

The Study of Diagnostic Methods and Sequencing for Trichomoniasis in Mongolia

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Objectives: The aim of this study was to determine the usefulness of a diagnostic method for trichomoniasis that uses sequencing of the Tv-E650 gene. **Methods:** Totally 109 patients visited Ulaan Tuuz Hospital, National Center of Communicable Disease, Ulaanbaatar and were examined for trichomoniasis using wet mount examination, Papanicolaou (Pap) smear, culture and PCR. A family of 650 base pair (bp) long repeats from the *T. vaginalis* genome, designated as the Tv-E650 family, was sequenced for five Mongolian strains. **Results:** As a result, 21.1% (23/109) were positive by wet mount, 18.3% (20/109) were positive by Pap smear, 28.4% (31/109) were positive by culture and 36.6% (40/109) were positive by PCR. The differences among the strains are single-nucleotide and 2-nucleotide substitutions and insertions or deletions. The sequence uniformity of the strains as well as the presence of identical mutations in different isolates suggest efficient sequence homogenization mechanisms. In blast results, the Tv-E650 repeat family is conserved in all *T. vaginalis* strains examined, regardless of their diverse geographical origin. **Conclusion:** The Tv-E650 repeat family of *T. vaginalis* is a simple, rapid sensitive and specific accurate method for identification and diagnosis of trichomoniasis. **Keywords:** Sexually Transmitted Diseases, Trichomonas Vaginalis, Vaginitis, Polymerase Chain Reaction

Introduction

Trichomoniasis is the most common sexually transmitted infection (STI), caused by protozoan species *Trichomonas vaginalis* [1]. The extracellular parasite resides in the urogenital tract of both sexes and can cause vaginitis in women and urethritis and prostatitis in men [2-5]. Emerging epidemiological

data have implicated *T. vaginalis* infection in a higher incidence of human immunodeficiency virus (HIV) infection. The adhesion of *T. vaginalis* to vaginal epithelial cells plays an important role in the pathogenesis of trichomoniasis [3]. Disruption of urogenital epithelial monolayers by *T. vaginalis* could facilitate passage of HIV to underlying layers. Acute infections are associated with pelvic inflammatory disease and the increase

the risk of HIV infection by two to threefold [6, 7]. In pregnant women, trichomonads are implicated in the premature rupture of membranes, premature delivery, and the delivery of low-birth-weight infants [8, 9].

The World Health Organization estimated 276.4 million new cases of trichomoniasis infection among people aged 15-49 in 2008, a significant increase over around 170.0 million cases in 2005 [10, 11]. In the same year, 17648 STI cases were reported in Ulaanbaatar, Mongolia, which accounted for 40.3% of all communicable diseases, 5419 more cases than were reported than in 2007. Among reported STI cases, trichomoniasis accounted for 39.5% [12].

The number of newly infected cases are increasing year by year in Mongolia and all over the world. The prevalence of *T. vaginalis* is likely to be underestimated in our region, because there are no guidelines for screening such infection, and clinicians often rely upon insensitive diagnostic methods. In our country various methods have been used to diagnose trichomoniasis, such as wet mount, the Papanicolaou test, culture and serological test. Wet mount examination is straightforward and rapid, but more than 10^3 /mL of live protozoa are required for detection [13, 14], and cultures require inocula of 300-500 trichomonads/mL and specialized medium, as well as 2-5 days to make a diagnosis [15, 16]. During recent years, molecular biological techniques have provided new approaches to the diagnosis of *T. vaginalis* infection [17-20]. Therefore, the genetic epidemiology and characterization of isolate which can be found to be specific in population and geographical region of interest can provide potential leads for the development of easy detection and sorting out of the infection source upon medical inspection. The objective of this study was to compare diagnostic methods for trichomoniasis including sequencing of Tv-E650 gene.

Materials and Methods

1. Clinical sample collection

Vaginal discharges were sampled from women who visited the Ulaan Tuuz Hospital in the National Center of Communicable Diseases, Ulaanbaatar, Mongolia. All patients (109) with contingent indication the symptoms of vaginal discharge were assessed by performing wet mount, culture, Pap smear and PCR on their samples. The study was approved by the Medical Research Ethics Committee of the Mongolian National University

of Medical Sciences and informed consent was obtained from all patients (10/1A).

2. Wet mount

Two sterile, cotton-tipped applicators were used for the swab of vaginal discharge for each patient, and one applicator was gently agitated in one drop of normal saline on a slide for wet mount.

3. Cultivation of *T. vaginalis*

One applicator was put into a tube containing 5 mL of Feinberg-Whittington medium (HiMedia). *T. vaginalis* positive isolates were transferred from Feinberg-Whittington medium to TYM medium (Trypticase-Yeast extract-Maltose, Diamond 1982) to obtain a sufficient amount of trophozoites. Of the culture in Feinberg-Whittington medium, 1-2 drops were transferred to 5 mL of prepared TYM medium, cultivated in an incubator at 37°C for 2-5 days, and the presence of parasites was confirmed with a microscope. Trophozoites were supported with fresh TYM medium 3 times a week.

4. Microscopic examination

Samples were observed at daily intervals for 5 days. Wet mount and culture samples were examined microscopically using the low (x100) and high power (x400) magnification.

5. Papanicolaou smears

Pap smears were done by scraping the cervix with a cytobrush and spatula.

6. *T. vaginalis* isolates and DNA extraction

DNA material from clinical isolates was extracted by G-Spin Genomic DNA Extraction Kit (Intron Biotech). DNA concentration for each isolate was quantified by optical density readings at the wavelength of 260 nm and was checked by agarose gel electrophoresis.

7. PCR amplification of Tv-E650 repetitive sequence

PCR was performed with Gene ALL (Exgene) PCR premix, in 50 µl of total reaction volume. The Primers specific for *T. vaginalis* Tv-E650 repetitive sequence used for PCR have been described previously [22], and are as follows: forward 5'-GAGTTAGGGTATAATGTTTGATGTG 3' and reverse

5'-AGAATGTGATAGCGAAATGGG 3'. The desired product length was 330 bp. The amplification was obtained by having 5 minutes of denaturation at 94°C followed by 32 cycles each consisting 45 seconds of denaturation at 94°C, 40 seconds of annealing at 59°C, 45 seconds of extension at 72°C, and 5 minutes of final extension. The PCR product was analyzed by electrophoresis in 1.5% agarose gel containing 0.5-µg/mL ethidium bromide in TAE buffer. The sizes of the amplified products were assessed by comparing with commercial 100 bp DNA marker (Intron Biotech).

8. Sequencing and analysis

The PCR product was purified by QIAquick PCR Purification Kit (QIAGEN), and the purified product was used in sequence preceding reaction by Big Dye Terminator Cycle v.3.1 Reagent (Amersham Biosciences), according to specified protocol and conditions. Primers used in the reaction are the same as reported in the PCR condition. The samples were applied for sequencing using ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). The data was analyzed by online available CLUSTAL 2.1 multiple sequence analysis and the results were confirmed by MEGA 6.06

molecular evolutionary genetics analysis and MultAlin sequence alignment software.

9. Statistical analysis

The PCR was used as the "gold standard" diagnostic method to study the vaginal swab specimens. The study evaluation was done by using SPSS 17. The results were expressed as means ± SD of three to four independent experiments. The McNemar's Chi-square test and Cohen's kappa was used for statistical analysis, and a p-value of <0.05 was considered statistically significant.

Results

1. Trichomonads isolation

T. vaginalis positive samples were transferred from Feinberg-Whittington medium to TYM medium to obtain a sufficient amount of trophozoites. Taking into consideration the characteristic morphological features of the *T. vaginalis* organisms, all isolates were observed with the microscopic method (Figure 1).

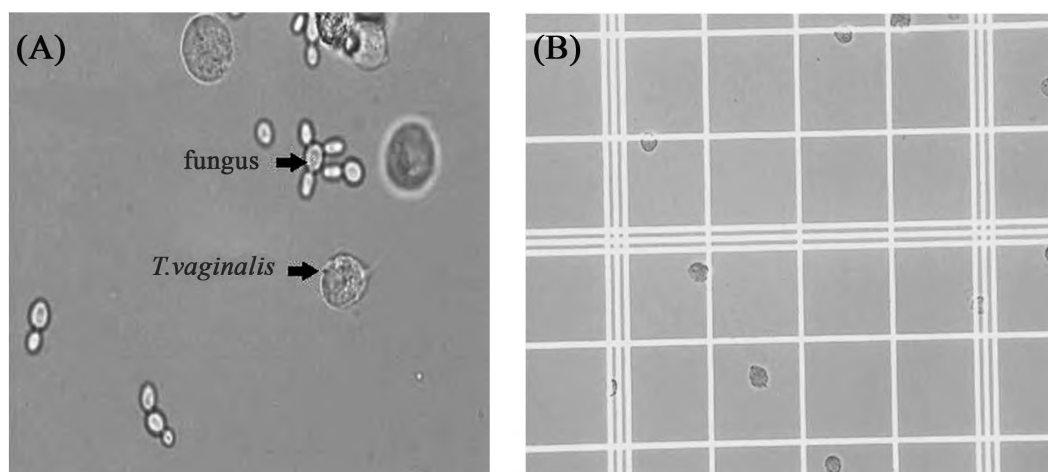


Figure 1. Culture of *T. vaginalis*: (A) Live *T. vaginalis* with fungus in Feinberg-Whittington medium culture, (B) Live *T. vaginalis* in TYM medium culture.

2. Comparison of the detection rate of PCR with other tests

Detection rates of wet mount, Pap smear, culture and PCR were 21.1% (23/109) were 18.3% (20/109), 28.4% (31/109) and 36.6% (40/109), respectively. Out of 109 samples 17 (15.5%) had positive by all detection methods. In total 40 (36.6%) cases were positive by PCR, however 6 of these cases were negative

by wet mount, 20 cases were missed by Pap smear and 9 were cases negative by culture. PCR for *T. vaginalis* showed a higher detection rate compared with conventional wet mount, Pap smear or culture. Therefore, results of wet mount, culture and Pap smear were compared in relation to the 'gold standard' PCR test results and sensitivity, specificity and kappa (k) were calculated (Table 1, 2, 3).

Table 1. Comparison between PCR and wet mount for diagnosis of *T. vaginalis*

| Wet mount | PCR ^a | | Total |
|-----------|------------------|----------|-------|
| | Positive | Negative | |
| Positive | 22 | 1 | 23 |
| Negative | 18 | 68 | 86 |
| Total | 40 | 69 | 109 |

^aPCR was used in the study as a reference test (gold standard); specificity 98%, sensitivity 55%, $k=0.58$, $p<0.005$

Pap smears were interpreted as positive for trichomonads in 20 (18.3%) of 109 patients, but one of these 20 cases was not confirmed by PCR and culture, and 3 cases were also missed by wet mount (Tables 2).

Table 2. Comparison between PCR and Pap smear for diagnosis of *T. vaginalis*

| Pap smear | PCR ^a | | Total |
|-----------|------------------|----------|-------|
| | Positive | Negative | |
| Positive | 19 | 1 | 20 |
| Negative | 21 | 68 | 89 |
| Total | 40 | 69 | 109 |

^aPCR was used in the study as a reference test (gold standard); specificity 98.5%, sensitivity 47.5%, $k=0.51$, $p<0.005$

For Diamond’s culture compared with PCR, 31 (28.4%) cases showed positive culture and all of these positive results were consistent with that of PCR. The overall proportions of agreement between culture and PCR were $k=0.81$, $p<0.005$ (Table 3).

Table 3. Comparison between culture and PCR for diagnosis of *T. vaginalis*

| Culture | PCR ^a | | Total |
|----------|------------------|----------|-------|
| | Positive | Negative | |
| Positive | 31 | 0 | 31 |
| Negative | 9 | 69 | 78 |
| Total | 40 | 69 | 109 |

^aPCR was used in the study as a reference test (golden standard); specificity 100%, sensitivity 77.5%, $k=0.81$, $p<0.005$

3. Studies on the sensitivity and specificity of PCR

To determine the sensitivity of the reaction, PCR was undertaken with clinical samples. The *T. vaginalis* were counted with a hemocytometer and the number of trophozoites with PBS was

adjusted to different cells. 50 µl of PCR reaction mixture were added to each tube containing a different number of trophozoites such as 1-3156; 2-1578; 3-100; 4-50; 5-12; 6 and 7-3; 8, 9 and 10- 1 cells, respectively (Figure 2). The expected product of 330 bp was obtained from as few as one organism. The negative control did not show any reactions (Figure 2). We checked the specificity of PCR using DNA extracted from *Candida albicans*. When DNA from the different organism *Candida albicans* was analyzed by PCR, no specific amplification was obtained.

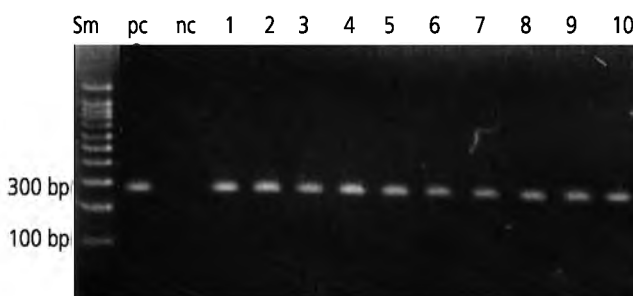


Figure 2. Agarose gel electrophoresis of PCR products with increasing number of *T. vaginalis*.

4. Symptoms of patients with suspected vaginitis

All patients showed various symptoms of vaginitis that included vaginal discharge, pruritus, erythema, edema on vaginal wall, dyspareunia, postcoital bleeding, burning sensation and strawberry patch (Table 4).

Table 4. Symptoms of patients with suspected vaginitis

| Characteristics | Ratio of patients with symptoms |
|---------------------|---------------------------------|
| Vaginal discharge | 85.3% (93/109) |
| Pruritus | 43.1% (47/109) |
| Erythema | 66.9% (73/109) |
| Postcoital bleeding | 65.1% (71/109) |
| Dyspareunia | 30.7% (33/109) |
| Burning sensation | 34.8% (38/109) |
| Strawberry patch | 42.2% (46/109) |

There was no correlation between these variables and the results by PCR for the diagnosis of trichomoniasis (Table 5).

Table 5. Comparison of symptoms between women with positive results and negative results by PCR for *T.vaginalis*

| Symptoms | PCR ^a | | | |
|---------------------|------------------|---------|----------|---------|
| | Positive | | Negative | |
| Vaginal discharge | 77.5% | (31/40) | 89.8% | (62/69) |
| Pruritus | 55.0% | (22/40) | 36.2% | (25/69) |
| Erythema, edema | 70.0% | (28/40) | 65.2% | (45/69) |
| Postcoital bleeding | 75.0% | (30/40) | 59.4% | (41/69) |
| Dyspareunia | 22.5% | (9/40) | 34.7% | (24/69) |
| Burning sensation | 40.0% | (16/40) | 31.8% | (22/69) |
| Strawberry patch | 65.0% | (26/40) | 28.9% | (20/69) |

^ar=0.001

5. Molecular characterization

We selected five Mongolian strains having good genomic DNA including MnTv-1, MnTv-2, MnTv-6, MnTv-26 and MnTv-27 for sequencing of the Tv-E650 gene (Figure 3).

The nucleotide sequence is A+T-rich (73.3% A+T in the consensus sequence) and highly conserved among the 5 strains analyzed. The sequence uniformity of the isolates as well as the presence of identical mutations in different isolates suggests efficient sequence homogenization mechanisms. These results showed that all five Mongolian strains tested have the same sequence of E650 gene.

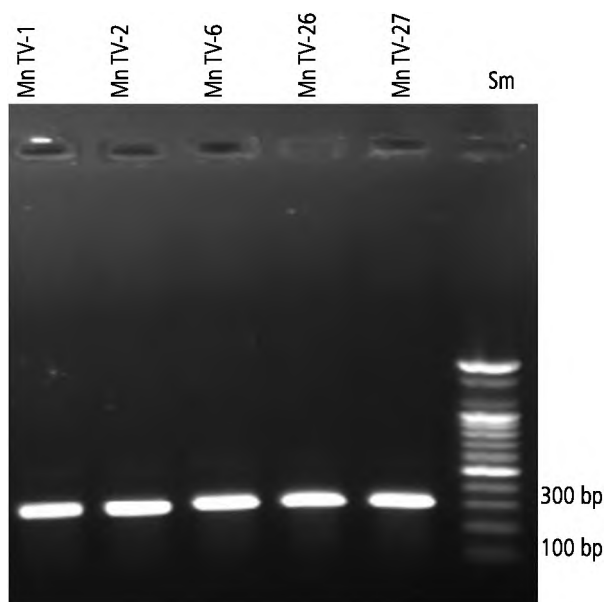


Figure 3. PCR for T.v-E650-1 gene detection.

The five strains of Mongolian DNA sequence parts were compared with online available similar parts of DNA sequence derived from isolates of local patients from a single study in Czech Republic [21]. The blast results showed at most single nucleotide and 2-nucleotide substitutions, insertions or deletions. Overall, as shown in former studies, the sequence part is shown to be homogenic between patients in Ulaanbaatar and patients from diverse geographical origin in the Czech Republic (Figure 4). In Figure 4, (*) nucleotides are specific to all samples, (-) indicates the absence of a nucleotide on given location on at least one of isolates, (.) indicates a single nucleotide difference among isolates on the same location, and underlined sequences refer to the double repeated short sequence in M864872.1-M864879.1 isolates, which is absent in MnTv isolates from Mongolia.

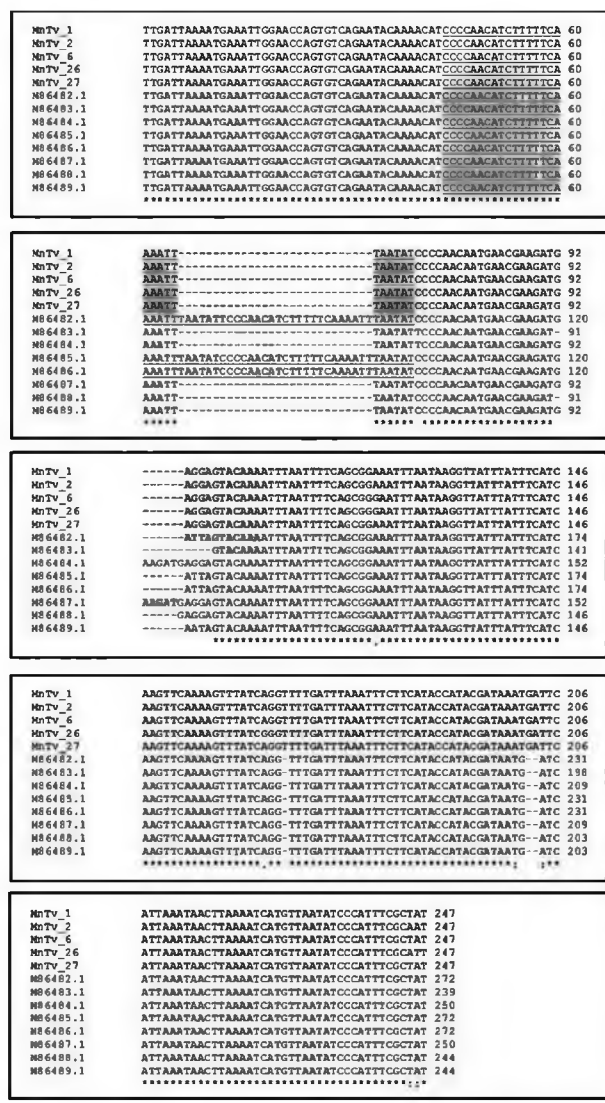


Figure 4. Comparison of Tv-E650 gene sequencing by DNA blast.

We checked if the potential difference in characterization between isolates could be attributed to different regions. This was accomplished by comparing our data with online available data by drawing UPGMA phylogenetic tree. The amount of isolates and their data statistically cannot fulfill the clear diversity

and distance (Figure 5). These results show that it is impossible to identify trichomoniasis isolates from different geographical regions based on their Tv-E650 repetitive conservative DNA sequence. Our results cannot fully support the difference between two different isolates.

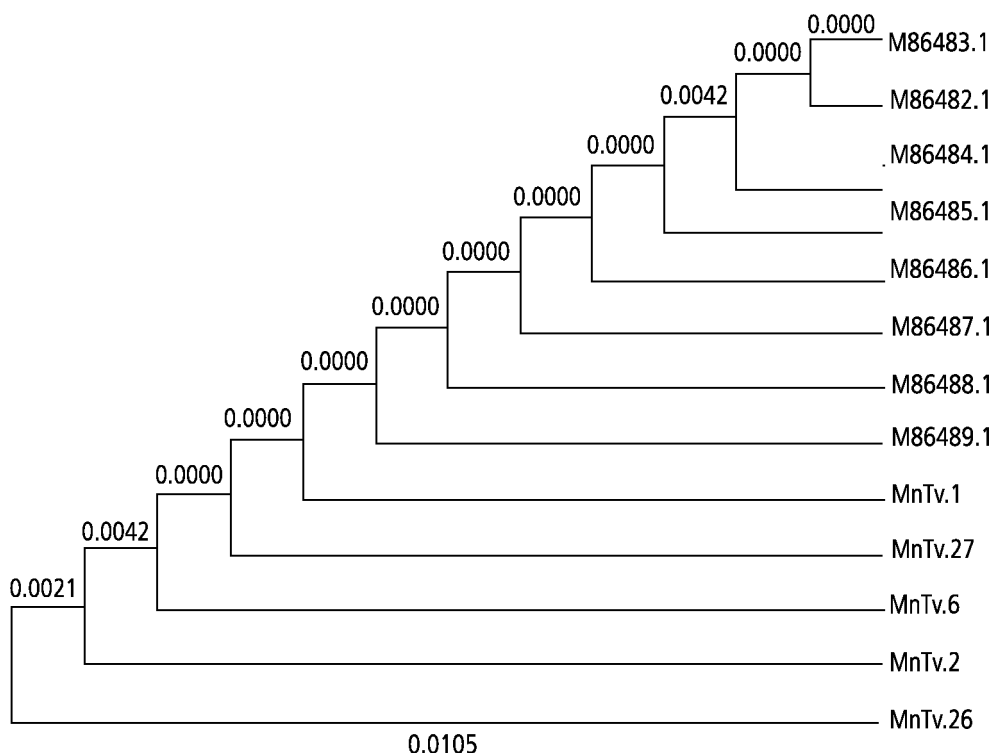


Figure 5. Inter-species type of phylogenetic tree showing the potential distance between isolates based on Tv-E650 DNA sequences. Each 0.0001 unit shows single nucleotide difference in every 10000 nucleotides between samples.

Discussion

In this study, we examined trichomonads using wet mount examination, Pap smear, Diamond’s culture, and PCR on samples from 109 women examined at Ulaan Tuuz Hospital, National Center of Communicable Disease, Ulaanbaatar. Of 109 samples, 17 (15.5%) were positive by all detection methods. Among the listed methods, PCR is shown to have highest detection rate and sensitivity (36.6%, 40 of 109 isolates).

Accurate diagnosis of *T. vaginalis* can be affected by patient factors, clinician’s experience, specimen sampling, processing and test interpretation as well as the skill set and expertise of those doing microscopic assessments. On the other hand, the

sensitivity, specificity, cost, ease of use, and time to results for the different methods for diagnosing *T. vaginalis* infection should be also considered [17]. Wet mount is the most available and commonly used in clinical practice, as it is inexpensive, and easy to perform. However, *T. vaginalis* can produce an overwhelming inflammatory response, concealing the parasites, or the number of organisms may be very low. Thus, even with skilled diagnosticians, the diagnostic sensitivity of wet mount is only 60% [22]. Our results showed that wet mount method’s specificity is 98% and sensitivity is 55%.

Therefore, previous studies recommended the culture method, advised by many authors who reported sensitivity approaches 100% and that as few as one parasite in the sample

may be detected [23]. Unfortunately, culture is not available in most clinical laboratories and our results showed that although the culture method's specificity is 100%, the sensitivity is only 77.5%. Pap smears were interpreted as positive for trichomonads in 20 (18.3%) of 109 suspected vaginitis patients, but one of these 20 cases was not confirmed by PCR and culture, 3 cases were negative by wet mount. An additional, 20 patients were identified as positive by PCR, but of these there were no positive patients by Pap smear. PCR for infection diseases such as gonorrhea and chlamydia is shown to have greater sensitivity than culture methods [24, 25]. Our results show the clinical usefulness of PCR in the diagnosis of trichomoniasis by comparing with other conventional tests.

Moreover, we investigated noncoding repetitive DNA sequence known as Tv-E650 for the potential characterization of local or endemic isolates in Mongolia. Our results showed high sequence uniformity among five isolates that were checked from Mongolia. The further blast with the same part of Tv-E650 repetitive DNA sequence of isolates from different regions in Czech Republic showed high uniformity between isolates from Mongolia and the Czech Republic by its similar high conserved characteristics [21]. The only possible remark is the absence of 28 nt part from the repetitive DNA part in Tv-E650 sequence we checked. The deletion of specific 28 nt part is found to be uniform among all five checked isolates from Mongolia. The data of isolates from the Czech Republic show that not all, but five of eight samples have this similar absence.

Studying genetic polymorphisms of the pathogen and using the results for identification of specific antibiotic-resistant or strong disease-causing isolates are being done in several types of STIs [26-28]. A few published reports using multilocus sequence typing RFLP and RAPD showed clear polymorphisms on its genomic parts such as 5.8S ribosomal gene, Hps70 and actin genes, between isolates from different countries or different cities in the same country [29-31]. In our further study, we are planning to use PCR for detection of trichomonads in male urine specimens, to compare strains with more variation in geographic areas, genetic differences and less conservative genomic regions of *T. vaginalis*. Therefore, we will investigate inflammatory responses in *T. vaginalis* infection and interaction between host-parasite relationships.

In conclusion, PCR-based analysis on the repetitive sequence Tv-E650 could detect *T. vaginalis* in vaginal discharge at a

concentration as low as one trophozoite. The Tv-E650 repeated sequences seem to be applicable for the identification and detection of *T. vaginalis* in Mongolia.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

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