EVALUATION OF THE ANTIBODY RESPONSE AGAINST *T. VAGINALIS* DURING FOLLOW-UP VISITS OF PHARMACOLOGICALLY TREATED PATIENTS

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Abstract

Objective: The protist *Trichomonas vaginalis* is the most common non-viral, curable, sexually transmitted disease (STD) agent worldwide. The overall objective of this study is to evaluate the antibody response against *T. vaginalis* during follow-up visits of pharmacologically treated patients. **Materials and methods:** The study included 46 women affected by trichomoniasis and 8 male sexual partners. All women were subjected to standard clinical examination, vaginal samples were collected for identification of *Trichomonas vaginalis* by wet mount and cultivation in specific media. Serological reactivity of patients affected by trichomoniasis was studied for a 5 months period after pharmacological treatment in order to estimate the persistence of anti-trichomonas antibodies after eradication of protozoan infection by ELISA assay and western blotting. **Results:** Serological follow-up by ELISA showed the trending line of sera *T. vaginalis* IgG antibody going down after 4-5 months in the group of recovered patients; while those from the unrecovered/re-infection patients kept the high level of IgG antibody, a marker of infection persistence. Results from Western blot analysis showed very good correlation with those from ELISA assay and clinical symptoms during the course of follow-up periods. There were persistence of antibody to *T. vaginalis* antigen weighted 84 -115kDa in men partners and recovered patients. **Conclusions:** Host immunological response in trichomoniasis patients were IgG antibody, and dependent on the individual reaction. The titer of IgG decrease during the recovery time and become normal after 4-5 months. However, there were the persistence of antibody to *T. vaginalis* antigen 84 -115kDa in men partners and recovered patients.

Key words*: *T. vaginalis, ELISA, Western blot.

1. INTRODUCTION

Trichomoniasis is a sexually transmitted disease caused by the parasitic protozoan *Trichomonas vaginalis*. It is the most common nonviral curable sexually transmitted disease. According to WHO there were 248 million cases of *T.vaginalis* infection in women each year worldwide [1,2,3]. Trichomoniasis has been implicated in causing adverse pregnancy outcomes [4] and has been associated with an increased risk of human immunodeficiency virus (HIV) transmission [5].

Trichomonas vaginalis induces humoral, secretory, and cellular immune responses in infected individuals [7]. Serological techniques therefore seem to be advantageous for diagnosing infections [8]. Many serological techniques (for example, haemagglutination, complement-fixation, immunofluorescence, and radioimmunoassay) have been used to detect antibody to *Trichomonas vaginalis*. An enzyme-linked immunosorbent assay (ELISA) for detecting antibody to antigenic *Trichomonas vaginalis* macromolecules has

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*- Received: 20/5/2013 * Revised: 2/6/2013 * Accepted: 15/6/2013*

been identified using whole cells of *T.vaginalis* had good sensitivity [9]. Otherwise, the efforts to getting more understand the pathogenesis and immunogen of trichomoniasis disease for prevention and control especially vaccination are emphasized. Nowadays, research into the development of a vaccine for *T. vaginalis* has shown some promise, elucidating a number of mechanisms by which protection could potentially be achieved. However, there are not much study about the persistence of the antibody against *T. vaginalis* in prospective studies.

Therefore, our research has been carried out with the objective of evaluation the antibody response against *T. vaginalis* during follow-up visits and determine the kinetic of antibody disappearance in sera of pharmacologically treated patients.

2. MATERIALS AND METHODS

2.1. Materials

This was a prospective cohort study, conducted from September 2010 to June 2012 at following settings:

- Gynecological Clinic of Hue University Hospital (GCHUH)
- Reproductive Healthcare Centre of Thua Thien Hue Province (RHC TTHP)
- Parasitology Department, Hue University Hospital
- Carlo Urbani Centre, Department of Microbiology, Hue University of Medicine and Pharmacy
- Division of Experimental and Clinical Microbiology, Department of Biomedical Sciences – University of Sassari.

2.2. Study population

All individuals were provided information and signed informed consent on study procedure.

In total, 52 trichomoniasis patients were diagnosed by gynecological examination and wet mount microscopic examination, among them 46 patients agreed to participate into the study of evaluating the immuno-response against *T. vaginalis* during the follow up visits. Their blood and vaginal discharge samples were collected before pharmacological treatment. Sera were collected for detecting anti - *T. vaginalis* specific antibody by ELISA assay. The vaginal discharge samples were collected for *T. vaginalis* culture. They were also given the appointments for the follow up period of 5 months by 1-month interval. Every time, patients were clinically examined to evaluate the improvement of clinical symptoms. The blood samples were again collected for monitoring the titers of anti - *T. vaginalis* specific antibody by ELISA assay and Western blotting analysis. The vaginal discharge samples were collected for evaluation of *T.vaginalis* presence or eradication by direct examination, culture.

2.3. Treatment and follow-up

Trichomoniasis patients on the basis of wet mount direct examination on microscopy were provided free treatment, health education about STDs and subjected to follow-ups for examining titers of anti - *T. vaginalis* specific antibody by ELISA assay and Western blotting analysis.

Treatment regimens for trichomoniasis women and her partner were prescribed according to US Center for Disease Control's Sexually Transmitted Diseases treatment guidelines 2010, with metronidazole administered orally 500mg twice a day for 7 day. Alternative regimens were Tinidazole 2g orally in a single dose or Tinidazole 2g orally once daily for 2 days or Tinidazole 1g orally once daily for 5 days [10].

2.4. Recovered and unrecovered/re-infectious patients

Recovered patients were defined by the improvement of clinical symptoms and the eradication of *T.vaginalis* by microscopic examination, culture and PCR assay, confirmed during the course of 5 months of follow-up.

Unrecovered or re-infectious patients were defined by the persistence of clinical symptoms and/or *T. vaginalis* by microscopic examination, culture and PCR assay at any time during the course of 5 months of follow-up.

2.5. ELISA plate

The ELISA assay was carried out following a method described by Alderete P.J. (1984)[9], and Mason P. R. (2001)[11] using the G3 strain of *T. vaginalis*. This isolate originated from United Kingdom, and is characterized by being free from mycoplasma infection. Long-term cultures in T25 flask (Becton Dickinson England No 353014) using standard protocol were maintained using mycoplasma-free Diamond's medium. Parasites in logarithmic growth were harvested, washed

three times in phosphate buffered saline (PBS) and suspended at 1-1.5 x106 cells/ml. Aliquots (50 µl) were added to the wells of microtitre plates. The plate was incubated overnight at room temperature (RT). The well were added 50µl /well of methanol, fix for 10minutes at RT. After removing methanol from the plate by aspiration, the well were washed 3 times by PBS-tween 20 0,05% (200µl/well). After adding 100µl/well of PBS – bovine serum albumin (BSA) (A 2153-Sigma Aldrich) 1%, the well were incubated for 2 hours at RT or overnight at 4^0C. Then the well were washed with distilled water. After drying in air about 1-2hours, the well were stored at 4^0C until use.

2.6. ELISA assay

All sera samples were collected were tested by ELISA assay using the whole cell of *T. vaginalis* as antigen for detecting anti – *T. vaginalis* IgG antibody.

Each ELISA plates had the positive, negative sera control and one white well with PBS alone. Positive sera control were obtained from trichomoniasis patient, of whom had an active *T. vaginalis* infection, based on microscopy and culture, at the time of blood collection. Negative sera control were obtained from children sera 2-10 years old confirmed from the patients without the blood transmitted diseases in Pediatric sesion.

Wells were blocked by the addition of 100µl PBS - 0,5%Tween-20 (PBS-T) containing BSA 1%. Sera were diluted 1:100 in PBS with 5% of tween 20 and BSA 1% solution, and 100µl were added to each well and the plate were incubated for 90 minutes at RT. Washing the well 3 time by 200µl/ well of PBS with 5% of tween 20 (250µl tween 20 in 500ml PBS). Anti-human IgG Fc specific alkaline phosphatase conjugate (No. A9544 of Sigma Aldrich, USA) were diluted 1:30,000 in PBS-BSA 1% immediately before adding 100µl into each well. After incubating for 60 min at RT, the well were washed 3 times by 200µl/well of PBS with 5% of tween 20. One tablet of ELISA substrate (p-Nitrophenyl phosphate, product No.N2765 of Sigma Aldrich USA) were diluted in 20ml AP buffer pH 9,5. After adding 100µl/ well of substrate solution, the well were read the optical density (OD) at 405 nm in 15-30 minutes by ELISA reader of Biorad 680.

2.7. Western blot to find specific antibody against *T. vaginalis*

Total *T. vaginalis* SS-22 protein preparations were obtained by trichloroacetic acid precipitation, electrophoresed by SDS - PAGE, and transferred onto a nitrocellulose membrane. Vietnamese sera of following up trichomoniasis patients affected by trichomoniasis were diluted 1 : 200. As a control, the sera from Vietnamese women negative for *T. vaginalis* by both ELISA and wet-mount. After incubation, membranes were washed and incubated with antihuman IgG immunoglobulins conjugated with alkaline phosphatase (Sigma, St. Louis). Bound antibodies were detected with chromogenic substrates.

2.8. Ethical issue

Study protocols were approved by Hue University of Medicine and Pharmacy Institutional Review and Ethical Board.

2.9. Data analysis

Statistical analysis was performed using Microsoft Excel 2010 and Medcalc software.

Comparison of two mean of following up sera antibody titers were calculated by Kruskal – Wallis test because of the small number of patients.

ROC analysis were also used to evaluate the relation between the following up sera antibody titers and clinical symptoms. Levene's test for equality of variances, ANOVA test for evaluation the relationship, Student-Newman-Keuls test for all pairwise comparisons.

All reported confidence intervals were two-sided 95% confidence intervals and P - values < 0.05 were regarded as statistically significant.

3. RESULTS

3.1. Clinical features of trichomoniasis patients

From 46 trichomoniasis patients diagnosed by clinical and microscopic examination, 91.3% of patients were in reproductive age, with the mean age 37±9 (20-60). There were 4 (8.7%) menopausal patients.

The most prevalent symptoms were vaginal erythema (80.4%), malodorous vaginal discharge (73.9%), profuse vaginal discharge (60.9%); cervicitis (58.7%), and yellowish-green frothy discharge (54.3%), and 10.9% asymptomatic cases.

Follow-up of selected patients

Antibody response against *T. vaginalis* during follow-up visits

Before pharmacological treatment, there were 46 trichomoniasis patients. However, only 30 of them attended the follow-up visits. Because of several reasons, there were only 6 patients attended 5 follow-up visits during 5 months. The interval periods were show on the table 1.

In recovered patients, who are defined by improvement of clinical symptoms and the eradication of *T.vaginalis* by microscopic examination, culture and PCR assay, confirmed during the course of 5 months of follow-up, the symptom improved gradually over time. The malodor, yellowish-green frothy discharge, serious vaginal discharge were noted that improved by 1 month. In the first follow-up visit after 1 month, there were 13/28 patients still having vaginal erythema and cervicitis by speculum examination and, at the second follow-up visit there were 6/17 patients having vaginal erythema and cervicitis symptoms by speculum examination. At the third visit, even though some cases were still having OD ratio greater than 1, 100% of clinical symptoms improved.

In unrecovered/reinfections patients (described in Materials and Methods), the symptoms remained unchanged during the follow up visits.

OD ratio of IgG antibody against *T. vaginalis* of patients and OD of negative control (NC) was used as baseline to compare the titers of antibody IgG against *T. vaginalis* in follow-up patients.

Table 1. OD ratio of patients (Pa)/negative control (NC) during 5 -month follow-up

	OD ratio of Pa/NC					
Patient number	Pretreat.	1 month	2 months	3 months	4 months	5 months
28	**0.95**	0.95	0.95			
66	**2.01**	**2.17**	**1.63**	1.6	0.95	1.13
68	**2.76**	**1.7**				
69	**1.89**	**1.98**	**2.83**	**1.94**	**3.59**	**1.27**
79	**2.64**	**4.57**	**3.35**	**6.95**	**2.55**	**2.94**
85	**2.9**	**4.28**		1.33	1.35	1.09
84	**1.4**	**1.4**	0.82	0.88	0.86	0.88
227	**1.91**	**1.01**	**1.04**	0.98	0.93	0.107
360	**2.16**	**1.92**	**1.87**		1.73	1.73
361	**1.27**			1.1		
362	**1.65**	**1.4**	**1.28**	**1.3**	**1.35**	**0.65**
363	**1.12**	**1.13**	**1.18**	1.19	0.84	
365	**1.1**	**1.13**		0.79		0.72
368	**1.08**	**1.16**		0.82		
370	**0.99**	**1.12**	0.72			
372	**1.8**	**1.51**	0.75	0.51		
374	**1.37**	0.88				
376	**0.92**	0.98	**1.34**	1.29		0.71
378	**1.03**	0.68				
379	**1.16**	0.97		0.83		
380	**1.1**	0.71				
383	**1.17**	0.66	0.57			0.65
384	**1.36**	**1.19**	**1.18**		0.62	0.58
386	**1.1**	0.95				
387	**1.22**	**1.21**			1.11	0.94
388	**1.13**	1.2				
389	**0.81**	0.86				
392	**1.16**			0.84	0.77	0.68
574	**0.98**	1.43	0.85		0.76	0.72
961	**0.93**	0.89	0.98			

Number in bold: presence of clinical symptoms; non-bold: improvements of clinical symptoms

The clinical symptoms of patients included in follow up studies were also shown on the table 1. The numbers in bold show clinical symptoms and the non-bold shows the improvement of clinical symptoms during the follow up periods.

In un-recovered/reinfections patients, the antibody titer stay at high level during the follow-up. In recovered patients, the antibody titer decrease over time.

3.2. Host immunological response in different groups

Table 2. Comparison of the OD ratio during follow-up and between the recovery and unrecovery group (Kruskal – Wallis test).

	Pretreat.	1m	2ms	3ms	4ms	5ms
Recovered group	1.37±0.5	1.28±0.7	1.07±0.4	0.95±0.4	0.99±0.3	0.83±0.4
	n = 27	n = 25	n = 13	n = 12	n = 10	n = 12
P	0.06	0.03	0.03	0.03	0.02	0.04
t	3.6	4.6	4.9	5.3	5.2	4.0
Unrecovered group	2.06±0.5	2.65±1.7	2.49±1.1	3.40±3.1	2.50±1.1	2.11±1.2
	n = 3	n = 3	n = 3	n = 3	n = 3	n = 2

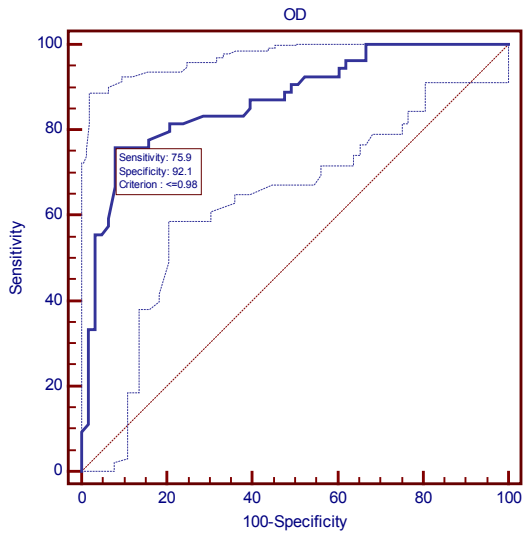
The mean of OD ratio of Pa/NC in recovered patients gradually decrease over time and became lower than 1 at the third month of follow–up. In contrast, the mean of OD ratio of Pa/NC in un-recovered/reinfections patients go slightly down at the fifth month but maintains a high level.

A general decrease of the antibody response was observed after pharmacological treatment in patients that do not show symptoms or *T.vaginalis* infection. On the contrary, drug resistant (or re-infected) cases do not show antibody titre decrease, confirming that presence of antigenic stimulus is mandatory to induce and maintain antibody response.

In contract, 8 cases of male partners of trichomoniasis patients did not show the immunological response with OD ratio all of them were minor than 1.

Related to the kinetics of antibody titre, data from this study also demonstrated that the "shelf life" of specific antibody response is 5-7 months in trichomoniasis female patients.

3.3. Relation between antibody IgG against *T. vaginalis* titers with clinical symptoms



Graph 1. ROC of the relation between titers of IgG against T. vaginalis with clinical symptoms. Levene's test for equality of variances ($P < 0.001$), ANOVA test for evaluation the relationship ($p < 0.001$), Student-Newman-Keuls test for all pairwise comparisons.

Table 3. Cut-off values and coordinates of the ROC curve

Cut-off	Sensitivity%	Specificity%
<=0.94	59.26	93.65
<=0.95	66.67	92.06
<=0.97	68.52	92.06
<=0.98*	**75.93**	**92.06**
<=0.99	75.93	90.48
<=1.01	75.93	88.89
<=1.03	75.93	87.30

This data showed that the decreasing of antibodies titers (0.98) related to the healing of clinical symptoms (Se%=75.93%, Sp%=92.06%).

Western blotting analysis specific antibody during follow-ups

Figure 1. Immunoblot patterns of patient number 79 (case number 2: recurrent exposure) during the follow up periods and her husband. Representative sera with high IgG response obtained by probing a total Trichomonas vaginalis protein preparation. The number from 1 to 5 represent months after treatment and the last band is serum samples from male sexual partner. Molecular weight (WM) markers are shown on the right.

Figure 2. WB analysis of 2 recovering patients (No 227 and No 84) and re-infected patient (patient number 362) during follow-up periods. The number from 1 to 5 are months after treatment. Molecular weight (WM) markers are shown on the right.

There are the significant correlations between Western blotting, ELISA assay and clinical symptoms. The high OD ratio of Pa\NC of current trichomoniasis and unrecovery patients produced many more bands than the low OD ratio of Pa\NC cured patients at all MW ranges particularly evident in the high-molecular weight range. The OD ratio of Pa\NC of cured patients decreases over time and is confirmed by the disappearing of antibody to Tvaginalis in WB analysing. However, antibody to T. vaginalis antigen between 84-115KDa are still present. The same results can be observed in the band pattern on WB analysing of male partners' sera.

4. DISCUSSION

In our patients, the main reasons leading them to attend the gynecological clinic were malodorous vaginal discharge (73.9%) and the profuse vaginal discharge (60.9%). The typical signs of trichomoniasis were yellowish-green frothy discharge (54.3%) and strawberry cervix (30.4%). There were 10.9% asymptom and 8.7% case of menopausal patients. However, all of 8 cases of male partners were asymptom.

In general, the clinical features of this disease are variable. Therefore, a combination of clinical and microbiological examination should be performed. In our study, monitoring the clinical symtom was useful to record the recovery\ unrecovery patients

The result of evaluation of sera antibody titer during the five months of follow up period was given on table 1. There are two statistically different trending line of human IgG antibody titer to T. vaginalis in recovering and unrecovering or re-infection groups follow-up since the line of is going down after 4-5 months in the group of recovering patients while those from the unrecovering\reinfection patients maintain a high level of antibody during all the period. This can be considered as a good marker of persistent infection.

In addition, in recovering patients, the symptoms improved gradually over time, and at the third follow-up 100% of clinical symptoms show improvement. The existence of high level of antibody and clinical symptom in unrecovering\ reinfections patients during the follow up time suggest a close relationship between clinical

symptoms and antibody level.

Immunobloting confirms the correlation between clinical symptoms, *T. vaginalis* identification, ELISA antibody titer and specific reactive antigen. All sera, both male and female recognize common antigen molecules of about 100kDa MW (84 - 115kDa). This is consistent with previous finding (Addis et al. 1999, Wos SM et al. 1986, Gaber et al 1986) [17, 18, 19] and makes the search for common immunogens particularly appealing with possible application for a more sensitive serologic test or as a possible vaccinogen and for studies of pathogenicity.

In general, eventhough, most of antibody disappeared during the recovery time, there were the persistence of antibodies weighting 84 -115 kDa in both of trichomoniasis patients and their partners.

5. CONCLUSION

Clinical features of *T. vaginalis* infection showed a wide spectrum. The most prevalent symptoms were vaginal erythema, malodorous vaginal discharge, profuse vaginal discharge, cervicitis, and yellowish-green frothy discharge. There was 8.7% asymptomatic patients.

Serological follow-up by ELISA showed the trending line of sera *T. vaginalis* IgG antibody going down after 4 - 5 months in the group of recovered patients; while those from the unrecovered or re-infection patients kept the high level of IgG antibody, a marker of infection persistence.

Detection of specific antibody response in sera could be considered as a good marker for therapy success.

Results from Western blot analysis showed significant correlation with those from ELISA assay and clinical symptoms during the course of follow-up periods.

There were the persistence of antibody to *T. vaginalis* antigen 84 -115kDa in men partners and recovery patients.

REFERENCES

1. World Health Organization Prevalence and incidence of selected sexually transmitted infections, Chlamydia trachomatis, Neisseria gonorrhoeae, syphilis and Trichomonas vaginalis: methods and results used by WHO to generate 2005 estimates. Geneva: the Organization; 2011. [cited 2012 Jan 31]. http://whqlibdoc.who.int/publications/2011/9789241502450_eng.pdf.
2. Coleman JS, Gaydos CA, Witter F.,Trichomonas vaginalis vaginitis in obstetrics and gynecology practice: new concepts and controversies., Obstet Gynecol Surv. 2013 Jan;68(1):43-50
3. Mason PR, Fiori PL, Cappuccinelli P, Rappelli P, Gregson S, Seroepidemiology of *Trichomonas vaginalis* in rural women in Zimbabwe and patterns of association with HIV infection, Epidemiol. Infect. (2005), 133, 315–323.
4. Johnson HL, Ghanem KG, Zenilman JM, Erbelding EJ, Sexually transmitted infections and adverse pregnancy outcomes among women attending inner city public sexually transmitted diseases clinics, Sex Transm Dis. 2011 Mar;38(3):167-71.
5. Mirmonsef P, Krass L, Landay A, Spear GT, The Role of Bacterial Vaginosis and *Trichomonas* in HIV Transmission Across The Female Genital Tract, Curr HIV Res. 2012 Apr 1;10(3):202-10.
6. Thurman AR, Doncel GF, Innate immunity and inflammatory response to *Trichomonas vaginalis* and bacterial vaginosis: relationship to HIV acquisition, Am J Reprod Immunol. 2011 Feb;65(2):89-98
7. Ackers J.P., Trichomonads, Principles and Practice of Clinical Parasitology, John Wiley and Sons Ltd, 2001: 243-268.
8. Mason PR, Serodiagnosis of *Trichomonas vaginalis* infection by the indirect fluorescent antibody test. J. Clin. Pathol. 1979. 32:1211–1215
9. Alderete JF, Enzyme linked immunosorbent assay for detecting antibody to *Trichomonas vaginalis*: Use of whole cells and aqueous extract as antigen, Br J Vener Dis 1984; 60:164-70.
10. Workowski KA, Berman S; Centers for Disease Control and Prevention (CDC), Sexually transmitted diseases treatment guidelines, 2010, MMWR Recomm Rep. 2010 Dec 17;59(RR-12):1-110.
11. Mason PR, Gregson S, Gwanzura L, Cappuccinelli P, Rapelli P, Fiori PL, Enzyme immunoassay for urogenital trichomoniasis as a marker of unsafe sexual behavior, Epidemiol. Infect. (2001), 126, 103±109.
12. Diaz N, Dessì D, Dessole S, Fiori PL, Rappelli P,

Rapid detection of coinfections by *Trichomonas vaginalis, Mycoplasma hominis*, and *Ureaplasma urealyticum* by a new multiplex polymerase chain reaction, Diagn Microbiol Infect Dis. 2010 May;67(1):30-6.
13. Carr PL, Felsenstein D, Friedman RH, Evaluation and management of vaginitis, JGIM volume 13, May, 1998,p.335-346.
14. Dan M, Sobel JD, Trichomoniasis as Seen in a Chronic Vaginitis Clinic, Infectious Diseases in Obstetrics and Gynecology 4:77-84 (1996).
15. Heine P., and MacGregor JA, *Trichomonas vaginalis*: a re-emerging pathogen. Clin. Obstet. Gynecol. 1993. 36:137–144.
16. Fouts A C, and Kraus SJ, *Trichomonas vaginalis*: reevaluation of its clinical presentation and laboratory diagnosis. J. Infect. Dis.1980. 141:137– 143.
17. Wos SM, Watt RM, Immunoglobulin Isotypes of Anti-*Trichomonas vaginalis* Antibodies in Patients with Vaginal Trichomoniasis, J Clin Microbiol. 1986 Nov;24(5):790-5.
18. Addis MF, Rappelli P, Andrade AMP, Rita FM, Colombo MM, Cappuccinelli P, and Fiori PL, Identification of *Trichomonas vaginalis* a-Actinin as the Most Common Immunogen Recognized by Sera of Women Exposed to the Parasite, The Journal of Infectious Diseases 1999;180:1727–30. Mason PR, Serodiagnosis of *Trichomonas vaginalis* infection by the indirect fluorescent antibody test. J. Clin. Pathol. 1979. 32:1211–1215
19. Garber GE, Proctor EM, Bowie WR**.** Immunogenic proteins of *Trichomonas vaginalis* as demonstrated by the immunoblot technique. Infect Immun 1986;51:250–3.