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RESEARCH ARTICLE

GENETIC VARIATION OF *TRICHOMONAS VAGINALIS* ISOLATES FROM IRAQI WOMEN: A NEW SEQUENCE TYPE

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Abstract

The *Trichomonas vaginalis* genome was found to be approximately 160 megabases in size and the developed genotyping tools allow better understanding of its population genetics.

This study aimed to determine the genetic variation among *T.vaginalis* isolates by Multilocus sequence typing (MLST) method for six housekeeping genes.

A total of 154 Iraqi women (age range 15-54 years) who were attending the Gynecology out -patient department in Baghdad province during the period from February 2013 to April 2014, were recruited for this study.

High vaginal swabs were collected then diagnosed the infection by culturing method. DNA of *T.vaginalis* was extracted from the cultured swabs. MLST method for six housekeeping genes, were used for studying the genetic variations among *T.vaginalis* isolates.

Fifty three (34.41%) women were positive for *T.vaginalis*. MLST method for housekeeping genes resulted in different alleles and there was a new allele in four loci based on the database of GenBank. Furthermore, a new sequence type of *T.vaginalis* was found based on the Trichomonas MLST website.

It can be concluded that there are a wide genetic diversity among local *T.vaginalis* isolates which influence the clinical outcome, and their local strains have a characteristic allele compared with a universal

Introduction:-

Trichomonas vaginalis is a flagellated protozoan parasite found in the human vagina and urethra (Donne, 1836). The parasite causes the most common, non-viral, sexually transmitted disease in the world, infecting 248 million people annually according to the World Health Organization (WHO) estimates (WHO, 2011).

Trichomonas spp. genome is highly repetitive with 65% of the genome consisting of repeated sequences, including 59 repeat families of transposable elements representing 25% of genome (El-Nazer et al., 2013). The new methods of diagnosing and treating the disease come to light with the cracking of the genome of the *T. vaginalis*, and identification of 26,000 confirmed genes (Carlton et al., 2007). There is no "gold standard" method for genotyping isolates of *T. vaginalis* (McClelland, 2008), and multiple approaches to typing Trichomonas isolates have been described; Pulsed field gel electrophoresis, Random amplified polymorphic DNA analysis (RAPD), Restriction fragment length polymorphism (RFLP), Multilocus enzyme electrophoresis (MLEE), Microsatellite polymorphism and Multilocus sequence typing (MLST) (Crucitti, et al. 2008; Tibayrenc,2009; Conrad et al., 2011). These studies produced different results, even when using similar techniques.

MLST is a simple technique, requiring only the ability to amplify DNA fragments by PCR and to sequence the fragments. In MLST, the direct assignment of alleles based on nucleotide sequence determination of internal fragments from multiple housekeeping genes MLST schemes and strain databases are available for a growing number of prokaryotic and eukaryotic organisms. Sequence data are ideal for strain characterization and strains can readily be compared between laboratories via the Internet (Hanage et al., 2004 & Aanensen and Spratt, 2005). The adoption of MLST as a genotyping method for *T. vaginalis* could provide the gateway for better understanding of the epidemiology, genetic diversity, and population structure of *T. vaginalis* (Cornelius et al., 2012).

Materials and methods:-

One hundred fifty four samples were collected from females attending the Gynecology out -patient department in Baghdad during the period from February 2013 to April 2014. High vaginal swabs were taken from females at different ages from 15-54 years.

Swab was pressed between the In-Pouch TV System media (Biomed Diagnostics, Inc. USA). The culture was examined every day for three days before being considered negative (Sood et al., 2007). Positive culture then 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 every day 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 harvested by centrifugation for 15 min at 500 xg at 4°C, in a 1.5 ml microcentrifuge tube. The supernatant was discarded carefully so as not to disturb the cell pellet. Pelleted cells were washed twice with phosphate-buffered saline (PBS pH 7.4). Washed pellets were stored in -20°C prior to DNA extraction (Cornelius et al., 2012).

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

PCR amplification and nucleotide sequence determination: - Six single-copy housekeeping genes of *T.vaginalis*, were genetically characterize based on their diversity. Primers for MLST loci were described by Cornelius et al. (Cornelius et al., 2012). These primers were designed to amplify gene fragments of 450 to 500 bp (Table 1). The absolute chromosomal location of each MLST gene is unknown. The primers used were designed from Eurogene kit primers (Eurogene, UK).

Table (1): Primers used for *T. vaginalis* MLST method.

Locus (abbreviation)	Primer Sequences(5'–3')		Tm	Amplicon Size (bp)
Tryptophanase (P1) tryp	F	CGTCAACATCGGTGGCTTCA	62	489
	R	GCGACAGCGACGACATTCAT	62	
Glutaminase (P3) glut	F	GTGCCATTACAACAGCATCG	60	451
	R	CCAAGTATAGCTCCGCTGAC	62	
Family T2 aparaginase-like threonine peptidase (P6) ft2a	F	GAACAGGAGCACCAGCAGAA	62	412
	R	TCTCTAGCAACGCAGCCAAC	62	
Alanyl tRNA synthetase (P8) alts	F	TCTGTCCAGGATGGTGTCTT	60	494
	R	ACGCCTTCCTCCTTCATCTT	60	
DNA mismatch repair protein (P13) dmrp	F	TCATCGGCCAATGGAACCAA	60	491
	R	TCCGTGCGGACAATTCCAAG	62	
Mannose 6-phosphate isomerase (P16) m6pi	F	AGCCAGTTGGCTTCTGAGTT	60	459
	R	AACAATTCCGCAAGCTGGAG	60	

Tm: Melting temperature

Sixteen isolate were typed. All genes were amplified at 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, 2.5mMMgCl2, and 0.5µg of template DNA. Amplifications were performed using the Applied BioSystem thermal cycler (Applied BioSystem USA). The 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 amplification. The remaining 47 µl of PCR product were purified prior to sequencing using Charge Switch PCR clean-up kit according to the manufactures' instructions.

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

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

Nucleotide sequences analysis software:-
Sequence Alignment searches were conducted between the sequencing results of PCR products and the sequence of standard gene by BLAST program (details of *Trichomonas vagialis* G3 (ATCC Pra98 [http://trichdb.org /trichdb/]), which is available at the national center biotechnology information (NCBI) online at (http://www.ncbi.nlm.nih.gov)).

The alleles at each of the loci were assigned and the sequence type (ST) defined using the publically accessible *Trichomonas* MLST website (http://Trichomonas.mlst.net/).

Results:-

Out of 154 women patients examined at the age ranged between 15 and 54 years with mean of 34.5 year, 53 (34.4%) were found to be infected with *T. vaginalis* by the use of In-Pouch TV culture, The isolates have a range of morphologies. Sixteen isolate were typed, 7 of them were not typed because the original primers failed to generate an amplicon.

Analysis of the loci sequence data:-
MLST software package program for detecting the allele and allele profile (ST) number of the strain revealed the number of alleles of six studied loci as shown in table 2.

Table (2): Allele numbers of studied housekeeping loci.

Locus	No. of alleles	No. of mutations	No. of changes	
			Synonymous	Nonsynonymous
tryp (P1)*	5	2	1	1
glut (P3)*	3	53	37	16
ft2a (P6)*	1	1	-	1
alts (P8)*	1	1	-	1
drmp (P13)	10	1	1	-
m6pi (P16)	2	1	-	1

* new allele

In the present study, the ST at the level of 5 loci of isolates was assigned as 'a', and the ST at the level of 3 loci of other isolates was assigned as 'b' (Table 3).

Table (3): Allele numbers of studied housekeeping genes referred to ST 'a' and ST 'b'.

ST	tryp	glut	ft2a	alts	dmrp	shmt	m6pi
a'	**5**	**3**	**-**	**1**	**10**	**-**	**2**
b'	**5**	**-**	**1**	**-**	**-**	**-**	**2**

The nucleotide sequence data of current studied alleles are available in the GenBank database under accession numbers LC036655 to LC036664.

Regarding the locus *glut* (P3), there was a higher number of mutations comparing to other studied genes. The sequence traces (electropherograms) indicated the presence of multiple alleles by the presence of 3 double nucleotide peaks.

Discussion:-

Several factors; biological, sociocultural and economical along with the poor access to information may place women at high risk of infection with *T. vaginalis* (Miller et al., 2008). We have introduced the first MLST markers for investigating the genetic diversity of *Trichomonas vaginalis* in Iraq.

MLST typically involves sequencing internal fragments of six single copy housekeeping genes per strain (Spratt, 1999). *T. vaginalis* may possess certain unique features of gene expression, such as strict differential transcription rate even for housekeeping genes (Clark, et al. 2010).

Nucleotide mismatches or a lack of primer target sites (Miah, 2009) and sequence diversity (Cornelius et al., 2012) may lead to the failure of particular primers to amplify gene fragments.

Analysis of Sequences Data for Allelic and Allelic Profile (ST):-

Although the study is limited by the fact that *T.vaginalis* isolates were not combined in more than one or two genes, these data may provide a putative assignment to a known clonal lineage as mentioned by Maiden (Maiden, 1998) by using only two or three loci. The present data suggest a 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 number of alleles for studied 6 loci and presence of a new allele in 4 loci as shown in table 2.

The ST at the level of 5 loci ‘a’, was differ from all 60 ST of isolates in a study done by Cornelius et al. (Cornelius et al., 2012), that’s mean this is a new ST, while ST at the level of 3 loci which was assigned as ‘b’ (Table 3), resemble the ST 56 in its allele number of the 3 loci (Appendix 1).

Presence of double nucleotide peaks in the locus *glut* (P3), could possibly have resulted from: (i) the presence of multiple strains (mixed infection) or clones (Cornelius et al., 2012). *T.vaginalis* have meiosis-specific proteins. Thus, *T.vaginalis* contains either recent evolutionary relics of meiotic machinery or genes functional in meiotic recombination (Carlton et al., 2007). Some karyological studies suggested the occurrence of meiosis in a fraction of *T. vaginalis* cells in cultures (Drmota and Král, 1997). There was an expectation that even a very rare occurrence of sex in the population of this species, i.e., the existence of a weak gene flow among different *T. vaginalis* strains, could obscure the phylogenetic signal in the molecular data (Tibayrenc, 1998); (ii) the presence of a hybrid genotype; (iii) intragenomic variation in the single copy housekeeping gene; or a combination of these (Gelanew et al., 2010).

Presence of a range of *T. vaginalis* morphologies when examined microscopically, may be explained by presence of multiple strains which infect the same individual or may be due to intragenomic variation in the certain one or more single copy housekeeping gene causing morphological variation to a certain strain.

High variability of the locus *glut* (P3) in recent study, could be attributed to the highly polymorphism and variation in *T. vaginalis* isolates.

It is important to note that further MLST studies are needed to compare a larger number of isolates from different localities.

Thus, the degree at which the perceived importance and stability of essential metabolic genes equate with their phylogenetic consistency, remains an open question, despite the fact that MLST genes were chosen on the basis that they are likely to represent the stable (core) of the genome (Fei et al., 2003).

The occurrence of common mutants might be responsible for the phylogenetic variation among the strains (Olorunfemi et al., 2005) and the intragenetic diversity may be associated with clinical outcome (Stensvold et al., 2013). Advances in genetic characterization of *T. vaginalis* isolates showed that the extensive clinical variability in trichomoniasis and its disease sequelae are matched by significant genetic diversity in the organism itself, suggesting a connection between the genetic identity of isolates and their clinical manifestations (Meade and Carlton, 2013). This suggestion is agree with recent results of this study and this has important implications for genetic research and control the disease.

Previous molecular methods for *T. vaginalis* characterization had approved the diversity (Merdaw et al., 2014; Conrad et al., 2012; Meade et al., 2009; Matini et al., 2014; Matini et al., 2012). The diversity also can be shown at the level of protein expression, that there is a substantial variation between isolates (De Jesus et al., 2009; de Miguel et al., 2010; AL-Mudhaffar, 1995).

Despite the limited number of patients involved, this study showed a wide spectrum of clinical features of *T. vaginalis* infection and documented the genetic diversity for this parasite in Iraqi isolates.

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Appendix 1:-

Trichomonas vaginalis - 60 ST's and allele numbers of housekeeping genes by Cornelius et al. (Cornelius et al., 2012).

ST	tryp	glut	ft2a	alts	dmrp	shmt	m6pi
1	**1**	**1**	**1**	**1**	**1**	**1**	**1**
2	**1**	**1**	**1**	**1**	**2**	**2**	**1**
3	**1**	**1**	**1**	**1**	**3**	**1**	**2**
4	**1**	**1**	**1**	**2**	**2**	**1**	**1**
5	**1**	**1**	**1**	**2**	**2**	**1**	**4**
6	**1**	**1**	**1**	**2**	**4**	**2**	**2**
7	**1**	**1**	**1**	**2**	**5**	**1**	**2**
8	**1**	**1**	**1**	**2**	**5**	**2**	**1**
9	**1**	**1**	**1**	**2**	**6**	**2**	**2**
10	**1**	**1**	**1**	**2**	**8**	**1**	**1**
11	**1**	**1**	**1**	**4**	**1**	**2**	**2**
12	**1**	**1**	**1**	**7**	**3**	**2**	**2**
13	**1**	**1**	**1**	**7**	**7**	**2**	**2**
14	**1**	**1**	**1**	**8**	**2**	**1**	**1**
15	**1**	**1**	**1**	**9**	**5**	**1**	**2**
16	**1**	**1**	**1**	**11**	**2**	**1**	**2**

17	**1**	**1**	**1**	**11**	**7**	**1**	**1**
18	**1**	**1**	**1**	**12**	**6**	**1**	**2**
19	**1**	**1**	**1**	**17**	**12**	**1**	**1**
20	**1**	**1**	**6**	**4**	**5**	**1**	**2**
21	**1**	**2**	**6**	**4**	**5**	**1**	**2**
22	**1**	**3**	**1**	**1**	**9**	**2**	**1**
23	**1**	**3**	**1**	**4**	**5**	**1**	**2**
24	**1**	**3**	**1**	**4**	**6**	**1**	**2**
25	**1**	**3**	**1**	**4**	**6**	**2**	**2**
26	**1**	**3**	**1**	**13**	**5**	**1**	**1**
27	**1**	**3**	**1**	**15**	**5**	**1**	**1**
28	**1**	**3**	**3**	**4**	**10**	**1**	**2**
29	**1**	**3**	**4**	**5**	**5**	**1**	**1**
30	**1**	**3**	**5**	**6**	**6**	**2**	**2**
31	**1**	**3**	**6**	**2**	**5**	**2**	**3**
32	**1**	**3**	**6**	**4**	**5**	**1**	**2**
33	**1**	**3**	**6**	**4**	**5**	**2**	**1**
34	**1**	**3**	**6**	**4**	**6**	**1**	**2**
35	**1**	**3**	**6**	**6**	**4**	**1**	**2**
36	**1**	**3**	**6**	**6**	**6**	**1**	**2**
37	**1**	**3**	**6**	**6**	**6**	**2**	**2**
38	**1**	**4**	**1**	**4**	**5**	**1**	**2**
39	**1**	**4**	**1**	**13**	**4**	**1**	**2**
40	**1**	**4**	**6**	**7**	**11**	**1**	**1**
41	**1**	**5**	**1**	**4**	**2**	**1**	**2**
42	**2**	**1**	**1**	**1**	**4**	**1**	**2**
43	**2**	**1**	**1**	**2**	**2**	**1**	**2**
44	**2**	**1**	**1**	**3**	**1**	**1**	**2**
45	**2**	**1**	**1**	**9**	**6**	**1**	**2**
46	**2**	**1**	**1**	**9**	**8**	**1**	**2**
47	**2**	**1**	**1**	**11**	**6**	**1**	**2**
48	**2**	**1**	**1**	**13**	**5**	**1**	**1**
49	**2**	**1**	**1**	**14**	**2**	**1**	**1**
50	**2**	**1**	**1**	**16**	**4**	**1**	**2**
51	**2**	**1**	**2**	**2**	**2**	**1**	**4**
52	**2**	**3**	**1**	**10**	**10**	**1**	**1**
53	**2**	**3**	**6**	**4**	**5**	**1**	**2**
54	**3**	**1**	**1**	**12**	**5**	**1**	**2**
55	**4**	**4**	**1**	**4**	**5**	**1**	**2**
56	**5**	**1**	**1**	**2**	**8**	**1**	**2**
57	**5**	**1**	**6**	**4**	**5**	**2**	**2**
58	**5**	**3**	**6**	**4**	**5**	**1**	**2**
59	**5**	**3**	**6**	**4**	**6**	**1**	**2**
60	**5**	**4**	**6**	**4**	**5**	**1**	**2**