Short communication

Molecular detection of norovirus in sheep and pigs in New Zealand farms

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Abstract

Human norovirus (NoV) is reportedly the major cause of non-bacterial gastroenteritis outbreaks worldwide and is commonly associated with water- and food-borne transmission via the faecal–oral route. Aside from humans, norovirus has been detected in pigs, cattle and mice. The close relatedness of some human and animal noroviruses has raised concerns about potential zoonotic transmission. Our laboratory recently reported the development of a multiplex real-time RT-PCR for the detection and genotyping of norovirus of genogroups I–III. Here we report a study of 56 faecal specimens from pigs and sheep that were collected and screened for noroviruses using this assay. Norovirus was found in 2/23 (9%) of porcine specimens (all were genogroup II) and in 8/33 (24%) of ovine specimens (all were genogroup III). Samples tested positive for norovirus were verified by conventional RT-PCR with different primer sets. Genomes of representative porcine and ovine norovirus strains underwent partial sequence analysis (343 and 2045 bases, respectively). This is the first report describing norovirus in sheep.

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1. Introduction

The genus Norovirus (NoV) is a member of the family Caliciviridae and has been recognized as the leading viral agent causing gastroenteritis worldwide in humans (Fretz et al., 2005; Greening et al., 2001; Höhne and Schreier, 2004; Lopman et al., 2003) and has also been found to infect other mammals. The transmission pathways of NoV are from person to person, animal to animal, or via contaminated food and water (Glass et al., 2000; Hewitt et al., 2007; Lopman et al., 2006). NoV are small non-enveloped viruses of

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about 27–35 nm and possess a positive-sense single-stranded RNA genome that ranges from 7.2 to 7.7 kb, that encodes for three open reading frames (Hardy, 2005). Based on genetic analysis, NoV have been subdivided into five distinct genogroups (GI–V) (Zheng et al., 2006) and have been found in humans (HuNoV; GI, II and IV), pigs (PoNoV; GII), cattle (BoNoV; GIII) and mice (GV). PoNoV have been identified in Europe, Japan and the United States and can be further subdivided into three genotypes (GII.11, GII.18 and GII.19) (Reuter et al., 2007; Sugieda et al., 1998; van der Poel et al., 2000, 2003; Wang et al., 2005). BoNoV have been found in the above countries plus South Korea and New Zealand, and genetic analysis suggests two distinct genotypes (GIII.1, or Jena-like and GIII.2, or Newbury2-like) (Ando et al., 2000; Costantini et al., 2006; Ike et al., 2007; Oliver et al., 2003, 2007; Park et al., 2007; Smiley et al., 2003; Wise et al., 2004; Wolf et al., 2007). Our group recently published a multiplex real-time reverse transcription (RT)-PCR method for the simultaneous detection and differentiation of NoV GI–III (Wolf et al., 2007). Using this assay we detected BoNoV in cattle from two New Zealand farms. The assay was also designed to be sensitive for PoNoV but had not been tested against porcine faecal specimens at the time of publication. The aim of this study was to apply the multiplex NoV assay to a broader range of farm animals by screening faecal material from pigs and sheep. Positive findings were confirmed by conventional RT-PCR using previously published and newly designed primers. New NoV strains were characterized by analysis of nucleotide sequences.

2. Materials and methods

2.1. Specimens

A total of 56 faecal specimens were collected between May 2006 and August 2007 from two pig farms (farms A and B; 10 and 13 specimens, respectively) and two sheep farms (farms C and D; 27 and 6 specimens, respectively) situated in New Zealand’s North Island. The pig farms were about 50 km and the sheep farms about 150 km apart from each other. All sampled pigs and sheep were <20 weeks and >2 years old, respectively.

2.2. RNA extraction and real-time RT-PCR

A pea-sized portion of faecal material was resuspended in 2 mL PBS and 200 μL chloroform and clarified by centrifugation at 12,000 × g for 10 min at 4 °C. Viral RNA was extracted from the concentrates (200 μL) using the high pure viral nucleic acid kit (Roche Molecular Biochemicals Ltd., Mannheim, Germany) as per the manufacturer’s instructions. Multiplex two step real-time RT-PCR for the detection of NoV genogroup I–III was carried out as described elsewhere (Wolf et al., 2007). All real-time assays were carried out in a Rotor-Gene™ 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia).

2.3. Confirmation of NoV-positive specimens by RT-PCR

Pig faecal specimens that were positive for NoV were retested using a semi-nested RT-PCR with the Mon431 ‘region B’ (Vinje et al., 2003) forward primer and the ‘region C’ (Kojima et al., 2002) reverse primer for the primary PCR and the ‘region C’ forward and reverse primers for the semi-nested PCR. One of the ovine specimens positive for NoV by real-time RT-PCR underwent several successive overlapping RT-PCR reactions in the RNA-dependent RNA polymerase (RdRp) and capsid regions with degenerate primers that were based on aligned GIII NoV sequences retrieved from GenBank, and successive primers that were designed after the nucleotide sequence of the previous RT-PCR products had been analyzed: J4549f (5'-TTYTCCTTCTATGGBGATGATGA) and J5041r (5'-TCAGTCATCTTCATTACAAXATC), J4972f (5'-CGCTCCATGTCCCCCCGTAAGTTT), J5468r (5'-ATCAACATGGRGAAACTG), J5411f (5'-GTCTTTTGCAATTTCAACCAATC), J5988r (5'-TCGAAAAATCTGGAAAGGG), J5938f (5'-GTTTACACCGTTTAAC), J5938f (5'-GTCTTTTGCAATTTCAACCAATC), J5988r (5'-TCGAAAAATCTGGAAAGGG), J5938f (5'-GTTTACACCGTTTAAC), J5938f (5'-GTCTTTTGCAATTTCAACCAATC), J5988r (5'-TCGAAAAATCTGGAAAGGG), J5938f (5'-GTTTACACCGTTTAAC). The total nucleotide sequence of the RT-PCR reactions covered a stretch of 2045 bases (ca. 28%) of the ovine NoV genome.

The 10-μL RT reactions comprised 100 units SuperScript III reverse transcriptase, 10 units RNase inhibitor (RNaseOUT™, Invitrogen, CA, USA), 100 nM of reverse primer, 1 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1 × first strand RT buffer...
(50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂) and 5-µL viral RNA. RT was carried out at 50 °C for 30 min followed by 95 °C for 4 min. PCR was carried out using a Taq PCR master mix kit (Qiagen Inc., Germany). Cycling conditions were 95 °C for 2 min then cycling at 94 °C for 30 s, annealing for 30 s and 72 °C for 60 s with a final extension step for 7 min at 72 °C. Cycle numbers (25–40) and annealing temperatures (50–59 °C) varied between assays depending on the primer set. PCR products were electrophoresed on a 2% (w/v) agarose gel and were either directly purified or, in case of multiple bands, the expected product size bands were excised. In both cases purification was carried out using the QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions.

2.4. Nucleotide sequencing and analysis

DNA sequencing was carried out in both directions using the Big Dye-Terminator cycling methodology (Applied Biosystems Corp., USA) and an automated sequencer ABI 3130XL (Applied Biosystems). Sequences were assembled using BioNumerics, version 4.6. After primer sequences were removed, sequences were aligned using ClustalW, implemented in MEGA4 (Tamura et al., 2007). Phylogenetic analysis was performed with 2000 replicates for bootstrapping by using the neighbor-joining method of the tree-builder tool of the same software. Sequence similarities were calculated using Geneious, version 3.0.6. The sequences of the porcine and ovine NoV strains, Po/NLV/0711/2007/NZL and Ov/NLV/ Norsewood30/2007/NZL, have been submitted to the GenBank database under accession numbers EU193659 and EU193658, respectively.

2.5. Electron microscopy

The ovine faecal specimen that produced the lowest-cycle threshold (C₅) value for NoV GIII in the real-time PCR was screened for caliciviruses by electron microscopy. For this 10 mL of faecal suspension were clarified as described above. The supernatant was centrifuged at 100,000 × g for 2 h at 4 °C. The sample was analyzed under the electron microscope (Phillips CM100, Eindhoven, The Netherlands) after negative staining with 2% potassium phoshotungstate.

3. Results

Eight out of 33 (24%) sheep faecal specimens tested positive for NoV GIII and tested negative for NoV GI and II by real-time RT-PCR. All positive specimens were from farm C and came from animals that showed

![Fig. 1. Electron micrograph showing norovirus particles present in a sheep faecal specimen stained with 2% potassium phosphotungstate.](image-url)
no obvious clinical signs. The specimen with the
highest virus load, as defined by the lowest-C<sub>t</sub> value
data not shown), was analyzed by electron microscopy
(EM) and revealed the presence of particles with
characteristic norovirus size and morphology (Fig. 1).
In order to genetically characterize the virus, RNA of
this specimen underwent several successive over-
lapping RT-PCR reactions and subsequent nucleotide
sequencing. The assembled sequences covered a total
of 2045 bases of the partial RdRp and complete capsid
genes (nucleotide 4582 to nucleotide 6631 in Bo/NLV/
Newbury2/UK; AF097917). A BLAST query of the
nucleotide sequence confirmed that the ovine strain is
related to other NoV GIII strains. The sequence shared
71.8% (77.1% amino acid [aa]) and 72.2% (76.3% aa)
pairwise similarity with the NoV GIII prototype strains
GIII.1-Jena and GIII.2-Newbury2, respectively. If
compared separately with the GIII prototype strains,
the pairwise similarity of the partial RdRp and the
complete capsid genes of the ovine NoV were 79.1%
(91.4% aa) and 69.8% (72.6% aa) to GIII.1-Jena and
77.7% (91.4% aa) and 70.7% (71.5% aa) to GIII.2-
Newbury2. The phylogenetic analysis suggests that the
strain is genetically different to both GIII.1-Jena-like
and GIII.2-Newbury/2-like clusters (Fig. 2). The
sequenced region of the ovine NoV showed 100%
homology to the probe and forward primer sequence
and one mismatching nucleotide position to the reverse
primer sequence of the multiplex real-time RT-PCR
assay originally used for the detection of the virus.

Two out of 23 (9%) pig faecal specimens gave
positive results for NoV GII by real-time RT-PCR, but
were negative for NoV GI and III. The two positive
porcine faecal specimens originated from 20-weeks
old clinically normal pigs on farm A, and were
confirmed by RT-PCR, producing amplicons of 343 bp
as defined by the ‘region C’ primers (Kojima et al., 2002). Sequence analysis using BLAST showed the sequences (both identical) with 93% nucleotide identities most closely related to Sw/NLV/Sw43/1997/JP (AB074892).

4. Discussion

Worldwide, farmed animals, including pigs and cattle, have been reported to be susceptible to NoV infections (Park et al., 2007; Reuter et al., 2007; Scipioni et al., 2008; Smiley et al., 2003; Sugieda et al., 1998; van der Poel et al., 2003; Wang et al., 2006; Wolf et al., 2007). The results presented here add sheep to the list of susceptible animals, and show PoNoV to be present in the Australasian region, thus confirming its global presence.

In our study, no obvious clinical signs were observed in pigs and sheep positive for NoV GII and III. This is in concordance with other reports for pigs, where NoV GII was exclusively found in faecal specimens of pigs without clinical signs (Wang et al., 2005, 2006) or any history of diarrhoea (Reuter et al., 2007). NoV GIII has been found in calves and cattle with diarrhoea but also in clinically normal animals (Han et al., 2005; Smiley et al., 2003; van der Poel et al., 2003). Generally, NoV in infected bovines are considered to be benign pathogens that could facilitate or complicate gastroenteritis (Scipioni et al., 2008). NoV infections in sheep may therefore produce similarly mild symptoms or be subclinical.

Previously NoV strains of genogroup III have been known to only infect cattle. Both cattle and sheep belong to the same family Bovidae and are ruminants with a similar diet and digestive system. This may be analogous to humans and pigs which, although genetically less closely related than sheep and cattle, have a similar digestive system and can be infected by genetically closely related NoV GII strains. Further, sheep and cattle may share a common reservoir for NoV GIII.

Phylogenetic analysis based on a 2045 bases long stretch of the partial RdRp and the complete capsid genes in the ovine NoV, suggests that the strain may belong to a currently uncharacterized cluster or genotype within NoV GIII. The nucleotide and amino acid similarity of the aligned sequences were <73% and <78% to both NoV GIII.1 and GIII.2 prototype strains. The nucleotide and amino acid sequences of the RdRp of the ovine NoV showed a greater similarity to both prototype strains than the capsid sequences. This may be due to the fact that the sequenced region of the RdRp is relatively short and is mainly located in the overlapping ORF1/ORF2 region, which is highly conserved in NoV. The analysis of the complete RdRp gene may reduce the percentage of similarity to other NoV GIII strains. The proposal of a new NoV genotype, NoV GIII.3, with Ov/NLV/Norsewood30/07/NZL as a prototype strain may be considered. However, a more comprehensive sequence analysis to include the whole genome of the ovine NoV is desirable and we plan to address this in a future study.

In conclusion, we provided the first evidence of NoV presence in faecal samples of sheep. Genetically the ovine strain is distinct from both the Jena and Newbury2 clusters and may represent a new NoV GIII genotype. Additionally NoV GII was identified in pigs for the first time in Australasia. The screening of larger sample numbers and a broader spectrum of animal faeces for NoV may help to both confirm actual carriage rates in animals and disclose potential reservoirs for zoonotic transmission of NoV between animals and humans. Our finding of ovine NoV also supports our recent suggestion to apply NoV GIII-specific assays for microbial source tracking (Wolf et al., 2007), because so far this genogroup is known to contain only animal NoV strains.

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