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Transfer of maternal immunity to piglets is involved in early protection against *Mycoplasma hyosynoviae* infection

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Abstract

Mycoplasma hyosynoviae causes arthritis in pigs older than 12 weeks. The role of colostrum in protection of piglets against *M. hyosynoviae* infection is not clear. Our objective was therefore to investigate whether transfer of maternal immunity to piglets was involved in early protection against the infection. Experimental infections were carried out in three groups of weaners receiving different levels of *M. hyosynoviae*-specific colostrum components; Group NC derived from Mycoplasma free sows and possessed no specific immunity to *M. hyosynoviae*. Group CAb pigs, siblings of the NC group, received colostrum with *M. hyosynoviae*-specific antibodies immediately after birth. Group CCE pigs were born and raised by infected sows and presumably had the full set of colostrally transferred factors, including specific antibodies. When 4½ weeks old, all pigs were inoculated intranasally with *M. hyosynoviae*. The course of infection was measured through clinical observations of lameness, cultivation of *M. hyosynoviae* from tonsils, blood and synovial fluid and observation for gross pathological lesions in selected joints.

Specific immune status in the pigs was evaluated through detection of antibodies by immunoblotting and measurement of *M. hyosynoviae*-specific T-cell proliferation. The latter analysis may possibly indicate that *M. hyosynoviae* infection induces a T-cell response. The CCE piglets were significantly protected against development of lameness and pathology, as well as infection with *M. hyosynoviae* in tonsils, blood and joints, when compared to the two other groups. Raising the CCE pigs in an infected environment until weaning, with carrier sows as mothers, apparently made them resistant to *M. hyosynoviae*-arthritis when challenge-infected at 4½ weeks of age. More pigs in group NC had *M. hyosynoviae* related pathological lesions than in group CAb, a difference that was significant for cubital joints when analysed on joint type level. This finding indicates a partially protective effect of passively transferred *M. hyosynoviae*-specific colostral antibodies upon development of *M. hyosynoviae* related pathology. Thus, the level of passive immunity transferred from sow to piglet seems to provide, at least partial, protection against development of arthritis. It cannot be ruled out that the CCE pigs, by growing up in an infected environment, have had the chance to establish an active anti-*M. hyosynoviae* immune response that complements the maternally transferred immune factors. Evident from this study is that the general absence of *M. hyosynoviae* arthritis in piglets can be ascribed mainly to their immunological status.

Keywords

Mycoplasma hyosynoviae, arthritis, colostrum, antibody, pig, lymphocyte proliferation

Introduction

Mycoplasma hyosynoviae infection is a common cause of acute and severe lameness among Danish growing-finishing pigs (Nielsen et al., 2001). Herds with severely affected pigs experience increased use of antibiotics and workload as well as reduced animal welfare (Kobisch and Friis, 1996; Nielsen et al., 2001). The prevalence of *M. hyosynoviae* in the Danish swine industry has not been investigated thoroughly, however non-published experiences form Danish pig herds indicate that the majority of these are infected.

M. hyosynoviae is harboured in the tonsils of infected pigs (Ross and Spear, 1973; Friis et al., 1991). This carrier state is primarily established in pigs above ten weeks of age and infection is rarely transmitted from sows to piglets (Hagedorn-Olsen et al., 1999a). Via the blood stream the mycoplasmas may spread to the joints (Kobisch and Friis, 1996; Hagedorn-Olsen et al., 1999b) and cause arthritis in pigs above 12 weeks of age (Ross and Duncan, 1970; Hagedorn-Olsen et al., 1999a). A previous

84 experiment showed that 6-week-old pigs, immunologically naive with respect to *M.*
85 *hyosynoviae*, were able to develop acute joint infection within 2 to 13 days after
86 intranasal inoculation with the agent (Lauritsen et al., 2008). This indicated that the
87 absence of *M. hyosynoviae* related lameness in this age group under field conditions
88 must have another explanation than strictly age related factors.
89
90 Sow colostrum contains antibodies, which the newborn piglets absorb to the
91 circulation through the gut (Frenyo et al., 1981; Klobasa et al., 1981; Rooke and
92 Bland, 2002; Salmon et al., 2009; Bandrick et al., 2011; Nechvatalova et al., 2011),
93 as well as other immunological components such as cells of the immune system, e.g.
94 neutrophils and eosinophils, macrophages and lymphocytes (Evans et al., 1982;
95 Schollenberger et al., 1986a; Schollenberger et al., 1986b; Magnusson et al., 1991,
96 Nechvatalova et al., 2011). Live cells from sow colostrum are transferred across the
97 gut epithelium of the piglet and into the blood/lymphatics (Tuboly et al., 1988;
98 Williams, 1993; Salmon, 2000; Salmon et al., 2009; Nechvatalova et al., 2011) and it
99 has been discussed in several papers whether colostral cells actively could comprise a
100 pool of cellular immunocompetence that can be transferred from sow to the suckling
101 piglet (Salmon, 2000; Wagstrom et al., 2000; Salmon et al, 2009; Nechvatalova et al.,
102 2011). The role of colostrum in protection of piglets against *M. hyosynoviae* infection
103 has so far not been clarified, although the abovementioned results by Lauritsen et al.
104 (2008) may point in the direction of presence of maternally transferred immunity.

Antibodies specific for *M. hyosynoviae*, presumably originating from colostrum, have been shown to be present in suckling piglets (Blowey, 1993; Hagedorn-Olsen et al., 1999a). In the present study the hypothesis was that transfer of maternal immunity to piglets is involved in early protection against *Mycoplasma hyosynoviae* infection. The possible role of specific *M. hyosynoviae* antibodies was investigated by developing an experimental colostrum model. One group of piglets were isolated from the sow immediately after birth and fed cell-free colostrum containing significant levels of specific *M. hyosynoviae* antibodies (Colostrum antibody group - CAb group). Protection of this group after inoculation with *M. hyosynoviae* was compared to two other groups, one that had suckled infected sows (Complete Colostrum and Exposure group - CCE group) and one that had suckled sows that were immunologically naive to *M. hyosynoviae* (Naive Colostrum group - NC group).

Materials and methods

Animal material and housing conditions

Thirty-two pigs were allocated to three groups, subjected to different regimens of colostrum intake; *i)* the CAb group received *M. hyosynoviae*-specific antibodies via colostrum that had been frozen and thawed to destroy live cells, *ii)* the NC group suckled colostrum without *M. hyosynoviae*-specific immunity and was therefore immunologically naive, *iii)* the CCE group received complete colostrum containing

antibodies and cellular components from their *M. hyosynoviae* infected mothers, and was exposed to infected environment until weaning. The experimental design is illustrated in Fig. 1. All pigs included in the study were cross-breds (offspring from Danish Landrace/Yorkshire sows and Duroc or Hampshire boars). The CAb and NC groups were kept isolated under experimental conditions from birth, whereas the CCE group was transferred to the experimental facilities one week before inoculation (Fig. 1). All pigs of the study were weaned at 3-3½ weeks of age (Fig. 1). The pigs were kept loose in pens with concrete floors and abundant straw-bedding. Fresh water was supplied *ad libitum* through water nipples, and the pigs were fed factory-made pelleted standard swine feed without addition of any antimicrobials.

Preparation of the colostrum pool

The colostrum artificially fed to the CAb group (Fig. 1) was prepared from colostrum of four sows from a *M. hyosynoviae* infected herd, but not the same herd which supplied pigs for the CCE group. Within 24 hours after parturition 300-600 ml of colostrum was collected from each sow using the following method; Sows were prepared for colostrum collection by i.v. injection of 20 IU oxytocin (Oxytocin®, Leo Vet) and the udder was washed and disinfected (0.5% chlorhexidin in 70% ethanol). Colostrum was collected by hand stripping into sterile wide mouth glass bottles. After removing 2-4 ml for later cultivation for *M. hyosynoviae*, 200 mg tiamulin (Tiamutin

145 [R], Novartis) was added per 100 ml colostrum and the colostrum was stored at -20°C.
146 Further, the colostrum was thawed, pooled, filtered through sterile gauze, aliquoted
147 into sterile bottles and stored at -20°C until use. Before being fed to the newborn
148 pigs, the colostrum was thawed in a lukewarm water bath, and the temperature
149 adjusted to 38°C. Further details on the colostrum feeding to piglets are described in
150 Fig. 1. All colostra used were cultivation negative for *Mycoplasma spp* prior to
151 addition of Tiamulin.
152

153 **Inoculation with *M. hyosynoviae***

154 Between 4 and 4½ weeks of age all pigs were inoculated with a cloned field strain of
155 *M. hyosynoviae*, Mp927 (titres 10^7 to 10^8 colour changing units (CCU) per ml). The
156 method used for preparation of inoculum is described by Lauritsen et al. (2008). Pigs
157 were inoculated intranasally into the *dorsal meatus*, while in dorsal recumbency.
158 Inoculation dose was 1ml in each nostril.
159

160 **Mycoplasma cultivation**

161 Cultivation for *M. hyosynoviae* from heparin-stabilized blood samples was performed
162 on post inoculation day (PID) 4, 7, 9, 12 and 15. Tonsillar scrapings, obtained with a
163 sterile blunt steel scraper especially designed for the purpose, were collected two
164 days before inoculation and on PID 4, 7, 9 and 12. Cultivation for *M. hyosynoviae* in

colostrum was performed in 1:10 serial dilutions to 10^{-4} in modified Hayflick's medium (Kobisch and Friis, 1996). All other methods, used in this study for mycoplasma cultivation, including production of inoculation material, have been described by Lauritsen et al. (2008).

Clinical recordings and post mortem examinations

Prior to inoculation all pigs had a normal body condition and did not show any clinical signs of disease. Every day post inoculation, the pigs were observed for clinical signs of lameness and other signs of disease. The pigs were euthanized and autopsied on PID 12, 14 or 16, i.e. in the time period of expected occurrence of the acute infection phase (Kobisch and Friis, 1996; Hagedorn-Olsen et al., 1999c). The date of euthanasia for each pig was determined before inoculation and pigs from each group were evenly represented on the necropsy days. Euthanasia was performed by stunning with a captive bolt pistol followed by exsanguination. At autopsy, six joints per pig were examined for gross pathological lesions since we focused on cubital, stifle and tibiotarsal joints. For each joint the conditions of the synovial fluid and synovial membrane were evaluated by scoring the following seven variables: Synovial fluid colour, volume and transparency, Synovial membrane edema, hyperaemia, hypertrophy and discolouration. In addition the joint cartilages were examined for lesions and discolouration. A pathoanatomical diagnosis was made for

each joint based on the sum of all macroscopic findings recorded for the joint. Synovial fluid was collected aseptically for *M. hyosynoviae* cultivation from these joints and transferred to a sterile tube containing mycoplasma transport medium (Kobisch and Friis, 1996). The amount of synovial fluid used for cultivation varied depending on the amount obtainable from the joints - from one drop to one ml. The synovial fluid samples were also examined for the presence of *M. hyorhinis*, *M. hyopneumoniae* and *M. flocculare* by cultivation. From each pig, the tonsils were collected for *M. hyosynoviae* cultivation.

The procedures related to animal experimentation had been approved by the Danish Animal Experiments Inspectorate (Licence No. 1999/561-207).

M. hyosynoviae antigen for lymphocyte proliferation assay and immunoblots

Pelleted (1.6 g) *M. hyosynoviae* species type strain S16 (Ross and Karmon, 1970) resuspended in 10 ml sterile Milli Q water was subject to 10 repeated freeze-thaw cycles and finally centrifugated at 1000 x g, 30 min. The washed pellet was solubilized twice on ice in NP40 lysis buffer (2 % v/v Nonidet P40, 2 mM EDTA, 0.1 mM IAA and 1 mM PMSF in PBS), and centrifuged at 20000 x g after which the new pellet was boiled for 5 minutes in 3 ml lysis buffer with addition of 2 % w/v SDS and centrifuged at 20000 x g for 30 min. Free SDS was removed by ultrafiltration through an YM 10 (Amicon) membrane at 4°C. The resulting antigen solution was called

Mhyos-antigen. It was aliquoted and stored at -20°C. A protein concentration of 3.3 mg/ml was measured by the Micro BCA (bicinchoninic acid) Protein Assay (Pierce) using bovine serum albumin as standard. To assure that the antigen exerted no inhibitory effect on cell cultures, an MTT test (Mosmann, 1983) for antigen toxicity and non-specific stimulation was performed. The antigen preparation induced low non-specific activity at 20 μg/ml. However, at lower concentrations no non-specific activity was observed and the antigen was non-toxic at all concentrations.

BrdU lymphocyte proliferation assay

Antigen-specific lymphocyte proliferation in response to *M. hyosynoviae* challenge infection was investigated in blood samples from all pigs two days before inoculation and on PID 7 and 12. Proliferation was measured by flow cytometry, assessing cells that had incorporated the thymidine analog Bromo-deoxy-Uridine (BrdU) in newly synthesized DNA (Riber and Jungersen, 2007). Briefly, peripheral blood mononuclear cells (PBMCs, 3 x 10^6/ml) in cell culture medium (RPMI 1640 with GlutaMAX™ I, foetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml)) were incubated in 24 well, cell culture plates (Greiner Labortechnik GmbH, Germany): SEB-culture (Staphylococcal enterotoxin B, 5 μg/ml, Alexis, Grünberg, Germany), Ag-culture (Mhyos-antigen, 10 μg/ml), RPMI-culture (nil-stimulation). Incubation was performed for 5 days at 37°C in 5% CO_2, the last 18 hours with

addition of 5-Brom-2'-Deoxyuridine (BrdU 60 μM, Sigma-Aldrich, St. Louis, MO, USA).

Cells were harvested and stained with mAb against swine CD3 (clone PPT3, Yang et al 1996) and secondary R-phycoerythrin conjugated antibody (R0439, DAKO, Denmark). Then cells were fixed with BD-lysis-solution (BD biosciences) and permeabilized with BD-permeabilizing-solution (BD Biosciences) and stained with FITC conjugated Mab against BrdU containing DNAse (BD Biosciences). As control for BrdU staining, cells were incubated with isotype-control antibody (X0927, DAKO, Denmark). Cells were analysed on FACScan by use of CellQuest software (BD Biosciences).

20000 gated cells (interpreted as live lymphocytes) were acquired and CD3+BrdU+ double positive cells, i.e. T-cells that have proliferated, were measured (see supplementary material for details). Mhyos-antigen-specific lymphocyte proliferation was calculated as: %CD3+BrdU+ cells (Ag-culture) with subtraction of %CD3+BrdU+ (RPMI-culture).

Detection of antibodies

Sera from the pigs collected before inoculation (when pigs were 2-3 days, 2 and 4 weeks of age) and colostrum samples from sows no. 1-4 were tested by immunoblotting for the presence of specific antibodies against *M. hyosynoviae*; The Mhyos-antigen was diluted in sample buffer (4× NOVEX NuPAGE Sample Buffer,

San Diego, CA) containing 100mM β-mercaptoethanol. For the electrophoresis, NuPAGE 4-12% Bis-Tris gels (NOVEX) were used in running buffer (20× NOVEX NuPAGE MOPS SDS Running Buffer). SeeBlue PreStained Standards (NOVEX) were used as marker. Blotting was performed in NuPAGE Transfer Buffer (NP0006, NOVEX) and membranes were blocked with TBS + 0.5 % Tween-20. Nitrocellulose strips cut from the blots were incubated overnight with either serum or colostrum (dilutions 1:200 or 1:500 in TBS + 0.5 % Tween-20, respectively). HRP-conjugated rabbit anti-swine Ig antiserum (DAKO cat.no. P164, 1:2000 in TBS + 0.5 % Tween-20) was used as secondary antibody. Between each step, the strips were washed with TBS + 0.5 % Tween-20. Finally the strips were washed for 10 minutes in 50 mM sodiumacetate and the protein bands were developed in dioctyl sodium sulfasuccinate (DSS)/tetramethylbenzidine-solution for 1 to 15 minutes. The strips were then washed in a DSS-solution for maximum 15 minutes and finally dried, after which the presence of *M. hyosynoviae*-specific bands was evaluated. Defining the bands that were specific for *M. hyosynoviae* was performed by comparing Western blot band patterns obtained with serum of experimentally infected pigs (sera supplied by Dr. Niels Filskov Friis). Recognition of two bands at level with the 191kDa size marker (Fig. 2) was consistent in all expectedly positive pigs and was absent in naive pigs (data not shown) as well as in pigs infected with other swine specific mycoplasmas. These two bands were used for differentiating between seropositive and seronegative pigs (Fig. 2).

Additionally the total Ig content in serum from 2 to 5 days old pigs was measured in a non-competitive direct ELISA, using plates coated with rabbit anti-swine Ig antiserum (DAKO Z0139). As secondary antibody HRP-conjugated, rabbit anti-swine Ig antiserum (DAKO P0164) was used. Ig concentration in serum samples was calculated from a standard curve of two-fold dilutions of normal swine Ig fraction 20 mg/ml (DAKO X0906) (start dilution 3.125 ng/ml).

Statistical analysis

Multiple measurements on the same pig were, whenever possible without substantial loss of information, aggregated into a single measure reflecting the overall status of the pig. This approach facilitates the biological interpretation of the results and avoids complex modelling of discrete repeated measures outcomes (Diggle et al., 2002). Cultivation of blood samples and recordings of clinical signs of lameness during the period from challenge to autopsy were interpreted in parallel, that is, the pig was considered a positive reactor if at least one recording was positive. Cultivation of tonsillar samples at autopsy were positive for almost all pigs, and an additional analysis was therefore carried out for records of whether pigs had only positive samples (negative interpretation in parallel). Autopsy results (synovial fluid cultivations, pathological findings) for multiple joints were both interpreted in

parallel across joints and analysed separately for cubital, stifle and tibiotarsal joints because *M. hyosynoviae* arthritis seems to be more frequently observed in some joints than in others (Ross, 1973). As the majority of joints in two of the groups showed no pathological lesions, a presence/absence recording of lesions was preferred over using scores of the individual arthritis severity.

The statistical procedure used to compare the groups with respect to dichotomous outcomes at the pig level was a logistic regression controlling for confounding of experiment (1 or 2), litter and day of measurement (autopsy recordings only) by fixed effects. The potential confounders were omitted when statistically clearly non-significant ($p>0.10$) and without any substantial confounding effect (less than 20% change in odds-ratio (Dohoo et al., 2009)). The odds-ratio expresses roughly the factor by which the occurrence of *M. hyosynoviae* related findings was higher in one group (e.g., NC) relative to another group (e.g., CAb). The effectiveness of controlling for experiment was confirmed by additional Mantel-Haenszel analyses and Generalised Estimating Equation (GEE) logistic regression (Davis, 2002) with an exchangeable correlation structure. Analyses for the NC and CAb groups of synovial fluid and arthritis outcomes at multiple joints used a similar GEE logistic regression to account for two joints (of each type) being measured in each pig. In addition to the logistic regression analyses, Fisher's exact test was used for outcomes that were constant within at least one group. As described by Greenland et al. (2016), we

interpreted the p-values as continuous measures that express the compatibility between the data and the statistical model used. The significance level was set at $p<0.05$. Some findings close to statistical significance were noted as such because of their potential interest, but they were not treated as significant results in the discussion and conclusion. All analyses were carried out by the statistical software SAS, version 9.

T-cell proliferation was compared among the three groups by the non-parametric Kruskal-Wallis test, supplemented with comparisons between selected pairs of groups by the non-parametric Mann-Whitney test, using GraphPad Prism version 5.02, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Clinical signs

Among the pigs in the NC and CAb groups there were some registrations of lameness post challenge (Table 1). The CCE group had no lameness registrations and was found to differ significantly from the NC group ($p=0.007$) (Table 1). The difference between group NC and group CAb was not statistically significant ($p=0.22$).

Gross pathological findings

The observed gross pathological lesions were of varying severity ranging from slight serous arthritis (slightly increased volume of synovial fluid that might be discoloured and turbid. In synovial membrane: mild edema, hyperaemia and/or discolouration), hyperplastic arthritis (varying degree of increase in both synovial fluid volume, discolouration and turbidity. The synovial membrane showed pronounced hyperplasia, sometimes edema, and quite often hyperaemia and discolouration) or serofibrinous arthritis (significantly increased synovial fluid volume with some discolouration and pronounced turbidity, the synovial membrane had marked edema, and some discolouration). Several pigs in groups NC and CAb had *M. hyosynoviae* related pathological lesions, a marked difference to group CCE with no such findings (Table 1). For all joint types, more pigs in group NC had pathological lesions than in group CAb. The difference was statistically significant for cubital joints (odds-ratio=29, p=0.002) and close to significant for stifle joints (odds-ratio=6.4, p=0.095) (Table 2). For tibiotarsal joints, the day of autopsy had a significant effect; day 16 post challenge had a higher occurrence of pathological lesions than preceding days (odds-ratio=14, p=0.003).

Synovial fluid cultivations

Cultures of synovial fluid from the joints from all pigs in group CCE were negative for *M. hyosynoviae* while *M. hyosynoviae* was demonstrated in joints from the majority of pigs in both groups NC and CAb (Table 1). For both cubital, stifle and tibiotarsal joints, more pigs had positive cultures in group NC than in group CAb. These differences were not statistically significant (Table 2), even if the comparison between NC and CAb groups for stifle joints was close to significant (odds-ratio >1000, p=0.086). *M. hyorhinis*, *M. hyopneumoniae* and *M. flocculare* were not isolated from any of the synovial fluid samples.

Cultivation of *M. hyosynoviae* from tonsils and blood

Prior to inoculation tonsillar scrapings from all pigs were *M. hyosynoviae* negative except from one pig in group CCE. Contrary to this, all but one pig from the CCE group, were tonsil carriers at autopsy (Table 1). While almost all pigs in groups NC and CAb were positive on all repeated samplings post inoculation, the pigs in group CCE had any number between 0 and 5 (maximum) positive samplings. One interpretation of this pattern is that pigs in the CCE group tended to develop a carrier state later than pigs of the other groups. This was reflected in a significant difference between the CCE group and the two other groups but no significant difference

363 between the latter groups, when analysing positive cultivations on PID 4 (results not
364 shown).
365
366 Group CCE pigs had no positive blood samples during the experiment while all pigs
367 in groups NC and CAb experienced a haematogenous phase (Table 1). The majority
368 of pigs in the NC and CAb groups had the same pattern of cultivation positive blood
369 samples, with the first three samples (PID 4, 7 and 9) being culture positive.
370

371 **Lymphocyte proliferation**

372 The T-cell proliferation assay was implemented in a previous experiment including
373 four *M. hyosynoviae* inoculated pigs (13 weeks old) and one non-inoculated control
374 pig (unpublished data, pigs described by Lauritsen et al. (2008)). Signs of specific
375 lymphocyte proliferation against Mhyos-antigen were found on PID 11 in the four
376 inoculated pigs ($CD3^+BrdU^+$ cells: 1.4%; 2.2%; 7.2%; 7.6%), but not in the control
377 pig (0.81%). No differences in level of proliferation were observed between Ag-
378 cultures with either 2 μg/ml or 10 μg/ml of Mhyos-antigen.
379 In the present study $\%CD3^+BrdU^+$ in RPMI-cultures varied quite a lot, and
380 particularly on PID 7 high $\%CD3^+BrdU^+$ was measured in RPMI-cultures in some
381 pigs from all groups (Mean: 4.6%, Range 1.0-15.6%), which in some cases could be
382 related to positive cultivation of *M. hyosynoviae* in blood samples. Contrary to this,

most samples from PID 12 had a %$CD3^{+}BrdU^{+}$ in RPMI-cultures around 1% (Mean: 1.4%, Range 0.4-3.9%), and only one pig had a *M. hyosynoviae* cultivation-positive blood sample on this day. Therefore, comparison of antigen-specific proliferative response in the three treatment groups was only performed at PID 12. As shown in Fig. 3, there was a large variation in degree of proliferation on pig level seen as an individual variation within the groups, concerning the antigen-specific proliferative response that we have measured on PID 12. We found no significant differences between the three treatment groups (P=0.35, Kruskal-Wallis test). The medians of the CAb and CCE groups (i.e. the groups appearing most different in Fig. 3) were not statistically different (P=0.16, Mann-Whitney test). Likewise, the difference in the medians of the NC and CCE groups was statistically non-significant (P=0.37) on PID 12.

Antibody responses

Evaluating the serum antibody profiles of pigs in the three groups by immunoblotting revealed that the NC group possessed no bands specific for *M. hyosynoviae* at any time prior to inoculation (Fig. 2). Contrary to this, immunoblots from all pigs of groups CAb and CCE revealed bands specific for *M. hyosynoviae* prior to inoculation, but the general band patterns of these two groups differed from one to another (Fig. 2). In accordance with the findings in the pigs, colostrum from the

M. hyosynoviae immunologically naive sows (no. 1 and 2) had no *M. hyosynoviae* specific bands whereas specific bands were found in colostrum of sows no. 3 and 4 from the infected herds and in the colostrum pool used for feeding the piglets. The average total immunoglobulin content in serum from 2- to 5-day-old pigs, measured in ELISA, were for the CAb group 16 mg/ml, the NC group 30 mg/ml and the CCE group 32 mg/ml.

Discussion and conclusion

The result of the immunoblottings confirmed that the specific immune statuses of the pigs were the following on the day of inoculation with *M. hyosynoviae*; **i)** The pigs from sows that were immunologically naive (group NC) possessed no specific immunity against the agent. **ii)** The colostrum treated pigs (group CAb pigs) possessed specific antibodies as a consequence of the artificial colostrum administration. **iii)** The pigs from infected sows (group CCE) had received specific antibodies, and presumably the full set of maternally transferred factors from colostrum of their infected dams.

The course of infection after inoculation in the CCE group differed significantly from that of the two other groups for several parameters measured; no signs of clinical arthritis or gross pathological findings was found in the CCE group, and cultivation of *M. hyosynoviae* from tonsils, blood and joints of these pigs was significantly

reduced compared to the other groups. With respect to immunological status and *M. hyosynoviae*-infection status, the CCE group represent the population of newly weaned pigs in most Danish herds. They possess *M. hyosynoviae*-specific antibodies just as it has been shown to be the case for piglets from infected sows in Danish herds (Hagedorn-Olsen et al., 1999a). Also the limited effect of the experimental infection in the CCE pigs is in accordance with the observation that clinical *M. hyosynoviae* arthritis does not affect pigs below 30-40 kg in infected herds (Kobisch and Friis, 1996). Raising the CCE pigs in an infected environment until weaning, with carrier sows as mothers, apparently made these pigs resistant to *M. hyosynoviae*-arthritis, when challenge infected at 4 weeks of age. However observational studies performed in infected herds indicate that this, probably maternally-derived, protection is of limited duration and that pigs become susceptible to *M. hyosynoviae* infection and are at risk of developing *M. hyosynoviae*-related arthritis later in life (Ross and Spear, 1973; Hagedorn-Olsen et al., 1999a).

Previous intranasal inoculation experiments with *M. hyosynoviae*, performed in *M. hyosynoviae*-free pigs, have shown that 13 to 17-week-olds experienced a generalisation phase from PID 2, with no detectable mycoplasmas in blood after PID 9 (Hagedorn-Olsen et al., 1999b) and that 6-week-old pigs, immunologically naive with respect to *M. hyosynoviae*, primarily had bacteremia at PID 4, 6 and 8 (Lauritsen et al., 2008). This is in accordance with the findings in groups NC and CAb, where

positive blood cultivations were predominantly seen on PID 4, 7 and 9. The lack of positive blood cultivations in group CCE pigs during the experiment, i.e. no detectable generalisation from PID 4 and forth, indicates a very short period of, or maybe a total lack of haematogenous spread of *M. hyosynoviae* in this group.

The experimental infection resulted in occurrence of clinical *M. hyosynoviae*-arthritis both in pigs of group NC and group CAb. Also gross pathological findings compatible with *M. hyosynoviae*-infection, bacteremia and early tonsillar colonization were observed in these groups. The presence of an ongoing disease condition in the pigs of the NC group was also reflected by a drop in the negative acute phase protein, transthyretin, 7 days after infection (Heegaard et al. 2011). The higher frequency of *M. hyosynoviae* related pathological lesions in cubital joints upon challenge infection in group NC, as compared to group CAb, may indicate a partially protective effect of the intake of *M. hyosynoviae*-specific colostral antibodies upon development of *M. hyosynoviae* related pathology. The average total immunoglobulin content in serum from 2- to 5-day-old pigs, as measured in ELISA, showed that the colostrum treated pigs possessed the lowest concentration of total IgG in serum. Therefore the observed, partially protective, effect of the colostrum treatment on development of arthritis can be ascribed to *M. hyosynoviae* specific antibodies, and not to a generally higher level of non-specific maternally transferred immunoglobulins.

The role of specific antibodies in *M. hyosynoviae* infection is not clear, and could include elimination of the mycoplasmas (neutralisation, opsonisation, complement activation) as well as creation of pathology during infection (e.g. deposition of immune complexes). The partially protective effect of *M. hyosynoviae* specific antibodies on development of arthritis found in this study is contradictory to the findings in pigs above 10 weeks made by Blowey (1993). He found that the appearance of arthritis in gilts was independent of the level of specific antibodies in serum, indicating that the antibody level was of lesser importance for protection against the disease. Also conflicting herd observations, describing antibody levels in relation to bacteremia with *M. hyosynoviae*, have been reported; Hagedorn-Olsen et al. (1999a) found cases of pigs that had raised a specific serological response against *M. hyosynoviae*, after which they developed a generalisation phase with the agent. Contrary to this, Nielsen et al. reported that 3 to 5-month-old pigs with bacteremia had a lower level of *M. hyosynoviae* specific antibodies, than pigs with no demonstrable bacteremia (Nielsen et al., 2005). The general ability of mycoplasmas to vary their surface antigens to evade the host immune response (Razin et al., 1998) may make antibody-mediated elimination difficult to obtain and could be an explanation for the above mentioned ambiguous effect of antibodies. Thus the importance of antibodies in protection against *M. hyosynoviae* arthritis is equivocal.

It has not previously been investigated whether *M. hyosynoviae* infection induces a specific T-cell response. In this study we found a marked variation between individual pigs concerning specific T-cell responses post inoculation. However, T-cell responses are in general widely fluctuating over time and between individuals, so we would not expect all pigs to mount a synchronised and similar specific T-cell response post inoculation. The high percentage of specific proliferation in response to *M. hyosynoviae* antigen found in some pigs on PID 12 indicates that *M. hyosynoviae* infection has the potential of inducing an antigen-specific T-cell response. The involvement of this response in protection against the infection remains uncertain, however, it might be that a cell-mediated immune response participate in protection. We found in this study no statistically significant difference between the three treatment groups, with respect to percentages of *M. hyosynoviae* antigen-specific T-cell proliferation.

The design of the study did not take differences in *M. hyosynoviae* strains into account. Ross et al. (1978) demonstrated different *M. hyosynoviae* strains by serological and electrophoretic methods, and Kokotovic et al. (2002a; 2002b) found a pronounced genetic diversity in chromosomal fingerprints performed on Danish herd isolates of *M. hyosynoviae*. In our study several strains could be involved; 1) the inoculation material, 2) the strain(s) present in the herd that supplied the colostrum batch, and 3) the strain(s) present in the origin herd of the group CCE pigs. We found

differences in band pattern between immunoblots of pigs in the CAb and the CCE groups. However, the immunoblots reveal many bands of which we do not know how many are *M. hyosynoviae*-specific. Thus we do not know whether we worked with at homo- or heterologous system, or a mixture of the both, in this challenge experiment. Designing the study in a way so that all infected sows were infected with a strain similar to that of the inoculation material was neither economically nor practically feasible.

Based on the results of our study, we conclude that in contrast to immunologically naive piglets, piglets that have been raised in an infected environment and have suckled infected sows are protected against early infection with *M. hyosynoviae* and development of arthritis when challenge infected at 4½ weeks of age. We found indications that this protection is related to the level of passive immunity because *M. hyosynoviae*-specific maternally transferred antibodies provided at least partial protection against development of arthritis in otherwise immunologically naive pigs. For comparison, a similar protective effect of colostral antibodies against another mycoplasma, *Mycoplasma hyopneumoniae,* has been described by Rautiainen & Wallgren (2001), Wallgren et al. (1998) and Siblia et al. (2008). However the marked difference between groups CAb and CCE indicates that something more than maternally derived antibodies contributes to the protection. It cannot be ruled out that the CCE pigs, by growing up in an infected environment, have had the chance to

529 establish an active immune response against the agent that complements the
530 maternally transferred immune factors, although only one of the 13 pigs in this group
531 was cultivation positive in the tonsils prior to inoculation. Alternatively the observed
532 high level of resistance to the challenge infection in the pigs that had suckled infected
533 sows could be due to the uptake of cellular components, e.g. primed lymphocytes,
534 from the fresh sow colostrum. By using a model antigen, Nechvatalova et al. (2011),
535 showed that sows via colostrum were able to transfer antigen-specific lymphocytes to
536 the mesenteric lymph nodes and blood stream of their offspring. Likewise Hlavova et
537 al. (2014), found that colostrum contained high numbers of antigen-experienced
538 lymphocytes with a central/effector memory function, that might play a role as
539 passive immunity in offspring, besides having a local mucosal immune defence effect
540 in mammae of the sow. Oh et al. (2012) demonstrated passive transfer of maternally
541 derived PCV-2-specific cellular immune response to piglets from colostrum, by
542 measuring intradermal delayed type hypersensitivity responses and specific blood
543 lymphocyte proliferation in piglets from vaccinated sows. For the other swine
544 pathogenic mycoplasma, *Mycoplasma hyopneumoniae*, Bandrick et al. (2008, 2014)
545 have described the transfer of functional antigen-specific T-cells from sows to their
546 offspring that leaves the newborn piglet able to mount an antigen-specific secondary
547 immune response. They further stated that this transfer of *Mycoplasma*
548 *hyopneumoniae*-specific cellular immunity is dependent on the piglet suckling its

549 biological mother sow (Bandrick et al., 2011). Something similar could take place
550 between *M. hyosynoviae* infected sows and their offspring too.
551
552 We have earlier induced clinical *M. hyosynoviae* arthritis experimentally in 6-week-
553 old piglets from a mycoplasma free herd (Lauritsen et al., 2008), and the findings in
554 the NC group confirms the absence of a strictly age related insusceptibility to *M.*
555 *hyosynoviae* arthritis in young pigs. Regardless of whether it is primarily acquired
556 antibodies (and cells) or also an active immune response that causes the high level of
557 protection in the CCE group, it is evident that the general absence of arthritis in herd
558 piglets can be ascribed mainly to their immunological status with respect to *M.*
559 *hyosynoviae*. Protective immunity against the infection is apparently achievable in
560 piglets, a fact that is of importance e.g. when considering development of an effective
561 immune prophylaxis.
562

563 ## Conflict of interest

564 The authors declare that they have no competing interests.
565

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