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Detecção de agentes associados com doenças respiratórias de suínos por PCR em tempo real

Detection of agents associated with respiratory diseases of swine by real time PCR

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RESUMO

Complexo de Doenças Respiratórias de Suínos (CDRS) é um grupo de doenças que causam grandes perdas na indústria suína. Vários agentes infecciosos estão relacionados com a CDRS , entre eles o circovírus suíno 2 (PCV - 2), vírus da pseudo-raiva (SuHV -1) , *Haemophilus parasuis* (HP) , *Mycoplasma hypneumoniae* (MH) e *Pasteurela multocida* (PM). O objetivo com este estudo foi desenvolver PCR em tempo real (qPCR) para a detecção destes agentes infecciosos. Os oligonucleotídeos foram concebidos para cada agente infeccioso específico e marcado com fluoróforos diferentes para amplificar partes específicas do genoma em dois grupos de reacções , uma qPCR dúplex em SuHV -1 e PCV- 2 e uma qPCR multiplex para detectar as três bactérias simultaneamente. As reações foram testadas em 142 amostras de *pools* de linfonodos e pulmões de suínos com sinais clínicos de CDRS. Foram detectadas 135 amostras positivas para PCV- 2 , 61 para a HP, 29 para PM e 30 para MH e zero para SuHV -1, dentre esses foram registrados 76 casos de co-infecção . As qPCRs desenvolvidas neste estudo são ferramentas úteis no diagnóstico da CDRS.

Palavras-chave: biologia molecular, medicina veterinária preventiva, viroses

SUMMARY

Porcine Respiratory Disease Complex (PRDC) is a group of diseases that cause high losses in the swine industry. Several infectious agents are related to PRDC including porcine circovirus 2 (PCV-2), pseudorabies virus (SuHV-1), *Haemophilus parasuis* (HP), *Mycoplasma hypneumoniae* (MH) and *Pasteurela multocida* (PM). The aim of this study was to develop real-time PCRs (qPCR) for the detection of these infectious agents. Oligonucleotides were designed for each specific infectious agent and labeled with different fluorophores to amplify specific parts of the genome. This was done in two groups of reactions—a duplex qPCR for SuHV-1 and PCV-2 and a multiplex qPCR to detect the three bacteria simultaneously. The reactions were tested in 142 pooled samples of swine lymph nodes and lungs with clinical signs of PRDC. There were 135 samples that tested positive for PCV-2, 61 for HP, 29 for PM, 30 for MH and zero for SuHV-1. We recorded 76 cases of co-infection. The qPCRs developed in this study are useful tools in the diagnosis of PRDC.

Keywords: molecular biology, preventive veterinary medicine, viruses

INTRODUCTION

Porcine Respiratory Disease Complex (PRDC) is a group of diseases that cause high losses in the swine industry. It occurs 16-22 week old pigs and causes weight loss, anorexia, dyspnea, fever and cough (TACKER, 2001). The etiologic agents of PRDC are diverse and may be present together or separately in swine and include viruses and bacteria such as Aujeszky's disease virus, porcine circovirus 2, reproductive syndrome virus, swine respiratory virus, *Mycoplasma hypneumoniae*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae, Pasteurela multocida, Bordetella bronchiseptica* and *Streptococcus suis* (TACKER, 2001).

In particular, two DNA viruses are important causes of this syndrome in pigs. The porcine circovirus (PCV-2) is one the most common viruses found in herds around the world. It is associated with postweaning multisystemic wasting syndrome (PMWS) and PRDC (ELLIS et al., 2004). The suid herpesvirus 1 (SuHV-1) is the causative agent of Aujeszky's disease or pseudorabies. It causes clinical signs that vary according to the age of the affected pigs. Symptoms include neurological signs in younger animals to respiratory and reproductive signs in adults (KLUGE et al., 1999).

Three bacterial etiologic agents belong to the *Pasteurellaceae* family. They are of great importance to pigs. *H. parasuis* (HP), *M. hypneumoniae* (MH) and *P. multocida* (PM) participate alone or in tandem with PRDC and may cause various clinical signs. HP is the causative agent of Glasser's disease, swine arthritis and swine polyserositis (RAPP-GABRIELSON et al., 2006). MH is the primary agent of enzootic pneumonia, which is a respiratory disease that affects pigs in the finishing phase (SIBILA et al., 2009). PM causes atrophic rhinitis in swine and can infect other species including cholera in birds and haemorrhagic septicemia in cattle (GLISSON et al., 2003).

The diagnosis and identification of these three bacteria is laborious and is based on isolation and biochemical tools. The previous use of antibiotics, low analytical sensitivity (REGISTER & DEJONG, 2006) and errors in the biochemical processing (COSTA et al., 2004) are common problems that can be solved with molecular tests such as PCR.

For example, one common problem for the detection of viral agents is cell contamination (GIAMMARIOLI et al., 2008). Individual molecular tests used have excellent sensitivity, specificity and speed to diagnosis PRDC (CORNEY et al., 2007; ANGEN et al., 2007). Such methodologies can still be improved through use of real time PCR (qPCR) that facilitates more sensitive and specific diagnosis, but with low risk of contamination because the operator does not need to open the tubes to analyze the specimens.

The aim of this study was to develop two real-time PCRs for multiplex measurements of the following: 1) *H. parasuis*, *M. hypneumoniae* and *P. multocida* and 2) SuHV-1 and PCV-2.

MATERIAL AND METHODS

Primers and probes for qPCR were designed using PrimeTime qPCR assay (IDT, USA; Table 1). Each probe received a different marking to detect amplification. We used multiplex qPCR to detect the three bacteria (qPCR-Bac) with the following fluorophores: FAM for HP, JOE for MH, and Cy5 for PM.

We used a multiplex qPCR to detect the two viruses (qPCR-Vir) with the following fluorophes: FAM for SuHV-1 and Cy5 for PCV-2. DNA extraction and the presence of PCR inhibitors were evaluated using qPCR to detect the beta-actin gene according to a protocol described by Bielanski et al. (2009) with adaptations for DNA detection.

Both reactions were optimized for amplification using a Rotorgene 3000 instrument (Corbett, Australia). DNA extracted from cultured bacteria and viruses was used as the template. Positive controls for HP, PM and MH were gently provided by Embrapa Swine and Poultry Corporation. The Laboratory for the Diagnosis of Viral Diseases in Lanagro/MG kindly provided positive controls for SuHV-1; these were characterized in a previous work (FONSECA JUNIOR et al., 2010a). Positive controls for PCV-2 were donated by the Escola de Veterinária in the Universidade Federal de Minas Gerais. Reaction efficiency was measured using a dilution of bacteria or viral DNA submitted to qPCR in triplicate to produce a standard curve with five points.

The limit of detection (LOD) was determined using serial dilution in triplicate of bacterial and viral DNA. The lowest dilution that used only three amplifications was considered the LOD this was confirmed with 21 replicates and gave 95% confidence in the results.

The analytical specificity was tested with DNA extracted from *Mycoplasma mycoides*, *Mycoplasma bovigentalis*, *Mycoplasma arginini*, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyorhinis*, *Bordetella bronchiseptica*, *Streptococcus suis*, classical swine fever, African swine fever virus, porcine parvovirus and porcine circovirus 1.

Diagnostic sensitivity was tested using samples that were positive in bacterial or viral isolation. The qPCR-Bac was tested in 20 pooled samples containing lymph node and lung tissue from swine positive for MH, MP and HP using a bacterial isolation technique. The qPCR-Vir was tested on a sample of lung and 10 brain samples positive for swine SuHV-1 using the isolation technique. The DNA from each sample was extracted according to Fonseca Junior et al. (2010b).

The multiplex qPCR was tested in 142 clinical samples collected between 2006 and 2007 from pigs with respiratory signs. The DNA was extracted from pooled lymph node and lung as described by Fonseca Junior et al. (2010b).

RESULTS AND DISCUSSION

Both qPCRs were optimized to a volume of 25 µL divided into 12.5 µL of RealQ x2 PCR Master Mix (Ampliqon, Denmark), 1.0 µL of 50 mM $MgCl_2$, and 2 µL of DNA (300 – 600 ng/µL). Oligonucleotide concentrations are detailed in Table 1. The thermocycler was programmed with the following temperatures: 1) 50°C for 2min to allow UNG actitvity, 2) 95°C for 15 min for denaturation, DNA polymerase activation and UNG denaturation, 3) 45 cycles at 95°C for 15 s, 58°C for 35 s with fluorescence label, and 72°C for 20 s. The LOD experiments were different for each target (Table 1). Using the detection limit data, we found that samples with Ct above 42 could be considered negative.

Table 1. Primers and probes used for the detection of pathogens studied in this work

Pathogens	Gene	Oligonucleotides	Final concentration (pmol/µL)	PCR Efficiency	Limit of Detection
*H. parasuis**	*infB*	F: CCGCTTGCCATACCCTCTT	0.6	0.90	200 fg
		R: CGACTTACTTGAAGCCATTCTTCTT	0.6		
		FAM-ATCGGAAGTATTAGAATTAAGTGC-BHQ1	0.2		
P. multocida	*KMT1*	F: TTGTTTGCTCGAAATTGCTG	0.6	0.74	240 fg
		R: GTGCTTTGCTTGCCACACATTTA	0.6		
		Cy5-CCGCTTGATTCATCACGCCA-BHQ2	0.3		
M. hypneumoniae	*P216*	F: CCATCCACTGGTATTTGATCTTTG	0.4	0.90	80 fg
		R: AGAAATGAGTGCGGTTCCC	0.4		
		JOE-TGAGCAATATGATCCAAGAACCCCGC-BHQ2	0.2		
PCV-2	*ORF2*	F: CCTACGTGGTCTACATTTCCAG	0.4	1.0	Not calculate due to absence of a titatred virus isolate
		R: CATACCATAACCCAGCCCTTC	0.4		
		Cy5- CCCCAAACCTGTCCTAGATTCCACT - BHQ2	0.2		
SuHV-1	*gB*	F: CTC CTG CCG CAC CTG AAG	0.3	1.0	$10^{-1.3}$ $TCID_{50}$/mL
		R: GTC TGG AAG CGG TAG AAG CC	0.3		
		FAM-CGGAACTCGCTGACGCACCAATCGC-BHQ1	0.2		

*Primers and probe for detection of HP were described by Turni et al. (2010).

The results demonstrate that multiplex qPCR can significantly help diagnose these agents. The development was carefully performed to provide a qualitative diagnosis with high efficiency. The multiplex reaction was adjusted to amplify more than one pathogen simultaneously because co-infection is common during PRDC.

The efficiency of each qPCR was tested (Table 1). It is generally recommended that these tests use pure DNA, often with the PCR product inserted into a plasmid. In this work, we used clinical samples diluted to eliminate other interferences during amplification. The efficiency remained intact even when more than one agent was detected at a time. The LOD for detection of SuHV-1 was 10-fold more sensitive than a previously described qPCR using Sybr-Green (FONSECA JUNIOR et al., 2013). qPCR-Bac did not offer good results in tests of efficiency, however this result had no impact on qualitative tests.

The clinical data showed many positive samples for PCV-2, MH, HP and PM, but none to SuHV-1. Of the 142 samples tested, 4 were removed because they were negative in the qPCR for the detection of beta-actin gene indicating that the negative results were not reliable. Of the 138 remaining samples, multiplex qPCRs detected 135 samples positive for PCV-2, 61 for HP, 30 for MH, 29 for PM, and none for SuHV-1. We recorded 76 cases of co-infection mostly due to the presence of PCV-2 (Table 2).

The high prevalence of PCV-2 was expected. The virus is present in most pig herds and causes various problems (HA et al., 2008). There are very few studies on the prevalence of HP in Brazilian pig production, but studies in other countries cite a large number of samples infected by this bacterium (MAcINNES et al., 2008). The high number of positive results for PM was already expected because it is common even in healthy swine (ALWIS et al., 1990). Other agents like *Actinobacillus pleuropneumoniae* may be involved in the respiratory distress detected in the clinical exams, but they were not included in this study. There were no positive results for SuHV-1. In fact, there have been no reports of pseudorabies in Minas Gerais, Brazil since 1984 (FONSECA JUNIOR et al., 2010c). Due to the large number of pathogens that may be involved in PRDC, the development of other multiplex qPCR assays should aid in the detection of these agents.

Table 2. Number of samples positive for more than one infectious agent in the qPCRs

Infectious agents	Number of positive samples
PCV-2+HP	26
PCV-2+MH	11
PCV-2+PM	5
PCV-2+HP+PM	13
PCV-2+HP+MH	9
PCV-2+MH+PM	2
PCV-2+HP+PM+MH	8
HP+PM	1

Molecular diagnostic methodologies are of great importance to the swine industry. They allow a rapid and practical diagnosis of many diseases without reducing sensitivity and specificity. These methodologies are found in various forms including conventional PCR, nested PCR (FONSECA JUNIOR et al., 2010b) and real-time PCR (ANGEN et al., 2007). Of these, qPCR has the highest sensitivity and specificity.

The use of multiplex molecular diagnostics allows a rapid and practical diagnosis of animal diseases. The qPCRs described here detected four agents important to the pork industry. These bacteria and viruses are known to cause debilitating and severe clinical symptoms in pigs, but are easily confused with other illnesses. The presence of more than one type of agent in the PRDC is relatively common and can exacerbate the clinical signs (KIM et al., 2003).
The qPCRs developed here are useful tools in the analysis of PRDC. They facilitate rapid, sensitive and specific diagnosis of four pathogens associated with large losses in the swine industry.

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REFERENCES

ALWIS, M.C. de; WIJEWARDANA, T.G.; GOMIS, A.I.; VIPULASIRI, A.A. Persistence of the carrier status in haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:Binfection) in buffaloes. **Tropical Animal Health Production**, v.22, p.185–94, 1990.

ANGEN, O.; OLIVEIRA, S.; AHRENS, P.; SVENSMARK, B.; LESER, T.D. Development of an improved species specific PCR test for detection of Haemophilus parasuis. **Veterinary Microbiology**, v.119, n.2-4, p.266-76, 2007.

BIELANSKI, A.; ALGIRE, J.A.; LALONDE, S.; NADIN-DAVIS, S. Transmission of bovine viral diarrhea virus (BVDV) via in vitro-fertilized embryos to recipients, but not to their offspring. **Theriogenology**, v.71, p.499-508, 2009.

CORNEY, B.G.; DIALLO, I,S.; WRIGHT, L.L.; HEWITSON, G.R.; JONG, A.J. de; BURRELL, P.C.; DUFFY, P.F.; STEPHENS, C.P.; RODWELL, B.J.; BOYLE, D.B.; BLACKALL, P.J. *Pasteurella multocida* detection by 5' Taq nuclease assay: a new tool for use in diagnosing fowl cholera. **Journal of Microbiological Microbiol Methods**, v.69, n.2. p.376-80, 2007.

COSTA, M.M.; KLEIN, C.S.; BALESTRIN, R.; SCHRANK, A.; PIFFER, I.A.; SILVA, S.C. da; SCHRANK, I. S. Evaluation of PCR based on gene apxIVA associated with 16S rDNA sequencing for the identification of *Actinobacillus pleuropneumoniae* and related species. **Current Microbiology**, v.48, p.189-95, 2004.

ELLIS, J.; CLARK, E.; HAINES, D.; WEST, K.; KRAKOWKA, S.; KENNEDY, S.; ALLAN, G.M. Porcine circovirus-2 and concurrent infections in the field. **Veterinary Microbiology**, v.98, n.2, p.159-63, 2004.

FONSECA JUNIOR, A.A.; CAMARGOS, M.F.; D'AMBROS, R.M.; BRAGA, A.C.; CIACCI-ZANELLA, J.R.; HEINEMANN, M.B.; LEITE, R.C.; REIS, J.K. Diagnóstico e genotipagem do vírus da pseudoraiva por nested-PCR e análise de restrição enzimática. **Ciência Rural**, v.40, p.921-927, 2010a.

FONSECA JUNIOR, A.A.; DIAS, N.L.; HEINEMANN, M.B.; LEITE, R.C.; REIS, J.K.P. PCR duplex para diferenciação de amostras vacinais e

selvagens do vírus da doença de Aujeszky. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.62, p.1259-1262, 2010b.

FONSECA JUNIOR, A.A.; CAMARGOS, M.F.; OLIVEIRA, A.M.; CIACCI-ZANELLA, J.R.; PATRÍCIO, M.A.; BRAGA, A.C.; CUNHA, E.S.; D'AMBROS, R.; HEINEMANN, M.B.; LEITE, R.C.; REIS, J.K. Molecular epidemiology of Brazilian pseudorabies viral isolates. **Veterinary Microbiology**, v.141, p.238-245, 2010c.

FONSECA JUNIOR, A.A.; COTTORELLO, A.C.; DIAS, N.L.; D´AMBROS, R.M.; LEITE, R.C.; HEIMENANN, M.B.; REIS, J.K.P. Real time PCR for detection of Aujeszky's disease virus. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.65, n.3, p.801-808, 2013.

GIAMMARIOLI, M.; PELLEGRINI, C.; CASCIARI, C.; DE MIA, G.M. Development of a novel hot-start multiplex PCR for simultaneous detection of classical swine fever virus, African swine fever virus, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus and porcine parvovirus. **Veterinary Research Communications**, v.32, n.3, p.255-62, 2008.

GLISSON, J.R.; HOFACRE, C.L.; CHRISTENSEN, J.P. Fowl cholera. In: SAIF, Y.M.; BARNES, H.J.; GLISSON, J.R.; FADLY, A.M.; McDOUGALD, L.R.; SWAYNE, D.E. (Eds.). **Diseases of Poultry**. Ames, Iowa, USA: Iowa State University Press, 2003. p.658–676.

HA, Y; LEE, Y.H.; AHN, K.K.; KIM, B.; CHAE, C. Reproduction of postweaning multisystemic wasting syndrome in pigs by prenatal porcine circovirus 2 infection and postnatal porcine parvovirus infection or immunostimulation. **Veterinary Pathology**, v.45, n.6, p.842-8, 2008.

KIM, J.; CHUNG, H.K.; CHAE, C. Association of porcine circovirus 2 with porcine respiratory disease complex. **Veterinary Journal**, v.166, n.3, p.251-6, 2003.

KLUGE, J.P.; BERAN, G.W.; HILL, H.T.; PLATT, K.B. Pseudorabies (Aujeszky's Disease), p.233-146. In: STRAW B.E, D'ALLAIRE S., MENGELING W.L., TAYLOR D.J. (Eds.). **Diseases of Swine**. Ames, Iowa, USA: Iowa State University Press, 1999.

MAcINNES, J.I..; GOTTSCHALK, M.; LONE, A.G.; METCALF, D.S.; OJHA, S.; ROSENDAL, T.; WATSON, S.B.; FRIENDSHIP, R.M. Prevalence of *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Haemophilus parasuis*, *Pasteurella multocida*, and Streptococcus suis in representative Ontario swine herds. **Canadian Journal of Veterinary Research**, v.72, n.3, p.242-8, 2008.

REGISTER, K.B.; DEJONG, K.D. Analytical verification of a multiplex PCR for identification of Bordetella bronchiseptica and Pasteurella multocida from swine. **Veterinary Microbiology**, v.117, n.2-4, p.201-10, 2006.

RAPP-GABRIELSON, S.R.; OLIVEIRA, C.; PIJOAN. Haemophilus parasuis. In: STRAW, B., ZIMMERMAN, J.J.; D'ALLAIRE, S.; TAYLOR, D.J. (Eds.). **Diseases of Swine**. Williston, FL, USA: Blackwell Publishing, 2006. p.681–690,

SIBILA, M.; PIETERS, M.; MOLITOR, T.; MAES, D.; HAESEBROUCK, F.; SEGALÉS, J. Current perspectives on the diagnosis and epidemiology of Mycoplasma hyopneumoniae infection. **Veterinary Journal**, v.181, n.3, p.221-31, 2009.

THACKER, E.L. Immunology of the porcine respiratory disease complex. **Veterinary Clinics of North America: Food Animal Practice**, v.17, n.3, p.551-65, 2001.

TURNI, C.; PYKE, M.; BLACKALL, P.J. Validation of a real-time PCR for *Haemophilus parasuis*. **Journal of Appled Microbiology**, v.108, n.4, p.1323-1331, 2010.

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