Title: Bacterial histo-blood group antigens contributing to genotype-dependent removal of human noroviruses with a microfiltration membrane

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22 **Abbreviations:**

23 DALY: disability-adjusted life years

24 ELISA: enzyme-linked immunosorbent assay

25 EPS: extracellular polymeric substance

26 HBGA: histo-blood group antigen

27 HuNoV: human norovirus

28 IgG: immunoglobulin G

29 IgM: immunoglobulin M

30 LB-EPS: loosely-bound extracellular polymeric substances

31 LPS: lipopolysaccharide

32 MF: microfiltration

33 MBR: membrane bioreactor

34 MLSS: mixed liquor suspended solids

35 NoVLP: norovirus-like particle

36 SMP: soluble microbial product

37 TB-EPS: tightly-bound extracellular polymeric substance

38 TEM: transmission electron microscopy

39

40 **Abstract**

41 We demonstrated the genotype-dependent removal of human norovirus particles
42 with a microfiltration (MF) membrane in the presence of bacteria bearing histo-blood
43 group antigens (HBGAs). Three genotypes (GII.3, GI.4, and GI.6) of norovirus-like
44 particles (NoVLPs) were mixed with three bacterial strains (*Enterobacter* sp. SENG-6,
45 *Escherichia coli* O86:K61:B7, and *Staphylococcus epidermidis*), respectively, and the
46 mixture was filtered with an MF membrane having a nominal pore size of 0.45 μ m. All
47 NoVLP genotypes were rejected by the MF membrane in the presence of *Enterobacter* sp.
48 SENG-6, which excreted HBGAs as extracellular polymeric substances (EPS). This MF
49 membrane removal of NoVLPs was not significant when EPS was removed from cells of
50 *Enterobacter* sp. SENG-6. GI.6 NoVLP was not rejected with the MF membrane in the
51 presence of *E. coli* O86:K61:B7, but the removal of EPS of *E. coli* O86:K61:B7 increased
52 the removal efficiency due to the interaction of NoVLPs with the exposed B-antigen in
53 lipopolysaccharide (LPS) of *E. coli* O86:K61:B7. No MF membrane removal of all three
54 genotypes was observed when *S. epidermidis*, an HBGA-negative strain, was mixed with
55 NoVLPs. These results demonstrate that the location of HBGAs on bacterial cells is an
56 important factor in determining the genotype-dependent removal efficiency of norovirus
57 particles with the MF membrane. The presence of HBGAs in mixed liquor suspended
58 solids from a membrane bioreactor (MBR) pilot plant was confirmed by
59 immune-transmission electron microscopy, which implies that bacterial HBGAs can
60 contribute to the genotype-dependent removal of human noroviruses with MBR using MF
61 membrane.

62

63 **Keywords:** extracellular polymeric substance, histo-blood group antigen, human norovirus,
64 lipopolysaccharide, microfiltration, membrane bioreactor
65

66 **1. Introduction**

67 Noroviruses are single-stranded, non-enveloped, positive-sense RNA viruses that
68 belong to family *Caliciviridae* (Robilotti et al., 2015). Currently there are more than 40
69 genotypes, which are divided into 6 major genogroups (GI-GVI) with a proposed seventh
70 (Eden et al., 2014; Vinjé, 2014). The GI, GII, and GIV strains infect humans (Eden et al.,
71 2014). Human noroviruses (HuNoVs) are responsible for more than 90% of non-bacterial
72 epidemic gastroenteritis cases and attributable to 18% of acute gastroenteritis patients
73 worldwide with 15.1 million disability-adjusted life years (DALYs) (Ahmed et al., 2014;
74 Kirk et al., 2015; Patel et al., 2008). Outbreaks of HuNoV infection can occur in various
75 community settings such as hospital wards, cruise ships, and restaurants and can affect all
76 age groups, with particularly higher infection risks to organ transplant and
77 immune-compromised patients (Glass et al., 2009).

78 Wastewater is a major transmission source of HuNoVs (van den Berg et al.,
79 2005; Victoria et al., 2010). In 2012, a large outbreak of HuNoV infection (more than
80 10,000 gastroenteritis patients) in eastern Germany was likely caused by frozen
81 strawberries imported from China (Bernard et al., 2014). Multiple genotypes of HuNoVs
82 were found from gastroenteritis patients, which supported the speculation that the imported
83 strawberries were cultivated with untreated wastewater (Mäde et al., 2013). Other previous
84 studies also reported that reclaimed wastewater used for irrigation (Brugha et al., 1999)
85 and recreational activities (Hoebe et al., 2004) has caused HuNoV outbreaks. These cases
86 exemplify the importance of the efficient removal of HuNoVs in wastewater treatment for
87 reducing infection risks of HuNoVs through reclaimed wastewater.

88 The effects of the attachment of viral particles to a variety of wastewater solids,
89 in which one of the main components is organic matter including bacterial cells, on the
90 removal efficiency of enteric viruses from wastewater has been studied (Da Silva et al.,
91 2008; Imai et al., 2011; Miura et al., 2015; Nordgren et al., 2009; Sano et al., 2004).
92 Understanding the binding affinity and specificity of NoV particles to wastewater solids is
93 thus crucial for achieving efficient removal of this human pathogen in wastewater
94 treatment processes (Sano et al., 2010). However, the wide variety of wastewater solids
95 makes it difficult to characterize the attachment of HuNoVs in wastewater, and the factors
96 that affect the binding state of HuNoVs in wastewater have not been very well identified.

97 In our previous study, *Enterobacter* sp. SENG-6 bearing histo-blood group
98 antigens (HBGAs) was isolated and proven to specifically capture noroviral particles
99 (Miura et al., 2013). Human HBGAs are carbohydrates determining ABO and Lewis blood
100 types. They have been suggested to be important factors for HuNoV infection of intestinal
101 epithelial cells (Shirato et al., 2008; Tan and Jiang, 2005). Human HBGAs are found on
102 mucosal epithelial cells and excreted in body fluids, including the saliva of
103 secretor-positive (Se+) individuals (Tan and Jiang, 2005). The association between human
104 HBGAs and HuNoV infection has been extensively studied using in-vitro binding assays,
105 human volunteer challenge studies, and outbreak investigations (de Rougemont et al.,
106 2011; Marionneau et al., 2002; Tan et al., 2008). The binding of HuNoV particles with
107 non-human HBGAs has been also investigated, and it has been reported that the
108 genotype-dependent bioaccumulation of HuNoVs in oyster is caused by the attachment to
109 HBGA-like substances expressed on oyster tissue surface (Le Guyader et al., 2012, 2006).
110 Since *Enterobacter* sp. SENG-6 excretes HBGAs as extracellular polymeric substances

(EPS) (Miura et al., 2013), the binding of HuNoVs to bacterial HBGAs may affect the persistency of HuNoVs in environments. As an example of the effect of HBGA-positive bacteria on the persistent property of HuNoVs, Li et al. (2015) reported that HBGA-expressing *Escherichia coli* maintained the antigen integrity and mucin-binding ability of NoVLPs after heat treatment (Li et al., 2015). However, the effect of bacterial HBGAs on the life cycle of HuNoVs, including the environmental persistency and removal properties in water and wastewater treatment processes, has not been fully investigated.

In this study, we tested the removal efficiency of norovirus-like particles (NoVLPs) associated with HBGA-positive enteric bacteria using a microfiltration (MF) membrane. Two HBGA-positive bacteria and one HBGA-negative bacterium were separately mixed with three genotypes of NoVLPs and filtered by an MF membrane installed in a spin column. The reduction of NoVLP in the filtrate was detected with enzyme-linked immunosorbent assay (ELISA). The presence of HBGAs in mixed liquor suspended solids (MLSS) from a membrane bioreactor (MBR) pilot plant was analyzed by immuno-transmission electron microscopy (TEM), and the effect of the localization of bacterial HBGAs on the genotype-dependent removal of noroviral particles with an MF membrane was discussed.

2. Materials and Methods

2.1 Bacterial strains and norovirus genotypes

Three bacterial strains (*Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7, and *Staphylococcus epidermidis*) and three norovirus genotypes (GII.3, GII.4 and GII.6) were used in this study (Table S1). *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7 (ATCC

12701) have HBGAs in EPS and lipopolysaccharides (LPS), respectively (Miura et al., 2013; Yi et al., 2006). *S. epidermidis* (ATCC 35984) was used as an HBGA-negative control strain (Miura et al., 2013). Luria-Bertani (LB) agar and broth were prepared according to the manufacturer's instruction manual (Zimbro et al., 2009) for the cultivation of *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, whereas Reasoner's 2A (R2A) agar and broth were used for the cultivation of *S. epidermidis* (Nihon Pharmaceuticals, Japan). HBGA activity of these bacterial strains was tested with the ABOsphia (Kamakura Techno Science Inc., Japan) blood typing kit according to the manufacturer's instructions. NoVLPs of genotypes GII.3, GII.4, and GII.6 were prepared as described previously (Kitamoto et al., 2002). These genotypes are the prevalent genotypes among recent NoV GII outbreaks (Han et al., 2011; Iritani et al., 2010; Zeng et al., 2012).

2.2. Removal of EPS and LPS from bacterial cells

The removal of EPS from bacterial cells was performed in two steps: the separate removal of loosely-bound EPS (LB-EPS) and tightly-bound EPS (TB-EPS) (Zhang et al., 2009). Bacterial cells of *Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7 and *S. epidermidis* were cultivated in relevant broth overnight. On the following day, 25 ml of the bacterial cell suspension including the growth medium were centrifuged at $6000 \times g$ for 5 min to separate the supernatant and cell pellet. The supernatant includes soluble microbial products (SMP). The cell pellet was re-suspended in 5 ml of 0.1 M PBS (pH 6.5) and mixed for 2 min by a vortex machine. Then the cell suspension was shaken using a multi shaker for 10 min at 37 °C and again mixed for 2 min by a vortex machine. Finally, the cell suspension was centrifuged at $8000 \times g$ for 10 min, and the supernatant including

157 LB-EPS was removed. The cell pellet was re-suspended in 5 ml of 0.1 M PBS and mixed
158 for 3 min by a vortex machine. The cell suspension was then heated in a water bath at 80
159 °C for 30 min, and the final centrifugation at $12000 \times g$ for 20 min was performed to
160 remove TB-EPS in the supernatant. The heat treatment at 80°C does not lead to the loss of
161 antigen integrity and the leaching of bacterial intracellular components (Li et al., 2015).
162 Cells after the removal of LB- and TB-EPS were used for the filtration experiment.

163 A fraction of the cell pellet remained after the TB-EPS removal was used for
164 LPS extraction. LPS extraction was performed in four steps, namely lysis, LPS purification,
165 washing, and elution using the LPS extraction kit (Intron Biotechnology, Inc., Korea)
166 according to the manufacturer's instructions. Briefly, 1 ml of lysis buffer was added and
167 thoroughly mixed using a vortex machine until the cell clump disappeared. Then 200 µl of
168 chloroform was added and mixed vigorously for 10-20 sec using a vortex machine. The
169 mixture was then incubated at room temperature for 5 min and again centrifuged at $18700 \times g$
170 for 15 min at 4 °C using a Hitachi Rx series centrifuge with T15A33 rotor (Hitachi
171 Corporation, Japan). Next, 400 µl of the supernatant was transferred to a new 1.5 ml
172 centrifugation tube by carefully avoiding the transfer of white sediment, and 800 µl of
173 purification buffer was added. The mixture was incubated at -20°C for 10 min. The
174 incubated mixture was then centrifuged at $18700 \times g$ for 15 min at 4°C using the T15A33
175 rotor. An LPS pellet was obtained by removing the supernatant. The pellet was then
176 washed using 1 ml of 70% ethanol, and the mixture was centrifuged at $18700 \times g$ for 3 min
177 at 4°C using the T15A33 rotor. The supernatant was removed, and 60 µl of 10 mM
178 Tris-HCl buffer (pH 7.9) was added to the tube. This mixture was then boiled for 2 min

179 until the LPS pellet was completely dissolved. LPS samples were stored at -80°C until
180 further experiments.

181

182 **2.3 ELISA for the detection of bacterial HBGAs**

183 ELISA using a GII.6 NoVLP-immobilized microplate was performed to confirm
184 the presence of bacterial HBGAs in EPS and LPS of *E. coli* O86:K61:B7. GII.6 NoVLP
185 was used as an adsorbent because this genotype can recognize HBGAs (Shirato et al.,
186 2008) and is less adhesive to non-HBGA substances (Miura et al., 2013). Firstly, GII.6
187 NoVLP was serially diluted 100 times with PBS (0.1 M, pH = 6.5) and added in triplicate
188 to the relevant wells of a Microton 96 well flat bottom, high-binding ELISA plate (Greiner
189 Bio-One, Germany) and kept overnight at 4°C until the NoVLPs were attached to the wells.
190 On the following day, the NoVLP suspension was removed, and the wells were washed
191 twice using 200 μl of PBS (pH=6.5) per well. The well surface was blocked using a 5%
192 bovine serum albumin (BSA) (Wako Chemicals, Japan) in PBS for 2 h at room
193 temperature and washed twice with PBS. After the blocking and washing, 50 μl of LPS
194 solution was added to the well and incubated at room temperature for 1 h. The wells were
195 washed twice with PBS and 50 μl each of anti-A, B, and H mouse monoclonal antibodies
196 (sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc., USA)
197 serially diluted up to 100-fold with 5% BSA in PBS added to relevant wells. The plate was
198 incubated for 1 h at room temperature, and washed twice with PBS. Then, 50 μl of goat
199 anti-mouse immunoglobulin M (IgM) HRP-conjugated antibody (A90-101P; Bethyl
200 Laboratories, USA) diluted 500-fold with 5% BSA in PBS were added to each well. The
201 plate was incubated at room temperature for 1 h, and washed four times with PBS.

O-phenylenediamine (OPD) solution was prepared by dissolving 0.52 g of citric acid, 0.71 g of disodium hydrogen phosphate (Na_2HPO_4), one OPD tablet (P-7288; Sigma-Aldrich, Japan), and 30 μl of hydrogen peroxide in 100 ml of MilliQ water. One hundred microliters of the OPD solution was added to each well, and the plate was incubated for 30 min at room temperature. Then 50 μl of 2 M H_2SO_4 was added to stop the coloring reaction. Absorbance at 490 nm in each well was measured for 0.1 sec using a multi-label counter (ARVO MX; Perkin Elmer Inc., Japan). The signal/noise (S/N ratio) was calculated by dividing the absorbance value from GII.6 NoVLP-immobilized well by that from GII.6 NoVLP-negative well. Student's t test was performed using Microsoft Excel 2010 to compare the S/N ratio values between samples.

212

2.4 Membrane filtration of NoVLPs

All bacteria grown overnight were centrifuged at $3000 \times g$ for 15 min to obtain the cell pellet. The supernatant was removed, 10 ml of 0.1 M PBS was added to the tubes, and the cells were suspended by a vortex machine. Optical density was measured using a spectrophotometer (Bio-Rad Laboratories, USA) at 600 nm, and the value was adjusted to around 1.5 by careful addition of PBS (7.6×10^8 – 7.9×10^8 cells/ml). NoVLPs of human norovirus GII.3, GII.4, and GII.6 genotypes were prepared by the dilution of the original VLP suspension with PBS. Final particle numbers per milliliter were 2.0×10^{11} particles for GII.3, 1.2×10^{11} particles for GII.4, and 6.0×10^{11} particles for GII.6. Equal volumes of VLP and bacteria solutions were mixed for 15 min at 4 °C using a mechanical shaker. Then, 500 μL of the mixture of cells and NoVLP were filtered using NANOSEP MF GHP 0.45 μm filter with the effective filtration area of 0.28 cm^2 (PALL Corporation, Ann Arbor, MI,

225 USA) at $8,000 \times g$ until the total volume passed through the membrane. The filtrate was
226 collected and stored at 4 °C for further analysis.

227

228 **2.5. ELISA for the detection of NoVLPs**

229 The mixture of bacterial cells and NoVLP (before filtration) or NoVLP only and
230 its filtrate obtained in the membrane filtration test were added to relevant wells in triplicate
231 and kept overnight at 4 °C. Wells without any samples are considered as negative. On the
232 following day, the wells were washed twice with PBS. The well surface was blocked using
233 a 5% BSA in PBS for 2 h at room temperature. Wells were washed twice with PBS, and 50
234 μ l of anti-NoVLP serum from rabbit diluted with 5% BSA in PBS was added to relevant
235 wells. The plate was incubated for 1 h and washed twice with PBS. Goat Anti-Rabbit
236 immunoglobulin G (IgG) pAb HRP conjugate (ADI-SAB-300-J; Enzo Life Sciences Inc.,
237 USA) was used as the secondary antibody. The secondary antibody was diluted with 5%
238 BSA in PBS, and 50 μ l was added to each well. The plate was incubated for 1 h and
239 washed 4 times with PBS. The coloring reaction was performed using the OPD solution as
240 described above, 50 μ l of 2M H_2SO_4 was added to stop the coloring reaction, and
241 absorbance at 490 nm (A_{490}) in each well was measured for 0.1 sec using a multi-label
242 counter (ARVO MX; Perkin Elmer Inc., Japan). The filtration experiment and the ELISA
243 quantification is schematically explained in Figure S1 (supporting information). The
244 Mixture/Filtrate ratio was then calculated as the A_{490} of mixture/ A_{490} of filtrate. The
245 major focus of this study is the retention of NoVLPs on the MF membrane due to the
246 interaction with bacterial HBGAs, which can be reflected by the larger values of
247 Mixture/Filtrate ratio. All tests were performed in triplicates and the average data were

248 presented. Student's t-test was performed using Microsoft Excel 2010 to compare the
249 mixture/filtrate ratio values.

250

251 **2.6. Immuno-TEM observation of the localization of HBGAs in MLSS from MBR**

252 The localization of HBGAs in MBR MLSS was analyzed by immuno-TEM.

253 Mixed liquor was taken from an MBR pilot plant (Miura et al., 2015). One hundred
254 microliters of the mixed liquor were placed in a 2-mL tube for 30 min and resulting
255 supernatant was removed. Sedimented MLSS were re-suspended in 2 mL of PBS
256 containing 4% paraformaldehyde and 0.1% glutaraldehyde, and incubated at 4 °C for 2 h.
257 The fixed MLSS was collected by sedimentation. The sedimented MLSS were washed by
258 soaking in 2 mL of PBS at 4 °C for 10 min. This washing step was repeated twice. The
259 MLSS were dehydrated by soaking in 2 mL of increasing concentrations of ethanol (70, 80,
260 90, and 95%) and then mixed gently using a rotator at 4 °C for 15 min. The following
261 dehydration and infiltration reactions were also conducted on a rotator at 4 °C. For
262 complete dehydration, the MLSS were soaked in 2 mL of 100% ethanol for 20 min. This
263 final dehydration step was repeated twice. Next, the MLSS were infiltrated with 2 mL of
264 LR white resin (Medium grade; London Resin Company Ltd., England) and 100% ethanol
265 (1:2) mixture for 12 h, followed by 2 mL of LR white resin and 100% ethanol (2:1)
266 mixture for 12 h. The solution was replaced with 2 mL of pure LR white resin for 1 h,
267 followed by another 2 mL of pure LR white resin for 12 h. The MLSS and pure LR white
268 resin were polymerized using an ultraviolet polymerizer (TUV-100; Dosaka EM Co., Ltd.,
269 Japan) at 4 °C for 3 days. Ultrathin sections measuring 70 nm in thickness were prepared

270 from the embedded MLSS using a diamond knife on an ultramicrotome (RMC MTXL;
271 Boeckeler Instruments, Inc., USA). The sections were then placed on nickel grids.

272 The ultrathin sections on grids were washed by floating the grid upside down on
273 a drop of PBS at room temperature for 1 min. The following incubations were also
274 conducted by floating the grid upside down on drops of reagent at room temperature. The
275 sections were blocked with 4% BSA in PBS for 5 min and incubated with anti-blood group
276 A, B, or O(H) antibody diluted 1:30 in PBS containing 1% BSA for 2 h, followed by
277 washing with PBS (6 changes, 1 min each). The bound primary antibodies were localized
278 by incubating the sections on anti-mouse IgM antibody gold conjugate (10 nm, ab39613;
279 Abcam, Japan) diluted 1:10 in PBS containing 1% BSA for 1 h, followed by washing with
280 PBS (6 changes, 1 min each). The sections were fixed with 2% glutaraldehyde in PBS for
281 15 min, followed by washing with deionized distilled water (DDW; 7 changes, 1 min each).
282 Finally, the sections were stained with 5% uranyl acetate, followed by washing with 50%
283 ethanol for 1 min and DDW (4 changes, 1 min each). Stained sections were observed under
284 TEM. Ultrathin sections without the primary antibodies were used as controls.

285

286 **3. Results**

287 **3.1 Availability of bacterial HBGAs for NoVLP attachment**

288 HBGAs associated with bacterial cells were detected by the blood typing kit.
289 This kit detects A, B, and O(H) antigens on cells by forming an aggregate of cells and
290 anti-HBGA antibody-coated beads. *Enterobacter* sp. SENG-6 displayed the highest
291 response for all A, B, and O(H) antigens, whereas *E. coli* O86:K61:B7 and *S. epidermidis*
292 gave negative results for all tested cell densities (Table 1, Figure S1). These A, B, and

O(H) antigens of *Enterobacter* sp. SENG-6 were not detected when EPS was removed mechanically, which indicates that bacterial HBGAs of *Enterobacter* sp. SENG-6 were included mainly in EPS. On the other hand, the B antigen in LPS of *E. coli* O86:K61:B7 was detected after the EPS removal (Table 1, Figure S2), showing that the B antigen in LPS of *E. coli* O86:K61:B7 can be recognized by the anti-B antibody in the kit, but this antigen-antibody interaction is physically blocked by the presence of EPS covering cells of *E. coli* O86:K61:B7. We also confirmed that A and O(H) antigens of *E. coli* O86:K61:B7 were not detected even after the EPS removal (Table 1, Figure S2).

For confirming the interaction between LPS of *E. coli* O86:K61:B7 and NoV particles, ELISA using a GII.6 NoVLP-immobilized microplate was performed. Extracted LPS from *E. coli* O86:K61:B7 was applied to the GII.6 NoVLP-immobilized well, and bacterial HBGAs in the trapped LPS were detected by anti-HBGA antibodies. The negative control condition (without LPS) gave a signal/noise ratio value larger than 1.0 for the B antigen, but a significantly higher value of the signal/noise ratio was observed under the test condition (with LPS, $p < 0.01$, Figure 1). Although a signal/noise ratio value larger than 1.0 with LPS was also observed for the O(H) antigen, the statistically significant difference was not obtained between test and negative conditions. These results indicate that the B antigen is the most abundant in LPS of *E. coli* O86:K61:B7 and can bind the GII.6 NoVLP.

3.2 Membrane filtration of NoVLPs mixed with bacterial cells

NoVLP of GII.3, GII.4, or GII.6 was mixed with suspended cells of *Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7, or *S. epidermidis*. The mixture of NoVLPs

and bacterial cells was filtered using NANOSEP MF GHP 0.45 μm filter in a spin column. NoVLPs in the mixture with bacterial cells (before filtration) and those in filtrate (after filtration) were detected by ELISA. As a bacteria-negative control, only NoVLPs (without bacterial cells) were also filtered separately. The mixture/filtrate ratio of ELISA signal was around 1.0 (0.91-1.01) for all genotypes tested under the bacteria-negative control (Figure 2, VLP only), which explains that NoVLP (diameter: ca. 40 nm) has passed through the MF membrane pore (nominal pore size of 0.45 μm). The mixture/filtrate ratio values were always less than 1.0 when cells of *S. epidermidis* were mixed with NoVLPs (Figure 2), probably because of an inhibitory effect of *S. epidermidis* cells on the signal detection in ELISA. The presence of *S. epidermidis* did not affect the passing of the majority of NoVLPs through the MF membrane pore as there were no HBGAs to retain the NoVLPs.

When NoVLPs were mixed with cells of *Enterobacter* sp. SENG-6, the mixture/filtrate ratio of ELISA signal was significantly larger than 1.0 for all genotypes tested ($p < 0.01$), showing that a significant amount of NoVLPs were trapped by *Enterobacter* sp. SENG-6 cells and did not pass through the MF membrane (Figure 2). On the other hand, the behavior of NoVLPs was dependent on the genotype when mixed with cells of *E. coli* O86:K61:B7. The mixture/filtrate ratio of GII.4 NoVLP was significantly larger than 1.0 (Figure 2(B)) ($p < 0.01$), but those of GII.3 and GII.6 were not (Figure 2(A) and (C)), which means that GII.3 and GII.6 NoVLPs were not trapped by *E. coli* O86:K61:B7 cells very effectively.

3.3 Impact of the location of bacterial HBGAs on the cell attachment of noroviral particles

LB- and TB-EPS were removed from cells of *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, and the membrane filtration test was performed using NANOSEP MF GHP 0.45 μm filter in a spin column to analyze the effect of the localization of bacterial HBGAs on the removal property of noroviral particles. The mixture/filtrate ratio of ELISA signal was compared between bacterial cells before and after the EPS removal. As a result, the removal of EPS from *Enterobacter* sp. SENG-6 cells has been attributed to a significantly lower mixture/filtrate ratio ($p < 0.01$). The difference in the ratio values for *Enterobacter* sp. SENG-6 with EPS between Figure 2(C) and Figure 3 was caused by experimental random errors in both values of numerator (A490 of mixture) and denominator (A490 of filtrate) in the ratio calculation. A490 values of mixture in Figure 2(C) and Figure 3 were 1.50 ± 0.13 and 1.13 ± 0.01 , respectively, and those of filtrate were 0.24 ± 0.04 and 0.84 ± 0.08 . Although we used the same amount of NoVLP in these experiments, this level variation in ELISA signal can be observed. On the other hand, the EPS-removed *E. coli* O86:K61:B7 cells gave a significantly higher value of the mixture/filtrate ratio ($p < 0.01$) (Figure 3). These results demonstrate that the localization of bacterial HBGAs is an important factor for the norovirus removal with the MF membrane, and the HBGAs in LPS of *E. coli* O86:K61:B7 have to be exposed for the association with GII.6 NoVLP.

357

358 **3.4 Immuno-TEM confirmation of the localization of bacterial HBGAs**

Ultrathin sections of MBR MLSS labeled with gold nanoparticles bearing anti-mouse IgM after reacting with anti-blood group A, B, or O(H) antibody (used as the primary antibody) were examined by immuno-TEM to study the localization of HBGAs. A

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number of gold nanoparticles were observed in the EPS region of MLSS (Figure 4(A)) that had reacted with anti-A antibody. In contrast, the B and O(H) antigens were not detected in the EPS region, but a few gold nanoparticles carrying anti-B and O(H) antibodies were observed inside bacterial cells (Figure 4(B) and (C)). These immuno-TEM observations indicate that at least the A antigens can be present in the EPS region of MBR MLSS.

367

368 **4. Discussion**

We demonstrated the role of bacterial HBGAs on the MF membrane separation of HuNoVs. Two HBGA-positive bacteria, *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, or one HBGA-negative bacteria, *S. epidermidis*, were mixed with each genotype of NoVLP GII.3, GII.4, or GII.6, and the reduction of NoVLPs in the filtrate with an MF membrane (nominal pore size of 0.45 μm) installed in a spin column was compared. All results indicated that bacterial HBGAs in EPS and LPS could capture noroviral particles and contribute to the MF membrane removal, but the attachment of NoVLPs to HBGA-positive LPS is physically hindered by EPS covering bacterial cells.

The membrane used in this study is an MF membrane with a nominal pore size of 0.45 μm . MF membranes with a nominal pore size of 0.1 to 0.45 μm are very frequently employed in MBR systems for wastewater treatment (Kimura et al., 2015). A membrane pore size larger than 0.1 μm is commonly preferable because the membrane fouling associated with pore plugging is one of the most crucial problems in MBR operation (Guo et al., 2012). Noroviral particles have a diameter of around 40 nm (Koho et al., 2012) and mixture/filtration ratios around 1.0 is obtained in the absence of bacterial cells with all 3 types of NoVLPs (Figure 2, VLP only), which explains the sieve mechanism does not

385 appear to work in the removal of NoVLPs, as observed in the membrane filtration test of
 386 noroviral particles indigenous in wastewater (Sano et al., 2006). However, the significant
 387 reduction of NoVLPs of all genotypes was observed in the presence of *Enterobacter* sp.
 388 SENG-6 (Figure 2). *Enterobacter* sp. SENG-6 produced HBGAs in EPS (Table 1), which
 389 contributed to the efficient removal of all norovirus genotypes of GII.3, GII.4, and GII.6
 390 with the MF membrane. For the efficient removal of HuNoV in the MBR, the association
 391 of viral particles to mixed liquor suspended solids (MLSS) and/or the cake layer formed on
 392 the membrane surface is of primary importance (Miura et al., 2015; Simmons et al., 2011).
 393 MLSS and the cake layer are mainly composed of bacterial cells and EPS (Lin et al., 2014),
 394 and the immuno-TEM images show that the A antigen was present in the EPS region of
 395 MBR MLSS (Figure 4(A)). A thorough understanding about the prevalence and
 396 spatial/temporal (seasonal) variation of the expression level of these bacterial HBGAs and
 397 the binding property of noroviral particles to bacterial components including EPS is thus
 398 critical for discussing the contribution of bacterial HBGAs in MLSS on the removal of
 399 HuNoVs in MBR.

400 The removal efficiency of NoVLPs in the presence of *E. coli* O86:K61:B7 was
 401 genotype-dependent, in which GII.4 NoVLP was well removed but GII.3 and GII.6 were
 402 not (Figure 2). The NoV genotype GII.4 can recognize broad types of epitope compared to
 403 the other genotypes (Bally et al., 2012; Ruvoën-clouet et al., 2013; Tamura et al., 2004). It
 404 is likely that the interaction between the GII.4 NoVLPs and *E. coli* O86:K61:B7 (Figure
 405 2(B)) is caused by the broad spectrum of HBGA-epitope recognition of this genotype.
 406 Several phenomena have been proposed as the determination factors of virus removal
 407 efficiency with membrane filtration, including the attachment of viruses to biosolids

408 (Chaudhry et al., 2015b; Miura et al., 2015), the virus retention by cake layer on membrane
409 surface (Lu et al., 2013), the virus retention on membrane surface (Chaudhry et al., 2015b),
410 the initial concentration level of virus in feed water (Lu et al., 2016), and the virus types
411 (Chaudhry et al., 2015a). This study verified for the first time that the epitope-recognizing
412 capability of viral particle is one of the factors determining virus removal efficiency with
413 membrane filtration.

414 All results from the present study provide evidence that the removal efficiency of
415 NoV particles with the MF membrane is dependent on the location of bacterial HBGAs
416 and the norovirus genotype. Different norovirus genotypes have different HBGA-binding
417 profiles (Donaldson et al., 2008; Lindesmith et al., 2008; Shirato et al., 2008), which
418 means that norovirus genotypes other than those tested in the present study have different
419 removal properties by the MF membrane and HBGA-positive bacteria. Since a recent
420 report describes that the HBGA-binding profile can also vary within a genotype (Hoa-Tran
421 et al., 2015), a strain-dependent removal property can be observed when multiple strains
422 from an identical genotype are used to test the removal efficiency with the MF membrane.
423 Particularly, the rapid evolution of NoV GII.4 should be paid attention, because new
424 variants have appear every 2-3 years and increased relative affinity towards the A, B, O(H)
425 and Lewis antigens over the evolution has been observed (Ruvoën-clouet et al., 2013). The
426 GII.4 NoVLP used in this study is a variant strain of Den Haag 2006b (Imai et al., 2011),
427 which can recognize all the A, B, O(H), and Lewis antigens (Kroneman et al., 2011) as
428 well as the other GII.4 strains (Shirato et al., 2008). This means that the Den Haag 2006b
429 strain can be representative among GII.4 strains in terms of the MF membrane removal
430 with HBGA-positive bacteria. Recent studies have shown the emergence of NoV GII.17

431 strains and the replacement of the currently predominant GII.4 Sydney strain (De Graaf et
432 al., 2015; Zhang et al., 2015). It is expected that these GII.17 strains can be effectively
433 removed by MF membrane because of the broad HBGA binding spectrum (Zhang et al.,
434 2015), but it is necessary to conduct the filtration test as in this study for coming to a
435 conclusion about the removal efficiency of the emerging NoV strains with MF membrane.

436 Jones et al. reported that *Enterobacter cloacae* (ATCC 13047), a relative of
437 *Enterobacter* sp. SENG-6 (99.9% identity in the 16S rRNA gene sequence), bound
438 norovirus GII.4 Sydney strain through the bacterial O(H) antigen, which resulted in the
439 infection of the Sydney strain to cultivated B cells (Jones et al., 2014). Jones et al. (2014)
440 used whole cell components of *Enterobacter cloacae* and detected the H antigen by
441 western blotting. From the bacteriological viewpoint, even the 100% identity of 16S rRNA
442 gene sequence is not enough to identify bacterial species, and multiple phenotypes
443 including substrate availability must be tested for the species identification. Since enteric
444 bacteria are so diverse, it is highly likely that *Enterobacter* sp. SENG-6 is identified as a
445 new species in genus *Enterobacter*, which is different from *Enterobacter cloacae*. The
446 comparison of the HBGA expression profile among *Enterobacter* spp. and other enteric
447 bacteria is necessary to discuss the ubiquity of HBGA-positive bacteria in nature. Further
448 understanding of the prevalence of HBGA-positive bacteria in environments may lead to
449 better insights about norovirus survival and persistence in environmental waters and
450 removability in wastewater treatment processes, including MBR. A small-scale (500µl)
451 test for the membrane filtration was performed in this study to investigate the effect of
452 specific interaction between bacterial HBGAs and noroviral particles on the removal
453 efficiency, and the observations at a larger scale must be validated in the further study.

454

455 **5. Conclusions**

- 456 - The interaction with HBGA-positive bacteria contributes to the removal of HuNoV
 457 particles with MF membrane.
- 458 - Cell-associated and HBGA-positive EPS is important for removing HuNoV particles with
 459 MF membrane.
- 460 - The removal efficiency of HuNoV particles with MF membrane and bacterial HBGA is
 461 genotype-dependent.
- 462 - HBGAs are expressed in mixed liquor suspended solids from MBR, which can be
 463 available for the removal of NoVs in wastewater.

464

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470

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661 **Table 1.** Detection of bacterial histo-blood group antigens by a blood typing kit

Cells/ml	<i>Enterobacter</i> sp. SENG-6						<i>E. coli</i> O86:K61:B7 (ATCC 12701)						<i>Staphylococcus</i> <i>epidermidis</i> (ATCC 35984)		
	with EPS			w/o EPS			with EPS			w/o EPS					
	A	B	O(H)	A	B	O(H)	A	B	O(H)	A	B	O(H)	A	B	O(H)
10 ⁹	+++	+++	+++	–	–	–	–	–	–	–	+	–	–	–	–
10 ⁸	+++	+++	+++	–	–	–	–	–	–	–	+	–	–	–	–
10 ⁷	++	++	++	–	–	–	–	–	–	–	–	–	–	–	–

662 +++ , strong aggregation; ++ , moderate aggregation; + , weak aggregation; – , no aggregation.

663 **Figure Legends**

664

665 **Figure 1.** Detection of histo-blood group antigens (HBGAs) of *Escherichia coli*
 666 O86:K61:B7 (ATCC 12701). A plate was coated by GII.6 norovirus-like particles
 667 (NoVLPs), and HBGAs in lipopolysaccharide of *E. coli* O86:K61:B7 attached to NoVLP
 668 were detected by anti-A, B, and O(H) antibodies. **, $p < 0.01$.

669

670 **Figure 2.** Membrane separation of norovirus-like particles (NoVLPs) with bacterial cells.
 671 Reduction of (A) GII.3, (B) GII.4, and (C) GII.6 NoVLP by the filtration with a
 672 microfiltration membrane (nominal pore size of 0.45 μm) in the presence of *Enterobacter*
 673 sp. SENG-6, *Escherichia coli* O86:K61(B7) (ATCC 12701), and *Staphylococcus*
 674 *epidermidis* (ATCC 35984). NoVLP was filtered without bacterial cells in the condition of
 675 VLP only. **, $p < 0.01$.

676

677 **Figure 3.** Effect of extracellular polymeric substances on membrane separation of
 678 norovirus-like particles (NoVLPs). The mixture/filtrate ratio between the quantity of GII.6
 679 NoVLPs in the samples before and after the filtration with a microfiltration membrane
 680 (pore size: 0.45 μm) in the presence of *Enterobacter* sp. SENG-6 and *Escherichia coli*
 681 O86:K61(B7) (ATCC 12701) before and after removal of soluble microbial product,
 682 loosely-bound EPS, and tightly-bound EPS. **, $p < 0.01$.

683

684 **Figure 4.** Localization of histo-blood group antigens in mixed liquor suspended solids
 685 from a membrane bioreactor pilot plant. Ultrathin section was labeled with immuno-gold

686 after reacting with anti-blood group A, B, and O(H) antibodies (primary antibodies) and
687 observed under transmission electron microscopy. Ultrathin sections without primary
688 antibodies were used as controls. A) The A antigen, B) the B antigen, C) the O(H) antigen,
689 and D) control.

Figure 1

Figure 2

Figure 3

Figure 4