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Mycoplasma hyorhinis is a potential pathogen of porcine respiratory disease complex that aggravates pneumonia caused by porcine reproductive and respiratory syndrome virus

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ABSTRACT

The porcine respiratory disease complex (PRDC) caused by numerous bacterial and viral agents has a great impact on pig industry worldwide. Although *Mycoplasma hyorhinis* (Mhr) has been frequently isolated from lung lesions from pigs with PRDC, the pathological importance of Mhr may have been underestimated. In this study, 383 serum samples obtained from seven herds with a history of PRDC were tested for specific antibodies to Mhr, *Mycoplasma hyopneumoniae* (Mhp), and porcine reproductive and respiratory syndrome virus (PRRSV). Seropositive rates of PRRSV were significantly correlated with those of Mhr (correlation coefficient, 0.862; P-value, 0.013), but not with those of Mhp (correlation coefficient, −0.555; P-value, 0.196). *In vivo* experiments demonstrated that pigs co-infected with Mhr and PRRSV induced more severe lung lesions than pigs infected with Mhr or PRRSV alone. These findings suggest that Mhr is closely associated with pneumonia caused by PRRSV and provide important information on Mhr pathogenesis within PRDC. Therefore, effective PRDC control strategies should also consider the potential impact of Mhr in the pathogenesis of PRDC.

1. Introduction

Porcine respiratory disease complex (PRDC) is a major health concern in the pig industry worldwide (Brockmeier et al., 2002; Hansen et al., 2010). Pigs in growing and finishing stage (14–22 weeks) are most commonly affected with PRDC (Kim et al., 2003; Thacker, 2001). PRDC is characterized by problems with growth performance and feed efficiency as well as clinical signs such as fever, cough, anorexia, and dyspnea (Thacker, 2001). Prominent histopathologic findings are bronchopneumonia alone or in combination with interstitial pneumonia (Harms et al., 2002; Kim et al., 2003).

The most frequently detected pathogens in PRDC affected pigs were porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), *Pasteurella multocida* and porcine mycoplasmas, such as *Mycoplasma hyopneumoniae* (Mhp), *Mycoplasma hyorhinis* (Mhr), and *Mycoplasma flocculare* (Mflo) (Hansen et al., 2010). All these species of mycoplasma have been isolated from the porcine respiratory tract and are genetically related (Stemke et al., 1992). Mhp is the primary pathogen causing enzootic pneumonia and has been studied intensively (Maes et al., 2008). However, the epidemiology and pathogenesis of Mhr is less well understood.

Mhr is a commensal bacterium of the porcine respiratory tract (Christensen et al., 1999). However, Mhr has been proposed as a possible primary pathogen of a number of porcine diseases including arthritis, conjunctivitis, otitis media, eustachitis, and pneumonia (Duncan and Ross, 1973; Friis, 1976; Gois and Kuksa, 1974; Lin et al., 2006; Morita et al., 1995; Morita et al., 1999). Mhr has also been identified in co-infection with PRRSV or PCV2 in the porcine respiratory system (Gagnon et al., 2007; Kawashima et al., 2007, 1996; Kobayashi et al., 1996). Furthermore, synergistic effects of porcine mycoplasmas and PRRSV have been reported in the literature and several reports, have indicated that Mhr has been diagnosed in numerous cases of PRDC (Hansen et al., 2010; Kobayashi et al., 1996; Lin et al., 2006; Thacker et al., 1999). These

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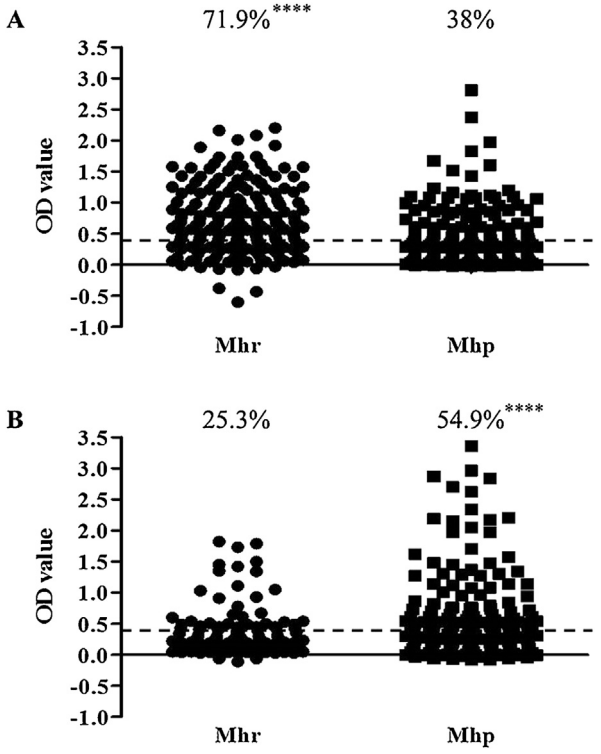

Fig. 1. Scatterplot of the enzyme-linked immunosorbent assay (ELISA) results for *Mycoplasma hyorhinis* (Mhr) and *Mycoplasma hyopneumoniae* (Mhp) from field serum samples. A) positive (n = 221) and B) negative (n = 162) for PRRSV. ****P < 0.0001.

results suggest that Mhr may play a significant role in PRDC not only as secondary infectious agent.

Although Mhr is easily isolated from bronchioalveolar lavage fluid and nasal swabs from pigs, and can be grown readily in culture, isolating and identifying mycoplasma species is generally time-consuming. Outbreaks of PRDC can be controlled with accurate diagnosis and appropriate treatment using antibiotics and vaccines. However, the multifactorial etiology and the environmental stressors involved in the pathogenesis of disease, complicate the implementation of appropriate control strategies, which require adequate use of vaccination and medication, as well as the reduction of potential stressors.

This study focused on the investigation of synergistic effects between mycoplasmas and PRRSV infections within PRDC, with a special focus on Mhr, as measured by the seroprevalence of Mhr, Mhp, and PRRSV in pig herds with a history of PRDC as well as in *in vivo* experiments of pigs inoculated with Mhr and/or PRRSV.

2. Materials and methods

2.1. Serum samples

Serum samples were collected from 383 pigs aged between 4 and 16 months from seven herds. The number of samples collected from pigs of herd 1 through 7 was 72, 69, 49, 40, 39, 48, 89, and 25 samples, respectively. None of the pigs in these herds was vaccinated against Mhp. Eleven sera each from 10-day-old colostrum-free and 6-week-old specific pathogen-free pigs were used as negative reference sera.

2.2. Enzyme-linked immunosorbent assay (ELISA) analysis

The Mhr Korean field strain EH5 was isolated from lung tissue of a pig showing mycoplasmal lesions. The EH5 strain was used to prepare sonicated antigen for Mhr serological testing. A 100 ml aliquot of freshly cultured mycoplasma cells was harvested by centrifugation at 15,000*g* for 20 min and washed three times with phosphate buffered saline (PBS). The cells were sonicated 10 times for 9 s with 9 s pause between each sonication step at 100 W. After an additional centrifugation at 15,000*g* for 20 min, the supernatant was stored at −70 °C until use. To detect the intensity of the background signal generated by components of the mycoplasmal culture medium (media-only antigen) the medium was treated in the same way as described above. Ninety-six well microtiter plates (SPL Life science, Daejeon, Korea) were coated with 100 μl Mhr antigen (0.625 μg/ml) or the media-only antigen diluted in coating buffer (0.1 M sodium carbonate) and were then incubated overnight at 4 °C. After blocking with 100 μl PBS containing 0.1% (v/v) Tween-20 (PBST) and 5% (w/v) skim milk powder at room temperature (RT) for 1 h, 100 μl of test sera diluted 1:40 in dilution buffer (2.5% [w/v] skim milk in PBST) was added to each mycoplasma antigen and media-only wells. After 1 h incubation at RT, a 100 μl aliquot of horseradish peroxidase-conjugated goat anti-porcine IgG (AbD Serotec, Raleigh, NC, USA) diluted 1:1000 in PBST was used as secondary antibody. Plates were incubated at RT for 1 h and developed with 100 μl (0.4 g/L) of 3,3′,5,5′-tetra-methylbenzidine (TMB) peroxidase substrate (KPL Inc., Gaithersburg, MD, USA) in the dark. The reaction was stopped after 15 min by adding 100 μl of 1 M H_2SO_4. The optical density (OD) of the solution was measured at 450 nm. The normalized OD value of each sample was calculated by subtracting the OD value of the media-only well from that of the mycoplasma antigen wells. The Mhr cut-off point was determined to be ≥0.35 which is three standard deviations above the mean OD value of the media-only wells (Beier et al., 1988; Van Loon and Van der Veen, 1980).

PRRSV- and Mhp-specific antibody levels were detected using commercial ELISA kits (HerdCheck; IDEXX Laboratories, Inc., Westbrook, ME, USA) following the manufacturer's protocol. Samples were considered positive if the calculated sample-to-positive control ratio was ≥ 0.4.

2.3. Animal experiment

Three-week-old pigs tested seronegative for Mhr, Mhp, and PRRSV were challenged with Mhr strain EH5 and PRRSV strain LMY. The two strains used in this study were Korean field isolates. One group of two piglets was intra-tracheally inoculated with 1×10^{10} color change units (CCU)/ml Mhr, while a second group of two piglets was intra-tracheally inoculated with 1×10^{6} median tissue culture infective dose ($TCID_{50}$)/ml of PRRSV (Labarque et al., 2003; Lin et al., 2006). A third group of two piglets was co-inoculated with Mhr and PRRSV using the same microbe dosage and route. Each group of piglets was housed separately and fed antibiotic-free. All animals were euthanized 3 weeks post-inoculation, and tracheal and lung samples were collected for histopathological analysis. The tissue samples were fixed in 10% formalin and processed by routine hematoxylin and eosin staining.

2.4. Statistical analysis

Statistical analyses were performed with GraphPad Prism ver. 5.0 software (GraphPad Software, San Diego, CA, USA). Fisher's exact test and Pearson's product-moment correlation coefficient analysis were conducted. Fisher's exact test was used for calculation of the Mhr and Mhp seropositive rate in PRRSV seropositive and seronegative sera, respectively. Pearson's product-moment corre-