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

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S tudies 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 UK1,2 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 Turkey17 and Tunisia18 and neboviruses have been detected in South Korea,19 France,16 Tunisia18 and the USA.12

Bovine coronaviruses are also associated with diarrhoea in cattle. These viruses belong to the order Nidovirales, family Coronaviridae, subfamily Coronavirusae, genus Betacoronavirus and are enveloped, positive sense RNA viruses.20 In Australia, bovine coronaviruses have been detected in association with diarrhoea21 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 2700g 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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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, 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, designed to hybridise to neboviruses in the same region as CBECU-F/R; p289/p290, 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’CTAACCGCCGGGCTGATGTTAC); and BCoV-fwd and BCoV-rev, 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’CTAACCGCCGGGCTGATGTTAC).

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 MgCl2, 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 MgCl2 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

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         | 4       | 26/06/2006         |
| C                  | 350  | 7         | 7       | 26/06/2006         |
| D                  | 500  | 21        | 21      | 26/06/2006         |
| E                  | 400  | 18        | 18      | 26/06/2006         |
| F                  | 400  | 16        | 16      | 26/06/2006         |
| G                  | 200  | 10        | 10      | 26/06/2006         |
| H                  | 600  | 13        | 13      | 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)                 |
|                    | SGS    | 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) |

the same region as CBECU-F/R; p289/p290, 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, 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’CTAACCGCCGGGCTGATGTTAC) 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 MgCl2, 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 MgCl2 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 XR+ 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.2/340_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 and 9.3% of faecal samples from a study in South Korea to as high as 49.6% in a study from Norway and 53.6% in a study from New Zealand. 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, although in a New Zealand study only GIII.1 bovine noroviruses were detected.

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

bp, base pairs.
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 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 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 norovirus-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.34–36 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
suggests it is a potential aetiologic 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 diarrheal disease in Australian cattle.

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**Conflicts of interest and sources of funding**

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

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