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1 **RAPD and VNTR analyses demonstrate genotypic heterogeneity**
2 **of *Mycoplasma hyopneumoniae* isolates from pigs housed in a**
3 **region with high pig density**

4
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18 Short Title: *Genotypic heterogeneity of *M. hyopneumoniae**

19

20 **Abstract**

21 Since differences in the virulence of *Mycoplasma (M.) hyopneumoniae* strains
22 have been described, the isolation of field strains followed by genotypic and
23 phenotypic characterisation has become a major goal in epidemiological studies.
24 The aim of this study was to compare various *M. hyopneumoniae* isolates from
25 different pig herds and numerous pigs within the same herd. Therefore, pigs of
26 109 herds located in North-Western Germany were sampled either on-farm or
27 during necropsies. Overall, 52 isolates of *M. hyopneumoniae* were recovered from
28 45 pigs originating from 21 herds. The identity of cultures was confirmed by PCR
29 targeting the 16S-23S intergenic spacer region. Typing of isolates was achieved
30 by random amplified polymorphic DNA (RAPD) analysis and multi-locus
31 analysis of variable number of tandem repeats (VNTR) and demonstrated a high
32 degree of heterogeneity of *M. hyopneumoniae* isolates. Differences among
33 isolates recovered from animals of the same herd or even from the same pig
34 revealed a grouping into different genotypic clusters. This outcome was observed
35 with both methods. It was concluded that more than one strain of
36 *M. hyopneumoniae* might be present in a pig herd and even in a single pig,
37 suggesting high genetic heterogeneity between isolates of the same
38 epidemiological source. These factors should be considered when applying
39 nucleic amplification techniques for characterising *M. hyopneumoniae* strains to
40 specify the epidemiology of infection and to evaluate virulence factors triggering
41 the corresponding disease.

42

43 **Keywords**

44 *Mycoplasma hyopneumoniae*, epidemiology, genotyping (RAPD, VNTR), 16S-23S

45 *IGS PCR*

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47 **1. Introduction**

48 *Mycoplasma (M.) hyopneumoniae* is the causative agent of porcine enzootic
49 pneumonia (EP) and contributes significantly to the porcine respiratory disease
50 complex. The development and severity of enzootic pneumonia is influenced by
51 various factors including management, hygiene, vaccination against
52 *M. hyopneumoniae*, etc. (Maes et al., 1999), and, moreover, by the virulence of
53 the *M. hyopneumoniae* strain affecting the pigs (Vicca et al., 2003). For decades,
54 the genetic and antigenic heterogeneity of *M. hyopneumoniae* strains have been
55 elaborated, but evidence for direct association between genetic heterogeneity and
56 virulence is still pending. An association between the presence of a 5,000 bp
57 fragment in the genome of *M. hyopneumoniae* and high virulence in pigs was
58 found in an experimental approach (Vicca et al., 2003), but was never confirmed
59 under field conditions. Further studies showed that genomic diversity and
60 proteomic variability of *M. hyopneumoniae* isolates are high within this species,
61 but only one clone circulates within a herd (Calus et al., 2007; Mayor et al., 2008).
62 Based on these findings, the genotypic characterisation and/or phenotypic
63 determination of isolates from different farms remained a major goal in
64 epidemiological studies, at least to assess routes of infection and, if possible, to
65 describe virulence markers (Calus et al., 2009; de Castro et al., 2006). The main
66 aim of our study was to apply established and easy to perform typing methods
67 with sufficient discriminatory power for comparing *M. hyopneumoniae* isolates,
68 namely RAPD and multi-locus VNTR analyses, to a large number of

69 epidemiologically well-defined *M. hyopneumoniae* isolates (de Castro et al., 2006;

70 Stakenborg et al., 2006a).

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72 2. Materials and Methods

73 2.1. *Mycoplasma* strains and isolates for validation purposes

74 The following *Mycoplasma* type strains were purchased from the National
75 Collection of Type Cultures (NCTC; Colindale, UK): *M. flocculare* Ms42^T
76 NCTC10143, *M. hyopharyngis* H3-6BF^T NCTC11705, *M. hyopneumoniae* J^T
77 NCTC10110, *M. hyorhinis* BTS-7^T NCTC10130, and *M. hyosynoviae* S16^T
78 NCTC10167. Field isolates (*M. flocculare* n=12, *M. hyopharyngis* n=6,
79 *M. hyopneumoniae* n=19, *M. hyorhinis* n=39, *M. hyosynoviae* n=34) previously
80 recovered from porcine samples using Friis broth (Friis, 1975) and identified to
81 species level by epi-immunofluorescence test were selected randomly from the
82 mycoplasma strain collection of the Institute of Bacteriology, Mycology and
83 Hygiene (IBMH) at the University of Veterinary Medicine, Vienna, Austria. All
84 strains and isolates were incubated aerobically in Friis broth at 37°C until a colour
85 change of the medium occurred. Broth cultures were submitted to species-specific
86 PCR (Stakenborg et al, 2006b) and 16S-23S PCR analysis as described below.

87

88 2.2. Animals and samples for the epidemiological study

89 Material for cultural examination was derived from three different sources: (a)
90 bronchoalveolar lavage fluid (BALF) from fattening pigs obtained by nasal route,
91 (b) lung tissue and BALF from nursery pigs obtained during necropsy and (c)
92 lung tissue and BALF from fattening pigs obtained after slaughter.

93 The BALF was sampled on-farm in herds either affected by enzootic pneumonia
94 (case herds) or free from clinical signs of respiratory disease (control herds). The

95 lavage was carried out with 20-60 mL of sterile physiological saline solution and
96 further equipment as described elsewhere (Nienhoff et al. 2004). On each farm,
97 ten fattening pigs weighing more than 100 kg were selected either by the
98 parameter “coughing” (case herds) or randomly (control herds). Subsequently,
99 after sampling, 1 mL of BALF was diluted 1:5 in Friis broth and stored at -70 °C
100 until further examination.

101 Lung tissue from nursery and slaughtered pigs was sampled from one of the
102 cranial lobes of the lung. A piece with each edge measuring 5 cm was sliced from
103 the cranial part of the lobe independently of visible lesions which might indicate
104 pneumonia. Afterwards, the lung tissue was prepared in two ways: First, a piece
105 0.3 cm in length including a visible airway (i.e. bronchus or bronchioli) was
106 placed in 2.5 mL of Friis broth. In a second step, a bronchoalveolar lavage was
107 performed by pipetting 300 µL physiological saline solution into one bronchus.
108 Approximately 100 µL of this BALF was recovered and subsequently placed in
109 2.5 mL of Friis broth. All samples were stored at -70 °C until further examination.

110 This study was performed under licence for experimenting on animals from the
111 German Federal State Veterinary Administration Offices in Lower Saxony
112 (LAVES, Oldenburg, license no. 33-42502-06A436) and in North-Rhine
113 Westphalia (BRMS, Muenster, license no. 50.0835.3.0/A71/2006).

114

115 **2.3. Isolation of *M. hyopneumoniae***

116 The frozen samples (broth containing BALF or lung tissue) were thawed and
117 gently mixed. Each time, 100 µL of sample was pipetted into 3 ml of fresh Friis

118 medium which was incubated aerobically at 37°C for 14 days or until a colour
119 change occurred. Afterwards, 100 µL of broth cultures were passaged onto Friis
120 agar, incubated for a further 14 days at 37°C in 7% CO₂ and 93% air, and plates
121 were daily checked for colony formation by stereomicroscopy. In those cases
122 where 16S-23S PCR on broth cultures (see below) revealed mixed cultures five
123 single colonies were selected and incubated in Friis medium. After propagation,
124 cultures were tested again for purity of *M. hyopneumoniae* by 16S-23S PCR.
125 Cultures were stored at -70°C until further usage.

126

127 **2.4. Nucleic acid purification and amplification of the 16S-23S** 128 **intergenic spacer region**

129 From 500 µL of broth cultures the genomic DNA was extracted and purified using
130 a silica-membrane-based spin kit (GenElute™ Mammalian Genomic DNA
131 Miniprep Kit, Sigma-Aldrich, Vienna, Austria) according to the manufacturer's
132 instructions.

133 PCR reaction mixes (25 µL) were prepared using 12.5 µL of a commercial PCR
134 reagent (REDTaq™ ReadyMix™, Sigma-Aldrich, Vienna, Austria) added with
135 10 pmol of primer F2A (5'-GTGGGGATGGATCACCTCCT-3') and 10 pmol of
136 primer R2 (5'-GCATCCACCAAAAACCTCTT-3'), 3 µL of DNA template and
137 water as described by Tang et al., 2000. The amplification protocol included an
138 initial step at 94°C for 30 sec. Afterwards, 35 cycles were performed (94°C for 30
139 sec, 55°C for 120 sec and 72°C for 120 sec) followed by a final extension step at
140 72°C for 120 sec. Reactions were carried out in single tubes in a thermocycler

141 (GeneAmp 9700, Applied Biosystems, Darmstadt, Germany). PCR products were
142 separated by electrophoresis using 1.5% agarose gel and 1 x Tris-Borat-EDTA
143 buffer, at 1 V/cm for 60 minutes. Amplicons were visualised after staining the gel
144 with ethidium bromide (0.1 μ M) for 30 minutes.

145

146 **2.4.1. RAPD analysis of *M. hyopneumoniae* isolates**

147 For RAPD analysis single colonies of *M. hyopneumoniae* isolates were
148 propagated in Friis medium, and DNA was extracted from obtained cultures. The
149 type strain *M. hyopneumoniae* J^T was included in the study.

150 RAPD analyses were performed using 37.5 pmol of the primer R6 (5'-
151 CCCGTCAGCA-3') (illustra Ready-To-Go RAPD Analysis Kit; GE healthcare,
152 Buckinghamshire, United Kingdom). To minimise the variability between
153 different runs, puRe Taq Ready-To-Go PCR Beads (GE Healthcare,
154 Buckinghamshire, UK) were used, and all samples were run simultaneously
155 during one single PCR. Prior to this study, discriminatory power as well as
156 reproducibility of banding patterns were determined by repeating RAPD analysis
157 of selected isolates. All in all, 10 randomly selected isolates were analysed in
158 three replicates employing 6 standard RAPD primers (illustra Ready-To-Go
159 RAPD Analysis Kit; GE healthcare, Buckinghamshire, United Kingdom). Primer
160 R6 showing 100% reproducibility of banding patterns and a sufficient
161 discriminatory power was selected for further analysis (data not shown). The
162 amplification conditions included an initial denaturation step at 95°C for 5 min,
163 followed by 45 cycles of 94°C for 1 min, 36°C for 1 min, a 2 min ramp time to

164 72°C (0.3°C/s) and 72°C for 2 min, followed by one final elongation step at 72°C
165 for 1 min. Reactions were carried out in single tubes in a thermocycler (GeneAmp
166 9700). After amplification, 10 µL of the PCR mixture were analysed by
167 electrophoresis using 1.5% high resolution agarose gel (Agarose NEEO Ultra-
168 Qualitaet, Carl Roth GmbH, Karlsruhe, Germany) at 1 V/cm for 60 minutes. The
169 DNA fragments were visualised using a gel documentation system and images
170 were stored in a file format maintaining a 16-bit-greyscale (Bio-Rad Laboratories
171 GmbH, Vienna, Austria).

172

173 **2.4.2. VNTR analysis of *M. hyopneumoniae* isolates**

174 Three different regions containing tandem repeats located within the genes of the
175 P97 adhesin (P97R1), a putative membrane protein (H4), and the adhesin like-
176 protein P146 (P146R3), previously shown to exhibit highly diverse numbers of
177 repeats in different *M. hyopneumoniae* strains (de Castro et al., 2006; Stakenborg
178 et al., 2006a), were amplified using primers listed in Table I. The amplification
179 protocol with primers P97 FOR and P97 REV included an initial step at 94°C for
180 2 min and 30 cycles each lasting 30 sec at 94°C, 30 sec at 54°C and 60 sec at
181 72°C. For final extension samples were heated for 2 min at 72°C. The
182 amplification protocol with primers H4 FOR/H4 REV and P146 FOR/P146 REV
183 included an initial step at 94°C for 2 min and 35 cycles of 60 sec at 94°C, 60 sec
184 at 60°C and 60 sec at 72°C, followed by a final extension step at 72°C for 2 min.
185 Reactions were carried out in single tubes in a thermocycler (GeneAmp 9700).
186 From each of the three runs, 10 µL of the PCR mixtures were analysed by

187 electrophoresis using 1.5% high resolution agarose gel (Agarose NEEO Ultra-
188 Qualitaet, Carl Roth GmbH, Karlsruhe, Germany) at 1 V/cm for 60 minutes. At
189 least twice for every 10 samples, a molecular weight marker (GeneRuler™ 100 bp
190 DNA ladder, Fermentans, Leon-Rot, Germany) was loaded onto the gel. The
191 DNA fragments were visualised using a gel documentation system and images
192 were stored in a file format maintaining a 16-bit-greyscale (Bio-Rad Laboratories
193 GmbH).

194

195 **2.5. Data analysis**

196 Files from gel-documentation were imported to Bionumerics (V3.5, Applied-
197 Maths, Sint-Martens-Latern, Belgium) for further data analysis. Bands annotated
198 by the software were visually controlled and fragments smaller than 100 bp were
199 omitted from further analysis.

200 Calculation of similarity coefficients applied by RAPD analysis was performed
201 using the Dice algorithm. The unweighted pair group method with arithmetic
202 means (UPGMA) was used for clustering with a band position tolerance and
203 optimisation setting of 1%.

204 The VNTR profiles were analysed in two steps. First, fingerprints from each gene
205 locus (P97, H4 and P146) were analysed and compared. Finally, the fingerprints
206 from all three experiments were combined and similarity values were calculated
207 in one single composite cluster analysis (multi-locus VNTR analysis).

208

209

210 3. Results

211 3.1. Identification of porcine mycoplasmas by 16S-23S IGS PCR

212 16S-23S IGS PCR allowed the distinguishing of cultured mycoplasma species
213 frequently isolated from the porcine respiratory tract by their size of amplicons.
214 Amplified products of type strains ranged in size from 260 bp (*M. hyosynoviae*),
215 320 bp (*M. hyorhinis*), 360 bp (*M. hyopharyngis*), 450 bp (*M. flocculare*) to 580
216 bp (*M. hyopneumoniae*) (Fig. 1A). No size variability of amplicons within a given
217 species occurred when field isolates were tested by this identification scheme.
218 Two or more PCR products were observed, if the medium contained more than
219 one mycoplasma species (Fig. 1B). During validation in the laboratory, PCR
220 results for 110 isolates of porcine mycoplasma species obtained from the
221 laboratory reference bank were 100% linked to traditional epi-immunofluorescence
222 test and species-specific PCR results (data not shown).

223

224 3.2. Detection rates and isolation of *M. hyopneumoniae*

225 Samples from 109 herds were included. In total, 52 isolates of *M. hyopneumoniae*
226 were recovered from samples originating from 21 different herds. In 47.6%
227 (10/21) of these herds only one isolate was recovered. Two and three isolates were
228 obtained from four and two herds, respectively. In 23.8% (5/21) of
229 *M. hyopneumoniae* positive herds the number of isolates was three or more; a
230 maximum of seven isolates per herd was seen twice. Of the 21 herds, 19 were
231 suffering from enzootic pneumonia in fattening pigs or coughing in nursery pigs.
232 In two herds, where overall three *M. hyopneumoniae* isolates had been recovered,

233 pigs were healthy in terms of respiratory symptoms at the time of sampling and
234 had had no history of respiratory disease within the preceding two years.

235 *M. hyopneumoniae* was also isolated from lung tissue of 39 pigs (3 nursery pigs
236 and 36 fattening pigs) and from BALF of 13 pigs (3 nursery pigs and 10 fattening
237 pigs). Taken together, in seven animals BALF and lung tissue were positive (3
238 nursery pigs and 4 fattening pigs).

239

240 **3.3. RAPD analysis of colony-derived *M. hyopneumoniae* isolates**

241 The 52 *M. hyopneumoniae* isolates tested showed RAPD patterns of high diversity
242 (Fig. 2). The band size assessed on the gels was between 180 and 1400 bp and the
243 number of bands per isolate varied from 7 to 13. Overall, 51 isolates of
244 *M. hyopneumoniae* exhibited a band of approximately 1,300 bp and 49 isolates
245 showed a band of approximately 530 bp.

246 The cluster analysis using Bionumerics resulted in a tree, where isolates from at
247 least four farms (K, L U and T) demonstrated a similarity with this method of less
248 than 80%. Differences among isolates which were recovered from animals of the
249 same herd, or even from the same pig resulted in grouping into two or more
250 different clusters (Farm L, isolate 7-7 from BALF vs. isolate 7-7 from lung tissue;
251 Farm U, isolate L06/050 from BALF vs. isolate L06/050 from lung tissue, etc.).

252

253 **3.4. VNTR analysis of *M. hyopneumoniae* isolates**

254 Amplifying P97 RR1, P146 RR3 and H4 of each isolate resulted in various
255 banding profiles (Fig 3). Among 52 isolates 8 different lengths of P97 RR1

256 amplicon were observed. Similarly, 6 different lengths of P146 RR3 and 9
257 different lengths of H4 amplicons were present indicating that herds and
258 individuals are colonised by several strains.

259 By summarising banding patterns into a single cluster analysis, a high genetic
260 heterogeneity of *M. hyopneumoniae* in single pigs, within pig herds and between
261 pig herds was evident. A total of 10 clusters were identified, if 80% was set as the
262 minimal identity of related isolates/strains of *M. hyopneumoniae*.

263

264 **4. Discussion**

265 *M. hyopneumoniae*, *M. flocculare*, *M. hyorhinis*, *M. hyosynoviae* and
266 *M. hyopharyngis* are fastidious mycoplasma species frequently colonising the
267 upper and lower respiratory tract of swine (Kobisch and Friis, 1996). In contrast
268 to previous reports (Kobisch and Friis, 1996; Stakenborg et al., 2006b), in our
269 study all these mycoplasma species were able to grow in the same isolation
270 medium, indicating therefore that reliable and rapid methods for differentiation of
271 all porcine respiratory mycoplasmas are required. While several species-specific
272 PCR systems for detecting *M. hyopneumoniae* exist (Baumeister et al., 1998;
273 Blanchard et al., 1996; Dubosson et al., 2004; Kurth et al., 2002; Mattsson et al.,
274 1995; Staerk et al., 1998; Strait et al., 2008; Verdin et al., 2000), only few
275 (*M. flocculare*, *M. hyorhinis*) or no (*M. hyopharyngis*) molecular diagnostic
276 methods for identifying economically less important porcine mycoplasmas are
277 available (Caron et al., 2000; Lin et al., 2006; Stakenborg et al., 2006b).
278 Identification however can be achieved by epi-immunofluorescence which is a

279 laborious identification method depending on specific porcine anti-mycoplasma
280 antisera and on high expertise of diagnostic personnel. In this study we were able
281 to establish an identification system based on length differences of the amplified
282 16S–23S rRNA IGS region of porcine mycoplasma species. The 16S–23S rRNA
283 IGS region was targeted as it has shown to exhibit a high degree of interspecies
284 heterogeneity in mycoplasmas varying in length and composition (Harasawa,
285 1999; Volokhov et al., 2006). The PCR described generated species-specific
286 lengths of amplicons which were easily distinguishable using standard gel
287 electrophoresis allowing simultaneous detection and identification of cultivated
288 mycoplasma species frequently colonising the porcine respiratory tract.

289 Genotypic and phenotypic characterisation of bacteria is often used to specify the
290 epidemiology of an infection and to evaluate virulence factors triggering the
291 corresponding disease (Horii et al., 1998; Kielstein et al., 2001; Knutton et al.,
292 2001; Taillon et al., 2008). In case of *M. hyopneumoniae* infection and enzootic
293 pneumonia in pigs, virulence factors have been proposed after analysing single
294 strains from various herds with different molecular techniques (Calus et al., 2007;
295 Vicca et al., 2003). However, detailed knowledge about virulence factors of
296 *M. hyopneumoniae* and laboratory methods predicting the virulence of strains are
297 still unavailable (Sibila et al., 2009).

298 Vicca and colleagues (2003) suggested RAPD analysis has the potential to
299 distinguish between low and high virulent strains of *M. hyopneumoniae* with a
300 sufficient power of discrimination. Characterising *M. hyopneumoniae* strains with
301 RAPD after virulence had been assessed in an experimental approach led to the

302 assumption that high virulence is connected to the presence of a dedicated 5,000
303 bp fragment in the genome of the bacteria. Using the same protocol as described
304 by Vicca and colleagues we were not able to detect a 5000 bp fragment, when this
305 RAPD analysis was applied to a subset of the 52 isolates of the present study. In
306 other studies examining field strains of mycoplasma species a low reproducibility
307 of RAPD analysis was reported (Hong et al., 2005; Stakenborg et al., 2006a), and
308 authors recommended the examination of different strains only within one
309 experiment/PCR run. The limited reproducibility of RAPD analysis also forfeits
310 the use of this method in postponed examinations for routine diagnostic purpose.
311 The utility as a diagnostic tool would require all isolates of interest to be
312 examined in one single experiment at the same time. Comparing strains from
313 different epidemiological and/or geographical sources, it was concluded that
314 mainly one clone of *M. hyopneumoniae* circulates at a specific point in time in a
315 single pig herd. Interestingly, this statement was generalised, although in the same
316 study *M. hyopneumoniae* strains from four herds demonstrated differences in the
317 band pattern from RAPD analysis (Stakenborg et al., 2006a). Similar conclusions
318 regarding the hypothesis of one strain per herd were also drawn after applying
319 MLST to a panel of *M. hyopneumoniae* strains (Mayor et al., 2008). The authors
320 found multiple strains in the swine population but only specific strains or clones
321 responsible for local outbreaks of EP. Since all field samples in this study came
322 from Switzerland, where *M. hyopneumoniae* has been eradicated and only
323 sporadic re-infections occur, cautious interpretation of the results is called for and
324 extrapolation to endemically infected pig populations is not recommended.

325 Addressing the limitations of the method, we applied RAPD analysis to 52
326 isolates of *M. hyopneumoniae* originating from 21 different herds from North
327 Western Germany. This region has the highest pig density in Europe, remarkable
328 trading of pigs (import of piglets and gilts from Denmark and the Netherlands)
329 and its pig population is known to be endemically infected with
330 *M. hyopneumoniae* (grosse Beilage et al., 2009). In the present study conducted in
331 this region, strong evidence was found that more than one strain of
332 *M. hyopneumoniae* might be present in a pig herd and even in a single pig.
333 Grounds for this hypothesis are based on the significant differences which were
334 observed when band patterns of isolates from the same animal or the same pig
335 herd were compared with each other.

336 VNTR analysis is a feasible and suitable method to characterise bacteria without
337 the necessity for any culture steps although results from VNTR analysis without
338 previous isolation may be confounded by mixed strains within a sample.
339 Additionally, VNTRs are mainly found in surface-expressed genes and
340 differences in these genes may or may not be reflective or even predictive of
341 differences in other regions of the genome. Nonetheless, VNTR analysis was
342 reported to be a useful tool for *M. hyopneumoniae* strain typing and EP diagnosis
343 (de Castro et al., 2006), and it was also demonstrated that particular VNTRs of
344 *M. hyopneumoniae* and their number, i.e. in P97 RR1, are directly linked to
345 functional properties (Hsu and Minion, 1998). These facts might approve VNTR
346 analysis as the method of choice when characterisation of large numbers of
347 *M. hyopneumoniae* strains is the aim. However, the discriminatory power of

348 single VNTRs of *M. hyopneumoniae* was shown to be low (Stakenborg et al.,
349 2006a), and, moreover, the number of *M. hyopneumoniae* genes containing
350 tandem repeat regions appears to be limited (Minion et al., 2004). Taking this into
351 account, the use of single VNTRs in characterising *M. hyopneumoniae* might be
352 of limited value. When examining P97 RR1 only 8 different amplicon sizes were
353 observed among the 52 isolates, these results tallying with previous reports
354 (Stakenborg et al., 2006a). To solve the problem of low discriminatory power,
355 results from three different VNTR loci were subjected to one single analysis
356 followed by comparison of similarity. This kind of multiple loci VNTR analysis
357 (MLVA) considerably enhances the resolution of the approach to disseminate
358 bacterial agents (van Belkum, 2007). Similar to RAPD, with MLVA a high degree
359 of genetic heterogeneity was found and isolates from the same pig herd and even
360 from the same pig were grouped into different clusters. However, results of the
361 two typing methods used in this study did not correlate 100% with each other.
362 Discrepancies may be attributed to divergent target sequences of typing methods
363 or the presence of quasispecies within single colony isolates which may type
364 differently.

365 To the authors' knowledge, this is the first report on high genetic heterogeneity of
366 *M. hyopneumoniae* between isolates of the same epidemiological source, i.e. pig
367 herd and/or single pigs. Since previous studies often included only one
368 strain/isolate per herd or strains from re-infected pig populations, this study,
369 which included up to eight isolates from the same epidemiological source, reflects
370 the situation in endemically infected pig herds and pig populations, respectively.

371 Obviously, the trading of piglets, growing pigs and gilts, combined with frequent
372 changing of trading networks, *M. hyopneumoniae* strains are able to spread within
373 pig populations. These facts should be considered when applying molecular
374 techniques to examine and to characterise strains of *M. hyopneumoniae* in terms
375 of virulence, protein expression or other factors involved in pathogen-host
376 interaction.

377

378

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383

384 **Conflict of interest statement**

385 None of the authors of this paper have a financial or personal relationship with
386 other people or organisations which could inappropriately influence or bias the
387 content of the paper.

388

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Figure 1: 16S-23S PCR of porcine mycoplasma type strains (A) and of field isolates examined in this study (B): A: lane 1: molecular weight marker, 2: *M. hyopneumoniae* J^T, 3: *M. flocculare* Ms42^T, 4: *M. hyopharyngis* H3-6BF^T, 5: *M. hyorhinis* BTS-7^T, 6: *M. hyosynoviae* S16^T. B: lane 1: molecular weight marker, 2, 3, 9 and 10: *M. hyorhinis*, 4 and 13: *M. hyorhinis* and *M. hyopneumoniae*, 5: *M. flocculare*, 6: *M. hyosynoviae*, 7: *M. hyosynoviae* and *M. hyopharyngis*, 8: *M. hyosynoviae* and *M. hyopneumoniae*, 11 and 12: *M. hyopneumoniae*

Figure 2: Genetic relationship among *M. hyopneumoniae* isolates based on comparison of RAPD patterns. The dendrogram was produced using the unweighted pair group method with arithmetic means (UPGMA) and the Dice similarity coefficient matrix. *a*, strain ID; *b*, farm ID.

Figure 3: Cluster analysis of VNTR patterns (H4, P146, P97) of *M. hyopneumoniae* isolates. *a*, strain ID; *b*, farm ID.

Table I: Primers used for VNTR analyses

Target Primer	Sequence (5'-3')	Reference
P97 RR1		
P97 FOR	GAAGCTATCAAAAAGGGGAAACTA	Stakenborg et al., 2006
P97 REV	GGTTTATTTGTAAGTGAAAAGCCAG	
H4		
H4 FOR	TCGGGATTTTTTCTCTTTCTTC	de Castro et al., 2006
H4 REV	AGGATCTTGTGGTTTTAGCG	
P146 RR3		
P146 FOR	AAAACCCAAAGTAGTGATTC	de Castro et al., 2006
P146 REV	TGTATCGGTTTCAGAAGAAG	

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