



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

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Published in:
Veterinary Immunology and Immunopathology

Link to article, DOI:
[10.1016/j.vetimm.2016.12.002](https://doi.org/10.1016/j.vetimm.2016.12.002)

Publication date:
2017

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Lauritsen, K. T., Hagedorn-Olsen, T., Jungersen, G., Riber, U., Stryhn, H., Friis, N. F., Lind, P., & Kristensen, B. (2017). Transfer of maternal immunity to piglets is involved in early protection against *Mycoplasma hyosynoviae* infection. *Veterinary Immunology and Immunopathology*, 183, 22-30.
<https://doi.org/10.1016/j.vetimm.2016.12.002>

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

3

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29

30 **Abstract**

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

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

64

65 **Keywords**

66 *Mycoplasma hyosynoviae*, arthritis, colostrum, antibody, pig, lymphocyte
67 proliferation

68

69 **Introduction**

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

77

78 *M. hyosynoviae* is harboured in the tonsils of infected pigs (Ross and Spear, 1973;
79 Friis et al., 1991). This carrier state is primarily established in pigs above ten weeks
80 of age and infection is rarely transmitted from sows to piglets (Hagedorn-Olsen et al.,
81 1999a). Via the blood stream the mycoplasmas may spread to the joints (Kobisch and
82 Friis, 1996; Hagedorn-Olsen et al., 1999b) and cause arthritis in pigs above 12 weeks
83 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 colostrum 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.

105 Antibodies specific for *M. hyosynoviae*, presumably originating from colostrum, have
106 been shown to be present in suckling piglets (Blowey, 1993; Hagedorn-Olsen et al.,
107 1999a). In the present study the hypothesis was that transfer of maternal immunity to
108 piglets is involved in early protection against *Mycoplasma hyosynoviae* infection. The
109 possible role of specific *M. hyosynoviae* antibodies was investigated by developing
110 an experimental colostrum model. One group of piglets were isolated from the sow
111 immediately after birth and fed cell-free colostrum containing significant levels of
112 specific *M. hyosynoviae* antibodies (Colostrum antibody group - CAb group).
113 Protection of this group after inoculation with *M. hyosynoviae* was compared to two
114 other groups, one that had suckled infected sows (Complete Colostrum and Exposure
115 group - CCE group) and one that had suckled sows that were immunologically naive
116 to *M. hyosynoviae* (Naive Colostrum group - NC group).

117

118 **Materials and methods**

119 **Animal material and housing conditions**

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

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

135

136 **Preparation of the colostrum pool**

137 The colostrum artificially fed to the CAb group (Fig. 1) was prepared from colostrum
138 of four sows from a *M. hyosynoviae* infected herd, but not the same herd which
139 supplied pigs for the CCE group. Within 24 hours after parturition 300-600 ml of
140 colostrum was collected from each sow using the following method; Sows were
141 prepared for colostrum collection by i.v. injection of 20 IU oxytocin (Oxytocin[®], Leo
142 Vet) and the udder was washed and disinfected (0.5% chlorhexidin in 70% ethanol).
143 Colostrum was collected by hand stripping into sterile wide mouth glass bottles. After
144 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 colostrum 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 10⁷ to 10⁸ 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

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

169

170 **Clinical recordings and post mortem examinations**

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

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

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

195

196 ***M. hyosynoviae* antigen for lymphocyte proliferation assay and immunoblots**

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

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

212

213 **BrdU lymphocyte proliferation assay**

214 Antigen-specific lymphocyte proliferation in response to *M. hyosynoviae* challenge
215 infection was investigated in blood samples from all pigs two days before inoculation
216 and on PID 7 and 12. Proliferation was measured by flow cytometry, assessing cells
217 that had incorporated the thymidine analog Bromo-deoxy-Uridine (BrdU) in newly
218 synthesized DNA (Riber and Jungersen, 2007). Briefly, peripheral blood
219 mononuclear cells (PBMCs, 3×10^6 /ml) in cell culture medium (RPMI 1640 with
220 GlutaMAX™ I, foetal calf serum (10%), penicillin (100 U/ml), streptomycin (100
221 µg/ml)) were incubated in 24 well, cell culture plates (Greiner Labortechnik GmbH,
222 Germany): SEB-culture (Staphylococcal enterotoxin B, 5 µg/ml, Alexis, Grünberg,
223 Germany), Ag-culture (Mhyos-antigen, 10 µg/ml), RPMI-culture (nil-stimulation).
224 Incubation was performed for 5 days at 37°C in 5% CO₂, the last 18 hours with

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

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

235 20000 gated cells (interpreted as live lymphocytes) were acquired and CD3+BrdU+
236 double positive cells, i.e. T-cells that have proliferated, were measured (see
237 supplementary material for details). Mhyos-antigen-specific lymphocyte proliferation
238 was calculated as: %CD3+BrdU+ cells (Ag-culture) with subtraction of
239 %CD3+BrdU+ (RPMI-culture).

240 **Detection of antibodies**

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

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

266

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

273

274 **Statistical analysis**

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

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

291

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

307 interpreted the p-values as continuous measures that express the compatibility
308 between the data and the statistical model used. The significance level was set at
309 $p < 0.05$. Some findings close to statistical significance were noted as such because of
310 their potential interest, but they were not treated as significant results in the
311 discussion and conclusion. All analyses were carried out by the statistical software
312 SAS, version 9.

313

314 T-cell proliferation was compared among the three groups by the non-parametric
315 Kruskal-Wallis test, supplemented with comparisons between selected pairs of
316 groups by the non-parametric Mann-Whitney test, using GraphPad Prism version
317 5.02, GraphPad Software, San Diego California USA, www.graphpad.com.

318

319 **Results**

320 **Clinical signs**

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

325

326 **Gross pathological findings**

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

343

344 **Synovial fluid cultivations**

345 Cultures of synovial fluid from the joints from all pigs in group CCE were negative
346 for *M. hyosynoviae* while *M. hyosynoviae* was demonstrated in joints from the
347 majority of pigs in both groups NC and CAb (Table 1). For both cubital, stifle and
348 tibiotarsal joints, more pigs had positive cultures in group NC than in group CAb.
349 These differences were not statistically significant (Table 2), even if the comparison
350 between NC and CAb groups for stifle joints was close to significant (odds-ratio
351 >1000, p=0.086). *M. hyorhinis*, *M. hyopneumoniae* and *M. flocculare* were not
352 isolated from any of the synovial fluid samples.

353

354 **Cultivation of *M. hyosynoviae* from tonsils and blood**

355 Prior to inoculation tonsillar scrapings from all pigs were *M. hyosynoviae* negative
356 except from one pig in group CCE. Contrary to this, all but one pig from the CCE
357 group, were tonsil carriers at autopsy (Table 1). While almost all pigs in groups NC
358 and CAb were positive on all repeated samplings post inoculation, the pigs in group
359 CCE had any number between 0 and 5 (maximum) positive samplings. One
360 interpretation of this pattern is that pigs in the CCE group tended to develop a carrier
361 state later than pigs of the other groups. This was reflected in a significant difference
362 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,

383 most samples from PID 12 had a %CD3⁺BrdU⁺ in RPMI-cultures around 1% (Mean:
384 1.4%, Range 0.4-3.9%), and only one pig had a *M. hyosynoviae* cultivation-positive
385 blood sample on this day. Therefore, comparison of antigen-specific proliferative
386 response in the three treatment groups was only performed at PID 12. As shown in
387 Fig. 3, there was a large variation in degree of proliferation on pig level seen as an
388 individual variation within the groups, concerning the antigen-specific proliferative
389 response that we have measured on PID 12. We found no significant differences
390 between the three treatment groups (P=0.35, Kruskal-Wallis test). The medians of the
391 CAb and CCE groups (i.e. the groups appearing most different in Fig. 3) were not
392 statistically different (P=0.16, Mann-Whitney test). Likewise, the difference in the
393 medians of the NC and CCE groups was statistically non-significant (P=0.37) on PID
394 12.

395

396 **Antibody responses**

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

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

409

410 **Discussion and conclusion**

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

419

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

424 reduced compared to the other groups. With respect to immunological status and
425 *M. hyosynoviae*-infection status, the CCE group represent the population of newly
426 weaned pigs in most Danish herds. They possess *M. hyosynoviae*-specific antibodies
427 just as it has been shown to be the case for piglets from infected sows in Danish herds
428 (Hagedorn-Olsen et al., 1999a). Also the limited effect of the experimental infection
429 in the CCE pigs is in accordance with the observation that clinical *M. hyosynoviae*
430 arthritis does not affect pigs below 30-40 kg in infected herds (Kobisch and Friis,
431 1996). Raising the CCE pigs in an infected environment until weaning, with carrier
432 sows as mothers, apparently made these pigs resistant to *M. hyosynoviae*-arthritis,
433 when challenge infected at 4 weeks of age. However observational studies performed
434 in infected herds indicate that this, probably maternally-derived, protection is of
435 limited duration and that pigs become susceptible to *M. hyosynoviae* infection and are
436 at risk of developing *M. hyosynoviae*-related arthritis later in life (Ross and Spear,
437 1973; Hagedorn-Olsen et al., 1999a).

438

439 Previous intranasal inoculation experiments with *M. hyosynoviae*, performed in *M.*
440 *hyosynoviae*-free pigs, have shown that 13 to 17-week-olds experienced a
441 generalisation phase from PID 2, with no detectable mycoplasmas in blood after PID
442 9 (Hagedorn-Olsen et al., 1999b) and that 6-week-old pigs, immunologically naive
443 with respect to *M. hyosynoviae*, primarily had bacteremia at PID 4, 6 and 8 (Lauritsen
444 et al., 2008). This is in accordance with the findings in groups NC and CAb, where

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

449

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

466

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

486

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

500

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

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

515

516 Based on the results of our study, we conclude that in contrast to immunologically
517 naive piglets, piglets that have been raised in an infected environment and have
518 suckled infected sows are protected against early infection with *M. hyosynoviae* and
519 development of arthritis when challenge infected at 4½ weeks of age. We found
520 indications that this protection is related to the level of passive immunity because *M.*
521 *hyosynoviae*-specific maternally transferred antibodies provided at least partial
522 protection against development of arthritis in otherwise immunologically naive pigs.
523 For comparison, a similar protective effect of colostral antibodies against another
524 mycoplasma, *Mycoplasma hyopneumoniae*, has been described by Rautiainen &
525 Wallgren (2001), Wallgren et al. (1998) and Sibia et al. (2008). However the marked
526 difference between groups CAb and CCE indicates that something more than
527 maternally derived antibodies contributes to the protection. It cannot be ruled out that
528 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

566 **Acknowledgements**

567 The project was funded by The Research Centre for the Management of Animal
568 Production and Health (CEPROS), Denmark (project no. CEP-SVS97-7). Hans

569 Skåning and staff in the animal facility at National Veterinary Institute are thanked
570 for their great help. Also laboratory technicians Ulla Amtoft, Parvin Faghan, Jesper
571 Juehl Hansen and Jan Lauritsen are thanked for assistance with *Mycoplasma*
572 cultivation, cell culture, antigen production, and serological testing. Associate
573 Professor N. C. Nielsen from the University of Copenhagen is thanked for invaluable
574 input on the experimental planning and design. John Lund and his staff from the
575 experimental herd related to Faculty of Health and Medical Sciences, University of
576 Copenhagen, are thanked for supplying colostrum.

577

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579

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