



ISOLATION AND GENOTYPING OF *TRICHOMONAS VAGINALIS* ISOLATES BY PCR-RAPD IN BAGHDAD CITY

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ABSTRACT

In spite of the wide prevalence of *Trichomonas vaginalis*, little is known about the genetic variation of this parasite due to the lack of appropriate tools. The aim of this study was to find genotypic diversity and relationships among the local isolates in Baghdad city by PCR-RAPD method. A panel of 4 random primers (OPD1, OPD2, OPD3, and OPD5) was used to determine the genotypic differences between the isolates of *T. vaginalis*. Numerical analysis of 76 RAPD amplified bands generated by these 4 primers was carried out with the unweighted pair group methods analysis (UPGMA) using Nei and Li to construct a dendrogram. Four main groups were distinguished by RAPD data, these groups coincide with four different patient categories (fertile, infertile, cervical abnormalities and noncervical abnormalities). The least number of bands were seen by using primer OPD2 and the most by using OPD5. By using primer OPD1 no band were found in isolates. A specific 754.5 bp marker was found to be specific for 75% of infertile isolates, while 812.5 bp was specific for 90.9% of cervical abnormalities. This is the first description of possible virulence markers for *T. vaginalis*. Further studies will be necessary to confirm the importance and function of these genetic markers in clinical infection.

KEYWORDS: *Trichomonas vaginalis*, RAPD, infertile, cervical abnormalities.

INTRODUCTION

T. vaginalis causes the most common, non-viral, sexually transmitted disease in the world, infecting 248 million people yearly according to WHO estimates (WHO, 2011). Although both men and women are infected, it causes disease almost exclusively in women (Saksirisampant *et al.*, 2009). 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, while men are often asymptomatic carriers of the parasite (Ryan and de Miguel, 2011; Kutikhin *et al.*, 2013). Trichomoniasis is also considered a biological marker trigger and on evaluation for other STD (Schwebke and Burgess, 2004). *Trichomonas* infected subjects may become more susceptible to higher risk of acquiring the HIV-1 and HSV-2 infection. (Kissinger *et al.*, 2009; Peterman *et al.*, 2006) and a 2-fold increased risk of cervical neoplasia, even after controlling for human papillomavirus (HPV) infection (Gram *et al.*, 1992). Potential sequelae of this STD in females include tubal factor infertility (Wiwanitkit, 2008) and the cervical cytological abnormalities can also result when the parasite move easily through the cervical mucus, (Donders *et al.*, 2013). Furthermore, low birth weight infants have been associated with *T. vaginalis* infection (Cotch *et al.*, 1997). There is no "gold standard" method for genotyping isolates of *T. vaginalis* (Cornelius *et al.*, 2012). Multiple methods for typing *Trichomonas* isolates have been used in previous attempts; antigenic characterization, isozyme

analysis, repetitive sequence hybridization, ribosomal gene and intergenic region sequence polymorphisms, pulsed-field gel electrophoresis, random amplified polymorphic DNA (RAPD) analysis, and restriction fragment length polymorphism (RFLP) (Crucitti *et al.*, 2008; Upcroft *et al.*, 2006; Tibayrenc, 2009; Conrad *et al.*, 2011). These methods produced different results, even when using similar techniques and have variable levels of reproducibility in determining the genetic relatedness of isolates (Cornelius *et al.*, 2012). RAPD is still a useful approach to identify and characterize novel DNA markers. Different studies suggest that this technique provides powerful markers to analyze the genetic diversity in *T. vaginalis* and is considered a simple method to detect DNA polymorphism in correlation with patient's records (Mkada-Driss *et al.*, 2014; Kaul *et al.*, 2004; Rojas *et al.*, 2004). The current study aimed to investigate the efficiency of PCR- RAPD in differentiation between complicated and uncomplicated trichomonas-related infection.

MATERIALS & METHODS

A total of 154 high vaginal swabs were collected from women in Baghdad city during the period from February 2013 to April 2014. Swabs were taken from the posterior fornix by sterile cotton swab (Fouts and Kraus, 1980). During this process examination for redness, congestion or cervical abnormalities was done. The isolates collected from 29 fertile and 24 infertile women; 31 no cervical abnormalities women and 22 with cervical abnormalities.

Swabs were inoculated into a *T. vaginalis* In-Pouch (Biomed Diagnostics, USA) culture. Only positive In-Pouch cultures were inoculated in *Trichomonas* Modified CPLM Medium (HIMEDIA) supplemented with 10% heat-inactivated horse serum and antibiotics (50 µg of gentamicin/ml, 40 µg of ciprofloxacin/ml, and 50 µg of miconazole/ml). The pH of the medium was adjusted to pH 6.2. *T. vaginalis* trophozoites were grown at 37°C until the culture populations were in the log phase of growth at an inoculating concentration of 10⁶ cells/ml. These cells were harvested by centrifugation for 15 min at 500 x g at 4°C. Pelleted cells were washed twice with phosphate-buffered saline (pH 7.4) prior to DNA extraction. DNA of samples was extracted by using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. The RAPD technique was used based on Jamali et al. (Jamali et al., 2006). Four primers OPD1, OPD2, OPD3 and OPD5 were found suitable for analyzing *T. vaginalis* isolates (Tab. 1). The DNA amplification was performed at final volume of 25µl using Hot Start Taq Master Mix (Qiagen, Hilden, Germany), 4µl of template DNA. Negative controls for each of 4 primers used contained all components except template DNA. One sample was chosen as reference sample and used in all RAPD PCR test for checking the whole steps of experiment. The amplification protocol consisted of an initial denaturation step at 94°C for 10 min followed by 40 cycle repetitions of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. The extension was 20 min at 72°C. PCR products were analyzed by electrophoresis in 1.5 % agarose gel in TBE buffer. The gels were then stained with ethidium bromide (0.5µg/ml) and viewed under the U.V transilluminator. By each primer the banding pattern size of each isolates was scored (bp) in compare with size marker in DNA ladder (GeneRuler™, Fermentas) and the data were correlated with patient's records.

TABLE 1: Primers used for RAPD analysis

Primer	Size (nt)	Sequence(5' to 3')
OPD1	10	ACCGCGAAGG
OPD2	10	GGACCCAACC
OPD3	10	GTCGCCGTCA
OPD5	10	TGAGCGGACA

Phylogenetic analyses

Phylogenetic analyses were achieved using RAPD data. A database was created consisting of every isolate and its corresponding RAPD band pattern. For this calculation, genetic distance (G.D) was estimated between all pairs of the isolates according to Nei and Li (Nei and Li, 1979). Based on the data matrix based on following formula:

$$G.D = 1 - \{2N_{ab} / (N_a + N_b)\}$$

Where N_a = the total number of fragments detected in individual 'a'; N_b = the total number of fragments shown by individual 'b' and N_{ab} = the number of fragments shared by individuals 'a' and 'b'. Cluster analysis was performed to construct genetic relationship tree diagrams among studied *T. vaginalis* isolates using an Unweighted Pair-Group Method with Arithmetic Average (UPGMA) (Sneath and Sokal, 1973). All computations were carried out using the Numerical Taxonomy and Multivariate Analysis System STATISTICA, Version 6 (STSTSOFT, 2003).

Concordance with fertility, cervical abnormalities were tested by assigning a "1" to isolates with them and a "0" to isolates without them.

RESULTS

Genotypic variations by RAPD analysis have demonstrated the presence of different strains. The isolates with similar banding pattern were assigned as a single type. By using primer OPD1 no amplified fragment were found in any isolate. OPD2 resulted least typing (20 types) while OPD5 gave highest typing (35 types) ability (Fig.1).

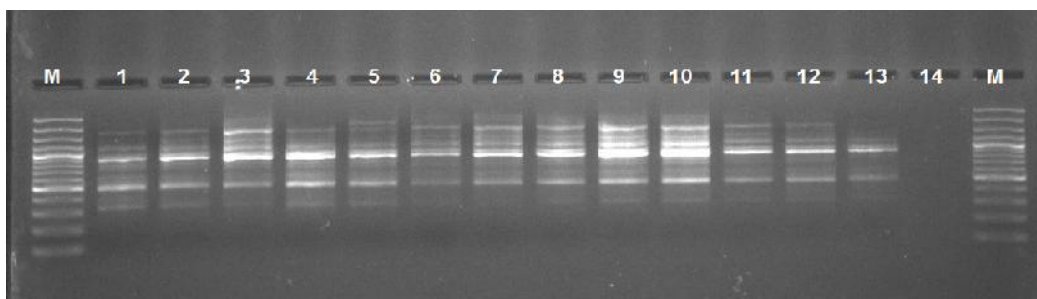


FIGURE 1.a-RAPD banding pattern obtained with OPD2 primer: M= 100bpDNA ladder, Lane 1-13 *T.vaginalis* isolates, Lane14: Negative control

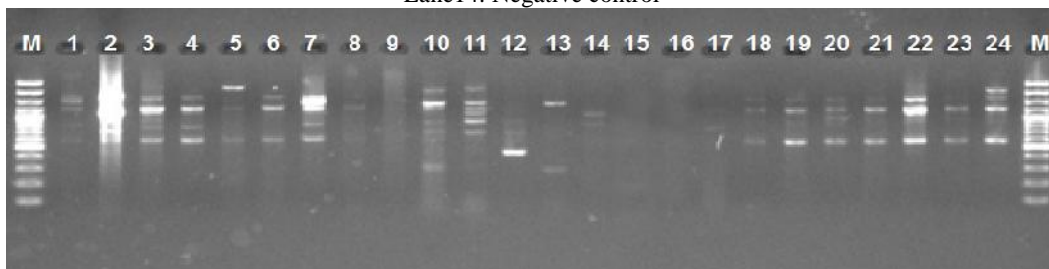


FIGURE 1.b-RAPD banding pattern obtained with OPD3 primer: M= 100bpDNA ladder, Lane 1-15 and 17-24

T. vaginalis isolates, Lane16: Negative control

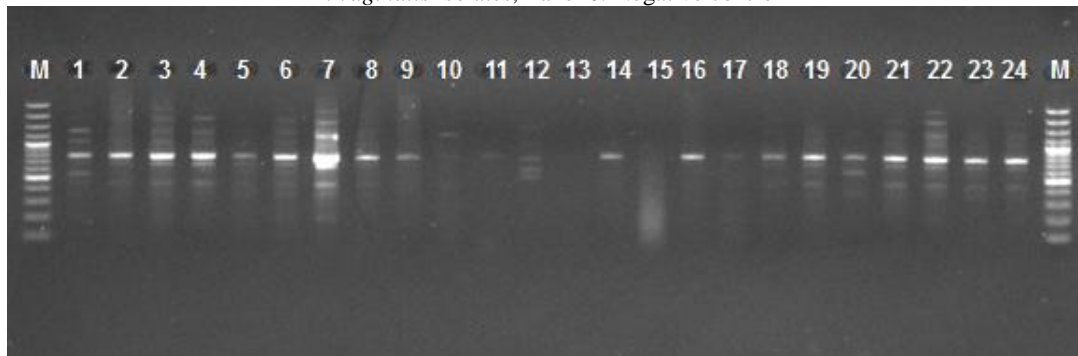


FIGURE 1: c-RAPD banding pattern obtained with OPD5 primer: M= 100bpDNA ladder, Lane 1-12 and 14-24 *T.vaginalis* isolates, Lane13: Negative control

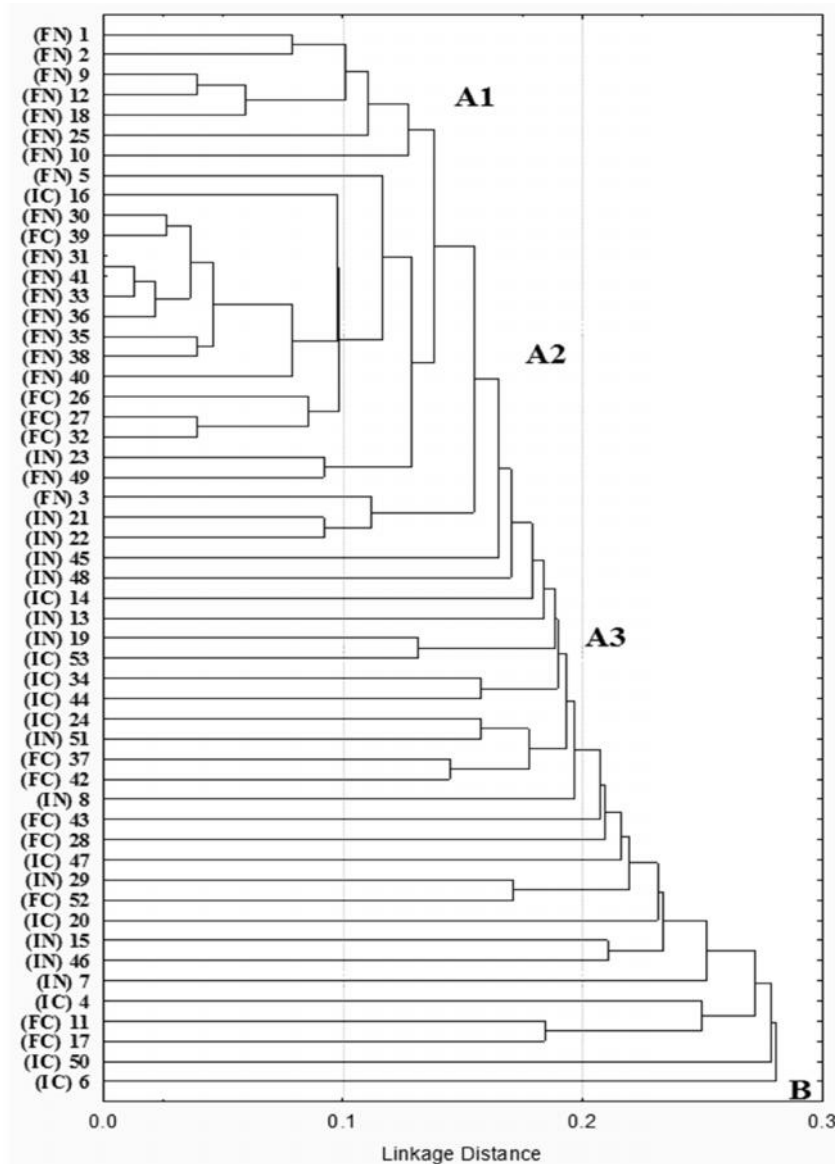


FIGURE 2: Constructed dendrogram by using UPGMA method based on data resulted from 53 isolates by RAPD method: F=Fertile, I=Infertile, C=Cervical abnormalities, N=No cervical abnormalities

A total of 76 fragments, sizing 350–1450 bp were scored and the data generated from the detection of polymorphic fragments were analyzed. The primers OPD2, OPD3 and OPD5 showed polymorphic bands in percentage 100%.

Four main groups could be distinguished by RAPD data. These groups coincide with four different patient categories (fertile, infertile, cervical abnormalities and no cervical abnormalities), but at this grouping isolates were

not completely separated from each other. Phylogenetic analysis using RAPD distance software indicated two clusters (A, B). Upper cluster consist of 52 isolates while lower cluster consisting of only one isolate (Fig. 2). The studied samples could be divided into three sub-clusters (A1, A2 and A3). 87.5% of the infertile samples and 72.7% of the cervical abnormalities samples were located in cluster A3. Fertile samples were distributed almost in all groups: 24.1% of them were in cluster A1, 48.2% in A2 and 27.5% in A3. A specific 754.5 bp marker was found for 75% of an infertile patients (18 infertile out of 24 samples), and 812.5 bp to be specific marker in 90.9% of cases with cervical abnormalities (20 cervical abnormalities case out of 22 samples).

DISCUSSION

Reports about the strain variation and phylogenetic polymorphisms of *T. vaginalis* are rare (Valadkhani *et al.*, 2011). The report from India by Kaul *et al.* indicated that OPD3 had least (nine types) while OPD4 had highest typing (18 types) ability (Kaul *et al.*, 2004). The least number of bands were seen in Iran by using primer OPD8 (13 types) and the most by using OPD3 (26 types) (Valadkhani *et al.*, 2011). In another study in Iran, Jamali *et al.* found that OPD1 had the least typing ability (32 types) and OPD5 had the highest typing ability (58 types) (Jamali *et al.*, 2006), however in this study no amplified fragment were found in any isolate by using primer OPD1, while OPD2 indicated least (20 types) and the OPD5 indicated highest typing (35 types) ability. Valadkhani *et al.* found specific amplified fragment with length 1300 base pair in only 8 isolates belonged to addicted women in Iran by using primer OPD1 (Valadkhani *et al.*, 2011), this result agree with our finding, because the OPD1 wasn't given any band in any isolate, and this indicates that there were no addicted women in our samples. This is also identical to the data obtained from patients in the questionnaire. OPD5 gave the highest typing as Jamali *et al.* in Iran while Valadkhani *et al.* found OPD3 as a highest typing primer, may be because they did not use OPD5 in their study, as we did with OPD4 which was the highest one in India (highest than OPD5) (Kaul *et al.*, 2004), but they found that OPD3 was the least typing ability not OPD1. Valadkhani *et al.* in Iran found that OPD8 was less typing than OPD1, and they found OPD3 as a highest one (not using OPD5). The compatibility between our results and Iranian studies was higher than Indian studies, this could be due to the low distance between sampling areas and frequent gene flow between neighboring isolates.

There are a close genetic relationship and certain gene polymorphism among the *T. vaginalis* isolates (Sheng Chong and Sheng Chong, 2004), as our results showed. Hampl *et al.* assayed the relationship between 20 strains of *T. vaginalis* from 8 countries using RAPD analysis and they found that the phylogenetic tree reproduces the pattern of virulence, geographic origin or infection by TVV (Hampl *et al.*, 2001), while Simoes-Barbosa *et al.* did not find any correlation between the genetic relatedness of *T. vaginalis* isolates and clinical phenotype (Simoes-Barbosa *et al.*, 2005). Rojas *et al.* found the association between genetic polymorphism of organism with clinical characterization when used RAPD technique in 40 isolates. Their results give emphasis to that the severity of

infection depends on the genetic type of *T. vaginalis* involved (Rojas *et al.*, 2004). The conflicting correlations in different reports from different geographical locations may be due to phenotypic and genotypic variations (Malla, 2012). Infertile isolates existing in a closely related group of isolates indicated that one or few mutations have occurred which may resulted in infertility, as well as cervical abnormalities isolates. It is suggested that the improvement of the capability of strains to cause these features in patients may be resulting from genetically predisposition of some genealogical line of *T. vaginalis*. RAPD technique may be suitable in discriminating between the different isolates of the same species resulting in different clinical profiles (Farooq, 2012). Our results confirmed this suitability for genealogical studies in *T. vaginalis*. This is the first description of a 754.5 bp possible marker for *T. vaginalis* in 75% of an infertile patients (18 infertile out of 24 samples), and a 812.5 bp possible marker in 90.9% of cases with cervical abnormalities (20 cervical abnormalities case out of 22 samples). Thus more study is needed to correlates these markers to an infertile women and patients with cervical abnormalities.

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