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Serodiagnosis of *Mycobacterium avium* infections in pigs

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Abstract

The aim of this study is the development and evaluation of a serodiagnostic assay for *Mycobacterium avium* (MA). After screening MA lipid fractions in an ELISA format, a polar lipid fraction was selected as antigen because of its superior recognition by serum antibodies in experimentally infected pigs. The resulting MA-ELISA was evaluated as an alternative for detection of MA infection by traditional pathological examination of pig lymph nodes for granulomatous lesions by meat inspectors. By comparing with bacteriological examination, the MA-ELISA showed significantly better sensitivity (69%) as compared to pathological examination (31%) in experimentally infected pigs. The MA-ELISA also appeared significantly more specific in a set of serum samples from MA negative pigs: only 1 out of these 153 serum samples reacted positive, whereas 99 (65%) of these had displayed false positive results by detection of lymph nodes lesions that appeared not to be associated with MA (Komijn et al., 2007).

The MA-ELISA was subsequently evaluated using serum samples from two farms with pigs known to be infected with MA. Bacteriological examination of the sub-maxillary and mesenteric lymph nodes showed that 56% (103/184) and 35% (41/117) of the pigs, respectively were positive for MA in these farms. In the first farm, 16% (29/184) of the pigs tested positive in MA-ELISA and 31% (57/184) by pathological examination. On the contrary, in the second farm, more pigs tested positive 17% (15/117) in MA-ELISA with 8% (9/117) positivity by pathological examination. Taking the results on both farms together, the sensitivity of the MA-ELISA was 14% and the specificity 83%, whereas the sensitivity of the pathological examination was 31% and the specificity 86%.

For practical reasons use of a serological test as the MA-ELISA may be preferred over pathological or bacteriological examination. Our studies in experimentally infected and negative “field“ sera indicate that the MA-ELISA is significantly more specific and more sensitive than detection by classical pathological examination. However, the studies in two MA infected farms show a variable picture with pathological examination overall performing better. Study in a wider range of “positive” farms will be needed to provide a more comprehensive view of the quality of both tests for detection of MA in infected farms. At the same time further optimization of MA-ELISA with use of lipid antigens from a broader range of serotypes may improve its performance in the face of infections with different MA serotypes.

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1. Introduction

Mycobacterium avium (MA) is an important pathogen in both animals and humans which belongs to the *Mycobacterium avium* complex (MAC). To date, 28 MAC serotypes have been identified from which the serotypes 1-6, 8-11 and 21 belong to *Mycobacterium avium* subsp. *avium* (MAA). The serotypes 1, 2 and 3 are isolated from birds and have been designated as the MAA "bird" serotypes whereas the serotypes 4-6, 8-11 and 21 are identified as the "non-bird" serotypes (Dvorska et al., 2004). The "non-bird" serotypes are isolated from humans and pigs and for these reasons Mijs et al., (2002) proposed to name these serotypes *M. avium* subsp. *hominissuis* (MAH). These "non-bird" serotypes are also found in the environment, especially in soil (Dvorska et al., 2004).

MA is a potential zoonotic pathogen as it can produce disease in immunocompromised individuals with acquired immunodeficiency syndrome and pulmonary disease in immunocompetent adults (Biet et al., 2005; Wagner and Young, 2004). An infection with MA in pigs develops mostly subclinically leading to granulomatous lesions in the lymph nodes. Most of the lesions are formed in the sub-maxillary and mesenteric lymph nodes and disseminated lesions are occasionally observed in the liver, spleen and lungs (Thoen, 1992).

Sources of MA infections in humans are still uncertain. Results of high resolution genotyping methods showed that humans and pigs can be infected with the same MAC strains (Komijn et al., 1999). The authors discussed the possibilities that humans and pigs share common sources of infection or that contaminated meat originating from pigs is a potential source for infection in susceptible humans. To exclude pigs as a reservoir for infection in man, regulations in European Union legislation (2004/854/EC) are prescribed for special handling of pig carcasses in slaughter houses in which evidence of an MA infection is observed.

Diagnosis of MA infections in pigs is based on detection of granulomatous lesions in lymph nodes of pigs by palpation and incision at slaughter (Brown and Neuman, 1979; Komijn et al., 2007). Because MA has been isolated from lymph nodes without lesions, and, on the other hand, non mycobacterial species such as *Rhodococcus equi* have been frequently isolated from granulomatous lesions, sensitivity and specificity of the current detection method have been questioned (Brown and Neuman, 1979; Dvorska et al., 1999; Komijn et al., 2007; Offermann et al., 1999). In this study, we describe a serodiagnostic test that may provide a more reliable tool for detecting MA infected pig herds.

Based on earlier observations that indicate that MA-derived lipid preparations contain dominant antigens of MA, we focused on the applicability of antigenic lipid fractions for the development of an antibody

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based immunodiagnostic test for detection of MA infections in 26-weeks old slaughter pigs (Brennan et al., 1981; Inderlied et al., 1993; Kitada et al., 2002, 2005). Using serum samples of pigs that were experimentally infected with MA, lipid fractions of MA were evaluated for applicability, and a polar lipid fraction was selected for developing an ELISA for MA. The ELISA was validated using serum samples from pigs, experimentally and naturally infected with MA. Bacteriological examination of the lymph nodes for MA was used as reference to indicate the true infection status and the outcome of the ELISA was compared to the traditional method of detecting lesions in lymph nodes (Komijn et al., 2007).

2. Materials and Methods

2.1 Bacterial strain

In this study MAH serotype 4 strain 17404 was used. This strain was isolated in the course of a study to the prevalence of MA in Dutch pig herds and it was selected as a representative of one of the most frequently isolated serotypes in the Netherlands (Komijn et al., 1999).

2.2 Experimental infection with MA

Thirty-two pigs, three weeks of age and crossbreeds of Yorkshire and Dutch landrace were obtained from a high health status herd of the Animal Sciences Group of Wageningen UR (Lelystad, The Netherlands). Pigs were allotted in four groups of eight pigs each and housed in separate boxes at the animal facilities of Central Veterinary Institute of Wageningen UR (Lelystad, The Netherlands).

For preparation of the inoculum for the experimental infection, MAH strain 17404 was cultured in Middlebrook 7H9 medium enriched with OADC (Becton Dickinson, Breda, The Netherlands) at 35°C to an Optical Density (OD) of 2 at 600 nm. The cells were harvested by centrifugation at 3000 x g for 30 minutes and washed twice in isotonic saline. Until use, aliquots of one ml were stored in Tryptone Soya Broth medium (Oxoid B.V., Badhoevedorp, The Netherlands) in the presence of 20% glycerol at – 70 °C. Viability of the inoculum was determined by subculture on Coletsos Osein (Bio-Rad, Veenendaal, The Netherlands) and Dubos Tween albumin medium (Becton Dickinson, Breda, The Netherlands). Purity of the inoculum was determined from smear specimens stained by Ziehl-Neelsen. Prior to administration to the pigs the stored MAH bacteria were revived by culturing one ml of the stored inoculum in four ml Middlebrook 7H10 supplemented with OADC for two days at 37 °C.

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To study the influence of an early and late infection with MA on bacteriological, serological and pathological status we infected pigs at different ages. Three of the four groups were infected at 2½, 4½ and 18 weeks of age, respectively. To increase the probability of infection, pigs in the fourth group were inoculated with MAH at three time points (Table 1). A syringe was used to deposite the inoculum in the caudal area of the pharynx. To promote the penetration of the bacteria, the pharynx epithelial tissue was scarified with a cotton-wool swab before inoculation.

Until the end of the experiment at 24 weeks of age, pigs were monitored once a week for clinical signs of disease. Furthermore, blood serum samples were collected weekly to monitor the immune response. White blood cells were counted, using a semi-cell bloodcounter (Sysmex, model F 800, Charles Goffin Medical Systems). The number of neutrophils was calculated after a differential count of Giemsa-stained blood smears. Three days before the end of the experiment, all pigs underwent an intradermal tuberculin test in the ear with 0.1 ml Avian Tuberculin PPD (25.000 I.U., ASG, Lelystad, The Netherlands). The presence at the injection site of erythema and induration was scored 24, 48 and 72 hours after injection. After reading, pigs were euthanized. Organs were examined pathologically for deviations and lymph nodes were examined pathologically for granulomatous lesions and bacteriologically for MA. For bacteriological examinations, the surface of the lymph node specimens was decontaminated for 6 to 8 seconds in boiling water and cut aseptically into small pieces of 1 cm^3. The specimens were sealed in polyethylene bags and squeezed in a Stomacher (Laméris Laboratorium B.V., Breukelen, The Netherlands). This material was treated for 10 min at room temperature with 3 volume parts of 6% H_2SO_4 and subsequently centrifugated for 10 min at 1000 g. The supernatant was discarded and the sediment was washed twice with an excess of phosphate-buffered saline (PBS), 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 2.79 KH_2PO_4 and resuspended in 0.5 ml PBS. This suspension was inoculated on Middlebrook 7H10 plates enriched with OADC, on Coletsos Osein and on Dubos Tween albumin medium, incubated for eight weeks at 35 °C and observed every week for growth. To identify colonies, Ziehl-Neelsen stain was performed for acid-fast bacilli and PCRs for the presence of IS*1245* and IS*901*, which are characteristic for MA and MAA respectively (Kunze et al., 1992; van Soolingen et al., 1998).

2.3 Collection of pig serum samples in the field

Field serum samples of pigs with a different MA health status were collected. Serum samples negative for infections with MA (n=153) were collected in the course of a previous study for the prevalence of MA infections in the Netherlands. The design and results of this study were described by Komijn et al. (2007).

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In order to obtain positive field serum samples in the period October 2006 till June 2008 two herds (A and B) were selected where recently cases of MAH infections were detected in slaughter pigs. Both farms were farrow-to-finish farms. From farm A four batches of pigs were sampled in the slaughterhouse (n=184) and from farm B two batches (n=117). Blood serum samples and the sub-maxillary and mesenteric lymph nodes were collected at slaughter. In the laboratory, serum samples were stored at -20 °C and the lymph nodes were examined pathologically for granulomatous lesions and bacteriologically for MA as described above.

2.4 Isolation of lipid fractions from MAH

In order to harvest lipids as antigen for the serodiagnostic ELISA assay, MAH strain 17404 was inoculated in Middlebrook 7H9 supplemented with OADC (Becton Dickinson, Breda, The Netherlands). The growth of MA was monitored by measuring weekly the OD at 600 nm in a spectrophotometer. The incubation was stopped at an OD of 1 and the MA cells were pelletted by centrifugation (15 min 5000g). Lipids were extracted from the MA cells according to a protocol of Dobson et al., (1985). Briefly, the apolar lipids were extracted from MA cells by mixing these cells with methanol and petroleumether. The mixture was centrifuged, the upper layer with apolar lipids was harvested and stored. The lower layer was treated with petroleumether to extract remaining apolar lipids. After centrifugation, the upper layer containing a second yield of apolar lipids was harvested and combined with the already stored apolar fraction. The combined fractions of apolar lipids were dried under a gentle flow of nitrogen. The polar lipids were further extracted by adding a mixture of chloroform and methanol to the lower layer. The mixture was mixed during one hour and centrifuged. Supernatant was mixed with chloroform for 5 minutes and centrifuged. The lower layer containing the polar lipids was dried under a gentle flow of nitrogen.

2.5 Selection of lipid fraction for ELISA

Polar and apolar lipid fractions of MAH strain 17404 were tested in indirect ELISA assays for selection of the most promising fraction for use. Each well of polysorb microtiterplates (Nunc, Roskilde, Denmark) was coated for 16 hours at 37°C with 1 µg polar or apolar lipids suspended in 50mM $NaHCO_3$, PH 9.6. Endpoint serum samples of eight pigs, 21½ weeks after an experimental infection with MAH strain 17404 (see above and Table 1 group 1) were tested in a twofold serial dilutions from 1:40 to 1:81920 in PBS containing 0.5% Tween 80. As conjugate monoclonal antibody IgL 27.2.1 was used. This monoclonal

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antibody recognizes the kappa chains of pig immunoglobulins (Sinkora et al., 2001). As substrate TMB (3, 3', 5, 5'-tetramethylbenzidine) was used. Development of the reaction was stopped by adding 0.5 M H_2SO_4. The OD was determined at 450 nm in an ELISA reader. Titers were expressed as the reciprocal of the ^{2}log of the highest dilution showing an absorbance of more than 50% of the positive control.

2.6 Execution of the ELISA

For evaluation of the ELISA basically the test was performed as described above. As positive control, endpoint serum samples of a pig, 21½ weeks after an experimental infection with MAH strain 17404 was used (see above and in Table 1). As negative control, a serum sample was used from a pig negative in bacteriological examination for MA in its sub-maxillary and mesenteric lymph nodes (Komijn et al., 2007). In each microtiterplate these positive and negative control serum samples were tested in twofold in a dilution of 1:200. Test results were calculated as percentage positivity (PP). To calculate the PP, the OD of the test serum samples was calculated as a percentage of the difference in OD of the negative and positive control serum samples. This results in a uniform and continuous scale from 0-100% calculated according to the following formula: PP = {OD (test sample) – OD (negative control)} / {OD (positive control) – OD (negative control)}.

2.7 Statistical analysis

The cut-off value of the MA-ELISA was determined as the mean plus three-fold standard deviation of the results on a set of serum samples (n=153) negative in bacteriological examination for MA (Komijn et al., 2007). Sensitivity and specificity with 95% confidence intervals (CI) from the MA-ELISA and from the pathological examination of the sub-maxillary lymph nodes were calculated considering the results of bacteriological examination of lymph nodes for MA as a gold standard. McNemar's test was used as a significance test for differences in sensitivity and specificity of both tests. *P* values below 0.05 were considered statistically significant.

3. Results

3.1 Development of MA-ELISA

We started the development of the MA-ELISA by selection of the most promising lipid fraction for use in an ELISA. For this purpose we used serum samples of eight pigs, collected 21½ weeks after an

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experimental infection with MAH. All these serum samples displayed a higher titer against the polar lipid fraction (range 290-1577) as compared to the apolar lipid fraction (range 20-250). Therefore the polar lipid fraction was selected for further use in an MA-ELISA.

Checkerboard titrations using a concentration range of the same set of serum samples and of the polar lipid fraction from MAH strain 17404 resulted in the selection of an optimal concentration of 2.4 µg/well of polar lipid and a dilution of 1:200 of pig serum samples for the MA-ELISA.

The development of the MA-ELISA was finalized with the determination of a cut-off value for negative and positive test results. For this purpose we tested serum samples of pigs (n=153) free of an MA infection (Komijn et al., 2007) in the MA-ELISA. The PP in 80 (52.3%) of these serum samples, was lower than 0% (Table 2). The highest titer detected in these 153 serum samples was 16 PP. The mean and standard deviation of the ELISA for these serum samples was 0.26 PP and 3.45 PP, respectively. The mean plus three standard deviations for the assay: 10.6 PP, was used as the cut-off value for the MA-ELISA.

3.2 Experimental infection with MAH

To evaluate the serodiagnostic ELISA test as an alternative for an inspection of lymph nodes by eye at slaughter we infected pigs experimentally with MAH strain 17404 and examined the correlation between the presence of MAH bacteria, the formation of lesions in lymph nodes of pigs and the antibody response. Four groups of eight pigs were infected, one group at 2½ weeks of age, one group at 4½ weeks of age, one at 18 weeks of age, and one group at all three time points (2½, 4½ and 18 weeks of age). The experiment was terminated when all pigs were 24 weeks old. During the experimental phase the pigs showed no clinical disease symptoms and their leucocyte counts were normal (results not shown). In tuberculin skin tests three days before necropsy 31 out of 32 pigs reacted positive; the skin of the ear at the site of injection showed redness and focal swelling. Size of reactions 72 hours after injection varied between 10 and 20 mm. Pigs were subsequently euthanized and from the results of the bacteriological examination all 32 pigs appeared to be infected with MAH. Irrespective of the age at which pigs were infected, MAH bacteria were detected in one or more of the lymph nodes, most frequently in the sub-maxillary and mesenteric lymph nodes (Table 3). Corresponding to the results of the bacteriological examination of the lymph nodes, pathological examination showed that most frequently the sub-maxillary and mesenteric lymph nodes were affected. However, bacteriological examination gave more positive results as compared to pathological examination. Sixteen (50%) pigs had granulomatous lesions in one or more of the lymph nodes and 10 (31%) of the pigs in the sub-maxillary lymph nodes (Table 3). Results of

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the MA-ELISA on longitudinally serum samples obtained during the experimental phase showed that titers (in PP) were low at the time of infection and started to increase 6 to 8 weeks after infection. At 10-12 weeks after infection the mean PP of groups of pigs infected at an age of 2½ and 4½ weeks was maximal. In serum samples of pigs infected at three time points (2½ , 4½ and 18 weeks of age) titers increased further and at necropsy, 21½ weeks after infection these titers were at a maximum (Figure 1). At that timepoint 69% of the pigs (22/32) were serologically positive (Table 4). Taken together, the sensitivity of the pathological examination as performed at slaughter was 31% and of the serological examination 69%. According to McNemar's test this difference in sensitivity was significant (p = 0.01). We determined whether the length of the infection period influenced the formation of granulomatous lesions and the anti MA antibody response. In groups of pigs infected at 2½ and 4½ weeks of age, 7 out of 8 pigs showed granulomatous lesions in their lymph nodes at necropsy when pigs were 24 weeks of age. No lesions were detected in lymph nodes of pigs infected at 18 weeks of age when they were slaughtered 6 weeks later (Table 3). The results of the MA-ELISA showed that most (20/24) of the pigs infected at 2½ and 4½ weeks of age had detectable anti-MA antibody responses at an age of 24 weeks whereas 2 out of 8 pigs infected at 18 weeks of age had a positive antibody titer (Table 4). In conclusion, these results indicated that lesions and antibody responses can generally be detected 19½ weeks after experimental infection, but are relatively inconsistent after 6 weeks of infection.

3.3 Natural infections with MA

In order to evaluate the serodiagnostic ELISA test for use under field conditions we collected a considerable number of serum samples from pigs from two farms (A and B) with a recent history of MAH infections and performed bacteriological, serological and pathological examinations of particular lymph nodes. Results of bacteriological examination of the sub-maxillary and mesenteric lymph nodes showed that 56% (103/184) of the pigs on farm A were infected with MAH and 35% (41/117) of the pigs on farm B (Table 5). Granulomatous lesions in the sub-maxillary lymph nodes were detected by pathological examination in 31% (57/184) of the pigs originating from farm A and in 8% (9/117) of the pigs from farm B. Using the MA-ELISA, on farm A, 16% (29/184) of the pigs tested serologically positive and on farm B, 17% (15/117) of the pigs. These results indicate that, compared to the results of the pathological and serological examination, the bacteriological examination for MA is the most sensitive diagnostic method for detection of MA infections in these two herds. Furthermore, that on farm A the pathological

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examination had more positive results than the serological examination, whereas on farm B the serological examination showed more positive results.

Sensitivity and specificity of the pathological and serological examination were calculated on the samples of pigs from these two farms using the results of the bacteriological examination as a reference. For the MA-ELISA, sensitivity was 14% (9% - 21% CI) and the specificity 83% (77% – 89% CI). For the pathological examination of the sub-maxillary lymph nodes, sensitivity was 31% (23% - 39% CI) and the specificity 86% (79% - 91% CI). According to McNemar's test pathological examination of the sub-maxillary lymph nodes for granulomatous lesions had a significantly (p = 0.005) higher sensitivity than the serological examination by the MA-ELISA whereas statistically no difference was found in specificity of both tests.

4. Discussion

Diagnosis of MA infection in pigs is traditionally based on the detection of granulomatous lesions in lymph nodes seen by eye at slaughter. Since sensitivity and specificity of this approach is questioned (Brown and Neuman, 1979; Komijn et al., 2007; Offermann et al., 1999) we developed as an alternative a serodiagnostic ELISA assay on the basis of a polar lipid fraction from MA. We evaluated the MA-ELISA and found in samples from experimentally infected pigs a significantly higher sensitivity for the MA-ELISA (69%) as compared to pathological examination (31%). The MA-ELISA seemed also more specific since on a selected set of sera from MA negative pigs (Komijn et al., 2007) only one out of 153 pigs was positive in the MA-ELISA whereas 99 (65%) pigs had granulomatous lesions. This shows that the MA-ELISA was highly specific even when a high number of serum samples originated from pigs with lesions. Results of the evalution of the MA-ELISA test on serum samples from pigs originating from two MA infected herds showed a variable picture. On samples of these pigs the sensitivity of the pathological examination (31%) was significantly higher than the MA-ELISA (14%). However, more positive results were obtained with the MA-ELISA on the first farm whereas on the second farm more pigs showed lesions in the sub-maxillary lymph nodes. A possible reason for the variation in results on the two MA infected farms may be related to antigenic variation between the MAH strain used for the preparation of polar lipid fraction for the MA-ELISA and the MAH strains circulating on both farms. Study in a wider range on MA infected farms will be needed to provide a more comprehensive view of the quality of both tests for detection of MA in infected farms. At the same time further optimization of MA-ELISA with use of

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lipid antigens from a broader range of serotypes may improve its performance in the face of infections with different MA serotypes.

Currently, diagnosis of MA infections in pigs is based on detection of granulomatous lesions in lymph nodes of pigs by incision and visual inspection at slaughter (Brown and Neuman, 1979; Komijn et al., 2007). In accordance with results of others we frequently detected MA bacteria whereas lesions were absent (Brown and Neuman, 1979; Offermann et al., 1999). Although the sensitivity we calculated on naturally and experimentally infected pigs was low (31%), it cannot be excluded that this sensitivity is still overestimated as we performed pathological examinations of the lymph nodes by multiple incisions and under optimal conditions in the facilities of the Central Veterinary Institute of Wageningen UR. Lesions in lymph nodes vary in size from pinpoint to pea size and especially mild lesions can easily be missed at slaughter due to the rapid line speed.

The specificity of the pathological examination of sub-maxillary lymph nodes on the two MA infected farms in this study was calculated to be 86%. Results of an earlier study of us on selected lymph node samples from nine pig herds in the Netherlands had given evidence about a low specificity since we found that granulomatous lesions in lymph nodes of slaughter pigs were not associated with the presence of MA bacteria but with *R. equi* (Komijn et al., 2007). The MA infection level of the two for this study selected herds was high but low in the nine herds in our earlier study. Therefore it is most likely that in highly MA infected herds the pathological examination of lymph nodes is better correlated with the presence of MA bacteria than in populations where MA infections are absent or low. In the Netherlands the prevalence of MA infections is low (Komijn et al., 2007), for this reason the specificity as calculated in this study is probably lower in the real field situation. Brown and Neuman (1979) calculated a specificity of 53%, which confirms the idea that specificity could be lower than the 86% we found on the two MA infected herds.

We developed the MA-ELISA with the aim to detect MA infected pigs at slaughter when they are 26 weeks of age. Results of the experimental infection with MA showed that titers started to increase 6-8 weeks after infection and stayed detectable till the end of the experiment, 21½ weeks after infection.

There is no clear evidence at which age pigs are most at risk for an infection with MA. Infections in herds are reported when peat or compost are provided as a source of iron and minerals to piglets soon after birth (Engel et al., 1978; Matlova et al., 2005) but infections later in on lifetime cannot be excluded. MA bacteria are present in bedding materials, sawdust and drinking water in the stables (Matlova et al., 2003; Thoen, 1992), to this materials are pigs constantly exposed during their whole lifetime. Taking the results

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of the MA-ELISA into account, serodiagnosis offers possibilities to detect MA infections during the whole lifetime of the pig, except when pigs are infected in the latest weeks before slaughtering.

It was unexpected that in the experimental infection pigs repeatedly infected with MAH had a lower number of lesions compared to pigs once infected (Table 3). No differences were seen between groups regarding tuberculin reactions and isolation of MAH bacteria, but the development of immune response differed between the group of repeatedly infected pigs and fthe groups of once infected pigs. Whereas from the other groups the mean anti MA antibody titer was maximal at 10-12 weeks after infection, in the repeatedly infected group the mean anti MA antibody titer still further increased. Apparently, the repeated MA infection leads to this diffrent immune response associated with a lack of lesion formation.

For immunediagnosis of human MA infections lipid antigens derived from MA are successfully used (Enomoto et al., 1998; Kitada et al., 2008, 2002, 2005), here we show that lipid fractions are also suitable for immunodiagnosis of MA infections in pigs. The polar lipid fraction showed a higher reactivity with sera from experimentally infected pigs than the apolar lipid fraction, for this reason the polar lipid fraction was chosen as antigen in the MA-ELISA. For detection of MA infections in humans, purified glycopeptidolipids (GPL) are used as antigen in ELISA tests with sufficient sensitivity and specificity (Kitada et al., 2008). GPLs are immunodominant antigens from MAC and are well-characterized polar lipids (Brennan et al., 1981; Inderlied et al., 1993). In preliminary experiments we found that GPLs are indeed present in the polar lipid fraction we used for the MA-ELISA assay (results not shown). Although there are no reports on the immunogenicity of GPLs in MA infections in pigs it can be speculated that in our ELISA test, GPLs contribute to the difference between reactivity of the polar and apolar lipid fraction of MAH.

It can be concluded that sensitivity and specificity of the use of granulomatous lesions in lymph nodes as read out criterion for a potential MA infection performs poorly. A more sensitive and objective test for routine diagnosis of MA infections in slaughter pigs is needed. The MA serodiagnostic test could meet these requirements and may be a valuable tool for screening herds to prevent and control MA infections in pigs.

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References

Biet, F., Boschiroli, M.L., Thorel, M.F., Guilloteau, L.A., 2005, Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). Vet. Res. 36, 411-436.

Brennan, P.J., Aspinall, G.O., Shin, J.E., 1981, Structure of the specific oligosaccharides from the glycopeptidolipid antigens of serovars in the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex. J. Biol. Chem. 256, 6817-6822.

Brown, J., Neuman, M.A., 1979, Lesions of swine lymph nodes as a diagnostic test to determine mycobacterial infection. Appl. Environ. Microbiol. 37, 740-743.

Dobson, G., Minnikin, D.E., Minnikin, S.M., Parlett, M., Goodfellow, M., Ridell, M., Magnusson, M., 1985, Systematic analysis of complex mycobacterial lipids. In Chemical Methods in Bacterial Systematics, pp. 237–265. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.

Dvorska, L., Matlova, L., Bartos, M., Parmova, I., Bartl, J., Svastova, P., Bull, T.J., Pavlik, I., 2004, Study of *Mycobacterium avium* complex strains isolated from cattle in the Czech Republic between 1996 and 2000. Vet. Microbiol. 99, 239-250.

Dvorska, L., Parmova, I., Lavickova, M., Bartl, J., Vrbas, V., Pavlik, I., 1999, Isolation of *Rhodococcus equi* and atypical mycobacteria from lymph nodes of pigs and cattle in herds with the occurrence of tuberculoid gross changes in the Czech Republic over the period of 1996-1998. Vet. Med. 44, 321-330.

Engel, H.W., Groothuis, D.G., Wouda, W., Konig, C.D., Lendfers, L.H., 1978, "Pig-compost" as a source of *Mycobacterium avium* infection in swine. Zbl. Vet. Med. B. 25, 373-382.

Enomoto, K., Oka, S., Fujiwara, N., Okamoto, T., Okuda, Y., Maekura, R., Kuroki, T., Yano, I., 1998, Rapid serodiagnosis of *Mycobacterium avium-intracellulare* complex infection by ELISA with cord factor (trehalose 6, 6'-dimycolate), and serotyping using the glycopeptidolipid antigen. Microbiol. Immunol. 42, 689-696.

Inderlied, C.B., Kemper, C.A., Bermudez, L.E., 1993, The *Mycobacterium avium* complex. Clin Microbiol Rev 6, 266-310.

Kitada, S., Kobayashi, K., Ichiyama, S., Takakura, S., Sakatani, M., Suzuki, K., Takashima, T., Nagai, T., Sakurabayashi, I., Ito, M., Maekura, R., 2008, Serodiagnosis of *Mycobacterium avium*-complex pulmonary disease using an enzyme immunoassay kit. Am. J. Respir. Crit. Care Med. 177, 793-797.

ACCEPTED MANUSCRIPT

Kitada, S., Maekura, R., Toyoshima, N., Fujiwara, N., Yano, I., Ogura, T., Ito, M., Kobayashi, K., 2002, Serodiagnosis of pulmonary disease due to *Mycobacterium avium* complex with an enzyme immunoassay that uses a mixture of glycopeptidolipid antigens. Clin Infect Dis 35, 1328-1335.

Kitada, S., Maekura, R., Toyoshima, N., Naka, T., Fujiwara, N., Kobayashi, M., Yano, I., Ito, M., Kobayashi, K., 2005, Use of glycopeptidolipid core antigen for serodiagnosis of *Mycobacterium avium* complex pulmonary disease in immunocompetent patients. Clin Diagn Lab Immunol 12, 44-51.

Komijn, R.E., de Haas, P.E., Schneider, M.M., Eger, T., Nieuwenhuijs, J.H., van den Hoek, R.J., Bakker, D., van Zijderveld, F.G., van Soolingen, D., 1999, Prevalence of *Mycobacterium avium* in slaughter pigs in The Netherlands and comparison of IS1245 restriction fragment length polymorphism patterns of porcine and human isolates. J. Clin. Microbiol. 37, 1254-1259.

Komijn, R.E., Wisselink, H.J., Rijsman, V.M., Stockhofe-Zurwieden, N., Bakker, D., van Zijderveld, F.G., Eger, T., Wagenaar, J.A., Putirulan, F.F., Urlings, B.A., 2007, Granulomatous lesions in lymph nodes of slaughter pigs bacteriologically negative for *Mycobacterium avium* subsp. *avium* and positive for *Rhodococcus equi*. Vet. Microbiol. 120, 352-357.

Kunze, Z.M., Portaels, F., McFadden, J.J., 1992, Biologically distinct subtypes of *Mycobacterium avium* differ in possession of insertion sequence IS901. J Clin Microbiol 30, 2366-2372.

Matlova, L., Dvorska, L., Ayele, W.Y., Bartos, M., Amemori, T., Pavlik, I., 2005, Distribution of *Mycobacterium avium* complex isolates in tissue samples of pigs fed peat naturally contaminated with mycobacteria as a supplement. J Clin Microbiol 43, 1261-1268.

Matlova, L., Dvorska, L., Bartl, J., Bartos, M., Ayele, W.Y., Alexa, M., Pavlik, I., 2003, Mycobacteria isolated from the environment of pig farms in the Czech Republic during the years 1996 to 2002. Vet. Med. 48, 343-357.

Mijs, W., de Haas, P., Rossau, R., Van der Laan, T., Rigouts, L., Portaels, F., van Soolingen, D., 2002, Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and '*M. avium* subsp. *hominissuis*' for the human/porcine type of M. avium. Int J Syst Evol Microbiol 52, 1505-1518.

Offermann, U., Bodmer, T., Audigé, L., Jemmi, T., 1999, The prevalence of Salmonella, Yersinia and Mycobacteria in slaughtered pigs in Switzerland. Schweiz Arch Tierheilkd 141, 509-515.

ACCEPTED MANUSCRIPT

Sinkora, J., Rehakova, Z., Samankova, L., Haverson, K., Butler, J.E., Zwart, R., Boersma, W., 2001, Characterization of monoclonal antibodies recognizing immunoglobulin kappa and lambda chains in pigs by flow cytometry. Vet Immunol Immunopathol 80, 79-91.

Thoen, C.O., 1992, Tuberculosis, In: Leman, A.D., Straw, B.E., Mengeling, W.L., d'Allaire, S., Taylor, D.J. (Eds.) Diseases of Swine, 7th edn. Iowa State University Press, Ames, pp. 617-626.

van Soolingen, D., Bauer, J., Ritacco, V., Leao, S.C., Pavlik, I., Vincent, V., Rastogi, N., Gori, A., Bodmer, T., Garzelli, C., Garcia, M.J., 1998, IS*1245* restriction fragment length polymorphism typing of *Mycobacterium avium* isolates: proposal for standardization. J Clin Microbiol 36, 3051-3054.

Wagner, D., Young, L.S., 2004, Nontuberculous mycobacterial infections: a clinical review. Infection 32, 257-270.

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Figure legend

Three groups of eight pigs each were experimentally infected with *M. avium* subsp. *hominissuis* strain 17404 at 2½ (◆), 4½ (■)and 18 (▲) weeks of age, the fourth group was infected three times at 2½, 4½ and 18 (X) weeks of age. All pigs were necropsied at 24 weeks of age. Longitudinal antibody responses were determined in ELISA tests against the polar lipid fraction of MAH strain 17404. Mean Percentage Positivity and Standard Deviation was calculated for each group and for each timepoint.

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Table 1

Design experimental infection of pigs with *Mycobacterium avium* subsp. *hominissuis*

Group	No. of pigs	Age (weeks)	
		Infected	Necropsied
1	8	2½	24
2	8	4½	24
3	8	18	24
4	8	2½, 4½, 18[a]	24

[a] Pigs were three times infected.

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Table 2
Distribution of PP obtained in the MA-ELISA assay on serum samples from pigs (n=153) negative for infections with *Mycobacterium avium* (MA) [a].

Percentage Positivity	Number (%)
< 0%	80 (52.3)
0 - 5%	60 (39.2)
5 - 10%	10 (6.5)
10 – 15%	2 (1.3)
15 – 20%	1 (0.7)
> 25%	0 (0)
Total	153 (100)

[a] Serum samples were collected in the course of a study for the prevalence of MA infections in the Netherlands. The design and the outcome of the study was previously described by Komijn et al. (2007).

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Table 3
Post-mortem results recorded in pigs after experimental infection with *Mycobacterium avium* subsp. *hominissuis*

Group[a]	No. of pigs with lesions at														Total no. of pigs with lesions	
	Tonsil		Md[b]		Mes[c]		Ing[d]		Trach-br (le)[e]		Trach-br (ri)[f]		Retro-phar[g]			
	Pt[h]	Bac[i]	Pt	Bac	Pt	Bac	Pt	Bac	Pt	Bac	Pt	Bac	Pt	Bac	Pt	Bac
1	1	2	3	5	6	8	0	0	2	2	2	3	3	7	7	8
2	0	1	7	8	7	7	0	2	0	0	2	1	4	6	7	8
3	0	8	0	8	0	5	0	0	0	0	0	0	0	6	0	8
4	0	3	0	6	2	7	0	0	0	1	0	0	0	3	2	8
Total	1	14	10	27	15	27	0	2	2	3	4	4	7	22	16	32

[a] Experimental design is described in Table 1A
[b]Lnn mandibularis
[c]Lnn mesenterialis
[d]Lnn inguinalis
[e]Lnn trachea-bronchialis
[f]Lnn retro-pharyngeal
[g]Lnn retro-pharyngeal
[h]Pathological lesions
[i]Isolation of *M. avium* subsp. *hominissuis* at necropsy

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Table 4

Results of the MA-ELISA assay on endpoint serum samples of pigs experimentally infected with *Mycobacterium avium* subsp. *hominissuis*.

Group	No. of pigs	Weeks after infection	Number of pigs	
			Negative	Positive
1	8	21.5	0	8
2	8	19.5	3	5
3	8	6	6	2
4	8	21.5, 19.5, 6[a]	1	7
Total	32		10	22

[a]Pigs were three times infected.

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Table 5

Results of bacteriological, pathological and serological examinations of pigs from two *Mycobacterium avium* infected herds for detection of *M. avium* infections.

Examination	Number of pigs (%) tested		
	Negative	Positive	Total (n)
Farm A			
Bacteriological[1]	81 (44)	103 (56)	184
Pathological[2]	127 (69)	57 (31)	184
Serological[3]	155 (84)	29 (16)	184
Farm B			
Bacteriological	76 (65)	41 (35)	117
Pathological	108 (92)	9 (8)	117
Serological	100 (85)	17 (15)	117

[1] Positive when *M. avium* bacteria were detected by bacteriological examination on sub-maxillary lymph nodes and mesenteric lymph nodes.
[2] Positive when granulomatous lesions were seen in the sub-maxillary lymph nodes.
[3] As detected by the MA-ELISA with a cut-off value of 10.6 PP.

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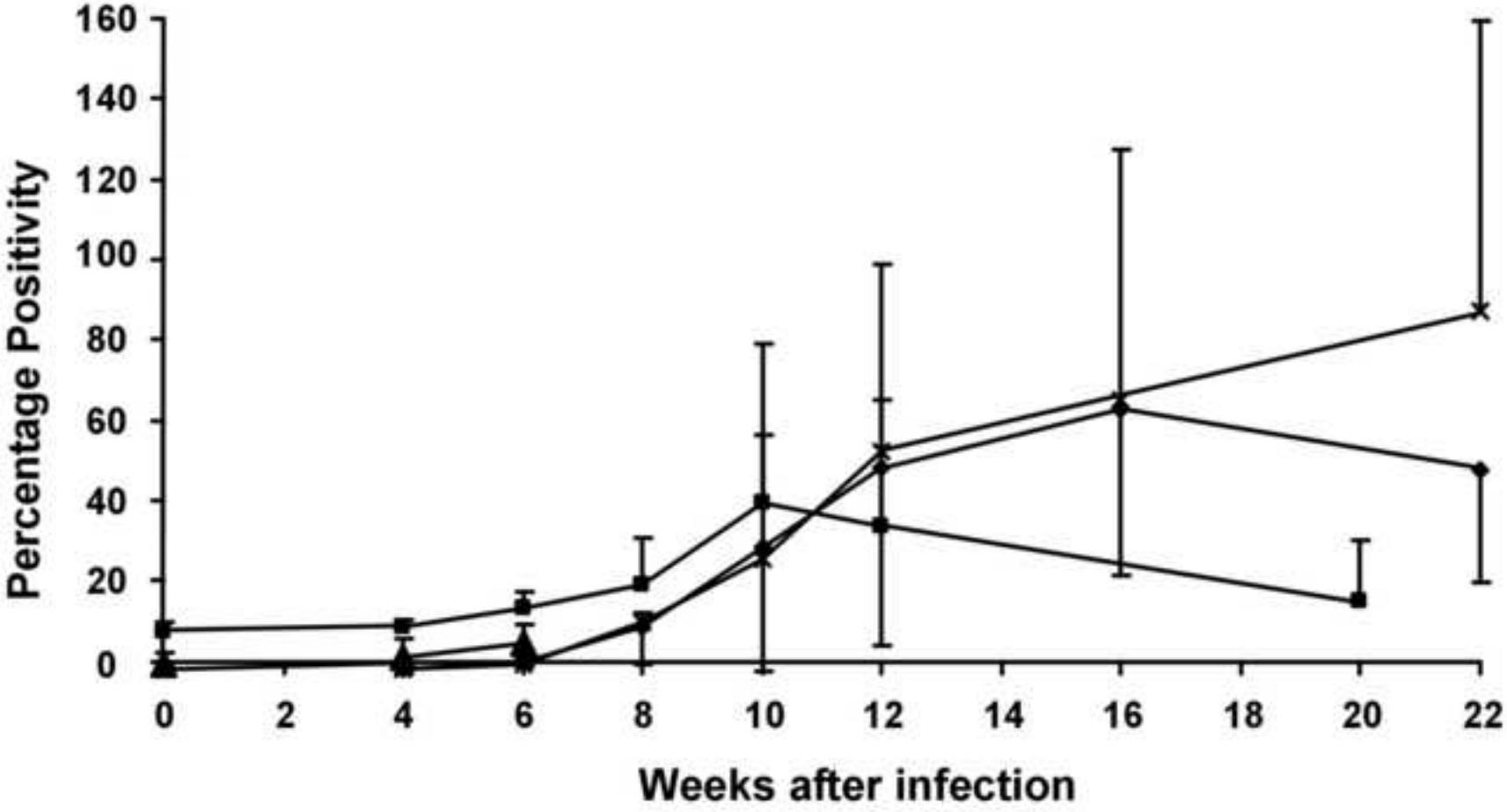
Figure


Percentage Positivity
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
Weeks after infection