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Original article

Comparison of PCR methods for detection of classical swine fever virus and other pestiviruses

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Abstract

Classical swine fever (CSF) is a notifiable, highly contagious disease of swine controlled mainly with costly administrative methods. Swine may be infected not only with classical swine fever virus (CSFV), but also with other, non porcine, genetically and antigenically related pestiviruses. Differentiation of infections with CSFV and other pestiviruses is a crucial element of diagnostics.

In the present study two real-time PCR methods and conventional one-tube nested PCR for specific detection of CSFV were compared. Additionally, two methods designed for detection of all pestivirus species real-time SYBR Green I and one-tube nested PCR were included into the study. Analyzed methods varied considerably regarding their sensitivity and specificity, what suggests that careful selection of diagnostic methods and their evaluation on a regular basis is necessary.

Key words: pestiviruses, CSFV, real-time PCR, nested PCR, diagnostics

Introduction

Classical swine fever (CSF) is an economically important pig disease notifiable within EU and listed by OIE. Most of European countries are free from CSF, however, during the last five years several episodes of CSF were identified in pigs (Lithuania, Serbia, Slovakia, Bulgaria, Romania) as well as in wild boars (Lithuania, Slovakia, Bulgaria, Germany, Hungary). The stamping out policy of CSF control adopted within EU resulted in fully susceptible population. Several examples have shown that introduction of the virus into naive population may cause tremendous economical losses (Ribbens et al. 2012). During the CSF epidemic in the Netherlands in 1997-1998 approximately 12 million pigs were slaughtered and the cost of disease eradication reached 2.3 billion USD (Stegeman et al. 2000).

The etiological agent of the disease is CSF virus (CSFV), classified in the genus *Pestivirus,* family *Flaviviridae* together with bovine viral diarrhoea virus 1 (BVDV-1), BVDV-2 and border disease virus (BDV) (Becher et al. 2003). BVDV and BDV cause non-notifiable infections in ruminants but they are also able to infect pigs, sometimes causing misinterpretations in diagnostic tests (de Smit et al. 1999, Oguzoglu et al. 2001, Loeffen et al. 2009). Therefore, rapid and effective detection of infections with pestiviruses and their differentiation is a crucial aspect in diagnosis of CSF.

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Currently, tests prescribed by OIE for international trade are neutralizing peroxidase-linked assay (NPLA), fluorescent antibody virus neutralization test and ELISA. For identification of the virus fluorescent antibody test on cryostat sections and virus isolation are recommended. However, ELISA methods widely used in monitoring of population for CSFV-specific antibodies are prone to non-specific results due to cross-reactions with antibodies specific to ruminant pestiviruses (de Smit et al. 1999, Bingham et al. 2010). During the CSF outbreak in the Netherlands in 1997/98 26.5% of samples initially diagnosed as CSFV-positive by ELISA were eventually recognized as infections with ruminant pestiviruses (de Smit et al. 1999). Also for NPLA, considered as a gold standard in detection of CSFV antibodies, incorrect results in double BVDV and CSFV infections were reported (Wieringa-Jelsma et al. 2006). Traditional virus isolation method is time-consuming and laborious. Moreover, it may be influenced by the presence of neutralizing antibodies. OIE manual also refers to RT-PCR as a method of increasing significance in preclinical diagnosis of CSF and screening of infected herds.

The aim of the study was to compare the usefulness of different PCR methods applied in differential diagnostics of pestiviruses. The sensitivity of two PCR methods detecting RNA of all pestiviruses species (pan-pestivirus) and three methods designed to detect CSFV RNA were compared. Methods evaluated in the study included real-time PCR-based on SYBR Green I intercalating dye and specific TaqMan probes as well as two conventional PCR methods.

Materials and Methods

Viruses

Strains representing all species within *Pestivirus* genus were used in the study. Two CSFV strains, Alfort/187 (genotype 1) and field strain 1795/94 (genotype 2), BVDV-1 strain NADL, BVDV-2 strain Short and BDV strain Moredun were propagated and titrated using PK15 (CSFV), MBDK (BVDV-1 and BVDV-2) and SFTR cell line (BDV).

RNA extraction and reverse transcription

Total RNA was extracted using Total RNA Prep Plus kit (A&A Biotechnology) according to the manufacturer's recommendations and eluted in 100 µl of RNase-free water. cDNA was synthesized using MMLV reverse transcriptase (Invitrogen) and 2.5 uM of random nonamers (Sigma) based on previously optimized method (Podgorska and Stadejek 2010), aliquotted and stored at -70ºC until further analysis.

Polymerase chain reaction

Real-time PCR reactions were carried out in MicroAmp Optical Tubes (Applied Biosystems) closed with Optical Caps (Applied Biosystems). Amplification was performed using Stratagene MxPro 3005P (real-time PCR) or Biometra (conventional PCR) equipment. All PCR reactions were run in triplicates. Thermal conditions of each test and sequences of oligonucleotides used for amplification are presented in Tables 1 and 2.

Two methods designed to detect all pestivirus species were applied (Table 1, 2). Real-time PCR based on SYBR Green I (Stadejek et al. 2006) was carried out in a volume of 25 µl containing 1x QuantiTect SYBR Green PCR Master Mix, 0.8 µM of csfv 6 and csfv 7 primers, 1 µl of cDNA and nuclease-free water. The PCR reaction was followed by analysis of the melting temperature of amplification product to control the specificity of reaction. The other pan-pestivirus method was a conventional two-step nested PCR (PP-nPCR) based on Vilcek et al. (1994) with further modifications (Stadejek and Pejsak 2000). First step was performed based on 50 µl reaction mix containing 0.4 µM of external primers (V324/V326), 2.5 mM $MgCl_2$, 0.4 mM of dNTPs, 1 µl of 10% Triton X-100, 2.5 U Taq polymerase (Fermentas), 1x Taq Buffer with KCl, RNAse-free water and 5 µl of cDNA. The reaction mix was covered with a layer of mineral oil and subjected to amplification. Next, the amplification tubes were thoroughly mixed to dissolve reagents previously dried in the lids in 22% trehalose (20 pmols of internal primers, 1 µl of a mixture of 10 mM dNTP and 1.25 U Taq polymerase) then briefly centrifuged and subjected to nested PCR reaction.

Two different TaqMan-based real-time PCR methods, TaqMan I (Stadejek et al., unpublished) and TaqMan II (Hoffmann et al. 2005), as well as conventional two-step nested PCR (CSFV-nPCR) (Katz et al. 1993) were used for specific detection of CSFV (Table 1, 2). Reactions were carried out in 25 µl volume containing 5 µl of cDNA, 0.4 µM (TaqMan I) or 0.8 µM (TaqMan II) of primers, 1x Quantitect – Probe RT-PCR Master Mix (Qiagen), 0.8 µM (TaqMan I method) or 0.1 µM (TaqMan II) of dually-labeled probe and RNAse-free water. CSFV-nPCR was based on external (A/D) and internal (B/C) pairs of primers. The concentration of reagents was the same as described above for PP-nPCR.

Table 1. Characteristics of the oligonucleotides used in the study.

Method		Oligonucleotides	Sequence (5'→3')	Amplified region of the genome	References
CSFV-specific	TaqMan I	→ csfv 6	CTA GCC ATG CCC IYA GTA GGA	5' UTR	Uttenthal et al. 2003
		← csfv 7	CTC CAT GTG CCA TGT ACA GCA		
		→ TaqMan probe	6FAM- CCC TGG GTG GTC TAA GTC CTG AGT ACA G-TAMRA	5' UTR	Stadejek et al. (unpublished)
	TaqMan II	→ CSF100-F	ATG CCC AYA GTA GGA CTA GCA	5' UTR	Hoffmann et al. 2005
		← CSF192-R	CTA CTG ACG ACT GTC CTG TAC		
		→ TaqMan probe	6FAM-TGG CGA GCT CCC TGG GTG GTC TAA GT -TAMRA		
	CSFV nPCR	→ A	ATA TAT GCT CAA GGG CGA GT	E2	Katz et al.1993
		→ B	CTG TGG CTA ATA GTG ACC TAC		
		← C	CAT TTC TTT ATG GGC TCA TC	E2/NS2	
		← D	ACA GCA GTA GTA TCC ATT TCT TTA		
Pan-pestivirus	SYBR Green I	→ csfv 6	CTA GCC ATG CCC IYA GTA GGA	5' UTR	Uttenthal et al. 2003 Stadejek et al. 2006
		← csfv 7	CTC CAT GTG CCA TGT ACA GCA		
	PP nPCR	→ V324 (324)	ATG CCC WTA GTA GGA CTA GCA	5' UTR	Vilcek et al. 1994
		← V326 (Pest2)	TCA ACT CCA TGT GCC ATG TAC		
		→ A11	AGT ACA GGG TAG TCG TCA GTG GTT CG		
		← A14	CAA CTC CAT GTG CCA TGT ACA GCA G		

Table 2. PCR reaction conditions.

Method	PCR reaction conditions			
	stage	step	duration (min)	temp. (°C)
TaqMan I	I	1	15:00	95
	II (40 cycles)	1	00:15	94
		2*	01:00	60
TaqMan II	I	1	15:00	95
	II (42 cycles)	1	00:30	95
		2*	00:30	56
		3	00:30	72
SYBR Green I	I	1	15:00	95
	II (42 cycles)	1	00:15	95
		2*	00:30	57
		3	00:30	72
	III	1	10:00	72
	IV	Melting temp. analysis*		
PP-nPCR, CSFV-nPCR	I	1	03:00	95
	II (35 cycles)	1	01:00	94
		2	01:00	52
		3	01:00	72
	III (30 cycles)	1	01:00	94
		2	01:00	52
		3	01:00	72
	IV	1	10:00	72
		2	05:00	22

* collection of the fluorescence data

Table 3. Summary results of amplification. Number of positive results per three repeats of every reaction is indicated.

Strain	Dilution	Titer ($TCID_{50}$/ml)	Method				
			CSFV-specific			Pan-pestivirus	
			TaqMan I	TaqMan II	CSFV-nPCR	SYBR Green I	PP-nPCR
CSFV Alfort/187 Genotype 1	N*	E+7	3/3	3/3	3/3	3/3	3/3
	10–1	E+6	3/3	3/3	3/3	3/3	3/3
	10–2	E+5	1/3	3/3	3/3	3/3	3/3
	10–3	E+4	1/3	3/3	3/3	–	3/3
	10–4	E+3	–	3/3	2/3	–	2/3
	10–5	E+2	–	3/3	2/3	–	1/3
	10–6	E+1	–	2/3	1/3	–	–
	10–7	E+0	–	–	–	–	–
CSFV 1795/94 Genotype 2	N	E+6.55	–	3/3	3/3	3/3	3/3
	10–1	E+5.55	–	3/3	3/3	3/3	3/3
	10–2	E+4.55	–	3/3	3/3	1/3	3/3
	10–3	E+3.55	–	3/3	3/3	–	3/3
	10–4	E+2.55	–	3/3	2/3	–	2/3
	10–5	E+1.55	–	3/3	2/3	–	1/3
	10–6	E+0.55	–	–	–	–	–
	10–7	E–1.55	–	–	–	–	–
BVDV-1 NADL	N	E+6.05	–	–	–	3/3	3/3
	10–1	E+5.05	–	–	–	3/3	3/3
	10–2	E+4.05	–	–	–	2/3	3/3
	10–3	E+3.05	–	–	–	–	3/3
	10–4	E+2.05	–	–	–	–	3/3
	10–5	E+1.05	–	–	–	–	3/3
	10–6	E+0.05	–	–	–	–	1/3
	10–7	E–1.05	–	–	–	–	–
BVDV–2 Short	N	E+5.8	–	–	–	3/3	3/3
	10–1	E+4.8	–	–	–	3/3	3/3
	10–2	E+3.8	–	–	–	1/3	3/3
	10–3	E+2.8	–	–	–	–	3/3
	10–4	E+1.8	–	–	–	–	2/3
	10–5	E+0.8	–	–	–	–	–
	10–6	E–1.8	–	–	–	–	–
	10–7	E–2.8	–	–	–	–	–
BDV Moredun	N	n.d.**	–	–	–	–	3/3
	10–1	n.d.	–	–	–	–	3/3
	10–2	n.d.	–	–	–	–	–
	10–3	n.d.	–	–	–	–	–
	10–4	n.d.	–	–	–	–	–
	10–5	n.d.	–	–	–	–	–
	10–6	n.d.	–	–	–	–	–
	10–7	n.d.	–	–	–	–	–

*N – not diluted
**n.d. – not determined

Sensitivity of all PCR methods was assessed by amplification of dilution series of individual strains of known (except BDV) concentration (Table 3).

Statistical analysis

The results of particular methods regarding individual strain detection was evaluated with McNemar's test. The level of significance was set at $p=0.05$. Also, the agreement between tests was assessed based on Cohen's kappa coefficient. Both statistical tests were performed using GraphPad statistical software (http://graphpad.com/).

Results

The results of this study have shown differences in sensitivity of the evaluated methods (Table 3). None

of the methods specific for CSFV detected other pestiviruses species. The highest sensitivity in detection of CSFV was obtained using the TaqMan II method which allowed to detect Alfort/187 strain in a concentration of 10^1 $TCID_{50}$/ml in 2 of 3 repeats and $10^{1,55}$ $TCID_{50}$/ml of the field strain 1795/94 in all 3 repeats. CSFV-nPCR gave comparable results detecting the same concentration of CSFV strains but in 1 of 3 and 2 of 3 repeats, respectively. There was no statistically significant difference between these two methods ($p>0.05$) and the kappa coefficient indicated substantial agreement regarding Alfort ($\kappa = 0.65$) and good agreement regarding 1795/94 ($\kappa = 0.8$) strain. Significantly lower performance was observed for TaqMan I method ($p<0.05$). The test detected 10^4 $TCID_{50}$/ml of genotype 1 strain Alfort/187 but failed to detect field CSFV strain 1795/94 of genotype 2. The agreement with the best working TaqMan II method regarding Alfort strain was poor ($\kappa = 0.18$). What was interesting, PP-nPCR method designed to detect all pestiviruses detected CSFV at a similar level as TaqMan II regarding both Alfort/187 and 1795/94 ($p<0.05$) with a moderate ($\kappa = 0.75$) and substantial ($\kappa = 0.71$) agreement, respectively. PP-nPCR detected Alfort strain in 1 log higher concentration compared to TaqMan II and CSFV-nPCR methods. Only slightly lower sensitivity was recorded for 1795/94 strain. In that case, PP-nPCR detected $10^{1.55}$ $TCID_{50}$/ml of 1795/94 CSFV in 1 per 3 reactions while TaqMan II was able to detect the virus in all 3 reactions. On the other hand, SYBR Green I detected only 10^5 $TCID_{50}$/ml of Alfort/187 and $10^{4.55}$ $TCID_{50}$/ml of 1795/94 strain. PP-nPCR gave significantly better results ($p<0.05$) in detection of BVDV-1 and BVDV-2 compared to SYBR Green I assay. The difference was as high as 4 and 2 logs for BVDV-1 and BVDV-2, respectively. BDV strain Moredun was detected only using PP-nPCR test.

Discussion

Despite strict and costly methods of CSF control within EU events of re-introduction of the disease still occur causing serious economic damage (Ribbens et al. 2012). Although most of the EU countries are free from CSFV, infections with other pestiviruses (BVDV-1, BVDV-2 and BDV) are present in ruminants (Loeffen et al. 2009). Poland is free from CSFV but BVDV infections in cattle are common (Polak and Zmudzinski 1999). Also, seroconversion to pestiviruses has been detected in free living ruminants in Poland (Fabisiak et al. in press). The status of BDV in Poland has not been determined. It was experimentally proved that ruminant pestiviruses may also infect swine, sometimes causing symptoms suggesting infection with low virulent CSFV (Paton and Done 1994). Such infections may also interfere with diagnostic and intervention programs, especially based on marker DIVA vaccines (Loeffen et al. 2009, Passler and Walz 2010). Influence on CSFV transmission in pig population and possibly delayed identification of CSF outbreak were also discussed (Wieringa-Jelsma et al. 2006, Loeffen et al. 2009). Therefore, constant monitoring of swine population not only for CSFV but also for the presence of ruminants pestiviruses is necessary.

PCR methods offer a promising alternative in the diagnosis of CSF and differentiation of the virus from other pestiviruses (Hoffmann et al. 2005). Amplification and sequencing of the specific regions in pestiviruses genome (5' untranslated region, E2, NS5B genes) gives an additional advantage of further phylogenetic analysis which is of great importance in epidemiology and tracing of the origin of CSF outbreaks (Paton et al. 2000).

In the present study 3 PCR methods specifically directed for detection of CSFV and 2 pan-pestivirus specific PCR methods were evaluated regarding a set of strains representing all pestiviruses species. Compared methods significantly differed in sensitivity to detect CSFV. Real-time PCR TaqMan II proved to be the most sensitive. It detected 1000 times less concentrated Alfort/187 compared to other CSFV-specific TaqMan I method (Table 3). Moreover, the latter assay failed to detect genotype 2 CSFV field strain 1795/94. However, 1795/94 was detected by SYBR Green I method based on the same primers (Table 1). Most probably the problem was associated with the specificity of a TaqMan probe which was not able to recognize genotype 2 strain. That false negative result underlines the necessity of an evaluation of applied RT-PCR methods regarding currently circulating CSFV strains on a regular basis.

Out of the two pan-pestivirus specific tests PP-nPCR gave significantly better results ($p<0.05$) regarding BVDV-1 and BVDV-2 compared to real-time SYBR Green I assay. Moreover, it was the only method that detected BDV strain. Although SYBR Green I allowed for elimination of electrophoresis stage and minimization of the risk of contamination, its low sensitivity suggest that conventional gel-based PP-nPCR should be a method of choice in detection of BVDV and BDV. This method could be used for monitoring of the swine population for the presence of infections with pestiviruses and collect data important for epidemiology and diagnosis of CSF. Subsequent use of CSFV-specific systems would allow further differentiation of detected pesitiviruses.

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What is important, both conventional PCR methods used in the study, PP-nPCR and CSFV-nPCR, have shown relatively high sensitivity in detection of CSFV. The results were comparable to the best-working TaqMan II method and much better than two other real-time tests used in the study. Although classical nested-PCR is a multistage method which increases the risk of laboratory contamination, not all laboratories can afford expensive real-time PCR equipment. The abovementioned results indicate that conventional PCR methods may be used as an alternative for CSFV detection without major impairment of sensitivity.

Our previous study reported that optimization of reverse transcription improved the sensitivity of RT-PCR for detection of CSFV about 1000 times (Podgorska and Stadejek 2010). In the present study we indicated that further improvement of similar magnitude may be obtained by a proper selection of diagnostic method. In the worst case scenario (TaqMan I and CSFV strain 1795/94) strain) the infection with CSFV may not be detected what can result in serious economic losses. Additionally, method for monitoring of pig population for the presence of ruminant pestiviruses infections was proposed.

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