Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
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

Article history:
Received 24 June 2008
Received in revised form 2 December 2008
Accepted 9 December 2008
Available online 21 January 2009

Abstract

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.

© 2008 Elsevier B.V. All rights reserved.

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 calcivirus, 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...
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, Cytoplasmolovirus, 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 H2O, 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 Lo f1 0 × PCR buffer 10 mM Tris–HCl (pH 8.3), 1.8 µL of MgCl2 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 MgCl2 concentration of 25 mM, 0.16 µL TaqStart™ Antibody (5 U/µL) (Clontech Laboratories Inc.), and 13.12 µL H2O.

### Table 1

| Primer, sequence, and their position on the genome. Expected product sizes of PCR products are indicated in base pairs (bp). ND, not done. |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Primer            | Nucleotide position | Sequence (5′–3′) | Expected product size (bp) | 
|                  |                   |                  | Vesican FOR             | CaCV FOR             | CaCV REV             | Vesi REV             | SAN1 REV             | SAN2 REV             |
| BR1              | 4294–4311          | CTGGGGWTGYGAYGTTGG | 501 bp | 329 bp | 311 bp | ND | ND | ND |
| FOR              | 4466–4485          | GTTGACTAYTCNAARTGGGA | 501 bp | 329 bp | 311 bp | ND | ND | ND |
| FCO             | 4778–4795          | TACGGGGATGATGGTGTC | 501 bp | 329 bp | 311 bp | ND | ND | ND |
| YGDD            | 4778–4795          | TATGGTGATGATGAGATT | 501 bp | 329 bp | 311 bp | ND | ND | ND |
| Vesi FOR         | 4778–4800          | TACGGCGACGACGGNGTNTACAT | 506 bp | 334 bp | 316 bp | ND | ND | ND |
| SAN1 REV         | 4781–4800          | GGYGACGACGGTGTCTACAT | 506 bp | ND | ND | ND | ND | ND |
| SAN2 REV         | 4778–4795          | TACGGCGACGACGGTGTC | 501 bp | ND | ND | ND | ND | ND |
| CaCV REV         | 5298–5317          | TTTGTACTTTCTGTTATGCC | 1023 bp | 851 bp | 833 bp | ND | ND | ND |
| 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 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 10^9 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, GI2, WR, GI2 SOV, GI3, GI4, GI5, GI6, GI7 and GI10, II (GI1, GI2, GI3, GI4, GI6, GI7 and GI10) and IV, 4 clinical samples of SaV (genotypes GI1, GI2, GI3 and GI1), 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, 229E, and NL63, RSV A and B, human metapneumovirus (hMPV), Chlamydia pneumoniae, Mycoplasma pneumoniae, clinical isolates of 10 human paroviruses 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 dideoxynucleotide 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 MagNApur 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 parovirus 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 × 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           | A48            | 1948                       | USA                            |
| VESV           | B1–34          | 1934                       | USA                            |
| VESV           | B51            | 1951                       | USA                            |
| VESV           | C52            | 1952                       | USA                            |
| VESV           | D53            | 1953                       | USA                            |
| VESV           | E54            | 1954                       | USA                            |
| VESV           | F55            | 1955                       | USA                            |
| VESV           | H54            | 1954                       | USA                            |
| VESV           | G55            | 1955                       | USA                            |
| VESV           | I55            | 1956                       | USA                            |
| VESV           | J56            | 1954                       | USA                            |
| VESV           | K54            | 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            | 1984                       | NK                             |
| FCV F9         | FCV            | 2000                       | IT                             |
| 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).

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.

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 $1.6 \times 10^7$ and $1.6 \times 10^6$ TCID50/mL. A PCR positive signal was obtained at $10^{-4}$ dilution for FCV F9, with melting temperatures ($^\circ$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 ($^\circ$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 $1.6 \times 10^3$ TCID50/mL for CaCV no. 48 and $1.6 \times 10^2$ 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 isolates had melting temperatures ($^\circ$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 $1.6 \times 10^3$ TCID50/mL for CaCV no. 48 and $1.6 \times 10^2$ 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.

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 x 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.

Acknowledgments

The authors thank Dr. Scott Reid, Dr. Karen Coyne, Dr. Vito Martella, and Dr. Masami Mochizuki for supplying the Vesivirus strains and Dr. Rob van Binnendijk, Dr. Adam Meijer, Dr. Berry Wilbrink, Dr. Harrie van Avoort for supplying the RNA from clinical samples. This work was supported financially by the European Commission, DG Research Quality of Life Program, 6th Framework (EVENT, SP22-CT-2004-502571).

References

Chen, R., Neill, J.D., Estes, M.K., Prasad, B.V., 2006. X-ray structure of a native calicivirus: structural insights into antigenic diversity and host specificity. Proc. Natl. Acad. Sci. USA 103, 8048–8053.
Clarke, I.N., Lambden, P.R., 1997. Viral zoonoses and food of animal origin: caliciviruses and human disease. Arch. Virol. Suppl. 13, 141–152.
Green, K.Y., Ando, T., Balayan, M.S., Berke, T., Clarke, I.N., Estes, M.K., Matson, D.O., Nakata, S., Neill, J.D., Studdert, M.J., Thiel, H.J., 2000. Taxonomy of the caliciviruses. J. Infect. Dis. 181 (Suppl. 2), S322–S330.
Guo, M., Chang, K.O., Hardy, M.E., Zhang, Q., Parwani, A.V., Saif, L.J., 1999. Molecular characterization of a porcine enteric calicivirus genetically related to Sapporo-like human caliciviruses. J. Virol. 73, 9625–9631.
Heijnen, M.L., Dorigo-Zetsma, J.W., Bartelds, A.J., Wilbrink, B., Sprenger, M.J., 1999. Surveillance of respiratory pathogens and influenza-like illnesses in general practices—The Netherlands, winter 1997–98. Euro. Surveill. 4, 81–84.
Herremans, M., Vennema, H., Bakker, J., van der Veen, B., Duizer, E., Benne, C.A., Waar, K., Hendriks, B., Schneeberger, P., Blauw, G., Kooiman, M., Koopmans, M.P., 2007. Swine-like hepatitis E viruses are a cause of unexplained hepatitis in the Netherlands. J. Viral. Hepat. 14, 140–146.
Liu, B.L., Lambden, P.R., Gunther, H., Otto, P., Elschner, M., Clarke, I.N., 1999. Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses. J. Virol. 73, 819–825.
O’Hara, T.M., House, C., House, J.A., Suydam, R.S., George, J.C., 1998. Viral serologic survey of bowhead whales in Alaska. J. Wildl. Dis. 34, 39–46.
Radford, A.D., Gaskell, R.M., Hart, C.A., 2004. Human norovirus infection and the lessons from animal caliciviruses. Curr. Opin. Infect. Dis. 17, 471–478.
Reid, S.M., Ansell, D.M., Ferris, N.P., Hutchings, G.H., Knowles, N.J., Smith, A.W., 1999. Development of a reverse transcription polymerase chain reaction procedure for the detection of marine caliciviruses with potential application for nucleotide sequencing. J. Virol. Methods 82, 99–107.
Reid, S.M., King, D.P., Shaw, A.E., Knowles, N.J., Hutchings, G.H., Cooper, E.J., Smith, A.W., Ferris, N.P., 2007. Development of a real-time reverse transcription polymerase chain reaction assay for detection of marine caliciviruses (genus Vesivirus). J. Virol. Methods 140, 166–173.
Sawyer, J.C., 1976. Vesicular exanthema of swine and San Miguel sea lion virus. J. Am. Vet. Med. Assoc. 169, 707–709.
Scheltinna, S.A., Templeton, K.E., Beersma, M.F., Claas, E.C., 2005. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. J. Clin. Virol. 33, 306–311.
Seal, B.S., House, J.A., Wherestone, C.A., Neill, J.D., 1995. Analysis of the serologic relationship among San Miguel sea lion virus and vesicular exanthema of swine virus isolates. Application of the western blot assay for detection of antibodies in swine sera to these virus types. J. Vet. Diagn. Invest. 7, 190–195.
Smiley, J.R., Chang, K.O., Hayes, J., Vinje, J., Saif, L.J., 2002. Characterization of an enteropathogenic bovine calicivirus representing a potentially new calicivirus genus. J. Virol. 76, 10085–10098.
Smith, A.W., Akers, T.G., 1976. Vesicular exanthema of swine. J. Am. Vet. Med. Assoc. 169, 700–703.
Smith, A.W., Berry, E.S., Skilling, D.E., Barlough, J.E., Poet, S.E., Berke, T., Mead, J., Matson, D.O., 1998a. In vitro isolation and characterization of a calicivirus causing a vesicular disease of the hands and feet. Clin. Infect. Dis. 26, 434–439.
Smith, A.W., Iversen, P.L., Skilling, D.E., Stein, D.A., Bok, K., Matson, D.O., 2006. Vesivirus viremia and seroprevalence in humans. J. Med. Virol. 78, 693–701.
Smith, A.W., Latham, A.B., 1978. Prevalence of vesicular exanthema of swine antibodies among feral mammals associated with the southern California coastal zones. Am. J. Vet. Res. 39, 291–296.
Smith, A.W., Prato, C.M., Skilling, D.E., 1977. Characterization of two new serotypes of San Miguel sea lion virus. Intervirology 8, 30–36.
Smith, A.W., Skilling, D.E., Cherry, N., Mead, J.H., Matson, D.O., 1998b. Calicivirus emergence from ocean reservoirs: zoonotic and interspecies movements. Emerg. Infect. Dis. 4, 13–20.
Sugieda, M., Nagaoka, H., Kakishima, Y., Osibita, T., Nakamura, S., Nakajima, S., 1998. Detection of Norwalk-like virus genes in the caecum contents of pigs. Arch. Virol. 143, 1215–1221.
Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veen, B., Dorrestein, B., Koopmans, M., 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in the Netherlands from 1994 through 2005. J. Clin. Microbiol. 45, 1389–1394.
van Binnendijk, R.S., van den Hof, S., van den Kerkhof, H., Kohl, R.H., Woonink, F., Berbers, G.A., Conyn-van Spaadonck, M.A., Kimman, T.G., 2003. Evaluation of serological and virological tests in the diagnosis of clinical and subclinical measles virus infections during an outbreak of measles in The Netherlands. J. Infect. Dis. 188, 988–993.
van der Sanden, S., de Bruin, E., Vennema, H., Swanink, C., Koopmans, M., van der Avoort, H., 2008. Prevalence of human parechovirus in the Netherlands in 2000 to 2007. J. Clin. Microbiol. 46, 2884–2889.
Waar, K., Herremans, M.M., Vennema, H., Koopmans, M.P., Benne, C.A., 2005. Hepatitis E is a cause of unexplained hepatitis in The Netherlands. J. Clin. Virol. 33, 145–149.
Wang, Q.H., Han, M.G., Cheetham, S., Souza, M., Funk, J.A., Saif, L.J., 2005. Porcine noroviruses related to human noroviruses. Emerg. Infect. Dis. 11, 1874–1881.
Yamashita, T., Sugiyama, M., Tsuzuki, H., Sakae, K., Suzuki, Y., Miyazaki, Y., 2000. Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the Picornavirus family associated with gastroenteritis in humans. J. Clin. Microbiol. 38, 2955–2961.