Mycoplasma suis Antigens Recognized during Humoral Immune Response in Experimentally Infected Pigs

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Today, serodiagnostic tests for Mycoplasma suis infections in pigs have low accuracies. The development of novel serodiagnostic strategies requires a detailed analysis of the humoral immune response elicited by M. suis and, in particular, the identification of antigenic proteins of the agent. For this study, indirect enzyme-linked immunosorbent assay (ELISA) and immunoblot analyses were performed using pre- and sequential postinfection sera from M. suis-infected and mock-infected control pigs. M. suis purified from porcine blood served as the antigen. Eight M. suis-specific antigens (p33, p40, p45, p57, p61, p70, p73, and p83) were identified as targets of the immunoglobulin G (IgG) antibody response during experimental infection, with p40, p45, and p70 being the preferentially recognized M. suis antigens. Besides the M. suis-specific antigens, porcine immunoglobulins were identified in blood-derived M. suis preparations. By immunoglobulin depletion, the specificity of the M. suis antigen for use in indirect ELISA was significantly improved. M. suis-specific Western blot and ELISA reactions were observed in all infected pigs by 14 days postinfection at the latest and until week 14, the end of the experiments. During acute clinical attacks of eperythrozoonosis, a derailment of the antibody response, determined by decreases in both the M. suis net ELISA values and the numbers of M. suis-specific immunoblot bands, was accompanied by peaking levels of autoreactive IgG antibodies. In conclusion, the M. suis-specific antigens found to stimulate specific IgG antibodies are potentially useful for the development of novel serodiagnostic tests.

Mycoplasma suis (formerly Eperythrozoon suis) belongs to a group of hemotrophic bacteria which have recently been reclassified within the genus Mycoplasma (16, 17, 18, 19, 22, 29). M. suis is an epcculinar hemoparasite that attaches to and causes deformity and damage to porcine erythrocytes (32). The resulting disease, traditionally called porcine erythrozoonosis (PE), has been reported worldwide and is considered a problem of feeder pigs, where it manifests as a febrile acute icteroanemia with low morbidity and high mortality (8). Chronic low-grade M. suis infections vary from asymptomatic infections to a range of clinical conditions, including (i) anemia, mild icterus, and general unthriftiness in newborns, (ii) growth retardation in feeder pigs, and (iii) poor reproductive performance in sows (3, 8, 34). Moreover, M. suis is suspected of suppressing the host’s immune response, leading to an increased proneness to infection with other infectious agents of porcine respiratory and enteric diseases (33).

The lack of an in vitro cultivation system is the crucial barrier to systematic analyses of the biology of M. suis as well as the development of valuable diagnostic procedures for, e.g., the accurate assessment of the prevalence and significance of M. suis in pig populations (5, 20). Hitherto, laboratory diagnosis of M. suis usually relies on the microscopic examination of chemically stained peripheral blood smears to directly visualize the microorganisms attached to erythrocytes (15). Recently established PCR assays for M. suis can detect acutely diseased animals and also asymptomatic carrier pigs and are therefore principally suitable tools for the diagnosis of PE (6, 10, 15). However, PCR-based methods are still restricted to specialized, well-equipped research laboratories.

Methods to detect carrier animals are important for investigating the epidemiology of M. suis infections. For these purposes, serological assays are still the methods of choice. A specific and sensitive serological assay based on defined M. suis antigens would allow extensive prevalence studies and be applicable as a matter of routine in diagnostic laboratories. However, attempts to analyze the humoral immune response of pigs to M. suis have been impeded by the poor sensitivities and specificities of current antibody assays, which comprise the complement fixation test, the indirect hemagglutination assay, and the enzyme-linked immunosorbent assay (ELISA) (2, 12, 25, 26, 27, 28). Serodiagnostic assays described so far have the intrinsic disadvantage of employing complex and undefined M. suis antigens obtained from the peripheral blood of experimentally infected pigs.

Analogous to M. pneumoniae respiratory infection in humans as well as M. synoviae and M. gallisepticum infections in chickens, M. suis is capable of transiently inducing in swine the expression of cold-reactive anterythrocyte autoantibodies known as cold agglutinins (CA) (21, 24, 33). CA are of the immunoglobulin M (IgM) isotype and are directed against carbohydrate antigens expressed on the erythrocyte surface (7, 21, 33). The biological activities of CA are considered directly responsible for pathogenic effects in PE such as acrocyanosis and pallor and probably also for a suppressive effect on T-lymphocyte blastogenic responses (33). In the blood of experimentally infected pigs, CA and M. suis-specific antibodies appear simultaneously (33). As a result, CA may interfere with the identification of M. suis-specific serum antibodies by ELISAs,
which are still dependent on using blood-derived \textit{M. suis} antigens. In these assays, CA bound to stray erythrocyte membrane residues in the blood-derived crude antigen would be targeted thereby masking \textit{M. suis}-specific reactivities (25). This fact may limit the validity of serological diagnostic methods. Moreover, the lack of knowledge about the \textit{M. suis} antigens which are recognized during infection has surely delayed the development of improved serodiagnostic tests.

The present study was aimed at detecting immunogenic proteins expressed by \textit{M. suis} during infection. For this purpose, we analyzed in detail the antibody response to \textit{M. suis} during experimental infections of pigs.

\section*{Materials and Methods}

\textit{Mycoplasma suis} strain and experimental inoculum. The \textit{M. suis} strain, 5496, used in this study originated from a pig suffering from strong acute PE. The strain was characterized as \textit{M. suis} using PCR (33) and was identified as \textit{M. suis} by subsequent artificial infection as described elsewhere (10). For use as an inoculum in experimental infections, blood was collected from the cranial vena cavae of \textit{M. suis}-infected pigs during heavily parasitemic clinical attacks of PE, as confirmed by microscopic examination of acridine orange-stained peripheral blood smears. Alsever’s solution was used as an anticoagulant (111.0 mM glucose, 27.2 mM sodium citrate, 71.8 mM sodium chloride, pH 6.1). Blood samples were diluted at a 1:1 ratio with Alsever’s solution and stored in 4.0-ml volumes at \(-80\)°C. Three piglets confirmed free of \textit{M. suis} by repeated microscopic examination of acridine orange-stained peripheral blood smears as well as by PCR according to established methods were used as blood donors for a negative control inoculum (10).

Animals and experimental design. The experimental study was approved by the government of Upper Bavaria under registration number 21-2531-7798 and was performed in compliance with animal care legal regulations. Sixty German White-landrace cross piglets (12 weeks old, 26 to 34 kg) were included in the study. Piglets were born from sows without any history of clinical PE and confirmed negative for \textit{M. suis} by means of PCR and peripheral blood microscopy (10). Animals were allocated into three groups (groups I to III; 20 piglets per group) on a randomly stratified basis. Groups were housed in isolated but identical pens. Animals were provided a commercial pellet diet and water ad libitum and were subjected to the same environmental and managerial conditions throughout the study. Animals in group I (negative controls) were sple Necutomized 21 days prior to inoculation with the \textit{M. suis}-negative control inoculum. Animals in group II were spleenectomized 21 days prior to inoculation with \textit{M. suis}, and group III consisted of unspleenectomized piglets inoculated with \textit{M. suis}. On day 0 (3 weeks after splenectomy), piglets were inoculated intramuscularly with 2.0 ml of either the \textit{M. suis}-specific antigen (groups II and III) as described elsewhere (10, 32, 33). Animals in group I (negative controls) were maintained in splenectomized piglets (10 to 12 weeks of age) by subsequent artificial infection as described elsewhere (10, 32, 33). Animals were randomly selected from group I animals according to the method of Tijssen (30).

Results

Antigen reactivity. To determine the antigenicities of \textit{M. suis} proteins, Western blot analyses were performed on a panel of convalescent-phase sera from \textit{M. suis}-infected pigs. Using \textit{M. suis} antigen, these sera exhibited distinct reactivities with 11 proteins ranging from 27 to 83 kDa in size (p27, p33, p40, p45, p56, p57, p61, p70, p73, p77, and p83). In contrast, only two proteins (p27 and p56) were detected in the control antigen (Fig. 1). Sera from noninfected pigs showed strong reactions with p27, p56, and p77 in the \textit{M. suis} antigen and weak reactions with p27 and p56 in the control antigen (Fig. 1). Notably, protein bands for p27, p56, and p77 were also reactive with the anti-pig IgG conjugate (H+L chain specific) in controls for nonspecific binding (no serum samples; Fig. 1). Using subclass-specific anti-pig IgG and IgM antibodies, p56 and p77 were identified as heavy chains of IgG and IgM, respectively. Band p27 was identified as the light chain of immunoglobulins based on its molecular mass and selective immunoreactivity: it was positive with the H+L-chain-specific conjugate and negative with subclass H chain-specific conjugates (data not shown). These findings confirm that 8 of the 11 seroreactive proteins are specific for \textit{M. suis}.

\textit{M. suis} ELISA. The results shown in Fig. 2A indicate that porcine immunoglobulins are a common impurity of the \textit{M. suis} antigen. In ELISAs, these immunoglobulins may display a marked ability to bind the secondary antibody, thus interfering with the detection of \textit{M. suis}-specific antibodies. Figure 2A illustrates that sera from \textit{M. suis}-negative control pigs (negative sera) as well as the anti-pig IgG conjugate alone exhibited
considerable ELISA reactivities with both the *M. suis* antigen and the control antigen (OD$_{405}$, 1.797 ± 0.083 to 1.870 ± 0.128 [mean values ± standard deviations]). ELISA reactivities of sera from *M. suis*-infected pigs (positive sera) with the control antigen ranged around the same level (1.909 ± 0.133). The differences in the mean values among these five groups were not statistically significant (Fig. 2A; *P* = 0.412). In contrast, ELISA reactivities of positive sera with *M. suis* antigen exhibited OD values with an average of 2.304 ± 0.166. These values significantly exceeded the cutoff value of 1.961 calculated from OD measures of negative sera with *M. suis* antigen as well as the OD mean values obtained for reactions of positive sera with the control antigen (*P* < 0.001). Taken together, the data indicate that the ELISA using *M. suis* and control antigen allows for the identification of *M. suis*-specific antibodies despite the substantial background levels due to the inherent binding of secondary antibody to porcine immunoglobulin residues in both antigens.

In order to determine the extent to which these immunoglobulin residues bind the secondary antibody, *M. suis* and control antigens were depleted of immunoglobulins and comparatively analyzed under the same ELISA conditions. Figure 2B demonstrates that the depletion of immunoglobulins from the antigens led to a significant reduction in nonspecific ELISA reactions. OD values for positive sera, negative sera, and the conjugate alone with the depleted control antigen declined by an average of 93.5% compared with OD values measured with the undepleted control antigen (*P* < 0.001). These results were paralleled by a significant drop of 93.9% (on average) in OD values for negative sera and the conjugate with the depleted *M. suis* antigen (*P* < 0.001). On the other hand, even though the mean ELISA response level of positive sera with the *M. suis* antigen was also significantly lowered due to depletion, by 17.1% (*P* < 0.05), the remaining mean OD level for positive sera with undepleted *M. suis* antigen (1.909 ± 0.216) was nearly 15-fold higher than the corresponding values for all control reactions (positive sera versus undepleted control antigen or negative sera and conjugate versus both undepleted antigens). These data support the idea that the *M. suis*-specific analytical ELISA sensitivity can be remarkably enhanced by a preceding depletion of immunoglobulins from the antigens. This appreciable improvement in sensitivity was also expressed by a significantly reduced cutoff OD value of 0.130 due to the depletion of immunoglobulins from the antigens, in contrast to 1.961 using undepleted antigens.

**Antibody response kinetics.** Western blotting and ELISA were performed on a total of 120 serum samples obtained from...
eight experimentally infected pigs between days 0 and 98. A representative Western blot is shown in Fig. 3. All preinfection sera were negative for *M. suis*-specific reactivities on the immunoblots. *M. suis*-specific antibodies were detected as early as 1 week postinfection, and all pigs were identified as antibody responders within 3 weeks postinfection. Band reactivities for the postinfection sera were highly variable (Table 1). Three proteins, i.e., p40, p45, and p70, were detected as early as 7 to 14 days postinfection and remained the preferentially recognized antigens even in the late stage of infection. These observations suggested that antibodies against these three antigens might serve as useful markers for a past infection with *M. suis*.

Further antigens recognized by most animals by days 56 to 63 were p33, p61, and p83.

Figure 4 shows representative kinetics of the antibody response obtained by ELISA. *M. suis* antibodies were not found in any of the sera taken at housing and prior to inoculation. After experimental infection, *M. suis*-specific antibodies significantly increased from week 1 to week 2. This significance was maintained for the remainder of the experiment compared to the day 0 control. For all pigs, ELISA OD values peaked around attacks of acute PE, and these increases were observed with both *M. suis* and control antigens. Strikingly, these markedly peaking ELISA values were consistently correlated with a

![FIG. 3. Kinetic immunoblot analysis of antibody response following experimental *M. suis* infection (pig no. 96/99). Western blots of blood-derived *M. suis* antigens were probed with sequential sera collected weekly before inoculation (lane P) and weeks after infection (lanes 1 to 14) with *M. suis*. Filled arrows on the right indicate the locations of the immunodominant *M. suis* antigens (p70, p45, and p40). The positions of coreactive immunoglobulins extracted from porcine blood during antigen processing are marked (empty arrows) and indicate bands of 27 and 56 kDa. Arrows on the bottom indicate the time points of clinical PE attacks (weeks 5 and 7).](http://cvi.asm.org/

### TABLE 1. Antibody reactions of experimental animals to *M. suis* antigens

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**Avg no. of bands detected**

| 0 | 1.5 | 2.4 | 4.1 | 4.0 | 3.5 | 3.0 | 3.8 | 4.4 | 6.1 | 5.1 | 6.5 | 6.5 | 5.5 | 5.5 |
substantial decrease in the number of seroreactive M. suis proteins on the immunoblots (Fig. 4). Figure 5 shows a detailed analysis of ELISA and Western blot results obtained for sera from eight pigs during clinical PE attacks compared to sera taken 1 week before and 1 week after the PE attacks. During PE attack, both the M. suis-specific antibodies detected by ELISA (net OD for M. suis = OD with M. suis antigen − OD with control antigen) and the number of M. suis-specific immunoblot bands were significantly decreased in comparison to the values found 1 week before and 1 week after the attack. In parallel, the concentration of antibodies highly cross-reacting with the control antigen significantly increased during the PE attack (P ≤ 0.001). Subsequent analyses showed that these antibodies belonged to the immunoglobulin subclasses IgG (95%) and IgM (5%).

Western blot and ELISA reactivities of clinically defined sera. To prove the diagnostic feasibility of Western blotting and ELISA, specified experimental porcine sera were investigated. Serum samples taken 3 weeks after experimental infection from 20 nonapparently M. suis-infected pigs (group III) and from 20 clinically diseased pigs (group II, acute PE at 6−10 days postinfection) consistently showed M. suis-specific Western blot reactivities with at least p40, p45, or p70. This M. suis-specific antibody response was also consistently detected by ELISA, using antigens depleted of residual immunoglobulins. OD values ranged between 0.339 and 2.215. Serum samples from mock-infected pigs (group I, n = 20) exhibited no M. suis-specific seroreactivities in Western blots and ELISAs (data not shown).

DISCUSSION

In order to understand more thoroughly the antibody response against M. suis, we sought to characterize M. suis-specific antigens in blood-derived preparations of the agent. For this, we modified our recently published protocol for gaining M. suis DNA by a density gradient centrifugation step that was described to efficiently separate hemotrophic mycoplasmas from erythrocytic membranes (1). The results of our Western blot studies show that M. suis expresses in vivo at least eight specific proteins, ranging from 33 to 83 kDa in size, which are recognized by convalescent-phase sera. To the best of our knowledge, this is the first analysis of M. suis-specific antigens. Due to the use of host-derived M. suis antigens, the antibody response measured is expected to exclusively target antigens expressed by M. suis within the porcine host. However, it is impossible at this time to conceive any precise function for these proteins.

Of particular note is the consistent detection of three protein bands (p27, p56, and p77) in immunoblots of sera from infected and uninfected pigs as well as in conjugate control blots. Using subclass-specific conjugates, p56 and p77 were identified as heavy chains of IgG or IgM, and p27 was identified as the light chain of immunoglobulins. These immunoglobulins are copurified from the blood and are components of the positive (p27, p56, and p77) and negative (p27 and p56) antigen preparations. Thus, M. suis antigen contains large amounts of IgM antibodies induced by acute M. suis infection which cannot be detected in control antigens derived from the blood of healthy pigs.

Using blood-derived antigens in a similar study of feline M. hemofelis infection, Alleman and coworkers (1) were able to identify a 56-kDa antigen which showed significant seroreactivities with both initial and follow-up serum samples. The
authors postulated that this p56 antigen originated from feline erythrocytes. However, it is plausible from our studies using commercially available immunoglobulins (data not shown) that p56 represents the heavy chain of the IgG molecule. Thus, immunoglobulins should be taken into consideration as contaminants of blood-derived M. suis preparations. Our attempts to remove immunoglobulins from the M. suis antigen by using a commercially available immunoglobulin depletion kit were successful. In contrast to the case with undepleted M. suis antigen, only slight ELISA reactivities due to anti-swine IgG conjugate binding (equivalent to normal ELISA background blank levels) remained after immunoglobulin depletion. As a consequence, we can conclude that the depletion of immunoglobulins is an important step forward to increase the specificity of host-derived M. suis antigens. This finding led us to further analyze the kinetics of the M. suis antibody response in experimentally infected pigs by means of an indirect ELISA with depleted antigens.

In representative kinetics of the M. suis antibody responses, as demonstrated by Western blotting, sequential sera from infected pigs reacted with M. suis-specific bands within 2 weeks postinfection, and this antibody response persisted until the termination of the experiment. From immunoblot result analysis, some features should be pointed out. First, the antibody response during PE involves multiple antigens and is characterized by temporal and animal-to-animal variations in antigen recognition. This highly heterogeneous antigen recognition may reflect either host immune response variability or differences in infection progression. These findings should be addressed in future studies. Second, it is noteworthy that three of eight seroreactive antigens, i.e., p40, p45, and p70, were preferentially involved in the antibody response since all infected pigs showed distinct seroreactivities with at least one of these proteins during the second week of infection, and these persisted until the end of the experiment. We therefore concluded that antibodies against these three antigens can serve as useful markers for a past infection with M. suis. Finally, it must be pointed out that compared to the case for serum samples from subclinical phases of PE, the number of seroreactive M. suis-specific protein bands was significantly reduced for serum samples taken during clinical PE attacks. Since p40 reactivity in the sera persisted even through the clinical PE attacks, we propose using this protein as an antigen in ELISAs.

In parallel, we monitored serum IgG antibody levels against either M. suis or control antigen by indirect ELISA. All animals developed a strong antibody response against M. suis by 8 to 10 days postinfection at the earliest and until the end of the experiment at 14 weeks. These findings are consistent with a functional humoral immune response, and consequently, we reasoned that the high levels of antibodies specifically targeting the M. suis antigen are strongly related to active M. suis infection. Most importantly, vigorous ELISA IgG peaks with both M. suis and control antigens were recorded for all animals during all clinical attacks of PE observed throughout the entire experiment. Thus, on the one hand, the ELISA using immunoglobulin-depleted blood-derived M. suis antigens has proven valid for detecting antibodies against M. suis during the asymptomatic stage of PE and is therefore suitable as a screening assay to identify carrier animals. However, on the other hand, the detection of M. suis-specific antibodies by indirect ELISA fails during clinical attacks of PE due to the fact that high levels of autoreactive antibodies mask lowered levels of M. suis antibodies. The immunoglobulin kinetics, as measured by ELISA, agree with earlier findings of the fundamental study of Zachary and Smith (33) showing that clinical attacks of PE go along with peaks of hyperglobulinemia in pig serum due to a polyclonal B-lymphocyte activation. Notably, our study extends the findings of Zachary and Smith (33), i.e., that acute PE is also associated with a polyclonal increase in IgG antibodies and that these antibodies are obviously autoreactive to normal constituents of the porcine blood. Moreover, parallel analyses of ELISA and Western blotting results evidenced a striking inverse correlation between IgG antibodies that are autoreactive with porcine blood components and M. suis-specific antibodies. Of course, the reasons for the temporal interrelation between the increase in the autoreactive antibody response and the significant drop in the number of seroreactive M. suis protein antigens, as recognized by immunoblotting, are clearly open to speculation at this point.

The increase in autoreactive IgG is directly associated with maximal bacteremia and clinical symptoms, whereas serum levels of anti-erythrocyte CA IgM increase afterwards (33). The M. suis-induced anti-erythrocyte CA IgM may arise in response to a modification of carbohydrate antigens on the erythrocytic cell surface as a result of intimate membrane interactions between M. suis and erythrocytes and is suggested to contribute to clinical symptoms, i.e., the appearance of pallor and acrocyanosis via cold agglutination (32). In contrast, the mechanism by which M. suis initiates the production of autoreactive IgG antibodies during acute clinical PE are currently unknown. Members of the genus Mycoplasma, including pathogenic species associated with diseases of animals, are known to present a variety of structures capable of engaging both innate and adaptive components of the immune system (4, 9). For instance, a series of studies have revealed that mycoplasmas possess potent B-cell mitogens capable of stimulating a polyclonal activation and proliferation of B cells, resulting in the production of polyclonal immunoglobulin, some of which may be autoreactive (4, 23). However, the development of M. suis-induced autoreactive IgG antibodies and their role in the pathogenesis of PE remain to be determined.

In conclusion, we provide here the first report of M. suis-specific protein antigens. Among these, three immunodominant proteins with apparent molecular masses of 40, 45, and 70 kDa were consistently recognized in Western blots by sera from experimentally infected animals. The finding that the specificities of blood-derived M. suis preparations increased significantly after the depletion of immunoglobulins could improve the serodiagnosis of PE by indirect ELISA. Our ELISA studies of the M. suis-induced antibody response revealed a distinct specific antibody response alongside a nonspecific response of probably autoreactive IgG antibodies due to polyclonal B-cell proliferation during clinical PE attacks. Finally, it is anticipated that the M. suis proteins demonstrated in this study will be evaluated in detail as antigens leading to the development of novel serodiagnostic tests for M. suis.

REFERENCES


