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Diagnostic Performance of Rose Bengal, Competitive ELISA, and Native Hapten (NH) Assays in S19 Mass-Vaccinated Cattle in an Endemic Brucellosis Environment

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ABSTRACT

Controlling bovine brucellosis requires reliable diagnostics and effective vaccination strategies. We evaluated the performance of three serological assays—the rose bengal test (RBT), competitive ELISA (cELISA), and agar gel immunodiffusion (AGID) with native hapten (NH)+lipopolysaccharide (LPS) antigen—following *Brucella abortus* S19 vaccination under different protocols. Serum samples were collected from two cattle populations in Costa Rica: (i) a brucellosis-free cohort of 25 heifers (Farm 1) vaccinated once with a reduced dose of S19 (5×10^9 CFU) via either the conjunctival ($n = 5$) or subcutaneous ($n = 20$) route, and (ii) a previously vaccinated herd with *B. abortus* RB51 (Farm 2) of 253 cattle, cleared of *Brucella* infection by test and slaughter, was then vaccinated with S19 (either 5×10^{10} or 5×10^9 CFU, conjunctival or subcutaneous). All sera were tested by RBT, cELISA, and AGID using standard procedures. In validation with control sera, RBT, cELISA, and AGID NH+LPS each correctly identified all 35 positive and all 35 negative sera, while AGID NH+LPS correctly identified 33 of 35 positive sera and all negative sera. In Farm 1, conjunctival vaccination induced only a transient antibody response: all heifers were seronegative by 7 weeks postvaccination, and none developed NH-specific precipitin lines. Subcutaneous vaccination produced stronger and longer-lasting responses: ~20% of animals remained RBT/cELISA-positive at 1 year (5% at 2 years), though none reacted to NH. In Farm 2, S19 vaccination elicited sustained RBT and cELISA seropositivity, especially after subcutaneous administration. About 5% of animals developed NH-specific precipitin lines over time, consistent with an anamnestic response to residual infection. These findings show that vaccination route affects serological outcomes: conjunctival S19 vaccination yields minimal long-term interference, while subcutaneous vaccination prolongs seropositivity. The AGID NH+LPS test helped identify actively infected animals in vaccinated herds. We recommend S19 mass vaccination via the conjunctival route and caution against using cELISA as a routine confirmatory test following RBT in endemic settings.

Wilson J. Bonilla-Machado and Andrés Aguilar-Chavarría contributed equally to this work.

1 | Introduction

Members of the genus *Brucella* cause bovine brucellosis. This disease is broadly distributed in the American, Eurasian, and African continents, mainly in the middle- and low-income countries, where it causes economic constraints and public health concerns due to its zoonotic potential [1]. The most common agent is *Brucella abortus*. However, when bovines are reared with infected caprine or ovine herds, *Brucella melitensis* may become predominant and perpetuated in the herds [1].

A handful of high-income countries in Europe, North America, and Oceania have eradicated bovine brucellosis through great efforts using *B. abortus* S19 vaccination combined with serological testing and culling the seropositive animals with compensatory actions [2–5]. This procedure, known as "test and slaughter," is commonly carried out alongside the S19 vaccination [2–5]. As expected, this is a long-lasting and expensive process that requires validated diagnostic tests, suitable vaccination protocols, significant economic investment, knowledge of the epidemiological settings, and an understanding of the performance of the diagnostic tools [2, 6].

Vaccination with *B. abortus* S19 is the best immunization approach to control bovine brucellosis [2–5]. Unfortunately, herd vaccination coverage is only moderate to low in middle- and low-income nations where bovine brucellosis is highly prevalent [7–9]. In many of these countries, the S19 vaccine is used simultaneously with the RB51 vaccine, and not infrequently, the latter is employed as the only vaccine against bovine brucellosis [2, 7, 8]. However, while S19 has been instrumental in controlling and eliminating bovine brucellosis in countries that have succeeded with eradication, there is no single country in which bovine brucellosis has been eradicated with the concourse of RB51 [2]. We emphasize that a strict comparison of RB51 and S19 efficacy or immunogenicity is beyond the scope of this study, which focuses exclusively on diagnostic performance in S19-vaccinated herds.

An effective strategy to control brucellosis in infected herds is to lower the prevalence by culling as many seropositive bovines as possible, followed by mass S19 vaccination of the remaining cows [2–6]. The test and slaughter approach, when accompanied by biosecurity practices, fair compensation for owners, and increased awareness of farm personnel, can also facilitate the release of herds from quarantine. However, in many endemic areas, culling a large proportion of animals is not economically feasible, and mass vaccination is primarily used to lower the prevalence. Although RB51 vaccination has been used in some endemic regions, no country has achieved eradication with RB51 alone, whereas S19 has consistently been the basis of successful eradication programs [2]. After mass vaccination in endemic areas, standard serological tests become challenging to interpret, and the results are frequently misunderstood, resulting in over-culling healthy vaccinated animals [6]. In Costa Rica, the prevalent species associated with bovine brucellosis is *B. abortus* biovar 1, which has been consistently isolated in outbreaks [10]. The National Service of Animal Health (SENASA) coordinates the official brucellosis control program in Costa Rica, which combines test and slaughter, subcutaneous S19 vaccination in calves and vaccinations of RB51, mostly in dairy farms [10]. However, vaccination coverage almost never exceeds 25%.

Native Hapten (NH) *Brucella* polysaccharides share the determinants with the lipopolysaccharide (LPS). It has been demonstrated that NH, which is intertwined in the outer membrane with the O chain of the LPS, can be identified as a separate molecule in agar gel immunodiffusion (AGID) tests where NH and LPS show different migration, resulting in distinct precipitation bands [6, 11, 12]. Despite their epitopic similarities, the behavior of NH and LPS in immunoprecipitation tests differs. While a proportion of S19-vaccinated animals persistently produce antibodies against LPS in assays such as the rose bengal test (RBT) and ELISAs, the NH does not react with sera of these bovines bled 2–3 months after vaccination [6, 11, 12]. Furthermore, these serological reactions are minimized when a reduced dose (5×10^9 CFU) of S19 is applied, mainly when administered through the conjunctival route [2, 13]. In contrast, NH reacts in the AGID test with a high proportion of sera from *Brucella*-infected bovines, correlating with bacterial shedding [11–13]. Following this, it has been proposed that the AGID with extracts rich in NH and LPS is a practical test for identifying the epidemiologically relevant cows (i.e., *Brucella*-infected animals shedding the bacterium) in infected herds submitted to S19 mass vaccination.

There is a knowledge gap in how the different serological diagnostic assays function in brucellosis endemic areas after different mass vaccination protocols. Here, we explore the performance of the RBT, competitive ELISA (cELISA), and AGID with NH-LPS in bovines immunized with *B. abortus* S19 through various vaccination protocols in brucellosis-free and *B. abortus*-infected herds in the tropics of Costa Rica.

2 | Materials and Methods

2.1 | Vaccination and Obtention of Sera

Quality control of the S19 vaccine batch used in all experiments (Antibrucelica, CDV, Argentine, Lot Number 136205688) was assessed according to WOAH protocols, including residual virulence in mice [1, 14]. The following groups of sera were used: Positive control sera (used for test validation) were from 35 naturally infected cows from Costa Rica showing a *B. abortus* positive culture [10, 14]. Negative control sera (used for test validation) were obtained from 35 nonvaccinated dairy cows from a brucellosis-free herd in a Costa Rica region with no brucellosis for at least 25 years. Sera from Farm 1 corresponding to 25 crossbred Angus-Zebu brucellosis-free heifers (8–11 months of age) from a *Brucella*-free environment in San Carlos, Alajuela, Costa Rica, were vaccinated with a reduced dose (5×10^9 CFU) of the *B. abortus* S19 applied either by the subcutaneous ($n = 20$ heifers) or conjunctival ($n = 5$ heifers) routes [2, 14]. Farm 1 was officially certified as brucellosis-free for over 25 years by SENASA, and all animals tested negative by RBT and cELISA before vaccination. The vaccinated animals of Farm 1 were maintained in the same brucellosis-free herd during the experiment and bled at intervals through a 1100-day observational period (Table 1).

Sera from Farm 2 correspond to a collection obtained from Holstein-Zebu crossbred bovines inhabiting a brucellosis endemic area in Guácimo, Limón (Caribbean, Huetar region), Costa Rica. During 2016–2017, the herd of Farm 2 was heavily *Brucella*-infected (individual prevalence ~47% assessed with both the RBT (ID.vet San José,

TABLE 1 | S19-vaccinated bovines from brucellosis-free (Farm 1) or infected (Farm 2) herds according to age group, vaccination protocol, and previous RB51 vaccination.

Facility	Reproductive status	S19 Vaccination and dose (CFU)[a]	Previous RB51 vaccination	Number of bovines
Farm 1	Heifers	Conjuntival 5×10^9	No	5
		Subcutaneous 5×10^9	No	20
Farm 2	Cows	Subcutaneous 5×10^9	Yes	32
			No	42
		Conjuntival 5×10^9	Yes	62
			No	44
	Heifers	Subcutaneous 5×10^{10}	No	19
		Subcutaneous 5×10^9	No	18
		Conjuntival 5×10^9	No	36
Total				278

[a]Conjuntival and subcutaneous S19 vaccinations were carried out as described elsewhere [4, 5, 15, 16].

Costa Rica, www.id-vet.com) and cELISA (BIONOTE, Gyeonggi-do, Korea) tests and high abortion rates (with a maximum of 30%), with recurrent isolation of *B. abortus* biovar 1 always of the same genetic cluster [10]. At the beginning of 2017, RBT and cELISA testing were initiated following SENASA's national procedures for Costa Rica [10] and the guidelines set by Bovina N° 34858-MAG, Government of Costa Rica. This resulted in the culling of a total of 593 animals by the end of 2018. Of these, 345 were culled due to positivity in brucellosis tests and 248 due to pregnancy loss/infertility. A remnant of 253 bovines of Farm 2 with no record of abortions, which tested negative in both RBT and cELISA and were negative for *Brucella* spp. by repeated culture of milk samples, were vaccinated with S19 following various protocols (Table 1). Ninety-four of these 253 animals had been previously vaccinated at 4–5 months of age and revaccinated as adults with a standard dose of RB51 (Colorado Serum Co., USA) during 2016–2017; however, the herd continued to show a high seroprevalence (~47%) and abortion rate (up to 30%), indicating that RB51 vaccination alone did not achieve a measurable reduction in infection or reproductive losses (Table 1). Following the S19 vaccination of the 253 bovines of Farm 2, blood samples were collected periodically over a 13-month observational period. Initial serological testing (RBT, cELISA, and AGID) was conducted after 13 months (day ~395), with follow-up analyses at 1000 days postvaccination.

During the observational period, 81 bovines (out of 253) were culled due to lack of pregnancy as determined by palpation (Table 2). Sera were kept under −80°C until tested. Sera were coded, and the identity of each serum was not revealed until all results were analyzed. Ethical approval and consent were not required for this study because it did not involve direct experimentation with animals, and samples were obtained following the mandate of the SENASA brucellosis surveillance program of the Ministerio de Agricultura y Ganadería, Government of Costa Rica, Decree N° 41219-MAG –N° 34858-MAG.

2.2 | Bacteriological Studies and Serological Assay

Searching for *Brucella* infection in milk, vaginal secretions, and fetuses was performed using the Farrell's and CITA culture media

TABLE 2 | The number of S19 vaccinated animals tested in Farm 2 and culled at different periods over 1000 days.

Sampling days after S19 vaccination	Number of bovines	
	Tested	Culled[a]
0	253	0
45	253	0
105	252	1
165	251	1
225	248	4[b]
285	243	5
345	215	28
405	211	4
1000	172	38

[a]The animals were culled due to infertility.
[b]One cow *B. abortus* culture-positive was culled due to abortion.

[10, 14]. Cultures were incubated in a 10% CO_2 atmosphere at 37°C for at least 2 weeks. The identification of suspected colonies was carried out by conventional bacteriological procedures and genetic analyses [10, 14].

The RBT was performed as described elsewhere [17]. The test was validated using our panel of control sera (35 culture-confirmed positive and 35 negative sera from a brucellosis-free herd >25 years free of infection), correctly identifying all 35 positive and 35 negative control sera under these controlled conditions. However, we recognize that in practical field applications, no serological test can be universally considered 100% sensitive and specific [1, 6, 18, 19]. RBT was considered positive when rendering any agglutination after 4 min [17]. cELISA was performed and standardized according to the procedures described before [10, 14]. The cutoff settled at 30%, coincident with the value currently accepted for diagnosing bovine brucellosis by the SENASA of Costa Rica [10]. Under controlled conditions, this cutoff correctly identified all positive and all negative control sera. These values reflect performance under controlled conditions and should not be interpreted as absolute across all epidemiological contexts.

The AGID test was performed as described elsewhere [11–13]. Briefly, the antigen preparation rich in NH and LPS was a soluble lyophilized extract from *B. melitensis* 16 M, obtained as described elsewhere [11]. Before use, the antigen preparation was reconstituted with deionized water. One percent of Noble Agar (Difco) in borate buffer (pH 8.3) (Merck & Co., Inc.) containing 10% NaCl was used for immunodiffusion. A volume of 11 mL of hot liquified agarose solution was placed in a plastic Petri dish of 100 mm × 15 mm and let solidify for 30 min at room temperature and 1 h at 4°C. The thickness of the gel in the plate was 1 mm, allowing the cutting of four rosettes of six wells around a central one inside the plate (3 mm-diameter wells set 3 mm apart). Each well was filled with 16 µL of the antigen (central well) or serum samples (external wells) for immunodiffusion [11]. The optimal antigen concentration for the AGID test was established at 1 mg/mL by serial dilutions against the control positive sera [11–13]. Gel diffusion plates were incubated at room temperature inside a wet chamber and read at 24, 32, and 48 h to test the reactivity of sera against NH and/or LPS [11–13]. Before the 48 h reading, the plates were soaked in a 5% sodium citrate solution (Merck & Co., Inc.) for 1 h to clear potential unspecific reactions [11–13]. The same person recorded the precipitin lines in all samples using a dark box with indirect light coming from the bottom. The AGID reaction generated the following possible results (Figure 1): (i) precipitation lines against only the LPS close to the antigen well (henceforth AGID/LPS positive) were interpreted as due to infection or vaccination; (ii) precipitation lines against both NH (close to the serum well) and LPS (henceforth AGID/NH + LPS positive) were interpreted as a very recent vaccination or as being infected with *Brucella* and possible shedding of the bacterium; and (iii) no precipitation lines (AGID negative).

2.3 | Statistical Analysis

Interrater reliability against different assays and between vaccinated animal populations was achieved by calculating Cohen's kappa coefficient (κ) [20], which quantifies agreement beyond chance, adjusted for the probability of random concordance. This metric is widely employed in veterinary diagnostic studies to evaluate the consistency of categorical test outcomes under field conditions [1, 20]. Calculations included 95% confidence intervals. The level of agreement according to the κ values and the corresponding proportion of reliable data (within parenthesis) followed the interpretation as follows [20]: none = 0–0.2 (0%–4%); minimal = 0.21–0.39 (4%–15%); weak = 0.4–0.59 (15%–35%); moderate = 0.6–0.79 (35%–63%); strong = 0.8–0.9 (64%–81%); almost perfect >0.9 (82%–100%).

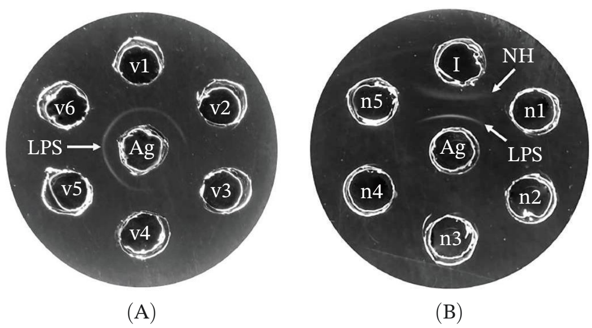
2.4 | Ethics

All methods followed the guidelines and regulations of SENASA Ministerio de Agricultura y Ganadería, according to the "Reglamento para la Intervención de la Brucellosis Bovina N° 34858-MAG Government of Costa Rica." The study was undertaken under the mandatory SENASA brucellosis surveillance program of the Ministerio de Agricultura y Ganadería, Government of Costa Rica, Decree N° 41219-MAG –N° 34858-MAG. SENASA Ministerio de Agricultura y Ganadería approved all the experimental protocols, according to the Reglamento para la Intervención de la Brucellosis Bovina N° 34858-MAG, Government of Costa Rica.

3 | Results

3.1 | Diagnostic Performance of Sera From *B. abortus*-Infected and Brucellosis-Free Cows

Under controlled conditions with well-characterized serum panels, the RBT, cELISA, and AGID/LPS assays correctly identified all 35 culture-confirmed positive and negative sera. The AGID/NH+LPS assay correctly identified 33 of 35 positive sera (94.3%) and all negative sera. However, the two sera from infected cows that resulted in negative AGID/NH+LPS were positive in the AGID/LPS. Precipitin lines against the NH alone, occasionally seen in infected animals in previous studies, were not observed. As stated, AGID/NH+LPS observable reactions positively correlate with active infection in the bovines and shedding of *Brucella* organisms [11–13].



(A) (B)

FIGURE 1 | AGID test in infected, S19-vaccinated and negative controls using LPS-NH-rich antigen. A total of 16 µL of 1 mg/mL of LPS-NH antigen (Ag) was tested against sera of S19-vaccinated, *Brucella*-positive, and *Brucella*-negative bovines. (A) The sera from S19-vaccinated bovines labeled v1–v6 were confronted against NH-LPS-rich antigen. (B) The sera from one *Brucella*-infected bovine (labeled "I") and five control sera from negative bovines (labeled n1–n5) were tested. (A) None of the S19-vaccinated bovines reacted against NH; however, sera from bovines v1, v2, and v4-v6 reacted against the LPS antigen (precipitation line). Although in v3, the precipitation line against LPS is not conspicuous, the neighboring precipitation lines bend toward the direction of the v3 well, indicating mild reactivity against the LPS antigen. (B) The serum of the *B. abortus*-infected bovine shows two precipitation lines: one corresponding to the LPS and the other to the NH polysaccharide. None of the nonvaccinated/noninfected bovines reacted against the LPS-NH antigen.

3.2 | Diagnosis After *B. abortus* S19 Vaccination in Brucellosis-Free Heifers

The evolution of reactors after S19 vaccination in brucellosis-free heifers (i.e., Farm 1) is shown in Figure 2. None of the vaccinated animals induced positive responses in the AGID/NH+LPS independently of the route of vaccination. As expected, conjunctival vaccination (Figure 2A) induced a significantly lower and shorter serological response than subcutaneous vaccination (Figure 2B). The conjunctively vaccinated heifers became negative in all tests 7 weeks after vaccination (Figure 2A). Moreover, no conjunctively vaccinated animal was either AGID/LPS positive or AGID/NH+LPS.

In contrast, the serological response induced in the subcutaneously vaccinated heifers was of high intensity and duration (Figure 2B), with 20% of the animals remaining positive after 1 year, 5% after 2 years and none after 3 years. Although the proportion of reactors in the RBT in the subcutaneously vaccinated group was slightly higher than the cELISA after day 70, the correlation of the grouped assays between both tests was similar throughout the experiment ($\kappa = 0.81$) (Table 3), which does not support the notion that cELISA performs better than RBT in distinguishing S19-vaccinated cattle (Figure 2B). A moderate to high proportion of the subcutaneously vaccinated heifers was AGID/LPS positive until day 80, but none reacted against the NH (Figure 2B).

3.3 | Diagnostic Performance After S19 Vaccination of the *B. abortus*-Infected Herd

The first RBT and cELISA analyses in Farm 2 were performed just 1 year after the S19 vaccination, while the AGID responses were studied 1000 days after the S19 vaccination. About 1 year after vaccination (day 345), 39 animals were culled. After this

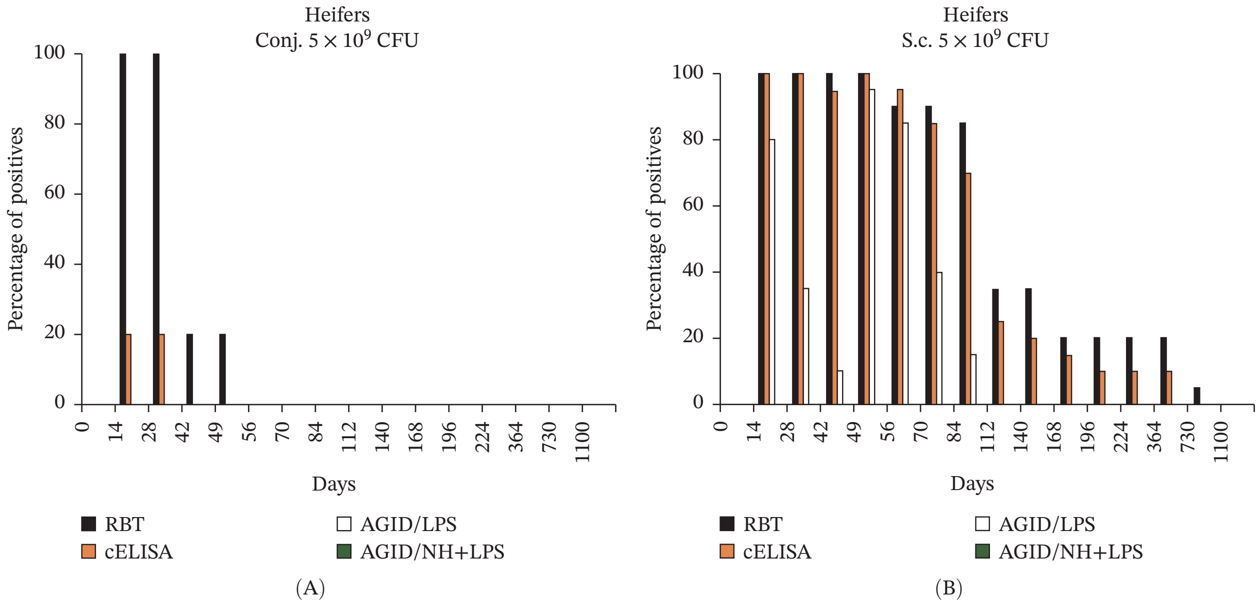


FIGURE 2 | Evolution of the proportion of conjunctivally (A) or subcutaneously (B) S19-vaccinated heifers reacting in different serological tests in the brucellosis-free farm (Farm 1). Notice that several heifers vaccinated subcutaneously were AGID/LPS positive, but none of the vaccinated heifers reacted positive in the AGID/NH+LPS. Day 0 corresponds to the bleeding before vaccination.

TABLE 3 | Interrater reliability κ values between RBT and cELISA tests of sera from bovines of Farm 1 and Farm 2, following different S19 vaccination protocols and tested at various intervals.

Facility	Reproductive status	S19-vaccination[a]	Number of bovines	RBT vs. cELISA
Farm 1	Heifers	S.c. 5×10^9 CFU	20	0.81 (1.00–0.65)
		S.c. 5×10^9 CFU	5	ND[b]
Farm 2	Cows	S.c. 5×10^9 CFU	74	0.55 (0.47–0.63)
		Conj. 5×10^9 CFU	106	0.27 (0.21–0.34)
	Heifers	S.c. 5×10^{10} CFU	19	0.61 (0.46–0.75)
		S.c. 5×10^9 CFU	18	0.55 (0.40–0.71)
		Conj. 5×10^9 CFU	36	0.16 (0.06–0.25)

Note: The confidence interval was 95%. The numbers within the parenthesis correspond to interval range values.
[a]S.c., subcutaneous; Conj., conjunctival.
[b]ND, not performed because values were nonsuitable for analysis.

time, 42 additional bovines were culled, with 172 bovines remaining after 1000 days (Table 2). Although all cows in this infected farm were serologically negative just before the S19 vaccination, they displayed positive antibody responses in the various tests after the S19 vaccination (Figure 3). Overall, the serological responses were higher and more persistent in cows and heifers vaccinated subcutaneously (Figure 3A,B,E). The evolution of the proportion of reactors in the cows vaccinated subcutaneously with the reduced doses (Figure 3A) was similar to that of the heifers vaccinated subcutaneously with the standard dose, with a high number of serologically positive animals (mainly in the cELISA) until the end of the observational period.

The subcutaneous vaccination produced the highest level of serological cross-reactivity across tests, with some animals positive even in the AGID/NH+LPS for a protracted interval after vaccination (Figure 3A,B). In contrast, the lowest serological reactivities were obtained in conjunctively vaccinated animals (Figure 3C,D). None of the heifers vaccinated through this route generated positive AGID/NH+LPS results (Figure 3D). Similar results were obtained in the cows vaccinated conjunctively (Figure 3C) (only one animal was positive in the AGID/NH+LPS). Moreover, in the conjunctival vaccinated bovines, the proportion of reactors in RBT was moderate to very low but not in cELISA. In the infected herd, cELISA yielded the highest proportion of positive results among vaccinated animals, making interpretation of postvaccination serological responses more difficult. Thirteen months after vaccination, no additional AGID/NH+LPS-positive animals were detected. The proportions of AGID/LPS and AGID/NH+LPS positive reactions (mainly the latter) were significantly lower than those observed in RBT and cELISA.

The only abortion registered occurred in a cow vaccinated subcutaneously with a reduced dose of S19 (arrow in Figure 3A), resulting in a positive culture for the *B. abortus* biovar 1 field strain, thus confirming the infection. As expected, the aborted cow displayed a positive AGID/NH+LPS response. After this abortion event, the proportion of AGID/NH+LPS reactors of

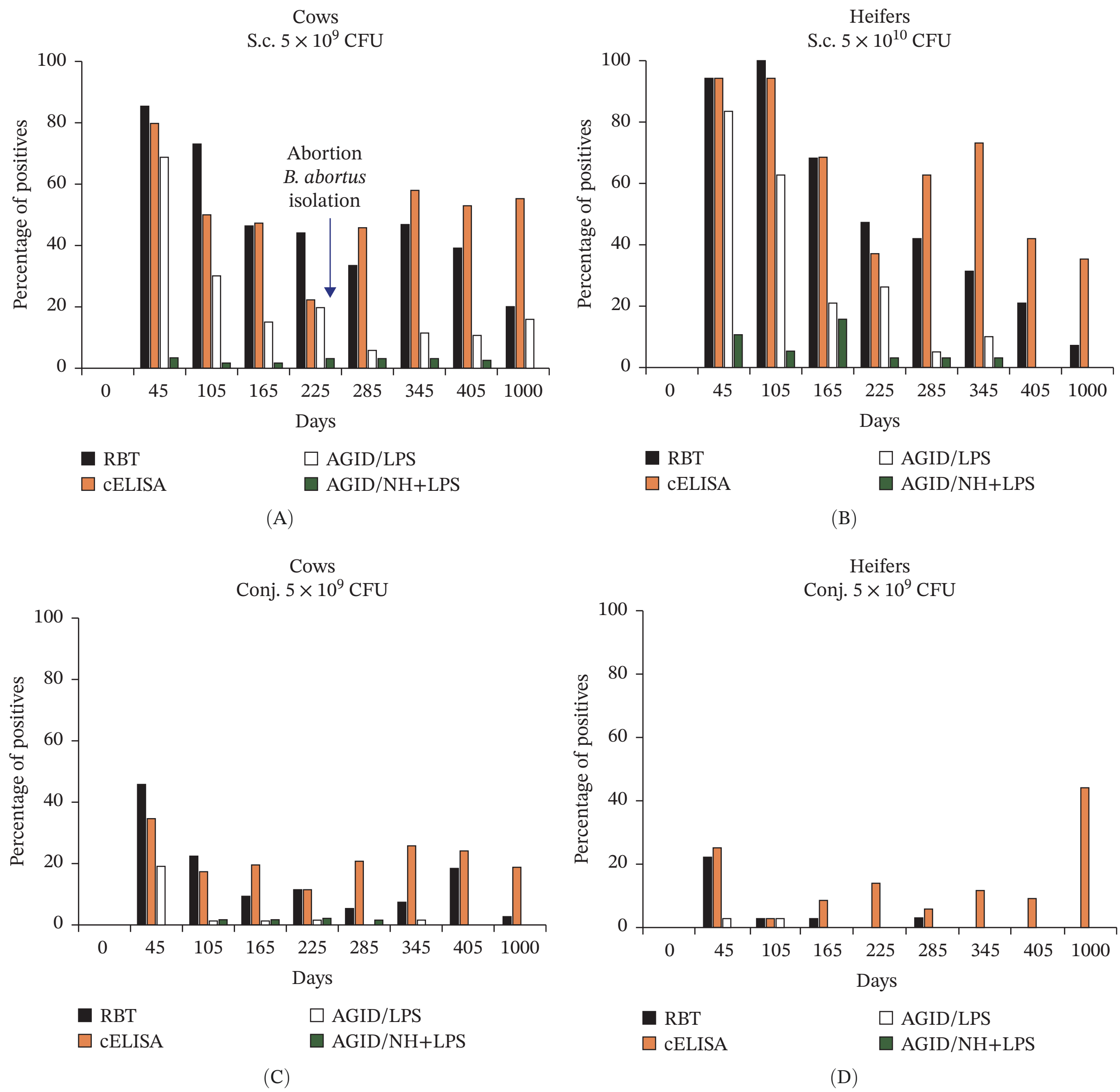
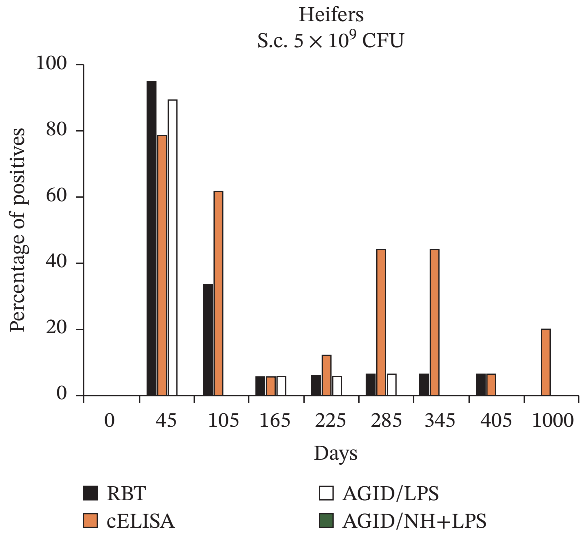
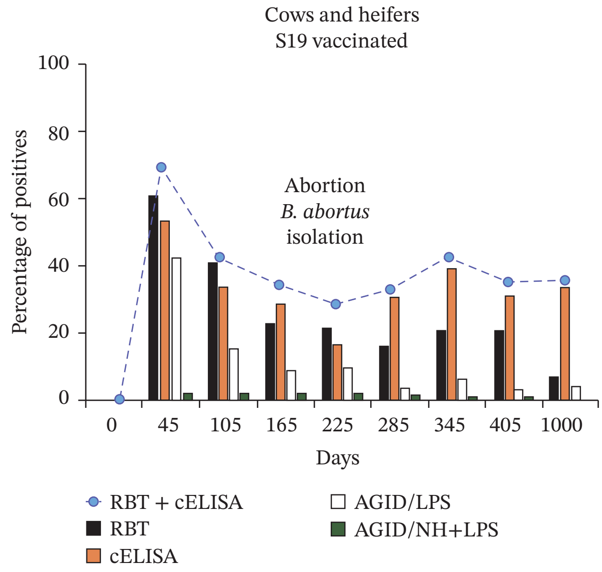


FIGURE 3 | (Continued)



(E)



(F)

FIGURE 3 | Proportion and evolution of S19-vaccinated bovines reacting against different serological tests in Farm 2. Evolution of the antibody response in time to different serological tests of cows (A and C) and heifers (B, D, and E) that were vaccinated subcutaneously (S.c.) (A, B, and E) or conjunctively (Conj.) (C and D) with S19. The evolution of all bovines of Farm 2 vaccinated with S19 (independently of the dose and route) was positive for RBT + cELISA (F). Notice that in (F), the antibody response shows biphasic kinetics due to vaccination (peak at 45 days) and boosting effect with field *B. abortus* (peak at 345 days). Vaccination with complete (5×10^{10} CFU) or reduced (5×10^{9} CFU) doses is indicated. The cELISA/RBT ratio in (F) from day 45 to 225 is 0.9% ± 0.2%, while from day 345 to 1000 corresponds to 2.5% ± 1.6%. All animals were kept together in infected Farm 2 for 1000 days.

Farm 2 increased (Figure 3F), attaining its maximum at 345 days, indicating an anamnestic response of the herd upon contact with field *Brucella*.

It is worth noting that, independently of the S19 vaccination scheme of bovines in Farm 2, a higher overall ratio of cELISA/RBT reactors was observed in the grouped values from days 285 to 1000 (2.5% ± 1.6%) (after the abortion event) than in preceding days (0.9% ± 0.2%). Likewise, the correlation values between the RBT and cELISA were lower from 285 to 1000 days ($\kappa < 0.50$) than on previous dates ($\kappa = 0.60$–0.79) (Figure 3F). The proportion of cELISA-positive bovines increased relative to RBT-positive animals after day 225, adding further doubts about the usefulness of the cELISA as a "confirmatory" test. While RB51 vaccination status was documented for contextual completeness, a systematic evaluation of its immunological or epidemiological implications falls outside the scope of this work. No other field, *Brucella* was isolated despite repeated attempts, and no other cow aborted during the study. However, a *B. abortus* RB51 was isolated from the milk of one RBT-negative but cELISA-positive cow, showing that the RB51 vaccine can be shed during a protracted period after immunization and induces positive cELISA responses.

The interrater reliability κ values between tests were estimated in cases where data allowed analysis (Table 3). The overall κ coefficients among the RBT and cELISA in the population of bovines from Farm 2 varied from none to barely moderate ($\kappa = 0.16$–0.61 [0%–35%]) levels (Table 3). Again, this inconsistent agreement reflects the poor reliability of the cELISA as a "confirmatory assay" in the S19-vaccinated animals of Farm 2 (Figure 3). Overall, there is no detectable influence of previous RB51 vaccination on the serological response against the AGID/NH+LPS after S19 vaccination (regardless of the route). Indeed, the κ coefficients among the various tests varied from none to weak ($\kappa < 0.55$).

The results of the animals reacting positively in the AGID (either AGID/LPS positive or AGID/NH+LPS positive) after vaccination in the infected farm are described in Figure 4. AGID/NH+LPS positive responses appeared throughout the serological follow-up in seven cows and five calves, corresponding to ~5% of the herd. All these animals were also RBT, cELISA, and AGID/LPS positive on all sampling dates after vaccination.

One subcutaneously vaccinated cow (574/5) and two calves (18161 and 18168) gave a positive AGID/NH+LPS reaction on day 45, probably due to immunoprecipitating antibodies produced early after vaccination that disappeared afterward. One cow (587/5) was AGID/NH+LPS positive on day 45 and again on day 345, indicating infection or reactivation close to 1 year after vaccination. Bovines (7338, 10517, 8044, 10002, 18162, 18174, and 18166) that precipitated NH in later days are considered infected. The cow 7338 was RBT, cELISA, and AGID/NH+LPS positive before and on the date it aborted. Since immunoprecipitating antibodies against NH correlate with the shedding of *Brucella* organisms [11–13], these are the most epidemiologically relevant animals.

4 | Discussion

We have demonstrated that under conditions of S19 mass vaccination, (i) the serial testing of RBT-positive bovines with cELISA as a "confirmatory assay" is not recommended due to confusion in interpreting the serological results; (ii) under conditions of S19 mass vaccination, conjunctival administration induced fewer persistent antibodies, suggesting it may minimize diagnostic interference in the application of control and eradication programs; (iii) the NH-test helps identify actively infected cows in brucellosis endemic areas subjected to S19 mass vaccination; and

	Identification	Vaccine route and dose	Age at day 0 (years)	AGID results at the indicated postvaccination days								
				0	45	105	165	225	285	345	405	1000
Cows	574/5	S.c. 5×10^9 CFU	14.20		++	++					ND	
	587/5	S.c. 5×10^9 CFU	3.80		++	++	++	++	++	++	ND	+,+
	7338	S.c. 5×10^9 CFU	11.80		++	++	++	Abort	ND	ND	ND	ND
	10517	S.c. 5×10^9 CFU	8.50		++	++	++	++	++	++	++	ND
	71/6	S.c. 5×10^9 CFU	13.20		++	++	++	++	++	++	ND	ND
	8044	S.c. 5×10^9 CFU	11.00		+	+	++	+	++	++	++	ND
	10002	Conj. 5×10^9 CFU	8.50		++	++	++	++	++	ND	ND	ND
Heifers	18161	S.c. 5×10^{10} CFU	0.30		++	++	++					ND
	18162	S.c. 5×10^{10} CFU	0.30		++	++	++	++	++	++	++	ND
	18168	S.c. 5×10^{10} CFU	0.25		++	++	++	++	++	++	++	
	18174	S.c. 5×10^{10} CFU	0.25		++	++	++	++	++	++	++	+
	18166	S.c. 5×10^{10} CFU	0.26		++	++	++	++	++	++	+	ND
Number of bovines												

FIGURE 4 | Characteristics of the animals immunoprecipitating in the AGID with the NH+LPS antigen after vaccination with S19 in the infected Farm 2. AGID negative reactions: empty boxes in gray color; AGID/LPS positive reactions: boxes in blue; AGID/NH+LPS positive reactions: boxes in orange; boxes in green: culled bovines; positive RBT (+ [in red]); positive cELISA (+ [in blue]); abortion (abort); no serum available (ND). None of the bovines included in the table were previously vaccinated with RB51. At the time of abortion, cow 7338 was positive in both RBT and cELISA tests. Conj., conjunctively; S.c., subcutaneously.

(iv) the previous vaccination with RB51 did not modify the serological results of S19-vaccinated bovines.

Most bovines are protected against brucellosis independently of the S19 vaccination protocol and vaccine dose [3–5, 15, 16, 21–26]. However, this protection is not absolute, as revealed by an abortion registered during the 1000-day trial in Farm 2. Still, the serological response varies depending on whether the vaccinated animals are calves, adults, or subcutaneously immunized with complete or reduced doses, or through the conjunctiva, and whether the bovines inhabit a brucellosis-free or a *Brucella*-endemic environment.

Conjunctival vaccination has advantages over other protocols since it induces lower and less persistent antibodies, indicating that this route is the most suitable for brucellosis control programs, either in combination with test and slaughter or after mass vaccination [2–5]. Although protective, subcutaneous S19 vaccination of brucellosis-free heifers is less suitable for eradication programs since a significant proportion of bovines remain positive in serological tests, leading to the slaughtering of false-positive protected animals.

Mass vaccination with S19 aims to interrupt the transmission cycle of brucellae and, with time, reduce the brucellosis prevalence to a minimum to initiate programs based on test and slaughter combined or not with the vaccination of young replacement heifers [2]. Although most vaccinated bovines are protected under this strategy [1–4, 14], interpreting serological tests becomes extremely difficult in endemic conditions where field *B. abortus* coexists with S19 vaccination. These findings highlight that the epidemiological situation—whether a herd is free of brucellosis or in an endemic region—directly affects cattle immune status and the apparent efficacy of vaccination, underscoring the need to interpret diagnostic results and control strategies within their specific epidemiological context. This problem is due to boosting effects in the antibody responses of vaccinated animals due to previous contact with field brucellae. In all likelihood, this was the case of Farm 2, regardless of the vaccination scheme. However, even under these conditions, the conjunctival route was the strategy that induced the lower antibody responses.

Proposals to interpret serology under problematic conditions similar to Farm 2 are based on feeble and unreliable assumptions about the so-called "confirmatory tests" for distinguishing vaccinated from *Brucella*-infected bovines [2]. Indeed, over the years, several investigators have argued that cELISA is a "confirmatory" test capable of differentiating vaccinated from infected cattle [18, 19, 27], an assumption reflected in the guidelines of at least some makers (e.g., https://www.svanova.com/; https://www.bionote.co.kr/). The terms "screening" and "confirmatory" must be used cautiously, as LPS-based assays cannot reliably distinguish infection from vaccination in S19-immunized adult herds. The cELISA, correctly standardized, identified all positive and negative control sera under controlled laboratory conditions. However, it was not fully specific even when testing sera from brucellosis-free heifers maintained in a brucellosis-free environment of Farm 1. Moreover, regardless of the S19 dose, route, or reproductive status of the bovines, the number of cELISA reactors in Farm 2 surpassed the RBT-positives after day 225. This should not be interpreted as cELISA yielding false positives, but rather as evidence that the disagreement between tests complicates the interpretation of serial testing strategies under conditions of S19 mass vaccination in endemic areas. Because this was an observational study, we cannot determine the intrinsic diagnostic performance of these assays, nor can we conclude on their definitive role in eradication programs. Instead, our findings underscore the interpretive challenges that arise when applying standard serological schemes in mass-vaccinated herds. A similar conundrum applies to other binding tests, such as iELISA, FPA, and complement fixation (CFT), claimed also to be "confirmatory" brucellosis assays [2, 6]. These diagnostic interpretation challenges, while common to many disease control programs, are particularly relevant in the context of brucellosis when mass vaccination with S19 is implemented in endemic areas [2, 6].

Under the conditions of mass S19 vaccination, the wiser approach is to procure a rational line of thought according to the epidemiological setting [2, 28]. Indeed, serological testing for a protracted time with conventional assays (e.g., RBT, iELISA, cELISA, FPA, and CFT) is not recommended in the scenarios of mass vaccination in endemic areas. This decision-making seems counterintuitive; however, the rationale is based on the assays' diagnostic confusion after mass vaccination in endemic areas for a protracted period. Nevertheless, AGID/NH+LPS precipitation tests may still be valuable in these epidemiological scenarios since bovines reacting against NH are the most hazardous animals from an epidemiological standpoint because immunoprecipitating NH correlates with an active infection and bacterial shedding [11–13]. Because AGID/NH+LPS reactivity is strongly associated with active infection and bacterial shedding (as seen in cow 7338 from Farm 2), this assay is suitable for identifying epidemiologically relevant animals in settings similar to Farm 2. It can support targeted removal of high-risk cows. This strategy has successfully been used in the brucellosis eradication program in Zaragoza, Spain, and was broadly tested in the USA at the end of the period of the eradication program [2, 12, 13, 29] and was recommended as a serological assay by the World Organization for Animal Health (OIE) [30]. Of note is that this culling strategy was not undertaken in this work because our study aimed to determine the evolution of the serological responses in the cumbersome epidemiological conditions of Farm 2, in which S19 vaccinated animals were maintained in an endemic environment.

The AGID/NH+LPS assay is valuable for identifying epidemiologically relevant animals (those likely to be actively infected and shedding *Brucella*) due to the distinct immunoprecipitation pattern of NH, which is absent in S19-vaccinated animals after 2–3 months but present in infected ones. This makes AGID/NH+LPS a surrogate marker of active infection. This performance pattern has supported its implementation in eradication programs to guide targeted culling and reduce unnecessary slaughter, reinforcing its value as a surveillance tool during mass S19 vaccination [2, 6, 11–13].

We did not find any systematic study describing the effects that RB51-immunized cattle have on S19 vaccination. However, RB51 vaccination did not improve the outcome of *Brucella* infection of the herd in Farm 2 living in an endemic environment. Indeed, before testing/slaughter and S19 vaccination were implemented in Farm 2, there was a high brucellosis prevalence and high abortion rates. Moreover, the fact that RB51 was isolated in one animal indicates that this rough vaccine was circulating in the herd for a prolonged period. This event is relevant because this rough vaccine may hamper the interpretation of diagnoses, induce abortions in cattle, and infect humans [31]. RB51 vaccination does not affect LPS-based assays (e.g., RBT/cELISA). However, as revealed by its shedding, the RB51 infection may induce non-LPS antibodies detectable in some binding tests, such as ELISAs, consistent with WOAH warnings [1, 6, 31]. These findings reinforce that vaccination alone is insufficient to eradicate brucellosis. Sustainable control requires integrated measures, including removal of infected animals, restriction of animal movements, and strict biosecurity practices [2, 10]. Moreover, although RB51 vaccination can occasionally induce abortions when administered to pregnant cattle, reported rates are low [1]. Finally, and most importantly, no single serological test should be solely relied upon for diagnosing brucellosis in S19- or RB51-vaccinated herds; interpretation must always be based on assays considering the epidemiological framework [1, 6].

5 | Conclusion

Overall, conjunctival S19 vaccination appears to be the most suitable protocol for minimizing long-term serological interference, as it induced fewer and less persistent antibody responses than subcutaneous vaccination. Although most vaccinated cattle remained protected, interpreting serology in endemic settings is challenging because antibody responses can be boosted by prior exposure to field *Brucella* spp. Under these conditions, serial testing with RBT followed by cELISA is challenging to interpret, as both assays yield discordant proportions of positive animals over time. In contrast, the AGID/NH+LPS assay proved helpful in identifying animals likely to be actively infected or shedding bacteria, making it a valuable complementary tool in endemic herds vaccinated with S19. While our findings are based on a single observational setting, they support the use of conjunctival vaccination, combined with targeted identification of epidemiologically relevant animals, to improve brucellosis management in similar high-prevalence regions. Broader field studies will be required to confirm the applicability of these findings to other endemic contexts.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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