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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Molecular characterization of *Trichomonas* infections in women of Ilam City, southwestern Iran

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Abstract

Trichomoniasis is a sexually transmitted infection (STI) caused by the flagellated protozoan *Trichomonas vaginalis*. Little information is available on the epidemiology and genetic diversity of *T. vaginalis* in Ilam City, southwestern Iran. A descriptive cross-sectional investigation was carried out between July 2017 and December 2018 on the suspected women patients referred to eight gynecology clinics of Ilam City for probable *Trichomonas* infection. They were undergone a set of clinical, parasitological, and molecular examinations. During clinical consultation, posterior vaginal fornix secretions and urine samples were gathered from the participants. For the reasons such as physical conditions and cultural and religious constraints, most of participating women, especially young girls due to their virginity, preferred to give urine samples instead of vaginal discharge. The presence of *Trichomonas* was diagnosed by microscopic examination and molecular detection using conventional PCR targeting ITS1-rDNA. A total of 1765 suspected individuals were examined clinically via vaginal secretions (495 specimens) and urine samples (1270 specimens). Of them, 21 (1.18%) cases, including 13 vaginal secretions and 8 urine samples, were positive for *Trichomonas* infection by microscopy. Slightly more than half of the patients (11/21, 52.4%) complained of vulvar itching, burning, and frequent urination. Cervical lesions, patchy erythema, and vaginal discharge were recorded in 28.6%, 23.8%, and 19% of the patients respectively. All patients with positive microscopic identification were confirmed by amplification of 450-bp fragment of ITS1-rDNA. Phylogenetic analysis revealed a high rate of genetic homogeneity in which all our isolates together with homologous sequences from China, Philippines, Austria, and USA were clustered within the same clade. A statistically significant relationship was recorded between the patients positive for trichomoniasis and the presence of chronic disease (e.g., diabetes, immune system deficiency).

Keywords *Trichomonas vaginalis* · Molecular characterization · Genetic diversity · ITS1-rDNA · Ilam

Introduction

Trichomoniasis is an infectious disease caused by a single-cell flagellated protozoan that lives in the human genito-urinary tract (Menezes et al. 2016). With 276.4 million new

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infections each year and a prevalence between 0.9% and 80%, *T. vaginalis* infection mainly affects the people living in developing countries (WHO 2012; Gatti et al. 2017). In the Americas, the disease affects 22% of women and 2.2% of men between 15 and 49 years old, while its prevalence in Africa is 20.2% and 2%, respectively (Bouchemal et al. 2017). Studies in India revealed an incidence ranging from 6.8 to 10% in different geographical areas (Kaur et al. 2008). A prevalence rate of around 4% was reported in Brazil (Alves et al. 2011). Strikingly, in Europe, the epidemiologic survey in Denmark, Great Britain, or France highlights a prevalence of 0.3 to 1.7% (Pereyre et al. 2017; Field et al. 2018). In Iran, a meta-analysis conducted recently on the *T. vaginalis* infection disclosed an infection rate of 8% among the general population of Iranian women (Hezarjaribi et al. 2015). Age, education, accessibility to health care, and the type of diagnostic methods are among the factors that may explain the difference in prevalence rates reported from various locations (Ambrozio et al. 2016). Trichomoniasis is the most common non-viral sexually transmitted disease (STD) in the world, particularly in populations with high-risk behaviors, such as low sexual hygiene or multiple sexual partners (Arbabi et al. 2018). *T. vaginalis* causes a variety of clinical manifestations in women such as abnormal malodorous vaginal discharge, vagina erythema, vaginitis, vulvar itching, inflammation, adverse pregnancy outcomes, infertility, premature birth, low birth weight, petechiae on the cervix, pelvic inflammatory disease, and cervical neoplasia (Swygard et al. 2004; Abramowicz 2004; Uneke et al. 2007; Kissinger 2015). These variable clinical manifestations may be associated with genotypic strain variation in *T. vaginalis* (Meade et al. 2009; Cornelius et al. 2010). The vaginal pH (typically pH 4.5) often rises above 5 or 6 during *T. vaginalis* infection (Petrin et al. 1998; Lin et al. 2021). Women are at higher risk of getting infections than men (WHO 2001; Daugherty et al. 2019). Disease in men tends to be "clinically asymptomatic," but dysuria, urethritis, epididymitis, and prostatitis are the recorded symptoms (Doxtader and Elsheikh 2017). *T. vaginalis* is often associated with acquisition and transmission of the human immunodeficiency virus (HIV) or human papillomavirus (HPV) infections (Kissinger and Adamski 2013; Masha et al. 2019). *T. vaginalis* infected patients were 1.5 times more likely to acquire HIV than individuals uninfected with *T. vaginalis* (Masha et al. 2019). Additionally, it may be a threefold increase in a potential risk factor to develop cervical cancer (Sayed el-Ahl et al. 2002).

Recent investigations on the genetic characterization of *Trichomonas* parasites reveal that the significant genetic diversity of the parasite has resulted in extensive clinical variability in trichomoniasis. Consequently, it implies an association between the genetic diversity of the parasite and the clinical manifestations exhibited by the patients (Meade and Carlton 2013). However, despite several investigations focusing on trichomoniasis in Iran, little is known about the prevalence, intraspecific variability, phylogenetic relationships, and polymorphisms within the *Trichomonas* species in Ilam City. Therefore, the present study was conducted to determine the prevalence and genetic diversity of *T. vaginalis* in women referred to gynecology clinics in Ilam City in southwestern Iran and to evaluate the epidemiological and clinical variables that govern the prevalence of diseases in this city.

Material and methods

Samples and clinics

This descriptive cross-sectional investigation was performed from July 2017 to December 2018 on the women referred to the Gynecology Clinics of Ilam City, southwestern Iran. At arrival, all participants were subjected to clinical examinations. The vaginal secretion from the posterior vaginal fornix was sampled separately using three sterile cotton swabs. One swab was used for wet mount preparation to search the presence of flagellated motile organisms by microscopic examination. A second swab was used for smear preparation, stained with Giemsa, and examined under the light microscope. The third swab was inoculated immediately into a sterile tube containing 1 ml of physiological saline buffer (NaCl) for in vitro cultivation and subsequent molecular analysis. In addition, 20 to 50 ml of urine was collected separately into a sterile container from the same individuals. Samples were centrifuged at 2000 rpm for 5 min. The pellet was used (i) to prepare Giemsa-stained smears for microscopic wet-mount examination and (ii) after washing with 500 µl of PBS for molecular analysis. For the reasons such as physical conditions and cultural and religious constraints, most of participating women, especially young girls due to their virginity, preferred to give urine samples instead of vaginal discharge, which led to an inequality in the number of samples processed. Furthermore, the epidemiological (e.g., age, sex, and marital status) and clinical (e.g., clinical symptoms, cause of referral, previous disease background, drug consumption, and pregnancy outcome) information were recorded for each patient using a questionnaire. The isolates positive in microscopy were further analyzed by molecular approach.

DNA extraction and PCR amplification

According to the manufacturer's instructions, DNA extraction was performed using a DNG-plus™ kit (Cinna Gene, Iran). DNA yield was quantified using NanoDrop (Thermo Scientific, USA) and then subjected to a conventional PCR targeting the ITS1 rDNA region, using species-specific

primers (forward: 5′-GTTAATGGCAGAATCTTTGCAG-3′ and reverse: 5′-CTC GCAGTCCTATTGATCCTAAC-3′). The reactions were set for a final volume of 25 µl, containing 12.5 µl Master mix RED (Gold-Amplitech), 1 µl (10 pmol) of each forward and reverse primers, 2 µl of DNA templates, and 8.5 µl of double-distilled water. PCR amplification included 35 reaction cycles, each cycle consisting of 10 s at 94 °C, 45 s at 45.2 °C, and 15 s at 72 °C with a final extension step for 5 min at 72 °C. A couple of previously extracted *Trichomonas* DNA and distilled water were used as positive and negative controls. Amplicons were visualized after electrophoresis in 1.5% agarose gel containing SYBR Safe (Invitrogen, USA).

Phylogenetic reconstruction and species assignation

Amplicons were purified using a BigDye XTerminator purification kit (Thermo Fisher, USA) and sequenced in both directions, with primers used for the primary PCR. Sequences were trimmed and compared with homologous sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST). Based on ≤ 99% identity with the sequence deposited in GenBank, the strains were identified at the species level. To evaluate the intraspecific variability, phylogenetic relationships, and polymorphisms within *Trichomonas* species, the ITS1 sequences of our specimens were aligned against the GenBank sequences using BioEdit v7.0.0 software (Hall 1999). The phylogenetic analysis was carried out using MEGA v.6 software. An inferred phylogenetic tree of *Trichomonas* strains, obtained in this study and GenBank sequences, was constructed based on the neighbor-joining (NJ) method with bootstrap values determined by 1000 replicates.

Statistical analysis

The Pearson chi-square test was used to evaluate the correlation of various clinical and epidemiological factors influencing the disease using SPSS v. 18 software.

Results

In total, 1765 women were referred to eight Gynecology Clinics in Ilam City, southwestern Iran. The posterior vaginal fornix secretions and urine samples were taken from 495 and 1270 women, respectively. Among these samples, 21 (1.18%) were positive for *T. vaginalis* infection after the microscopic examination. These included 2.6% (13/495) individuals after posterior vaginal fornix secretion examination and 0.6% (8/1270) positive after urine examination. The infected women ranged from 15 to 50 years old, with higher positivity between 25 and 34 (47.6%). Slightly more than half of the patients (11/21) complained of vulvar itching, burning, and frequent urination. The cervical lesion was recorded in 28.6% of patients, the most commonly reported clinical disorder, followed by patchy erythema (23.8%) and vaginal discharge (19%). No history of treatment was recorded in most of the patients examined (81%). Abortion and preterm birth were reported in 9.5% and 4.8% of the patients, respectively. More than half of the patients (61.9%) used contraceptive prevention pills, 19% used IUD (intrauterine device), whereas the remaining (19%) present no history of prevention pill consumption or IUD installation. All patients were married. Epidemiological and clinical information of the processed patients are given in Table 1. The analysis of the data collected during this survey discloses a statistically significant relationship (p-value < 0.05) between trichomoniasis and chronic disease history (such as diabetes and immunodeficiency). No correlation was observed between trichomoniasis and age groups, clinical symptoms, history of abortion, or the use of contraceptive tools (Table 1).

All results gathered after microscopic examinations were confirmed by amplifying the 450-bp fragment of ITS1-rDNA (Fig. 1). Direct sequencing of the PCR products allowed us to confirm *T. vaginalis*, with ≥ 99% identity with reference sequences from GenBank. Sequences obtained in the present study were deposited in GenBank under the accession numbers of MK953695 to MK953713. The phylogenic reconstruction demonstrates that all samples collected in the Ilam City fall within the *T. vaginalis* clade, genetically close to *T. vaginalis* sequences from other countries such as China, Philippines, Austria, or the USA (Fig. 2).

Discussion

During the last two decades, the incidence of sexually transmitted infections (STIs) has dramatically increased, being a significant public health problem in developing countries. In Iran, several investigations on the disease incidence have been conducted (Akhlaghi et al. 2005; Mazloumi Gavgani et al. 2008; Rezaeian et al. 2009), but the epidemiologic data are still missing in some regions, and surprisingly little is known about the genetic variability of *Trichomonas* species in Ilam City. Furthermore, the recognition of disease epidemiology in some areas of this province is still incomplete. In Ilam, a single investigation performed by Nikpay et al. (2020) revealed that 7 out of 481 (1.5%) women referred to a central laboratory and Shahid Mostafa laboratory for suspicious *Trichomonas* infection were found positive after direct microscopic examination of vaginal smears. The more extensive investigations were performed in our previous (Alikhani

Table 1 Detailed epidemiological and clinical information gathered from the patients infected by *Trichomonas vaginalis* in Ilam City, southwestern Iran

Epidemiological/clinical information		Number of processed individuals	Number of positive cases	Infection rate (%)	*p* value
Demographic information	‹15	12	0	0	0.44
	15–24	177	1	0.56	
	25–34	759	10	1.31	
	35–44	599	7	1.16	
	44–49	136	2	1.47	
	≤50	82	1	1.21	
Clinical symptoms	Gray color discharge	184	1	0.54	0.41
	Erythema and vaginal edema	310	1	0.32	
	Patchy erythema	219	5	2.28	
	Cervical lesions	442	6	1.36	
	White color discharge	330	4	1.21	
	No symptoms	280	4	1.43	
Drug use	Yes	144	4	2.78	0.06
	No	1621	17	1.05	
Pregnancy preventive method	Condom	207	0	0	0.28
	Contraceptive pills	842	13	1.54	
	IUD	256	4	1.56	
	Tubal ligation	113	0	0	
	No prevention	347	4	1.15	
Pregnancy outcome	Preterm infant birth	73	1	1.37	0.11
	Normal infant birth	1015	17	1.67	
	No pregnancy	565	1	0.18	
	Abortion	105	2	1.9	
	Death	7	0	0	
Disease background	Diabetes	17	4	23.5	0.04
	Immunodeficiency	9	2	22.2	
	No background	1739	15	0.86	

et al. 2021) and present studies encompassing the patients of eight gynecologic clinics across the city using two different approaches. These are the first molecular characterization and genetic diversity of *Trichomonas* parasites performed in this region of southwestern Iran.

Overall, *T. vaginalis* was detected by microscopic or molecular analyses in 21 out of 1765 (1.18%) patients. These results are consistent with two studies previously performed in Robat Karim or Ilam (Akhlaghi et al. 2005; Nikpay et al. 2020). In a meta-analysis carried out from 1992 to 2012 in Iran, the processed patients had an average age of 24.5 with a maximum and a minimum of 45 and 22.5 years old (Hezarjaribi et al. 2015). The minimum and maximum prevalence rates of *T. vaginalis* infection were recorded at Kermanshah (0.009%) and Tehran (38.8%) cities. In a study carried out by Bakhtiari et al. (2008), the prevalence of trichomoniasis was low (4%) in Babol City, north of Iran, with a decreased prevalence over the past decade. Considering the infection rate of women processed in the present study (1.2%), Ilam City appears to be a region with a low infection rate, agreeing with previous findings (Nikpay et al. 2020).

Various factors are suspected to affect trichomoniasis infection risk, including age, sexual activity, number of sexual partners, sexual behaviors, hygienic situation, educational level, socioeconomic condition, vulnerability in moral condition, cultural-religious beliefs, and methods of diagnosis (Van Der Pol 2007; Poole and McClelland 2013; Rowley et al. 2019). Therefore, low prevalence of *Trichomonas* infection we report here can be linked to (i) religious beliefs of processed people (Madani 2006), (ii) socio-cultural condition, and finally (iii) low population density of this city (194,030 inhabitants). Women between 25 and 34 years old are at the highest risk due to higher sexual activity in these young women (Schwebke and Burgess 2004; Ibáñez-Escribano et al. 2014; Hezarjaribi et al. 2015). Vaginal disorders, as one of the known symptoms observed among women infected by *T. vaginalis* (Taghavi et al. 2014; Nazari et al. 2015), were also recorded in our

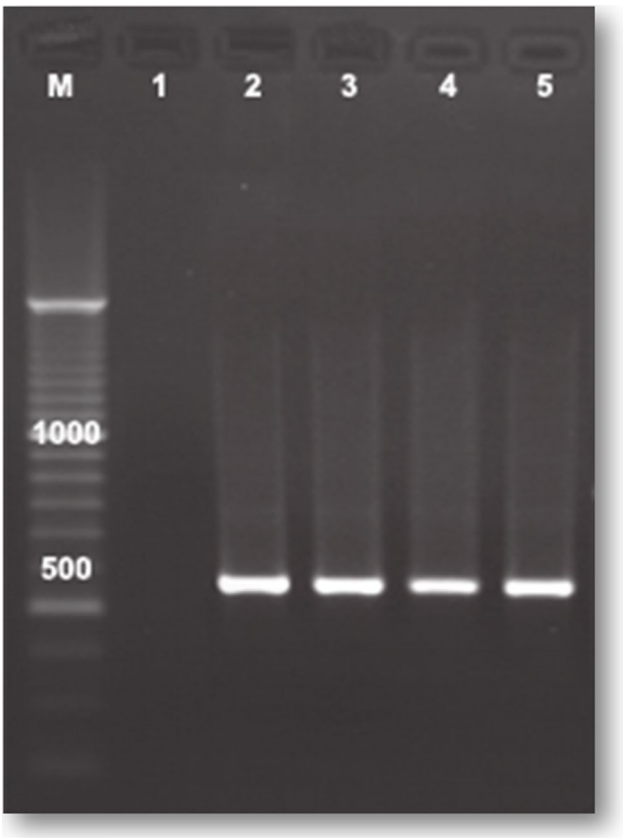

Fig. 1 ITS1-rDNA amplification of *T. vaginalis*. *M* molecular size marker (bp), *lane 1* negative control, *lane 2* positive control, *lanes 3 to 5* patients found to be positive for *T. vaginalis* infection

study. More than half of positive patients (11/21, 52.4%) complained of vulvar itching, burning, and frequent urination. Abortion and preterm delivery, as two common disorders among women with trichomoniasis, were recorded in 9.5% of women with *Trichomonas* infection. Considering that sexual contact is the primary mode of disease transmission, the use of condom will be the best option to reduce disease transmission (Moodley et al. 2002; Taghavi et al. 2014). In our study, none of the patients reported condom usage. Nineteen percent of the patients examined in this study had a history of chronic diseases (e.g., diabetes, immune system deficiency). Statistical analysis revealed a significant relationship between the patients with a history of these chronic diseases and trichomoniasis (p-value < 0.05). These findings agree with those reported by Younis and Elamami (2016) and Kalra and Kalra (2017).

Various methods such as wet mount, culture, Pap smear, polymerase chain reaction (PCR), and serological tests have been used for diagnosis of trichomoniasis (Garber 2005). The presence of parasites can be diagnosed in the Papanicolaou smear, but it is not a reliable method due to low sensibility (Bowden and Garnett 2000; Aslan et al. 2005). Moreover, the lack of sensitivity and specificity of serological examines is the major limitation for detecting *T. vaginalis* by indirect serological testing (Ryu and Min 2006). Wet mount preparation is the most commonly used diagnostic test (Van Der Pol 2016a, b). Although the culture of *T. vaginalis* from a clinical specimen is a reliable diagnosis way, it presents some limitations like (i) contamination issue and (ii) lag of up to 7 days for *T. vaginalis* identification (Van Der Pol 2016a, b). On the other hand, the diseases like candidiasis or bacterial-caused vaginitis present clinical manifestations similar to trichomoniasis. Considering the similarity in clinical manifestations, the accurate identification of the causative agent is a prerequisite to deliver a proper treatment (Schwebke and Burgess 2004; Ijasan et al. 2018). The successful molecular characterization of *T. vaginalis* by conventional PCR amplification of ITS1-rDNA was previously reported (Kim et al. 2016; Moradi et al. 2017). Herein, we confirm the molecular approach as a reliable method for identification and genetic studies in trichomoniasis.

An exciting feature of *T. vaginalis* is its high-level diversity. This heterogenetic protozoan has a specific population structure, including two different types reported worldwide (Conrad et al. 2012). Genotyping on 235 isolates from Mexico, Chile, India, Australia, Papua New Guinea, Italy, Africa, and the USA revealed two distinct lineages of *T. vaginalis* strains in equal proportions worldwide (Conrad et al. 2012). Another investigation in the Philippines displayed that all the ITS1 sequences clustered in a single clade, demonstrating low genetic polymorphism (Rivera et al. 2009). Ibáñez-Escribano et al. (2014) reported 99.7% ITS nucleotide sequence identity among the vaginal specimens isolated from women in Madrid, Spain. Based on CSGE (conformation-sensitive gel electrophoresis) and PCR analysis of the vaginal isolates in Tehran (Iran), 3.9% presented a nucleotide mutation in the ITS1 fragment (Kazemi et al. 2010). Using the same target gene (ITS1), Matini et al. (2012) reported two distinct reproducible banding patterns (types I, II) among the patients originating from Hamadan and Tehran in Iran. In Ilam, our earlier study demonstrated the presence of two genotypes (G and E) of *T. vaginalis* within amplified fragments of Actin gene in ten isolates (Alikhani et al. 2021). Nevertheless it was not able to obtain deep insights from the city of Ilam due to limited number of processed specimens while the current study, using a different set of primers (ITS1) in contrast to Actin, allowed more details to emerge from strains obtained from Ilam. Based on phylogenetic analysis, *T. vaginalis* isolates demonstrated a high genetic homogeneity which clustered in a well-differentiated clade, supported by a bootstrap value of 100%, indicating no hybrid or intraspecific taxa among processed isolates (Fig. 2). Consequently, a low genetic diversity was observed among *T. vaginalis* isolates of Ilam City.

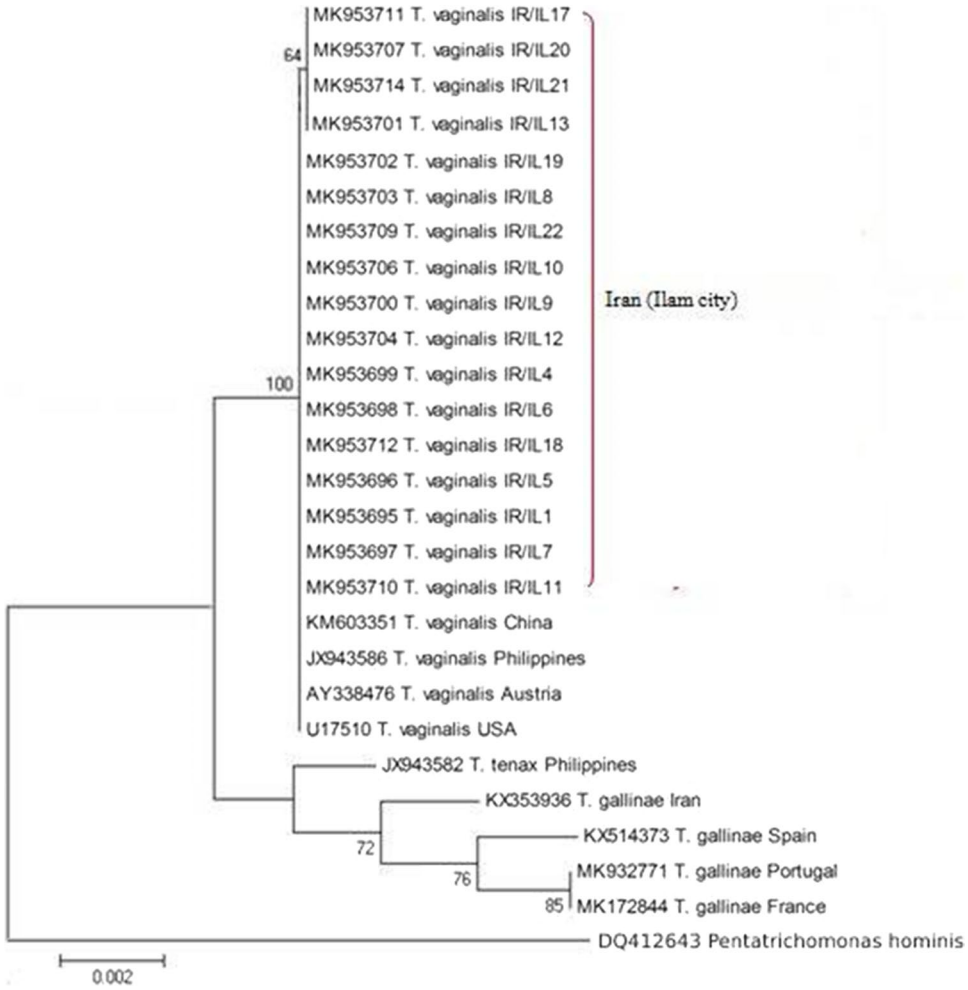

Fig. 2 Neighbor-joining phylogenetic tree constructed based on ITS1-rDNA sequences of *Trichomonas vaginalis* obtained in the present study (highlighted in red) together with those deposited in GenBank

Conclusion

The incidence of trichomoniasis is low in the city of Ilam (21/1765; 1.18%), and the disease affects primarily women aged from 25 to 34 years old (47.6%), associated significantly with chronic disease background (such as diabetes, immune system deficiency). *T. vaginalis* strain processed in this study presents high homogeneity clustered with the strains reported from other countries.

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Declarations

Ethics approval The study was approved by the Ethics Committee of Ilam University of Medical Sciences, with the Code of Ethics: IR.MEDILAM.REC.1394.124.

Conflict of interest The authors declare no competing interests.

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