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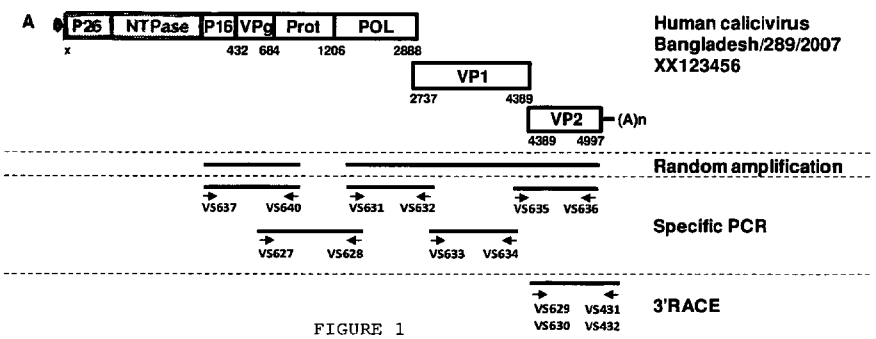


FIGURE 1

(57) Abstract: The present invention relates to a not previously known calicivirus being a causative agent for diarrhea. The present invention further relates to methods for the detection of the present calicivirus in a sample and to a kit of parts allowing the detection of the present calicivirus in a sample. Specifically, the present invention relates to caliciviruses characterised in the virus comprises a nucleic acid sequence encoding a VP1 capsid protein comprising an amino acid sequence as defined in SEQ ID No. 1, or encoding a VP1 capsid protein comprising an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 1, and in that said virus is a causative agent for diarrhea.

NEW CALICIVIRUS

Description

The present invention relates to a new calicivirus being a causative agent for diarrhea. The present invention further relates to (in vitro) methods for the detection of the present calicivirus in a sample and to a kit of parts allowing the detection of the present calicivirus in a sample.

Diarrhea is a common cause of morbidity and mortality and remains the second leading cause of death around the world for children under 5 years of age, especially in low and middle-income countries.

For otherwise healthy individuals suffering from diarrhea medical attention is not always necessary. However, weakened individuals suffering from diarrhea, such as individuals already suffering from other diseases and especially individuals with a compromised immune system, often require immediate medical attention or hospitalization.

Many causative agents of diarrhea have been identified including viruses, bacteria and parasites. Especially considering the divergent nature of diarrhea causing pathogens, establishing the nature of the pathogen is important for diagnosis and treatment. For example, if the pathogen is a virus, antibiotic treatment will inherently not be effective. Similar observations can be made for anti-parasite medicaments being not effective against viruses and bacteria. Especially with respect to viral diarrhea causing pathogens knowledge is limited.

In recent years, random amplification in combination with (next-generation) sequencing methods has

been employed in many types of samples such as human and animal blood, respiratory secretions, and fecal material, which resulted in the identification of many novel viruses. Despite these multiple viral metagenomic studies available, a large proportion of viral diarrhea cases, and especially human viral diarrhea cases, remains undiagnosed, i.e. the causative viral agent is not known.

Considering the potential morbidity and mortality of diarrhea, there is a need in the art to identify diarrhea causing pathogens, and especially diarrhea causing viruses, allowing (early) diagnosis, epidemic confinement and control, and/or appropriate treatment.

It is an object of the present invention, amongst other objects, to meet the above need in the art.

This object of the present invention, amongst other objects, is met by providing a new diarrhea causing calicivirus virus as defined in the appended claims.

Specifically, this object of the present invention, amongst other objects, is met, according to a first aspect, by providing a calicivirus wherein the virus comprises a nucleotide sequence encoding a VP1 capsid protein comprising an amino acid sequence as defined in SEQ ID No. 1, or encoding a VP1 capsid protein comprising an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 1, and wherein the present virus is a causative agent for diarrhea.

Caliciviruses are non-enveloped, positive-stranded RNA viruses with a ~6.4-8.4 kb non-segmented polyadenylated genome.

The family *Caliciviridae* consists of five recognized genera, Norovirus, Sapovirus, Lagovirus,

Vesivirus and Nebovirus, and two proposed genera, Recovirus and Valovirus.

Caliciviruses cause a wide spectrum of diseases in animals, among which pigs, reptiles, amphibians, cattle, cats and chickens and are an important etiological agent in viral gastroenteritis in humans.

The RNA genome of caliciviruses encodes a polyprotein precursor for non-structural proteins, and two structural capsid proteins, VP1 and VP2. The genomes of caliciviruses differ in genome organization and contain two or three open-reading frames (ORFs)

The present invention relates to nucleotide and amino acid sequences characterised by a percentage sequence identity. Sequence identity as used herein is defined as the number of identical consecutive aligned nucleotides, or amino acids, over the full length of the present sequences divided by the number of nucleotides, or amino acids, of the full length of the present sequences and multiplied by 100%. For example, a sequence with 80% identity to SEQ ID No. 1 comprises over the total length of 550 amino acids of SEQ ID No. 1 440 identical aligned consecutive amino acids, i.e., 440/550 * 100% = 80%.

According to a preferred embodiment of the present invention, the new calicivirus identified herein is further characterised by comprising a nucleotide sequence encoding a VP2 capsid protein comprising an amino acid sequence as defined in SEQ ID No. 2 or encoding a VP2 capsid protein comprising an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 2.

According to another preferred embodiment of the present invention, the new calicivirus identified herein is

further characterised by comprising a nucleotide sequence encoding a polyprotein comprising an amino acid sequence as defined in SEQ ID No. 3 or encoding a polyprotein comprising an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 3.

According to yet another preferred embodiment of the present invention, the new calicivirus identified herein is further characterised by a nucleotide sequence comprising SEQ ID No. 4 or a nucleotide sequence having at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 4

According to the present invention, the present calicivirus preferably is a human calicivirus more preferably belonging to the genus Recovirus.

Considering that the present viruses are a causative agent of diarrhea and the amino acid and nucleotide sequences disclosed herein allow identification of the present viruses using commonly known techniques for nucleotide (RNA or DNA) or protein detection, the present invention, according to a second aspect, relates to the use of one or more sequences selected from the group consisting of SEQ ID Nos. 1 to 4 for establishing the presence of a diarrhea causing calicivirus in a sample, preferably a stool sample, most preferably a human stool sample.

According to a preferred embodiment of this second aspect of the present invention, protein analysis comprises ELISA.

According to another preferred embodiment of this second aspect of the present invention, nucleotide analysis comprises PCR, rtPCR or NASBA.

Again considering that the present viruses are a causative agent of diarrhea and the amino acid and nucleotide sequences discloses herein allow identification of the present viruses, the present invention, according to a third aspect, relates to kits of parts for detecting the presence of a diarrhea causing calicivirus in a sample, preferably a stool sample, most preferably a human stool sample, the kits comprise:

means for detecting the presence of one or more sequences selected from the group consisting of SEQ ID Nos. 1 to 4 in said sample; and

instructions for use.

The present invention will be further detailed in the following example of preferred embodiments of the invention. In the example, reference is made to figures wherein:

Figure 1: shows a schematic outline of the strategies used for PCR amplification of human calicivirus Bangladesh/289/2007. (A) The upper panel shows a schematic representation of the human calicivirus Bangladesh/289/2007 genome. The boxes represent the ORFs encoding the calicivirus proteins. Indicated are the 5'-end with a cap (circle) and the poly(A)-tail (An). The boxes in gray extrapolated based on the genome composition of other caliciviruses. The lower panels show a schematic outline of the RT-PCR assays employed to amplify calicivirus Bangladesh/289/2007 sequences, using random amplification, degenerate PCR and 3'RACE PCR. The orientations and positions of the oligonucleotides on the calicivirus genome are shown. (B) This panel

shows the genome organization of caliciviruses in the genera Reco-, Vesi-, Valo-, Nebo-, Noro-, Sapo-, and Lagovirus for comparison with the new human calicivirus Bangladesh/289/2007. The numbers indicate the nucleotide positions according to the virus genome for which the Genbank accession number is indicated.

Figure 2: shows phylogenetic trees of the amino acid sequences of the partial polyprotein sequence (A), VP1 (B) and VP2 (C) capsid proteins of selected representative caliciviruses and the newly identified human recovirus Bangladesh/289/2007. Phylograms were generated using MEGA5, with the Neighbor-joining method with p-distance and 1,000 bootstrap replicates. Significant bootstrap values and Genbank accession numbers are shown.

Example

In an effort to identify unknown human viruses in the enteric tract, sequence-independent amplification was performed on partially purified viral nucleic acid from diarrhea samples from Bangladesh.

The samples included were 105 diarrheic stool specimens from patients enrolled in the 2% systematic routine surveillance system at the Clinical Research and Service Centre of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDRB) from 2007. These samples were pre-screened negative for adenovirus and rotavirus A, using Taqman EZ RT-PCR Core Reagents (Applied Biosystems) and rotavirus primers RVNSP3R and RVNSP3F and probe 5′-FAM-AGTTAAAAGCTAACACTGTCAAA- TAMRA-3′ and the

Taqman Universal Mastermix (Applied Biosystems) for the adenovirus primers according to the manufacturer's instructions.

Large scale molecular virus screening, based on host nucleic acid depletion, viral nucleic acid isolation, sequence-independent amplification and next-generation sequencing with a 454 GS Junior Instrument (Roche) was performed as described previously and by the manufacturer.

The pyrosequencing reads were trimmed for adaptor and primer sequences and more than 725,000 trimmed reads were assembled using de novo assembly in CLC Genomics Workbench 4.5.1 and analyzed according to BLASTn (contigs and singletons) and BLASTx searches (contigs).

Sequences were classified into eukaryotic viruses, phages, bacteria, and eukaryotes based on the taxonomic origin of the best-hit sequence using MEGAN 4.40. An E value of 0.001 was used as the cut-off value of significant virus hits. Most of the identified sequences were of eukaryotic or bacterial origin or did not have any significant hits to nucleotide or amino acid sequences in Genbank. The largest proportion of fecal virus-related sequences in human diarrhea samples from Bangladesh in 2007 was related to known bacteriophages and mammalian viruses.

A novel mammalian virus from the family Caliciviridae was identified in sample 289 by near full-length genome sequencing using random amplification in combination with next-generation sequencing, specific RT-PCRs, and 3'RACE PCR and was designated virus Bangladesh/289/2007. The genome organization of calicivirus Bangladesh/289/2007 is most closely related to that of viruses in the genus Norovirus, with ORF2 encoding VP1 overlapping with ORF1 (**Figure 1**).

The partial polyprotein precursor sequence and complete VP1 and VP2 capsid proteins were aligned to the corresponding sequences of selected representative caliciviruses. Divergence analysis using p-distance demonstrated that the human calicivirus Bangladesh/289/2007 was 43.2%, 57.8%, and 50.7% divergent on the amino acid level in the polyprotein, VP1, and VP2, respectively, from its most closest relative, Tulane virus, a recently characterized calicivirus from rhesus macaques in the proposed genus Recovirus (**Table 1**).

Table 1: *Percentage amino acid diversity of human calicivirus Bangladesh with representative caliciviruses of other genera.*

Calicivirus genus[a]	Strain	Accession no.	NS polyprotein[b]	VP1	VP2
Norovirus	Norwalk	M87661	72.7	74.9	80.0
	Southampton	L07418	74.1	85.0	81.3
Sapovirus	Manchester	X86560	80.2	82.9	85.3
	PEC Cowden	AF182760	83.1	81.8	86.7
Vesivirus	FCV CFI68	U13992	82.3	83.4	85.3
	SMSV 1	U15301	81.9	87.7	90.7
Lagovirus	RHDV FRG	M67473	82.6	84.0	88.0
	EBHSV GD	Z69620	83.2	84.0	93.3
Nebovirus	BEC NB	AY082891	81.0	84.0	90.7
	Newbury 1	DQ013304	80.6	84.0	89.3
Recovirus	Tulane	EU391643	43.2	57.8	50.7
Valovirus	AB104	FJ355930	65.3	61.5	81.3

[a] Valovirus and Recovirus are two tentative genera not yet accepted by the International Committee on Taxonomy of Viruses.

[b] All polyprotein sequences were aligned without the capsid protein encoding sequences.

Neighbor-joining phylogenetic trees were generated using the partial polyprotein and complete VP1 and VP2 proteins, which underlined the divergence analysis (**Figure 2**). Thus, it was concluded that human calicivirus Bangladesh/289/2007 belongs to the genus Recovirus.

To obtain insight in the prevalence of recoviruses in humans, a diagnostic real time human recovirus PCR was performed on 1614 diarrhea samples from patients enrolled in the 2% systematic routine surveillance system at the ICDDRB, Bangladesh from years 2007, 2008, and 2009. RT-PCR grade viral RNA was extracted from an aliquot of the samples using Magnapure LC total nucleic acid isolation kit (Roche), according to the manufacturer's instructions.

Human recoviruses were amplified by real time RT-PCR using primers VS665 (5'-CAAGGTGATTGTGAATGACCAACT-3), VS666 (5'-CGATTGGCCAGTTTACAGC-3') and probe VS664 (5' FAM-CACTGGATTGCCTCAAC-3' TAMRA), and the Taqman EZ RT-PCR Core Reagents (Applied Biosystems), using the cycling program 50°C for 2 min, 60°C for 30 min, 95°C for 5 min and 50 cycles of 95°C for 20 seconds and 59°C for 1 min.

Real time RT-PCR amplicons were recovirus-confirmed by sequence analysis. In addition to the sample positive for human calicivirus Bangladesh/289/2007, five other samples were positive for a human recovirus with high homology to human calicivirus Bangladesh/289/2007 (**Table 2**), indicating that human recovirus Bangladesh infects humans. The clinical data indicate that all six patients with a recovirus infection presented with 6 to >21 watery stools in the first 24h. Vomiting occurred in four patients and fever was present in two. The age of the patients ranged from 3 months to 50 years. Other clinical and demographic data are represented in Table 2.

Table 2: *Clinical data of patients positive for human Recovirus Bangladesh.*

Patient	Age	Sex	Collection	Diarrhea	Temperature	Duration[a]	No.stool[b]	Vomiting[c]	Abd. Pain[d]	Condition[e]	Pathogens[f]
201	23	M	2007	Watery	Fever	< 1 day	11-15	< 10	Yes	Lethargic	No
289[g]	25	M	2007	Watery	Normal	< 1 day	6-10	< 10	Yes	Lethargic	No
445	20	M	2007	Watery	Normal	1-3 days	11-15	0	Yes	Normal	No
507	50	F	2007	Watery	Normal	< 1 day	6-10	< 10	No	Drowsy	*Vibrio cholera*
809	0	M	2008	Watery	Normal	4-6 days	> 21	< 10	No	Lethargic	Rotavirus A
1084	1	M	2008	Watery	Fever	1-3 days	6-10	0	Yes	Normal	Adenovirus/*Salmonella*

[a] Disease duration in days.

[b] The number of passing stools in the previous 24 hours.

[c] Times of vomiting in the previous 24 hours.

[d] Abdominal pain.

[e] Overall condition of the pacient.

[f] The pathogens that were tested for are *Shigella*, *Aeromonas*, *Vibrio cholera*, *Campylobacter*, and *Salmonella* species. In addition, the samples were screened for *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium, Ascaris lumbricoides*, *Cyclospora cayetanensis*, and *Isospora belli*, adenoviruses and group A rotaviruses.

[g] The virus from patient 289 is the described human calicivirus Bangladesh/289/2007 in this study.

Three human recovirus-positive patients showed evidence for co-infecting pathogens that are known to cause diarrhea in humans, such as rotavirus A, adenovirus, *Vibrio cholera*, and/or *Salmonella* species, the other three did not, suggesting that human recovirus Bangladesh may be involved in causing diarrhea.

Conclusions

A large proportion of human diarrhea cases remains undiagnosed, despite multiple viral metagenomic studies in human stool aimed at identifying new etiological agents. This observation, in combination with the fact that it cannot be predicted when and where (re-)emerging viruses

will appear in the human population, urged to perform a metagenomic viral inventory in 105 diarrhea samples from Bangladesh, which led to the identification of a new calicivirus.

Although no species demarcation criteria have been defined for the family Caliciviridae by the International Committee on the Taxonomy of Viruses, the new virus was classified human calicivirus Bangladesh/289/2007 in the proposed genus Recovirus, based primarily on phylogenetic analyses.

The human calicivirus Bangladesh/289/2007 is considered a new type species of the genus Recovirus based on the facts that

(i) the genetic distance between Recovirus Tulane and Recovirus Bangladesh/289/2007 is larger than that between the two type species of the genus Lagovirus,

(ii) the genome organization of Recovirus Tulane and Bangladesh/289/2007 differs, and

(iii) Recovirus Tulane and Bangladesh/289/2007 were identified in different host species.

CLAIMS

1. Calicivirus characterised in that said virus comprises a nucleotide sequence encoding a VP1 capsid protein comprising an amino acid sequence as defined in SEQ ID No. 1, or encoding a VP1 capsid protein comprising an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 1, and in that said virus is a causative agent for diarrhea.

2. Calicivirus according to claim 1, wherein said virus further comprises a nucleotide sequence encoding a VP2 capsid protein comprising an amino acid sequence as defined in SEQ ID No. 2 or encoding a VP2 capsid protein comprising an amino acid sequence with at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 2.

3. Calicivirus according to claim 1 or claim 2, wherein said virus further comprises a nucleotide sequence encoding a polyprotein comprising an amino acid sequence as defined in SEQ ID No. 3 or encoding a polyprotein comprising an amino acid sequence having at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 3.

4. Calicivirus according to any of the claims 1 to 3, wherein said virus is further characterised by a nucleotide sequence comprising SEQ ID No. 4 or a nucleic acid sequence having at least 70%, preferably at least 80%, more preferably at least 95% and most preferably at least 99% sequence identity with SEQ ID No. 4

5. Calicivirus according to any of the claims 1 to 4, wherein said virus is a human calicivirus.

6. Calicivirus according to any of the claims 1 to 5, wherein said virus belongs to the genus Recovirus.

7. Use of one or more sequences selected from the group consisting of SEQ ID Nos. 1 to 4 for establishing the presence of a diarrhea causing calicivirus in a sample.

8. Use according to claim 7, wherein said sample is a stool sample.

9. Use according to claim 7 or claim 8, wherein said sample is a human sample.

10. Use according to any of the claims 7 to 9, wherein said establishing comprises protein analysis or RNA analysis.

11. Use according to claim 10, wherein said protein analysis comprises ELISA.

12. Use according to claim 10, wherein said RNA analysis comprises PCR, rtPCR or NASBA.

13. Use according to claim 10, wherein said DNA analysis comprises PCR or NASBA.

14. Kit of parts for detecting the presence of a diarrhea causing calicivirus in a sample, preferably a stool sample, most preferably a human stool sample comprising:

means for detecting the presence of one or more sequences selected from the group consisting of SEQ ID Nos. 1 to 4 in said sample; and

instructions for use.

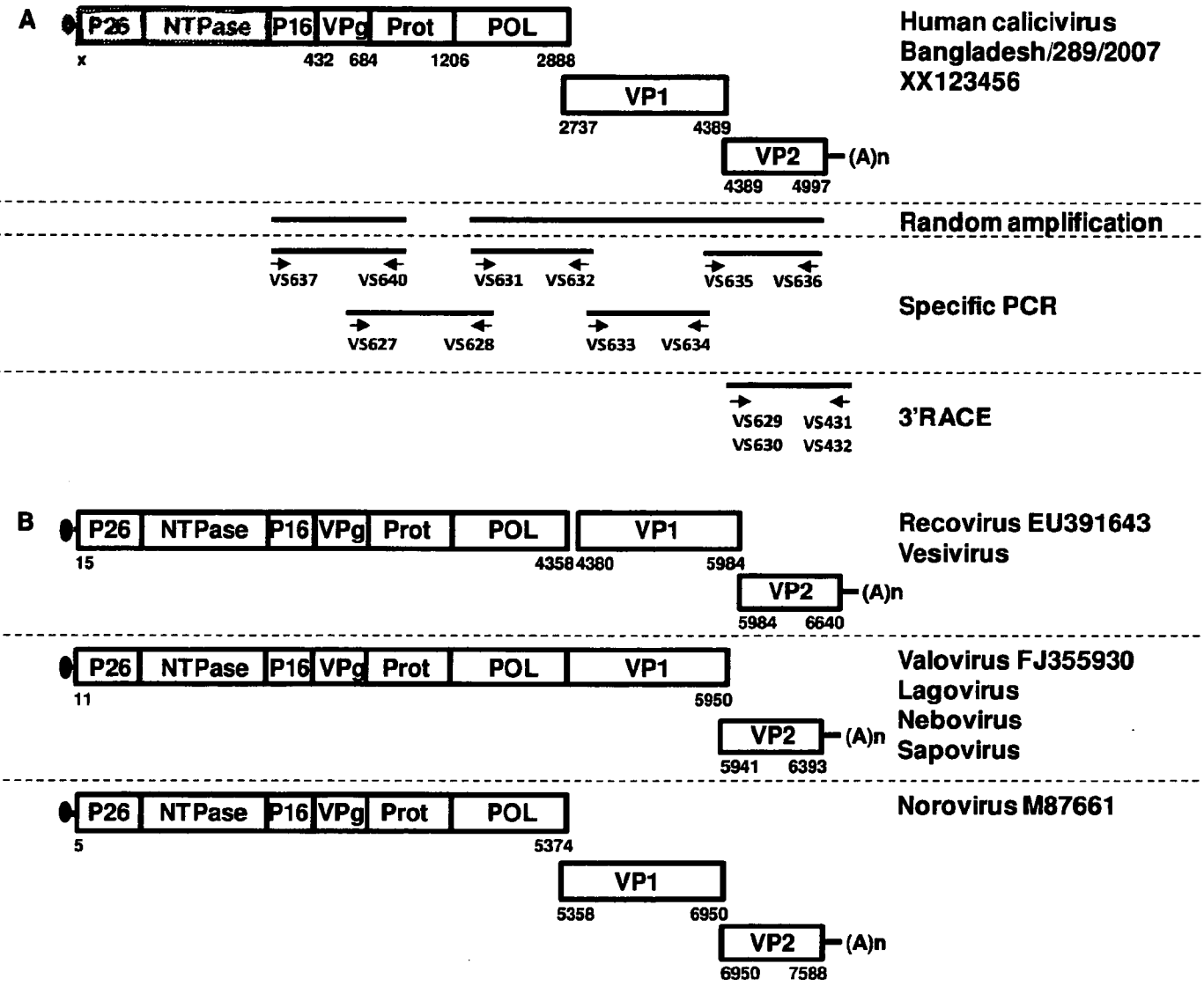
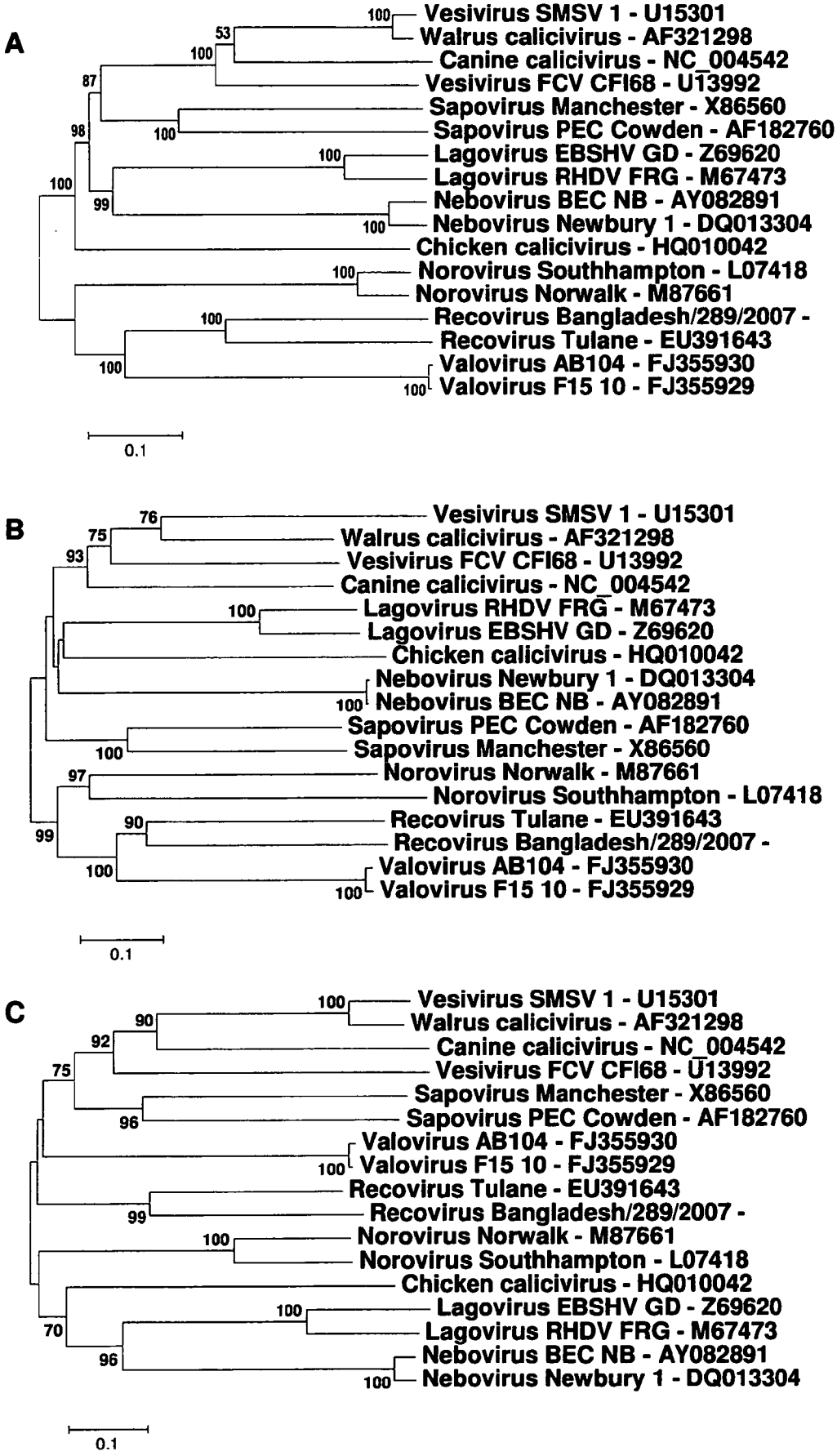

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Human calicivirus
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Random amplification
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VS634
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Norovirus M87661

FIGURE 1


A
Vesivirus SMSV 1 - U15301
Walrus calicivirus - AF321298
Canine calicivirus - NC_004542
Vesivirus FCV CFI68 - U13992
Sapovirus Manchester - X86560
Sapovirus PEC Cowden - AF182760
Lagovirus EBSHV GD - Z69620
Lagovirus RHDV FRG - M67473
Nebovirus BEC NB - AY082891
Nebovirus Newbury 1 - DQ013304
Chicken calicivirus - HQ010042
Norovirus Southhampton - L07418
Norovirus Norwalk - M87661
Recovirus Bangladesh/289/2007 -
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Nebovirus BEC NB - AY082891
Nebovirus Newbury 1 - DQ013304
0.1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/052706

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/00 C12N7/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	T. FARKAS ET AL: "Characterization of a Rhesus Monkey Calicivirus Representing a New Genus of Caliciviridae", JOURNAL OF VIROLOGY, vol. 82, no. 11, 1 June 2008 (2008-06-01), pages 5408-5416, XP55024651, ISSN: 0022-538X, DOI: 10.1128/JVI.00070-08 figures 1-5; tables 1-4; sequences B2Y6D0, B2Y6D1, B2Y6D2, EU391643	1-14
A	WO 2010/055134 A1 (RIBOXX GMBH [DE]; ROHAYEM JACQUES [DE]) 20 May 2010 (2010-05-20) sequence 5	3,7-14

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/052706

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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