

Enzyme immunoassay for urogenital trichomoniasis as a marker of unsafe sexual behaviour

P. R. MASON^{1,2*}, S. GREGSON¹, L. GWANZURA², P. CAPPUCCINELLI³,
P. RAPELLI³ AND P. L. FIORI³

¹ Biomedical Research and Training Institute, Harare, Zimbabwe

² Department of Medical Laboratory Sciences, University of Zimbabwe Medical School, Harare, Zimbabwe

³ Department of Biomedical Sciences, Division of Experimental and Clinical Microbiology, University of Sassari, Sassari, Italy

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SUMMARY

Enzyme immunoassay (EIA) was used to detect antibodies to *Trichomonas vaginalis* in sera from Zimbabwe. The EIA showed a sensitivity of 95 and 94% when compared with vaginal swab culture among women attending a family planning clinic (FPC) and female commercial sex workers (CSW) respectively. The specificity was 85 and 77% in the two groups. Culture-negative FPC women were sub-divided into high risk or low risk of exposure to trichomoniasis. The seroprevalence was 10% (6/61) among low risk women, 21% (10/48) among high risk women and 23% (9/39) among culture negative CSW. The EIA was positive in 46% (18/39) men with genital discharge but only 5% (2/37) healthy blood donors. None of 31 sera from prepubescent children was positive. The EIA may be useful for community surveys of trichomoniasis. Because *T. vaginalis* is a common sexually transmitted disease, the test may indicate behaviour that increases the risk of STD transmission.

INTRODUCTION

Infection with *Trichomonas vaginalis* is among the most frequently occurring causes of genital discharge in women, and studies in both urban and rural centres in Zimbabwe have shown a high prevalence of trichomoniasis in women attending genito-urinary clinics [1, 2]. Infection may lead to considerable morbidity, and has been associated with premature rupture of membranes, pre-term labour and low birth weight [3]. In men, trichomoniasis is found less frequently [4], and this may be due to infections being only transient with asymptomatic carriage and spontaneous resolution of infection [5]. Trichomoniasis is primarily sexually transmitted and there is an epi-

demiological association between urogenital trichomoniasis and other sexually transmitted diseases (STD) [3]. More recently an association has also been shown with HIV [6, 7] presumably because of the strong epidemiological relationship between STD and HIV [8, 9]. The epidemiology of trichomoniasis may therefore be an indicator of sexual behaviour that increases the risk of STD and HIV acquisition.

Data on the epidemiology of trichomoniasis are limited mainly to clinic-based studies because the detection of acute infection relies on microscopy of discharge, collected during a clinical examination. Diagnosis may be improved by culture in Diamond's or other media, and more recently, polymerase chain reaction (PCR) assays have been described [10, 11]. These methods still, however, require genital swabs and while swabs can be self-collected, in practice collections are nearly always made in the setting of a

* Author for correspondence: Director General, Biomedical Research and Training Institute, PO Box CY1753 Causeway, Harare, Zimbabwe.

clinic. Immunologic assays would have a number of benefits in investigating trichomoniasis at the community level, and while a variety of such assays have been described, they are characterized by low sensitivity and specificity [12–14].

The overall objective of this study was to identify surrogate markers of behaviour that may predict increased risk of STD and HIV acquisition. We examined the value of serology for identifying exposure to *T. vaginalis* with the intention that the test could be used subsequently in community studies of the interaction between HIV and STDs. In this paper we describe the application of an enzyme immunoassay (EIA) to detect antibodies to *T. vaginalis* in men, women and children in Zimbabwe. In order to evaluate the EIA, we used serum specimens from a number of defined patient groups. These included women with a proven trichomonal infection, men and women who could be regarded as at high risk or low risk of current or past trichomonal infection and children who were presumed to not having been exposed to trichomoniasis.

MATERIALS AND METHODS

The sample collection procedures were approved by both the Institutional Review Board of the Biomedical Research and Training Institute, and by the Medical Research Council of Zimbabwe, and all adults had agreed to serologic screening for STDs. All sera were labelled with a reference number, and tests were conducted anonymously.

Female family planning clinic attendees

The blood samples we used were from 150/1500 (10%) women attending a family planning clinic (FPC) in Harare who had enrolled into a study of STD infection and cervicitis. The women were attending the clinic for family planning advice, rather than because they had symptoms of infection, though some had a discharge on clinical examination. The demographic and clinical status of these women and the results of investigations for STD are to be published elsewhere. After clinical examination, specimens were collected for microbiologic investigations. For trichomoniasis, a high vaginal swab was inoculated into Diamond's medium and incubated at 36 °C for up to 72 h. The medium was examined daily by microscopy for the presence of *T. vaginalis*.

Gonococcal infection was diagnosed by culture of cervical and urethral swabs on Thayer–Martin medium, and chlamydial infection was detected using commercial antigen detection assays (Chlamydiazyme, Abbott Laboratories, USA). Gram-stained smears of cervical and high vaginal swabs were examined by microscopy and the presence of pus cells was noted in a semi-quantitative manner (score 0–3), and the presence of grade 2–3 pus cells in the vaginal swab smear was regarded as indicative of a purulent genital discharge. Bacterial vaginosis was detected on the basis of the presence of clue cells on microscopic examination of the high vaginal swab. Clotted blood was obtained from these women, and after testing for syphilis, the serum was stored at –20 °C.

Control positive sera were from the 41 women who had a microscopically proven *T. vaginalis* infection at the time of blood collection, though the duration of that infection was not known. Presumed negative sera were from women who were culture negative for *T. vaginalis*. They included 61 women who had no evidence of any STD at the time of examination, and who had no pus cells in examinations of Gram-stained vaginal smears. These were recorded as low risk negative sera. Sera from 48 women were recorded as high risk negative sera because while the women were culture negative for trichomoniasis, they had a purulent discharge, bacterial vaginosis, or chlamydial or gonococcal infection. A purulent discharge is one of the most frequent symptoms of trichomoniasis, and because there is an epidemiologic association between trichomoniasis and other STDs, these women were presumed at increased risk of having an undetected or past infection with *T. vaginalis*.

Commercial sex workers

As part of a programme to monitor STDs in female commercial sex workers (CSW) in Harare, enrolment sera were obtained from 61 CSWs, of whom 19 (31%) had an active *T. vaginalis* infection, based on microscopy and culture as above, at the time of blood collection. Again the duration of the infection was not known. Seven (34%) of these culture-positive women had at least one other STD, in addition to trichomoniasis.

Sera from 42 female CSWs who had no evidence of active trichomoniasis at the time of enrolment were all regarded as high-risk negative sera. These women described themselves as having multiple sex partners,

and 26 (67%) had evidence of a purulent discharge at the time of examination, or had laboratory evidence of chlamydial, gonococcal or treponemal infection.

Follow-up vaginal swab specimens were collected from the CSWs at varying intervals of 1–3 months for a period of 6–12 months. Although offered monthly testing, counselling and treatment for STD, the women were very unreliable in attending regularly. The swabs were examined for *T. vaginalis* as before. At the final visit, repeat blood samples were collected in addition to a vaginal swab and the sera were re-tested for IgG antibody to *T. vaginalis*.

High risk/low risk male sera

Because of the difficulty of identifying active trichomoniasis in men, we studied serum specimens from men belonging to high- or low-risk groups without seeking specific evidence of trichomonal infection. Serum samples were obtained from 40 male regular blood donors, who as part of the screening procedure for donation denied having multiple sexual partners, or having a genital infection since the last time they donated blood. Routine testing confirmed these men had no antibodies reactive with HIV 1 or HIV 2, and were negative for syphilis and hepatitis B under the routine screening procedures of the blood transfusion service. These men were regarded as at low risk of exposure to *T. vaginalis*.

Sera were also obtained from 61 men attending a genito-urinary clinic in Harare, 41 (67%) with a genital discharge. Routine examinations of these 41 men showed 20 men with a gonococcal infection and 4 men with either chlamydial or mixed gonococcal-chlamydial infections. No pathogens were detected in 17 men. The other 20 sera were from men with genital ulcer disease. Although no specific examination for trichomoniasis was made with any of these men, they were regarded as being at high risk of having been exposed to *T. vaginalis* because of their presenting symptoms and/or because of the epidemiologic association between *T. vaginalis* and other STDs.

Children's sera

Serum specimens were obtained from 31 infants and children under the age of 12 years, who had been admitted to hospital in Harare with a clinical diagnosis of a variety of infectious diseases, including malaria, pneumonia, septicaemia or meningitis. These sera

were regarded, on epidemiologic grounds, as being at low risk of having antibodies to *T. vaginalis* antigens.

Enzyme immunoassay

The EIA was carried out following a method similar to that described by Alderete [15] using the SS-22 strain of *T. vaginalis*. This isolate originated in Italy, and is characterized by being free from mycoplasma infection, and by phenotypic stability [16]. Long-term cultures 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 1×10^6 cells/ml. Aliquots (50 μ l) were added to the wells of microtitre plates. The plates were dried at 37 °C and 50 μ l ice-cold 95% ethanol was added to each well. After drying in air, the wells were washed with distilled water and stored at 4 °C until use. An aliquot of the suspension used for plate production was confirmed to be mycoplasma-free using PCR [17].

Wells were blocked by the addition of 100 μ l PBS-0.2% Tween-20 (PBS-T) containing 5% non-fat dried milk and incubating for 2 h at room temperature. Plates were thoroughly rinsed with PBS using an automated plate washer (Labsystems, UK). Sera were diluted 1/50 in PBS-T, and 100 μ l was added to the wells and plates were incubated at 37 °C for 1 h. After rinsing with PBS-T, 100 μ l of horseradish peroxidase labelled anti-human IgG (Sigma, USA) diluted 1/3000 with PBS-T was added to the wells. Plates were incubated again at 37 °C for 1 h, rinsed and 50 μ l of substrate (ortho-phenylenediamine in buffer) was added before incubating the plates in the dark at room temperature. The reaction was stopped by the addition of 50 μ l 2 M sulphuric acid and plates were read immediately at 450 nm using a Multiskan plate reader.

Treatment

All women who were found to have *T. vaginalis* by microscopy and culture were offered treatment according to national guidelines. We did not offer treatment on the basis of positive serology alone.

RESULTS

Control positive and negative sera from women

Comparison of the OD values obtained from sera from women with a confirmed trichomonal infection,

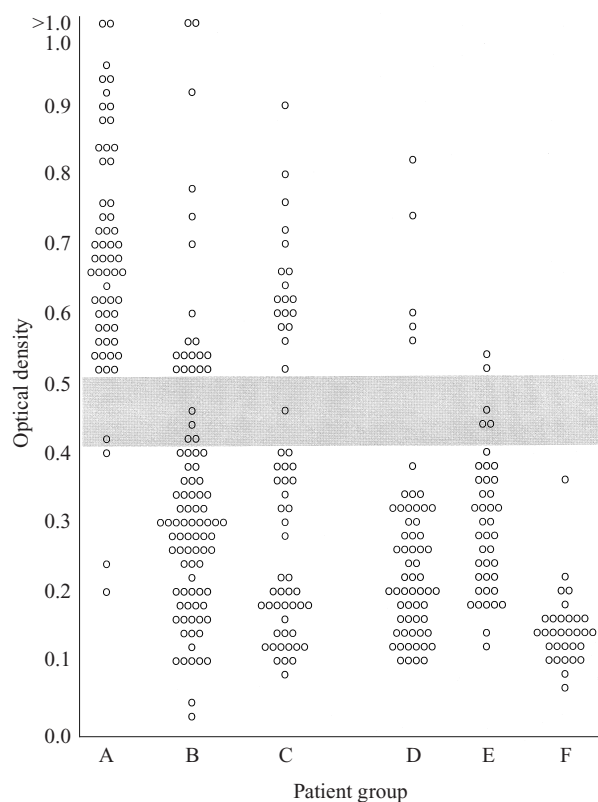


Fig. 1. Optical density in *T. vaginalis* EIA with sera from different patient groups. Each circle indicates the OD value of sera from an individual patient. The shaded area shows the area of indeterminate reactivity. Sera with an OD value above this range were positive and sera with an OD value below this range were negative. Group A, women with proven trichomoniasis. Group B, high-risk women with no evidence of trichomoniasis. Group C, symptomatic men attending a GU centre. Group D, low-risk women with no evidence of trichomoniasis. Group E, asymptomatic male blood donors. Group F, prepubescent children.

and from low risk women with no evidence of trichomoniasis are shown in Figure 1. ODs > 0.5 were strongly associated with current trichomonal infection, while ODs < 0.4 were characteristic of those with a low risk of infection. These OD values were used as the 'cut off' for positive and negative sera respectively. A few sera (8/343, 2%) repeatedly showed intermediate OD values under the test conditions and these sera were excluded from the analysis.

As shown in Table 1, 56/60 (93%) women with culture proven trichomonal infection had positive serology, compared with 6/61 (10%) of women with no evidence of infection, and having a low risk of infection as defined by an absence of other STDs or discharge, (χ^2 with Yates' correction = 90.5, $P < 0.001$). Amongst the high-risk women, the differ-

entiation was less clear. Nine (23%) of the 39 CSWs and 10/48 (21%) high risk family planning clinic attendees had a positive trichomonal serology in the absence of detectable trichomonal infection.

Sera from men and children

Only 2/37 (5%) sera from men with a low risk of trichomoniasis gave an OD > 0.5 compared with 18/57 (32%) men with a genital discharge (Table 1) (χ^2 , with Yates' correction = 9.1, $P < 0.005$). Two sera from men with genital ulcer disease had indeterminate reactivity, but sera from the other 18 men in this group were negative. All 31 samples from children gave ODs < 0.4 .

Sensitivity and specificity

Determination of the sensitivity and specificity of the EIA used only samples from women because microscopic demonstration of *T. vaginalis* in cultured vaginal swabs was the gold standard. On this basis, the EIA had an overall sensitivity of 95% (56/59) and a specificity of 83% (123/148). The sensitivity and specificity of the EIA was 94% (17/18) and 77% (30/39) respectively using sera from CSWs, compared with 95% (39/41) and 85% (93/109) for FPC attendees. The specificity of the EIA using sera from low risk women (56/61, 90%) was higher than with sera from high risk women (68/87, 78%) when using negative culture as the gold standard.

Inter-test variation

One hundred and twenty-two serum specimens from *T. vaginalis* positive and negative women were subjected to repeated testing (four repeats) using different antigen plates. Comparison of the individual OD values with the mean showed little variation in reactivity, except amongst those sera giving indeterminate results. Of the 8 indeterminate sera, 1 (12%) gave at least 1 negative OD and 4 (50%) gave at least 1 positive OD when the test was repeated. There were no negative sera that gave a positive OD during repeated tests, and no positive sera that gave a negative OD in repeated tests.

Follow-up sera

Ten CSWs who were seronegative at the time of enrolment had acquired an infection with *T. vaginalis*,

Table 1. Reactivity of sera with *T. vaginalis* antigens using EIA

Source of sera	No. (%) of sera			Total
	Positive	Negative	Indeterminate	
<i>T. vaginalis</i> -positive women				
FPC*	39 (95%)	2 (5%)	0	41
CSW†	17 (89%)	1 (5%)	1 (5%)	19
Total	56 (93%)	3 (5%)	1 (2%)	60
<i>T. vaginalis</i> -negative women				
FPC-low risk	6 (10%)	55 (90%)	0	61
FPC-high risk	10 (20%)	38 (78%)	1 (2%)	49
CSW-high risk	9 (21%)	30 (71%)	3 (7%)	42
Men				
STD‡ patients	18 (30%)	40 (65%)	3 (5%)	61
Blood donor	2 (5%)	35 (88%)	3 (8%)	40

* FPC, family planning clinic;

† CSW, commercial sex worker;

‡ STD, sexually transmitted disease.

High and low risk assessment was made on the basis of clinical, microbiological or behavioural data.

as demonstrated by microscopy, during the follow-up period. Eight of these had seroconverted at the time of final examination, 3 or more months after the infection had been detected. Two CSWs remained seronegative 2 months after the infection had been detected.

DISCUSSION

A number of serologic tests for trichomoniasis, using different methodologies, have been described [12–14] but in most cases, the specificity of the test has been low. The poor performance of serology may be due, in part, to the presence of mycoplasma in most trichomonal cultures [17]. Crude antigen preparations from such cultures include mycoplasma antigens and this may lead to false positive reaction when using sera from women with anti-mycoplasma antibodies. The use of a mycoplasma-free isolate of *T. vaginalis* for antigen preparation has improved the specificity of the serologic assay that we applied here.

We used sera from different sources, some which were classified at high risk and some at low risk of having a trichomonal infection. We based this definition of risk on epidemiologic, behavioural and clinical criteria, and on the association between trichomoniasis and other STDs. The serologic findings were compatible with these different group definitions. We found for example low reactivity with sera from children, and from regular blood donors who were at

low risk of having an STD. In Zimbabwe, where the risk of HIV infection is high, blood donors are carefully screened for a history of change in sexual behaviour and for genital tract infection. Both regular donors and children would be expected to have little risk of contact with trichomoniasis, and the EIA findings were consistent with this. By contrast most sera from women with a proven trichomonal infection were positive in the EIA. A number of women were *T. vaginalis* culture negative but positive by EIA. Most of these belonged to the high-risk group because they were sex workers, or they had symptoms of genital discharge or they had other STDs. These may have been women who had infections that were not detected by culture, or they may have been infected in the past and remained seropositive. The higher prevalence of seroreactivity in men with a genital discharge correlates with the increased risk of such men having trichomoniasis even though we did not obtain specific samples for laboratory detection of *T. vaginalis* in this study. The absence of positive reactivity using sera from men with genital ulcer disease suggests that seroreactivity was not a characteristic of all STDs, but was particularly associated with genital discharge. The sample size was, however, small.

Analyses of sensitivity and specificity are difficult to design in infections that may be asymptomatic and where the gold standard is itself known to have low sensitivity. Up to 50% of trichomonal infections may be asymptomatic [3]. Also, serological markers may

reflect the entire past history of exposure to trichomonal antigens, whereas microscopy and culture detect current infection only. The use of a specific IgM assay may have given better correlation with active infection, but the purpose of this assay was to use an EIA for epidemiological rather than diagnostic purposes and this was not investigated. In this study, the sensitivity and specificity of serology, using culture in Diamond's medium as the gold standard were 95% (39/41) and 85% (93/109) respectively for women attending a FPC. For CSWs, the sensitivity was similar, 94% (17/18), but the specificity was lower, 77% (30/39).

Possible reasons for low specificity have been discussed above. The absence of detectable antibody to *T. vaginalis* in women who harboured these parasites in their genital tract, the false negatives, may be explained in a number of ways. The simplest explanation is that the infection was only recently acquired, and so there was insufficient time to mount a systemic IgG response. Data on the kinetics of serological response to *T. vaginalis* are limited, but the longitudinal studies of the CSWs showed two (20%) women who acquired an infection after enrolment had not seroconverted 2 months later. These numbers are small, but indicate there may be considerable delay between acquisition of infection and the appearance of specific IgG antibody.

Antigenic variation, with alteration in epitopes expressed at the cell surface is known to occur in *T. vaginalis* [18], and geographic variation in surface expressed epitopes has been described [19]. It is possible that antibodies raised to the infecting variant of the parasite in Zimbabwe were not highly reactive with epitopes expressed by the Italian strain used for antigen preparation. It has recently been shown that the major immunogen of *T. vaginalis* is alpha-actinin [16], and the development of more specific assays, using this as antigen, may improve both sensitivity and specificity in a global context. Moreover, the use of a more specific antigen may also allow the design of a more robust assay, with a larger difference between positive and negative OD.

Because trichomoniasis is one of the most frequently occurring STDs in women, the incidence of infection with *T. vaginalis* may be a useful surrogate marker of unsafe sexual behaviour. It has, for example, been used as a marker of non-compliant use of the female condom [20]. We have demonstrated that positive trichomonal serology correlates with an active infection, and with other indicators of unsafe

sexual behaviour such as having another STD. The collection of blood samples may be more acceptable in community surveys than the collection of vaginal or urethral swabs from healthy individuals. Moreover, blood and serum can be stored and re-tested for quality control purposes. Seroconversion may be a useful surrogate marker that indicates unprotected sexual contact with an infected partner. The *T. vaginalis* EIA may be an effective and inexpensive additional tool that can be used in the evaluation of STD intervention strategies.

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