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Detection of economically important viruses in boar semen by quantitative RealTime PCR™ technology

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Abstract

The objective of this study was to develop quantitative real-time polymerase chain reaction (ReTi-PCR) tests for the detection of five economically important viruses in swine semen namely, pseudorabies virus (PRV), classical swine fever virus (CSFV), foot-and-mouth disease virus (FMDV), swine vesicular disease virus (SVDV), and porcine reproductive and respiratory syndrome virus (PRRSV). Each ReTi-PCR test was validated for specificity, analytical sensitivity (detection limits), and experimental infection studies were performed to compare the conventional virus isolation methods with the newly developed ReTi-PCR tests.

All five developed ReTi-PCR tests are very rapid compared to virus isolation, highly specific, and even more sensitive (lower detection limits) than conventional virus isolation methods for the detection of mentioned viruses in semen. In semen of experimentally infected boars, viruses were detected much earlier after infection and more frequently by ReTi-PCR tests than by virus isolations. The high throughput of these rapid ReTi-PCR tests makes it possible to screen large number of semen samples for the presence of viruses prior to insemination. This is a substantial advantage, in particular for boar semen the quality of which deteriorates quickly after storage.

In general, the newly developed ReTi-PCR tests are valuable tools for the early, reliable and rapid detection of five economically important viruses, namely PRV, CSFV, FMDV, SVDV, and PRRSV in boar semen. These ReTi-PCR tests will improve the control of viral diseases transmitted via semen.
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Keywords: ReTi-PCR; Swine; Semen

1. Introduction

The epidemiology of animal virus diseases has changed significantly by the introduction of artificial insemination (AI). The application of artificial insemination prevents the transmission and spread of virus by direct contact between males and females, and it has been proven that artificial insemination improves the control of many diseases. On the other hand, virus spread by contaminated semen by artificial insemination can be enormous, since contaminated semen could infect numerous farms, areas, or even countries within a few hours or days. Pseudorabies virus (PRV) has been detected in semen (Guerin et al., 1995), and foot-and-mouth disease virus (FMDV), swine vesicular disease virus (SVDV), and African swine fever virus (AFSV) have been isolated from porcine semen (Thacker et al., 1984). For SVDV and AFSV, transmission to sows by artificial insemination of contaminated semen has been reported (McVicar et al., 1977; Thacker et al., 1984). Under experimental conditions it was shown that transmission of CSFV by artificial insemination was possible and that semen contaminated with CSFV could cause an infection

Abbreviations: AI, artificial insemination; CSFV, classical swine fever virus; FMDV, foot-and-mouth disease virus; PRRSV, porcine reproductive and respiratory syndrome virus; PRV, pseudorabies virus; ReTi-PCR, real-time polymerase chain reaction; SVDV, swine vesicular disease virus

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in sows after artificial insemination (de Smit et al., 1999). Transmission of PRRSV to sows artificially inseminated with undiluted semen from experimentally infected boars has also been demonstrated (Yaeger et al., 1993). It has been suggested that the introduction of PRRSV in herds by semen contaminated with PRRSV might even be of high risk (LePotier et al., 1997).

All these reports demonstrated the potential risk of virus spread by contaminated semen through artificial insemination. Frequent monitoring, and all kinds of precautions intensively guards the health of boars in artificial insemination centers in order to prevent virus transmission via semen. But, the occurrence of viral diseases in the AI centers is still possible as introduction of virus into the centers cannot be excluded completely. Despite all precautions, virus spread via semen still could remain unnoticed.

To guarantee the safety of semen for artificial insemination, very rapid, sensitive and reliable tests for virus detection are crucial. Conventional methods for virus detection in semen, like virus isolation, are: (a) not very sensitive, (b) time-consuming, (c) very expensive, and (d) the application of the virus isolation technique is markedly reduced for semen due to cell toxicity. Furthermore, boar semen can only be stored temporarily, and consequently a rapid test is a prerequisite to guarantee the safety of boar semen prior to use.

The polymerase chain reaction (PCR) technique as a diagnostic method is generally known as a very sensitive, specific, and rapid tool for the detection of, e.g. viral genomic sequences. It has been shown that even matrices such as boar semen can be used in PCR-based tests (Shin et al., 1997). However, conventional PCR tests can be hampered by the high risk of contamination of previously amplified material. Recently, the quantitative ReTi-PCR technology has been developed with several advantages to the conventional PCR. The ReTi-PCR technique combines amplification and detection of amplified products in one closed tube. Therefore amplified material will not be released in the environment by reopening and other handlings of test tubes. Consequently, the chance of contamination of other test samples by previously amplified material is strongly reduced.

After the CSF outbreak in The Netherlands in 1997, it was decided to develop rapid and reliable assays for the detection of list-A diseases such as CSF, FMD and other economically important diseases. In this study, the development and validation results of ReTi-PCR tests are described for the detection of five important porcine viruses in semen; PRV, CSFV, FMDV, SVDV and PRRSV.

2. Materials and methods

2.1. Development of ReTi-PCR assays

2.1.1. Primers and probes

Primers and probes for the amplification and detection of PRV, CSFV, FMDV, SVDV and PRRSV were selected primarily in highly or even completely conserved regions of the respective virus genomes in order to detect all serotypes, subtypes, field strains, and/or vaccine strains. Additionally, the fundamental specificity of primers and probes were confirmed by nucleotide sequence alignments with known sequences of closely related viruses using blast search at NCBI (http://www.ncbi.nlm.nih.gov) (OMIGA). If necessary, primers and/or probes were degenerated at defined nucleotide positions in order to maximize the detection of all known strains and isolates. For each ReTi-PCR, two probes were designed to hybridize side by side on the PCR product. The 3′ end of the upstream probe was labeled with fluorescein acting as a fluorescence resonance energy transfer (FRET) donor for the acceptor dye (LC Red 640) coupled to the 5′ end of the downstream probe. The FRET signal is transmitted only if donor and acceptor hybridize to their respective specific regions and in the vicinity of each other.

2.1.2. Isolation of DNA or RNA

DNA or RNA was isolated from 200 μl of semen by column chromatography using commercially available kits. PRV-DNA was isolated from serum or semen with the High Pure Viral Nucleic Acid Kit and performed as recommended by the manufacturer (Roche, Germany). RNAs from CSFV, FMDV, SVDV or PRRSV were isolated with the High Pure RNA Isolation kit (Roche, Germany). Prior to the use of the High Pure RNA Isolation kit, 200 μl of semen was incubated for 10–15 min at room temperature with 10 μl of RNAse free DNAse. After addition of 400 μl lysis/binding buffer, the semen samples were centrifuged at 13,000 rpm for 1 min. Thereafter, the procedure was performed according to the protocol of the manufacturer. RNA or DNA was eluted from the column in a final volume of 50 μl RNAse-free water.

2.1.3. Optimizations of the ReTi-PCR tests

Reverse transcription (RT)-PCR, ReTi-PCR and the detection of amplified fragments were all performed on the LightCycler 2.0^{TM} (Roche, Germany) in a volume of 20 μl. For the five different ReTi-PCR tests, the LightCycler-Fast Start DNA Master hybridization probes kit (PRV), the LightCycler DNA Master hybridisation probes kit in combination with the C-therm kit (CSFV), the LightCycler RNA master hybridisation probes kit (FMDV and SVDV), and the LightCycler-RNA amplification Kit Hybridisation Probes kit (PRRSV) were used (Roche, Germany). Subsequently, conditions of the PCR tests were optimized by systematically varying conditions, e.g. annealing temperature, primer and $MgCl_2$ concentrations, and time intervals. Amplification was monitored real-time, using hybridisation probes specific for the amplification products. Samples were considered positive when the fluorescence signal increased above the background signal. After optimization, ReTi-PCR tests were validated for specificity, analytical sensitivity (detection limits) and for the detection of viruses in semen of experimentally infected boars. Details on NA-extraction procedures and ReTi-PCR tests are available on request.

2.1.4. Control samples

Dilutions of each virus strain (e.g. 1 μl of FMDV type O Taiwan containing 10^5 $TCID_{50}$ of infectious virus/ml) in negative semen served as positive control samples for the corresponding ReTi-PCR. In each run, 1–3 dilutions of positive control samples were tested, distributed randomly in the run and included from the start (isolation of the DNA or RNA). Negative semen samples served as negative controls. In each test run, the freshly prepared PCR mix, including 10 μl of H_2O, was added as an additional negative control.

2.1.5. Specificities of the ReTi-PCR assays

The specificities of the developed ReTi-PCR tests were investigated for cross-reactivity by testing related and non-related viruses. For SVDV, no other non-related viruses were studied for possible cross-reactivity. However, cross-reactivity with related picornaviruses such as FMDV was not expected based on the alignment of selected primers.

2.1.6. Analytical sensitivities of the ReTi-PCR assays

The analytical sensitivities (detection limits) of the PCR assays were studied by comparison with infectious virus titers as obtained by virus isolation. Therefore, dilutions of defined virus stocks were prepared in cell culture medium for PRV (NIA-3), CSFV (C112), FMDV (type O Taiwan), SVDV (Net92), and PRRSV strain Lelystad (LV, ter Huurne). Negative semen samples, spiked with the prepared virus dilutions in cell culture medium, were tested in order to determine the sensitivities of the ReTi-PCR tests in semen samples.

2.1.7. Precautions

To prevent contamination 3 separate rooms were used to prepare stock solutions of the ReTi-PCR premixes (PCR room 1), to isolate the DNA or RNA from the clinical samples (PCR room 2) and to run the ReTi-PCR tests (room 3). It was obligatory to wear gloves and a clean overcoat for each room. Furthermore, premixes were prepared before starting any other activities that involved contact with possible DNA or RNA materials that might cause contamination. DNA or RNA was isolated in a laminar flow cabinet or using the MagnaPure LC (Roche, Germany).

2.2. Experimental infection experiments on boars

In five independent animal experiments two to four boars, free of PRV, CSFV, FMDV, SVDV or PRRSV, were either infected intranasally (IN), intramuscularly (IM), intradermally in the bulb of the heel (IH), or intravenously (IV). Inoculation of each virus was as follows: for PRV NIA3 (2 ml $10^{5.5}$ pfu/ml IN), CSFV SW/NL/Venh/97 (2 ml $10^{4.7}$ $TCID_{50}$/ml IN and 1 ml $10^{4.3}$ $TCID_{50}$/ml IM), FMDV type O Taiwan (IH with 0.1 ml 10^3 $TCID_{50}$/ml or 0.1 ml 10^5 $TCID_{50}$/ml, and after 8 days with 5 ml 10^5 $TCID_{50}$/ml IV and IM), SVDV (2 ml Net92 10^5 $TCID_{50}$/ml IV), and for PRRSV (2 ml of strain Lelystad virus (LV) ter Huurne 10^5 $TCID_{50}$/ml IN). Boars were observed daily for clinical symptoms such as increased body temperatures, lameness, loss of appetite and libido. Blood samples for serology and/or virus isolation were taken during the whole experiment starting prior to the experimental infection. Blood was centrifuged for 10 min at 1500 rpm, and serum samples were stored at −70 °C. Semen was collected daily or every other day (if possible, since loss of libido or excruciating pains (for FMD) interfered with collection). Collection of semen samples was started 1 week prior to the experimental infection in order to collect virus-negative semen, and to obtain an impression of the libido of each boar. Semen samples were collected, during collection temporarily stored at 4 °C, and as soon as possible stored at −70 °C. Collection of semen was continued during the whole experiment or until euthanization.

2.3. Virus isolation

Virus isolations for PRV, CSFV, FMDV, and SVDV were performed by standard techniques, based on the OIE manual of standards for diagnostic tests and vaccines (OIE manual, 2000). Sensitive cell types, as recommended in the OIE manual, were chosen for virus isolation. Briefly, for PRV a swine kidney cell line (SK-6 cells) was used. SK-6 cells were plated (10^6 cells/ml in DMEM containing 5% fetal calf serum and antibiotics) and incubated with a volume of 125 μl of 1:10 and 1:100 diluted semen. After an incubation period of 2 days at 37 °C (5% CO_2), 100 μl of the supernatant was passed blindly to fresh SK-6 monolayer cells. After another 2 days of incubation at 37 °C (5% CO_2), monolayers were stained with amido-black and screened for the presence of plaques.

For CSFV, 300 μl of undiluted and 300 μl of BTS diluted semen were incubated on a 70% semi-confluent monolayer of SK-6 cells. After a wash procedure, EMEM supplemented with 5% foetal calf serum and antibiotics was added to the wells and the microplates were incubated for 4 days at 37 °C (5% CO_2). After incubation, cells were screened for the presence of CSFV antigens by immunoperoxidase monolayer assay (IPMA). Supernatants of negatively stained monolayers were passed again on fresh SK-6 cells, incubated for 4 days and screened again for CSFV antigens by IPMA.

For FMDV, 1-day-old monolayers of 10^6 secondary porcine kidney (PK-2) cells per micro plate well (6-well cell culture plates), plated in EMEM medium containing 5% fetal calf serum (FCS) and 2% antibiotics, were infected with 200 μl of 10 and 100 times diluted semen. Therefore, 1 ml of boar semen was centrifuged for 20 min at 2500 rpm. The cell fraction was re-suspended in EMEM containing 5% FCS and 2% antibiotics, and diluted 10 and 100 times in EMEM containing 5% FCS and 2% antibiotics. After incubation of 1 h, the inoculum was removed and the microplates were washed with PBS before fresh medium was added, and the incubation was continued for 2 days. Then, the microplates were frozen at −70 °C. After thawing, 200 μl

of the supernatant was blindly passed to fresh monolayers of PK-2 cells and incubated for 1 h. An overlay of methylcellulose was added, and the incubation was continued. After the second incubation period of 2 days, the cells were stained with amido-black and the plaques were counted.

For the isolation of SVDV IBRS-2 cells were plated in Eagles basal EHM containing 5% foetal calf serum and 2% antibiotics. A volume of 100 μl of semen, four times diluted in serum, was incubated on a monolayer of IBRS-2 cells for 4 days at 37 °C. Then, microplates were frozen at −70 °C. After thawing the plates, a volume of 400 μl of the supernatant was blindly passed on fresh IBRS-2 cells and incubated for a second period of 4 days. After a freeze/thaw step, both passages were screened for SVDV antigens by ELISA (Hamblin et al., 1984; Roeder and Le Blanc Smith, 1987).

PRRSV was isolated on primary alveolar lung macrophages as described (Wensvoort et al., 1991). Briefly, per microplate well (6-well cell culture plate) 10^6 cells in RPMI were seeded and incubated with 480 μl of 10 and 100 times diluted semen. Incubation and a blind passage were performed as described for FMDV (see above). Cells were screened for the presence of PRRSV antigens by IPMA using monoclonal antibodies. In addition, for a very limited number of samples a bioassay was performed. Therefore, a volume of 10 ml of semen was intra-peritoneally injected in 10-week-old pigs. Serum samples were collected for 2 weeks, and screened for the presence of PRRSV.

2.4. Serology

Serological tests were carried out to investigate whether the experimental viral infections were successful and resulted in humoral responses. Therefore, the following antibody detection assays were performed; CSFV-ab ELISA for the detection of CSFV antibodies (Colijn et al., 1997), the virus neutralization test (VNT) for FMDV antibodies against FMDV type O Taiwan (de Leeuw et al., 1979), the SVDV neutralization test for SVDV antibodies, the PRRSV and the PRV antibody test kits (Idexx, USA) for antibodies specific for PRRSV and PRV, respectively.

3. Results

3.1. Primers and probes selection

For the detection of PRV, CSFV, FMDV, SVDV, and PRRSV by ReTi-PCR tests, primers and probes were selected on the basis of completely or highly conserved sequences among strains and isolates in order to detect all known variants of the respective virus. For detection of PRV, the highly conserved gB gene was used as target. The highly conserved 5′-nontranslated region was used as target for detection of CSFV, and for the picornaviruses, FMDV and SVDV, the conserved gene encoding the RNA-dependent RNA polymerase served as template for amplification. Within this 3D encoding RNA-dependent RNA polymerase, primers and probes were selected for the detection of all FMDV serotypes; O, A, C, Sat 1, 2, 3 and Asia-1. The ORF-6 and ORF-7 genes, encoding the envelope-associated protein M, and nucleocapsid protein N (ORF-7), were selected for the detection of PRRSV by ReTi-PCR.

To enlarge the theoretical specificities of the ReTi-PCR tests, differences in the selected regions of non-related viruses were checked by nucleotide comparisons in blast searches at NCBI (http://www.ncbi.nlm.nih.gov). For each ReTi-PCR, blast searches showed no clear homology of the selected primer and probe regions with nucleotide regions of non-related viruses. Therefore, possible false-positive reactions after the amplification of DNA or RNA extractions from these viruses were not expected or may even be excluded.

3.2. ReTi-PCR optimizations

ReTi-PCR tests were preliminary optimized by selecting the best primer pair/probe combination using genetic material of well-defined virus strains as templates. Thereafter, several parameters were optimized, such as concentrations of primers, probes and Mg^{2+} or Mn^{2+}, temperatures and time intervals for reverse transcription, denaturation, annealing and elongation.

After optimization; for the amplification of PRV genomic sequences the final concentration of the LightCycler Fast-start PCR mix contained per 20 μl: 3 mM $MgCl_2$, 5% DMSO, 0.3 μM primer pairs, 0.15 μM of each probe and 10 μl of the DNA eluate. For the amplification of PRV genomic sequences, the optimized ReTi-PCR conditions were: denaturation at 95 °C for 10 min, followed by 45 cycles of: 5 s at 95 °C, 5 s at 60 °C, and 10 s at 72 °C.

For the amplification of CSFV, FMDV, SVDV or PRRSV genomic sequences, the RNA Master Hybridization probes kit (Roche), the LightCycler DNA Master hybridisation probes kit, or the LightCycler-RNA amplification Kit Hybridisation Probes were used. The PCR mix, in a final volume of 20 μl, contained $Mn(Oac)_2$ and/or $MgCl_2$, 0.2–0.5 μM of each pair of primers, 0.15 μM probes, and 9 or 10 μl RNA eluate. The optimized ReTi-PCR conditions were; reverse transcription for 20 min at 61 °C or 30 min at 50 °C, denaturation at 95 °C, followed by 45 PCR cycles with denaturation at 95 °C and elongation at 72 °C. The annealing temperatures were ReTi-PCR specific. After optimization, specificity, analytical sensitivity (detection limits) and its value in the detection of viruses in semen of experimentally infected boars were determined for each ReTi-PCR.

3.3. Specificities of the ReTi-PCR tests

3.3.1. PRV-gB ReTi-PCR

The PRV marker vaccines 783, Bartha and Begonia, and DNA isolated from 50 PRV field isolates from Denmark,

Italy, and The Netherlands reacted positively in the gB-based ReTi-PCR. Genomic sequences of viruses closely related to PRV, such as bovine herpes virus 1, Marek's disease virus, herpes simplex virus type 1, and equine herpes virus type 1 were not detected.

3.3.2. CSFV ReTi-PCR

The ReTi-PCR for CSFV was positive for 142 CSFV isolates collected from all over the world, including isolates of the CSF outbreak in 1997 in The Netherlands. In addition, 71 bovine viral diarrhea virus (including type 1), and border disease virus isolates (including border disease virus strain F) were tested as negative by the CSFV ReTi-PCR.

3.3.3. FMDV ReTi-PCR

All serotypes of FMDV: A10, A12, A22/Iraq, A24/Cruzei, A turkey (type A), C1, C3 (type C), O1 Geschure, O1 Kaufburen, O Taiwan, O Manisa, O BFS, O Campos (type O), and the types SAT-1, SAT-2, SAT-3 and Asia 1 were detected by the FMDV ReTi-PCR. Genomic sequences of related picornaviruses, such as SVDV, and Coxsackie B5 virus were not detected.

3.3.4. SVDV ReTi-PCR

The detection of SVDV strain Net92 genomic sequences was possible by the SVDV ReTi-PCR. According to the blast search, six SVDV strains showed highly conserved nucleotide sequences on primer and probes selected regions used for the detection of SVDV Net92. Therefore, detection of SVDV strains was expected, in contract to the amplification of previously tested FMDV strains.

3.3.5. PRRSV ReTi-PCR

With the ReTi-PCR for the detection of PRRSV, genomic sequences of ORF6-7 from 55 EU-PRRSV isolates or strains and 10 US-PRRSV isolates or strains were detected. Additionally, the ORF6-7 sequences of 27 Dutch EU-PRRSV strains, isolated from 1992 to 1999 were determined and no nucleotide differences were found within the ReTi-PCR selected primers and probes regions. No genomic sequences were detected of the closely related arteriviruses, equine arteritis virus, lactate dehydrogenase-elevating virus and simian hemorrhagic fever virus, and the less related swine coronavirus, transmissible gastroenteritis virus.

3.4. Analytical sensitivities (detection limits) of the ReTi-PCR tests

For all five viruses, the detection limits of the developed ReTi-PCR tests were lower than the detection limits obtained by the virus isolation techniques. The detection limit of the PCR tests were: 0.06 pfu/ml for PRV, 0.5 $TCID_{50}$/ml for CSFV, 0.8 $TCID_{50}$/ml for FMDV, between 0.07 and 0.7 $TCID_{50}$ for SVDV, and 0.08 $TCID_{50}$ per semen sample for PRRSV.

3.5. Experimental infection experiments (and bioassays)

For the animal experiments, healthy, well trained boars were obtained from artificial insemination centers, or if necessary (PRRSV and PRV) specific pathogen free (SPF) artificial insemination boars. Two to four boars were experimentally infected via a route depending on the virus. In some animals no clinical signs and no reduction in libido were observed, whereas for some of the experimentally infected boars the libido was strongly reduced or absent for more than 1 week. Consequently, in the absence of libido no semen was collected for testing. Nevertheless, infections of all experimentally infected boars were successful as seroconversion was recorded for all boars (data not shown).

3.5.1. PRV experimental infection

One of the two boars, infected via the intranasal route with PRV strain NIA3, showed severe clinical symptoms at day 2 post-infection (pi), and this boar was not able to deliver semen starting at day 2 pi (boar no. 2). Pseudorabies virus was detected in semen from this boar collected on day 1 pi by ReTi-PCR, but not by virus isolation. In oropharyngeal swabs from boar no. 2 PRV was detected by virus isolation on days 3 and 7 pi, and by ReTi-PCR on days, 1, 3, 7 and 10 pi. Collected semen of boar no. 1 was negative by virus isolation, whereas semen samples were PRV-positive with the ReTi-PCR test from day 2 till the end of the experiment, except for day 16 (Table 1). PRV was detected in oropharyngeal swabs from boar no. 1 by virus isolation on days 4, 6 and 7, and by ReTi-PCR on days 2–7 pi and swabs were intermittently positive on days 11, 13 and 16.

3.5.2. CSFV experimental infection

Three boars, A, B and C, were infected intranasally with CSFV strain SW/NL/Verh/97. Semen samples of boar no. B were CSFV-positive by virus isolation on days 7 and 9 pi, whereas semen of boars A and C remained VI negative after the first incubation period on cell cultures (Table 2). After one blind passage on SK-6 cells, CSFV was detected

Table 1
PRV detection in semen by virus isolation and ReTi-PCR

Days post-infection	Boar 1	
	Virus isolation	ReTi-PCR
0	−	−
2	−	+
4	−	+
12	−	+
13	−	+
14	−	+
15	−	+
16	−	−
19	−	+
21	−	+

Semen from boar 2 was collected on day 1 only (virus isolation revealed to be negative and ReTi-PCR was positive), due to severe sickness.

Table 2
Detection of CSFV in semen from experimentally infected boars by virus isolation and ReTi-PCR

Days post-infection	Boar A		Boar B		Boar C	
	Virus isolation	ReTi-PCR	Virus isolation	ReTi-PCR	Virus isolation	ReTi-PCR
0	−	−	−	−	−	−
3	−	−	−	+	−	−
5			+*	+		
6	−	−			−	−
7			+	+		
8	+*	−			+*	+
9			+	+		
10	+*	−			−	+
11			+*	+		
12	−	+			−	−
13			−	−		
14	−	−			−	−
15			−	+		
16	−	+			−	+
17			−	+		
18	−	−			−	+

Only sampled on the indicated days post-infection. +: Positive result in the ReTi-PCR or virus isolation; +*: positive result in the virus isolation after one blind passage.

in five semen samples that were scored previously as virus isolation negative (boar no. A, on days 8 and 10 pi, boar no. B on days 5 and 11 pi, and boar no. C on day 8 pi). The CSFV ReTi-PCR confirmed all positive results obtained by virus isolation, except for 2 which became positive after one blind passage. In total, of all 3 boars, 13 semen samples were scored positively by ReTi-PCR, whereas 7 samples were positive by virus isolation (including those that became positive after 1 blind passage). These experimental infection data showed that CSFV was excreted in semen as early as 3 days pi, and was excreted intermittently till the end of the experiment (day 18 pi) (Table 2).

3.5.3. *FMDV experimental infection*

For FMDV, the boars infected in the bulb of the heel with 0.1 ml of a virus suspension containing 10^3 or 10^5 $TCID_{50}$ FMDV type O Taiwan did not develop clinical signs of FMD. Eight days after this IH inoculation, all boars were re-infected intravenously (1 ml) and intramuscularly (4 ml) with 10^5 $TCID_{50}$/ml of FMDV strain O Taiwan. To check whether FMDV could be detected in organs at an early stage of infection, boar no. 1 was killed at 4 days pi. Due to severe clinical signs of FMDV boar no. 2 was killed at 10 days pi, and boar nos. 3 and 4 at 11 days pi. Post-mortem, tissue samples, e.g. tonsils, were collected to determine virus spread after the experimental infection of the boars. Ten percent (w/v) tissue suspensions were prepared for virus isolation. Virus isolation was performed on PK cells as based on the OIE descriptions (OIE manual, 2000).

No FMDV was detected in semen by virus isolation, whereas seven semen samples of three out of the four boars, nos. 2–4, were positive by ReTi-PCR. As soon as (1 day) after infection, the semen of boar 2 was positive in the ReTi-PCR (Table 3). Boars 3 and 4 were also positive in the

Table 3
Detection of FMDV in semen from experimentally infected boars by virus isolation and ReTi-PCR

Days post-infection	Boar 1		Boar 2		Boar 3		Boar 4	
	Virus isolation	ReTi-PCR	Virus isolation	ReTi-PCR	Virus isolation	ReTi-PCR	Virus isolation	ReTi-PCR
0	−	−	−	−	−	−	−	−
1	ns	ns	−	+				
2	−	−	ns	ns	−	+	−	+
3	−	−	−	−				
4					−	−	−	−
5	#	#	−	−				
6					−	−	−	+
7			ns	ns				
9					−	−	−	+
10			#	#	−	+	−	−
11					#	#	−#	+#

Only sampled on the indicated days post-infection. +: Positive result in the ReTi-PCR or virus isolation; ns: no semen collected; #: boar killed because of severe clinical signs of FMD.

Table 4
Detection of SVDV by virus isolation and ReTi-PCR in semen from boars experimentally infected with SVDV NET92

Days post-infection	Boar A		Boar B		Boar C	
	Virus isolation	ReTi-PCR	Virus isolation	ReTi-PCR	Virus isolation	ReTi-PCR
0	−	−	−	−	−	−
1	−	−	−	−	−	−
2	−	−	−	−	−	−
3	−	−	−	−	−	−
4	ns	ns	ns	ns	−	+
5	−	−	ns	ns	−	−
6	−	−	ns	ns	ns	ns
7	−	$+^{w}$	ns	ns	ns	ns
8	ns	ns	ns	ns	ns	ns
9	−	−	ns	ns	−	−
10	−	$+^{w}$	ns	ns	−	−
11	−	$+^{w}$	ns	ns	−	$+^{w}$
12	−	$+^{w}$	ns	ns	−	−
13	−	−	ns	ns	−	−
14	$+^{1}$	−	ns	ns	$+^{1}$	−
15	−	−	−	−	$+^{1}$	−
16	−	−	−	−	−	$+^{w}$
17	−	−	ns	ns	−	−
18	−	$+^{w}$	−	−	−	−

$+^{w}$: Weak positive by ReTi-PCR; $+^{1}$: positive by virus isolation after one blind passage; ns: no semen collected.

first semen sample collected on day 2 pi. Semen samples collected from boar 1 were neither positive by ReTi-PCR nor by virus isolation. However, only two semen samples from this boar were available for testing (collected on days 2 and 3 pi). The post-mortem collected tonsil of boar no. 1 was positive by ReTi-PCR (data not shown).

In this experimental infection, oropharyngeal swabs and serum were collected as well from the three remaining boar nos. 2–4. The FMDV infection in these three infected boars was confirmed by the detection of FMDV in oropharyngeal swabs and by seroconversion. Organs and tissues collected post-mortem, e.g. tonsils, were also positive for FMDV by ReTi-PCR and/or virus isolation (data not shown).

3.5.4. SVDV experimental infection

The three boars, A–C, intravenously infected with 2 ml of SVDV strain Net92 seroconverted as expected. Boar B lost appetite after a few days post-infection, and showed a strongly reduced libido starting from day 4 till day 14 pi (Table 4). Boar C also showed a strongly reduced libido as observed on days 6–8 pi. None of the collected semen samples were SVDV positive by virus isolation in the first passage. However, after one blind passage on IBRS-2 cells, a few samples became SVDV positive. Only one semen sample was clearly positive by ReTi-PCR, whereas seven semen samples were weakly positive suggesting low number of SVDV RNA. Remarkably, no correlation was found between results obtained by virus isolation and ReTi-PCR. Duplicate analyses of the SVDV suspected semen samples confirmed the results obtained in the first ReTi-PCR run. The weak positive results obtained by virus isolation and ReTi-PCR indicated that SVDV strain net92 is excreted in semen, however, most likely to a very low extent.

The SVDV neutralization test revealed the development of neutralizing antibodies against SVDV starting on day 9 pi for boar A and on day 7 pi for the boar nos. B and C (data not shown).

3.5.5. PRRSV experimental infection

To investigate PRRSV excretion in semen, three boars were infected with PRRSV strain LV ter Huurne, and serum and semen samples were collected for ReTi-PCR and virus isolation (Tables 5 and 6). In general, serum samples were positive by virus isolation and ReTi-PCR within comparable periods after infection. Virus isolation was positive from day 8 pi till day 22 pi (boar no. 1), and from day 6 pi till day 13 pi for boar nos. 2 and 3. In two boars, nos. 1 and 3, the presence of PRRSV in serum was detected earlier by ReTi-PCR than by virus isolation. Serum of boar no. 1 was virus isolation positive at day 22 pi, whereas the ReTi-PCR was negative.

For semen samples, PRRSV detection by ReTi-PCR was positive as early as 6 days pi for boar nos. 1 and 3. Only one semen sample from boar no. 2 was scored as positive by ReTi-PCR (day 31 pi). A positive reaction was obtained again after retesting this semen sample by ReTi-PCR.

Whereas 17 samples were positive in the ReTi-PCR test, only 2 semen samples were PRRSV-positive by virus isolation. Semen collected from boar 2 remained negative by virus isolation during the entire experiment. PRRSV was detected intermittently in semen from boar nos. 1 and 3, and prolonged up to 45 days pi, whereas viraemia was not detected by ReTi-PCR after 20 days pi in the respective boars (Tables 5 and 6). Apparently, the excretion period of PRRSV via semen is not similar to the viraemic period.

A bioassay was performed to compare this sensitive method with the PRRSV ReTi-PCR. The bioassay showed

Table 5
Analysis of serum samples for PRRSV antibodies by ELISA, and for the presence of PRRSV in serum by virus isolation (VI) and ReTi-PCR

Boar no.	Days post-infection																					
	−1	0	1	3	6	8	10	13	15	17	20	22	24	27	29	31	34	36	38	41	43	45
Boar 1																						
Elisa	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VI	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
PCR	−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
Boar 2																						
Elisa	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VI	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
PCR	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Boar 3																						
Elisa	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VI	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
PCR	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table 6
Analysis of semen samples for the presence of PRRSV by virus isolation (VI) and ReTi-PCR

Boar no.	Days post-infection																					
	−1	0	1	3	6	8	10	13	15	17	20	22	24	27	29	31	34	36	38	41	43	45
Boar 1																						
VI	−	−	ns	−	+	ns	−	−	ns	ns	−	−	−	−	ns	−	−	−	−	ns	ns	−
PCR	−	−	ns	−	+	ns	−	−	ns	ns	+	+	+	+	ns	−	+	−	−	ns	ns	−
Bioassay					+						+		−									
Boar 2																						
VI	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
PCR	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Bioassay														−		−						
Boar 3																						
VI	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+
PCR	−	−	−	−	+	−	−	+	+	−	+	+	−	−	−	+	−	+	+	−	+	+
Bioassay											+	+	−									

ns: no semen collected.

that pigs injected with semen samples collected at day 20 pi from boar no. 1, and at day 22 pi from boar no. 3 became infected with PRRSV (Table 6).

4. Discussion

This study shows that ReTi-PCR tests, for the detection of PRV, CSFV, FMDV, SVDV, and PRRSV in boar semen, are highly specific and very sensitive (low limits of detection) with respect to virus isolation. Furthermore, ReTi-PCR tests proved to have several advantages in the detection of viruses in semen above the conventionally used virus isolation methods. Due to its sensitivity and rapidity, ReTi-PCR tests can be used for the sensitive screening of semen for the presence of viruses during outbreaks, for the rapid screening of semen for the presence of e.g. PRRSV prior to artificial insemination, and these tests offer opportunities for the early diagnosis of list A-diseases. The ReTi-PCR technique makes it possible to develop highly specific PCR tests mainly based on the strategic choice of primers in combination with specific probes. Herein, the blast search is a powerful tool in the search for conserved regions with respect to related and non-related viruses. Due to strict regimes of sample preparation, and the use of a closed system (amplification and analyses in one closed capillary) strongly reduces the spread of previously amplified product and consequently the risk of contamination as observed by conventional PCR techniques.

The use of artificial insemination has increased enormously in the swine industry. One disadvantage of this increase might be that one infected boar can rapidly infect multiple sows in more than one area or country. As viral infections can run a subclinical course, not all virus infections in boars will result in clinical symptoms, such as fever or reduced libido. This indicates that boars can shed virus in semen without showing clinical signs. This theory was confirmed by some of the boars infected experimentally. PRV, CSFV, FMDV, PRRSV, and to a lesser extent SVDV, were excreted in semen of experimentally infected boars, and furthermore, excretion could occur intermittently. For PRRSV it was shown that excretion in semen was prolonged and

PRRSV could be detected for a longer time in semen than in blood. This means that PRRSV test results of blood samples not always reflect the situation in semen, and that, despite intensive monitoring of boars with regular tests on blood samples, the safety of semen cannot be guaranteed for 100%. Although we did not use infected semen on recipient sows, others have shown that intra-vaginal infection by artificial insemination with contaminated semen is possible (Thacker et al., 1984; Yaeger et al., 1993; de Smit et al., 1999).

Based on the experimental data, we conclude that artificial insemination could be a potential risk for the transmission of PRV, CSFV, FMDV, SVDV and PRRSV to pig producing farms. To minimize the risk of introducing viral infections by artificial insemination as much as possible, semen may be screened for the presence of infectious viruses or viral genomic sequences by very sensitive tests. Due to the fact that undiluted semen is often toxic for cultures of many cell types, virus isolation can often not be used for the detection of viruses in semen or its sensitivity is too low. In addition, virus isolation is laborious and expensive. But most important, virus isolation is time consuming and therefore not applicable for boar semen, since this can only be stored temporarily, or for the rapid diagnosis of list-A diseases. Most conventional methods, used for the diagnosis of list-A diseases, take several days depending on the virus of interest. In contrast to virus isolation, the newly developed ReTi-PCR tests are rapid assays as results can be obtained within 4 h. This also offers opportunities to screen fresh semen prior to insemination for the presence of viruses such as PRRSV, as semen has been suspected to be an important source in the transmission of PRRSV (LePotier et al., 1997).

Besides rapid detection, the ReTi-PCR tests also detected more frequently virus positive semen samples from experimentally infected boars than virus isolation. For PRRSV, the sensitive bioassay confirmed two positive results of the ReTi-PCR, whereas virus isolations of these semen samples were negative. The presence of neutralizing antibodies may partly interfere with the low virus recovery by virus isolation. The presence of neutralizing antibodies might also explain the low recovery of CSFV in semen collected after day 12 pi by virus isolation, as antibodies were detected in all boars experimentally infected with CSFV. Furthermore, our study also shows that the presence of viruses in semen was detected earlier by ReTi-PCR than by virus isolation, but also earlier than most antibody detection assays. For example, the ReTi-PCR was able to detect FMDV at 1 day pi. Early diagnosis and rapid measures to reduce the transmission and spread of list-A diseases, such as CFS, FMD and SVD, are of great importance. However, the ReTi-PCR tests may also have their limitations as some semen samples were scored as negative whereas virus isolation became positive, although only after one blind passage. A more extensive validation study needs to be performed to determine the real percentage of false negative samples by ReTi-PCR, and whether these ReTi-PCR tests have their limitations.

Due to its high sensitivity, these rapid ReTi-PCR tests can be used for the detection of viruses in the early stage of infection or disease outbreak. It might also offer new opportunities for the screening of probably also other materials, besides semen, for the presence of viruses. It is appreciated that the PRV, CSFV, and the SVDV ReTi-PCR tests still have to prove their value in outbreak situations, and that the PRRSV ReTi-PCR has to prove its value for export and in the delivery of PRRSV-negative semen to PRRSV-free herds. To date, only the FMDV ReTi-PCR has proven its value in an outbreak situation. Their study showed that for the validation of other samples, such as plasma and tissues samples, only the DNA/RNA isolation procedure for that particular sample had to be optimized, whereas the conditions for amplification and detection remained unchanged (Moonen et al., 2003).

In conclusion, the high specificities and sensitivities of the ReTi-PCR tests, in combination with a strong reduction of time for detecting amplified products, make it possible to improve the early and rapid detection of five economically important viruses; PRV, CSFV, FMDV, SVDV and PRRSV, and to reduce the risk of spreading these viruses by artificial insemination. Although, the presented ReTi-PCR tests have not been accepted yet by the OIE as reference tests, these ReTi-PCR tests have several advantages above the routinely used virus isolation techniques, and therefore these PCR tests can be used as valuable supplementary assays in the early and rapid detection of mentioned viruses in semen and for surveillance purposes.

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References

Colijn, E.O., Bloemraad, M., Wensvoort, G., 1997. An improved ELISA for the detection of serum antibodies directed against classical swine fever virus. Vet. Microbiol. 59, 15–25.

de Smit, A.J., Bouma, A., Terpstra, C., van Oirschot, J.T., 1999. Transmission of classical swine fever virus by artificial insemination. Vet. Microbiol. 67 (4), 239–249.

Guerin, C., Allietta, M., Guerin, B., Thibier, M., 1995. Detection of pseudorabies virus in semen of boars by the polymerase chain reaction. Vet. Res. 26, 140–144.

Hamblin, C., Armstrong, R.M., Hedger, R.S., 1984. A rapid enzyme-linked immunosorbent assay for the detection of foot-and-mouth disease virus in epithelial tissues. Vet. Microbiol. 9 (5), 435–443.

de Leeuw, P.W., Tiessink, J.W.A., Frenkel, S., 1979. Vaccination of pigs with formaldehyde-inactivated aluminium hydroxide foot-and-mouth disease vaccines, potentiated with diethylaminoethyldextran (DEAE-D). Zbl. Vet. Med. Reihe B 26, 85–97.

LePotier, M.-F., Blanquefort, P., Morvan, E., Albina, E., 1997. Results of a control programme for the porcine reproductive and respiratory syndrome virus in the French “Pays de la Loire” region. Vet. Microbiol. 55, 355–360.

McVicar, J.W., Eisner, R.J., Johnson, L.A., Pursel, V.G., 1977. Foot-and-mouth disease and swine vesicular disease viruses in boar semen. Proc. Annu. Meeting US Anim. Health Assoc. 81, 221–230.

Moonen, P., Boonstra, J., Hakze-van der Honing, R., Boonstra-Leendertse, C., Jacobs, L., Dekker, A., 2003. Validation of a LightCycler-based reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus. J. Virol. Meth. 113, 35–41.

Roeder, P.L., Le Blanc Smith, P.M., 1987. The detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. Res. Vet. Sci. 43, 225–232.

Shin, J., Torrison, J., Choi, C.S., Gonzalez, S.M., Crabo, B.G., Molitor, T.W., 1997. Monitoring of porcine reproductive and respiratory syndrome virus infection in boars. Vet. Microbiol. 55, 337–346.

Thacker, B.J., Larse, R.E., Joo, H.S., Leman, A.D., 1984. Swine diseases transmissible with artificial insemination. J. Am. Vet. Med. Assoc. 185, 511–516.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J.M., Moonen, P.L.J.M., Zetstra, T., de Boer, E.A., Tibben, H.J., de Jong, M.F., van't Veld, P., Groenland, G.J.R., van Gennep, J.A., Voets, M.T.h, Verheijden, J.H.M., Braamskamp, J., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Quart. 13, 121–130.

Yaeger, M.J., Prieve, T., Collins, J., Christopher-Hennings, J., Nelson, E., Benfield, D., 1993. Evidence for the transmission of porcine reproductive and respiratory syndrome (PRRS) virus in boar semen. Swine Health Prod. 1, 7–9.