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Proteomic and Genetic Diversity of *Mycoplasma hyopneumoniae* Isolates from South Korea

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The purpose of the present study was to evaluate the diversity of *Mycoplasma hyopneumoniae* (Mhp) isolates from South Korea. Overall, 42 field isolates recovered from 36 herds were analyzed using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and random amplification of polymorphic DNA (RAPD). Fifteen highly virulent strains were identified based on the presence of a 181 kDa band, a protein band associated with high virulence. A variability of 25.3% and 35.5% was noticed between field strains and J strain at protein and genomic level, respectively. Among the field strains, a variability of 22.2% and 31% was observed at protein and genomic level, 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 South Korea.

Key words: Enzootic swine pneumonia, *Mycoplasma*, Typing, SDS-PAGE, RAPD.

Mycoplasma hyopneumoniae (Mhp) is the etiologic agent of enzootic pneumonia (EP) in pigs. There is evidence that Mhp is a highly heterogeneous species; SDS-PAGE (protein pattern) heterogeneity[1-3] and RAPD (genetic pattern) heterogeneity have been reported previously[4-6]. Individual strains of Mhp also differ in their virulence potential and have been classified into three virulence groups: low, moderate and high virulence. In general, disparity between the vaccine strain and local field strains can result in insufficient protection by vaccine [7]. Available reports on Mhp variability include typing studies performed on isolates from Belgium, Denmark, the U.S.A., the U.K., Germany and Serbia[4, 5, 8, 9]. However, until now, there has been no report addressing mycoplasma heterogeneity in Asia.

The present study aimed to evaluate the diversity of Mhp field isolates from South Korea and to compare the local isolates with Mhp J strain, the strain present in most commercial vaccines[10].

MATERIALS AND METHODS

The reference strain used in this study was Mhp J (ATCC 25934) (American Type Culture Collection (ATCC), USA). Forty-two field isolates were obtained from the lung lesions of 4-5month old pigs randomly selected from each of 36 herds. These herds were not closely related and there was no mingling of pigs from one herd with pigs of other herds. Mhp isolates were confirmed using a multiplex PCR [11]. The isolates were named in the format Mhp7.25, where 7 represents the herd

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number and 25 indicates the pig's number. Isolates used in this study were passaged one time except for Mhp 1.4 and Mhp 13.3 that were passaged 2 and 3 times, respectively.

The *in vitro* growth rates of isolates were determined by performing color-changing unit (CCU) titrations[12]. To prepare the total protein from each sample, isolates grown in Friis broth were harvested by centrifugation. The protein content was measured by using the Pierce® BCA Protein Assay Kit (Thermo Scientific, USA). Total protein from the isolates was processed and subjected to SDS-PAGE as described previously [2]. Curve-based Pearson similarity coefficients were calculated using Bionumerics V5.1 (Applied-Maths, Belgium) as previously reported [2].Genomic DNA of the isolates was extracted using Genomic DNA Extraction Mini Kit (RBC Biosciences, Taiwan). DNA concentrations were quantified using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA). RAPD was performed as follows on a PTC100 Thermal Cycler (MJ Research, USA): 45 amplification cycles of 1 min at 95°C, 1 min at 37°C, and 2 min at 72°C. The 25 μl reaction mixture was optimized to contain 30 ng of purified genomic DNA, 2 mM MgCl2, 20 pmol of OPA-3 primer (Bioneer, South Korea), 1X AccuPower®PCR Premix (Bioneer), 1X Stoffel Buffer and 2 U AmpliTaq DNA polymerase Stoffel Fragment (Applied Biosystems, USA). The amplified fragments were separated using standard 1% gel electrophoresis. Amplification bands annotated by Bionumerics software V5.1 were visually assessed, and fragments smaller than 500 bp were omitted prior to further analysis. This assay was done in triplicate for reproducibility. The calculation of similarity coefficients was performed using the Dice algorithm. The unweighted pair group method with arithmetic means (UPGMA) was used for clustering, and a band position tolerance and optimization setting of 1% was implemented.

RESULTS AND DISCUSSION

SDS-PAGE patterns of isolates revealed high protein variability (Fig. 1). Two molecular weight ranges of particularly high variability were detected, from 72 to 130 kDa and 55 to 34 kDa.

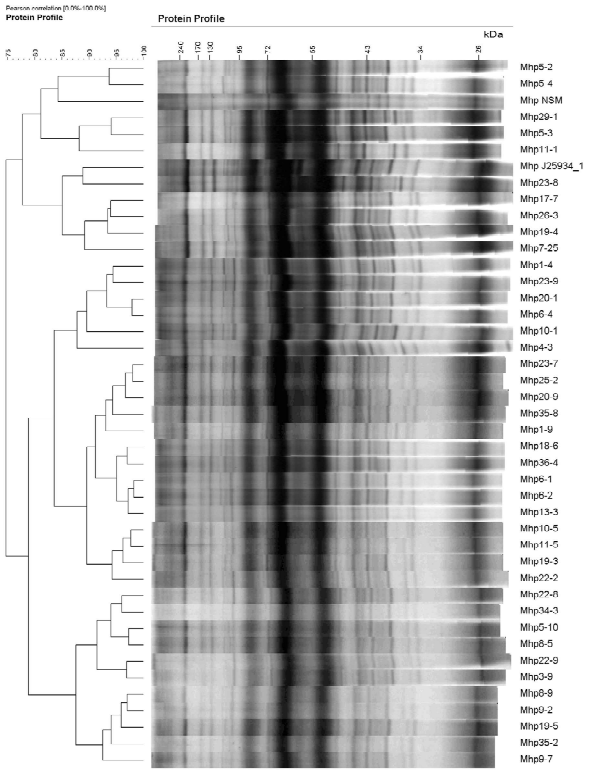

Fig. 1. Cluster analysis of Mhp protein patterns using the curve-based Pearson similarity coefficients

Additionally, regions of low variability were detected from 130 to 240 kDa, from 72 to 55 kDa and below 34 kDa. These results are in agreement with previous reports of Mhp protein patterns[1-3]. A maximal proteomic variability of 25.3% was found when the J strain was compared to field isolates. This finding is similar to the 30% protein variability reported in a previous study [2]. Among the field isolates, a maximum of 22.2% divergence was observed; moreover, isolates from the same herd showed non-identical patterns (Fig. 1). In 2007, identical protein profiles were reported for same-herd isolates by visually comparing their protein profiles[2]. To avoid any potential individual error, the protein patterns of isolates in this study were compared and clustered only with the help of computer software (Bionumerics). Based on the presence of a 181 kDa band [2], 15 highly virulent strains were identified from hosts with high CCU titers (data not shown) as reported previously [13]. In this study, we did not observe the 181 kDa band for J strain isolates; however, this absence could be due to loss of protein expression during the course of frequent *in vitro* passaging[14-17]. The results obtained from these isolates were confirmed by repeated electrophoresis. To avoid the problem of reproducibility in RAPD, all the samples were analyzed in a single run in triplicate. All attempts to reproduce the 5,000 bp amplicon using the standard protocol were unsuccessful[6]. Therefore, we optimized conditions by varying $MgCl_2$ concentration, dNTPs concentration, DNA polymerase and even the PCR machine used for amplification. Low reproducibility using this method has been previously reported [4, 5, 18]. A very faint band of approximately 5,000 bp could be obtained for a few isolates using our modified RAPD protocol; however, this band did not always correlate with the protein pattern, suggesting that it may represent a different amplicon than previously reported (Fig. 2). Two bands of 1,300 bp and 500 bp in length were present in most isolates, and many isolates contained two other intense bands of 1,700 bp and 900 bp in length. In this study, 35.5% variability was detected between the South Korean field isolates and the J strain. RAPD diversity of 50% and 45% has been previously reported for field isolates; the higher genetic diversity observed in these former studies could be the result of strains originating from diverse geographical areas [5] or the endemic nature of disease and high volumes of animal trade [4]. Similar to protein patterns, non-identical RAPD patterns were observed for isolates from the same herd, indicating the presence of more than one Mhp strain per swine herd. These findings are similar to a recent study that observed different patterns for isolates from single herd [4]. Neither protein pattern nor RAPD pattern Bionumerics

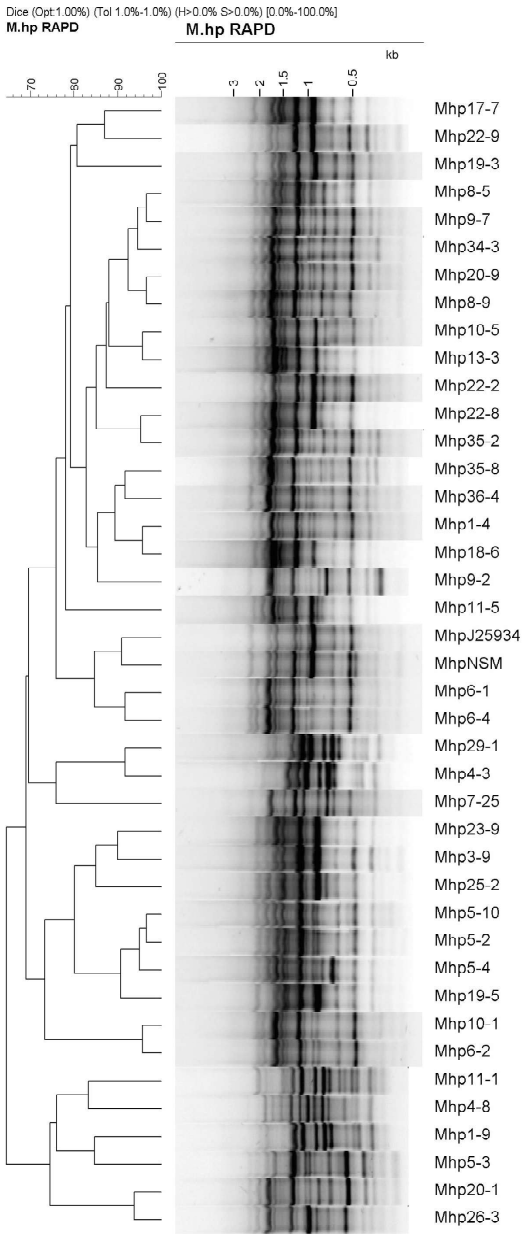

Fig. 2. Cluster analysis of Mhp RAPD patterns using the Dice coefficient

could cluster the isolates according to virulence as reported previously [2]. RAPD typing method is based on short arbitrary primers that bind DNA at random sites and a single base pair change can even alter the band pattern. On the other hand depending upon the *in vivo* pressure protein expression in mycoplasma is specifically altered by mechanisms such as epitope masking, turning on or off of particular gene, size variation, phase variation, protein cleavage [19, 20]. Thus, the two methods studied give different clustering results.

CONCLUSION

Results of this study reveal that Mhp field isolates with very diverse genetic types and protein variability circulate in the swine herds of South Korea. Also there is variability between the field isolates and Mhp-J, the strain mostly used in commercial vaccines. This study lays the foundation for preventive measures and molecular typing of Mhp in South Korea.

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