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Research paper

Molecular detection of bovine Noroviruses in Argentinean dairy calves: Circulation of a tentative new genotype

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ABSTRACT

Bovine noroviruses are enteric pathogens detected in fecal samples of both diarrheic and non-diarrheic calves from several countries worldwide. However, epidemiological information regarding bovine noroviruses is still lacking for many important cattle producing countries from South America. In this study, three bovine norovirus genogroup III sequences were determined by conventional RT-PCR and Sanger sequencing in feces from diarrheic dairy calves from Argentina (B4836, B4848, and B4881, all collected in 2012). Phylogenetic studies based on a partial coding region for the RNA-dependent RNA polymerase (RdRp, 503 nucleotides) of these three samples suggested that two of them (B4836 and B4881) belong to genotype 2 (GIII.2) while the third one (B4848) was more closely related to genotype 1 (GIII.1) strains. By deep sequencing, the capsid region from two of these strains could be determined. This confirmed the circulation of genotype 1 (B4848) together with the presence of another sequence (B4881) sharing its highest genetic relatedness with genotype 1, but sufficiently distant to constitute a new genotype. This latter strain was shown *in silico* to be a recombinant: phylogenetic divergence was detected between its RNA-dependent RNA polymerase coding sequence (genotype GIII.2) and its capsid protein coding sequence (genotype GIII.1 or a potential norovirus genotype). According to this data, this strain could be the second genotype GIII.2_GIII.1 bovine norovirus recombinant described in literature worldwide. Further analysis suggested that this strain could even be a potential norovirus GIII genotype, tentatively named GIII.4. The data provides important epidemiological and evolutionary information on bovine noroviruses circulating in South America.

1. Introduction

Noroviruses are a major cause of epidemic and sporadic gastroenteritis in humans (Green, 2013). They were also identified in cattle and other animal species with reports of lesions and clinical signs of gastroenteritis (Bridger et al., 1984; Hsu et al., 2007; Martella et al., 2007; Ntafis et al., 2010; Pinto et al., 2012; Scipioni et al., 2008a; Sugieda and Nakajima, 2002; Wang et al., 2007; Wolf et al., 2009). Noroviruses belong to the genus *Norovirus*, within the Caliciviridae family. They are non-enveloped viruses with a diameter of approximately 27–40 nm and possess a positive-sense, single stranded RNA genome of around 7.5 kb composed of three open reading frames (ORF) (Green, 2013). The ORF1 encodes a large polyprotein that is further cleaved into at least six non-structural proteins by the viral 3C-like protease, including the RNA-dependent RNA polymerase (RdRp, P). ORF2 and ORF3 encode the major (VP1) and minor (VP2) capsid proteins, respectively (Green, 2013). The VP1 capsid protein is organized into two domains: a shell domain (S domain, N-terminal part) and a protruding domain (P domain, C-terminal part, exhibiting two sub-domains: P1 and P2) (Prasad et al., 1999). The P2 is a hypervariable domain and has an external localization, compatible with its function of both cell receptor ligand and immunogenic determinant (Tan et al., 2004).

Noroviruses are genetically and antigenically diverse (Green, 2013; Zheng et al., 2006). As no cell culture system has been established for growing Norovirus (with the exception of murine strains), currently reverse transcription-polymerase chain reaction (RT-PCR) and genomic sequencing are used for characterizing viruses and understanding the

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phylogenetic relationships among strains detected in animals and humans worldwide. Routinely, Noroviruses are genotyped by analysis of partial capsid (VP1) or polymerase (RdRp) gene sequences. However, genotyping based exclusively on one region of Norovirus genome is not an accurate representation of viral epidemiology, especially due to frequent recombination within Noroviruses (Bull et al., 2007). Recombination commonly occurs at the ORF1–ORF2 junction although other recombination sites have been reported (Bull et al., 2005, 2007; Chhabra et al., 2010; Eden et al., 2013). To address the inconsistencies in Norovirus genotyping, a nomenclature system which incorporates both polymerase and capsid regions has been proposed (Kroneman et al., 2013). At least six genogroups (GI to GVI) have been described in the genus *Norovirus* and a tentative genogroup VII has been proposed (Green, 2013; Vinjé, 2015).

Currently, bovine Norovirus sequences described so far cluster within GIII. This genogroup is further subdivided into genotypes: GIII.1 and GIII.2 (Di Martino et al., 2014; Green, 2013; Mauroy et al., 2009a; Oliver et al., 2003) including all bovine Norovirus strains and a proposed GIII.3 containing ovine Noroviruses (Wolf et al., 2009). The bovine Norovirus strains Bo/DE/1980/GIII.1/Jena (Gunther and Otto, 1987; Liu et al., 1999; Otto et al., 2011) and Bo/UK/1976/GIII.2/Newbury2 (Dastjerdi et al., 1999; Woode and Bridger, 1978) are the prototypes for GIII.1 and GIII.2, respectively, while Ov/NZL/2007/GIII.3/Norsewood30 is the prototype strain for GIII.3 (Wolf et al., 2009). Genetic studies on bovine Noroviruses have been hampered by the small number of partial sequences available and, more importantly, the paucity of complete genome sequences (Di Martino et al., 2014; Mauroy et al., 2012, 2014).

Viral RNA recombination is a major evolutionary mechanism for viruses. It can affect phylogenetic grouping, virulence, prophylactic and diagnostic approaches. Bull et al. defined recombinant Norovirus as those that cluster with two distinct groups of Norovirus strains when two different regions (normally, the capsid and the RdRp) of the genome are phylogenetically analyzed (Bull et al., 2005). Studying all available sequences in 2007, Bull et al. reported the existence of two recombinant prototypes for bovine Noroviruses: GIII.P1_GIII.2 and GIII.P2_GIII.1 with Bo/UK/2000/GIII.P1_GIII.2/Thirsk10 and Bo/US/2003/GIII.P2_GIII.1/B-1SVD as reference strains, respectively (Bull et al., 2007; Oliver et al., 2004). Since then, molecular characterization of bovine Noroviruses from diverse geographical settings have revealed the circulation of a number of recombinant strains genetically related to Bo/UK/2000/GIII.P1_GIII.2/Thirsk10 (Di Martino et al., 2014; Han et al., 2004; Jor et al., 2010; Mauroy et al., 2009b). However, to our knowledge no other bovine Norovirus strains related to Bo/US/2003/GIII.P2_GIII.1/B-1SVD have been reported.

Livestock industry is a major economic activity in Argentina. Among cattle infectious diseases, neonatal calf diarrhea is considered an important sanitary problem of both beef and dairy cattle (Virtala et al., 1996). Molecular detection of bovine Norovirus sequences has been frequently associated with calf diarrhea (Scipioni et al., 2008b). Their pathogenicity is believed to be low or moderate (Jung et al., 2014), although studies analyzing their economic impact (weight loss, health status, veterinary treatment cost) as co-infecting agent in combination with other enteric pathogens have not yet been conducted. Molecular characterizations of bovine Noroviruses circulating in cattle from different regions of the world have been performed but still there is scarce data regarding South American countries, where large cattle populations are living, such as Argentina or Brazil. The aim of this study was to investigate the presence and genetic diversity of bovine Noroviruses in fecal samples from Argentinean diarrheic dairy calves.

2. Materials and methods

2.1. Fecal samples

Between 2008 and 2012, a total of 90 fecal samples from dairy calves suffering from diarrhea were collected from herds belonging to the major dairy regions of Argentina. The provinces included were Buenos Aires, Córdoba, Santa Fe and Entre Ríos. The ages of the sampled calves ranged from 1 to 90 days old. From these, 69 samples were received at the Diagnosis Service of the Virology Institute of the National Institute of Agriculture Technology (INTA) between 2008 and 2012. These 69 samples tested negative for Group A Rotavirus (RVA) and Bovine Coronavirus (BCoV). Screening for bovine RVA was performed using an antigen capture ELISA (Cornaglia et al., 1989) while screening for BCoV was done using an ELISA based on monoclonal antibodies previously described (Smith et al., 1996). These results were reported elsewhere (Badaracco et al., 2012; Bok et al., 2015).

The other 21 samples were selected from 11 dairy herds included in a statistically designed survey carried out in Southern Buenos Aires province to study the microbiology of calf diarrhea in dairy herds from the dairy region named “Cuenca Lechera Mar y Sierras” between 2008 and 2009, These 21 samples tested negative for RVA, BCoV, *Salmonella* ssp. and *Cryptosporidium* ssp.

2.2. RNA extraction and bovine Norovirus one step RT-PCR

For the viral assays, fecal suspensions of samples were prepared by diluting feces 1:4 in distilled water. The suspensions were then vortexed for 15 s, centrifuged at 2500*g* for 5 min, and supernatants were collected and stored at −80 °C for further testing. The RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) following manufacturer's instructions from a 200 μl starting volume of the centrifuged fecal suspensions. The recovered RNA was eluted in 40 μl of elution buffer and stored at −80 °C until further use.

For the amplification of the partial RdRp coding region, a One Step RT-PCR kit was used (Qiagen) according to manufacturer's instructions. Primers used were CBECu-F (5′-AGTTAYTTTCCTTYTAYGGBGA-3′) and CBECu-R (5′-AGTGTCTCTGTCAGTCATCTTCAT-3′), with an expected amplicon size of 532 nt (Smiley et al., 2003b). The mixture was incubated for 30 min at 50 °C for reverse transcription, followed by 15 min at 95 °C for initial PCR activation. Then, 35 amplification cycles (1 min at 94 °C, 1 min at 46 °C and 2 min at 72 °C) were performed followed by a final extension step at 72 °C for 10 min. The amplification products were analyzed by 2% agarose gel electrophoresis and visualized under UV light. Agarose gel purification was performed on RT-PCR amplicons of the expected size using the QIAquick Gel Extraction kit (Qiagen). The RT-PCR products were Sanger sequenced directly in both directions obtaining a region spanning the primer binding areas.

2.3. Sample preparation for next generation sequencing

Fecal suspensions were homogenized for 1 min at 3000 rpm with a MINILYS homogenizer (Bertin Technologies, France) and filtered consecutively through 100 μm, 10 μm and 0.8 μm membrane filters (Millipore). The filtrate was then treated with a cocktail of Benzonase (Norovirusagen) and Micrococcal Nuclease (New England Biolabs.) at 37 °C for 2 h in a homemade buffer (1 M Tris, 100 mM $CaCl_2$ and 30 mM $MgCl_2$) to digest free-floating nucleic acids. RNA and DNA were extracted using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions, but without carrier RNA in the lysis buffer. First and second strand synthesis and random PCR amplification for 20 cycles were performed using a slightly modified Whole Transcriptome Amplification (WTA) Kit procedure (Sigma-Aldrich), with a denaturation temperature of 95 °C instead of 72 °C to allow for the denaturation of dsDNA and dsRNA. This modification leads to the amplification of both DNA and RNA. WTA products were purified with MSB Spin PCRapace spin columns (Stratec) and were prepared for Illumina sequencing using the KAPA Library Preparation Kit (Kapa Biosystems). Libraries were quantified with the KAPA Library Quantification kit (Kapa Biosystems) and sequencing of the samples was performed on a HiSeq 2000 platform (Illumina) for 201 cycles (100 bp paired ends).