies had been done on the genotype of *T. vaginalis* and the relationship between its genotype and the clinical presentation of trichomoniasis, including metronidazole susceptibility, the presence of *T. vaginalis* virus (TVV) and *Mycoplasma hominis* (*6-9*). In this study, all the samples were collected from Xinjiang and the low

migration rate of local people in this province may contribute to genetic conservativation of *T. vaginalis*. Our results demonstrated that the genetic diversity of Chinese *T. vaginalis* isolates was associated with geographic distance based on the 18S ribosomal RNA gene. The unique genetic feature of our isolates may suggest a different metronidazole susceptibility, TVV or *Mycoplasma* co-infection characteristics. The results provided insight into the genetic features of *T. vaginalis* isolates and differences in genetic distance among China and other countries in different continents.

PCR analysis of the 18S ribosomal RNA gene sequence of *T. vaginalis* in 73 females and 28 males identified 110 sequences. The majority of these sequences formed a group that differed from most sequences reported in Genbank. These data suggest that *T. vaginalis* isolates from patients living in Xinjiang Province differ genetically from those of patients in other locations. Greater awareness of the molecular epidemiology of *T. vaginalis* in Xinjiang may be beneficial for disease diagnosis and treatment.

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