Genetic Variation of *Trichomonas vaginalis* Isolates from Iraqi Women: Association with Fertility and Cervical Abnormalities

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Abstract

Background: *Trichomoniasis*, is one of the most common non-viral sexually transmitted diseases caused by the parasite *Trichomonas vaginalis*. Little is known about the genetic diversity and population structure of this parasite. This study aimed to determine the genetic diversity of *T. vaginalis* isolated from Iraqi women and its association with the fertility and the cervical abnormalities.

Methods: Overall, 154 Iraqi women attending the Gynecology Outpatient departments in Baghdad Province, Iraq from February 2013 to April 2014, were enrolled in this study. DNA of *T. vaginalis* isolates was extracted from the culture of

high vaginal swabs. Multilocus sequence typing (MLST) method for six housekeeping genes was done in studying the genetic variations.

Results: Fifty-three women (34.41%) were positive for *T. vaginalis*. MLST method resulted in different alleles. With glutaminase gene, the highest degree of variation was found among the six genes.

Conclusion: The genetic diversity in the organism itself in Iraqi isolates can associate with clinical outcome. Further MLST studies are needed to compare a larger number of isolates from different localities and correlate the certain mutations in housekeeping genes to infertile women and patients with cervical abnormalities.

Introduction

Trichomonas vaginalis is a flagellated parasite found in the human vagina and urethra [1]. It causes the most common, non-viral, sexually transmitted disease in the world. Overall, 160 million cases of infection are acquired yearly worldwide [2]. Population of developing countries is the majority of affected cases by this parasite [3].

Symptoms in women range from malodorous vaginal discharge, inflammation, and swelling of the urogenital tract to increased risk for cervical cancer and adverse pregnancy outcomes [4, 5]. “In addition, the risk of tubal infertility was almost twice as high in women who recalled previous trichomoniasis, compared with women with no such infection” [6]. The incidence rate of trichomonal infections among infertile couples is higher than that among fertile couples [7]. The World Health Organization (WHO) defines infertility by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected intercourse. Primary infertility is inability to get pregnancy. Secondary infertility is a failure to conceive following a previous pregnancy [8, 9]. Trichomoniasis is suggested by

punctate hemorrhages over the vagina and cervix, the so-called strawberry cervix [10].

The new methods of diagnosing and treating the disease are shown with the cracking of the genome of the *T. vaginalis* [11]. Genetic diversity caused by selection and different mutational and sexual events rests on genome changes ranging from a single base-pair exchange to changes of entire chromosomes [12]. Multiple methods for typing *Trichomonas* isolates have been designated, and these studies produced different results, even when using similar techniques [13,14,15].

"MLST method, requiring only the ability to amplify DNA fragments by PCR and to sequence the fragments"._Using single-copy gene for this purpose has several advantages over gene with multiple copies. It allows highly accurate diversity measurement and phylogenetic relationships [16]. The present study aimed to search this intragenomic heterogeneity of *T. vaginalis* isolates and its association with the fertility and the cervical abnormalities.

Materials and Methods

One hundred fifty- four samples were collected from women attending the Gynecology out -Patient departments in Baghdad, Iraq during the period from February 2013 to April 2014. Based on availability of full clinical information (A questionnaire sheet was filled out) about each patient, high vaginal swabs were taken from 80 fertile and 74 infertile (29 primary infertile and 45 secondary infertile) women at different ages from 15-54 yr. During this process, examination by a gynecologist for cervical abnormalities was done. Ethical clearance was obtained the committee of ethical standards in the Collage of Medicine/ University of Al-Nahrain, and informed consent was obtained from patients.

The swab was pressed between the In-Pouch TV System media (Biomed Diagnostics, Inc. USA). The culture was examined daily for three days before being considered negative. Then positive culture inoculated into culture tubes containing *Trichomonas* modified medium (CPLM), incubated at 37 °C supplemented with 10% inactivated horse serum and antibiotic solution (50 µg of gentamicin/ml, 40 µg of ciprofloxacin/ml, and 50 µg of miconazole /ml). The culture was examined daily until the culture populations were in the log phase of growth at an inoculating concentration of 10^6 cells/ml by using Neubauer chamber slide. These cells were collected by centrifugation for 15 min at 500 xg at 4 °C. Pelleted cells were washed twice with phosphate-buffered saline (PBS pH 7.4). Washed pellets were stored in -20 °C prior to DNA extraction [17].

DNA extraction: for DNA extraction from *Trichomonas* cultured cells, a ready QIAmp DNA mini kit (Qiagen Catalog no. 51304) was used according to the manufacturer's instructions.

PCR amplification and nucleotide sequence determination: Six single-copy housekeeping genes of *T. vaginalis*, were characterized based on their diversity. Primers for MLST loci were mentioned [17]. These primers were designed from Eurogene kit primers (Eurogene, UK) to amplify gene fragments of 450 to 500 bp for the six genes; Tryptophanase (P1) *tryp*, Glutaminase (P3) *glut,* Family T2 asparaginase-like threonine peptidase (P6) *ft2a,* Alanyl tRNA synthetase (P8) *alts,* DNA mismatch repair protein (P13) *dmrp* and Mannose 6-phosphate isomerase (P16) *m6pi* also mentioned in part I of this article [18].

Sixteen isolate were typed. The six genes were amplified at a final volume of 50µl using Hot Start Taq Master Mix. The master mixture contained 0.2 µM

concentrations of forward primer, 0.2 µM concentrations of reverse primer, 2.5 mMMgCl2, and 0.5µg of template DNA. Amplifications were performed using the Applied Biosystems thermal cycler (Applied Biosystems USA). Thermocycler program conditions were the same for all loci as follows: 95 °C for 5 min; followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min; followed in turn by 72 °C for 10 min. Three µl of each reaction was visualized on a 1.5% agarose gel to verify the amplification. The remaining 47 µl of PCR product were purified prior to sequencing using Charge Switch PCR clean-up kit according to manufacturers‟ instructions.

After purification of PCR products and determine the product quality using gel electrophoresis, the purified products were used as a template sequence cycle using ABI big dye terminator ready reaction kit.

After setup for sequencing, the plate was placed in the 3730 sequencers. Then the data were examined by using the Mutation Surveyor software.

Nucleotide sequence analysis software:

A sequence Alignment search was conducted between the sequencing results of PCR products and the sequence of standard gene by BLAST program (details of *T. vagialis* G3 (ATCC Pra98 [http://trichdb.org /trichdb/]), available at the national center biotechnology information (NCBI) online at (http://www.ncbi.nlm.nih.gov)).

Results

By the use of In-Pouch TV culture, out of 154 examined women patients at the age ranged between 15 and 54 yr with a mean of 34.5 yr, 53 (34.4%) were infected with *T. vaginalis*. The isolates have a range of morphologies.

The women participated in this study were categorized according to the baseline of fertility and infection with *T. vaginalis* as in (Table 1). Then a comparison between the two classes of infertility and fertility cases were shown in (Table 2). Cervical abnormalities in women represented 41.5% (22/53) according to the examination of the gynecologist for infected women.

Table 1: Comparing between fertile and infertile women according to the percentage of infected cases.

Studied group	No. of tested cases (154)	No. of infected cases and %	χ^2	P- value
Fertile	80	29 (36.25%)	1.028	0.392 NS
Infertile	74	24 (32.43%)		

NS: Non significant

Table 2: Comparing among fertile, primary infertile and secondary infertile women according to the percentage of infected cases.

Studied group	No. of tested cases (154)	No. of infected cases (53) and %	χ^2	P- value
Fertile	80	29 (54.71%)	5.269	0.0271*
Primary infertile	29	7 (13.20%)		
Secondary infertile	45	17 (32.07%)		

***(*$P \leq 0.05$*)**

Sixteen isolates were typed, seven of them were not typed because the original primers failed to generate an amplicon.

The obtained sequences of the six housekeeping genes and the mutations were denoted by the first letter indicating the nucleotide or amino acid present in the genome sequence of reference strain G3 (ATCC Pra98 [http://trichdb.org /trichdb/]), the number indicating the position of the change relative to the open reading frame, and the second letter indicating the variant nucleotide or amino acid.

Analysis of the Loci Sequence Data

The results reflect P1 locus (*tryp* gene) showed the mutation *tryp* A48328G which belong to isolates of secondary infertile patients with and without cervical abnormalities as shown in (Table 3).

Table 3: The sequencing result of *tryp* (P1) locus

Mutation	Type	Position	Wild type codon	Mutated codon	Change of amino acid	Effect on translation	No. of isolates (4)
A > G	Transition	c.48328	CC**A**	CC**G**	P16110 P	Silent	4
C > A	Transversion	c.48349	TT**C**	TT**A**	F 16117 L	Missense	2

The missense mutation *tryp* C48349A was appeared in isolates belong to cervical abnormalities patients only in addition to the mutation *tryp* A48328G. A new allele found in two isolates' sequence results belong to secondary infertile patients had cervical abnormalities, with sequence closest similarity (99%) since there was only one change at base number 48349 bp (C to A).

Many mutations were obtained from the locus *glut* (P3) including presence of double peaked mutations referred to primary infertile women with no cervical abnormality (Fig. 1, 2), which do not occur in other isolates that belong to fertile and secondary infertile women. A number of alleles of this locus in our studied isolates were three, and a new allele was found.

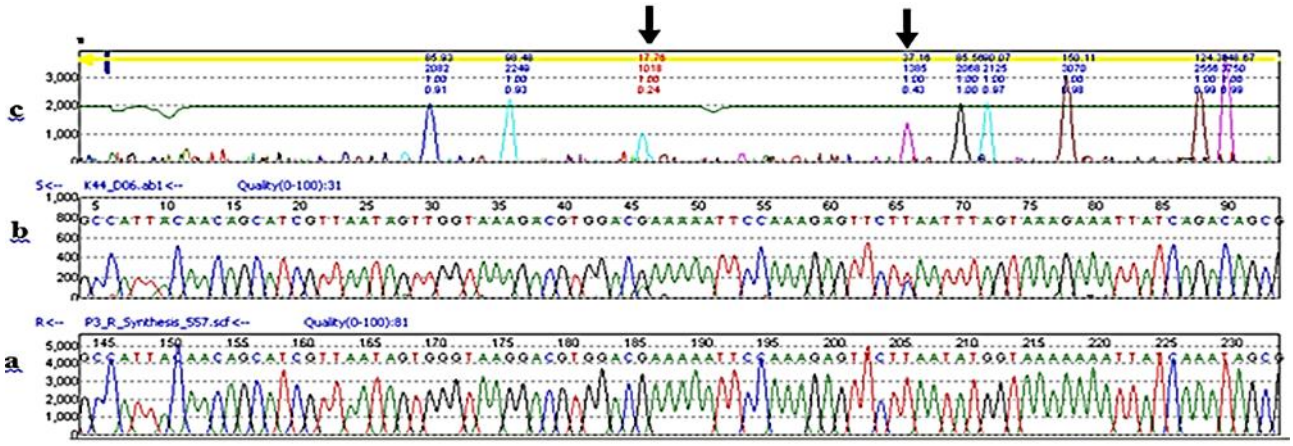

Fig. 1: Sequencing results of *glut*. belong to primary infertile patient with no cervical abnormality a- wild type of *glut* gene, b- the transition of the wild type alleles and c- the mutation surveyor. The arrow refers to the position of double peaked mutation.

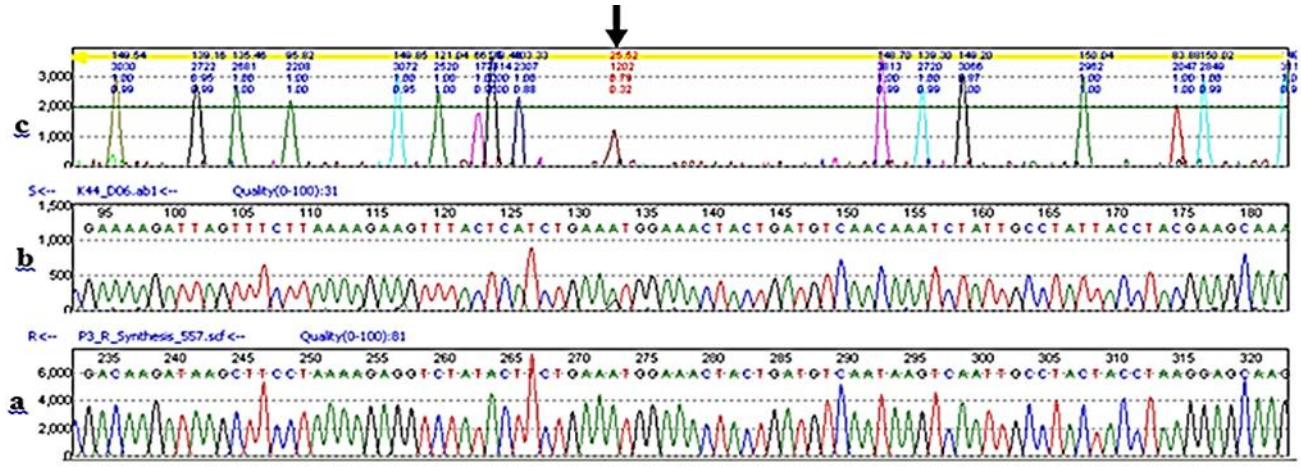

Fig. 2: DNA sequencing results (continue)

As regard to the locus *ft2a* (P6), there was only one missense mutation (Fig. 3) belong to secondary infertile women did not have cervical abnormality (*ft2a* G85398A). Allele number of the P6 locus was one with sequence closest similarity (99%) and there was maybe a new allele (G to A) at base number 85398 bp.

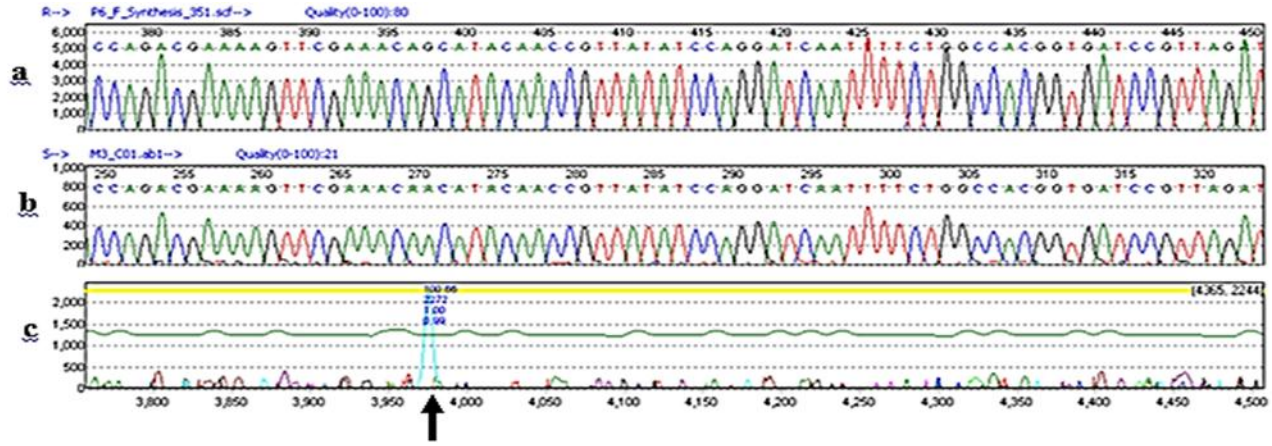

Fig. 3: Sequencing results of *ft2a*. referred to secondary infertile patient did not have a cervical abnormality. a- wild type of *ft2a gene* b- the transition of the wild type alleles and c- the mutation surveyor. The arrow refer to the position of mutation.

Analysis of the locus *alts* (P8) revealed one missense mutation (Fig. 4) *alts* T81244C, which belong to secondary infertile women with cervical abnormalities. MLST software package program to analyze this locus revealed the allele number 1 with closest similarity 99%. A new allele probably was found (T to C) at position 81244bp.

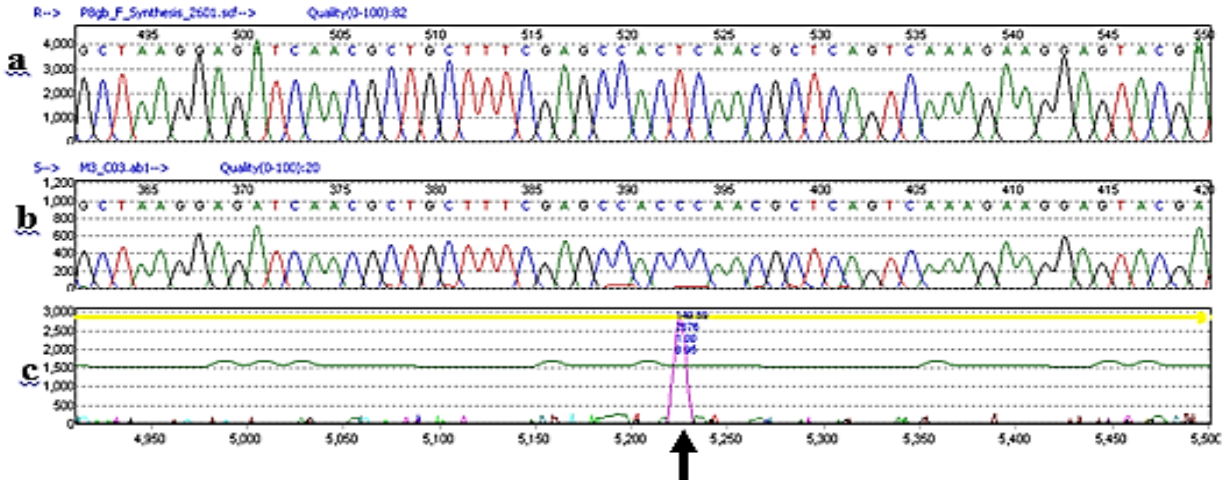

Fig. 4: Sequencing results of *alts* referred to secondary infertile patient have a cervical abnormality.

Locus *dmrp* (P13) showed one silent mutation *dmrp* C14179T referred to isolates of secondary infertile patient with cervical abnormalities (Fig. 5). Search results of this locus revealed allele number 10 to studied isolates.

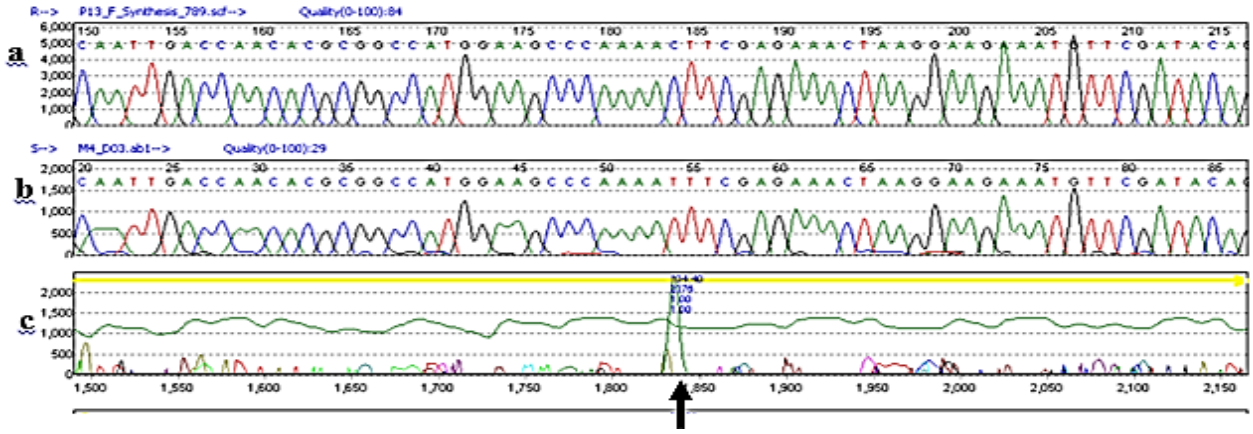

Fig. 5: Sequencing results of *dmrp*.referred to secondary infertile patient with cervical abnormalities.

Finally, the locus *m6pi* (P16) showed one missense mutation *m6pi* G54771T. The mutation belongs to isolates of secondary infertile patients with and without cervical abnormalities (Fig. 6). Allele number of this locus in studied isolates was two.

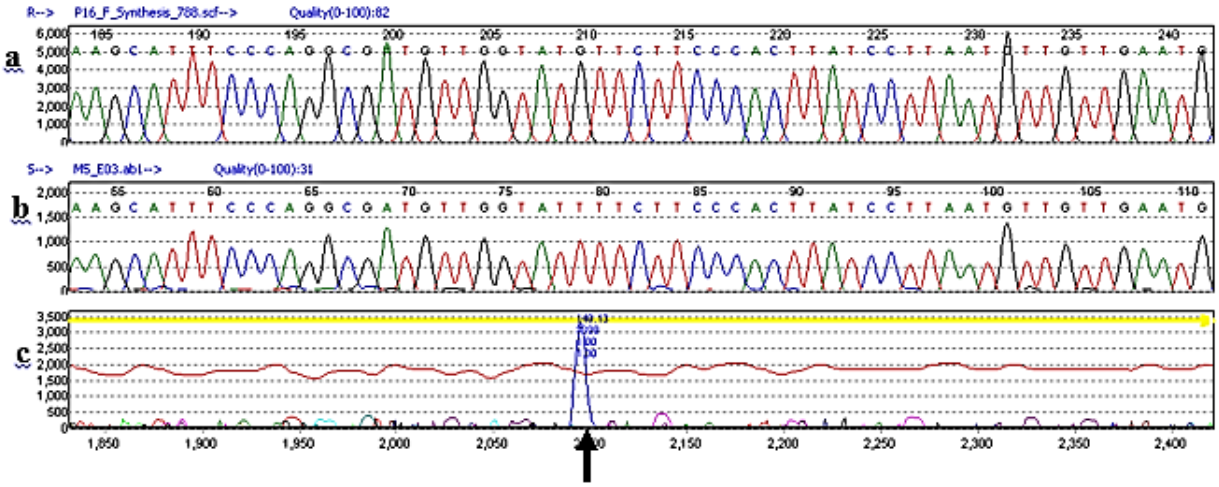

Fig. 6: Sequencing results of *m6pi* referred to secondary infertile patient with and without cervical abnormalities.

Discussion

No significant difference in infection rates was found between the percentages of fertile and infertile patients (Table 1), but there was a significant difference at the level P≤0.05 between primary and secondary infertile patients (Table 2). This was in line with previous study, that women with secondary infertility are more likely to have pathogenic organisms than women with primary infertility [18]. This may referred to infections acquired during a previous delivery especially when the women are delivered in homes by unskilled birth attendants, or lack of asepsis during insertion of Intrauterine devices (IUD) as a type of birth control methods.

We have introduced the first MLST method for investigating the genetic diversity of *Trichomonas vaginalis* in Iraq.

Failure of particular primers to amplify gene fragments may cause by nucleotide mismatches or lacking of primer target sites [19] and the sequence diversity [17].

Sequence analysis of the locus P1 was identified by MLST. Yet one missense mutation in this locus with similarity of 99%, (*tryp* C 48349 A) belong to secondary infertile patients who had cervical abnormalities and did not appear in isolates of cases without cervical abnormalities. This may be of great diagnostic value. On the other hand, *tryp* A 48328 G, which was silent mutation associated with isolates of secondary infertile patients who had cervical abnormalities and who did not have cervical abnormalities cases.

Analysis of P3 gene showed a higher number of mutations comparing to other studied genes with similarity of (87%, 88%, and 98%) to the isolates with a standard subject in BLAST. Sixteen missense mutation scattered across this gene resulted in alteration in encoded amino acids, while the other mutations were silent. The sequence traces (electropherograms) indicated the presence of multiple alleles by the presence of 3 double nucleotide peaks as shown in figure 1 and figure 2; (*glut* G 1189

A), (*glut* T 1169 C) and (*glut* A 1102 G) in two isolates belong to primary infertile women which do not exist in other isolates that referred to fertile and secondary infertile women. The first one (*glut* G 1189 A) was the most important because it can alter the amino acid Glutamic acid to Lysine and this change implies changing from acidic polar to basic polar (negative to positive side chain charge) which would be expected to result in changes in peptide structures and inter and intralineage catalytic/phenotypic differences [20]. The third one was also missense (Methionine to Valine), but those amino acids are similar in most properties. Although the second one was silent, it provides a reasonable biological mechanism for why it would be associated with primary infertile isolates.

There was another double nucleotide peak (*glut* G 871 A) that appeared in two isolates belong to secondary infertile patients who had cervical abnormalities but not in other patients. This mutation altered the translated amino acid from alanine to threonine (nonpolar or hydrophobic to polar), that is mean from amino acid involved in proteins' tertiary structure to amino acid usually found at the surface of proteins. This mutation may have a diagnostic value for cervical abnormality in trichomoniasis patients.

The arginine dihydrolase metabolism is a major pathway for energy production for the parasite. Glutamate dehydrogenase and aminotransferases have a central role in this regard. These pathways are likely catabolic but may be reversible to allow the parasite to synthesize glutamate, alanine, aspartate, glutamine, and glycine [11]. High variability and polymorphism resulted by MLST method in current study, could be attributed to the high variability of this locus [18].

Housekeeping gene *ft2a* (P6) analysis showed one mutation (*ft2a* G 85398 A) which was missense and changed the amino acid from Alanine to Threonine

(nonpolar to polar) in isolates belong to secondary infertile patients. This mutation also expected to result in significant changes in peptide structures as above.

Analyses of alts (P8) gene revealed also one missense mutation (alts T 81244 C) in isolates belong to secondary infertile patients who had cervical abnormalities.

One mutation had been shown in the gene *drmp* (P13) which is (*drmp* C 14179 T). This silent mutation was belonging to secondary infertile patients who had cervical abnormalities. The gene showed 99% similarity. Although the effect of these two mutations (in P8 and P13) cannot be compared with fertile, primary infertile women and no cervical abnormalities women, they can provide an assignment for secondary infertile and cervical abnormalities patients at the same time.

The sixth gene P16 with similarity 99%, exhibited one missense mutation (*m6pi* G 54771 T) in isolates belonged to secondary infertile, cervical and no cervical abnormalities patients simultaneously. This mutation alters the amino acid from Methionine (non-polar sulfur containing) to Isoleucine (non-polar).

Variation in the morphology may cause due to presence of multiple strains or "may be due to intragenomic variation in the certain one or more single copy housekeeping gene causing morphological variation to a certain strain" [18].

Although the study is restricted by the fact that *T. vaginalis* isolates were not shared in more than one or two genes, these data may offer a putative assignment to a known clonal lineage [21] by using only two or three loci. The present data suggest different degrees of conservation in *T. vaginalis* housekeeping genes.

MLST software package program for detecting the allele and allele profile (ST) number of the strain revealed the 22 different alleles for studied six loci and presence of a new allele in four loci [18].

The intragenic diversity in the organism itself may be associated with clinical outcomes [22, 23], and this agrees with recent results of this study and this has important implications for genetic research and controls the disease.

Conclusions:

Based on the findings of the present study, the genetic diversity in the *Trichomonas vaginalis* in Iraqi isolates can associate with clinical manifestations and this suggests that different genotypes are prevalent in Iraq. More researches are needed to compare a larger number of isolates from different areas and correlate the certain mutations in housekeeping genes to understand and prevent the adverse impact of *T.vaginalis* on the reproductive health of women.

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التغاير الوراثي لعزلات طفيلي المشعرات المهبلية المعزول من النساء العراقيات: علاقته مع الخصوبة وتشوهات عنق الرحم

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الخلاصة

الخلفية/ الهدف: داء المشعرات المهبلية هو واحد من أكثر الأمراض غير الفيروسية المنقولة جنسيا, يسببه طفيلي *Trichomonas vaginalis*. لا يعرف سوى القليل عن التنوع الوراثي والهيكل المعيشي لهذا الطفيلي. هدفت هذه الدراسة إلى تحديد التنوع الوراثي لعزلات طفيلي المشعرات المهبلية وارتباطه بالخصوبة وتشوهات عنق الرحم.

الأساليب: تم تعيين مجموعة من ١٥٤ امرأة عراقية ممن يراجعن قسم الولادة والامراض النسائية في عدد من مستشفيات محافظة بغداد خلال الفترة من شباط ٢٠١٣ إلى نيسان ٢٠١٤ ، لهذه الدراسة. تم استخراج الحمض النووي لعزلات الطفيلي من مزروع المسحات المهبلية. تم إجراء طريقة التنميط التتابعي المتعدد المواقع (MLST) لستة من الجينات المرجعية لدراسة التغيرات الجينية.

النتائج: ثلاث وخمسون امرأة (٣٤.٤١ ٪) قد أعطت نتيجة موجبة للأصابة بالطفيلي *T.vaginalis*. أظهرت طريقة MLST وجود اليلات مختلفة. وكان للمورث *glut* أعلى درجة من التغايرات بين المورثات الستة.

الاستنتاج: يمكن الاستنتاج أن التنوع الوراثي في الكائن الحي نفسه في عزلات العراق ، يمكن أن يرتبط بالنتائج السريرية. هناك حاجة إلى مزيد من الدراسات MLST لمقارنة عدد أكبر من العزلات من مختلف المواقع ودراسة الطفرات المحددة في المورثات المرجعية لنساء اللاتي يعانين من العقم ومن تشوهات عنق الرحم.

الكلمات المفتاحية: طفيلي المشعرات المهبلية. التغاير الوراثي, الخصوبة, تشوهات عنق الرحم, طريقة التنميط التتابعي المتعدد المواقع.