Use of Recombinant Antigens To Detect Antibodies against *Mycoplasma suis*, with Correlation of Serological Results to Hematological Findings

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Porcine eperythrozoonosis is a disease with worldwide distribution caused by the unculturable hemotrophic bacterium *Mycoplasma suis*. Current serological testing utilizes crude *M. suis* antigens purified from the blood of experimentally infected pigs. These antigens show high variability and are restricted to specialized laboratories. We evaluated a novel serological assay based on two recombinant *M. suis* antigens (rMSG1 and rHspA1). Antigen specificity was proven by means of sera raised against nonhemotrophic mycoplasma and other relevant bacteria. Using experimental and convalescent-phase sera, rMSG1 and rHspA1 enzyme-linked immnosorbent assays (ELISAs) demonstrated sensitivities, specificities, and predictive values (94.0 to 100.0%) equal to or higher than those of the *M. suis* whole-cell ELISA. Field samples from 120 weaning piglets grouped by quantitative PCR results were used to evaluate the diagnostic capability of the new ELISA systems in comparison to that of the whole-cell ELISA. Assuming a 100.0% specificity of the PCR, the whole-cell ELISA, rHspA1 ELISA, and rMSG1 ELISA showed specificities of 84.8%, 83.8%, and 90.6% and sensitivities of 61.5%, 74.0% and 58.1%, respectively. Cohen's kappa coefficients comparing the recombinant ELISAs to the whole-cell ELISA indicate moderate to substantial agreement. The detection of anti-MSG1 and/or anti-HspA1 antibodies in pigs was significantly correlated with decreased hematocrit, erythrocyte numbers, and hemoglobin concentrations, indicating that a single seropositive result is connected with clinical and etiological significance. In conclusion, rMSG1 and rHspA1 are sensitive and specific serological and infection markers which are for the first time used independently of animal experiments. They are especially fit to be used in routine diagnosis, pathogenesis studies, and large-scale epidemiological investigations.

*Mycoplasma suis* is the etiological agent of porcine eperythrozoonosis (PE), a bacterial infection reported worldwide that manifests as a severe and often life-threatening acute febrile icteroanemia mainly in piglets, pregnant sows immediately after the parturition event, even where the application of specific serological tests would allow for a cost-effective diagnosis on a herd basis, follow-up investigations, and extensive prevalence studies. Previous studies applied three different methods: (i) a complement fixation assay (1, 14, 16), (ii) an indirect hemagglutination assay (15), and (iii) whole-cell enzyme-linked immunosorbent assays (ELISAs) (5, 9, 14). All these tests require animal experiments for the acquisition of partially purified antigens from the blood of experimentally infected pigs. Although previous studies have shown that immunoglobulin (Ig) depletion of blood-derived antigens increased the specificity of the *M. suis* ELISA significantly (5), the application of serological assays for the routine diagnosis of *M. suis* remained difficult. All serodiagnostic antigens described to date share the intrinsic disadvantage of extreme variability among batches and restriction to specialized laboratories because of the necessity of animal experiments. Therefore, an accurate adoption and standardization of diagnostic serological procedures with whole-cell *M. suis* antigens is impossible. Hence, recombinant antigens seem to be a good alternative substitute for blood-derived *M. suis* antigens and may overcome the difficulties encountered in using experimental animals as a source of *M. suis*. The recombinant *Escherichia coli* expression of proteins would allow the production of reproducible and characterized antigenic proteins for unculturable mycoplasmas.

Recently two immunodominant *M. suis* proteins (p40 and p70) were identified as promising serological markers (5). Detailed identification and characterization of these proteins (p40 and p70) were achieved using serological proteome analysis and genomic library screening procedures: p70 was identified as HspA1, a surface-localized DnaK-analogous protein (6),
and p40 was identified as MSG1, a surface-localized adhesion protein with glyceraldehyde-3-phosphate dehydrogenase properties (7).

The aim of this study was to develop and evaluate the first recombinant serological assay for detecting M. suis-specific antibodies. To this effect, we investigated the antibody responses and kinetics of experimentally infected pigs with immunoblotting and indirect ELISA, using the purified recombinant HspA1 and MSG1 proteins (rHspA1/rMSG1) produced in Escherichia coli. Furthermore, we evaluated the diagnostic application of rMSG1 and rHspA1 and the combination of both for the detection of antibodies against M. suis in field samples and compared them with a whole-cell M. suis ELISA (5), PCR results, and hematological parameters.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and control sera.** M. suis strain 54/96 was obtained from experimentally infected pigs as described previously (4, 5). E. coli K12 strains Top10 and LMG1994 (Invitrogen, Basel, Switzerland) were grown in Luria-Bertani broth containing 100 μg/ml ampicillin and used to clone and express the M. suis a1 and msg1 genes. The arabinose-inducible expression plasmid pBAD/his (N-terminal his- and myc tag; Invitrogen) was used for protein expression. Experimental sera from rabbits and pigs immunized/infected with nonhemotropic Mycoplasma spp. and pig-specific antibodies were selectively Table 1. Experimental sera used for antigen specificity testing

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Animal species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycoplasma hyopneumoniae</td>
<td>Pig</td>
<td>J. Frey*</td>
</tr>
<tr>
<td>Mycoplasma hyorhinis</td>
<td>Pig</td>
<td>J. Frey</td>
</tr>
<tr>
<td>Actinobacillus pleuropneumoniae</td>
<td>Pig</td>
<td>C. Baums*</td>
</tr>
<tr>
<td>Chlamydia suis</td>
<td>Pig</td>
<td>IVB</td>
</tr>
<tr>
<td>Chlamyaphila abortus</td>
<td>Pig</td>
<td>IVB</td>
</tr>
<tr>
<td>Salmonella enterica subs. enterica</td>
<td>Rabbit</td>
<td>IVB</td>
</tr>
</tbody>
</table>

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* Institute for Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany.
* Institute of Veterinary Bacteriology, University Zurich, Zurich, Switzerland.

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**DNA extraction and PCR assay.** DNA was extracted from 200 μl of EDTA-anticoagulated blood using the Bacterial Genomic DNA Kit (Sigma, Buchs, Switzerland). M. suis-specific real-time PCR (LC PCR) was carried out based on an assay described previously (8).

**Hematological analysis.** Hematological parameters of the pigs of group 5 and group 6 were evaluated with EDTA-anticoagulated blood, using the Scil Vet ABC tool (Scil Animal Care Company GmbH; Viernheim, Germany); biochemical parameters of blood sera were analyzed by means of the Hitachi 911 chemist analyzer (Roche, Mannheim, Germany).

**Production of recombinant proteins MSG1 and HspA1.** To circumvent the *Mycoplasma*-specific translational barrier of the UGA codon, the genes encoding HspA1 (gene a1) and MSG1 (gene msg1) were adapted to the codon usage of *E. coli* and de novo synthesized as described previously (6, 7). Recombinant E. coli clones were grown in RM base medium (Invitrogen) supplemented with 100 μg/ml ampicillin to an optical density at 600 nm (OD600) of 0.6 at 37°C. Recombinant protein synthesis was induced by the addition of 0.02% arabinose, and *E. coli* cultures were incubated for a further 2 h at 37°C. His-tagged fusion proteins were purified as described previously (6, 7). The purities of the recombinant proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Protein concentrations were determined using a Bradford assay kit (Bio-Rad, Reinach, Switzerland).

**SDS-PAGE and immunoblotting.** SDS-PAGE was performed according to standard procedures (11). Separated proteins were transferred onto nitrocellulose membranes, using a semidyed electrophoretic transfer cell (Trans Blot; Bio-Rad, Reinach, Switzerland). Immunoblots were probed with *E. coli* absorbed sera. The experimental pigs were inoculated with 1:100 in phosphate-buffered saline (PBS), with 2% skim milk powder. Immunoreactive proteins were visualized using horseradish peroxidase-labeled goat anti-pig IgG (Sigma) as secondary antibodies and 4-chloro-1-naphthol as the chromogenic reagent.

**Recombinant-antigen ELISA.** Polystyrene microtiter plates (Greiner, Nurtin, Germany) were coated at 4°C overnight with rMSG1, rHspA1, or the purified *E. coli* control, 0.5 to 300 ng/well suspended in 0.05 M sodium carbonate (pH 9.6). The plates were washed three times with PBS-0.05% Tween 20. Goat anti-pig IgG (Sigma) was added to the plates, blocked with PBS containing 1% Protease Peptone (Difco-Brunschwig, Basel, Switzerland) for 1 h at room temperature, and after three washes with PBST, stored at −20°C until use. All incubation steps listed below were performed in a 100-μl volume for 1 h at room temperature and under agitation. Wells were incubated with sera, diluted 100-fold in blocking solution, and then washed three times with PBST and incubated with anti-pig IgG conjugated to horseradish peroxidase (heavy plus light chains; Sigma). The plates were then washed thrice with PBST and incubated with 2,2'-azino-bis(3-ethylbenzthiazolin-sulfonic acid) (Roche Diagnostics, Rotkreuz, Switzerland) for 30 min in the dark. The absorbance at 405 nm was measured using a computer-assisted microplate reader (Tecan, Maenndorf, Switzerland).

**Initial assays** were performed to determine the antigen concentration that best discriminated between ELISA-positive and ELISA-negative sera from samples serum from *M. suis*-infected pigs and those of samples from mock-infected control pigs. Checkboard titrations were performed with serial twofold antigen dilutions (starting concentration, 1 μg) and with twofold serum dilutions (25-, 50-, 100-, and 200-fold). In subsequent assays, the plates were coated with the predetermined optimal antibody concentration and *E. coli* control antigen. Incubations were performed with 100- and 10,000-fold dilutions of sera and secondary antibody conjugate, respectively. Individual sera were tested in duplicate with recombinant proteins and the *E. coli* control. The means of the net OD values (ODEcoli−ODbackground − ODcontrol) were calculated for analysis. Paired measurements differing by more than 10% were tested only once again. Positive and negative control sera were included on each plate and in duplicate. To determine the serostatus of the slaughterhouse pig groups (groups 5 and 6), separate ELISAs for each recombinant protein were performed and results from the individual ELISAs were combined.

**Cutoff values** for each recombinant protein were calculated as the mean net OD value for the sera from 25 mock-infected animals plus three times the standard deviation. Test specificity (the minimum number of false-negative results) was assessed with sera from PCR-negative, mock-infected experimental pigs (“true negative”). The percent specificity was calculated from the following equation: [number of true negatives/(number of true negatives + number of false positives)] × 100. The test sensitivity was assessed using sera from experimentally *M. suis*-infected pigs (“true positive”). The percent sensitivity was calculated using the following equation: [number of true positives/(number of true positives + number of false negatives)] × 100. Diagnostic efficiency was defined as follows: (number of true positives + number of true negatives)/all test
results. The negative predictive value was calculated as follows: number of true negatives/(number of true negatives + number of false negatives). The positive predictive value was calculated as follows: number of true positives/(number of true positives + number of false positives).

Data analysis. Parameters (ELISA OD values, PCR results, and blood parameters) were compiled and analyzed using Excel (Microsoft, Wallisellen, Switzerland), Origin 7.5 (Redacom), and SigmaStat 3.5 (Statcon) software. Parameters were considered to be significant if P values of <0.05 were considered to be significant. Cohen's kappa coefficients were calculated to compare results of whole-cell ELISA with those of recombinant ELISAs. Interpretation of the kappa values was performed as suggested by Landis and Koch (12). Kappa values of 0.20 or less indicate a slight compliance; 0.21 to 0.40, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80, substantial agreement, and 0.81, almost perfect agreement, and 1.00, perfect agreement between tests.

To estimate the efficiencies of the three ELISAs and the PCR tests with field samples, a latent class model using maximum-likelihood techniques was used. It was assumed that the PCR test had a diagnostic specificity of 100% but that the sensitivities of the PCR, the sensitivity and specificity of the ELISA tests, and the number of true-positive samples from the 120 field samples were unknown. Correlation dependence on the sensitivities and specificities of the tests was assumed and included in the model (3). With a known specificity of the PCR, the model was definable even in the presence of multiple dependencies between the diagnostic tests. Starting values of each test, sensitivity and specificity, the population prevalence, and the correlation values of the tests were determined. Each animal was assigned a probability calculated from these starting values and the combination of test results seen for each animal. Each probability was converted to a negative logarithm and summed to give the negative logarithm of the likelihood of the data. Parameters were varied using the solver function of Excel to calculate the parameter values that minimized the negative log likelihood. To estimate confidence intervals, a likelihood profile was calculated using the PopTools add-on (CSIRO) of Excel.

RESULTS

Setting-up of recombinant serological assay. SDS-PAGE and Coomassie brilliant blue staining confirmed a high purity of recombinant protein preparations with only marginal unspecific protein bands (Fig. 1A). Immunoblot analysis with pooled sera from experimentally infected pigs confirmed the immunoreactivities of both proteins: the serum pool recognized the expected recombinant proteins rHspA1 and rMSG1 with molecular masses of approximately 40 kDa and 70 kDa, respectively, but did not react with the noninduced control preparation (Fig. 1B). Pooled sera from negative-control pigs did not react with rHspA1 or with rMSG1 (Fig. 1C).

All ELISA procedures were optimized with regard to antigen concentrations and serum dilution. Antigen titration experiments revealed that 0.6 µg/ml of rHspG1, 0.3 µg/ml of rHspA1, and a serum dilution of 1:100 produced the best resolution between the ODs of the positive and negative serum pools. The ELISA cutoff value for each recombinant protein was calculated as the mean net OD of the mock-infected group sera plus thrice the standard deviation (Table 2).

Serum samples from pigs experimentally infected with M. suis (n = 25; group 1), from naturally infected pigs with symptoms typical for PE (n = 25; group 3), and from M. suis-free, mock-infected animals (n = 25; group 2) and samples from clinically healthy, PCR-negative pigs (n = 25; group 4) were used to test the performance of the M. suis recombinant serology assays. To exclude antibody reactions against E. coli components in the purified recombinant antigens, all sera were adsorbed with E. coli cells and additionally tested against E.

![FIG. 1. (A) SDS-PAGE of recombinant MSG1 and HspA1, showing the purity after affinity chromatography preparation. (B and C) Immunoblot analysis of recombinant MSG1 and HspA1; blots were detected with a serum pool of experimentally M. suis-infected pigs (B) or with a serum pool of M. suis-negative pigs (C). MW, molecular mass standard; Ec, E. coli control preparation.](http://cvi.asm.org/)

### TABLE 2. Diagnostic performance of recombinant ELISAs in comparison with that of M. suis whole-cell ELISA

<table>
<thead>
<tr>
<th>Serological test</th>
<th>Cutoff value&lt;sup&gt;b&lt;/sup&gt; (OD&lt;sub&gt;405&lt;/sub&gt;)</th>
<th>Diagnostic sensitivity&lt;sup&gt;c&lt;/sup&gt; (%)</th>
<th>Diagnostic specificity&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Diagnostic efficiency&lt;sup&gt;e&lt;/sup&gt; (%)</th>
<th>PPV&lt;sup&gt;f&lt;/sup&gt; (%)</th>
<th>NPV&lt;sup&gt;g&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rMSG1 ELISA</td>
<td>0.213</td>
<td>94.0</td>
<td>100.0</td>
<td>97.0</td>
<td>100.0</td>
<td>94.3</td>
</tr>
<tr>
<td>rHspA1 ELISA</td>
<td>0.259</td>
<td>100.0</td>
<td>94.0</td>
<td>97.0</td>
<td>94.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Whole-cell ELISA</td>
<td>0.273</td>
<td>94.0</td>
<td>96.0</td>
<td>93.0</td>
<td>95.9</td>
<td>94.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> These results were obtained from experimentally infected acutely diseased pigs and from mock-infected experimental animals and healthy pigs.

<sup>b</sup> Cutoff values were calculated as the mean OD value for the sera from mock-infected animals plus three times the standard deviation.

<sup>c</sup> Sensitivity was calculated as follows: [number of true positives/(number of true positives + number of false negatives)] × 100.

<sup>d</sup> Specificity was calculated as follows: [number of true negatives/(number of true negatives + number of false positives)] × 100.

<sup>e</sup> Diagnostic efficiency was defined as follows: [number of true positives + number of true negatives] × 100.

<sup>f</sup> Positive predictive value (PPV) was defined as follows: [number of true positives/(number of true positives + number of false positives)] × 100.

<sup>g</sup> Negative predictive value (NPV) was calculated as follows: [number of true negatives/(number of true negatives + number of false negatives)] × 100.
coli as the antigen. All adsorbed sera showed no reactions with E. coli antigens in immunoblots and low OD values in the ELISA (data not shown). Different adsorption profiles were tested for their application suitability. An adsorption procedure using 100 μl serum with a serum predilution of 1:25, an E. coli cell pellet volume of approximately 50 μl, and an adsorption time of 2 h was found to produce a sufficient adsorption effect.

The ELISA reactivities of each individual experimental serum sample with the two recombinant proteins are shown in Fig. 2. For comparison purposes, the results obtained with the whole-cell M. suis ELISA (5) are also shown. A comparison of the performances of the rMSG1 and rHspA1 ELISAs compared with those of the whole-cell ELISA is summarized in Table 2. Overall, the test sensitivities (the minimum number of false-negative results in groups 1 and 3) of the recombinant-antigen ELISAs were equal to or higher than the whole-cell ELISA sensitivity (94.0% and 100.0%, respectively, compared to 94.0%). In terms of the test specificities (the minimum number of positive results in groups 2 and 4), both recombinant ELISAs were also comparable to the whole-cell ELISA (100.0% and 94.0%, respectively, compared to 96.0%). The well-to-well variations of the rMSG1 and rHspA1 ELISAs were determined to be 6.5% and 8.2%, respectively. Furthermore, both recombinant ELISAs were shown to have a mean interassay variation of 20.6%. To determine the antigen specificity with control animals, experimental sera from animals immunized or infected with nonhemotrophic M. spp. and other pig-related bacteria (Table 1) were tested. Both recombinant antigens showed an antigen specificity of 100.0%, since antibodies raised against other Mycoplasma species and other pig-related bacteria did not react with rHspA1 or MSG1 in any case.

**Antibody kinetics measured against M. suis recombinant proteins.** To analyze the kinetics of the antibody responses to rMSG1 and rHspA1, ELISAs were performed with a total of 120 serum samples obtained from 8 experimentally infected pigs (group 1 animals) between days 0 and 98. Representative rHspA1 and rMSG1 antibody kinetics are shown in Fig. 3. All preinfection sera were negative for rMSG1 and rHspA1 reactivities. HspA1- and MSG1-specific antibodies were detected as early as 2 weeks postinfection, and all pigs were identified as antibody responders within 3 weeks postinfection until the end of the experiment. Only at the time of the major acute clinical PE symptoms did the ELISA OD values with both antigens and the pig-derived M. suis antigen decline significantly and partly below the cutoff values.

**Diagnostic applicability of rMSG1 and rHspA1 ELISAs.** Applicability and discriminatory efficacy of the recombinant serology assays were evaluated using serum samples randomly collected from 120 weaning piglets at a slaughterhouse. Prior to serological testing, the pigs were grouped into PCR-positive (n = 60) and PCR-negative (n = 60) animals. The results of the ELISAs (whole cell, rMSG1, rHspA1, and the combination of rMSG1 plus rHspA1) are shown in Table 3.

**Table 3. Diagnostic application of recombinant ELISAs in investigation of field samples.**

<table>
<thead>
<tr>
<th>Animal group (n)</th>
<th>rMSG1</th>
<th>rHspA1</th>
<th>rMSG1 + rHspA1</th>
<th>Whole cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-positive pigs (60)</td>
<td>35 (58.3)</td>
<td>45 (75.0)</td>
<td>48 (80.0)</td>
<td>37 (61.7)</td>
</tr>
<tr>
<td>Bacterial load of ≥10⁶/ml blood (15)</td>
<td>11 (73.3)</td>
<td>13 (86.6)</td>
<td>14 (93.3)</td>
<td>11 (73.3)</td>
</tr>
<tr>
<td>Bacterial load of &lt;10⁵/ml blood (45)</td>
<td>24 (53.3)</td>
<td>32 (71.1)</td>
<td>34 (75.5)</td>
<td>26 (57.7)</td>
</tr>
<tr>
<td>PCR-negative pigs (60)</td>
<td>8 (13.3)</td>
<td>12 (20.0)</td>
<td>16 (26.7)</td>
<td>11 (18.3)</td>
</tr>
</tbody>
</table>

* Results were obtained by investigating 60 sera from PCR-positive animals and 60 sera from PCR-negative animals.

b n, no. of pigs.

M. suis whole-cell antigen.
Analysis of the results obtained with all four different ELISAs showed a clearly higher detection rate for the group of PCR-positive pigs than for the group of PCR-negative pigs. In detail, 35 (58.3%) sera out of the 60 PCR-positive pigs tested positive with rMSG1 ELISA. Forty-five (75.0%) tested positive with rHspA1, and 48 (80.0%) tested positive using both rMSG1 and rHspA1 as antigens. By means of the whole-cell ELISA, 37 of 60 PCR-positive animals were judged to be positive (61.7%). In greater detail, 11 (73.3%) of the 15 animals showing an M. suis load by PCR of $\pm 10^5$ per ml blood tested positive with rMSG1, 13 (86.6%) tested positive with rHspA1, and 14 (93.3%) revealed a positive serological result using both rMSG1 and rHspA1 as antigens. In this group of animals, 11 pigs (73.3%) were tested as M. suis antibody responders by means of the whole-cell ELISA. In the group of PCR-positive pigs showing bacterial loads of less than $10^2$ per ml blood (n = 45), 24 (53.3%) samples reacted positively with rMSG1, 32 (71.1%) reacted positively with rHspA1, and 34 (75.5%) reacted positively using rMSG1 in combination with rHspA1. Twenty-six (57.1%) reacted positively in the whole-cell ELISA. In the group of PCR-negative pigs (n = 60), eight pigs (13.3%) were identified as M. suis antibody positive with the same ELISA and 12 pigs (20%) tested positive in the rHspA1 ELISA, and in the combinatorial rMSG1/rHspA1 ELISA, 16 pigs (26.6%) were positive. Using the whole-cell ELISA, samples from 11 pigs (18.3%) reacted as serologically positive.

**Comparison of the different ELISAs.** Diagnostic values of the serological assays were obtained by comparison of the different ELISA results with PCR results. If quantitative LC PCR is considered the reference test, the estimated M. suis prevalence in the 120 abattoir samples was 54.0% (95% confidence interval [CI], 50.0% to 65.0%), with 65 pigs having a positive infection status (95% CI, 60 to 78 pigs). Then, the quantitative LC PCR had a sensitivity of 93.6% (95% CI, 77.0 to 100.0%), assuming that the specificity was 100.0%. Out of all three different ELISAs, the rHspA1 ELISA showed the highest sensitivity at 74.0% (95% CI, 61.7 to 84.2%) and the rMSG1 ELISA the highest specificity at 90.6% (95% CI, 79.2 to 98.0%). In comparison, sensitivity and specificity of the whole-cell ELISA were estimated to amount to 61.5% (95% CI, 49.2 to 72.9%) and 84.8% (95% CI, 72.0 to 95.0%), respectively. Combined testing with both antigens revealed dependence on the sensitivity of the rMSG1 and rHspA1 ELISA of 0.09 (95% CI, 0.043 to 0.145).

When the results of the rMSG1 ELISA were compared to those from the whole-cell ELISA, we observed an agreement of 80.0% and a Cohen’s kappa coefficient of 0.52, indicating a moderate agreement. The comparison of the rHspA1 ELISA results to those from the whole-cell ELISA revealed an agreement of 82.5% and a Cohen’s kappa coefficient of 0.71, indicating a substantial agreement.

**Hematological characteristics of pigs testing serologically positive for M. suis.** Correlation between the presence of anti-HspA1 and anti-MSG1 antibodies and the hematological status of pigs was analyzed. Of the hematological parameters evaluated, three parameters were significantly associated with a serologically positive status for M. suis. M. suis-serologically positive pigs had significantly lower packed cell volume, hemoglobin, and red-blood-cell values ($P < 0.05$). The mean values, 95% confidence intervals, and ranges for these parameters are provided in Table 4. No significant association between leukocyte indices, i.e., mean cellular volume, mean cellular hemoglobin, and mean cellular hemoglobin concentration, and the serological status of the pigs was found. Likewise, no significant association could be evaluated between leukocyte, serum glucose, bilirubin, and iron values and the serological status of the pigs examined.

### DISCUSSION

Serological assays are inexpensive, easy-to-perform diagnostic tests which are applied in a great range of microbiological routine laboratories. Their overall quality depends on the choice and high class of the antigens used for antibody detection. All serological M. suis assays described formerly have the intrinsic disadvantage of employing complex and undefined M. suis antigens obtained from the peripheral blood of experimentally infected pigs that contains uncontrollable amounts of porcine components (e.g., porcine Igs) (1, 5, 9, 14, 15, 16). Serological assays based on recombinant antigens are the only possibility for circumventing these drawbacks. Recombinant protein-based serological tests may achieve high sensitivity and

### TABLE 4. Hematological findings for serologically M. suis-positive and -negative pigs

<table>
<thead>
<tr>
<th>Blood parameter</th>
<th>Value for pigs with serological status of:</th>
<th>Reference range</th>
<th>P value&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Median 95% CI</td>
<td>Median 95% CI</td>
<td></td>
</tr>
<tr>
<td>PCV&lt;sup&gt;f&lt;/sup&gt; (%)</td>
<td>32.9 31.9–33.9</td>
<td>34.6 33.3–35.9</td>
<td>32–42</td>
</tr>
<tr>
<td>Hemoglobin (mmol/liter)</td>
<td>6.3 6.1–6.5</td>
<td>6.7 6.4–7.0</td>
<td>6.6–8.3</td>
</tr>
<tr>
<td>Erythrocytes (10&lt;sup&gt;6&lt;/sup&gt;/μl)</td>
<td>6.3 6.1–6.6</td>
<td>6.9 6.6–7.1</td>
<td>5.8–7.8</td>
</tr>
<tr>
<td>Leukocytes (10&lt;sup&gt;3&lt;/sup&gt;/μl)</td>
<td>26.8 24.6–28.7</td>
<td>28.9 26.1–31.6</td>
<td>13–24</td>
</tr>
<tr>
<td>Serum glucose (mmol/liter)</td>
<td>3.7 3.3–4.1</td>
<td>4.1 3.7–4.4</td>
<td>3.6–6.7</td>
</tr>
<tr>
<td>Bilirubin (μmol/liter)</td>
<td>3.4 2.5–4.4</td>
<td>3.4 2.6–4.3</td>
<td>&lt;4.3</td>
</tr>
<tr>
<td>Iron (μmol/liter)</td>
<td>17.0 15.2–18.7</td>
<td>17.3 15.1–19.5</td>
<td>&gt;17.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Pigs from the slaughterhouse groups (group 5 and group 6).

<sup>b</sup> Samples from serologically positive animals reacted positively with rMSG1 or rHspA1 or with both recombinant antigens.

<sup>c</sup> Samples from serologically negative animals were negative with both rMSG1 and rHspA1.

<sup>d</sup> Reference ranges according to Kixmoeller and coworkers (10).

<sup*e</sup> Asterisks indicate P values of <0.05, which were considered significant.

<sup>f</sup> PCV, packed cell volume.
specificity because of the high concentration of immunoreactive antigens and the lack of nonspecific moieties found in whole-cell preparations from the blood of experimentally infected pigs. An ideal antigen would be a principal target of the host immune response (serodiagnostic markers of infection), expressed by *M. suis* during acute and chronic disease and conserved among *M. suis* isolates. In addition, differentiation between autoreactive serological reactions directed against porcine constituents and agent-specific seroreactivities should be easily possible given the fact that the type and intensity of the immune response (*M. suis*-specific versus autoreactive) obviously play a major role in the course of the hemoplasma infection and the development of disease (5, 18).

In previous studies, we identified two *M. suis* proteins with such features, i.e., HspA1 and MSG1 (5, 6, 7). Within the scope of this study, we evaluated serological assays based on these two proteins expressed in *E. coli*. Purity and seroreactivity of both recombinant proteins were proven in SDS-PAGE and immunoblot studies. The analytical specificity of the two recombinant antigens was confirmed by testing rabbit and pig sera induced against nonhemotropic mycoplasma species of a different origin but related to *M. suis* and against other microorganisms with clinical relatedness. We did not detect any cross-reactivity in immunoblot and ELISA analyses in any of the aforementioned cases.

The investigation of sera from experimentally infected pigs with the novel recombinant ELISA demonstrated high specificities and sensitivities and a high agreement with the whole-cell *M. suis* ELISA. In addition, the kinetics of the immune response in the course of experimentally induced PE was confirmed using rHspA1 and rMSG1. Typically, during acute clinical PE, a derailment of the *M. suis*-specific antibody response was accompanied by peaking levels of autoreactive IgG antibodies (5). The derailment of *M. suis*-specific antibodies was also observed using recombinant proteins as antigens. Therefore, the problem of detecting *M. suis*-specific antibodies during clinical acute PE was not resolved by means of using recombinant antigens. This phenomenon is obviously to be traced back not to the antigen used but to a derailment of the immune response during acute PE (5).

Our results demonstrate that both antigens are suitable for diagnostic assays. In comparison, rHspA1 showed a higher sensitivity with a lower specificity, whereas rMSG1 showed a higher specificity combined with a lower sensitivity. Therefore, an assay combining both antigens could prove to be a good alternative: in our study, the parallel application of both antigens to classify pigs as positive or negative enhanced the sensitivity. Moreover, this antigen combination is considered to be appropriate because investigations of the *M. suis*-specific immune response in the course of infection revealed a very heterologous antibody profile by means of the detected antigens (5). Nevertheless, the combination of two immunodominant *M. suis* antigens (HspA1 and MSG1) allowed us to detect both acutely and chronically infected pigs.

A reference test (gold standard) for the detection of antibodies directed against *M. suis* with which the ELISA results could be compared is not available. Therefore, the establishment of the infectious status of the pigs was performed on the basis of quantitative PCR results. Negative sera were collected from experimental pigs free of *M. suis* as tested by PCR. Positive sera were collected from pigs infected experimentally that tested positive for infections (PCR and clinical investigation). The examination of these well-defined groups by means of both recombinant ELISAs yielded high specificities and sensitivities (94.0 to 100.0%). To evaluate the diagnostic applicability, field sera from slaughtered pigs were grouped by investigating the corresponding anticoagulated blood specimens using real-time PCR (8). In the group of PCR-positive piglets, the ratio of serologically positive piglets was significantly higher (58.3 to 75.0%) than that in the group of PCR-negative piglets (13.3 to 26.6%).

When the results of recombinant ELISAs were compared to those of the whole-cell ELISA, we observed considerable levels of agreement (80.0% and 82.5%; kappa values, 0.52 and 0.71, respectively). It must be noted that the use of both recombinant ELISA results as a diagnostic criterion means that the two tests are not conditionally independent (both tests measure anti-*M. suis* antibodies). Combinatorial testing with rMSG1 and rHspA1 as diagnostic antigens revealed a correlation of sensitivity of 0.09 (CI, 95%; range, 0.043 to 0.145) and the highest sensitivity (80.0% positive in the group of PCR-positive animals).

The significance of a positive serological result with rHspA1 or/and rMSG1 as antigens could be proved by a significant correlation between the result of the serology and the clinical hematological parameters, i.e., pigs showing antibodies against *M. suis* also revealed significantly lowered values of packed cell volume, hemoglobin, and RBC numbers. The absence of significant differences in erythrocytic indices (mean cellular volume, mean cellular hemoglobin, and mean cellular hemoglobin concentration) are indicative for a normochromic, normocytic anemia due to intravascular hemolysis. However, at this point, we do not know whether intra- or extravascular hemolysis predominates during *M. suis* infections (18), and the analysis of hemolysis mechanisms during *M. suis* infections should be the goal of further studies. In addition, we discovered a greater ratio of pigs with bacterial loads higher than 10^7 per ml in the group of seropositive piglets.

Remarkably, antibodies against *M. suis* were detected in approximately 20% of the piglets in the PCR-negative group. At first sight, these results seem to speak in favor of a false-positive serology. However, all sera were reanalyzed using immunoblots against whole-cell *M. suis* antigens and against the recombinant proteins, and the seropositivity could thus be confirmed in practically all cases (data not shown). Therefore, it is obvious that our serological assay enables the detection of seropositive animals after clearance of the infection or the detection of chronic low-grade infections which cannot be recognized by PCR or by microscopy. These carrier pigs are of major economic significance, as well as being important with regard to epidemiology, surveillance, and the eradication of PE. Altogether, the new *M. suis* serological assays are a very valuable completion of our diagnostic possibilities for *M. suis* infections, especially in addition to PCR.

To date, no serological test for PE has achieved integration into routine diagnostic laboratories. The serological assays currently available are inadequate for routine work because they can be carried out only in those few laboratories able to propagate *M. suis* in splenectomized pigs and therefore able to prepare diagnostic antigens. Our new recombinant ELISA will contribute to rectifying this problem. The robustness of our
serological assays, reflected by the low intra- and interassay coefficients of variation of 6.5 to 8.2% and 20.6%, respectively, provide further evidence for this. Arguments against the application of recombinant antigens in serology are protein stability, lack of sensitivity due to only a few antigens of the agent being used, and E. coli impurities which can be traced back to insufficient purification procedures. Within the scope of our work, the stability of the recombinant proteins proved to be very high (approximately 6 months at −80°C). We determined a similar sensitivity in comparison to results with whole-cell antigens, and as mentioned, our rHspA1 and rMSG1 antigens were quite pure. Nevertheless, an adsorption of the investigated sera with E. coli was unavoidable. Because an adsorption of the prediluted sera (1:25) over a period of about 2 h proved to be sufficient to remove cross-reacting antibodies, it should be possible to perform this additional task in routine laboratories.

In summary, this article describes for the first time the development of a serodiagnostic assay for PE based on antigens produced in the laboratory. The use of the recombinant proteins MSG1 and HspA1 has important advantages, i.e., (i) improved specificity, sensitivity, reproducibility, and reliability of PE serodiagnosis, (ii) replacement of animal experiments necessary to propagate M. suis for antigen production, (iii) the possibility of extensive prevalence and surveillance studies determining the actual impact of M. suis infection in pig populations, and (iv) studies on the connection between the M. suis immune response and PE pathogenesis.

REFERENCES