
Bovine Norovirus: Carbohydrate Ligand, Environmental Contamination, and Potential Cross-Species Transmission via Oysters

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Abstract:

Noroviruses (NoV) are major agents of acute gastroenteritis in humans and the primary pathogens of shellfish-related outbreaks. Previous studies showed that some human strains bind to oyster tissues through carbohydrate ligands that are similar to their human receptors. Thus, based on presentation of shared norovirus carbohydrate ligands, oysters could selectively concentrate animal strains with increased ability to overcome species barriers. In comparison with human GI and GII strains, bovine GIII NoV strains, although frequently detected in bovine feces and waters of two estuaries of Brittany, were seldom detected in oysters grown in these estuaries. Characterization of the carbohydrate ligand from a new GIII strain indicated recognition of the alpha-galactosidase (α-Gal) epitope not expressed by humans, similar to the GIII.2 Newbury2 strain. This ligand was not detectable on oyster tissues, suggesting that oysters may not be able to accumulate substantial amounts of GIII strains due to the lack of shared carbohydrate ligand and that they should be unable to contribute to select GIII strains with an increased ability to recognize humans.

1 INTRODUCTION

2 Environmental sources of animal pathogens, and most specifically of RNA viruses, may
3 constitute substantial risk factors for cross-species transmission to humans (14). In this context,
4 noroviruses infecting cattle could be of importance owing to the high densities of cows bred in
5 areas of human activities. The ability of shellfish to concentrate pathogens released in seawater
6 raises questions about the transmission of animal NoVs to humans through oyster consumption,
7 but so far very few studies compared water and shellfish contamination. One of the first such
8 studies, conducted more than thirty years ago, comparing enterovirus by cell culture in water and
9 oysters yielded about the same frequency of positive water (59%) and shellfish samples (35%)
10 (12). More recently, phages of *B. fragilis* and *Salmonella* detected in sewage effluents were also
11 detected in receiving waters and oysters (6). Human NoVs were detected in 75% of river water
12 samples and in 60% of oyster beds (38). Only one study reported the detection of porcine
13 norovirus in 15% of shellfish collected from the US market without any information from the
14 surrounding water (8).

15 Noroviruses (NoVs) are small non enveloped viruses approximately 30 nm in diameter
16 with a positive-sense-single-stranded RNA genome. They belong to the *Caliciviridae* family and
17 in humans, they are the most frequent cause of diarrhea outbreaks in all age groups (11, 28). They
18 are classified in five genogroups, with human strains belonging to genogroups I, II and IV, GIII
19 strains infecting cattle and murine strains classified in GV (46). Recently, two new genogroups
20 (VI and VII) infecting animals have been proposed (29). Based on analysis of the ORF2 sequence
21 encoding the capsid protein, high diversity has been observed so that genogroups have been
22 subdivided into clusters, up to nineteen for GII strains. Porcine NoVs have been classified into
23 three clusters of GII (GII.11, GII.18, GII.19), whilst all bovine strains of NoV described so far
24 belong to GIII (25, 29, 41, 46). The first bovine strain Bo/Newbury2/1976/UK was isolated from

1 calves with diarrhea in the UK (43). Later, another distinct genotype of bovine NoV,
2 Bo/Jena/1978/GER was identified in Germany (21). These two strains represent the prototypes of
3 the GIII.2 and GIII.1 genotypes respectively.

4 Although many gaps persist in our understanding of human NoV infections and
5 pathogenesis, recent advances demonstrated a genetically determined host-susceptibility based on
6 histo-blood group antigens diversity. Various human NoV strains attach to distinct carbohydrates
7 of the ABH and Lewis histo-blood group family and evidence accumulated from volunteer
8 studies and outbreaks indicate that binding to these carbohydrates is required for infection (19,
9 35). In addition, it was recently shown that the prototype bovine GIII.2 strain binds to a related
10 carbohydrate structure which is absent from human tissues (45). Similarly it was also
11 demonstrated that some strains of either GI or GII specifically attach to oysters tissues through
12 recognition of histo-blood group antigens (HBGAs) (17, 22, 36). This finding could contribute to
13 explain observations such as rapid contamination of oysters, long persistence of viral particles
14 and consequently shellfish-borne outbreaks (3, 16). It additionally suggests that oysters may not
15 merely act as passive filters randomly accumulating virus particles, but instead, may act as
16 selective filters specifically concentrating strains by recognition of carbohydrate epitopes shared
17 with humans. As shellfish are grown in coastal waters frequently exposed to contamination from
18 bovine in neighboring fields, they may be contaminated by these animal strains. This raises the
19 issue of the potential role of oyster in the emergence of bovine NoVs into the human population.

20 The aim of our study is to provide quantitative data on the presence of GIII NoV strains in
21 bovine feces, river or estuarine waters as well as shellfish from an area of both high cattle and
22 high oysters breeding by comparison with GI and GII strains. The possibility of GIII strains
23 specific binding to carbohydrate ligands of oyster tissues that may be shared with cows and
24 humans is additionally examined. The results are discussed in the context of the environmental

- 1 data in order to provide a first appreciation of the risk of GIII NoVs transmission to humans
- 2 through oyster consumption.
- 3

1 MATERIALS AND METHODS

2

3 **Reagents.** The lectin from *Griffonia simplicifolia* B4 isolectin 1 (GS1-B4) either
4 peroxidase or fluorescein isothiocyanate (FITC) conjugated, which recognizes α 1,3-linked
5 terminal galactosyl residues, was purchased from Vector Laboratories (Burlingame, CA) and
6 from EY Laboratories (San Mateo, CA USA), respectively. Alpha-galactosidase from green
7 coffee beans was purchased from Sigma (St Louis, MO). The anti-B mAb ED3 was a kind gift
8 from Dr. A. Martin (CRTS, Rennes, France). The anti-GIII rabbit polyclonal antiserum was
9 prepared at the Veterinary School of Nantes by immunizing a rabbit with VLPs from the GIII.2
10 Bo/Newbury2/76/UK strain (45). This antiserum reacts nearly equally well with the BEC28
11 VLPs (see below) and the immunizing Newbury2 VLPs. The anti-GI rabbit antiserum was
12 prepared similarly using VLPs from the NV strain. Synthetic oligosaccharides as polyacrylamide
13 conjugates were kindly provided by Dr. N. Bovin (Moscow, Russia). Oligosaccharides coupled to
14 HSA (human serum albumin) were obtained from IsoSep AB (Tulligen, Sweden). The structure
15 of all oligosaccharides used is given on Table A1 of the Supplemental Material.

16 **Stool samples.** Bovine stool samples were collected from cattle in the surrounding area of
17 environmental sampling site in Brittany (North west of France). For analysis, a 10% suspension
18 was prepared and then nucleic acids (NA) were extracted and purified with a QIAmp Viral RNA
19 kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (1).

20 **Environmental samples.** Two small estuaries, sites A and B, located in northwestern
21 France (Brittany) were selected based on high bovine breeding since cows represent 8.86×10^6
22 and 1.84×10^6 inhabitant equivalents in terms of bacterial shedding in these two areas,
23 respectively, and based on shellfish productions classified as B areas (European Regulation

1 54/2004/EC) (Fig. 1). Streams or small rivers impacted by farms and then flowing into the
2 estuaries were identified and sampled during the study under a volume of one liter. Oyster
3 samples, each one constituted by at least 12 individuals, located downstream of potentially
4 contaminated water were collected.

5 **Viral elution and concentration:** One liter water samples without any pretreatment were
6 concentrated to 40 mL by using an ultrafiltration membrane (Vivaflow 50, Sartorius) after
7 addition of a known amount of Mengovirus (see below), and then by polyethylene glycol 6000
8 (PEG 6000) precipitation (Sigma, St Quentin, France) overnight at 4°C, before centrifugation for
9 1.5h at 1,500 x g (9). For oysters, all 12 individuals of a sample were dissected and digestive
10 tissues were pooled together to homogenize the sample and then frozen under 6 g portions. For
11 analysis, one portion was thawed and Mengovirus was added as a viral extraction control. Then
12 tissues were homogenized using beads (Fast Prep), extracted by vortexing with an equal volume
13 of chloroform-butanol for 30 s, and treated with cat-floc T (Calgon, Ellwood City, PA) for 5 min
14 at room temperature before centrifugation for 15 min at 13,500 x g. The resulting suspension was
15 precipitated with PEG 6000, for 1h at 4°C and centrifuged for 20 min at 11,000 x g at 4°C (2).

16 **Nucleic acid extraction:** For nucleic acid extraction and purification, the Nuclisens
17 extraction kit (BioMerieux, Lyon France) was used according to the manufacturer's instructions,
18 with minor modifications (18). PEG pellets, from water or oyster samples, were suspended in 1
19 mL of RNase-free water, mix with the lysis buffer (2 mL), and incubated for 30 min at 56°C.
20 After a brief centrifugation to eliminate particles (if needed), 50 µl of paramagnetic silica was
21 added and incubated for 10 min at room temperature. All washes were performed using the
22 magnetic ramp, and viral nucleic acids were recovered in 100 µl of elution buffer (BioMerieux,
23 Lyon, France). Extracts were directly analyzed or frozen at -80°C until they were used.

1 **Real-time RT-PCR.** All amplifications were conducted using a one step real time RT-
2 PCR (*r*RT-PCR) kit (Ultrasens, Invitrogen) with previously described primers and probes for
3 **Mengo virus (30)** or NoV (18, 42). Briefly, for NoV GI QNIF4 (5'-
4 CGCTGGATGCGNTTCCAT-3' with n: a/c/g/t), NV1LCR (5'-
5 CCTTAGACGCCATCATCATTTAC-3') and NV1LCpr (5'-TGGACAGGAGAYCGCRATCT-3'
6 with y: c/t and r: a/g); for GII, QNIF2d (5'- ATGTTCAAGRTGGATGAGRTTCTCWGA-3' with r:
7 a/g and w: a/t), COG2R (5'-TCGACGCCATCTTCATTCACA-3'), QNIFs (5'-
8 AGCACGTGGGAGGGCGATCG-3'); and for GIII, SW GIII forw (5'-
9 CGCTCCATGTTYGCBTGG-3' with y: c/t), SW GIII rev (5'-
10 TCAGTCATCTTCATTTACAAAATC-3') and the probe SWGIII (5'-
11 TGTGGGAAGGTAGTCGCGACRYC-3' with r: a/g and y: c/t).

12 All samples were analyzed in duplicate on 5 µl of undiluted or ten-fold diluted RNA
13 extracts. Two negative amplification controls (water) were included in each amplification series
14 and no more than six samples were analyzed in a *r*RT-PCR assay. Precautions such as isolated
15 rooms for various steps and the usage of filter tips were taken to prevent false positive results.

16 The cycle threshold (Ct) was defined as the cycle at which a significant increase in
17 fluorescence occurred (i.e. when fluorescence became distinguishable from background). The
18 number of NoV RNA copies present in positive samples was estimated using GI, GII and GIII
19 standard curves. To be included in the quantitative analysis, all wells had to yield a Ct value ≤ 41
20 **which was considered as the quantification threshold (Qt)**. The final concentration was then
21 determined based on the NA volume analyzed (5 µL of 100 µL of NA extract) and measured
22 weight of DT (6 g analyzed) (18, 30).

23 The efficiency of virus extraction procedures was determined for each extraction by seeding
24 10⁴ 50% tissue culture-infective doses of mengovirus prior to sample processing and

1 determining mengovirus recovery by *r*RT-PCR, as previously described (7, 18). NoV
2 concentrations were then corrected for virus loss during extraction by dividing the final norovirus
3 concentration (uncorrected) by the mean mengovirus extraction efficiency.

4 **Preparation of VLPs.** The entire capsid region was amplified from the intestinal content of a
5 calf infected with strain BEC/28/03IT (Acc. n°. GQ397857) identified in a survey performed in
6 Northern Italy as described in the Supplemental Material. It was then cloned into the pFastBac
7 vector which was used to prepare the recombinant bacmid BAC BEC28 according to the
8 manufacturer's instructions (Invitrogen). The sequence of the vector obtained confirmed the
9 proper frame of the ORF2 and the close relationship, 92% identity, of the Italian strain with a
10 previously described dutch BEC CH131-like strain (39). The bacmid was transfected into Sf9
11 insect cells to prepare the high titre BACBEC28 virus stock solution. VLPs were produced by
12 infection of Sf9 cells with the stock baculovirus solution and purified by ultracentrifugation
13 through a 30% sucrose cushion (wt/vol), followed by a CsCl (1.362 g/cm³) density gradient (13).
14 Quality of the purified BEC28 VLPs was analyzed by Western blotting and electron microscopy.

15 VLPs from the GIII.2 Bo/Newbury2/76/UK (NB2) strain prepared as previously described
16 (44) were a kind gift from Dr. D. Poncet (CNRS, Gif/Yvette, France). VLPs from the GI.1
17 Norwalk strain were prepared as previously described (24).

18 **Bioaccumulation of VLPs.** GI.1 (Norwalk) (0.8 µg) and GIII.2 (Newbury2) (1.28 µg)
19 VLPs were added to 500 mL of clean sea-water and homogenized for 5 minutes to obtain 3×10^7
20 and 7×10^7 VLPs/mL for GI and GIII, respectively. Two clean live oysters were added to the
21 seawater and incubated for 24 hours at 15°C (water temperature) under oxygenation. The
22 bioaccumulation experiment was conducted twice.

23 **Tissue samples and immunohistochemical analysis.** Bovine and porcine tissues samples
24 from the gastroduodenal junction were obtained from healthy animals autopsied at the National

1 Veterinary School of Nantes. Animals were handled in strict accordance with good animal
2 practice as defined by the French national guidelines. Human gastroduodenal junction samples
3 had been obtained from organ donors before the law 88-1138 of December 20, 1988 concerning
4 resection of human tissues after death for scientific investigations. Oysters tissues were prepared
5 from a batch of clean oysters (tested negative for NoV contamination) or from the VLPs
6 bioaccumulated oysters so as to visualize all organs on a single section. Animal tissues were
7 fixed in formalin and human tissues were fixed in ethanol 95% for 48h, and paraffin embedded.
8 Sections (5 μm) were rehydrated in graded ethanol and washed in PBS. Endogenous peroxidase
9 was inhibited by a 20 min incubation with methanol/ H_2O_2 0.3%. Sections were then washed in
10 PBS/bovine serum albumin (BSA) 1% for 30 min at room temperature. After washing in PBS,
11 sections were incubated with the peroxidase- conjugated GS1-B4 lectin at 10 $\mu\text{g}/\text{mL}$ at 4°C
12 overnight. The attachment of VLPs to tissue sections was tested with BEC28 VLPs at 10 $\mu\text{g}/\text{mL}$
13 or NB2 VLPs at 1 $\mu\text{g}/\text{mL}$, followed by sequential incubations with a rabbit anti-GIII.2 antiserum,
14 peroxidase-conjugated anti-rabbit IgG and the 3-amino-9-ethylcarbazol peroxidase substrate as
15 previously described (45). Detection of Norwalk or Newbury2 VLPs from bioaccumulated
16 oysters was performed by directly the anti-GI or anti-GIII rabbit antisera. Periodate treatment at 1
17 and 10 mmol/L for 30 min and alpha-galactosidase treatment were performed immediately after
18 the endogenous peroxidase quenching as previously described (17).

19 **ELISA-based carbohydrate microtiter plate assays.** Oysters located in the same
20 geographic area, under the same climatic conditions, yet in a clean area, were collected during the
21 same period of time as those from the field study. Tissue extracts were prepared according to
22 Maalouf et al. (22). For each tissue (digestive tissue, gills, mantle), extracts were prepared from 7
23 samples, each constituted by at least 8 oysters, collected throughout the year. Tissue extracts at

1 40 µg/mL were coated onto NUNC Maxisorp immunoplates (ThermoFischer Scientific) in 100
2 mmol/L carbonate buffer pH=9.6 by overnight incubation at 4°C. After blocking with 10%
3 defatted dried cow's milk in PBS for 1 hour, NB2 VLPs (1 µg/mL) or NV VLPs (1 µg/mL) in
4 PBS 5% milk were added and incubated for 1 h at 37°C. Plates were then incubated with rabbit
5 anti-GIII.2 serum or anti-GI.1 serum at 1/1000 dilution in PBS 5% milk for 1 hour at 37°C. Then,
6 peroxidase anti-rabbit IgG (Uptima, Montluçon, France) at a 1/2000 dilution in PBS 5% milk
7 were added and incubated for 1 hour at 37°C. Between each step, the plates were washed 3 times
8 with PBS 5% Tween 20. The enzyme signals were detected with TMB (3, 3', 5, 5'
9 tetramethylbenzidine) as substrate (BD Bioscience, San Jose, CA) and then read at 450 nm.

10 Oligosaccharides as PAA and HSA conjugates were coated at 10 µg/mL at 37°C in a wet
11 atmosphere. After blocking with 5% defatted dried cow's milk in PBS for 1 hour, BEC28 VLPs
12 (9,2 µg/mL) in PBS 5% milk were added. After incubation for 2 hours at 4°C, binding of VLPs
13 was detected as above using rabbit anti-GIII.2 serum and then peroxidase anti-rabbit IgG with 1
14 hour incubations at 4°C.

15 **Cell transfection and flow cytometry analysis.** The complete coding sequence of the
16 *Ggtal* gene encoding the rat α 1,3galactosyltransferase was cloned as previously described (37)
17 and inserted into the pCR3.1 eukaryotic expression vector (InVitrogen, Paisley, UK). It was used
18 to transfect Human Embryonic Kidney (HEK293) cells with lipofectAMINTM (InVitrogen). Forty
19 eight hours later, cells were labeled with either FITC-labelled GS1-B4 lectin at 10 µg/mL or
20 BEC28 VLPs at 9.2 µg/mL as previously described (45). In the former case, fluorescence
21 analysis was performed immediately following washes. In the latter case, cells were sequentially
22 incubated with the rabbit anti-GIII.2 serum at a 1/1000 dilution and with FITC-labeled anti-rabbit

- 1 IgG (Sigma) for 30 min. After final washing, fluorescence analysis was performed on a
- 2 FACSCalibur (Becton Dickinson, Heidelberg, Germany) with the CELLQUEST program.

1 RESULTS

2 **Detection of NoVs in feces and environmental samples.**

3 GIII NoV was detected in 25 samples out of 136 stool samples analyzed (18%). A total of 70
4 water samples were collected (29 from site A and 41 from site B) on June, September, November
5 2008, January, March, April, May, July, August and September 2009 and tested for the presence
6 of GI, GII and GIII NoVs. The three genogroups were detected in water samples from both sites.
7 GI NoVs were detected in two samples from site A and three from site B. GII NoVs were
8 detected in four samples from site A and 13 samples from site B, and GIII NoVs in two samples
9 from site A and eight samples from site B. A total of 47 samples of oysters (constituting at least
10 564 oysters) were collected over the study. Thirty eight samples were collected from site A and
11 among these, one and seven were positive for GI and GII NoVs, respectively. From site B, over
12 the nine oyster samples collected, one was found positive for GI, three for GII and one for GIII
13 NoVs. Multiple contaminations by GI and GII NoVs were detected in three water samples.
14 Contaminations with both GII and GIII were detected in three additional water samples. The only
15 oyster sample positive for GIII NoV was also found contaminated with a GII NoV. The
16 frequencies of either waters or oysters NoV contamination did not differ significantly between
17 the two sites for any of the genogroups ($p < 0.2$, Fisher's exact test). The results from both areas
18 were thus pooled for further analysis as shown in [Table 1](#).

19 Quantitative analysis of NoV contamination of water samples indicated that far less GI
20 than either GII or GIII RNA copies were detected during the study period. Yet, the mean values
21 of contaminated water samples did not differ between the three genogroups owing to the high
22 variation in the number of detected RNA copies and to the lower number of GI contaminated
23 samples. Likewise, the mean numbers of RNA copies detected in contaminated oyster samples

1 were not significantly different among genogroups. For both GI and GII NoVs, the percentages
2 of contaminated water and oyster samples were quite similar (7% vs 4% for GI and 24% vs 21%
3 for GII). Yet, in the case of GIII NoV, the percentage of positive water samples was higher than
4 that of oyster samples (14% and 2%, respectively), suggesting GIII NoVs may not accumulate as
5 efficiently as either GI or GII into oysters. However, owing to the small number of contaminated
6 oyster samples, this difference did not reach statistical significance. When calculating the ratio of
7 total RNA copies detected in waters over copy numbers detected in oysters, a striking difference
8 appeared between GI and either GII or GIII NoVs. Indeed, this ratio was 39 and 53 fold lower for
9 GI than for GII and GIII, respectively, suggesting a highly efficient GI accumulation as compared
10 to GII and GIII accumulations in oysters (Table 1).

11 **Characterization of the BEC28 VLPs ligand.** We previously used
12 immunohistochemistry to show the binding of VLPs from several calicivirus strains, including
13 the bovine GIII.2 Bo/Newbury2/76/UK norovirus strain (NB2), to carbohydrates expressed on
14 epithelial digestive cells (17, 24, 32, 45). Within genogroup, variations in carbohydrate
15 specificity have been reported for human GII.4 strains (20). Thus, to determine if the ligand
16 specificity of NB2 is also variable among GIII.2 strains, we sought to characterize a potential
17 carbohydrate ligand of the newly described strain BEC28. This strain is clearly distinct from
18 NB2, yet it can be classified as GIII.2 (see Figure A1 of the Supplemental Material). To this aim,
19 BEC28 VLPs were incubated on tissue sections from the gastroduodenal junction of bovine,
20 porcine and humans. Distinct results were obtained for each species. In bovine tissues, we
21 observed a staining on both endothelial and epithelial cells from the gut. In porcine tissues, the
22 BEC28 capsids attached exclusively to non epithelial cells of the digestive tract, mainly vascular
23 endothelial cells, whereas staining was completely absent on human tissue sections tested in the
24 same experiments (Fig.2 A-D). In similar experimental conditions, GI.1 and GII.4 VLPs readily

1 stained human epithelial cells of serial tissue sections from the same individuals (data not
2 shown). To determine if the binding involved carbohydrates, bovine tissue sections were
3 pretreated with sodium periodate prior to incubation with the VLPs. At a 10 mmol/L
4 concentration, but not at 1 mmol/L, the staining was completely lost, suggesting that BEC28
5 VLPs recognize a neutral glycan structure expressed both on bovine digestive surface epithelial
6 cells and vascular endothelial cells (Fig.2 E and F). Since these results were reminiscent of what
7 we previously observed with VLPs from the bovine NB2 strain which bind to the α Gal epitope
8 (see below), the effect of α -galactosidase treatment was tested. We observed that following
9 treatment, the staining completely disappeared, indicating recognition of glycans with a terminal
10 galactose residue in alpha linkage.

11 To define more precisely their carbohydrate specificity, binding of BEC28 VLPs was
12 tested on a set of HBGA-related oligosaccharides by ELISA. A signal five times above
13 background, was observed on a single structure terminated by a galactose in α 1,3 linkage, the
14 Gal α 3Gal β 4GlcNAc trisaccharide called the α Gal or Galili epitope (Fig. 3). We next transfected
15 human HEK 293 cells with the rat *Ggtal* cDNA to allow expression of the α Gal antigen as
16 detected by flow cytometry with the GS1-B4 lectin. Control HEK cells that lacked the α Gal
17 antigen were not recognized by BEC28 VLPs. In contrast, a clear binding was observed on the
18 *Ggtal* transfected human cells (Fig. 4). Collectively these results indicate that BEC28 VLPs
19 have a carbohydrate specificity similar to that of NB2 VLPs (45), specifically recognizing the
20 α Gal antigen.

21 **GIII.2 ligands from oyster tissues.** Searching for the α Gal epitope on oyster tissues,
22 tissue sections allowing analysis of all organs were covered by the GS1-B4 lectin. No staining
23 was observed, contrasting with the strong staining obtained on bovine gut tissue sections (Fig.5 A

1 and B). The α Gal epitope is closely related to the B blood group antigen (compare α Gal
2 trisaccharide and B type 2 in Table A1 of Supplemental Material), we looked for the presence of
3 the B antigen in oyster tissues. An anti-B mAb that strongly binds to human tissue sections from
4 B blood group persons did not react with oyster tissues (data not shown). Since oysters may
5 present a GIII ligand distinct from the α Gal epitope that could be recognized by the VLPs, we
6 tested the binding of the NB2 recombinant capsids to oyster soluble tissue extracts. No specific
7 signal could be detected by ELISA on seven soluble extracts of three types of tissues (mantle,
8 gills and digestive tissues) each prepared from at least eight oysters, whereas in the same assays,
9 these VLPs attached to the synthetic α Gal epitope and GI.1 VLPs, used as positive controls,
10 readily attached to oyster digestive tissues soluble extracts (Fig. 6). Likewise, although a clear
11 binding of both NB2 and BEC28 VLPs was observed on bovine duodenum tissue sections used
12 as positive controls in parallel assays, only a slight occasional labeling of some digestive tubules
13 surface by either type of VLPs could be detected (Fig.5 C-F). In control experiments no staining
14 could be observed in absence of GIII VLPs, whereas strong unambiguous stainings were obtained
15 by using GI.1 VLPs detected with a specific antiserum, consistent with previous reports (data not
16 shown). These results indicate that oyster tissues do not express the α Gal epitope in detectable
17 amounts, but may nevertheless either express small amounts of another ligand yet to be
18 characterized or bind small amounts of GIII VLPs in a non specific manner.

19 Next, the ability of GI.1 and GIII.2 VLPs to be bioaccumulated in oysters was compared.
20 Both types of VLPs were seeded in water at similar concentrations (3×10^7 and 7×10^7
21 particle/mL, respectively). After 24h oyster tissues were fixed and the presence of VLPs was
22 searched by immunohistology using anti-GI and anti-GIII antisera that have identical titers

1 against their respective VLPs by ELISA. In these conditions GI.1 VLPs, used as positive
2 controls, were readily detected. In contrast, GIII VLPs could not be detected (Fig. 6 G and H).
3

1 **DISCUSSION**

2 Tracing viral contamination in the open environment is quite difficult due to several
3 factors such as source identification, dilution, currents and finally sampling strategy (15, 31).
4 Cows are bred at very high densities in some areas such as the west coast of France (Brittany),
5 which is also a major area of shellfish production. The present study represents a first approach to
6 evaluate the prevalence of bovine NoVs in environmental waters and in shellfish. Two sites from
7 Brittany were selected based on their bovine population at least a hundred fold superior to their
8 human population in terms of sewage input (10^6 inhabitants equivalents for bovine compared
9 with 10^4 and 10^3 inhabitants equivalents for humans at sites A and B, respectively). The shellfish
10 growing areas of these two sites (mainly oysters) are classified as B areas (European Community
11 regulation, 54/2004/EC). Eighteen percent of the bovine stool samples tested were positive for
12 GIII NoV, a value comparable with those from other studies which range widely from 2% to 50%
13 (25-27, 33, 39). Bovine NoVs were detected in a total of 14% water samples. By comparison GI
14 and GII NoVs were detected in 7% and 24% of water samples, respectively. Moreover the sum of
15 GI RNA copies detected in water during the whole study period was 28 times lower than that of
16 GII, in accordance with epidemiological data which show a large predominance of GII strains in
17 the human population (34). The sum of GIII RNA copies detected was only twice lower than that
18 of GII Human NoVs, showing the high impact of bovine production on water contamination. In
19 Brittany, bovines are bred in open fields most of the year and thus feces may directly be dragged
20 along towards small nearby rivers. Considering the substantial percentage of water positive
21 samples and the long persistence of human NoVs in contaminated oysters, we were surprised to
22 find only one GIII shellfish positive sample. Because extraction and inhibitor controls were used,
23 we trust that negative samples were truly negative (18). The primers and probe set that was used
24 is another factor that could have influenced the results. However, [the primers and probe used,](#)

1 selected from the literature, readily detected GIII viruses in bovine stools and water samples
2 collected in the same area.

3 Specific binding of human NoV strains to oysters tissues was previously shown to occur
4 through interaction with carbohydrate ligands shared with humans, suggesting that oysters may
5 select NoV strains present in the environment, based on the presence of specific carbohydrate
6 ligands (17, 36, 40). Contamination of seawater by animal NoVs could thus potentially lead to a
7 selection of strains recognizing shared carbohydrate motifs, thereby facilitating cross-species
8 transmission to humans. We show here, that similar to the GIII.2 prototype NB2 strain (45),
9 BEC28 VLPs recognize the α Gal epitope ($\text{Gal}\alpha 3\text{Gal}\beta 4\text{GlcNAc}\beta\text{-R}$) expressed on surface
10 epithelial cells of bovine stomach and duodenum. Specificity for this ligand therefore appears to
11 be conserved among GIII.2 strains despite the genetic distance between the BEC28 and NB2
12 strains. Synthesis of the α Gal epitope requires an $\alpha 1,3$ galactosyltransferase which is lacking in
13 all human cells since the *GGTA1* gene encoding this enzyme has been inactivated and is therefore
14 completely absent from human tissues (23). In addition, the GIII ligand was not detectable on
15 porcine digestive epithelial cells making it unlikely that either humans or swine could be infected
16 by these NoV strains since binding to HBGAs appears necessary for infection by various human
17 strains (10, 19, 35). Moreover, we observed that the α Gal epitope and the closely related B blood
18 group antigen are not expressed on oyster tissues, suggesting that oysters should not be able to
19 specifically concentrate bovine strains through binding to these carbohydrate structures which
20 share a terminal $\text{Gal}\alpha 3\text{Gal-R}$ motif. Nevertheless, a weak binding of both NB2 and BEC28 VLPs
21 to unidentified structures of oysters digestive epithelial cells was detectable, consistent with the
22 rare and quantitatively weak detection of GIII sequences in oyster samples compared with the
23 frequent water contamination. In bioaccumulation experiments no GIII VLPs was detected in

1 oyster tissues, suggesting that the lack of specific ligand in these tissues may contribute to
2 explain the rare occurrence of GIII oyster contamination despite frequent water contamination. In
3 contrast, GI VLPs, which have a specific carbohydrate ligand in oyster digestive tract, were
4 efficiently bioaccumulated as previously described (22). This is in accordance with the low ratio
5 of GI RNA copies between water and oyster samples found in our environmental study.
6 Alternatively, we cannot exclude the possibility that GIII strains, as well as GII strains, may not
7 accumulate and persist in oysters as efficiently as GI strains because of a lower stability. Thus, GI
8 NoVs could accumulate and persist in oysters because of high environmental stability and of the
9 presence of a specific ligand. In contrast, GII and GIII strains would accumulate and persist less
10 well because of the absence of one of these two characteristics. Overall, our results indicate that
11 while largely present in the coastal environment, GIII strains are not so frequently found in
12 oysters. Because of the absence of a specific carbohydrate ligand, selection of variants that would
13 be able to cross-recognize human carbohydrate receptors would unlikely occur. Nevertheless,
14 some contamination of oysters by GII through nonspecific interactions may occur. Thus, it is
15 unlikely that oyster consumption would facilitate GIII NoV infections in humans.

16 Human NoV strains of the GI and GII genogroups use distinct binding sites on the P2
17 domain of the capsid protein to attach to HBGAs (4, 5). The binding site of GIII strains and their
18 mode of recognition of the α Gal epitope is not known as yet, but to more precisely evaluate the
19 risk of cross-species transmission, it will be important in future studies to determine the structural
20 changes necessary for GIII strains to recognize HBGA motifs expressed on the human gut and
21 potentially shared with cows. Likewise, bioaccumulation experiments with virions, rather than
22 with VLPs, will be necessary to precisely determine the ability of oysters to accumulate and
23 maintain GIII NoV strains. Stability in the environment of the different genogroups also needs to

1 be compared. In conclusion, we demonstrated that a new approach combining *in vitro* studies
2 and environmental samples analysis brings valuable information to investigate the possible
3 transmission of animal viruses to humans through oyster consumption.

4

5

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10

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- 47

1
2 **Table 1.** Detection and quantification of NoVs in water and oysters samples.

3

| | Water samples (N=70) ^a | | | Oyster samples (N=47) ^a | | | Water/Oysters ^e |
|------|-----------------------------------|---------------------------|------------------|------------------------------------|---------------------------|------------------|----------------------------|
| | N (%) ^b | Mean (range) ^c | Sum ^d | N (%) ^b | Mean (range) ^c | Sum ^d | |
| GI | 5 (7%) | 2,302 (120-4,900) | 11,510 | 2 (4%) | 190 (71-310) | 381 | 30 |
| GII | 17 (24%) | 19149 (410-110,000) | 325,530 | 10 (21%) | 45 (5-115) | 278 | 1,171 |
| GIII | 10 (14%) | 14,222 (130-120,000) | 142,220 | 1 (2%) | 90 | 90 | 1,580 |

4 ^aTotal number of samples

5 ^bNumber (and percentage) of positive samples

6 ^cNoVs geometric mean concentrations expressed as number of RNA copies/liter of water or g of
7 digestive tissue. The means between the three NoV genogroups, either in water or in oyster
8 samples, are not significantly different (Mann-Whitney two-tailed test).

9 ^dSum of NoV RNA copies/liter of water or g of tissue detected during the study period

10 ^eRatio of the sum of RNA copies detected in water and oyster samples during the study

11

FIGURE LEGENDS

1

2

3 **Figure 1:** Localization of the two sampling areas. The two sites (A and B) are located in Brittany,

4 western France. The dark grey areas represent the catchment basins impacting water quality at

5 these sites.

6 **Figure 2:** Analysis of BEC28 binding to tissues. VLPs were incubated on tissue sections and

7 detected as described in the materials and methods section. Binding to bovine pyloric surface

8 mucosa (A) and to the vascular endothelium of a bovine artery (B). Lack of binding to human

9 pyloric surface mucosa (C). Binding to porcine duodenal surface mucosa with lack of staining of

10 epithelial cells is shown by a star and staining of vascular endothelial cells of the lamina propria

11 is shown by an arrow (D). Effect of α -galactosidase treatment prior to VLPs incubation on a

12 bovine duodenum section (E) and serial control section incubated in the same condition in

13 absence of enzyme (F).

14 **Figure 3:** Binding of BEC28 VLPs to immobilized synthetic oligosaccharides. A panel of

15 neoglycoconjugates was coated on ELISA plates and the binding of BEC28 VLPs was detected

16 as described in the materials and methods section. Results for a selected set of

17 neoglycoconjugates with a terminal galactose in alpha linkage are shown as signal to noise ratios

18 from one experiment representative of three independent ones. None of the other

19 neoglycoconjugate tested showed a signal to noise ratio above 1 (see the list and structures in

20 Table A1 of the Supplemental Material).

21 **Figure 4:** Flow cytometric analysis of the binding of BEC28 VLPs and the GS1-B4 isolectin to

22 rat Ggta1 transfected or control mock transfected human HEK 293 cells. The respective negative

23 controls correspond to either cells incubated in the absence of the lectin or to cells incubated in

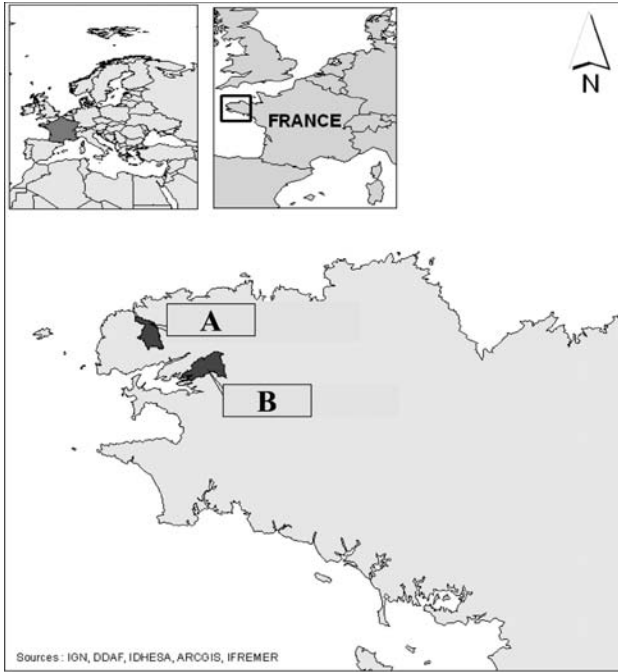
1 the presence of the VLPs followed by incubation with an irrelevant rabbit antiserum and FITC-
2 labeled anti-rabbit IgG. The log of fluorescence intensities in arbitrary units is plotted against the
3 cell number.

4 **Figure 5:** Expression of GIII ligands in oysters digestive tract and detection of bioaccumulated
5 VLPs. Staining of bovine pyloric mucosa by the GS1-B4 isolectin (A), BEC28 VLPs (C), NB2
6 VLPs (E) and comparison with oyster digestive tract. Absence of staining of oyster tissues by
7 GS1-B4 (B), and slight staining of the lumen of oyster digestive tubules by BEC28 VLPs (D) and
8 NB2 VLPs (F) shown by arrows. Presence of GI.1 (NV) VLPs in hemocytes and digestive
9 epithelial cells following bioaccumulation (G). Absence of GIII.2 (NB2) in oyster tissues
10 following bioaccumulation (H).

11 **Figure 6:** Binding of recombinant GI.1 (NV) and GIII.2 (NB2) VLPs to oyster tissue extracts.
12 Binding of GI.1 (black bars) and GIII.2 (white bars) VLPs to extracts of digestive tissues, gills
13 and mantle (x axis), expressed as signal to noise ratio +/- SD (y axis). The positive threshold is
14 indicated by a dashed line. Binding of NB2 VLPs to the α Gal trisaccharide coupled to
15 polyacrylamide and of NV VLPs to the H type 1 trisaccharide were used as positive controls on
16 each plate (not shown).

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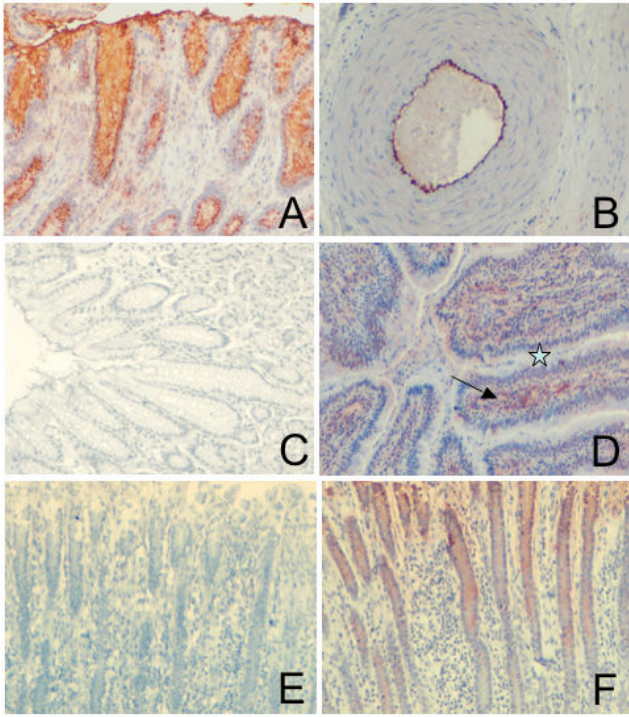
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2 Figure 1

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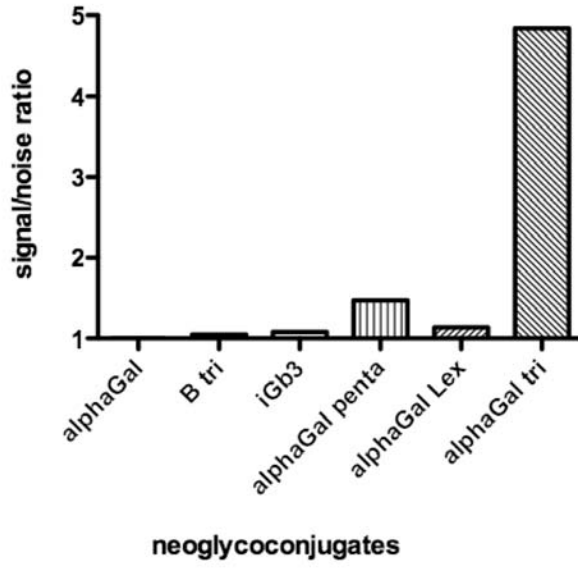


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4 Figure 2

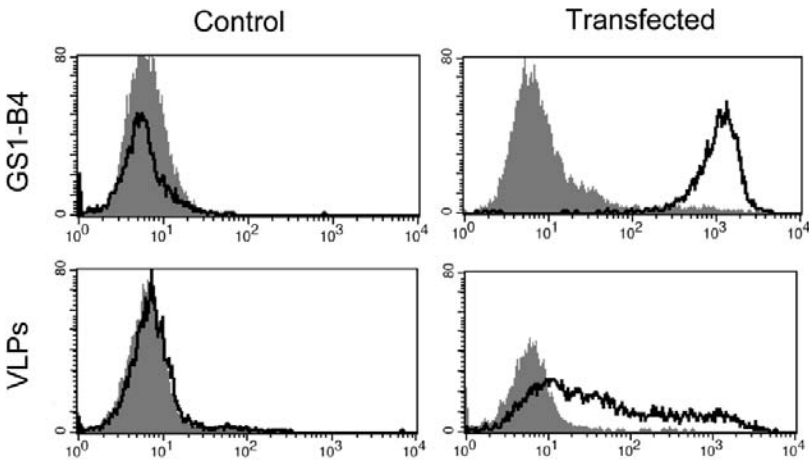
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3 Figure 3

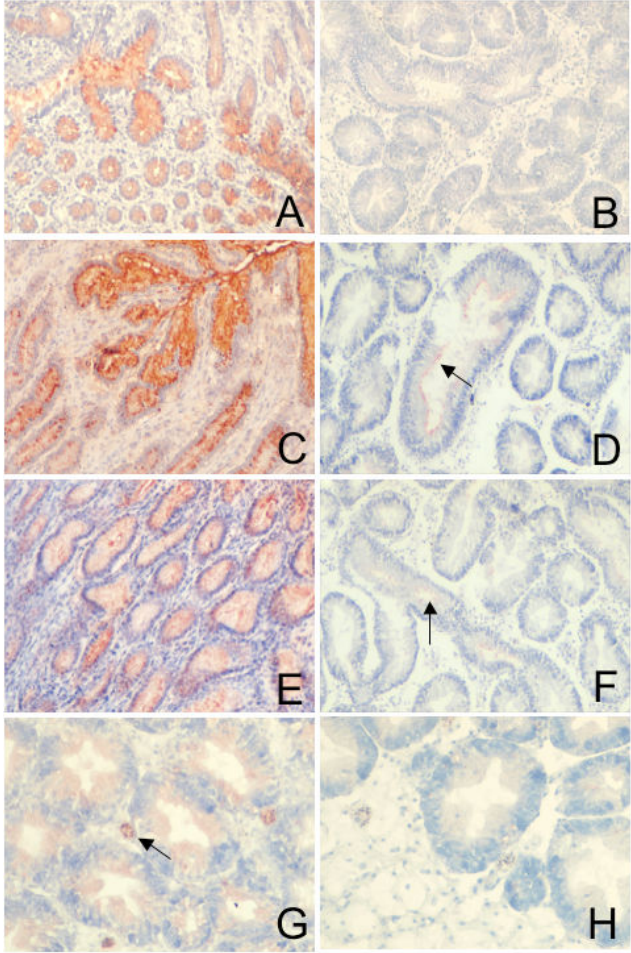
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3 Figure 4

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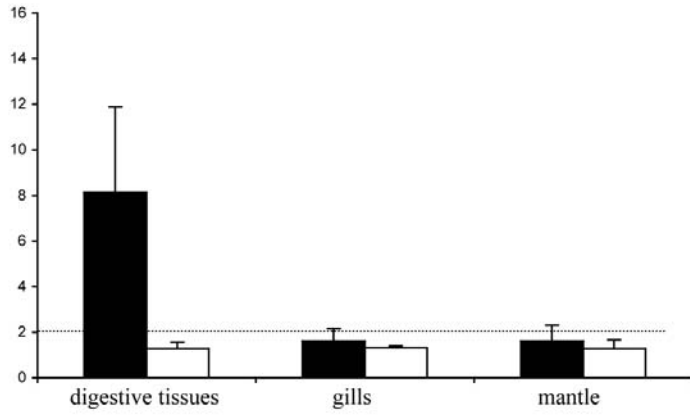


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4 Figure 5

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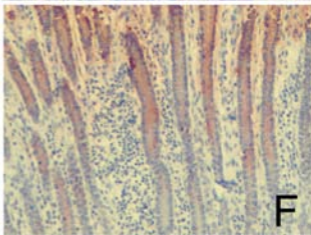
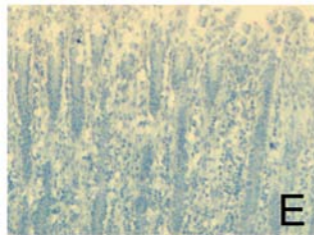
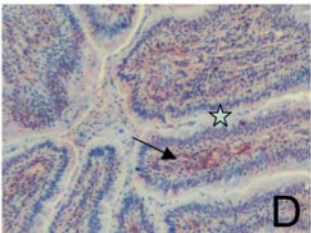
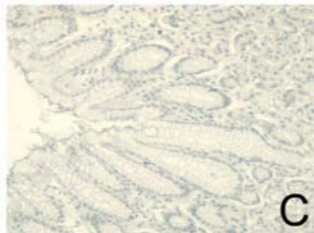
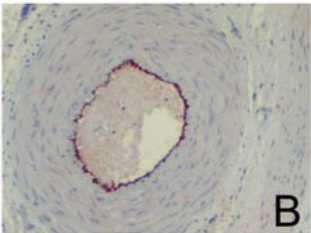
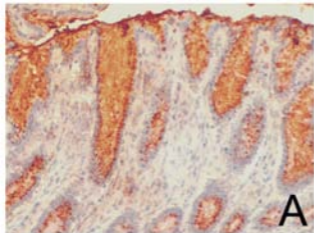
3 Figure 6

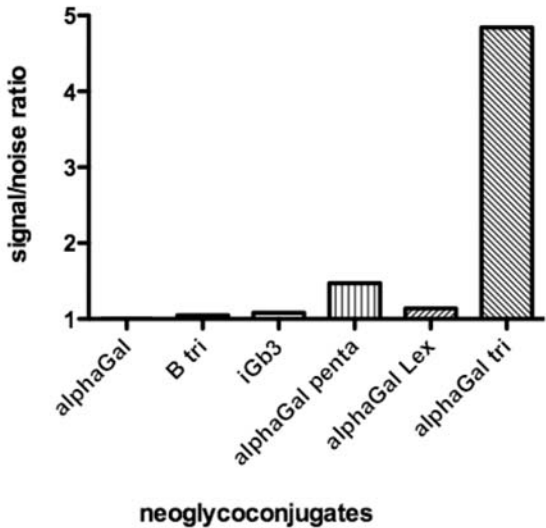
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Sources : IGN, DDAF, IDHESA, ARCOIS, IFREMER

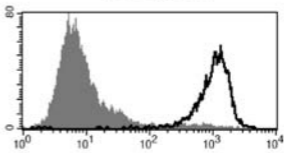
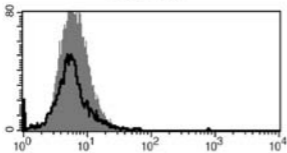




Control

Transfected

GS1-B4



VLPS

