Characterization of *Mycoplasma hyopneumoniae* strains in vaccinated and non-vaccinated pigs from Spanish slaughterhouses

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25 **Keywords**: *Mycoplasma hyopneumoniae*; genetic variability; lung lesions; vaccination;
26 slaughterhouse

Abstract

This study aimed to describe *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) genetic variability in vaccinated (V) and non-vaccinated (NV) slaughtered pigs showing cranio-ventral pulmonary consolidation (CVPC). Ten V and 10 NV fattening farms with respiratory problems associated to *M. hyopneumoniae* were selected. Lung lesions of one batch per farm were scored at slaughterhouse and the enzootic pneumonia (EP)-index was calculated. Moreover, three lungs showing the most extensive CVPC per farm were sampled and tested for *M. hyopneumoniae* detection by real-time (rt)-PCR. Positive samples with cycle threshold ≤30 were selected to be genotyped by sequencing of four loci (P97, P146, H1 and H5). Typing profiles (TP) were assigned considering the four or two (P97, P146) loci. Five commercial vaccines for *M. hyopneumoniae* (VS) and two reference strains (RF) were also genotyped. The EP-index (mean±SD) in NV farms (3.8±1.9) was not significantly different from V ones (2.2±1.3). From the 60 selected lungs, 46 (76.7%) were *M. hyopneumoniae* positive by rt-PCR (25/30 and 21/30 from NV and V farms, respectively), and 43 (93.5%) of those were successfully genotyped. A total of 24 different TP (12 in V and 12 in NV farms) or 17 TP (9 in V and 9 in NV farms) were identified by analyzing the four or two loci, respectively. One to three TP per farm were detected, being different from VS and RF. Interestingly, farms with same breeding origin had the same TP using two loci, but such link was not found using four loci. Therefore, high *inter*-farm and limited *intra*-farm *M. hyopneumoniae* genetic variability were detected, but variability depended on the number of studied loci.

1. Introduction

Mycoplasma hyopneumoniae (M. hyopneumoniae) is the primary etiological agent of enzootic pneumonia (EP) and plays an important role in porcine respiratory disease complex (PRDC) (Maes et al., 2018). *M. hyopneumoniae* infection mainly affects growing and finishing pigs, and is clinically characterized by a non-productive cough and causes cranio-ventral pulmonary consolidation (CVPC). The clinical signs and lesions cause reduced growth rate and increase feed conversion ratio (Thacker and Minion, 2012). Despite all efforts performed to reduce economic losses caused by this pathogen, *M. hyopneumoniae* continues to be an important concern for worldwide swine herds (Maes et al., 2018).

The severity of this respiratory disease depends on the presence of co-infections, management and housing conditions, and the virulence of the *M. hyopneumoniae* strain involved in the infection (Vicca et al., 2002, 2003; Maes et al., 2008). Strain genetic diversity has been studied using different genotyping techniques such as random amplified polymorphic DNA (RAPD; Artiushin and Minion, 1996), amplified fragment length polymorphism (AFLP; Kokotovic et al., 1999), pulsed-field gel electrophoresis (PFGE; Stakenborg et al., 2005), PCR combined with restricted fragments length polymorphism (PCR-RFLP; Stakenborg et al., 2006; Charlebois et al., 2014), individual locus sequencing (Mayor et al., 2007), multi-locus sequencing typing (MLST; Mayor et al., 2008) and multiple-locus variable number of tandem repeats (VNTR) analysis (MLVA; Vranckx et al., 2011). The importance of these variable VNTR regions relies on its capability to recombine (Torres-Cruz and van der Woude, 2003), increasing the genomic and proteomic variability of *M. hyopneumoniae* (Calus et al. 2007; Vranckx et al. 2011; Galina-Pantoja et al. 2016). The use of many different techniques covering different targets of detection, different power of discrimination and reproducibility has

complicated the comparison and interpretation of results published to date (Stakenborg et al., 2006). However, in the latest peer-reviewed studies, the more frequently used techniques are based on MLVA of loci related with adhesion to the host cells (Minion et al., 2000; Bogema et al., 2012).

Up to now, *M. hyopneumoniae* genetic diversity has been detected at farm, batch, pig and even at sample level, with variable *inter* and *intra* farm genetic variability (Nathues et al., 2011; Vranckx et al., 2012a; Charlebois et al., 2014; Dos Santos et al., 2015; Michiels et al., 2017a). Variability of *M. hyopneumoniae* has been studied in different scenarios including non-vaccinated (Vranckx et al., 2012a; Overesch et al., 2017) and vaccinated pigs (Michiels et al., 2017a, Charlebois et al., 2014; Tamiozzo et al., 2015), as well as in pigs with unknown vaccination status (Nathues et al., 2011). Additionally, some of the *M. hyopneumoniae* strain used as bacterins have also been genotyped (Vranckx et al., 2011; Charlebois et al., 2014; Tamiozzo et al., 2015). However, a contemporaneous comparison of *M. hyopneumoniae* variability between non-vaccinated and vaccinated animals, and the strain of the vaccine used in the vaccinated farms has not been assessed. Therefore, the aim of the present study was to compare, using conventional sequencing of different loci, the *M. hyopneumoniae* typing profiles (TP) detected in vaccinated and non-vaccinated slaughtered pigs showing CVPC lesions, as well as in the vaccines used in such farms.

2. Materials and methods

2.1 *Farm selection*

Ten vaccinated (V) and ten non-vaccinated (NV) fattening farms experiencing clinical respiratory signs compatible with *M. hyopneumoniae* infection (dry cough and presence of animals with CVPC in lungs at slaughterhouse) were selected. A fattening farm was

included as V when pigs were vaccinated against *M. hyopneumoniae* at weaning. Pigs from NV fattening farms did not receive *M. hyopneumoniae* vaccination at any point in the production cycle. Information on farm batch, vaccine products used as well as authorization for the slaughter checks were obtained from the practitioner and/or the producer. Farms included in the study were located in north-eastern Spain.

2.2 *Lung lesion scoring and sample collection at slaughterhouse*

Twenty lung batches (from 10 NV and 10 V farms) of finishing pigs with *M. hyopneumoniae* compatible lung lesions (purple to grey pulmonary consolidation areas, generally located bilaterally in the cranio-ventral areas; Maes et al., 2008) were individually scored at slaughterhouse. The scoring system used to quantify the *M. hyopneumoniae*-like lung lesions was the Ceva Lung Program (CLP), a lung scoring software based on two methods previously described (Christensen et al., 1999; Madec and Kobisch, 1982). In the CLP system, each lobe was scored from 0 to 4 points according to the following classification: 0) no lesion, 1) lesion affecting <25% of the lobe surface, 2) lesion affecting ≥25% to <50% of the lobe surface, 3) lesion affecting ≥50% to <75% of the lobe surface and 4) lesion affecting ≥75% of the lobe surface (Madec and Kobisch, 1982). Each lobe score was finally normalized by its relative volume (Christensen et al., 1999). Moreover, an additional point to the total lung score was considered when a scar was present in any lung lobe. The EP-index of each farm was calculated as the mean score of all evaluated lungs.

Three lungs showing the most extensive CVPC lesions within each batch were selected for *M. hyopneumoniae* strain variability detection and characterization. From those lungs, a portion of each lobe (including affected and healthy tissue) were collected

to increase the rate of bacterium detection. Samples were transported in refrigeration to the laboratory where they were stored at -80ºC until used.

2.3 *Vaccines and reference strains*

Five commercial vaccines (bacterins) against *M. hyopneumoniae* (A, B, C, D and E) used in V farms were included for genotyping. All the vaccine strains (VS) were genotyped from its corresponding commercial product, except for the strain coming from the vaccine E, which was directly genotyped from a bacterial culture (kindly provided by manufacturer E), due to the impossibility of being directly amplified from the vaccine product. Moreover, the two reference strains (RF), the strain 11 (ATCC® 25095™) and the type strain J (ATCC® 25934™), were included in the study as controls. In order to test the RF in the same conditions as the strains detected in lung samples, different lung tissue portions negative to rt-PCR were spiked with each RF culture *in vitro* and subsequently processed as slaughterhouse lung portions.

2.4 *DNA extraction and M. hyopneumoniae detection*

Approximately 1 cm^3 including affected and healthy lung tissue from slaughtered pigs or the lung portion spiked with *M. hyopneumoniae* RF strain was homogenized in plastic tubes with 600 µL of PBS and glass beads. These lung homogenates were disrupted using TissueLyser (Qiagen GmbH, Germany) by shaking for 10 min and centrifuged at 11,000 g for 1 min. After centrifugation, 200 µL of tissue supernatant was collected for DNA extraction. Likewise, 200 µL of each vaccine product was directly used for extraction.

DNA extraction from tissue supernatant, vaccines and *M. hyopneumoniae* culture (vaccine E strain) was performed by MagMax™ DNA Multi-Sample Kit (Life

148 Technologies, USA) according to the manufacturer's instructions on the BioSprint 96
149 workstation (Qiagen GmbH, Germany). Two different positive extraction controls were
150 used: 1) a lung tissue portion spiked with *M. hyopneumoniae* strain 11
151 (ATCC®25095™) was added to each extraction plate; and 2) a commercial internal
152 positive control (Xeno™, included in qPCR Master Mix kit, VetMax™-Plus, Life
153 Technologies, USA) was added to every tissue sample. Negative controls (PBS) were
154 also included to assess potential contamination during extraction.

155 Extracted DNA was tested by a commercial real time PCR (rt-PCR) for *M.*
156 *hyopneumoniae* detection: VetMax™-Plus qPCR Master Mix (Life Technologies, USA)
157 with VetMax™ *M. hyopneumoniae* Reagents (Life Technologies, USA) according to
158 the manufacturer's instructions. A positive DNA control for amplification of *M.*
159 *hyopneumoniae* (VetMax™ *M. hyopneumoniae* Controls) was also included in the rt-
160 PCR procedure. All rt-PCR runs were carried out in ABIPRISM® 7500 machine
161 (Applied Biosystems, Singapore). The rt-PCR threshold was set at 10% of the
162 maximum fluorescence value of the commercial DNA positive control. Samples with
163 cycle threshold (Ct) values lower than 40 were considered positive. Only samples with
164 *M. hyopneumoniae* positive rt-PCR with Ct values ≤30 were selected to be
165 characterized by sequencing of different loci (Galina-Pantoja et al., 2016).

166

167 2.5 *Characterization of M. hyopneumoniae strains*

168 Characterization of *M. hyopneumoniae* strains was based on the VNTR count of four
169 different loci related with the adhesion: P97 (repeat region 1, RR1), P146 (RR3), H1
170 (complete loci) and H5 (RR2; Vranckx et al., 2011). For the genotyping assay, the four
171 loci were individually amplified in a final volume of 50 μL. Reaction mixtures
172 contained 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide

triphosphate, 0.4 µM of each primer, 1.5 U of GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, USA), and finally, 6 µL of extracted DNA diluted 1:10. Cycling conditions were 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C; then, a final extension step of 7 min at 72°C was performed.

The PCR products from each locus were analyzed by electrophoresis on 2% agarose gel in Tris-Acetate-EDTA (TAE)-buffer and stained with ethidium bromide. Afterwards, products were purified by ExoSAP-IT® (Isogen Life Science, The Netherlands) according to manufacturer's instructions and sequenced using ABI PRISM 3130xl (Applied Biosystems, Singapore) genetic analyzer.

2.6 *Data analysis*

Nucleotide sequences were aligned to obtain the consensus sequence obtained using FingerPrinting II Informatix software (Applied Maths, Saint-Martens-Latem, Belgium). The translation of consensus sequences to protein sequences was performed using the Expasy web tool (http://web.expasy.org/translate). Subsequently, VNTR counts per locus were performed, according to the following amino acid repetitions: AAKP[EV] for P97, S for P146, QTTQ(KD) for H1 and Q for H5 (Vranckx et al., 2011). All translated sequences obtained for each loci were first aligned using MUSCLE (v3.8.31) and, then, concatenated to obtain all the loci per sample. Such analysis was performed in two different fashions, considering 4 loci, P97-P146-H1-H5 (Michiels et al., 2017a; Vranckx et al., 2011), or 2 loci, P97-P146 (Dos Santos et al., 2015; Galina-Pantoja. et al., 2016; Takeuti et al., 2017). Afterwards, a TP was defined for each different combination of VNTR when 4 (TP4) or 2 loci (TP2) were considered. Minimum spanning trees, constructed considering the origin and type of the sample (V and NV

197 farms, RF and VS) together with the TP detected, were performed in PHYLOViZ 2.0
198 (Ribeiro-Gonçalves et al., 2016).
199
200 2.7 *Statistical analyses*
201 Mean of continuous variables (EP-index and mean of Ct rt-PCR values) between NV
202 and V farms was compared by unpaired *t*-test. All statistical analyses were performed
203 using GraphPad Prism 6.07 software (GraphPad software Inc., San Diego, USA). The
204 significance level was set to $p<0.05$.
205
206 **3. Results**
207 3.1 *Lung lesion scoring*
208 A total of 3,069 lungs were scored with an average (min. – max.) number of lungs
209 evaluated per batch of 153 (93 – 212). The EP-index of each batch is shown in Table 1.
210 The mean of EP-index (mean ± standard deviation [SD]) in NV farms (3.8±1.9) was
211 numerically higher, but not significantly different, than that of V farms (2.2±1.3).
212
213 3.2 *M. hyopneumoniae detection by rt-PCR*
214 From the 60 (30 from V and 30 from NV farms) tested lung samples, 46 (76.7%, 25
215 from NV and 21 from V herds) were positive by rt-PCR (Table 2). However, there were
216 4 farms (V3, V4, V10 and NV4) with all tested samples negative to rt-PCR and one
217 farm (NV5) with only one rt-PCR positive sample. The Ct mean value (±SD) of rt-PCR
218 positive lungs from V farms (25.5±2.9) was slightly higher than that of NV farms
219 (24.5±3.8), although this difference was not statistically significant.
220 The rt-PCR Ct values for the five commercial vaccines against *M. hyopneumoniae*
221 (A, B, C, D and E) and RF are detailed in Table 3.

3.3. *PCR amplification and number of VNTR.*

From the 46 samples that resulted positive by rt-PCR, the 43 (93.5%) having a Ct≤30 were selected to be genotyped. Although the 4 loci were amplified in 43 samples, in only 31 (72.1%) the 4 loci were sequenced appropriately and, therefore, the samples were typeable. However, when only 2 loci (P97 and P146) were considered, 38 (38/43, 88.4%) samples were typeable. Variations with respect to the amino acid sequence of the VNTR previously described were detected (i.e. TTKP[EV] instead of AAKP[VE]). Such repetition was counted but marked with a star (*) in the final number of repetitions of such loci (Table 2).

Only one TP per sample was detected and one to three TP per farm were identified. Considering NV and V farms, different VNTR were identified for each locus, i.e. 7 for P97, 10 for P146, 9 for H1, and 11 for H5. The number of repetitions detected in each locus ranged between 2 to 12 for P97, 13 to 46 for P146, 2 to 18 for H1 and 13 to 27 for H5 (Table 2).

Considering the four loci, 24 different *M. hyopneumoniae* TP were identified: 12 in NV and 12 in V farms (Table 2). In contrast, when only 2 loci (P97 and P146) were considered, 17 different TP were detected, 9 TP in NV and 9 in V farms, because TP2-6 was present in NV and V farms (Table 2).

Regarding VS, vaccine B was considered non-typeable in all four genes due to the failure in the amplification of loci P97 and H5. Thus, in the five VS, a total of four different TP were identified using either four or two loci (Table 3). As expected, detected TP in vaccinated and non-vaccinated pigs were different from the tested VS either using four or two loci (except in the case of vaccine E and one of the pigs from farm NV6, which had the same TP when only two loci were considered). Likewise, in the *M. hyopneumoniae* RF, one TP was identified in each culture, being different from

247 TPs detected in vaccinated pigs and VS, regardless the number of loci considered for
248 the analysis. The minimal spanning trees depicting the similarity of the detected TP
249 according to four loci (A) and two loci (B) are shown in Figure 1.
250
251 **4. Discussion**
252 This study attempted to describe the *M. hyopneumoniae* genetic variability in lungs
253 showing EP compatible lesions from various NV and V Spanish farms. In addition,
254 variability was compared with the strain of the vaccine used in each of the V farms, as
255 well as with two RF. To reach the goals, previously described regions from four loci
256 related with adhesion (Vranckx et al., 2011) were sequenced, VNTR were counted and
257 used to define the TP of each strain and the number of TP per farm.
258 Although the assessment of the vaccine efficacy was not included in the objective
259 of the present study, the differences in EP-index between V and NV farms were
260 calculated. Generally, lungs from NV farms showed a numerically higher EP-index
261 (more severe lesions) than V farms. The fact that the differences between V and NV
262 farms were not statistically significant might be explained by the different disease status
263 of evaluated farms, the different vaccines used, the sample size, as well as the potential
264 failure or inefficiency of vaccines. Interestingly, V farms with vaccine B (V10) or with
265 vaccine E (V3 and V4) in which *M. hyopneumoniae* was not detected in the tested
266 samples, showed the lowest EP-index. This finding would be in favor of the previously
267 suggested capability of vaccines to decrease the bacterial load implying a lung lesion
268 reduction (Vranckx et al., 2012b; Woolley et al., 2012; Michiels et al., 2017b).
269 However, similar results (no *M. hyopneumoniae* detection by rt-PCR) were detected in
270 farms NV4 and NV5. This can be explained by the fact that the lesions were on a
271 resolution phase or were caused by other respiratory pathogens (i.e. *swine influenza*

virus; Maes et al., 2008). Therefore, considering that, in this study the presence of other CVPC-causing pathogens was not investigated, no definitive conclusions regarding the effect of the vaccine on lung lesions reduction can be reached in the farms where *M. hyopneumoniae* was not detected.

In the present study, only one *M. hyopneumoniae* TP per sampled lung was able to be detected by Sanger sequencing. This result would differ from those previous studies, in which MLVA has been used, and in which co-infections with more than one *M. hyopneumoniae* strain (or TP) at pig level were described (Nathues et al., 2011; Vranckx et al., 2012a; Charlebois et al., 2014; Michiels et al., 2017a). These divergent results could be derived from intrinsic limitations of the different techniques. While the observation of different peaks in MLVA informs on the presence of different strains, Sanger sequencing most probably identifies the predominant one (Vranckx et al., 2011, 2012a, Michiels et al., 2017a). Indeed, the effect of multiple strain infection at the level of lung lesion is still controversy. Whereas Michiels et al. (2017a) linked the number of different strains detected with the severity of lesions, other authors have not observed such association (Vranckx et al., 2012a; Charlebois et al., 2014).

The use of different genotyping techniques influences the number of TP profiles obtained and, thus, the conclusions on *M. hyopneumoniae* genetic variability. In Sanger sequences the number of VNTR is visually and directly counted from each sequence per locus allowing a clear definition of the obtained TP (unique combination of loci; Falde et al., 2018). On the contrary, in MLVA, the number of VNTR is estimated from the height of the peak. Therefore, in cases of multiple infections, multiple peaks per loci would be obtained, and the exact number of VNTR for each strain would not properly ascertained.

296 An interesting conclusion from the data is that the number of TP obtained varied
297 according to the number of loci used: the higher the number of tested loci, the higher the
298 heterogeneity, which is especially evident in the minimum spanning tree. This finding
299 would explain the results obtained in previous studies in terms of genetic diversity.
300 While high genetic diversity was detected at *inter* and *intra* farm level when four loci
301 were considered (Nathues et al., 2011; Tamiozzo et al., 2015; Michiels et al., 2017a), a
302 limited variability has been detected when only two loci were studied, specially within
303 the same herd (Charlebois et al., 2014; Dos Santos et al., 2015; Galina-Pantoja et al.,
304 2016). In the present study, genotyping with two loci allowed to detect the same TP in
305 pigs from farms with the same breeding origin. Although this finding suggests that the
306 sows could be the origin of infection (Sibila et al., 2007), when 4 loci were used, such
307 link was not found. Thus, the slightly difference on TPs using 4 loci could indicate TPs
308 might vary independently during nursery and fattening period.
309 The comparison of VNTR between *M. hyopneumoniae* field samples, VS used in
310 each farm, and RF strains revealed different TP. Interestingly, vaccine A had a different
311 TP compared to that of RF type strain J, from which is originated. This difference could
312 be explained by the effect of serial bacterium passages and/or the inactivation process in
313 the case of vaccine manufacturing. Likewise, in agreement with previous studies, a low
314 similarity between field strains and VS was also found (Charlebois et al., 2014;
315 Tamiozzo et al., 2015). In fact, TPs detected in V farms were different from the VS of
316 corresponding vaccines used in each V farm. It is not known whether this difference
317 among field and vaccine TPs implies differences at antigenic level. If this would be the
318 case, these differences might potentially explain the variable effect of vaccination
319 observed under field conditions (Maes *et al.*, 2008). However, a previous study did not

detect differences on protective efficacy using homologous or heterologous strains as bacterins in experimentally inoculated pigs (Villareal et al., 2012)

Another important point to be considered for *M. hyopneumoniae* genotyping is locus selection. In the present study, four loci previously used in the literature were selected (Vranckx et al., 2011). Among them, the loci most frequently used are the P97 and P146 (Kuhnert et al., 2011; Nathues et al., 2011; Charlebois et al., 2014; Dos Santos et al., 2015; Galina-Pantoja et al., 2016; Takeuti et al., 2017), which encode adhesins involved in binding to cilia (Minion et al., 2000; Bogema et al., 2012). The variability and ranges of VNTR for these loci were in agreement with the previously described in Spanish field isolates (Dos Santos et al., 2015), showing greater variability in P146 than in P97. On the contrary, in other countries such as Brazil, Mexico and United States, P97 has shown more variability than P146, although the ranges of P146 of Brazilian isolates were similar to the Spanish samples (Dos Santos et al., 2015). The other two selected loci, H1 and H5, encode for hypothetical proteins related to adhesion (Vranckx et al., 2011; Tamiozzo et al., 2015; Michiels et al., 2017a). The use of H1 and H5, from which previous information on VNTR was not available, resulted in a high number of non-typeable samples. The lack of success for sequencing has been previously associated with potential *M. hyopneumoniae* mutations in primer-binding sites or with insufficient DNA quantity or quality (Kuhnert et al., 2011; Vranckx et al., 2011; Tamiozzo et al., 2015).

In summary, a high inter-farm *M. hyopneumoniae* genetic variability in slaughtered pigs from V and NV Spanish farms was detected. Interestingly, detected TP in V farms were different from the strain of the corresponding vaccine used either four or two loci. Likewise, the analysis using two loci showed that pigs from farms with the same breeding origin harbored the same *M. hyopneumoniae* TP, but this link was not

observed if four loci were considered. *M. hyopneumoniae* diversity at intra-farm level was limited and the number of TP detected per farm varied according to the number of the loci considered.

Conflicts of interest

Laura Garza-Moreno, Marta Carmona and Roman Krejci are employees of Ceva Santé Animale.

Funding

This work was funded by Ceva Santé Animale, *Secretaria d'Universitats i Recerca* del *Departament d'Economia i Coneixement de la Generalitat de Catalunya* (2015DI078). The CERCA Programme (*Generalitat de Catalunya*) to IRTA is also acknowledged.

Acknowledgments

The authors thank Eva Huerta, Ana del Valle and Javier Mozota for technical collaboration. We are also grateful to practitioners, farmers and slaughterhouse personnel involved in this study. CERCA Programme (*Generalitat de Catalunya*) is also acknowledged.

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