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ORIGINAL ARTICLE

Discovery and genetic characterization of novel caliciviruses in German and Dutch poultry

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Abstract Caliciviruses (CV) were identified in the intestinal contents of five chickens and one turkey from various regions in Germany between 2009 and 2011 by degenerate reverse transcription PCR. The full 7,656-nt-long genomic sequence of the turkey CV L11043 was determined. Partial nucleotide sequences were determined for nine chicken strains. Phylogenetic analysis based on partial deduced amino acid sequences of the protease and RNA polymerase and the complete VP1 capsid sequence identified two distinct clusters of avian CVs, the first of which contained chicken CVs that were closely related to strains found in German chickens in Bavaria and that had been proposed to form a novel CV genus (proposed name: Bavovirus). In contrast, the turkey CV strain L11043 and three chicken CV strains formed a genetically distinct second cluster. Distance analysis suggested that the strains of the second cluster may represent members of two distinct genogroups of another novel CV genus (proposed name: Nacovirus). Based on the newly obtained sequence information, two real-time RT-PCR assays were developed and used to identify bavovirus and nacovirus in pooled intestinal contents from 24 chicken farms in Germany and the Netherlands. Of these, 20 (83 %) were positive for bavovirus, 11 (46 %) were positive for nacovirus, and nine (38 %) were positive for both bavovirus and nacovirus. Attempts were made to propagate chicken and turkey CVs from both the bavovirus and nacovirus clusters in primary chicken cecal cells, embryonal liver cells and fibroblast cells, but these attempts were not successful.

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Introduction

Caliciviruses (CV) are nonenveloped, icosahedral viruses of approximately 27-42 nm in diameter belonging to the family *Caliciviridae*. These viruses have a single-stranded, positive-sense, polyadenylated RNA genome of about 6.4 to 8.5 kb and exhibit a characteristic morphology in which their capsids have 32 distinct cup-shaped depressions (Latin *calix* = cup, chalice). The CVs include members of five recognized genera (*Norovirus, Sapovirus, Lagovirus, Vesivirus and Nebovirus*) [1, 6] and several unclassified CV strains, including the Tulane virus and the St-Valérien-like viruses [5, 10]. Recently, our group reported the detection and genetic analysis of novel enteric CVs from intestinal contents of German chickens from 2004 (strains Bavaria/04V0013, Bavaria/04V0021 and Bavaria/04V0027), which may represent yet another CV genus [18]. These viruses could not be directly associated with a disease, although one virus-positive chicken suffered from

runting and stunting syndrome (RSS). However, to date, no further data on genetic variability and epidemiology of these viruses are available. Recently, CV sequences were detected in turkeys by analyzing the gut metagenome of pooled turkey intestinal contents using a high-throughput pyrosequencing approach [4]. Although the turkeys originated from flocks with a history of enteric disease, a direct link to disease could not be established. Initial molecular data obtained by sequencing a 937-nucleotide (nt)-long fragment of turkey CV strain MD-2010 indicated that the virus was distinct from other CVs but clustered most closely with sapoviruses. To date, no other reports on turkey CVs are available, and thus information on the phylogeny and genetic variability of these viruses is limited.

In this study, we report on the detection and phylogenetic analysis of CVs of chicken and turkey origin collected from German and Dutch farms between 2009 and 2011. The partial genomic sequences of nine chicken CVs and the full genomic sequence of a turkey CV were determined. Attempts were made to propagate chicken and turkey CVs in cell culture using different cell lines.

Materials and methods

Specimens

In the first round, intestinal contents from five chickens at the age of 9 to 25 days and a turkey at the age of 14 days were collected. Two chicken specimens (F10026 and F10034) originating from one farm were collected from a slaughterhouse in the state of Brandenburg (Germany) in 2010. Weight loss, enteritis and liver enlargements with foci of intrahepatic necrosis and inflammation were observed in these two specimens. Three chicken specimens (L11038, L11039 and L11041) originated from different flocks in the state of Saxony (Germany) in 2010, of which two chickens had diarrhea with the clinical symptoms of enteritis. Failure to thrive was common to all cases. The turkey specimen (L11043) was collected in 2009 from a separate farm in Saxony. The turkey had clinical symptoms of enteritis and rickets and originated from a flock with slightly increased mortality.

In a second round, a total of 155 intestinal contents from broiler chickens were collected in 2011 from 24 farms in northern Germany and from the Netherlands, and the samples from each farm were pooled (2–10 chickens per farm, average 6.5). The chickens were 3 to 28 days old and suffered mostly from diarrhea and/or growth retardation or showed clinical signs of RSS. Specimens were screened for chicken CV by two newly developed real-time RT-PCR assays that were designed based on published chicken CV nt sequences and on sequence information obtained from the first round of sampling from RT-PCR positive specimens.

The intestinal contents were suspended at a 1:5 ratio in phosphate-buffered saline (PBS, pH 7.2). Liver tissues of specimens F10026 and F10034 were cut into small pieces and homogenized using a pestle and sea sand by adding 2 to 3 ml of PBS, pH 7.2. The suspensions of intestinal contents or liver tissues were clarified at 3,345 × *g* for 20 min. The supernatants were stored at −20 °C until RNA preparation.

RNA extraction

Viral RNA was extracted from a 200-µl suspension of clarified intestinal contents or liver tissue using a High Pure Viral RNA Kit (Roche Molecular Biochemicals Ltd, Mannheim, Germany) according to the manufacturer's instructions and eluted in 50 µl elution buffer.

Design of degenerate reverse transcription (RT)-PCR

Two novel degenerate RT-PCR assays with increased sensitivity and specificity were designed to identify CVs. For this purpose, two degenerate primers (pYGDD_c and pYGDD_d) specific for the RNA-dependent RNA polymerase (NS^{pol}) YGDD-motif were designed to generate cDNA with an adapter sequence at the respective 5′ ends (Table 1). Two sets of degenerate primers (set A: p289CVa/p289CVb and set B: p289CVc/p289CVd) targeting the conserved NS^{pol} motif D(Y/F)(S/T)(K/A/R/G)WDS were designed to be used as forward primers in two separate PCR assays (PCR A and PCR B), each using the adapter sequence as a reverse primer (p_adapter_UP). The forward primer sets differ in the last two nt positions at the 3′ end, accounting for differences in the first two codon-positions of the C-terminal serine (TC and AG, respectively). Furthermore, each set contains at primer positions seven to nine either WCI, coding for ACN-threonine and TCN-serine, or AGY, coding for serine. Altogether, the primer pools p289CVa/b and p289CVc/d are each 64-fold degenerate and allow for a maximum of two mismatches to any CV that encodes the D(Y/F)(S/T)(K/A/R/G)WDS motif. None of the conceivable mismatches are located within the last five nucleotides at the 3′ end of the primers. Primers were tested for their melting temperatures, potential hairpins, self-annealing sites and primer-primer interactions using OligoAnalyzer 3.1 (Integrated DNA Technologies, http://www.idtdna.com/ANALYZER/Applications/OligoAnalyzer/). Compared to the single degenerate RT-PCR published previously by us [18], the two separate RT-PCR assays using the modified primer sets were shown to be more specific and more sensitive to a broad range of CVs, including human sapovirus, feline CV,

Table 1 Oligonucleotides used in this study

Target virus	Name	Sequence (5′-3′)	Sense	Target gene
Generic calicivirus	pYGDD_c	GTTACAGCTTCGCAGTTATCYCRTCRTNCCRTA[a]	–	NS^{pol}
	pYGDD_d	GTTACAGCTTCGCAGTTATCCARTCRTNCCRTA[a]	–	NS^{pol}
	p289CVa	GAYTAYWCIRGGTGGGAYTC	+	NS^{pol}
	p289CVb	GAYTAYAGYRGGTGGGAYTC	+	NS^{pol}
	p289CVc	GAYTAYWCIRGGTGGGAYAG	+	NS^{pol}
	p289CVd	GAYTAYAGYRGGTGGGAYAG	+	NS^{pol}
	p_adapter_UP	GTTACAGCTTCGCAGTTATC	–	–
Bavovirus	qBavofor	GATTTCTCCGCAAGCCTTAG	+	NS^{pol}
	qBavorev	ACCCTCCATAACATAATCAACCTT	–	NS^{pol}/VP1
	qBavoprobe	AGTGTTGTTATGTCTCCTTCCGCAGTG[b]	–	NS^{pol}
Nacovirus	qNacofor	CTTCTGGAACYCCTGCAAC	+	NS^{pol}
	qNacorev	CTTCYTGAAGKGGTTGCGG	–	NS^{pol}
	qNacoprobe	TGGCTTGCGCARGAYCAGGTTGG[c]	+	NS^{pol}
Generic calicivirus	p774	ACCACACCAGGNGAYTGYGG	+	NS^{pro}
	CVp774	ACCAAGCAAGGNGAYTGYGG	+	NS^{pro}

[a] Non-complementary adapter sequence underlined

[b] 5′-CFG540-BHQ1-3′ and [c] 5′-FAM-BHQ1-3′ labelled

human and bovine norovirus and chicken CV strains Bavaria/04V013, Bavaria/04V021 and Bavaria/05V027 (data not shown).

Calicivirus detection by degenerate RT-PCR

The 10-µl RT reaction consisted of 100 units of Super-Script III reverse transcriptase, 10 units of RNase inhibitor (RNaseOUT™, Invitrogen, Darmstadt, Germany), 0.5 mM each dNTP (dATP, dCTP, dGTP and dTTP), 1x first-strand RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 5 mM DTT, 1 µM each primer pYGDD_c/d and 5 µl of viral RNA. RT was carried out at 46 °C for 30 min and was terminated at 70 °C for 15 min. Each 25-µl PCR reaction contained 1 µl of cDNA, 1x PCR buffer without $MgCl_2$, 2 mM $MgCl_2$, 0.2 mM each dNTP, 1 U Platinum Taq polymerase (Invitrogen), 0.6 µM each primer p289CVa and p289CVb (RT-PCR A) or p289CVc and p289CVd (RT-PCR B) and 0.4 µM primer p_adapter_UP. Cycling conditions were 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s.

PCR products were separated by electrophoresis on a 2 % (w/v) agarose gel and purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCR products were cloned (CloneJet™ PCR cloning Kit, Fermentas Life Science, St. Leon-Rot, Germany), and DNA sequencing was carried out by GATC Biotech AG (Konstanz, Germany), using an ABI 3730xl sequencer.

Sequence extension

In order to obtain more sequence information, ~1.1-kb amplicons of the 5′-region adjacent to the degenerate RT-PCR target site were generated for all CV strains from the first round of sampling using gene-specific reverse primers and forward primer p774 [5] or a modified p774 forward primer, primer CVp774 (Table 1), targeting the conserved GDCG-coding region in the CV protease (NS^{pro}) gene. PCR products were sequenced in both directions. In addition, the 3′-end genomic sequence of chicken strain F10026 was amplified using strain-specific primers and the T_{25}-VN primer (5′-T_{25}-A/G/C-A/T/G/C) in a semi-nested RT-PCR 3′-RACE. Additionally, the whole genome of the putative turkey CV strain L11043 was amplified in separate overlapping RT-PCR reactions and sequenced in both directions using a primer-walking strategy as described elsewhere [18]. Chicken and turkey nucleotide sequences were submitted to the GenBank database under accession numbers JQ347522 through JQ347530 (Table 2).

Chicken-calicivirus-specific real-time RT-PCR assays

To confirm the positive CV results of the specimens from the first round of sampling and to screen for chicken CV in specimens of the second round of sampling, two chicken-CV-specific TaqMan real-time RT-PCR assays were designed (Table 1). The assays were based on the sequence data from RT-PCR-positive specimens of the first round of

Table 2 Virus strains, tree notations, and partial NS^{pro}/NS^{pol} and complete VP1 amino acid sequence lengths and locations used for phylogenetic comparison

Calicivirus strain	GenBank accession number	Tree notation[a]	Partial NS^{pro}/NS^{pol} [b]	VP1[c]
Norwalk virus	M87661	NoV Norwalk	341 (1241-1581)	530
Bovine enteric norovirus strain Jena	AJ011099	BoNoV GIII Jena	340 (1134-1473)	519
Murine norovirus 1	DQ285629	MNV-1	347 (1135-1481)	541
Sapovirus Manchester	X86560	HuSaV GI Manchester	337 (1171-1507)	559 (1722-2280)
Sapovirus Mc10	NC_010624	HuSaV GII Mc10	336 (1176-1511)	556 (1726-2281)
Porcine enteric calicivirus strain Cowden	AF182760	PoSaV GIII PEC	335 (1165-1499)	542 (1713-2254)
Feline calicivirus strain Urbana	NC_001481	FCV	349 (1195-1543)	668
VESV-like calicivirus strain Pan-1	AF091736	Primate Pan-1	351 (1308-1658)	709
Walrus calicivirus	AF321298	WCV	351 (1307-1657)	708
European brown hare syndrome virus	NC_002615	EBHSV	347 (1207-1553)	574 (1761-2334)
Rabbit hemorrhagic disease virus MIC-7	EU871528	RHDV MIC-7	347 (1209-1555)	578 (1763-2340)
Newbury agent 1 virus	NC007916	BEC/Newbury-1	326 (1126-1451)	550 (1668-2217)
Calicivirus strain BEC/NB/80/US	AY082891	BEC/NB/80/US	326 (1126-1451)	550 (1668-2217)
St-Valérien calicivirus pig/AB90/CAN	NC_012699	St-Valerien	323 (958-1280)	517 (1463-1979)
Tulane virus	EU391643	Tulane virus	318 (938-1255)	534
Bavaria/04V0021	HQ010042	Bavaria/04V0021b	337 (1180-1516)	578
Bavaria/04V0013	HQ616593	Bavaria/04V0013	337	N/A
Bavaria/04V0027	HQ616594	Bavaria/04V0027	337	N/A
Turkey calicivirus strain L11043	JQ347522	Turkey L11043	340 (1017-1356)	584 (1721-2304)
Chicken calicivirus strain F10026n	JQ347523	Chicken F10026n	339	582
Bavaria/04V0021n	JQ347524	Bavaria/04V0021n	339	N/A
Chicken calicivirus strain F10026b	JQ347525	Chicken F10026b	337	N/A
Chicken calicivirus strain F10034b	JQ347526	Chicken F10034b	337	N/A
Chicken calicivirus strain F10034n	JQ347527	Chicken F10034n	339	N/A
Chicken calicivirus strain L11038	JQ347528	Chicken L11038	337	N/A
Chicken calicivirus strain L11039	JQ347529	Chicken L11039	337	N/A
Chicken calicivirus strain L11041	JQ347530	Chicken L11041	337	N/A

N/A: no sequence information

[a] Calicivirus notation used in phylogenetic trees (Fig. 1)

[b] Lengths and, if known, locations (in brackets) of aligned amino acid sequences in their respective ORF-1

[c] Lengths of aligned amino acid sequences in their respective ORF-1 (locations in brackets) and ORF-2, respectively

sampling and used in a duplex real-time RT-PCR format generating 83-bp and 122-bp amplicons, respectively. The 10-µl RT reaction contained 100 units of SuperScript III, 10 units RNaseOUT™ (Invitrogen), 100 nM reverse primers qBavorev and qNacorev, respectively, 1 mM each dNTP (dATP, dCTP, dGTP and dTTP), 1x first-strand RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) and 5 µl viral RNA. RT was carried out at 50 °C for 30 min, followed by 70 °C for 15 min. For real-time PCR, each 20-µl reaction contained 2 µl of cDNA, 10 µl of 2x ABsolute Blue QPCR Mix (ABgene®UK, Epsom, Surrey, UK), 0.4 µM each forward and reverse primer and 0.2 µM each probe. Initial activation at 95 °C for 15 min was followed by a two-step cycling protocol consisting of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s over 40 cycles.

Standard anticontamination procedures were employed for all RNA and DNA procedures. RNase-free reagents and procedures were used to minimize contamination. Negative and positive controls were included in each run.

DNA plasmids

Fragments of target virus PCR products cloned into DNA plasmids were used to evaluate the sensitivity of each real-time RT-PCR assay and for semi-quantitation of virus titers in intestinal contents. Real-time RT-PCR products of a bavovirus and a nacovirus were cloned into pJET1.2/blunt cloning vectors and used to transform One Shot® TOP10 cells. Plasmids were purified using a PureLink™ Quick Plasmid Miniprep Kit (Invitrogen), and plasmid concentrations were determined using a NanoDrop ND-1000

UV-Vis spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA). Serial dilutions of prequantified plasmids were subsequently used to evaluate the sensitivity of the real-time RT-PCR assays and for semi-quantitation of virus titers in the intestinal contents.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analysis of multiple sequence alignments of deduced amino acid (aa) sequences of the partial NS^{pro} and NS^{pol} genes and the complete VP1 capsid protein gene was conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.05 [15]. Amino acid alignments were completed with the CLUSTALW algorithm (Gap opening penalty 12, Gap extension penalty 0.2, Protein weight matrix: BLOSUM). From the initial alignment, a 318- to 351-aa-long region that is flanked by conserved motifs was realigned and then corrected manually. Lengths and locations of the partial NS^{pro} and NS^{pol} proteins used and of the VP1 proteins are shown in Table 2. Phylogenetic trees were constructed using the maximum-likelihood method based on the JTT matrix-based model. For each analysis, phylogeny was tested using 1,000 bootstrap replications. Evolutionary distances between sequences and groups were computed using the JTT matrix-based model with standard errors being obtained by a bootstrap procedure (1,000 replicates). Molecular weights from deduced aa sequences were calculated using the Molecular Weight Calculator of the Protein Information Resource (PIR), Georgetown University Medical Center (http://pir.georgetown.edu/pirwww/search/comp_mw.shtml). Isoelectric points of deduced proteins were calculated using the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/).

Attempts to isolate virus particles in cell culture

A 50 % (v/v) suspension of intestinal contents from three broiler chickens that were shown to be positive for CV from either the bavovirus or the nacovirus cluster and from the turkey-CV-positive specimen were prepared in Hanks solution with 500 µg/ml gentamycin (Sigma, Taufkirchen, Germany) and incubated for 1 h at room temperature. The suspensions were used directly as inocula or stored for up to two months at −80 °C. The confluent monolayers of primary chicken cecal (PCC) cells derived from three-week-old chickens, embryonal liver and fibroblast cells were rinsed with Hanks solution. For the PCC cells, virus inocula were activated by addition of trypsin (Serva, Heidelberg, Germany) to a final concentration of 0.4 IU/ml for 1 h at 37 °C and centrifuged onto the cells at 1,000 x *g* for 1 h at 22-24 °C. Previous experiments showed that liver and fibroblast cells did not tolerate trypsin at these concentrations and detached from the glass substrate. Here, the suspensions were inoculated directly onto the liver and fibroblast cells, which were then incubated for 1 h at 37 °C. The inocula were removed and replaced with fresh serum-free DMEM supplemented with 1 % non-essential amino acids (Sigma) and 50 µg of gentamycin per ml. Cells were incubated rolling (PCC cells) or stationary (embryonal liver and fibroblast cells) at 37 °C in 25-ml glass tubes or 25-ml glass flasks. Inocula for each of the subsequent two passages on PCC cells and nine passages on liver or fibroblast cells, respectively, were prepared by two freeze-thaw cycles. The cells were examined daily for the appearance of a cytopathic effect (CPE). Cell suspensions were further analyzed by transmission electron microscopy (TEM) using procedures described previously [18] and by degenerate RT-PCR and real-time RT-PCR as described above.

Results

Detection of chicken and turkey CV in intestinal contents by degenerate RT-PCR

Products of the expected size were obtained with intestinal specimens from all chickens and turkeys from the first round of sampling using RT-PCR A. No PCR products were obtained from any of the intestinal specimens using RT-PCR B or from the corresponding liver specimens from chickens F10026 and F10034 with either RT-PCR A or B. All PCR-positive specimens were cloned (six clones per specimen) and sequenced. Chicken specimens L10038, L10039 and L10041 and turkey specimen L10043 contained one CV sequence each, whereas in chicken specimens F10026 and F10034, two distinct CV sequences could be found. All sequences contained a nucleic acid sequence encoding the GLPSG motif within the NS^{pol} region, which is conserved in all known CVs.

Phylogenetic analysis

A phylogenetic tree of the deduced aa sequences from a 1.1-kb region that spans the partial NS^{pro} and NS^{pol} genes of all chicken and turkey strains from the first round of sampling, including the Bavaria/04V0021 chicken strain, was constructed (Fig. 1). Strains from specimens F10026 (F10026b), F10034 (F10034b), L11038, L11039 and L11041 clustered closely with the Bavaria/04V0021 strain (Bavaria/04V0021b), henceforth referred to as the bavovirus cluster. In contrast, strains from specimens F10026 (F10026n), F10034 (F10034n), Bavaria/04V0021 (Bavaria/04V0021n) were rooted with the turkey strain L11043 in

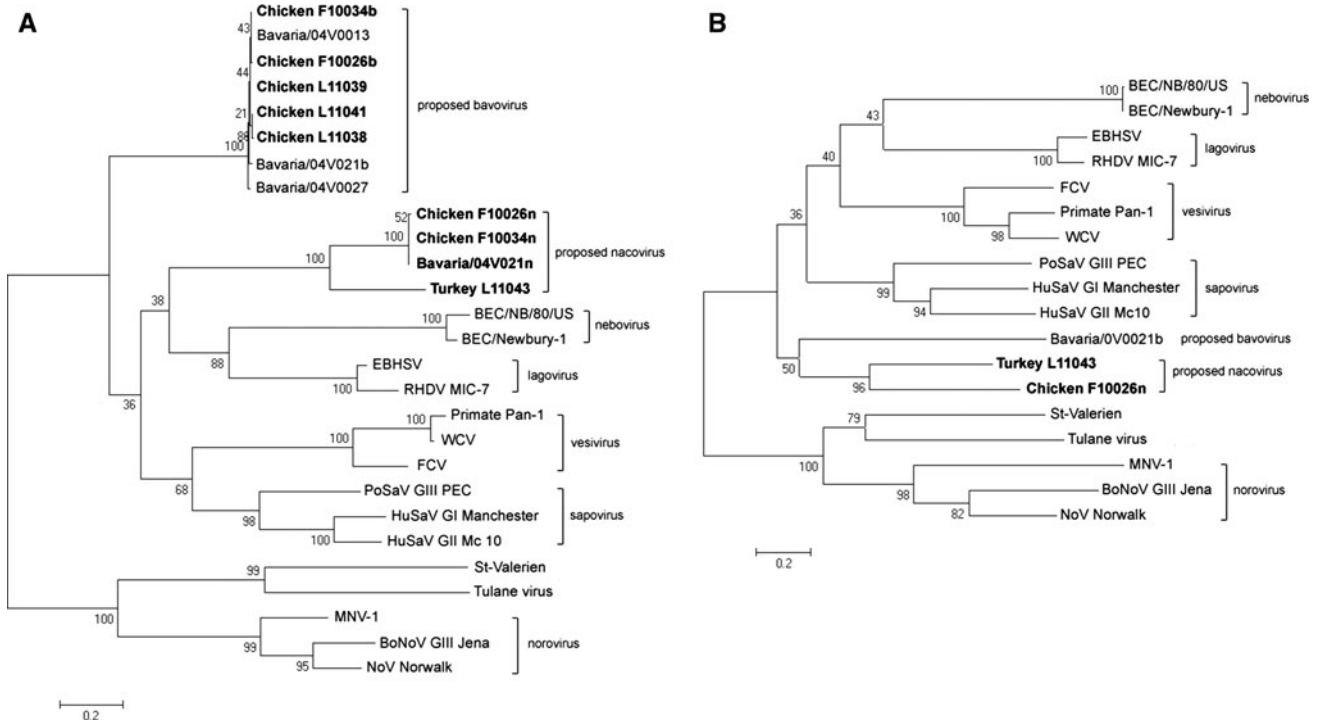

Fig. 1 Molecular phylogenetic analysis of caliciviruses on the basis of the partial NS^{pro} and NS^{pol} (**A**) and complete VP1 major capsid (**B**) amino acid sequences. Strains in boldface are described in the present study. The length and locations of the amino acid sequences used in ClustalW alignments are shown in Table 2. The trees were constructed using the maximum-likelihood method based on the JTT matrix-based model in MEGA5. The percentages of replicate trees in which the associated viruses clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site

two subclades and were placed on a separate branch in the tree (henceforth referred to as the nacovirus cluster), although bootstrap values of 38 and 36 indicate low significance for the branching points that separate this cluster from other CVs, including the bavovirus cluster. However, a bootstrap value of 100 indicates a high significance for the branching point that separates the F10026n/F10034n/Bavaria/04V0021n subclade from the turkey L11043 subclade within the nacovirus cluster.

Phylogenetic analysis of the complete CV VP1 multiple alignment using the maximum-likelihood method predicted a similar tree (Fig. 1). Chicken strain F10026n and turkey strain L11043 were rooted together with high bootstrap support. In contrast to the NS^{pro}/NS^{pol} tree, in the VP1 tree, the F10026n/L11043 cluster was rooted together with the Bavaria/04V0021b strain in a monophyletic clade. However, low bootstrap support of the branching points for most CV genera compared to the F10026n/L11043 cluster suggests the need for a cautious interpretation of the significance of these results.

Evolutionary divergence in the NS^{pro}/NS^{pol} and VP1 regions was estimated between members of established CV genera and those of the bavovirus and nacovirus clusters. The estimated distance of the bavovirus cluster was shortest in the NS^{pro}/NS^{pol} region to lagoviruses (substitutions per site/standard error s_e: $1.217/s_e = 0.116$) and in the VP1 capsid to the nacovirus cluster ($1.640/s_e = 0.124$), whereas the distance of the nacovirus cluster was shortest in the NS^{pro}/NS^{pol} region to sapoviruses ($1.393/s_e = 0.115$). These distances are as long as or longer than the distances between some of the established CV genera, e.g., lagovirus/nebovirus (NS^{pro}/NS^{pol}: $1.209/s_e = 0.111$; VP1: $1.613/s_e = 0.140$) or vesivirus/sapovirus (NS^{pro}/NS^{pol}: $1.230/s_e = 0.094$; VP1: $1.536/s_e = 0.116$), suggesting that the bavovirus and the nacovirus clusters may represent two separate novel genera within the family *Caliciviridae*. The distances between turkey strain L11043 and the chicken CV strains within the nacovirus cluster (NS^{pro}/NS^{pol}: $0.573/s_e = 0.055$; VP1: $0.998/s_e = 0.087$) were comparable to distances between strains that represent different genogroups within some of the established genera, e.g., HuSaV GI Manchester/PoSaV GIII PEC (NS^{pro}/NS^{pol}: $0.680/s_e = 0.067$; VP1: $0.963/s_e = 0.077$) or BoNoV GIII Jena/MNV-1 (NS^{pro}/NS^{pol}: $0.575/s_e = 0.060$; VP1: $1.325/s_e = 0.120$), suggesting that the turkey and chicken subclades may represent two separate genogroups within the nacovirus cluster.

Genomic organization of strains L11043 and F10026n

The complete sequence of the RNA genome of the L11043 turkey CV was determined. It consists of 7,656 nt, excluding the poly-A tail. It has a genome base composition of 22.5 % G, 24.1 % A, 25.9 % U, and 27.5 % C. The genome is predicted to contain two main coding ORFs, which is similar in organization to the Bavaria/040021 chicken CV, as well as to sapo-, lago- and neboviruses and the St-Valérien-like viruses. A first AUG (predicted start codon of ORF1) is observed at nt 9. ORF1 is 6,915 nt long and encodes a polypeptide of 2,304 aa with a calculated molecular weight of 250.6 kDa. ORF1 of turkey strain L11043 codes for a continuous polyprotein including non-structural (NS) proteins and the VP1 capsid protein. Conserved CV NS-protein aa motifs within ORF1 were identified. These included the NTPase (NS^{NTPase}) motif GXPGXGKT at position 529, the NS^{pro} motif G(D/Y)CGXP at position 1,172, and the NS^{pol} motifs DYSKWDST, GLPSG and YGDD at positions 1,456, 1,511 and 1,559, respectively. Using published cleavage maps of lagoviruses and of the porcine sapovirus strain PEC/Cowden [7, 11, 14], a putative NS^{pro} cleavage site for the VP1 capsid protein between positions 1,720/1,721 (E/G) was identified. The putative VP1 protein is predicted to be 584 aa long and to have a molecular weight of 61.6 kDa, which is similar to those of other CVs. It contains the conserved PPG motive and a FXXLXXP (FCLLKEP) hinge segment between the shell (S) and protruding (P) domains, which is similar to other CV VP1 proteins. ORF1 is terminated by an UAA and followed directly in the same frame by the start codon of ORF2 (GA**UAA**AUGGC; ORF1 stop codon in bold and ORF2 start codon underlined). The 624-nt-long ORF2 is initiated at nt 6,924 and encodes a polypeptide of 207 aa with a calculated molecular weight of 21.5 kDa, which is 8.9 kDa smaller than that of the Bavaria/04V021 chicken CV protein but similar to that of other CVs. The predicted protein has a calculated isoelectric point of 9.8. The 3′ non-translated region (NTR) is 109 nt long.

A total of 4,472 nt of the 3′end of chicken CV strain F10026n were sequenced. The sequence consisted of the 3,453-nt 3′ end of an ORF (ORF1), one complete ORF (ORF2), and a 3′ NTR of 409 nt, which is considerably longer than the 3′ NTR of other previously described CVs, including the Bavaria/04V021b chicken strain and the L11043 turkey strain. As in the turkey L11043 strain, conserved major NS-protein motifs could be identified in ORF1 of the chicken F10026n strain, including the motifs G(D/Y)CGXP, DYSKWDST, GLPSG and YGDD. A potential NS^{pro} cleavage site for the VP1 capsid protein was predicted between positions 583 and 582 (E/G) from the C-terminus of the ORF1 polyprotein. The putative VP1 protein is 582 aa long and has a calculated molecular weight of 61.4 kDa. The PPG motif is present, and the hinge segment between the S and P domains (FCLLKEP) is identical to that in the L10043 turkey strain. Between ORF1 and ORF2, a +1 frameshift was observed with three potential initiation sites for ORF2: the first AUG (38-nt overlap with ORF1), followed by a second in-frame AUG (32-nt overlap with ORF1) and a third in-frame AUG (1 nt downstream of ORF1), resulting in predicted ORF2 lengths between 648 nt (215 aa) and 609 nt (202 aa) and calculated molecular masses between 22.7 and 21.3 kDa. All three of these possible ORF2 proteins are very basic, with a calculated isoelectric point of 10.5.

Sequence comparison of turkey CV strains L11043 and MD-2010

A pairwise alignment of turkey CV strain L11043 (this study) and the 936-nt partial non-structural polyprotein gene (putative $NS3^{NTPase}$-NS4-$NS5^{VPg}$ region) of turkey CV strain MD-2010 (GenBank accession no. HM803966) revealed 84.7 % nt sequence identity. At position 810 of the MD-2010 sequence, a potential sequencing error was detected, which leads to a stop codon (frame 1) at nt positions 934 to 936 within the putative ORF1 polyprotein gene. At the conflicting position, strain L11043 has an "A" insertion, which leads to a +1 frame shift, allowing for a continuous ORF. After pairwise alignment of the deduced aa sequences of strain L11043 and the corrected strain MD-2010, only four aa differences were observed over the entire alignment (=98.7 % aa identity).

Detection of chicken CV in intestinal contents by specific real-time RT-PCR assays

Two specific real-time RT-PCR assays, targeting chicken CVs from the bavovirus and nacovirus clusters, were designed to confirm chicken CV-positives in the intestinal specimens and the absence of chicken CVs in the liver homogenates from the first round of sampling and to screen for chicken CVs in intestinal contents from the second round of sampling. Serial dilutions of prequantified plasmids (10^7 to 10 copies per reaction) revealed that the real-time RT-PCR assays reliably detect 10 copies per reaction of bavovirus and nacovirus. Using the formula $E = (10^{-1/\text{slope}})$-1 [9], the calculated efficiency of the assay was 0.98 for bavoviruses and 1.02 for nacoviruses. Positive real-time RT-PCR signals were obtained with the intestinal contents of all chicken specimens from the first round of sampling. Specimens F10026 and F10034 were positive in both the bavovirus and nacovirus assays, confirming a double infection. In specimens L10038, L10039 and L10041, real-time RT-PCR signals were obtained with the bavovirus assay, whereas the nacovirus assay did not generate any

detectable qPCR signals. Using the DNA plasmid standards, the concentrations of genome equivalents (gen. eq.) per reaction were estimated to be 3.4 x 10^4 and 1.0 x 10^5 (nacovirus) and 4.9 x 10^2 and 1.4 x 10^4 (bavovirus), respectively. Disregarding all losses and inefficiencies during the extraction steps, RT reaction and real-time PCR, this is equivalent to 4.3 x 10^7 and 1.3 x 10^8 (nacovirus) and 6.1 x 10^5 and 1.7 x 10^7 (bavovirus) gen. eq./ml fresh feces. Furthermore, no real-time RT-PCR signals were obtained with either of the two assays in the liver specimens, confirming the absence of chicken CVs in the enlarged chicken livers of specimens F10026 and F10034. Additionally, the stored intestinal contents of the chicken in which the 2004 chicken CV strain Bavaria/04V0021 was initially detected was also re-examined using the bavovirus and nacovirus real-time RT-PCR assays. Most interestingly, here too, specific real-time RT-PCR signals could be generated with both assays, suggesting a double infection that was not detected in the original examination (nacovirus 2.7 x 10^6 gen.eq./ml fresh feces and bavovirus 2.6 x 10^9 gen.eq./ml fresh feces). No cross-reactivity of the assays could be observed with various CV and non-CV strains, including feline CV, human sapovirus GII, human norovirus GI and GII, rotavirus strain WA, human astrovirus and hepatitis A virus. After this initial round of evaluation to determine its sensitivity and specificity, the real-time RT-PCR assays were used for the screening of 24 pooled field samples that were obtained from separate farms in northern Germany and the Netherlands. Of these, 11 (46 %) were positive for nacovirus, 20 (83 %) were positive for bavovirus and 9 (38 %) tested positive for bavovirus and nacovirus. Two (8 %) specimens were negative for nacovirus or bavovirus. The average estimated virus titers in the positive specimens were 5.9 x 10^8 (min 1.2 x 10^5 max 2.5 x 10^9) and 1.8 x 10^8 (min 4.9 x 10^4 max 1.4 x 10^9) gen.eq./ml fresh feces for nacoviruses and bavoviruses, respectively.

Tissue culture propagation of chicken and turkey CVs

No virus-dependent CPE was observed in the PCC cells after passages 1 to 5 or in the liver or fibroblast cells after passages 1 to 10, and real-time RT-PCR, RT-PCR and TEM results did not suggest tissue culture propagation of chicken and turkey CVs.

Discussion

Partial genomic sequences of CVs from intestinal contents of five chickens and the full genomic sequence of a turkey CV were generated. Based on phylogenetic analysis, these CVs represent members of two distinct genetic clusters. The first cluster so far consists exclusively of very closely related strains, detected in chickens from various farms in different geographic regions in Germany between 2004 and 2010. Calculated evolutionary distances support the creation of a novel CV genus with strain Bavaria/04V0021 as the prototype strain. The second cluster consists of three genetically closely related chicken CV strains and one turkey CV strain, which genetically split into two distinct subclades or genogroups. Phylogenetic analysis of two major genomic regions (partial NS^{pro}/NS^{pol} and complete VP1) and the long evolutionary distances to the members of the other CV genera suggest that these strains are members of a separate genus, for which we propose the name nacovirus (*n*ovel *a*vian *C*V). So far, all strains of the nacovirus cluster have been found in Germany and the Netherlands. However, the ~99 % aa sequence identity in the partial putative $NS3^{NTPase}$-NS4-$NS5^{VPg}$ region between the German turkey strain L11043 and the American strain MD-2010 suggests a global presence of these viruses.

RT-PCR and real-time RT-PCR assays specific for bavoviruses and nacoviruses revealed a high incidence of CV infections in farms in Germany and the Netherlands. Semi-quantification cautiously suggests titers for bavovirus and nacovirus in the intestinal contents of infected animals of up to 10^9/ml fresh feces, which is in the middle range of reported titers from other enteric viruses, including CVs [2, 8]. Most of the examined pooled intestinal contents were collected from birds with various clinical symptoms ranging from enteric disease, growth retardation and RSS. Two of the chicken specimens in this study had clinically enlarged livers, but CVs could not be detected in the liver homogenates by RT-PCR and real-time RT-PCR, respectively. The fact that some of the pooled specimens that tested positive for bavovirus or/and nacovirus were from farms with clinically healthy chickens (data not shown) and that differential diagnostic control was not carried out show that further investigations are necessary to uncover the etiologic role and the clinical relevance of bavoviruses and nacoviruses in chickens. In chickens, CVs have been associated with enteric disease, RSS, poor feathering, sticky vents and other symptoms and have been shown to cause disease in specific-pathogen-free day-old chicks [3, 12, 18]. CVs have been detected in diarrheic turkeys [4], but avian reoviruses and astroviruses were present in the same samples. The absence of disease in CV-infected animals, such as pigs and cattle, is not uncommon [16, 17]. However, there is evidence that multiple infections with CVs and other enteric pathogens may synergistically result in a more severe disease [13]. It remains to be seen if this observation is true for chicken and turkey CVs, and further research is required to identify carriage rates of CVs in clinically normal birds and birds with disease with associated clinical data in a more comprehensive follow-up study. For this purpose, and to elucidate their etiologic role,

sensitive and specific tools have been presented in this study. However, due to limited sequence information, a broadly reactive turkey CV real-time RT-PCR has not been developed.

Unfortunately, we were not able to grow chicken and turkey CVs in cell culture. Although the successful propagation of CV-like particles in primary chicken embryo fibroblast cells was reported more than 25 years ago [3], to the best of our knowledge no other reports of a successful cell culture system for chicken CVs have been published. Although the results reported in the article by Cubitt and Barrett were encouraging [3], it seems that they were not able to passage the virus or grow it to high titres. It is possible that the chicken virus strain that was propagated previously may have been genetically different from the chicken strains in this study and thus may have a different tropism.

Although many CVs appear to be host specific, chickens and turkeys may share a common reservoir for CVs. Furthermore, a potential transmission of these viruses to other animals or humans cannot be excluded at this stage, and this also warrants further research.

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