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Latex particles sensitized with proteins of *Leptospira interrogans* for application in immunoagglutination assays

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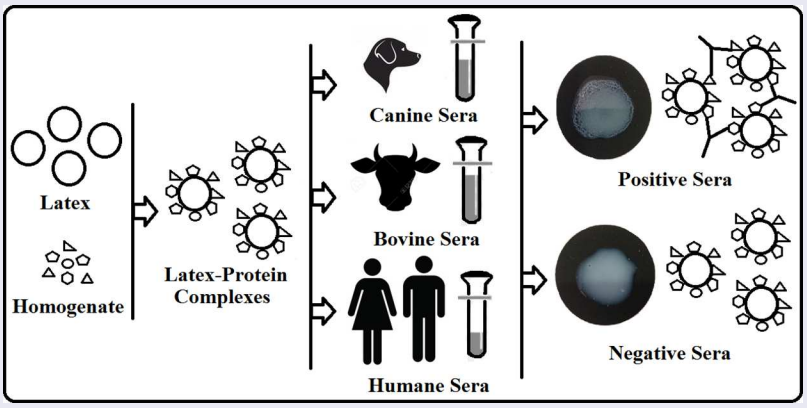
ABSTRACT

Physical adsorption and chemical coupling of recombinant proteins of *Leptospira interrogans* onto polystyrene and core-shell carboxylated particles were, respectively, investigated with the aim of producing latex–protein complexes to be used in immunoagglutination assays able to detect the leptospirosis disease in either humans or animals. To this effect, a protein lysate of crude extracts was evaluated, and sensitizations were carried out at different pHs, with the antigenic proteins approach to the particle surface favored at pH close to their isoelectric point. In the covalent coupling experiments, high fractions of proteins were chemically bound to carboxyl groups on the particle surface and higher densities of linked proteins were obtained for particles exhibiting greater carboxyl group densities. The produced latex–protein complexes were tested in immunoagglutination assays, by turbidimetry and a visual method, from a panel that included positive and negative bovine, canine, and human sera. The area under receiver operating characteristic curves was used as an index of accuracy. The complexes obtained by covalent coupling of proteins on the latex of higher density of carboxyl groups allowed an acceptable discrimination between the studied positive and negative sera.

GRAPHICAL ABSTRACT



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1. Introduction

Leptospirosis, caused by the bacterium *Leptospira interrogans*, is one of the most widespread and underdiagnosed zoonosis in the world, affecting humans and a wide range of animals [1,2]. Transmission occurs indirectly by contact with contaminated water or soil or by direct contact with urine from carriers [3]. Detection methods can be classified in direct methods (which demonstrate the presence of the causative agent or its genetic material) and indirect or serological methods [which detect the antibody (Ab) response generated by the host against infection]. The direct methods include culture, dark-field microscopy, inoculation of experimental animals, (immuno) staining, and different variants of polymerase chain reaction [3–7]. The main indirect detection methods used are the microscopic agglutination test (MAT) and the enzyme-linked immunoassay, which involve either lysates of crude extracts or recombinant antigens [2,3,8,9]. In recent years, various immunological techniques called "rapid detection tests" (RDT) for clinical diagnostics and detection of chemical and biological agents were developed [10–14]. Most of these RDT are based on particle agglutination principles [15].

The typical immunoagglutination assays (IA) are based on latex microspheres with Ag molecules bound to their surface (latex–protein complexes; LPC). An aqueous dispersion of these LPC is mixed with a sample containing Ab molecules from whole blood or serum. The Ab molecules normally bind

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Ag molecules situated on different micro/nanospheres and cause agglutination of latex particles. To produce LPC (process called sensitization), the biomolecules can either be physically or chemically fixed to the surface of the polymer particles. The covalent coupling of proteins on the particle surface, unlike physical adsorption, prevents the partial desorption of the bonded protein and maintains the native conformation of the protein [16]. Main advantages of latex agglutination test are its rapidness, simplicity, and low cost. Also, it does not require specific conjugates (i.e., it may be used in both different animal species and humans) and can be simply determined convenient determination by direct visual or instrumental methods [16–18].

Based on the problems observed in the diagnosis of leptospirosis and their implications in the treatment of the disease, latex particles were sensitized in this study by physical adsorption and covalent coupling of total bacteria homogenate of *L. interrogans*, with the aim of producing immunodiagnosis kits as a valuable tool for detecting leptospirosis. The reaction of agglutination was followed both by measuring the changes in the optical absorbance through turbidimetry (T) and by the visual method. The diagnostic test evaluation was realized on the basis of receiver operating characteristic (ROC) curves using bovine sera. Then, the complexes that produce the best discrimination between positive and negative sera were tested with canine and human control sera previously typified.

Table 1. Main characteristics of the latex particles.

	PS	C1	C2
D_{DLS}(nm)[a]	300	340	354
σ (μC cm^{-2})[b]	25.0	78.5	161.7
δ_{SO_4} Z(mEq cm^{-2})[c]	3×10^{-7}	2.4×10^{-7}	3.8×10^{-7}
δ_{COOH} (mEq cm^{-2})[d]	—	5.7×10^{-7}	13.0×10^{-7}
CCC_{vis} (mM KBr)[e]	250	650	>2000
CCC_{DLS} (mM KBr)[f]	100	250	>2000

[a]Mean particle diameter by dynamic light scattering, DLS.
[b]Surface charge density.
[c,d]Surface density of functional groups (sulfate and carboxyl).
[e,f]Critical coagulation concentration, by visual observation and by DLS.

2. Materials and methods

Latex particles previously produced in our laboratory were used. Polystyrene (PS) particles were synthesized by emulsion polymerization of styrene (St) in the presence of the MA-80 emulsifier. Particles with carboxyl functionality (C1 and C2) were obtained by emulsifier-free semibatch emulsion copolymerization of St and methacrylic acid onto a uniform PS latex seed [19]. Main characteristics of the latex particles are shown in Table 1 and Figure 1 [20].

The total bacteria homogenate (TBH) was obtained from a culture of *L. Interrogans* (Hardjoprajitno strain) and produced by the staff of the National Institute of Respiratory Diseases "Emilio Coni" of Santa Fe, Argentina. The culture was centrifuged 20 min at 10,000*g*, washed three times with phosphate-buffered saline pH 7.2, and resuspended in the same buffer. Finally, the culture was sonicated two times for 2 min (42 kHz, 100 W) to obtain the TBH. The isoelectric point (Ip) of TBH was determined by isoelectrofocusing analyses and it was in the range 5.0–5.4.

The following buffers were used: glycine (0.1M—pH 3); acetate (0.1M—pH 5); phosphate (0.1M—pH 5 and 7); borate (0.002M—pH 8); and carbonate (0.1M—pH 9).

The water solution of *N-N*-(3-dimethylamine propyl) *N'*-ethyl carbodiimide activator (EDC) was from Fluka. The emulsifier 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (Triton X-100), the bovine serum albumin (BSA), and the polyethylene glycol (PEG 8000) were all from Sigma.

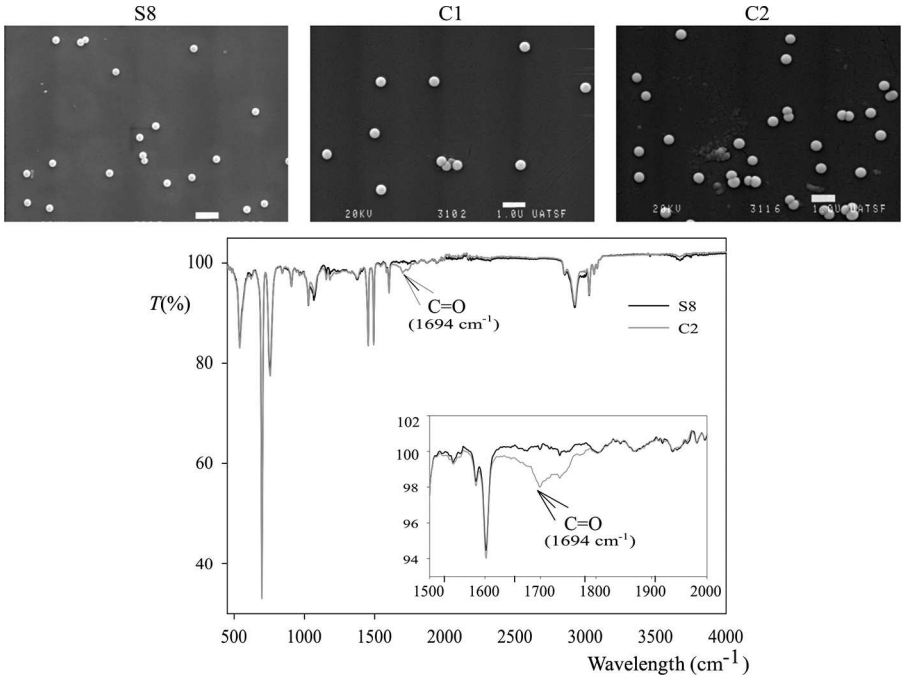

Figure 1. ■.

The concentrations of dissolved protein were determined through the copper reduction/bicinchoninic acid (BCA) method [21]. The BCA was from Pierce Reagents. Serum samples from *L. interrogans*-infected patients or animals and from noninfected ones were obtained from the National Institute of Respiratory Diseases "E. Coni." The serum samples were analyzed and classified by the reference technique MAT.

2.1. Latex–protein complexes

The LPC were obtained by physical adsorption (PA) and covalent coupling (CC) of the TBH onto PS and carboxylated particles (C1 and C2), respectively.

In PA experiments, antigenic proteins of increasing concentrations (0.1–4.0 mg mL^{-1}) were added to the latex samples (0.2 m^2 of latex surface) in 1.5 mL microcentrifuge tubes, and the mixtures were gently shaken during 5 h at room temperature. The LPC were isolated from the solution by ultracentrifugation during 30 min at 11,852*g*, and the concentration of dissolved protein was quantified by the BCA method. Finally, the LPC were redispersed in the borate buffer (pH 8). The adsorbed protein per unit surface ($\Gamma_{TBH,\ pa}$) was determined from the difference between the total added protein (C^{o}_{TBH}) and the protein remaining in solution ($C_{TBH,\ sol}$). The buffers used in the adsorption were glycine (pH 3), acetate (pH 5), phosphate (pH 7), and carbonate (pH 9). The final ionic strength was 0.002M. For each experiment, a blank without protein was prepared.

In CC experiments, increasing concentrations of antigenic proteins (0.3–1.2 mg mL^{-1}) were added to the latex samples (0.2 m^2) in the presence of EDC activator in 1.5 mL microcentrifuge tubes, and they were shaken during 5 h at room temperature. The LPC were isolated by ultracentrifugation during 30 min at 11,852*g* and the protein remaining in solution was quantified by the BCA method. The total linked protein (Γ_{TBH}) was determined from the difference between C^{o}_{TBH} and $C_{TBH,\ sol}$. The buffer used in the chemical coupling was phosphate at pH 5 and pH 7. The final ionic strength was 0.002M. For each experiment, a blank without protein was prepared.

After reactions, the physically adsorbed protein was desorbed by employing Triton X-100 1% emulsifier during 24 h under gentle agitation at room temperature. Finally, the resulting LPC were isolated by ultracentrifugation and redispersed in borate buffer at pH 8.0, and the concentration of desorbed protein was determined by the BCA method. The surface density of covalently bound protein ($\Gamma_{TBH,\ cov}$) was calculated from the total-linked protein and the desorbed protein that is present in solution.

2.2. Immunoagglutination assay

2.2.1. Determination of the optimal conditions

Positive (containing Abs) and negative (without Abs) bovine control serum samples were used. The following variables were analyzed: (i) reaction time; (ii) sensitization mechanism; and (iii) Ag concentration. The search of optimal conditions was carried out by varying only one variable at a time, and keeping all the other variables constant. The experiments were carried out at ambient temperature to simulate the field conditions in which the immunoagglutination reagents will be employed. The LPC were conditioned before the agglutination test. To this effect, complexes were first centrifuged and then redispersed in buffer borate (pH 8.0) with BSA, glycine, and PEG 8000, the so-called "immunoaglutination buffer," which was used in all the assays to work at a pH near the physiological value [22].

2.2.2. Instrumental method

Nine hundred and fifty microliters of the dispersion containing the LPC at a known concentration was mixed with 50 µL of the serum (positive or negative). The immunoagglutination was detected by T, measuring the optical absorbance (*A*) at 570 nm. The increment in *A* (ΔA) was determined by subtracting the absorbance of a blank (the complex without serum) to the absorbance measured for the (complex + serum) sample. The absence of changes in *A* of a blank, in the same dispersion medium where immunological reactions take place during the period of analysis, was indicative of "colloidal stability."

2.2.3. Test of the latex–TBH complex at optimal conditions

A panel of 20 positive and 20 negative bovine sera was assayed using the optimal conditions previously obtained. The values of ΔA were plotted through the scatter graphic software (GraphPad Prism) and used to build ROC curves by means of the graphic software MedCald. In general, area under the curve (AUC) values from 0.5 to 0.7 represent low accuracy and do not allow disease–health discrimination; AUC values from 0.7 to 0.8 represent acceptable ability of the test to discriminate positive from negative sera; and for AUC values ≥0.9 the test can discriminate between positive and negative sera [22–24]. Then, the complex that produced the largest AUC was tested with negative and positive canine and human control sera previously typified.

2.2.4. Visual method

On a slide of black background, 50 µL of LPC and 50 µL of positive serum (or the negative serum) were mixed, registering the time at which the process of agglutination was visualized.

3. Results and discussion

3.1. Latex–protein complexes

Figure 2 shows the adsorption isotherms obtained in PA experiments of the TBH onto the PS particles. In general, differences in adsorption isotherms for a given protein at different pH values are normally explained by variations of this net charge in the different media. Close to the Ip, the protein has zero net charge, both the intra- and intermolecular repulsions decrease, and the amount of adsorbed protein is maxima [17]. Such charge decrement is convenient, because it increases the stability of the adsorbed protein and it avoids the protein denaturalization [25,26]. At pH values different from the Ip, the protein becomes charged and its electrostatic charge may affect the adsorption process. Furthermore, with increasing net charge, proteins tend to undergo structural changes, which

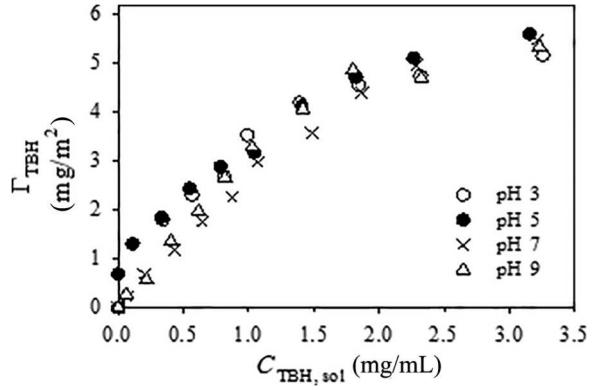

Figure 2. ■.

increase the surface area per molecule and decrease the adsorbed amount.

At pH values below the pI (pH 3), proteins have positive surface charge and this tend to favor the adsorption by electrostatic attraction to the latex negative surface. However, the surface charge of the protein increases the intermolecular repulsion, counteracting the attractive effect.

At pH values above the pI (pH 7 and 9), proteins have negative surface charge. In this case, not only the intra- and intermolecular repulsion are increased, but also the electrostatic repulsion between proteins and the particle surface.

Figure 2 shows that while the maximum amount of bound protein only varied slightly with pH, the protein affinity for the particle surface was greater for pH 3 and 5 (higher initial slopes of the isotherms). This may be because the TBH is a mixture of proteins of different sizes and amino acid compositions, and this produce a shielding of pH effect on the adsorption of proteins onto the particle surface.

The results corresponding to the CC experiments of the TBH onto the carboxylated particles C1 and C2 are presented in Table 2 and in Figure 3. Both the total-linked and the chemically coupled proteins increase with C^{o}_{TBH}, and high fractions of chemically bound proteins ($f_{CC,t}$) were observed. In all cases, the amount of linked protein increases with the concentration of the added protein. Nevertheless, the fraction of total linked and covalently bound proteins with respect to the added protein ($f_{CC,i}$) both decreased when increasing C^{o}_{TBH} (Table 2). Notice that, when the concentration of the added protein was low, a high percentage of protein was linked to the latex particles; and when the added protein concentration was increased, a higher protein fraction remained in solution. This could be due to the saturation of the particle surface by the protein.

It is known that the medium pH may influence the chemical coupling of proteins, and this effect was mainly observed in the $f_{CC,t}$ of protein onto latex C2. The highest density of covalently linked protein occurred at pH 5 (close to the protein Ip), where the covalently bound protein represented more than 95% of the total-linked protein (Table 2). At this pH, the repulsion between the particle surface and the protein was decreased, and the repulsion between neighboring molecules was also reduced. At pH 7 the protein is negatively charged, thus producing both the repulsion between neighboring biomolecules, and the repulsion between proteins and the particle surface that also presents negative charge (compare Figures 3b and 3d). However, the amount of bound protein to the latex C1 at different pH values was similar. This may be due to the lower density of carboxyl groups, which results in reduced electrostatic repulsion between biomolecules and the particle surface (Figures 3a and 3c).

Regarding the effect of the density of surface functional groups on the amount of bound protein, in general, a greater amount of protein covalently bound to latex C2 was observed, which exhibits higher density of carboxyl groups. This result could be explained by the increased amount of available functional groups on the particle surface, allowing a greater number of protein molecules to react with such groups.

Table 2. Covalent coupling of the TBH onto the carboxylated latex particles. Surface density of covalently bound protein ($\Gamma_{TBH,\,cov}$); percentages of covalently bond protein with respect to the total bound protein ($f_{CC,t}$); and percentages of covalently coupled protein with respect to the initially added protein ($f_{CC,i}$).

			Sample			
			1	2	3	4
		C^{o}_{TBH} (mg mL^{-1})	0.3	0.6	0.9	1.2
C1	pH 5	$\Gamma_{TBH,\,cov}$ (mg m^{-2})	1.75	2.43	3.34	3.85
		$f_{CC,t}$ (%)	100	94	99	98
		$f_{CC,i}$ (%)	84	59	54	46
	pH 7	$\Gamma_{TBH,\,cov}$ (mg m^{-2})	1.12	1.22	3.21	3.92
		$f_{CC,t}$ (%)	89	68	89	95
		$f_{CC,i}$ (%)	54	29	52	47
C2	pH 5	$\Gamma_{TBH,\,cov}$ (mg m^{-2})	1.98	3.22	4.40	5.60
		$f_{CC,t}$ (%)	95	95	97	97
		$f_{CC,i}$ (%)	95	77	70	67
	pH 7	$\Gamma_{TBH,\,cov}$ (mg m^{-2})	1.03	1.95	2.39	3.15
		$f_{CC,t}$ (%)	89	91	93	88
		$f_{CC,i}$ (%)	49	47	38	38

TBH, total bacteria homogenate.

3.2. Immunoagglutination assay

3.2.1. Determination of the optimal conditions

Reaction time. The reaction time (t) is defined as the time interval between the mixture of the serum with the LPC and the absorbance reading. Because the immunoagglutination

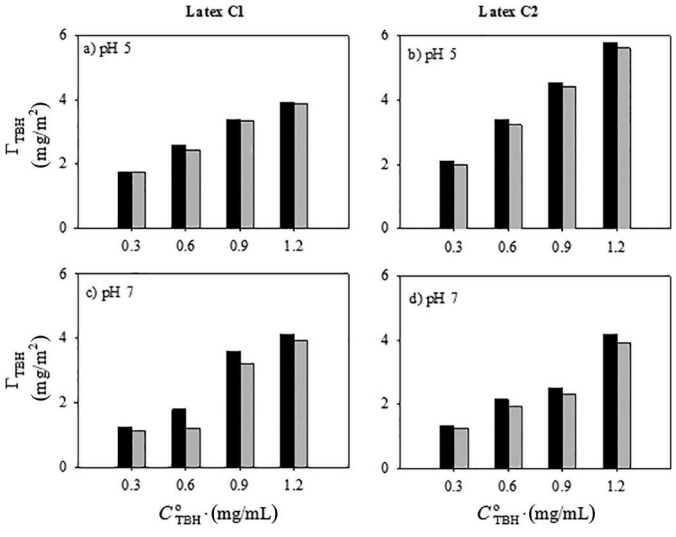

Figure 3. ■.

assay does not reach an end point, reaction time analysis is an important factor to consider when optimizing an assay. In a previous work [22] it was determined that at $t = 5$ min a good discrimination between positive and negative sera was obtained. When analyzing the evolution of ΔA during the first 5 min of reaction for the complexes and sera studied here, it was observed that the greater differentiation between positive and negative sera occurs at $t = 1$ min.

Influence of the sensitization mechanism. The effect of the sensitization mechanism was considered by comparing the results obtained in IA after 1 min of reaction when the Ag was physically adsorbed (PS-TBH) or covalently coupled (C1-TBH and C2-TBH) to the particle surface. Bovine control sera and LPC with similar amount of bound protein were used. Similar results were obtained with the three LPC, with a slight increase in the differentiation between positive and negative sera with the C2–TBH complex. This is probably due to the lower hydrophobicity of the particle resulting in a smaller number of nonspecific interactions. Also, it should be noted that the LPC obtained by physical adsorption have a limited applicability in immunodiagnosis because of the partial desorption of Ag that normally occurs during its storage [22].

Influence of the antigen concentration. To study the influence of the amount of the antigenic protein present on the particle surface in the IA, complexes with varying amounts of covalently bound protein were used. Although not shown there was a range of amount of bound protein (3–4 mg m^{-2}) that produced a greater differentiation between positive and negative sera. In the regions of Ag excess (5.6 mg m^{-2}) and Ab excess (1.98 mg m^{-2} of bound protein) the system seemed to lose reactivity because $\Delta A(+)/\Delta A(-)$ diminished. These results are consistent with those of the precipitine curve proposed by Heidelberger and Kendall [23]. When Ag is in great excess, all Abs are complexed to individual Ag molecules, so no aggregation occurs. Otherwise when Ab is in excess there is insufficient Ag to form an aggregate. This results in formation of small Ag–Ab complexes.

3.3. Test of the latex–TBH complex at optimal conditions with the instrumental method

The LPC obtained at pH 5 with a similar density of linked protein (3 mg m^{-2}) were employed. The complexes were tested on the basis of a panel of 20 negative bovine sera and 20 positive bovine sera previously typified, and its performance was evaluated through ROC curves (Figure 4).

It is observed that the C2–TBH complex allowed a greater differentiation between positive and negative sera, possibly due to the increased antigenicity of covalently coupled protein (higher mean value of the distribution of positive sera: mv = 0.50), and the greater hydrophilicity of the latex C2, which decreased the nonspecific interactions (low mean value of the distribution of negative sera: mv = 0.32). Considering the AUC values, the PS–TBH (AUC = 0.58) and C1–TBH (AUC = 0.67) complexes did not provide an adequate discrimination power between positive and negative sera. However, the TBH–C2 complex (AUC = 0.92) exhibited a good discrimination power (Figure 4).

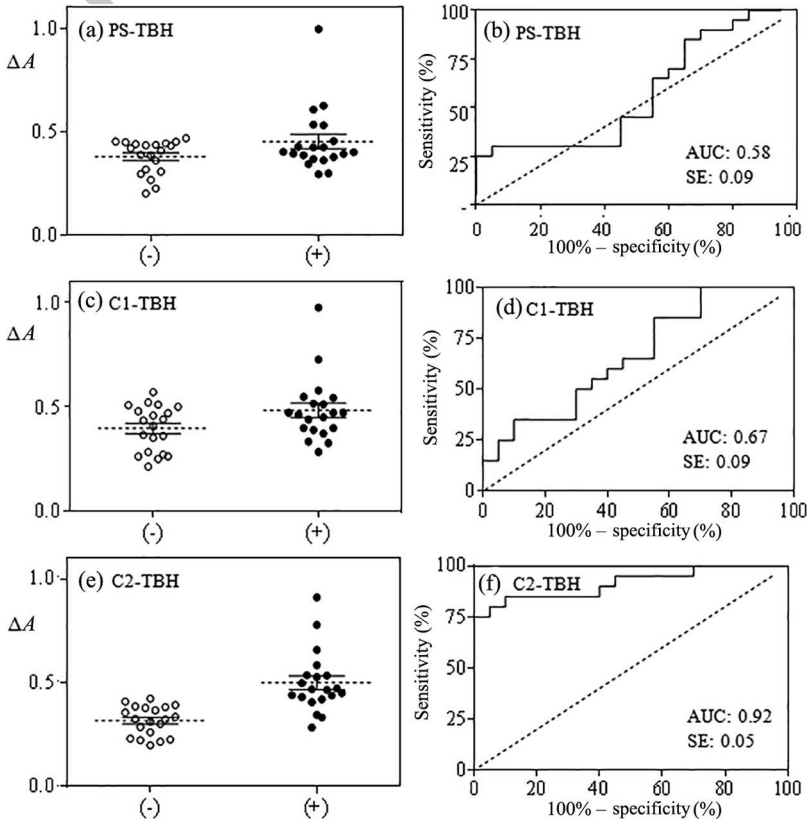

Figure 4. ■.

Finally, the C2–TBH complex was tested with negative and positive canine and human control sera previously typified. Figure 5 shows the results obtained from different species sera. The C2–TBH complex allowed a good discrimination between positive and negative sera in all cases, showing the IA another advantage, which is its ability for testing sera from different origins (e.g., bovine, canine, and human).

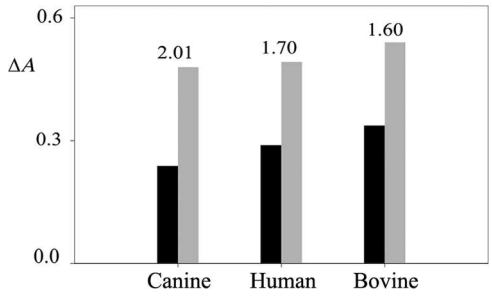

Figure 5. ■.

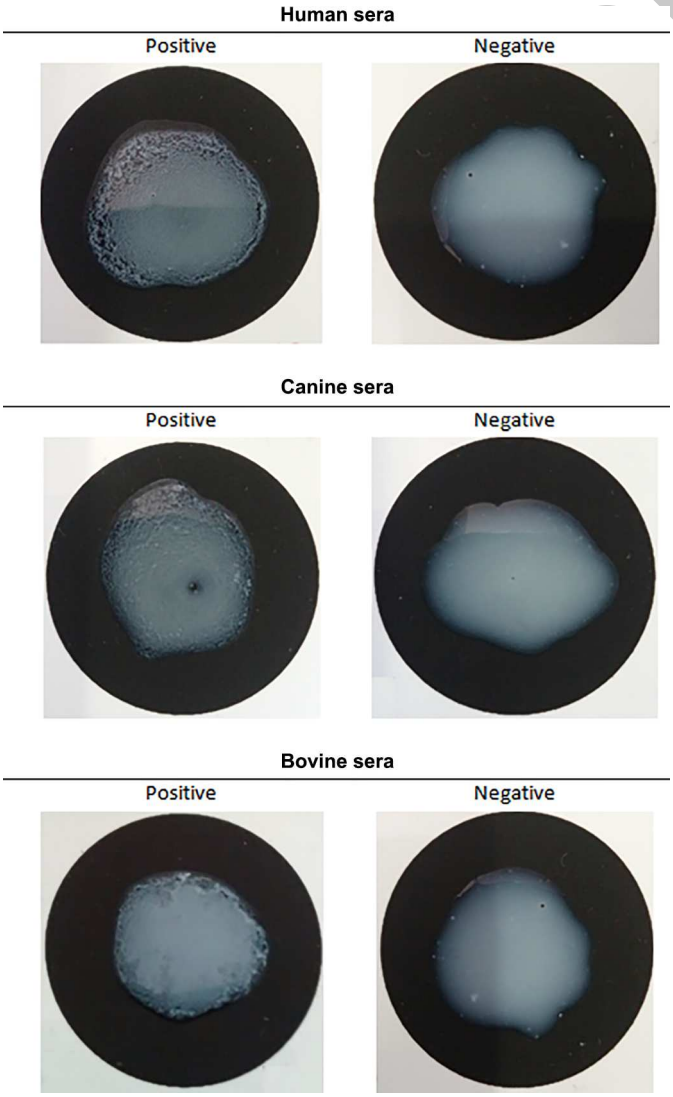

Figure 6. ■.

3.4. Test of the latex–TBH complex at optimal conditions with the visual method

Figure 6 shows photographs of the tests performed with negative and positive canine, human, and bovine control sera by the visual method. In all cases, a good discrimination between positive and negative sera was observed. The agglutination with the positive sera was visible from 60 to 80 s, and the difference between positive and negative sera could be observed for about 10 min.

4. Conclusion

Latex–protein complexes were obtained by physical adsorption and covalent coupling of the total homogenate of *L. interrogans* onto latex particles.

The approach of the protein to the surface of the latex particles was favored at a pH close to the protein Ip. In these conditions the intra- and intermolecular repulsions and also the electrostatic repulsion between proteins and the surface of the latex particles decrease. In CC experiments, high levels of covalent coupling of the antigenic proteins on the carboxylated particle surface were observed. The highest density of linked protein occurred onto latex C2, with greater density of carboxyl groups.

The IA conditions to detect leptospirosis, based on latex–TBH complexes, were studied using bovine serum. A reaction time of 1 min proved to be adequate for the immunoassays, and the best discrimination between positive and negative sera was reached with LPC obtained by CC. Complexes with a density of bound protein of about 3 mg m^{-2} produced the highest discrimination between positive and negative sera.

In the IA against a panel of bovine sera, only the C2–TBH complex allowed a good discrimination between positive and negative sera (AUC = 0.92). This is possibly due to the increased antigenicity of covalently coupled protein, and the greater hydrophilicity of the latex C2, which decreased the nonspecific interactions. In addition, the IA performed for a small number of human sera also showed a better performance of the C2–TBH complex.

Under the obtained optimal conditions, leptospirosis positive human, canine, and bovine sera and negative sera were tested by both instrumental and visual methods. It was observed that the negative sera response was clearly different from that of the leptospirosis positive sera, and was noticed that the same complex allowed the diagnosis from different species sera.

Finally, the immunoagglutination tests based on the complexes obtained from carboxylated latexes and TBH could be evaluated as a screening method for detecting leptospirosis disease. But, even though this test is rapid, easy to implement, and could be used under field conditions, its results should be confirmed by reference techniques.

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