Development of a Recombinant Antigen for Antibody-Based Diagnosis of Mycoplasma bovis Infection in Cattle

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Mycoplasma bovis induces various clinical manifestations in cattle, such as mastitis, arthritis, and pneumonia. We have evaluated the immunoreactivity of three variable surface proteins (Vsps) of M. bovis, namely VspA, VspB, and VspC, with sera collected from herds with mycoplasmosis or from cattle experimentally infected with M. bovis. Western blot analysis revealed that the Vsps are the predominant antigens recognized by the host humoral response during M. bovis infection. The immunoreactivity of VspA, VspB, and VspC with host antibodies was dependent on the clinical manifestations, the geographical origin of the M. bovis isolates, the mode of infection, and the animal’s history. Moreover, the results showed that Vsp-specific host antibodies can be detected about 10 days after experimental infection and for up to several months. The full-length or truncated versions of the VspA product were overexpressed in Escherichia coli as fusion proteins (FP-VspA). Reombinant products showed strong immunoreactivity with the Vsp-specific monoclonal antibodies 1A1 and 1E5, with the corresponding epitopes localized at the VspA N-terminal and C-terminal ends, respectively. Anti-M. bovis sera of cattle naturally or experimentally infected also strongly recognized the full-length FP-VspA. The seroreactivity of sera collected from cattle between 6 and 10 days after experimental infection was weaker with truncated versions of VspA lacking the 1E5 epitope than with the full-length VspA or the truncated versions lacking the 1A1 epitope. Overall, the results indicate that the Vsps, despite their inter- and intraclonal variability, may be applied as target antigens in serodiagnostic assays for epidemiological studies.

Mycoplasma bovis is considered one of the most pathogenic bovine mycoplasmas (18). While mycoplasmosis induced by this pathogen is spread worldwide, it occurs predominantly in Europe and North America, resulting in significant economic losses in areas with intensive dairy and meat production (18, 30). In cattle, M. bovis is associated with diverse clinical manifestations, such as mastitis in cows and arthritis and pneumonia in young animals, as well as genital disorders, abscess, conjunctivitis, otitis, and meningitis (11–13, 18, 28, 32). In most cases, fatal outcomes are due to coinfection with other bacterial pathogens, such as pasteurellas (8, 31). M. bovis may be asymptotically present as commensal organisms in the upper respiratory tracts of older animals, where the mycoplasmas form a constant source of infection for young animals that are more susceptible to developing clinical symptoms (17, 31). In the absence of an effective antibiotic therapy or vaccination, the only strategy currently available to control infection is the use of effective antibiotic therapy or vaccination, because they can identify animals which have been infected within a large herd even in the absence of shedding organisms.

In a preliminary study, serum antibodies from animals naturally infected with M. bovis originating from Northern Germany were shown to predominantly recognize major epitopes carried by a family of abundant, variable surface lipoproteins, designated as Vsps (25). So far, three Vsps, VspA, VspB, and VspC, have been characterized in clonal variants derived from M. bovis type strain PG45. Detailed analysis revealed that each Vsp undergoes high-frequency variation in expression and size, generating extensive surface diversification in a given M. bovis strain or isolate (3). This phenomenon may profoundly affect the outcome of serodiagnostic assays, because their sensitivity may vary, depending on the choice of the target antigen (26).

Development of sensitive and specific serologic tests for the rapid detection of infected animals is bound to the identification of a specific antigen. In this study, we have evaluated the reactivity of M. bovis antigens, and more specifically of Vsp epitopes, with sera obtained from animals experimentally or naturally infected with M. bovis. We describe the expression of recombinant VspA products in Escherichia coli which contain immunodominant epitopes strongly reacting specifically with sera from naturally infected cattle as well as with sera collected 6 days after experimental infection with M. bovis.

MATERIALS AND METHODS

Mycoplasmas, bacterial strains, and plasmids. M. bovis 1067 was originally isolated from an animal with mastitis in 1983 and propagated as a filter-cloned culture (22). This strain and a clonal variant derived from M. bovis type strain PG45, which expressed a 67-kDa version of VspA (see below) (3) designated VspA 67, were used for experimental infections. Clonal variants used for West-
TABLE 1. Characteristics of bovine sera selected from four experimental M. bovis infections

<table>
<thead>
<tr>
<th>Expt (yr)a</th>
<th>Strain</th>
<th>Route of infectionb</th>
<th>Clinical manifestation and pathology</th>
<th>Designation of animal sera tested</th>
<th>No. of days postinoculation</th>
<th>Presence of anti-Vsp antibodiesc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1989)</td>
<td>1067</td>
<td>Endobronchial</td>
<td>Lung lesions</td>
<td>J004</td>
<td>10 and 30</td>
<td>+ and +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Contactd</td>
<td>Lung lesions</td>
<td>J005</td>
<td>10 and 30</td>
<td>+ and +</td>
</tr>
<tr>
<td>2 (1997)</td>
<td>Clonal variant derived from PG45e</td>
<td>Endobronchial</td>
<td>Lung lesions</td>
<td>A009</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>3 (1998)</td>
<td>1067</td>
<td>Endobronchial</td>
<td>Lung lesions</td>
<td>M</td>
<td>6 and 14</td>
<td>+ and +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H</td>
<td>6 and 14</td>
<td>+ and +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vc</td>
<td>6 and 14</td>
<td>+ and +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vd</td>
<td>6 and 14</td>
<td>+ and +</td>
</tr>
<tr>
<td>4 (1986)</td>
<td>1067</td>
<td>Endobronchial</td>
<td>Abortions</td>
<td>F006</td>
<td>62 and 524</td>
<td>+ and +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>E095</td>
<td>101 and 532</td>
<td>+ and +</td>
</tr>
</tbody>
</table>

a Experiments were conducted as described in Materials and Methods.
b Inoculation was performed with 50 ml of an M. bovis culture containing 10^8 to 10^10 CFU/ml.
c All sera were tested by Western blot as described in Materials and Methods and were shown to predominantly contain antibodies directed toward Vspa, Vspb, and Vspc.
d Animal J005 (expt 1) was infected by contact with a previously inoculated animal, J004.
e Clonal variant derived from strain PG45 expressing a single Vsp, 67-kDa VspA.
f Sera from animals Y and V were also taken at day 9 and 21 and shown to react with the fusion protein FP-Vspa-I and its truncated versions as described in Results.

TABLE 2. Characteristics of bovine sera selected from natural M. bovis outbreaks

<table>
<thead>
<tr>
<th>Outbreak designation</th>
<th>Geographical location and date(s)</th>
<th>Clinical manifestation</th>
<th>No. of animal serum samples testedd</th>
<th>No. of serum samples containing predominantly anti-Vsp antibodiese</th>
<th>No. of serum samples lacking M. bovis antibodiesf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outbreak 1</td>
<td>France, August 1994</td>
<td>Arthritis</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Outbreak 2</td>
<td>France, April 1997</td>
<td>Arthritis and pneumonia</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Outbreak 3</td>
<td>France, January 1988</td>
<td>Arthritis, pneumonia, and mastitis</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Outbreak 4</td>
<td>Switzerland, April 1997</td>
<td>Mastitis</td>
<td>10</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Field sampling 5g</td>
<td>France, 1988 and 1990; Switzerland, 1997</td>
<td>No pathology</td>
<td>19</td>
<td>17</td>
<td>2</td>
</tr>
</tbody>
</table>

Values correspond to one serum sample per animal.
b Serum samples from three different animals were tested twice at 20-day intervals and showed a constant similar reaction to Vsp.
c Serum samples tested of antibodies directed toward Vsp was assessed by Western blot analysis.
d In a previous study, 200 healthy herds were randomly screened by IHA for the presence of antibodies directed to M. bovis antigens. Among those, 10 were shown to contain several asymptomatic animals presenting a strong reaction by IHA, suggesting an M. bovis infection. From these 10 herds, 17 serum samples of animals positive for VspA and 2 serum samples of animals negative by IHA were selected.
e The 17 serum samples were all positive by IHA.
f The two serum samples were negative by IHA.
similar procedure and was shown to react with VspA and VspC, but not with VspB (3, 15, 25). Both MABS were shown not to react with ruminant mycoplasma species other than M. bovis. Rabbit hyperimmune sera were raised against those mycoplasma species which are most frequently isolated from cattle (M. bovigenitalium, M. bovirhinis, M. arginini, Achopleus laidawai, and Ureaplasma diversum) and against M. agalactiae, which is closely related to M. bovis, as previously described (21). The serum PAL, kindly provided by D. Bergonier (Ecole Vétérinaire de Toulouse, France), was collected from a sheep naturally infected with M. agalactiae and shown to strongly react with M. agalactiae surface components.

1HIA and Western blot analysis. The IHA was performed as described elsewhere (5, 20) with the type strain PG45 as an antigen. The procedures for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of mycoplasma proteins have previously been described (25). For Western blot analysis, nitrocellulose membranes were blocked for 50 min with Tris-buffered saline (TBS [0.01 M Tris-HCl, 0.15 M NaCl, pH 7.2]) containing 10% (vol/vol) horse serum, washed once with TBS containing 0.05% (vol/vol) Tween 20 (TBS T20) and once with TBS only, and then incubated for 2 h at 33°C with the primary antibodies diluted in TBS supplemented with 5% (vol/vol) horse serum (bovine sera diluted 1:75, MAb 1E5 diluted 1:100, MAb 1A1 diluted 1:2,000; rabbit hyperimmune sera diluted 1:1,000). After three washes with TBS T20 and one with TBS, the blots were incubated for 1 h at 33°C with the appropriate secondary antibodies. The peroxidase-conjugated rabbit anti-bovine IgGs (Dako, Glostrup, Denmark), sheep anti-bovine IgM and IgG (Bethyl Laboratories, Inc., Montgomery, Tex.), and goat anti-mouse IgM and IgG (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) were diluted in phosphate-buffered saline as recommended by the manufacturer.

Expression of Vsp in E. coli as a fusion protein. The fusion protein FP-VspA-I was generated by using the pMAL-c2 protein fusion system of New England Biolabs. Briefly, oligonucleotides P-5 (5'-GGA TCC TGT GGT GAG ACC AAA G-3') and P-3 (5'-TAT TAA GCT TAA GAA CTT GTT GGT ATT TT-3') (boldface letters indicate engineered restriction sites, and underlined letters indicate the codon encoding the first amino acid of the VspA mature protein), which span the exported, mature coding sequence of VspA (Fig. 1A), were used to produce by PCR a DNA fragment from the cloned VspA gene template (16). Engineered BamHI and HindIII restriction sites located in primers P-5 and P-3, respectively, were used to insert the PCR product in frame with the malE gene, which encodes the maltose binding protein (MBP), into the pMAL-c2 plasmid by standard procedures. The fusion protein FP-VspA-I encoded by the resulting recombinant plasmid pFP-VspA-I was overexpressed in transformed E. coli DH10B cells (GIBCO BRL, Life Technologies, Inc., Grand Island, N.Y.) and was purified by affinity chromatography by using maltose binding properties, as prescribed by the manufacturer (New England Biolabs, Inc.). Cleavage of the VspA product from the MBP by the protease factor Xa was achieved as instructed by the manufacturer.

Three truncated versions of the FP-VspA fusion protein (Fig. 1C), namely FP-VspA-I, FP-VspA-II, and FP-VspA-III, and FP-VspA-V, were obtained by the same procedure. For this purpose, primers complementary to the junction of two distinct repeated units or to unrepeated sequences located between two blocks of repeated elements were designed: R3-5 (5'-CCC AGG ATC CCC GCA TGA T-3'), R3-3 (5'-CCT GAA GCT TGT GGT GAG TTA G-3'), and R3-3 (5'-GTG TTC TCT CTC AAG CTT TTT AAT TTT C-3'). These primers were used in combination with P-5 (5') or P-3 (3'), as shown in Fig. 1A. Boldface letters represent engineered BamHI (5'-end primer) and HindIII (3'-end primer) restriction sites for in-frame insertion of the PCR fragment into the pMAL-c2 vector downstream of the malE sequence. Finally, the FP-VspA-I-V fusion protein was obtained by subcloning the EcoRI DNA fragment of the plasmid pFP-VspA-I, which contained the 3' end of the pMAL-c2 polylinker and the first 313 nucleotides of the vspA gene, into EcoRI-pMAL-c2. Expression of the truncated fusion proteins was performed as described above. PCR fragments cloned into pMAL-c2 were sequenced by deoxy terminator cycle sequencing with infrared.
Humoral response to M. bovis epitopes in naturally infected cattle. To further investigate whether the results obtained with sera from experimentally infected animals reflected the situation occurring in the field, similar experiments were performed with sera collected from animals displaying diverse clinical manifestations during natural M. bovis outbreaks in geographically distant herds. As illustrated in Fig. 2G to K, immunoprofiles obtained with sera representative of outbreaks 1, 2, and 4 (Table 2) were identical to that obtained with MAb 1E5 or with sera collected from animals experimentally infected with M. bovis (Fig. 2). In a few cases, immunoprofiles obtained with field sera were more complex than that presented in Fig. 2, because several mycoplasma components other than the VspBs were also weakly reacting with the host antibodies (data not shown). Sera obtained from asymptomatic animals that were strongly reacting with M. bovis in the IHA displayed a pattern similar to that obtained in Fig. 2, because they only reacted with Vsp epitopes (Table 2, field sampling). In some cases, we observed that the reactivity of the sera with 46-kDa VspB was weaker than that observed with 64-kDa VspA (Fig. 2H, lane 2, and data not shown) indicating that recognition of VspB epitopes by the immune system may vary from one animal to another, while all sera reacted strongly with the 67-kDa VspA product (Fig. 2G to K, lane 3). Finally, hyperimmune sera against other mycoplasmas frequently isolated from cattle (M. bovigenitalium, M. bovirhinis, M. arginini, Achoro- leplasma laidlawii, and Urealyticum diversum) and against M. agalactiae, a mycoplasma that is phylogenetically closely related to M. bovis and occasionally found in cattle (21), did not react with M. bovis antigens (except for a very weak reaction with M. arginini and uncharacterized antigens of M. bovis whole-cell extract).

Reactivity of VspA overexpressed in E. coli with hyperimmune sera of cattle experimentally and naturally infected with M. bovis. To define whether the VspA product would be a suitable tool for serodiagnostic purposes, for instance, epidemiological studies, the VspA product of M. bovis PG45 was expressed in E. coli as a nondenatured recombinant product. This was achieved by inserting the DNA sequence encoding the VspA gene (16) into the pMAL-c2 vector to create an in-frame fusion with the malE gene, which encodes the MBP (Fig. 1). In SDS-PAGE, the resulting recombinant fusion protein, designated FP-VspA-I, had an apparent molecular mass of about 107 kDa (Fig. 3) which corresponds to the mature VspA sequence (67 kDa without the signal peptide) (16) fused to the C-terminal region of the MBP (36 kDa for the MBP). Western blot analysis showed that the FP-VspA-I was antigenically comparable to the native VspA, because it reacted both with the VspA-specific MAb 1E5 and with sera collected from experimentally infected cattle (Fig. 2F). As shown in Fig. 2, circulating host antibodies directed toward Vsp epitopes were detected in sera as early as 6 days after infection (Fig. 2C and E), but were also detected 532 days postinoculation (Fig. 2F).

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Humoral response to M. bovis epitopes in experimentally infected cattle. To evaluate the host antibody reactivity to M. bovis antigens, and more specifically to VspA, identical immunoblots representing whole-cell extracts of selected clonal variants derived from M. bovis type strain PG45 were independently immunostained with MAb 1E5 (A), with sera collected from experimental infection (B to F), or with sera collected from natural outbreaks (G to K). The sera used in this experiment are described in Tables 1 and 2 and correspond to animal A009 before (B) and after (C) infection; animal M before (D) and after (E) infection; sera E095 (F), 30 (G), and 56 (H) from two animals of outbreak 1; and serum 283/17 (K) from the field sampling. Lanes 1 through 3 represent clonal variants expressing 79-kDa VspC (lane 1), 64-kDa VspA plus 46-kDa VspB (lane 2), and 67-kDa VspA (lane 3).

RESULTS

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epitopes was achieved by Western blot analysis. This showed that the first 106-amino-acid sequence contains the 1A1 epitope (Fig. 3C, lane 1), while the C-terminal region from amino acid 155 to amino acid 325 encodes the 1E5 epitope (Fig. 3C, lane 2). The four truncated fusion proteins reacted with sera 56 and 30, collected from outbreak 4, and with serum 6248, collected from outbreak 2. Interestingly, serum A009, collected 6 days after experimental infection with the VspA 67 clonal variant (experiment 2), only reacted with fusion proteins (FP-VspA-I and -II) that contain the 1E5 epitope (Fig. 1C and 3C, lane 3). Similarly, sera collected 9 days after infection with M. bovis 1067 (experiment 3, calves Y and V [Table 2]) only recognized the FP-VspA-I and -II, while sera taken at day 21 from the same calves (experiment 3, calves Y and V) did react well with FP-VspA-III (data not shown).

DISCUSSION

The results presented in this report show that the Vsps represent those components that predominantly elicit the bovine humoral immune response in cattle after experimental or natural infection with M. bovis, independently of the clinical manifestations, the geographic location and origin of the agent, the mode of infection, and the animal’s history. In experimentally infected calves, circulating host antibodies directed toward Vsp epitopes appeared within an average of 10 days following inoculation with M. bovis, but also as early as 6 days, and were still detectable for several months after infection. Results obtained with contact-infected animals indicated that a similar situation is likely to occur in the field. Serum antibodies collected from cattle naturally infected with M. bovis of unknown Vsp phenotype and genotype were shown to recognize the three Vsps expressed by the type strain PG45. Inoculation of animals with strain 1067 also resulted in the appearance of antibodies that cross-reacted with the Vsps of strain PG45. This implies that despite their clonal variability, the Vsps or at least some members of the Vsp family are persistently expressed by M. bovis in the bovine host during infection and that immunodominant epitopes are highly conserved among strains and isolates. The presence of anti-VspB and anti-VspC antibodies in addition to anti-VspA antibodies during infection with a clonal variant expressing VspA (experiment 2) indicated that common epitopes shared by the three Vsps (VspA, VspB, and VspC) are strongly immunogenic in the host and/or that oscillation in Vsp expression occurs in vivo, generating subpopulations expressing VspB and VspC. In some cases, the reactivity of bovine serum antibodies was stronger with the VspA and VspC products than with VspB. This can be explained by (i) the absence of the 1A1 epitope on VspB which is shared by both the VspA and the VspC proteins and (ii) the fact that the number of repeated elements which constitute 80% of the molecule and are thought to contain the immunodominant epitopes is lower in the 46-kDa VspB product than in the 64-kDa VspA and 79-kDa VspC molecules. On the other hand, previous data suggested that the VspA and the VspC proteins may be the products of two distinct allelic versions of the same vsp gene (16), explaining their similar reactivity with the MAbs 1A1 and 1E5 and the animal sera.

In light of these findings and the proven nonreactivity of the Vsps to sera raised against closely related mycoplasmas commonly isolated from cattle, the surface-exposed VspA product of M. bovis was overexpressed in E. coli as a recombinant protein. This product was shown to be antigenically comparable to the native VspA, because it reacted with two MAbs directed to VspA, 1A1 and 1E5, as well as with all sera used in

4). The immunoreactivity of FP-VspA-I was also tested with (i) serum collected from sheep infected with M. agalactiae and (ii) serum obtained from an animal which was shown to be free of M. bovis. As shown in Fig. 3A, lanes 5 and 6, none of these sera reacted with FP-VspA-I. Moreover, no reaction was obtained with hyperimmune serum raised against M. arginini (data not shown), which was shown to react weakly with uncharacterized antigens of M. bovis whole-cell extract. Immunoblot analysis of FP-VspA-I digested with the factor Xa revealed that the cleaved 67-kDa VspA product reacted with (i) the MAbs 1E5 (Fig. 3B, lane 2) and 1A1 (data not shown) and (ii) all positive bovine sera corresponding to outbreaks 1, 2, 3, and 4 shown in Table 1 (see Fig. 3B, lanes 8 and 9 for two representative serum samples). By the same procedure, host antibodies to VspA were detected in IHA-negative serum of animal J005 (Table 1, experiment 1) collected at day 13 or 17 following contact with the infected animal J004 (Fig. 3B, lanes 10 and 11). Since M. bovis was first isolated in BALs of animal J005 at day 6, these results support our previous findings that showed the early appearance of circulating host antibodies to Vsp epitopes in animals infected by endobronchial inoculation (Table 1, experiments 2 and 3). Serum samples collected from animal J005 after day 23 were all positive in IHA and reacted with the VspA product. Similar results obtained with sera collected from two other contact-infected animals (data not shown) indicated that host antibodies to M. bovis are detectable within 10 days with FP-VspA-I, however, not with the IHA.
this study, collected from cattle experimentally or naturally infected with M. bovis. Interestingly, recognition of the VspA immunodominant domains by the host immune system was slightly different, because truncated recombinant Vspa products lacking the R1,4 repeated region, FP-Vspa-III, FP-Vspa-IV, and FP-Vspa-A-V, failed to react with sera taken between 6 to 10 days, but were recognized by sera of the same animals collected at a later stage. As shown in this study, the R4 region contains the target epitope of MAb 1E5, which is an IgM isotype, while the N-terminal R4,1 repeated motif, encoded by the genes coding for all of the truncated Vspa products, is recognized by MAb 1A1, which is an IgG isotype. This suggests that detection of the N-terminal region of Vspa, which contains the R4,1 motif, may require the seroconversion of IgM to IgG, due to either a low concentration of IgM reacting with the 1A1 target epitope or due to a conformational structure that temporarily masks the target epitope. Nevertheless, these data indicate that the recombinant product truncated recombinant Vsps revealing the location of the 1A1 epitope or due to a conformational structure that temporarily masks the target epitope.

The presence of vsp gene homologues in field isolates or strains other than the PG45 type strain was recently assessed in 250 M. bovis field isolates collected in France, Germany, Italy, Spain, and Switzerland (23). All were shown to contain DNA sequences homologous to vsp genes and to express, to various degrees, epitopes that reacted with either the 1A1 or the 1E5 MAb. Interestingly, the few isolates that did not react with MAb 1E5 failed to react in Southern blot analysis with the oligonucleotide probe corresponding to the R4,4 motif. In contrast, all isolates contained multiple copies of the sequence encoding the motif R4,1 and were reacting with MAb 1A1 (23). This corresponds to the results obtained in this study with the truncated recombinant Vsps revealing the location of the 1A1 and 1E5 epitopes within the R4,1 and R4,4 repeated motifs, respectively.

Even though the Vsp proteins were shown to participate in adhesion to the host cell (27), their exact role during the process of the disease remains to be elucidated. However, if the presence of Vsp epitopes at the surface of the mycoplasma depends on the on and off status of the corresponding gene or genes, it also depends on the number of vsp genes that dictate the Vsp repertoire in a given strain. For the type strain PG45, which contains eight distinct vsp genes (24) that may all be transcribed, the Vsp repertoire in a given strain. For the type strain PG45, which contains eight distinct vsp genes (24) that may all be transcribed, the Vsp repertoire in a given strain.

REFERENCES


