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1 *Mycoplasma hyopneumoniae* **variability*****:*** **Current trends and proposed terminology for**

2 **genomic classification**

3

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RUNNING TITLE: *M. HYOPNEUMONIAE* VARIABILITY AND CLASSIFICATION

Summary

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the etiologic agent of enzootic pneumonia in swine, a prevalent chronic respiratory disease worldwide. *Mycoplasma hyopneumoniae* is a small, self-replicating microorganism that possesses several characteristics allowing for limited biosynthetic abilities, resulting in the fastidious, host-specific growth, and unique pathogenic properties of this microorganism. Variation across several isolates of *M. hyopneumoniae* has been described at antigenic, proteomic, transcriptomic, pathogenic, and genomic levels. The microorganism possesses a minimal number of genes that regulate the transcription process. Post-translational modifications (PTM) occur frequently in a wide range of functional proteins. The PTM by which *M. hyopneumoniae* regulates its surface topography could play key roles in cell adhesion, evasion, and/or modulation of the host immune system.

The clinical outcome of *M. hyopneumoniae* infections is determined by different factors, such as housing conditions, management practices, co-infections, and also by virulence differences among *M. hyopneumoniae* isolates. Factors contributing to adherence and colonization as well as the capacity to modulate the inflammatory and immune responses might be crucial.

Different components of the cell membrane (i.e. proteins, glycoproteins and lipoproteins) may serve as adhesins and/or be toxic for the respiratory tract cells. Mechanisms leading to virulence are complex and more research is needed to identify markers for virulence.

The utilization of typing methods, and complete or partial-gene sequencing for *M. hyopneumoniae* characterization has increased in diagnostic laboratories as control and elimination strategies for this microorganism are attempted worldwide. A commonly employed molecular typing method for *M. hyopneumoniae* is Multiple-Locus Variable number tandem repeat Analysis (MLVA). The

agreement of a shared terminology and classification for the various techniques, specifically MLVA, has not been described, which makes inferences across the literature not suitable. Therefore, molecular trends for *M. hyopneumoniae* have been outlined and a common terminology and classification based on VNTR types has been proposed.

Keywords: genomic classification, *Mycoplasma hyopneumoniae*, terminology, VNTR, variability

1. **Introduction**

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the etiologic agent of enzootic pneumonia (EP) in swine (Mare & Switzer, 1965; Goodwin et al., 1965), a worldwide prevalent, chronic respiratory disease (Maes et al., 2018). Similar to other mycoplasmas*, M. hyopneumoniae* is a small, self-replicating microorganism that possesses several characteristics allowing for limited biosynthetic abilities, resulting in the fastidious, host-specific growth, and unique pathogenic properties of this microorganism (Razin et al., 1998). The pathogenesis of *M. hyopneumoniae* is complex and not yet fully elucidated. It is perceived that the level of colonization and initial development of EP depend on the ability for *M. hyopneumoniae* to adhere to ciliated epithelium within respiratory airways, which is mediated through receptor-ligand interactions and surface adhesion proteins (Ross, 1999; Minion et al., 2000). Once adherence occurs, the epithelium becomes deciliated and bronchial epithelial and goblet cells are lost, leading to the impairment of the mucociliary tract apparatus and the invasion of secondary respiratory pathogens to induce disease (DeBey et al., 1992; Thacker & Minion, 2012). The development of EP is assumed to occur from antigenic variation (Wise, 1993; de Castro et al., 2006), influx of pro-inflammatory cytokines to the site of infection due to alteration in the host immune response (Thacker et al.,

69 2000; Rodriguez et al., 2004), and potentially the formation of biofilms in the airways (Raymond
70 et al., 2018). Clinical signs suggestive of *M. hyopneumoniae* infection consist of dry cough,
71 labored breathing, and reduced growth rate (Thacker and Minion, 2012), which usually occur after
72 six to eight weeks post-exposure (Sibila et al., 2009). Economical losses result from the clinical
73 manifestations of EP, and the increased costs of prevention and eradication, that are implemented
74 to combat further disease and occurrence of secondary bacterial pathogens infections (Pointon et
75 al., 1985; Straw et al., 1990; Maes et al., 2018). Control strategies for *M. hyopneumoniae,* including
76 antimicrobials, vaccines, and management practices, have been employed to reduce the economic
77 losses derived from the clinical manifestations of EP (Pieters and Fano, 2016; Maes et al., 2018;
78 Garza-Moreno et al., 2018).

79 Several laboratory techniques have been utilized to characterize *M. hyopneumoniae* and have
80 shown differences at antigenic, proteomic, transcriptomic, pathogenic, and genomic levels
81 (Assunçao et al., 2005; Calus et al., 2007; Minion et al., 2004). Understanding *M. hyopneumoniae*
82 at the molecular level would eventually aid in the advancement of disease control strategies.
83 However, discrepancies regarding the molecular characterization of *M. hyopneumoniae* are present
84 across the literature, and inferences based on such information can be challenging to agree upon.
85 Common methods for genomic classification and interpretation would provide clarity on disease
86 and outbreak investigations for this microorganism. Therefore, this review article aims to
87 summarize published information on *M. hyopneumoniae* variability at the antigenic, proteomic,
88 transcriptomic, pathogenic, and genomic levels, while highlighting methods employed for the
89 characterization and classification at the genomic level, proposing commonality among
90 investigations.

91 ## 2. ***Mycoplasma hyopneumoniae* variability**

92 *Mycoplasma* species have been suggested to have phylogenetically evolved from gram-positive,
93 walled eubacteria, allowing for unique, taxonomic characteristics (Maniloff, 1983; Razin, 1985).
94 Studies have demonstrated the ability of *M. hyopneumoniae* to undergo phenotypic switching,
95 antigenic variation and proteome alterations that may contribute to the versatility and variations
96 between host-pathogen interactions (Minion et al., 2004; Assunçao et al., 2005; Calus et al., 2007).
97 Variability has been demonstrated across several *M. hyopneumoniae* strains and isolates at the
98 antigenic, proteomic, transcriptomic, pathogenic, and genomic level. For the purpose of this study,
99 the difference between a bacterial strain and an isolate is that the former has been characterized
100 (classified and/or identified) and the latter has been obtained from single colony cloning using
101 pure culture.

102 *2.1. Antigenic variability*

103 Antigenic variability is described as the ability of a microorganism to alter its surface components
104 (i.e. flagella, microvilli and/or membrane proteins, among others), causing differences in the host
105 immune response against those structures (Razin et al., 1998). Among *M. hyopneumoniae* strains,
106 antigenic variability, has been evaluated in several studies using different serologic and proteomic
107 assays, such as immunoblotting and two-dimensional gel electrophoresis (Ro & Roos, 1983;
108 Scarman et al., 1997; Assunçao et al., 2005). Techniques used, antigenic proteins identified, and
109 the most relevant findings of each study are summarized in Table 1.
110 *Mycoplasma hyopneumoniae* has a relatively small number of proteins with identified antigenic
111 properties (Table 1), including cytosolic (i.e. P36), membrane (i.e. P44, P46, P50, P65, P70, P74
112 and P76, among others) or adhesion proteins (i.e. P97). The cytosolic protein P36, named lactate
113 dehydrogenase (LDH; Hadimann et al., 1993), was detected in 13 *M. hyopneumoniae* field strains
114 from Switzerland, Hungary, France and Canada (Stipkovits et al., 1991). In contrast, a subsequent

115 report concluded that this protein was not detected in the 18 studied *M. hyopneumoniae* field strains
116 from Spain (Assunção et al., 2005). However, it remains unknown whether this lack of detection
117 is the result of a true absence or is derived from the lack of expression due to unfavorable culture
118 conditions, as previously described for other bacteria such as *Escherichia coli* (Jang et al., 2001),
119 or a detection failure due to substantial sequence variability between different *M. hyopneumoniae*
120 strains.
121 Information regarding membrane protein immunoreactivity is scarce. For example, P46 was
122 identified in all tested strains (Assunção et al., 2005) and it was considered useful for the
123 development of an ELISA test for *M. hyopneumoniae* antibody monitoring (Okada et al., 2005).
124 Another membrane protein (P74) with well-known antigenic properties is currently used as antigen
125 for a commercial ELISA test to detect antibodies against the pathogen (Bereiter and Young, 1990).
126 In addition, P44, P50, P65 and P70 are also membrane proteins that confer different proteolytic
127 epitope maps (Wise & Kim, 1987a, 1987b). Similarly, antigenic variability of adhesins such as
128 P97, involved in the adherence of *M. hyopneumoniae* to the cilia*,* was detected in all studied strains
129 (Zhang et al., 1995; Assunção et al., 2005).
130 Differences of antigenic profiles has been related to the variation in the size (Assunção et al., 2005;
131 Wise & Kim, 1987b; Zhang et al., 1995), the expression of the proteins (Rosengarten & Yogev,
132 1996; Razin et al., 1998), and adaptation mechanisms of *M. hyopneumoniae* against environmental
133 changes, e.g. culture passages (Razin et al., 1998; Assunção et al., 2005). Such variability has been
134 proposed as a pathogenic mechanism to evade the porcine immune response (Zhang et al., 1995).
135 Although *Mycoplasma hyopneumoniae* antigenic diversity has been shown, a close relationship of
136 antigenic profiles between different strains has also been described (Ro and Ross, 1983). In fact,
137 comparable antigenic profiles (immunoreactivity against proteins of molecular weight from 36 to

138 200 kDa) were detected in different *M. hyopneumoniae* strains from Australia, the United
139 Kingdom and the United States using sodium dodecyl sulfate polyacrylamide gel electrophoresis
140 (SDS-PAGE) as well as immunoblotting techniques (Scarman et al., 1997). This information
141 suggests that limited antigenic variability among several *M. hyopneumoniae* strains obtained from
142 different geographical locations can also occur, proposing the presence of conserved antigens
143 related to the survival of the species (Scarman et al., 1997).
144 Additionally, the existence of cross-reactive antigens between *M. hyopneumoniae* and other swine
145 mycoplasmas like *M. hyorhinis, M. hyosynoviae* and *M. flocculare* has been documented (Table
146 1). The closest antigenic relationship was described between *M. hyopneumoniae* and *M. flocculare*
147 strains (Ro & Ross, 1983; Petersen et al., 2016). In fact, one recent study indicated that only 3
148 *(mhp182*, *mhp638* and *mhp684)* out of 39 tested *M. hyopneumoniae* proteins were exclusively
149 present in *M. hyopneumoniae* and did not cross-react with *M. hyorhinis, M. hyosynoviae and M.*
150 *flocculare* (Petersen et al., 2016). Similarly, a 43 kDA protein was identified as reactive with *M.*
151 *hyopneumoniae* but not with *M. flocculare* or *M. hyorhinis* (Scarman et al., 1997).
152 The *M. hyopneumoniae* antigenic variability described in the previously mentioned studies may
153 have several implications. Although the mechanisms of *M. hyopneumoniae* pathogenesis have not
154 been fully elucidated, surface protein variation might play a crucial role in *M. hyopneumoniae*
155 adhesion to the ciliated epithelium (Wise & Kim, 1987) and to evade the host immune system
156 (Maes et al., 2018). Indeed, antigenic differences could modify the ability to induce immune
157 responses among *M. hyopneumoniae* strains as it has been observed under experimental (Vicca et
158 al., 2002; Strait, 2003) and natural conditions (Ameri et al., 2006). Likewise, it is still unclear
159 whether putative antigenic variations may imply differences in the antibody profiles generated by
160 field strains when compared to those used in bacterins (Villarreal et al., 2012). Furthermore, the

161 cross-reactivity detected between *M. hyopneumoniae* and other swine mycoplasmas may interfere
162 with the results of serological diagnostic methods (Rosengarten and Yogev, 1996; Assunçao et al.,
163 2005; Petersen et al., 2016). Therefore, these findings indicate the need to identify conserved *M.*
164 *hyopneumoniae* antigens as targets to develop new vaccines against this bacterium, as well as
165 highly accurate serological diagnostic tests.

166 *2.2. Proteomic and transcriptomic variability*

167 Under adverse conditions, *M. hyopneumoniae,* like other bacteria, launches "stress responses" that
168 improve its chances of a successful adaptation to the challenges posed by unfavorable
169 environments. Such adaptation is reflected in protein diversification by means of different
170 mechanisms, thus allowing the microorganism to survive, colonize, and cause disease.

171 One mechanism of protein diversification is gene expression regulation. Several transcriptomic
172 studies have aimed to identify differences in gene expression, under different environmental
173 conditions, such as heat shock (Madsen et al., 2006), iron deprivation (Madsen et al., 2006),
174 oxidative stress (Schafer et al., 2007), and norepinephrine exposure (Oneal et al., 2008). Most of
175 the referenced studies agree that, in face of adverse conditions, an increase in the regulation of
176 metabolic related genes occurs, while there is an expression decrease in transcription and
177 translation of related genes.

178 Using different approaches, high variability of total proteins among field and reference *M.*
179 *hyopneumoniae* strains has been observed (Assunção et al., 2005; Calus et al., 2007; Pinto et al.,
180 2007; Pinto et al., 2009). In fact, the analysis of *M. hyopneumoniae* field isolates, obtained from
181 several herds located in the same or different countries with various degrees of virulence,
182 demonstrated high protein variability without relation to isolate source (Assunção et al., 2005;
183 Calus et al., 2007). High protein heterogeneity has been already observed between pathogenic (i.e.

184 232 and 7448) and non-pathogenic (i.e. J) *M. hyopneumoniae* strains (Pinto et al., 2007; Pinto et
185 al., 2009; Li et al., 2009). Moreover, Paes et al. (2017) compared the secretomes of *M.*
186 *hyopneumoniae* strain 7448 and the commensal *M. flocculare*. They identified that a higher
187 number of proteins were secreted by the virulent *M. hyopneumoniae* strain 7448 (62) compared to
188 *M. flocculare* (26). Overall, 15 virulence-related proteins were identified in *M. hyopneumoniae*
189 strain 7448 and only four in *M. flocculare*, of which two proteins were shared with *M.*
190 *hyopneumoniae*. However, evidence suggests that protein variability is not necessarily related to
191 virulence (Calus et al., 2007; Pinto et al., 2007; Pinto et al., 2009). Pinto et al. (2009) showed
192 differential expression in approximately one-third of the proteome between two strains of similar
193 virulence. Furthermore, an overexpression of proteins related to heat-shock and oxidative stress
194 responses was observed in the pathogenic *M. hyopneumoniae* strains 7448 and 7422, compared to
195 the non-pathogenic *M. hyopneumoniae* strain J.

196 *Mycoplasma hyopneumoniae* possesses a minimal number of genes that regulate the transcription
197 process (Vasconcelos et al., 2005), thus, the regulation of post-translational processes is relevant
198 for this pathogen. Post-translational modifications (PTM) occur frequently in a wide range of
199 functional proteins of *M. hyopneumoniae* (Tacchi et al., 2016). Endoproteolytic cleavage has been
200 identified in cell surface proteins as lipoproteins, multitasking proteins (Tacchi et al., 2016) and
201 P97/P102 adhesins (Bogema et al., 2011; Bogema et al., 2012; Djordjevic et al., 2004; Deutscher
202 et al., 2010, Deutscher et al., 2012; Raymond et al., 2013; Raymond et al., 2015; Seymour et al.,
203 2012; Tacchi et al., 2014; Wilton et al., 2009). The PTM by which *M. hyopneumoniae* regulates
204 its surface topography could play key roles in cell adhesion and evasion and/or modulation of the
205 host immune system.

206 Finally, secreted proteins from both, swine respiratory cells and *M. hyopneumoniae,* play
207 important roles between the host-pathogen interaction. *Mycoplasma hyopneumoniae* strain 7448
208 secretes higher number of adhesins and other proteins with known function than *M.*
209 *hyopneumoniae* J strain and *M. flocculare* (Leal Zimmer et al., 2018; Paes et al., 2017), which may
210 be related to bacterial pathogenicity. On the other hand, swine tracheal cells showed different
211 secretion profiles in response to the infection with *M. hyopneumoniae* strains or with *M. flocculare*
212 (Leal Zimmer et al., 2018). Some proteins secreted in response to cell injury and death, as Damage-
213 associated molecular patterns (DAMPs) proteins and extracellular proteasome proteins, have been
214 detected only in swine cells infected with the *M. hyopneumoniae* 7448 (Leal Zimmer et al., 2018).
215 These results suggest differences in the secretome composition from *M. hyopneumoniae* according
216 to the virulence of the strain.

217 *2.3. Pathogenic variability*

218 The pathogenicity of an organism refers to its ability to cause disease or lesions to the host.
219 Commensals and opportunistic pathogens lack this inherent ability. Virulence refers to the degree
220 of disease or lesion severity caused by the organism.

221 *2.3.1. Differences in virulence*

222 The clinical outcome of *M. hyopneumoniae* infections is determined by different factors such as
223 housing conditions, management practices, infections with other pathogens, and also by
224 differences among *M. hyopneumoniae* strains.

225 Previous studies have shown that *M. hyopneumoniae* field isolates may induce different levels of
226 disease and lesions upon infection. The first and most extensive study was conducted by Vicca et
227 al. (2003) in which the virulence of six field isolates of *M. hyopneumoniae* was compared using

228 challenge infections in conventional pigs followed by necropsy four weeks later (commonly
229 accepted as the standard infection model). The isolates were classified as highly, moderately, or
230 low virulent based on severity of clinical signs and macroscopic and microscopic lung lesions
231 produced. The virulence of some of these *M. hyopneumoniae* isolates was confirmed in subsequent
232 studies (Meyns et al., 2004; Villarreal, 2009; 2011). Woolley et al (2012) also observed differences
233 between two Australian *M. hyopneumoniae* field isolates (i.e. Hillcrest and Beaufort), with the first
234 one being superior to experimentally induce more severe coughing and pneumonic lesions than
235 the second isolate.

236 Although *M. hyopneumoniae* is primarily a respiratory pathogen, it has also been isolated (Le
237 Carrou et al., 2006; Marois et al., 2007; Marchioro et al., 2014) or detected by PCR (Woolley et
238 al., 2012) in liver, spleen, and kidneys of experimentally infected and contact pigs. However, this
239 spread within the body appears to be transient and is likely not involved in the development of
240 disease (Marois et al., 2007; Woolley et al., 2012). Woolley et al. (2012) could only detect the
241 highly virulent isolate in internal organs and not the low virulent isolate. Interaction with surface
242 accessible actin on the epithelial cells and the causation of cytoskeletal rearrangements would
243 allow this microorganism to be phagocytosed. It has been hypothesized that *M. hyopneumoniae*
244 can survive within and escape the phagolysosome and reside within the cytoplasm (Tacchi et al.,
245 2016). Therefore, it can not only evade the immune system but could disseminate to internal
246 organism and persist within the host without causing disease. Recent work by Raymond et al.
247 (2018) showed that approximately 8% of *M. hyopneumoniae* cells reside intracellularly, which
248 demonstrates the need to further explore this topic.

249 *2.3.2. Virulence factors*

250 Little is known about the mechanisms involved in these differences and the virulence factors of
251 *M. hyopneumoniae*. It is also unclear why in certain pigs, *M. hyopneumoniae* might remain in the
252 respiratory tract without causing clinical signs, or which factors are involved in progressing from
253 presence of the pathogen to infection. Factors contributing to adherence and colonization, as well
254 as the capacity to modulate the inflammatory and immune response might play a role. Meyns et
255 al. (2007) showed that the difference between the highly and low virulent isolates was associated
256 with a faster *in vitro* growth, a higher capacity to multiply in the lungs and the induction of a more
257 severe inflammation process (e.g. higher TNF-alpha and IL-1β concentration in BAL fluid) by the
258 highly virulent isolate. Woolley et al. (2012) also detected significantly higher levels of IL-1β and
259 IL-6 in BAL fluid in pigs challenged with a highly virulent strain. A faster *in vitro* multiplication
260 of a highly virulent isolate during the logarithmic phase was confirmed by Calus et al. (2010),
261 suggesting a higher capacity to multiply in the lungs. In contrast, García-Morante et al. (2018)
262 described the fastest growing strain to be *M. hyopneumoniae* strain J using color changing units.
263 Villarreal et al. (2011) demonstrated that contrary to a highly virulent strain, a low virulent strain
264 required more than 4 weeks post-infection to reach maximum infection levels and clinical signs.

265 Different components of the cell membrane (i.e. proteins, glycoproteins and lipoproteins) may
266 serve as adhesins and/or be toxic for the respiratory tract cells. Some of the molecules are post-
267 translationally processed and cleaved. It is possible that highly virulent strains have a different cell
268 surface composition and/or a different proteolytic processing. It has been proposed that a minimum
269 of eight repeat region 1 (RR1) units located at the carboxy terminus of the *mhp183* gene as the
270 cilium- and antibody-binding sites of P97, is required for cilium binding (Minion et al., 2000).
271 Whilst the amino acid sequence of P97 is highly conserved amongst *M. hyopneumoniae* strains
272 (Wilton et al., 1998) the number of tandem 5-amino acid repeats in RR1 varies considerably

273 between isolates with the *M. hyopneumonia*e J strain, possessing only nine tandem copies in
274 contrast to the fifteen copies present in *M. hyopneumoniae* strain 232 (Wilton et al., 1998).
275 However, adherence by *M. hyopneumoniae* to epithelial cilia is a multifactorial process involving
276 an array of adhesins including those which lack RR1, and therefore the number of repeats in RR1
277 as such may not be suitable as a virulence marker.

278 It has been described that *M. hyopneumoniae* pathogenic strains were able to increase intracellular
279 calcium concentrations in porcine ciliated epithelial cells. However, such increase was not
280 observed in non-pathogenic *M. hyopneumoniae* and *M. flocculare* strains (Park et al., 2002).
281 Additionally, *M. hyopneumoniae* strains characterized as highly virulent were able to induce
282 higher cytoplasmic calcium concentrations in neutrophils, compared to low virulent ones,
283 suggesting that signal transduction mechanisms in neutrophils are altered by virulent *M.*
284 *hyopneumoniae* strains (Chen et al., 1992).

285 *Mycoplasma hyopneumoniae* strain passaging *in vitro* or *in vivo* may alter the virulence quickly.
286 DeBey and Ross (1994) showed that after 20 to 40 *in vitro* passages compared to only 1 or 2
287 passage(s), *M. hyopneumoniae* strain 232 exhibited a lower capacity to induce ciliary damage.
288 However, when the same previously passaged strain was passaged in gnotobiotic pigs *in vivo*, the
289 strain was reversed to original virulence (DeBey and Ross, 1994). The reference strain J,
290 commonly used in commercial vaccines, was originally a virulent and pathogenic strain causing
291 mild pneumonia in sows. Nevertheless, after continued *in vitro* passaging, *M. hyopneumoniae*
292 strain J lost its pathogenic capacity to adhere and to cause disease in pigs (Zielinski & Ross, 1993).

293 In addition, a 23 kb region was also observed to be similar to a previously described Integrative
294 Conjugal Element (ICE) of *M. fermentans* in the genetic material of *M. hyopneumoniae* strains
295 7448 and 232, that was absent in J-strain (Pinto et al., 2007). This ICE is generally recognized to

296 be involved in virulence in other Mycoplasma species. Therefore, the authors suggested that these
297 putative ICE's might also be implicated in the virulence of *M. hyopneumoniae.* Liu et al. (2013)
298 not only detected this element in the virulent *M. hyopneumoniae* strain 168, but also in its
299 attenuated variant.. Thus, the role of the ICE in the virulence of *M. hyopneumoniae* remains unclear
300 and warrants further research (Maes et al., 2018). Using MLVA testing on lungs from slaughtered
301 pigs, Charlebois et al. (2014) showed that *M. hyopneumoniae* strains lacking the detection of Locus
302 1 protein exhibited significantly less severe lesions and lower numbers of bacteria. The gene
303 amplified by this locus encodes for a hypothetical protein of 77078 kDa, and the authors suggested
304 that this Locus 1 protein might be a potential virulence factor for *M. hyopneumoniae*.

305 Mycoplasmas in general lack classical virulence factors like toxins. Nevertheless, the production
306 of a community-acquired respiratory distress syndrome toxin was identified for *M. pneumoniae,*
307 which has been shown to potentially contribute to this microorganism's pathogenesis (Kannan et
308 al., 2005; Medina et al., 2012). Therefore, the ability to detect the presence of unidentified toxins
309 for other Mycoplasma species may be possible in the future. Ferrarini et al. (2018) recently
310 demonstrated that pathogenic strains of *M. hyopneumoniae* are able to use glycerol as a carbon
311 source thereby enabling the production of the toxic metabolite hydrogen peroxide. In a previous
312 study, Ferrarini et al. (2016) showed that only *M. hyopneumoniae,* but not the commensal *M.*
313 *flocculare,* was able to assimilate myo-inositol, and remained viable when this was the primary
314 energy source. Therefore, mechanisms leading to virulence are complex and more research is
315 needed to identify markers for virulence (Maes et al., 2018).

316 *2.3.3. Effect of the number of different strains on the severity of lung lesions*

317 Several *M. hyopneumoniae* strains can be detected in a herd, and even in one individual pig from
318 bronchoalveolar lavage fluid or lung tissue (Nathues et al., 2011; Vranckx et al., 2011; Dos Santos

et al., 2015; Michiels et al., 2017). Although some studies point in the direction that co-infection with more than one strain in a pig or batch of pigs might result in more severe lung lesions (Villarreal et al., 2009; Vranckx et al., 2011; Michiels et al., 2017), other studies did not detect such a relationship (Charlebois et al., 2014). Michiels et al. (2017) investigated the genetic diversity of *M. hyopneumoniae* strains in different successive batches of ten pig herds using Multiple-Locus Variable number tandem repeat Analysis (MLVA). Multivariable analyses accounting for the potential effect of risk factors for respiratory disease were performed to assess associations between the number of different Variable Number Tandem Repeats (VNTR) variants per batch, and lung lesions as outcome variables. Batches of slaughtered pigs with several *M. hyopneumoniae* strains showed a significantly higher prevalence and severity of *Mycoplasma*-like lung lesions at slaughter, implying that reducing the number of different strains may lead to less lung lesions at slaughter. The exact mechanisms leading to this phenomenon remain to be elucidated.

2.3.4. Acquired antimicrobial resistance

Antimicrobial susceptibility testing of *M. hyopneumoniae* is not routinely performed due to the fastidious isolation of the pathogen. In addition, established breakpoints are lacking for result interpretation and for identifying strains as sensitive or resistant. *Mycoplasma hyopneumoniae* is intrinsically resistant to antibiotics which interfere with the polymerization of cell wall precursors, such as beta-lactam antibiotics, and to polymyxins, 14-membered ring macrolides (such as oleandomycin and erythromycin), trimethoprim and sulfonamides. *In-vitro,* acquired resistance has been documented for tetracyclines, 15-membered ring macrolides (tulathromycin, gamithromycin), 16-membered ring macrolides (tylosin, tilmicosin), lincosamides (lincomycin) and fluoroquinolones (Vicca et al., 2004; Tavio et al., 2014; Klein et al., 2017; Felde et al., 2018).

342 It was shown that mutations in the 23S rRNA gene were responsible for resistance to macrolides
343 and lincosamides (Stakenborg et al., 2005). Isolates that were resistant against quinolones harbored
344 mutations in the quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC* or *parE*.
345 In four out of five resistant isolates, there was only one-point mutation (C → A) in *parC*, resulting
346 in an amino acid change from serine to tyrosine at position 80 (*E. coli* numbering). These isolates
347 showed a minimum inhibitory concentration (MIC) of enrofloxacin of 0.5 µg/mL, while for
348 sensitive isolates the MIC of enrofloxacin was ≤ 0.06 µg/mL. One resistant isolate had an extra
349 mutation (C → T) in *gyrA* resulting in an amino acid change from alanine to valine at position 83
350 (*E. coli* numbering), correlated with an increase in the MIC of enrofloxacin (> 1 µg/mL). Felde et
351 al. (2018) showed that single nucleotide polymorphisms in *parC* correlated with a decrease in
352 fluoroquinolone susceptibility. No mutations resulting in an amino acid change were detected in
353 the QRDR of the *gyrB* and *parE* genes of the selected isolates (Thongkamkoon et al., 2013; Felde
354 et al., 2018). As summarized by Gautier-Bouchardon (2018), *M. hyopneumoniae* strains isolated
355 from 2000 to 2016 showed higher *in vitro* MIC levels for tetracyclines, macrolides, lincosamides,
356 and fluoroquinolones compared to those isolated prior to 2000.

357 *2.4. Genomic variability*

358 Through the employment of several molecular techniques, genomic variation has been evident in
359 several *M. hyopneumoniae* strains and isolates at different geographic and population levels (Table
360 2). The *M. hyopneumoniae* genome has been fully sequenced for six strains and has been described
361 to be composed of 892,758-964,503 base pairs and contain a low G + C content of 28.4-28.6%
362 (Minion et al., 2004; Vasconcelos et al., 2005; Liu et al., 2011; Siqueira et al., 2013; Han et al.,
363 2017). In addition, 679-692 predicted protein coding sequences have been described, in which
364 approximately 44%, 38%, and 12% encode for hypothetically functional, conserved, and unique

365 proteins, respectively (Minion et al., 2004; Vasconcelos et al., 2005; Liu et al., 2011; Siqueira et
366 al., 2013; Han et al., 2017). Like other mycoplasmas, *M. hyopneumoniae* has the ability to modify
367 gene and antigenic expression, which primarily occurs during DNA replication (Minion et al.,
368 2004). Within many lipoprotein and adhesion encoding genes, tandem repetitive regions of DNA,
369 known as VNTRs are present and undergo phase variation, recombination, and slipped strand
370 mispairing during replication, thus resulting in adherence and antigenic variation from
371 modifications in cell surface structure (Razin et al., 1998; Rosengarten et al., 1999; Minion et al.,
372 2000; de Castro et al., 2006). Differences in VNTR length of several adhesins and chromosomal
373 restriction patterns have been described across *M. hyopneumoniae* strains and isolates using
374 complete or individual gene sequencing (Hsu et al., 1998; Vasconcelos et al., 2005; Garza-Moreno
375 et al., 2019), field inversion gel electrophoresis (Frey et al., 1992), and random amplified
376 polymorphism DNA analysis (Artiushin et al., 1996).

377 From a geographical standpoint, increased genomic heterogeneity has been demonstrated across
378 *M. hyopneumoniae* isolates and strains originating from diverse regions (i.e. different countries)
379 compared to those originating from a more localized region (Frey et al., 1992; Dos Santos et al.,
380 2015). The identification of potential drivers (e.g. temporal changes) for *M. hyopneumoniae*
381 genomic heterogeneity across different geographical locations has not been investigated. Several
382 *M. hyopneumoniae* VNTR types have been identified (Vranckx et al., 2011) within a country and
383 individual state, suggesting a wide circulation of *M. hyopneumoniae* VNTR types in swine
384 populations (Vranckx et al., 2011; Dos Santos et al., 2015; Takeuti et al., 2017). Among all VNTR
385 types detected in different countries (i.e. United States, Mexico, Brazil, and Spain), a common *M.*
386 *hyopneumoniae* VNTR type has not been detected (Dos Santos et al., 2015). In comparison, a
387 similar circulation of VNTR types for *M. pneumoniae* has been shown across different countries

388 (Dégrange et al., 2009). Furthermore, in swine dense geographical areas, multiple *M.*
389 *hyopneumoniae* VNTR types has been identified in endemically infected herds and even in
390 individual pigs (Vranckx et al., 2011; Nathues et al., 2011; Michiels et al., 2017). Greater
391 homogeneity and/or identical *M. hyopneumoniae* VNTR types in herds that are of close
392 geographical proximity, or herds that belong to the same production flow, has also been shown
393 (Mayor et al., 2007; Charlebois et al., 2014; Pantoja et al., 2016; Takeuti et al., 2017; Rebaque et
394 al., 2018). In all cases, one distinct *M. hyopneumoniae* VNTR type along with multiple clonal
395 types (i.e. similar VNTR types) have been described. It could be hypothesized that the degree of
396 *M. hyopneumoniae* genetic variability among herds might be influenced by herd management.
397 However, the potential drivers and mechanisms of such heterogeneity have been poorly explored
398 and defined.

399 ## 3. **Molecular characterization methods of mycoplasmas**

400 ### *3.1. Mycoplasma spp.*

401 With the advancement of molecular technology and more comprehensive knowledge of
402 mycoplasmas, the repertoire of molecular diagnostics and characterization methods have expanded
403 from nucleic acid amplification to the identification of single nucleotide polymorphism and
404 variation in VNTR and house-keeping gene length and composition (Diaz & Winchell, 2016). The
405 employment of sequencing and typing methods have allowed for a higher level of discrimination
406 among strains and isolates to further understand *Mycoplasma spp.* epidemiology and pathogenesis
407 (Diaz & Winchell, 2016).

408 ### *3.2. M. hyopneumoniae*

409 Several techniques have been standardized to molecularly characterize *M. hyopneumoniae* using
410 nucleic acid amplification, including Amplified Fragment Length Polymorphism (AFLP;
411 Kokotovic et al., 1999; Stakenborg et al., 2006), Restriction Amplified Polymorphic DNA analysis
412 (RAPD; Artiushin & Minion, 1996; Vicca et al., 2003), Pulse-Field Gel Electrophoresis (PFGE;
413 Stakenborg et al., 2006), Random Fragment Length Polymorphism (RFLP; Stakenborg et al.,
414 2006), and DNA Microarrays (Madsen et al., 2007). However, variations in reproducibility,
415 feasibility, and discriminatory power exist among these molecular methods (Stakenborg et al.,
416 2006; Sibila et al., 2009). To provide further discrimination, Multiple-Locus Sequence Typing
417 (MLST; Mayor et al., 2008), MLVA (Vranckx et al., 2011; Nathues et al., 2011; Dos Santos et al.,
418 2015; Tamiozzo et al., 2015), partial gene sequencing (Tsungda & Minion, 1998; Mayor et al.,
419 2007; Garza-Moreno et al., 2019), and complete genome sequencing (Minion et al., 2004;
420 Vasconcelos et al., 2005; Liu et al., 2011; Siqueira et al., 2013; Han et al., 2017) have been recently
421 employed. While each molecular method has their strengths and disadvantages, it is important to
422 consider the question at hand, resources available, and capabilities that each method can provide
423 prior to the utilization of such techniques.

424 Thus far, the utilization of molecular characterization methods for *M. hyopneumoniae* has been
425 commonly employed in research settings. However, the application of *M. hyopneumoniae* genomic
426 characterization in field investigations has increased in the United States to provide veterinarians
427 additional insight related to the transmission and control of this microorganism. Although
428 complete genome sequencing has been proposed as a highly descriptive and thorough method, the
429 routine application of this technique may represent a more futuristic approach based on its current
430 practicality and feasibility as it applies to disease investigations. Therefore, the utilization of typing
431 methods (i.e. MLVA), complete or partial-gene sequencing for *M. hyopneumoniae*

432 characterization has increased in veterinary diagnostic laboratories as the control and elimination
433 of this microorganism is widely attempted worldwide (Maes et al., 2018).

434 *3.2.1 M. hyopneumoniae typing methods*

435 The most commonly employed genomic typing methods for *M. hyopneumoniae* are MLST and
436 MLVA. The MLST assay has been utilized for many bacterial species and was standardized for
437 the molecular characterization of *M. hyopneumoniae* by Mayor et al (2008). Several loci have been
438 targeted, including putative and house-keeping genes (i.e. *efp, metG, pgiB, recA, adk, rpoB, tpiA,*
439 *gyrB, and gmk*). As identified in *Mycoplasma spp.,* house-keeping genes have been described to
440 be highly conserved, resulting in limited variation among strains (Dumke et al., 2003). The MLVA
441 assay is a typing method that identifies the number of VNTRs within surface proteins and has been
442 employed for the molecular characterization of *M. hyopneumoniae* in Spain, Argentina, Belgium,
443 United States, Mexico and Brazil (Table 3; de Castro et al., 2006; Vranckx et al., 2011; Dos Santos
444 et al., 2015; Tamiozzo et al., 2015; Takeuti et al., 2017; Michiels et al., 2017). Several techniques
445 have described the *M. hyopneumoniae* variability using different loci numbers (e.g. 2-4) and types
446 (i.e. P97, P146, H1, H4, H5; Table 3). The targeted loci have been selected for their presumed
447 bacterial adhesive capability to ciliated epithelium, presence of repetitive, tandem repeats, or high
448 degree of variability (Vranckx et al., 2011; Dos Santos et al., 2015; Tamiozzo et al., 2015; Rebaque
449 et al., 2018). Currently, MLVA assay is commonly performed due to its high discriminatory
450 power, reproducibility, and use of clinical samples has been widely implemented for
451 epidemiological investigations (Vranckx et al., 2011; Dos Santos et al., 2015). Despite this, current
452 discrepancies regarding the genomic characterization of *M. hyopneumoniae* and interpretation
453 currently exist. Like other typing methods, MLVA evaluates few genomic areas. Therefore, other
454 areas within the genome that may influence variability may be unrepresented.

455 *3.2.2 M. hyopneumoniae sequencing methods*

456 Genomic sequencing methods have been developed to evaluate the entire genome or individual
457 genes of *M. hyopneumoniae.* To further explore how the genomic structure of *M. hyopneumoniae*
458 can influence pathogenesis and host-pathogen interaction, complete genome sequencing has been
459 attempted (Minion et al., 2004; Vasconcelos et al., 2005; Liu et al., 2011; Siqueira et al., 2013;
460 Han et al., 2017). While this molecular technique offers descriptive and thorough information, the
461 feasibility to routinely perform complete genome sequencing may be limited due to cost, difficulty
462 of interpretation, computational requirements, and the need for sophisticated equipment.
463 Therefore, complete or partial sequencing of individual *M. hyopneumoniae* genes has been
464 attempted for the purpose of strain typing (Mayor et al., 2007; Felde et al., 2018). Due to the
465 paralogous nature of P146 with P97, and the presence of distinct motifs and consensus regions (i.e.
466 PQ, S, PS for locus P146), partial sequencing of the encoded gene *Mhp-684* (3954 bp) has been
467 previously described (Mayor et al., 2007; Bogema et al., 2012). Throughout the employment of
468 sequencing methods, the number of VNTRs have also been evaluated for *M. hyopneumoniae*
469 (Felde et al., 2018; Garza-Moreno et al., 2019). However, as well with MLVA, there is limited
470 knowledge on the interpretation of sequencing methods and the insight gained from the
471 comparison of molecular assays that evaluate nucleotide differences at the consensus or individual
472 gene level. To provide further understanding, a comprehensive review of the *M. hyopneumoniae*
473 genome is necessary to identify regions that can be utilized for classification, as well as for
474 prevention and control efforts.

475 4. **Proposed terminology for the study of *M. hyopneumoniae* genomic variability**

476 To provide clarity on disease investigations, defining a commonality for *M. hyopneumoniae*
477 genomic classification using MLVA is proposed based on current knowledge. One of the reasons

478 why a genomic classification criterion for *M. hyopneumoniae* has not been previously established
479 for MLVA is due to the fact that the agreement of a common technique and terminology has not
480 been made. However, a systematic and scientific approach to genetically classify *M.*
481 *hyopneumoniae* should be made in congruency to help promote and drive scientific knowledge
482 advancement on this microorganism.

483 Across the literature, different terms such as strains, variants, types, and typing profiles have been
484 used to define *M. hyopneumoniae* strains according to the VNTRs obtained by different typing or
485 sequencing methods. In this review, the term 'VNTR types' is suggested since the terminology
486 'strain' is often defined as a pathogen that has been isolated and characterized and a 'variant' refers
487 to any genomic modification of a pathogen. In addition, the term 'VNTR types' is proposed as it
488 can be incorporated for MLVA and sequencing assays that are employed to evaluate the number
489 of VNTRs within specific loci.

490 Classification of determining a unique or different *M. hyopneumoniae* VNTR type compared to
491 other types varies and continues to be arbitrary. Such variation across several MLVA techniques
492 may be due to differences in the type and number of loci targeted as well as analytic method. For
493 example, one method for classifying a unique *M. hyopneumoniae* VNTR type may be based on
494 one tandem repeat difference in surface adhesin proteins (*e.g.* 16-17 to 17-17). In comparison, this
495 minute difference in VNTR length might be perceived from a genomic modification, probably
496 without biological significance and related to host-pathogen adaptations during evolutional
497 pressures. Currently, the biological importance of variations in VNTR length and translation of
498 repeat motifs remains unclear. Due to these reasons, the ability to make inferences from previously
499 published literature to understand the genomic variability of *M. hyopneumoniae* becomes
500 challenging, especially when different techniques are employed. Therefore, it is proposed that a

501 minimum of two loci (i.e. P97 and P146) should be included in published literature papers. On the
502 other hand, minimum spanning trees and unweighted pair group method with arithmetic mean
503 (UPGMA) dendrograms have been employed to illustrate differences between VNTR types;
504 however, the relationship and/or difference among types is not easily portrayed as each locus is
505 unequally weighted. Therefore, the need to identify a bioinformatics tool that can portray
506 differences between VNTR types in a systematic and numerical manner is highlighted.
507 Furthermore, there is a need to create a database of *M. hyopneumoniae* VNTR types worldwide,
508 with a curator and free access. In doing so, this knowledge can be applied towards the development
509 of a genomic classification method for *M. hyopneumoniae.*

510 ## 5. **Conclusions**

511 *Mycoplasma hyopneumoniae* variation across several strains and field isolates has been described
512 at the antigenic, proteomic, transcriptomic, pathogenic, and genomic levels. This microorganism
513 may undergo adaptations that may drive variation and differences between host-pathogen
514 interactions. One approach to provide further insight on this topic is the molecular characterization
515 of *M. hyopneumoniae* using genomic sequencing and typing methods, which has been commonly
516 implemented in research and clinical investigations. However, the agreement of a shared
517 terminology and classification for the various techniques, specifically MLVA, has not been
518 described and thus causes discrepancies and the inability to make inferences across the literature.
519 Therefore, current molecular trends for *M. hyopneumoniae* have been outlined and common
520 terminology and classification based on VNTR types has been proposed.

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