# Diagnostic performance of Rose Bengal, competitive ELISA, and Native Hapten (NH) assays in S19 mass-vaccinated cattle in endemic brucellosis environment.

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## Abstract

Vaccination with S19 is the best alternative to control bovine brucellosis, but depending on the immunization protocol, it may induce long-lasting antibodies interfering with diagnostic tests. Conjunctival vaccinated brucellosis-free heifers (Farm 1) produced antibodies detected in RBT and cELISA that disappeared <50 days after immunization. In contrast, heifers of Farm 1 subcutaneously vaccinated with S19 reduced dose produced antibodies detected in 10% of the bovines for up to one year. Sera of brucellosis-free vaccinated heifers did not immunoprecipitate native hapten polysaccharide (NH). In contrast, sera of *Brucella*-infected bovines readily immunoprecipitated NH. A *Brucella abortus* infected herd (Farm 2), previously vaccinated with RB51 and subjected to regular tests and slaughter until reaching negative serology, was mass vaccinated with S19 through either the conjunctival or subcutaneous routes with reduced and complete doses. Bovines of Farm 2 displayed sustained and increasing RBT and cELISA positive reactions for a protracted period, with ~5% bovines reacting against NH, indicating an anamnestic response upon contact with field *Brucella*. After an abortion and isolation of field *B. abortus* at day 225 in one cow of Farm 2, a significantly higher ratio of cELISA/RBT reactors was detected (2.5±1.6%) than in preceding days (0.9±0.2). Previous vaccination with RB51 did not modify the herd brucellosis situation or the serological results of S19-vaccinated bovines. We conclude that serial testing of RBT-positive animals with cELISA is not an adequate diagnostic strategy and that the NH immunoprecipitation test helps identify actively infected cows in herds submitted to mass vaccination with S19.

**Keywords**: *Brucella*, brucellosis, *B. abortus*, S19, vaccination, native hapten, NH, RBT, cELISA, bovine.

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## 1. Introduction

Facultative intracellular Gram-negative alpha-proteobacteria 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 since it is also a relevant zoonosis (Moreno et al. 2022). The most common etiological agent is *Brucella abortus*. However, when bovines are reared with infected caprine or ovine herds, *Brucella melitensis* may become predominant and perpetuated in the affected herds if no sanitary actions are undertaken (Aliyev et al. 2022; Verger et al. 1989).

A handful of high-income countries in Europe, North America, and Oceania have eradicated bovine brucellosis through great effort and high costs using *B. abortus* S19 vaccination combined with serological testing and culling the seropositive animals with compensatory actions (Blasco et al. 2020; 2023). This procedure, known as "test and slaughter," is commonly carried out alongside the S19-vaccination (Blasco et al. 2020; 2023). As expected, this is a long-lasting and expensive process that requires well-validated diagnostic tests, suitable vaccination protocols, significant economic investment, knowledge of the epidemiological settings, and a proper understanding of the performance of the diagnostic tools (Blasco et al. 2020; Ducrotoy et al. 2018).

Vaccination with *B. abortus* S19 is the best immunization approach to control bovine brucellosis. Unfortunately, herd vaccination coverage is only moderate to low

in middle- and low-income nations where bovine brucellosis is highly prevalent (Hernández-Mora et al. 2017; Quinteros-Zurita A. 2022). 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 (Hernández-Mora et al. 2017; Blasco et al. 2020; 2023). However, while S19 has been instrumental in controlling and eliminating bovine brucellosis in the countries succeeding with eradication, there is no single country in which bovine brucellosis has been eradicated with the concourse of RB51 (Blasco et al. 2020; 2023).

The most effective strategy to control brucellosis in heavily infected herds is to lower the prevalence by culling as many seropositive bovines as possible, followed by mass S19 vaccination of the remaining cows (Nicoletti 1990). In many endemic circumstances, culling a large proportion of animals is not economically feasible, and mass vaccination is primarily used to lower the prevalence. However, after mass vaccination in endemic areas, standard serological tests become challenging to interpret, and the results are frequently misunderstood, resulting in over-culling of healthy vaccinated animals.

Native Hapten (NH) *Brucella* polysaccharides share the N-formyl perosamine sugar determinants with the lipopolysaccharide molecule (LPS). It has been demonstrated that NH, which in the bacterium 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 (Aragón et al. 1996; Moreno et al. 1981,1987). In addition, 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 (Díaz et al. 1979; Jones et al. 1980; Moreno et al. 1981). Furthermore, these serological reactions are minimized when a reduced dose (5 x $10^9$ CFU) of S19 is applied, mainly when administered through the conjunctival route (Alonso-Urmeneta et al. 1998; Díaz-Aparicio et al. 1993; Ducrotoy et al. 2018; Marin et al. 1999; Moreno 1981; Muñoz et al. 2005; Blasco et al. 2021). In contrast, NH reacts in the AGID test with a high proportion of sera from Brucella-infected bovines, correlating with bacterial shedding (Alonso-Urmeneta et al. 1988a; 1988b; Díaz et al. 1979; Díaz-Aparicio et al. 1993; 1994; Ducrotoy et al. 2018; Jones et al. 1980; Marín et al. 1999; Moreno 1981; Muñoz et al. 2005; WOAH 2023). 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 that are likely shedding the bacterium) in infected herds submitted to S19 mass vaccination. Here, we present a descriptive study on the performance of the RBT, competitive ELISA (cELISA), and AGID with NH-LPS-rich extracts in bovines immunized with *B. abortus* S19 through various vaccination protocols in brucellosis-free and *B. abortus* infected herds.

## 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 by CFU

121 counting, absence of dissociation, and determination of residual virulence in mice
122 following WOAH protocols (Grillo et al. 2000; WOAH 2023). The following groups of
123 sera were used: Positive control sera (used for test validation) were from 35 naturally
124 infected cows from Costa Rica showing a *B. abortus* positive culture (Hernández-
125 Mora et al. 2017). Negative control sera (used for test validation) were obtained from
126 35 non-vaccinated dairy cows from a brucellosis-free herd in a Costa Rica region
127 where no brucellosis has been recorded for at least 25 years. Sera from Farm 1
128 corresponding to a total of 25 crossbred Angus-Zebu brucellosis-free heifers (8 -11
129 months of age) located in a *Brucella*-free environment in San Carlos, Alajuela, Costa
130 Rica which were vaccinated with a reduced dose (5 x$10^9$ CFU) of the *B. abortus* S19
131 vaccine applied either by the subcutaneous (n= 20 heifers) or conjunctival (n=5
132 heifers) routes, as described before (Blasco et al. 2020; Chacon-Díaz et al, 2020).
133 The vaccinated animals of Farm 1 were permanently maintained in the same
134 brucellosis-free herd during the experiment and bled at intervals after vaccination
135 through an 1100-day observational period (Table 1).

136 Sera from Farm 2 corresponds to a collection obtained from Holstein-Zebu
137 crossbred bovines inhabiting a brucellosis endemic area in Guácimo, Limón
138 (Caribbean, Huetar region), Costa Rica. During 2016-2017, the herd of Farm-2 was
139 heavily *Brucella*-infected with a high individual prevalence (with a maximum of 47%
140 assessed with both the RBT and cELISA tests) and high abortion rates (with a
141 maximum of 30%), with recurrent isolation of *B. abortus* biovar 1 always of the same
142 genetic cluster (Hernandez-Mora et al. 2017). A significant proportion of bovines
143 from Farm 2 were previously vaccinated and revaccinated with standard doses of
144 the RB51 vaccine (Colorado Serum Co, USA) applied at 4-5 months of age and

145 revaccinated as adults vaccine (Colorado Serum Co, USA) without obtaining any
146 significant reduction in the seroprevalence or abortion rate. At the beginning of 2017,
147 RBT and cELISA testing was initiated following the national mandatory procedures
148 of SENASA, Costa Rica (Hernandez-Mora et al. 2017), resulting in the culling of a
149 total of 593 at the end of 2018. Of these, 345 were culled due to positivity in
150 brucellosis tests and 248 due to pregnancy loss/infertility. A remanent of 253 bovines
151 of Farm 2 with no record of abortions, which tested negative in both RBT and cELISA
152 and were negative for *Brucella* spp. by a repeated culture of milk samples, were
153 vaccinated with S19 following various protocols (Table 1). Ninety-four of these 253
154 animals had been previously vaccinated during 2016-2017, with RB51 (Table 1).
155 Following the S19 vaccination of the 253 bovines of farm 2, blood samples were
156 taken at intervals over 13 months. Sera was just tested after 13 months of S19
157 vaccination with RBT, cELISA, and AGID and, after that, at 1000 days. During the
158 observational period, 81 bovines (out of 253) were culled due to lack of pregnancy
159 as determined by palpation (Table 2). One cow aborted at day 225 of pregnancy,
160 yielding a positive culture for *B. abortus* biovar 1 field strain.

161 The sera collections were kept under -80ºC at the Bacteriology Laboratory at
162 the National Service of Animal Health (SENASA) of Costa Rica until tested. All sera
163 were coded, and the identity of each serum was not revealed until all results were
164 analyzed.

165

166 ***2.2 Bacteriological studies and serological assay.***

167 Searching for *Brucella* infection in milk, vaginal secretions and fetuses was
168 performed using the Farrell's and CITA media and the culture as described (De-

169 Miguel et al. 2011). Cultures were incubated in a 10% $CO_2$ atmosphere at 37°C for
170 at least two weeks. The identification of suspected colonies was carried out by
171 conventional bacteriological procedures and genetic analyses (Hernández-Mora et
172 al. 2017; Suárez-Esquivel et al. 2020).

173 The RBT (ID.Vet, France) was performed as described elsewhere (Alton et
174 al. 1988). The test was validated (showing 100% diagnostic sensitivity and
175 specificity) with the control positive and negative serum populations (see above) and
176 considered positive when rendering any agglutination after four minutes (Alton et al.
177 1988). Competitive ELISA (BioNote. Inc., Korea) was performed and standardized
178 according to the procedures described before (Moreno et al. 1998), and the cut-off
179 established at 30%, coincident to the value currently accepted for diagnosing bovine
180 brucellosis by the National Service of Animal Health (SENASA) of Costa Rica
181 (Hernández-Mora et al. 2017), which resulted in 100% diagnostic sensitivity and
182 specificity when using the control sera.

183 The AGID test was performed as described elsewhere (Marín et al. 1999).
184 Briefly, the antigen preparation rich in NH and LPS was a soluble lyophilized extract
185 from *B. melitensis* 16M, obtained as described elsewhere (Aragón et al. 1996).
186 Before use, the antigen preparation was reconstituted with deionized water. One
187 percent of Noble Agar (Difco) in borate buffer (pH 8.3) containing 10% NaCl was
188 used for immunodiffusion. A volume of 11 ml of hot liquified agarose solution was
189 placed in a plastic Petri dish of 100 mm x 15 mm and let solidify for 30 min at room
190 temperature and 1 h at 4ºC. The thickness of the gel in the plate was 1 mm, allowing
191 to cut four rosettes of six wells around a central one inside the plate (3 mm-diameter
192 wells set 3 mm apart). Each well was filled with 16 µl of the antigen (central well) or

193 serum samples (external wells) for immunodiffusion (Moreno et al. 1981). The
194 optimal antigen concentration for the AGID test was established at 1mg/ml by serial
195 dilutions against the control positive sera (Muñoz et al. 2005). Gel diffusion plates
196 were incubated at room temperature inside a wet chamber and read at 24, 32, and
197 48 hours to test the reactivity of sera against NH and/or LPS (Marín et al. 1999).
198 Before the 48h reading, the plates were soaked in a 5% sodium citrate solution for
199 1 hour to clear potential unspecific reactions (Muñoz et al. 2005). The same person
200 recorded the precipitin lines in all samples using a dark box with indirect light coming
201 from the bottom. The AGID reaction generated the following possible results (Fig 1):
202 i) precipitation lines against only the LPS close to the antigen well (henceforth
203 AGID/LPS positive) were interpreted as due to infection or vaccination; ii)
204 precipitation lines against both NH (close to the serum well) and LPS (henceforth
205 AGID/NH+LPS positive) were interpreted as a very recent vaccination or as infected
206 with *Brucella* and possible shedding of the bacterium, and; iii) no precipitation lines
207 (AGID negative).
208
209 ***2.3. Statistical analysis***
210 Interrater reliability against different assays and between vaccinated animal
211 populations was achieved by calculating Cohen's kappa coefficient (κ) (McHugh
212 2012), with a Confidence Interval of 95%. The level of agreement according to the κ
213 values and the corresponding proportion of reliable data (within parenthesis)
214 followed the interpretation as follows (McHugh 2012): none=0-0.2 (0-4%);
215 minimal=0.21-0.39 (4-15%); weak=0.4-0.59 (15-35%); moderate=0.6-0.79 (35-
216 63%); strong=0.8-0.9 (64-81%); almost perfect>0.9 (82-100%).

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## 3. Results

### *3.1. The diagnostic sensitivity and specificity of sera from B. abortus infected and brucellosis-free cows.*

With positive and negative control sera, the RBT, cELISA and AGID/LPS scored 100% diagnostic specificity and 100% diagnostic sensitivity. The AGID/NH+LPS resulted in 100% diagnostic specificity and 94.3 % diagnostic sensitivity. However, the two sera from infected cows that resulted negative in 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.

### *3.2. Diagnostic performance after B. abortus S-19 vaccination in brucellosis-free heifers*

The evolution of the proportion 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 (Fig 2A) induced a significantly lower and shorter serological response than subcutaneous vaccination (Fig 2B). The conjunctively vaccinated heifers became negative in all tests seven weeks after vaccination (Fig 2A). Moreover, no conjunctively vaccinated animal was either AGID/LPS positive or AGID/ NH-LPS positive. In contrast, the serological response induced in the subcutaneously vaccinated heifers was of high intensity and duration (Fig 2B), with 20% of the animals remaining positive after one year, 5% after two

241 years and none after three years. Although the proportion of reactors in the RBT in
242 the subcutaneous vaccinated group was slightly higher than the cELISA from day 70
243 on, the correlation of the grouped assays between both tests was similar throughout
244 the experiment ($\kappa$=0.81) (Table 3), inquiring about the alleged higher specificity of
245 the cELISA for discriminating the S19 vaccinated cattle (Fig 2B). A moderate to high
246 proportion of the subcutaneously vaccinated heifers was AGID/LPS positive until day
247 80, but none reacted against the NH (Fig. 2B).
248
249 ***3.3. Diagnostic performance after S-19 vaccination of the B. abortus-infected***
250 ***herd***
251 The first RBT and cELISA analyses in Farm 2 were performed just one year
252 after the S19 vaccination, while the AGID responses were studied 1000 after the
253 S19 vaccination. About one year after vaccination (day 345), 39 animals were culled.
254 After this time, 42 additional bovines were culled, with 172 bovines remaining after
255 1000 days (Table 2). Although all cows in this infected farm were serologically
256 negative just before S19 vaccination, they displayed positive antibody responses in
257 the various tests after S19 vaccination (Fig. 3). Overall, the serological responses
258 were higher and more persistent in cows and heifers vaccinated subcutaneously (Fig
259 3A, B and E). The evolution of the proportion of reactors in the cows vaccinated
260 subcutaneously with the reduced doses (Fig 3A) was closely similar to that of the
261 heifers vaccinated subcutaneously with the standard dose, with a high number of
262 serologically positive animals (mainly in the cELISA) until the end of the
263 observational period. The subcutaneous vaccination route caused the lowest
264 diagnostic specificity of all serological tests, with some animals positive even in the

AGID/NH+LPS for a protracted interval after vaccination (Fig 3A and B). In contrast, the lowest serological reactivities were obtained in conjunctively vaccinated animals (Fig 3 C and D). None of the heifers vaccinated through this route generated positive AGID/NH+LPS results (Fig 3D). Similar results were obtained in the cows vaccinated conjunctively (Fig 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. This last test showed the lowest specificity to interpret the serological response of the vaccinated animals in the infected herd. 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 cows vaccinated subcutaneously with a reduced dose of S19 (see arrow in Fig 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 reactors of Farm 2 increased (Fig 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 day 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 in previous dates ($\kappa$=0.60-0.79) (Fig 3F). This cELISA increase related to the RBT assay after day 225 adds further doubts on

289 the usefulness of the cELISA as a "confirmatory" test. No other field *Brucella* was
290 isolated despite repeated attempts, and no other cow aborted during the study.
291 However, a *B. abortus* RB51 vaccine strain was isolated from the milk of one RBT-
292 negative but cELISA-positive cow, showing that RB51 can be shed during a
293 protracted period after vaccination and induces positive cELISA responses.

294 The interrater reliability κ values between tests was estimated in cases where
295 data allowed analysis (Table 3). The overall κ coefficients among the RBT and
296 cELISA in the population of bovines from Farm-2 varied from none to barely
297 moderate (κ=0.16-0.61 [0-35%]) levels (Table 3). Again, this inconsistent agreement
298 reflects the poor reliability of the cELISA as a "confirmatory assay" in the S19-
299 vaccinated animals of Farm 2 (Fig 3). Overall, there seems not to be a significant
300 influence of previous RB51 vaccination on the serological response against the
301 AGID/NH+LPS after S19 vaccination, regardless of the route. Indeed, the κ
302 coefficients among the various tests varied from none to weak (κ <0.55).

303 The results of the animals reacting positively in the AGID (either AGID/LPS
304 positive or AGID/NH+LPS positive) after vaccination in the infected farm are
305 described in Table 4. AGID/NH+LPS positive responses appeared throughout the
306 serological follow-up in 7 cows and 5 calves, corresponding to ~5% of the herd. All
307 these animals were also RBT, cELISA and AGID/LPS positive at all sampling dates
308 after vaccination. One subcutaneously vaccinated cow (574/5) and two calves
309 (18161, 18168) gave a positive AGID/NH+LPS reaction on day 45, probably due to
310 immunoprecipitating antibodies produced early after vaccination that disappeared
311 afterward. One cow (587/5) was AGID/NH+LPS positive on day 45 and again on day
312 345, indicating infection or reactivation close to one year after vaccination. Bovines

(7338, 10517, 8044, 10002, 18162, 18174 and 18166) precipitating 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 (Alonso-Urmeneta et al. 1988a; 1988b; Díaz et al. 1979; Díaz-Aparicio et al. 1993; 1994; Ducrotoy et al. 2018; Jones et al. 1980; Marín et al. 1999; Moreno 1981; Muñoz et al. 2005; WOAH 2023), these are the most epidemiological relevant animals.

## 4. Discussion

Independent of the S19 vaccination protocol and dose, most bovines become protected against brucellosis (Nicoletti 1990a; 1990b). Only one abortion due to field *B. abortus* was registered during the 1000 trial period in Farm 2, and no other bacterial isolation was noted during this lapse. However, 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.

As shown here, conjunctival vaccination has significant advantages over other protocols since it induces lower and less persistent antibodies detected by diagnostic serological assays, indicating that this vaccination method is the most suitable for programs aimed to control brucellosis, either in combination with test and slaughter or after mass vaccination (Blasco et al. 2020; Nicoletti 1990a; 1990b). In contrast, subcutaneous S19 vaccination of brucellosis free-heifers induced antibody responses detected by serological assays in some animals for a protracted period.

Accordingly, this vaccination method, although protective, is less suitable for eradication programs that combine S19 vaccination of heifers or cows with tests and slaughter since a significant proportion of the animals remain positive in diagnostic serological tests.

Mass vaccination of cattle with S19 aims to interrupt the transmission cycle brucellae and, with time, to reduce bovine brucellosis prevalence to a reasonable minimum to allow initiating programs based on test and slaughter combined or not with the vaccination of young replacement heifers (Blasco et al. 2021). Although most vaccinated animals are protected under this strategy, interpreting serological tests becomes extremely difficult in endemic conditions where field *B. abortus* coexists with S19 vaccination. This problem is due to previous vaccinal or post-vaccinal contacts with field *Brucella* and boosting effects in the antibody responses of vaccinated animals. In all likelihood, this was the case of Farm 2, regardless of the vaccination scheme. However, even under these conditions, the conjunctival route was again the strategy that induced the lower antibody responses.

Proposals to interpret serological tests under problematic conditions similar to Farm 2 are based on feeble and unreliable assumptions on the existence of the so-called "confirmatory tests" for distinguishing vaccinated from *Brucella*-infected bovines (Blasco et al. 2020; Moreno et al. 2022). Indeed, over the years, several investigators have argued that cELISA is a "confirmatory" test capable of differentiating vaccinated from infected cattle (Nielsen et al. 1995; 2007; 2008), an assumption reflected in the guidelines of at least some makers (e.g. (https://www.svanova.com/ ; https://www.bionote.co.kr/). However, it was clear that cELISA 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 vaccination dose, route, or reproductive status of the bovines, the number of cELISA reactors in Farm-2 surpassed the RBT-positives after day 225, which contradicts the confirmatory potential of this test, arguing against using cELISA after "screening" with the RBT. Indeed, if cELISA is intended as a "confirmatory" assay, should the RBT-negative/cELISA-positive be considered infected or non-infected? If estimated infected, there is the danger of over-culling protected animals. Alternatively, If considered non-infected, there is the risk of maintaining infected cows in the herd. A similar conundrum applies to other binding tests, such as iELISA, FPA and complement fixation (CFT), claimed also to be "confirmatory" brucellosis tests (Ducrotoy et al. 2018; Moreno et al. 2022).

Under the conditions of mass S19 vaccination, the wiser approach is procuring a rational line of thought according to the epidemiological setting (Blasco et al. 2020; Moreno et al. 2022; Khurana et al. 2021). For instance, serological testing for a protracted time with conventional assays (e.g., RBT, ELISAs, FPA, 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 there is clear evidence that bovines reacting against NH are the most hazardous animals from an epidemiological standpoint. Indeed, antibodies immunoprecipitating NH correlate with an active infection and bacterial shedding (Alonso-Urmeneta et al. 1998; Díaz et al. 1979; Díaz-Aparicio et al. 1993; Ducrotoy et al. 2018; Jones et al. 1980; Marín

et al. 1999; Moreno 1981; Muñoz et al. 2005). Since the diagnostic specificity of the NH tests for detecting actively infected bovines is high (as in the case of cow 7338 of Farm 2), the AGID/NH+LPS test is suitable under epidemiological conditions similar to that of Farm 2 for the serial testing and culling of the most hazardous cows. This strategy has been successfully applied to eradicate bovine brucellosis in some regions (Blasco et al. 2021). It is important to emphasize 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.

We did not find any systematic study describing the effects that RB51-immunized cattle have on S19 vaccination. Overall, the previous immunization of Farm 2 cows with RB51 did not improve the outcome of infection since, before the test and slaughter and S19 vaccination were implemented, 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 since this rough vacccine may hamper the diagnoses' interpretation, induce abortions in cattle and infect humans (Blasco et al. 2021).

Overall, we conclude that S19 conjunctival vaccination is the most suitable immunization protocol to combat bovine brucellosis, that serial testing of RBT-positive animals with cELISA is not an adequate diagnostic strategy under any epidemiological conditions and that the NH immunoprecipitation test helps identify actively infected cows in herds living in endemic areas and subjected to mass vaccination with S19.

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## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Ethical approval

The authors confirm that the ethical policies of the journal have been followed.

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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 | Nº bovines |
|---|---|---|---|---|
| Farm 1 | Heifers | Conjuntival $5x10^9$ | No | 5 |
| | | Subcutaneous $5x10^9$ | No | 20 |
| Farm 2 | Cows | Subcutaneous $5x10^9$ | Yes | 32 |
| | | | No | 42 |
| | | Conjuntival $5x10^9$ | Yes | 62 |
| | | | No | 44 |
| | Heifers | Subcutaneous $5x10^{10}$ | No | 19 |
| | | Subcutaneous $5x10^9$ | No | 18 |
| | | Conjuntival $5x10^9$ | No | 36 |
| **Total** | | | | **278** |

[a] Conjuntival and subcutaneous S19 vaccinations were carried out as described elsewhere (Balsco et al. 2020, 2023; Chacón-Díaz 2021, WOAH 2023).

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

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. $5x10^9$ CFU | 20 | 0.81 (1.00-0.65) |
| | | S.c. $5x10^9$ CFU | 5 | ND[b] |
| Farm 2 | Cows | S.c. $5x10^9$ CFU | 74 | 0.55 (0.47-0.63) |
| | | Conj. $5x10^9$ CFU | 106 | 0.27 (0.21-0.34) |
| | Heifers | S.c $5x10^{10}$ CFU | 19 | 0.61 (0.46-0.75) |
| | | S.c $5x10^9$ CFU | 18 | 0.55 (0.40-0.71) |
| | | Conj. $5x10^9$ CFU | 36 | 0.16 (0.06-0.25) |

[a] Subcutaneous: S.c; Conjunctival: Conj. The confidence interval was 95%. The numbers within the parenthesis correspond to interval range values.

[b] ND, not performed because values were non-suitable for analysis

Table 4. Characteristics of the animals immunoprecipitating in the AGID with the NH+LPS antigen after vaccination with S19 in the infected Farm 2.

| | 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. 5x10$^9$CFU | 14.20 | | + + | + + | | | | | ND | |
| | 587/5 | S.c. 5x10$^9$CFU | 3.80 | | + + | + + | + + | + + | + + | + + | ND | +,+ |
| | 7338 | S.c. 5x10$^9$CFU | 11.80 | | + + | + + | + + | Abort | ND | ND | ND | ND |
| | 10517 | S.c. 5x10$^9$CFU | 8.50 | | + + | + + | + + | + + | + + | + + | + + | ND |
| | 71/6 | S.c. 5x10$^9$CFU | 13.20 | | + + | + + | + + | + + | + + | + + | ND | ND |
| | 8044 | S.c. 5x10$^9$CFU | 11.00 | | + | + | + + | + | + + | + + | + + | ND |
| | 10002 | Conj. 5x10$^9$CFU | 8.50 | | + + | + + | + + | + + | + + | ND | ND | ND |
| Heifers | 18161 | S.c. 5x10$^{10}$CFU | 0.30 | | + + | + + | + + | | | | | ND |
| | 18162 | S.c. 5x10$^{10}$CFU | 0.30 | | + + | + + | + + | + + | + + | + + | + + | ND |
| | 18168 | S.c. 5x10$^{10}$CFU | 0.25 | | + + | + + | + + | + + | + + | + + | + + | |
| | 18174 | S.c. 5x10$^{10}$CFU | 0.25 | | + + | + + | + + | + + | + + | + + | + + | + |
| | 18166 | S.c. 5x10$^{10}$CFU | 0.26 | | + + | + + | + + | + + | + + | + + | + | ND |
| Number of bovines | | | | | | | | | | | | |

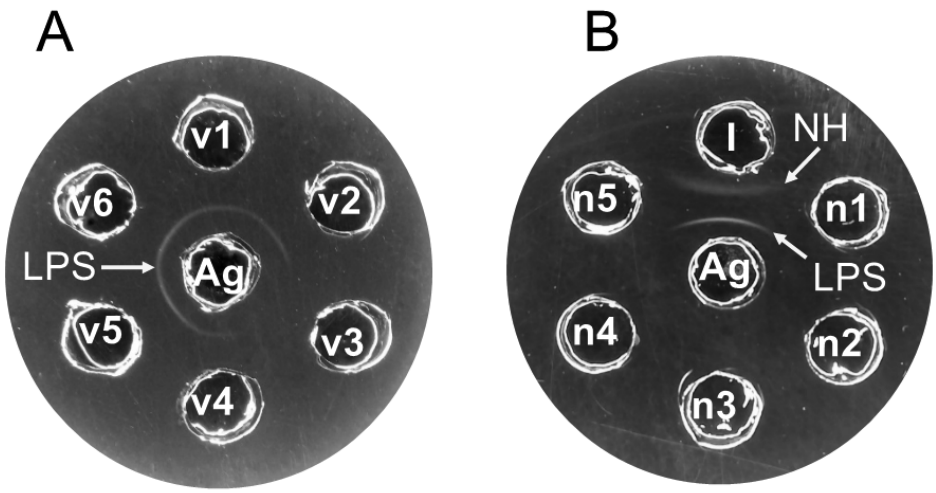
Subcutaneously (S.c.); conjunctively (Conj); AGID negative reactions: empty boxes in gray color; AGID/LPS positive reactions: boxes in blue; AGID/ NH+LPS positive reactions: boxes in orange; boxes in green: culled bovines; positive RBT( **+** ); positive cELISA ( **+** ); abortion (Abort); no serum available (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.

Figure legends,

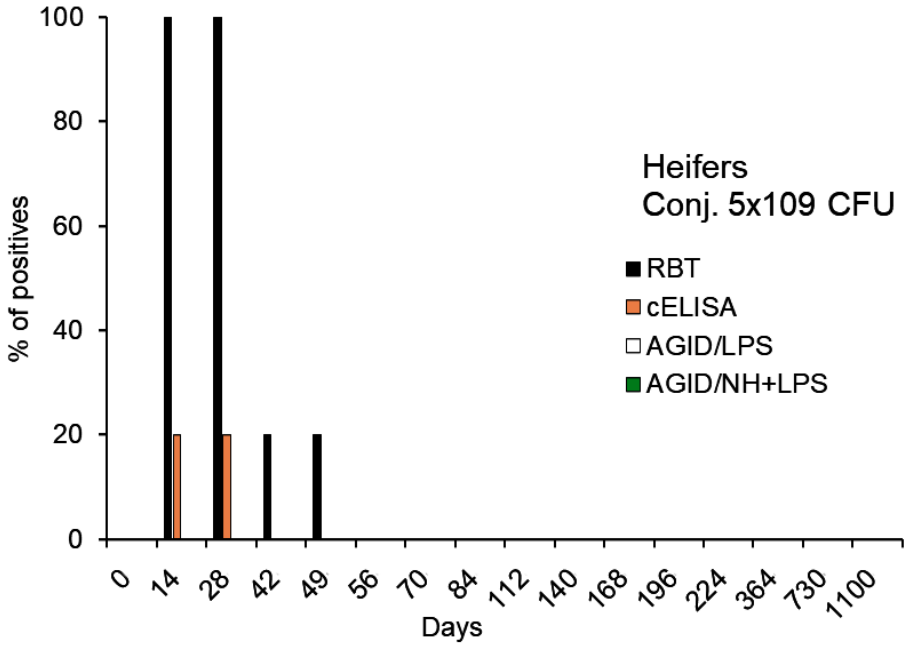
**Fig 1**. **AGID test in infected, S19 vaccinated and negative controls using LPS-NH rich antigen.** A total of 16 μl of 1mg/ml of LPS-NH antigen (Ag) was tested against sera of S19-vaccinated, *Brucella*-positive, and *Brucella*-negative bovines. The sera from S19-vaccinated bovines labeled v1 to v6 were confronted against NH-LPS-rich antigen (A). The sera from one *Brucella*-infected bovine (labeled "I") and five control sera from negative bovines (labeled n1 to n5) were tested (B). In panel A, five S19-vaccinated bovines were AGID/LPS positive but AGID/LPS-NH negative, while one vaccinated animal (v3) was AGID negative. In panel B, the serum of the *Brucella*-infected bovine (I) showed two lines of precipitation (i.e., was AGID/NH+LPS positive) corresponding to the LPS (line closer to the antigen well) and NH (line closer to the serum well).

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

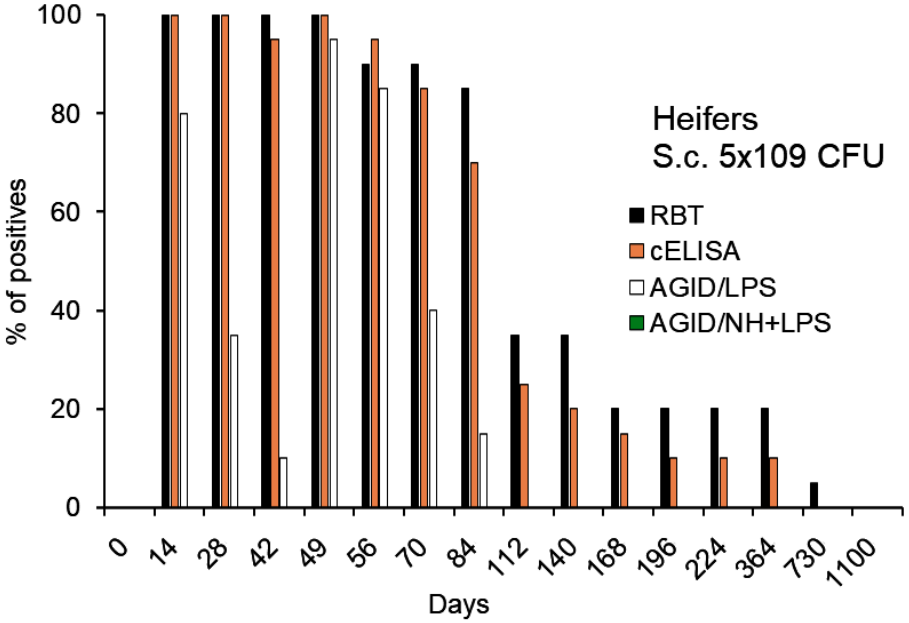
**Fig 3**. **Proportion and evolution of S19-vaccinated bovines reacting against different serological tests in Farm 2**. Evolution of the antibody response in time to different serological tests of cows (A and C) and heifers (B, D and E) that were vaccinated subcutaneously (S.c) (panels A, B and E) or conjunctively (Conj.) (C and D) with S19. Evolution of all bovines of Farm 2 vaccinated with S19 (independently of the dose and route) 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 ($5x10^{10}$ CFU) or reduced ($5x10^{9}$ 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.

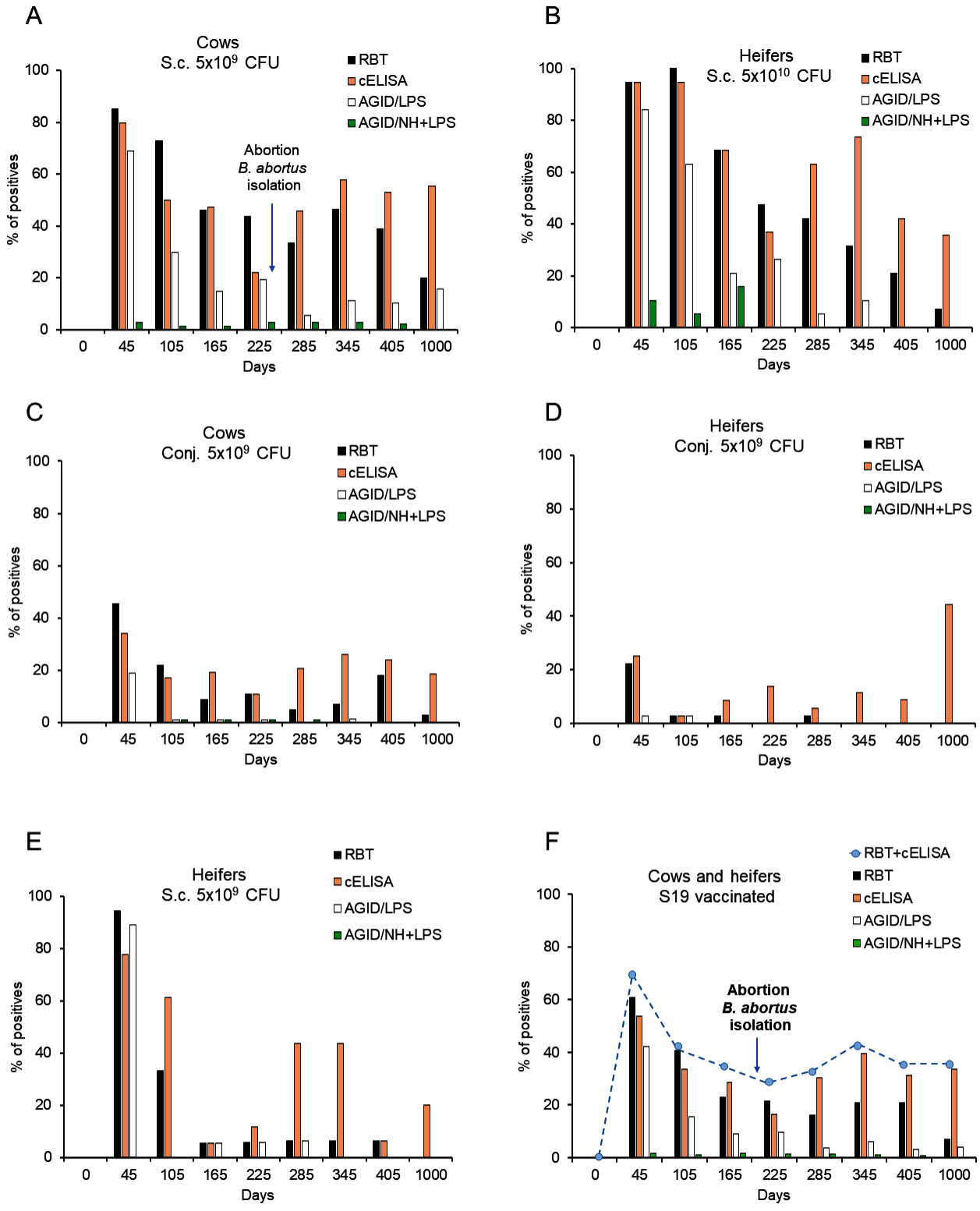

A
v1
v6
v2
LPS
Ag
v5
v3
v4
B
I
NH
n5
n1
Ag
LPS
n4
n2
n3

A


100
80
60
40
20
0
% of positives
Heifers
Conj. 5x109 CFU
RBT
cELISA
AGID/LPS
AGID/NH+LPS
0
14
28
42
49
56
70
84
112
140
168
196
224
364
730
1100
Days

B


100
80
60
40
20
0
% of positives
Heifers
S.c. 5x109 CFU
RBT
cELISA
AGID/LPS
AGID/NH+LPS
0
14
28
42
49
56
70
84
112
140
168
196
224
364
730
1100
Days


A
Cows
S.c. 5x10⁹ CFU
RBT
cELISA
AGID/LPS
AGID/NH+LPS
Abortion
B. abortus
isolation
% of positives
Days
B
Heifers
S.c. 5x10¹⁰ CFU
C
Cows
Conj. 5x10⁹ CFU
D
Heifers
Conj. 5x10⁹ CFU
E
Heifers
S.c. 5x10⁹ CFU
F
Cows and heifers
S19 vaccinated
RBT+cELISA
Abortion
B. abortus
isolation