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Lauritsen, Klara Tølbøl; Hagedorn-Olsen, Tine; Jungersen, Gregers; Riber, Ulla; Stryhn, H.; Friis, N.F.; Lind, Peter; Kristensen, B.

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K. Tølbøll Lauritsen [1,2,*], T. Hagedorn-Olsen [1], G. Jungersen [1], U. Riber [1], H. Stryhn [3], N. F. Friis [1^], P. Lind [1] and B. Kristensen [2]

Addresses of authors:

[1] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark

[2] Department of Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 4, 1870 Frederiksberg C, Denmark

[3] Department of Health Management, University of PEI, Charlottetown, Prince Edward Island, Canada. C1A 4P3 Canada

* Corresponding author: Section for Diagnostic and Scientific Advice, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark
E-mail: ktla@vet.dtu.dk, Tel.: +45 35 88 63 72, FAX; +45 35 88 62 30

Tine Hagedorn-Olsen present address: Dako Denmark A/S, Produktionsvej 42, 2600 Glostrup, Denmark

24

25   Birte Kristensen present address: Klinik for Veterinær Dermatologi, Skovalleen 31, 2880

26   Bagsværd, Denmark

27

28   [^] Deceased. N.F. Friis approved the manuscript before he passed away.

29

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

68

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.

77

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.

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 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

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

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 x 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% CO_2, the last 18 hours with

225 addition of 5-Brom-2'-Deoxyuridine (BrdU 60 μM, 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× 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).

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

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,

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 Siblia 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**

577

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579

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