



RAPD and SDS-PAGE analysis of *Mycoplasma hyorhinis* isolates of South Korea

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Received: 3 April, 2013; Accepted: 29 September 2013

Key words: *Mycoplasma hyorhinis*, Porcine enzootic pneumonia, RAPD, SDS-PAGE, Typing

Mycoplasma hyorhinis (*Mhr*) is part of the normal microflora in the respiratory tract of pigs. This organism is implicated in the development of polyserositis, arthritis, otitis and other symptoms (Thacker 2006). Furthermore, using a challenge test, it was reported that *Mhr* is a primary aetiological agent of the economically important disease porcine enzootic pneumonia (PEP, Lin *et al.* 2006). Molecular typing studies provide information on the strain level differences in microbes that may help in defining the virulence factors. In the past, whole cell protein (WCP) typing (Calus *et al.* 2007) and random amplified polymorphic DNA (RAPD) typing (Vicca *et al.* 2003) have been used to identify and classify the mycoplasma strains into three virulence types. Given the importance of *Mhr* in PEP and other diseases, information about its heterogeneity is prerequisite. Nevertheless, until now there are no published articles on *Mhr* typing. The purpose of this study was to use the WCP and RAPD typing to evaluate the diversity of *Mhr* field isolates from South Korea, if any.

The reference strain used in this study was *Mhr* (ATCC27717) (American Type Culture Collection, ATCC). A total of 25 isolates were obtained from the lung lesions of 4–5 month old pigs randomly selected from each of 14 herds. In these herds, the pigs were maintained in two-site system facilitated with controlled ventilation under the temperate climatic conditions of South Korea. These herds were not closely related and there was no mingling of pigs from one herd to another. The identity of these isolates was confirmed with a multiplex PCR (Barate *et al.* 2012). The isolates were named in the format *Mhr*11.2, in which 11 represents the number of the herd and 2 indicates the pig number. Isolates used in this study were passaged one time except for *Mhr* 9.1 and *Mhr* 2.8, those were passaged three times after isolation. The *in vitro* growth rates were determined by

performing colour-changing unit (CCU) titrations in triplicate for each isolate (Stemke and Robertson 1982).

To prepare the WCP from the samples, isolates grown in Friis broth (3.12% 10X Hanks' Balanced salt solution, 0.54% Mycoplasma broth base, 0.51% Brain heart infusion, 0.62% yeast extract, 0.41% of 2% thalium acetate, 10% porcine serum, 10% horse serum, 100 µg/ml ampicillin, 100 µg/ml glucose and 40 µg/ml phenol red) were harvested by centrifugation. The pellets were washed three times with phosphate-buffered saline (PBS) and then resuspended in PBS. The protein content was measured using the BCA protein assay kit. For SDS-PAGE, 7.5 µg proteins from each sample were boiled with sample buffer (0.1 M Tris/HCl (pH 6.8), 2% SDS (w/v), 20% glycerine (w/v), 5% 2-mercaptoethanol (v/v) and 0.025% bromophenol blue (w/v)) for 5 min (Calus *et al.* 2007). Curve-based Pearson similarity coefficients were calculated using Bionumerics V5.1 as described by Calus *et al.* (2007). The genomic DNA of the isolates was extracted using a Genomic DNA extraction mini kit. RAPD was performed using the following protocol: 45 amplification cycles (each consisting of 1 min at 95°C, 1 min at 37°C and 2 min at 72°C) were run on a PTC100 Thermal Cycler. The 25 µl reaction mixture was optimised containing 30 ng of purified genomic DNA, 2 mM MgCl₂, 20 pM of OPA-3 primer, 1X AccuPower® PCR, 1X Stoffel Buffer and 2 U AmpliTaq DNA polymerase, Stoffel Fragment. Initial denaturation step of 5 min at 95°C and a final extension of 10 min at 72°C were used in the RAPD protocol. The RAPD fingerprints were analysed using Dice similarity coefficient and unweighted pair group method with arithmetic means (UPGMA) clustering method (Fig. 1B).

Bionumerics analysis of *Mhr* WCP revealed a proteomic variability of 44.9% when the standard strain *Mhr* (ATCC 27717) was compared with the isolates (Fig. 1A). Among the isolates, a maximal divergence of 34.2% was observed. Two regions of high variability were detected in the molecular weight ranges 170 to 95 kDa and 72 to 34 kDa. The remaining regions, in the molecular weight range 170 to 240 kDa and below a molecular weight of 34 kDa, showed low variability.

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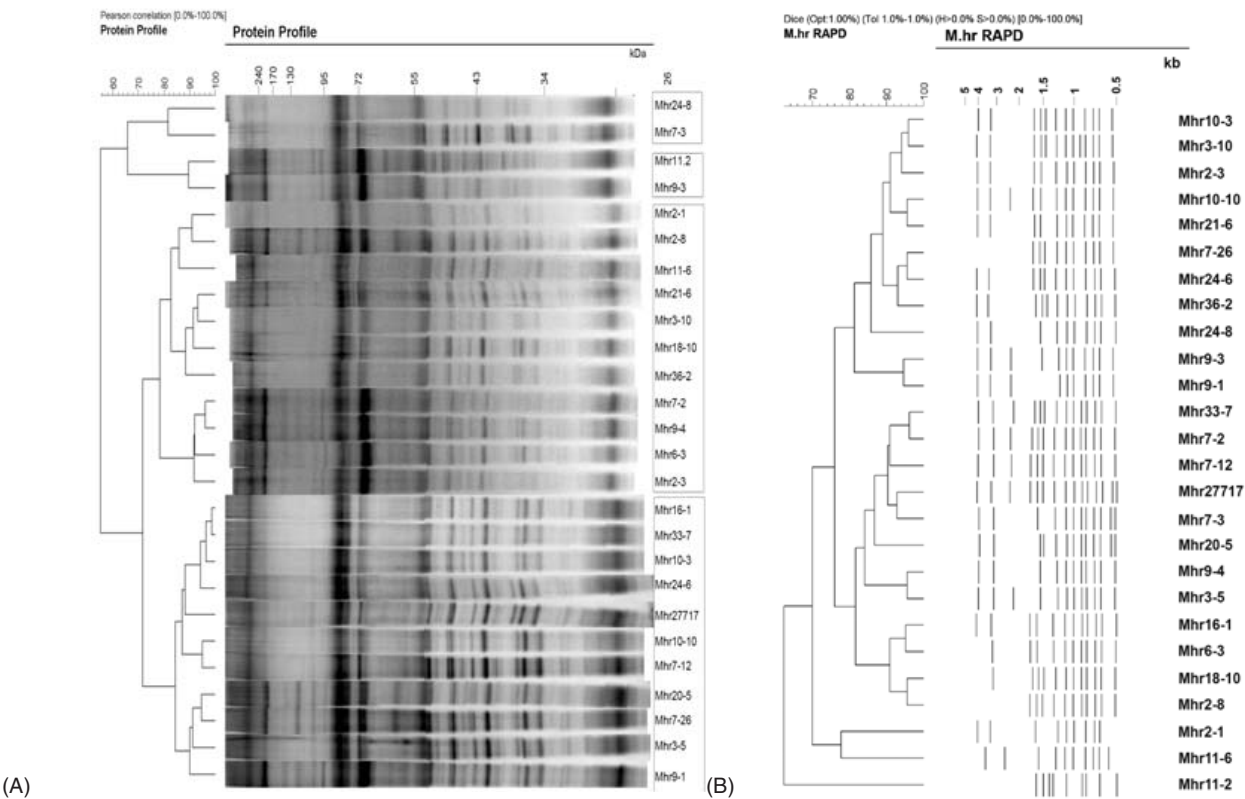


Fig. 1 A. cluster analysis of *Mhr* protein patterns using the curve-based Pearson similarity coefficient. B . Cluster analysis of *Mhr* RAPD patterns using the Dice coefficient.

These results are similar to the protein pattern differences in the region of 95 to 120 kDa reported previously for *Mycoplasma hyopneumoniae* isolates by Calus *et al.* (2007). Isolates from one herd were sometimes placed closely in clustering but did not show identical protein profiles. None of the isolates evaluated in this study had any unique protein bands.

Based on the RAPD patterns the *Mhr* isolates could be divided into five major clusters (Fig. 1B). Overall, 37.7% variability was detected when the field strains were compared with the *Mhr* (ATCC 27717) reference strain. The RAPD pattern of the isolate *Mhr*11.2 showed high variation compared with the remaining *Mhr* isolates (Fig. 1B). Three bands of sizes 1,100 bp, 800 bp and 700 bp were present in all of the isolates (except *Mhr*11.2). A high-intensity band of size 1,700 bp was observed only in strain *Mhr*11.6. Two isolates (*Mhr*6.3 and *Mhr*18.10) exhibited a low-intensity, high-molecular-weight band of 3,200 bp, and 20 isolates exhibited low-intensity, high-molecular-weight bands of sizes 4,000 bp and 3,200 bp. Strain *Mhr*11.6 showed slightly different molecular bands, viz., 3500 bp and 2,800 bp. Similar to protein patterns, non-identical RAPD patterns were observed for isolates from the same or different herd. This indicates the presence of more than one *Mhr* strain in one swine herd. These findings are similar to a recent report that

found many mycoplasma strains in one herd (Nathues *et al.* 2011). The field strains not only differed in typing but they also had different growth rates, estimated by using CCU titrations (data not shown). No correlation was observed between the *Mhr* WCP and RAPD clusters.

To conclude, this study reveals that *Mhr* field isolates on comparison with the standard *Mhr* (ATCC 27717) as well as among field isolates, exhibits very diverse RAPD and SDS-PAGE protein profile circulated in the swine herds of South Korea. To our knowledge, this article is the first report on *Mhr* WCP and DNA variability. These results will be helpful in clinical studies and typing of *Mhr*.

SUMMARY

In this study, 25 field isolates recovered from 14 herds were analysed using SDS-PAGE and RAPD. Among the field strains, variability of 34.2% and 30% were observed at the protein and genomic levels, respectively. Also, variability of 44.9% and 37.7% were noticed between the field strains and a reference strain at the protein and genomic levels, respectively. Furthermore, isolates from the same herd demonstrated differences in protein and RAPD patterns. These results reveal high proteomic and genetic diversity among the field isolates of *Mhr* in South Korea.

ACKNOWLEDGEMENTS

This research was supported by the Technology Development Programme for Agriculture and Forestry, Ministry of Agriculture, Food and Rural Affairs, 94 Dasom2-ro, Government Complex-Sejong, Sejong-si 339-012, South Korea.

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