Detection of *Mycoplasma hyopneumoniae* by ELISA and nested PCR from blood samples and nasal swabs from pigs in Slovakia

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Abstract

The aim of our study was to map the situation of swine mycoplasmoses on four farms in the region of Eastern Slovakia. The primary agent of Enzootic pneumonia of swine is *Mycoplasma hyopneumoniae*. After reviewing the health status of conventional herds and evaluation of clinical symptoms, paired samples of nasal swabs and venous blood samples were collected from 38 pigs with clinical signs of respiratory disease. Nasal swab samples were tested by nested PCR, while blood samples were used to detect antibodies against *M. hyopneumoniae* by blocking ELISA. The presence of *M. hyopneumoniae* was confirmed by nested PCR in four pigs (10.5%) and by blocking ELISA in 16 pigs (42.1%) of all four farms. This work presents for the first time comparison of different methods to diagnose *M. hyopneumoniae* infection on pig farms in Eastern Slovakia.

Diagnosis, respiratory diseases, Enzootic pneumonia, antibodies

Respiratory diseases can cause great distress to individual animals. They also have a serious effect on performance and production in affected groups of pigs. They are a major problem for the pig industry, and the potential cost of respiratory disease outbreaks is high. Various causes of respiratory diseases have been identified, including viruses, mycoplasmas, bacteria, and parasites. Secondary bacterial infection of lung tissue already compromised by primary pathogens frequently occurs (Jackson and Cockcroft 2007). Respiratory syndrome in swine, porcine respiratory disease complex (PRDC) has been described as an important cause of decreased productivity in swine. *Mycoplasma hyopneumoniae* is one of the most important pathogens associated with PRDC (Calsamiglia et al. 1999; Thacker et al. 1999; Silin et al. 2001). *M. hyopneumoniae*, the causative agent of enzootic porcine pneumonia (EPP) in pigs, is often associated with bacterial species, including *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* or with viruses including porcine reproductive and respiratory syndrome virus (Verdin et al. 2000).

Enzootic pneumonia is a very important disease of pigs that causes severe economic losses through clinical disease and an adverse effect on food conversion and weight gain. This disease has widespread occurrence throughout the world (Siugzdaite and Garlaite 2002; Jackson and Cockcroft 2007). Chronic pneumonia of swine has worldwide distribution and occurs in virtually all herds. Previous surveys have indicated that 30–80% of market swine have chronic pneumonia. Serologic surveys indicated that approximately 20% of breeding animals and 60% of herds have complement fixing antibodies to *M. hyopneumoniae* (Young et al. 1983). Pointon et al. (1985) and Jackson and Cockcroft (2007) reported that rate of weight gain may be decreased by as much as 16–30% and feed conversion by 14–20%.

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The aim of our study was to detect *M. hyopneumoniae* and antibodies against *M. hyopneumoniae* in pigs with respiratory diseases and to suggest preventive precautions.

Materials and Methods

Sampling

The samples were collected in July 2008 on four conventional pig farms in the region of Eastern Slovakia with an "all in, all out" production system. The number of samples was proportional to the number of pigs on each farm (0.5% on farm). A total of 38 nasal swab samples and 38 blood samples were collected from 4-month-old growing pigs from a population of the White Improved breed with signs of respiratory disease. We observed a dry and non-productive cough as a typical symptom of enzootic pneumonia, low body condition, decreased appetite, dyspnoea and tachypnoea. The respiratory disease score described by Halbur et al. (1996), was 4. The herds were not treated with antibiotics at that time and after detection of *M. hyopneumoniae*. In pigs, clinical signs of other bacterial infections were not observed. They were also not vaccinated against infectious diseases. Table 1 summarizes sampling data.

Table 1. Characteristic of samples taken from pigs with signs of respiratory disease

Sampling location	District	Number of samples	Symbols of samples
Klokočov	Michalovce	3	KL1 – KL3
Pláne	Michalovce	15	PL1 – PL15
Grajciar	Košice surroundings	10	GR1 – R10
Odorín	Spišská Nová Ves	10	OD1 – OD10

Blood samples from pigs were obtained by jugular vein puncture. The samples were individually identified, and delivered to the laboratory for processing. Serum was obtained by centrifugation for 10 min at 3500 × *g* and stored at –20 °C until subsequent analysis. Serum was analysed for the presence of antibodies to *M. hyopneumoniae* using a blocking ELISA.

Nasal swabs were inserted deeply into the nostrils of pigs, then rotated and moved up and down gently. We used commercially manufactured sampling swabs with a Stuart Amies transport medium. Nasal swabs were frozen and kept at –80 °C until analysis. Nested PCR was used to detect the presence of *M. hyopneumoniae* in the nasal swabs.

ELISA detection

Mycoplasma hyopneumoniae ELISA (OXOID Ltd, UK) is a blocking assay that has a significantly improved performance compared with traditional assays and is rapid and simple to use even with high numbers of samples. The specificity of the test is improved over other methods by the use of a highly specific monoclonal antibody against a conserved epitope of the *M. hyopneumoniae* 74KDa protein which does not cross react with *Mycoplasma flocculare*. Porcine serum samples were diluted and incubated in microwells pre-coated with *M. hyopneumoniae* antigen. Monoclonal antibody to *M. hyopneumoniae* was then added to the wells containing the diluted samples. After 15 min, wells were washed and a chromogenic substrate was added. The colour reaction was stopped by the addition of acid and the intensity of the colour was measured at 450 nm and compared with the absorbance obtained from buffer controls. Specimens with a mean optical density (OD) value less than 50% of the OD buffer control were interpreted as positive. Specimens with a mean OD value ≥ 65% of the OD buffer control were interpreted as negative. Specimens with a mean OD value 50% and 65% of the OD buffer control were interpreted as equivocal.

Nested PCR

DNA from clinical samples (nasal swab) was isolated using the NucleoSpin® Tissue kit (MACHEREY-NAGEL, GmbH & Co. KG, Germany).

The primer sequences used for amplification of the 16S rRNA gene for the detection of *M. hyopneumoniae* were as follows: Hp1 (5′TTT TAG TTC GCT AAA ATA TTT AGT AGC A 3′), Hp3 (5′ TCT GTC ATC TCG TTA GCC TCG 3′), Hp4 (5′ TTT TAT TCA AAG GAG CCT TCA 3′) and Hp6 (5′ GTC TTA GTC ACT TTT GCC ACC 3′). The primer combination was Hp1/Hp3 for the first round. The nested primers (Hp4/Hp6) amplified a 660 bp fragment. In the PCR reaction we used Taq-Purple DNA Polymerase PCR Master Mix with 5mM $MgCl_2$ (PPP master mix from Top-Bio). All samples were tested in standardized 20 µl PCR reactions containing 0.5 µl of each primer, 7 µl ddH_2O, 10 µl PPP master mix and 2 µl DNA. One microlitre of undiluted PCR product was transferred to a new tube for nested PCR. The cycling protocol used for the first step PCR was 5 min denaturation at 95 °C followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s and extension at 72 °C for 90 s. The reaction was then incubated at 72 °C for 5 min to complete the extension reaction. The cycling protocol for the second step consisted of 4 min denaturation at 95 °C followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 60 s. The reaction was then incubated at 72 °C for 5 min to complete the extension reaction. The reference strain (MEVAK, Slovakia) was used as positive

control for the PCR detection of *M. hyopneumoniae*. Amplified samples were loaded and electrophoresed on a 1% agarose. The gels were stained with ethidium bromide and viewed under ultraviolet (UV) illumination to assess the product.

Statistical analysis

Relationship between the results of serological and nested PCR tests and significance of correlations were calculated ($P < 0.094$). Statistical analyses were carried out using statistical Excel software.

Results

Using ELISA assay we detected 42.1% (16/38) serum samples positive for the presence of antibody against *M. hyopneumoniae*. Antibodies against *M. hyopneumoniae* were present in pigs from all four farms. Only 10.5% (4/38) of the nasal samples from pigs with clinical signs of respiratory disease were found positive by nested PCR. A 660-bp PCR product was observed in samples positive for *M. hyopneumoniae* (Plate I, Fig. 1). Comparison of the results obtained by ELISA and nested PCR is summarized in Table 2. Correlations between the results of serological and nested PCR tests were not significant. This dependence was expressed by the value R = 0.27 and $P < 0.094$.

Discussion

The results obtained by ELISA and nested PCR confirmed the presence of *M. hyopneumoniae* infection on all four pig farms. The PCR detects the presence of *M. hyopneumoniae* in the respiratory tract while ELISA detects indirectly the passage of the mycoplasma in pigs (Verdin et al. 2000). According to Feld et al. (1992), Le Potier et al. (1994) and Morris et al. (1995), the time required for seroconversion is at least 3–5 weeks after infection and seroconversion is more commonly observed after 11

Table 2. Comparison of results obtained by ELISA from blood samples and by nested PCR from nasal swabs of pigs with signs of respiratory disease

Sample	ELISA	nested PCR	Sample	ELISA	nested PCR
KL1	+	-	GR2	+	-
KL2	+	-	GR3	-	-
KL3	-	-	GR4	-	-
PL1	+	-	GR5	+	-
PL2	+	+	GR6	+/-	-
PL3	+	-	GR7	+	+
PL4	+	-	GR8	+	-
PL5	+	-	GR9	+/-	-
PL6	-	-	GR10	+	-
PL7	-	-	OD1	+/-	-
PL8	-	-	OD2	-	-
PL9	-	-	OD3	+	-
PL10	-	-	OD4	-	-
PL11	+	-	OD5	+	+
PL12	-	-	OD6	+/-	-
PL13	-	-	OD7	+	-
PL14	+/-	-	OD8	-	-
PL15	+/-	-	OD9	-	-
GR1	-	-	OD10	+/-	+

Positive (+), negative (-) and dubious (+/-) samples

weeks, so the difference in sensitivity may reflect the time of sampling. Compared with the complement fixation test (KFT), the ELISA method is more sensitive and does not give false positive reactions as KFT (Pilipčinec et al. 1998). This PCR test should be a useful tool for the control of enzootic pneumonia and detection of healthy carriers in pig herds. Tracheobronchial brushes and bronchial alveolar lavage fluids are the best sites for detection of *M. hyopneumoniae* with a sensitivity of 95%–100%, while lung tissues and nasal swabs were not reliable sites for sampling (Kurth et al. 2002). The sample from lung lesions is perhaps too small, so in many cases this subsection of the lesion may not have contained these organisms.

We found that in region of Eastern Slovakia, the presence of antibodies against *M. hyopneumoniae* in pigs is on average 42.1%. Our results are comparable with findings from the neighbouring states, Europe and beyond. In the Ukraine, the seroprevalence ranged from 5.7%–57% (Lysenko et al. 1980), in Poland it was 91.3% (Dors et al. 2012). In Germany, seropositivity was 65% from 2578 tested sows (Grosse-Beilage et al. 2009). In Japan, the prevalence of antibodies against *M. hyopneumoniae* in sows and fattening pigs of different ages from convention farms ranged from 2.9% to 79.1% (Yagihashi et al. 1993). In Thailand, Damrongwatanapokin et al. (2004) found 58% blood samples positive in ELISA in 22 of 23 pig breeding farms and 74.4% in 80 of 84 fattening farms. In China, among 12 intensive farms, only two of them showed no infection of *M. hyoneumoniae* and the seroprevalence ranged from 0% to 90%, with an average prevalence of 45.7% (He et al. 2011). In Sweden, Mattsson et al. (1995) detected antibodies against *M. hyopneumoniae* in 90% of pigs by ELISA. Simultaneously, nasal swabs of pigs were tested by PCR. The positivity was 16%. Positivity of 4% was the result of the second examination conducted three weeks later in the same group. In our study in region of Eastern Slovakia, the total average percentage of positive pigs within the herds was 10.5% (4/38). Our results are comparable with a large study conducted by Villarreal et al. (2010) in different EU countries. According to these authors 3% of pig were positive in Denmark, 6% in Belgium, 9% in Spain, 14% in The Netherlands, 17% in Poland, 18% in Hungary, 23% in Germany, 38% in France and 40% in Italy. In total, 10.7% of suckling pigs and 67.3% of sampled herds were positive.

This study demonstrated the presence of a pathogenic strain of *M. hyopneumoniae* in the nasal swab and presence of antibodies against *M. hyopneumoniae* in pigs with respiratory disease. The presence of this bacteria and enzootic porcine pneumonia had a negative impact on the health and productivity of farm pigs. Avoiding a direct contact of newborn piglets with the sow and other infected pigs, or contact between different age groups of animals belong to preventive measures. In the remediation or elimination program, vaccination has important implications, because according to Lehner et al. (2008) increasing seropositivity and typical lung lesions confirmed infection with *M. hyopneumoniae* at the end of the fattening period. Effects of infection became most evident in non-vaccinated pigs or those from vaccinated sows that received the vaccine in the 1st week of life. We recommend the use of complex measures, involving immunoprophylaxis and appropriate antibiotic therapy.

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Plate I
Prokeš M. et al.: Detection of ... pp. 327-331

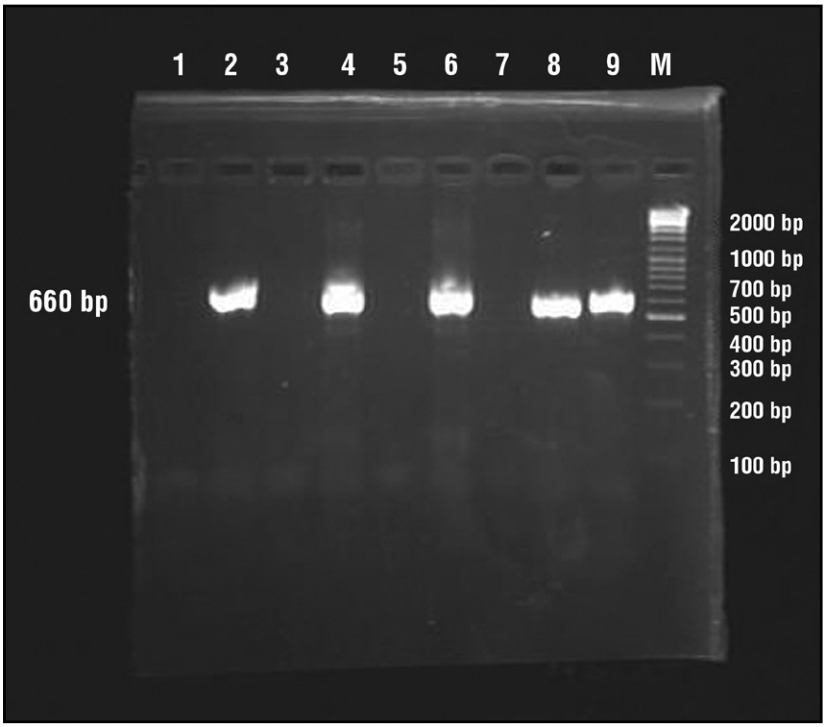

Fig. 1. Agarose gel electrophoresis of nested PCR products obtained from four pigs with signs of respiratory disease

Lane 1 – negative control, lane 2 – positive control of *Mycoplasma hyopneumoniae* (660 bp), lanes 4,6,8,9 – positive samples (nasal swab), lane M – 100 bp DNA molecular-size marker