Vet Res Commun (2008) 32:255–262
DOI 10.1007/s11259-007-9026-6

ORIGINAL ARTICLE

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

Monica Giammarioli · Claudia Pellegrini · Cristina Casciari · Gian Mario De Mia

Accepted: 16 October 2007 / Published online: 2 November 2007
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Abstract A novel hot-start multiplex PCR (mPCR) assay was developed and subsequently evaluated for its effectiveness in simultaneously detecting multiple viral infections of swine. Specific primers for each of five virus genomes, namely classical swine fever virus (CSFV), African swine fever virus (ASFV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine parvovirus (PPV) were used. Combined nucleic acid purification was carried out using a commercial RNA/DNA extraction kit. The mPCR consisted of a two-step procedure which included reverse transcription and PCR amplification. This mPCR and the corresponding separate assays were evaluated comparatively on serial ten-fold dilutions of each virus. Analysis of the sensitivity in comparison to the corresponding single PCR (sPCR) for the detection of each of the five targets was identical for CSFV, PCV2 and PPV, 1 log lower for PRRSV and 2 logs lower for ASFV. No spurious PCR amplification reactions among all five pathogens were noticed with various amounts of DNA and RNA mixtures. All the uninfected controls were scored negative. The relative efficiency of the mPCR developed in this study compared to performing sPCR for each virus, suggests its potential application for routine molecular diagnostic purposes.

Keywords African swine fever · Classical swine fever · Differential diagnosis · Porcine circovirus type 2 · Porcine parvovirus · Porcine reproductive and respiratory syndrome · Multiplex PCR

Abbreviations

ASFV	African swine fever virus
BVDV	bovine viral diarrhea virus
BDV	border disease virus
CSFV	classical swine fever virus

M. Giammarioli · C. Pellegrini · C. Casciari · G. M. De Mia (✉)
Perugia 06126, Italy
e-mail: gm.demia@izsum.it

DEPC diethylpyrocarbonate
mPCR multiplex PCR
PCV porcine circovirus
PPV porcine parvovirus
PRRS porcine reproductive and respiratory syndrome
sPCR single PCR

Introduction

Under typical conditions of intensive swine production it is common for swine to be simultaneously infected with two or more pathogens. Furthermore, there are situations where a differential diagnosis is required. For example acute and suacute forms of classical swine fever (CSF) are characterized by hemorrhages and can be easily confused with those observed in African swine fever (ASF) infection. This fact makes the differential diagnosis essential especially in areas where CSF and/or ASF are endemic therefore haemorrhagic symptoms and fever are frequently observed. Although CSF has been known for decades, laboratory diagnosis remains difficult because of the need for extensive differential diagnosis, further complicated by the emergence of other infectious diseases such as porcine reproductive and respiratory syndrome (PRRS) and porcine circovirus infections. During the CSF epizootics reported in the UK in 2000, there was a serious problem differentiating the clinical signs and lesions of porcine dermatitis and nephropathy syndrome (PDNS) from CSF. The same is true for those infections associated with abortion and/or stillbirth which can be easily confused with low-virulence CSFV infections. The standard laboratory methods for diagnosis of viral diseases were mainly based on viral isolation in cell culture, which is time consuming. The PCR is an alternative rapid virus detection method and several sPCR-based methods have been reported for a number of porcine pathogens. However, in those cases where clinical signs are variable and thus fail to provide a clear indication of the most appropriate means of testing, a number of costly individual virus specific tests are sometimes performed initially to expedite the testing procedure. In several previous studies multiplex PCR (mPCR) has been used to simultaneously identify and differentiate multiple viruses in a single sample on the basis of amplicon size (Ouardani *et al*., 2000; Agüero *et al*., 2004; Cao *et al*., 2005; Huang *et al*., 2004) However, to the authors' knowledge, there is no report on the use of a mPCR to detect five viral infections of swine in a single reaction. In this study a novel hot-start mPCR assay was developed and evaluated for simultaneous detection of nucleic acid from five viruses: classical swine fever virus (CSFV), African swine fever virus (ASFV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine parvovirus (PPV).

Materials and methods

Viruses and cells

ASFV strain Torino 83 was propagated in pig leukocytes and virus isolation and haemadsorption tests were performed as previously described (Malmquist and Hay, 1960). ASFV strain Lisbon 60 was propagated in Vero cell line. CSFV strain Alfort 187 was grown in PK15 cell line following standard procedures. PRRSV was grown in porcine alveolar macrophages (Lelystad strain) or MARC-145 cell line (American strain VR2332,

Ingelvac PRRS MLV, Böhringer Ingelheim, St. Joseph, USA). PPV was isolated from aborted foetuses and further propagated in swine testis (ST) cells. PCV2 was isolated from postweaning multisystemic wasting sindrome affected pigs, propagated in circovirus-free PK15 and confirmed by fluorescent antibody test or directly used as lymph node homogenate. A PK15 persistently PCV1 infected cell line was used as PCV1 stock virus. Uninfected PK15 cell line as well as bovine viral diarrhoea (BVD type 1 and 2) and border disease (BD) reference viruses were also used in the specificity assays.

Clinical specimens

In order to evaluate the efficiency of the extraction method and for the assessment and optimization of the mPCR reaction, a collection of 7 blood and 65 tissue samples from natural ASFV infections as well as a collection of blood and tissue samples from CSFV experimentally infected pigs were artificially spiked with mixtures containing identical amounts of pure culture of each other virus. Both collections came from the National Swine Fever Laboratory of IZS Umbria e Marche (Perugia, Italy). Virus negative organs from clinically healthy animals were also included.

Extraction of RNA and DNA

RNA and DNA extraction from tissues from animal tissues and from supernatants of infected cell cultures, was simultaneously carried out with Qiagen RNA/DNA Mini Kit according to the manufacture's protocol (Qiagen RNA/DNA handbook, Quiagen, Hilden, Germany).

Oligonucleotide primers

Five previously described sets of primers that specifically amplify CSFV, ASFV, PCV2, PRRS, PPV (Gradil *et al*., 1994; Suarez *et al*., 1994; Vilček *et al*., 1996; Ellis *et al*., 1999; Agüero *et al*., 2003) (Table 1) were obtained from a commercial source (Invitrogen, Life Sciences Corporation, Carlsbad, CA, USA).

Optimization of multiplex conditions

The reactions were optimised by varying primer concentrations of each target. Optimization was carried out by methodical variation of each test parameter under standard PCR

Table 1 Specific primer pairs used to amplify each target gene

Virus	Target gene	Primer sequence (5′–3′)	Expected Product (bp)
CSFV	NS5B	HC33: GACACAAGYGCAGGCAAYAG HC34: AGTGGGTTCCAGGARTACAT	449
ASFV	VP73	PPA1: AGTTATGGGAAACCCGACCC PPA2: CCCTGAATCGGAGCATCCT	257
PCV2	ORF2	1443: CGGATATTGTAGTCCTGGTCG 150: ACTGTCAAGGCTACCACAGTC	481
PRRSV	ORF7	PRRSF: GGGAATGGCCAGCCAGTCAATCAACTG PRRSR: TGTAGAAGTCACGCGAATCAGGCGCACT	312
PPV	VP2	PPVF: GCAGTACCAATTCATCTTCT PPVR: TGGTCTCCTTCTGTGGTAGG	158

conditions. The primer concentrations tested ranged from 0.5 to 50 pmol. Optimum amplification profiles were obtained with a primer concentration of 5 pmol for all sets of primers. Different $MgCl_2$ concentrations ranged from 1.5 to 3.5 mM were evaluated and the most efficient selected. The annealing temperature and number of cycles were also determined experimentally. The best results were obtained with a temperature of 56°C for 35 cycles.

Single PCR and RT-PCR assay

CSFV, ASFV, PCV2, PRRS and PPV single PCR were performed as previously described (Gradil *et al*., 1994; Suarez *et al*., 1994; Vilček *et al*., 1996; Ellis *et al*., 1999; Agüero *et al*., 2003).

Reverse transcription and mPCR reaction

The mPCR consists of a two-step procedure which includes reverse transcription (RT) and PCR amplification. The RT-reaction was performed in 20 μl volumes, in which the reaction mixture contained 5X Strand buffer, 25 mM each deoxynucleoside triphosphate (dNTP) (Amersham Biosciences Corp., Piscataway, NJ, USA), 2.5 U RNase inhibitor (Promega Corporation, Madison, WI, USA), 50 pmol/ml random hexamers, MMLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA), 5 μl of mix (RNA + DNA) and DEPC-water. RT was carried out at 37°C for 90 min and 99°C for 5 min. The mPCR reaction was performed in a 25 μl volume using the Gene Amp PCR kit and AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). The reaction contained 2.5 mM $MgCl_2$, 1x PCR Buffer II (500 mM KCl, 100 mM Tris HCl, pH 8.3), 200 μM each dNTP, 5 pmol of each primer, 2.5 Units AmpliTaq Gold. This mixture was added to the RT reaction tubes along with 100 ng of template and DEPC-water. The cycling protocol consisted of an initial denaturation at 94°C for 5 min, then 35 cycles which consisted of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72 °C for 1 min. The sample was then heated at 72°C for 10 min for a final extension. Negative controls were run with each test. Agarose gel electrophoresis was used to detect mPCR products.

Sensitivity and specificity of single and multiplex RT-PCR assays

The sensitivity of each sPCR has been previously reported (Molitor *et al*., 1991; Gradil *et al*., 1994; Suarez *et al*., 1994; Vilček *et al*., 1996; Paton *et al*., 2000; Agüero *et al*., 2003).

The sensitivity of the mPCR and the corresponding single PCRs was evaluated comparatively on serial ten-fold dilutions of selected spiked samples containing all the target viruses. The specificity of primer pair for each virus was analyzed in single PCR. Specificity of mPCR was assessed in clinical specimens. The identity of amplified fragments was confirmed by restriction enzyme digestion.

Restriction enzyme analysis

Analysis with Bstx I, Hind III, EcoR I, Taq I and Bgl II restriction endonuclease of the respective CSFV, ASFV, PCV2, PRRSV and PPV amplicons was performed in a 20 μl volume reaction, using 10 μl of the amplification products and 15 to 30 U of enzyme. Reactions were incubated for at least 30 min at 37°C (Hind III, EcoR I, Bgl II), 55°C (Bstx I)

and 65°C (Taq I) respectively. Restriction fragments were analyzed by electrophoresis on a 2% agarose gel.

Prevention of carry-over contamination

To prevent carry-over contamination within the laboratory, the following precautions were taken. All purchased reagents were stored in small single-use aliquots. Preparation of PCR reagents, extraction of nucleic acids from clinical samples and amplification were conducted in three different rooms. Tips equipped with sealing filters were used for pipetting reagents introduced into the PCR. All areas and equipment were decontaminated with an alcoholic disinfectant and/or by irradiation with UV-light for 30 min prior to and after pipetting.

Results

Sensitivity of single and multiplex RT-PCR assays

Five PCR primer sets were selected for optimization and standardization of the mPCR. Different amplification assays were also performed to optimize the multiplex reaction conditions. Analysis of the PCR products by agarose gel electrophoresis after staining with ethidium bromide allowed, depending upon the virus, as little as 0.2 to 200 pcg of each viral target DNA detected in sPCR and 20 to 200 pcg in mPCR. PCR experiments were performed on serial ten-fold dilutions of selected spiked samples containing all the target viruses in order to determine the multiplex RT-PCR's detection limit. The sensitivity of the mPCR in comparison to the corresponding sPCR for the detection of each of the five targets was identical for CSFV, PCV2 and PPV, 1 log lower for PRRSV and 2 logs lower for ASFV.

Specificity of single and multiplex RT-PCR assays

The specificity of primer pair for each virus was analyzed in sPCR. As shown in Fig. 1, each viral target gene could be specifically amplified using its defined primer pair and the PCR products showed the expected size ranges. All the amplicons showed the expected pattern after restriction endonuclease analysis (Fig. 2). Specificity of mPCR was assessed in the clinical samples previously identified as negative for the five pig pathogens. The analysis of blood and tissue positive ASFV and CSFV samples showed positive amplification in any clinical sample yielding the corresponding product when analyzed by both the specific single and multiplex reactions (data not shown). Amplicons with molecular size different than expected were not obtained in any of the known positive samples. Neither were non-specific reactions observed with any of the negative clinical samples included in the study. Different representative pestiviruses such as BVDV types 1 or 2 and BDV, PCV1 and PK15 uninfected cell suspensions did not generate amplicons (Fig. 3).

Discussion

Under typical conditions of intensive swine production, several clinically similar viral diseases can occur which require laboratory differential diagnosis. Diagnostic laboratories

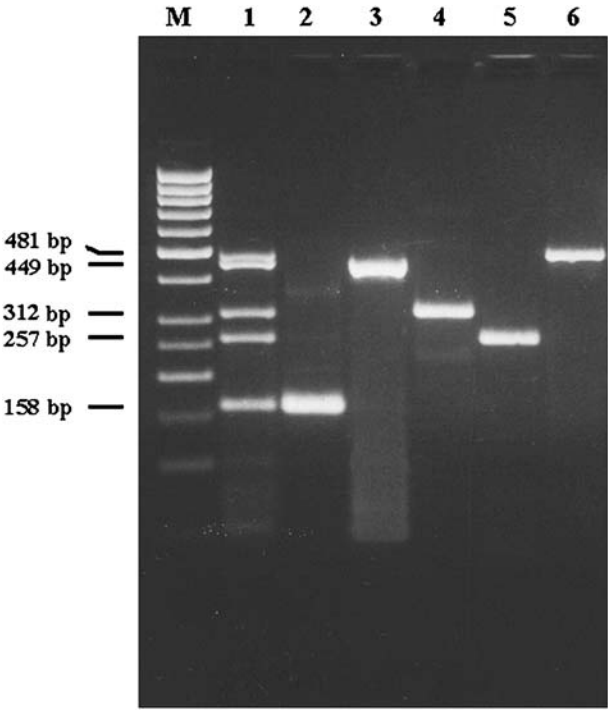

Fig. 1 Electrophoretic profiles of DNA amplification products obtained by uniplex or multiplex PCR. Lane M: 50 bp DNA ladder (Fermentas Life Sciences, Hanover, USA). Lane 1: multiplex PCR with mixed viral DNA's using all mixed primer pairs. Uniplex PCR for PPV (lane 2), CSFV (lane 3), PRRSV (lane 4), ASFV (lane 5) and PCV2 (lane 6) viral DNA was carried out using single specific primer pair to each target gene

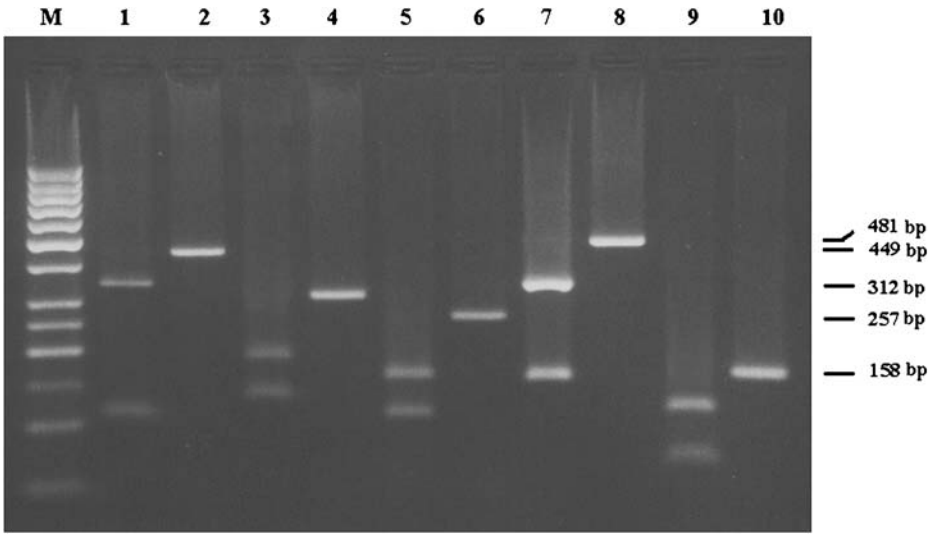

Fig. 2 Restriction endonuclease analysis of amplification products. Lane M: 50 bp DNA ladder (Fermentas Life Sciences, Hanover, USA). Lane 1: CSFV amplicon digested with *Bstx I* into fragments of 342 and 107 bp. Lane 3: PRRSV amplicon digested with *Taq I* into fragments of 128 and 184 bp. Lane 5: ASFV amplicon digested with *Hind III* into fragments of 102 and 155 bp. Lane 7: PCV2 amplicon digested with *EcoR I* into fragments of 327 and 149 bp. Lane 9: PPV amplicon digested with *Bgl II* into fragments of 55 and 103 bp. Lanes 2, 4, 6, 8, 10: CSFV, PRRSV, ASFV, PCV2, PPV amplicons

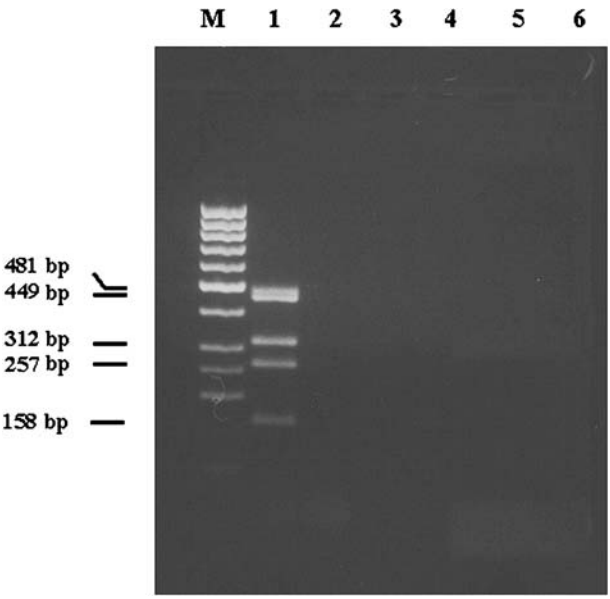

Fig. 3 Multiplex PCR specificity. Primers specificity was tested by running a multiplex PCR reaction with mixed viral DNA's from all virus targets (lane 1) and with several negative controls: BVDV1 (lane 2), BVDV2 (lane 3), BDV (lane 4), PCV1 (lane 5) and PK15 uninfected cell line (lane 6). Lane M: 50 bp DNA ladder (Fermentas Life Sciences, Hanover, USA)

must have rapid and accurate procedures for use in the surveillance campaigns and/or eradication programs, since the rapid detection of infected animals would reduce the potential transmission of the viruses to uninfected herds avoiding the spread of the diseases.

In this work, a novel sensitive and specific RT-PCR assay is described and applied for simultaneously and differential detection of five important swine viruses in one reaction.

In general, the development of a mPCR assay is not an easy task. Optimization of a multiplex reaction requires compromises in concentrations of reagents, annealing temperature, and/or cycling conditions in order to obtain the best overall strategy for amplification of more than one target sequence. Such compromises may contribute to less than optimal conditions for some primers (Henegariu *et al*., 1997). The most common problem is that some of the primers used in the same reaction tube may interact with each other, thus blocking the reaction (Elnifro *et al*., 2000). However, variability observed among viruses in a multiplex reaction is more likely a result of primer design. Indeed different primers can alter the sensitivity of a PCR significantly, and in some instances, changing a primer by as little as a few base pairs can have a dramatic effect on the sensitivity. The choice of primer sets that would exhibit equal sensitivity for their target sequences in a multiplex assay would require extensive testing. A further problem may be the reliable identification of the various PCR products. To overcome these problems, a very careful primer selection was performed here, which yielded amplified products that could clearly be differentiated by agarose-gel electrophoresis. One of the main keys to setting up the single step mPCR protocol was the use of an enzyme mix containing hot start DNA polymerase. Since template could be DNA or RNA genomes, and a first step of reverse transcription had to be performed at low temperature, the use of a hot start Taq DNA polymerase was essential to avoid non-specific amplifications caused by primer annealing at low temperature.

In this study, no spurious amplification reactions among all five pathogens were noticed with various amounts of DNA and RNA mixtures and all the uninfected controls as well as other porcine viruses were scored negative.

It still remains an open question, that is what results of screening investigations would be in herds with co-infections where a single pathogen is high prevalent. Might the putative presence of high copy numbers, for instance of PCV2, compromise the detectability of another target virus? Unfortunately, according to the present study that cannot be definitively excluded. Further studies have to be done in order to advance our understanding in this respect. In conclusion, although the sensitivity of some viral targets could be potentially reduced, the improvement of both turnaround time and cost effectiveness would compensate for this minor reduction suggesting its potential application as a useful screening method for routine molecular diagnostic purposes.

References

Agüero, M., Fernandez, J., Romero, L.J., Sanchez Mascaraque, C., Belak, S., Arias M. and Sanchez-Vizcaino, J.M., 2003. Highly sensitive PCR assay for routine diagnosis of African swine fever in clinical samples, *Journal of Clinical Microbiology*, 41, 4431–4434

Agüero, M., Fernandez, J., Romero, L.J., Sanchez, C., Arias, M. and Sanchez-Vizcaino, J.M., 2004. A highly sensitive and specific gel-based multiplex RT-PCR assay for the simultaneous and differential diagnosis of African swine fever and classical swine fever in clinical samples, *Veterinary Research*, 35, 551–563

Cao, S., Chen, H., Zhao, J., Lu, J., Xiao, S., Jin, M., Guo, A., Wu, B. and He., Q., 2005. Detection of porcine circovirus type 2, porcine parvovirus and porcine pseudorabies virus from pigs with postweaning multysistemic wasting syndrome by multiplex PCR, *Veterinary Research Communications*, 29, 263–269

Ellis, J., Krakowka, S., Lairmore, M., Haines, D., Bratanich, A., Clark, E., Allan, G., Konoby, C., Hassard, L., Meehan, B., Martin, K., Harding, J., Kennedy S. and McNeilly, F., 1999. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets, *Journal of Veterinary Diagnostic Investigation*, 11, 3–14

Elnifro, E.M., Ashshi, M., Cooper, R.J., and Clipper, P.E., 2000. Multiplex PCR: optimisation and application in diagnostic virology, *Clinical Microbiology Reviews*, 13, 559–570

Gradil, C.M., Harding, M.J. and Lewis, K., 1994. Use of polymerase chain reaction to detect porcine parvovirus associated with swine embryos, *American Journal of Veterinary Research*, 55, 344–347

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.K. and Vogt, P.H., 1997. Multiplex parameters and step-by-step protocol, *BioTechniques*, 23, 504–511

Huang, C., Hung, J.-J., Wu C.-Y. and Chien, M.-S., 2004. Multiplex PCR for rapid detection of pseudorabies virus, porcine parvovirus and porcine circoviruses, *Veterinary Microbiology*, 101, 209–214

Molitor, T.W., Oraveerakul, K., Zhang, Q.Q., Choi, C.S. and Ludemann, L.R., 1991. Polymerase chain reaction (PCR) amplification for the detection of porcine parvovirus, *Journal of Virological Methods*, 32, 201–211

Malmquist, W.A. and Hay, D., 1960. Hemadsorption and cytophathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures. *American Journal of Veterinary Research*, 21, 104–108

Ouardani, M., Wilson, L., Jette, R., Montpetit, C. and Dea, S., 2000. Multiplex PCR for detection and typing of porcine circoviruses, *Journal of Clinical Microbiology*, 37, 3917–3924

Paton, D.J., McGoldrick, A., Bensaude, E., Belak, S., Mittelholzer, C., Koenen, F., Vanderhallen, H., Greiser-Wilke, I., Scheibner, H., Stadejek, T., Hoffmann, M. and Thuer, B., 2000. Classical swine fever viruss: a second ring test to evaluate RT-PCR detection methods, *Veterinary Microbiology*, 77, 71–81

Suarez, P., Zardoya, R., Prieto, C., Solana, A., Tabares, E., Bautista, J.M. and Castro, J.M., 1994. Direct detection of the porcine reproductive and respiratory syndrome (PRRS) virus by reverse polymerase chain reaction (RT-PCR), *Archives of Virology*, 135, 89–99

Vilček, S., Stadejek, T., Ballagi-Pordany, A., Lowings, J.P., Paton, D.J. and Belak, S., 1996. Genetic variability of classical swine fever virus, *Virus Research*, 43, 137–147