Development of a Sensitive and Specific Enzyme-Linked Immunosorbent Assay Based on Recombinant Antigens for Rapid Detection of Antibodies against *Mycoplasma agalactiae* in Sheep\(^*\)†

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We developed a new recombinant enzyme-linked immunosorbent assay (rELISA) for serodiagnosis of contagious agalactia (CA), a disease caused by *Mycoplasma agalactiae* in sheep and goats. The assay is based on two *M. agalactiae* surface proteins, namely, P80 and P55. Identification of these immunodominant and common antigens was accomplished by examining the antibody response elicited in sheep during experimental infection and comparing it to the protein expression profiles of 75 *M. agalactiae* field strains. Our rELISA was tested with 343 sera, collected from sheep with a laboratory-confirmed diagnosis of CA (n = 223) and from healthy animals (n = 120). All sera had previously been tested by Western blotting (WB) for reactivity against *M. agalactiae*. In addition, our rELISA was compared with a commercial routine ELISA based on inactivated antigens (CHEKiT). Among the 223 samples that were WB positive for *M. agalactiae*, 209 (93.7%) tested positive for rP80-P55 with our ELISA, whereas only 164 (73.8%) tested positive with the CHEKiT ELISA. Among the 120 samples tested that were WB negative for *M. agalactiae*, 96.7% were confirmed as negative with our rELISA, while only 75.8% were confirmed as negative with the CHEKiT ELISA. A comparison of the results with receiver operating characteristic curves indicated that the differences observed between our rELISA and the CHEKiT ELISA are statistically significant. The use of recombinant peptides instead of inactivated antigens could significantly improve the discrimination of positive and negative animals, bringing significant advantages in controlling the import/export of live animals and helping in eradication of this economically detrimental disease.

*Mycoplasma agalactiae* is a cell wall-less bacterium belonging to the class *Mollicutes*, and it has been identified as the etiological agent of contagious agalactia (CA) in small ruminants. This disease, characterized primarily by agalactia, mastitis, arthritis, and sometimes keratoconjunctivitis and pneumonia, has a worldwide diffusion but is prominent in the Mediterranean basin (1). *M. agalactiae* can cause serious economic losses in areas where the economy is based largely on shepherding, since it causes a reduction of milk and cheese production and leads to increased mortality in lambs. For these reasons, vaccination is widely used as a highly effective and practical measure for preventing the disease in sheep and goats. In Italy, the inactivated vaccine has the most widespread use. Several methods have been reported for detection of the serological response to this pathogen, including a growth inhibition test (2), enzyme-linked immunosorbent assay (ELISA) (11, 12), and immunoblotting (19). These tests are used to detect, but also to discriminate, the antibody responses elicited in both vaccinated and infected sheep. Although the growth inhibition test allows discrimination of an antibody response elicited by infection from one elicited by vaccination (21), it is cumbersome and time-consuming. The Western blot (WB) assay is extremely sensitive (it was used as the “gold standard” test in our study), but it is expensive and does not allow quantification of the antibody levels. On the other hand, ELISA is the test of choice among existing serological procedures because it has the potential for high sensitivity and specificity, and in addition, it is simple and allows testing of a large number of samples in a short time (i.e., it has a high throughput). In a previous work, we identified and characterized the surface membrane proteins involved in the immunological response of sheep (21). Among the proteins we characterized at the molecular level, some are stable proteins, such as P80, P48, and P30 (3, 14, 22), while others belong to a family of variable surface lipoproteins which undergo high-frequency phase and size variation (6, 16); one of them (P40), however, plays an important role in attachment to the host cell (4). These proteins are expressed with remarkable variability among *M. agalactiae* strains and isolates (17, 19). Therefore, a good ELISA should be based on antigens that are both highly immunogenic and expressed during the entire course of infection in the highest possible number of field isolates. In order to assess which and how many antigens we should use in our ELISA, we studied antibody production during experimental infection of sheep and analyzed the protein expression of 75 *M. agalactiae* field strains. The antigenicity of the two selected proteins and their usefulness as recombinant antigens were established after their expression in *Escherichia coli*. We also evaluated the applicability of the recombinant ELISA (rELISA), using sera collected from ex-

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Recombinant protein expression. The M. agalactiae genes and oligonucleotide primers used for production of the truncated proteins are indicated in Table 1. The primer sets were designed for the directional cloning of genes and for amplification of the gene fragments without TGA codons. The 1,014-bp (P80) and 576-bp (P55) gene fragments encode proteins of 337 and 196 amino acids, with calculated molecular masses of 37,543 and 21,123 kDa, respectively. The PCR products were digested with the BamHI and KpnI restriction enzymes and inserted in frame into the pQE-30 expression vector (QIAGEN, Chatsworth, CA), using engineered restriction sites located in the forward and reverse primers. Each construct was used to transform E. coli DH5α cells containing the pREP-4 repressor plasmid (Invitrogen). Cloning into pQE-30 makes it possible to produce recombinant proteins linked to a polyhistidine (His) stretch that binds strongly to nickel-chelated columns (QIAGEN). pQE-30 derivatives were selected on LB agar plates supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. Plasmid DNAs were extracted with a QIAJET Plasmid Mini kit. The sequences of the resulting clones were confirmed by automated DNA sequencing. Recombinant E. coli clones were grown in LB medium containing 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 18 h. Bacteria were harvested by centrifugation at 4,000 g for 15 min and resuspended in buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0) at room temperature for 1 h, with agitation. The His-tagged proteins were then extracted with a QIAJET kit following the manufacturer’s instructions. Truncated proteins were examined by WB with naturally infected sera and specific anti-P80 and anti-P55 sera.

ELISA. All 343 serum samples were tested with both rELISA and CHEKIT ELISA. For the rELISA, antigens diluted in 0.2 M boric acid buffer at pH 9.6 were used to sensitize 96-well plates (Pro-Bind assay plate; Falcon Becton Dickinson, NJ) overnight at 4°C. The concentrations of the two antigens were optimized in order to obtain the best discrimination between positive and negative samples. Concentrations of 10 μg/ml of rP80 and 3.125 μg/ml of rP55 gave the best discrimination between the high and low optical density values. After three washes with PBS containing 0.1% Tween 20 (PBST), the plates were blocked with 1% bovine serum albumin (Sigma) in PBS for 1 h at room temperature. The plates were then washed with PBST, and 200 μl of a serum sample diluted 1:100 in PBST was applied to each well. The plates were incubated for 30 min at 37°C and washed, and then 200 μl of peroxidase-conjugated donkey anti-sheep whole-molecule immunoglobulin G (IgG) (Sigma) was added to each well at a dilution of 1:1,000. The plates were incubated for 30 min at 37°C and washed, and then 100 μl of 0.05 M disodium phosphate-0.02 M citric acid buffer at pH 5.0 containing 0.2 m M of o-phenylenediamine dihydrochloride per ml and 0.03% hydrogen peroxide was added to each well. The reaction was terminated by adding 100 μl of 2 N sulfuric acid to each well. The absorbance at 492 nm was then read. For the CHEKIT ELISA, antibodies against M. agalactiae were measured as described by the manufacturer.

Statistical analysis. The performance evaluation and the comparison of rELISA and CHEKIT ELISA were carried out by the receiver operating characteristic (ROC) curve method (7, 8). ROC analysis is a powerful statistical method used to evaluate a diagnostic test by calculating its sensitivity and specificity with a wide range of cutoff points and by plotting sensitivity against (1 − specificity) to form the so-called “ROC curve.” Graphic visualization and calculation of the area under the curve (AUC), which is the main indicator of a good test, were performed with AccuROC software (Accuretmetric).

RESULTS

WB analysis of serum sets from experimental infections. As illustrated by the WB in Fig. 1, the antibody response of sheep experimentally infected with pooled M. agalactiae strains changed during the course of infection. Eight days after infection, the IgG response of the host was directed against three antigens in the 40- to 55-kDa range. From the 9th to the 17th

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession no.</th>
<th>Primer set sequences (5’ to 3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mp81 gene</td>
<td>AF299294</td>
<td>ATGGAAAAATGGTAAAG and TTTACCTGGAACAGGA</td>
<td>1,014</td>
<td>22</td>
</tr>
<tr>
<td>avgC (vpmaU)</td>
<td>AF205062 (AF248865)</td>
<td>ACAGAACCACCCAGGAAAACCA and TTCTACTTTCCGTGACTTAATT</td>
<td>576</td>
<td>6(5)</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

Strains and growth conditions. Seventy-five M. agalactiae strains (see the supplemental material) were isolated from milk samples from sheep with contagious agalactia in different regions of Italy, namely, Sardinia, Lazio, Sicily, and Puglia. All strains were cloned and identified by PCR assay (18); isolates NU-2697, SS-440, OR-352, and NU-658 were used for experimental infection. Each mycoplasma strain was grown at 37°C in modified Hayflick medium containing 10% horse serum, whereas NU-2697, SS-440, OR-352, and NU-658 were cultured together in 100 ml of Hayflick medium. In both cases, viable cell numbers were determined by the last positive dilution in liquid culture, according to standard procedures (13). Cells were harvested from logarithmic-phase broth cultures by centrifugation at 20,000 g for 30 min and were washed twice with phosphate-buffered saline (PBS) (0.1 M, pH 7.4). The final pellet was resuspended in 1/10 the original culture volume and used immediately or stored at −80°C. The protein concentrations of washed whole-cell suspensions were determined using the DC protein assay reagent (Bio-Rad, Richmond, CA) according to the manufacturer’s instructions.

Serum samples collected from experimentally and naturally infected animals. Two Sarda milking ewes of 3 years of age were used for the experimental infection. Before infection, the two ewes were clinically examined in order to rule out past or ongoing pathologies, especially those involving the mammary glands. In addition, to also rule out any previous contact with M. agalactiae, serum samples and milk samples collected from the right and left teats were also tested. A PCR assay was performed on milk samples (20), while WB was performed on samples and milk samples collected from the right and left teats were also tested. The protein concentrations of washed whole-cell suspensions were determined using the DC protein assay reagent (Bio-Rad, Richmond, CA) according to the manufacturer’s instructions.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and WB. Twenty micrograms of total protein from each strain or from pooled samples was electrophoresed in 12% (wt/vol) polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS), according to the method of Laemmli (10). The apparent molecular weights of mycoplasma proteins were determined using appropriate molecular weight markers (Kaleidoscope prestained standards; Bio-Rad). Electrophoresed proteins were transferred to nitrocellulose membranes in a Trans-Blot semidyTW7F apparatus (Bio-Rad) as described by the manufacturer. Blots were incubated for 1 h at 37°C with sheep sera diluted 1:100 in PBS-2% skim milk. After several washings with PBS-2% skim milk, blots were incubated for a further hour at 37°C with alkaline phosphatase-conjugated anti-sheep antibodies (Sigma). After five more washes, blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega, Madison, WI) in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris, pH 9.5).

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day postinfection, the number of immunogenic proteins recognized by the antibodies increased, also including high- and medium-molecular-weight antigens. Additionally, from the 18th day, an antibody response against low-molecular-weight antigens was also detected. Among the three early proteins which appeared on the eighth day after infection, we decided to exclude the antigen with an apparent molecular mass of about 41 kDa, considering that it was not detected on the external surface of *M. agalactiae* by the immunoprecipitation technique (21). Concerning the two remaining early proteins, of 55 kDa (P55) and 48 kDa (P48), the idea of including P48 in the assay was abandoned after the antigenic profile analysis of the 75 *M. agalactiae* isolates, as described below. Among the other proteins expressed later, we focused on the 81-kDa surface membrane protein (P80) since it was stable and expressed in the largest number of isolates (22).

**Polyclonal sera against P80 and P55.** The bands corresponding to P80 and P55 were electroeluted from the Coomassie blue-stained gels, concentrated (Fig. 2A), and inoculated into two different lambs, as described by Tola et al. (22). As shown

FIG. 1. WB of serum samples from experimentally infected sheep. Whole-cell antigens from a pool of four cultured *M. agalactiae* strains were resolved in a 12% polyacrylamide gel under denaturing conditions. Following electrotransfer, membranes were incubated at 37°C with pooled sera collected daily from sheep infected with $10^4$ CCU and $10^7$ CCU of mycoplasmas. Blots were developed as described in Materials and Methods. No antigens were recognized by the sera collected from 0 (infection day) to 7 days after infection. Proteins with molecular masses spanning from 40 kDa to 80 kDa were recognized by sera collected from 8 to 17 days. Starting from the 18th day after infection, the range of immunodominant proteins also included low-molecular-weight antigens. The arrows on the right indicate the 81-kDa and 55-kDa antigens used in this study.

FIG. 2. Purification and concentration of P80 and P55 from total *M. agalactiae* proteins. P80 and P55 were sliced from gels, electroeluted, and concentrated. (A) Purified P80 (lane 1), purified P55 (lane 3), and whole *M. agalactiae* cells (lane 2) were subjected to 12% SDS-PAGE and stained with Coomassie brilliant blue. (B) WB analysis showing reactivities of anti-P80 (lane 2), anti-P55 (lane 3), and pooled sera from naturally infected sheep with clinical symptoms (lane 1) with whole-cell *M. agalactiae* antigens. M, M1, and M2, different Kaleidoscope prestained standards. Molecular masses are indicated on the right.
agalactiae DNA and cloned into a prokaryotic expression vector (pQE-30; Invitrogen) inserted in E. coli indicated on the right.

from naturally infected sheep with clinical symptoms. P80 and P55 are lane 7, Bul/SS) and the PG2 reference strain (lane 3) with pooled sera B17; lane 2, 11LO; lane 4, Ag19; lane 5, 9631/358; lane 6, Ag23; and sera and with the sera from infected sheep (Fig. 4). nant proteins reacted strongly both with the polyclonal specific hyperimmune serum directed against electroeluted P55 while P55 was detected in 85% of strains. We detected the was detected in 69 strains, corresponding to 92% of strains, was detected in 45 strains (60%), which is why we chose not to pursue it further, as indicated in Materials and Methods.

by the WB in Fig. 2B, the IgG antibodies produced against P80 recognized only this antigen; in contrast, the lamb monospecific hyperimmune serum reacted against electroeluted P55 also recognized other antigens of middle and low molecular weight (Fig. 2B), suggesting that all these proteins might share common antigenic determinants.

Analysis of M. agalactiae isolates. In order to evaluate the expression of P80 and P55 among M. agalactiae isolates, 75 strains were analyzed by WB (Fig. 3). Using the same pooled sera from naturally infected sheep with clinical symptoms, P80 was detected in 69 strains, corresponding to 92% of strains, while P55 was detected in 85% of strains. We detected the 48-kDa protein in only 45 strains (60%), which is why we chose not to pursue it further, as indicated in Materials and Methods.

Production of recombinant P80 and P55 peptides. A 1,014-bp fragment of the M. agalactiae mp81 gene and a 576-bp fragment of the avgC gene were amplified from genomic M. agalactiae DNA and cloned into a prokaryotic expression vector (pQE-30; Invitrogen) inserted in E. coli DH5α. As expected, the apparent molecular masses of both expressed fusion proteins were larger than the predicted masses, at approximately 56 and 35 kDa, respectively. The two recombinant proteins reacted strongly both with the polyclonal specific sera and with the sera from infected sheep (Fig. 4).

Serodiagnosis of M. agalactiae infection by rELISA. To evaluate whether rP80 and rP55 could be suitable candidate antigens for diagnosis of M. agalactiae infection, different concentrations of recombinant proteins were tested by ELISA, using the same panel of 33 sera from experimentally infected sheep as that used for Fig. 1. rELISA plates containing 10 μg/ml of rP80 and 3.125 μg/ml of rP55 were used to test 343 sera. At the same time, the same sera were analyzed with CHEKIT ELISA. The results are shown in Table 2 and Fig. 5. A total of 209 samples (93.7%) were confirmed to be positive by our rELISA, compared to 73.6% by CHEKIT ELISA. A large number of sera (n = 56), corresponding to 25.1% of samples, were doubtful by CHEKIT ELISA, in contrast to 5.4% by our rELISA. Two sera gave false-negative results by our rELISA, and three sera gave false-negative results by CHEKIT ELISA. Of 120 WB-negative sera, 116 samples (96.7%) were confirmed to be negative by rELISA, whereas only 91 (75.8%) were negative by CHEKIT. Few samples (n = 3) were doubtful by our rELISA, and only one gave a false-positive result. In contrast, with CHEKIT ELISA, 23 sera (19.2%) were doubtful, and 6 (5%) gave false-positive results.

A hypothesis test aimed to assess if the differences observed between the two tests (rELISA and CHEKIT ELISA) were statistically significant was performed. The critical ratio (AUC1 − AUC2)/√(SE1^2 + SE2^2 − 2SE1 * SE2), where AUC1 and AUC2 are the areas under the first (CHEKIT ELISA) and second (rELISA) ROC curves, SE is the standard deviation, and r is the correlation coefficient between the two areas, produced a z value of 3.42. The r value was obtained using a specific table that links the averages of the two AUCs with the average correlation between the two “true positive” and “true negative” groups (7). Under the null hypothesis that the underlying area is the same for both methods, the ratio z has a standard normal distribution, so values of z above a given cutoff value are taken as evidence that the “true” ROC areas are different. We obtained a value of z of >1.96, with a 0.95 confidence level. This result is a strong indicator that the differences observed between the two methods are statistically significant.

TABLE 2. Percentages of positive, negative, and doubtful sera from infected and uninfected sheep with the two ELISAs

<table>
<thead>
<tr>
<th>Sera</th>
<th>No. (%) of samples by CHEKIT ELISA</th>
<th>No. (%) of samples by rELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Confirmed outbreak (n = 223)</td>
<td>164 (73.6)</td>
<td>3 (1.3)</td>
</tr>
<tr>
<td>Negative farms (n = 120)</td>
<td>6 (5)</td>
<td>91 (75.8)</td>
</tr>
</tbody>
</table>
DISCUSSION

Many studies have now reported that P30, P48, P55, and P80 are the immunodominant surface proteins of *M. agalactiae* (3, 14, 16, 22). The immunoreactivities and suitability of these proteins as diagnostic antigens have been demonstrated. Rosati et al. (15) developed an ELISA based on recombinant P48. However, in a comparative analysis using other conventional serological tests, the P48 assay displayed a low sensitivity, corresponding to 56.7% (9). In this study, we analyzed the immunological response elicited during the course of an experimental infection and combined it with the protein expression profiles of different *M. agalactiae* field strains. This led to the conclusion that the combination of two antigens might lead to a more effective detection strategy. After a careful evaluation of both aspects, P55 and P80 were chosen as the most effective and conserved antigens. P55 is a strongly immunoreactive protein and presents very early after infection, although it was expressed in 85% of the strains analyzed. Using the hyperimmune anti-P55 serum against whole-cell *M. agalactiae* proteins, we observed not only that it reacts with the protein band corresponding to P55 but also that it reacts strongly with a protein of about 26 kDa (Fig. 2). This last protein is expressed starting from the 18th day postinfection and remains steadily expressed during disease progression (Fig. 1). This might suggest that P55 shares antigenic similarity with P26. In a previous study, Santona et al. (16) showed by N-terminal sequencing that P55 was identical to the AvgC protein encoded by the *avgC* (vmpaU) gene. The *avgC* (vmpaU) gene is 717 bp long and encodes a protein with an expected molecular mass of approximately 26 kDa. In this study, we amplified and cloned a 576-bp fragment from the *avgC* (vmpaU) gene. The truncated protein rP55 displays strong reactivity with sera from naturally infected animals (Fig. 4) and with anti-P55 and anti-P26 hyperimmune sera (data not show). In contrast, P80 is a stable lipoprotein conserved among isolates from different Italian regions (22). The conservation of this major outer membrane protein among *M. agalactiae* strains makes it an extremely interesting candidate for the development of an effective serodiagnostic assay. In this study, we observed that P80 is expressed later than P55, but it is always present during the course of experimental infection. To overcome this problem, rP80 and rP55 were combined in our rELISA. The combined rELISA strategy was then compared with a routine ELISA (CHEKIT ELISA), using positive and negative sera. The rELISA was highly sensitive, since it detected 93.7% of the naturally infected sheep sera, in contrast to 73.6% of samples detected with the CHEKIT ELISA test. Furthermore, 96.7% of the negative sera were confirmed to be negative with our rELISA, versus 75.8% with the CHEKIT ELISA.

In conclusion, we demonstrated that P80 and P55 consistently induce the production of antibodies in sheep infected both naturally and experimentally with *M. agalactiae*. The rELISA developed here is a specific, sensitive, and reliable assay for the detection of serum antibodies against *M. agalactiae*.

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