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DNA Banding Patterns of *Trichomonas vaginalis* Strains Isolated from Symptomatic and Asymptomatic Subjects

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ABSTRACT

Restriction digestion pattern analysis has emerged as a useful tool in assessing strain variation at the genetic level. The present study was aimed at exploring its utility in assessing strain variation in *Trichomonas vaginalis* and correlating these with the clinical presentation. Vaginal swabs and urine specimens obtained from 500 women were processed for isolation of *T. vaginalis* by wet smear examination and culture technique. Twenty seven (5.4 percent) specimens were found to be positive for *T. vaginalis*. Twelve strains could be axenised out of 19 isolated by culture technique. Parasite DNA was extracted in both early and late stage culture of strains both from symptomatic and asymptomatic subjects. Parasite DNA was isolated by three different methods. Proteinase K digestion was found to be the most suitable method compared to the methods using guanidinium isothiocyanate or diethylpyrocarbonate. Different strains of *T. vaginalis* could not be differentiated by banding patterns following restriction endonuclease digestion. Although the study did not reveal any genotypic variation among different strains, the pathogenic potential of the parasite might parallel the phenotypic variation as a result of differences in the expression of various genes. It may well be that host factors might be playing a critical role in the pathogenesis of trichomoniasis.

INTRODUCTION

In recent years, *Trichomonas vaginalis* has emerged as the most common cause of sexually transmitted disease of parasitic origin (Levine, 1991). Trichomoniasis results in significant worldwide morbidity, primarily among sexually active women (Honigberg, 1978; Muller, 1983). Its average incidence in the normal population is about 10 percent with a range of 0-65 percent among different groups (Lossick, 1990). An earlier study from PGI,

Chandigarh, India, revealed an incidence of 6.8 percent (Sharma et al, 1988). The clinical presentation of trichomoniasis shows wide variation ranging from complete absence of symptoms to severe inflammatory manifestations as urethritis, vulvovaginitis, cervicitis and the associated complications in females (Kiviat et al, 1985) and urethritis and prostatitis in males (Latif et al, 1987). Over and above, trichomoniasis is believed to enhance susceptibility to HIV infection (Krieger et al, 1992).

Despite the vast literature available on epidemiological, clinical and diagnostic aspects of trichomoniasis, little is known about the virulence factors of the organism which could explain the diverse symptomatology of the disease. To elucidate strain differences, there is need for a reliable, reproducible typing method, which in turn might reflect the physical or biochemical correlates of virulence. Attempts have been made earlier to differentiate *T. vaginalis* strains by antigenic characterization (Alderete et al, 1986) and isoenzyme analysis (Vohra et al, 1991) but without much success.

In recent years, DNA-typing, a new tool as a sensitive measure of strain variation at the genetic level, has proved valuable in differentiating pathogenic from nonpathogenic protozoa (Nash et al, 1985). One approach to DNA-typing involves agarose gel electrophoresis of restriction enzyme digested DNA. This reliable method of typing has not been put to test, to assess its utility in typing of *T. vaginalis* strains. The present study was undertaken to examine the possibility of differentiating symptomatic and asymptomatic clinical isolates of *T. vaginalis* based on their DNA banding patterns.

MATERIALS AND METHODS

Subjects: Five hundred women in the child-bearing age-group, attending the Obstetrics and Gynaecology Out Patient Department of the Nehru Hospital, attached to the Postgraduate Institute of Medical Education and Research, Chandigarh were selected for the study. These patients were attending the clinic for gynaecological complaints, antenatal/postnatal check up and family planning advice. The study period extended from March 1991 to May 1993. History and clinical findings were recorded for all the subjects on a preplanned proforma.

Specimens: Midstream urine samples were collected from female patients and male contacts of women positive for *T. vaginalis*. At the time of per vaginum examination, two vaginal swabs were collected using sterile cotton-tipped applicators. The samples were immediately transported to the laboratory for further processing.

Processing: One vaginal swab and a drop of the centrifuged sediment of urine were inoculated into TPS-I culture medium for initiation and maintenance of *T. vaginalis* strains (Diamond, 1970). The other vaginal swab and another drop of the urine sediment were used to prepare wet smears which were examined for the presence of *T. vaginalis*. The strains isolated in culture were axenized using penicillin (1000 U/ml) and streptomycin (1000 ug/ml) in the culture medium during first 3-5 sub-cultures. Axenic strains were further cultivated in bulk for the isolation of DNA in sufficient quantity. Large scale cultivation was done twice, once after 3-5 subcultures and again after 40-50 subcultures to study the effect of in vitro propagation as well. The parasites in mid-log phase growth were separated by

chilling and centrifugation followed by washing of trophozoites in phosphate-buffered saline (PBS, pH 7.2) and resuspended in 1 ml PBS. The cell pellets of about $1x10^9$ trophozoites/ml were stored at -70°C until further use.

Isolation of DNA: Isolation of intact *T. vaginalis* DNA was attempted by three different methods as it is relatively difficult to get intact DNA owing to the release of endogenous nucleases (Wang and Wang, 1985). Among the proteinase-K method (Rubino et al, 1991), guanidinium isothiocynate method (Paces et al, 1992) and diethylpyrocarbonate method (Riley & Krieger, 1992), the proteinase-K method was selected as it was found to be superior to the other two methods in isolating undegraded *T. vaginalis* DNA. To the cell pellet, 10 times its volume of lysis buffer containing 1 X SSC (150 mM NaCl, 15 mM sodium citrate), 300 mM NaCl, 0.2% sodium dodecyl sulphate (SDS) and 100 ug/ml proteinase-K (prepared fresh) was added and incubated in a 65°C waterbath for 30 mins. After checking for completion of lysis, phenol-chloroform extraction of the DNA was performed according to previously described methods (Sambrook et al, 1989). The DNA obtained was precipitated with ethanol and the pellet suspended in 500 ul of Tris-EDTA buffer (TE, pH 7.6). RNA contamination was removed by treatment with 20 ug/ml RNase. The DNA suspension was subjected to one more cycle of phenol-chloroform extraction and ethanol precipitation. The DNA isolated was quantitated by the spectrophotometric method and the quality was assessed by agarose gel electrophoresis (Sambrook et al, 1989). The concentration of DNA was adjusted to 0.5 ug/ul.

Restriction Digestion and Banding Pattern Analyses : In the initial experiment the DNA was digested using *EcoRI* alone, *Hind III* alone and both enzymes simultaneously to select the enzyme giving clear bands following digestion. *Hind III* enzyme was selected for restriction digestion and 3 ul of the enzyme was added to 20 ul of DNA sample. The mixture was incubated overnight at 37°C in a waterbath followed by agarose gel electrophoresis after 16 hours.

DNA isolates were digested and completion of digestion was checked on a mini-gel electrophoresis apparatus with 0.8% agarose slab. *Lambda* DNA/*Hind III* & ϕX174 DNA/*Hinc II* digest was used as standard marker. A random sample of uncut DNA was also run to demonstrate the relative integrity of DNA used. The DNA bands were photographed on a Polaroid film (type 665, Polaroid Corpn. USA) and analysed.

RESULTS

It was possible to isolate *T. vaginalis* from 27 subjects (5.4 percent) out of a total of 500 subjects screened. Strains isolated from patients complaining of vaginal discharge and/or pruritus, dysuria and dyspareunia were considered as symptomatic strains (8 such strains were obtained) while the isolates from patients without any such complaints (4 such strains were obtained) were considered as asymptomatic isolates (Table 1). The 12 axenized strains were cultured on a large scale at two stages, once after 3-5 subcultures (early stage strains) and again after 40-50 subcultures (late stage strains). DNA was isolated from 6 each of early and late stage cultures from symptomatic patients and 4 early stage strains and 3 late stage strains from asymptomatic subjects (Table 2).

Table 1: Isolation of *Trichomonas vaginalis* in the study population

Method of isolation	Symptomatic Women (16)	Asymptomatic Women (11)
VS only	1	4
US only	-	1
VC only	5	-
VS + US	1	1
VC + UC	1	-
VS + UC	1	-
VS + US + VC	2	-
VS + US + UC	-	2
VS + VC + UC	2	2
VS + US + VC + UC	3	1

VS, Vaginal smear; US, Urine smear; VC, Vaginal culture; UC, Urine culture

Table 2: Source of strains used for Restriction Digestion Pattern analysis.

Strain Number	Patient Status	Subculture Stage
211/91	S	L only
34/91	S	L only
G/92	S	E only
44G/92	S	E only
11/92	A	E & L
12/92	A	E & L
19/92	S	E & L
44/92	S	E & L
71/92	S	E & L
124/92	A	E & L
158/92	S	E & L
5/93	A	E only

S, symptomatic; A, asymptomatic; E, Early stage (3 to 5) subculture; L, Late stage (40 to 50) subculture.

DNA isolation from three strains of *T.vaginalis* using three different mehtods indicated that the proteinase K method was best suited for obtaining intact, undegraded DNA. The DNA obtained by lysis with guanidinium isothiocyanate and diethylpyrocarbonate showed marked degradation (Fig. 1, lanes 3,4 and 5,6 respectively) and hence the samples were considered unsuitable for further use in digestion pattern studies. However, the proteinase K lysis buffer method showed minimum smearing and a

distinct band of intact DNA (Fig. 1, lanes 1,2), so all the strains were further subjected to this method of DNA isolation.

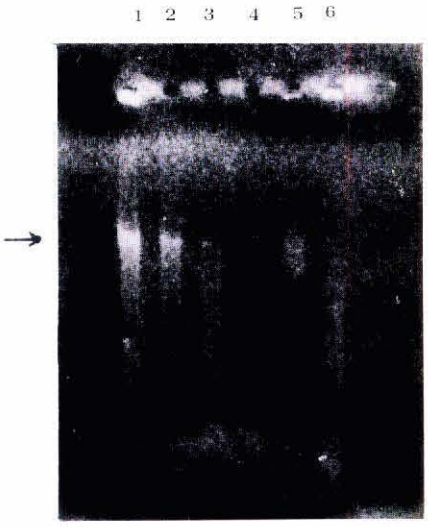

Figure 1: Comparison of 3 different methods used for *T. vaginalis* DNA isolation. Figure shows AGE of undigested DNA (strain 211/91) isolated following lysis with proteinase K (lanes 1&2); guanidinium isothiocyanate (lanes 3&4) or diethylpyrocarbonate (lanes 5 &6).

The uncut DNA revealed a major band with mobility equivalant to a DNA size greater than 23.1 kb (Fig. 2, lane 21) as was observed in earlier experiments as well. The digested DNA samples of different strains showed uniform smearing of DNA, indicating some amount of degradation of DNA. This could not be avoided despite repeated attempts to isolate DNA in an intact form.

Multiple bands were observed in both symptomatic and asymptomatic strains (Fig. 2). The bands were seen more clearly in lanes with less background smearing. Wherever the bands were visible, these were similar irrespective of whether the isolate was from a symptomatic or asymptomatic case and early or late stage strain. Most of the bands were common in several isolates and no isolate-specific band could be identified.

DISCUSSION

Emergence of trichomoniasis as a major sexually transmitted disease has enhanced the need to elucidate various aspects of the molecular biology of *T. vaginalis* so as to understand the potential mechanisms involved in disease pathogenesis. So far it is not clear why only a proportion of infected individuals become symptomatic while others do not. Some workers believe that this may be attributed to the inherent virulence of the parasite while others implicate various host factors (Honigberg et al, 1978).

The differences in the morphology, experimental virulence, enzymes, surface carbohydrates and proteins of different isolates have not been found useful for classifying

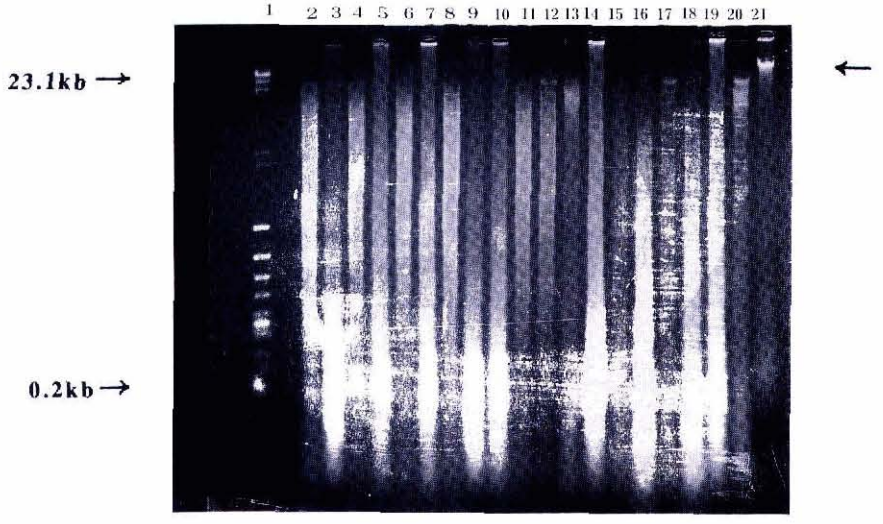

Figure 2: Restriction digestion pattern analyses of *T. vaginalis* DNA from symptomatic and asymptomatic, early and late stage isolates digested with *Hind III* restriction endonuclease. Lane 1, *Lambda* DNA/*Hind III* & ϕ X174 DNA/*Hinc II* digest; Lanes 2 to 13 symptomatic isolates; Lanes 14 to 20, asymptomatic isolates; Lane 21, undigested *T. vaginalis* DNA (23.1kb band of uncut DNA); Lane 2&3, strain 19/92, early and late stages; Lanes 4&5, strain 71/92, early and late stages; Lanes 6&7, strain 44/92, early and late stages; Lanes 8&9, strain 158/92, early and late stages; Lane 10, strain 211/91, late stage; Lane 11, strain G/91, early stage; Lane 12, strain 44G/91, early stage; Lane 13, strain 34/91, late stage; Lanes 14&15, strain 11/92, early and late stages; Lanes 16&17, strain 12/92, early and late stages; Lanes 18&19, strain 124/92, early and late stages; Lane 20, strain 5/93, early stage.

the *T. vaginalis* strains (Honigberg, 1978). Similarly, immunological methods and zymodeme pattern analysis have failed to differentiate symptomatic from asymptomatic strains (Alderete, 1986, Vohra et al, 1991). As DNA banding pattern (schizodeme) analysis has been found to be useful for typing various other protozoan parasites (Nash et al, 1985), we have attempted to utilize this technique for distinguishing and matching diverse or related strains of *T. vaginalis*.

The major limitation in the study of *T. vaginalis* genome is the difficulty to obtain relatively intact DNA. This nucleolytic degradation of genomic DNA, has been attributed to the endogenous nucleases and should be inhibited during DNA isolation, which would be successful only if the rate of denaturation of nucleases exceeds that of DNA hydrolysis (Wang and Wang 1985; Riley and Krieger, 1992). In our experimental set up proteinase-K lysis buffer method proved to be relatively better than lysis with either guanidinium isothiocyanate or in presence of diethylpyrocarbonate.

Though it is clear that DNA degradation would have considerable impact on the quality of restriction digestion patterns, it may not be a major problem with *T. vaginalis* as this parasite contains multiple repetitive sequences (Paces et al, 1992). Moreover, the size of the largest DNA fragments after restriction digestion is not very large, being just over 23 Kb. Despite all precautions, some degree of background smearing due to degraded DNA appeared in AGE, which obscured the clear bands of the digested *T. vaginalis* DNA.

In the *Hind III* restriction digestion banding pattern of *T. vaginalis* DNA, the banding pattern was similar in all the strains tested, irrespective of whether they were from asymptomatic or symptomatic patients and whether from early or late stage strains. The results do not indicate any correlation between the clinical presentation and the genetic make up of the parasite, at least which could be detected using this approach. Similarly, absence of any clear-cut difference between the digested DNA of early and late stage strains of *T. vaginalis* suggests that the DNA is stable and unaffected by repeated sub-culture. It was not possible to isolate *T. vaginalis* from male contacts of infected females and therefore the digestion pattern difference in these strains causing asymptomatic, self-limiting disease in men could not be analyzed.

It is possible that difference in the genetic make-up of various strains exists only in a few minor bands obtained after AGE. Unfortunately, these could not be visualized due to predigestion fragmentation and background smearing of the lanes during AGE. Similarly, the difference in ethidium bromide staining of sheared DNA and the separated DNA band could be attributed to difference in the yield of intact DNA from trophozoites in mass culture. Restriction fragment length polymorphism (RFLP) study using a standard probe may provide a better means of differentiating strains of *T. vaginalis* from patients with varying clinical spectrum or from different geographical areas. However, the present study indicates that the variation in pathogenic potential of *T. vaginalis* strains may be expressed phenotypically rather than genotypically. Furthermore, host factors may also be important in the pathogenesis of trichomoniasis, influencing the biology of the parasite markedly.

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