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Characterization of a highly immunogenic *Mycoplasma hyopneumoniae* lipoprotein Mhp366

identified by peptide spot array

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Abstract

Enzootic Pneumonia (EP) in pigs caused by *Mycoplasma hyopneumoniae* is a highly prevalent, chronic respiratory disease, which causes considerable economic losses in the swine industry. Most herds are vaccinated, but classical bacterin vaccines do not prevent colonization and it is not possible to detect flourishing *M. hyopneumoniae* infections in vaccinated herds since commonly used commercial ELISAs can not differentiate infected from vaccinated animals. To solve this problem, new immunogenic proteins, up-regulated or solely expressed during infection, need to be identified. For this purpose a peptide spot array was constructed which presents 105 potential linear B-cell epitopes identified by *in-silico* analysis in 35 putative lipoproteins encoded on the genome of *M. hyopneumoniae* type strain 232. Subjecting this array to immunoblotting using porcine convalescent serum revealed a single strongly immunoreactive epitope on the Mhp366 protein which did not react with serum from bacterin-immunized pigs. In addition, it was not possible to detect Mhp366 in total cell lysates of *in-vitro* grown *M. hyopneumoniae* strains, using a polyclonal rabbit serum raised against a recombinant GST-Mhp366 fusion protein. To investigate the possibility of using an Mhp366-based ELISA in the field for differentiating vaccinated herds with and without a flourishing infection it was shown that i) homologues of the corresponding *mhp366* gene were present in all 17 *M. hyopneumoniae* strains and porcine lung samples tested from different geographic origins and ii) an ELISA based on epitope-specific synthetic peptides as solid phase antigen allowed a classification of field samples. Therefore, Mhp366 might be the first antigen identified which facilitates the detection of flourishing *M. hyopneumoniae* infections even in vaccinated herds.

Keywords: *Mycoplasma hyopneumoniae*, immunogenic proteins, peptide-spot array, lipoprotein
1. Introduction

Enzootic Pneumonia (EP) in pigs caused by *Mycoplasma hyopneumoniae* is a highly prevalent, chronic respiratory disease affecting pigs worldwide (Ross, 1999). Pure *M. hyopneumoniae* infections are characterised by high morbidity and low mortality, but are often complicated by secondary infections with other bacterial or viral pathogens (Maes et al., 2008).

The control of *M. hyopneumoniae* infections critically requires suitable vaccines and diagnostic tools. Two commercial ELISAs are commonly used for monitoring the health status of pig herds, a monoclonal antibody-based blocking ELISA (*M. hyopneumoniae* ELISA, Oxoid; (Feld et al., 1992)) and an indirect whole cell ELISA (IDEXX HerdChek *M. hyopneumoniae*, IDEXX Laboratories). Comparison of ELISA results revealed a high specificity of both assays whereas the sensitivity strongly depends on the vaccination status and the time point of sampling (Erlandson et al., 2005; Ameri-Mahabadi et al., 2005). Most importantly, none of the tests can be used to differentiate infected from vaccinated animals. The development of new diagnostic tools requires the identification of new immunogenic proteins, preferentially those which are up-regulated or solely expressed during infection.

An important group of proteins found in all Mycoplasma species are surface-exposed lipoproteins which mediate different interactions with the host cells, like adherence (Sachse et al., 2000) or damage of host tissue (Hopfe and Henrich, 2008) and which are major targets of the host’s immune response (Zuo et al., 2009). Since the genome sequences of three *M. hyopneumoniae* strains have recently been published (Minion et al., 2004; Vasconcelos et al., 2005), the cellular localization of all annotated proteins can be predicted to define sub-proteomes like membrane proteins or lipoproteins.

In the present study, a peptide spot array representing predicted immunogenic epitopes of putative *M. hyopneumoniae* lipoproteins was constructed; immunoblotting resulted in the identification of Mhp366 as an infection-related protein which might facilitate the detection of flourishing *M. hyopneumoniae* infections even in vaccinated herds.
2. Material and Methods

2.1. Bacterial strains and culture conditions.

All *M. hyopneumoniae* strains (Table 1) were cultured in modified Friis medium (Friis, 1975) at 37°C and 5% CO₂. Cells were grown to late exponential growth phase as determined by measurement of color-changing units (CCU), harvested by centrifugation at 20,000 x g for 30 min and washed three times in phosphate-buffered saline (PBS). *Escherichia coli DH5αF⁻* (Raleigh et al., 1989) was grown at 37°C on LB agar plates or in LB medium supplemented with ampicillin (100 µg/ml), D-glucose (0.5%, w/v) or IPTG (1 mM) as required.

2.2. Synthesis and identification of immunogenic epitopes.

Putative lipoproteins were identified by analysing the genome of *M. hyopneumoniae* strain 232 (acc. no. NC_006360) using the programs Lipo (Berven et al., 2006) (http://www.bioinfo.no/tools/lipo) and LipoP 1.0 (www.cbs.dtu.dk/services/LipoP; Juncker et al., 2003). Putative lipoproteins recognized by both programs (Table 2) were used for prediction of potential B-cell epitopes using the ABCpred program (www.imtech.res.in/raghava/abcpred; Saha and Raghava, 2006) with a window of 20 amino acid residues as predicted epitope length and a threshold setting of 0.7.

For each putative lipoprotein, three epitopes showing the highest predictive scores were selected, and overlapping 15-mer peptides initiating at every third amino acid were synthesized as an array of spots on an aminopegylated cellulose membrane (AIMS Scientific Products, Braunschweig, Germany; (Frank, 2002)). Immunoblot analyses of peptide spot arrays was essentially done as described (Frank and Overwin, 1996). Briefly, the ethanol-wetted membrane was washed three times with Tris-buffered saline [pH 7.0] (TBS) for 10 min and incubated overnight at 4°C in membrane blocking solution (MBS; 80% TBS-T [TBS with 0.05% Tween 20], 20% [vol/vol] casein-based blocking buffer concentrate [Thermo Scientific 37532], 5% [w/v] sucrose [pH 7.0]).
After washing 10 min with T-TBS, the membrane was incubated for 2 h at room temperature with porcine convalescent or hyperimmune serum against *M. hyopneumoniae* (Meens et al., 2006) diluted 1:200 in MBS. After washing three times with T-TBS, alkaline phosphatase-conjugated goat anti-swine immunoglobulin G (Dianova, Hamburg, Germany) 1:2000 diluted in MBS was applied for 1 h. Blots were washed with T-TBS and citrate-buffered saline [pH 7.0] (CBS) and developed using BCIP (5-bromo-4-chloro-3-indolylphosphate) and MTT (thiazolyl blue tetrazolium, Sigma M2128) as chromogenic substrates. The reaction was stopped by washing blots twice in PBS. For repeated use, the membrane was stripped as described (Frank and Overwin, 1996).

### 2.3 DNA techniques, plasmids and sequencing

Genomic DNA from culture-grown *M. hyopneumoniae* was isolated as described (Caron et al., 2000). DNA modifying enzymes were purchased from NEB (Bad Schwalbach, Germany), preparation of plasmid DNA, DNA ligation, transformation, and agarose-gel electrophoresis were done following standard procedures (Sambrook et al., 1989). Expression vector pGEX5x3 was obtained from Amersham Biosciences (Freiburg, Germany). Taq polymerase, cloning vector pCR® 2.1-TOPO and TopoTA cloning kits were purchased from Invitrogen (Karlsruhe, Germany).

Standard PCR reactions were performed as described earlier (Meens et al., 2006). DNA from lung lesions was isolated from tissue pieces (20–25 mg) by digestion in 180 μl of lysis buffer (20 ml 0.1 M Tris-HCl [pH 6.4]; 4.4 ml 0.2 M EDTA [pH 8.0]; 1 g/ml of guanidinium thiocyanate; 0.5 ml Triton X-100; 20 μl 600 mAU/ml proteinase K) for 5 min at 65°C, followed by silica DNA extraction, essentially as described by Boom et al. (Boom et al., 1990). In a nested PCR approach, 5 μl of the extracted DNA were first amplified using 0.2 mM of primers oMhp366A/H, followed by the second PCR, performed with primers oMhp366A/B and 1 μl of the amplification product from the first PCR as a template. Chromosomal DNA from strain *M. hyopneumoniae* 232 was used as positive control. Plasmid constructs were confirmed by DNA sequencing, performed by SeqLab GmbH (Goettingen, Germany). The BLAST program (Altschul et al., 1990) was used for nucleotide
and protein homology search. The Clustal W program (Thompson et al., 1994) was used for
multiple-sequence protein alignments.

2.4 Construction of GST-Mhp366 fusion protein expression vector

The recombinant expression of the mhp366 gene in E. coli required the replacement of 4 nonsense
TGA codons at amino acid positions 279, 323, 422 and 483 of the mature protein by tryptophan-
encoding TGG triplets. Using chromosomal DNA of the M. hyopneumoniae type strain 232 and
mutagenic primer pairs oMhp366A-H (Table 1) with class IIS restriction sites at their 5´ends
(Shigaki and Hirschi, 2001), four PCR fragments 366-AB (811 bp), 366-CD (152 bp), 366-EF (345
bp) and 366-GH (401 bp) were generated, restricted with BsmBI and ligated. After reamplification
using primers oMhp366A and oMhp366H, the resulting PCR product was ligated into the SmaI and
NotI digested plasmid pGex5x3, yielding pMhp366-501. Successful construction of the expression
vector was confirmed by DNA sequencing and small-scale expression of the GST fusion protein.

2.5 Expression and purification of recombinant GST-Mhp366 fusion protein.

Protein expression using E. coli DH5αF/pMhp366-501 was essentially done as described earlier
(Meens et al., 2006). The resulting aggregated fusion protein was isolated as previously described
(Gerlach et al., 1992), followed by further purification by preparative 16 x 18 cm SDS-PAGE on a
SE600 unit (Amersham Biosciences, Freiburg, Germany). After Coomassie staining, the GST-
Mhp366 band was cut from the gel and isolated by electro-elution.

2.6 Protein analyses and preparation of sera.

Protein concentrations were determined using a MicroBC™ Assay (Uptima Interchim, Montlucon
Cedex, France). Analysis of protein samples on 1D SDS-PAGE and Western blotting were done
following standard procedures (Sambrook et al., 1989). Broad range prestained protein marker
(NEB, Frankfurt, Germany) and LMW calibration kit (Amersham Biosciences, Freiburg, Germany) were used as molecular weight standards.

Rabbit antibodies were raised against the GST-Mhp366 protein; 100 µg fusion protein dissolved in 0.5 ml PBS were emulsified with 10% (final concentration) of Emulsigen®-Plus (MVP Inc., Ralston, NE). Immunisation of a New Zealand white rabbit and preparation serum was done as described (Meens et al., 2006). A porcine convalescent serum pool from five non-vaccinated pigs infected with M. hyopneumoniae, which was used as a positive reference serum in all experiments, and porcine hyperimmune serum have been described earlier (Meens et al., 2006). The porcine negative control serum was pooled from 10 non-vaccinated, non-infected pigs.

2.7. ELISA

Two commercial ELISAs, an indirect ELISA (IDEXX HerdChek Mycoplasma hyopneumoniae®, IDEXX Laboratories, Westbrook, Maine) and a blocking ELISA (Oxoid Mycoplasma hyopneumoniae ELISA, Oxoid Ltd, Cambridgeshire, UK) were used according to the manufacturers’ instructions.

Putative immunogenic epitopes (nonamers) were synthesized with an amino-terminal biotinylation linked by a 2-aminohexanoic acid linker and purified by high-performance liquid chromatography. Lyophilized peptides were resuspended in distilled water to obtain stock solutions of 10 mg/ml. For the Mhp366-specific peptide-based ELISA, Nunc Immobilizer Streptavidin F96 microtiter plates were coated with 100 µl of the corresponding synthetic, biotinylated peptide at a concentration of 0.1 µg/ml in coating buffer (2.83 g/l NaHCO₃; 1.59 g/l Na₂CO₃ [pH 9.6]) and incubated at 4°C overnight. Serial twofold dilutions of the sera were added and incubated for 1 h at room temperature. Goat anti-pig or goat anti-rabbit peroxidase conjugates (Dianova, Hamburg, Germany) were each incubated for 1 h at room temperature. The ELISA was developed using 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS; Roche Diagnostics, Mannheim, Germany) as a substrate.
3. Results

3.1. Construction and analysis of M. hyopneumoniae lipoprotein peptide array

The genome of the M. hyopneumoniae type strain 232 (Minion et al., 2004) was analysed in-silico using the programs Lipo (Berven et al., 2006) and LipoP 1.0 (Juncker et al., 2003) to identify putative lipoproteins. A total of 35 ORFs were detected by both programs (Table 2) and chosen for further analysis using the ABCpred server (Saha and Raghava, 2006) which is widely used to predict linear B-cell epitopes. Based on these results, three peptide sequences showing the highest predictive scores were chosen for each putative lipoprotein. If equal scores for more than three epitopes were obtained, sequences evenly distributed within the protein were selected. Since all epitope sequences were represented by three overlapping 15-mer peptides initiating at every third amino acid, a total of 315 peptides (supplemental material Table 1) were synthesized and spotted onto cellulose membranes (Frank, 2002) for further analysis by immunoblotting.

The spot membrane was blotted using a porcine convalescent serum pool and a porcine hyperimmune serum raised against a commercial M. hyopneumoniae vaccine and the signal intensity of corresponding spots was compared. Predicted epitopes showing a positive reaction in only one or two out of three peptides were not considered because the core sequence of the corresponding B-cell epitope was obviously not completely covered. As shown in Fig. 1A, the porcine convalescent serum recognized, in particular, the peptide spots 82-84 corresponding to the first epitope predicted from the mhp366 gene. In addition, epitopes of the proteins Mhp164 (spots 16–18), Mhp293 (spots 37–39), Mhp364 (spots 73–75), Mhp682 (spots 301–306) and Mhp695 (spots 310–312) were also recognized, but to a lesser extent. As shown in Fig. 1B, the Mhp366 epitope was not recognized by the hyperimmune serum, whereas Mhp164 (spots 16–18) and Mhp293 (spots 37–39) again showed a positive reaction, comparable to the convalescent serum.

None of the peptide spots was recognized by the negative control serum (data not shown).
specific recognition of the Mhp366 epitope by convalescent serum but not by hyperimmune serum led us to examine its properties in more detail.

The mhp366 gene has a length of 1,665 bp (555 amino acid residues), starting at position 432,758 in the genome of *M. hyopneumoniae* strain 232 (acc. no. AE017332; (Minion et al., 2004)). It encodes the putative lipoprotein Mhp366 (acc. no. AAV27851) with a calculated molecular mass and IP of 64,394 Da and 9.90; respectively. The predicted epitope recognized by the convalescent serum covers the amino acid positions 68 to 88 within the Mhp366 protein (68QKENSQKNDVVNSQNTEKTE88), the overlapping sequence of the three peptides which should define the actual B-cell epitope, is located at position 74 to 82 (74KNDVVNSQN82). Immediately following this epitope, a region of 4 tandem repeats of the sequence motif [KR]TE starts which has recently been characterized by de Castro et. al. (de Castro et al., 2006).

### 3.2. Antigenic specificity of immunogenic Mhp366 protein.

The plasmid pMhp366-501 encodes a GST-Mhp366 fusion protein composed of the amino-terminal GST followed by the mature part of Mhp366 starting at Cys22. The purification of the recombinant fusion protein was controlled by SDS-PAGE analysis and Coomassie staining, which confirmed the homogeneity of the protein preparation and the apparent molecular mass of around 90 kDa (Fig. 2A), corresponding to the value calculated from the deduced amino acid sequence (89,869 Da). As expected, the GST-Mhp366 fusion protein and the GST protein were recognized by GST-specific antibodies (Fig. 2B). Using the porcine convalescent serum, the GST-Mhp366 fusion protein was clearly recognized and this reaction was solely based on the Mhp366 part of the fusion protein, since the GST protein was not recognized by the convalescent serum (Fig. 2C, lane 1). In contrast, the porcine hyperimmune serum showed only a very weak reaction with the GST-Mhp366 fusion protein (Fig. 2D), thus confirming the results obtained on the peptide spot array. In addition, a Mhp366-specific signal in the expected size of 62 kDa (mature Mhp366 protein) could not be detected in total cell lysates of *M. hyopneumoniae* type strains J and 232
grown in Friis medium using a polyclonal rabbit serum raised against the GST-Mhp366 fusion
protein (Fig. 3B) although a broad spectrum of immunogenic bands was visible when porcine
convalescent serum was used (Fig. 3C). Two protein bands, 95 kDa and 45 kDa in size, recognized by the Mhp366-specific polyclonal rabbit serum (Fig. 3B, lanes 1 and 2) were also
detected by rabbit pre-immune serum (data not shown) and, therefore, the reaction was
considered as non-specific.

3.3. Sequence analysis of the immunogenic epitope and adherent VNTR regions in various
M. hyopneumoniae strains and diagnostic samples
A possible future application of the immunogenic epitope identified in Mhp366 as a diagnostic
marker facilitating the differentiation of infected and vaccinated animals required a more
detailed examination of its distribution and sequence variations among M. hyopneumoniae
isolates from different geographic origins. For this purpose, a PCR fragment (800 bp) was amplified
using primers oMhp366A / oMhp366B and chromosomal template DNA prepared from a total of
fourteen M. hyopneumoniae strains from Denmark (11), Belgium (2) and Germany (1) (Table 1).
In addition, three porcine lung samples from Ukraine were also analysed directly by nested PCR.
The sequencing revealed the presence of mhp366 homologous genes in all strains and samples
tested. The alignment of the obtained amino acid sequences confirmed the presence of the
immunogenic epitope whose core sequence is highly conserved, showing only two variations at
amino acid position 75 (N/K) and 82 (N/D), respectively (Fig. 4). The VNTR region is also present
in all samples. The number of repeats varies between 3 and 5, with most sequences containing 4
copies of the [KR]TE motif.

3.4. ELISA
The alignment of immunogenic epitopes (Fig. 4) revealed the presence of four different core
sequences. Synthetic peptides corresponding to these sequences were synthesized (Table 1) and
tested as coating antigens. A porcine convalescent serum pool from five non-vaccinated pigs identified as infected with *M. hyopneumoniae* was used as a positive reference serum. Plates were coated with 0.1 μg/ml of the particular peptides (as determined by checkerboard titration using the porcine convalescent serum) or with an equal mixture of all peptides, each at a concentration of 0.025 μg/ml. As shown in Fig. 5a, the four single peptides and the peptide mixture showed very similar reactivity with the convalescent serum pool, and a strong discrimination between positive and negative control serum (pooled serum from 10 non-vaccinated, non-infected pigs).

A total of 80 field serum samples from 9 herds were first tested using two commercial ELISA kits. The results of IDEXX HerdChek *M. hyopneumoniae* were determined on the basis of the sample–positive (S/P) ratio, resulting in 17 positive (S/P > 0.4), 61 negative (S/P < 0.3) and 2 suspect samples (S/P from 0.3 to 0.4). The monoclonal antibody-based blocking ELISA (Oxoid *M. hyopneumoniae* ELISA) revealed 17 positive samples (OD < 50% of buffer control OD), 60 negative (OD > 65% of buffer control OD) and 3 suspect samples (50 to 65 % of buffer control OD). The overall agreement between both ELISAs was calculated to be 92.5% (data not shown), which is in good agreement with recently published comparison of both tests (Ameri-Mahabadi et al., 2005).

The relative strength of field serum samples reactivity towards the immunogenic epitope was determined on ELISA plates coated with the equal mixture of epitope-specific peptides. The results, given as OD% with respect to the positive reference serum pool are shown in Fig. 5b. The field samples could be clearly separated into two groups. The first group includes four samples showing ELISA activities ≥ 100 OD% compared to the positive reference serum pool. The second group combines the remaining 76 samples with OD% values between 67.7% and 14.7%, with a majority of 59 samples showing activities ≤ 40 OD%. The negative control serum always resulted in OD% values ≤ 5% of the positive reference serum. None of the field serum samples showed comparably low values.
4. Discussion

Vaccination of piglets against *M. hyopneumoniae* is an important prophylactic measure to control Enzootic Pneumonia. Commercial bacterin vaccines are applied in many countries in more than 70% of the pig herds (Maes et al., 2008); in Germany, it is the most frequently applied vaccination in pigs with more then 85% of the herds being vaccinated. Although the beneficial effects of vaccination are well documented (Haesebrouck et al., 2004; Maes et al., 2008), the failure of currently used vaccines to prevent colonization (Sibila et al., 2008) or to reduce transmission significantly (Meyns et al., 2004) are major drawbacks. Due to the high vaccination coverage using inactivated whole cells, the detection of ongoing *M. hyopneumoniae* infections in vaccinated herds is strongly impeded. The construction of DIVA marker vaccines, which is based on the absence of one immunogenic protein (van Oirschot, 2001) to bypass this problem, is not possible since *M. hyopneumoniae* is not amenable to genetic manipulation.

In order to identify new infection-specific serological markers, we used an *in-silico* approach to predict immunogenic epitopes within putative lipoproteins. In the first step, the genome sequence of strain 232, which is commonly used to study virulence and vaccination regimens of *M. hyopneumoniae* (Minion et al., 2004), was analysed using the programs Lipo (Berven et al., 2006) and LipoP 1.0 (Juncker et al., 2003). A total of 35 ORFs were detected by both programs (Table 2). In a second step, potential linear B-cell epitopes were predicted using the ABCpred program (Saha and Raghava, 2006).

The epitope length window for predicted epitopes was set at 20 amino acid residues, corresponding to most linear B-cell epitopes which are 5 to 30 amino acid residues in length (Saha and Raghava, 2006). On the peptide array, all predicted epitopes were represented by three overlapping 15-mer peptides initiating at every third amino acid, thereby covering 21 amino acid residues. The immunoblot experiments revealed only one epitope from the Mhp366 protein which was clearly differentially recognized by convalescent and hyperimmune serum, respectively (Fig. 1). Epitopes
from at least five additional proteins reacted with both sera, indicating that the selection of a limited
number of B-cell epitopes to identify immunogenic proteins was successful.

Homology analysis of the \textit{mhp366} gene using the BLAST program revealed no significant
homology to genes or proteins from other bacterial species, but within the \textit{M. hyopneumoniae}
genome the \textit{mhp366} gene has four paralogs (\textit{mhp367}, \textit{mhp445}, \textit{mhp037} and \textit{mhp414}), all except
\textit{mhp414} being putative lipoproteins. The sequence identity of this paralogous gene family relating
to \textit{mhp366} varies between 50\% (\textit{mhp367}) and 29\% (\textit{mhp445}), but the epitope identified in our study
is unique to \textit{mhp366}. None of these proteins has yet been functionally characterized.

The \textit{mhp366} gene has recently been described in two publications. De Castro and colleagues
analysed the distribution of variable number of amino acid repeats (VNTARs) in five
\textit{M. hyopneumoniae} strains (de Castro et al., 2006) and found three to five copies of a conserved
[\text{KR}]\text{TE} motif in \textit{mhp366}-homologous genes, located immediately downstream of the immunogenic
epitope. Our analysis of 17 additional strains and diagnostic samples confirmed these results. Most
strains contain four copies of the [\text{KR}]\text{TE} motif and the sequence of the immunogenic epitope is
highly conserved showing only two amino acid variations (Fig. 4).

A microarray-based study to search for transcriptional changes during infection revealed a 2.43 fold
up-regulation of the \textit{mhp366} gene in \textit{M. hyopneumoniae} cells collected from bronchial alveolar
lavage fluid of infected pigs (Madsen et al., 2008). The values for transcript level differences
observed in this study are in general comparably low, but the up-regulation of \textit{mhp366} is the third-
highest among all genes of \textit{M. hyopneumoniae} strain 232. These results are in good agreement with
our findings. The Mhp366-specific epitope is strongly recognized by convalescent serum, but not
by hyperimmune serum (Fig. 1), which was produced using a commercial bacterin vaccine
containing inactivated \textit{M. hyopneumoniae} cells. The weak reactivity might be due to low or
negligible amounts of Mhp366 protein in \textit{M. hyopneumoniae} cells grown under laboratory
conditions as it is strongly supported by the results of our Western blotting experiments.
The strong reaction of the GST-Mhp366 fusion protein with the convalescent serum pool and the weak recognition by the hyperimmune serum (Fig. 2C/D) clearly reflects a different concentration of Mhp366-specific antibodies in both sera. For recombinant antigens, it has already been shown that higher antigen levels induce a stronger and more rapid immune response (Himmelrich et al., 2000) and this might also be true for native bacterial antigens. In total cell lysates of both M. hyopneumoniae strains J and 232 grown in Friis medium, it was not possible to detect a protein signal using a Mhp366-specific polyclonal rabbit serum (Fig. 3B). Nevertheless, very low amounts of Mhp366 below the detection limit of the rabbit serum might be present even in in-vitro grown cells because the hyperimmune serum still shows a weak positive reaction with GST-Mhp366.

Based on our initial results, synthetic peptides corresponding to the four different epitope core sequences (Fig. 4) were tested as coating antigens. A strong ability to discriminate between positive and negative reference sera from non-vaccinated infected or healthy pigs, respectively, could be demonstrated (Fig. 5a). The sequence variations observed in M. hyopneumoniae strains and diagnostic samples did not influence the recognition, since all four peptides showed a similar reactivity (Fig. 5a). The examination of 80 field serum samples supported our hypothesis. Thus, whereas 17 samples were recognized as positive by two commercial ELISA kits, only four samples showed an ELISA activity ≥ 100 OD% compared to the positive reference serum pool on our peptide based ELISA. The lower number of pigs reacting in the Mhp366 antigen ELISA most likely mirrors the absence of a prime-boost effect due to vaccination with respect to this antigen. This is supported by the gap observed in the range of ELISA results. None of the samples ranged from 70 to 100 OD%, the vast majority of samples ranged from 10 to 40 OD%. Most likely, this large group represents background levels caused by vaccination or unspecific reactions. To support this hypothesis, a more detailed examination of hyperimmune sera raised against different commercial vaccines, as well as larger numbers of field serum samples obtained from herds with a defined M. hyopneumoniae vaccination status would be necessary. These experiments should not only confirm our present results but also allow us to define a threshold value to discriminate between
background levels and positive signals. Additional experiments will also be necessary to characterise the expression pattern of the mhp366 gene in-vivo and in-vitro and the time course of Mhp366-specific antibody formation during infection to support our hypothesis, that Mhp366 might be the first antigen identified to facilitate the detection of flourishing M. hyopneumoniae infections even in vaccinated herds.

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Immune responses induced by recombinant BCG strains according to level of production of a foreign antigen: malE. Vaccine 18, 2636-2647.


Legends to figures

Fig. 1
Peptide spot array developed with porcine convalescent serum (A) or porcine hyperimmune serum (B), both diluted 1:100. Peptides 82 – 84 corresponding to epitope 1 of protein Mhp366 are enframed. The other peptides correspond to predicted immunogenic epitopes of other lipoproteins as listed in supplementary Table 1.

Fig. 2
Total cell lysates of *E. coli* DH5α/pGex5x2 (lane 1) and *E. coli* DH5α/pMhp366-501 (lane 2) as well as the purified GST-Mhp366 fusion protein (Lane 3 / 2 μg per lane) were separated on a 10.8% SDS-PAGE and stained with Coomassie blue (A) or blotted onto nitrocellulose membranes. Western blots were developed using polyclonal rabbit anti-GST serum (B; 1:1000) and porcine convalescent (C; 1:250) or hyperimmune (D; 1:250) sera. M, molecular mass standard.

Fig. 3
Total cell lysates of *M. hyopneumoniae* type strains J (lane 1), and 232 (lane 2) and the purified GST-Mhp366 fusion protein (Lane 3 / 2 μg per lane) were separated on 10.8% SDS-PAGE and stained with Coomassie blue (A) or blotted onto nitrocellulose membranes. Western blots were developed using polyclonal rabbit anti-Mhp366 serum (B; 1:1000) and porcine convalescent (C; 1:250) serum. M, molecular mass standard.

Fig. 4
Alignment of partial amino acid sequences of Mhp366-homologous proteins from different *M. hyopneumoniae* strains and porcine lung samples (VB-1A/2A/3A). The predicted B-cell epitope
is written in bold letters. The sequences of *M. hyopneumoniae* strains J (AE017243), 232
(AE017332), 7448 (AE017244), PMS (DQ386461) and 7442 (DQ386460) were obtained from
GenBank.

Fig. 5

ELISA results using epitope-specific synthetic peptides. Reactivity of four single synthetic peptides
(366-A/D) with a positive convalescent serum pool and comparison of an equal mixture of all four
peptides with positive and negative control serum (pooled serum from 10 non-vaccinated, non-
infected pigs) (A). Field serum samples tested on ELISA plates coated with the peptide mixture.
Results were given as OD% with respect to the positive reference serum pool and grouped into
classes of 10 OD% (B). Peptides were coated on Nunc Immobilizer Streptavidin F96 microtiter
plates.
Figure 2
Figure 3
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<th>Peptid</th>
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<td>84</td>
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FMS     | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 3       |

7442    | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 3       |

Mp18    | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 3       |

Mp47    | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 3       |

Mp73    | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mp88    | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mp182   | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mp195   | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mp263   | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mp473   | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mp529   | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mp612   | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mp654   | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

Mhyop3873 | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

MhyopB2V2W20 | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

MhyopOef91 | PTKESKVSPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

VB-1A   | PTKESKVGPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

VB-2A   | PTKESKVGPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |

VB-3A   | PTKESKVGPVEENTQKNDVVNSQNKTEKTEKTERTE------NQTTDNNFWSEST      | 4       |
Table 1  Bacterial strains, plasmids, primers and peptides used in this study

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<td><strong>Strains and plasmids</strong></td>
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<td><em>M. hyopneumoniae</em> strain J</td>
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<td><em>M. hyopneumoniae</em> strain 232</td>
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<td><em>M. hyopneumoniae</em> field strains Mp18, Mp47, Mp73, Mp88, Mp182, Mp195, Mp263, Mp473, Mp529, Mp612, Mp654</td>
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<td><em>M. hyopneumoniae</em> field strains Mhyop.B2V2W20, Mhyop.Oef91</td>
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<td><em>M. hyopneumoniae</em> field strain Mhyop.3873</td>
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<td>F&lt;sup&gt;+&lt;/sup&gt;/endA1 hsdR17 (r&lt;sup&gt;K&lt;/sup&gt; K&lt;sup&gt;K&lt;/sup&gt;) supE44 thi-1 recA1 gyrA (Nal&lt;sup&gt;R&lt;/sup&gt; relA1 Δ(lacZYA-argF)U169 deoR [φ80dlacΔ(lacZ)M15])</td>
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<td>pGex5x2</td>
<td><em>E. coli</em> cloning vectors carrying an ampicillin resistance determinant, the lac&lt;sup&gt;I&lt;/sup&gt; gene and the glutathione S-transferase gene</td>
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<td>pMph366-501</td>
<td>pGex5x3 containing the N-terminal truncated <em>mhp366</em> gene, Pos. 70-1665</td>
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Table 2

Putative lipoproteins identified within the genome of *M. hyopneumoniae* type strain 232

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Putative lipoproteins were predicted using the programs Lipo (Berven et al., 2006) and LipoP 1.0) (Juncker et al., 2003). Starting and endpoint as well as the number of amino acids, molecular weight, pI and functional description are taken from http://mycoplasma.genome.uab.edu/genomes.asp.
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