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Comparison of Trichomoniasis Diagnosis by Microscopic Methods and Indirect ELISA Technique in a Sample of Iraqi Women

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Abstract

Trichomonas vaginalis is an eukaryotic parasite that causes the most common non-viral sexually transmitted infection, trichomoniasis. This disease is responsible for many serious health problems such as preterm birth. More than half of the infected women do not develop symptoms, which makes it difficult to diagnose the disease. In this study, a specific indirect ELISA method was developed to detect anti-*Trichomonas vaginalis* IgM and IgG immunoglobulins in the sera of infected females. The aim of this study was to investigate the sensitivity of a simple ELISA procedure in comparison to the classical urine examination and vaginal wet mount preparation for the diagnosis of *T. vaginalis*. The sensitivity of the indirect ELISA was compared with the classical vaginal discharge swab and urine microscopic examination, and the results showed sensitivities of 65.5% and 57.2%, respectively. Furthermore, the infection was measurable as acute or chronic with the refined test.

Keywords: *Trichomonas vaginalis*, Trichomoniasis, indirect ELISA, IgM, IgG

مقارنة بين تشخيص داء المشعرات المهبلية بطرق الفحص المجهري وتقنية الممتز المناعي المرتبط بالانظيم غير المباشرة في عينة من النساء العراقيات

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الخلاصة

طفيلي المشعرات المهبلية *Trichomonas vaginalis* هو طفيلي حقيقي النواة ويسبب الاصابة بداء المشعرات الذي يعد المرض الجنسي غير الفايروسي الاكثر انتشارا. إن هذا الداء مسؤول عن العديد من المشاكل الصحية مثل الولادة المبكرة. إن اكثر من نصف النساء المصابات لا يعانين من أعراض سريرية مما يصعب عملية تشخيص المرض. في هذه الدراسة تم تطوير طريقة متخصصة للكشف عن الغلوبيولينات المناعية IgM و IgG للطفيلي في امصال النساء المصابات وهي طريقة الممتز المناعي المرتبط بالانظيم غير المباشرة. إن الهدف من هذه الدراسة هو التحقق من حساسية هذه الطريقة بالمقارنة مع الطرق التقليدية للتشخيص كفحص الادرار و فحص الافرازات المهبلية. تمت مقارنة حساسية فحص الممتز المناعي المرتبط بالانظيم غير المباشرة مع طريقة فحص المسحة المهبلية التقليدي و الفحص المجهري للادرار وتبين ان حساسية فحص الافرازات المهبلية و فحص الادرار هي 65.5% و 57.2% على التوالي عند مقارنتهم مع طريقة الممتز المناعي المرتبط بالانظيم غير المباشرة. بالإضافة الى ذلك كان من الممكن معرفة ما اذا كانت الاصابة حادة او مزمنة باستخدام الطريقة المذكورة اخيرا.

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Introduction

Trichomonas vaginalis is a very important extracellular, flagellated, single celled anaerobic eukaryotic protozoan parasite, transmitted mainly through unsafe intercourse [1- 3]. Trichomoniasis is probably the most common non-viral sexually transmitted infection. It accounts for almost 180 million infections worldwide every year [4, 5]. *T. vaginalis* infection is associated with many adverse health problems, such as preterm birth and the delivery of a low birth-weight baby [6]. Even though both sexes can be infected, the disease occurs almost exclusively in women while men keep asymptomatic [7]. Most infected women are asymptomatic, but sometimes trichomoniasis may cause mild symptoms, such as itching, frothy yellow greenish vaginal discharge, maybe with blood, burning sensation while urinating, and strawberry cervix [8, 9]. The most traditional method used worldwide to diagnose the infection with *T. vaginalis* is the direct microscopic examination of wet vaginal swab or urine examination. However, it is subjective method that requires experience and the hands of a trained observer. In addition, the sensitivity of this method is below 60% and can only be used for symptomatic women [10, 11]. The attempt to diagnose trichomoiasis infection using ELISA has been the focus of many recent studies, since the detection of anti-*Trichomonas* serum antibodies using ELISA showed a sensitivity of up to 73.3%, with the prevalence of detection of undiagnosed infections because the parasite antibodies can be detected in the patients' sera [12, 13].

Epidemiologically, in Iraq, a study tested 200 samples from symptomatic and asymptomatic low educated women in Baghdad, and 17% were detected positive using RT-PCR [14]. A similar study compared between the efficiency of three detection methods (wet mount, staining and culture) and detected 15 (6%) positive samples out of 250 samples collected. Moreover 2012, the infection rate in Baghdad reached 26% [13, 15]. While in 2019, Hassan *et al.* (2019), tested 157 samples among which 100 (63.7%) samples were detected positive by PCR[16]. However, worldwide estimates may not be specific since they've been largely based on wet mount direct microscopy, which has moderate sensitivity when compared to the more sensitive methods [17, 18].

Materials and Methods

During the period from October 2018 to April 2019, high vaginal swabs and urine samples were collected from symptomatic married women, suffering from itching and/or vaginal discharge, attending different hospitals; Gynecology Clinics of Al-Imamain Al-Kadhemain Teaching Hospital, Al-Yarmuk Teaching Hospital and Al-Shahid Al-Hakim General Hospital, as well as a private Gynecology Clinic in Al-Hurria city in Baghdad. From each patient, vaginal discharge samples were collected from the posterior fornix using a sterile metal speculum and a disposable swab [19]. Urine samples were collected in sterile urine cups. Also, 5 ml of venous blood was collected in gel tubes from 114 patients. The blood was centrifuged and stored at -20 for further examination [20]. Furthermore, 20 venous blood samples were collected from healthy, with no chronic diseases or illnesses, married and un-married females from different age groups to be considered as controls.

Sample examination

Vaginal discharge was directly examined within few minutes after collection under light microscope on a sterile slide. The swab was agitated in a sterile tube filled with 500 µl of normal saline or distilled water, and then a drop of the mixture was placed on a clean glass slide with a cover slip and examined under 10x then 40x objective lenses for the visualization of motile pear-shaped Trichomonads [21, 20]. Moreover, some samples were stained using Giemsa stain, according to Khatoon, 2014 [22], to improve the visualization process.

Urine samples examination

Each urine sample was centrifuged at 2000 round per minute (rpm) for 5 minutes. Next, the supernatant was discarded and a drop of the sediment was placed on a clean glass slide then covered with a clean cover slip. The slides were examined following the same protocol for wet mount examination [20].

Detection of anti-*Trichomonas* IgM and IgG by indirect ELISA

There is no available commercial kit for detection of specific immunoglobulins of *T. vaginalis* in the blood. In this study and in collaboration with Bio-Rad Company (UK), it was attempted to develop a specialized ELISA kit to detect specific anti *Trichomonas vaginalis* antibodies, IgM and IgG, in patient serum. IgM detection in serum samples represents acute infection while IgG detection represents chronic infection. This kit was made for a universal *T. vaginalis* species and was successfully tested on the Iraqi strain of the same parasite.

Trichomonas vaginalis antigen and all buffers and solutions needed for this indirect ELISA kit were ordered from Bio-Rad Company, UK. These included the *Trichomonas vaginalis* antigen, anti-human IgM:HRP and anti-human IgG:HRP, 5x ELISA Coating buffer, 10x ELISA Wash buffer that contained PBS 0.05% Tween 20, ELISA BSA Block, and TMB CORE+ substrate. Also, a stop solution was prepared in the laboratory by diluting concentrated sulphuric acid (H_2SO_4) to 0.2M or 1.5M, which is the usually used stop solution with TMB substrate ELISA kits [23].

The detection of anti-*Trichomonas vaginalis* IgM and IgG was performed according to Sharma, 1991, Yadav, 2005, and Kaur, 2008 [24- 26] with one step of modifications according to Bio-Rad indirect ELISA protocol [23].

Different concentrations were tested to find those giving the best results. Different concentrations of serum samples were prepared by dilution in wash buffer (1 "undiluted", 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256 µl/ml) [27, 12, 28, 25, 26]. Antigen concentrations were 5, 3 and 1.5 µg/ml, selected according to BIO-RAD indirect ELISA protocol (1-10 µg/ml) and similar papers [23, 25, 26, 29]. Also, different dilutions of IgM and IgG secondary Abs (1/4000, 1/6000 and 1/8000) in PBS were tested according to Yadav, 2005 and Kaur, 2008 [25, 26].

Indirect ELISA procedure

1- The first step was the coating of the high binding, 96-well, flat bottom ELISA plate wells with 100 µl of the Ag solution diluted in 1x Coating Buffer.

2- The plate was covered and incubated at 4°C overnight in the dark.

3- The solution was discarded and the plate was washed 4 times with 1x Wash Buffer, with agitation to ensure thorough washing.

4- Blocking solution, 150 µl, was added to each coated well and incubated at 37°C for 90 minutes, then washed 4 times with agitation.

5- Serum samples (Primary detection antibody) of 100 µl were added to each well then incubated for 1 hour at 37°C. Next, they were washed 4 times with agitation to get rid of any non-specific binding. Furthermore, the serum was substituted with wash buffer and placed in few wells for the Blank measurement.

6- HRP: IgM or IgG (enzyme conjugated secondary Ab) of 100 µl diluted in PBS was added to all wells and incubated at 37°C for 1 hour then washed 4 times as mentioned before.

7- Substrate of 100 µl was added to all wells in the dark and incubated for 10 minutes or less until blue color development.

8- Stop solution of 50 µl (0.2M H_2SO_4) was added and gentle tapping was applied to ensure mixing thoroughly.

9- The blue colored substrate turned yellow immediately, and the absorbance was read within few minutes at 450 nm.

All reagents were at room temperature before routine-work.

Results

Out of an overall of 114 samples tested, the parasite was detected by wet mount preparations of vaginal discharge in 19 patients (16.6%), with the aid of staining with Giemsa stain, while the number of patients diagnosed by urine sample examination was only 10 (8.7%). As for the detection by indirect ELISA, after optimization trials, it was possible to select the concentrations which were giving the best results with specificity for the detection of anti-*Trichomonas vaginalis* IgM and IgG. Those concentrations were 1.5 µg/ml for the Ag, diluted in coating buffer, while serum samples were best undiluted, and 1/8000 µl/ml in PBS for the secondary Abs (IgM and IgG:HRP).

The highest IgM Control OD was 0.975 and, thus, the cut-off value adopted was 1.0, with lower values being considered negative whereas equal or higher values considered positive. Out of all 114 serum samples tested, 24 (21%) patients were "parasite specific" IgM positive. As for IgG results, the highest Control OD was 1.05, so the cut-off value was adopted as 1.1.

Out of 114 serum samples, 22 (19.3%) patients were detected as IgG positive. Six patients were positive for both IgM and IgG, implying that a total of 40 (35%) out of 114 patients were positive for *Trichomonas vaginalis* infection in general, and 46 cases were diagnosed by this kit.

Analyzing the above results, the sensitivity of the vaginal swab wet mount examination and urine examination compared with the indirect ELISA technique were 65.5% and 57.2% respectively. The sensitivity was calculated according to the formula (Sensitivity = True Positive/ True Positive + False Negative) [30].

Figure-1 represents the numbers of acute (IgM) and chronic (IgG) cases of trichomoniasis diagnosed by the same kit, while Figure-2 represents a comparison between the numbers of positively diagnosed cases by the three methods used in this study.

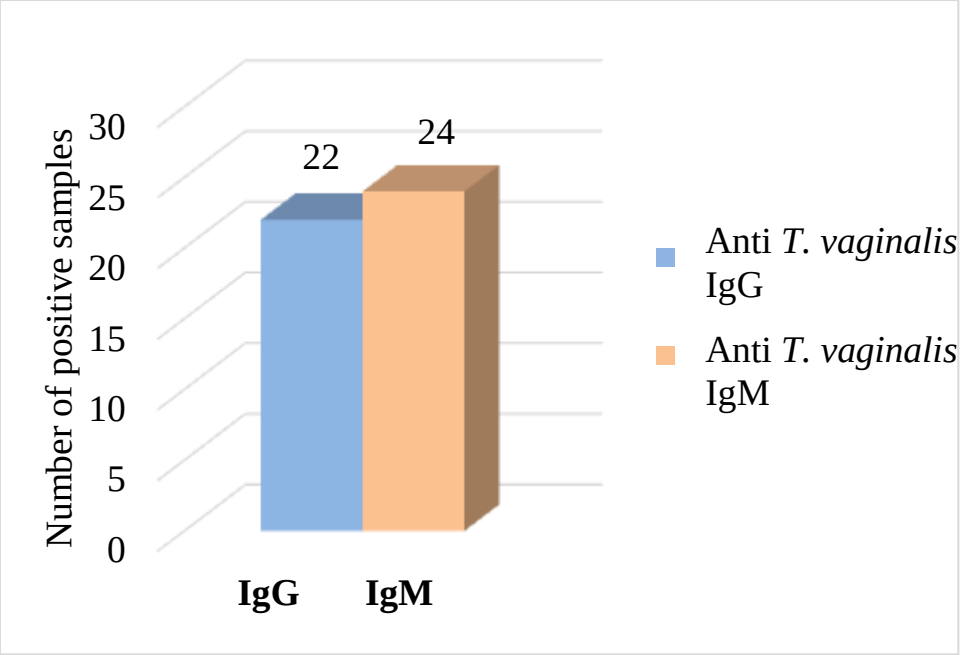

Figure 1-The numbers of acute and chronic cases of Trichomoniasis.

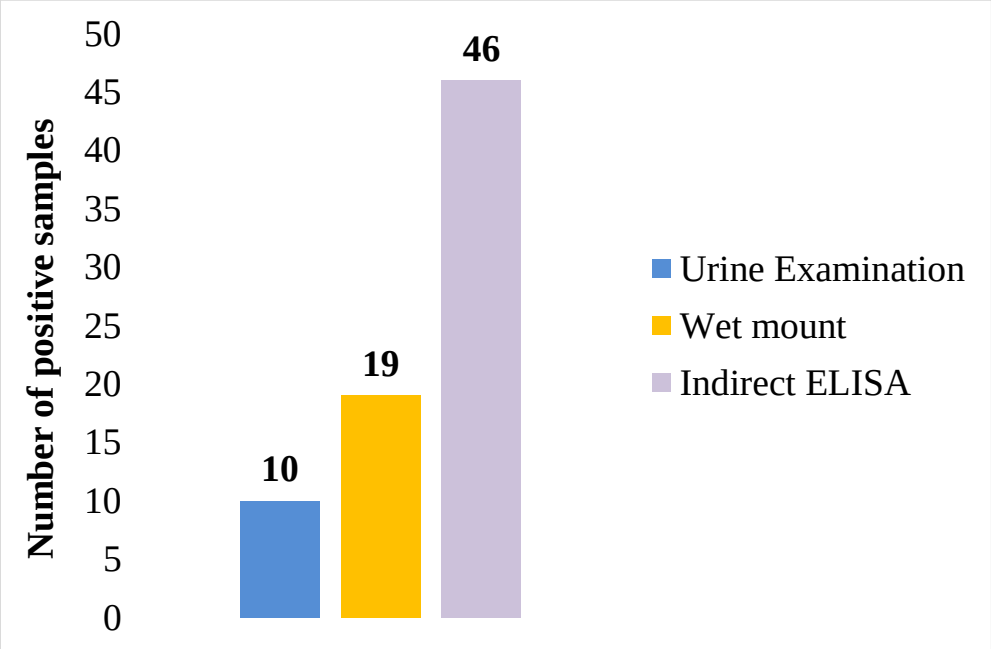

Figure 2-Comparison between the three methods of Trichomoniasis diagnosis.

Discussion

According to the results obtained from all 3 different methods, ELISA is clearly the most accurate method of diagnosis. These results are in agreement with many other recent studies. The most common methods used to diagnose trichomoniasis in Iraq are by clinical symptoms and sometimes urine examination. Clinical symptoms cannot be guaranteed as an evidence of infection, since only 2% of patients can develop strawberry cervix and 12% may have abnormal vaginal discharge. From this information, 88% of infected women may not be diagnosed nor treated, and more than 25% of women can be infected with any other type of sexually transmitted diseases or sexually transmitted infections

but mistakenly diagnosed with trichomoniasis [31, 13]. Other studies proved that ELISA is more accurate in diagnosis of trichomoniasis infection than the traditional wet mount preparations of vaginal discharge and urine examination. This technique can even be used for asymptomatic women. Different studies in Iraq also compared between different diagnostic methods, such as that of Khalaf (2013) [32], which compared between PCR technique, vaginal discharge wet preparation and urine examination, where PCR showed the highest sensitivity of 100% by detecting three false negative patients, while wet preparation of vaginal discharge showed 88.4% and urine examination gave the lowest sensitivity with 1.9%. There is a general difference between the diagnosis depending on vaginal discharge and urine, as proved by Lawing *et al.* (2000) [33] who compared between direct examination, culture and PCR between vaginal discharge and urine samples. The vaginal swab culture was the most sensitive (94%) followed by vaginal discharge PCR (88.7%), while wet preparation was the least sensitive 58.5% for both.

A comparison was also made, by Street *et al.*, 1982 [27], between the detection of anti-*Trichomonas vaginalis* antibodies using ELISA on sera and vaginal secretions. Serum IgG antibodies were detected in 68% of tested women, while 50% had IgG antibodies in their secretions. IgM was detected in the sera of 21.9% of patients while it wasnot detected in vaginal secretions; instead IgA antibodies were detected by ELISA in 50% of infected woman's discharge [27]. A recent study in Ghana also compared between the three most popular diagnostic methods, i.e., high vaginal swab wet mount, urine culture and ELISA. The latter performed best compared to the other methods, with the highest sensitivity (88.9%), while the other two methods' sensitivities were 33.3% and 11.1%, respectively [20]. It is very important to diagnose the infection with trichomoniasis accurately, especially in pregnant women since treatment provides an opportunity to improve the health outcomes of women and their infants [34].

Trichomoniasis poses a public health threat to pregnant women and neonatal health. The infection causes lower abdominal pain and pre-term labors and abortions. It may also cause reversible infertility and upper reproductive tract post-caesarian infections. The infection with *T. vaginalis* has been an indicator for many other common sexually transmitted pathogens such as *Chlamydia trachomatis*, *Neiserria gonorrhoeae* and even HIV [35-37]. In a study that used molecular tests, a striking result was that almost half of the pregnant women treated for *T. vaginalis* had a positive result. Treated individuals continued to test positive after treatment beyond the period of infectivity because molecular tests detected nucleic acid from organisms whether they were alive or dead after treatment [38].

In this study, a developed ELISA technique was conducted for tricomoniasis of an Iraqi strain of *T. vaginalis* and it is recommended to be used for high sensitive diagnosis of this parasite, in comparison with the classical used methods.

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