AUSTRALIAN VETERINARY JOURNAL
AUSTRALIA'S PREMIER VETERINARY SCIENCE TEXT
PRODUCTION ANIMALS

First detection of bovine noroviruses and detection of bovine coronavirus in Australian dairy cattle

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Background and objective Noroviruses have been recognised as a significant cause of neonatal enteritis in calves in many countries, but there has been no investigation of their occurrence in Australian cattle. This study aimed to establish whether bovine noroviruses could be detected in faecal samples from Australian dairy cattle. It also sought to determine whether bovine coronaviruses, also associated with neonatal enteritis in calves, could be detected in the same faecal samples.

Methods A selection of faecal samples that were negative for rotaviruses from dairy farms located in three geographically distinct regions of Victoria were pooled and tested by reverse transcription-PCR for the presence of noroviruses (genogroup III), neboviruses and bovine coronaviruses.

Results and conclusion Genetically distinct genogroup III noroviruses were detected in two sample pools from different geographic regions and bovine coronavirus was detected in a third pool of samples. This is the first report of bovine norovirus infection in Australian cattle and suggests that future work is required to determine the significance of these agents as a cause of bovine enteric disease in Australia.

Keywords bovine coronavirus; bovine norovirus; *Caliciviridae*; *Coronaviridae*; cattle; diarrhoea; enteritis

Abbreviations GIII.1, genogroup III, genotype 1; ORF, open reading frame; RT, reverse transcription; VLP, virus-like particle

Aust Vet J 2018;96:203–208 doi: 10.1111/avj.12695

Studies of viral causes of neonatal enteritis in Australia have focussed on rotaviruses, but several other enteric viruses, including caliciviruses and bovine coronavirus, have been identified in cattle in other countries. Bovine caliciviruses were first detected in the 1970s and 1980s in faeces from diarrhoeic calves in the UK[1,2] and Germany.[3] However, it was not until later that these viruses were confirmed to belong to the family *Caliciviridae*, based on their genomic sequences, and were found to belong to two separate genera, *Norovirus* (within genogroup III)[4,5] and *Nebovirus*.[6] The genogroup III noroviruses were further divided into genotypes,[7] with this division based on phylogenetic relatedness. The prototype strain for bovine genogroup III genotype 1 noroviruses is Jena virus, (GIII/Bo/DE/1980/GIII.1/Jena) and for bovine genogroup III genotype 2 noroviruses the prototype strain is Newbury agent-2 (GIII/Bo/UK/1976/GIII.2/Newbury2). In humans, noroviruses are one of the most important aetiological agents of gastroenteritis,[8] but our understanding of their significance as a cause of diarrhoea in cattle is limited.

Since their initial detection, a number of studies have used reverse transcription (RT)-PCR-based molecular detection methods to ascertain the presence and prevalence of these agents in cattle in a limited number of countries. In addition to the UK and Germany, bovine noroviruses have been detected in the Netherlands,[9,10] the USA,[11,12] New Zealand,[13] South Korea,[14] Norway,[15] France,[16] Turkey[17] and Tunisia[18] and neboviruses have been detected in South Korea,[19] France,[16] Tunisia[18] and the USA.[12]

Bovine coronaviruses are also associated with diarrhoea in cattle. These viruses belong to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae,* genus *Betacoronavirus* and are enveloped, positive sense RNA viruses.[20] In Australia, bovine coronaviruses have been detected in association with diarrhoea[21] and respiratory disease in cattle.[22]

As no bovine caliciviruses from either genus have been detected in Australia, the aim of this study was to search for Australian bovine noroviruses and neboviruses in faeces from diarrhoeic calves and, if they were detected, to compare them with strains characterised in other countries.

Materials and methods

In 2006, faecal samples were collected from calves with diarrhoea on dairy farms in three Victorian regions: South Gippsland, Northern Victoria and the Western District. Table 1 summarises the farms sampled in each of these regions. Faecal samples were classified based on the severity of diarrhoea, on a scale from 1 to 3. For this study, samples that were scored as most severe (3) but were negative for rotavirus, based on polyacrylamide gel electrophoresis of phenol/chloroform extracted RNA (data not shown), were selected for analysis. These samples were pooled by region, as detailed in Table 2.

Pooled faecal samples were diluted 1 in 5 and homogenised in phosphate-buffered saline. Samples were centrifuged at 2700*g* for 10 min to remove larger particulate matter before the nucleic acid was extracted with the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For RT, 5 μL of the extracted nucleic acid was mixed with 100 ng of random oligonucleotide hexamers and incubated at 80°C for 5 min before being placed on ice. After the addition of 1 × first strand buffer (Life Technologies, Carlsbad, CA, USA), 1.5 mmol/L dNTP, 10 mmol/L DTT, 20 U RNaseOUT (Life Technologies) and 100 U SuperScript

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Table 1. Faecal samples collected in 2006 from calves with diarrhoea on dairy farms in three regions of Victoria, Australia: South Gippsland, Northern Victoria and the Western District

Region	Farm	Herd size	Samples	Rotavirus positive	Date of collection
South Gippsland	1	130	3	0	27/07/2006
	1a	–	3	0	27/07/2006
	2	–	18	1	27/07/2006
	3	–	15	1	27/07/2006
	4	–	9	2	27/07/2006
	5	–	12	0	27/07/2006
	6	–	13	1	27/07/2006
Northern Victoria	A	–	18	0	24/08/2006
	B	–	14	0	24/08/2006
	C	–	9	0	24/08/2006
	D	–	27	0	24/08/2006
	E	–	16	1	24/08/2006
	F	–	18	0	24/08/2006
	G	–	10	0	24/08/2006
	P	–	9	3	24/08/2006
	Q	–	19	0	24/08/2006
	X	–	12	0	24/08/2006
Western District	A	240	16	0	26/06/2006
	B	300	4	0	26/06/2006
	C	350	7	0	26/06/2006
	D	500	21	1	26/06/2006
	E	400	18	5	26/06/2006
	F	400	16	1	26/06/2006
	G	200	10	6	26/06/2006
	H	600	13	1	26/06/2006
	L	–	1	1	26/06/2006

Table 2. Pooled faecal samples from dairy farms across three regions of Victoria, Australia, with a diarrhoeal severity score of 3, excluding rotavirus positive samples

Region	Pool	Farm (no. of samples)
South Gippsland	SG2	2 (5)
	SG3	3 (4)
	SG5	5 (7)
	SG6	6 (4)
Northern Victoria	NVD	D (7)
	NVF	F (7)
	NV-Mix	B (2), C (1), G (2), P (1), Q (1), X (1)
Western District	WD-Mix	B (2), D (1), F (1), G (1), H (1)

III (Life Technologies), reactions were incubated at 50°C for 50 min, then at 70°C for 15 min.

PCR reactions used the following primer sets: CBECU-F/R,[23] designed to hybridise with the conserved YGDD polymerase motif and the open reading frame (ORF)1/2 junction of bovine noroviruses (genogroup III); NBU-F/R,[23] designed to hybridise to neboviruses in the same region as CBECU-F/R; p289/p290,[24] designed to hybridise to the polymerase region of the genome of human caliciviruses, but believed to be broadly reactive; and BCoV-fwd and BCoV-rev,[25] designed to target the gene encoding the nucleocapsid protein of the Nebraska strain of bovine coronavirus, with modification of the forward primer (BCoV-fwd alt: 5′ CTAACAAGCAGGCTGATGTTAATACC) that allowed detection of equine coronavirus. A positive control was not available for the bovine caliciviruses, but a coronavirus positive control was included (an 87-bp BCoV-fwd alt/BCoV-rev equine coronavirus amplicon cloned into pGEM-T; Promega, Madison, WI, USA). Negative controls (sterile water) were included in all reactions. Reactions for the calicivirus assays included 1 × GoTaq Flexi buffer (Promega), 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.4 µmol/L of each of the forward and reverse primers and 1 U GoTaq polymerase (Promega). Reactions for the coronavirus assays were identical except that 2.0 mmol/L of $MgCl_2$ was used. The same incubation conditions were used for all reactions: 1 cycle at 94°C for 1 min; 30 cycles of 94°C for 30 s, 45°C for 30 s and 68°C for 40 s; and 1 cycle of 68°C for 7 min. For PCR product visualisation, 5 µL of each reaction was electrophoresed through a 2% (w/v) agarose gel containing SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) in 0.5 × TBE buffer (1 × TBE is 89 mmol/L Tris, 89 mmol/L

boric acid, 2 mmol/L EDTA, pH 8.3) and an image of the gel was captured with a Molecular Imager ChemiDoc XRS+ imaging system (Bio Rad, Hercules, CA, USA) using transillumination with ultraviolet light.

Amplified DNA from the RT-PCR assay identified as positive on the gel was purified using QIAquick Gel Extraction kits (Qiagen) prior to sequencing using the BigDye version 3.1 cycle sequencing kit (Life Technologies). Bioinformatic analyses of the resulting sequences were performed using the program Geneious (Biomatters: http://www.geneious.com).

Results

A product of the expected size was amplified from the pooled samples SG5 and WD-mix with CBECU-F/R primer set, which targeted the genogroup III noroviruses, and from the SG6 sample with the BCoV-fwd alt/BCoV-rev primer set (Table 3). DNA sequencing was used to confirm the similarity of the products with the sequences of genogroup III noroviruses and the BCoVs, respectively.

A comparison of the 271 nucleotides (equivalent to nucleotides 4777–5047 of GIII/Bo/UK/1976/GIII.2/Newbury2, AF097917) for which high-quality sequence obtained from the norovirus products amplified from the SG5 and WD-mix pools detected 11 nucleotide differences (Figure 1) and resulted in a nucleotide sequence identity of 95.9%. None of the nucleotide changes in this 271 nucleotide region equated to amino acid differences between SG5 and WD-mix. However, SG5 and WD-mix showed a single amino acid difference from Newbury2 and 11 amino acid differences from Jena across the 90 deduced amino acids. A search of GenBank using BLAST revealed that the virus with the highest nucleotide sequence identity with the SG5 product was GIII/Bo/NOR/2006/GIII.2/340_1235 (FM242185), with 94.8% nucleotide identity, and the most similar viruses to that detected in the WD-mix were GIII/Bo/NOR/2006/GIII.2/216_0114 (FM242188) and GIII/Bo/NOR/2006/GIII.2/340_1235 (FM242185), both with 95.2% nucleotide sequence identity. These strains were detected in Norway in 2006 and belong to genotype GIII.2, with the prototype strain being GIII/Bo/UK/1976/GIII.2/Newbury2.

Genotyping into norovirus GIII.2 was confirmed by phylogenetic analysis with high bootstrap support (Figure 2), with the two Australian viruses grouping most closely with each other and the Norwegian viruses.

Discussion

The prevalence of bovine noroviruses has been determined in several countries, but the data are not always comparable because the different studies have had different designs. These differences include the detection method and the primer pairs used, the samples collected (diarrhoeal, non-diarrhoeal or both), pooled or individual samples and inclusion or exclusion of samples in which other pathogens have been detected. Reported detection rates for the bovine noroviruses range from as low as 8.6% of diarrhoeic faecal samples in Turkey[17] and 9.3% of faecal samples from a study in South Korea[14] to as high as 49.6% in a study from Norway[15] and 53.6% in a study from New Zealand.[13] In the current study, bovine noroviruses were detected in two of the eight pooled samples from two different geographical regions.

Because of the pooled nature of the samples tested, it was not possible to determine the prevalence of these viruses. However, given that the bovine noroviruses were detected in two geographically distinct regions of Victoria (South Gippsland and the Western District) and that the sequences obtained were different from each other at the nucleotide level, suggests that the GIII bovine noroviruses may be common in Australian cattle.

Both noroviruses detected in this study were from the GIII.2 genotype. This is consistent with the observation that in more recent years the GIII.2 genotype has predominated over the GIII.1 genotype,[14–18] although in a New Zealand study only GIII.1 bovine noroviruses were detected.[13]

As a primary focus of this study was to detect and describe bovine caliciviruses in Australian cattle for the first time, faecal samples were pooled. However, to reduce the dilution effect of pooling too many samples, some samples were excluded from the study. Samples were excluded that had previously tested positive for rotaviruses or

Table 3. Amplification of enteric viruses from pooled faecal samples from dairy farms across three regions of Victoria, Australia

Region	Pool	Primer set (expected size)			
		CBECU-F/R (532 bp)	NBU-F/R (549 bp)	p289/p290 (~319–331 bp)	BCoV-F/R (87 bp)
South Gippsland	SG2	–	–	–	–
	SG3	–	–	–	–
	SG5	+	–	–	–
	SG6	–	–	–	+
Northern Victoria	NVD	–	–	–	–
	NVF	–	–	–	–
	NV-mix	–	–	–	–
Western District	WD-mix	+	–	–	–

bp, base pairs.

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                                                                      60
                                                                      |
SG5         TCAATACTCCGACAGCTCTATTGGACTCGTGGACCCAACCATGATGACCCGAGTGAGACC
WD-mix      ................T.....................C..................
Newbury2    ..C........G.....T..C...........G..T.................C..A...
Jena        ..C..CT.G..C.....T...........C..CT................ATCA....G.

                                                                     120
                                                                     |
SG5         CTGGTCCCCCACAGCAACCGAAAAGTCCAGCTGCTTTGCCTCCTTGGCGAGGCGGCCCTC
WD-mix      ......................G..T.....................A.........
Newbury2    ..................G.....T...T....C........C..T..A..T......
Jena        A...........TCT.....C..GA..........CA..T.G..A..T..A..CT.....

                                                                     180
                                                                     |
SG5         CATGGCGAGAAGTTCTACCGCCGAGTTGGCTCTATGGTGACTGCAGAGGCCAAAGAAGGG
WD-mix      ..............T..T.......................................
Newbury2    ..C..T.....A..T..T.....G..GA....C.....C..............G......
Jena        .....G..A..............C........GC.......C..C..A...CGCAGC..C

                                                                     240
                                                                     |
SG5         GGGATGGAGATTTTTGTACCATCGCACCGCTCCATGTTTGCTTGGATGAGATTTCATGAT
WD-mix      ..................G..A.....................................
Newbury2    ..A........C.....................................................
Jena        ..A.................T..TTT..............C......C.T.........

                                    271
                                    |
SG5         TTGTCGTTGTGGGAAGGTAGTCGCGACACTC
WD-mix      C.....C........................
Newbury2    .....A.......................C.
Jena        ......C.....................TC.
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Figure 1. Nucleotide alignment between bovine norovirus sequences from Jena virus (GIII/Bo/DE/1980/GIII.1/Jena, GenBank accession AJ011099), Newbury agent-2 (GIII/Bo/UK/1976/GIII.2/Newbury2, GenBank accession AF097917) and pools SG5 and WD-mix. Dots represent identical residues when compared with SG5 as a reference. Alignment spans the nucleotides equivalent to 4777–5047 of Bo/Newbury2/1976/UK.

that scored less than 3 on the diarrhoea severity score. The decision to assay the more clinically severe diarrhoeal samples was supported by the observation that bovine noroviruses have been found to be more commonly associated with watery faeces.[12] Although samples testing positive for rotavirus were excluded from the pools in our study, mixed infection with bovine noroviruses and bovine rotaviruses have been described previously,[12] so these results likely underestimate the frequency of bovine noroviruses in our sample set.

In addition, there are a number of ways in which the detection of viruses in these samples could have been increased. These include assaying individual samples (rather than pooled samples), additional primer sets (to encompass more of the genetic diversity of noroviruses) and increasing the number of cycles in the PCR screening step. The faecal samples in this study were collected in 2006 and stored at −70°C. The viral extractions were performed 7 years later, in 2013. The length of time spent in storage may have affected the integrity of the viral RNA in the samples, which could explain why only short sequences were recovered.

The bovine norovirus sequences in this study were obtained from the relatively highly conserved polymerase-encoding region of the genome, as the conservation of this area makes the initial amplification more likely. However, in the future it would be beneficial to also determine the sequence of the capsid-encoding region, because this region provides important information about the phylogenetic grouping of these viruses. The capsid sequence could also be used to establish whether the isolates in this study had undergone recombination, which in the caliciviruses most commonly occurs between the polymerase and capsid-encoding regions of the genome.[26]

To date, there have been no reports of bovine noroviruses associated with disease in humans. However, there has been speculation about the possibility of zoonotic transmission.[27–29] Veterinarians in the Netherlands, particularly those with exposure to cattle, were more frequently found to have IgG antibodies against recombinant bovine norovirus virus-like particles (VLP) than the general population, who are less likely to come into contact with cattle.[27] In addition, a study looking at the acquisition of antibodies to different norovirus genogroups in children in India found antibodies against bovine noroviruses, using recombinant VLPs.[30] However, cross-reactivity could not be ruled out in these studies. In fact, cross-reactive epitopes have been found in bovine and human norovirus capsids.[31,32]

Given that bovine noroviruses have not been reported in humans and human noroviruses have only been detected very infrequently in cattle,[33] if zoonotic transmission is possible, it is likely to be an uncommon occurrence. Recombination, including between genogroups, has been reported for noroviruses and other caliciviruses.[34–38] Norovirus recombination most commonly occurs at the junction of the first two ORFs.[26,39,40] This region of the genome is highly conserved and it is thought that the RNA

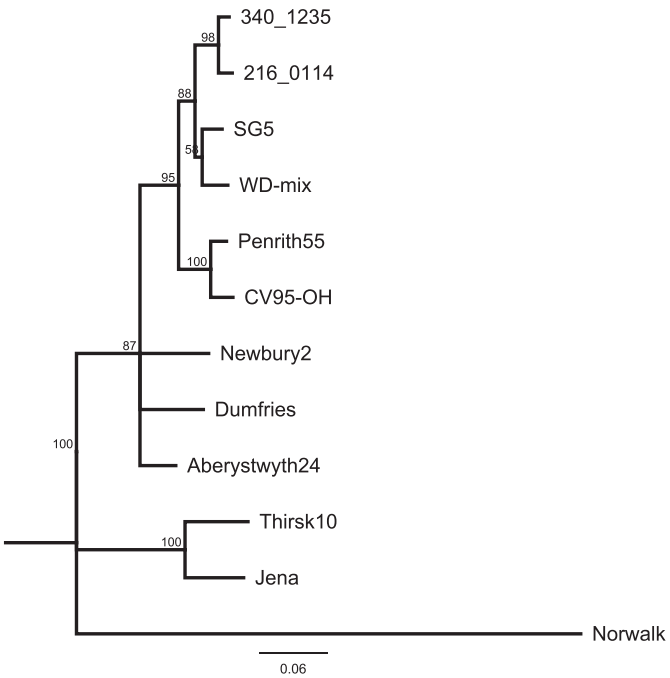

Figure 2. Neighbour joining phylogenetic tree with 1000 bootstrap replicates showing the relationship of two Australian GIII noroviruses to other bovine noroviruses. The human GI.1 Norwalk (GI/Hu/USA/1968/GI.1/Norwalk, M87661) virus was used as an outgroup. Reference GIII bovine noroviruses include the GIII.1 viruses: Jena (GIII/Bo/DE/1980/GIII.1/Jena, AJ011099) and Thirsk10 (GIII/Bo/UK/2000/GIII.1/Thirsk10, AY126468); and the GIII.2 viruses: Newbury2 (GIII/Bo/UK/1976/GIII.2/Newbury2, AF097917), Dumfries (GIII/Bo/UK/1994/GIII.2/Dumfries, AY126474), Penrith55 (GIII/Bo/UK/2000/GIII.2/Penrith55, AY126476), CV95-OH (GIII/Bo/USA/2002/GIII.2/CV95-OH, AF542083), Aberystwyth24 (GIII/Bo/UK/2000/GIII.2/Aberystwyth24, AY126475), 340_1235 (GIII/Bo/NOR/2006/GIII.2/340_1235, FM242185) and 216_0114 (GIII/Bo/NOR/2006/GIII.2/216_0114, FM242188). The tree is based on the alignment of 271 base pairs from the polymerase region of the genome. Bootstrap values are expressed as percentages and are shown at the branch points. Scale bar represents substitutions per site.

secondary structure in this region is involved in the recombination process.[39] As the majority of noroviruses cannot be cultured, the precise mechanisms of recombination have not been extensively studied and it is not known how much conservation of the RNA sequence or secondary structure conservation is required to allow two strains to recombine.

Although the primary aim of this study was to investigate bovine noroviruses and neboviruses in Australian samples of calf diarrhoea, as neither of these agents has been detected in this country, a secondary aim was to investigate other viral agents of diarrhoea. The samples were previously screened for rotavirus and only samples that were negative for rotavirus were included in further screening. Bovine coronaviruses, which are known to be associated with diarrhoea in Australian calves,[21] were also investigated. Although one pooled sample was found to be positive for bovine coronavirus by RT-PCR and confirmed by sequencing, the length of the amplicon was insufficient to provide any meaningful sequence comparison with other bovine coronavirus sequences. However, it does support the association of this virus with diarrhoea in Australian cattle and suggests it is a potential aetiological agent. Further sequence information from this virus in the future would be informative.

This study is the first report of bovine noroviruses in Australia. Bovine norovirus was detected in faecal samples from two geographically distinct regions and sequence analysis found that these viruses both clustered with the GIII.2 bovine noroviruses. Although this report establishes that bovine noroviruses are present in Australian dairy calves, further investigations are required to fully understand the role of bovine norovirus in diarrhoeal disease in Australian cattle.

Acknowledgment

We thank Cynthia Brown, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, for excellent technical assistance.

Conflicts of interest and sources of funding

The authors declare no conflicts of interest for the work presented here.

Funding for this study was provided by a Special Virology Fund.

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(Accepted for publication 22 August 2017)