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A new generic real-time reverse transcription polymerase chain reaction assay for vesiviruses; vesiviruses were not detected in human samples

Sanela Svraka[a,*], Erwin Duizer[a], Herman Egberink[b], Jojanneke Dekkers[a], Harry Vennema[a], Marion Koopmans[a,c]

[a] *Laboratory for Infectious Diseases and Screening, Center for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, The Netherlands*
[b] *Faculty of Veterinary Medicine, University Utrecht, Utrecht, The Netherlands*
[c] *Erasmus Medical Center, Rotterdam, The Netherlands*

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A B S T R A C T

Different viruses belonging to the genus *Vesivirus* infect a broad range of animals, and cause gastroenteritis, vesicular skin lesions, hemorrhagic disease, respiratory diseases and other conditions. A recent report on *Vesivirus* viremia, as detected by PCR, in samples from patients with hepatitis of unknown etiology in the USA suggested a zoonotic potential for vesiviruses. These results have not been confirmed by another laboratory. In order to do so, a generic PCR assay on the RNA polymerase region was developed, and validated with RNA from 69 different *Vesivirus* species. Except SMSV serotype-8, all species tested were detected, including the ones that were suggested to be involved in zoonotic transmission in the USA (SMSV serotype-5).

The generic *Vesivirus* assay was used on RNA extracted from serum samples from patients with hepatitis, stool samples from patients with gastroenteritis, throat-swab specimens of patients with rash illnesses, throat-swab and nose-swabs of patients with acute respiratory diseases, and cell cultures with cytopathologic effect from enterovirus surveillance in which no pathogen was found. None were found positive. In this study a generic *Vesivirus* assay was developed and it was concluded that vesiviruses are an unlikely cause of common illnesses in humans in the Netherlands.

1. Introduction

The *Caliciviridae* are a family of positive-sense single-stranded RNA viruses comprising both human and animal pathogens (Clarke and Lambden, 1997). The four genera of the *Caliciviridae*, i.e. *Norovirus*, *Sapovirus*, *Lagovirus* and *Vesivirus*, have been classified on the basis of their genome organization and structure (Green et al., 2000). A fifth genus has been suggested following discovery of a bovine enteric calicivirus, which is genetically most similar to sapoviruses and lagoviruses, based on genomic organization of two open reading frames (ORF's) (Chen et al., 2006; Liu et al., 1999; Smiley et al., 2002; Sugieda et al., 1998). Noroviruses and sapoviruses are common causes of acute viral gastroenteritis in humans (Chen et al., 2006; Liu et al., 1999; Smiley et al., 2002; Sugieda et al., 1998), and are also found in swine, cattle, mice and possibly companion animals (cats and dogs) (Guo et al., 1999; Wang et al., 2005). Viruses in the genus *Lagovirus* infect lagomorphs (rabbits and brown hares) and the viruses in the genus *Vesivirus* are animal pathogens with a clearly broader host range (Radford et al., 2004).

Vesiviruses infect many different animal species, and cause a range of diseases (Smith et al., 1977). Infections with vesiviruses in humans have been reported to cause vesicular exanthema on hands after a laboratory incident, and on the face of a field biologist working with marine mammals. The strain recovered from the laboratory worker was related most closely to San Miguel sea lion virus (SMSV) serotype-5, which has several natural hosts (Chen et al., 2006; Smith et al., 1998a,b). The zoonotic potential of some *Vesivirus* strains has further been suggested by reports of SMSV antibodies in humans (Chen et al., 2006; Smith et al., 1998a,b). A high prevalence of anti-*Vesivirus* antibody together with *Vesivirus* viremia was observed among patients with hepatitis of unknown etiology in the USA (Smith et al., 2006). These findings indicate a potential for *Vesivirus* infection, and possibly illness, in humans. It was suggested that vesiviruses, with their broad host range, might

* Corresponding author at: National Institute for Public Health and the Environment, Centre for Infectious Disease Control, P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Tel.: +31 30 274 4073; fax: +31 30 274 4418.
E-mail address: Sanela.Svraka@RIVM.NL (S. Svraka).

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manifest themselves by causing hepatitis and possibly other diseases in humans.

In the Netherlands studies are conducted currently to decrease the diagnostic gap that exists for several illnesses. Samples from hepatitis patients that have been tested negative for hepatitis A, B, C viruses, Cytomegalovirus, and Epstein Barr virus were collected. From this cohort, over 90% remains unexplained after testing for hepatitis E (Herremans et al., 2007; Waar et al., 2005). Furthermore, in outbreaks of acute gastroenteritis, 12% remains unexplained after elaborate testing (Svraka et al., 2007). In samples of respiratory diseases, 50% remains unexplained (Heijnen et al., 1999), about 12% of samples of unexplained rash illnesses (van Binnendijk et al., 2003), and 5% of the isolates from enterovirus surveillance (van der Sanden et al., 2008) currently remain without any pathogen being detected.

To determine if vesiviruses have caused infections in humans in the Netherlands a generic RT-PCR for vesiviruses is described and the results of this assay on the samples described above are reported.

2. Materials and methods

2.1. Design of synthetic oligonucleotide primers

In order to develop a *Vesivirus* generic PCR a complete search of GenBank was done and 300 *Vesivirus* entries were identified. For the primer development 37 complete genome and partial polymerase gene sequences of vesiviruses were used. These sequences were imported as GenBank files into BioEdit Sequence Alignment Editor and aligned using the ClustalW multiple alignment tool, which is included in BioEdit software. The following *Vesivirus* genome sequences were used: Feline calicivirus (FCV) isolates (AY560118, AY560117, AY560116, AY560115, AY560114, AY560113 and AF098932) and strains (NC_001481, D31836, DQ424892, L40021, AF479590, M86379 and AF109465); Canine calicivirus (CaCV) (NC_004542, AB070225 and AF053720); Skunk calicivirus (U14668, U14670, U14672 and U18743); Vesicular exanthema of swine virus (VESV) strains (NC_002551, AF091736 and U76874); Rabbit vesivirus (AJ866991); various SMSV serotypes (U15301, U52094, U52093, U52089, U52087, U52088 and U52090); Walrus calicivirus (NC_004541); Reptile calicivirus (U52092); Mink calicivirus (AF338405); Cetacean calicivirus (U52091); Primate calicivirus Pan-1 (U52086). Subsequently, conserved genomic regions of different *Vesivirus* species were selected and primers were developed (Table 1).

2.2. Reverse transcription and PCR amplification

This generic assay is based on SybrGreen chemistry and uses a set of primers targeting the polymerase region. The concentrations and the conditions as described below were used for the most optimal primer set and were the same throughout. Annealing with the reverse primer was done using 1.5 μL (50 μM) of the reverse primer, 5 μL H_2O, and 2.5 μL of the extracted RNA for 2 min at 94 °C followed by cooling for at least 2 min. Six microliters of reverse transcription mix containing 1.5 μL of 10× PCR buffer 10 mM Tris–HCl (pH 8.3), 1.8 μL of $MgCl_2$ concentration of 25 mM, 1.5 μL of dNTPs (10 mM each), 0.5 μL of 10 U AMV-RT (Promega, Leiden, the Netherlands) were added and incubated for 60 min at 42 °C. Two microliters of the RT-mix were added to 18 μL of a LightCycler (LC) PCR-mix containing 2 μL of the DNA SybrGreen Mastermix Solution (Roche Diagnostics GmbH, Mannheim, Germany), 0.12 μL forward primer with concentration of 50 μM, 2.6 μL of $MgCl_2$ concentration of 25 mM, 0.16 μL TaqStart™ Antibody (5 U/μL) (Clontech Laboratories Inc.), and 13.12 μL H_2O.

Table 1
Primer sequences, and their position on the genomes. Expected product sizes of PCR products are indicated in base pairs (bp). ND, not done.

Primer	Nucleotide position	Sequence (5′–3′)	BR1	Vesi FOR	Vesican FOR	CaCV FOR
			4294–4311[a]	4466–4485[a]	4484–4501[a]	5095–5114[b]
			CTGGGGWTGYGAYGTTGG	GTTGACTAYTCNAARTGGGA	GACTCNACCCAACCNCCA	GTGGTGTGTCCTTCAAAAC
FCO YGDD	4778–4795[a]	TACGGCGATGATGGTGTC	501 bp	329 bp	311 bp	ND
VVrev	4778–4795[a]	TACGGRGATGATGGTGTC	501 bp	329 bp	311 bp	ND
YGDD	4778–4795[a]	TATGGTGATGATGAGATT	501 bp	329 bp	311 bp	ND
Vesi REV	4778–4800[a]	TACGGCCACGACGGNGTNTACAT	506 bp	334 bp	316 bp	ND
SAN1 REV	4781–4800[a]	GGYGACGACGGTGTCTACAT	506 bp	ND	ND	ND
SAN2 REV	4778–4795[a]	TACGGCCACGACGGTGTC	501 bp	ND	ND	
CaCV REV	5298–5317[b]	TTTGTACTTTCTGTTATGCC	1023 bp	851 bp	833 bp	

[a] NC_001481: Feline calicivirus, complete genome.
[b] NC_004542: Canine calicivirus, complete genome.

The PCR amplifications were performed on a LightCycler apparatus (Roche Diagnostics GmbH, Mannheim, Germany). Samples were denatured for 1 min at 95 °C, subjected to 40 amplification cycles with 0.1 s denaturing at 95 °C, 55 °C annealing for 5 s, and 72 °C elongation for 20 s with fluorescence acquisition in single mode. The first-derivative melting curve analysis was performed by heating the mixture to 95 °C for 0.1 s and then cooling to 55 °C for 5 s and heating back to 95 °C at increments of 0.5 °C. Detection was performed at 530 nm. Identification of PCR products of vesiviruses was done using the first-derivative melting curve analysis. Additionally, the PCR products were run on a 2% agarose-gel in order to define product size, and visualized using SybrSafe solution according to manufacturer's instructions.

2.3. Evaluation of the generic Vesivirus assay

2.3.1. Detection limit of the assay

Feline calicivirus strain F9 (from the collection of Faculty of Veterinary Medicine, Virology Department, Utrecht University) and Canine calicivirus strain no. 48 (kindly provided by Dr. M. Mochizuki, Laboratory of Clinical Microbiology, Tsukuba Central Laboratories, Kyoritsu Seiyaku Corporation, Japan) were chosen to evaluate the detection limit of the real-time PCR. Tenfold dilutions from 10^0 to 10^{-6} were made from both virus suspensions. Each dilution was subjected to reverse transcription, PCR amplification, and the first-derivative melting curve analysis in order to determine the detection limit. Additionally, PCR products of the dilution series were analyzed on a 2% agarose-gel.

2.3.2. Sensitivity and specificity of the assay

Sensitivity of the assay was evaluated against a diverse range of *Vesivirus* strains (Table 2). This includes strains with various origins of FCVs, e.g. 20 FCV isolates from across the UK (kindly provided by Dr. K. Coyne, University of Liverpool Veterinary Teaching Hospital, United Kingdom), 10 field isolates from Italy, and FCV 2280 (kindly provided by Dr. V. Martella, University of Bari, Bari, Italy), 5 FCV field isolates from across the Netherlands (Faculty of Veterinary Medicine, Virology Department, Utrecht University), 3 strains of FCV F9 (University of Utrecht, the Netherlands, Dr. S. Reid, and Dr. V. Martella), 1 strain of CaCV no. 48 (kindly provided by Dr. M. Mochizuki, Laboratory of Clinical Microbiology, Tsukuba Central Laboratories, Kyoritsu Seiyaku Corporation, Japan). Other *Vesivirus* isolates (kindly provided by Dr. S. Reid; Institute for Animal Health, Surrey, United Kingdom) were SMSVs serotypes 1–13 and 11 VESV strains (A48, C52, D53, E54, G55, I55, J56, B1, B51, F55, H54 and K54), and other VESV strains such as primate (isolated from a gorilla), Cetacean (dolphin) and Skunk CV, and Bovine Tillamook virus (BCV Bos-1) strain (Reid et al., 2007).

Specificity was evaluated using clinical samples containing different NoV genogroups I (GI.1, GI.2 WR, GI.2 SOV, GI.3, GI.4, GI.5, GI.6, GI.7 and GI.10), II (GII.1, GII.2, GII.3, GII.4, GII.6, GII.7 and GII.10) and IV, 4 clinical samples of SaV (genotypes GI.1, GI.2, GI.3 and GII.1), clinical isolates from astrovirus types 1–8, adenovirus types 40 and 41, 34 clinical serum samples of HEV, and 15 samples from HAV infected patients, clinical samples of Influenza viruses A and B, rhinoviruses, corona viruses OC43, 229^E, and NL63, RSV A and B, *human metapneumovirus* (hMPV), *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, clinical isolates of 10 *human parvoviruses B19*, 14 rubella viruses, 5 measles viruses, 3 mumps viruses, and 5 enteroviruses (Human enterovirus 71, coxsackieviruses B3 and A9, echoviruses 9 and 30). *Rotavirus* isolates (WA, DS1, K8, NCDV, 69M, SA11, ST3, B223), and strains G10P[11], G9P6, G9P[23]) were used, and Aichi virus strain A846/88 (Yamashita et al., 2000).

2.3.3. Spiking of fecal samples with CaCV and FCV isolates

Fecal samples, in which no viruses were detected, were spiked with CaCV strain no. 48 and FCV F9 strain. Five hundred microliters of the stool homogenates were spiked with 500 μL of 10-fold dilutions of CaCV no. 48 strain (1.6×10^7 TCID50/mL) and FCV F9 strain (1.6×10^6 TCID50/mL). Tenfold dilutions were added to the stool homogenates prior to centrifugation and RNA extraction was performed on 210 μL of the supernatant, with MagNAPure LC Total Nucleic Acid Isolation kit using the Total NA External Lysis protocol and eluted in a volume of 50 μL according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany). Five microliters of the extracted RNA were subjected to amplification. Melting curve analysis of PCR products was performed. Subsequently, the PCR products were visualized on agarose-gel.

2.3.4. Sequencing

PCR products were purified from gel using the QIAgen PCR purification kit according to the manufacturer's instructions and confirmed by sequencing using a fluorescence-labeled dideoxynucloetide technology from Applied Biosystems (Applied Biosystems, Foster City, USA). Sequence reactions were analyzed on an ABI 3700 automated sequencer. The sequences obtained were assembled using Seqman and Editseq software (DNAStar, Konstanz, Germany).

2.4. Sample panels

In order to determine if vesiviruses have caused infections in humans in the Netherlands, the following patient samples were collected systematically and tested.

2.4.1. Samples from patients with acute clinical hepatitis of unknown etiology

A panel of 412 serum samples was used, collected from patients with acute hepatitis and sent to the RIVM in 2005 and 2006. The sera were serologically negative for hepatitis A, B, C, and E viruses, Cytomegalovirus, Epstein Barr virus and the available volumes exceeded 500 μL (Herremans et al., 2007; Waar et al., 2005). The serum samples had been stored at −20 °C prior to testing. RNA was extracted from these samples with MagNAPure LC Total Nucleic Acid Isolation kit using the Total NA External Lysis protocol and eluted in a volume of 50 μL according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany).

2.4.2. Samples from gastroenteritis outbreaks with unexplained etiology

In order to narrow the diagnostic gap in the viral gastroenteritis surveillance all unexplained outbreaks collected from 1994 through 2006 (Svraka et al., 2007) were tested. In total, 605 fecal samples from 153 unexplained outbreaks of gastroenteritis were tested for the presence of vesiviruses.

2.4.3. Samples from patients with unexplained rash illnesses

The RNA isolated from throat-swabs of 131 patients with unexplained rash illnesses was used. These samples had previously been tested using PCR for Human parvovirus B19, rubella virus, and measles virus (van Binnendijk et al., 2003) and were negative using these assays. The RNA samples had been stored at −70 °C prior to testing for vesiviruses. Throat-swab specimens from patients with rash illnesses were collected in viral transport medium. Cells from the swabs were sedimented (10 min of centrifugation at $350 \times g$), and 20% of the cells were reconstituted in 200 μL of PBS, from this RNA was extracted using the High Pure Viral Nucleic Acid RNA isolation kit (Roche Diagnostics) according to manufacturer's recommendations, and modified by adding poly[A] to the lysis

Table 2
Vesivirus strains used in this study.

Virus	Strain/isolate	Year of original isolation	Country or region(s) of origin
VESV	A_{48}	1948	USA
VESV	B1–34	1934	USA
VESV	B_{51}	1951	USA
VESV	C_{52}	1952	USA
VESV	D_{53}	1953	USA
VESV	E_{54}	1954	USA
VESV	F_{55}	1955	USA
VESV	H_{54}	1954	USA
VESV	G_{55}	1955	USA
VESV	I_{55}	1956	USA
VESV	J_{56}	1954	USA
VESV	K_{54}	1972	USA
SMSV	SMSV-1	1972	USA
SMSV	SMSV-2	1972	USA
SMSV	SMSV-3	NK	USA
SMSV	SMSV-4	1973	USA
SMSV	SMSV-5	NK	USA
SMSV	SMSV-6	1975	USA
SMSV	SMSV-7	1976	USA
SMSV	SMSV-8	1975	USA
SMSV	SMSV-9	1975	USA
SMSV	SMSV-10	1977	USA
SMSV	SMSV-11	1977	USA
SMSV	SMSV-12	1977	USA
SMSV	SMSV-13	1984	USA
Primate CV	Primate CV	1978	USA
Cetacean CV	Cetacean CV	NK	USA
Skunk CV	Skunk CV	NK	USA
Bovine CV (Bos-1)	Bovine CV (Tillamook)	1981	USA
CaCV strain no. 48	CaCV	NK	JP
FCV F9	FCV	NK	NL
FCV F9	FCV	1984	NK
FCV F9	FCV	2000	IT
FCV 2280	FCV	2000	IT
FCV (10 isolates)	FCV	2000–2005	IT
FCV (20 isolates)	FCV	2005–2006	UK
FCV (5 isolates)	FCV	NK	NL

VESV, Vesicular exanthema swine viruses; SMSV, San Miguel Sea Lion virus; CV, Calicivirus; FCV, Feline Calici Virus; CaCV, Canine Calici virus; NK, not known; USA, United States of America; JP, Japan; NL, the Netherlands; IT, Italy; UK, United Kingdom.

buffer (0.04 mg/mL) to improve the recovery of specific RNA (van Binnendijk et al., 2003).

2.4.4. Samples from patients with unexplained respiratory disease

In total, 286 RNA samples extracted from combined throat-swab and nose-swab samples from patients with acute respiratory diseases of unknown etiology from 2006 were used. These samples previously tested negative for Influenza viruses A and B, rhinoviruses, enteroviruses, coronaviruses, adenoviruses, respiratory syncytial viruses (RSV) A and B, *human metapneumovirus*, *C. pneumoniae*, and *M. pneumoniae*. These clinical samples were spiked with a known amount of Equine arteritis virus (EAV). This internal control was co-extracted and co-amplified in the reaction (Scheltinga et al., 2005). A 10^{-4} dilution of EAV was used for spiking of patient material. Inhibition was not detected. The RNA samples of unexplained respiratory diseases had been stored at −70 °C prior to testing. The combined nose-swabs and throat-swabs (200 μL) from patients with respiratory illnesses were added to 300 μL lysis buffer. The RNA of all these samples was extracted with MagNAPure LC Total Nucleic Acid Isolation kit using the Total NA External Lysis protocol and eluted in a volume of 50 μL according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany).

2.4.5. Stool culture isolates of unknown etiology from enterovirus surveillance

Enterovirus surveillance in the Netherlands involves screening of stool samples from children with systemic viral infection, varying from meningitis to gastrointestinal disorders (van der Sanden et al., 2008). One of the objectives of this surveillance is to exclude poliovirus. These samples were cultured previously on tertiary monkey kidney (tMK) cell lines and tested negative for enteroviruses, parechoviruses, and adenoviruses by PCR. The culture isolates had been stored at −70 °C prior to RNA extraction. The RNA of 29 cell cultures collected between 2000 and 2006 was extracted with MagNAPure LC Total Nucleic Acid Isolation kit using the Total NA External Lysis protocol and eluted in a volume of 50 μL according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany).

3. Results

3.1. Design of synthetic oligonucleotide primers

A global alignment of 37 *Vesivirus* genome sequences was used for development of *Vesivirus* generic primers. In total, four forward and seven reverse primers were developed (Table 1) on the polymerase region. These primers were used in different combinations in order to select the most optimal primer pair. This was done using serial 10-fold dilutions of CaCV no. 48 and FCV F9. The combination of the primers BR-1 5′-CTGGGGWTGYGAYGTTGG-3′ and VVrev 5′-GACACCATCATCRCCGTA-3′ generated a 501 base pairs (bp) PCR product and was found to be the most optimal, when assessing at the melting temperatures and bands on an agarose-gel of the dilu-

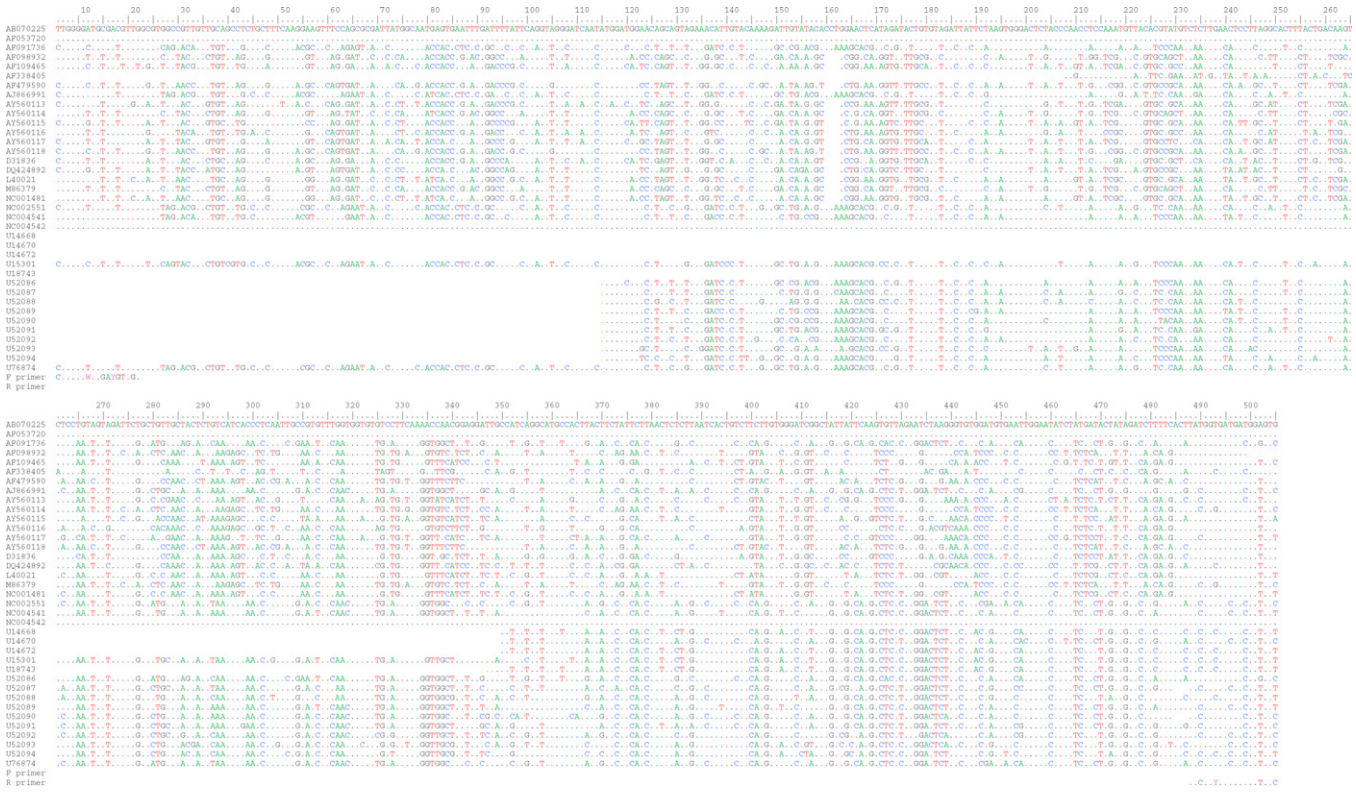

Fig. 1. Alignment of the 37 *Vesivirus* genome sequences with accession numbers used for primer development.

tion series, for the detection of the two strains tested (Figs. 1 and 2 and Table 1).

3.2. Evaluation of the generic Vesivirus assay

3.2.1. Detection limit of the assay

The detection limit of the generic assay was determined using serial 10-fold dilutions of CaCV no. 48 and FCV F9 stocks with respective titers of 1.6×10^7 and 1.6×10^6 TCID50/mL. A PCR positive signal was obtained at 10^{-4} dilution for FCV F9, with melting temperatures (°C) of 89.04 for undiluted FCV F9, 88.89 for 10^{-1}, 88.72 for 10^{-2}, 88.39 for 10^{-3}, and 88.54 for 10^{-4} FCV F9. For CaCV no. 48 a signal at 10^{-4} dilution was observed when the first-derivative melting curve analysis was performed. This isolate had melting curve temperatures (°C) of 86.01 for 10^0 CaCV no. 48, 86.09 for 10^{-1}, 86.18 for 10^{-2}, 86.43 for 10^{-3}, and 86.14 for 10^{-4} CaCV no. 48. Detection limits were determined at 1.6×10^3 TCID50/mL for CaCV no. 48 and 1.6×10^2 TCID50/mL for FCV F9. The dilution series were visualized on 2% agarose-gel in order to compare the results with the melting curve analysis. A PCR positive signal on agarose-gel was observed at 10^{-4} dilution for both isolates.

3.2.2. Sensitivity and specificity of the assay

The sensitivity of the assay was tested using different *Vesivirus* species, as described in Section 2.3.2. All of the isolates generated a PCR product and the melting temperatures were determined. All *Vesivirus* isolates were also visualized on an agarose-gel, and all

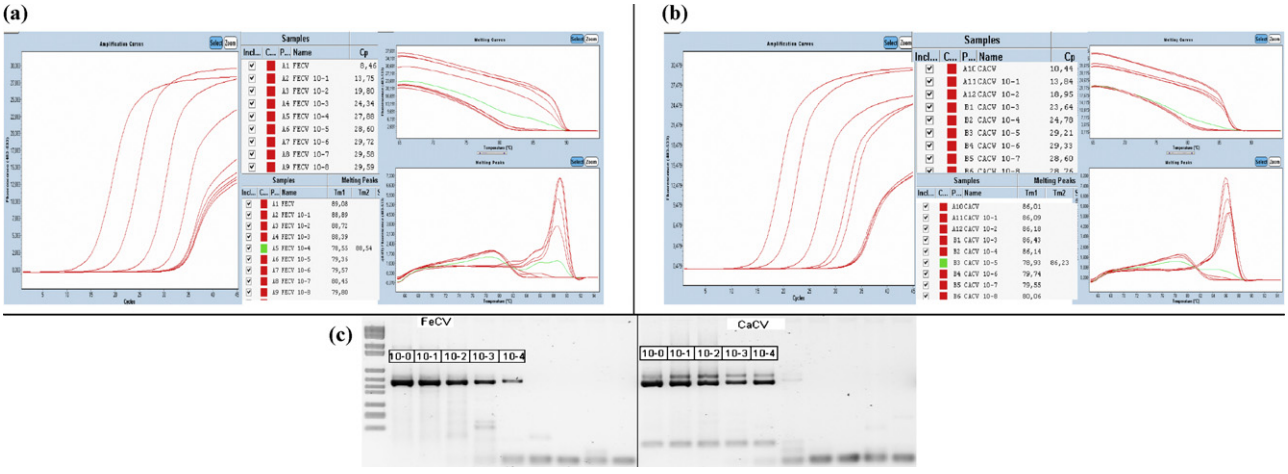

Fig. 2. Standard amplification curves, melting curves, melting peaks and gel electrophoresis (c) picture for 10-fold serial dilutions of Feline calicivirus (a), Canine calicivirus (b).

isolates generated a PCR product of the expected size, except SMSV serotype-8. The PCR products were sequenced to confirm specificity of the methods. All SMSV, VESV, CaCV, and FCV isolates were identified correctly.

The specificity of this assay was tested using a panel of viruses as described in Section 2. This assay yielded products with several viruses, however the melting temperatures of PCR products of noroviruses, adenoviruses, astroviruses, Aichi virus, HEVs, HAVs, enteroviruses, Influenza virus A and B, rhinoviruses, coronaviruses, adenoviruses, RSV A and B, hMPV, *C. pneumoniae*, and *M. pneumoniae* did not match the melting temperatures of vesiviruses. These products were also analyzed on an agarose-gel and no PCR product was visible. The amplicons/products of two clinical *Sapovirus* samples and a *Rotavirus* 69M positive sample had a melting temperature of 87.94 and 86.37 °C approached that of the FCV and CaCV products, respectively. On agarose-gel these three samples yielded three visible PCR products, which differed in size from the *Vesivirus* amplicon. The products were sequenced and confirmed as one *Sapovirus*, one bacterial sequence with a highest homology of 88% with *Pectinatus frisingensis* and a *Rotavirus* product. The sequence of the product of the *Rotavirus* 69M containing sample confirmed the presence of *Rotavirus* RNA when the sequence obtained was subjected to BLAST. Furthermore, viruses belonging to 12 different norovirus genogroups developed a PCR product on agarose-gel. The melting temperature of these viruses did not match those of vesiviruses, however the samples were sequenced for confirmation and matched to noroviruses. The specificity calculated (after melting curve and gel electrophoresis analysis) for the generic *Vesivirus* assay for detection of vesiviruses was 90%.

3.2.3. Spiking of fecal samples with CaCV and FCV isolates

RNA loss in extraction and possible inhibition by fecal components of the RT-PCR were measured in fecal samples spiked with 10-fold dilutions of CaCV no. 48 and FCV F9 isolates. CaCV and FCV dilutions were detected down to 0.8×10^3 TCID50/mL.

3.3. Sample panels

To determine if vesiviruses have caused infections in humans in the Netherlands, samples collected from patients with five different clinical syndromes for which common pathogens had been ruled out, were tested ($n = 1493$). None of the samples from unexplained etiologies yielded a specific product in the *Vesivirus* generic assay. None of the products formed were compatible with *Vesivirus* characteristics, based on melting curve analysis or expected product size of 501 bp by agarose-gel analysis. However, 244 clinical samples did generate a non-specific PCR product around 300 bp, as described below. PCR of 99 clinical samples from patients with gastroenteritis outbreaks of unexplained etiology generated a non-specific PCR product around 300 bp. Sequencing of these bands did not yield *Vesivirus* sequences when subjected to BLAST, but sapoviruses ($n = 1$), bacteroides spp. ($n = 19$) and the other products did not yield a sequence. The *Sapovirus* positive sample had two bands on gel, of which the upper band was approximately 500 bp. This sample was confirmed as *Sapovirus* using a *Sapovirus*-specific assay (Svraka et al., 2007). From the samples of patients with unexplained rash illnesses 16 generated a product of 300 bp. When sequenced, these products matched *Staphylococcus aureus*. One hundred and thirty-six samples (46%) from patients with unexplained respiratory disease generated non-specific products of 300 bp, of these samples 40 were sequenced and all of them matched *S. aureus*. Three stool culture isolates of unknown etiology from enterovirus surveillance generated a PCR product of 300 bp, when visualized on agarose-gel. The sequences of these products yielded a mammalian orthoreovirus 1.

4. Discussion

In this study a sensitive broad range real-time reverse transcriptase *Vesivirus* PCR assay was developed. The primer set of BR-1 and VV-rev targets is a highly conserved motif in the RNA polymerase region.

The sensitivity of the assay was evaluated using 69 various *Vesivirus* strains. It successfully detected all available *Vesivirus* strains, except SMSV serotype-8. It was reported previously that SMSV serotype-8 may be different antigenically and antigenetically from other marine calicivirus serotypes and this type was found to be negative in other PCR assays. In contrast with previously published *Vesivirus* assays, SMSV serotype-12 was detected by our assay (Reid et al., 1999, 2007; Seal et al., 1995).

The specificity of the primers was tested using a wide variety of viruses. None of the samples generated a product of 501 bp using the *Vesivirus* assay. Several non-specific products of 300 bp were obtained for sapoviruses. However, 244 non-specific products of approximately 300 bp were detected in clinical samples, what necessitated in sequencing of 10.6% (158 of 1493) of clinical samples, in order to exclude vesiviruses. SybrGreen was used because development of a generic assay was aimed, which would be able to detect all (or almost all) *Vesivirus* isolates. It was not possible to design a generic probe, which would be able to detect all vesiviruses. Development of different probes for different strains of *Vesivirus* genus could be an option for this and will be considered.

Competition in clinical samples between *Vesivirus* RNA and large amounts of viral or bacterial nucleic acids from other pathogens was assessed by spiking of the fecal samples with cultured CaCV and FCV strains. Although no noticeable competition was noticed, large amounts of other nucleic acid could interfere with the detection of vesiviruses, if those are present in much lower amounts. However, presence of other caliciviruses, like NoVs and SaVs, was excluded before testing with the *Vesivirus* generic assay and therefore little competition is expected.

Inhibition is not expected since no inhibition was detected in respiratory clinical samples that were spiked with EAV as internal control. Furthermore, EAV was included in 533 fecal samples to measure the inhibition. These samples were tested for common gastroenteritis viral pathogens, in none of these samples inhibition was measured (Svraka et al., manuscript in preparation. Syndromic detection of acute viral gastroenteritis by use of random primers and internally controlled multiplex real-time PCR assays). Vesiviruses are known as animal pathogens, and infect a broad range of species causing different clinical syndromes. In addition, cross-species infections have been documented for several vesiviruses (Smith et al., 1998a). Recently, Smith and co-workers detected *Vesivirus* RNA by PCR in 9.8% (11 of 112) of serum samples from a group of blood donors in the USA, suggesting that such infections are widespread. Ten of the RNA positive serum samples originated from blood donors with elevated blood liver alanine aminotransferase levels. Smith et al. (2006) also found a high seroprevalence of anti-*Vesivirus* antibodies in patients with acute hepatitis of unknown etiology (5 of 26), and the highest seroprevalence was detected in patients with acute hepatitis associated with blood transfusion and dialysis (7 of 15). Furthermore, VESV and SMSV have been described in swine, sea lions, and other animals in the USA (O'Hara et al., 1998; Sawyer, 1976; Smith and Akers, 1976; Smith and Latham, 1978; Smith et al., 1977). This may imply that these vesiviruses are endemic on the Northern American continent. In contrast, no reports on any vesivirus infection in either animals or humans in Europe have ever been published. In view of these findings, it was decided to assess the possibility of vesiviruses as causes of illness in the Netherlands. Therefore, unexplained hepatitis serum samples, stool samples of unexplained etiologies from patients with gastroenteritis, throat-swab speci-

mens of patients with rash illnesses, throat-swab and nose-swabs of patients with acute respiratory diseases, and unexplained cultures from enterovirus surveillance were tested. No evidence of any *Vesivirus* involvement was found in these samples.

Since the study described in this paper does not include serological testing, but is limited to detection of viral RNA in patient specimens, it cannot be concluded with certainty that *Vesivirus* infections do not play a role in the Netherlands. However, since we have tested a larger number of acute hepatitis patients with PCR than Smith and co-workers ($n = 412$ versus 112), with a *Vesivirus* assay with a detection limit of 1.6×10^3 TCID50/mL or better, detection of a *Vesivirus* species as causative pathogen in patients with acute hepatitis would have been very likely.

In summary, a rapid and sensitive real-time reverse transcriptase PCR assay for the broad detection of vesiviruses was developed successfully. This generic assay was validated using RNA from 69 *Vesivirus* strains and was shown to detect all strains of the *Vesivirus* genus, except SMSV serotype-8. *Vesivirus* RNA was not found in any of the clinical samples tested. Therefore, it can be concluded that vesiviruses are an unlikely cause of acute hepatitis and gastroenteritis, rash illnesses, respiratory and intestinal diseases in humans in the Netherlands.

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