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1 **EPIDEMIOLOGICAL STUDY OF BOVINE NOROVIRUS INFECTION BY RT-PCR**
2 **AND A VLP-BASED ANTIBODY ELISA**

3

4 Axel Mauroy^a, Alexandra Scipioni^a, Elisabeth Mathijs^a, Claude Saegerman^a, Jan Mast^b, Janice
5 C. Bridger^c, Dominique Ziant^a, Christine Thys^a, Etienne Thiry^{a,*}.

6

7 ^a Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University
8 of Liège, Belgium

9 ^b Veterinary and Agrochemical Research Centre, Department Biocontrole, Research Unit
10 Electron Microscopy, Brussels, Belgium

11 ^c Department of Pathology and Infectious Diseases, Royal Veterinary College, London,
12 United Kingdom

13

14 * Corresponding author. Tel.: +3243664250; fax: +3243664261, etienne.thiry@ulg.ac.be

15

16 **ABSTRACT**

17

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

36

37 **Keywords : norovirus/ epidemiology/seroprevalence/PCR /bovine**

38

39 1. Introduction

40

41 Noroviruses (NoV) belong to the family *Caliciviridae* and have been identified in the human
42 being and in several animal species including the bovine (Scipioni et al., 2008b; Green, 2007).

43 Noroviruses are small, round, non-enveloped viruses, with a 25-40 nm diameter and have a
44 positive-sense, single-stranded RNA genome with three open reading frames (ORF). The non
45 structural proteins, including the viral RNA dependent RNA polymerase (RdRp), the single
46 capsid protein (VP1) and the minor structural protein are encoded by ORF1, 2 and 3
47 respectively (Green, 2007). Ninety dimers of VP1 form the capsid which presents an
48 icosahedral symmetry under electron microscopy (EM). Capsid protein VP1 has a molecular
49 weight of around 55-60 kDa and is organised into two domains: a shell (S) domain (N-
50 terminal part of VP1) and a protruding (P) domain (C-terminal part of VP1) (Prasad et al.,
51 1999). The P domain supports receptor recognition and immunogenic functions (Lochridge et
52 al., 2005). Noroviruses are difficult to grow in cell culture but abundant quantities of virus-
53 like particles (VLP) can be obtained by expressing VP1 in the baculovirus protein expression
54 system. These VLP are morphologically and antigenically similar to the native strain (Jiang et
55 al., 1992).

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

64 Their relative environmental stability, their main faecal-oral transmission route, their low
65 infectious dose and their large strain diversity increase the risk of cattle infection with these
66 viruses (Green, 2007). In gnotobiotic calves, BoNoV induce non-hemorrhagic enteritis, mild
67 diarrhoea, transient anorexia and malabsorption (Woode and Bridger, 1978). But the real
68 impact of these viruses in the field is currently poorly evaluated. The two genotypes of
69 BoNoV were recently shown to be endemic in Great Britain and Germany (Oliver et al.,
70 2007b). They were also isolated in all continents and thus seem to be widespread (Scipioni et
71 al., 2008b).

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

78

79 2. Materials and methods

80

81 2.1. Bovine stool samples and genetic analysis

82 Stool samples from calves aged 1 week to 6 months were systematically received from a
83 Belgian diagnostic laboratory each month through the year 2007. Samples came from
84 different places out of five provinces of Belgium (Hainaut, Namur, Liege, Luxembourg,
85 Walloon Brabant). Their origin was either from diarrhoeic samples (n=74) submitted for
86 etiologic diagnosis of gastroenteritis or from necropsy samples (n=59) where gastroenteritis
87 lesions were found. This stool bank (n=133) was screened by one step RT-PCR for the
88 presence of BoNoV genome sequences with Quick Access kit (Promega, Madison, WI, USA).
89 Four different primer pairs were used to amplify segments of the polymerase gene and the
90 beginning of the capsid region: CBECu (Smiley et al., 2003), JV12/13 (Vinje et al., 2003),
91 P289/290 (Jiang et al., 1999) and BEC (Ike et al., 2007). These primers were selected in order
92 to perform the broadest detection of NoV including a possible zoonotic transmission. Out of
93 the BoNoV specific primers (CBECu and BEC), CBECu pair was seen to detect not only
94 genotypes 2 but also genotypes 1 BoNoV with an apparent sensitivity of about 83% relative
95 to the p289/290 pair in a previous study (Smiley et al., 2003). RT-PCR program consisted in a
96 reverse transcription step of 45 min at 45°C followed by the PCR reaction. It consisted in an
97 initial denaturation step of 2 min at 94°C followed by 40 PCR cycles (30 sec denaturation step
98 at 94°C; 1 min annealing step at 48°C for CBECu, JV12/13 and BEC primers, 51°C for
99 p289/290 primers; 2 min extension step at 68°C) and a final extension step of 10 min at 68°C.
100 Positive amplicons were purified from agarose gel and cloned into pGEMt Easy (Promega,
101 Madison, WI, USA). Sequencing reactions were carried out on three clones of each amplicon
102 with the BigDye terminator kit version 3.1 (AppliedBiosystem, Foster City, CA, USA) and
103 resolved with an ABI 3730 automatic capillary sequencer (AppliedBiosystem, Foster City,

104 CA, USA). Nucleotidic sequences were analysed with the BioEdit Sequence Editor version
105 7.0 software. Nucleotidic similarity with the NCBI genetic database was assessed using the
106 BLAST tool (available at <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Phylogenetic
107 inference was performed with the MEGA version 4 software package (Tamura et al., 2007).
108 Phylogenetic tree was constructed by neighbor-joining analysis where evolutionary distances
109 were computed using the Maximum Composite Likelihood method. The confidence values of
110 the internal nodes were calculated by performing 1,000 replicates bootstrap values.

111 **2.2. Construction of a recombinant baculovirus for the BoNoV capsid protein**

112 A long fragment product (RdRp region to the poly-A tail) from the genome of a genotype 2
113 BoNoV strain previously isolated in the laboratory (named B309) was generated by RT-PCR
114 and cloned into pTopoXL (Invitrogen, Carlsbad, CA, USA). The insert was sequenced by
115 primer walking. The sequence coding for the ORF2 was isolated and mutations in this
116 sequence were evaluated with the vector NTI 6 software (InforMax, North Bethesda, MD,
117 USA). Specific forward and reverse PCR primers to amplify the ORF2 were selected using
118 the Vector NTI program (InforMax, North Bethesda, MD, USA). These primers include the
119 *EcoRI* (B309_ *EcoRI*, aaagaattcaatgaagatgact), and *HindIII* (B309_ *HindIII*,
120 cccaagctttcagaagccatcaagacggggaaggcg) restriction site upstream and downstream of the start
121 and stop codon of the ORF2 respectively. The PCR product was cloned into the
122 *EcoRI/HindIII* digested pFastBac Dual plasmid of the bac-to-bac system (Invitrogen,
123 Carlsbad, CA, USA). A recombinant plasmid was used to transform MAX Efficiency
124 DH10bac competent cells (Invitrogen, Carlsbad, CA, USA) where transposition between the
125 recombinant pFastBac Dual and a baculovirus shuttle vector (bacmid) occurred. Recombinant
126 bacmid purification was performed from positive clones with the Plasmid Purification Mega
127 kit (Qiagen, Valencia, CA, USA). The transposition was controlled by PCR with M13 primer
128 pair. Monolayers of *Spodoptera frugiperda* (Sf9) insect cells were grown in Sf-900 II medium

129 (Invitrogen, Carlsbad, CA, USA) supplemented with a 10% association of penicillin
130 (5,000 U/ml)-streptomycin (5 mg/ml) (Invitrogen, Carlsbad, CA, USA) and subcultured when
131 they reached confluence. Transfection of Sf9 cells was conducted in 6-wells according to the
132 manufacturer protocol to generate a recombinant baculovirus for the BoNoV capsid protein.

133 **2.3. Expression and purification of the capsid protein of a bovine norovirus**

134 A recombinant baculovirus stock was generated and monolayers of Sf9 insect cells were
135 infected at a multiplicity of infection of 10. Five days post-infection, cells and supernatants
136 were harvested and submitted to three freezing/thawing cycles. It was followed by low speed
137 centrifugation to remove cell debris and by high speed centrifugation through 5% sucrose
138 cushion in a Beckman SW28 rotor at 100,000 x g for 2 h. The pellet was suspended in
139 phosphate buffered saline (PBS) and centrifuged through a 35% to 65% sucrose gradient at
140 100,000 x g for 18 h in a Beckman SW28 rotor. The gradient was fractionated and each
141 fraction was dialysed separately against PBS in 25 kDa porous Spectra/Por membrane (VWR,
142 Leuven, Belgium). Protein concentration in each gradient fraction was evaluated by BCA
143 (Pierce, Rockford, IL, USA). Fractions were then subjected to verification for the presence of
144 either capsid protein as VLP.

145 **2.4. Validation of the production of the bovine norovirus-like particles**

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

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

166 **2.5. Serum sampling**

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

178 2.6. Evaluation of the seroprevalence against BoNoV by ELISA

179 Positive gradient fractions by both SDS-PAGE analysis and EM examination were selected
180 for the ELISA. Ninety-six well Microlon plates (Greiner Bio-one, Piscataway, NJ, USA) were
181 coated with 100 μ l of a VLP solution in PBS (2 ng/ μ l) and incubated 1 h at 37°C. They were
182 washed once with PBS and then blocked with PBS complemented with 0.1% tween20 (PBST
183 0.1%) and with a 4% casein hydrolysate (PBST/HC). Plates were washed once with PBS and
184 then incubated with the 100-fold diluted bovine serum samples in triplicate at 37°C. After 1 h
185 and three washes with PBST 0.1%, 10,000-fold diluted, HRP conjugated rabbit IgG
186 anti-bovine IgG were applied to each well. After incubation at 37°C for 1 h, wells were
187 washed three times with PBST 0.1% and 50 μ l of the substrate of HRP, tetramethylbenzidine
188 (Sigma-Aldrich, Salt Lake city, UT, USA), were added. The reaction was stopped after 5 min
189 by addition to each well of 50 μ l of H₂SO₄ 2M. Optical densities (OD) at 450 nm were
190 measured with a Thermo Labsystems Multiskan microplate reader and analysed with Ascent
191 Software for Multiskan version 2.6 (Thermo Labsystems, Waltham, MA, USA). All sera were
192 analysed on the same day. The cut-off value for the indirect ELISA test was obtained from the
193 serum samples of the 30 colostrum deprived calves (mean + 1.96 standard deviation) and the
194 apparent prevalence was determined on the 439 bovine serum samples with a 95% confidence
195 interval assuming a binomial exact distribution. All statistical analyses were carried out with
196 the STATA/SE software (Stata Corp., College Station, TX, USA). The OD means in the
197 different age groups of animals (unequal variance and non normal distribution) were
198 compared with the Wilcoxon rank-sum test (Dagnelie, 1998). The limit of statistical
199 significance of the conducted test was defined as $P \leq 0.05$.

200

201 3. Results

202

203 3.1. Molecular prevalence of bovine norovirus infection

204 A molecular prevalence of 7.5% (CI 95% : [3.7 ; 13.4%]) for BoNoV infection was found in
205 the stool samples of diarrhoeic calves. Twelve potentially positive amplicons were generated
206 by use of the different primer pairs: 9 with the CBECu pair, 1 with both the CBECu and the
207 BEC pairs, 1 with both the CBECu and the JV12/13 pairs and 1 only with the BEC pair. No
208 amplicon was generated with the p289/290 pair. By BLAST and phylogenetic analysis, 10
209 amplified polymerase sequences were genetically related to NoV genogroup III genotype 2
210 strains (Fig. 1). The two other ones, amplified with the CBECu pair at a lower molecular size
211 than expected (about 418 bp), matched with about 90% identity with bovine rotavirus
212 polymerase (VP1) gene sequence. These two samples were also confirmed rotavirus-positive
213 by ELISA based on the rotavirus VP6 detection.

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

219 3.2. Virus-like particles of the bovine norovirus isolate B309

220 The B309 BoNoV isolate was previously related to the genotype 2 BoNoV in its RdRp
221 region. By BLAST after the sequencing of its C-terminal part, the B309 isolate shared 89% of
222 nucleotide and 98% of amino acid identity with the NB2 in the entire capsid gene. The
223 B309_ *EcoRI*/B309_ *HindIII* amplified ORF2 sequence was compared to the original sequence
224 and two mutations were found. The first mutation was a transition in the 5th codon and
225 modified the composition in amino acid (Asp₅ → Asn₅). The second mutation was located in

226 the 97th codon and was a silent mutation. A recombinant bacmid for the B309 ORF2 was
227 generated and 72 hours after recombinant baculovirus infection, proteins of about 57 kDa
228 could be detected by SDS-PAGE analysis in several fractions obtained by the gradient applied
229 on harvested cells and supernatants (Fig. 2). Bioinformatic analysis with the Vector NTI
230 software predicted a similar molecular weight for the B309 capsid protein. Particles from 30
231 to 40 nm range diameter with typical calicivirus cup-shaped depressions were observed under
232 EM examination of some positive fractions of the gradient (Fig. 3).

233 **3.3. Western blot analysis**

234 By western blot analysis, fractions containing B309 VLP reacted at about 57 kDa with a
235 serum resulting from the immunization of a calf with NB2 strain. A weak positive band was
236 also obtained in the lane containing Sf9 cells infected by a wild type baculovirus (Fig. 4A).
237 Western blot performed under non denaturing conditions revealed a band at about 120 kDa
238 with VLP but not against mock infected or Sf9 cell components recovered after wild type
239 baculovirus infection (Fig. 4B).

240 **3.4. Seroprevalence against BoNoV genotype 2 in the Belgian cattle**

241 Only fractions positive to EM examination (visualization of VLP) were used in the ELISA
242 format. Analysis of sera from colostrum deprived newborn calves allowed the definition of a
243 cut-off value of 0.3 for the ELISA. Optical density values obtained for the bovine anti-rabbit
244 IgG serum (mean: 0.1) were similar to those of colostrum deprived newborn calves. By
245 statistical analysis, a high seroprevalence of 93.2% (CI 95% : [90.4 ; 95.3%]) was found in
246 Belgian cattle sera. Significant statistical differences were found between the mean OD value
247 of different age groups of animals: between the colostrum deprived calves and the 1 week to 1
248 month old calf groups, the 1 to 6 months old and 6 months to 1 year old groups, and the 6
249 months to 1 year old group and the 1 to 3 years old group. In the boxplot graph, median value
250 of the “1 week to 1 month old” class rose dramatically compared to the median value of the

251 newborn class. This value was maintained during the “1 month to 6 months old” period and
252 was followed by a new rise in the “6 months to 1 year old” age class. A drop in the values of
253 medians was shown between this last class and the “1 to 3 year old” class. Medians were
254 maintained thereafter in the adult period (Fig. 5). Remarkably, during this period, some OD
255 values were below the cut-off (dots below the line at 0.3 OD value in fig. 5) and were similar
256 to the values recorded during the colostral period.

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258 **4. Discussion**

259

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

269 The molecular prevalence of BoNoV in samples coming from the Belgian diagnostic
270 laboratory was in accordance with studies in Great Britain (Milnes et al., 2007) and Korea
271 where Park *et al.* (Park et al., 2007) report also a majority of genotype 2 infection. The
272 genotype of the strains detected in the British study was not clarified. The prevalence
273 determined by RT-PCR analysis in this study is also similar to the results obtained by an
274 antigen capture ELISA in Germany and developed using a genotype 1 JV strain (Deng et al.,
275 2003). As genotypes 1 and 2 BoNoV are antigenically distinct (Oliver et al., 2006), the true
276 BoNoV prevalence could be higher or a preferential geographic distribution could exist. In
277 contrast, Mattison et al. (2007) detected only 1.6% positive in stool samples from a random
278 study in Canadian cattle but Smiley et al. (2003) detected up to 72% of samples positive in
279 two farms in USA. In a longitudinal study in The Netherlands, prevalences of 31.6% and
280 4.2% were found in farm stool samples and individual samples of dairy cattle respectively
281 (Van Der Poel et al., 2003). Thus, prevalence results by RT-PCR seem to depend on the
282 continent, the country, the sampling strategy and especially on the test conditions (e.g.

283 annealing temperature, primers, use of internal control as mentioned in Scipioni et al., 2008a).
284 Also the type of study, cross-sectional versus longitudinal as a part of the Dutch study, could
285 explain some differences. BoNoV infection seems to be strongly related to the age with a
286 higher rate in the first year of the animal life. Positive results were obtained along all the
287 sampling period (data not shown) but further research is needed to investigate a potential
288 seasonality for the infection unlike as described for HuNoV (Mounts et al., 2000). In our
289 results, we detected more isolates with the CBECu pair than with all the other pairs. Also, the
290 CBECu primers allowed the detection of two bovine rotavirus infections with however a
291 small difference in the molecular weight for the amplicon (418 bp versus 532 bp). To our
292 knowledge, such observation has never been reported and it could account for some
293 differences between RT-PCR prevalence results depending on the primers used. Nucleotide
294 similarities were found between the CBECu primers and the prototype bovine rotavirus
295 sequence to which they matched by BLAST. On the 23 nt of the CBECu-F sequence, 14 nt
296 including the last five nt matched exactly with the sequence of the bovine rotavirus
297 polymerase (VP1, genbank accession number: J04346). Nucleotide similarity for CBECu-R
298 was smaller. The CBECu-F primer was designed to match the widely conserved YGDD motif
299 in RNA-dependent RNA polymerases of RNA viruses (Bruenn, 1991) and this could explain
300 the detection of bovine rotavirus. Some strains were detected with primers designed to detect
301 HuNoV (JV pair) but at a low rate, as previously reported (Van Der Poel et al., 2003). No
302 HuNoV sequences were identified despite the use of two primer sets designed for their
303 detection, suggesting at least a very low prevalence of these human viruses in cattle. Despite
304 the variety of the primers used in the present study, the lack of detection of the genotype 1
305 BoNoV could be explained by suboptimal primer use for the RT-PCR (Oliver et al., 2007b).
306 The age of the cattle population studied was also suggested as an explanation for the lower
307 detection of genogroup III genotype 1 NoV (Oliver et al., 2007b). As RT-PCR primers used

308 in this study only target the viral polymerase region and thus miss potential recombinant
309 strains, the possibility that some positive samples contained chimaeric bovine noroviruses
310 could be another explanation for the low apparent prevalence of GIII.1 noroviruses.

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

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

331 A high seroprevalence (93.2%) against genotype 2 BoNoV was found by ELISA. Genotypes
332 1 and 2 in the BoNoV genogroup, although antigenically distinct, were shown to cross-react

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

346 The finding that cattle aged 3 to 9 years still had antibodies suggests either a long lasting
347 immunity against BoNoV or frequent re-infections. Several characteristics of NoV
348 (environmental resistance, fecal-oral transmission) and farming practices in developed
349 countries could increase the risk of re-infection in cattle, whereas such re-infection was not
350 observed during a prospective RT-PCR study in The Netherlands (Van Der Poel et al., 2003)
351 and during a cross-sectional RT-PCR study performed by ourselves on stool from up to 6
352 months old cattle (n=47) housed in two different premises (Mauroy and Thiry, unpublished
353 results). However in a retrospective study in Great Britain, Milnes et al. (2007) have
354 amplified norovirus sequences by RT-PCR in adult stool samples. The detection of high
355 levels of antibodies in the adult also suggests their presence in the colostrum. It could explain
356 the antibody rise in neonates after colostrum intake. Remarkably, in contrast to the 1 week to
357 1 year old group, some OD values of adult cattle were below the cut-off of the test. These data

358 suggest that antibody levels decrease in some adult cattle reinforcing the hypothesis of a short
359 lived immunity.

360 The zoonotic risk associated with animal NoV is widely discussed but is hypothetical.
361 However, several recent data showed that HuNoV replication occurred experimentally in
362 gnotobiotic animals including calves (Souza et al., 2008). Furthermore, Mattison et al. (2007)
363 have identified HuNoV sequences in swine and cattle. Shellfish like oysters are known as a
364 main route of food-associated HuNoV outbreaks. As they bind specifically HuNoV to their
365 digestive tissues (Le Guyader et al., 2006) and as BoNoV sequences were also detected in
366 these molluscs (Costantini et al., 2006), new recombinant strains could appear through mixed
367 infection. Such event was already reported with human strains (Symes et al., 2007). Mixed
368 infections could occur in countries with high population densities of human and production
369 animals and with high prevalence of BoNoV in premises. However, this study did not detect
370 any contaminations of cattle with HuNoV.

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

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

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

387

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389

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398

399 **Conflict of interest statement**

400

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

402

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- 507

508 **FIGURE LEGENDS**

509

510 Figure 1. Neighbor-joining phylogenetic tree based on the RNA dependent RNA
 511 polymerase/capsid protein coding region (429 nt) of bovine noroviruses and the
 512 Norwalk virus. Bovine norovirus isolates identified in this study (in bold face) are
 513 genetically compared to bovine norovirus reference strains (underlined) and
 514 previously isolated strains. Scale bar represents the phylogenetic distances expressed
 515 as units of expected nucleotidic substitutions per site. Bootstrap values (1,000
 516 replicates) are reported. Genbank accession numbers of strains used : AF097917
 517 (Bo/NV/Newbury2/1976/UK) ; AJ011099 (Bo/NV/Jena/1980/DE) ; AY12674
 518 (Bo/NV/Dumfries/94/UK) ; EU877966 (Bo/NV/BV9/2007/BE) ; EU877967
 519 (Bo/NV/BV15/2007/BE) ; EU877969 (Bo/NV/BV24/2007/BE) ; EU877970
 520 (Bo/NV/BV52/2007/BE) ; EU877971 (Bo/NV/BV99/2007/BE) ; EU877972
 521 (Bo/NV/BV119/2007/BE) ; EU877973 (Bo/NV/BV120/2007/BE) ; EU877974
 522 (Bo/NV/BV129/2007/BE) ; EU877975 (Bo/NV/BV133/2007/BE) ; M87661
 523 (Hu/NV/Norwalk 8FIIa/1968/USA). The tenth amplicon, Bo/NV/BV18/2007/BE,
 524 (Genbank accession number EU877968), was not introduced in the tree because its
 525 sequence was shorter.

526

527 Figure 2. Supernatants of the baculovirus expression of the capsid protein of the B309 isolate
 528 were submitted to purification in sucrose gradient. SDS-PAGE analysis was
 529 performed on each fraction and the membrane was stained with Coomassie blue.
 530 Results for some fractions are reported here (lanes 1 to 9). Proteins of about 57 kDa,
 531 the expected molecular weight for the B309 capsid protein, were shown. Supernatant

532 of Sf9 cells infected by a wild type baculovirus (lane WT) was used as negative
533 control. MwM : molecular weight marker.

534

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

542

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

550

551 Figure 5. Seroprevalence against bovine noroviruses in the Belgian cattle. The OD means of
552 the different age groups of animals, collected in the ELISA format (unequal variance
553 and non normal distribution), were compared with the Wilcoxon rank-sum test. The
554 distribution of the optical densities obtained with the indirect ELISA is represented in
555 a box plot format where optical densities are reported in function of age classes. An
556 asterisk highlights significant statistical differences between means of the different age

557 groups. The cut-off is represented by the line at 0.3 and the dots represent extreme
558 values. OD: optical density, negative control: optical density value obtained for the
559 bovine negative serum (anti-rabbit IgG), positive control: optical density value
560 obtained for the positive control (calf experimentally infected with the Newbury2
561 strain (Bridger et al., 1984)), mo: month, wk: week, yr: year.
562

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Figure 1

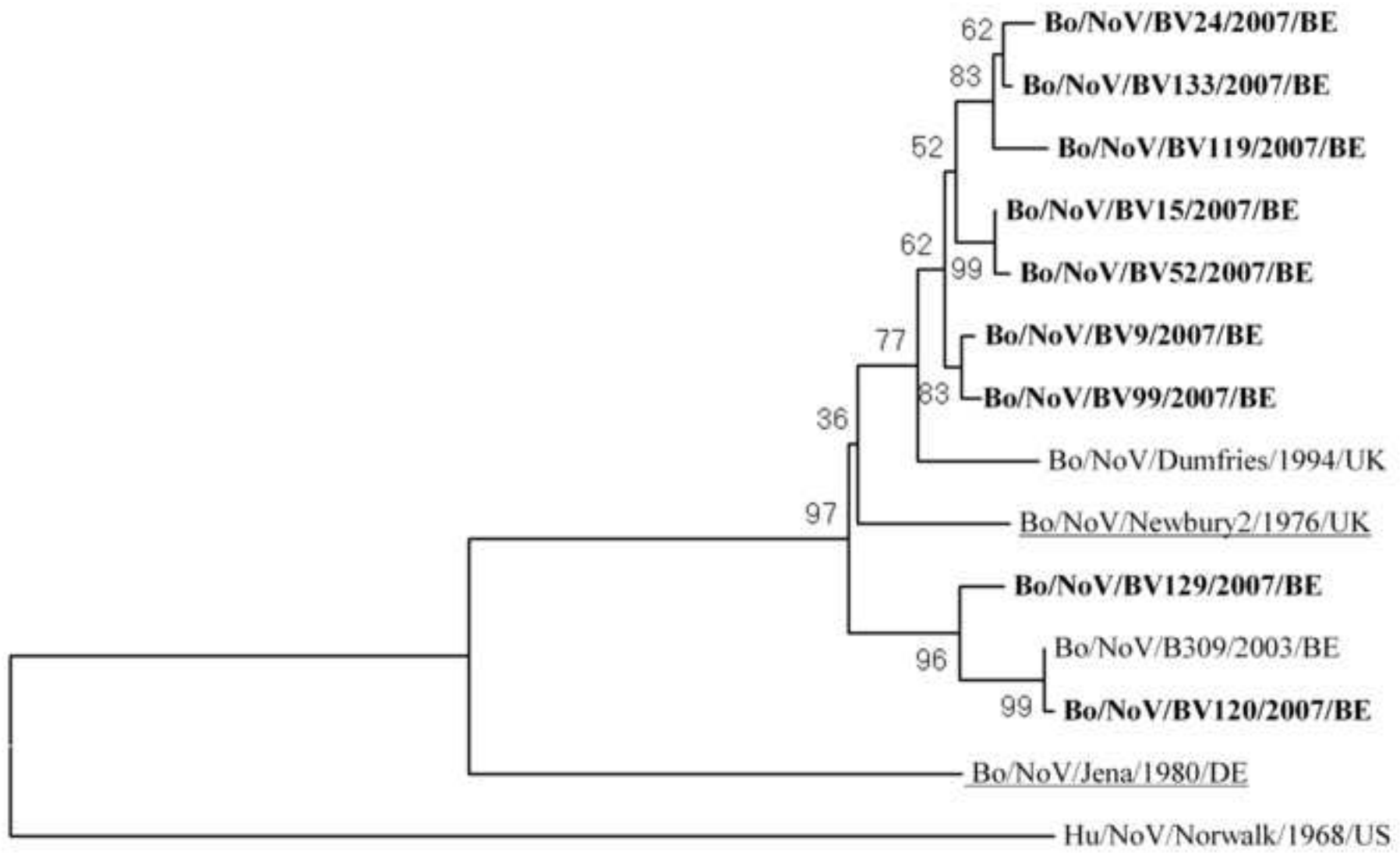
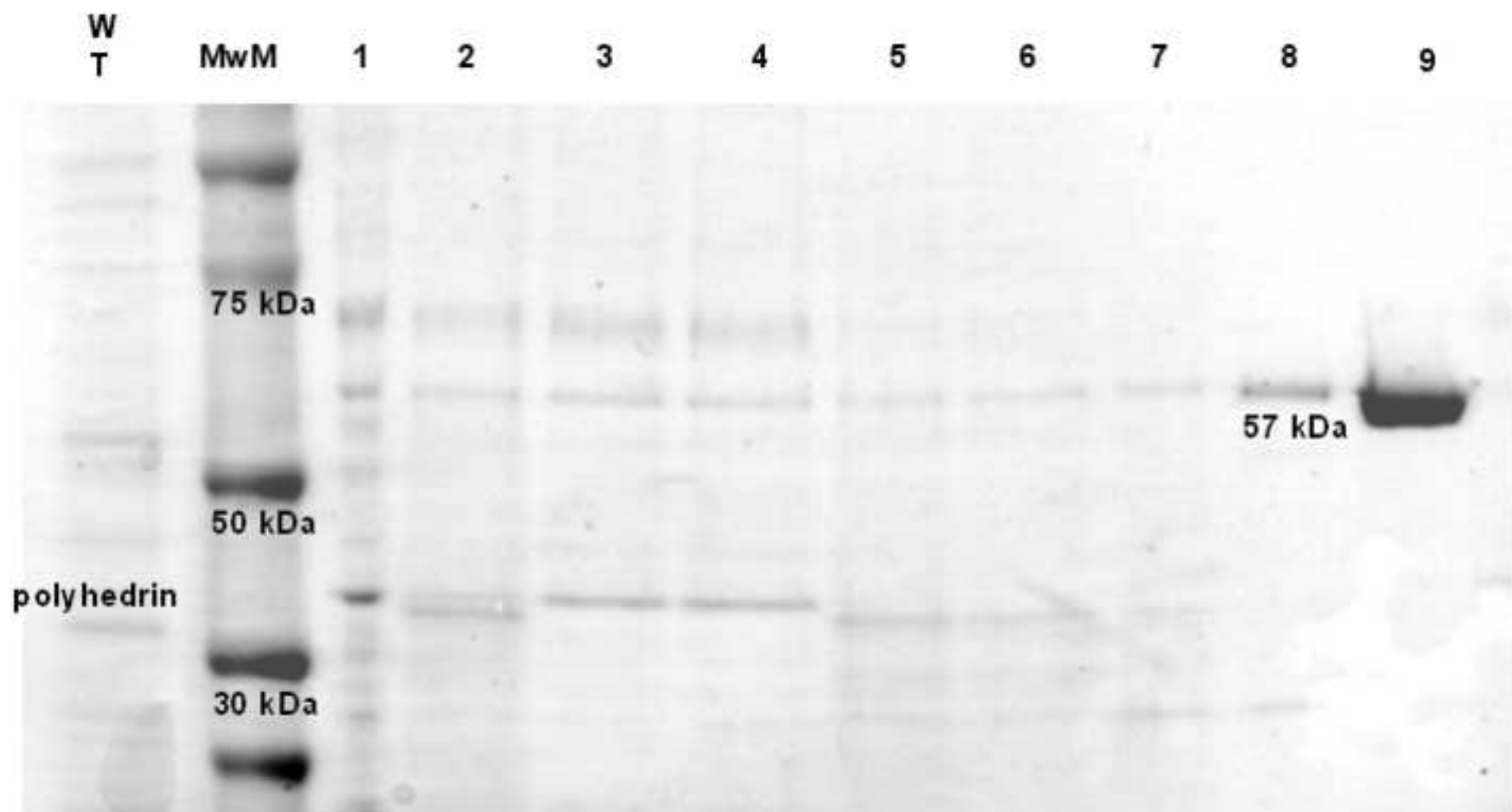
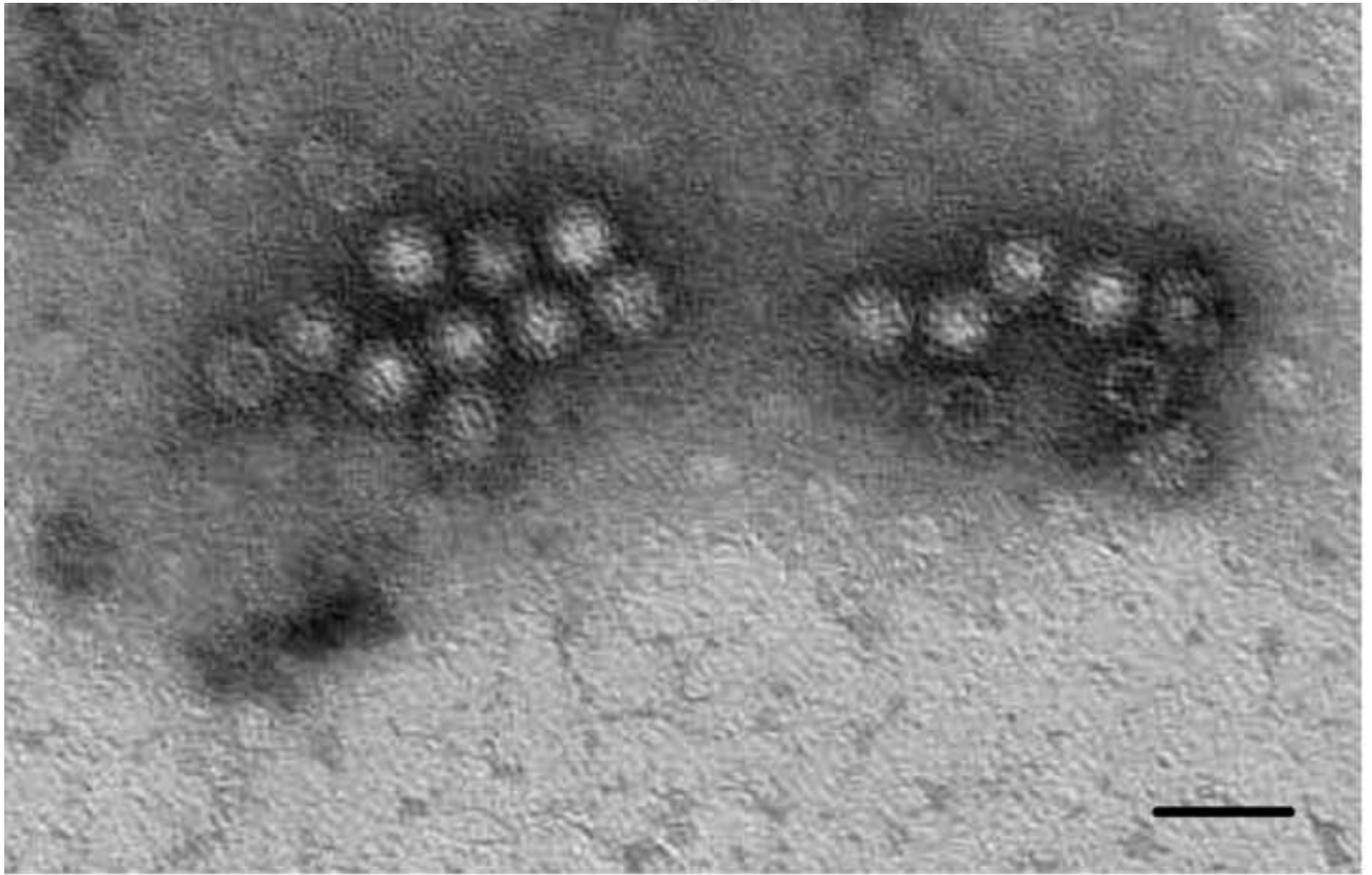


Figure 2





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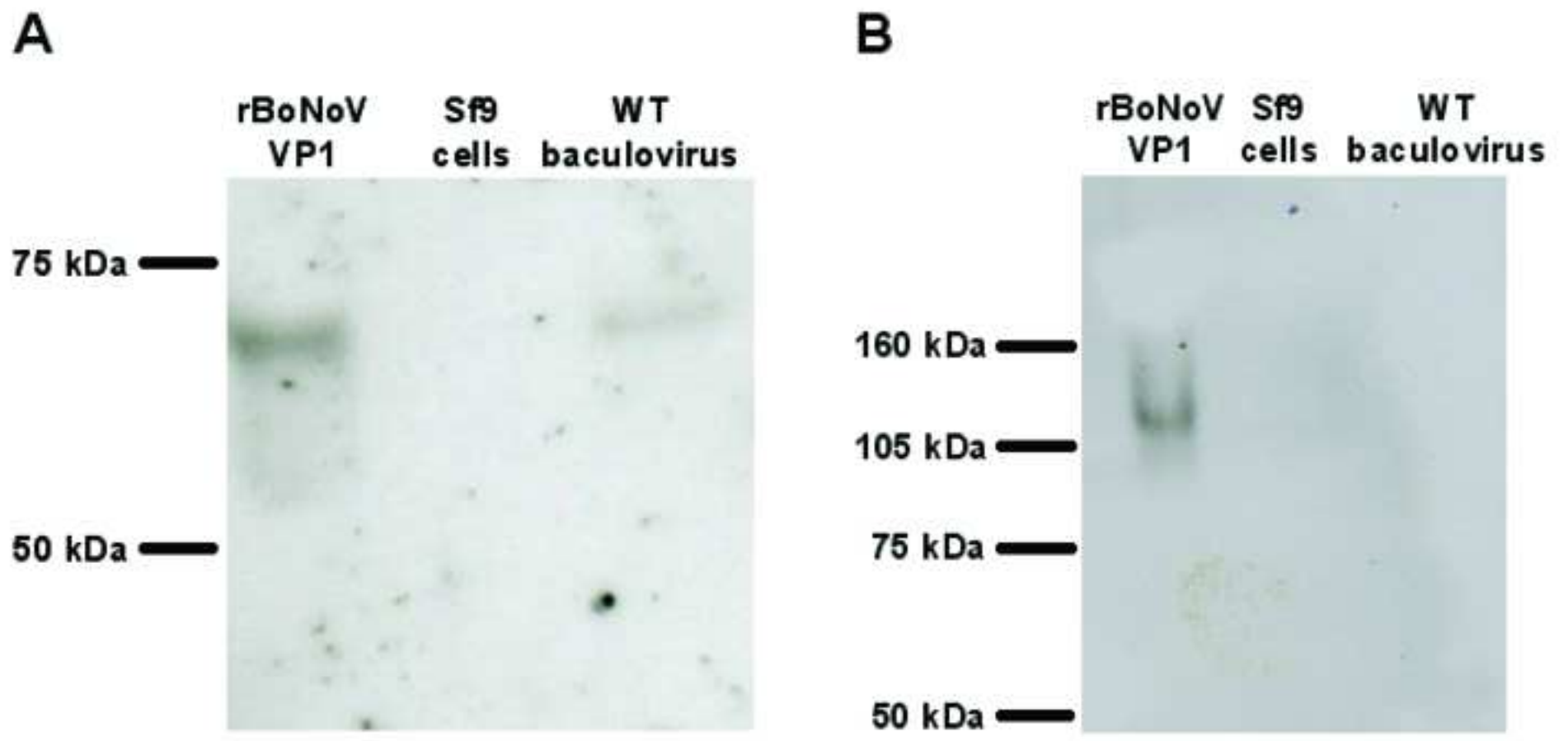
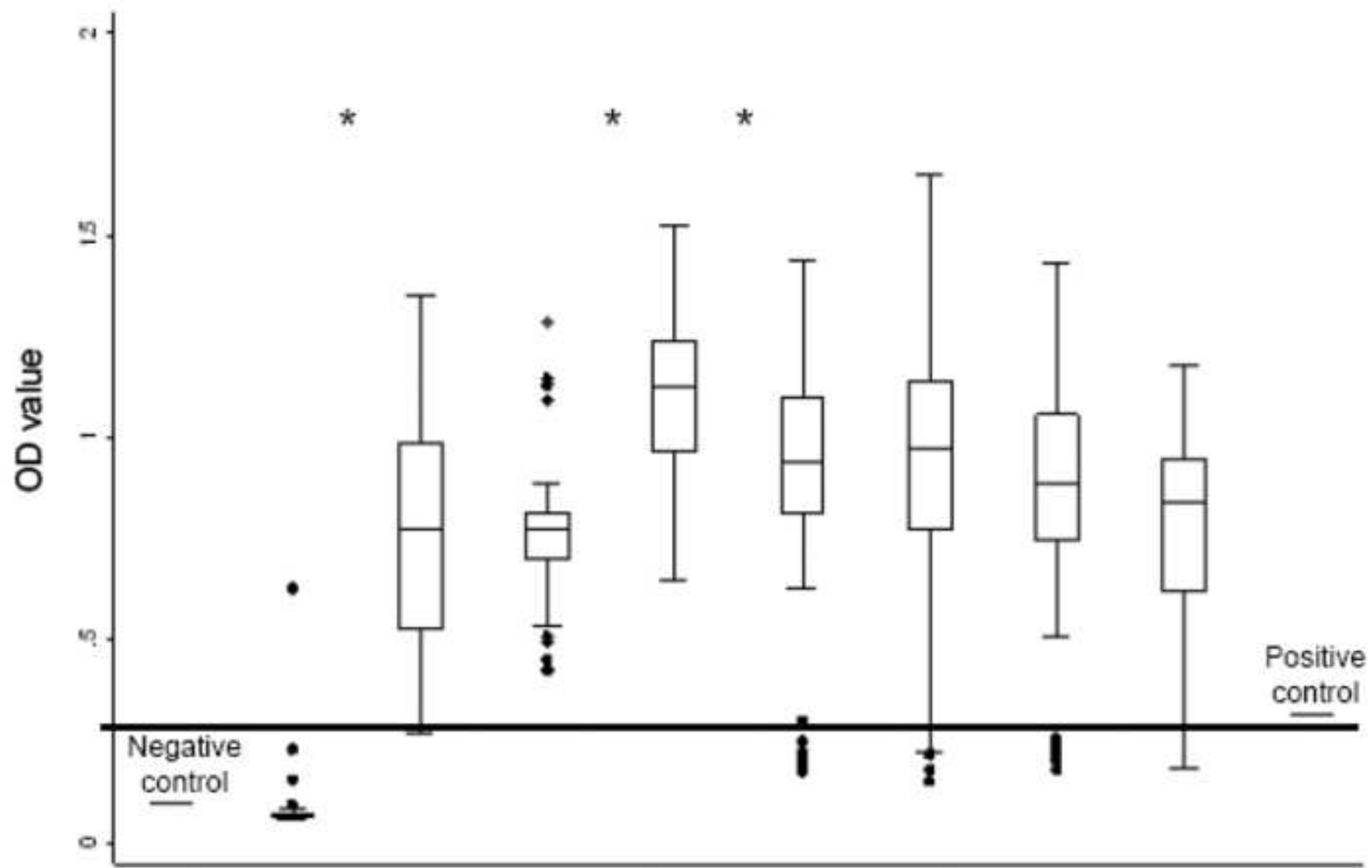


Figure 5

Preprint



Age =	Newborns	1wk -1 mo	1-6 mo	6 mo-1 yr	1-3 yr	3-6 yr	6-9 yr	Up to 9 yr
N =	30	20	29	76	105	123	59	27