Identification of a calicivirus isolate of unknown origin

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Chinese hamster ovary (CHO) cells manifesting striking cytopathogenic changes in culture were investigated to determine the causative agent. Electron microscopic analyses revealed viral particles of about 40 nm in diameter, displaying typical calicivirus morphology. To date, this virus, designated isolate 2117, exclusively replicates in CHO cells, achieving only moderate titres. After cloning, the coding region of 7928 nucleotides, the 3′ non-coding region and the poly(A) tail were sequenced. The genome consists of three open reading frames (ORFs), with the first and second ORF having the same reading frame. The overall genomic organization as well as the nucleotide sequence of isolate 2117 is most similar to that of a recently described canine calicivirus, but also shows significant similarity to the sequences of mink calicivirus and other caliciviruses within the genus Vesivirus. In Western blots, using antibodies against the viral protease, a stable, unprocessed 3CD protein of 68 kDa was identified in homogenates of 2117-infected CHO cells. Furthermore, antibodies raised against ORF 3 reacted with the respective protein in 2117-virions, demonstrating that this predicted 9 kDa protein is a minor structural component of the virion. In addition, an RT-PCR assay was established to detect 2117 viral RNA in biological products such as foetal bovine serum, which will aid the discovery of the origin and host of the virus.

INTRODUCTION

The isolation of caliciviruses from diverse species has been reported by many investigators. These viruses have been classified into four genera: Vesivirus, e.g. vesicular exanthema of swine virus (VESV), feline calicivirus (FCV) and San Miguel sea lion virus (SMSV); Lagovirus, e.g. rabbit haemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV); the human genera Norovirus and Sappovirus (Capucci et al., 1996; Green et al., 1994, 2000; Ohlinger et al., 1990; Schaffer et al., 1980, Smith et al., 1979; Studdert, 1978; Wirblich et al., 1994). In addition, caliciviruses have been isolated from mink, dog, cattle and non-human primates (Dastjerdi et al., 1999, 2000; Guo et al., 2001; Liu et al., 1999a; Mochizuki et al., 1993; Smith et al., 1985).

In humans, caliciviruses represent the main cause of non-bacterial gastroenteritis (Clarke & Lambden, 1997b), whereas animal caliciviruses can cause vesicular lesions in swine and sea lions, respiratory illness and conjunctivitis in cats, and severe haemorrhagic liver diseases in rabbits and hares (Neill et al., 1998; Smith et al., 1973). While VESV, SMSV or FCV can easily be propagated in cell culture and cause a cytopathogenic effect within a few hours (Studdert, 1978), there is no cell culture system available for lagoviruses or human caliciviruses (Konig et al., 1998; White et al., 1996). Caliciviruses consist of non-enveloped virions 32–40 nm in diameter. Electron microscopy has revealed that several, but not all species, display typical cup-shaped surface depressions, the characteristic ‘calyx’ morphology, from which the family derives its name (calix, Latin for cup) (Granizow et al., 1996; Prasad et al., 1994, 1999; Schaffer et al., 1980).

Caliciviruses have a polyadenylated plus-stranded RNA genome of 7·3–8·3 kb with a viral VPg protein of 10–15 kDa covalently attached to its 5′ end and a coterminating subgenomic RNA of 2·2–2·4 kb. The non-structural proteins are encoded in the 5′ end, the major capsid protein VP1 and the minor, basic structural protein VP2 in the 3′ end of the genome (Clarke & Lambden, 1997a, 2000).

Based on sequence homologies to picornavirus proteins, the putative function of several non-structural proteins of
caliciviruses has been proposed and, by analogy, they have been designated 2C-like helicase, 3C-like protease and 3D-like polymerase (Neill, 1990; Sosnovtseva et al., 1999; Wei et al., 2001; Wirblich et al., 1996). These proteins are expressed as a polyprotein and subsequently processed by the viral protease into mature, non-structural proteins (Konig et al., 1998; Liu et al., 1996, 1999a; Meyers et al., 2000; Sosnovtsev et al., 1998, 1999; Wirblich et al., 1996).

The aim of the present study was to characterize a virus, designated isolate 2117, which caused cytopathogenic effects in Chinese hamster ovary (CHO) cells. Electron microscopic analyses of infected cell suspensions revealed viral particles with typical calicivirus morphology. The viral genome sequence and potential ORFs within it were identified and the presence of the 2117 protease and VP2 was shown. Furthermore, a diagnostic RT-PCR assay for rapid and sensitive viral RNA detection was developed.

**METHODS**

**Cell culture and virus.** Chinese hamster ovary (CHO-K1) and Crandell-Reese feline kidney (CRFK) cells were obtained from ATCC and propagated in Dulbecco’s modified Eagle’s medium (DMEM), with non-essential amino acids (complete medium; Life Technologies), supplemented with 5–10 % foetal bovine serum (FBS). Infected cells were then incubated for 20 min with 25 % Formalin/5 % BSA for 45 min. Grids were washed with PBS/0.5 % BSA and negatively stained with 1 % uranyl acetate. Rabbit preimmune serum served as a negative control.

**Preparation of 2117-virions and electron microscopy.** 2117-virions were isolated from the medium of infected CHO cells 24–72 h post-infection (p.i.) and centrifuged at 10 min, 800 g and viral particles in the supernatant concentrated by either crude ultracentrifugation (1.5 h, 104 000 g, 4 °C) or centrifugation through a cushion of 17% sucrose in TEN-buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5; 1 mM EDTA) (3 h, 104 000 g, 4 °C). 2117-virions were then resuspended in 0.05–0.1 ml medium or TEN-buffer, respectively, for 12–24 h at 4 °C. The virus was then purified from the pellet by layering on a sucrose gradient (60–30–20% sucrose in TEN-buffer) and centrifugation for 5 h at 126 000 g at 4 °C. The virus-containing band was visualized and collected under scattered light.

For electron microscopy, an aliquot of the virus sample was adsorbed to grids and negatively stained with 1% uranyl acetate.

For immune electron microscopy the virus on the grid was incubated with anti-2117-virion polyclonal rabbit serum, diluted 1:1000 in PBS with 0.5% BSA for 45 min. Grids were washed with PBS/0.5% BSA, then incubated with the secondary antibody (goat-anti rabbit IgG) (Biocell) and conjugated to colloidal gold particles (10 nm). After 45 min grids were washed in PBS/0.5% BSA and H2O2 and negatively stained with 1 % uranyl acetate. Rabbit preimmune serum served as a control.

**RNA preparation.** QIAamp RNA isolation kits (Qiagen) were used to extract RNA from medium as well as 2117-infected cells, according to the manufacturer’s instructions. Otherwise, prior to the isolation of RNA, viral VPr was digested with 0.1 mg proteinase K (Roche) in a sample of either 10^6 cells or culture supernatant collected from 10^7 cells in 15 mM Tris, pH 6.8, 0.2-0.5% SDS at 56 °C. After 1 h, 1 ml TRIzol (Life Technologies, originally described by Chomczynski & Sacchi, 1987) was added and the protocol followed according to the manufacturer’s instructions. For the isolation of polyadenylated RNA, PolyAtract mRNA isolation systems III/IV from Promega were used.

**cDNA cloning.** For analysis of the 2117 nucleotide sequence, polyadenylated RNA was isolated from 5 × 10^6 virus-infected CHO cells by using the PK/TRIzol RNA preparation method and the PolyAtract mRNA isolation system. The polyadenylated RNA was used for the synthesis of a cDNA library by means of the ZAP Express cDNA Synthesis-/ZAP Express cDNA Gigapack II/III Gold Cloning kit (Stratagene). The phage library was screened by plaque hybridization with a 2117-specific PCR-fragment, labelled with [32P]dCTP, random primers and Klenow enzyme (Rediprime DNA labelling system, Amersham Life Science) (Feinberg & Vogelstein, 1983). Subcloning of pBK-CMV plasmids was carried out by in vivo excision using a helper-phae, as recommended by the supplier.

**RT-PCR.** For the RT reaction, RNA and 1.25–12 µM reverse primer (rev) were mixed in a volume of 10 µl, heated for 10 min at 65 °C and cooled on ice. 4 µl 5 × RT-buffer (Roche), 2 µl dNTP-mix (2.5 mM each dNTP; Invitrogen) and 12.5 U avian myeloblastosis virus (AMV) reverse transcriptase (Roche) were then added and incubated for 1 h at 42 °C in a total volume of 20 µl, followed by 10 min at 85 °C. For the PCR reaction, the RT sample was mixed with 10 µl 10 × PCR-buffer (Roche), 0.25 µM of each primer (reverse and forward), 2 µl dNTP-mix (2.5 mM each dNTP; Invitrogen) and 0.5 U Taq DNA polymerase (Roche) or 1–7.5 U Expand enzyme mix (Roche) to a total volume of 100 µl. Standard PCR amplification protocol: 3 min 95 °C, 25–35 cycles [(30 s 95 °C, (30 s Tm–3 °C), (60 s/1000 bp 72 °C)] and 8 min 72°C. All PCR amplifications were carried out in a Trio-Thermocycler (Biometra).

PCR samples were analysed by agarose gel electrophoresis and either extracted or purified using the QIAquick gel extraction or PCR purification kit (Qiagen), respectively. PCR products were either inserted into the pCR2.1-TOPO-TA cloning vector (Invitrogen), according to the instructions of the supplier, or cloned into the EcoRV site of pBS SK (Stratagene), according to standard procedures.

To demonstrate the presence of caliciviral RNA in 2117-infected cells, total RNA from 2117-infected CHO cells was prepared and RT-PCR carried out by using the PCR Optimizer kit (Invitrogen), according to the manufacturer’s instructions. The primers, derived from the polymerase-coding region (1arev: 5’-TAMACRCCATCATC-RCCATAMGT; 1afor: 5’-TGGGGCGTGTGAYGTYGGYGGYCCG), amplified a fragment that was then gel-purified and inserted into pBS SK. As a control, RNA from FCV-infected CRFK-cells was analysed in parallel.

To confirm the nucleotide sequence of the middle part of the genome and to obtain the 5′ end of the coding sequence of the
2117-virus, the following primers were selected: for the midgenome-PCR (midrev: 5'-TCACAAGAATGTCTCAGG; 2117-position 4106-4123; midfor: 5'-TGGCAGACACTCTCTTGG; 2117-position 1542-1559) and for the 5' end of the genome as well as for a nested PCR (endrevpcr: 5'-GATTCCGGATTAGTGCT; 2117-position 1668-1685; endrevnest: 5'-TGATGGCAGACATTGG; 2117-position 1148-1165 and endfor: 5'-GTGTTTGTAGGCT). The resulting PCR-fragments were cloned using the pCR2.1-TOPO-TA cloning-kit (middle fragment) or the pBS SK- vector (5' end fragment), respectively.

Evaluation of PCR sensitivity for detection of 2117 RNA in FBS. To test the sensitivity of a 2117-specific nested PCR (PCR primer: 2117polrev, 5'-GCATCACATCAGAAGTTGTG; 2117-position 5219-5239 and 2117polfor, 5'-GCATCCCAGAGTGCCACTAA; 2117-position 4777-4795; and nested-primer: 2117polrevnest, 5'-AATTCGGTGTGCTCTTCAC; 2117-position 5086-5106 and 2117polfornest, 5'-ACCATCTGTATGATGCTGC; 2117-position 4912-4930), aliquots of the 2117-virus stock, ranging from 1 to 500 TCID₅₀ were mixed into 0.5 or 60 ml FBS, respectively. The 0.5 ml samples were used directly for the isolation of RNA (QIAamp viral RNA Mini kit), while the 60 ml samples were first concentrated to 5 ml and then RNA isolation performed. One-third of the RNA was taken for RT-PCR, and 1 μl of the PCR product for the subsequent nested PCR.

Nucleotide sequencing and sequence analyses. All 2117-specific DNA sequences were analysed on both strands in opposite directions, with an ABI PRISM 377 DNA sequencer (Perkin Elmer) using Big Dye Terminator Cycle Sequencing (Perkin Elmer). Computer analysis of the sequence data was performed using Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison. The 2117 virus sequence has been deposited in GenBank with accession no. AY343325.

CLUSTALX was used for the phylogram, excluding positions with gaps, corrected for multiple substitutions and run with 1000 bootstrap trials. The compared region comprises the amino acid sequence of ORF 2 of 2117 as well as the canine calicivirus CaCV (NP_777374), SMSG-4 (P36285), VESV (NP_066256), the primate calicivirus PCV Pan-1 (AAC61759), FCV (AAL93148), EBHSV (NP_068828), RHDV (AA69514), the porcine enteric calicivirus PECV (NP_051035) and a human calicivirus, isolate Manchester (CAA06262).

Bacterial protein expression, protein purification and immunization of rabbits. Parts of the putative 2117-protease (pro) coding region, as well as the gene encoding the minor structural protein (VP2), were amplified in RT-PCRs by primers with a restriction site (rev: HindIII; for: BamHI) at the 5' end (small) and 2117-specific sequences at the 3' region (capital letters) (prorev 5'-cagctaaatagctGTCACAAGGATTTCCTCCCG; 2117-position 3749-3766 and profor 5'-ctagcttctgctgATCCCTAAGACGGGTCTCC; 2117-position 3475-3492; VP2rev 5'-cagctaaatagctTTTGAATTTATGTGATT; 2117-position 7908-7925 and VP2for 5'-ctagctttctgctgATTTGAGATGGCT; 2117-position 7527-7544). The PCR products and the bacterial expression vector pQE9 (Qiagen) were double-digested with HindIII and BamHI, gel purified, ligated and transformed into E. coli XL-1 Blue (Stratagene) according to standard protocols.

For expression of the protease and VP2, overnight cultures of the transformed E. coli cells were diluted (1:4) and incubated at 37°C with shaking at 200 r.p.m. until the optical density at 600 nm was approximately 0.6. At this time IPTG (Sigma) was added to a final concentration of 1 mM. After 4 h, cells were harvested by centrifugation, the bacterial pellet resuspended in 4:1 PBS:sample buffer (0.25 M Tris/HCl, pH 6.8, 20 % β-mercaptoethanol, 20 % glycerol, 8 % SDS, 0.04 % bromophenol blue), shaken for 20 min at 200 r.p.m. and 65°C, and sonicated three times for 20 s.

For analysis and purification of proteins, the samples were subjected to SDS-PAGE (Laemmli, 1970). After electrophoresis on 12–5–17.5 % gels, proteins were visualized by staining for 20 min in 0.1 % Coomassie brilliant blue (in fixer) and destaining for 1–3 h in fixer (30 % ethanol, 10 % acetic acid).

For protein purification, the band of the expressed viral protein was excised and electroeluted overnight at 100 V in Tris/glycine buffer (25 mM Tris, 192 mM glycine) containing 0.025 % SDS, using an elution device (Bio-Trap, Schleicher & Schüll). The SDS-PAGE and electroeluation were repeated twice; the eluted protein was dialysed (dialysis tubes, Roth) for 48 h at 4°C in PBS and analysed by SDS-PAGE.

To obtain 2117-specific antibodies, 200 μg of the dialysed protein was mixed with PBS to a total volume of 250 μl. For the first immunization of rabbits, the sample was mixed with the same volume of complete Freund's adjuvant (Difco laboratories). For the first, second and third boost the sample was mixed with incomplete Freund's adjuvant (Difco laboratories) and emulsified. The injections were delivered subcutaneously in the back every 2 weeks. Blood was taken from the ear vein before the first immunization and 2 weeks after the second and third boost. The same immunization procedure was performed with rabbits immunized with 2117-virions purified from 2117-infected CHO cells.

Western blotting. To identify structural proteins, the medium of infected cultures only was centrifuged for 10 min at 800 g and concentrated by centrifugation through a 17 % sucrose cushion in TEN-buffer for 3 h at 104000 g and 4°C. The pellet was resuspended in medium for 12–24 h at 4°C and sample buffer added. For the non-structural proteins the cells were harvested and homogenized in PBS: sample buffer 4:1. The samples were shaken at 200 r.p.m. for 20 min at 65°C and sonicated three times for 20 s. The proteins were subjected to PAGE on 12–5–17.5 % SDS gels (Laemmli, 1970) and transferred onto Protran nitrocellulose transfer membrane (Schleicher & Schüll) in Tris/glycine buffer with 20 % methanol for 40–60 min at 25 V (Trans-blot semi-dry transfer cell; Bio-Rad). The nitrocellulose was incubated overnight at 4°C in TBS (150 mM NaCl, 10 mM Tris/HCl, pH 7.5), supplemented with 1 % BSA. The membrane was then shaken at room temperature in TBS-T (500 mM NaCl, 20 mM Tris/HCl, pH 7.5; 0.05 % Tween 20 for 2 h) with virus-specific antibodies (1:1000) and 1 h with peroxidase-conjugated IgG anti-rabbit antibody (Nordic Immunology). After incubation with the antibodies the membrane was washed three times for 10 min in TBS-T. The blot was developed for 1 min in a 1:1 ECLI/ECL2 mixture (Amersham) and exposed to X-ray film (BioMax; Kodak) for 0.5 to 7 min.

RESULTS

Propagation of 2117-virus in CHO cells

During the investigation of CHO cells for a cytopathogenic agent, the 2117-virus was identified by electron microscopy. The cytopathogenic effect and isolation of the virus were reproducible by incubating CHO cells with the original cell lysate. The titres of the virus stock were between 1 × 10⁵ and 1 × 10⁶ TCID ml⁻¹. At an m.o.i. of 0.02, about 20 % of the cells became rounded or detached from the cellular monolayer within approximately 24 h p.i. After 48 h, about 50 % of the cells were affected, and at 72 h p.i. most of the
cells were lysed. Propagation of the 2117-virus was only possible in CHO cells. Other cells, tested to support replication of 2117-virus, including Vero (monkey kidney), MDBK (bovine kidney), MDCK (canine kidney), CRFK (feline kidney), BHK (hamster kidney), PK15 (porcine kidney), and human HeLa and CaCo2 (colon carcinoma) cells, displayed no evidence of cytopathogenicity.

Electron microscopy of 2117-virions

Supernatant from 2117-infected CHO cells was concentrated through a sucrose cushion, purified by density-gradient centrifugation and analysed by electron microscopy. Viral particles were about 40 nm in diameter with icosahedral symmetry. These virions displayed a structured surface consisting of regularly arranged cup-shaped depressions (Fig. 1a). Size and morphology were consistent with classical caliciviruses. No virus particles were observed in a matching fraction of samples from uninfected CHO cells.

2117-virions were also visualized by immunoelectron microscopy, using 2117-specific antibodies and secondary antibodies conjugated to colloidal gold. The results demonstrated the reactivity and specificity of the 2117-antibody for VP1 in virions (Fig. 1b). Virions contained several bound gold particles, recognizable as black dots near or on the capsid, with only a few non-specific gold particles in the background. No specific binding was observed with preimmune serum.

Analysis of the coding sequence of the 2117-virus

In order to demonstrate the presence of calicivirus RNA in 2117-infected CHO cell cultures, RT-PCR was performed using a vesivirus-specific primer pair 1arev and 1afor, specific for the RNA polymerase. The sequence of the 508 bp 2117-fragment revealed nearly 80 % identity with the nucleotide sequence of the mink calicivirus MCV/13/1980/US. In a second step, a 2117-specific cDNA library was established and screened using the 508 bp 2117-specific PCR fragment. From two independent cDNA libraries several 2117 positive clones were isolated and sequenced. Since most of the viral sequences had a poly(A) tail and mapped to the 3’ part of published calicivirus genomes, the cDNA library was screened with a second probe specific for the 5’ region of the 2117 genome. Two clones covering the 5’ region of the 2117 genome were isolated.

To verify the results of this additional screening, RT-PCR spanning the region of the middle part of the genome was performed. Each of the sequences of four PCR-fragments, spanning 2581 nucleotides, from four independent RT-PCRs were determined.

The nucleotide sequence of the putative 5’ end of the 2117 genome was also obtained by RT-PCR and a following nested reaction. The comparison of 5’ ends of genomic and subgenomic RNAs of several caliciviruses, as well as the putative 5’ end of the 2117 subgenomic RNA, led to the selection of the endfor primer, used in the PCR and nested PCR. The primer contains an ATG sequence as a potential start codon of the first ORF of the 2117 genome. In the nested PCR, a DNA fragment of 1174 bp was amplified and 13 different fragments were analysed.

The entire coding nucleotide sequence of the 2117 genome has been assembled from the totality of all clones from the different reactions. From the adenine of the postulated start codon of ORF 1 up to the polyadenylated 3’ end, the 2117 genome is composed of 8091 nucleotides, excluding the poly(A) tail. Three ORFs were predicted from computer analysis of the 2117 nucleotide sequence and by comparing the results with the genomic organizations and ORFs of other caliciviruses (Clarke & Lambden, 1997a; Thiel & König, 1999). ORF 1 extends to nucleotide (nt) 5451, and encodes a putative polyprotein of 1816 amino acids (aa). ORF 2 ranges from nt 5455 to 7527 and ORF 3 from nt 7524 to 7928, and they encode proteins of 690 and 134 aa, respectively. The 3’-untranslated region consists of 163 nt.

With regard to genomic organization, ORFs 1 and 2 are in the first frame, with the ORF 1 stop codon (5449–5451) followed by 3 nucleotides and then the start codon of ORF 2 (5455–5457). ORF 3 is in the third frame, overlapping by 1 nt with ORF 2 (Fig. 2).

Within the 2117 sequence, conserved motifs typical for NTPase (GPAGCGKT, 1528–1551), VPg (KGKTK, 2992–3006), 3C-like protease (GDCGLP, 3718–3735) and 3D-like polymerase (KDEL at 4375–4386, GDD at 4918–4926) could be identified in ORF 1, and the motifs

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**Fig. 1.** (a) Ultrastructure of 2117-virions. The bar represents 100 nm. (b) Ultrastructure of immunogold-labelled 2117-virions. The bar represents 100 nm.
FRAES (5917–5931) and PPG (6277–6285) in ORF 2 (Fig. 2). However, compared to other caliciviruses, two remarkable differences were discovered in ORF 2. ORF 2 can be subdivided into six different regions, A–F, referring to amino acid sequences in VP1. In SMSV-1, VESV-A48, FCV-F9 and BCV-Tillamook, region D consists of exactly 15 conserved amino acids (Neill et al., 1998). In 2117 there is an insertion of 7 aa (KTIKSQV) in the conserved region D, which to date has only been described in the canine caliciviruses. With regard to this comparison, the 2117-virus seems to be more related to the canine calicivirus than to other vesiviruses. Also in SMSV, VESV, FCV and BCV, the hypervariable region E, probably the receptor binding site, contains a conserved sequence of 16 aa (Neill et al., 1998), which is not present in 2117 (data not shown). Comparisons of the nucleotide sequence of the 2117 genome with published sequences revealed significant similarity to the canine calicivirus CaCV (AF053720). Within the entire coding sequences of these two viruses, 72.5 % similarity was detected. Furthermore, the BLAST search showed similarities to the mink calicivirus MCV/13/1980/US, SMSV and other members of the genus Vesivirus. In addition, Fig. 3 illustrates the relationships between 2117 and other caliciviruses based on sequence comparisons in ORF 2 at the amino acid level.

**Unprocessed protease–polymerase protein in 2117-infected CHO cells**

For the investigation of viral non-structural proteins, 2117-infected CHO cells were analysed on Western blots with antibodies directed against the putative 2117 protease (anti-pro). Antibodies were raised against parts of the protease protein, mainly the region containing conserved amino acids motifs. Since total cell lysate and polyclonal serum were used for the protein gel and Western blot, respectively, multiple proteins were found to be immunoreactive (Fig. 4). However, these proteins were present in non-infected cells and were also seen with preimmune serum, indicating that they were non-specific. The only protein, detected in 2117-infected cells with anti-protease antibodies, has an approximate mass of 68 kDa. This size suggests that this molecule consists of the unprocessed, stable protease and polymerase protein.
VP2 as the minor structural protein in 2117-virions

To evaluate the location of VP2, 2117-virions were gradient-purified from the supernatant of 2117-infected CHO cells and analysed by Western blots with antibodies directed against ORF 3 (anti-bas). In these samples, the presence of intact viral particles was verified by electron microscopy. Uninfected CHO cells and preimmune serum were used as controls. A 9 kDa protein was detected only in purified virus preparations (Fig. 5). No specific signal was obtained with antisera against ORF 1 proteins (data not shown). This finding indicates that VP2 represents a minor structural protein of the 2117-virus.

Detection of 2117-RNA in FBS

Since the 2117-virus was isolated from CHO cells in culture, FBS, as a component of the culture medium, was used for investigations to detect 2117-RNA. For that purpose, a 2117 nested PCR was established with primer sequences specific for the conserved polymerase-coding region of the 2117-virus. To test the possibility of isolation of RNA from FBS and the sensitivity of the nested reaction, variable TCIDs were mixed in different volumes of FBS. RNA was isolated and RT-PCR and nested reactions were performed. After PCR, 500 TCID ml⁻¹ could be detected out of 60 ml and up to 100 out of 0.5 ml FBS. With nested PCR it was possible to detect at least one infectious particle in 60 ml FBS (Fig. 6).

DISCUSSION

The contamination of CHO cells with an as yet unknown calicivirus has been clarified by morphological analysis of the virion and molecular characterization of the viral RNA.

In addition, two viral proteins were identified and a diagnostic RT-PCR established for sensitive detection of viral RNA in biological material.

The calicivirus, designated 2117, was initially isolated from CHO cells showing apparent cytopathogenic effects. Electron microscopic analysis of the cell lysate revealed virions with typical calicivirus morphology. These kind of virions have been described for vesiviruses as well as lagoviruses and classical human caliciviruses (Prasad et al., 1994; Schaffer et al., 1980).

The analysis of the 2117 genome revealed considerable similarities to sequences of the canine and the mink calicivirus, as well as to SMSV and FCV strains. The relationship to the calicivirus family was also evident in the conservation of several amino acid motifs distributed throughout the genome, with the same linear arrangement and relative distances. For example, the non-structural proteins, for example NTPase, protease and polymerase motifs, are in the 5' end (ORF 1) and the motifs for the structural protein VP1 (ORF 2) in the 3' end. Additionally, through the amino acid motif FRAES in ORF 2, the predicted processing of the primary translation product to
the mature 2117 VP1 appears consistent, since in other
vesiviruses this motif has already been reported as the
potential (Matsuura et al., 2000; Neill, 1992; Neill et al.,
1998) or actual (Sosnovtsev et al., 1998) processing site of
the protein through the viral protease.

Regarding genomic organization, a rare feature was
observed in the 2117 genome. The separation of the first
and second ORF by a stop-codon resembles the organization
of vesiviruses and noroviruses. However, the location of the
coding sequences for the non-structural and the major
capsid protein within a single frame is more typical for the
lagoviruses and sappoviruses. This kind of arrangement has
so far been described only for the canine calicivirus (Roerink
et al., 1999).

The viral protease of 2117 has been identified as part of the
non-structural polyprotein. As known from other calici-
viruses, the non-structural proteins are expressed as a
polyprotein and cotranslationally processed to the indi-
vidual mature proteins by the viral protease. For the
Southampton virus, for example, initially a 113 kDa
C-terminal protein was observed in the rabbit reticulocyte
lysate system. Whereas in expression studies in E. coli the
protein was further processed to 22 and 16 kDa proteins,
in addition to the 19 kDa protease and 57 kDa polymerase
(Liu et al., 1996, 1999b). In RHDV, the translation of
viral RNA in rabbit reticulocyte lysates resulted only in a
protease–polymerase protein of 69 kDa (Wirblich et al.,
1996); however, the 15 kDa protease and 58 kDa poly-
merase could additionally be detected in cultures of RHDV-
infected primary rabbit liver cells (Konig et al., 1998) and
also after transient expression of RHDV cDNA in cell
culture (Meyers et al., 2000). In FCV, only a stable protease–
polymerase precursor protein of 78 kDa has been found so
far (Sosnovtsev et al., 2002; Sosnovtseva et al., 1999). Only
one immunoreactive protein of 68 kDa could be detected
in 2117-infected CHO cells, with antibodies against the
protease generated in this study. Since the C terminus of
the polymerase is defined by the stop codon of ORF 1 and
a predicted polyprotein of 68 kDa includes all known
amino acid motifs of both proteins, this protein is con-
cluded to be a stable protein of protease and polymerase.
Consequently, there is efficient processing of the precursor
protein at the N terminus of the protease in infected CHO
cells, but no detectable processing at the 3C–3D boundary.

Concerning structural proteins, the major capsid protein
VP1 and the minor, basic structural protein VP2 have both
been described as components of calicivirus particles (Glass
et al., 2000; Sosnovtsev & Green, 2000; Wirblich et al.,
1996). For VP2, proteins of 9 and 8.5 kDa have been identified
in RHDV and FCV, respectively, while a 35 kDa protein,
as well as multiple higher molecular mass proteins, was
characterized from stool samples of Norwalk virus-infected
volunteers (Glass et al., 2000; Sosnovtsev & Green, 2000;
Wirblich et al., 1996). In the present study, a protein with
a molecular mass of about 9 kDa was present in 2117-
virions. The function of this protein remains unclear. In

The origin of the virus, as well as the route of entry of the
virus or viral RNA into cell culture, is still unknown. Aside
from the cells, FBS as a culture supplement is the most likely
source of viral contamination. In order to detect the
presence of viral RNA in biological material, a 2117 specific
RT-PCR was developed. The PCR enabled the detection
of at least one infectious unit mixed into FBS, indicating
the sensitivity of the PCR and also the capability of iso-
lating RNA from FBS. This test will enable the screening
of cell culture material, especially FBS, for 2117 RNA.
Contamination of FBS with other viruses, especially bovine
diarrhoea virus, but also parainfluenza 3 virus, or
infectious bovine rhinotracheitis virus has already been
published (Erickson et al., 1991). In addition, transplacental
passage of caliciviruses has already been described for cats
and sea lions, where FCV and SMSV, respectively, have
been detected in aborted foetuses (Smith et al., 1973; van
Vuuren et al., 1999). Recently, Smith et al. (2002) published
the detection of a vesicular exanthema of swine-like calici-
virus in tissues from a naturally infected, spontaneously
aborted bovine foetus.

However, the spread of the 2117-virus in nature or its
association with any naturally occurring symptoms has not
yet been reported. Future studies will involve tests of several
FBS samples for the presence of 2117 RNA by RT-PCR and
infections of CHO cells with FBS under different conditions.
Further research on the origin of the 2117-virus will entail
screening of dogs and cattle for 2117-antibodies as well as
examinations for a potential link with diseases in different
species.

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REFERENCES

Detection and preliminary characterization of a new rabbit
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