Molecular diagnosis of respiratory diseases of swine - Klein C.S.[1*], Morés N.[1], Oliveira Filho J.X.[2], Rebelatto R.[1], Bellaver F.A.V.[1], Silva G.B.[3]

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The respiratory problems on intensive pig farming have a complex etiology, caused by the interaction of multiple infectious agents and therefore the term "Porcine Respiratory Diseases Complex" (PRDC) has been used. Among the bacterial agents, *Mycoplasma hyopneumoniae* (Mhyo), *Pasteurella multocida* (Pm), *Actinobacillus pleuropneumoniae* (App) and *Haemophilus parasuis* (Hps) are the most commonly agents found in lung lesions. Our focus is to develop molecular and immunological methods along with traditional microbiological assays to diagnose respiratory diseases in pigs. We have standardized and validated a nested PCR for Mhyo detection and implemented a multiplex PCR for Mhyo, *Mycoplasma hyorhinis* and *Mycoplasma flocculare*. From 2001 to 2009, we have standardized and implemented PCR for detection of virulence factors genes of App (*cpx*, *apxI*, *apxII*, *apxIII*, *apxIV* and *sodC*). Currently, we are implementing and validating the genotypic characterization of Pm by multiplex PCR in order to detect virulence factors, capsular typing and species-specific genes portions, following by sequencing. The Pm samples containing virulence factors detected by PCR analysis will be submitted to fingerprint DNA by pulsed-field gel electrophoresis (PFGE) and restriction endonuclease analysis (REA). Samples with greater pathogenic potential will have sequence of the complete genome. Finally, we are implementing and validating PCR for Hps/tbpA gene. We also intend to characterize the serotype of the Hps isolates using the Restriction Polymorphism Length Fragment (RFLP). The use of molecular diagnosis allows a faster diagnosis of the bacterial agents involved in PRDC in pigs as well as the characterization of serotypes and virulence factors of respiratory bacteria. The knowledge of such serotypes and virulence factors will be important to the development of target vaccines as well as guidance for epidemiological studies.

Key-words: molecular diagnostic, respiratory diseases, swine

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Molecular Diagnosis of Respiratory Diseases of Swine

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INTRODUCTION

The respiratory diseases in swine, also so called "Porcine Respiratory Diseases Complex" (PRDC) in intensive pig farming have a complex etiology, caused by the interaction of multiple infectious agents. Among the bacterial agents, *Mycoplasma hyopneumoniae* (Mhyo), *Pasteurella multocida* (Pm), *Actinobacillus pleuropneumoniae* (App), and *Haemophilus parasuis* (Hps) are the most commonly agents found in lung lesions. Our focus is to develop molecular and immunological methods along with traditional microbiological assays to diagnose the agents of PDRC in pigs.

METHODOLOGY

We have standardized and validated a nested PCR to Mhyo detection (YAMAGUTI et al., 2008) and we have implemented a multiplex PCR for Mhyo, *Mycoplasma hyorhinis* (Mh) and *Mycoplasma flocculare* (Mf) (STAKENBORG et al., 2006).

Since 2001, we have standardized and implemented simple PCR for detection of genes encoding virulence factors of the App (*cpx, apxI, apxII, apxIII, apxIV* and *sodC*) (COLLARES, 2002; KLEIN et al., 2003; COSTA et al., 2004; SOUZA et al., 2008).

Currently, we have been implementing and validating the genotypic characterization of Pm by multiplex PCR in order to detect virulence factors, capsular typing and species-specific genes portions, following by sequencing (TOWSEND *et al.*, 2001). From Pm samples containing virulence factors, DNA will be submitted to fingerprint by pulsed-field gel electrophoresis (PFGE) with the restriction enzyme *ApaI*, and restriction endonuclease analysis (REA) using *HpaII*. The results will be analyzed using the program Bio Numerics version 6.0 (ApliedMaths, Belgica). Sequencing of the complete genome of the highly pathogenic Pm samples will be performed.

Finally, we have proposed the implementation and validation of a PCR test to detection of *tbpA* gene from *H. parasuis*. We also intend to characterize the serotype of the Hps isolates using the Restriction Polymorphism Length Fragment (RFLP) with the restriction enzymes *TaqI*, *AvaI* and *RsaI* (DE LA PUENTE REDONDO et al., 2003).

RESULTS & DISCUSSION

M. hyopneumoniae: Two PCR tests have been established. Simple PCR, the length of the amplified fragment is 649 bp and a nested PCR is 352 bp (data not shown). N-PCR was optimized on the basis of three variables: different sample locations, transmission of the sample, and methods of DNA extraction. **Multiplex to Mhyo, Mh and Mf:** Three specific forward primers have been used based on the 16S rDNA sequences of Mhyo, Mf and Mh. A common reverse primer was used in a conserved region of the 16S rRNA genes. Based on these sequences, were amplified fragments length 1000bp (Mhyop), 754bp (Mf) and 1129bp (Mh) (Figure 1).

RESULTS & DISCUSSION

P. multocida: We have been establishing a multiplex PCR for detection of virulence associated genes *pfhA* (275bp), *hgbB* (499bp), *tbpA* (728bp) and *toxA* (846bp) and another PCR for detection of *hyaD-hyaC* (1044bp), *dcbF* (657bp) and *febD* (851bp) genes for capsular typing A, D, and F, respectively, and the specific portion *kmt1* (460bp) for Pm. ***A. pleuropneumoniae:*** The specific 715bp cpx gene DNA fragment were amplified from all of *A.pleuropneumoniae* (data not shown) isolates from lesions of acute cases of swine pleuropneumonia, from herds with clinical symptoms both serotypable and nonserotypable. The PCR method applied is highly sensitive for serotypable strains and for nonserotypable strains isolated from acute cases of swine pleuropneumoniae in Brazil. ***H. parasuis:*** The specific DNA fragments of 1.9 kb were amplified for the *tbpA* 15 serotypes Hps standard (Figure 2).

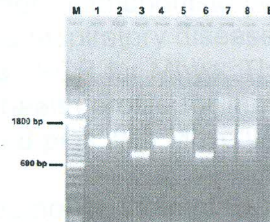

Figure 1. Multiplex PCR to detection of *M. hyopneumoniae*, *M. hyorhines* and *M. flocculare*. DNA ladder 100bp (M), Multiplex PCR of *Mycoplasma hyopneumoniae* DNA, specific primer Mhyo (1), *M. hyorhines* DNA, specific primer Mh (2), *M. flocculare* DNA, specific primer Mf (3), *Mycoplasma hyopneumoniae* DNA, mix from 3 primers forward (Mhyo, Mh and Mf, 4), *M. hyorhines* DNA, mix from 3 primers forward (5), *M. flocculare* DNA, mix from 3 primers forward (6), DNA Mix from Mhyo, Mh and Mf bacterial culture, mix from 3 primers forward (7), DNA Mix from Mhyo, Mh and Mf, mix from 3 primers forward (8) and without DNA (B)

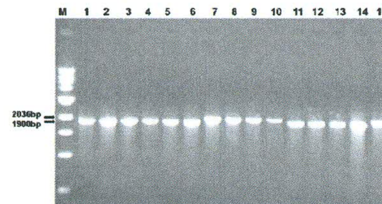

Figure 2. Simple PCR to detection of *Haemophilus parasuis* DNA gene *tbpA*. DNA ladder 100bp (M), *Haemophilus parauis* serovar 1 to 15 (1 to 15, respectively).

CONCLUSION

The use of molecular diagnostic tools for animal disease allows a faster diagnosis of bacterial agents involved in PRDC in pigs, as well as the characterization of serotypes and virulence factors of respiratory bacteria. The knowledge of such serotypes and virulence factors will be very important to the development of target vaccines as well as guidance for epidemiological studies.

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