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Brucellosis surveillance in Bavarian wild boar: Evaluation of a novel rLPS/sLPS-based ELISA compared to OIE-listed serological tests

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ABSTRACT

Introduction: Brucellosis is a globally significant zoonotic disease, caused by *Brucella spp.*, with wildlife reservoirs such as wild boars posing a potential threat to brucellosis-free livestock populations and public health. Despite eradication of brucellosis in domestic animals in Germany, the disease persists in wildlife. Reliable and specific diagnostic tools are essential for effective surveillance.

Material and methods: In this study, 149 serum samples from wild boars hunted during the 2023/2024 season in Bavaria (Germany) were analyzed using four serological tests: 1.the rose bengal test (RBT), 2. a conventional sLPS-based ELISA (BMS),3. a novel biwell ELISA (BSI) using both sLPS and rLPS antigens and 4. the complement fixation test (CFT) as gold standard. Sensitivity, specificity, and accuracy of all assays were calculated in comparison to the CFT.

Results: Of the 149 samples analyzed, 9 tested positive by CFT, resulting in a seroprevalence of 6.0 % [3.1 %, 11.2 %]in the sampled wild boar population. The BMS-ELISA demonstrated the highest sensitivity (100 %) but moderate specificity (85.0 %), whereas the BSI ELISA showed improved specificity (94.3 %) and accuracy (92.6 %) through combined detection of antibodies against sLPS and rLPS, albeit with lower sensitivity (66.7 %). The RBT performed least favorably with a sensitivity of 55.6 % and specificity of 92.7 %.

Discussion: The findings confirm that brucellosis remains endemic among wild boars in Bavaria. While the BSI ELISA shows promise due to its high specificity, its lower sensitivity limits its utility as a stand-alone diagnostic. Cross-reactions in sLPS-based assays highlight the importance of combining antigens for improved test reliability. The varying seroprevalence compared to previous studies underscores the dynamic nature of infection in wildlife populations.

Conclusion: A dual-step diagnostic approach – utilizing a sensitive ELISA for screening followed by CFT for confirmation – remains the most effective strategy for wildlife surveillance. The BSI ELISA may serve as a viable alternative in clinical or resource-limited settings. Continued monitoring is crucial to mitigate the zoonotic risk posed by wildlife reservoirs.

1. Introduction

Brucellosis is the most widespread zoonotic infection globally, with a true incidence of 5–12,5 million new cases reported annually (Hull and Schumaker, 2018). The disease is caused by Gram-negative, nonmotile, non-spore-forming and facultative intracellular bacteria belonging to the *Brucellaceae* family (Whatmore et al., 2016; Yagupsky et al., 2019). The host spectrum is broad and includes various domestic and wild

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animals as well as humans (Corbel, 2006). In Germany there have 762 human cases been notified to the public health authorities in the period from 2001 to 2025 (the proportion of imported cases is not separately documented). Among the primary pathogenic species, *Brucella melitensis*, *Brucella abortus* and *Brucella suis* are particularly relevant. They are the main cause of human brucellosis due to their high zoonotic potential (Di Bonaventura et al., 2021). Human infection usually occurs through the consumption of contaminated animal products, direct contact with infected animals or exposure to aerosols in occupational settings (Corbel, 2006). Brucellosis is considered a potential B-agent and is listed by the U.S. Centers for Disease Control and Prevention (CDC) as a Category B bioterrorism agent (Centers for Disease Control and Prevention (CDC) 2000; Doganay et al., 2019). The disease primarily affects regions with high livestock dependence, such as the Mediterranean basin, the Middle East, sub-Saharan Africa, and Latin America, where socioeconomic conditions often limit effective management and surveillance (Franco et al., 2007; Yagupsky et al., 2019). Despite considerable progress, brucellosis remains a diagnostic challenge due to its nonspecific clinical presentation in both humans and animals. This also contributes to inadequate reporting and the absence of comprehensive global control measures (Colmenero et al., 1990; Franco et al., 2007). The clinical symptoms are unspecific, highly variable and may range from mild flu-like symptoms to severe systemic complications affecting multiple organ systems. *Brucella spp.* has the potential to establish chronic infections (Di Bonaventura et al., 2021; Franco et al., 2007; Ulu Kilic et al., 2013). Laboratory diagnosis is therefore crucial for the identification of cases in humans and animals. (Di Bonaventura et al., 2021). The livestock population in Germany is currently classified as brucellosis-free after extensive eradication programs in recent decades (Godfroid and Kasbohrer, 2002; Macias Luaces et al., 2023). However, wild animals such as wild boar, hares and voles are still regarded as possible natural reservoirs and therefore pose a potential risk to pathogen-free populations or humans (Macias Luaces et al., 2023). Understanding the role of wildlife reservoirs is therefore of paramount importance for the formulation of effective control strategies. In this context serological monitoring of wildlife populations is an fundamental tool. Various serological assays such as the complement fixation test (CFT), the rose bengal test (RBT), fluorescence polarization assay (FPA) and different enzyme-linked immunosorbent assay (ELISA) formats are available for this purpose (Friedrich-Loeffler-Institut FLI, 2025; World Organisation for Animal Health (OIE), 2022). The CFT, which is also recommended by the World Organization for Animal Health (WOAH), is widely used as a reference method in comparative studies due to its excellent specificity and broad international acceptance, enabling reliable comparability across investigations (Meirelles-Bartoli et al., 2020; Nielsen and Yu, 2010; Pilo et al., 2015). However, the performance and handling of the CFT is complex. It requires on the one hand, well-equipped laboratory facilities and appropriately trained personnel, and on the other hand, reagents of high and consistent quality (World Organisation for Animal Health (OIE), 2022). ELISA formats are much easier to handle and have significantly lower laboratory infrastructure requirements. A major limitation of many ELISA formats is the high susceptibility to cross-reactions with antibodies against other Gram-negative bacteria, such as *Yersinia enterocolitica* O:9, *Francisella tularensis*, or *Escherichia coli*. These cross-reactions can lead to false-positive results and complicate the interpretation of diagnostic findings (Corbel, 2006; Yagupsky et al., 2019). Conventional ELISA tests typically use the smooth lipopolysaccharide (sLPS) of *Brucella* as the target antigen, which is particularly prone to such cross-reactivity. In contrast, a recently developed assay employs the rough lipopolysaccharide (rLPS), which offers improved specificity and greater resistance to cross-reacting antibodies (McGiven et al., 2012; Touloudi et al., 2022). In this study, we determined the seroprevalence of brucellosis in wild boar from a known endemic area in Bavaria, specifically the Franconia region, where *Brucella suis* biovar 2 is endemic. We used the Complement Fixation Test (CFT), an established OIE-compliant reference method, and further evaluated the performance of a newly available commercial ELISA (BSI, coated with rLPS and sLPS) by comparing it with the CFT and two additional serological tests – the Rose Bengal Test (RBT) and the conventional sLPS ELISA (BMS).

2. Material and methods

2.1. Serum samples

Samples came from wild boar hunted during the regular 2023/2024 hunting season in the Franconia region (Bavaria, Germany), see Fig. 1 for details. A serum sample was taken from the visceral cavity of all wild boar in serum separation tubes. The samples were centrifuged on site using a mobile centrifuge in serum separation tubes, stored at 4 °C and transported to the laboratory within 24 h. There, sera were separated and stored in the freezer at −20 °C until further use.

2.2. Complement fixation test (CFT)

All samples were additionally tested in the complement fixation test (CFT) according to the protocol provided by the German Federal Research Institute for Animal Health (FLI), which is based on the exemplary standard procedure outlined by the OIE (Friedrich-Loeffler-Institut FLI, 2025; World Organisation for Animal Health OIE, 2022). All sera were heat-inactivated at 60 °C for 30 min before testing. The CFT was carried out on standard 96-well microtiter plates, each containing positive and negative serum controls, as well as dedicated control wells for antigen, complement, and the hemolytic system in each test cycle. Sera were tested in serial dilutions and included an anti-complementary control. Plates were incubated overnight at 5 °C, followed by a 30-minute incubation at 37 °C after addition of the hemolytic system, after which results were evaluated. Samples were considered positive if the CFT was $\geq$ 20 sensitive units/mL. If the CFT result was invalid or the serum could not be tested due to unacceptable quality, the sample was excluded from the study.

2.3. Rose Bengal test (RBT)

All samples were also tested with the RBT according to the OIE protocol (Friedrich-Loeffler-Institut FLI, 2025; World Organisation for Animal Health OIE, 2022). A 1:1 ratio was used, mixing 30 µL of serum with an equal volume of Rose Bengal antigen. Samples with visible colored agglutination were evaluated as positive.

2.4. ELISA-assays

A conventional multi-species ELISA (BSM, ID Screen® Brucellosis Serum Indirect Multi-Species, ID Vet, Grabels, France) was used according to the manufacturer's instructions. This test was developed for the detection of antibodies against *Brucella abortus*, *melitensis* or *suis* in serum and plasma of cattle, sheep, goats and pigs. The measured optical density (OD) was converted into a ratio (S/P %) based on the calibrator values. Samples with a ratio of less than 110 were considered negative for *Brucella*-specific IgG antibodies, samples with a ratio between 110 and 120 were categorized as borderline, whereas samples with a ratio of 120 and greater were classified as positive. In addition, all samples were tested in a new biwell ELISA for the detection of antibodies against *Brucella suis* in porcine serum or plasma (BSI, ID Screen® *Brucella suis* Indirect, ID Vet, Grabels, France) also according to the manufacturer's specifications. This ELISA is designed as a biwell format and is coated with both sLPS (odd columns) and rLPS (even columns). Sera were examined in one well of an even-numbered column and one well of an odd-numbered column. The measured optical density (OD) was converted into a ratio (S/P %) based on the calibrator values. Samples with a ratio of less than 60 (sLPS) or 45 (rLPS) were considered negative and samples with a ratio of 60 (sLPS) or 45 (rLPS) and greater were classified

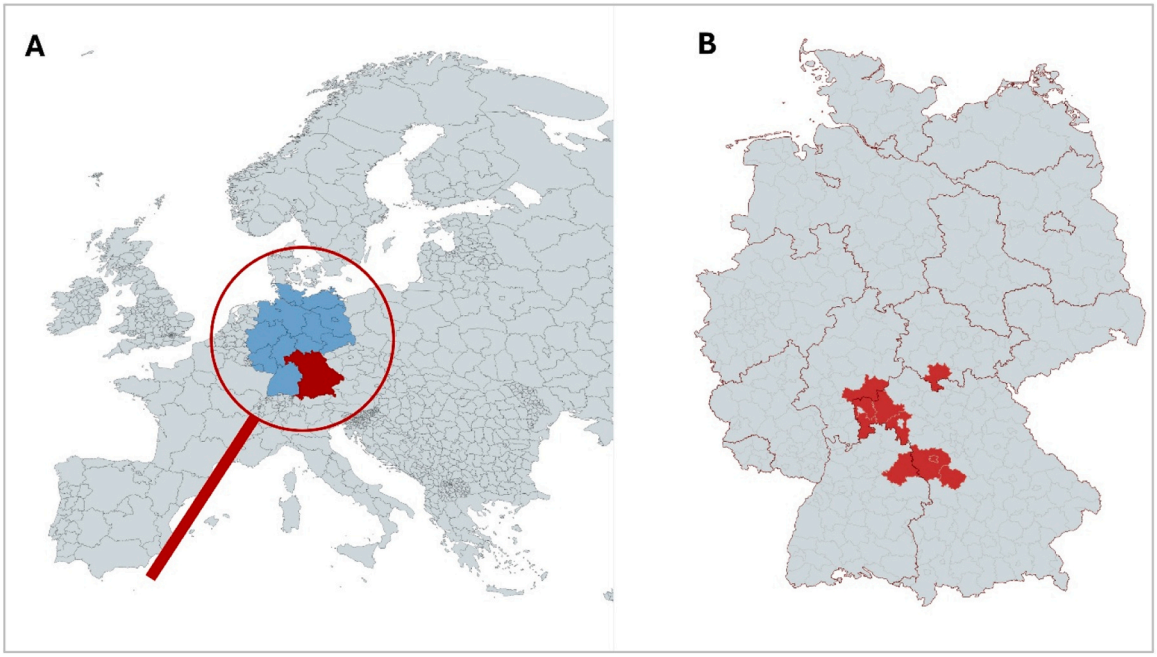

Fig. 1. (A) Overview of the study location. (B) Map of Germany showing the districts (in red) where the hunting areas are located from which the sampled wild boars originated. Some of the hunting areas and forest sections extend beyond the borders of Franconia into neighboring federal states such as Baden-Württemberg, Hesse, and the Upper Palatinate; therefore, these adjacent districts are also marked accordingly. Maps were created with mapchart.net.

as reactive; only sera that were reactive in both wells were evaluated as positive for *Brucella suis* - specific IgG antibodies.

2.5. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA). Seroprevalence was calculated as the proportion of positive samples among the total number of tested samples. The corresponding 95 % confidence intervals (CI) were estimated using the Modified Wald method, which provides reliable interval estimates for binomial proportions, especially in studies with moderate sample sizes. To assess the relationship between quantitative CFT units and ELISA values (S/P %), Spearman's rank correlation coefficient (ρ) was calculated for all positive samples. The analysis included the 95 % confidence interval and a two-tailed p-value to evaluate statistical significance. Separate correlations were performed for the BMS-ELISA, as well as for the BSI-ELISAs.

3. Results

3.1. Serum samples

A total of 156 serum samples were collected from hunted wild boar. Of these, 7 samples were excluded due to non-adequate quality. The remaining 149 sera were analyzed in parallel in all test procedures as described above.

3.2. Complement fixation test (CFT)

In the CFT 6.0 % (9/149) of the sera tested positive, with results ranging from 31.2 to 353.6 (mean 112.1) sensitive units/mL. All other sera were classified as negative with a result of less than 20 sensitive units/mL. See for overview Table 1.

3.3. Rose Bengal test (RBT)

In the RBT 30 sera could not be evaluated due to unspecific reactions. These sera were excluded for the subsequent calculation of the test performance in comparison with the CFT. Of the remaining 119 sera, 13 tested positive (10.9 %). Of the 9 CFT positive sera, 5 were also correctly identified in the RBT, which corresponds to a sensitivity of 55.6 %. Of the 110 CFT negative sera, 102 were correctly recognized as negative, resulting in a specificity of 92.7 %. The accuracy of the RBT was 89.9 % in our comparison.

3.4. ID Screen® Brucellosis serum indirect multi-species (BMS)

In the BMS-ELISA, 30 sera (20.1 %) showed a positive result. In comparison with the CFT, all 9 positive sera and 119 of 140 negative sera were correctly identified. This results in a sensitivity of 100 %, a

Table 1
Overview of the test performance of all assays tested against the CFT as reference standard. In the RBT, 30 sera could not be analyzed due to unspecific reactions. These sera were excluded for the subsequent calculation of the test performance in comparison to the CFT (number of CFT-negative sera used for comparison in brackets).

Serum Samples *n*		**RBT**		**BMS**		**BSI** (sLPS)		**BSI** (rLPS)		**BSI** (combined)	
		Pos	***Neg***	***Pos***	***Neg***	***Pos***	***Neg***	***Pos***	***Neg***	***Pos***	***Neg***
149	(Total)	13	106	30	119	36	113	20	129	14	135
9	(CFT positive)	5	4	9	0	9	0	6	3	6	3
140 (110)	CFT negative)	8	102	21	119	27	113	14	126	8	132
Sensitivity [%]		**55.6**		**100.0**		**100.0**		**66.7**		**66.7**	
Specificity [%]		**92.7**		**85.0**		**80.7**		**90.0**		**94.3**	
Accuracy [%]		**89.9**		**85.9**		**81.88**		**88.6**		**92.6**	

specificity of 85.0 % and an accuracy of 85.9 %. The performance of the ELISA compared to the CFT is shown graphically in Figs. 2 and 3.

3.5. ID Screen® Brucella suis Indirect (BSI)

A total of 36 of the 149 sera (24.2 %) analyzed showed reactions against sLPS while only 20 samples (13.4 %) tested positive for rLPS-specific antibodies. Only serum samples that were reactive against both sLPS and rLPS were classified as positive for *Brucella suis*-specific antibodies in the BSI, which was the case for only 14 serum samples (9.4 %). The performances for sLPS, rLPS and the combined score were calculated separatly to get a more differentiated insight into the test performance. A sensitivity of 100 % (9/9), a specificity of 80.7 % (113/140) and an accuracy of 81.2 % were obtained for the isolated sLPS component. For the rLPS component, the sensitivity was 66.7 % (6/9), the specificity 90.0 % (126/140) and the accuracy 90.6 %. In the combined version, as recommended by the manufacturer, the BSI achieved a sensitivity of 66.7 % (6/9), a specificity of 94.3 % (132/140) and an accuracy of 92.6 %. For the graphical comparison of ELISA and CFT see Figs. 2 and 3.

4. Discussion

Brucellosis remains a significant zoonotic disease and continues to pose a considerable challenge in both veterinary and human medicine worldwide. While many EU countries, including Germany, have achieved officially brucellosis-free status in livestock through intensive control programs, this success remains fragile. Wildlife species such as wild boars and hares serve as persistent reservoirs for Brucella spp., posing a continuous risk of spillover into domestic animal populations and potentially to humans (Gonzalez-Espinoza et al., 2021; Melzer, 2018). Recent studies have emphasized that wildlife-associated brucellosis is an increasingly relevant issue across Europe, particularly in wild boars, and warrants heightened attention in surveillance and control strategies (Jamil et al., 2022). This highlights the need for ongoing monitoring, especially in endemic areas, to better understand transmission dynamics and mitigate the risk of reintroduction into livestock populations. In this context, serological methods remain the cornerstone for monitoring and diagnosing brucellosis, particularly in wildlife, where direct detection is often limited. In this study, we determined the seroprevalence of *Brucella*-specific IgG antibodies in wild boar from a known Bavarian endemic area and analyzed the performance of three different test systems against the CFT as gold standard. The serological diagnostic tests used here for indirect pathogen detection, CFT, RBT and BMS-ELISA are listed and recommended as standard methods in the WOAH manual for the diagnosis of brucellosis (World Organisation for Animal Health (OIE), 2022). As described in numerous studies, these serological tests are characterized by relatively high sensitivity rates of 89 % and up to 100 %, but reach at the same time there are moderate specificity rates of 84 % (RBT) and 96 % (CFT) (Getachew et al., 2016; Praud et al., 2012). The limitations of the serological test systems with regard to their specificity are mainly due to a high number of false-positive results, which can be caused by cross-reactions with antibodies against other Gram-negative bacteria; notably cross-reactions with *Yersinia enterocolitica*, *Francisella tularensis*, *Escherichia coli* and others have been described before (Corbel, 2006; Getachew et al., 2016; Khurana et al., 2021). In the absence of a universally accepted gold standard for serological testing of brucellosis, the selection of an appropriate reference method remains a challenge. We chose the CFT as a reference in this study due to its widespread use and long-standing role in official diagnostic frameworks. The WOAH)lists the CFT as a suitable method for seroprevalence studies in pigs (World Organisation for Animal Health (OIE), 2022). Indeed, numerous studies have demonstrated that the CFT offers a very high specificity, comparable to that of modern indirect ELISAs, also in swine populations, where false-positive serological reactions can otherwise complicate interpretation (Meirelles-Bartoli et al., 2020; Nielsen and Yu, 2010; Pilo et al., 2015). While limitations such as lower sensitivity are well recognized, the test's robust performance in terms of specificity and its status as a widely established benchmark in comparative research support its continued use, especially in settings where regulatory comparability and interpretive consistency are critical. We therefore decided to use the CFT as a reference test as well. However, a major disadvantage of the CFT is its complex implementation, which requires a very good laboratory infrastructure and highly trained laboratory personnel. ELISA-based test systems, on the other hand, are much easier to handle. Therefore, a highly sensitive ELISA is often used as a screening test in *Brucella* diagnostics and only ELISA-positive sera are examined in a second step for confirmation in the CFT (Macias Luaces et al., 2023). Conventional ELISA formats as the BMS use the smooth *Brucella* lipopolysaccharide

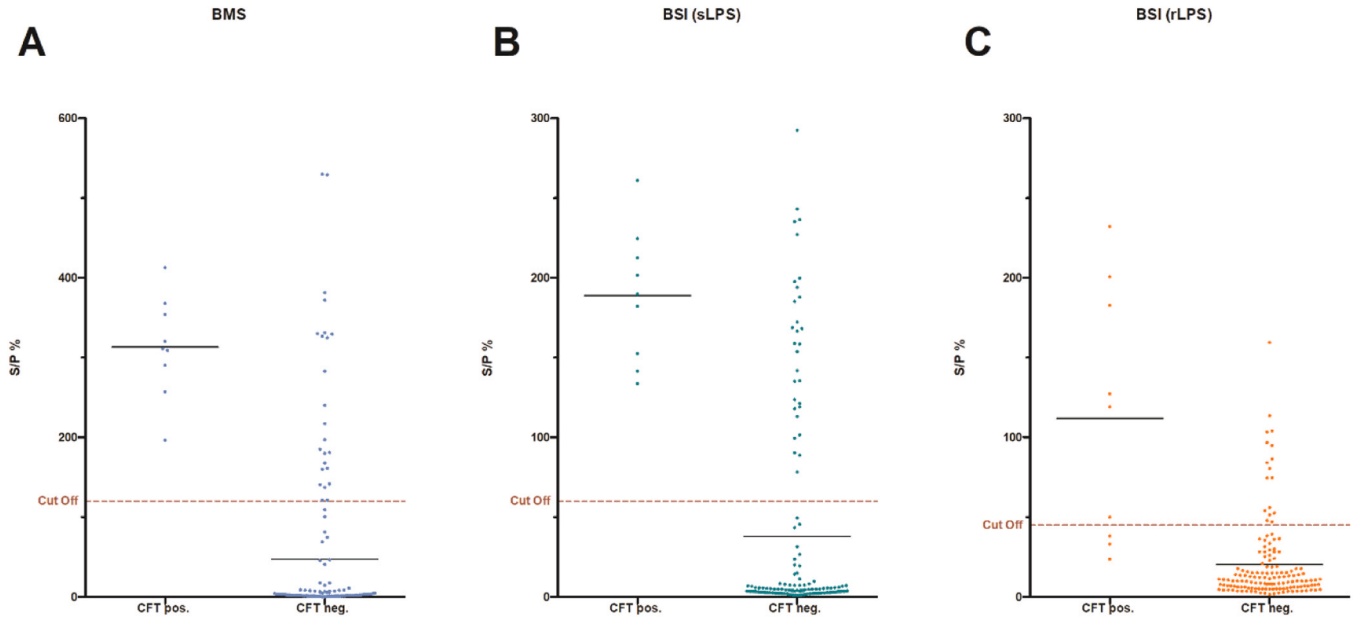

Fig. 2. Distribution of Units (S/P %) determined by BMS-ELISA (A), BSI-sLPS-ELISA (B) and BSI-rLPS-ELISA (C) for negative and positive CFT-results. The cut-off of each assay is shown as a red dashed line.

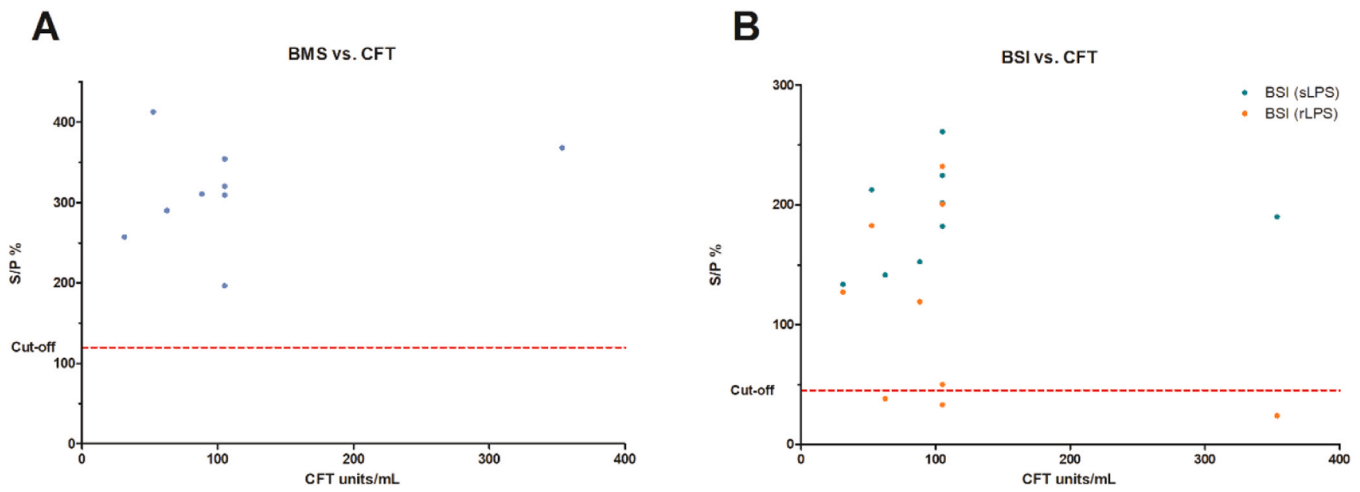

Fig. 3. Spearman correlation of the positive samples shows no correlation between CFT-units and ELISA- units (S/P %). A Spearman correlation (95 % confidence interval) was performed. The two-tailed P values show no significant correlation, neither for the BMS-ELISA (A, P = 0.5517) nor for the BSI-ELISAs (B, P = 0.1777 for sLPS and P = 0.4101 for rLPS). Thus, no conclusion about CFT units can be drawn from ELISA values. The cut-off of each ELISA is shown as a red dashed line.

(sLPS) as the target antigen. However, sLPS appears to be the main target for cross-reactive antibodies (Rao et al., 2015). Recent studies show that the alternative use of rough lipopolysaccharide (rLPS) significantly increases the specificity of test systems for *Brucella suis* and reduces the occurrence of false positive results due to cross-reacting antibodies (McGiven et al., 2012; Touloudi et al., 2022). The commercially available BSI-ELISA that was applied in this study is based on this approach and promises improved specificity through the combination of sLPS and rLPS. As described above, this assay is reactive on antibodies against sLPS and rLPS in different wells in one approach and reactions are only considered positive if there is reactivity against both *Brucella* LPS forms. Of all the assays tested, the BSI-ELISA achieved the highest specificity of 94.3 % and clearly outperformed the conventional BMS-ELISA (specificity 85.0 %). In terms of accuracy, the BSI-ELISA was also the best test in our group with 92.6 % and came closest to the CFS in its results. However, the sensitivity shown (66.7 %) was significantly below that of the conventional BMS-ELISA (100 %); only the RBT showed an even lower sensitivity performance with 55.6 % (Table 1). Notably, the RBT exhibited the highest proportion of samples that had to be excluded due to unreadable or non-specific reactions. This unexpectedly high rate may be attributed to suboptimal sample quality, which in turn could result from the challenging conditions of blood collection in the field. Furthermore, prolonged transport times may have negatively affected sample integrity. It is important to note that all RBTs in this study were performed and evaluated by the same experienced technician with decades of expertise in this test system. In routine diagnostic settings using freshly obtained blood from live animals, analyzed by the same individual, the proportion of unreadable RBT results is significantly lower. Therefore, we assume that the observed proportion in this study reflects the specific field-related constraints rather than issues with test execution or interpretation. Regarding the ELISA cutoff values, the results from our sample set suggest that raising the cutoff for the BMS-ELISA might slightly improve its specificity, as a few false-positive samples lie just above the current threshold (see Fig. 2A). However, the potential gain appears limited, given that the majority of CFT-negative samples are already clearly below the cut-off. In contrast, for the BSI-ELISAs (Fig. 2B and 2C), particularly the rLPS variant, increasing the cutoff would lead to a notable loss in sensitivity. More comprehensive studies with larger sample sizes are needed to evaluate whether adjusted cutoff values could improve overall diagnostic performance without compromising sensitivity or specificity.

The seroprevalence of the wild boar tested in this study was 6.0 % [3.1 %, 11.2 %], assuming that only the CSF-positive sera are truly positive. In a recently published study by Luaces et al. that also included the investigation of *Brucella* antibodies in Bavarian wild boars, an average seroprevalence of 17.9 % was found, with district-level variations ranging from 11.9 % to 31.2 % (Macias Luaces et al., 2023). The seroprevalence determined in this study was significantly higher than in our study, although the same CFT performed in the same laboratory (Bavarian Health and Food Safety Authority) was used as the final confirmatory test. In other studies, seroprevalence rates show greater variation, ranging from 0.2 % (Baden-Württemberg) to 22.0 % in Germany and up to nearly 60 % in other European countries (Al Dahouk et al., 2007; Cvetnić et al., 2009; Grégoire et al., 2012; Halli et al., 2012; Miller, 2018; Ruiz-Fons et al., 2006; Zurovac Sapundzic et al., 2022). As Franconia is localized at the frontier between Baden-Württemberg and Bavaria our results might reflect an intermediate epidemiological situation. Also, temporal variation might be responsible for the lower antibody prevalence rate in our study. Although the seroprevalence observed in our study is lower than that reported by Luaces et al., it is still within the range of published values and is probably explained by the typical fluctuations associated with studies on wild animal populations.

Our study also has a few limitations: With a total of 149 serums, our group is rather small. Due to the random and uncontrolled composition of the study group, only nine positive serum samples were found. Further studies are warranted to substantiate the reliability of the sensitivity and specificity estimates obtained in this investigation. Nevertheless, the data collected in this study are of considerable value, as our approach reflects the typical sample influx generated in the context of hunting activities. This is particularly relevant for the target group involved, as there is a growing need for novel methodologies that facilitate simplified laboratory processing and thereby enable broader surveillance in the field. A further limitation is the lack of bacterial isolation or molecular detection. As is generally recognized, it is not possible to achieve identification at the species or biovar level with serological tests alone. Isolation and molecular characterization remain the gold standard for definitive diagnosis and epidemiological tracing of Brucella infections (Gupta, 2014). However, due to the retrospective nature of our study and the exclusive availability of serum samples, no tissue or whole blood specimens were available to enable culture or PCR. In addition, PCR from serum is known to have limited sensitivity, due to the typically low bacterial load and the intracellular localization of Brucella (Keid et al., 2010). Future investigations using targeted sampling of fresh tissue or whole blood would be essential to complement serological surveillance with molecular confirmation and strain typing.

In conclusion, the findings of this study indicate that while the novel BSI ELISA demonstrates good specificity, its sensitivity remains inferior

compared to the CFT. For epidemiological studies, a two-tiered approach—consisting of a highly sensitive screening ELISA followed by a highly specific CFT as a confirmatory test—remains the most effective solution. For clinical use, where antibody titres may rise over the course of infection and could help compensate for lower sensitivity, or in regions where the implementation of a CFT is technically unfeasible, the BSI ELISA may offer a feasible alternative.Once again, it has been demonstrated that *Brucella* is endemic in wild boar populations in Bavaria, constituting a persistent reservoir of infection. However, the level of seroprevalence appears to vary considerably, as evidenced by comparisons with other studies conducted in the same region.

CRediT authorship contribution statement

Julia M. Riehm: Writing – review & editing, Validation, Methodology. **Gerhard Dobler:** Writing – review & editing, Conceptualization. **Johannes P. Borde:** Validation, Data curation. **Philipp Girl:** Writing – review & editing, Writing – original draft, Validation, Data curation, Conceptualization. **Leonard Limpinsel:** Writing – review & editing, Methodology.

Declaration of Competing interest

The authors declare no conflicts of interest.

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