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A bacterial engineered glycoprotein as a novel antigen for diagnosis of bovine brucellosis

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ABSTRACT

Brucellosis is a highly contagious zoonosis that affects livestock and human beings. Laboratory diagnosis of bovine brucellosis mainly relies on serological diagnosis using serum and/or milk samples. Although there are several serological tests with different diagnostic performance and capacity to differentiate vaccinated from infected animals, there is still no standardized reference antigen for the disease. Here we validate the first recombinant glycoprotein antigen, an *N*-formylperosamine *O*-polysaccharide-protein conjugate (OAg-AcrA), for diagnosis of bovine brucellosis. This antigen can be produced in homogeneous batches without the need of culturing pathogenic brucellae; all characteristics that make it appropriate for standardization. An indirect immunoassay based on the detection of anti *O*-polysaccharide IgG antibodies in bovine samples was developed coupling OAg-AcrA to magnetic beads or ELISA plates. As a proof of concept and to validate the antigen, we analyzed serum, whole blood and milk samples obtained from non-infected, experimentally infected and vaccinated animals included in a vaccination/infection trial performed in our laboratory as well as more than 1000 serum and milk samples obtained from naturally infected and S19-vaccinated animals from Argentina. Our results demonstrate that OAg-AcrA-based assays are highly accurate for diagnosis of bovine brucellosis, even in vaccinated herds, using different types of samples and in different platforms. We propose this novel recombinant glycoprotein as an antigen suitable for the development of new standard immunological tests for screening and confirmatory diagnosis of bovine brucellosis in regions or countries with brucellosis-control programs.

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1. Introduction

Brucellosis is a highly contagious zoonosis caused by Gram-negative bacteria of the genus *Brucella* that affects livestock, wild animal species and humans. The main pathogenic species worldwide are *B. abortus*, responsible for bovine brucellosis; *B. melitensis*, the major etiologic agent of ovine and caprine brucellosis; and *B. suis*, responsible for swine brucellosis (Corbel, 1997). These three *Brucella* species remain an important cause of veterinary morbidity and mortality. Brucellosis causes important economic losses not only because it affects animal production (reduced milk, abortion, delayed conception and impaired fertility) but also because detection of the disease in a region or country imposes, due to international veterinary regulations, restrictions on animal movements and trade (Seleem et al., 2010). Brucellosis in humans can be severely debilitating and disabling and remains an important public health concern (Young, 1995). In the absence of a human brucellosis vaccine, prevention of the disease depends predominantly on control of brucellosis in animals that constitute the natural disease's reservoirs (Godfroid et al., 2010). For these reasons, many programs have been implemented worldwide to control and/or eradicate brucellosis mainly in cattle, small ruminants, and pigs.

Control of bovine brucellosis depends on vaccination and/or detection of infected animals and slaughter. Due to the lack of pathognomonic signs for the clinical diagnosis of brucellosis in animals, laboratory confirmation of bovine brucellosis by isolation of the pathogen or demonstration of specific antibodies is essential. Bacteriological isolation of the microorganism confirms the diagnosis; however, slow growth of brucellae primary cultures (up to 7 days), the risk involved in their handling and poor sensitivity makes diagnosis based exclusively on isolation of brucellae not always feasible and effective. Therefore, laboratory diagnosis mainly relies on serological diagnosis using serum and/or milk samples (Godfroid et al., 2010; Saegerman et al., 2010).

The most commonly used serological tests for diagnosis of bovine brucellosis are the agglutination tests such as the buffered plate agglutination test (BPAT), rose Bengal plate agglutination test (RBPT) and serum agglutination test (SAT), and the complement fixation test (CFT) (Aznar et al., 2012; DILAB-SENASA, 2009; Godfroid et al., 2010; OIE, 2012). Indirect enzyme-linked immunosorbent assays (iELISA) have also been developed for serum and m. For analyzing individual and bulk tank milk samples the milk ring test (MRT) is the most widely used test (Gall and Nielsen, 2004). All these assays use as antigen the whole bacteria, bacterial extracts containing high concentrations of smooth lipopolysaccharide (sLPS) or purified sLPS. These tests suffer from false positive reactions and, because S19 vaccine is antigenically similar to virulent strains, does not allow a precise differentiation of vaccinated from infected animals. Consequently, other tests have been developed including the competitive ELISA (CELISA) and the fluorescence polarization assay (FPA), which have eliminated most reactions due to cross-reacting antibodies and residual antibodies produced in response to S19 vaccination (McGiven et al., 2003; Nielsen, 1990; Nielsen et al., 1995, 1996). Both assays measure specific antibodies against the immunodominant *O*-polysaccharide section of the LPS (Caroff et al., 1984b). Additionally, a common problem to all serological tests currently used for diagnosis of brucellosis is the lack of a standardized reference antigen, and one of the main reasons for this is the source of the antigens as well as the preparation methods used (Al Dahouk et al., 2003). Therefore, for harmonization of the serological diagnosis of brucellosis there is a need to develop a new reference antigen easy to standardize.

Previously, we have produced and characterized a recombinant glycoprotein consisting of a homopolymer of *N*-formylperosamine, the *O*-polysaccharide of *B. abortus* (OAg) covalently linked to the carrier protein AcrA (hereinafter OAg-AcrA) (Iwashkiw et al., 2012). OAg-AcrA was produced using an in vivo engineered bacterial glycosylation system based on the combination of the LPS biosynthesis pathway of *Yersinia enterocolitica O:9*, whose O-polysaccharide is identical to the *B. abortus* OAg, and the *N*-glycosylation pathway of *Campylobacter jejuni* (Feldman et al., 2005). OAg-AcrA was applied to the development and validation of a new indirect immunoassay for diagnosis of human brucellosis using magnetic beads (Ciocchini et al., 2013). We have demonstrated that the assay has an excellent diagnostic performance allowing the detection of infection caused by the three main human brucellosis agents; *B. abortus*, *B. melitensis* and *B. suis.*

Here we validate for the first time a recombinant glycoprotein antigen for diagnosis of bovine brucellosis using serum, whole blood and milk samples under two different platforms. Our results demonstrate that OAg-AcrA is a new standardizable antigen that allows the development of highly accurate glycoprotein-based immunodiagnostics for bovine brucellosis.

2. Materials and methods

2.1. Production and purification of the O:9-polysaccharide-protein conjugate (OAg-AcrA)

Production and purification of the recombinant glycoprotein OAg-AcrA was performed as previously described (Iwashkiw et al., 2012). *Y. enterocolitica O:9* wild type strain transformed with the plasmids pMAF10 (encoding the *C. jejuni* oligosaccharyltransferase OTase PglB) and pMH5 (encoding the *C. jejuni* carrier protein AcrA fused to an histidine tag) was grown overnight at 37 °C in LB media, grown at 37 °C for 2.5 h ($OD_{600} \sim 0.5$) and PglBCj expression was induced with arabinose 0.2% (w/v). Four hours after induction at 37 °C, PglBCj was re-induced by a second addition of arabinose to maximize glycosylation of AcrA. Cells were harvested by centrifugation after 20 h of induction and periplasmic extracts were prepared by lysozyme treatment as described in Feldman et al. (2005) and subjected to Ni^{2+} affinity chromatography.

2.2. OAg-AcrA magnetic beads-based immunoassay (glyco-beads assay) development and optimization

Superparamagnetic COOH-modified microbeads (Bangs Laboratories, Inc.) were activated in one step

with EDAC [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride] and NHS [N-hydroxy succinimide] in 100 mM MES (2-[N-Morpholino] ethanesulfonic acid) pH 5.5 buffer. Activated beads were washed with 0.01 M phosphate-buffered saline pH 7.2 (PBS) and incubated with different amounts of OAg-AcrA. The resulting functionalized magnetic beads were washed with quenching buffer (35 mM glycine, 1% gelatin from cold water fish skin) and incubated overnight at 4 °C with the same buffer. Finally, OAg-AcrA-beads were washed with storage buffer (1% gelatin from cold water fish skin, 2.25% Tween 20, 0.01% sodium azide), resuspended in the same buffer and stored at 4 °C until use.

To perform the assay, OAg-AcrA-functionalized microbeads were incubated with serum, whole blood or milk samples diluted in PBS containing 0.1% Tween 20 (PBST-0.1%), washed two times with PBS containing 0.2% Tween 20 (PBST-0.2%) and bound antibodies were detected incubating the beads with Cy5-conjugated goat anti bovine IgG antibodies (Jackson ImmunoResearch Laboratories). After washing twice with PBST-0.2%, fluorescence was determined using a plate fluorometer (DTX 880 Multimode Detector, Beckman Coulter, Inc.). All washes were done using a magnetic rack. To determine the optimal antigen concentration, sample and antibody dilutions and incubation times, a checkerboard titration analysis was carried out using high-, medium-, low-positive and negative serum and milk samples, and checking for strong vs. low background. The optimal amount of antigen was 2 μg per reaction and the optimal sample dilutions were 1/100 and 1/10 for serum and milk samples, respectively. The incubation time with the samples and the conjugate was reduced to 5 min at room temperature.

2.3. OAg-AcrA indirect ELISA (glyco-iELISA) development and optimization

Microtiter plates (Corning® #3590) were coated with 50 μl of OAg-AcrA at 2.5 μg/ml. The antigen was diluted in 0.05 M carbonate buffer, pH 9.6 and incubated for 18 h at 4 °C. The plates were washed three times in PBST-0.1% and blocked with 5% bovine skim milk in PBST-0.1% (blocking buffer) for 1 h at 37 °C. Serum and milk samples were diluted in blocking buffer and PBST-0.1% respectively, added in duplicate and incubated for 1 h at 37 °C. Positive- and negative-control samples were included in each plate. Subsequently, horseradish peroxidase-labeled goat anti-bovine IgG (H + L, Jackson ImmunoResearch Laboratories) antibodies diluted in blocking buffer was added and incubated for 1 h at 37 °C. Between each reaction step, the plates were washed three times with PBST-0.2% to remove excess reagents. After adding the substrate (0.36% H_2O_2 and 0.01% 3,3′,5,5′-tetramethylbenzidine [TMB] in 0.1 M citric acid, pH 4.2) the plates were incubated for 15 min at room temperature, the reaction was stopped with 2 N H_2SO_4 and the absorbance was determined at 405 nanometers using a plate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc.). Optimization of the assay was established in preliminary experiments through a checkerboard titration analysis using high-, medium-, low-positive and negative serum and milk samples, and checking for strong vs. low background. The optimal antigen concentration was 2.5 μg/ml (125 ng/well), and the optimal sample dilutions were 1/100 and 1/10 for serum and milk samples, respectively.

2.4. Other tests

The buffered plate agglutination test (BPAT), competitive ELISA (CELISA), fluorescence polarization assay (FPA), complement fixation test (CFT) and milk ring test (MRT) were performed by the National Brucellosis Reference Laboratory (DILAB-SENASA) from Argentina as previously described (DILAB-SENASA, 2009; OIE, 2012). For the BPAT, any degree of agglutination was considered positive. An agglutination titer of ≥8 (dilution 1/8) for the CFT was considered a positive result. For the CELISA, a percentage of inhibition ≥40% was considered positive, and for FPA a reactivity value ≤94 millipolarization units (mP) was considered negative, between 95 and 105 mP indeterminate and >105 mP positive. For the MRT, any blue layer at the interface of milk and cream was considered positive. The commercial indirect ELISA SVANOVIR® Brucella-Ab I-ELISA (Boehringer Ingelheim Svanova) was performed following the manufacturer's instructions. Results were expressed as percent positivity (PP) relative to the positive control. Serum samples were analyzed at 1/25 dilution and milk samples were tested neat. The antigen used in this assay is a heat inactivated phenol treated whole cell lysate of *B. abortus* strain 99. Cut-off values determined by the manufacturer are ≥40% and ≥10% for serum and milk samples, respectively.

2.5. Bovine samples

To validate the OAg-AcrA antigen for diagnosis of bovine brucellosis two different sample panels were analyzed. *Panel I.* A total of 102 serum samples were obtained from three different groups of cattle included in a controlled vaccination/challenge trial performed in our laboratory. Group 1 includes non-vaccinated pregnant heifers experimentally infected with the wild-type strain *B. abortus* 2308 (nine animals, 18 samples); pre- and post-infection serum samples were obtained. Group 2 consists of S19-vaccinated pregnant heifers challenged with *B. abortus* 2308 (nine animals, 54 samples). Samples were obtained pre-vaccination, at different times post-vaccination and post-challenge. Group 3 consists of pregnant heifers vaccinated twice 16 months apart with the rough vaccine strain *B. abortus* Δ*pgm* (Ugalde et al., 2000, 2003) and challenged with *B. abortus* 2308 (five animals, 30 samples). Serum samples were obtained pre-vaccination, at different times post first and second vaccination and post-challenge with the wild-type strain. *B. abortus* Δ*pgm* is a live attenuated strain lacking the phosphoglucomutase gene (*pgm*) (Ugalde et al., 2000). This strain synthesizes an *O*-polysaccharide with an approximate size of 45 kDa but it is unable to assemble the *O*-side chain into the complete LPS, in consequence is a rough-phenotype strain (Ugalde et al., 2003). *Panel II.* A total of 774 serum and 302 milk samples obtained from naturally infected and non-infected/S19-vaccinated bovines from Argentina were

provided by the National Brucellosis Reference Laboratory (DILAB-SENASA). Serum samples: the sera of 667 S19-vaccinated animals from officially certified brucellosis-free herds were used as negative reference samples. These samples were serologically negative by the standard screening test BPAT or were BPAT-positive but negative by CELISA, FPA and CFT. The sera of 107 animals positive by BPAT and confirmed by at least two positive results of CFT, CELISA and/or FPA were used as positive reference samples. These samples were obtained from brucellosis-positive herds confirmed by serology and bacteriology. Milk samples: serologically negative milk samples obtained from 208 S19-vaccinated cows from officially certified brucellosis-free dairy farms were used as negative reference samples. Milk samples of 94 cows positive by MRT and serologically positive by BPAT, CELISA, FPA and CFT were considered as positive reference samples. These samples were obtained from brucellosis-positive dairy herds confirmed by serology and bacteriology.

Positive and negative control sera included in each assay run. The positive control serum was obtained from a non-vaccinated animal, coming from a certified free-brucellosis herd, experimentally infected with the wild-type strain *B. abortus* 2308 (serological tests results: BPAT, positive; CELISA, 100%; FPA, 287 mP; CFT, 1/8). The negative control serum was obtained from a non-infected/non-vaccinated animal (serological tests results: BPAT, negative; CELISA, 21%; FPA, 49 mP; CFT, negative) coming from a certified free-brucellosis herd. The same controls were used for serum and milk assays.

OIE ELISA and SENASA standard sera. The strong positive, weak positive and negative OIE_ELISA (international reference standards; OIE_ELISA$_{SP}$SS, OIE_ELISA$_{WP}$SS and OIE_ELISA$_{NEG}$SS) and SENASA (national reference standards; SENASA$_{SP}$SS, SENASA$_{WP}$SS and SENASA$_{NEG}$SS) standard sera were provided by DILAB-SENASA of Argentina. The reactivity of the OIE_ELISA$_{SP}$SS should represent a point on the linear portion of a typical dose–response curve just below the plateau. The OIE_ELISA$_{WP}$SS should consistently give a positive reaction just above the cut-off, and the OIE_ELISA$_{NEG}$SS should give reactions that are always below the cut-off (OIE, 2012).

2.6. Western blotting

The carrier protein AcrA in its non-glycosylated form and the glycoprotein OAg-AcrA were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed with bovine serum samples at 1/500 dilution in blocking buffer (5% bovine skim milk in PBST-0.1%). Bound antibodies were visualized using horseradish peroxidase-labeled goat anti-bovine IgG (H + L, Jackson ImmunoResearch Laboratories) secondary antibodies and enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce Chemical Co.), according to the manufacturer's instructions.

2.7. Data analysis

Glyco-beads and glyco-iELISA assays. Results were expressed as percentage of reactivity of the mean fluorescence units (FU) or absorbance at 450 nanometers (Abs_{450}) of the positive control serum included in each assay run. Percentage of reactivity was calculated as follows: % of reactivity = (FU or Abs_{450} of the test sample/mean FU or Abs_{450} of the positive control) × 100. Dot, receiver-operating characteristic (ROC) (Greiner and Gardner, 2000; Swets, 1988), two-graph ROC (TG-ROC, plot of the diagnostic sensitivity and specificity of the assay as a function of the cut-off values) (Greiner et al., 1995) and Mann–Whitney test analyses were performed using the GraphPad Prism software (version 5.01 for Windows, San Diego California USA, [http://www.graphpad.com]). Comparison of areas under the ROC curves (AUC/s) was performed using the MedCalc Statistical Software version 13.0.6 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) (Hanley and McNeil, 1983). The compared two AUC/s were considered significantly different when the P value was less than 0.05 ($P < 0.05$). Repeatability of the assays was evaluated considering the reactivity values of the positive and negative controls included in each assay run and calculating the variation coefficient (CV) for n repetitions of the assays ($n = 10$). In all the assays (glyco-beads and glyco-iELISA using serum and milk samples) the % CV was <20% (CV < 20%).

3. Results

3.1. OAg-AcrA glycoprotein as a novel antigen for diagnosis of bovine brucellosis

As a proof of concept, and to demonstrate the usefulness of OAg-AcrA glycoprotein for diagnosis of bovine brucellosis, serum, whole blood and milk samples obtained from three different groups of animals included in a controlled vaccination/challenge trial performed in our laboratory (manuscript in preparation) were analyzed. The first group were non-vaccinated pregnant heifers infected with the wild-type strain *B. abortus* 2308. The second group were pregnant heifers vaccinated with the smooth-phenotype strain *B. abortus* S19 and challenged with the wild-type strain *B. abortus* 2308. The third group included pregnant heifers that were twice vaccinated with the rough strain *B. abortus* Δ*pgm* and then challenged with the wild-type strain *B. abortus* 2308.

An indirect immunoassay based on the detection of anti *O*-polysaccharide IgG antibodies was developed using as antigen the recombinant glycoprotein OAg-AcrA as previously described (Iwashkiw et al., 2012). To perform the assay, super-paramagnetic beads functionalized with the antigen were incubated with the samples and detection was performed directly by reading the fluorescence after incubating the beads with Cy5-conjugated anti-bovine IgG antibodies (hereinafter glyco-beads assay). Analysis of serum samples obtained pre-infection and 180 days post-infection with the wild-type strain *B. abortus* 2308 indicated that using OAg-AcrA antigen it is possible to clearly differentiate infected from non-infected animals (Fig. 1A). Similar results were observed when whole blood samples, also obtained at 180 days post-infection from the same group of animals, were analyzed (Fig. 2A). To determine the performance of this assay on vaccinated

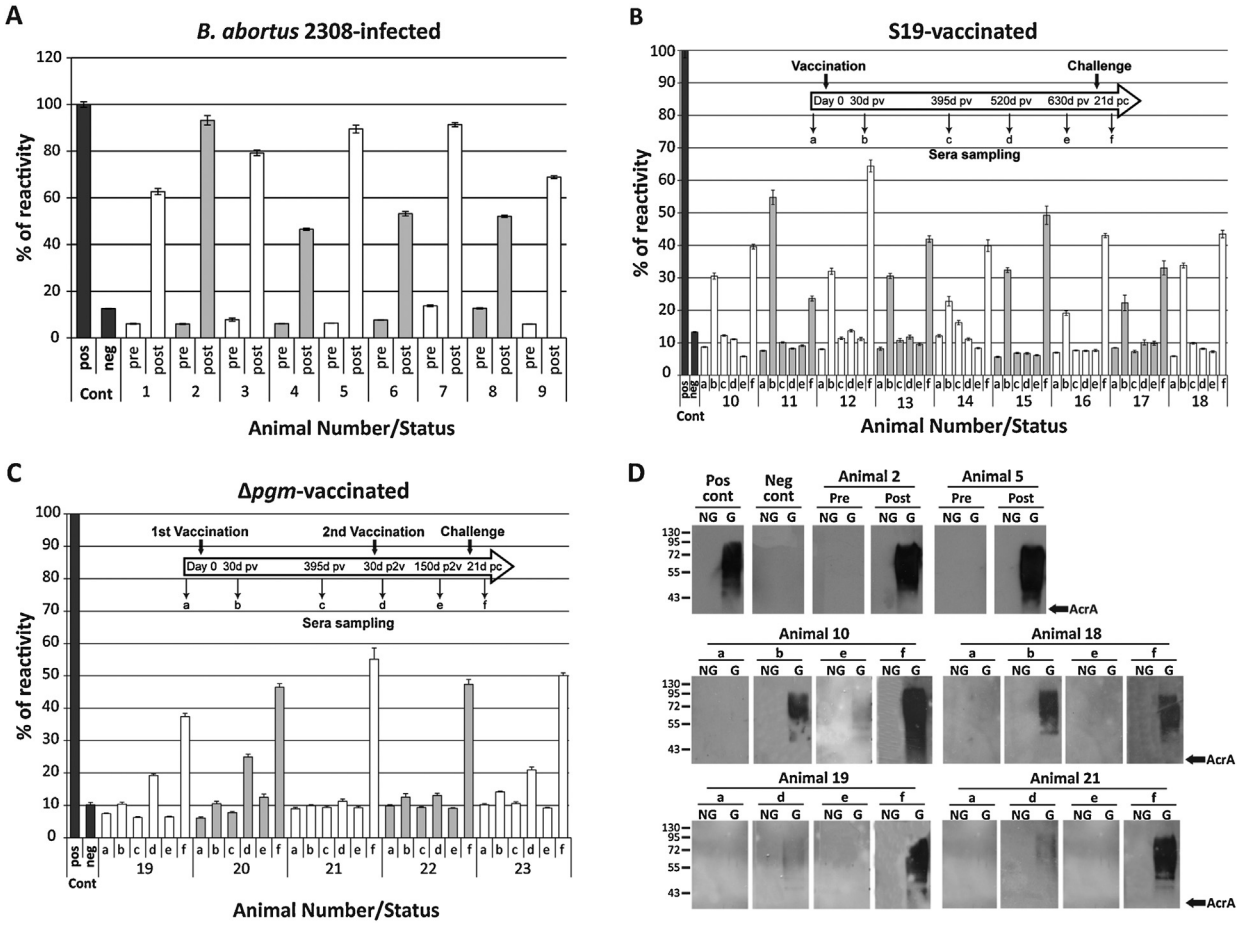

Fig. 1. Analysis of serum samples obtained from animals included in the controlled vaccination/challenge trial. (A) Glyco-beads assay analysis of serum samples obtained from bovines infected with the wild-type strain *B. abortus* 2308. Pre-infection (pre) and 180 days post-infection (post) samples were analyzed. Results are expressed as percentage of reactivity of the positive control serum. The bar graph data represents the means and standard deviation for two separate determinations. (B) Glyco-beads assay analysis of serum samples obtained from S19-vaccinated animals and challenged with the wild-type strain *B. abortus* 2308. Serum samples obtained pre-vaccination (a), at different times post-vaccination (b–e) and 21 days post-challenge (f) were analyzed. (C) Glyco-beads analysis of serum samples obtained from animals vaccinated with the rough strain *B. abortus* Δ*pgm* and challenged with *B. abortus* 2308. Serum samples obtained pre-vaccination (a), at different times post first (b and c) and second vaccination (d and e), and 21 days post-challenge (f) were analyzed. (D) Western Blot analysis of the indicated serum samples. NG, non-glycosylated AcrA; G, glycosylated AcrA (Oag-AcrA). The position of molecular mass standards (in kDa) is indicated on the left. The arrows on the right indicate the migration position of non-glycosylated AcrA. Pos and Neg cont; bovine sera included as positive and negative controls in each assay run (see Section 2).

animals, we analyzed serum samples obtained from experimentally vaccinated animals with the smooth vaccine strain S19 (Fig. 1B). Six samples were analyzed for each animal including a pre-vaccination sample, four samples obtained at different times post-vaccination and a sample obtained at 21 days post-challenge with *B. abortus* 2308. In all the analyzed animals, we were able to detect an increase in the reactivity against the antigen at 30 days post-vaccination that decreased to values similar to the negative control at 395 days post-vaccination (Fig. 1B). As it was expected, the reactivity increased post-challenge with the smooth wild-type strain. These results demonstrate that with this assay it is possible to differentiate S19-vaccinated from infected animals at least after 395 days post-vaccination. Furthermore, in this group of animals we analyzed milk samples that were taken post-delivery or post-abortion (between 60 and 90 days post-challenge), and in all of them reactivity values were significantly higher than those obtained with the serum and milk negative controls (Fig. 2B). In order to determine if this assay interferes with the diagnosis of animals vaccinated with a rough *Brucella* strain, we analyzed serum samples obtained from experimentally Δ*pgm* vaccinated animals (Fig. 1C). Unlike to S19-vaccinated group, no reactivity was observed after first vaccination (indeed at 30 days post-vaccination); this result is coherent with the rough phenotype of the Δ*pgm* strain. However, in three of the five animals tested, the reactivity increased at 30 days post second vaccination decreasing to negative levels at 150 days post second vaccination. This behavior could be explained by the fact that Δ*pgm*, although a rough strain, is able to synthesize a 45 kDa *O*-polysaccharide that is not assemble into the complete LPS and remains cytoplasmic (Ugalde et al., 2003). As in the other groups, the reactivity raised to positive levels after challenge with the smooth virulent strain. These results indicate that with OAg-AcrA

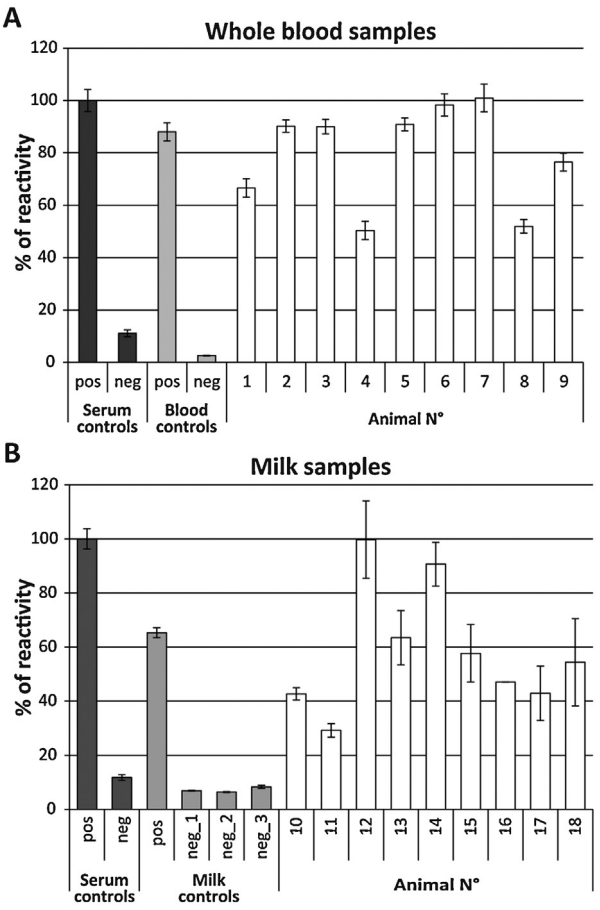

Fig. 2. Glyco-beads assay analysis of whole blood and milk samples obtained from animals included in the vaccination/challenge trial. (A) Analysis of whole blood samples obtained 180 days post-infection with the wild-type strain *B. abortus* 2308. Dilution tested, 1/250. (B) Analysis of milk samples obtained from S19-vaccinated animals and challenged with the wild-type strain *B. abortus* 2308. Milk samples were collected post-delivery or post-abortion between 60 and 90 days post-challenge. Dilution tested, 1/10. Results are expressed as percentage of reactivity of the positive control serum. The bar graph data represents the means and standard deviation for two separate determinations. Serum controls; bovine sera included as positive and negative controls in each assay run (see Section 2). Positive blood and milk control samples were obtained from animals naturally infected with *B. abortus*. Negative blood and milk control samples were sampled from an officially certified brucellosis-free animal.

antigen it is possible to differentiate animals vaccinated with a rough-phenotype strain Δ*pgm* from infected ones even at 30 days post first vaccination or 150 days after second vaccination.

To evaluate the specificity of the reaction, all serum samples were analyzed by Western Blot against the non-glycosylated and glycosylated forms of AcrA (results of two animals are shown as representative of the entire group). As can be observed in Fig. 1D, none of the sera reacted against the non-glycosylated form of AcrA indicating that the detected IgG response was directed specifically toward the carbohydrate moiety of the antigen.

Finally, all the serum samples were analyzed with different serological tests commonly used for screening and confirmatory diagnosis of bovine brucellosis such as BPAT, CELISA, FPA and CFT (Table S1) and the results were in agreement with those obtained by the glyco-beads assay.

Supplementary Table S1 can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.04.014.

Taken together, these results demonstrate the potential of the recombinant glycoprotein OAg-AcrA for diagnosis of bovine brucellosis allowing to differentiate infected from non-infected animals as well as vaccinated from natural infected ones. Moreover, OAg-AcrA antigen could be used to diagnose the disease using whole blood and milk samples, making it suitable for the development of diagnostic tests for field use.

3.2. OAg-AcrA antigen validation using serum samples

To validate this novel antigen, a total of 774 serum samples obtained from S19-vaccinated animals of certified brucellosis free herds (NEG, negative reference samples) and confirmed brucellosis-positive animals (POS, positive reference samples) were tested using the glyco-beads assay as well as immobilizing OAg-AcrA antigen to microtiter plates (glyco-iELISA; see Section 2). The NEG group included samples that were serologically negative by the standard screening test BPAT or were BPAT positive but negative by CELISA, FPA and CFT (NEG_BPAT+). In addition, all the samples were evaluated in parallel with a commercial iELISA that uses as antigen a whole cell lysate of *B. abortus* strain 99. The reactivity values obtained with the glyco-beads, glyco-iELISA and commercial iELISA assays are outlined in the dotplot diagram shown in Fig. 3A. For the NEG and POS samples, the reactivity values of the glyco-beads and glyco-iELISA assays showed a minimal overlap between the two sets of samples. Instead, a greater overlap among NEG, NEG_BPAT+ and POS samples was observed with the commercial iELISA. These results indicate that using the OAg-AcrA antigen immobilized to different platforms it is possible to discriminate between brucellosis-positive animals and non-infected/S19-vaccinated ones. Using the glyco-based assays, unlike to what was observed with the commercial iELISA, animals with a BPAT positive result but negative by CELISA, FPA and CFT were clearly distinguished from confirmed positive ones (Fig. 3A).

To evaluate the diagnostic performance of OAg-AcrA-based assays a receiver-operating characteristic (ROC) analysis was performed. ROC analysis was carried out considering as negative reference samples sera obtained from S19-vaccinated animals from herds without history of brucellosis since S19 strain is the most widely used vaccine in control programs in Argentina (Aznar et al., 2012). Based on the ROC results, the area under the ROC curve (AUC) values were 0.9998 (95% CI, 0.9994–1.000) and 0.9994 (95% CI, 0.9987–1.000) for the glyco-beads and glyco-iELISA assays, respectively, indicating that these tests are highly accurate for the diagnosis of bovine brucellosis. The AUC was significantly lower for the commercial iELISA (glyco-beads vs. commercial, $P = 0.0021$; glyco-iELISA vs. commercial, $P = 0.0144$), reaching a value of 0.9941 (95% CI, 0.9899–0.9982). Furthermore, a plot of the diagnostic Se and Sp as a function of the

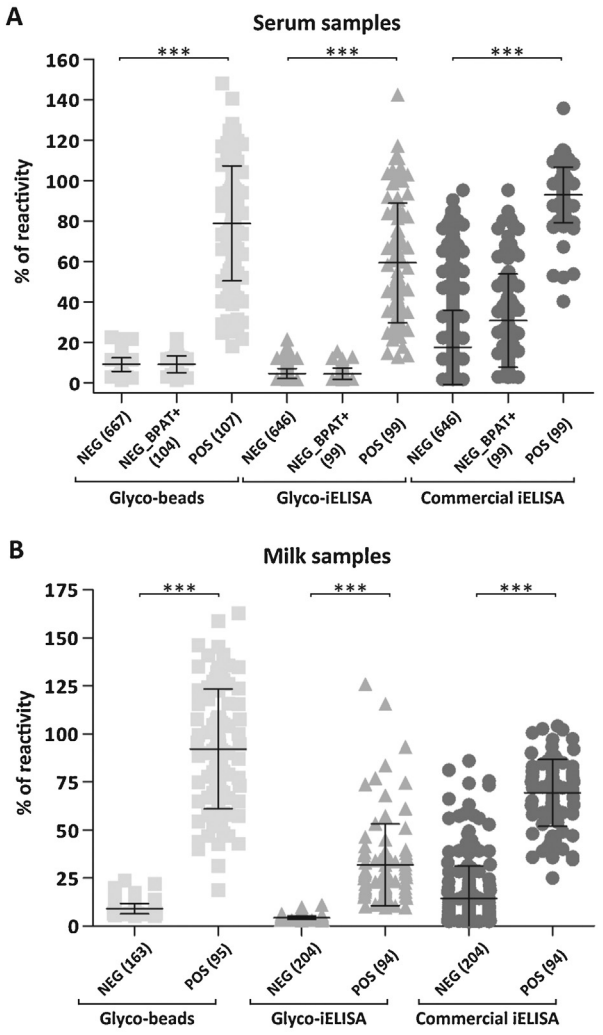

Fig. 3. Dotplot analysis of glyco-beads, glyco-iELISA and commercial iELISA results. (A) Analysis of serum samples. (B) Analysis of milk samples. Serum and milk samples obtained from naturally infected (POS, positive reference samples) and non-infected/S19-vaccinated (NEG, negative reference samples) bovines were tested as indicated in Section 2. The NEG group included samples that were serologically negative by the standard screening test BPAT or were BPAT-positive but negative by CELISA, FPA and CFT (NEG_BPAT+). Numbers in parentheses indicate the number of serum and milk samples analyzed for each group. The mean and standard deviation for each group are indicated; glyco-beads$_{sera}$: NEG (9.1 ± 3.4), NEG_BPAT+ (9.2 ± 4.1), POS (78.8 ± 28.4); glyco-iELISA$_{sera}$: NEG (4.6 ± 2.5), NEG_BPAT+ (4.5 ± 2.7), POS (59.3 ± 29.5); commercial iELISA$_{sera}$: NEG (17.6 ± 18.4), NEG_BPAT+ (30.8 ± 23.0), POS (92.9 ± 13.8); glyco-beads$_{milk}$: NEG (9.0 ± 2.7), POS (92.1 ± 31.2); glyco-iELISA$_{milk}$: NEG (4.2 ± 1.1), POS (31.8 ± 21.3); commercial iELISA$_{milk}$: NEG (13.9 ± 17.1), POS (69.3 ± 17.4). $^{***}P < 0.0001$, Mann–Whitney test.

cut-off values (TG-ROC plot) was performed for each assay in order to select different cut-off points so that the desired operating characteristics of the test in terms of diagnostic sensitivity (Se) and specificity (Sp) can be adjusted. This analysis allowed us to determine for each assay the cut-off value that concurrently maximizes Se and Sp, the cut-off value for which the diagnostic Se is 100% and the cut-off value that gives a 100% Sp (Fig. 4A and Table 1). These two last cut-off values represent the bounds of an intermediate range (IR) of reactivity values whose width correlates with the degree of overlapping between the positive and negative reference samples (see Fig. 3A and areas marked in gray in Fig. 4A). The IR of a test is wider when the overlap between the reactivity values of the two reference groups is higher. Analysis of the results obtained with the glyco-beads and glyco-iELISA assays showed very high values of diagnostic sensitivity and specificity at the selected cut-off values (Fig. 4A and Table 1). For the commercial iELISA, at a cut-off value of 40.1%, similar to the one established by the manufacturer (40%), the diagnostic Se was optimal but the Sp drops to 89.2%, a value significantly lower than the Sp values reached by the glyco-beads and glyco-iELISA assays (98.2% and 97.5%, respectively). Moreover, at the cut-off value corresponding to 100% Sp the Se drops to a very low value (35.4%) but at the cut-off that concurrently maximizes Se and Sp (75.2%) the diagnostic Se and Sp reaches values of 94.9% and 98.5%, respectively. These results indicate that the glyco-based immunoassays, which are based on detection of specific antibodies against the *O*-polysaccharide, present a better diagnostic performance than assays that use a complex mixture of antigens such as the commercial iELISA used in this study.

Finally, using the glyco-iELISA, we analyzed the strong positive, weak positive and negative standard sera provided by the World Organization for Animal Health (OIE) and by the National Brucellosis Reference Laboratory (DILAB-SENASA) from Argentina. The use of the OIE_ELISA international reference standards has been previously established to set the minimum diagnostic requirements for a wide range of commercially available and locally produced ELISAs (McGiven et al., 2006). As shown in Table 2, all the positive standard sera showed a positive reaction even at the highest dilution tested with exception of the OIE_ELISA$_{WP}$SS. Considering a cut-off value of 12.8%, this standard serum presented a negative reaction at 1/200 dilution but positive at 1/100, the optimum dilution previously established by us for the glyco-iELISA assay. The negative standard sera OIE_ELISA$_{NEG}$SS and SENASA$_{NEG}$SS showed a negative reaction at any of the tested dilutions.

Taken together, these results demonstrate that recombinant glycoprotein-based assays are highly accurate for diagnosis of bovine brucellosis using serum samples, even in S19-vaccinated herds, and comply with the international and national directives regarding the minimum accepted requirements established using standardized sera.

3.3. OAg-AcrA antigen validation using milk samples

To evaluate the performance of the OAg-AcrA antigen for diagnosis of brucellosis using milk, a total of 302 samples obtained from S19-vaccinated animals of certified brucellosis-free dairy farms and from animals naturally infected by brucellae were tested using the glyco-beads, glyco-iELISA and commercial iELISA assays. Reactivity values were outlined in a dotplot diagram and, as shown in Fig. 3B, a minimal overlap between negative and positive samples was observed with the glyco-beads and glyco-iELISA assays. As with serum samples, a greater overlap between positive and negative samples was obtained with the commercial iELISA.

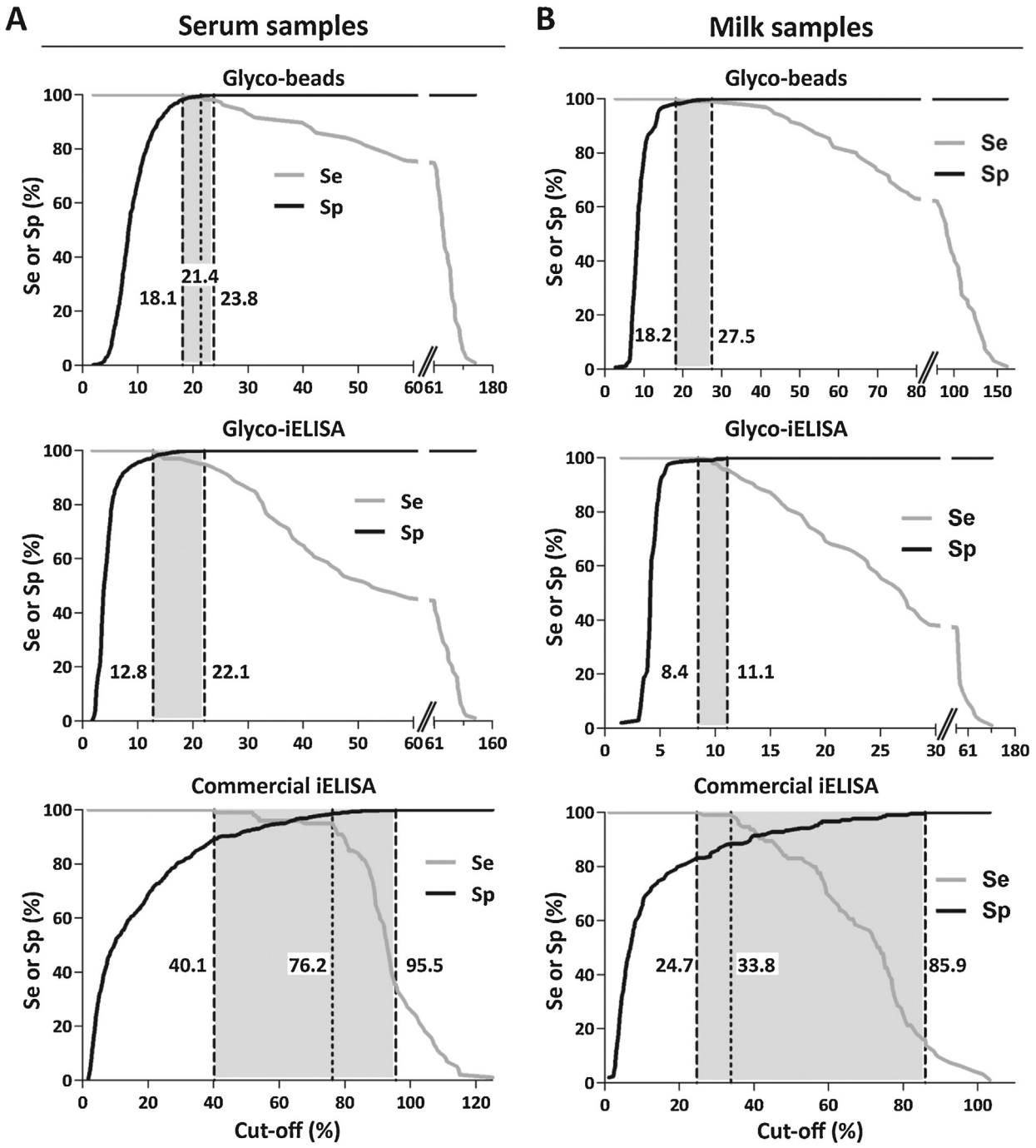

Fig. 4. Receiver-operating characteristic (ROC) analysis of glyco-beads, glyco-iELISA and commercial iELISA results. (A) TG-ROC plot of the results obtained with serum samples. (B) TG-ROC plot of the results obtained with milk samples. The analysis was carried out considering as positive reference samples sera and milk of animals naturally infected by brucellae, and as negative reference samples sera and milk of S19-vaccinated animals from herds without history of brucellosis. The dash vertical lines in the TG-ROC plots indicate the cut-off values for which maximal diagnostic sensitivity (Se) or specificity (Sp) is achieved for each assay. These two cut-off values represent the bounds of an intermediate range (IR) of reactivity values (area marked in gray). The dot vertical lines indicate the cut-off value that concurrently optimizes Se and Sp; for the glyco-iELISA/s (with serum and milk samples) these values coincide with the cut-off values (12.8% and 8.4%) for which maximal Se is achieved; for the glyco-beads (with milk samples) this value coincides with the cut-off value (27.5%) for which maximal Sp is reached.

To evaluate the performance of the assays, a ROC analysis was performed and the calculated AUC values were 0.9998 (95% CI, 0.9993–1.000), 0.9997 (95% CI, 0.9991–1.000) and 0.9695 (95% CI, 0.9526–0.9863) for the glyco-beads, glyco-iELISA and commercial iELISA, respectively. The AUC was significantly lower for the commercial iELISA (glyco-beads vs. commercial and glyco-iELISA vs. commercial, $P = 0.0004$). Based on the TG-ROC plot,

Table 1
Sensitivity, specificity and Youden's index of the tests calculated for different cut-off values.[a]

Assay	Cut-off (%)	Se (%)[b]	Sp (%)[b]	J[c]	n[d] (pos)	n[d] (neg)	TP	TN	FP	FN
Sera										
Glyco-beads	>18.1	**100**[e] (96.7–100)	98.2 (96.9–99.1)	0.982	107	667	107	655	12	0
	>21.4	99.1 (94.9–100)	99.6 (98.7–99.9)	**0.987**			106	664	3	1
	>23.8	98.1 (93.4–99.8)	**100** (99.4–100)	0.981			105	667	0	2
Glyco-iELISA	>12.8	**100** (96.3–100)	97.5 (96.0–98.6)	0.975	99	646	99	630	16	0
	>22.1	94.9 (88.6–98.3)	**100** (99.4–100)	0.949			94	646	0	5
Commercial iELISA	>40.1	**100** (96.3–100)	89.2 (86.5–91.5)	0.892	99	646	99	576	70	0
	>76.2	94.9 (88.6–98.3)	98.5 (97.2–99.3)	**0.934**			94	636	10	5
	>95.5	35.4 (26.1–45.6)	**100** (99.4–100)	0.354			35	646	0	64
Milk										
Glyco-beads	>12.8	**100**[d] (96.2–100)	98.2 (94.7–99.6)	0.982	95	163	95	161	3	0
	>27.5	98.9 (94.3–100)	**100** (97.8–100)	**0.989**			93	163	0	1
Glyco-iELISA	>8.4	**100** (96.1–100)	99.0 (96.5–99.9)	**0.990**	94	204	94	202	2	0
	>11.1	95.7 (89.5–98.8)	**100** (98.2–100)	0.957			90	204	0	4
Commercial iELISA	>24.7	**100** (96.1–100)	82.8 (77.0–87.7)	0.828	94	204	94	169	35	0
	>33.8	98.9 (94.2–99.8)	88.2 (83.0–92.3)	**0.871**			93	180	24	1
	>85.9	16.0 (9.2–24.9)	**100** (98.2–100)	0.160			15	204	0	79

[a] Analysis was performed using serum and milk samples from naturally infected and non-infected/S19-vaccinated bovines from Argentina (see Section 2).
[b] Se, sensitivity (TP/TP + FN) × 100; Sp, specificity (TN/TN + FP) × 100. Values in parentheses indicate the 95% confidence interval. TP, true positive; TN, true negative; FP, false positive; FN, false negative.
[c] *J*, Youden's index (Se + Sp − 1).
[d] *n*, number of positive (pos) or negative (neg) reference samples analyzed.
[e] Maximum values for Se, Sp and J are indicated in bold.

different cut-off values were selected and very high values of diagnostic Se and Sp were obtained for the glyco-based assays (Fig. 4B and Table 1). For the commercial iELISA, the diagnostic Sp values were significantly lower and a broader IR was obtained that correlates with the high level of overlapping between negative and positive samples (Figs. 3B and 4B).

In the interests of international harmonization, the OIE established that iELISAs for milk samples should be standardized so that the OIE_ELISA$_{SP}$SS, when diluted 1/125 in negative serum and further diluted 1/10 in negative milk, consistently tests positive (OIE, 2012). The OIE_ELISA$_{SP}$SS diluted as established by the OIE gave a percentage of reactivity of 17.2%, a value significantly higher than the cut-off values (8.4% and 11.1%) selected for the glyco-iELISA (see Fig. 4B and Table 1).

Taken together, these results demonstrate that the glyco-based assays are highly accurate for diagnosis of bovine brucellosis using milk samples, even in S19-vaccinated dairy herds, complying with the international guidelines regarding standardization of milk indirect ELISAs.

4. Discussion

Control programs of bovine brucellosis depends on vaccination, most frequently with *B. abortus* S19 strain, and/or detection of infected animals by testing samples combining different serological tests either in series or in parallel and slaughter. Since the humoral immune response to "smooth" brucellae is dominated by antibodies to the *O*-polysaccharide section of sLPS (Caroff et al., 1984b), and because the most effective serological tests for differentiation between animals infected with virulent *Brucella* spp. field strains and S19-vaccinated animals are based on detection of anti *O*-polysaccharide antibodies (McGiven et al., 2003; Nielsen, 1990; Nielsen et al., 1995, 1996), we decided to explore the application of the bacterial engineered OAg-AcrA in the diagnostics of bovine brucellosis.

Table 2
Glyco-iELISA analysis of OIE and SENASA standard sera.

Standard[a]	Glyco-iELISA results (%R)[b]			Interpretation of test results[c]		
	1/50	1/100	1/200	1/50	1/100	1/200
OIE_ELISA$_{SP}$SS	86.8	**70.3**	52.7	+	+	+
OIE_ELISA$_{WP}$SS	30.3	**14.3**	7.2	+	+	–
OIE_ELISA$_{NEG}$SS	5.2	**3.6**	2.9	–	–	–
SENASA$_{SP}$SS (1323 IU/ml)	95.4	**89.5**	84.6	+	+	+
SENASA$_{WP}$SS (288 IU/ml)	107.3	**102.0**	98.7	+	+	+
SENASA$_{NEG}$SS	3.4	**2.7**	2.6	–	–	–

[a] Strong positive (SP), weak positive (WP) and negative (NEG) standard sera provided by OIE and SENASA. IU/ml, international complement fixation test units per ml.
[b] The standard sera were tested in the same way as any field sample under routine diagnostic conditions. Dilutions were performed in 5% bovine skim milk in PBST-0.1%. %R, percentage of reactivity of the positive control serum included in each assay run. The %R values obtained at 1/100 (the dilution used in our assay) are indicated in bold.
[c] Considered cut-off value: 12.8%.

In the present work, we validated the recombinant glycoprotein OAg-AcrA for diagnosis of bovine brucellosis using different types of samples and coupling the antigen to different platforms (magnetic beads and ELISA plates). The glyco-beads and glyco-iELISA indirect immunoassays were developed and optimized as indicated in Section 2 and validated using two different sample panels. The first panel included serum, whole blood and milk samples obtained from a bovine vaccination/challenge trial performed in our laboratory. For this first panel, pre-infection, post-infection, post-vaccination (with a smooth or a rough strain) and post-challenge samples were analyzed. The second panel included serum and milk samples obtained from naturally infected and non-infected/S19-vaccinated bovines from Argentina provided by the National Brucellosis Reference Laboratory. The analysis of the first panel's samples allowed us to evaluate the performance of the OAg-AcrA antigen using a set of samples obtained from animals whose status of infection/vaccination was completely characterized. The results indicated that with this antigen it is possible to clearly discriminate between infected and non-infected animals even if they were vaccinated with a smooth or rough vaccine strain. This is in complete agreement with the results obtained with BPAT, CELISA, FPA and CFT. In addition, we confirmed by immunoblot that the detected antibody response in positive serum samples is specifically directed toward the *O*-polysaccharide moiety of the glycoprotein, and showed that the OAg-AcrA antigen can be used to diagnose the disease using whole blood and milk samples. The second sample panel allowed us to evaluate the performance of the glycoprotein-based assays using serum and milk samples from different herds in order to validate the antigen under the actual epidemiological situation of bovine brucellosis in Argentina. To evaluate the performance of the assays, a receiver-operating characteristic (ROC) analysis was performed (Greiner and Gardner, 2000; Greiner et al., 1995; Swets, 1988). For the glyco-beads and glyco-iELISA assays, using either serum or milk samples, the area under the ROC curve values indicated that the glyco-based assays are highly accurate tests to diagnose bovine brucellosis. Based on the ROC analysis, the cut-off values that determine a diagnostic Se and Sp of 100% were selected for each assay. These cut-off points represent the boundaries of an intermediate range (IR) of reactivity values. Therefore, samples with reactivity values below the lower cut-off should be considered as non-reactive, samples with reactivity values above the upper cut-off as reactive, while samples with values that fall in the IR as indeterminated. For the glyco-based assays, high values of diagnostic Se and Sp were obtained as well as a narrow IR that correlates with the low level of overlapping between the reactivity values obtained with the negative and positive reference samples. Comparison of the results obtained with the glyco-based assays and the commercial iELISA indicates that a better performance of the test can be obtained in an indirect immunoassay format using as antigen the recombinant glycoprotein OAg-AcrA instead of a total bacterial extract. Finally, we have shown that the glyco-iELISAs, using either milk or serum samples, comply with the minimum diagnostic requirements established by the national and international directives.

Currently, most serological tests for diagnosis of bovine brucellosis use as antigen whole "smooth" *Brucella* cells, total bacterial extracts containing high concentrations of sLPS, purified sLPS or a small molecular weight fragment of the *O*-polysaccharide of *B. abortus* sLPS (Godfroid et al., 2010; Saegerman et al., 2010). In all these cases, level-3 biosafety facilities are required for culturing the bacterium and production of the antigens. Instead, the recombinant OAg-AcrA antigen can be produced in large quantities under biosafety level-2 facilities without the need of culturing pathogenic brucellae. Additionally, the antigen can be purified in one step from the periplasm of the bacteria by affinity chromatography. This novel technology solves two problems: on one hand the cost of production is significantly reduced, on the other it eliminates the risk of infection during the production process. In comparison with other *O*-polysaccharide-protein conjugates produced by chemical traditional methods (Jacques et al., 1991), the production of OAg-AcrA does not require purification of sLPS or chemical treatments to obtain the *O*-polysaccharide from it. In addition, no chemical cross-linking of the carbohydrate to the protein is required, allowing the production of glycoproteins with a defined and reproducible sugar pattern, since the synthesis of the *O*-polysaccharide and the glycosylation process are controlled in vivo. Therefore, this novel technology allows the production of large homogeneous batches of antigen that may facilitate in the future the production of a standardized reference antigen with important implications not only for the diagnosis of bovine brucellosis but also for diagnosis of "smooth" brucellosis in other species. Additionally, the characteristics of the glyco-beads assay make it suitable for adapting it for point-of-care use.

B. abortus and *Y. enterocolitica O:9* share an almost identical *O*-polysaccharide structure. *B. abortus O*-polysaccharide contains both A (α-1,2-linked homopolymer of N-formylperosamine) and M (pentasaccharide with four α-1,2 and one α-1,3-linked polymers of the same sugar) epitopes with approximately 98% of A epitope, while *Y. enterocolitica O:9 O*-polysaccharide is comprised solely of α-1,2-linked *N*-formylperosamine (Bundle and Perry, 1985; Caroff et al., 1984a; Meikle et al., 1989). Albeit this structural similarity, in this study we observed an excellent performance of the test in terms of diagnostic specificity. Further work will be required to evaluate the specificity of the glyco-based assays using samples obtained from "known false positive brucellosis reactors" as well as samples coming from other regions or countries. Finally, the whole bacteria- and sLPS-based assays currently used to diagnose brucellosis, with the exception of FPA and the CELISA assays, that specifically detect anti *O*-polysaccharide antibodies, not only suffers from false-positive results due to these *O*-chains structural similarities but also due to the presence of cross-reactive antibodies against the common core and lipid A as well as other antigens. Therefore, glyco-based assays may be more specific than these tests since it uses as antigen only the *O*-polysaccharide fraction of the sLPS.

5. Conclusion

Here we validate the first recombinant glycoprotein antigen, an *N*-formylperosamine *O*-polysaccharide-protein conjugate, for the diagnosis of bovine brucellosis. This antigen can be produced in large homogeneous batches making it suitable for standardization. The glycoprotein-based assays developed, optimized and validated in this work are highly accurate for diagnosis of bovine brucellosis using different types of samples from both vaccinated and unvaccinated herds. For these reasons, we propose the glyco-assays as the tests of choice for screening and confirmatory diagnosis of bovine brucellosis in regions or countries with brucellosis-control programs. Further work will be required to validate the glyco-antigen in other regions with lower or higher incidence of bovine brucellosis than in Argentina, to evaluate the specificity in sera from known false positive reactors and its usefulness for diagnosing pigs and goats.

Conflict of interest statement

A patent has been filed regarding the diagnostic application of recombinant glycoproteins.

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