

**Genetic variability and limited clonality of *Mycoplasma hyorhinis* in pig herds**

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## 21    **Abstract**

22    *Mycoplasma hyorhinis* is a common inhabitant of the upper respiratory tract and tonsils of pigs. Its  
23    role as a possible pathogen remains controversial. In order to gain more insight into the  
24    epidemiology and population structure of *Mycoplasma hyorhinis* we genetically characterized 60  
25    isolates by multi locus sequence typing (MLST). The *M. hyorhinis* strains originated from Swiss  
26    and German pig herds with knowledge on the clinical background. The MLST scheme of  
27    Tocqueville *et al.* (J.Clin.Microbiol. 2014) was optimized, primers for the six MLST gene  
28    fragments were newly designed to allow amplification and sequencing with a single protocol. A  
29    total of 27 ST were observed with the 60 strains, 26 of those were previously unknown types.  
30    Generally identical genotypes were observed within a farm but they differed between farms. The  
31    identical genotype was also observed in three different Swiss farms. On the other hand different  
32    genotypes within a farm were found with three German farms. The Swiss isolates formed a distinct  
33    cluster but otherwise there was no geographical nor a clinical association with specific clusters  
34    observed. Data shows a high variability of *M. hyorhinis* comparable to what is observed for  
35    *Mycoplasma hyopneumoniae*. Similar to this pathogen the population structure of *M. hyorhinis* also  
36    shows some limited clonality with predominant genotypes within an animal and a single farm but  
37    different ones between farms. The comparable population structure of *M. hyopneumoniae* and *M.*  
38    *hyorhinis* could indicate a similar evolution of the two species in the common pig host.

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40    Keywords: MLST, genotyping, pig disease, enzootic pneumonia, coughing

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## 42    **1. Introduction**

43    *Mycoplasma hyorhinis*, a common inhabitant of the upper respiratory tract and tonsils of pigs, was  
44    first described in the fifties by Switzer (1955). It is generally regarded as a commensal, however, a  
45    role of *M. hyorhinis* as a potential pathogen has also been discussed. Infections with this organism  
46    are subclinical, even though arthritis, polyserositis, conjunctivitis and otitis as well as abortions are  
47    clinical disorders that may be observed in an infected pig (Friis and Feenstra, 1994; Kobisch and  
48    Friis, 1996; Shin et al., 2003). *Mycoplasma hyorhinis* has also been found associated with  
49    pneumonia, however, rather as a secondary pathogen (Falk et al., 1991; Kawashima et al., 1996;  
50    Kobayashi et al., 1996; Lin et al., 2006). Nevertheless, an increase in the detection of *M. hyorhinis*  
51    in recent years has been observed and the relevance of this pathogen as an emerging issue causing  
52    significant economic losses is suspected (Dos Santos et al., 2015).

53    Genotyping allows epidemiological investigation and population studies. This can help to clarify  
54    the role of a possible pathogen, identify specifically involved strains and their transmission.  
55    Multilocus sequence typing (MLST) is a highly selective and specific method which has been  
56    utilized for the characterization of many bacterial species including *Mycoplasma hyopneumoniae*  
57    and *M. hyorhinis* (Mayor et al., 2008; Tocqueville et al., 2014). Genotyping of *M. hyorhinis* using  
58    MLST is based on sequences of internal fragments from six housekeeping genes, i.e. the  
59    chromosomal replication initiation protein (*dnaA*), the RNA polymerase  $\beta$  subunit (*rpoB*), the DNA  
60    gyrase subunit B (*gyrB*), the glutamyl-tRNA synthetase (*gltX*), the adenylate kinase (*adk*) and the  
61    guanylate kinase (*gmk*).

62    In this study, we optimized the MLST scheme of six loci by defining completely new primer sets.  
63    They were used to genetically characterize 60 *M. hyorhinis* isolates belonging to twenty-five Swiss  
64    and German herds. Information about the herd health status, i.e. clinical respiratory disease, of the  
65    fattening units from where strains came from were gathered as well. The MLST data of the  
66    *M. hyorhinis* isolates was used to elucidate genetic variability of the species and was related to the  
67    clinical presentation of the pig in order to define possible virulent strains.

## 68 2. Materials and methods

### 69 2.1. Strains and template preparation

70 A total of 60 *M. hyorhinis* isolates with 47 from Germany and 13 from Switzerland previously  
71 included in a transnational cross sectional study determining the prevalence of *M. hyorhinis* were  
72 analyzed (Luehrs, 2015). For every source of strains, relevant pathological conditions in the  
73 corresponding animals were available from databases. This includes presence of (enzootic)  
74 pneumonia varying from mild, moderate to severe assessed during lung scoring after dissection, as  
75 well as arthritis and/or polyserositis. Moreover, the simultaneous infection with *M. hyopneumoniae*  
76 was examined in every sample using real-time PCR (Dubosson et al., 2004).

77 Strains of *M. hyorhinis* were kept at -80°C and cultured fresh in 1 ml liquid medium (Mycoplasma  
78 Experience, Bletchingley, Great Britain) for 1-2 days at 37°C. Once medium color change from red  
79 to yellow occurred the culture was centrifuged for 10 min. at 5000xg. The pellet was dissolved in  
80 70µl ddH<sub>2</sub>O and boiled for 15 min. at 95°C. This crude lysate was then kept at -20°C until being  
81 used for PCR.

82

### 83 2.2. Optimization of MLST

84 The MLST scheme of Tocqueville et al. (2014) using the six housekeeping genes *dnaA*, *rpoB*, *gyrB*,  
85 *gltX*, *adk* and *gmk* served as a basis to optimize the protocol. Primers with identical annealing  
86 temperatures were defined, allowing amplification of all targets with a single PCR protocol instead  
87 of 3 different ones as described by Tocqueville et al. (2014). For this purpose target genes were  
88 extracted from available genome sequences of *M. hyorhinis* strains DBS 1050, HUB-1, GDL-1,  
89 MCLD, SK76 as well as the type strain ATCC17981<sup>T</sup> (=BTS7<sup>T</sup>) and sequences were aligned.  
90 Consensus sequence stretches as close as possible to the starting sequence used for typing were  
91 defined and primers with similar annealing temperature selected (Table 1). Each PCR was  
92 performed in a 30-µl total volume containing 12 pmol of each primer, 1 x FIREPol® Master Mix  
93 Ready to Load (Solis BioDyne, Tartu, Estonia) and 1 µl lysate at the following cycling conditions:

94 3 min denaturation at 95°C, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 45  
95 s. A final extension step for 7 min at 72°C was included. Subsequently, the amplification products  
96 of the six genes from one strain were pooled and then purified using the “High pure PCR  
97 purification Kit” (Roche Diagnostics, Rotkreuz, Switzerland). Finally the purified pooled PCR  
98 products was used for sequencing with the BigDye® Terminator v3.1 Cycle Sequencing Kit  
99 (Thermo Fisher, Reinach, Switzerland), using the same primers as for PCR (Table 1). For this  
100 purpose 96-well plates (Thermo Fisher) were prepared with corresponding primers (5 pmol/well)  
101 using a multichannel pipette. The plates allowed batch-wise analysis of 8 strains and could be kept  
102 at -20°C until being used for sequencing (Korczak et al., 2009).

103

### 104 2.3. Data analysis

105 Sequences were edited using the Sequencher program v5.1 (GeneCodes, Ann Arbor, MI, USA) and  
106 imported into Bionumerics v7.5 (AppliedMaths, Sint Marten, Belgium). Sequence types (ST) were  
107 determined using the MLST plugin allowing direct query of the PubMLST database  
108 ([www.pubmlst.org](http://www.pubmlst.org)). New alleles and allele combinations were submitted to the curator of the  
109 database to assign allele and ST numbers. The MLST sequences of the 33 strains from the study of  
110 Tocqueville et al. (2014) as well as the ones of the type strain BTS7<sup>T</sup> (GenBank accession  
111 ARTL000000000) were also imported to the database and included in the analyses. The resulting 94  
112 entries were used for creating a minimal spanning tree based on allele numbers and a UPGMA tree  
113 based on allele sequences in Bionumerics.

114 The START2 program ([pubmlst.org](http://pubmlst.org)) was used to calculate the ratio of synonymous and  
115 nonsynonymous substitutions as well as the index of association ( $I_A$ ). The index of discrimination  
116 ( $D$ ) was calculated using the formula of Hunter and Gaston (1988). LIAN  
117 ([guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl](http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl)) was used to calculate the genetic diversity ( $H$ ).

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119

### 120 3. Results

#### 121 3.1. Genetic diversity of *M. hyorhinis*

122 All 60 isolates could be analyzed with the optimized MLST scheme. There were two new alleles  
123 observed for *dnaA* (totaling 12) and *gyrB* (totaling 8), as well as three new alleles for the targets  
124 *rpoB* (totaling 10), *gltX* (totaling 10), *adk* (totaling 7) and *gmk* (totaling 8). With the additional new  
125 alleles the genetic diversity ( $H$ ) of the six loci varies from 0.4155 (*gltX*) to 0.7596 (*gyrB*). The ratios  
126 of nonsynonymous and synonymous substitutions varied from 0.1303 (*dnaA*) to 0.6785 (*gmk*). The  
127 index of association ( $I_A$ ) was 0.026, indication no linkage disequilibrium. A total of 27 ST were  
128 defined, 26 of them were new resulting in a total of 55 different *M. hyorhinis* genotypes based on  
129 MLST. Including all strains, i.e. also those from the same herd and same animals, the index of  
130 discrimination ( $D$ ) was 0.982. When only single representatives of such repetitive isolations were  
131 included in the analysis the index of discrimination ( $D$ ) was 0.995.

132 All the isolates from our study belong to the clonal complex 1 as defined by at least 4 common  
133 alleles (Tocqueville et al., 2014). The clonal relationship of STs is graphically shown by the  
134 minimal spanning tree in Fig. 1. Six of the seven previously observed singletons remained while  
135 ST-28 was integrated into complex 1 (Tocqueville et al., 2014). There is only one common ST (ST-  
136 13) between our study and the latter one as shown in Fig.2.

#### 138 3.2. Limited clonality of *M. hyorhinis*

139 There are no clear and discrete geographic clusters present, even though some grouping can be  
140 observed. The type strain BTS7<sup>T</sup> originating from the USA was determined as ST-16 based on *in*  
141 *silico* analysis of its genome sequence. This ST was also observed in France. The Chinese isolate  
142 represents an out-group while most of the branches consist of isolates from more than one country.  
143 Noteworthy, all Swiss isolates can be found on a single cluster containing only isolates from one  
144 German farm where polyserositis was diagnosed in piglets. Another discernable cluster is formed  
145 by only German isolates. This cluster contains isolates from farm DE\_03 where two slightly

different strains based on a single base-pair change in *gmk* could be detected. Clearly different strains were observed in farm DE\_16. In all other cases isolates from a single farm had the same genotype, while isolates from different farms were also of different genotype. Identical genotypes were also found when multiple isolates from single animals were analyzed (indicated by C2, C3). Identical genotypes were observed in three Swiss farms. In German farms, *M. hyorhinis* was isolated from animals that were in most cases (32 of 44) affected by *M. hyopneumoniae* as was diagnosed by real-time PCR indicated by ABC/REP in Fig. 2. In one case with a clear clinical status of enzootic pneumonia (strain numbers 10003) a follow up was done a few months later (strain numbers. 10025). While at both stages *M. hyorhinis* could be isolated from broncho-alveolar lavage fluid (BALF) and showed identical genotypes in different animals, the isolates from the two different time points differed in genotypes, indicating a switch of *M. hyorhinis* population over time.

There were no clusters or genotypes associated with certain clinical manifestations.

#### 4. Discussion

This study analyzed the genetic variability of *M. hyorhinis* from pig farms in Germany and Switzerland. Genotypes were put in relation to available clinical data and were also compared to isolates contained in the PubMLST database. For this purpose the MLST scheme of Tocqueville et al. (2014) was optimized to allow a uniform PCR amplification and sequencing protocol. The new protocol, based on a completely new set of primers for the six target genes, proved to be robust. All 60 isolates could be genotyped. The genetic diversity of *M. hyorhinis* increased by 26 genotypes to a total of 55 different STs based on MLST. While in the previous study by Tocqueville et al. (2014) mainly isolates from France were genotyped, we analyzed strains from Germany and Switzerland. Except from one farm, where the previously identified ST-13 was found, all other were new STs. Generally the same ST was observed within a farm and also within a single animal. The same ST

could be observed even within three different Swiss farms. On the other side different ST were observed in three German farms (DE\_16, DE\_03 and DE\_05). From farm DE\_05 one sample resulted in an ambiguous base call at two positions what made a genotyping impossible but showed that it contained a mix of at least two different strains (data not shown). This was an indirect indication that indeed more than one strain can be present in a single animal, even though it might not be the rule. Nevertheless, the observation that within a farm and individual animal the same genotype is generally found is a fact that would allow for direct genotyping of clinical material. This approach is successfully used with *M. hyopneumoniae* (Kuhnert and Overesch, 2014; Kuhnert et al., 2011; Mayor et al., 2008) and could therefore also be applied for *M. hyorhinis*. This allows a rapid straight forward genotyping eliminating the culture step.

The high variability but still limited clonality of *M. hyorhinis* is very similar to what can be observed with *M. hyopneumoniae* (Kuhnert and Overesch, 2014; Mayor et al., 2008; Mayor et al., 2007; Nathues et al., 2011). Given this observation and fact that *M. hyorhinis* is mainly a commensal inhabiting the same niche as the more pathogenic *M. hyopneumoniae* could point to a similar selection pressure if not co-evolution of the two species. Nevertheless, while whole genome comparisons between these two species showed that rearrangements are common, clear differences in gene composition indicate that a direct interaction and dependency of the two is rather unlikely.

Clinical information was gathered from all cases including diagnostic results on enzootic pneumonia (EP) caused by *M. hyopneumoniae*. While *M. hyopneumoniae* was detected in more than 70% of German cases, all Swiss cases were negative for this pathogen (Fig.2). This reflects the different situation in these two countries. While in Switzerland only a few sporadic cases of EP are observed per year due to a successful eradication program, EP is still endemic in Germany (Nathues et al., 2014; Stark et al., 2007). Furthermore, clinical data is also available for strains contained in the PubMLST database (Tocqueville et al., 2014). No correlation or association of specific genotypes or clusters with clinical manifestations could be discerned. Similar observations were made by Dos Santos et al. (2015) using a multi-locus variable-number tandem-repeat analysis



198 (MLVA) that is based on more variable genes than the house-keeping genes used for MLST. These  
199 authors also could not find an association between isolates and clinical manifestations. The MLVA  
200 approach was less discriminating than MLST with a diversity index of 0.814 compared to 0.982,  
201 respectively. However, the MLVA is based on only 2 genes compared to the six genes used for  
202 MLST and is therefore less work intensive and less expensive than the MLST. Nevertheless, the  
203 MLST is highly reproducible with all six targets successfully analyzable in all strains whereas with  
204 MLVA one of the targets could not be amplified in about 2% of strains and more than 40% of  
205 clinical specimens (Dos Santos et al., 2015). Moreover, we could improve the MLST by designing  
206 new primers that allow a single PCR amplification and sequencing protocol. The PCR purification  
207 step using pooled samples as well as the pre-preparing of 96-well microtiter plates containing all  
208 sequencing primers for 8 strains further reduce hands on time and costs. This makes MLST the  
209 method of choice if comparing efforts and outcome.

210 In conclusion the MLST scheme for *M. hyorhinis* was optimized and the number of isolates  
211 analyzed by this method was extended. Thereby the variability of *M. hyorhinis* in farms and related  
212 clinical data of the host to genetic data of the potential pathogen was systematically investigated. A  
213 limited clonality of *M. hyorhinis* was observed, i.e. a high variability of genotypes was  
214 accompanied by identical genotypes within a farm and individual animal. No specifically virulent  
215 genotypes could be discerned, indicating the commensal nature of *M. hyorhinis* in the pig lung.

216

217

## 218 **5. Acknowledgements**

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221

222   **6. References**

223

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284

## 285 **Figure legends**

286

287 Fig. 1 Minimal spanning tree of *M. hyorhinis* sequence types (ST) based on allele numbers built in  
 288 Bionumerics v7.6. A clonal complex relation is defined as a minimum of 4 identical alleles out of  
 289 the six. Branch length and intensity reflects number of different alleles. The circle size represents  
 290 the number of isolates belonging to the specific ST which is indicated by the corresponding number.

291

292 Fig. 2 Cluster analysis and strain information of *M. hyorhinis* isolates. A UPGMA tree was built in  
 293 Bionumerics v7.6 based on the six partial gene sequences used for MLST. The isolate name,  
 294 sequence type (ST), country and year of isolation, clinic and source are indicated. For the strains  
 295 analyzed in this study the real-time PCR results for *M. hyopneumoniae* (ABC/REP) are included as  
 296 well. The bar indicates the genetic distance.

297

298

299 Table 1. Primers used for uniform MLST of *Mycoplasma hyorhinis*

300

301	Gene	Primer	Sequence	seq. (bp) <sup>a</sup>
302	<i>dnaA</i>	dnaA-F2	CAGAAGTCTTAGGTGGTTTTG	459
303		dnaA-R2	TGTGGAATGATCCTTGCCTC	
304				
305	<i>rpoB</i>	rpoB-F2	TCAAGCTGTTCCATTAATTACTAC	509
306		rpoB-R2	GCACTAACTTCTGATCCAATAC	
307				
308	<i>gyrB</i>	gyrB-F2	GATTCTGATGGTTCACATATTAG	358
309		gyrB-R2	GTCTAGGTTTTTTGCATATTTTGC	
310				
311	<i>gltX</i>	gltX-F2	CTGAAAGACTCTCAAAATCACC	448
312		gltX-R2	TTACAAGCCTTTTTTGAAATTAGTTC	
313				
314	<i>adk</i>	adk-F2	CGATGGCATCTAATTCTTTTAAAG	437
315		adk-R2	TACTCAGGCAAAGTTTTTAGAAC	
316				
317	<i>gmk</i>	gmk-F2	GCGCCTGTTTCTGTTAATATTG	452
318		gmk-R2	AAGAGACAAAAGACCTAATGAAG	

319 <sup>a</sup> length of the informative sequence, i.e. of the PCR product without primer sequences

320

321

Table 1. Primers used for uniform MLST of *Mycoplasma hyorhinis*

Gene	Primer	Sequence	seq. (bp) <sup>a</sup>
<i>dnaA</i>	dnaA-F2	CAGAAGTCTTAGGTGGTTTTG	459
	dnaA-R2	TGTGGAATGATCCTTGCCTC	
<i>rpoB</i>	rpoB-F2	TCAAGCTGTTCCATTAATTACTAC	509
	rpoB-R2	GCACTAACTTCTGATCCAATAC	
<i>gyrB</i>	gyrB-F2	GATTCTGATGGTTCACATATTAG	358
	gyrB-R2	GTCTAGGTTTTTTGCATATTTTGC	
<i>gltX</i>	gltX-F2	CTGAAAGACTCTCAAAATCACC	448
	gltX-R2	TTACAAGCCTTTTTGAAATTAGTTC	
<i>adk</i>	adk-F2	CGATGGCATCTAATTCTTTTAAAG	437
	adk-R2	TACTCAGGCAAAGTTTTAGAAC	
<i>gmk</i>	gmk-F2	GCGCCTGTTTCTGTTAATATTG	452
	gmk-R2	AAGAGACAAAAGACCTAATGAAG	

<sup>a</sup> length of the informative sequence, i.e. of the PCR product without primer sequences

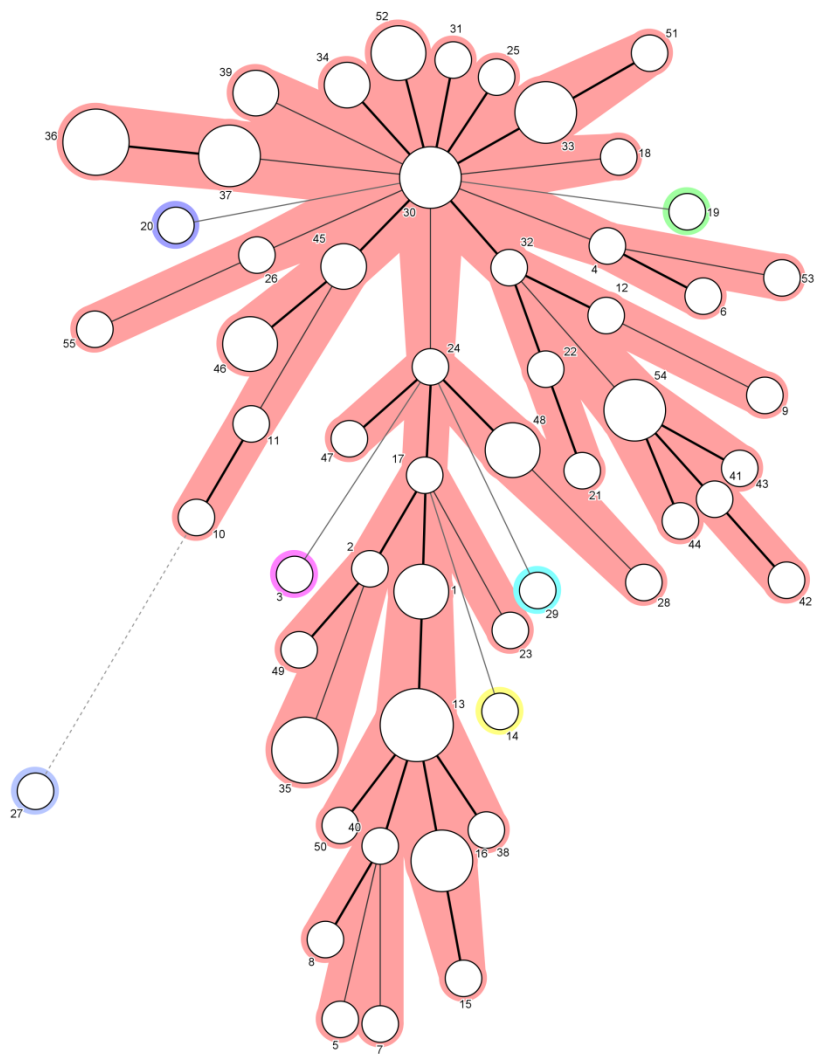


Fig. 1 Minimal spanning tree.

