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Comparison between Enzyme Linked Immunosorbent Assay (ELISA) and Immunochromatographic Test (ICT) Methods for the Screening of Syphilis among Blood Donors

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ABSTRACT

Blood transfusion is one of the most important routes for syphilis transmission, which is an infectious systemic disease caused by the *Treponema pallidum*. The aim of this study was to compare between two methods used commonly in detecting syphilis among blood donors. The immune chromatographic test (ICT), a rapid chromatographic immunoassay for only the qualitative detection of antibodies or antigens in whole blood, serum and plasma versus the enzyme-linked immunosorbent assay (ELISA), which is both a qualitative and/or quantitative technique used for detection of antibodies or antigens in human serum or plasma. This comparative study was carried out in 200 samples collected from 200 blood donors in central blood bank, Wad Medani, Sudan during August 2015. The samples were subjected to syphilis test using both ICT and ELISA techniques. The study clearly showed that out of the 200 samples tested using the ICT test 191(95.5%) gave negative results with only 9(4.5%) samples reported positive. However, when the same 200 blood samples were tested using ELISA test 187(93.5 %) samples gave negative results and 13(6.5%) samples reacted positively. The 191 negative samples obtained by ICT gave 6 positive samples after tested by the ELISA, and the 9 positive samples obtained by ICT gave 7 positive samples and 2 negative samples after tested by ELISA. Thus, the outcome of this comparison clearly indicated that ICT gave 2 false positive samples and 6 false negative samples compared to the ELISA test, a finding which reflects the possible hidden risk among the blood donors and can have a great impact in the case of blood transfusion. The study recommends the use of ICT in screening of syphilis but should be confirmed with other sensitive diagnostic tests. Further studies should be done in order to identify more sensitive, reliable techniques for syphilis detection in blood banks to avoid risks of false results.

INTRODUCTION

Syphilis is a systemic disease caused by *Treponema pallidum*. The disease has been divided into stages based on clinical findings, helping to guide treatment and follow up. Persons who have syphilis might seek treatment for signs or symptoms of primary syphilis infection (i.e, ulcers or chancre at the infection site), secondary syphilis (i.e., skin rash, mucocutaneous lesions and lymphadenopathy), or tertiary syphilis (i.e., cardiac, gummatous lesions, tabes dorsalis and general paresis). Latent infections (i.e., those lacking clinical manifestations) are detected by serologic testing. Latent syphilis acquired within the preceding year is referred to as early latent syphilis; all other cases of latent syphilis are late latent syphilis or syphilis of unknown duration. *Treponema pallidum* can infect the central nervous system and result in neurosyphilis, which can occur at any stage of syphilis. Early neurologic clinical manifestations (i.e., cranial nerve dysfunction, meningitis, stroke, acute altered mental status and auditory or ophthalmic abnormalities) are usually present within the first few months or years of infection. Late neurologic manifestations (i.e., tabes dorsalis and general paresis) occur 10–30 years after infection (CDC, 2015).

Transmission occurs by sexual contact, Transplacental infection of a fetus may occur during the pregnancy of an infected woman. Fetal infection occurs with high frequency in untreated early infections of pregnant women and with lower frequency later in the disease or in late latency. Syphilis is also transmitted by transfusion of blood from infected individuals (Schmid, 2004).

An ELISA is a test used to determine if a particular protein is present in a sample and if so, how much. There are two main variations of this method: it can determine how much antibody is in a sample, or how much protein is bound by an antibody. The distinction is whether you are trying to quantify an antibody or the protein (Gao *et al*., 2009).

The immunochromatographic assays, also known as lateral flow immunochromatographic assays, are simple devices intended to detect the presence (or absence) of a target analyte in sample without the need for specialized and costly equipment, though many lab based applications exist that are supported by reading equipment the first of which was developed in 1956 by singer and plot strip tests ideal for applications such as home testing, rapid point of care testing, and testing in the field for various environmental and agricultural analyses. In

addition, they provide reliable testing that might not otherwise be available to third world countries (Yetisen, 2013).

RATIONALES:

- Syphilis is a serious infectious disease; late syphilis is a slowly progressive inflammatory stage in which granulomatous lesions (gummas) develop in skin, bones, liver, stomach and other organs, and degenerative changes occur in the central nervous system causing meningovascular syphilis, and general paralysis with cerebral atrophy, psychosis, Visual problems, deafness and dementia. Cardiovascular syphilis may lead to aortic aneurysm and aortic valve insufficiency.

- No vaccine is available now, so prevention is the cornerstone in the management strategy. Since blood transfusion is the route of transmission all healthy donors must be screened accurately for syphilis.

General objective:

To compare between ICT technique and ELISA technique in the detection of Syphilis.

Specific objectives:

- To compare the sensitivity of both ICT and ELISA.

- To find out the appropriate diagnostic tool to diagnose Syphilis.

MATERIALS AND METHODS

Study design:

In vitro comparative study, conducted in August 2015 at Central blood bank, Gezira state, Sudan. 200 samples were collected from 18-40 blood donors in central blood bank.

Materials:

Test tubes, syringes, cotton, gloves, Micropipette, centrifuge.

Methodology:

1. Enzyme Linked Immunosorbent Assay (ELISA):

ELISA was used for screening of blood donors, and diagnosis and management of clinical conditions of syphilis.

1.1. Principle of ELISA:

The detection of anti-TP antibodies will be achieved by antigen sandwich enzyme linked immune sorbent assay, where the microwells were coated with recombinant *Treponema pallidum* antigens expressed in *Escherichia coli*. The samples were incubated in the microwells together with recombinant TP antigens conjugated to HRP conjugate antigens, but are expressed in different hosts. In case of presence of anti-TP in the sample, during incubation the pre-coated and conjugated antigens were bound to the two variable domains of the antibody and the specific antigen-antibody immune complex is captured on the solid phase.

After washing to remove sample and unbound conjugates, chromogen solution containing TMB and urea peroxidase was added to the wells. In presence of the antigen-antibody sandwich complex, the colorless chromogen was hydrolyzed by the bound HRP conjugate to a blue colored product, which turns yellow upon addition of the stop solution. This color is then red photometrically and is directly proportional to the amount of the antibody in the sample. Wells containing samples negative for anti-TP remain colorless.

1.2. Kits contents:

Kit contents	**Volume**
Microwell plate 96 test	1 plate (12x8/8x12 well strips per plate)
Negative control	1x0.5 ml
Positive control	1x0.5 ml
HRP- Conjugate reagent	1X13 ml
Stock wash buffer	1x50 ml (Dilute 1 to 20 with distilled water before use. Once diluted stable for two weeks at 2-8 C)
Chromogen solution A	1x7 ml (Ready to use and once open stable for one month at 2-8 C)
Chromogen solution B	1x7 ml (Ready to use and once open stable for one month at 2-8 C)
Stop solution	1x7 ml
Plastic sealable bag	1 unit
Plate cover	1sheet
Package insert	1 copy

1.3. Samples collection:

Fresh serum samples were used for this assay. Blood collected by venipuncture was allowed to clot naturally and completely, the serum was separated from the clot as early as possible to avoid hemolysis on the RBC. Care was taken to ensure that the serum samples are clear and not contaminated by microorganisms. Any visible particulate matters in the sample were removed by centrifugation at 3000 rm for at least 20 minutes at room temperature, but highly lipaemic, icteric, or hemolyzed samples were not be used as they could give erroneous results.

1.4. Stability of reagent:

The components of the kit were stored between 2-8ºC and were not freeze. To assure maximum performance of this anti-TP ELISA kit, during storage was protected the reagents from contamination with microorganism or chemicals.

1.5. Assay procedure:

Step1 Reagents preparation:

The reagents and samples were allowed to reach room temperature (18-30°C) for at least 15-30 minutes.

Step 2 Wells numbering:

The strips needed were Seted in strip-holder, and numbered sufficient number of wells including three negative controls (e.g.B1, C1 D1), tow positive control (e.g., E1, F1) and one blank (e.g.A1, neither samples nor HRP-Conjugate) was added into the blank well.

Step 3 Added HRP conjugate:

100 µl HRP conjugate was added to each well except the blank well.

Step 4 Added sample:

20 µl of Positive Control, negative Control and Specimen was added to their respective wells. Upon addition of the sample, the HRP Conjugate-sample was mixed will appear blue. A separate disposable tip was used for each specimen, Negative control and positive control to avoid cross-contamination.

Step 5 Incubating:

The plate was mixed by tapping gently; the plate was covered with the plate cover and incubated for 60 minutes at 37ºC.

Step 6 Washing:

At the end of the incubation, the plate cover was removed and discards; each well was washed 6 times with diluted wash buffer.

Step 7 Colouring:

50 µl of chromogen A and 50 µl chromogen B were dispensed into each well including the Blank, and mixed by tapping the gently, the plate was Incubated at 37^0C for 15 minutes avoiding light. The enzymatic reaction between the chromogen solutions and the HPR Conjugate produced blue color in positive Control and anti – TP positive sample wells.

Step 8 Stopping reactions:

50 µl stop solution was added to each well and mixed gently. Intense yellow color develops in positive control and anti-TP positive sample wells.

Step 9 Measuring the absorbance:

The plate reader was calibrated with the blank well and the absorbance was read at 450 nm. The Cut - off value was calculated and evaluated the results (Note: The absorbance was read within 5 minutes after stopping the reaction).

1.6. Interpretation of results:

Each microplate was considered separately when calculated and interpreted result of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample's optical density (OD) value to the Cut- off value (C.O) of the plate. (S = the individual absorbance (OD) of each specimen)

Negative results (S/C.O.<1): Samples giving an absorbance less than the cut-off value were considered negative, which indicates that anti-TP antibodies were detected with this anti-HP ELISA kit. And there are no serological indications for past infection with TP.

Positive results (S/C.O. ≥ 1): Samples giving an absorbance great than or equal to the cut-off value were considered initially reactive, which indicates that anti-TP antibodies were detected. Repeatedly reactive samples were considered positive for antibodies to anti-TP; there for three are serological indication for current or past infection with TP. any blood unit containing antibodies to *T. pallidum* was immediately discarded.

Borderline (S/C.O = 0.9-1.1): Samples with absorbance to cut-off ratio between 0.9 and 1.10 are considered borderline samples and retesting is recommended, repeatedly positive samples can be considered positive for anti- TP antibodies.

1.7. Calculation of cut-off value:

(C.O.) =*NC+0.18:

*Nc= the mean absorbance value of three negative controls.

1.8. Quality control range:

- The OD value of the blank well, which contains only chromogens and stops solution, is less than 0.080 at 450 nm.

- The OD value of the positive control was equal to or greater than 0.800 at 450/630 nm or at 450 nm after blanking.

- The OD value of the negative control was less than 0.100 at 450/630 nm or at 450 nm after blanking.

2. ICT:

2.1. Intended use:

The Syphilis Ultra Rapid Test Strip (whole blood/ serum/ plasma) is a rapid chromatographic immunoassay for the qualitative detection of antibodies (IgM and IgG) to *Treponema Pallidum* (TP) in whole blood, serum or plasma to aid in the diagnosis of Syphilis.

2.2. Principle:

The Syphilis Ultra Rapid Test Strip (whole blood/serum/plasma) is a qualitative membrane strip based immunoassay for the detection of TP antibodies (IgG and IgM) in whole blood,

serum or plasma, in this test procedure, recombinant syphilis antigen is immobilized in the test line region of the strip. After a specimen is added to the specimen pad it reacts with syphilis antigen coated particles that have been applied to the specimen pad, this mixture migrates chromatographically along the length of the test strip and interacts with the immobilized syphilis antigen, the double antigen test format can detect both IgG and IgM in specimens, if the specimen contains TP antibodies, a red line will appear in the test line region, indicating a positive result, if the specimen does not contain TP antibodies, a red line will not appear in this region, indicating a negative result. To serve as a procedural control, a pink line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

2.3. Reagents:

The test strip contains syphilis antigen-coated particles and syphilis antigen coated on the membrane.

2.4. Storage and stability:

Store as packaged in the sealed pouch either at room temperature or refrigerated (2-30^{0}C), the test strip is stable through the expiration date printed on the sealed pouch. The test strip must remain in the sealed pouch until use.

2.5. Specimen collection and preparation:

- Fresh serum samples were used for this assay. Blood collected by vein puncture was allowed to clot naturally and completely the serum was separated from the clot as early as possible as to avoid hemolytic on the RBC. Testing was performed immediately after specimens have been collected.

- Bring Specimens to room temperature prior to testing.

2.6. MATERIALS:

Materials were used:

- Test Strips.

- Disposable Specimen droppers.

- Buffer.
- Test cards.
- Packages insert.
- Specimen collection container.
- Centrifuge.
- Timer.

2.7. Directions for use:

- The test strip, specimen, and buffer were allowed to equilibrate to room temperature (15-30^{0}C) prior to testing.
- The test strips were removed from the sealed foil and used it as soon as possible.
- The tape from the test card was peeled off and stuck the test strip in the middle of the test card with arrows pointing downwards as illustrated.
- For Serum or Plasma specimens: the dropper was held vertically and 2 drops of serum or Plasma (approximately 50 µl) was transferred onto the specimens pad of the test strip, then 1 drop of buffer (approximately 40 µl) was added and the timer was started.
- Wait for the red line (s) to appear. The result was read at 10 minutes.

2.8. Interpretation of results:

POSITIVE: Two distinct red lines appeared. One line in the control line region (C) and another line in the test line region (T).

*Note: The intensity of the red color in the test line region (T) will vary depending on the concentration of TP antibodies present in the specimen. Therefore, any shade of red in the test line region (T) was considered positive.

NEGATIVE: One red line was appeared in the control line Region (C). No apparent red or pink line appears in the test line region (T).

INVALID: Control line fails to appear, insufficient specimen volume or incorrect procedural techniques are the most likely reasons for control line failure.

2.9. Quality control:

A procedural control was included in the test. A red line appearing in the control line Region (C) is considered an internal procedural control. It confirms sufficient specimen volume and correct procedural technique.

2.10. Expected values:

The Syphilis Ultra Rapid Test Strip (whole blood/serum/plasma) was compared with a leading commercial TPHA Syphilis test, demonstrating an overall accuracy greater than or equal to 99.7%.

RESULTS AND DISCUSSION

1. RESULTS:

1.1. Positive and negative cases for ELISA and ICT tests:

The number of cases that were used for running ELISA test was 200 blood samples, as same as that for running by ICT test. The negative cases were 187 for ELISA test, while they were 191 cases for ICT test 6 cases from these positive by ELISA test (False negative)). The positive cases were 13 for ELISA test, while they were 9 cases for ICT test (2 cases from these negative by ELISA test (False positive)).

Table (-1) Positive and negative cases of syphilis using ELISA and ICT test

Test type	Cases					
	Tested		Negative		Positive	
	N	Percent	N	Percent	N	Percent
ELISA	200	100.0%	187	93.5%	13	6.5%
ICT	200	100.0%	191	95.5%	9	4.5%

Table (-2) Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
ELISA* ICT	200	100.0%	0	0%	200	100%

Table (-3) ELISA* ICT tests cross tabulation

	ICT		Total
	Positive	Negative	
ELISA Positive	7	6	13
Negative	2	185	187
Total	9	191	200

Table (-4) Symmetric Measures

	Value	Approx. significant
Nominal by nominal Contingency Coefficient	0.532	0.000
N of valid cases	200	

Prob. Value ≤ 0.05 (Significant) *

Prob. Value ≤ 0.01 (Highly Significant) **

Prob. Value > 0.05 (No Significant)

The ICT test detected 6 cases false negative and 2 cases false positive when compared with the confirmatory ELISA test. There was highly significant difference between the ELISA and ICT test.

2. DISCUSSIONS:

The study clearly showed that out of the 200 samples tested using the ICT test 191 (95.5%) gave negative results with only 9(4.5%) samples reported positive. However, when the same 200 blood samples were tested using ELISA test 187 (93.5 %) samples gave negative results

and 13 (6.5%) samples reacted positively. The 191 negative samples obtained by ICT gave 6 positive samples after tested by the ELISA, and the 9 positive samples obtained by ICT gave 7 positive samples and 2 negative samples after tested by ELISA. Thus, the outcome of this comparison clearly indicated can that, ICT gave 2 false positive samples and 6 false negative samples compared to the ELISA test, a finding which reflects the possible hidden risk among the blood donors and can have a great impact in the case of blood transfusion. These findings agreed with

Hasab Elrasoul and Nafi (2014) enrolled 90 blood donors in their study they found 6 out of the 90 samples were positive by ICT and 4 positives by ELISA.

Elagib and Abdelmaged (2009), studied 451 blood donors, the ELISA test showed that 23.5 % from blood donors were positive for syphilis; while the ICT test showed that only 16.4 % from the blood donors were positive for syphilis. ELISA test showed that 106 individuals were syphilis positive, whereas ICT test showed that 74 individuals were syphilis positive. The ICT test detected 32 individuals false negative when compared with the confirmatory ELISA test. There was significant difference between the ELISA and ICT test.

Kilany *et al*., (2015), found that; depending on donor selection criteria, voluntary non-remunerated 7267 blood donors (26 females (0.36%, median age of 28) and 7241 males (99.64%, median age of 30) when selected to donate their blood , the serological screening of the samples resulted in positivity of many different markers. Two (0.028%) positive cases of anti-*Treponema pallidum* antibodies, one is 33 years old and the second is 36 years old both with positive markers for HBcAb.

PCR can also be used to detect *T. pallidum* genetic material, most often the PolA gene. To date, it is most commonly used to diagnose congenital syphilis. However, it has also been shown to be effective at diagnosing primary syphilis with sensitivities between 73% and 95% and specificities 95%. For the diagnosis of congenital syphilis, frozen tissue and formalin-fixed, paraffin-embedded tissue and, for the diagnosis of primary syphilis, swabs of the ulcer can be submitted to the CDC for processing with prior approval from the local health department. Other specimen types are acceptable but have less diagnostic value. Sensitivities of blood samples in primary syphilis are as low as 18% (Chaurasia *et al*., 2014).

CONCLUSION:

A positive result for syphilis antibodies gotten with an immune-chromatographic rapid strip test does not warrants that treatment should begin. False positive and negative results are common. Therefore, the presence of the disease should be investigated further using a more sensitive and specific assay prior to treatment. Although PCR assays are very expensive to be incorporated, an ELISA which is less expensive and more affordable can be implemented to give more valid results. A negative result too does not exclude the presence of the infection. If symptoms persist, then the infection should be investigated further with a PCR assay. It is important that diagnosis should be done together with the patient medical history since the major risk factors for infection syphilis.

Recommendations:

- ICT is not perfect to diagnose syphilis because the false results are probable.
- To confirm the diagnosis of syphilis ELISA must be used.
- ELISA test should be used in blood bank to avoid any probability of false blood transfusion and spreading the disease.
- Further studies should be done in order to identify more sensitive and reliable techniques for syphilis detection in blood banks.

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