Multiplex Screening of Surface Proteins from *Mycoplasma mycoides* subsp. *mycoides* Small Colony for an Antigen Cocktail Enzyme-Linked Immunosorbent Assay

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A recombinant antigen cocktail enzyme-linked immunosorbent assay (ELISA) for diagnosis of contagious bovine pleuropneumonia (CBPP) was developed after careful selection of antigens among one-third of the surface proteome proteins of the infectious agent *Mycoplasma mycoides* subsp. *mycoides* small colony (*M. mycoides* SC). First, a miniaturized and parallelized assay system employing antigen suspension bead array technology was used to screen 97 bovine sera for humoral immune responses toward 61 recombinant surface proteins from *M. mycoides* SC. Statistical analysis of the data resulted in selection of eight proteins that showed strong serologic responses in CBPP-affected sera and minimal reactivity in negative control sera, with *P* values of <10⁻⁶. Only minor cross-reactivity to hyperimmune sera against other mycoplasmas was observed. When applied in an ELISA, the cocktail of eight recombinant antigens allowed a fivefold signal separation between 24 CBPP-affected and 23 CBPP-free sera from different geographical origins. No false-positive results and only two false-negative results were obtained. In conclusion, the selected recombinant mycoplasma antigens qualified as highly specific markers for CBPP and could be employed in both a suspension bead array platform and a cocktail ELISA setting. This set of proteins and technologies therefore offers a powerful combination to drive and further improve serological assays toward reliable, simple, and cost-effective diagnosis of CBPP.

Contagious bovine pleuropneumonia (CBPP) is a severe infectious disease in cattle caused by *Mycoplasma mycoides* subsp. *mycoides* small colony (*M. mycoides* SC). Because of its potential for rapid spread with resulting massive losses of livestock and thereby severe socioeconomic consequences, an official declaration of disease to the World Organization for Animal Health is required. Vigorous and costly eradication programs involving mass slaughter, quarantine, and strict control of animal movements have been successful in eradicating CBPP from the United States, Japan, and Australia (13). In Western Europe, the disease has reappeared almost every decade in the 20th century, in spite of expensive eradication efforts, demonstrating its constant threat (13, 22). In sub-Saharan Africa, where the disease is endemic, this stamping-out procedure is not economically feasible. Vaccination is a better option for these countries, although existing vaccines so far give insufficient immunity and severe side effects (23). One of the main challenges within CBPP control is diagnosis of the pathogen, and incubation periods of up to several months (7) hinder contact tracing. Today, there are two diagnostic tests prescribed for international trade: the Campbell and Turner complement fixation test (CFT), established in 1953 (6) and based on whole-cell antigens, and the competitive enzyme-linked immunosorbent assay (ELISA), published in 1998 (14) and based on whole-cell antigens in combination with a monoclonal antibody toward Pts-G (9). Although the two serological tests supplement each other in sensitivity, they still do not allow adequate diagnostic certainty (8).

The complete genome sequence of *M. mycoides* SC (26) has paved the way for new diagnostics based on subcellular components. Methods including PCR have been shown to be successful (21) but put high demands on sampling procedures. Surface lipoproteins are of high interest both for diagnostic purposes and for studies regarding the pathogenicity of the bacterium (23). To date, only a few of the surface lipoproteins from *M. mycoides* SC have been studied thoroughly. LppA (p72) (17, 18), LppB (25), and LppC (23) are highly conserved lipoproteins that are present in closely related species within the *Mycoplasma mycoides* cluster. Pts-G is a variably expressed glucose phosphotransferase system permease (9), and Vmm is a small surface protein shown to have a variable expression pattern (20). LppQ is a highly antigenic lipoprotein specific to *M. mycoides* SC (1). Thorough characterization studies of LppQ (1, 4) and the development of a recombinant ELISA built upon LppQ as the antigen (5) show that it is suitable as a diagnostic marker. However, of the 187 predicted surface proteins of *M. mycoides* SC (10), more antigens than just LppQ should trigger antibody-mediated immune responses useful in diagnostic applications. Combinations of such antigens could thereby offer a higher specificity and sensitivity than existing methods by adding discriminative power to the current LppQ-based ELISA while circumventing cross-reactivity compared to whole-cell antigen-based methods.

The aim of this study was to identify the most potent...
diagnostic surface antigens and to test the performance of recombinant versions in combination in an ELISA format. The selection of targets was enabled by a recently developed multiplex suspension bead array assay that allowed high-throughput screening of a large set of sera for humoral immune responses to a large number of recombinant surface proteins (10). As a result, a cocktail ELISA was developed with the selected antigens, and its capacity was evaluated.

**MATERIALS AND METHODS**

**Recombinant surface proteins.** All proteins included in this study are listed in Table S1 in the supplemental material. The production of recombinant mycoplasma proteins was described previously (11) and included the selection of surface proteins specific to *M. mycoides* SC, design of full-length recombinants excluding a signal peptide and including the largest extracellular domain for transmembrane proteins, PCR and cloning into the vector pAff8c, mutagenesis to change TGA codons to TGG, expression in *Escherichia coli*, and, finally, purification by immobilized metal-ion chromatography. All recombinant proteins were equipped with a fusion tag consisting of a 17-kDa albumin binding protein with six N-terminal histidines (His6ABP) for enhanced solubility and purification purposes.

For the ELISA setup, relevant recombinant proteins were also produced without the fusion protein ABP. New forward PCR primers were designed to replace the N-terminal His6ABP fusion tag with a hexahistidine tag only. Sequence-specific regions were identical to those of previously used PCR primers, but the primer handles contained a 5′-terminal NcoI restriction site followed by a hexahistidine tag. A biotinylated primer complementary to this handle was used in a secondary PCR step to enable automated solid-phase restriction of the amplified mycoplasma gene fragments. Amplifications were performed previously described, using the sequence-verified clones with His6ABP gene fusions as templates. After solid-phase restriction, clones were ligated into NcoI/AceI-digested pAff8c vector. Proteins were expressed in *E. coli* strain BL21 (DE3) and purified using immobilized-metal-ion affinity chromatography. All proteins were stored at −20°C in phosphate-buffered saline (PBS) containing 1 M urea and were named after their corresponding open reading frames in the genome sequence.

**Serum collection.** The serum collection consisted of 97 bovine sera from different geographical origins as well as 17 *hypermun* sera (Table 1). Included in the collection were two CBPP-positive reference samples from Portugal, 50 CBPP-positive field samples from Spain, Namibia, and Tanzania, 24 CBPP-negative field samples from Kenya and Tanzania, 21 negative control samples from Sweden, and 17 *hypermun* sera from Denmark and Sweden. The bovine sera had well-characterized CBPP statuses, but some diagnostic test results from Sweden, and 17 *hypermun* sera from Denmark and Sweden. The bovine sera had well-characterized CBPP statuses, but some diagnostic test results from Sweden, and Namibia were CBPP-positive field samples from Spain, Namibia, and Tanzania, 24 CBPP-negative field samples from Kenya and Tanzania, 21 negative control samples from Sweden, and 17 *hypermun* sera from Denmark and Sweden. The bovine sera had well-characterized CBPP statuses, but some diagnostic test results from Sweden, and Namibia were CBPP-positive field samples from Spain, Namibia, and Tanzania, 24 CBPP-negative field samples from Kenya and Tanzania, 21 negative control samples from Sweden, and 17 *hypermun* sera from Denmark and Sweden. The bovine sera had well-characterized CBPP statuses, but some diagnostic test results from Sweden, and Namibia were CBPP-positive field samples from Spain, Namibia, and Tanzania, 24 CBPP-negative field samples from Kenya and Tanzania, 21 negative control samples from Sweden, and 17 *hypermun* sera from Denmark and Sweden.

**Screening by suspension bead array assay.** The suspension bead array assay was performed as previously described (10) on a total of 134 samples with a 62-plex surface protein array in suspension. The array consisted of 61 recombinant proteins with a predicted surface location on *M. mycoides* SC type strain PG1 as well as closely related *Mycoplasma* species to study potential cross-reactivity.

**Cocktail of the most immunogenic antigens.** A cocktail of the most immunogenic antigens was created by combining selected recombinant proteins in equal amounts in PBS. ELISA conditions were optimized by varying the protein coating density (10 to 1,000 ng/well), plate type (high-binding or medium-binding half-well-area 96-well plates; Greiner), reagent volume (50 to 100 μl), serum dilution (1:10 to 1:1,600), secondary antibody concentration (80 to 160 ng/ml), incubation time (10 to 60 min), and washing procedures (rounds, volumes, and addition of Tween 20 to PBS). The final assay was conducted as follows. ELISA microplates (high-binding half-well-area plates; Greiner) were coated with protein cocktail (100 μl; 1 μg/ml) and incubated overnight at 4°C. After being washed three times in 140 μl PBS, plates were blocked with 140 μl of BRE (blocking reagent for ELISA; Roche) at room temperature (RT) for 1 h, followed by three washes of 140 μl PBST (PBS with 0.1% Tween 20). Sera were diluted 1:1,000 in BRE, and 100 μl was dispensed into each well and incubated for 1 h at RT. After three washes, 100 μl anti-bovine immunoglobulin G conjugated to horseradish peroxidase (80 ng/ml; Jackson Immunoresearch) was added and incubated for 1 h at RT. After plate washing, the enzymatic color reaction was performed by the addition of 100 μl one-step ABTS [2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; Pierce], and the optical density was measured at 405 nm (Sunrise absorbance reader; Magellan Software, Tecan) after 30 to 60 min.

**RESULTS**

**Recombinant production of surface proteins.** Sixty-one proteins with a predicted surface location on *M. mycoides* SC were produced as recombinant proteins with the fusion tag His6ABP. Protein yields from 100 ml of culture after affinity purification ranged from 0.2 to 10 mg. For nine selected antigens, ABP-free recombinants were produced for the final ELISA. Protein yields were similar to those having the ABP fusion and ranged from 0.6 to 7.3 mg. However, for recombinant R0816, expression of a tag-free version failed, and it was therefore excluded.

**Screening of sera against 61 recombinant proteins.** In order to select the most immunogenic among 61 surface proteins from *M. mycoides* SC, a screening of sera to monitor the humoral immune response was performed. By a suspension bead array assay, a multiplex and simultaneous analysis of immunoglobulins with reactivity to the recombinant surface proteins was achieved for every serum sample. The signal intensities from all recombinant proteins formed specific and individual patterns that were shown to be reproducible for each serum in our previous work (10). Signal intensities in technical replicates varied with an average intra-assay coefficient of variation (CV) of 21%. The serum-free control, used to monitor unspecific binding of secondary antibodies to the protein-coated beads, displayed signals that peaked at 3 to 20 AU, the intensities equivalent to the intrinsic autofluorescence of the beads. The control beads carrying the tag protein His6ABP displayed a median MFI of 45 AU, reflecting binding of serum antibodies to the fusion partner of the proteins. To distinguish between noise and antibody-mycoplasma antigen signal, an MFI threshold of 142 AU was set. This threshold was based on the 95th percentile for the distribution of signal intensities from the His6ABP beads. Intensity levels exceeding this threshold value were judged as representing specific binding of serum antibodies to the recombinant mycoplasma proteins.

Distinct differences in serum patterns were seen between CBPP-affected and CBPP-free sera. For 19 proteins, the signal median for CBPP-positive sera was significantly above the threshold value of 142 AU by a Wilcoxon rank sum test, compared to 2 proteins in the CBPP-negative sera. For the majority of the proteins, however, signals were below the threshold.

**Antigen cocktail ELISA.** A cocktail of the most immunogenic antigens was created by combining selected recombinant proteins in equal amounts in PBS. ELISA conditions were optimized by varying the protein coating density (10 to 1,000 ng/well), plate type (high-binding or medium-binding half-well-area 96-well plates; Greiner), reagent volume (50 to 100 μl), serum dilution (1:10 to 1:1,600), secondary antibody concentration (80 to 160 ng/ml), incubation time (10 to 60 min), and washing procedures (rounds, volumes, and addition of Tween 20 to PBS). The final assay was conducted as follows. ELISA microplates (high-binding half-well-area plates; Greiner) were coated with protein cocktail (100 μl; 1 μg/ml) and incubated overnight at 4°C. After being washed three times in 140 μl PBS, plates were blocked with 140 μl of BRE (blocking reagent for ELISA; Roche) at room temperature (RT) for 1 h, followed by three washes of 140 μl PBST (PBS with 0.1% Tween 20). Sera were diluted 1:1,000 in BRE, and 100 μl was dispensed into each well and incubated for 1 h at RT. After three washes, 100 μl anti-bovine immunoglobulin G conjugated to horseradish peroxidase (80 ng/ml; Jackson Immunoresearch) was added and incubated for 1 h at RT. After plate washing, the enzymatic color reaction was performed by the addition of 100 μl one-step ABTS [2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; Pierce], and the optical density was measured at 405 nm (Sunrise absorbance reader; Magellan Software, Tecan) after 30 to 60 min.
value of 142 AU. Sera categorized as CBPP positive showed a higher overall average intensity (484 AU) than did CBPP-negative sera (106 AU). Swedish negative control sera were found to yield an even lower overall average signal intensity (64 AU) than the CBPP-free African field sera (151 AU).

The screening data were summarized in a heat map in combination with a cluster analysis on both the serum and protein levels (Fig. 1). The sera separated into five main clusters, denoted clusters I to V. All technical replicate samples clustered together, except for one specimen of serum B079. Clusters I, II, and V consisted of 25, 20, and 16 samples, respectively, whereof 25, 13, and 16 samples, respectively, were from CBPP-positive sera. These clusters contained most of the high-signal-intensity data points. All hyperimmune sera, except for the M. mycoides SC PG1 antiserum, clustered together in cluster III, which displayed particularly low signals, together with five sera from Swedish bovines infected with Mycoplasma bovis (B094 to B098), one Swedish negative control (B084), and one CBPP-positive sample (B028). Cluster IV consisted of 50 samples, whereof 45 were CBPP negative (including 10 replicates

| Serum type (n) | Serum ID | Diagnosis | Origin | Comment |
|---------------|----------|------------|--------|---------|
| CBPP-positive sera (52) | B001 | + | NA | NA | Portugal<sup>a</sup> | CBPP reference serum 845 from OIE Reference Laboratory for CBPP |
| | B002 | + | NA | NA | Portugal<sup>a</sup> | CBPP reference serum 840 from OIE Reference Laboratory for CBPP |
| | B003 to B008 | NA | NA | NA | Spain<sup>d</sup> | Field samples from outbreaks in Spain |
| | B009 | + | + | + | - | Namibia | Field sample from CBPP outbreak (2004) |
| | B010 | + | + | + | - | Namibia | Field sample from CBPP outbreak (2004) |
| | B011 | + | + | + | - | Namibia | Field sample from CBPP outbreak (2004) |
| | B012 | - | - | + | - | Namibia | Field sample from CBPP outbreak (2004) |
| | B013 | - | + | + | - | Namibia | Field sample from CBPP outbreak (2004) |
| | B014 | + | ? | + | - | Namibia | Field sample from CBPP outbreak (2004) |
| | B015 | + | + | ? | + | Namibia | Field sample from CBPP outbreak (2004) |
| | B016 | + | + | ? | + | Namibia | Field sample from CBPP outbreak (2004) |
| | B017 | + | +<sup>c</sup> | NA | NA | Tanzania | From acute CBPP case (1990); diluted 1:4 for use as reference serum |
| | B018 to B021 | + | +<sup>c</sup> | NA | NA | Tanzania | Field samples from CBPP outbreak (1990) |
| | B022 | - | -<sup>c</sup> | NA | NA | Tanzania | Field sample from CBPP outbreak (1990) |
| | B023 to B027 | - | +<sup>c</sup> | NA | NA | Tanzania | Field samples from CBPP outbreak (1990) |
| | B028 to B052 | + | NA | NA | NA | Tanzania | Field samples from CBPP outbreak |
| CBPP-negative sera (46) | B053 to B060 | NA | - | - | NA | Kenya | CBPP-free members of vaccine trial, before start of trial (1998) |
| | B061 to B076 | - | -<sup>c</sup> | NA | NA | Tanzania | From CBPP-affected region (1990) |
| | B077 to B092 | NA | NA | NA | NA | Sweden | From CBPP-free region (2007) |
| | B093 | NA | NA | NA | NA | Sweden | Pool of B081 to B092 |
| | B094 to B098 | NA | -<sup>c</sup> | NA | NA | Sweden | From CBPP-free region, with M. bovis infection (1988) |
| Hyperimmune sera (17) | H099 | NA | NA | NA | NA | Denmark<sup>e</sup> | Rabbit immunized with M. mycoides SC PG1 |
| | H100 | NA | NA | NA | NA | Sweden | Rabbit immunized with M. mycoides LC Y-goat |
| | H101 | NA | NA | NA | NA | Sweden | Rabbit immunized with M. mycoides capri PG3 |
| | H102 | NA | NA | NA | NA | Sweden | Rabbit immunized with M. capricolum CK |
| | H103 | NA | NA | NA | NA | Sweden | Rabbit immunized with M. capripneumonia F38 |
| | H104 | NA | NA | NA | NA | Sweden | Rabbit immunized with Mycoplasma sp. group 7 strain PG50 |
| | H105 | NA | NA | NA | NA | Denmark<sup>e</sup> | Rabbit immunized with M. bovis PG45 |
| | H106 | NA | NA | NA | NA | Sweden | Rabbit immunized with M. bovirhinis PG43 |
| | H107 | NA | NA | NA | NA | Sweden | Rabbit immunized with M. arginini G230 |
| | H108 | NA | NA | NA | NA | Denmark<sup>e</sup> | Rabbit immunized with M. californicum ST-6 |
| | H109 | NA | NA | NA | NA | Denmark<sup>e</sup> | Rabbit immunized with M. bovoculi M165/69 |
| | H110 | NA | NA | NA | NA | Denmark<sup>e</sup> | Rabbit immunized with M. canadense 275c |
| | H111 | NA | NA | NA | NA | Sweden | Rabbit immunized with M. bovigenitalium PG11 |
| | H112 | NA | NA | NA | NA | Sweden | Calf immunized with A. laidlawii |
| | H113 | NA | NA | NA | NA | Sweden | Calf immunized with M. arginini |
| | H114 | NA | NA | NA | NA | Sweden | Calf immunized with M. fermentans |
| | H115 | NA | NA | NA | NA | Sweden | Calf immunized with M. orale |

<sup>a</sup> B, bovine sera; H, hyperimmune sera.
<sup>b</sup> CFT, complement fixation test; cELISA, competitive ELISA; LAT, latex agglutination test; +, positive test result; -, negative test result; ?, inconclusive test result; NA, not available.
<sup>c</sup> Indirect ELISA (3).
<sup>d</sup> OIE Reference Laboratory for CBPP, Lisbon, Portugal (J. Regalla).
<sup>e</sup> Laboratorio de Sanidad Animal, Santa Fe, Granada, Spain (F. Garrido Abellan).
<sup>f</sup> WHO/FAO Collaborating Centre for Animal Mycoplasmas, University of Aarhus, Aarhus, Denmark (E. A. Freundt).
of a negative control pool, B093), 4 were CBPP positive, and 1 was the M. mycoides SC PG1 antiserum (H099). In the vertical protein dendrogram, the top cluster, consisting of nine proteins, appeared to have potentially diagnostic importance. Large amounts of antibodies were detected in response to these proteins in CBPP-positive sera. One of them, R1046 (LppQ), yielded particularly high signals in CBPP-positive sera.

Selection of antigens. Selection of the most immunogenic antigens among the 61 recombinant proteins was based on Welch’s two-sample t test. This test was performed on a log-transformed data set where blanks, replicates, hyperimmune sera, and Swedish control sera were removed. Thus, the statistical evaluation involved 24 CBPP-negative field samples, 50 CBPP-positive field samples, and 2 positive reference samples. The test revealed that among the 61 recombinant mycoplasma...
candidates for an antigen cocktail ELISA. Based on this test, the top eight proteins were selected as having statistically significant positive-to-negative signal ratios of \(>3\), with \(P\) values ranging from \(10^{-6}\) to \(10^{-18}\) (Fig. 2). These proteins were R1046, R0136, R0653, R0431, R0816, R0813, R0240, and R0397, all belonging to the protein cluster with apparent diagnostic importance (Fig. 1). As the theoretically most discriminative and therefore most potent diagnostic antigens among the investigated surface proteins, these eight were selected as candidates for further evaluation (Table 2). Additionally, four recombinant proteins, R0051, R0711, R0570, and R0322, appeared with \(P\) values below the significance threshold of 0.01, but their discriminating power, reflected by both the \(P\) value and the difference in antibody response, was not as good as that of the above eight proteins. An additional cluster analysis was performed on the screening data, employing all bovine sera with positive sera alone, thus supporting that an antigen cocktail could offer an improved alternative for building a diagnostic system.

From the associated study (10), three additional mycoplasma surface proteins became available at a later stage and increased the number of mycoplasma proteins investigated to 64. One of these antigens, denoted R0079, displayed potential as a CBPP marker (data not shown) and was therefore included in the following evaluation and ELISA development.

Among the proteins corresponding to the nine selected recombinants, only LppQ, corresponding to R1046, had previously been described as immunodominant. The protein corresponding to R0816 is annotated as a putative variable surface protein and has been used in our previous work to study immune responses (10, 11). The protein corresponding to R0397 is a putative lipoprotein whose gene was indentified in a screening to find candidate genes for a DNA vaccine against CBPP (15), and a protective effect was shown for the protein in a mycoplasmemia mouse model. There is no information published on the remaining six proteins except for the bioinformatic annotation of the genomic sequence. The protein corresponding to R0813 is annotated as a putative variable surface protein and shows resemblance to R0816, as a ClustalW alignment gave a pairwise score of 48. Three of the proteins, corresponding to recombinants R0240, R0431, and R0653, are putative lipoproteins, while the remaining two, corresponding to R0079 and R0136, are annotated as the putative phospho-nate ABC transporter PhnD and a putative protein, respectively. The MSC_0136 gene, corresponding to recombinant R0136, has previously been described as immunodominant. The protein corresponding to R0397 is a putative lipoprotein and has been used in our previous work to study immune responses (10, 11). The protein corresponding to R0816 is annotated as a putative variable surface protein and has been used in our previous work to study immune responses (10, 11). The protein corresponding to R0397 is a putative lipoprotein that was previously described as immunodominant. The protein corresponding to R0136, has previously been used in a phylogenetic study of the Mycoplasma mycoides cluster (24). The Interpro motif IPR005046, found in predicted surface proteins of many bacteria, is shared by six proteins, corresponding to R0079 and R0136, are annotated as the putative phospho-

![FIG. 2. Statistical selection of immunogenic antigens. In a display of the results of the significance test of log_{10}-transformed screening data, eight proteins (black) show signal changes between the CBPP-positive and CBPP-negative populations that are positive-to-negative signal ratios of \(>3\), with \(P\) values ranging from \(10^{-6}\) to \(10^{-18}\). Additionally, four proteins (gray) displayed changes with significance exceeding the \(P\) value limit of 0.01, but with less discriminative importance than the top eight. Based on this test, the top eight proteins were selected as candidates for an antigen cocktail ELISA.](http://cvi.asm.org/)

### TABLE 2. Selected recombinant mycoplasma antigens

| Recombinant protein | Native protein* | \(P\) value from suspension bead array screening | Included in cocktail ELISA | Protein size (kDa) |
|---------------------|-----------------|-----------------------------------------------|---------------------------|-------------------|
| R1046               | LppQ            | \(5.4 \times 10^{-18}\)                       | Yes                       | 49.7              |
| R0136               | Putative protein| \(2.5 \times 10^{-15}\)                       | Yes                       | 32.7              |
| R0431               | Put lipoprot    | \(1.5 \times 10^{-9}\)                       | Yes                       | 38.4              |
| R0653               | Put lipoprot    | \(1.6 \times 10^{-9}\)                       | Yes                       | 42.3              |
| R0813               | Put vsp         | \(3.4 \times 10^{-8}\)                       | Yes                       | 58.6              |
| R0816               | Put vsp         | \(1.2 \times 10^{-7}\)                       | No                        | 45.1              |
| R0240               | Put lipoprot    | \(8.6 \times 10^{-7}\)                       | Yes                       | 62.5              |
| R0397               | Put lipoprot    | \(7.6 \times 10^{-6}\)                       | Yes                       | 21.5              |
| R0079               | ABC transporter | Not included in screening                     | Yes                       | 49.3              |

* Put lipoprot, putative lipoprotein; Put vsp, putative variable surface protein.
R0653, R0813, R0816, and R1046. The sizes of the selected recombinant proteins, excluding His6ABP, range from 20 to 60 kDa, and those of their native counterparts range from 26 to 64 kDa. A summary of the selected antigens is presented in Table 2.

Cross-reactivity. In the process of evaluating the recombinant proteins as antigens in a diagnostic system, the cross-reactivity to closely related *Mycoplasma* species was investigated. Hyperimmune sera from rabbits and calves immunized with closely related species were included in the study, and their immune responses toward all 61 proteins were analyzed. In the suspension bead array assay, these samples showed serum patterns distinctly different from those for the bovine sera. Patterns consisted of signals of 10 to 20 AU for almost all proteins, within the range of bead intrinsic autofluorescence and below the noise level threshold of 142 AU. Only a few proteins gave rise to higher signals, of 200 to 5,000 AU. Among these samples, the highest signals were noted for the positive control *M. mycoides* SC type strain PG1 antiserum. The hyperimmune sera from calves had the same profiles as the rabbit sera, thus excluding that results were caused by the anti-rabbit secondary antibody. Five bovines from Sweden with *Mycoplasma bovis* infections were included (B094 to B098) to evaluate cross-reactivity to *M. bovis* antigens and were more comparable to the other bovine sera than to the hyperimmune rabbit and calf sera. These samples displayed no sign of cross-reactivity, as all signals were below the noise level of 142 AU. Cross-reactivity intensities for the eight selected antigens in the hyperimmune sera are summarized in Table 3. A minor cross-reactivity was observed for some antisera from the *M. mycoides* cluster (H099 to H104).

Effect of ABP in suspension array assay. A diagnostic system to monitor bacterial infection should preferably not be built on recombinant proteins with components of bacterial origin that could cause false-positive results. To verify that our screening could be relevant for tag-free recombinant proteins as well,
eight proteins with and without ABP, as well as the His6ABP control, were tested with 47 sera on the suspension bead array. Hereby it was shown that the separation between CBPP-positive and CBPP-negative sera was not affected by the presence or absence of ABP (data not shown). Generally, an increase in signal intensity was observed for recombinant proteins without the fusion tag, possibly associated with bead coating properties.

Development of antigen cocktail ELISA. A protein cocktail of the eight ABP-free proteins was used in an ELISA setup, and optimization experiments were performed to establish a robust protocol. Antigen coating concentrations and serum dilutions were varied to obtain signal intensities with the largest possible signal change between CBPP-positive and CBPP-negative sera. It was found that a protein coating concentration of 100 ng/well and a serum dilution of 1:1,000 allowed the most discriminative power. The surface and quality of the ELISA plate were crucial to obtain a robust assay. The intra-assay CV was reduced from 17% to 3% by changing the plastic from a particularly hydrophobic medium-binding-level plate to one spiked with polar groups and referred to as high binding. The most important parameter to achieve high signal intensity levels, and thus large signal changes, was the incubation time for developing the colorimetric substrate reaction. With the approach used, a signal separation between positive and negative sera was visible after 10 min, but the discrepancy increased with time. After 1 hour, signals stabilized, and this incubation time was therefore selected. The colorimetric signals were stable enough for the same relative results to be determined by remeasuring on the following day.

Performance of antigen cocktail ELISA. In the final setup of the eight-antigen cocktail ELISA, sera were analyzed in comparison to the reference serum B001 (16), and signals were reported in percent positivity (27). Evaluation of assay reproducibility with two experiments of triplicates of 15 sera showed an average intra-assay CV of 3% and an average interassay CV of 8% (Fig. 5). In a proof-of-concept study with 47 sera performed twice (Fig. 6), the assay allowed a fivefold signal ratio separation between CBPP-affected sera (105% average) and CBPP-free sera (22% average). The receiver operator characteristics of the assay showed a high diagnostic capability, since the area under the curve was calculated to 0.991. An initial cutoff in percent positivity of 50% resulted in no false-positive

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### TABLE 3. Cross-reactivity of recombinant antigens

| Serum ID | MFI (AU) with protein | R0146 | R0136 | R0653 | R0431 | R0816 | R0813 | R0240 | R0397 | His6ABP |
|----------|-----------------------|-------|-------|-------|-------|-------|-------|-------|-------|----------|
| H099     | 5,827                 | 16    | 14    | 20    | 14    | 22    | 285   | 2,074 | 20    | 20       |
| H100     | 58                    | 14    | 25    | 16    | 31    | 26    | 39    | 20    | 16    | 14       |
| H101     | 2,601                 | 77    | 30    | 36    | 41    | 172   | 280   | 1,523 | 31    | 31       |
| H102     | 47                    | 15    | 13    | 8     | 11    | 46    | 18    | 514   | 16    | 16       |
| H103     | 1,184                 | 18    | 19    | 19    | 17    | 28    | 1,465 | 119   | 18    | 18       |
| H104     | 2,812                 | 23    | 24    | 1,510 | 19    | 18    | 7     | 1,446 | 20    | 20       |
| H105     | 29                    | 15    | 13    | 8     | 10    | 16    | 7     | 6     | 16      |
| H106     | 101                   | 16    | 20    | 15    | 116   | 29    | 16    | 23    | 17      |
| H107     | 52                    | 15    | 24    | 10    | 17    | 19    | 12    | 8     | 20      |
| H108     | 33                    | 13    | 14    | 10    | 11    | 17    | 30    | 6     | 15      |
| H109     | 57                    | 19    | 25    | 27    | 21    | 25    | 41    | 114   | 30      |
| H110     | 50                    | 22    | 42    | 21    | 19    | 25    | 10    | 11    | 19      |
| H111     | 32                    | 23    | 20    | 15    | 17    | 27    | 98    | 11    | 18      |
| H112     | 69                    | 15    | 15    | 16    | 11    | 17    | 6     | 12    | 17      |
| H113     | 91                    | 21    | 17    | 21    | 23    | 48    | 40    | 13    | 20      |
| H114     | 94                    | 32    | 24    | 27    | 16    | 33    | 10    | 12    | 26      |
| H115     | 138                   | 19    | 42    | 33    | 75    | 34    | 13    | 15    | 21      |

* Screening results with hyperimmune sera for different mycoplasma species (H099 to H115) to detect reactivity to the eight selected recombinant proteins and the His6ABP control. Bold values indicate intensities greater than the noise threshold of 142 AU, thus predicting cross-reactivity in a diagnostic setup. Some cross-reactivity was detected for samples within the M. mycoides cluster (H099 to H110).
DISCUSSION

In the presented study, a bead-based suspension array assay was applied to discover the most immunogenic surface antigens from *M. mycoides* SC among 64 recombinant surface proteins, corresponding to one-third of the bacterium’s predicted surface proteome. Large-scale screening of bovine sera resulted in the selection of nine proteins to be antigen candidates for the development of enhanced serological diagnosis of CBPP. Eight of these were combined in an antigen cocktail and used to build an ELISA for diagnosis of *M. mycoides* SC infection.

The bead-based suspension array assay proved to be a powerful tool to monitor a multitude of humoral immune responses. Fingerprint-like immune response patterns for sera of CBPP-affected cattle appeared to reflect the individuality of humoral responses to different mycoplasma antigens and supported the idea of using multiple antigens in diagnostic tests. Importantly, objective numerical data were obtained, making it possible to apply a statistical significance test to select the antigens with the most discriminative power between CBPP-positive and -negative populations. The Swedish control samples needed to be excluded because the statistical analyses showed significant differences between the Swedish negative controls and the African negative controls. We could thereby conclude that in using the suspension bead array assay for selection of proteins that discriminate between sample populations, it is important to minimize differences between the groups and preferably to use samples with similar origins and collection procedures. In this case, the Swedish sera appeared to have lower general antibody titers than the African field sera, probably due to differences in exposure to infectious agents and other environmental factors. Still, the 21 Swedish control sera were important as technical controls to monitor assay-induced artifacts. It is noteworthy that the same top candidate diagnostic proteins were identified whether or not the Swedish samples were included.

The His$_6$ABP tag of the recombinant proteins, used for purification and to enhance solubility, played an important role in the suspension bead array assay, as its presence made it possible to measure the coating density of the beads with an anti-ABP antibody. By including a bead carrying the fusion partner His$_6$ABP per se, cross-reactivity to the His$_6$ABP tag was monitored in the suspension bead array assay. An option in the data analysis was to use the signal from this negative control to normalize signals between wells, but no correlation between response toward ABP and specific antibody signals was observed. The negative control signal was instead used to define a threshold value, with intensity levels exceeding this threshold judged as signals from serum antibodies binding to the recombinant mycoplasma proteins. During optimization of the ELISA, though, the fusion protein ABP was excluded because the experimental layout would require one to additionally coat wells with His$_6$ABP, as in other antigen ELISAs based on a fusion protein (2).

For an adequate diagnosis of CBPP, minimal cross-reactivity to related mycoplasmas is crucial. Surprisingly, the suspension bead array profiles for the 17 hyperimmune sera were found to be different from those for the African bovine sera, and signals were also obtained from antibody recognition of only a few...
recombinant proteins for the positive control anti-PG1 serum. However, those proteins that did appear were also among the most immunopotent surface proteins selected from the analysis of CBPP-positive sera. It therefore seems reasonable that any high signal intensity is representative for a case of infection, and “artificial” immunization appears to give a more selective immune response. Therefore, field samples from outbreaks of other mycoplasma infections would offer a preferable cohort in studies like this one. Nonetheless, the native protein corresponding to protein R1046, known as LppQ, has previously been shown to cross-react with antisera to Mycoplasma mycoides subsp. capri, Mycoplasma capricolum subsp. capripneumoniae, and Mycoplasma sp. bovine group 7 (1), and these were the same species found to cross-react with R1046 in this investigation. In a study of the antigenic specificity of LppQ (1), it was found that antisera raised against the C terminus of LppQ showed reactivity to proteins present in closely related species, while no cross-reactivity was detected for the N-terminal part of LppQ. This opens the possibility for more extended future studies that include thorough domain analysis of the selected antigens. Subcloning of the proteins could then offer a valuable strategy to adapt the target antigens to increase the specificity in the presented diagnostic application.

All serological diagnostic methods are influenced by alterations in the immune responses of infected animals. Antibody titers are highly individual and have been reported to lack a relationship to the severity of lesions (19), and chronically infected animals may additionally not be detectable with serological tests. This is why results from serological tests are most confident on a herd level. It is a challenge to determine the specificity of a new serological test when existing methods give inconsistent results, and it is therefore required to link experimental data directly to clinical data. The serum collection available for this study was limited to include sera from bovines that had adequate diagnostic information. As observed in our analysis, several transport events and storage times might have affected sample quality, and this could affect, among other things, the discovery of false-negative results. Since none of today’s serological methods are sufficiently sensitive per se, it is difficult to fully evaluate the performance of the antigen cocktail ELISA. One reason for the false-positive results discovered in our analysis could be a superior sensitivity of the bead-based array assay compared to traditional serology systems. Results from the well-characterized reference sera B001 and B002 were in clear concordance to expected and reported results (16) in both the bead-based assay and the cocktail ELISA.

The final antigen cocktail ELISA of this investigation had a discriminatory power that distinguished sera from CBPP-affected and CBPP-free bovines. Thus, a promising ELISA setup has successfully evolved from the screening assay. However, to become a solid diagnostic ELISA, future evaluation on larger cohorts of preferably fresh bovine sera from herds of various geographical origins is needed to optimize positivity thresholds in order to determine the sensitivity and specificity. It is preferred to conduct future studies in facilities at the sites of livestock affected by CBPP to allow a direct comparison between the cocktail ELISA and other available tests.

In conclusion, the selected recombinant mycoplasma antigens qualified as highly specific markers for CBPP and could be employed in both a suspension bead array platform and a cocktail ELISA setting. This set of proteins and technologies therefore offers a powerful combination to drive and further improve serological assays toward reliable, simple, and cost-effective diagnostics of CBPP.

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