Specificity dependence between serological tests for diagnosing bovine brucellosis in *Brucella*-free farms showing false positive serological reactions due to *Yersinia enterocolitica* O:9

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Abstract — When brucellosis false positive serological reactions happen in cattle, the serial use of pairs of specificity-correlated serological tests (rose bengal, complement fixation, competitive ELISA) results in specificities lower than expected. In this situation, highly specific tests, such as the indirect ELISA used alone, may be more adequate than serial testing.

Résumé — Dépendance de la spécificité entre les tests sérologiques pour le diagnostic de la brucellose bovine dans les fermes exemptes de *Brucella* obtenant de fausses réactions sérologiques positives attribuables à *Yersinia enterocolitica* O:9 En présence de réacteurs faux positifs à la brucellose chez le bovin, l'utilisation de paires de tests sérologiques qui présentent une corrélation en spécificité (rose bengal, fixation du complément et ELISA compétitif) a comme résultat une spécificité inférieure à celle escomptée. Dans ce cas, l'utilisation unique de tests à spécificité élevée, comme l'ELISA indirect, est plus recommandable que l'analyse en série.

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Brucellosis is a disease of worldwide distribution affecting animals and humans. The eradication of brucellosis is based on the serological testing of animals and the subsequent culling of those that are seropositive for antibodies to *Brucella* spp. Thus, the specificity of the serological tests used is of paramount importance, particularly during the final stages of an eradication program once vaccination has been discontinued and for surveillance purposes in brucellosis-free areas. In these circumstances, tests with the highest possible specificity are required (1,2); the use of at least 2 tests applied serially is usually recommended for maximal specificity, and it is generally accepted that a combination of the rose bengal (RBT) and complement fixation (CFT) tests is the most suitable serial testing scheme (3). In these serial testing strategies, an animal should react as positive in both tests to be considered infected. If test specificities are conditionally independent (the specificity of the 2nd test does not depend on that of the 1st), the resulting expected specificity (Sp) after serial testing should be always higher than the corresponding individual specificities of each test (4). In this case, the expected specificity is expressed as $Sp_{exp} = 1 - (1 - Sp_1)(1 - Sp_2)$, where Sp_1 and Sp_2 are the individual specificities of the 2 tests used. By contrast, if tests are showing specificity dependence, the final specificity obtained is expressed by the formula $Sp_{dep} = 1 - (1 - Sp_1)(1 - Sp_2) - \gamma_{Sp}$, where γ_{Sp} is an estimation of the specificity dependence between the 2 tests (see below for further explanation). Therefore, the serial use of tests showing specificity dependence will result in an overall specificity lower than what it would be if tests were conditionally independent.

Serological tests to detect antibodies to *Brucella* spp. are generally based on the determination of similar immunological events (5). Accordingly, when multiple tests are used, their corresponding sensitivity, specificity, or both, results would be showing some degree of dependence (6). However, there are subtle differences among the different serological tests for antibodies to *Brucella* spp. and it seems relevant to assess the degree of specificity dependence between tests to obtain an unbiased

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Table 1. Percentages of sensitivity (Se) and specificity (Sp), and their 95% confidence intervals (CI) of the 4 serological tests used for serial testing analyses

Test	Antigen	Cut-off value[a]	Se (95% CI)	Sp (95% CI)
RBT	*B. abortus* whole cells	—	100 (96.7–100)	86.4 (79.1–91.9)
CFT	*B. abortus* whole cells	—	100 (96.7–100)	94.4 (88.8–97.7)
cELISA	*B. abortus* S-LPS	> 31.66	88.3 (82.7–92.6)	86.4 (79.1–91.9)
iELISA	*B. melitensis* S-LPS	> 93.11	98.9 (96.2–99.8)	100 (97.1–100)

RBT — rose bengal test; CFT — complement fixation test. All sera having ≥ 20 international CFT units were considered as positive; cELISA — competitive enzyme-linked immunosorbent assay; iELISA — indirect enzyme-linked immunosorbent assay; B — *Brucella*; S-LPS — *Brucella* smooth lipopolysaccharide
[a]Cut-offs for ELISAs were chosen to result in the maximum sum of sensitivity and specificity

estimate of what the final specificity of serial testing should be (6). The eradication and surveillance programs for bovine brucellosis are strongly influenced by the presence of false positive serological cross-reactions (FPSR) due to other gram-negative bacteria sharing antigenic determinants with the *Brucella* O-chain. These bacteria include *Vibrio cholerae* O1, *Escherichia coli* O:157, some strains of *Escherichia hermanni* and *Stenotrophomonas maltophila, Salmonella* group N (O:30), and *Yersinia enterocolitica* O:9, but only the FPSR due to *Y. enterocolitica* O:9 seem to be relevant in the routine diagnosis of bovine brucellosis (7). Although several serological tests can be applied for the diagnosis of bovine brucellosis, only the RBT/CFT combination and the indirect (iELISA) or competitive (cELISA) enzyme-linked immunosorbent assays are widely used in eradication campaigns or for international trade testing purposes (3). However, the RBT/CFT combination, the most widely used serial scheme, has been shown to lack specificity for differentiating brucellosis-infected animals from FPSR animals (2,7,8).

The aim of this paper is to determine the specificity dependence among dual combinations of the RBT, CFT, iELISA, and cELISA and to calculate their final serial specificities in brucellosis-free herds affected by FPSR.

The blood sera of 189 cows naturally infected with *Brucella* spp. (assessed by a *Brucella* sp.-positive culture in all cases) were used as the positive control population. Sera of 125 cows from several herds that were brucellosis-free, but affected by the FPSR problem, were used as negative controls. Detailed information on the origin of the above sera has been given elsewhere (2,4). The RBT and CFT were carried out according to standard procedures (9). The iELISA and cELISA that were used have been also described in detail elsewhere (2). The cut-off values selected for both ELISAs were those resulting in the maximum sum of the sensitivity (calculated with the sera from the culture positive cattle) and specificity (calculated with the sera from the *Brucella*-free cattle affected by the FPSR) (10). The sensitivity and specificity results of these tests with both control populations are given in Table 1. Both the RBT and CFT showed 100% sensitivity to detect antibodies in sera from culture-positive animals but lacked specificity when testing the sera from *Brucella*-free cattle belonging to herds affected by FPSR. As discussed elsewhere (2), this unusually high sensitivity reflects the origin of the particular set of control positive sera used in this study and does not invalidate meaningful comparisons. The cELISA resulted in the lowest sensitivity and specificity results of all the tests compared (Table 1). It has been reported by others that this cELISA was not specific for differentiating *Brucella* spp. from *Y. enterocolitica* O:9 infections in cattle (11). On the other hand, at the selected cut-off, the iELISA resulted in 100% specificity, while maintaining an acceptable sensitivity.

Specificity dependence between 2 tests can be assessed through the estimation of the conditional covariance for specificity (γ_{Sp}), which is calculated as $\gamma_{Sp} = p - Sp_1Sp_2$, where p is the observed proportion of noninfected animals that are negative on both tests, and Sp_1 and Sp_2 are the individual specificities of the 2 tests used (6). When test specificities are conditionally independent, the γ_{Sp} is zero. We estimated these γ_{Sp} values for each pair combination along with their corresponding 95% confidence intervals using an appropriate software template. Conditional dependence between specificities is considered significant if 0 is excluded from the γ_{Sp} 95% confidence interval. Because covariance values for specificity are highly influenced by the value of the corresponding individual specificities and do not provide a direct measure of the magnitude of the dependence, it is more useful to use the degree of specificity dependence (ψ), calculated as a proportion of the γ_{Sp} obtained with respect to the maximal γ_{Sp} value possible (6). When (ψ) is equal to 1, a complete dependence exists between tests. Once dependence is demonstrated to exist between tests, the final specificity of the corresponding serial combination is calculated as described above.

Because serial testing is required only when the individual specificity of tests is not 100%, we excluded serial analysis with the iELISA and analyzed only the RBT/CFT, RBT/cELISA, and CFT/cELISA combinations (Table 2). The 3 test combinations that were analyzed showed significant positive specificity dependence, and their corresponding expected (Sp_{exp}) and final (Sp_{dep}) specificities are shown in Table 2. The RBT/CFT serial testing, currently recommended by the Office International des Épizooties (OIE) (3), showed a γ_{Sp} of 0.04 and resulted in the highest degree of dependence (ψ = 0.83). After adjusting for this specificity dependence, the RBT/CFT serial testing scheme resulted in a final specificity considerably lower than the expected specificity if tests were conditionally independent (95.2% versus 99.2%, respectively) (Table 2). This high specificity dependence seems logical, since both tests use the same *B. abortus* whole cells as antigen and detect mainly antibodies of the IgG isotype (5). The dependence

Table 2. Specificity covariance (γ_{Sp}) and its 95% confidence interval (CI), degree of specificity dependence (ψ), and final (Sp_{dep}) and expected (Sp_{exp}) specificities for each of the serial testing combinations analyzed

Test combinations	γ_{Sp} (95% CI)	ψ[a]	Sp_{dep}[b]	Sp_{exp}[c]
RBT-CFT	0.04 (0.02–0.05)	0.83	95.2	99.2
RBT-cELISA	0.03 (0.004, 0.06)	0.25	95.2	98.2
CFT-cELISA	0.03 (0.01, 0.05)	0.67	96.2	99.2

[a]Calculated as the ratio: γ_{Sp} obtained /maximal possible γ_{Sp} value
[b]Calculated as $Sp = 1 - (1 - Sp_1)(1 - Sp_2) - \gamma_{Sp}$
[c]Calculated as $Sp = 1 - (1 - Sp_1)(1 - Sp_2)$ (tests' specificities assumed to be conditionally independent)
RBT — rose bengal test; CFT — complement fixation test. All sera having ≥ 20 international CFT units were considered as positive; cELISA — competitive enzyme-linked immunosorbent assay; iELISA — indirect enzyme-linked immunosorbent assay

observed for this serial testing scheme was in the same range as that observed by others when similar test combinations were applied for the diagnosis of swine brucellosis (6), suggesting that the specificity dependence for these 2 serological tests remains similar regardless of the animal species considered. The cELISA/CFT combination resulted in a higher degree of specificity dependence than the cELISA/RBT serial testing scheme (0.67 versus 0.25, respectively), and also in a more significant specificity covariance when considering the 95% confidence interval (Table 2). However, both combinations had a similar impact on reduction of the expected specificity. In both cases, once adjusted for dependence, the final specificity was considerably lower (a 3% decrease) than that expected if tests were independent (Table 2). This positive dependence was likely to happen, because, again, all tests being compared detect mostly antibodies of the IgG isotype directed against the same antigen (the *Brucella* smooth lipopolysaccharide [S-LPS]). A possible explanation for the lower degree of specificity dependence of the cELISA/RBT and cELISA/CFT combinations could be in the ability of these tests to detect antibodies of differing degrees of avidity. While the RBT and the CFT detect antibodies of either low or high avidity, the cELISA detects mostly antibodies of high avidity (5,11). Interestingly, although a lower degree of specificity dependence (0.25) was found for the RBT/cELISA serial testing combination, the final specificity obtained with this scheme was similar to that obtained with the other combinations presenting a higher degree of specificity dependence (Table 2). Accordingly, it is the overall balance between specificity dependence and individual specificities that makes a combination of tests more or less appropriate for a given situation.

Since the iELISA is based on the use of *Brucella* S-LPS antigen and the detection of antibodies, mostly of the IgG isotype, a similar dependence would have been expected between the iELISA and the other 3 tests. However, iELISA allows the selection of a wide range of cut-off values, resulting in 100% specificity (2); thus, the γ_{Sp} was always zero when the iELISA was combined with any other test. If the individual specificity of a test to be used in serial testing is overestimated during the assessment of test accuracy, it may obscure evidence of dependence between tests, giving a false assumption of test independence. For example, if a *Brucella*-free population not subject to FPSR had been used as the negative control, the scenario would have been quite different, since all tests compared, with the exception of the cELISA, would have resulted in 100% specificity (2). Thus, the evaluation of test accuracy must always be performed on populations representative of the real context in which the tests have to be used (2,12). Accordingly, one should be cautious when recommending selected serial testing schemes for the diagnosis of bovine brucellosis, without consideration of different epidemiological situations. In some epidemiological scenarios, like that considered here with the existence of the FPSR problem, certain test combinations can result in a considerable increase in the number of false positive reactors in eradication programs. The assumptions on which serial testing are classically based (a rapid or simple/cheap test used first for screening and a more sophisticated and specific confirmatory test performed only on samples that are positive on the screening test) may not be adequate for brucellosis eradication or maintenance of brucellosis-free areas when the FPSR problem appears. The iELISA used here has been reported as a very sensitive and specific test for the diagnosis of brucellosis (2,13). When applied as a unique test, this iELISA would result in better performance than the classical RBT/CFT or any other combinations in the epidemiological context analyzed. In conclusion, when serial testing is considered in low-prevalence or brucellosis-free areas, the specificity dependence of tests should be determined by using the epidemiologically appropriate control sera.

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