Technical Reports

EVALUATION OF SYPHILIS BIOMARKERS Tpp17 AND Tpp47 BY USING AN IN HOUSE THIRD GENERATION DAS-ELISA

THE AIM OF THE RESEARCH PRESENTED IN THIS TECHNICAL REPORT IS THE EVALUATION OF SOME SYPHILIS RECOMBINANT ANTIGENS BY USING AN IN HOUSE THIRD GENERATION ELISA ASSAY. WITH THIS PURPOSE, THE RECOMBINANT ANTIGENS Tpp17 AND Tpp47, WERE BIOTINYLATED *IN VIVO* (MONOBIOTYNILATED) AND *IN VITRO* (POLYBIOTINYLATED) ALLOWING THE PERFORMANCE OF A DOUBLE-SANDWICH ANTIGEN ELISA ASSAY.

Syphilis is a multistage progressive disease caused by the spirochete *Treponema pallidum* subsp. *pallidum* and is characterized by localized, disseminated, and chronic stages. Manifestations include the development of a localized lesion called a chancre during the primary stage and disseminated skin lesions and meningovascular syphilis during the secondary stage (followed by a period of latency lasting from months to decades). It can also be transmitted from mother-to-child in utero or during birth.

The disease can be divided into stages based on clinical findings. The primary and secondary stages of disease are characterized by initial skin manifestations. If undetected, syphilis enters the lengthy latent period, with serologic proof of infection without symptoms of disease. If left untreated, the infection can progress to the symptomatic tertiary stage with subsequent systemic involvement and potentially serious complications.

Since direct microscopy is possible only when lesions are present, and this is not the case in the majority of patients, detection of antibodies against *T. pallidum* is the most effective method for the diagnosis of syphilis. There are many diagnostic tests available for syphilis; however, a commonly accepted gold standard is still lacking.

A confirmed serological test result is indicative of the presence of treponemal antibodies but does not indicate the stage of disease and, depending on the test, may not differentiate between past and current infection. Despite their shortcomings and the complexity of interpretation, serological tests are the mainstay in the diagnosis and follow-up of syphilis.

High specificity, especially in potentially cross-reactive samples, is required in order to prevent potential false-positive results, minimizing the requirements for re-testing and reducing patient anxiety.

High sensitivity is also required to minimize the likelihood of missing *T. pallidum* infections at all disease stages and assure an early detection of infection due to an appropriate treatment is extremely important.

In recent years, the double-antigen sandwich assay has been used for detection of antibodies to identify several diseases which require IgG + IgM detection. Those third generation IVD assays are able to achieve a high specificity thanks to the formation of double-antigen sandwich immune complexes during the immunoassay performance.

There are several reasons why nowadays a DAS-ELISA is preferred over an indirect ELISA: (i) it is possible to detect at the same time IgG + IgM, increasing the **sensitivity** of the assay and (ii) due to exist two points of interaction between Ag-Ab, increases also the **specificity** of the procedure.

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In the double-antigen sandwich ELISA system, serum specific IgG or IgM antibody binds to the antigen on the solid phase (capturer) by one of the two binding sites of Fab and to the conjugated antigen (detector) in the liquid phase with the other. Even if the nonspecific IgG/IgM molecules attached to the solid phase, they would not capture the conjugated antigen. Thus, double-antigen ELISA generally exhibits low backgrounds.

Moreover, an indirect ELISA generally requires preparation of high dilutions of test sera, which is inconvenient and prone to dilution error or might decrease sensitivity, whereas the double-antigen sandwich ELISA can be performed without sample dilution, which increases sensitivity and simplifies the procedure.

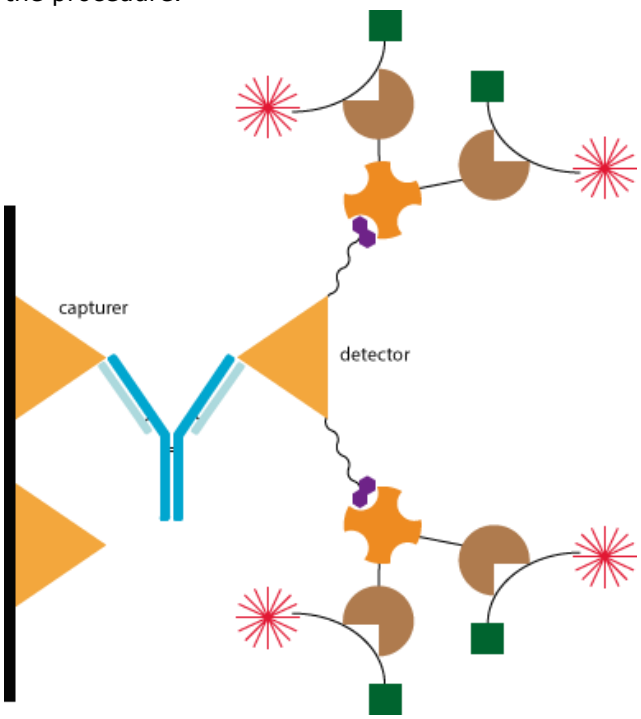

Figure 1. DAS-ELISA scheme

Several problems can arise in a DAS immune test like for instance: (i) the multi-site attachment of the conjugated molecule to the protein blocking the Ab binding site(s), (ii) the steric hindrance between capturer and detector and (iii) the improper orientation of the capturer at the plate. Regarding (i), it is necessary to consider the specific folding of the protein in addition to the conjugation chemistry used to obtain the detector antigen and, if known, the location of the potential antigenic determinants. For this reason, we have evaluated as detectors in a DAS system developed *in house*, site-directed mono-biotinylated (mb) antigens and randomly poly-biotinylated (pb) antigens to compare their accurate.

In the present study, several DAS-ELISA assays have been performed with Rekom Biotech biomarkers Tpp17 (RAG0008)/Tpp17-mb (RAG0008BIOT) *vs* Tpp17 (RAG0008)/Tpp17-pb and Tpp47 (RAG0010)/Tpp47-mb (RAG0010BIOT) *vs* Tpp47 (RAG0010)/Tpp47-pb. The poly-biotinylated antigens have been obtained by a biotinylation *in vitro*, the mono-biotinylated antigens have been obtained by biotinylation *in vivo* by a site-directed biotinylation procedure.

Both proteins have been produced as recombinant antigens in the heterologous system *Escherichia coli*, obtaining the mature proteins pure to homogeneity. These proteins have been analyzed in SDS-PAGE (figure 2).

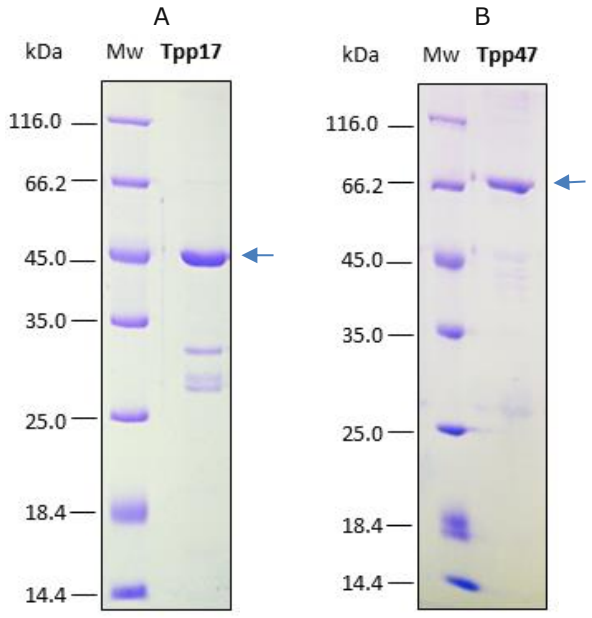

Figure 2. SDS-PAGE analysis of A) Tpp17 RAG0008; B Tpp47 RAG0010 which are Rekom Biotech recombinan biomarkers for syphilis

Firstly, an *in house* indirect ELISA were performed with the biomarkers, just to know that both antigens were able to discriminate between positive and negative specimen sera by being coated to the plates.

The assays were performed by using positive and negative syphilis specimen sera pre-validated with

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an ELISA (Abbott: Architeck); a TPHA (Spin React) and RPR (Becton Dickinson).

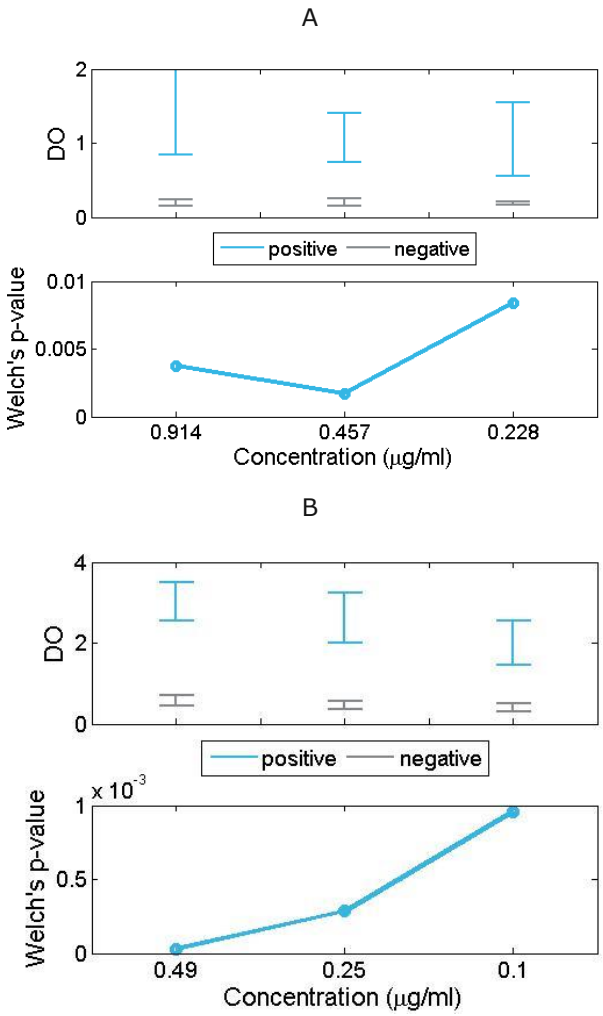

Figure 3. Indirect IgG ELISA. In these plots, the optical density at 450/620 nm for positive (blue) and negative (gray) sera are compared for each concentration of the recombinant antigens. A) indirect IgG ELISA with Tpp17; B) indirect IgG ELISA with Tpp47.An appropriate statistical test of significance for the comparison of means between both groups, the Welch's test, is employed. Eligible concentrations for the use of the antigen should present statistically significant differences between positive and negative sera. This happens when the intervals at the top do not overlap and, equivalently, when the p-value at the bottom is below 0.05. In the present figure, all p-values are below 0.05 and thus the intervals do not overlap. Therefore, any of the showed concentrations can be used to distinguish between positive and negative sera.

Also, the four detector antigens were analyzed with a western blot with HRP-streptavidin to be sure about the biotinylated nature of these antigens (figure 4A and 4B):

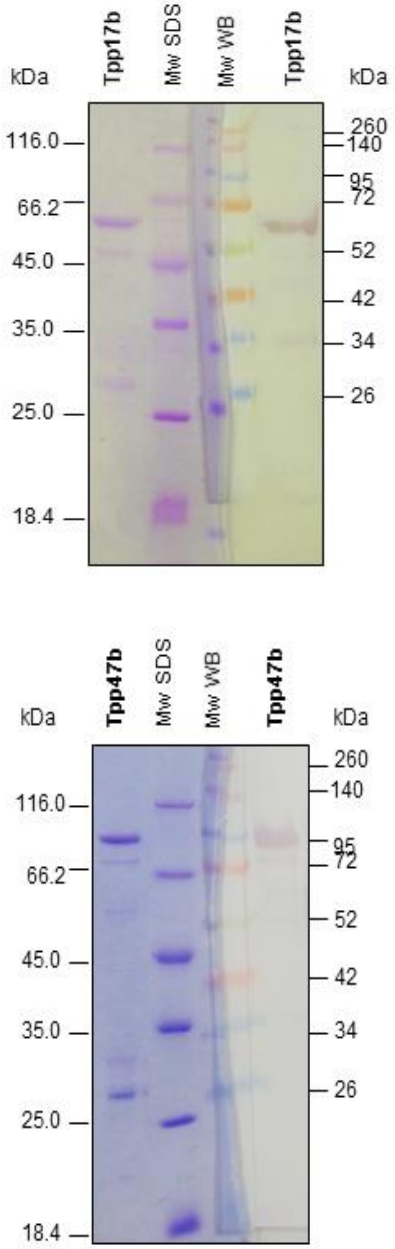

Figure 4A. Mono-biotinylated Tpp17 (RAG0008BIOT) and Tpp47 (RAG0010BIOT) were analyzed by western blot with streptavidin-HRP.

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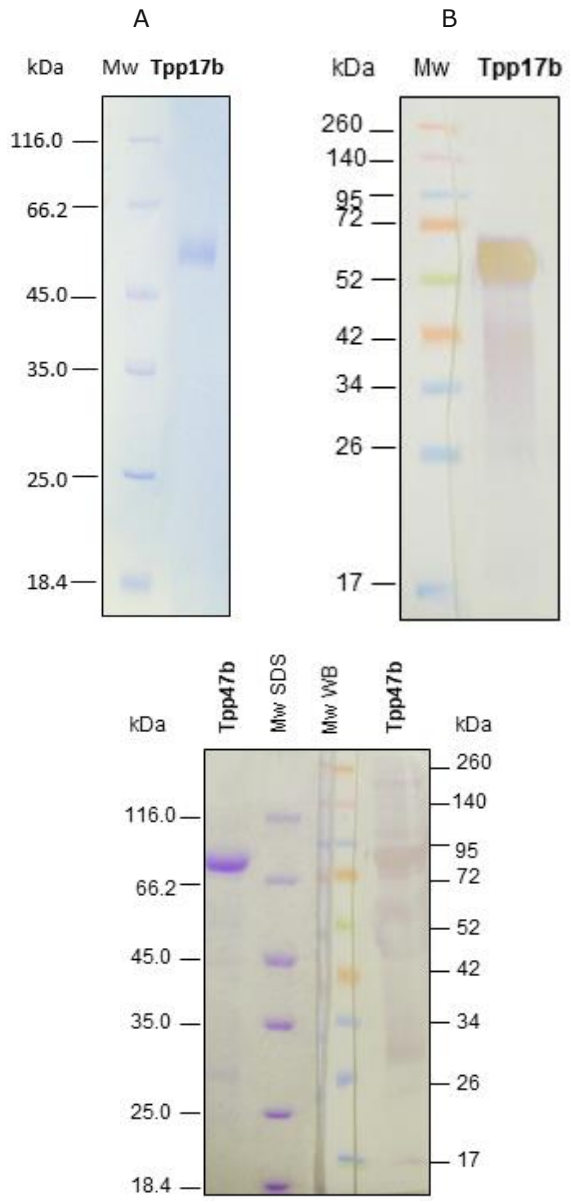

Figure 4B. Poly-biotinylated Tpp17 (A) SDS-PAGE and (B) western blot and Tpp47 were analyzed by western blot with streptavidin-HRP.

Finally, a spectrophotometric detection of the number of biotins were performed with the Pierce™ Biotin Quantitation Kit (Thermo Scientific™) HABA (4´-hydroxyazobenzene-2-carboxylic acid). This is a reagent that enables an estimation of the mole-to-mole ratio of biotin to protein, obtaining the following results for our detectors:

REFERENCE	EXPERIMENTAL Nº OF BIOTINS/MOLECULE
Tpp17-**poly**-biotinylated	11
(RAG0008BIOT) Tpp17-**mono**-biotinylated	1
Tpp47-**poly**-biotinylated	3
(RAG0010BIOT) Tpp47-**mono**-biotinylated	1

Once the biotinylated antigens were characterized, the DAS-ELISA assays were performed (figure 5A and 5B).

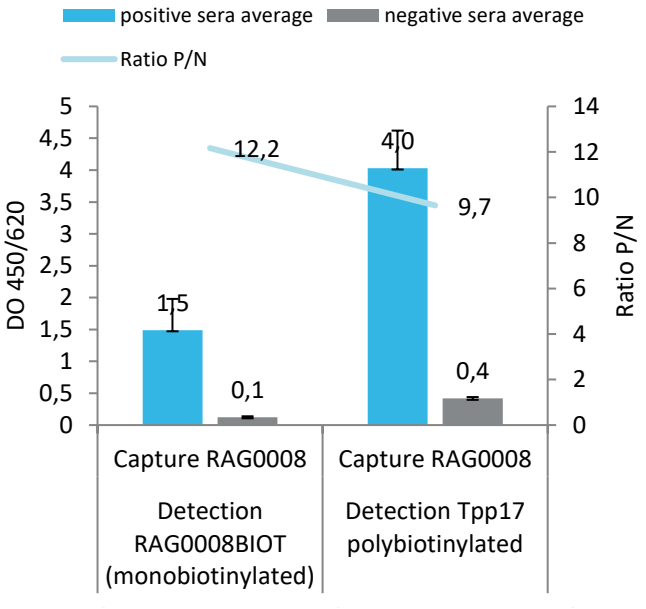

Figure 5A. The plates were coating with Rekom Tpp17 RAG0008 and the detection was performed with Rekom biotinylated Tpp17 (RAG0008BIOT and Tpp17 pb). In this plot, the optical density at 450/620 nm obtained in a DAS ELISA assay for several positive (blue) and negative (gray) sera were compared. Also, the positive/negative signal ratio were calculated for the signal average of positive and negative specimen sera. The plates were coating with 0.25 µg/ml of RAG0008, the detection was performed with 0.5 µg/ml, and the development was carried out with a 1:5000 dilution of strep-HRP.

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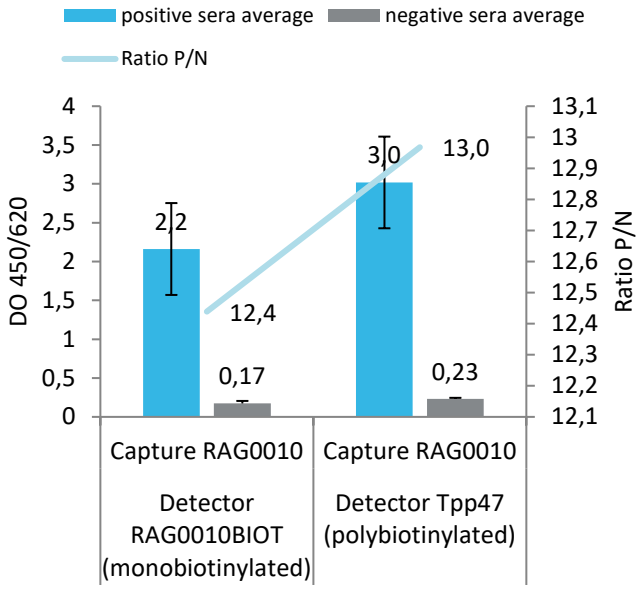

Figure 5B. The plates were coating with Rekom Tpp47 RAG0010 and the detection was performed with Rekom biotinylated Tpp47 (RAG0010BIOT and pb). In this plot, the optical density at 450/620 nm obtained in a DAS ELISA assay for several positive (blue) and negative (gray) sera were compared. Also, the positive/negative signal ratio were calculated for the signal average of positive and negative specimen sera. The plates were coating with 0.25 µg/ml of RAG0010, the detection was performed with 0.5 µg/ml, and the development was carried out with a 1:5000 dilution of strep-HRP.

CONCLUSION

In this study, we have determined that Rekom Biotech antigens Tpp17 (RAG0008) and Tpp47 (RAG0010) are able to develop a DAS immunoassay test. We have tried both bio-markers coated in plates and the mono-biotinylated and poly-biotinylated same antigens as detectors. Several conclusions can be discussed:

Regarding the poly-biotinylated antigens, both can bind effectively to their paratopes despite the biotins present around their sequences. Initially, we decided to test also mono-biotinylated antigens in case that the antibody binding sites may be blocked due to the biotin binding around the primary sequence.

In the specific case of Tpp17, the poly-biotinylated antigens contained 11 biotins *vs* 1 biotin detected in the *in vivo* biotinylated antigen. We can see that the signal with positive specimen sera is nearly 3 times higher when the poly-biotinylated antigen is used (probably due to the amplification effect of the 11 biotins), therefore the poly-biotinylation process does not reduce the accessibility to the antigenic determinants of the bio-marker. However, as the signal with negative specimen sera is also 4 times higher, the P/N ratio is better in the case of the mono-biotinylated detector (12.2 *vs* 9.7; mono *vs* poly).

In the case of the Tpp47, only 3 biotins were bound to the poly-biotinylated antigen, therefore the differences between mono and poly-biotinylated detectors are not thus different (P/N ratio of 13 *vs* 12.4; poly *vs* mono).

Finally, one consideration. As the P/N ratio is very similar between poly and mono-biotinylated antigens used as detectors, even in the case of the mono-biotinylated detector the background is lower, we recommend the usage of mono-biotinylated recombinant antigens as detectors for two clear reasons: (i) one biotin per protein molecule is always easier to control and reproduce and (ii) IgM detection is more dependent on the presence of conformational and/or discontinuous epitopes and using just one site-directed biotin assure us that the introduction of the biotin is always far away from the Ab binding site(s), increasing the possibility of IgM detection.

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