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Short note

Diversity of *Mycoplasma hyopneumoniae* in pig farms revealed by direct molecular typing of clinical material

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Abstract – *Mycoplasma hyopneumoniae* is the etiological agent of enzootic pneumonia in swine. Various reports indicate that different strains are circulating in the swine population. We investigated the variety of *M. hyopneumoniae* strains by a newly developed genetic typing method based on the polyserine repeat motif of the LppS homolog P146. PCR amplification using *M. hyopneumoniae* specific, conserved primers flanking the region encoding the repeat motif, followed by sequencing and cluster analysis was carried out. The study included strains isolated from different geographic regions as well as lysates from lung swabs from a series of pig farms in Switzerland. High diversity of *M. hyopneumoniae* was observed but farms being in close geographic or operative contact generally seemed to be affected by the same strains. Moreover, analysis of multiple samples from single pig farms indicated that these harbored the same, farm-specific strain. The results indicate that multiple strains of *M. hyopneumoniae* are found in the swine population but that specific strains or clones are responsible for local outbreaks. The method presented is a highly reproducible epidemiologic tool allowing direct typing of *M. hyopneumoniae* from clinical material without prior isolation and cultivation of strains.

enzootic pneumonia / epidemiology / LppS / P146

1. INTRODUCTION

Porcine enzootic pneumonia (EP) caused by *Mycoplasma hyopneumoniae* accounts for one of the most important disease-associated losses in swine production [11]. The disease has almost no mortality but a high morbidity, and infection with *M. hyopneumoniae* is characterized by a sporadic, dry and non-productive cough, retarded growth rate and inefficient utilization of feed [15]. Infection occurs through direct contact with respiratory secretions from carrier animals, but an airborne transmission is also strongly suspected [6, 10, 11]. Secondary infections, especially with porcine reproductive virus and influenza virus can intensify the clinical picture of EP [12, 13, 16].

Bacteriological diagnosis of *M. hyopneumoniae* is cumbersome, since the organism is difficult to grow and growth takes several weeks. PCR has therefore become a method of choice for detection

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of the organism. We recently published a highly specific and sensitive real-time PCR targeting two different sequences of *M. hyopneumoniae*, one a repetitive element (REP) the other a putative ABC-transporter gene (ABC) [3]. Analysis of clinical samples from lung or nasal swabs of infected herds showed that about 60% of the samples were positive for both, REP and ABC, 30% were only positive for ABC and 10% gave a result with REP alone [3, 17]. This indicates that different *M. hyopneumoniae* strains are circulating in the swine population. The occurrence of different strains has been demonstrated by others as well [1, 4, 14]. Classical typing methods described for *M. hyopneumoniae* like amplified-fragment-length-polymorphism (AFLP) or pulsed-field-gel-electrophoresis (PFGE) are cumbersome and generally require isolation and cultivation of *M. hyopneumoniae* [8,9]. An accurate and rapid typing tool that could directly be applied on clinical material without isolation of *M. hyopneumoniae* would therefore be highly welcomed for epidemiological investigations, especially in the context of sanitation and eradication of the disease.

The LppS protein of *Mycoplasma conjunctivae* is a lipoprotein showing a variable domain of serine repeats in its carboxyterminal end. DNA sequence determination of the region encoding this part of the protein proved to be a valuable approach for molecular epidemiology of *M. conjunctivae* [2]. The variability in encoded polyserine repeats as well as the flanking region was used for phylogenetic clustering of strains from different geographical origin and allowed the conclusion that the pathogen can be transmitted between sheep and wild caprinae. We therefore adapted and evaluated this epidemiological approach for *M. hyopneumoniae* and investigated clinical samples from affected pig farms as well as strains from different countries. The DNA sequence of the polyserine encoding region of the *p146*, a homologue of the *lppS*, was determined and a cluster analysis was carried out. This was complemented with results from the real-time PCR for REP and ABC targets.

2. MATERIALS AND METHODS

2.1. Samples

All the 115 samples analyzed and their origins are given in Figure 1. For the study, lung swabs of slaughtered pigs from 11 farms with epidemiologically clearly defined sanitary status regarding EP were used. In addition to the ten farms indexed by an "R" as a first letter, 14 samples from a multisite production ring, designated farm 11 ("Ue"), where animals are exchanged on a regular basis, were tested. Between 2–14 samples per farm were taken (average 6). Further, single case samples of 23 farms from routine diagnosis, indicated by "P", were included. Most of the samples were collected in the context of a study on diagnosis of EP and are described there [3]. Only samples which were previously shown positive for *M. hyopneumoniae* in the real-time PCR were used for amplification of a part of the *p146* gene. Material taken with bronchial swabs was lysed in a 500 μL mixture containing 0.1M Tris-HCl pH 8.5, 0.05% of Tween 20, 240 μg/mL Proteinase K, and 5 mg/mL N-Acetyl-L-Cystein. The tubes were heated to 60 ˚C for 1 h, and then boiled at 95 ˚C for 15 min to inactivate the Proteinase K. The lysed samples were kept at –20 ˚C. Several international strains as well as data from the 3 genome sequenced strains were included. Strains originated from Switzerland, Australia, France (obtained from M. Kobisch, Ploufragan, Bretagne), Canada, Brazil and the USA.

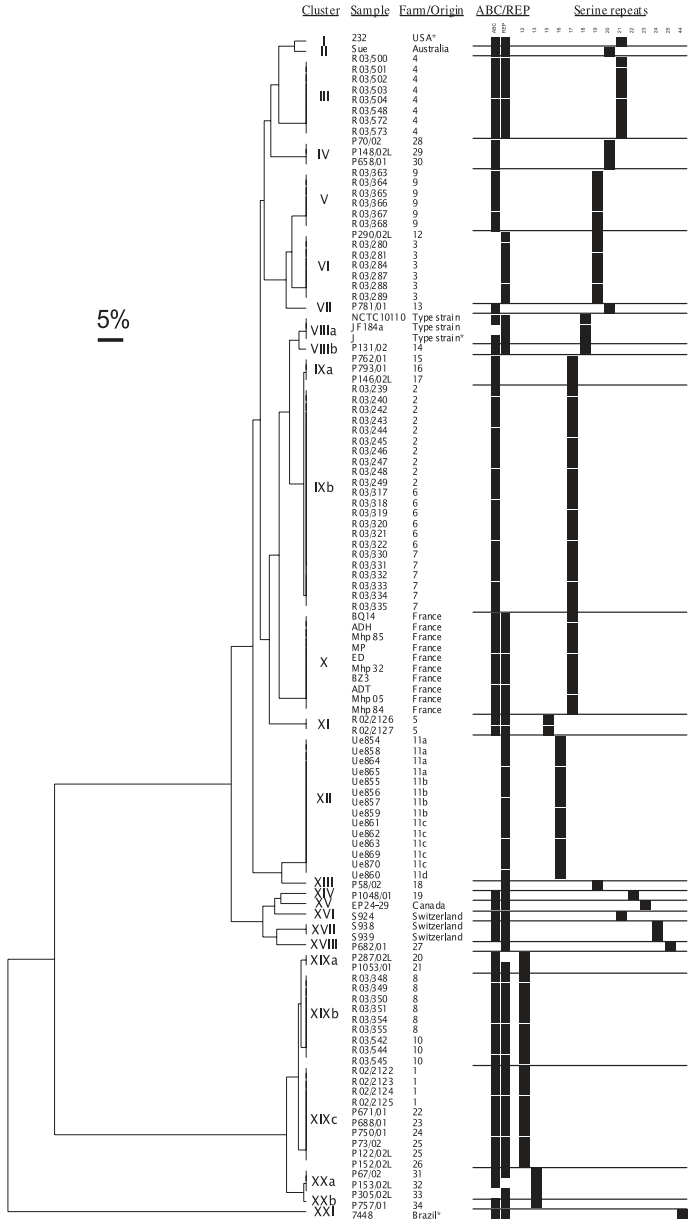

Figure 1. Graphic representation of clusters found with sequencing of the serine repeat encoding region of the *p146* gene. The sample names, their origin as well as results from real-time PCR of ABC and REP targets and the absolute number of serine repeats is given. Horizontal lines indicate the clusters. Column "Farm/Origin": samples that were directly from pigs without passages contain solely a number indicating the farm that was sampled. All other strains were collection or type strains. Collection strains from Switzerland and strain J had a low number of passages (< 5) while all other collection strains or the type strain underwent a high number of passages (> 20). The different sources of the type strain are the following: National Collection of Type Cultures (NCTC 10110^T); strain J obtained directly from Dr P. Whittlestone [5] and kept for 20 years as frozen culture; JF184a, obtained via the former *Mycoplasma* reference centre Aarhus, Denmark, that was subcultured for many passages and subcloned. Sequences of strains with an asterisk were taken from the genome sequence of these strains. The bar indicates 5% sequence difference.

2.2. PCR amplification and sequencing

Primers P146MH-L TCCAAGACGAAGATCTTGACTATC and P146MH-R TTAGAACTTGCAAGATAAAGCTTG targeting a conserved flanking region of the serine repeats were chosen from the consensus sequence of the three *M. hypneumoniae* genome sequences available at GenBank. All PCR amplifications were performed in 30 μL total volume containing 12 pmol of each primer, 1 mM dNTP, 1× reaction buffer B (supplied with FIREPol® DNA polymerase), 2.5 mM Mg Cl_2 and 2.5 U of FIREPol® polymerase (Solis BioDyne, Tartu, Estonia). About 100 ng genomic DNA or 2 μL lysate was added. Cycling conditions on a GeneAmp PCR System 9800 (Applied Biosystems, Foster City, CA, USA) were 3 min denaturation at 94 ˚C, followed by 35 cycles at 94 ˚C for 30 s, 50 ˚C for 30 s and 72 ˚C for 30 s. A final extension step for 7 min at 72 ˚C was included. PCR products were purified with the High Pure PCR Purification Kit (Roche Applied Science, Rotkreuz, Switzerland). Finally, about 30 ng of the purified PCR product was used for sequencing with the BigDye Terminator cycle sequencing kit (Applied Biosystems), using the same primers as for the amplification. Products were analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and edited using the Sequencher program (GeneCode, Ann Arbor, MI, USA).

2.3. Multiplex real-time PCR

Detection of the REP and ABC target genes by PCR was described previously [3]. For this study a multiplex assay on the ABI 7500 (Applied Biosystems) was used. Amplification for multiplex reactions was performed in a total of 25 μL of reaction mixture containing 12.5 μL of 2× TaqMan® Universal PCR Mastermix (Applied Biosystems), 300 nM of each primer MHPTM950-L, MHPTM950-R, MHABCTM-L, MHABCTM-R1, MHABCTM-R2, 250 nM of each probe MHPTM950-CB, and MHABCTM-MGB. The Exogenous Internal Positive Control (Applied Biosystems) with the IPC mix diluted 1:20 and the IPC template diluted 1:100 was included in every reaction. For testing, 2.5 μL of the lysate or DNA was added as the template. The probe MHABCTM-MGB used for the ABC assay was labeled with FAM-dye as reporter at the 5' end and has a minor groove binder (MGB) as a nonfluorescent quencher at the 3' end of the oligonucleotide. In contrast, the MHPTM950-CB developed for REP in the multiplex assay has a Cy-5 label at the 5' end and the Dark Quencher BHQ-2 at the 3' end. The sequence of the probe is identical to the one described for the classical individual REP assay.

2.4. Cluster analysis

Sequences of the *p146* gene fragment were entered into Bionumerics v. 4.5 (Applied Maths, Kortrjik, Belgium). Number of repeats as well as the real-time PCR results were added to the entries as character types. A pairwise alignment of sequences was done with 100% gap penalty. Cluster analysis was performed using UPGMA.

The discriminatory power of the method was calculated as the Simpson's index [7].

3. RESULTS

3.1. Specificity, sensitivity and reproducibility of the typing method

The selected primers for the amplification were shown to be specific for *M. hyopneumoniae* and did not amplify genomic DNA of *Mycoplasma hyorhinis* (BTS-7^T), *Mycoplasma flocculare*

(MS-42^T), *Mycoplasma hyosynoviae* (S-16^T), *Mycoplasma conjunctivae* (HRC^T), *Acholeplasma laidlawii* (PG8^T), *Pasteurella multocida* (CCUG17976^T), *Bordetella bronchiseptica* (CCUG219^T), *Actinobacillus pleuropneumoniae* (4070^T), *Haemophilus parasuis* (Bakos A9), *Streptococcus suis* (P145/00), *Staphylococcus hyicus* (P815), *Staphylococcus aureus* (V21179), *Arcanobacterium pyogenes* (JF2818) or *Escherichia coli* K88 (JF1264) (data not shown). The sensitivity of the assay was estimated in relation to the semiquantitative results from the real-time PCR assays performed on the same samples. Thereby a detection limit of 10^2-10^3 genome copies per reaction was determined corresponding to 5×10^4–5×10^5 *M. hyopneumoniae* per mL lysate.

An identical *p146* gene segment sequence of 202 bp (Fig. 1, cluster VIIIa) was determined analyzing *M. hyopneumoniae* type strain cultures obtained from different sources. The serine in the repeat region is encoded by three different codons TCT, TCA and TCC.

3.2. Cluster designation

A cluster analysis based on the DNA sequence covering the polyserine region of *p146*, the results of the real-time PCR, as well as the number of serine repeats are shown in Figure 1. The resulting clusters containing samples of identical sequence were assigned roman numbers. In cases where clusters differed only by minor variation they were defined as subclusters indicated by the cluster number followed by a letter. Thereby, these subclusters contained sequences of equal length and differed in less than four nucleotides. Thus, 21 main clusters (I-XXI), of which four were further divided into subclusters were assigned, resulting in a total of 26 clusters.

Only three different groups could be determined by the PCR based clustering (ABC, REP, and ABC/REP), whereas 14 groups were found with the serine repeat number, ranging from a minimum of 12 to a maximum of 44 repeats.

The Simpson's index was calculated with a total of 26 clusters (Fig. 1), once with regarding all samples as individual strains and once with regarding samples from the same farm as one strain (including the French cluster) resulting in a value of 0.92 and 0.97, respectively.

3.3. Direct analysis of lung swabs of pigs with EP

Lysates of lung swabs from a total of 11 EP-positive farms were used to investigate the variability of the serine-rich repeat region within and between farms. The results presented in Figure 1 show that within a farm the same type of *M. hyopneumoniae* was present. This is true for all three typing approaches. The results of the real-time PCR correlated with the number of serine repeats as well as the phylogenetic clusters.

Eight different clusters of *M. hyopneumoniae* were observed with the 11 defined farms. Cluster XIXb contained samples from 2 farms and cluster IXb from 3 farms. Looking at the geographic location of the farms, those in the same cluster were in close proximity. The farms 8 and 10 were in neighbor villages with less than 3 km distance. The farms 2, 6 and 7 were within a perimeter of less than 10 km. Within a farm the cluster was in every case highly conserved with 100% sequence match between the individual samples. The multisite production ring consisted of four participating farms in close geographic and operational proximity. All animals of this multisite production ring had an identical, very typical strain (cluster XII).

In order to get an idea of the spectrum of putative clusters that could be found, single cases from routine diagnosis were analyzed and investigated whether they match previously found clusters or if they form

new clusters. As seen from Figure 1 these cases indexed by "P" can be found associated to clusters already established with the defined farms (e.g. VI), they formed new clusters containing single samples from different farms (e.g. IV) or single samples formed a cluster of their own (e.g. VII), resulting in a heterogeneous picture for these single cases. Cluster XXa contained 3 samples, each with a different REP and ABC pattern. The same situation was found in cluster XIXa with 2 samples.

3.4. Analysis of collection strains

To get an even further picture, strains from different countries including the type strain as well as strains from which the entire genome sequence is available were included. A similar picture as seen with the diagnostic isolates was observed. One major cluster was formed by several strains from France (X). As mentioned before, all the type strain derivatives obtained from 3 different sources formed a single cluster (VIIIa). Subclustering within the cluster of the type strain, is a sample of a diagnostic case from Switzerland, showing a 3 bp difference to the sequence of the *M. hyopneumoniae* type strain.

Generally the clusters were mixed with samples from different countries. This holds true for the major branch (clusters I-XVIII). A second branch only contained field samples from Switzerland (clusters XIX and XX). Strain 7448 formed an outgroup in the tree with a high number of serine repeats. This *M. hyopneumoniae* strain is the only one from the South American continent.

4. DISCUSSION

Molecular typing of *M. hyopneumoniae* is useful to trace infection sources, an important step in combating enzootic pneumonia. It also allows to carry out epidemiological investigations and learn more about the biological basis of *M. hyopneumoniae* infections. Moreover, hypotheses on the clonality or variability of *M. hyopneumoniae* strains can be verified with an accurate typing method. Our study provides such a tool using PCR and sequencing of the region encoding the serine repeats of the *p146* gene. The determined sequence contains 148 additional bases (35 bp upstream and 113 bp downstream of the serine repeat encoding part), which were used for a cluster analysis. This together with the silent base changes in the repeat encoding region increased the resolution of the DNA sequence based analysis compared to the determination of the number of serine repeats alone. This can be seen with various samples having the same number of serine repeats but which are located on very different branches in the tree (Fig. 1). The cluster analysis carried out showed several characteristics of *M. hyopneumoniae* strains circulating in the swine population. There is high variability with samples from 34 farms being distributed over 18 clusters and subclusters (Fig. 1). On the other side certain strains (identical or closely related) can be found in different farms. In most of these cases a close geographical or operational proximity was observed between the farms, indicating that strains can be distributed from one farm to the other.

In all farms from which we analyzed more than a single sample we always found the same strain being present. This indicates that once established, a single strain is responsible for a local outbreak, suggesting a limited clonality of *M. hyopneumoniae*. Nevertheless, no strong geographic association of strains was observed when looking at international isolates. A clear outgroup was found with the genome sequenced strain 7448 from Brazil. This was due to the high number of serine repeats (44) which is far above the maximum

number found in other samples. Taken together these findings suggest a high genetic diversity of *M. hyopneumoniae* with herd associated clones. Similar observations have been made using PFGE [9].

Within a strain, the repeat number is relatively stable as can be seen with the often passaged type strain derivate JF184a, that not only shows the same number of repeats but a 100% identical DNA sequence (including the flanking regions) compared to the original type strain NCTC 10110T. This is noteworthy since changes over many passages might happen and are e.g. also indicated by the fact that the ABC target can get lost, as seen with JF184a. This indicates that the corresponding gene segment used in our molecular epidemiological study is stable in *M. hyopneumoniae* under standard *Mycoplasma* culture conditions and after storing over several decades. The fact that a field sample from a single farm clustered in proximity to the type strain with only 3 bp sequence difference is an indication that strains very similar or maybe even identical to the type strain are still circulating in the swine population.

Based on the different combination of REP/ABC results which were included as a complement in this study, we hypothesized earlier that different strains are present in Switzerland [3]. This study further supports this, since all three combinations were found. Whereas in a certain farm always the same REP/ABC type was found, our results also show that strains from the very same cluster can actually differ in the REP/ABC patterns. This is most impressively shown in cluster XXa where all three samples showed a different REP/ABC pattern. Therefore, REP/ABC results alone are not a good typing method, not only because the resolution is anyway very low but since most similar strains can vary in their REP/ABC pattern as is seen with the type strain cluster (VIII) as well as with cluster XIXa. Thereby results from real-time PCR of REP/ABC could in certain cases complement and further resolve identical clusters into subclusters, since patterns are stable within a farm but might be different with samples from different farms. However, this will not significantly increase the discrimination power, that is already quite high.

In conclusion, the study showed heterogeneity of *M. hyopneumoniae* in field samples and isolated strains with no strong geographic association, but suggests that clones are responsible for local outbreaks. The described molecular typing approach either alone or in combination with REP/ABC types therefore provides a highly discriminating and specific epidemiological tool to test clinical material without the need of culturing and DNA isolation.

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REFERENCES

[1] Artiushin S., Minion F.C., Arbitrarily primed PCR analysis of *Mycoplasma hyopneumoniae* field isolates demonstrates genetic heterogeneity, Int. J. Syst. Bacteriol. (1996) 46:324–328.

[2] Belloy L., Janovsky M., Vilei E.M., Pilo P., Giacometti M., Frey J., Molecular epidemiology of *Mycoplasma conjunctivae* in caprinae: Transmission across species in natural outbreaks, Appl. Environ. Microbiol. (2003) 69:1913–1919.

[3] Dubosson C.R., Conzelmann C., Miserez R., Boerlin P., Frey J., Zimmermann W., Häni H., Kuhnert P., Development of two real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples, Vet. Microbiol. (2004) 102:55–65.

[4] Frey J., Haldimann A., Nicolet J., Chromosomal heterogeneity of various *Mycoplasma hyopneumoniae* field strains, Int. J. Syst. Bacteriol. (1992) 42:275–280.

[5] Goodwin R.F., Pomeroy A.P., Whittlestone P., Characterization of *Mycoplasma suipneumonia*: a mycoplasma causing enzootic pneumonia of pigs, J. Hyg. (1967) 65:85–96.

[6] Hege R., Zimmermann W., Scheidegger R., Stark K.D., Incidence of reinfections with *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* in pig farms located in respiratory-disease-free regions of Switzerland–identification and quantification of risk factors, Acta Vet. Scand. (2002) 43:145–156.

[7] Hunter P.R., Gaston M.A., Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity, J. Clin. Microbiol. (1988) 26:2465–2466.

[8] Kokotovic B., Friis N.F., Jensen J.S., Ahrens P., Amplified-fragment length polymorphism fingerprinting of *Mycoplasma* species, J. Clin. Microbiol. (1999) 37:3300–3307.

[9] Stakenborg T., Vicca J., Butaye P., Maes D., Peeters J., de Kruif A., Haesebrouck F., The diversity of *Mycoplasma hyopneumoniae* within and between herds using pulsed-field gel electrophoresis, Vet. Microbiol. (2005) 109:29–36.

[10] Stark K.D.C., Nicolet J., Frey J., Detection of *Mycoplasma hyopneumoniae* by air sampling with a nested PCR assay, Appl. Environ. Microbiol. (1998) 64:543–548.

[11] Straw B.E., D'Allaire S., Mengeling W.L., Taylor D.J., Diseases of swine, 8th edition, Iowa State University Press, Ames, 1999.

[12] Thacker E.L., Halbur P.G., Ross R.F., Thanawongnuwech R., Thacker B.J., *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia, J. Clin. Microbiol. (1999) 37:620–627.

[13] Thacker E.L., Thacker B.J., Janke B.H., Interaction between *Mycoplasma hyopneumoniae* and swine influenza virus, J. Clin. Microbiol. (2001) 39:2525–2530.

[14] Vicca J., Stakenborg T., Maes D., Butaye P., Peeters J., de Kruif A., Haesebrouck F., Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates, Vet. Microbiol. (2003) 97:177–190.

[15] Whitford H.W., Rosenbusch R.F., Lauerman L.H., Mycoplasmosis in animals: laboratory diagnosis, Iowa State University Press, Ames, USA, 1994.

[16] Yazawa S., Okada M., Ono M., Fujii S., Okuda Y., Shibata I., Kida H., Experimental dual infection of pigs with an H1N1 swine influenza virus (A/Sw/Hok/2/81) and *Mycoplasma hyopneumoniae*, Vet. Microbiol. (2004) 98:221–228.

[17] Zeeh F., Kuhnert P., Miserez R., Doherr M.G., Zimmermann W., Field validation of a real-time PCR test for the detection of *Mycoplasma hyopneumoniae* in nasal swabs of live pigs, Schweiz. Arch. Tierheilkd. (2005) 147:373–379.

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