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Original Article

Role of pH on Adhesion of Trichomonas Vaginalis Isolated from Symptomatic & Asymptomatic Women to Vaginal Epithelial Cells *in Vitro*

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Abstract

Background: Vaginal epithelium is the most important site for initial contact of the parasite infection in humans.

Objective: To investigate the effect of Lactobacillus acidophilus (LA) and pH on attachment of *T. vaginalis* isolated from symptomatic and asymptomatic patients to vaginal epithelial cells (VECs).

Methods: Following 1-4 hrs contact of parasite with VECs, wet mounts were prepared and used for measuring cell adherence in vitro. This was done by addition of whole LA, its excretory secretory product (ESP) and changing the pH of the media either by HCl or LA. The mean of the three readings of the experiments was taken to compare the isolates and their controls.

Results: The exposure of VECs to whole LA for one hour led to enhancement of adhesion and then a gradual fall with ultimately non-viability *T.vaginalis* after 4 hrs of incubation with *T. vaginalis*. In the presence of ESP a gradual decreased of adhesion was observed. The number of VECs attached by *T.vaginalis* was higher in symptomatic than asymptomatic women's isolates when pH was reduced to 4.5 by HCl, when pH was reduced to a similar level by LA this ratio was 47% and 35% respectively.

Conclusion: The presence of LA enhanced the attachment of parasite at initial steps of infection, but its effect was deleterious thereafter. This study should be paralleled by appropriate in vivo experiments and if substantiated can be used for effective therapy and prevention of human trichomoniasis.
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Keywords ● Trichomoniasis ● adhesion ● lactobacillus ● pH

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Introduction

Trichomonas vaginalis (*T. vaginalis*), the causative agent of trichomoniasis is a microaerophilic protozoan. Human trichomoniasis is now widely recognized as a prevalent sexually transmitted disease, capable of causing considerable morbidity. The infection can result not only in varying degrees of vaginitis,[1] but also in infertility, premature labor, premature rupture of placental membranes, and low birth weight infants.[2,3] Relative risk of developing invasive cervical cancer and six fold higher probability of infection by human immuno deficiency

virus are linked to this disease, which have led to increasing interest in studies in relation to its pathogenesis.[4,5]

For the establishment of infection in humans, importance of initial adhesion followed by invasion and persistence are well accepted.[6] The mechanisms thought to be involved at different stages of urogenital trichomoniasis are based on their adhesive and colonization ability, production of cell detaching factors, pore forming proteins, soluble hemolysins and extra cellular proteinases.[7] Initial adhesion to vaginal epithelial cells (VECs) and to different cell lines is reported to be mediated by four adhesion proteins (AP) comprising AP65, AP51, AP33 and AP23.[8] Expression of these adhesion molecules, which is time, temperature, and pH dependent,[9] increases the ensuing contacts with VECs.[10] Upon contact and colonization of the parasite, the cell monolayers are destroyed through elaboration of a 200 KDa protein Cell Detaching Factor.[11] Previous reports have indicated that in mice, the sustained infection could only be induced in the presence of Lactobacillus acidophilus (LA).[12] However, the precise role of LA in persistent infection with trichomoniasis is not yet understood. This might be due to the reduction of pH in vaginal environment.[12]

For a better understanding of the host/ parasite relationship, it is thus desirable to study the interaction of the parasite with the host specific target cells. This study, therefore, was planned to compare the *T.vaginalis* strains, isolated from symptomatic and asymptomatic women, in relation to their adhesion to VECs in vitro, in the presence and / or absence of LA and low pH in order to elucidate the *T.vaginalis* pathogenesis.

Materials and Methods

T. vaginalis was isolated from clinical samples of 500 symptomatic and asymptomatic female patients attending to the department of Obstetrics and Gynecology of Post Graduate Institute of Medical Education and Research Center (PGIMER) of Chandigarh, India. Isolates obtained from those patients complaining of vaginal discharge and/or pruritis, dysuria and dyspareunia were considered as symptomatic patients isolate (SPI). Isolates from patients attending the clinic for routine checkup, infertility or some other gynecological problems without aforementioned complaints were considered as asymptomatic patients isolates (ASPI). Midstream urine samples and two sterile cotton vaginal swabs were used for isolation of the parasite. These were employed for wet smear examination and axenic cultivation in Tripticase Yeast extract Iron Serum 33 (TYI-S-33) medium at pH.6.2.[13] VECs were collected by two sterile cotton vaginal swabs from healthy women who attended to the outpatients Obstetrics and Gynecology department for family planning advice or routine postnatal/ antenatal checkups. One swab was used for examination of TV, and the other was kept in sterile phosphate buffer saline (PBS) at pH 7.2. Cells were washed thrice in PBS and centrifuged at 250 Xg for 5 minute for cyto-adherence assay. Fresh VECs were used in all experiments.

Cyto-adherence assay

Optimization of conditions for measuring vaginal epithelial cell adherence by *T. vaginalis* was done in microfuge tubes by incubating 100µl of VECs ($4/10^4$xml) with 100 µl of live motile trichomonads ($2/10^5$/ml) isolated from SPI and ASPI patients at 37 ºC. The concentration of parasite and VEC used in the present study was based on the earlier report.[6] Wet mounts were prepared at hourly intervals following incubation. Percentage of VECs attached by *T.vaginalis* as well as the number of parasites attached to VEC was determined by counting 100 VECs in various fields. Each isolate was subjected three times to adhesion assay and the mean reading of three such experiments was taken.

Effect of L. acidophilus on attachment of T. vaginalis to VECs

TV strains isolated (2×10^5/ml) from SPI and ASPI patients incubated at 37°C in the presence of LA (2×10^6/ml) for 1 to 4 hrs. Thereafter, VECs (4×10^4/ml) 100µl were added to each tube at time intervals of 1, 2, 3 or 4 hrs and incubated for 30 minutes at 37°C. The percentage of VECs attached by *T.vaginalis* was then counted, under microscope, after each time interval by wet smear examination. Controls without LA were also processed simultaneously with each isolate.

Effects of ESP of L. acidophilus on the attachment of T. vaginalis to VECs

LA was incubated at 2×10^6/ml in Man Rogosa Sharp (MRS) medium in different tubes at 37°C in 5% CO_2. After one to four hrs of incubation, the content of each tube were centrifuged at 500×g for 10 minutes and the EP was used subsequently. To the suspension of *T. vaginalis* at 2×10^5/ml and VECs at 4×10^4/ml, EP of L .acidophilus (100 µl) was added and incubated at 37°C for 30 minutes. Percentage of VECs attached by *T. vaginalis* was counted along with the control tubes (*T.vaginalis*, VECs and MRS media) without Lactobacilli.

Table 1: Mean attachment of *T. vaginalis* obtained from symptomatic patients (SPI) and Asymptoatic patients isolates (ASPI) in the presence of L. acidophilus (LA) and excretory secretory products (ESP) of LA to VECs. (10 strains in each group)

Time period	% age of VECs attached by *T. vaginalis*			
	1 hr	2 hrs	3 hrs	4 hrs
SPI + LA	56	48	28	NV
CNT - LA	39	37	40	37
ASPI + LA	52	36	23	NV
CNT - LA	31	33	34	30
SPI + ESP	15	20	21	NV
CNT- ESP	40	36	42	30
ASPI + ESP	10	5	3	NV
CNT - ESP	34	31	37	30

CNT = Control; NV = Non viable

Effect of acidic pH on the attachment ofT.vaginalis to VEC's

After adjusting the pH of TYI-S-33 medium to 4.5 by adding 10% HCl or LA, it was added to the tubes each containing a mixture of 100µl of *T.vaginalis* (2×10^5/ml) and VECs (4×10^4/ml) and incubated for 30 minutes at 37°C. In control tubes the pH was 6.2 and the rest were processed similarly. Then the VECs attached by *T.vaginalis* were counted.

Statistical analysis

Results were analyzed statistically by applying Student's *t* test to compare the significant differences between variables of both groups.

Results

Twenty two *T.vaginalis* isolated from vaginal swabs and/or urine samples of 500 women. From 272 symptomatic patients 12 (4.4%) and from 228 asymptomatic patients 10 *T.vaginalis* (4.39%) was isolated. The results showed that there was no significant difference between the isolation of *T.vaginalis* of symptomatic and asymptomatic patients. The isolates from each group were then maintained in the culture media and used for further experiments. Table 1 shows the attachment of *T.vaginalis* in the presence of LA. In the presence of LA, after one and two hrs of incubation, a large number of VECs were attached by *T.vaginalis* isolated from SPI (56, 48) as well as ASPI (52, 36) as compared to their own controls (without LA). However, the difference was only significant at one hr ($p<0.01$). At 3 hrs of incubation a significant fall in the number of adherent *T.vaginalis* was observed in both groups of isolates as compared to their own controls. However,

Table 2: Effect of pH reduction on attachment of T. vaginalis to VECs

Isolates	% age of VECs attached by TV			
	HCl (pH 4.5)	LA (pH 4.5)	Control (pH 6.2)	p value
SPI	62	47	-	p<0.05
CNT	-	-	33	
ASPI	52	35	-	NS
CNT	-	-	27	

T.vaginalis became non-viable after four hrs of incubation in both groups as indicated by trypan blue dye exclusion test, and differences were not significant. The results of the effect of ESP of LA on the attachment of parasite are also shown in Table 1. There was a significant reduction in the number of VECs attached by *T.vaginalis* in SPI ($p<0.01$) as well in ASPI ($p<0.001$) in comparison to their own controls. As the period of incubation increased, the number of VECs attached by *T.vaginalis* decreased after four hrs with no detectable parasites.

The effect of pH on the attachment of parasite to VECs is shown in Table 2. Reduction of the media pH to 4.5 either by adding HCl or LA increased the number of VECs attached by *T. vaginalis*. The number of VECs attached by *T. vaginalis* isolated from SPI was higher at pH 4.5 (62%) in the presence of HCl, as compared to the same pH induced by LA (52%) respectively. However, no statistically significant differences were observed between the results of the isolates obtained from SPI and ASPI patients. Fig 1 shows the number of *T. vaginalis* attached per VEC when pH was reduced by LA (whole cells), and HCl and excretory products of LA. The results showed that in the presence of LA, the average number of *T. vaginalis* per VEC was 8 in SPI as compared to 5 in ASPI ($P<0.001$).

When pH was reduced to 4.5 with HCl the number of *T. vaginalis* attached per VEC was less. However *T. vaginalis* isolated from SPI (2-6) showed more attachment than that of ASP isolates (2-4) When excretory secretory products of LA was added before incubation of VECs with *T. vaginalis*, the number of *T. vaginalis* attached per VECs decreased that was more in ASPI (1-3) than SPI (2-4).

Discussion

Trichomoniasis is one of the commonest sexually transmitted disease capable of causing considerable morbidity in infected patients.[4] The infection can occur both in males and females. Most often males may be asymptomatic

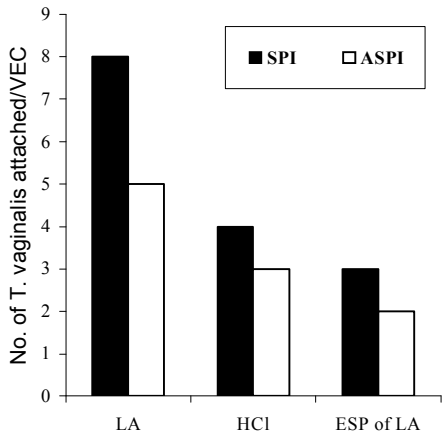

Fig 1: Comparative T. vaginalis per VEC in the presence of L. acidophilus whole cells (LA), HCl and excretory secretory products of LA (EPS of LA). SPI and ASPI = Symptomatic and Asymptomatic Patient Isolates

while females are known to be symptomatic as well as asymptomatic.[11]

Positive cultures were only obtained from 10 out of 22 urine samples. Mohamed et al reported that urine is a poor specimen for the detection of *T.vaginalis* particularly in women and recommended that the examination of vaginal secretion should be included.[14] It is not clear why in the infection in some women is symptomatic in others is asymptomatic.[11] In the present study, S was 54.4% and AS was 45.6% in patients seeking hospital service for gynecological problems. In a study performed by Alderete et al. suggested the existence of two strains of virulent and less virulent *T.vaginalis* which differed in their morphologic characteristics and intrinsic virulence that cause variable symptoms.[15] Different strains of *T. vaginalis* have been found to vary with respect to surface carbohydrates and proteins.[16,17] Asymptomatic infection is particularly important in terms of epidemiological investigation since it is a major source of transmission.

Both host and the virulence factors of the infecting parasites may involve in symptomatology of the disease.[16] Vaginal epithelium is the most important site for initial contact of the parasite. The present study showed that the amoeboid was converted to pear-shaped parasite during or after attachment to VEC. Heath et al also reported the appearance of amoeboid morphology following interaction with epithelial cells.[18] Another study showed that morphological changes of parasite was related to a phenotypically variable 270-KDa surface protein.[19] In our study, fresh vaginal epithelial cells were pooled from healthy women at various times during menstrual cycle because of variations in receptor availability. These cells were used for adhesion studies in order to simulate the prevailing in vivo conditions that minimize the variations in attachment experiments.

The process of adhesion and colonization is well recognized and precedes the establishment of mucosal infection.[6] In a study reported by Addis et al, LA was found to be an important contributor to the establishment and persistent *T.vaginalis* infection in mice.[20] In the present study, the use of live LA increased the attachment of *T. vaginalis* to VECs in both SPI and ASPI up to two hrs which was more significant regarding SPI ($p<0.01$). In contrast, the attachment of *T.vaginalis* to VECs in the presence of EP of LA was significantly reduced in both groups. Previous reports indicated that *T. vaginalis* was unable to grow well when a higher concentration of LA was present.[21] However, little is known about comparisons between adherence of *T.vaginalis* from SPI and ASPI to VECs. The present finding may explain the reasons for increasing the number of *T.vaginalis* after the reduction of LA as a normal flora in vaginal environment as found at the end of menstrual cycle and during menopause. This is coupled with increasing symptoms of trichomoniasis.[22] LA is also known to provide vaginal acidity with a pH of 4-4.5, due to the breakdown of glycogen into high level of lactic acid.[23] This is known to provide natural protection against colonization by genital pathogens like *Mycoplasma homonic* and *Gardnerella vaginalis*.[23] In the present study, the capacity of LA in lowering pH to 4.5 was compared with similar reduction in pH by HCl, in terms of adhesion of parasite to VECs of both SPI and ASPI. The parasite attachment to target cells may modify the local conditions, such as pH and Ca^{2+} concentration which creates an ideal environment for release and function of the pore forming proteins.[24] Formation of microenvironment is therefore fundamental to pathogenicity. LA is also known to produce many other biological compounds like acidolin and hydrogen peroxide.[25] Therefore, further studies are needed to define the initial process of adhesion which leads to infection by *T.vaginalis* facilitated by LA.

The results of the present study thus indicate that the enhancing effects of L. acidophilus on adhesive ability of symptomatic *T. vaginalis* to vaginal epithelial cells is probably due the reduction in vaginal pH; peaking only during the initial phase of the attachment.

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