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1 **Characterization of a highly immunogenic *Mycoplasma hyopneumoniae* lipoprotein Mhp366**
2 **identified by peptide spot array**

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1 **Abstract**

2

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

23

24 **Keywords:** *Mycoplasma hyopneumoniae*, immunogenic proteins, peptide-spot array, lipoprotein

25

1 1. Introduction

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

6 The control of *M. hyopneumoniae* infections critically requires suitable vaccines and diagnostic
7 tools. Two commercial ELISAs are commonly used for monitoring the health status of pig herds, a
8 monoclonal antibody-based blocking ELISA (*M. hyopneumoniae* ELISA, Oxoid; (Feld et al.,
9 1992)) and an indirect whole cell ELISA (IDEXX HerdChek *M. hyopneumoniae*, IDEXX
10 Laboratories). Comparison of ELISA results revealed a high specificity of both assays whereas the
11 sensitivity strongly depends on the vaccination status and the time point of sampling (Erlandson et
12 al., 2005; Ameri-Mahabadi et al., 2005). Most importantly, none of the tests can be used to
13 differentiate infected from vaccinated animals. The development of new diagnostic tools requires
14 the identification of new immunogenic proteins, preferentially those which are up-regulated or
15 solely expressed during infection.

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

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

1 2. Material and Methods

2

3 2.1. Bacterial strains and culture conditions.

4 All *M. hyopneumoniae* strains (Table 1) were cultured in modified Friis medium (Friis, 1975) at
5 37°C and 5% CO₂. Cells were grown to late exponential growth phase as determined by
6 measurement of color-changing units (CCU), harvested by centrifugation at 20,000 x g for 30 min
7 and washed three times in phosphate-buffered saline (PBS). *Escherichia coli* DH5 α F' (Raleigh et
8 al., 1989) was grown at 37°C on LB agar plates or in LB medium supplemented with ampicillin
9 (100 μ g/ml), D-glucose (0.5%, w/v) or IPTG (1 mM) as required.

10

11 2.2. Synthesis and identification of immunogenic epitopes.

12 Putative lipoproteins were identified by analysing the genome of *M. hyopneumoniae* strain 232
13 (acc. no. NC_006360) using the programs Lipo (Berven et al., 2006)
14 (<http://www.bioinfo.no/tools/lipo>) and LipoP 1.0 (www.cbs.dtu.dk/services/LipoP; (Juncker et al.,
15 2003). Putative lipoproteins recognized by both programs (Table 2) were used for prediction of
16 potential B-cell epitopes using the ABCpred program (www.imtech.res.in/raghava/abcpred;
17 (Saha and Raghava, 2006) with a window of 20 amino acid residues as predicted epitope length and
18 a threshold setting of 0.7.

19 For each putative lipoprotein, three epitopes showing the highest predictive scores were selected,
20 and overlapping 15-mer peptides initiating at every third amino acid were synthesized as an array of
21 spots on an aminopegylated cellulose membrane (AIMS Scientific Products, Braunschweig,
22 Germany; (Frank, 2002)). Immunoblot analyses of peptide spot arrays was essentially done as
23 described (Frank and Overwin, 1996). Briefly, the ethanol-wetted membrane was washed three
24 times with Tris-buffered saline [pH 7.0] (TBS) for 10 min and incubated overnight at 4°C in
25 membrane blocking solution (MBS; 80% TBS-T [TBS with 0.05% Tween 20], 20% [vol/vol]
26 casein-based blocking buffer concentrate [Thermo Scientific 37532], 5% [w/v] sucrose [pH 7.0]).

1 After washing 10 min with T-TBS, the membrane was incubated for 2 h at room temperature with
2 porcine convalescent or hyperimmune serum against *M. hyopneumoniae* (Meens et al., 2006)
3 diluted 1:200 in MBS. After washing three times with T-TBS, alkaline phosphatase-conjugated goat
4 anti-swine immunoglobulin G (Dianova, Hamburg, Germany) 1:2000 diluted in MBS was applied
5 for 1 h. Blots were washed with T-TBS and citrate-buffered saline [pH 7.0] (CBS) and developed
6 using BCIP (5-bromo-4-chloro-3-indolylphosphate) and MTT (thiazolyl blue tetrazolium, Sigma
7 M2128) as chromogenic substrates. The reaction was stopped by washing blots twice in PBS. For
8 repeated use, the membrane was stripped as described (Frank and Overwin, 1996).

9

10 2.3 DNA techniques, plasmids and sequencing

11 Genomic DNA from culture-grown *M. hyopneumoniae* was isolated as described (Caron et al.,
12 2000). DNA modifying enzymes were purchased from NEB (Bad Schwalbach, Germany),
13 preparation of plasmid DNA, DNA ligation, transformation, and agarose-gel electrophoresis were
14 done following standard procedures (Sambrook et al., 1989). Expression vector pGEX5x3 was
15 obtained from Amersham Biosciences (Freiburg, Germany). Taq polymerase, cloning vector pCR[®]
16 2.1-TOPO and TopoTA cloning kits were purchased from Invitrogen (Karlsruhe, Germany).
17 Standard PCR reactions were performed as described earlier (Meens et al., 2006). DNA from lung
18 lesions was isolated from tissue pieces (20–25 mg) by digestion in 180 µl of lysis buffer (20 ml 0.1
19 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
20 Triton X-100; 20 µl 600 mAU/ml proteinase K) for 5 min at 65°C, followed by silica DNA
21 extraction, essentially as described by Boom et.al. (Boom et al., 1990). In a nested PCR approach, 5
22 µl of the extracted DNA were first amplified using 0.2 mM of primers oMhp366A/H, followed by
23 the second PCR, performed with primers oMhp366A/B and 1 µl of the amplification product from
24 the first PCR as a template. Chromosomal DNA from strain *M. hyopneumoniae* 232 was used as
25 positive control. Plasmid constructs were confirmed by DNA sequencing, performed by SeqLab
26 GmbH (Goettingen, Germany). The BLAST program (Altschul et al., 1990) was used for nucleotide

1 and protein homology search. The Clustal W program (Thompson et al., 1994) was used for
2 multiple-sequence protein alignments.

3

4 *2.4 Construction of GST-Mhp366 fusion protein expression vector*

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

14

15 *2.5 Expression and purification of recombinant GST-Mhp366 fusion protein.*

16 Protein expression using *E. coli* DH5 α F'/pMhp366-501 was essentially done as described earlier
17 (Meens et al., 2006). The resulting aggregated fusion protein was isolated as previously described
18 (Gerlach et al., 1992), followed by further purification by preparative 16 x 18 cm SDS-PAGE on a
19 SE600 unit (Amersham Biosciences, Freiburg, Germany). After Coomassie staining, the GST-
20 Mhp366 band was cut from the gel and isolated by electro-elution.

21

22 *2.6. Protein analyses and preparation of sera.*

23 Protein concentrations were determined using a MicroBCTM Assay (Uptima Interchim, Montlucon
24 Cedex, France). Analysis of protein samples on 1D SDS-PAGE and Western blotting were done
25 following standard procedures (Sambrook et al., 1989). Broad range prestained protein marker

1 (NEB, Frankfurt, Germany) and LMW calibration kit (Amersham Biosciences, Freiburg, Germany)
2 were used as molecular weight standards.

3 Rabbit antibodies were raised against the GST-Mhp366 protein; 100 µg fusion protein dissolved in
4 0.5 ml PBS were emulsified with 10% (final concentration) of Emulsigen®-Plus (MVP Inc.,
5 Ralston, NE). Immunisation of a New Zealand white rabbit and preparation serum was done as
6 described (Meens et al., 2006). A porcine convalescent serum pool from five non-vaccinated pigs
7 infected with *M. hyopneumoniae*, which was used as a positive reference serum in all experiments,
8 and porcine hyperimmune serum have been described earlier (Meens et al., 2006). The porcine
9 negative control serum was pooled from 10 non-vaccinated, non-infected pigs.

10

11 2.7. ELISA

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

16 Putative immunogenic epitopes (nonamers) were synthesized with an amino-terminal biotinylation
17 linked by a 2-aminohexanoic acid linker and purified by high-performance liquid chromatography.
18 Lyophilized peptides were resuspended in distilled water to obtain stock solutions of 10 mg/ml. For
19 the Mhp366-specific peptide-based ELISA, Nunc Immobilizer Streptavidin F96 microtiter plates
20 were coated with 100 µl of the corresponding synthetic, biotinylated peptide at a concentration of
21 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
22 overnight. Serial twofold dilutions of the sera were added and incubated for 1 h at room
23 temperature. Goat anti-pig or goat anti-rabbit peroxidase conjugates (Dianova, Hamburg, Germany)
24 were each incubated for 1 h at room temperature. The ELISA was developed using 2,2-azino-di-[3-
25 ethylbenzothiazoline sulfonate] (ABTS; Roche Diagnostics, Mannheim, Germany) as a substrate.

1 3. Results

2

3 3.1. Construction and analysis of *M. hyopneumoniae* lipoprotein peptide array

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

14 The spot membrane was blotted using a porcine convalescent serum pool and a porcine
15 hyperimmune serum raised against a commercial *M. hyopneumoniae* vaccine and the signal
16 intensity of corresponding spots was compared. Predicted epitopes showing a positive reaction in
17 only one or two out of three peptides were not considered because the core sequence of the
18 corresponding B-cell epitope was obviously not completely covered. As shown in Fig. 1A, the
19 porcine convalescent serum recognized, in particular, the peptide spots 82-84 corresponding to the
20 first epitope predicted from the *mhp366* gene. In addition, epitopes of the proteins Mhp164 (spots
21 16–18), Mhp293 (spots 37–39), Mhp364 (spots 73–75), Mhp682 (spots 301–306) and Mhp695
22 (spots 310–312) were also recognized, but to a lesser extent. As shown in Fig. 1B, the Mhp366
23 epitope was not recognized by the hyperimmune serum, whereas Mhp164 (spots 16–18) and
24 Mhp293 (spots 37–39) again showed a positive reaction, comparable to the convalescent serum.
25 None of the peptide spots was recognized by the negative control serum (data not shown). The

1 specific recognition of the Mhp366 epitope by convalescent serum but not by hyperimmune serum
2 led us to examine its properties in more detail.

3 The *mhp366* gene has a length of 1,665 bp (555 amino acid residues), starting at position 432,758 in
4 the genome of *M. hyopneumoniae* strain 232 (acc. no. AE017332; (Minion et al., 2004)). It encodes
5 the putative lipoprotein Mhp366 (acc. no. AAV27851) with a calculated molecular mass and IP of
6 64,394 Da and 9.90; respectively. The predicted epitope recognized by the convalescent serum
7 covers the amino acid positions 68 to 88 within the Mhp366 protein
8 (⁶⁸QKENSQKNDVVNSQNKTEKTE⁸⁸), the overlapping sequence of the three peptides which
9 should define the actual B-cell epitope, is located at position 74 to 82 (⁷⁴KNDVVNSQN⁸²).
10 Immediately following this epitope, a region of 4 tandem repeats of the sequence motif [KR]TE
11 starts which has recently been characterized by de Castro et. al. (de Castro et al., 2006).

12

13 3.2. Antigenic specificity of immunogenic Mhp366 protein.

14 The plasmid pMhp366-501 encodes a GST-Mhp366 fusion protein composed of the amino-terminal
15 GST followed by the mature part of Mhp366 starting at Cys²². The purification of the recombinant
16 fusion protein was controlled by SDS-PAGE analysis and Coomassie staining, which confirmed
17 the homogeneity of the protein preparation and the apparent molecular mass of around 90 kDa
18 (Fig. 2A), corresponding to the value calculated from the deduced amino acid sequence
19 (89,869 Da). As expected, the GST-Mhp366 fusion protein and the GST protein were recognized
20 by GST-specific antibodies (Fig. 2B). Using the porcine convalescent serum, the GST-Mhp366
21 fusion protein was clearly recognized and this reaction was solely based on the Mhp366 part of the
22 fusion protein, since the GST protein was not recognized by the convalescent serum (Fig. 2C,
23 lane 1). In contrast, the porcine hyperimmune serum showed only a very weak reaction with the
24 GST-Mhp366 fusion protein (Fig. 2D), thus confirming the results obtained on the peptide spot
25 array. In addition, a Mhp366-specific signal in the expected size of 62 kDa (mature Mhp366
26 protein) could not be detected in total cell lysates of *M. hyopneumoniae* type strains J and 232

1 grown in Friis medium using a polyclonal rabbit serum raised against the GST-Mhp366 fusion
2 protein (Fig. 3B) although a broad spectrum of immunogenic bands was visible when porcine
3 convalescent serum was used (Fig. 3C). Two protein bands, 95 kDa and 45 kDa in size,
4 recognized by the Mhp366-specific polyclonal rabbit serum (Fig. 3B, lanes 1 and 2) were also
5 detected by rabbit pre-immune serum (data not shown) and, therefore, the reaction was
6 considered as non-specific.

7

8 *3.3. Sequence analysis of the immunogenic epitope and adherent VNTR regions in various* 9 *M. hyopneumoniae strains and diagnostic samples*

10 A possible future application of the immunogenic epitope identified in Mhp366 as a diagnostic
11 marker facilitating the differentiation of infected and vaccinated animals required a more
12 detailed examination of its distribution and sequence variations among *M. hyopneumoniae*
13 isolates from different geographic origins. For this purpose, a PCR fragment (800 bp) was amplified
14 using primers oMhp366A / oMhp366B and chromosomal template DNA prepared from a total of
15 fourteen *M. hyopneumoniae* strains from Denmark (11), Belgium (2) and Germany (1) (Table 1).

16 In addition, three porcine lung samples from Ukraine were also analysed directly by nested PCR.
17 The sequencing revealed the presence of *mhp366* homologous genes in all strains and samples
18 tested. The alignment of the obtained amino acid sequences confirmed the presence of the
19 immunogenic epitope whose core sequence is highly conserved, showing only two variations at
20 amino acid position 75 (N/K) and 82 (N/D), respectively (Fig. 4). The VNTR region is also present
21 in all samples. The number of repeats varies between 3 and 5, with most sequences containing 4
22 copies of the [KR]TE motif.

23

24 *3.4. ELISA*

25 The alignment of immunogenic epitopes (Fig. 4) revealed the presence of four different core
26 sequences. Synthetic peptides corresponding to these sequences were synthesized (Table 1) and

1 tested as coating antigens. A porcine convalescent serum pool from five non-vaccinated pigs
2 identified as infected with *M. hyopneumoniae* was used as a positive reference serum. Plates were
3 coated with 0.1 µg/ml of the particular peptides (as determined by checkerboard titration using the
4 porcine convalescent serum) or with an equal mixture of all peptides, each at a concentration of
5 0.025 µg/ml. As shown in Fig. 5a, the four single peptides and the peptide mixture showed very
6 similar reactivity with the convalescent serum pool, and a strong discrimination between positive
7 and negative control serum (pooled serum from 10 non-vaccinated, non-infected pigs).

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

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

26

1 4. Discussion

2

3 Vaccination of piglets against *M. hyopneumoniae* is an important prophylactic measure to control
4 Enzootic Pneumonia. Commercial bacterin vaccines are applied in many countries in more than
5 70 % of the pig herds (Maes et al., 2008); in Germany, it is the most frequently applied vaccination
6 in pigs with more than 85 % of the herds being vaccinated. Although the beneficial effects of
7 vaccination are well documented (Haesebrouck et al., 2004; Maes et al., 2008), the failure of
8 currently used vaccines to prevent colonization (Sibila et al., 2008) or to reduce transmission
9 significantly (Meyns et al., 2004) are major drawbacks. Due to the high vaccination coverage using
10 inactivated whole cells, the detection of ongoing *M. hyopneumoniae* infections in vaccinated herds
11 is strongly impeded. The construction of DIVA marker vaccines, which is based on the absence of
12 one immunogenic protein (van Oirschot, 2001) to bypass this problem, is not possible since
13 *M. hyopneumoniae* is not amenable to genetic manipulation.

14 In order to identify new infection-specific serological markers, we used an *in-silico* approach to
15 predict immunogenic epitopes within putative lipoproteins. In the first step, the genome sequence of
16 strain 232, which is commonly used to study virulence and vaccination regimens of
17 *M. hyopneumoniae* (Minion et al., 2004), was analysed using the programs Lipo (Berven et al.,
18 2006) and LipoP 1.0 (Juncker et al., 2003). A total of 35 ORFs were detected by both programs
19 (Table 2). In a second step, potential linear B-cell epitopes were predicted using the ABCpred
20 program (Saha and Raghava, 2006).

21 The epitope length window for predicted epitopes was set at 20 amino acid residues, corresponding
22 to most linear B-cell epitopes which are 5 to 30 amino acid residues in length (Saha and Raghava,
23 2006). On the peptide array, all predicted epitopes were represented by three overlapping 15-mer
24 peptides initiating at every third amino acid, thereby covering 21 amino acid residues. The
25 immunoblot experiments revealed only one epitope from the Mhp366 protein which was clearly
26 differentially recognized by convalescent and hyperimmune serum, respectively (Fig. 1). Epitopes

1 from at least five additional proteins reacted with both sera, indicating that the selection of a limited
2 number of B-cell epitopes to identify immunogenic proteins was successful.

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

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

16 A microarray-based study to search for transcriptional changes during infection revealed a 2.43 fold
17 up-regulation of the *mhp366* gene in *M. hyopneumoniae* cells collected from bronchial alveolar
18 lavage fluid of infected pigs (Madsen et al., 2008). The values for transcript level differences
19 observed in this study are in general comparably low, but the up-regulation of *mhp366* is the third-
20 highest among all genes of *M. hyopneumoniae* strain 232. These results are in good agreement with
21 our findings. The Mhp366-specific epitope is strongly recognized by convalescent serum, but not
22 by hyperimmune serum (Fig. 1), which was produced using a commercial bacterin vaccine
23 containing inactivated *M. hyopneumoniae* cells. The weak reactivity might be due to low or
24 negligible amounts of Mhp366 protein in *M. hyopneumoniae* cells grown under laboratory
25 conditions as it is strongly supported by the results of our Western blotting experiments.

1 The strong reaction of the GST-Mhp366 fusion protein with the convalescent serum pool and the
2 weak recognition by the hyperimmune serum (Fig. 2C/D) clearly reflects a different concentration
3 of Mhp366-specific antibodies in both sera. For recombinant antigens, it has already been shown
4 that higher antigen levels induce a stronger and more rapid immune response (Himmelrich et al.,
5 2000) and this might also be true for native bacterial antigens. In total cell lysates of both
6 *M. hyopneumoniae* strains J and 232 grown in Friis medium, it was not possible to detect a protein
7 signal using a Mhp366-specific polyclonal rabbit serum (Fig. 3B). Nevertheless, very low amounts
8 of Mhp366 below the detection limit of the rabbit serum might be present even in *in-vitro* grown
9 cells because the hyperimmune serum still shows a weak positive reaction with GST-Mhp366.

10 Based on our initial results, synthetic peptides corresponding to the four different epitope core
11 sequences (Fig. 4) were tested as coating antigens. A strong ability to discriminate between positive
12 and negative reference sera from non-vaccinated infected or healthy pigs, respectively, could be
13 demonstrated (Fig. 5a). The sequence variations observed in *M. hyopneumoniae* strains and
14 diagnostic samples did not influence the recognition, since all four peptides showed a similar
15 reactivity (Fig. 5a). The examination of 80 field serum samples supported our hypothesis. Thus,
16 whereas 17 samples were recognized as positive by two commercial ELISA kits, only four samples
17 showed an ELISA activity ≥ 100 OD% compared to the positive reference serum pool on our
18 peptide based ELISA. The lower number of pigs reacting in the Mhp366 antigen ELISA most likely
19 mirrors the absence of a prime-boost effect due to vaccination with respect to this antigen. This is
20 supported by the gap observed in the range of ELISA results. None of the samples ranged from 70
21 to 100 OD%, the vast majority of samples ranged from 10 to 40 OD%. Most likely, this large group
22 represents background levels caused by vaccination or unspecific reactions. To support this
23 hypothesis, a more detailed examination of hyperimmune sera raised against different commercial
24 vaccines, as well as larger numbers of field serum samples obtained from herds with a defined
25 *M. hyopneumoniae* vaccination status would be necessary. These experiments should not only
26 confirm our present results but also allow us to define a threshold value to discriminate between

1 background levels and positive signals. Additional experiments will also be necessary to
2 characterise the expression pattern of the *mhp366* gene *in-vivo* and *in-vitro* and the time course of
3 Mhp366-specific antibody formation during infection to support our hypothesis, that Mhp366 might
4 be the first antigen identified to facilitate the detection of flourishing *M. hyopneumoniae* infections
5 even in vaccinated herds.

6 7 **Acknowledgments**

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9 Haesebrouck, Ghent, and Dr. B. Kokotovic, Kopenhagen, for providing *M. hyopneumoniae* strains.
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11

12

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2

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- 21
22

1 **Legends to figures**

2

3 Fig. 1

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

8

9 Fig. 2

10 Total cell lysates of *E. coli* DH5 α /pGex5x2 (lane 1) and *E. coli* DH5 α /pMhp366-501 (lane 2) as
11 well as the purified GST-Mhp366 fusion protein (Lane 3 / 2 μ g per lane) were separated on a
12 10.8% SDS-PAGE and stained with Coomassie blue (A) or blotted onto nitrocellulose
13 membranes. Western blots were developed using polyclonal rabbit anti-GST serum (B; 1:1000)
14 and porcine convalescent (C; 1:250) or hyperimmune (D; 1:250) sera. M, molecular mass
15 standard.

16

17 Fig. 3

18 Total cell lysates of *M. hyopneumoniae* type strains J (lane 1), and 232 (lane 2) and the purified
19 GST-Mhp366 fusion protein (Lane 3 / 2 μ g per lane) were separated on 10.8% SDS-PAGE and
20 stained with Coomassie blue (A) or blotted onto nitrocellulose membranes. Western blots were
21 developed using polyclonal rabbit anti-Mhp366 serum (B; 1:1000) and porcine convalescent (C;
22 1:250) serum. M, molecular mass standard.

23

24 Fig. 4

25 Alignment of partial amino acid sequences of Mhp366-homologous proteins from different
26 *M. hyopneumoniae* strains and porcine lung samples (VB-1A/2A/3A). The predicted B-cell epitope

1 is written in bold letters. The sequences of *M. hyopneumoniae* strains J (AE017243), 232
2 (AE017332), 7448 (AE017244), PMS (DQ386461) and 7442 (DQ386460) were obtained from
3 GenBank.

4

5 Fig. 5

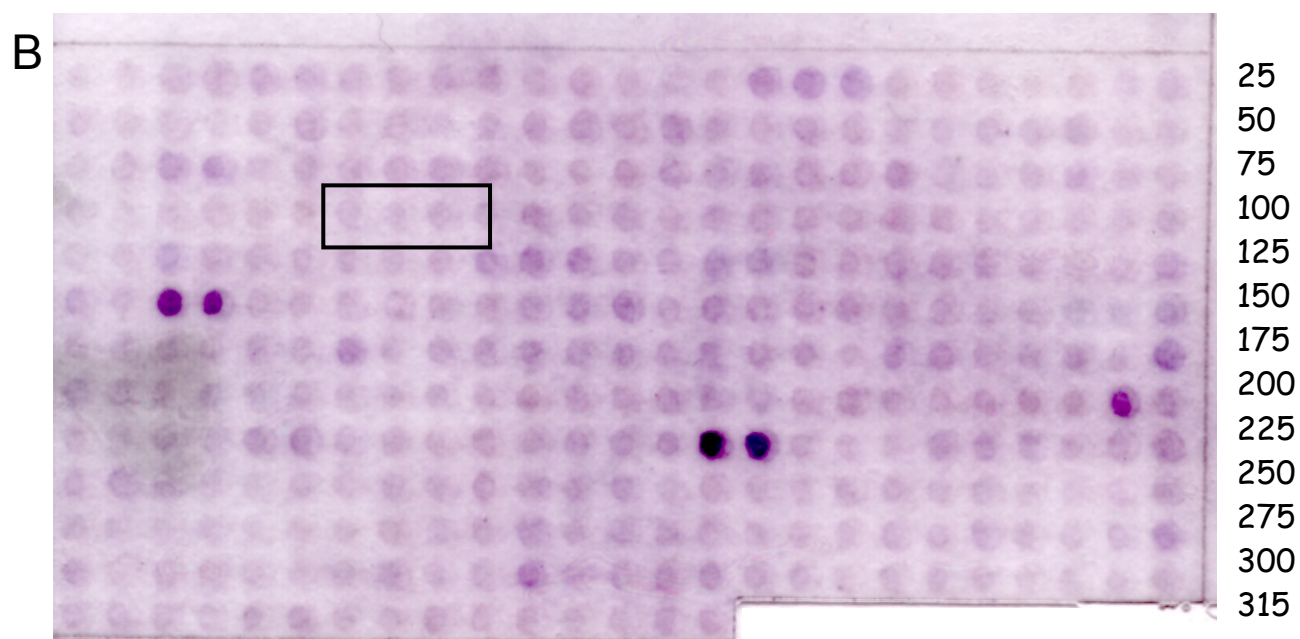
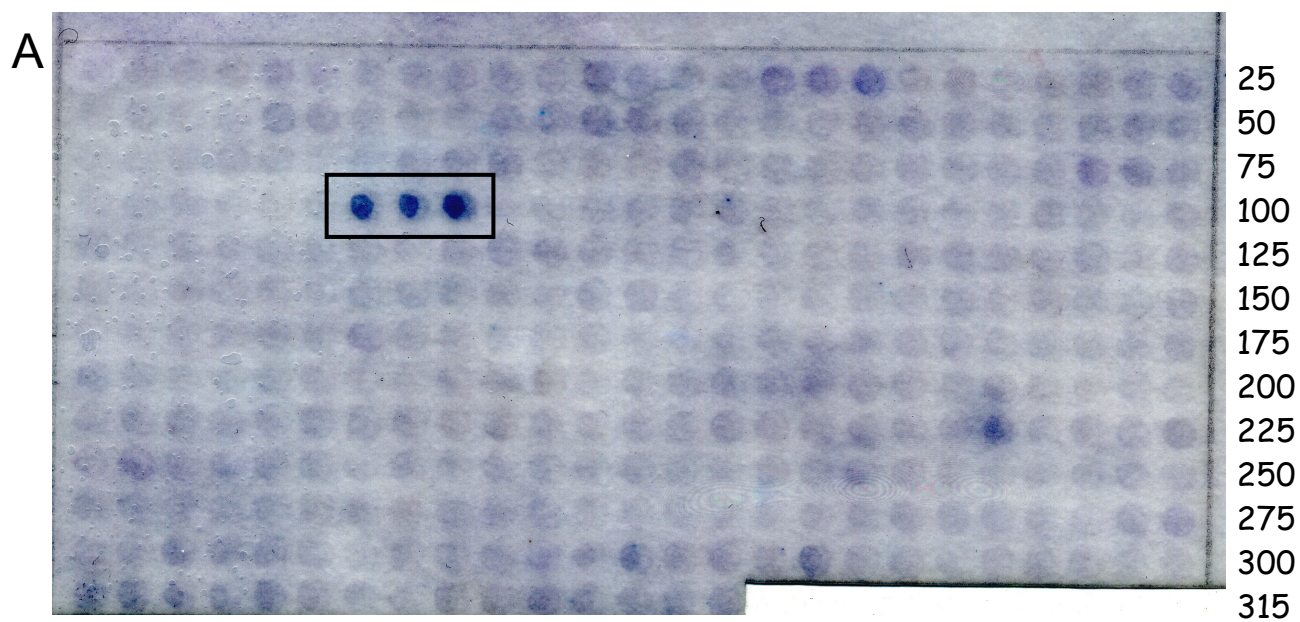
6 ELISA results using epitope-specific synthetic peptides. Reactivity of four single synthetic peptides
7 (366-A/D) with a positive convalescent serum pool and comparison of an equal mixture of all four
8 peptides with positive and negative control serum (pooled serum from 10 non-vaccinated, non-
9 infected pigs) (A). Field serum samples tested on ELISA plates coated with the peptide mixture.
10 Results were given as OD% with respect to the positive reference serum pool and grouped into
11 classes of 10 OD% (B). Peptides were coated on Nunc Immobilizer Streptavidin F96 microtiter
12 plates.

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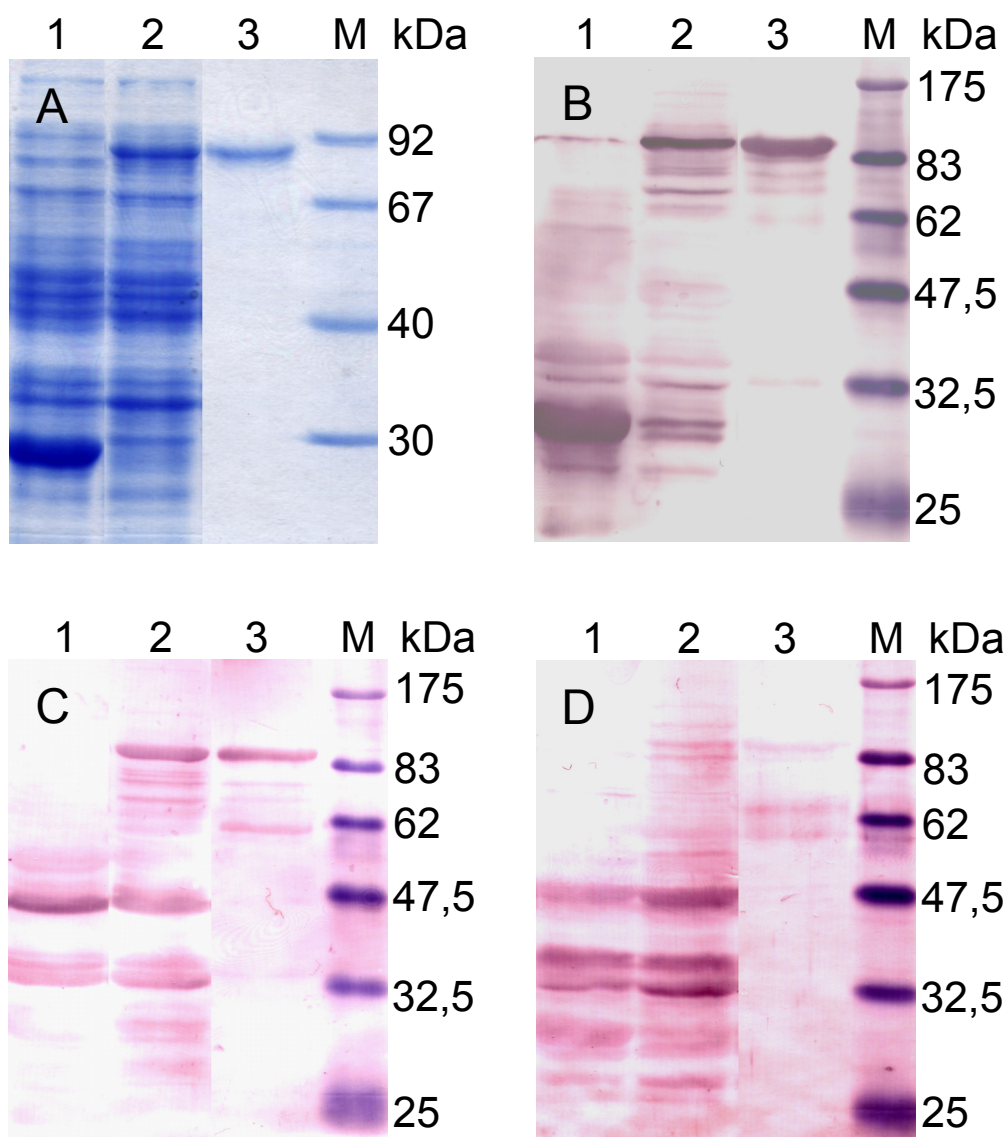


Figure 3

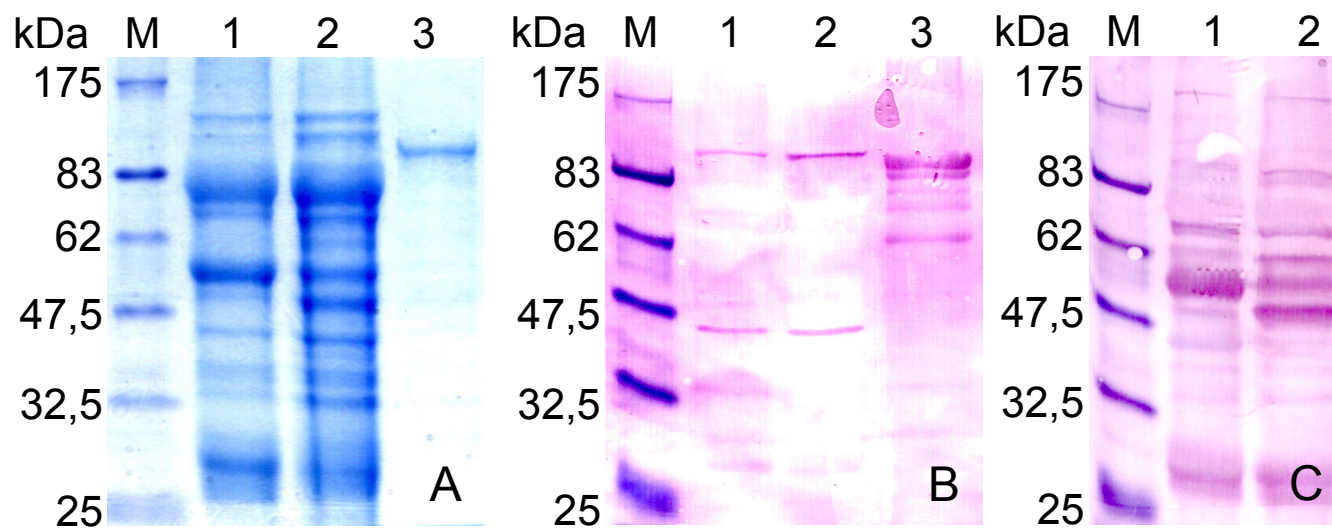
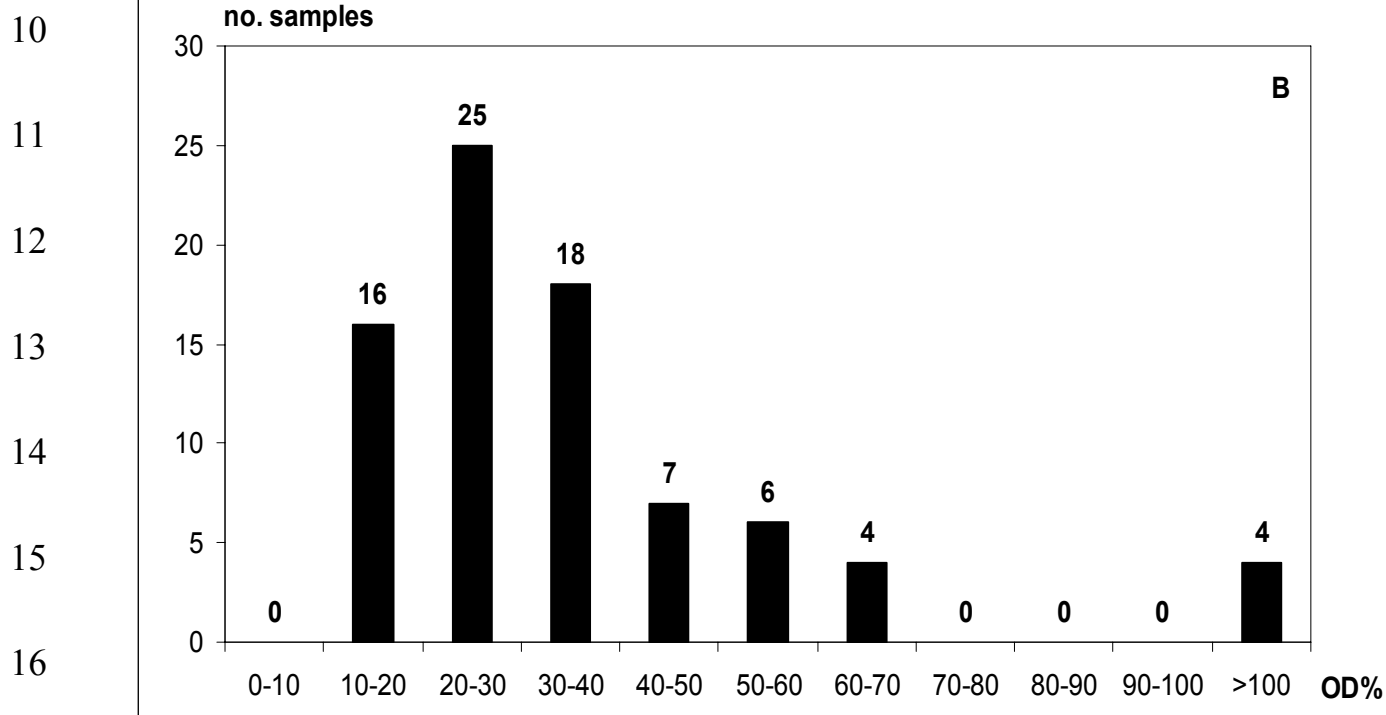
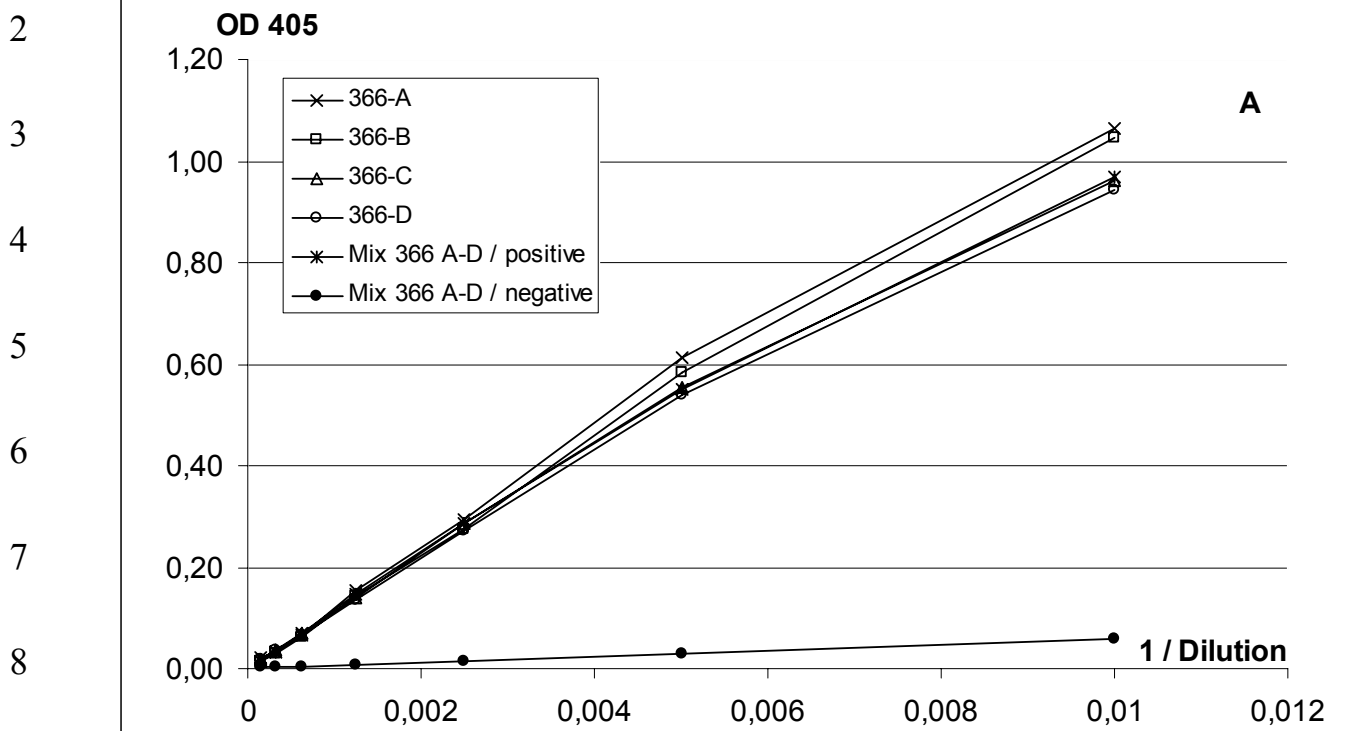


Figure 4

Peptid	Sequence	Repeats
Peptid 82	QKENSQKNDVVNSQN	
Peptid 83	NSQKNDVVNSQNKTE	
Peptid 84	KNDVVNSQNKTEKTE	
232	PTKESKVSPVEENTQKENSQKNDVVNSQNKTEKTEKTEQOQTE----NQTTDNNFWSEST	4
J	PTKESKVSPVEENT-----QKKDVVNSQNKTEKTERTE-----NNQTDSNFWSEST	3
7448	PTKESKVSPVEKNT-----QKKDVVNSQNKTEKTEKTEKTERTE-NQTTDNNFWSDEST	5
PMS	PTKESKVSPVEENT-----QKKDVVNSQNKTEKTERTE-----NQTTDSNFWSEST	3
7442	PTKESKVSPVEENT-----QKKDVVNSQNKTDKTERTE-----NQTTDSNFWSEST	3
Mp18	PTKESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp47	PTKESKVSPVEENT-----QKKDVVNSQNKTEKTERTE-----NNQTDSNFWSEST	3
Mp73	PTNESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp88	PTKESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp182	PTNESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp195	PTKESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp263	PTKESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp473	PTNESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp529	PTKESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp612	PTKESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mp654	PTKESKVSPVEENT-----QKNDVVNSQDKTEKTEKTERTE---NNQTDSNFWSEST	4
Mhyop3873	PTKESKVSPVEKNT-----QKKDVVNSQNKTEKTEKTERTE----NQTTDNNFWSDEST	4
MhyopB2V2W20	PTKESKVSPVEKNT-----QKKDVVNSQNKTEKTEKTERTE----NQTTDNNFWSDEST	4
MhyopOef91	PTKESKVSPVEKNT-----QKKDVVNSQNKTEKTEKTERTE----NQTTDNNFWSDEST	4
VB-1A	PTKESKVGPEENT-----QKNDVINSQNKTERTEKTERTE---NNQTDSNFWSEST	4
VB-2A	PTKESKVGPEENT-----QKNDVINSQNKTERTEKTERTE---NNQTDSNFWSEST	4
VB-3A	PTKESKVGPEENT-----QKNDVINSQNKTERTEKTERTE---NNQTDSNFWSEST	4
	** :***** :** ** :***** :** :*:**: *****.*****:**	

1 Fig. 5



1 Table 1 Bacterial strains, plasmids, primers and peptides used in this study

	Characteristics	Reference or source
Strains and plasmids		
<i>M. hyopneumoniae</i>	<i>M. hyopneumoniae</i> strain J	ATCC 25934
	<i>M. hyopneumoniae</i> strain 232	provided by D. Maes, Ghent
	<i>M. hyopneumoniae</i> field strains Mp18, Mp47, Mp73, Mp88, Mp182, Mp195, Mp263, Mp473, Mp529, Mp612, Mp654	provided by B. Kokotovic, Copenhagen
	<i>M. hyopneumoniae</i> field strains Mhyop.B2V2W20 , Mhyop.Oef91	provided by D. Maes, Ghent
	<i>M. hyopneumoniae</i> field strain Mhyop.3873	Collection of the Institute for Microbiology, Hannover
<i>E. coli</i> DH5 α F'	F' <i>endA1 hsdR17</i> (<i>r_K⁻ m_K⁻ supE44 thi-1 recA1 gyrA</i> (Nal ^r <i>relA1</i> Δ (<i>lacZYA-argF</i>))U169 <i>deoR</i> [ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15]	(Raleigh et al., 1989)
pGex5x2	<i>E. coli</i> cloning vectors carrying an ampicillin resistance determinant, the <i>lacI^q</i> gene and the glutathione S-transferase gene	Amersham Biosciences
pMph366-501	pGex5x3 containing the N-terminal truncated <i>mhp366</i> gene, Pos. 70-1665	This work

Primers		
oMhp366A	5'-CCG <u>CCCCGGGG</u> GCTGTA CTTATAAAAATTGAATCTAGC -3' (downstream primer binding at position 70 to 87 in <i>mhp366</i> ; SmaI recognition site underlined)	This work
oMhp366B	5'-ATCTGCGGCGCG <u>CGTCTC</u> GACTTTC CAAATGGGCCACCGTTATG -3' (upstream primer binding at position 817 to 834 in <i>mhp366</i> ; BsmBI recognition site underlined)	This work
oMhp366C	5'-AGGACGTCTCAAAGTAATGAATACCAA AAATTA -3' (downstream primer binding at position 838 to 859 in <i>mhp366</i> ; BsmBI recognition site underlined)	This work
oMhp366D	5'-GGC <u>CGTCTC</u> GCCATTTTATTTCCCAAAGACGAAAAAGAATCCG-3' (upstream primer binding at position 937 to 970 in <i>mhp366</i> ; BsmBI recognition site underlined)	This work
oMhp366E	5'-GGC <u>CGTCTC</u> AAATGGCTCAAAGATAATGGCAAAGA ACTTTTCCCGG -3' (downstream primer binding at position 965 to 1000 in <i>mhp366</i> ; BsmBI recognition site underlined)	This work
oMhp366F	5'-CCTTC <u>GTCTCT</u> GTTTAAATTTTGA AATTTTCCCAATATTTTAAATTCATTA AAATTTG-3' (upstream primer binding at position 1238 to 1290 in <i>mhp366</i> ; BsmBI recognition site underlined)	This work
oMhp366G	5'-GGAACGTCTCAAACCAACTAAAATTTCTGAACAAAGTTGGGATGATCAAGACTTTGATTATACAACAAA-3' (downstream primer binding at position 1285 to 1344 in <i>mhp366</i> ; BsmBI recognition site underlined)	This work
oMhp366H	5'-TTCTGCGGCGCGTTCCTTTTTTAAATAAACTTTATTGATTTG-3' (upstream primer binding at position 1636 to 1665 in <i>mhp366</i> ; NotI recognition site underlined)	This work

Peptides		
366-A	K NDVVNSQ N KTEKTE (amino acids varying between peptides are in bold letters)	This work
366-B	KK DVVNSQ N KTEKTE (amino acids varying between peptides are in bold letters)	This work
366-C	KK DVVNSQ N K T D K TE (amino acids varying between peptides are in bold letters)	This work
366-D	K NDVVNSQ D KTEKTE (amino acids varying between peptides are in bold letters)	This work

1

1 Table 2 Putative lipoproteins identified within the genome of *M. hyopneumoniae* type strain 232

Mh Gene	Strand	Start	Finish	AA	Mol.Weight	pI	Description
mhp037	+	47479	49488	670	78283	10.48	hypothetical protein
mhp164	-	195113	192438	892	101902	9.49	conserved hypothetical protein
mhp170	-	207565	206516	350	41382	9.22	hypothetical protein
mhp268	-	294598	293372	409	49094	9.74	conserved hypothetical protein
mhp293	+	334609	336078	490	58040	8.76	hypothetical protein
mhp312	+	357410	358159	250	29029	10.38	hypothetical protein
mhp321	+	369714	375638	1975	228056	9.79	hypothetical protein
mhp336	-	394824	393355	490	58123	9.74	hypothetical protein
mhp364	+	430738	432333	532	61225	8.25	conserved hypothetical protein
mhp366	-	434422	432758	555	64394	9.90	conserved hypothetical protein
mhp367	-	436110	434539	524	61399	7.60	conserved hypothetical protein
mhp371	+	440806	442068	421	48246	9.37	protein p37 precursor
mhp377	+	447568	449778	737	86449	9.69	putative lipoprotein
mhp378	+	449787	451910	708	80477	10.00	putative lipoprotein
mhp379	+	451940	452869	310	36013	9.90	putative lipoprotein
mhp390	-	469458	467647	604	68407	9.37	putative lipoprotein B precursor
mhp413	-	491140	490616	175	20292	9.19	hypothetical protein
mhp424	-	503969	502824	382	43899	9.28	conserved hypothetical protein
mhp436	-	517300	515393	636	74191	6.24	conserved hypothetical protein
mhp445	-	527462	525624	613	71098	5.57	conserved hypothetical protein
mhp465	-	576942	576004	313	37487	10.13	conserved hypothetical protein
mhp466	-	579010	576950	687	82333	6.49	conserved hypothetical protein
mhp502	-	633310	630497	938	107289	9.76	conserved hypothetical protein
mhp511	+	642797	644053	419	46347	9.18	46kd surface antigen precursor
mhp535	-	672303	670630	558	65896	8.99	hypothetical protein
mhp555	-	702834	702001	278	31521	9.56	conserved hypothetical protein
mhp597	-	747519	746389	377	42587	10.09	membrane nuclease A
mhp623	+	778502	779854	451	49776	8.30	conserved hypothetical protein

mhp640	+	805652	808117	822	93936	9.07	putative membrane lipoprotein
mhp650	-	822291	820486	602	69033	9.91	conserved hypothetical protein
mhp651	-	824498	822357	714	80935	6.74	5'-nucleotidase
mhp677	+	850938	852818	627	71128	8.69	surface lipoprotein
mhp681	+	857326	859008	561	65534	10.17	conserved hypothetical protein
mhp682	+	859075	860637	521	59625	5.83	hypothetical protein
mhp695	-	887557	886481	359	41969	9.91	hypothetical protein

1

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

Peptide No. on Spot Array	Gene / Epitope	Peptide Sequence
1	mhp037/1	KPQKPKKIEKKAINK
2		KPKKIEKKAINKFDI
3		KIEKKAINKFDISKL
4	mhp037/2	PMWTFFGYPFILFYF
5		TFFGYPFILFYFNNR
6		GYPFILFYFNNRINF
7	mhp037/3	PRHIAAKNMFKGVYI
8		IAAKNMFKGVYIRGQ
9		KNMFKGVYIRGQEAT
10	mhp164/1	LNLPGISFELYKTSL
11		PGISFELYKTSLPSS
12		SFELYKTSLPSSSGN
13	mhp164/2	GTDLSKFMTNGPFDI
14		LSKFMTNGPFDITEI
15		FMTNGPFDITEINWG
16	mhp164/3	TQNKVFDFIVIFEKI
17		KVFDFIVIFEKIIRE
18		DFIVIFEKIIREFVP
19	mhp170/1	AMQLKWFLNKSSLID
20		LKWFLNKSSLIDLSQ
21		FLNKSSLIDLSQEEK
22	mhp170/2	NNKNWRIKAPNQKPV
23		NWRIKAPNQKPVTL
24		IKAPNQKPVTLRLH
25	mhp170/3	EPFIRLVSQSFAIPF
26		IRLVSQSFAIPFDGE
27		VSQSFAIPFDGEITD
28	mhp268/1	EVIVKFFNTLENKDN
29		VKFFNTLENKDNFEK
30		FNTLENKDNFEKKEQ
31	mhp268/2	YLFLEEKFINEEKQW
32		LEEKFINEEKQWNPV
33		KFINEEKQWNPVIKL
34	mhp268/3	DLRVNQLAFNKFFS
35		VNNQLAFNKFFSFFT
36		QLAFNKFFSFFTKNL
37	mhp293/1	EFEDKFLKGELLEKV
38		DKFLKGELLEKVLEK
39		LKGELLEKVLEKNNI
40	mhp293/2	KISTSSPTFMFNIID
41		TSSPTFMFNIIDFSQ
42		PTFMFNIIDFSQEIN
43	mhp293/3	FDKKVSRLELIKDLK
44		KVSRLELIKDLKNNY