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Genotypes Diversity and Virulence Factor screening of *Trichomonas vaginalis* Isolated from Pregnant Women in Mosul (North of Iraq)

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Abstract:

Trichomonas vaginalis is a causative agent of trichomoniasis , one of the most common non-viral sexually transmitted disease (STD) over all the world, especially in immunocompromised women such as pregnant. Wet smear and Giemsa stain are the current methods used in hospital to diagnosis trichomoniasis. DNA based diagnosis is still to be validated to diagnose the local isolates, the objective of the present study was to compare the conventional methods of disease diagnosis with the DNA-based method to diagnose *Trichomonas* incidence in local isolates. In the present study, 105 samples were collected from outpatient women (18-45 years) of Maternity hospital in Mosul who showed a classical presentation of *Trichomonas* infection including foul-smelling vaginal discharge with a pH exceeding 4.5. Samples underwent microscopic examination and nucleic acid detection of *AP65-1* gene, the wet smear test showed that 16.18% of the collected specimen were positive while the parasite appeared only in 8.9% of the samples stained with Giemsa dye, the molecular screening for *AP65-1*gene that encodes for the surface attachment protein showed high sensitivity level of 100 at 50 specificity level in compare with other routine methods, the algorithm was evaluated according to specificity, sensitivity, and predictive values. Random Amplification Polymorphic DNA (RAPD) was achieved to estimate genetic indices among isolated strains. Phylogenetic analysis was performed using PAST (Paleontological statistics software) and dendrogram with neighboring clusters was constructed. According to the outcomes of research, we recommended to utilize a probiotic vaccines and molecular silencing vaccine (like miRNA sense and antisense oligonucleotides) as a treatment in.

Keywords: PCR, Phylogenetic tree, RAPD-PCR, *T. vaginalis*.

Introduction:

Sexually transmitted diseases (STDs) are spreading across the world, especially among immunocompromised women and women in their reproductive years who have sexual partners. Sexual activity continues to be a major source of infection in heterosexuals, with potentially dangerous bacterial, viral, fungal, and protozoal causative agents [1]. Recently, it has confirmed that uterus tissues are usually contaminated with unfavorable microorganisms (MOs) categories, like *Gardenerlla sp*., *Mycoplasma sp*., uncultivated bacteria, *Candida sp*., in addition to *T. vaginalis* as an irritant protozoan. Previous MOs were required to alter the vaginal milieu physiologically in order to produce a favorable setting for viral infection, such as HIV, HPV, and other causative agents [2-7].

T. vaginalis is one of the most pathogenic species, causing lysis of the genital tract's mucoid layer and irritating living tissue, particularly epithelial cells. This causes invasion of neighboring cells with certain causatives, which leads to increased complications, such as duct thickness and the development of papilloma and neoplasia. Asymptomatic infection is more common in men, and it's associated with urethral infection and dysuria. Several symptoms in an infected woman include a yellowish-green foamy discharge, purities, dysuria, and the strawberry cervix [8,9].

P65 is refers to surface protein of *T. vaginalis*, it is a prominent functional protein that's also bound to both the surface and its ligand to influences host cell binding [10,11], In comparison to other adhesion proteins, Garcia *et al.* [12], found that *T. vaginalis*

AP65 is still a crucial adherent protein of trichomonads [12].

RAPD markers are a non-directed technique for generating polymorphic PCR products from genomic templates utilizing monomer oligonucleotides that anneals with numerous sites in diverse genomic regions and simultaneously amplifies several genetic loci for each sample. These markers have also been shown to be effective genetic markers for a variety of eukaryotic organisms [13]. Many studies were carried out in this field for diagnose and classification of *T. vaginalis* based on classical and molecular techniques [14-17]. Our objective was included detection of *T. vaginalis* in pregnant women samples, then diagnosed it by routine methods and confirmed this investigation by conventional PCR technique and classification via molecular utilizing RAPD-PCR application to determine the genetic variation among the detected genera of this protozoan.

Material and Methods:

Subjects

Duplicated high vaginal swabs (HVS) were collected by gynecologist from 105 pregnant women admitted to Mosul maternity hospitals during July-December 2019.

Morphological diagnosis

Swabs were impregnated in 1ml of sterile saline solution and then used for wet mount and Giemsa staining technique to diagnose *T. vaginalis*. A drop of the sample was placed on the slide and covered with a coverslip. Prepared smears were examined at 40x lenses to detect the protozoan motility, which was diagnosed by its Jerky movement as a wave-like actions. In addition, wet mount is often used to show the presence of clue cells in vaginal secretions, these epithelial cells were surrounded by masses of bacteria. Concerning Giemsa staining method, the dried smears were fixed by methanol for 5minutes, and stained for 20 minutes at dilution 1:20. For parasite identification, scanning has been performed at 100x magni-fication level, pear shaped trophozoites have been recognized [18,19]. For conduct whiff test, vaginal discharge was collected by cotton swab, then a drop of vaginal discharge was mixed with a drop of KOH(10%) and placed onto a strip of pH paper (Qualigen, India), the outcome was compared with a standardized colorimetric reference chart to estimate the actual pH [20].

Molecular profile

Genomic(g) DNA was extracted from the parasite using Wizard gDNA purification kit according to manufacturer instruction Promega, USA [21], gDNA concentration (34-39ng/ml.) and purity were achieved by nano-spectrophotometer and their template integrity was evaluated by electrophoresis for conventional and RAPD-PCR application. The primer set *AP65-1*-Forward (5'-3') and Reverse (5'-3'), was designed in this study (AccessionNo.U18346), more information was detailed in Tab.1.

Table 1. Primer sets for molecular screening of T. *vaginalis*

Gene		Sequence 5'- 3'	Reference	Tm°C	Template length (bp.)
AP65-1	F	ATGTTCGATCACCGTGGCAT	This study	67	1751
	R	TTGACTTGTTCGGCTGGGAA			

Amplification program for detection of *AP65-1* gene included; 95°C as DNA unwinding for 3min and 35cycles of each of denaturation 94°C, annealing temperature 62°C, extension 72°C, the periods were 30sec for each step, in addition to final extension 72°C for 5min. The molecular diversity of *T. vaginalis* was investigated using the following random primers; OPAX-01(GTGTGCCGTT), OP AV-08 (GGACGGCGTT) and OPV-10 (GGACCTGCTG) which were designed and supplied by (Bioneer, S. Korea). Amplification tubes were transported to Applied Bio-system gradient thermo cycler (USA) for achieve following program: initial incubation 95°C for 3min, and 35 cycles of 95°C for 30s as initial denaturation, 34-39°C for 30sec as gradient annealing temperature and 72°C for 30sec as an extension and final extension 72°C for 180sec. Amplification premix (Bioneer, S. Korea) consist of lyophilized Taq-DNA polymerase 1U, dNTPs 250Mm (dATP, dCTP, dGTP, dTTP), 1.5mM. of MgCl, 30mM of KCl, 10mM of Tris-HCl (pH 9.0), in addition to methylene blue as a loading dye. Whereas the amplification mixture was included 1µl for each primer, 5µl as gDNA (template) and 13µl represent nuclease free water (total volume 20µl). DNA marker was 100bps, Bioneer, S. Korea [22].

Outcomes documentation and analysis

All amplification products were detected and photographed using Gel Documentation System (ATTA, Japan). By each primer, the banding pattern size of each isolates was scored (bp) in comparison with the size of DNA marker for AP65-1 gene (Bioneer, S. Korea). All random bands were classified to polymorphic and single, depending on the DNA marker (100bp.). The bands size was calculated using CS analyzer software (ATTA,

Japan), then the bands were converted into two digital data (1,refere to band; 0,absence). After that, a simple matching (SM) coefficient was investigated via Jaccard algorithm to determine a dendrogram and using PAST software [23]. The sensitivity of all results were also compared and calculated by applying the following equations: sensitivity = positive result/total positive result X 100 [24].

Ethical approval

The current study was carried out in accordance with the Human Rights Health Protection Guidelines governed by the ethics committee at Mosul maternity facilities, Iraq, and in accordance with the Helsinki Declaration on Ethical Principles.

Results and Discussion:

Of all 105 (100%) samples, 16(15%) was diagnosed morphologically as *T. vaginalis* via wet smear, 11(10.5%) was detected by Giemsa stain and 8(7%) by molecular screening depending PCR technique. Our findings, were nearly to those of Rajonic *et al.* [25], who diagnosed the parasite by wet mount in 14 (7.0%) cases and Giemsa stain 11(5.5%) in 21(10.5%) women. Point-of-care testing, such as vaginal pH assessment, microscopic investigation of vaginal fluid, and the wiff test, is routinely performed. According to this study, low rates of assessment for prior testing were seen among women admitted to maternity hospitals [26].

All phenotypically suspect samples 16 of *T. vaginalis* were genetically verified using PCR method, with eight of them giving a negative result for the presence of the *AP65-1* gene, and the other eight yielding a positive result. The *AP65-1* gene (Fig. 1, Tab.2) was screened for two purposes. The first was to validate the presence of *T. vaginalis*, the second aim was to evaluate the existence of an essential virulence factors that help this protozoan attach to the surface of host cells, which is an extremely specialized process mediated by adhesion proteins and is an early and critical phase in colonization [27]. The proteins of AP65 could be secreted out of trophozoite of *T. vaginalis* and attach to the surface of trophozoites and host cells [12].

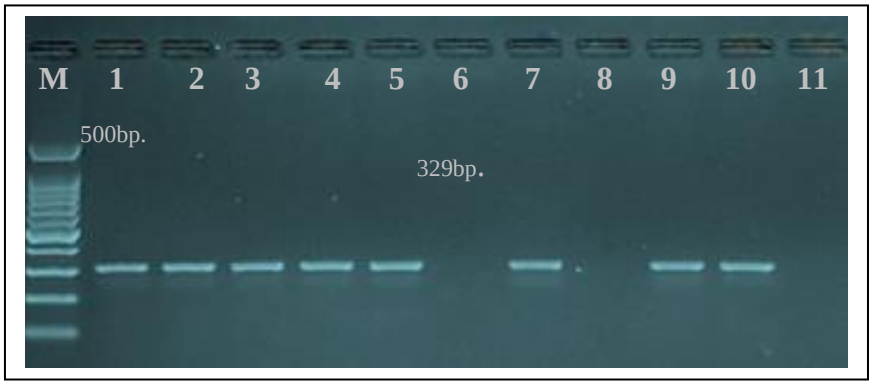

Figure 1. Amplicons of *AP65-1* gene. M is a molecular marker (100 bp),lines 1-5,7,9 and 10 were a positive results(329bp.) and lines 6,8 and 11 were represented some of negative results(electrophoresis conditions was 1.5% agarose concentration at 120 volt).

Table 2. Relationship between phenotypic and genotypic screening of *T. vaginalis* .

Tests		PCR				Total
		Positive		Negative		
		No.	%	No.	%	
Morphological diagnosis	+ve 16	8	50	8	50	105
	-ve 89	0	0	89	100	

PCR technique sensitivity was 7.6% for confirmation of *T. vaginalis*, in comparison with Giemsa staining technique (10.5%) and wet smear (15.2%). as that shown in the Tab.3. The incidence of *T. vaginalis* was 9% in members of the Malawian population who had urethritis, but only 3.5% in those without urethritis, according to Kaydos-Daniels *et al.* [28], who used PCR in first-catch urine.

Table 3. Evaluate of diagnostic methods sensitivity utilized for detection of *T. vaginalis*

Test Evaluate criterion	Wet smear	Giemsa stain	PCR (AP65-1)
Sensitivity (100%)	15.2	10.5	7.6

Genetic diversity of *T. vaginalis*

The eight samples that tested positive for the AP65-1 gene were chosen for random amplification to explore genetic diversity across *T. vaginalis* strains (Fig. 2), which was represented by a similarity and dissimilarity chart and a dendrogram. Due to the use of the same random primers with

many species or genera, RAPD is an essential confirmatory technique at the molecular level [13]. Random primers are made up of ten nucleotides that anneal to various locations on the targent gDNA choromosome based on repeating nucleotides. As a result, a variety of random bands emerged [29].

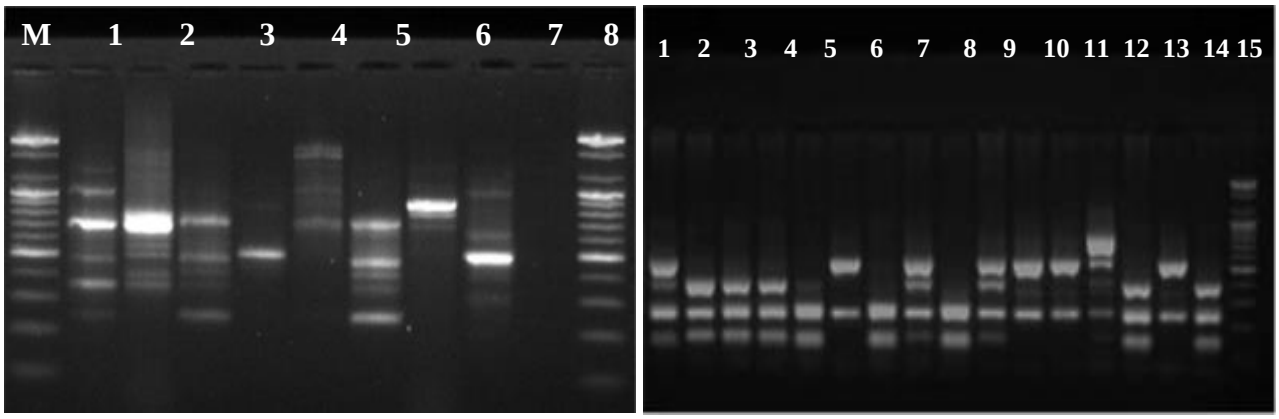

Figure 2. Random amplification of *T.vaginalis*. At left, Lines 1-8 are positive results(OPV-08), lines 9 is a negative control and Line(M)is a DNA marker(100bp).At right, Random amplicons of OPAX-01 (lines1-8), OPV-10(lines 9-16) and line(M)is a DNA marker,100bp. Electrophoresis conditions was 2% agarose concentration at 120 volt).

Similarity and distance indices

The observative data, involeved the highest similarity percent between 2&6 (0.38462), and the lowest similarity percent between 2&7(0.071429), whereas the other relationships were founded between those values, more detailes in Tab. 4, our results agree with that reported data [30] who discovered significance difference among *T. vaginalis* at molecular level using RAPD application, the outcomes were reported in the Tab. 4.

Table 4. Similarity and distance indices of *T. vaginalis* isolates.

Sample No.	1	2	3	4	5	6	7	8
1	1							
2	0.2069	1						
3	0.37037	0.38462	1					
4	0.074074	0.23077	0.25	1				
5	0.083333	0.17391	0.095238	0.095238	1			
6	0.27586	0.071429	0.076923	0.076923	0.34783	1		
7	0.13793	0.071429	0.076923	0.23077	0.17391	0.16421	1	
8	0.14286	0.074074	0.16	0.08	0.090909	0.12485	0.12374	1

On the other hand, phylogenetic tree categorized the parasites under research into two major classes (F&E), E line was represented by no.8, F was split into two neighbouring clusters (G&D), D subdivision included two isolates (5&6), while G subclass revealed C and A, in turn C has two subbranches 4 and 7, and finally A was clustered into subbranches B (2&3) and isolate no.1 line (Fig. 3). There are a close genetic relationship and certain gene polymorphism among the *T. vaginalis* isolates, thus geographical origin plays little role to the genetic characteristics [15].

Figure 3. Phylogenetic of *T. vaginalis* classification.

Future directions:

The development of a common framework for genotyping *T. vaginalis* is essential to provide the necessary tools for addressing potential

correlations between *T. vaginalis* the organism, and trichomoniasis the disease. The DNA-based techniques, MLST and MS genotyping, are most suitable for these investigations, but the relationship between the two population types identified in each of those approaches needs to be clarified. Analysis by both MLST and MS genotyping of a common set of clinically annotated *T. vaginalis* isolates has recently been initiated by the authors. This will facilitate investigations into the relationships between mixed infections, *T. vaginalis* genetic identity, the unique two-type population structure, and clinical manifestations of the disease. The interaction between the vaginal microbiome, patient immune response, and *T. vaginalis* genetic diversity can then be investigated in order to obtain a comprehensive understanding of trichomoniasis and its associated disease sequelae. The extensive genetic diversity and two clade population structure in *T. vaginalis* will also need to be carefully considered in other investigations of trichomoniasis. The use of proteomic and genomic-based techniques to elucidate genetic factors controlling clinical manifestations and severity, association with other disease entities, and drug resistance will require *T. vaginalis* isolates which, other than the relevant trait of interest, are as genetically similar as possible in order to maximize the discovery of the relevant genetic elements and avoid differences rooted in genetic diversity. By contrast, future vaccine design and development of antigen-based rapid diagnostic tests for trichomoniasis will need to focus on antigenic determinants which are broadly representative of the entire *T. vaginalis* population. The existence of a two-type population structure, and the association of particular clinical and phenotypic traits with genetic identity also highlight the need for genome sequencing of additional *T. vaginalis* strains. To date, the sequence of only a single *T. vaginalis* strain, G3 (American Type Culture Collection reference number Pra98), has been generated, and it is imperative that the genome sequence of additional clinically relevant isolates representative of both *T. vaginalis* population types be determined.

Conclusion:

Despite the fact that *T. vaginalis* is one of the most common STIs, it remains an orphan disease that relies on outdated technology, clinical suspicion, and empirical care. The disease appears to have a wide-ranging effect on the transmission of other sexually transmitted infections as well as the unborn child. The accuracy of a trichomoniasis diagnosis based solely on complaints of vaginal discharge is low. It's a crucial diagnostic test that's especially useful for detecting women who don't have clinically obvious disease. To date, screening approaches such as molecular analysis at a qualitative level using polymerase chain reaction play an essential role in reducing the complications associated with asymptomatic infection and progression of prevalent STIs among reproductive-age women.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee in University of Mosul.

Authors' contributions statement:

F S. I. A assisted with data analysis and manuscript preparation, as well as conducting the experiments. Firas M. B. Ahelped create the approach and conduct the experiments. The article was finally approved by two writers after thorough consideration.

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التباين الوراثي والتحري عن عامل الضراوة في المشعرات المهبلية المعزولة من النساء الحوامل في الموصل (شمال العراق)

فؤاد سالم إسماعيل الربيعي **فراس محمد بشير الخشاب**

كلية التربية للبنات، جامعة الموصل، الموصل، العراق

الخلاصة:

تعد المشعرات المهبلية احد مسببات الامراض المنتقلة جنسياً خصوصاً لدى النساء المثبطات مناعياً مثل الحوامل، جمعت خلال البحث الحالي تم تحديد النساء بعمر (18-45) كمصدر للعينات ، تم جمع (105) عينة بوساطة مسحات ثنائية من عمق المهبل احدها للفحص المجهري المباشر والأخرى للفحص الجزيئي ، تم تقييم الدالة الحامضية للعينات لبيئة المهبل والتي كانت اقل من 4.5 وذلك بمزج قطرة من الافرازات المهبلية مع قطرة من هيدروكسيد البوتاسيوم 10% (فحص ويف)، واعتمد الفحص المجهري كتشخيص مظهري لتحديد وجود الطفيل باستعمال الشريحة الرطبة (16) وصبغة كمزا (11)، من ناحية أخرى اظهر التحري عند المستوى الجزيئي باستعمال تقنية التفاعل التسلسلي لانزيم البلمرة تحديد المورث *AP65-1* المسؤول عن وجود بروتين الالتصاق السطحي لدى (8) عينات وبنسبة تحسس (7.5) وتم استعمال تطبيق التضخيم العشوائي للحمض النووي RAPD للكشف عن قيم التباعد والتقارب الوراثي لعينات الحمض النووي المستخلص للطفيلي من خلال تحليل الترحيل الكهربائي لنواتج التضخيم العشوائي ورسم معالم الشجرة الوراثية باعتماد برنامج PAST الاحصائي، توصي الدراسة الحالية بمزيد من الفحوصات ضمن جدول زمني محدد لتقييم إصابات الجهاز التناسلي الغير مرتبطة بأعراض ظاهرة لدى النساء بما في ذلك مخاطر تفاقم الإصابات الثانوية خصوصاً منها الإصابات الفيروسية الخطيرة واعتماد استحداث اللقاحات للمسببات المرضية وايضا استحداث التوجه لاستحداث اللقاح الجزيئية المثبطة للجينات الاساس والمؤثرة في حدوث الاصابة .

الكلمات المفتاحية : التفاعل التسلسلي لأنزيم البلمرة النوعي، شجرة التقارب والتباعد الوراثي، التفاعل التسلسلي لأنزيم البلمرة العشوائي، المشعرات المهبلية .