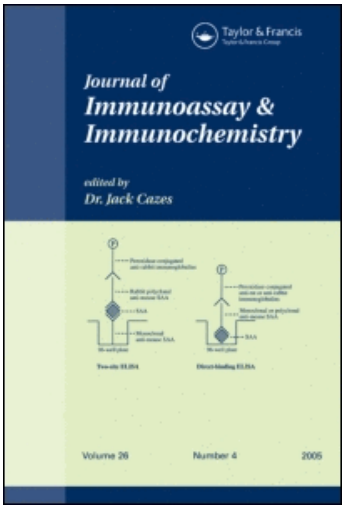

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Abstract: Immunogenic glycolipids from the cell wall of *Mycobacterium tuberculosis* are potential capture antigens in enzyme-linked immunosorbent assays (ELISAs) for the serodiagnostis of tuberculosis. Typically, washing steps in ELISAs are performed with buffers containing a detergent. However, Tween-20, the most commonly added detergent, was reported to be able to remove the coating of certain glycolipid antigens from microtitre wells. In order to determine the influence of the washing buffer composition on the results, we measured serum immunoglobulin G (IgG) against three mycobacterial glycolipids by ELISA, conducting three separate experiments with three different buffers: Tris-buffered saline (TBS), TBS plus 0.02% Tween-20 (TBS-Tween), or TBS plus 0.3% bovine serum albumin (TBS-BSA). The capture antigens applied were lipoarabinomannan with the basic

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arabinose-containing motif (AraLAM), the mannose-capped version of lipoarabinomannan (ManLAM), and trehalose-6,6′-dimycolate (cord factor).

All ELISAs achieved acceptable specificities around 95%. The sensitivities, however, varied widely, depending upon the sort of washing buffer used. In 38 patients with sputum smear-positive pulmonary tuberculosis and control groups of 79 patients with non-tuberculosis lung disease and 92 healthy volunteers, the anti-cord factor ELISA achieved 100%, 31.6%, and 60.5% with TBS, TBS-Tween, and TBS-BSA, respectively. Corresponding sensitivity values for AraLAM were 39.5%, 26.3%, and 23.7%, and for ManLAM 94.7%, 65.8%, and 55.3%. We conclude that Tween-20 or BSA should be omitted from the washing buffer in ELISAs, when the capture antigen is of lipid nature.

Keywords: Enzyme-linked immunosorbent assay, Tuberculosis, Cord factor, Lipoarabinomannan, Tween-20

INTRODUCTION

Enzyme-linked immunosorbent assays (ELISAs) for the serodiagnosis of tuberculosis (TB) have been widely explored, since they are rapid to perform, relatively inexpensive, and non-invasive.[1] Along with a variety of purified or recombinant peptide antigens, a number of mycobacterial glycolipids have been tested for their utility as capture antigen in ELISAs.[1–8] However, the test sensitivities obtained varied widely and this has been attributed to the use of blocking agents in the assays.[9]

Tween-20 (polyoxyethylene sorbitan monolaurate), a non-ionic detergent frequently added to the washing and diluting buffer to avoid non-specific binding, has been suspected to interfere in glycolipid-based assays.[10,11] Recently, Julián and co-workers impressively showed, that Tween-20 is able to detach certain glycolipids from microtitre wells.[9] Another author questioned the use of bovine serum albumin (BSA) because high background levels were observed.[5]

The objective of the present study was to compare the diagnostic sensitivity values in dependency on the washing buffer composition of an ELISA for serum immunoglobulin G (IgG) against glycolipid antigens of Mycobacterium tuberculosis. The ELISA was performed in a collective of patients with confirmed TB, non-TB pulmonary disease and healthy volunteers conducting three separate experiments with three different solutions for washing and serum dilution. As capture antigens we applied trehalose 6,6′-dimycolat (cord factor, CF), and the two forms of lipoarabinomannan (LAM): LAM with the basic arabinose-containing motif (AraLAM) and LAM capped with mannosyl residues (ManLAM). For comparison, samples of all study participants were tested with a commercially available ELISA kit in common use for measurement of IgG against antigen A60, the thermostable component of Purified Protein Derivative (PPD) from Mycobacterium bovis BCG.[1]

EXPERIMENTAL

Study Groups

In total, 209 study participants (median age 35 [12–90] years, 121 male, 88 female) were allocated into three groups.

Group I comprised 38 patient from the Chest Hospital, Al Medinah, Saudi Arabia, with smear-positive pulmonary TB confirmed by culture. Sera were taken prior to the initiation of any anti-tuberculous treatment.

Group II included 79 patients with a defined non-tuberculous respiratory disease: acute bacterial pneumonia (n = 61), lung cancer (n = 6) and various forms of non-infectious chronic inflammation (n = 12). The presence of tuberculosis was excluded by demonstration of negative sputum smears and negative culture for *Mycobacterium tuberculosis.* All pneumonia patients responded promptly to beta-lactam antibiotics and/or macrolides.

Group III consisted of 92 healthy individuals with no clinical or radiographic evidence of active TB.

All patients and volunteers were HIV sero-negative. Written informed consent was obtained from each study participant. Samples from patients were aliquots of routinely drawn blood. Sera were stored at −20°C until use.

Antigens

ManLAM and AraLAM from the virulent strain *Mycobacterium tuberculosis* H37Rv was a gift from Professor Patrick J. Brennan, Colorado State University.

Trehalose 6,6′-dimycolate from *Mycobacterium tuberculosis* and all other reagents were purchased from Sigma-Aldrich, Steinheim, Germany. The commercially available ELISA kit for the measurement of IgG against antigen A60 was obtained from Anda Biologicals, Strasbourg, France.

ELISA Protocol

Glycolipid antigens were dissolved 20 μg/mL in n-hexane (for CF) or ethanol (for LAMs). 50 μL of the solution (1 μg lipid) were added to each flat-bottomed well of a polystyren microtitre plate with unmodified surface (Greiner, Austria) and air dried. Plates were then saturated for 1 h with 200 μL/well 1% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS, 0.02 mol/L Tris-HCL, 0.5 mol/L NaCl, pH 7.5).

Washing steps, as well as serum dilution, were performed with one of the following solutions: TBS, TBS-Tween (0.02% wt/v Tween-20 in TBS) or TBS-BSA (0.3% wt/v BSA in TBS).

After washing three times 50 μL/well of serum, diluted 1/200 in the corresponding washing buffer, was added and incubated for 2 h at room

temperature. After another three washings 50 μL/well goat anti-human IgG alkaline phosphatase conjugate, diluted 1/10,000, was added, followed by a 2 h incubation period. After final three washings, colour reaction was started by addition of 100 μl/well p-nitrophenyl phosphate (1 mol/L in diethanolamine buffer, ph 9.8). The reaction was stopped after 30 min with 50 μL/well NaOH (3 mol/L). Optical density (O.D.) values were read at 405 to 620 nm.

Samples were measured in duplicate and for each sample one equally treated well, but without antigen coating, was included into the plate as negative control. Additionally three previously determined sera with low, medium and high antibody concentrations were measured in each plate to observe day-to-day variations. All ELISAs were carried out in three separate experiments according to the three types of washing buffer. The anti-A60 ELISA was performed according to the manufacturer's instruction. (The kit includes a washing buffer which contains no detergens).

Expression of Results and Statistical Methods

The difference between absorbance of tested serum and that of the corresponding negative control was taken and the arithmetic mean of two results for each sample was calculated. A result was considered positive if the corrected O.D. was higher than the mean plus 2 standard deviations of the corrected O.D.s of healthy controls. Anti-A60 ELISA results were calculated from a plot of values of standard sera, which are included by the manufacturer. Sensitivity and specificity for each antigen were calculated using standard formula. Additionally, the test performance was pictured by means of the receiver operating characteristic (ROC) curve.

Statistical analysis was performed using the statistical package SPSS 11.5 for Windows (SPSS Inc., Chikago, IL). Groups were compared by Mann-Whitney test. $p < 0.05$ was considered significant.

RESULTS

Mean O.D.s of the blank wells (without antigen coating) were not significantly different among the three sorts of washing buffer ($p > 0.3$). TB patients (group I) had significantly higher O.D.s than non-TB patients (group II) and healthy controls (group III) with all antigens and methods ($p < 0.001$). Mean O.D.s $\pm$ standard deviations are given in Table 1.

Sensitivity and Specificity

The efficiency of the ELISAs in all three methodological variations are pictured by means of the receiver operating characteristic (ROC) curves in

Table 1. Optical densities, sensitivity and specificity of anti-glycolipid ELISAs using three different washing buffers

		AraLAM			ManLAM			CF		
Washing buffer	Study group (n)	No. pos.	Sens./ Spec. (%)	O.D. (mean ± SD)	No. pos.	Sens./ Spec.(%)	O.D. (mean ± SD)	No. pos.	Sens./ Spec. (%)	O.D. (mean ± SD)
TBS	I (38)	15	39.5	0.289 ± 0.075[a]	36	94.7	0.472 ± 0.083[a]	38	100	0.506 ± 0.073[a]
	II (79)	10	87.3	0.155 ± 0.046	9	88.6	0.168 ± 0.050	5	93.7	0.229 ± 0.051
	III (92)	2	97.8	0.180 ± 0.060	5	94.6	0.142 ± 0.036	4	95.6	0.214 ± 0.047
TBS + Tween	I (38)	10	26.3	0.218 ± 0.117[a]	25	65.8	0.354 ± 0.124[a]	12	31.6	0.102 ± 0.125[a]
	II (79)	5	93.7	0.093 ± 0.057	2	97.5	0.099 ± 0.065	4	94.9	0.048 ± 0.013
	III (92)	3	96.7	0.153 ± 0.098	6	93.5	0.146 ± 0.082	3	96.7	0.046 ± 0.014
TBS + BSA	I (38)	9	23.7	0.189 ± 0.133[a]	21	55.3	0.306 ± 0.148[a]	23	60.5	0.247 ± 0.106[a]
	II (79)	3	96.2	0.111 ± 0.058	5	93.7	0.117 ± 0.076	2	97.5	0.090 ± 0.052
	III (92)	2	97.8	0.155 ± 0.072	6	93.5	0.132 ± 0.059	4	95.6	0.127 ± 0.041

LAM, lipoarabinomannan; CF, cord factor; Sens., Sensitivity; Spec., Specificity; O.D., optical density; TBS, Tris-buffered saline; BSA, bovine serum albumin. Sensitivity applies to group I (TB patients), specificity applies to group II (non-TB patients) and III (healthy controls); cut-off value: mean + 2SD of group III.

[a] $p < 0.001$.

Figs. 1 A–C. The ROC curve describes the corresponding sensitivity and specificity for different cut-off values. The nearer the curve approaches the "northwest" of the graph (were both sensitivity and specificity is 100%), the better the test efficiency.

Admixture of Tween-20 or BSA to the washing and serum diluting buffer drastically reduced the sensitivities of all three anti-glycolipid assays (Figs. 1 A–C and Table 1). As demonstrated by the ROC curves, the strongest deterioration was caused by TBS-Tween on the anti-CF ELISA and by TBS-BSA on both anti-LAM ELISAs.

The commercial ELISA kit for IgG against antigen A60 achieved 84.2% sensitivity and 73.4% and 78.3 specificity in groups II and III, respectively, with the cut-off value according to the manufacturer's recommendation (200 units/mL).

DISCUSSION

The decreased sensitivity values of our ELISAs when performed with TBS-Tween indicate that Tween-containing solutions partly remove glycolipid antigens from the well. The ability of Tween to dissociate glycolipids from polystyren surfaces was described by Julián and co-workers for acyltrehaloses, sulfolipids and phenolic glycolipids which too are possible lipid capture antigens for TB diagnostic.[9] There is evidence that the deteriorating effect of washing with Tween-20 might be compensated by an exceptional high amount of glycolipid applied for coating. Maekura and co-workers performed an anti-CF ELISA with 2.5 μg CF per well and washing steps with 0.05% Tween-20 in phosphate-buffered saline and achieved 81.1% sensitivity in smear-positive TB patients.[4] Contradictorily to our results, coating of wells with CF was not affected by washing with TBS-Tween in Julián's study.[9]

A single initial blocking step with 1% BSA in TBS was performed in all our anti-glycolipid ELISAs. However, repeated washing with TBS-BSA, similar to TBS-Tween, strikingly reduced the sensitivity of the assays. Albumin is a well known transport protein for lipophilic compounds.[12] Thus, it is conceivable that washing with BSA-containing solutions either detach glycolipids from the well or that BSA somehow disguises the epitopes. As shown in Fig. 1 A, CF with its long-chained branched mycolic acids, which are the immunogenic determinants,[13] appeared to be more resistant to the effects of BSA than to that of Tween-20. Simonney and co-workers reported the use of BSA-containing buffers in certain glycolipid-based ELISAs.[5] They noted high background levels leading to high cut-off values and suspected anti-BSA antibodies in their TB patients, which have previously been described in the literature.[14] We were not able to confirm the assumption, because in our study absorbance values in all groups were lower with TBS-BSA than with TBS ($p < 0.01$), and we did not observe elevated absorbance in the blank wells.

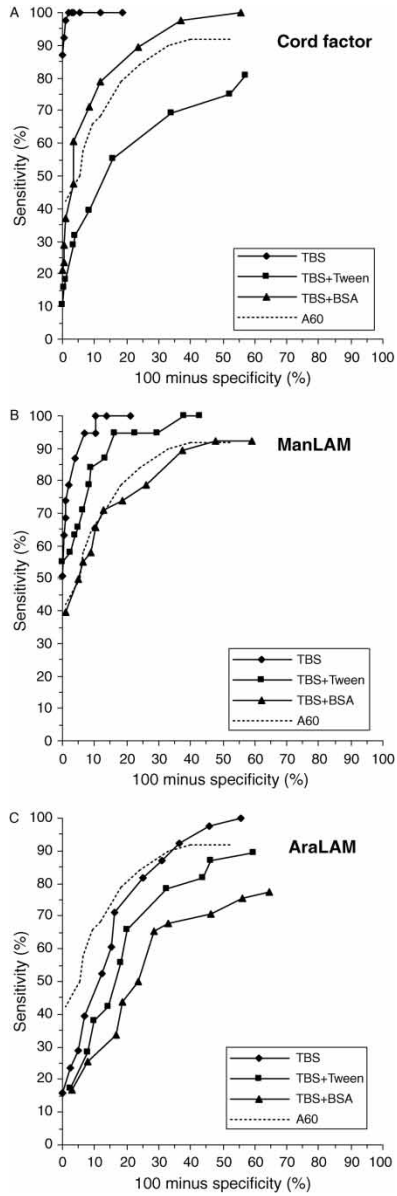

Figure 1. Receiver operating characteristic (ROC) curves of ELISAs for measurement of serum IgG against cord factor (A), ManLAM (B), and AraLAM (C). Assays were performed in 38 patients with smear positive pulmonary tuberculosis and 171 controls using one of the following three different washing buffers: Tris-buffered saline (TBS), TBS plus 0.05% Tween-20 (TBS-Tween), and TBS plus 0.3% bovine serum albumin (TBS-BSA). The dotted line represents the ROC curve of a commercially available ELISA kit for serum IgG against antigen A60.

With 84.2% and 76.1% for sensitivity and specificity, respectively, the diagnostic characteristics of the commercial anti-A60 ELISA, the "reference test" in our series, were similar to that in other reports.[15,16] The cut-off value is the most important parameter for the interpretation of serological results. However, there is a variety of recommended procedures for the establishment of a cut-off value. The ROC curve invalidates individual methods of calculating the cut-off value because it includes all possible choices. We therefore regard it as the most accurate way to present diagnostic tests.

It should be emphasized that we did not focus on the clinical utility per se of the ELISAs described in the present study. This ought to be determined in a larger trial including patients with more distinctive forms of pulmonary or extrapulmonary TB, immuno-compromised patients and patients with non-TB mycobacterial infections. In spite of the limitations it is worth noting that twice as many TB patients were positive for anti-ManLAM IgG than for anti-AraLAM IgG. Moreover the anti-ManLAM assay gave higher absorbance values than the anti-AraLAM assay in the TB patient group ($p < 0.001$). ManLAM is known to be the predominant LAM of disease-inducing strains of mycobacteria and elicits a specific antibody response.[17] The control groups with mixed ethnic background are representative of the patient collective admitted to our institution. Analysis of the control groups after disaggregation by nationality revealed, that mainly sera of refugees or immigrants from countries with high prevalence of TB (Eastern Europe, Africa and Middle East) gave O.D.s near the cut-off value or even false positive results. On the other hand, disaggregation by PPD status did not reveal a relationship between skin test reactivity and antibody titres against mycobacterial glycolipids (data not shown). The latter finding is in agreement with previous results.[18,19] Since elevated anti-glycolipid IgG concentrations in serum may persist for more than ten years after completion of anti-tuberculous chemotherapy,[4] the diagnostic value of corresponding ELISAs may be limited in patients with a history of TB and the role of these assays in monitoring compliance is questionable.

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