EVALUATION OF THE CO-INFECTION CAUSED BY SWINE CIRCOVIRUS 2 AND Mycoplasma hyopneumoniae IN LUNG SAMPLES OBTAINED FROM A SLAUGHTERHOUSE IN THE MATA MINEIRA REGION, BRAZIL

AVALIAÇÃO DA COINFECÇÃO DO CIRCOVÍRUS SUÍNO 2 COM Mycoplasma hyopneumoniae EM AMOSTRAS DE PULMÕES COLETADAS EM ABATEDOURO NA REGIÃO DA ZONA DA MATA MINEIRA

J. E. BEZERRIL[1*], J. P. MACHADO[1], F. M. F. DA SILVA[1], L. H. S. BULOS[1], M. I. V. VILORIA[1], M. R. ALMEIDA[1]

SUMMARY

The aim of this study was determine the association between PCV2 and *M. Hyopneumoneae* in pig lung samples. Thirty two lung fragments, from which 16 had macroscopic lesions (CLM) and the other 16 had no macroscopic lesions (SLM), were analyzed by histopathology, immunohistochemistry assays and polymerase chain reaction in real time (qPCR). No positive correlation was observed among the histology and the immunohistochemistry assay (IHC). There was also no positive correlation between the number of samples with viral pneumonia type of lesions and PCV2 viral load measured by qPCR. Analysis of the data obtained by detection of agents and associated microscopic findings showed that PCV2 and *M. Hyopneumoneae* co-infection was found in four samples (12,5%). This result reveals a possible association of these two agents. Further studies are required to determine additional potential pathogens involved in the pathogenesis of the PRDC in the Mata Mineira region, Brazil.

KEY-WORDS: Histopathology. Immunohistochemistry. Polymerase chain reaction in Real Time (PCRrt).

RESUMO

Este trabalho teve como objetivo pesquisar a associação do PCV2 e *M. Hyopneumoneae* em amostras de pulmão de suínos. 32 fragmentos de pulmões, sendo 16 com lesões macroscópicas (CLM) e 16 sem lesões macroscópicas (SLM) foram analisados pela histopatologia, pelo teste de imuno-histoquímica (IHC) e pela reação de polimerase em cadeia em tempo real (qPCR). Não foi observada correlação positiva entre a histopatologia e a imuno-histoquímica. Não houve também, correlação positiva entre número de amostras com lesões características de pneumonia viral e a carga viral de PCV2 medida por qPCR. Analisando os dados obtidos da detecção dos agentes e lesões microscópicas associadas, constatou-se que em 4 amostras analisadas (12,5%) houve uma co-infecção entre o PCV2 e *M. Hyopneumoneae*. Este resultado demonstra uma possível associação deste dois agentes. Novos estudos deverão ser realizados para determinação de outros possíveis patógenos envolvidos na patogenia da PRDC na região da Zona da Mata Mineira.

PALAVRAS-CHAVE: Histopatologia. Imuno-histoquímica. Reação de polimerase em cadeia em tempo real (qPCR).

[1] Universidade Federal de Viçosa – UFV.* julianaevb@gmail.com

Submitted: 16/04/2012 Accepted: 22/08/2012

INTRODUCTION

The Porcine Respiratory Diseases Complex (PRDC) is a set of respiratory diseases that affect pigs during the growing and finishing phases. The disease is caused by the association of viral (porcine circovirus 2, PRRSV, swine influenza virus) and bacterial (*Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Pasteurella multocida, Haemophilus parassuis, Streptococcus suis*) pathogens (HANSEN et al.,2010; FACHINGER et al., 2008; KIM et al., 2003, CHOI et al., 2003). Affected animals show dyspnea, cough, anorexia, fever, lethargy, decreased feeding efficiency and slow growth (KIM et al., 2003). Several studies show that PCV2 and *M. hyopneumoniae* are among the most prevalent pathogens in PRDC (HANSEN et al., 2010; KIM et al., 2003).

Mycoplasma hyopneumoniae is the main agent responsible for the Swine Enzootic Pneumonia (SIBILA et al., 2009). The disease is characterized by a chronic pneumonia with high morbidity and low mortality, and the economic losses are related to drugs and reduced performance (SARRADEL et al., 2003; MAES et al., 2008; SIBILA et al., 2009).

PCV2, in addition to causing respiratory diseases, is listed as the agent of Porcine Multi-systemic Wasting Syndrome (PMWS), Porcine Dermatitis and Nephropathy Syndrome (PDNS), as well as reproductive disorders and enteritis (SEGALES et al., 2005).

Some studies have demonstrated the synergism of PCV2 with other pathogens. Given how endemic the infection by *M. Hyopneumoneae* and PCV2 is in several pig farms in Brazil (KICH e PONTES, 2001), this study aimed to investigate the association between PCV2 and *M. Hyopneumoneae* in lung samples from pigs.

MATERIAL AND METHODS

Sampling

Lung samples of 32 pigs in termination phase, aged between 150 and 180 days, which came from pig farms positive for M. Hyopneumoneae and PCV2 in the Mata Mineira region, were examined macroscopically. Fragments of skull-ventral region of the cranial lobes were collected and divided into two parts. One portion was kept in a freezer at -80ºC, to be used later for performing polymerase chain reaction in real time (qPCR), while the second portion was fixed in 10% buffered formalin for histopathological and ICH analysis.

Histopathological analysis

The samples were fixed in 10% buffered formalin phosphate, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Subsequently, 4-µm thick cuts were stained using hematoxylin and eosin. The histological cuts examined were classified according to the microscopic changes observed, following the classification proposed by Sobestiasnky et al. (1999). Four categories were defined as follows: Stage 1- early acute, lymphocyte peribronchiolar infiltration and the presence of cellular infiltrate in the lumen of the alveoli and bronchioles; Stage 2- late acute, lymphocytic proliferation, extending through the lamina propria of the bronchioles and absence of cellular infiltrate in the lumen of the alveoli and bronchioles; Stage 3- sub-acute, discreet to moderate hyperplasia of peribronchial and peribronchiolar lymphoid follicles, absence of infiltrate cellular in the lumen of the alveoli, bronchi, bronchioles, as well as alveolar wall thickening; Stage 4- chronic, severe hyperplasia of peribronchial and peribronchiolar lymphoid follicles, with areas of atelectasis and absence of infiltrate into the lumen of the alveoli (Figure 1).

DNA isolation and Real time Polymerase chain reaction

Total DNA was extracted from the samples using the Wizard®* kit extraction, according to the manufacturer instructions. The final elution volume for tissue samples was quantified by measuring the density. PCV2 DNA was quantified by qPCR (OLVERA et al., 2004). The reactions were performed in 96-well plates, including samples and control samples in triplicate. Amplification and fluorescence detection were performed using the sequence detection system ABI Prism 7500 SV Genomic DNA Purification System (PROMEGA) (Applied Biosystems, Foster City, California). Each well contained 12.5 ml of TaqMan Universal Master Mix, 200 nM of each primer, 200 nM of PCV2 probe and 2.5 µL of sample, totaling 25 µL. The qPCR program was: 10 minutes at **95**ºC and 2 minutes at 50ºC, followed by 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. Controls and samples were tested together in each reaction. The viral load was expressed as the mean number of log10 copies per 500 ng of total DNA per sample.

Immunohistochemistry

IHC was performed by the streptavidin-biotin-peroxidase technique (LSAB). A solution of 3% hydrogen peroxide in absolute methanol was used to block endogenous peroxidase. Subsequently, the samples were immersed in citrate buffer, pH 6, at **90ºC** temperature during 20 minutes for antigen retrieval. Non-specific proteins were blocked out with 10% skim milk in distilled water for 30 minutes. The tissue was then incubated for 12 hours with primary antibody (rabbit IgG anti *Mycoplasma hyopneumoniae* from Embrapa Suínos e Aves – Concórdia-SC), diluted 1:100 in PBS (pH 6.0). The chromogen used was DAB and counter-staining was performed with Harris hematoxylin. Positive and negative controls were included in each sample processing. Based on the color intensity, the fragments were classified as: (1) absent immunostaining and (2) present immunostaining (Figure 2).

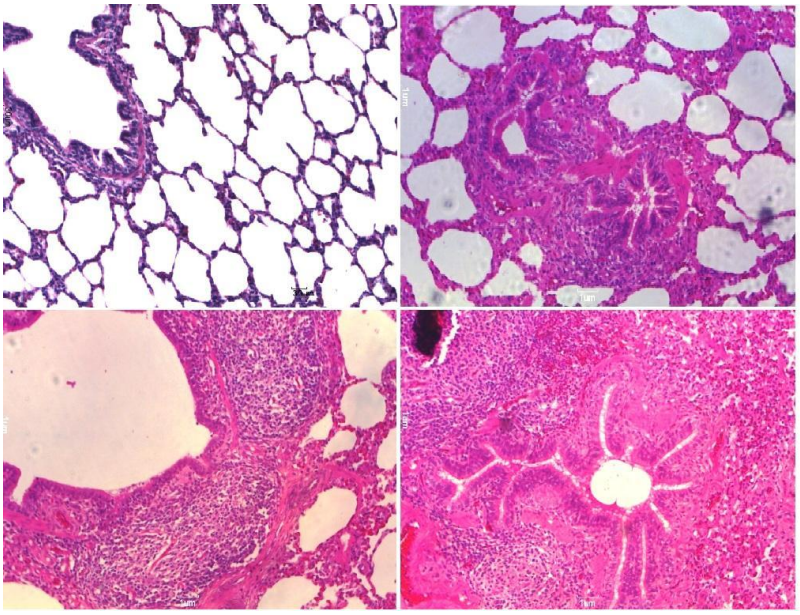

Figure 1 - Photomicrograph of histological analysis of pig lung tissues. A: histological section of fragment without significant lesion (phase 0). Black arrow: thin alveolar walls and no inflammatory infiltrate. B: Tissue samples showing late acute lesions (stage 2). Black arrow: lymphocytic proliferation, extending through the lamina propria of the bronchioles. Red Arrow: absence of cellular infiltration in the lumen of the alveoli and bronchioles. C: fragment of lung tissue lesion evidencing the late subacute stage (stage 3). Black Arrow: moderate hyperplasia of peribronchiolar lymphoid follicles, Red Arrow: absence of cellular infiltration in the lumen of the alveoli, bronchioles and bronchi. Green Arrow: thickening of the alveolar wall. D: Tissue samples showing chronic stage lesion (stage 4). Black Arrow: severe hyperplasia of peribronchiolar lymphoid follicles causing stenosis. Red Arrow: areas of atelectasis. Green Arrow: absence of infiltration into the lumen of the alveoli.

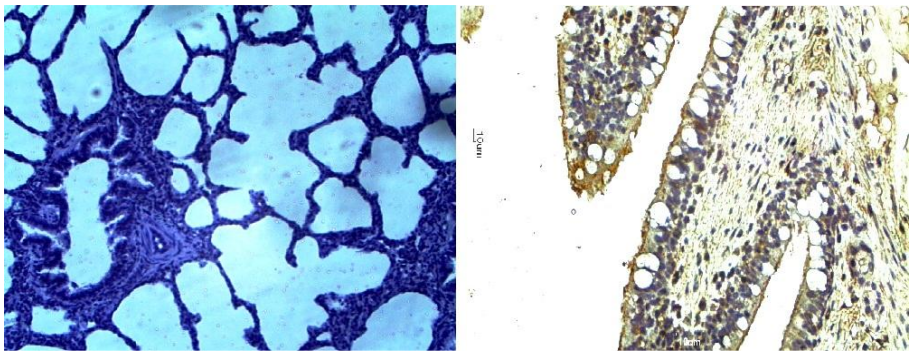

Figure 2 - Presence of *M. Hyopneumoniae* in lung tissue samples of pigs detected by immunohistochemistry (IHC). (A) negative sample. (B) positive sample (Arrow shows immunostaining presence).

Table 1 . Distribution of lung tissues according to the degree of microscopic lesion observed.

Pneumonia stage	Prevalence
0	4 (12,5%)
1	0 (0%)
2	6 (18,75%)
3	18 (56,25%)
4	4 (12,5%)

Tabela 2 - Distribuição das amostras de acordo com o grau de lesão para *M. hyopneumoniae* infectados pelo PCV2 e carga viral medida pela qPCR.

Lesion degree	Number of affected animals	Viral load
0	3	$0,702 \pm 0,319^{A}$
1	0	-
2	1	$1,024^{A}$
3	10	$0,936 \pm 0,366^{A}$
4	3	$0,773 \pm 0,261^{A}$
Total	**17**	-

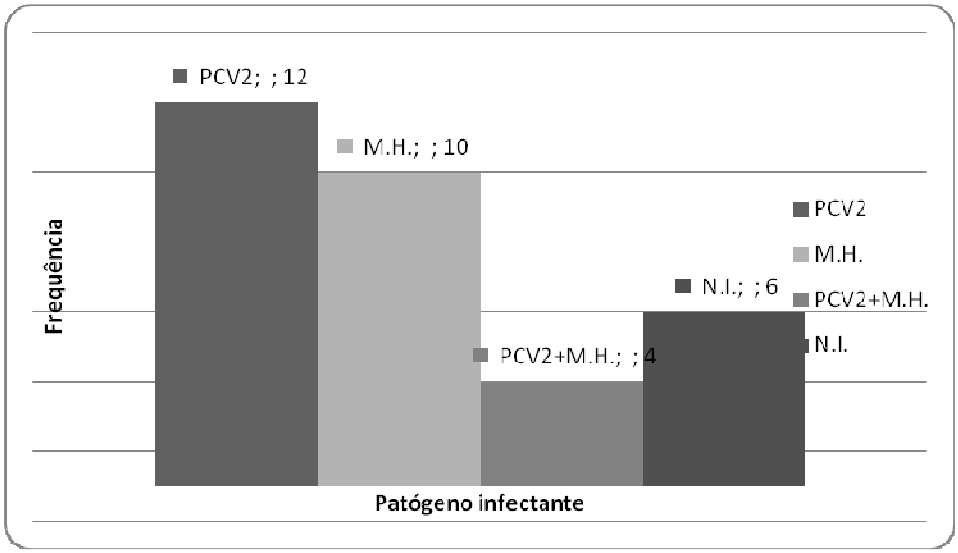

Figure 3 - Groups of animals according to the infecting pathogen. NI: studied pathogens not identified in the tissues**.**

RESULTS

M. hyopneumoniae detection x histopathological analysis

The microscopic lesions found in 28 (87.5%) samples were classified as stages 2, 3 and 4 (Figure 1) according to classification proposed by Sobestiansky et al (1999). No sample was classified as stage 1. Non-significant microscopic lesions were present in 4 (12.5%) samples (Table 1).

Histopathological analysis showed that 27 (84.37%) of 32 lungs had lesions characteristic of *M. hyopneumoniae*. Among the samples with lesions suggestive of infection, 14 (43.75%) were positively marked for IHC. In most samples, the IHC marking was homogeneous, which did not allow classifying into the different stages.

PCV2 detection x histopathological analysis

There was no positive correlation between the microscopic examination of lesions suggestive of infection by PCV2 (interstitial pneumonia) and viral DNA detection. Interstitial pneumonia, characteristic of viral infections, was observed in 20 (62.5%) of 32 samples. Whereas PCV2 was detected in only 11 (55.5%) of the 20 lung samples that had interstitial pneumonia.

M. hyopneumoniae detection x PCV2

IHC and qPCR analysis detected 12 samples infected exclusively by PCV2. Ten samples were positive for *M. Hyopneumoniae* only, and four samples were infected by both. Six samples were negative for the studied pathogens (Figure 3).

PCV2 viral load within different categories of lesions caused by *M. Hyopneumoniae* was found to be consistent in all tested samples; there was no relationship between the degree of lung injury and the number of copies of the viral genome (Table 2).

Statistical Analysis

The data were statistically analyzed by Pearson and Tukey tests in order to assess the correlation between diagnoses techniques used. The statistical analysis of the qPCR results were performed using the software Prism vs 5.02 (Graph pad software Inc., USA). The copies of the PCV2 genome from the lung samples were submitted to analysis of variance (ANOVA) ($p < 0.01$).

DISCUSSION

Most of the samples displayed injuries consistent with infection by *M hyopneumoniae*. From those, 84.37% had histopathological lesions, but only 43.75% were marked by the *M. Hyopneumoneae* detection test. The samples were not representative size wise. These results agree with Cai et al. (2007), who reported that there is a chance of getting false-negative results since the tissue fragment used in the test is very small.

Interstitial pneumonia was detected in 62.5% of the samples. However, PCV2 was detected in 55.5%. There was no positive correlation between the number of positive samples and the mean viral load determined by qPCR in the analyzed tissues. These results are consistent with the results reported by Brunborg et al. (2004) and Silva et al. (2011), who reported a higher viral load in lymph nodes compared to kidneys, intestine, spleen and liver. These results may be explained by the virus physiology that targets lymphoid organs (CHANG et al., 2006).

The interstitial pneumonia detected in the analyzed lung tissues is a pathologic change often caused by viruses (SORENSEN et al., 1997). In addition to PCV2, porcine respiratory coronavirus (PRCV), porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (H1N1) are among the main causes of respiratory diseases in pigs. PRRSV has not been detected in the Brazilian pig herd (CIACCI-ZANELLA, J.R., 2004; PESCADOR et al., 2007). Brentano et al. (2002) conducted a seroepidemiological survey for PRCV and H1N1 in the Brazilian swine herd, and reported only the presence of antibodies against H1N1. Therefore, the interstitial lesions in the lung tissues of animals that did not have PCV2 in their lungs may have been caused by another viral agent such as H1N1.

The analysis of the results to detect agents and the associated microscopic lesions showed that in four analyzed samples (12.5%) there was a co-infection caused by both PCV2 and *M. Hyopneumoneae*.

CONCLUSION

This result suggests a possible association between these two agents. Further studies are recommended to determine other possible pathogens involved with PRDC that might be present in the Mata Mineira region.

ACKNOWLEDGMENTS

Thanks are due to Professor Abelardo Silva Júnior (Departamento de Veterinária, Universidade Federal de Viçosa), for helping us to design the experiment and to write the manuscript.

REFERENCES

BRENTANO, L.; CIACCI-ZANELLA, J. R.; MORES, N.; PIFFER, I. A. Levantamento Soroepidemiológico para Coronavírus Respiratório e da Gastroenterite Transmissível e dos Vírus de Influenza H3N2 E H1N1 em Rebanhos Suínos no Brasil. **Comunicado Técnico, Embrapa Aves e Suínos, Ministério da Agricultura, Pecuária e Abastecimento**. 2002.

BRUNBORG, I. M.; MOLDAL, T.; JONASSEN, C. M.. Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR**. Jornal of Virological Methods** v.122, p.171–178, 2004.

CAI, H. Y.; VAN DREUMEL, T.; MCEWEN, B.; HORNBY, G.; BELL-ROGERS, P.; MCRAILD, P.; JOSEPHSON, G.; MAXIE, G. Application and field validation of a PCR assay for the detection of Mycoplasma hyopneumoniae from swine lung tissue samples. **Journal of Veterinary Diagnostic Investigation**, v.19, p.91–95, 2007.

CHANG, H. W.; JENG, C. R.; LIN, T. L.; LIU, J. J.; CHIOU, M. T.; TSAI, Y. C.; CHIA, M. Y.; JAN, T. R.; PANG, V. F. Immunopathological effects of porcine circovirus type 2 (PCV2) on swine alveolar macrophages by *in vitro* inoculation. **Veterinary Immunology and Immunopathology.** V.110, p.207-219, 2006.

CHOI, Y. K.; GOYAL, S. M.; JOO, H. S. Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. **The Canadian Veterinary Journal**, v.44, p.735–737, 2003.

CIACCI-ZANELLA J. C.; TROMBETTA C.; VARGAS I.; COSTA D. E. M. Lack of evidence of porcine reproductive and respiratory syndrome virus (PRRSV) infection in domestic swine in Brazil. **Ciência Rural**, v,34, n.2, p.449-455, 2004.

DOMINGO, M. Quantification of porcine circovirus type 2 (PCV2) DNA in serum and tonsillar, nasal, tracheo-bronchial, urinary and faecal swabs of pigs with and without postweaning multisystemic wasting syndrome (PMWS). **Veterinary Microbiology**. v.111, n.3-4, p.223-229, 2005.

FACHINGER V.; BISCHOFF, R.; JEDIDIA, S. B.; SAALMÜLLER, A.; ELBERS K.. The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. **Vaccine**, v.26, p.1488-1499, 2008.

HANSEN, M. S.; PORS S. E. V.; BILLE-HANSEN, S. K. J.; KJERULFF, N. O. L An Investigation of the Pathology and Pathogens Associated with Porcine Respiratory Disease Complex in Denmark**. Journal of Comparative Pathology**. v.143, p.120-131, 2010.

KICH, J. D.; PONTES, A. P. Análise da situação atual das doenças respiratórias no Brasil. In: Congresso Brasileiro de Veterinários Especialistas em Suínos, X, Porto Alegre. **Anais**. **Concórdia: Embrapa Suínos e Aves**, v.1, p.58-67, 2001.

KIM, J.; CHUNG, H. K.; CHAE, C.. Association of porcine circovirus 2 with porcine respiratory disease complex. **The Veterinary Journal**, v.166, p.251–256, 2003.

MAES, D.; SEGALÉS, J.; MEYNS, T.; SIBILA, M.; PIETERS, M.; HAESEBROUCK, F. Control of Mycoplasma hyopneumoniae infections in pigs. **Veterinary Microbiology**, v.126, p. 297–309, 2008.

OLVERA, A.; SIBILA, M.; CALSAMIGLIA, M.; SEGALÉS, J.; DOMINGO, M. Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. **Journal of Virological Methods**.v.80, p.117-75, 2004.

PESCADOR, C. A.; BANDARRA, P. M.; CASTRO, L. A,; ANTONIASSI, N. A. B.; RAVAZZOLO, A. P.; SONNE, L.; DRIEMEIER, C. E. F. C. D. Co- infection by porcine circovirus type 2 and porcine parvovirus in aborted fetuses and stillborn piglets in southern Brazil. **Pesquisa Veterinária Brasileira**. v.27, p.425-429, 2007.

SARRADELL, J.; ANDRADA, M.; RAMÍREZ, A. S.; FERNÁNDEZ, A.; GÓMEZ-VILLAMANDOS, J. C.; JOVER, A.; LORENZO, H.; HERRÁEZ, P. F. A; RODRÍGUEZ, A. Morphologic and Immunohistochemical Study of the Bronchus-associated Lymphoid Tissue of Pigs Naturally Infected with Mycoplasma hyopneumoniae. **Veterinary Pathology**, v**,** 40,p, 395–404, 2003.

SEGALÉS, J.; DOMINGO, M. (2005) Post-weaning multisystemic wasting syndrome (PMWS) in pigs. A review. **Veterinary Quarterly**, v.24, n.3, p.109-124, 2002.

SIBILA M., PIETERS M., MOLITOR T. , MAES D., HAESEBROUCK F., SEGALÉS JCurrent perspectives on the diagnosis and epidemiology of Mycoplasma hyopneumoniae infection. **The Veterinary Journal**, v, 181 p, 221–231, 2009.

SILVA, F. M. F.; JUNIOR, A. S.; VIDIGAL, P. M. P.; OLIVEIRA, C. R.; VIANA, V. W.; SILVA, C. H. O.; VARGAS, M. I.; FIETTO, J. L. R.; ALMEIDA, M. R. **Porcine Circovirus-2 Viral Load Versus Lesions in Pigs: Perspectives for Post-weaning Multisystemic Wasting Syndrome**. **Journal of Comparative Pathology**, v.144, p.296-302, 2011.

SOBESTIANSKY, J. Pneumonia enzoótica. **Clínica e patologia suína**. 2.ed. Goiânia, Goiás: Art 3 Impressos Especiais, p.359, 1999.

SORENSEN, V.; AHRENS, P.; BARFOD, K.; FEENSTRA. A. A.; FELD, N. C.; FRISS, N. F.; BILLE-HANSEN, V.; JENSEN, N. E.; PEDERSEN,

M. W. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and evaluation of four diagnostic assays, **Veterinary Microbiology**, v.54, p.23-34, 1997.