Epidemiological study of bovine norovirus infection by RT-PCR and a VLP-based antibody ELISA

Axel Mauroy [a], Alexandra Scipioni [a], Elisabeth Mathijs [a], Claude Saegerman [a], Jan Mast [b], Janice C. Bridger [c], Dominique Ziant [a], Christine Thys [a], Etienne Thiry [a,*]

[a] Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium
[b] Veterinary and Agrochemical Research Centre, Department Biocontrole, Research Unit Electron Microscopy, Brussels, Belgium
[c] Department of Pathology and Infectious Diseases, Royal Veterinary College, London, United Kingdom

ABSTRACT

Noroviruses, belonging to the family *Caliciviridae*, have been identified in human beings and in several animal species including cattle. The distribution of bovine norovirus infections was investigated by both RT-PCR to detect norovirus genomes and a virus-like particles-based ELISA to detect genotype 2 bovine norovirus antibodies. During a 1-year systematic study, a virus prevalence of 7.5% (CI 95%: [3.7; 13.4%]) (10 out of 133 samples) was found in stool samples from diarrhoeic calves screened by RT-PCR. Nucleotide sequencing performed on the polymerase region classified all the norovirus amplicons in the bovine norovirus genotype 2. Rather surprisingly, some rotavirus sequences were also detected. On the basis of the polymerase region, genotype 1 bovine norovirus was not identified. Other enteropathogens were found in all samples. By ELISA, a genotype 2 seroprevalence of 93.2% (CI 95%: [90.4; 95.3%]) was found from calves and adult cattle. Antibody levels against genotype 2 bovine noroviruses rose in the first 6 months of life and were maintained in adults. Together the results of virus prevalence and seroprevalence studies suggest that bovine norovirus infection occurs early in life and that re-infection with serologically related bovine noroviruses strains could occur in adult cattle as reported for rotaviruses. The antibody rise against genotype 2 bovine noroviruses in the adult cattle also suggests a short lived and/or strain specific immunity as already shown in human noroviruses. Genotype 2 bovine noroviruses are endemic in the region investigated.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Noroviruses (NoV) belong to the family *Caliciviridae* and have been identified in the human being and in several animal species including the bovine (Scipioni et al., 2008b; Green, 2007). Noroviruses are small, round, non-enveloped viruses, with a 25–40 nm diameter and have a positive-sense, single-stranded RNA genome with three open reading frames (ORF). The non-structural proteins, including the viral RNA-dependent RNA polymerase (RdRp), the single capsid protein (VP1) and the minor structural protein are encoded by ORF1, 2 and 3, respectively (Green, 2007). Ninety dimers of VP1 form the capsid which presents an icosahedral symmetry under electron microscopy (EM). Capsid protein VP1 has a molecular weight of around 55–60 kDa and is organised into two domains: a shell (S) domain (N-terminal part of VP1) and a protruding (P) domain (C-terminal part of VP1) (Prasad et al., 1999). The P domain supports receptor recognition and immunogenic functions (Lochridge et al., 2005). Noroviruses are difficult to grow in cell culture but abundant quantities of virus-like particles (VLP) can be obtained by expressing VP1 in the baculovirus protein expression system. These VLP are morphologically and antigenically similar to the native strain (Jiang et al., 1992).

---

* Corresponding author. Tel.: +32 43664250; fax: +32 43664261.
 E-mail address: etienne.thiry@ulg.ac.be (E. Thiry).

Bovine norovirus (BoNoV) prototype strains identified so far are Newbury2 strain (NB2), previously known as Newbury Agent 2, and the Jena virus (JV). The NB2 and JV were identified by EM in the faeces of diarrhoeic calves in 1978 in Great Britain (Woode and Bridger, 1978) and in 1980 in Germany (Gunther and Otto, 1987). They were later genetically identified as belonging to the genus *Norovirus* (Liu et al., 1999; Oliver et al., 2003) where all BoNoV strains fall into the genogroup III (Ando et al., 2000). Two genotypes are described in the BoNoV genogroup: viruses genetically related to JV were assigned to the genotype 1 and viruses genetically related to NB2 to the genotype 2 (Ando et al., 2000).

Their relative environmental stability, their main faecal-oral transmission route, their low infectious dose and their large strain diversity increase the risk of cattle infection with these viruses (Green, 2007). In gnotobiotic calves, BoNoV induce non-hemorrhagic enteritis, mild diarrhoea, transient anorexia and malabsorption (Woode and Bridger, 1978). But the real impact of these viruses in the field is currently poorly evaluated. The two genotypes of BoNoV were recently shown to be endemic in Great Britain and Germany (Oliver et al., 2007b). They were also isolated in all continents and thus seem to be widespread (Scipioni et al., 2008b).

In this work, an original combined virological and serological approach was used to characterise the epidemiology of BoNoV infection in cattle. In a first virological approach, the molecular prevalence was investigated in diarrhoeic calf samples during a 1-year study. In a second serological approach, a VLP-based ELISA was developed and the cattle seroprevalence against BoNoV was estimated in the same region. Together the results suggest an association of BoNoV infections with the age of the animals.

2. Materials and methods

2.1. Bovine stool samples and genetic analysis

Stool samples from calves aged 1 week to 6 months were systematically received from a Belgian diagnostic laboratory each month through the year 2007. Samples came from different places out of five provinces of Belgium (Hainaut, Namur, Liege, Luxembourg, Walloon Brabant). Their origin was either from diarrhoeic samples ($n = 74$) submitted for etiologic diagnosis of gastroenteritis or from necropsy samples ($n = 59$) where gastroenteritis lesions were found. This stool bank ($n = 133$) was screened by one step RT-PCR for the presence of BoNoV genome sequences with Quick Access kit (Promega, Madison, WI, USA). Four different primer pairs were used to amplify segments of the polymerase gene and the beginning of the capsid region: CBECu (Smiley et al., 2003), JV12/13 (Vinje et al., 2003), P289/290 (Jiang et al., 1999) and BEC (Ike et al., 2007). These primers were selected in order to perform the broadest detection of NoV including a possible zoonotic transmission. Out of the BoNoV specific primers (CBECu and BEC), CBECu pair was seen to detect not only genotypes 2 but also genotypes 1 BoNoV with an apparent sensitivity of about 83% relative to the p289/290 pair in a previous study (Smiley et al., 2003). RT-PCR program consisted in a reverse transcription step of 45 min at 45 °C followed by the PCR reaction. It consisted in an initial denaturation step of 2 min at 94 °C followed by 40 PCR cycles (30 s denaturation step at 94 °C; 1 min annealing step at 48 °C for CBECu, JV12/13 and BEC primers, 51 °C for p289/290 primers; 2 min extension step at 68 °C) and a final extension step of 10 min at 68 °C. Positive amplicons were purified from agarose gel and cloned into pGEMt Easy (Promega, Madison, WI, USA). Sequencing reactions were carried out on three clones of each amplicon with the BigDye terminator kit version 3.1 (AppliedBiosystem, Foster City, CA, USA) and resolved with an ABI 3730 automatic capillary sequencer (AppliedBiosystem, Foster City, CA, USA). Nucleotidic sequences were analysed with the BioEdit Sequence Editor version 7.0 software. Nucleotidic similarity with the NCBI genetic database was assessed using the BLAST tool (available at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic inference was performed with the MEGA version 4 software package (Tamura et al., 2007). Phylogenetic tree was constructed by neighbor-joining analysis where evolutionary distances were computed using the Maximum Composite Likelihood method. The confidence values of the internal nodes were calculated by performing 1000 replicates bootstrap values.

2.2. Construction of a recombinant baculovirus for the BoNoV capsid protein

A long fragment product (RdRp region to the poly-A tail) from the genome of a genotype 2 BoNoV strain previously isolated in the laboratory (named B309) was generated by RT-PCR and cloned into pTopoXL (Invitrogen, Carlsbad, CA, USA). The insert was sequenced by primer walking. The sequence coding for the ORF2 was isolated and mutations in this sequence were evaluated with the vector NTI 6 software (InforMax, North Bethesda, MD, USA). Specific forward and reverse PCR primers to amplify the ORF2 were selected using the Vector NTI program (InforMax, North Bethesda, MD, USA). These primers include the EcoRI (B309_EcoRI, aaagaattcaatgaagatgact), and HindIII (B309_HindIII, cccaagctttcagaagccatcaagacggggaaggcg) restriction site upstream and downstream of the start and stop codon of the ORF2, respectively. The PCR product was cloned into the EcoRI/HindIII digested pFastBac Dual plasmid of the bac-to-bac system (Invitrogen, Carlsbad, CA, USA). A recombinant plasmid was used to transform MAX Efficiency DH10bac competent cells (Invitrogen, Carlsbad, CA, USA) where transposition between the recombinant pFastBac Dual and a baculovirus shuttle vector (bacmid) occurred. Recombinant bacmid purification was performed from positive clones with the Plasmid Purification Mega kit (Qiagen, Valencia, CA, USA). The transposition was controlled by PCR with M13 primer pair. Monolayers of *Spodoptera frugiperda* (Sf9) insect cells were grown in Sf-900 II medium (Invitrogen, Carlsbad, CA, USA) supplemented with a 10% association of penicillin (5000 U/ml)–streptomycin (5 mg/ml) (Invitrogen, Carlsbad, CA, USA) and subcultured when they reached

confluence. Transfection of Sf9 cells was conducted in 6-wells according to the manufacturer protocol to generate a recombinant baculovirus for the BoNoV capsid protein.

2.3. Expression and purification of the capsid protein of a bovine norovirus

A recombinant baculovirus stock was generated and monolayers of Sf9 insect cells were infected at a multiplicity of infection of 10. Five days post-infection, cells and supernatants were harvested and submitted to three freezing/thawing cycles. It was followed by low speed centrifugation to remove cell debris and by high speed centrifugation through 5% sucrose cushion in a Beckman SW28 rotor at $100,000 \times g$ for 2 h. The pellet was suspended in phosphate buffered saline (PBS) and centrifuged through a 35–65% sucrose gradient at $100,000 \times g$ for 18 h in a Beckman SW28 rotor. The gradient was fractionated and each fraction was dialysed separately against PBS in 25 kDa porous Spectra/Por membrane (VWR, Leuven, Belgium). Protein concentration in each gradient fraction was evaluated by BCA (Pierce, Rockford, IL, USA). Fractions were then subjected to verification for the presence of either capsid protein as VLP.

2.4. Validation of the production of the bovine norovirus-like particles

An aliquot of each fraction was analysed by Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) with the NuPAGE kit (Invitrogen, Carlsbad, CA, USA) to select fractions containing proteins at a molecular weight of about 55–60 kDa. These fractions were analysed under 80 kV EM examination (Philips EM208 transmission electron microscope). Prior to the examination, samples were submitted to ultracentrifugation on grids with increased hydrophilicity by Alcian blue pre-treatment and subjected to negative staining by phosphotungstic acid or uranyl acetate.

Serum from a calf experimentally infected with NB2 (Bridger et al., 1984) was used in western blot to detect the B309 BoNoV capsid protein. Briefly, a positive fraction, non-infected Sf9 cells and wild type baculovirus infected Sf9 cells were blotted to a nitrocellulose membrane after electrophoresis under denaturing or non-denaturing conditions. The membrane was blocked overnight at room temperature with PBS complemented with 0.05% tween 20 (PBST 0.05%) and 20% horse serum. The second day, blocking solution was removed and the membrane was incubated for 1 h at room temperature in PBS containing 20% horse serum and the 1000-fold diluted calf serum. After three washes with PBST 0.05%, the membrane was incubated for 1 h in PBS containing 20% horse serum and a horseradish peroxidase (HRP) conjugated, 10,000-fold diluted, anti-bovine immunoglobulins (Ig) G rabbit IgG. After three washes with PBST 0.05%, the chemiluminescent peroxidase substrate (Sigma–Aldrich, Salt Lake city, UT, USA) was added on the membrane and it was revealed in dark room on Kodak BioMax Light film (Sigma–Aldrich, Salt Lake City, UT, USA).

2.5. Serum sampling

Blood samples were taken from 439 calves and adult cattle from different premises in Belgium. Serum samples originated from the same area that stool samples but from different premises. The sera were grouped according to the age of the animals. Seven age classes were determined: 1-week to 1-month old ($n = 20$), 1–6 months old ($n = 29$), 6 months to 1-year old ($n = 76$), 1–3 years old ($n = 105$), 3–6 years old ($n = 123$), 6–9 years old ($n = 59$) and older than 9 years old ($n = 27$). In the addition to the 439 sera, a collection of 30 serum samples of colostrum deprived newborn calves from the same region was used as a negative serum control. A commercial bovine anti-rabbit IgG antibody (Bethyl laboratories, Montgomery, TX, USA), purified by affinity chromatography, was also used to test the specificity of the ELISA. The serum of the NB2 experimentally infected calf P131 was used as a positive control (Bridger et al., 1984).

2.6. Evaluation of the seroprevalence against BoNoV by ELISA

Positive gradient fractions by both SDS-PAGE analysis and EM examination were selected for the ELISA. Ninety-six well Microlon plates (Greiner Bio-one, Piscataway, NJ, USA) were coated with 100 µl of a VLP solution in PBS (2 ng/µl) and incubated 1 h at 37 °C. They were washed once with PBS and then blocked with PBS complemented with 0.1% tween20 (PBST 0.1%) and with a 4% casein hydrolysate (PBST/HC). Plates were washed once with PBS and then incubated with the 100-fold diluted bovine serum samples in triplicate at 37 °C. After 1 h and three washes with PBST 0.1%, 10,000-fold diluted, HRP conjugated rabbit IgG anti-bovine IgG were applied to each well. After incubation at 37 °C for 1 h, wells were washed three times with PBST 0.1% and 50 µl of the substrate of HRP, tetramethylbenzidine (Sigma–Aldrich, Salt Lake city, UT, USA), were added. The reaction was stopped after 5 min by addition to each well of 50 µl of H_2SO_4 2 M. Optical densities (OD) at 450 nm were measured with a Thermo Labsystems Multiskan microplate reader and analysed with Ascent Software for Multiskan version 2.6 (Thermo Labsystems, Waltham, MA, USA). All sera were analysed on the same day. The cut-off value for the indirect ELISA test was obtained from the serum samples of the 30 colostrum deprived calves (mean + 1.96 standard deviation) and the apparent prevalence was determined on the 439 bovine serum samples with a 95% confidence interval assuming a binomial exact distribution. All statistical analyses were carried out with the STATA/SE software (Stata Corp., College Station, TX, USA). The OD means in the different age groups of animals (unequal variance and non-normal distribution) were compared with the Wilcoxon rank-sum test (Dagnelie, 1998). The limit of statistical significance of the conducted test was defined as $P \leq 0.05$.

3. Results

3.1. Molecular prevalence of bovine norovirus infection

A molecular prevalence of 7.5% (CI 95%: [3.7; 13.4%]) for BoNoV infection was found in the stool samples of

diarrhoeic calves. Twelve potentially positive amplicons were generated by use of the different primer pairs: 9 with the CBECu pair, 1 with both the CBECu and the BEC pairs, 1 with both the CBECu and the JV12/13 pairs and 1 only with the BEC pair. No amplicon was generated with the p289/290 pair. By BLAST and phylogenetic analysis, 10 amplified polymerase sequences were genetically related to NoV genogroup III genotype 2 strains (Fig. 1). The two other ones, amplified with the CBECu pair at a lower molecular size than expected (about 418 bp), matched with about 90% identity with bovine rotavirus polymerase (VP1) gene sequence. These two samples were also confirmed rotavirus-positive by ELISA based on the rotavirus VP6 detection.

BoNoV were found equally in diagnostic and necropsy samples. Another etiology (rotavirus, coronavirus, *Cryptosporidium parvum* or *Escherichia coli*) was found in all the 10 BoNoV positive samples by different tests performed in a diagnostic laboratory. Remarkably, the sample BV119 was positive by ELISA for bovine rotavirus but no rotavirus nucleotidic sequence was amplified with the CBECu pair in this sample.

3.2. Virus-like particles of the bovine norovirus isolate B309

The B309 BoNoV isolate was previously related to the genotype 2 BoNoV in its RdRp region. By BLAST after the sequencing of its C-terminal part, the B309 isolate shared 89% of nucleotide and 98% of amino acid identity with the NB2 in the entire capsid gene. The B309_EcoRI/B309_HindIII amplified ORF2 sequence was compared to the original sequence and two mutations were found. The first mutation was a transition in the 5th codon and modified the composition in amino acid ($Asp_5 \rightarrow Asn_5$). The second mutation was located in the 97th codon and was a silent mutation. A recombinant bacmid for the B309 ORF2 was generated and 72 h after recombinant baculovirus infection, proteins of about 57 kDa could be detected by SDS-PAGE analysis in several fractions obtained by the gradient applied on harvested cells and supernatants (Fig. 2). Bioinformatic analysis with the Vector NTI software predicted a similar molecular weight for the B309 capsid protein. Particles from 30 to 40 nm range diameter with typical calicivirus cup-shaped depressions were observed under EM examination of some positive fractions of the gradient (Fig. 3).

3.3. Western blot analysis

By western blot analysis, fractions containing B309 VLP reacted at about 57 kDa with a serum resulting from the immunization of a calf with NB2 strain. A weak positive band was also obtained in the lane containing Sf9 cells infected by a wild type baculovirus (Fig. 4A). Western blot performed under non-denaturing conditions revealed a band at about 120 kDa with VLP but not against mock
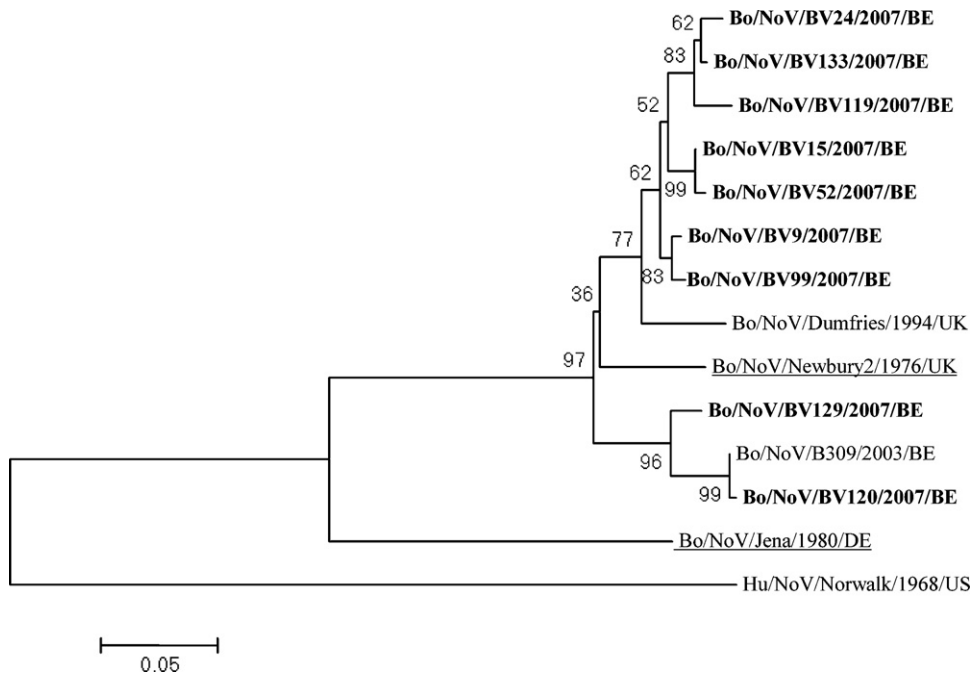
Fig. 1. Neighbor-joining phylogenetic tree based on the RNA-dependent RNA polymerase/capsid protein coding region (429 nt) of bovine noroviruses and the Norwalk virus. Bovine norovirus isolates identified in this study (in bold face) are genetically compared to bovine norovirus reference strains (underlined) and previously isolated strains. Scale bar represents the phylogenetic distances expressed as units of expected nucleotidic substitutions per site. Bootstrap values (1000 replicates) are reported. Genbank accession numbers of strains used: AF097917 (Bo/NV/Newbury2/1976/UK); AJ011099 (Bo/NV/Jena/1980/DE); AY12674 (Bo/NV/Dumfries/94/UK); EU877966 (Bo/NV/BV9/2007/BE); EU877967 (Bo/NV/BV15/2007/BE); EU877969 (Bo/NV/BV24/2007/BE); EU877970 (Bo/NV/BV52/2007/BE); EU877971 (Bo/NV/BV99/2007/BE); EU877972 (Bo/NV/BV119/2007/BE); EU877973 (Bo/NV/BV120/2007/BE); EU877974 (Bo/NV/BV129/2007/BE); EU877975 (Bo/NV/BV133/2007/BE); M87661 (Hu/NV/Norwalk 8FIIa/1968/USA). The tenth amplicon, Bo/NV/BV18/2007/BE, (Genbank accession number EU877968), was not introduced in the tree because its sequence was shorter.
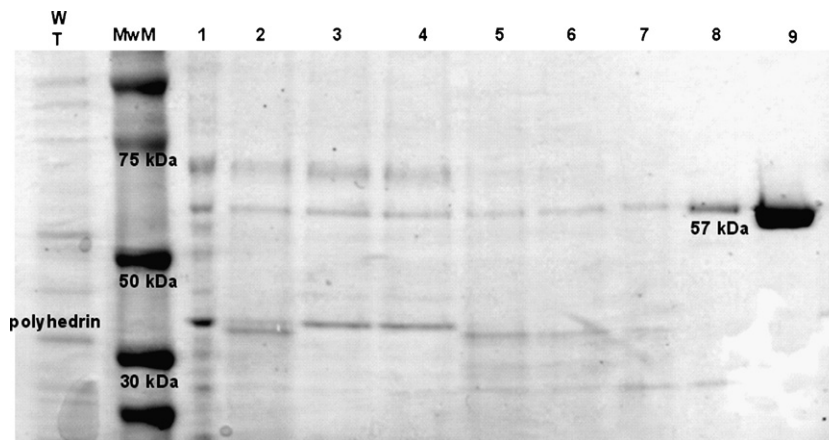
Fig. 2. Supernatants of the baculovirus expression of the capsid protein of the B309 isolate were submitted to purification in sucrose gradient. SDS-PAGE analysis was performed on each fraction and the membrane was stained with Coomassie blue. Results for some fractions are reported here (lanes 1–9). Proteins of about 57 kDa, the expected molecular weight for the B309 capsid protein, were shown. Supernatant of Sf9 cells infected by a wild type baculovirus (lane WT) was used as negative control. MwM: molecular weight marker.

infected or Sf9 cell components recovered after wild type baculovirus infection (Fig. 4B).

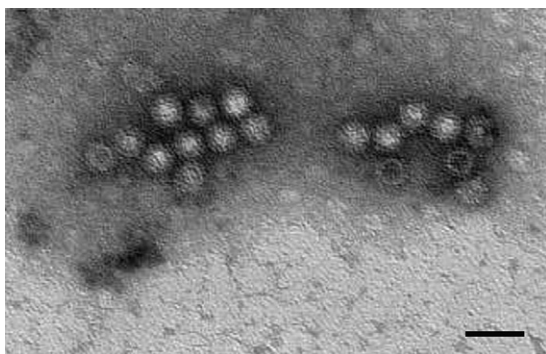### 3.4. Seroprevalence against BoNoV genotype 2 in the Belgian cattle

Only fractions positive to EM examination (visualization of VLP) were used in the ELISA format. Analysis of sera from colostrum deprived newborn calves allowed the definition of a cut-off value of 0.3 for the ELISA. Optical density values obtained for the bovine anti-rabbit IgG serum (mean: 0.1) were similar to those of colostrum deprived newborn calves. By statistical analysis, a high seroprevalence of 93.2% (CI 95%: [90.4; 95.3%]) was found in Belgian cattle sera. Significant statistical differences were found between the mean OD value of different age groups of animals: between the colostrum deprived calves and the 1-week to 1-month-old calf groups, the 1–6

Fig. 3. Electron micrographs of virus-like particles (VLP) of the bovine norovirus B309 isolate. The VLP were produced by expressing the capsid protein in the baculovirus protein expression system and purified in a sucrose gradient. The photograph was taken from the fourth fraction. After uranyl acetate negative staining on Alcyan blue treated greads, particles with the specific size (approximately 40 nm) and morphology of calicivirus-like virions were readily observed. The nucleocapsids were isometric, showed icosahedral symmetry and the border has cup-shaped depressions. Bar, 50 nm.

months old and 6 months to 1-year-old groups, and the 6 months to 1-year-old group and the 1–3 years old group. In the boxplot graph, median value of the "1 week to 1 month old" class rose dramatically compared to the median value of the newborn class. This value was maintained during the "1 month to 6 months old" period and was followed by a new rise in the "6 months to 1 year old" age class. A drop in the values of medians was shown between this last class and the "1 to 3 year old" class. Medians were maintained thereafter in the adult period (Fig. 5). Remarkably, during this period, some OD values were below the cut-off (dots below the line at 0.3 OD value in Fig. 5) and were similar to the values recorded during the colostral period.
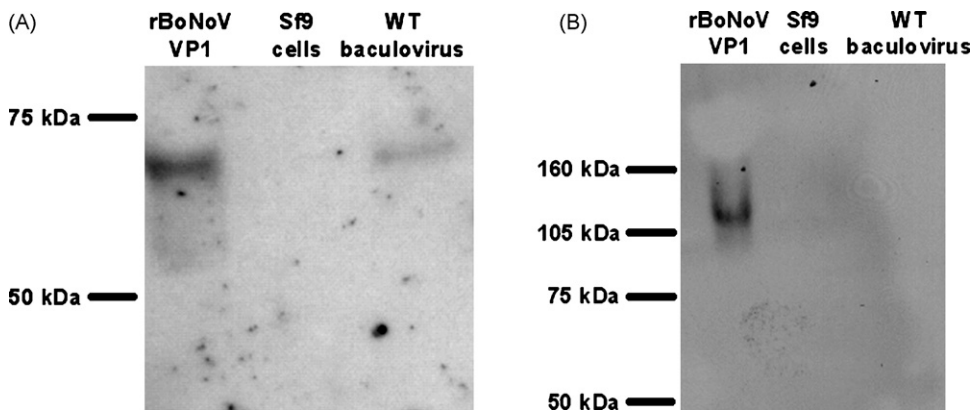
4. Discussion

Although HuNoV are known to be widely distributed and already recognized as a threat to public health, the impact of BoNoV is still poorly studied. In Europe, very few countries have performed epidemiological studies. The 1-year prospective study reported in the present paper endorsed that genotype 2 BoNoV are endemic in European cattle. A BoNoV prevalence of 7.5% was found in stool samples of diarrhoeic calves. In the serological approach, a strong seroprevalence (93.2%) against genotype 2 BoNoV was determined with a VLP-based antibody ELISA in cattle of the same region. Together, the results of prevalence and seroprevalence allowed the description of the evolution of the antibody levels against genotype 2 BoNoV infection during the life of the cattle.

The molecular prevalence of BoNoV in samples coming from the Belgian diagnostic laboratory was in accordance with studies in Great Britain (Milnes et al., 2007) and Korea where Park et al. (2007) report also a majority of genotype 2 infection. The genotype of the strains detected in the British study was not clarified. The prevalence determined by RT-PCR analysis in this study is also similar to the results obtained by an antigen capture ELISA in Germany and developed using a genotype 1 JV strain (Deng et al., 2003). As genotypes 1 and 2 BoNoV are antigenically

Fig. 4. Western blotting of the expressed capsid protein of B309, mock infected Sf9 cells and wild type baculovirus infected Sf9 cells. (A) After electrophoresis under denaturing conditions, a fraction of the applied gradient reacted at about 57 kDa with the serum of a calf experimentally infected with Newbury2 strain, a genotype 2 genogroup III norovirus, but also against Sf9 cells infected by wild type baculovirus. (B) After electrophoresis under non-denaturing conditions, a reaction was obtained at about 120 kDa only against the fraction resulting from purification.

distinct (Oliver et al., 2006), the true BoNoV prevalence could be higher or a preferential geographic distribution could exist. In contrast, Mattison et al. (2007) detected only 1.6% positive in stool samples from a random study in Canadian cattle but Smiley et al. (2003) detected up to 72% of samples positive in two farms in USA. In a longitudinal study in The Netherlands, prevalences of 31.6 and 4.2% were found in farm stool samples and individual samples of dairy cattle, respectively (Van Der Poel et al., 2003). Thus, prevalence results by RT-PCR seem to depend on the continent, the country, the sampling strategy and especially on the test conditions (e.g. annealing temperature,

primers, use of internal control as mentioned in Scipioni et al., 2008a). Also the type of study, cross-sectional versus longitudinal as a part of the Dutch study, could explain some differences. BoNoV infection seems to be strongly related to the age with a higher rate in the first year of the animal life. Positive results were obtained along all the sampling period (data not shown) but further research is needed to investigate a potential seasonality for the infection unlike as described for HuNoV (Mounts et al., 2000). In our results, we detected more isolates with the CBECu pair than with all the other pairs. Also, the CBECu primers allowed the detection of two bovine rotavirus
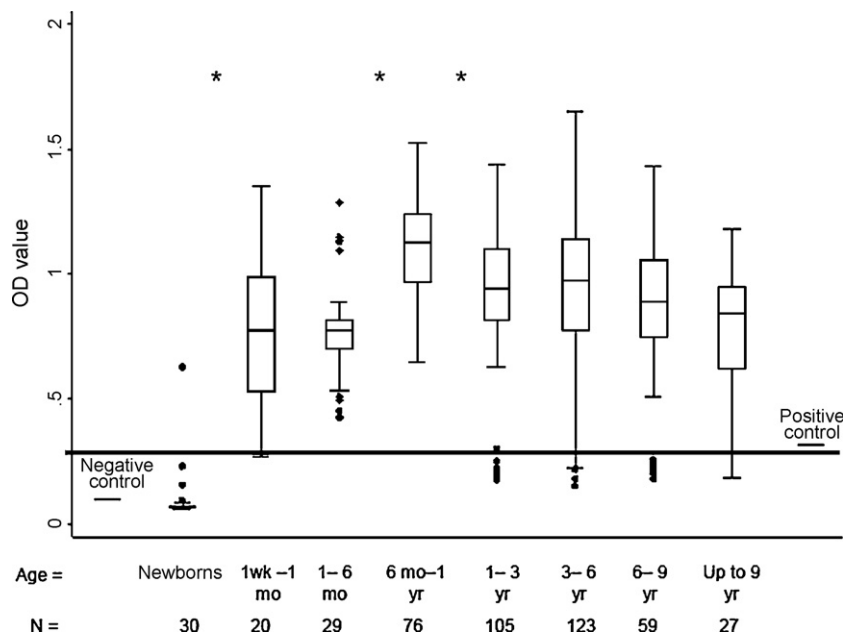
Fig. 5. Seroprevalence against bovine noroviruses in the Belgian cattle. The OD means of the different age groups of animals, collected in the ELISA format (unequal variance and non-normal distribution), were compared with the Wilcoxon rank-sum test. The distribution of the optical densities obtained with the indirect ELISA is represented in a box plot format where optical densities are reported in function of age classes. An asterisk highlights significant statistical differences between means of the different age groups. The cut-off is represented by the line at 0.3 and the dots represent extreme values. OD: optical density, negative control: optical density value obtained for the bovine negative serum (anti-rabbit IgG), positive control: optical density value obtained for the positive control (calf experimentally infected with the Newbury2 strain, Bridger et al., 1984), mo: month, wk: week, yr: year.

infections with however a small difference in the molecular weight for the amplicon (418 bp versus 532 bp). To our knowledge, such observation has never been reported and it could account for some differences between RT-PCR prevalence results depending on the primers used. Nucleotide similarities were found between the CBECu primers and the prototype bovine rotavirus sequence to which they matched by BLAST. On the 23 nt of the CBECu-F sequence, 14 nt including the last five nt matched exactly with the sequence of the bovine rotavirus polymerase (VP1, genbank accession number: J04346). Nucleotide similarity for CBECu-R was smaller. The CBECu-F primer was designed to match the widely conserved YGDD motif in RNA-dependent RNA polymerases of RNA viruses (Bruenn, 1991) and this could explain the detection of bovine rotavirus. Some strains were detected with primers designed to detect HuNoV (JV pair) but at a low rate, as previously reported (Van Der Poel et al., 2003). No HuNoV sequences were identified despite the use of two primer sets designed for their detection, suggesting at least a very low prevalence of these human viruses in cattle. Despite the variety of the primers used in the present study, the lack of detection of the genotype 1 BoNoV could be explained by suboptimal primer use for the RT-PCR (Oliver et al., 2007b). The age of the cattle population studied was also suggested as an explanation for the lower detection of genogroup III genotype 1 NoV (Oliver et al., 2007b). As RT-PCR primers used in this study only target the viral polymerase region and thus miss potential recombinant strains, the possibility that some positive samples contained chimaeric bovine noroviruses could be another explanation for the low apparent prevalence of GIII.1 noroviruses.

Interestingly, few amino acid mutations were detected between the native B309 strain sequence (year of isolation: 2003) and the NB2 strain sequence (year of isolation: 1976) (Scipioni et al., submitted for publication). Nearly all mutations were located in the P domain with surprisingly relatively few mutations in the P2 sub-domain, the most variable region of VP1 known to support antigenic determinants and drift-induced antigenic variation in NoV strains (Allen et al., 2008; Lochridge and Hardy, 2007).

The two mutations detected in the VLP constructed in the baculovirus system compared to the native B309 strain, did not affect the antigenicity by their effect and their localisation in the amino acid sequence. Oliver et al. (2007a) reported that the P131 calf serum did not react in western blotting with BoNoV genotype 2 VLP in denaturing reaction but react at a higher molecular weight than expected under non-denaturing conditions. In our conditions this serum reacts against a fraction containing VLP but also with infected Sf9 cells, in denaturing conditions. To test the specificity of this reaction against infected Sf9 cells, western blots were carried out with foetal calf serum and serum from a colostrum deprived newborn calf used in this study. With these two sera, a positive band at the same molecular weight was observed with baculovirus infected Sf9 cells under denaturing conditions whereas no reaction was detected in non-denaturing conditions (Mauroy and Thiry, unpublished results). These data confirm the non-specific binding of bovine immunoglobulins on denatured

protein extracts from baculovirus infected Sf9 cells. In non-denaturing conditions, the serum only reacts against VLP. Thus, B309 VLP are representative of the genotype 2 BoNoV.

A high seroprevalence (93.2%) against genotype 2 BoNoV was found by ELISA. Genotypes 1 and 2 in the BoNoV genogroup, although antigenically distinct, were shown to cross-react in ELISA to the heterologous VLP but at low serum dilution ($\log_{10}$ 1.7) (Oliver et al., 2006). However the true genotype 2 seroprevalence should still remain high, even corrected for a lower sensitivity and specificity of the test. The OD values obtained with the serum raised against rabbit IgG in bovine confirmed the selected cut-off. Almost all colostrum deprived calves did not have antibodies against BoNoV as previously described (Deng et al., 2003). It suggests that BoNoV infection is mainly localised in the intestine, according to the observations with HuNoV (Mattner et al., 2006), and that vertical transmission of the virus is rare. The rise in antibody levels in 1 or 2 weeks old calves suggests an acquisition of antibodies through colostrum absorption or from early infection. Information on prevalence brought by the RT-PCR analysis suggests that the risk of infection during the first 6 months of life is high. The antibody levels determined by ELISA rise again in the post-colostral period (6 months to 1-year old). These results could be explained by a large exposure during this period and the development of natural immunity.

The finding that cattle aged 3–9 years still had antibodies suggests either a long lasting immunity against BoNoV or frequent re-infections. Several characteristics of NoV (environmental resistance, fecal-oral transmission) and farming practices in developed countries could increase the risk of re-infection in cattle, whereas such re-infection was not observed during a prospective RT-PCR study in The Netherlands (Van Der Poel et al., 2003) and during a cross-sectional RT-PCR study performed by ourselves on stool from up to 6 months old cattle ($n = 47$) housed in two different premises (Mauroy and Thiry, unpublished results). However in a retrospective study in Great Britain, Milnes et al. (2007) have amplified norovirus sequences by RT-PCR in adult stool samples. The detection of high levels of antibodies in the adult also suggests their presence in the colostrum. It could explain the antibody rise in neonates after colostrum intake. Remarkably, in contrast to the 1-week to 1-year-old group, some OD values of adult cattle were below the cut-off of the test. These data suggest that antibody levels decrease in some adult cattle reinforcing the hypothesis of a short lived immunity.

The zoonotic risk associated with animal NoV is widely discussed but is hypothetical. However, several recent data showed that HuNoV replication occurred experimentally in gnotobiotic animals including calves (Souza et al., 2008). Furthermore, Mattison et al. (2007) have identified HuNoV sequences in swine and cattle. Shellfish like oysters are known as a main route of food-associated HuNoV outbreaks. As they bind specifically HuNoV to their digestive tissues (Le Guyader et al., 2006) and as BoNoV sequences were also detected in these molluscs (Costantini et al., 2006), new recombinant strains could appear through

mixed infection. Such event was already reported with human strains (Symes et al., 2007). Mixed infections could occur in countries with high population densities of human and production animals and with high prevalence of BoNoV in premises. However, this study did not detect any contaminations of cattle with HuNoV.

Experimental infection of calves with BoNoV has shown their low pathogenicity (Bridger et al., 1984) but their impact in association with classic enteric pathogens in superinfections and in economic losses (weight gain, health status, veterinary treatment cost) has not been studied. In this study, BoNoV were found both in diagnostic samples and necropsy samples and always in association with other pathogens. The role of BoNoV as pathogens in the bovine species should be considered, taking into account their endemicity in the investigated situation and their presence in diarrhoeic calves.

More specific primers, primers targeting both the ORF1-end and the ORF2 regions, a broader sampling strategy, in particular regarding the age of the animals, and an ELISA including VLP of the genotype 1 could refine epidemiological data for BoNoV infection. An Ig discriminative ELISA format (IgA, IgM, IgG1 and 2) could define the status of the animals during their first year of life.

Together, the results of prevalence and seroprevalence show the endemicity of the genotype 2 BoNoV in the studied region. The first 1-year period of life is critical for BoNoV infection. During this period, the immunological status of cattle against BoNoV varies both by colostrum intake and by natural infection.

Conflict of interest statement

The authors ensure that there are no conflict of interest with any person or organisation.

Acknowledgements

References

Allen, D.J., Gray, J.J., Gallimore, C.I., Xerry, J., Iturriza-Gomara, M., 2008. Analysis of amino acid variation in the P2 domain of the GII-4 norovirus VP1 protein reveals putative variant-specific epitopes. PLoS ONE 3, e1485.

Ando, T., Noel, J.S., Fankhauser, R.L., 2000. Genetic classification of Norwalk-like viruses. J. Infect. Dis. 181 (Suppl. 2), S336–S348.

Bridger, J.C., Hall, G.A., Brown, J.F., 1984. Characterization of a calici-like virus (Newbury agent) found in association with astrovirus in bovine diarrhea. Infect. Immun. 43, 133–138.

Bruenn, J.A., 1991. Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerases. Nucleic Acids Res. 19, 217–226.

Costantini, V., Loisy, F., Joens, L., Le Guyader, F.S., Saif, L.J., 2006. Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. Appl. Environ. Microbiol. 72, 1800–1809.

Dagnelie, P., 1998. Statistique théorique et appliqué. Tome 2. Inférence statistique à une et à deux dimensions. De Boek & Larcier, p. 659.

Deng, Y., Batten, C.A., Liu, B.L., Lambden, P.R., Elschner, M., Gunther, H., Otto, P., Schnurch, P., Eichhorn, W., Herbst, W., Clarke, I.N., 2003. Studies of epidemiology and seroprevalence of bovine noroviruses in Germany. J. Clin. Microbiol. 41, 2300–2305.

Green, K.Y., 2007. Caliciviridae: the noroviruses. In: Knipe, D.M. (Ed.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, USA, pp. 949–979.

Gunther, H., Otto, P., 1987. Studies on diarrhoea in young calves. 7. Serrated virus (Jena-Agent 117/80)—a new diarrhoea pathogen in calves. Archiv fur Experimentelle Veterinarmedizin 41, 934–938.

Ike, A.C., Roth, B.N., Bohm, R., Pfitzner, A.J., Marschang, R.E., 2007. Identification of bovine enteric Caliciviruses (BEC) from cattle in Baden–Wurttemberg. Dtsch. Tierarztl. Wochenschr. 114, 12–15.

Jiang, X., Wang, M., Graham, D.Y., Estes, M.K., 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J. Virol. 66, 6527–6532.

Jiang, X., Huang, P.W., Zhong, W.M., Farkas, T., Cubitt, D.W., Matson, D.O., 1999. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J. Virol. Methods 83, 145–154.

Le Guyader, F., Loisy, F., Atmar, R.L., Hutson, A.M., Estes, M.K., Ruvoen-Clouet, N., Pommepuy, M., Le Pendu, J., 2006. Norwalk virus-specific binding to oyster digestive tissues. Emerg. Infect. Dis. 12, 931–936.

Liu, B.L., Lambden, P.R., Gunther, H., Otto, P., Elschner, M., Clarke, I.N., 1999. Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses. J. Virol. 73, 819–825.

Lochridge, V.P., Jutila, K.L., Graff, J.W., Hardy, M.E., 2005. Epitopes in the P2 domain of norovirus VP1 recognized by monoclonal antibodies that block cell interactions. J. Gen. Virol. 86, 2799–2806.

Lochridge, V.P., Hardy, M.E., 2007. A single-amino-acid substitution in the P2 domain of VP1 of murine norovirus is sufficient for escape from antibody neutralization. J. Virol. 81, 12316–12322.

Mattison, K., Shukla, A., Cook, A., Pollari, F., Friendship, R., Kelton, D., Bidawid, S., Farber, J.M., 2007. Human noroviruses in swine and cattle. Emerg. Infect. Dis. 13, 1184–1188.

Mattner, F., Sohr, D., Heim, A., Gastmeier, P., Vennema, H., Koopmans, M., 2006. Risk groups for clinical complications of norovirus infections: an outbreak investigation. Clin. Microbiol. Infect. 12, 69–74.

Milnes, A.S., Binns, S.H., Oliver, S.L., Bridger, J.C., 2007. Retrospective study of noroviruses in samples of diarrhoea from cattle, using the Veterinary Laboratories Agency's Farmfile database. Vet. Rec. 160, 326–330.

Mounts, A.W., Ando, T., Koopmans, M., Bresee, J.S., Noel, J., Glass, R.I., 2000. Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses. J. Infect. Dis. 181 (Suppl. 2), S284–S287.

Oliver, S.L., Dastjerdi, A.M., Wong, S., El-Attar, L., Gallimore, C., Brown, D.W., Green, J., Bridger, J.C., 2003. Molecular characterization of bovine enteric caliciviruses: a distinct third genogroup of noroviruses (Norwalk-like viruses) unlikely to be of risk to humans. J. Virol. 77, 2789–2798.

Oliver, S.L., Batten, C.A., Deng, Y., Elschner, M., Otto, P., Charpilienne, A., Clarke, I.N., Bridger, J.C., Lambden, P.R., 2006. Genotype 1 and genotype 2 bovine noroviruses are antigenically distinct but share a cross-reactive epitope with human noroviruses. J. Clin. Microbiol. 44, 992–998.

Oliver, S.L., Asobayire, E., Charpilienne, A., Cohen, J., Bridger, J.C., 2007a. Complete genomic characterization and antigenic relatedness of genogroup III, genotype 2 bovine noroviruses. Arch. Virol. 152, 257–272.

Oliver, S.L., Wood, E., Asobayire, E., Wathes, D.C., Brickell, J.S., Elschner, M., Otto, P., Lambden, P.R., Clarke, I.N., Bridger, J.C., 2007b. Serotype 1 and 2 bovine noroviruses are endemic in cattle in the United Kingdom and Germany. J. Clin. Microbiol. 45, 3050–3052.

Park, S.I., Jeong, C., Kim, H.H., Park, S.H., Park, S.J., Hyun, B.H., Yang, D.K., Kim, S.K., Kang, M.I., Cho, K.O., 2007. Molecular epidemiology of bovine noroviruses in South Korea. Vet. Microbiol. 124, 125–133.

Prasad, B.V., Hardy, M.E., Dokland, T., Bella, J., Rossmann, M.G., Estes, M.K., 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science 286, 287–290.

Scipioni, A., Bourgot, I., Mauroy, A., Ziant, D., Saegerman, C., Daube, G., Thiry, E., 2008a. Detection and quantification of human and bovine noroviruses by a TaqMan RT-PCR assay with a control for inhibition. Mol. Cell. Probes 22, 215–222.

Scipioni, A., Mauroy, A., Vinje, J., Thiry, E., 2008b. Animal noroviruses. Vet. J. 178, 32–45.

Smiley, J.R., Hoet, A.E., Traven, M., Tsunemitsu, H., Saif, L.J., 2003. Reverse transcription-PCR assays for detection of bovine enteric caliciviruses (BEC) and analysis of the genetic relationships among BEC and human caliciviruses. J. Clin. Microbiol. 41, 3089–3099.

Souza, M., Azevedo, M.S., Jung, K., Cheetham, S., Saif, L.J., 2008. Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4-HS66 strain of human norovirus. J. Virol. 82, 1777–1786.

Symes, S.J., Gunesekere, I.C., Marshall, J.A., Wright, P.J., 2007. Norovirus mixed infection in an oyster-associated outbreak: an opportunity for recombination. Arch. Virol. 152, 1075–1086.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Van Der Poel, W.H., van der Heide, R., Verschoor, F., Gelderblom, H., Vinje, J., Koopmans, M.P., 2003. Epidemiology of Norwalk-like virus infections in cattle in The Netherlands. Vet. Microbiol. 92, 297–309.

Vinje, J., Vennema, H., Maunula, L., von Bonsdorff, C., Hoehne, M., Schreier, E., Richards, A., Green, J., Brown, D., Beard, S.S., Monroe, S.S., De Bruin, E., Svensson, L., Koopmans, M.P., 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. J. Clin. Microbiol. 41, 1423–1433.

Woode, G.N., Bridger, J.C., 1978. Isolation of small viruses resembling astroviruses and caliciviruses from acute enteritis of calves. J. Med. Microbiol. 11, 441–452.