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Title: Histo-Blood Group Antigen-Like Substances of Human Enteric Bacteria as Specific Adsorbents for Human Noroviruses

Running title: Bacterial HBGAs Specifically Bind to Human Norovirus

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Abstract

Histo-blood group antigens (HBGAs) have been suggested to be receptors or co-receptors for human noroviruses (HuNoVs) expressed on the intestinal epithelium. We isolated an enteric bacterium strain (SENG-6), closely related to *Enterobacter cloacae*, bearing HBGA-like substances from a fecal sample of a healthy individual using a biopanning technique with anti-HBGA antibodies. The binding capacities of four genotypes of norovirus-like particles (NoVLPs) to *Enterobacter* sp. SENG-6 cells were confirmed by enzyme-linked immunosorbent assay (ELISA). Transmission electron microscopy visualized that NoVLPs bound mainly to extracellular polymeric substances (EPS) of *Enterobacter* sp. SENG-6, where the HBGA-like substances were localized. EPS that contained HBGA-like substances extracted from *Enterobacter* sp. SENG-6 was shown by ELISA to be capable of binding to NoVLPs of GI.1 wild-type strain (8fIIa) and GII.6 that can recognize A antigen, but not to the NoVLP GI.1 mutant strain (W375A) that loses the binding capability to A antigen. Enzymatic cleavage of terminal N-acetylgalactosamine residues in the bacterial EPS weakened bacterial EPS binding to the GI.1 wild strain (8fIIa). These results indicate that A-like substances in the bacterial EPS play a key role in binding to NoVLPs. Since the specific binding of HuNoVs to HBGA-positive enteric bacteria is likely to affect the transmission and infection processes of HuNoVs in their hosts and in the environments, further studies of human enteric bacteria and their binding capacity to HuNoVs will provide a new scientific platform for understanding interactions between two types of microbes that were previously regarded as biologically unrelated.

Introduction

Human noroviruses (HuNoVs) are major causative agents of nonbacterial acute gastroenteritis in humans, which constitute a substantial disease burden worldwide (1). Noroviruses belong to the genus *Norovirus* in the family *Caliciviridae*. The genus *Norovirus* is divided into 5 genogroups, i.e., GI, GII, GIII, GIV, and GV, and the strains in each genogroup can be further divided into genotypes (2). GI, GII, and GIV NoVs, which include at least 15 genotypes, 18 genotypes, and 1 genotype, respectively (3), infect humans of all age groups, causing symptoms such as nausea, vomiting, diarrhea, abdominal cramps, headache, and fever (4). Human-to-human infection is the main transmission route of HuNoVs, but contaminated water and sea products such as oysters are reported to be sources or vehicles of infection (5, 6) because of environmental contamination with domestic wastewater (7, 8).

The lack of tissue cells for replicating HuNoVs has impeded the study of the life cycle of this important human pathogen. One major finding related to productive infections with HuNoVs is the interaction with histo-blood group antigens (HBGAs), which have been proposed to be receptors or co-receptors of human small intestinal epithelial cells for HuNoVs (9, 10). HBGAs comprise ABH and Lewis antigens, which are structurally related oligosaccharides, and each HuNoV genotype or strain has its own HBGA recognition pattern profile (11-13). For example, virus-like particles (VLPs) of Norwalk virus (NV/68), a genotype 1 strain in genogroup I (GI.1) and the prototype strain of norovirus, bind to HBGAs in saliva from secretor-positive individuals and preferentially bind to H type 1, Le^b, and type A carbohydrates (11, 14). Furthermore, VLPs of GII.4 (r104) can recognize a broader range of blood group carbohydrates than other genotypes (12), although the ligand binding patterns have changed over time (15). The importance of the HBGA

recognition pattern for HuNoV infections has been emphasized because GII.4 strains are the most prevalent etiological agents of infectious diseases caused by norovirus, probably because of their broad HBGA recognition profile.

In this study, we focused on a group of human enteric bacteria that produce HBGA-positive extracellular polymeric substances (EPS). EPS comprise organic macromolecules such as polysaccharides, proteins, nucleic acids, lipids, and other polymeric compounds located on or outside the cell surface (16). Humans possess immunoglobulin M (IgM) antibodies against non-self HBGAs in the blood, which is attributable to the presence of enteric bacteria with blood group activity (17). This led us to speculate that human enteric bacteria may capture HuNoV particles via specific interactions with HBGA-like bacterial substances. To elucidate the specific interaction between HuNoV particles and HBGA-like bacterial substances, we screened blood group-active human enteric bacteria from human feces using a biopanning technique with anti-HBGA antibodies. We tested the binding capacity of four genotypes to norovirus-like particles (NoVLPs) for bacterial cells using enzyme-linked immunosorbent assay (ELISA). NoVLP binding to bacterial cells was observed by transmission electron microscopy (TEM), and the localization of HBGA-like bacterial substances was analyzed by immuno-TEM. EPS, surface-retained organic matter (SOM), and lipopolysaccharide (LPS) were extracted from bacterial cells, and the interactions between HBGA-like substances in the extracted bacterial polymers and NoVLPs were examined by ELISA. The specific interactions between HBGA-like substances and NoVLPs were evaluated further by the quartz crystal microbalance (QCM) method.

Materials and Methods

Isolation of human enteric bacteria bearing HBGA-like substances. We screened enteric bacteria bearing HBGA-like substances from human feces using anti-HBGA antibodies. Fifty microliters of anti-blood group A, B, or O(H) mouse monoclonal antibodies (sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc., USA) were added to each well of an ELISA plate and kept at room temperature (RT) for 1 h to coat the well. The wells were washed two times with 0.1 M phosphate-buffered saline (PBS), and a diluted human fecal suspension derived from a healthy adult was added to the wells. After incubation at RT for 1 h, the wells were washed two times with PBS and 2% brilliant green bile broth (Kanto Chemical Co., Inc., Japan), *E. coli* broth (Nihon Pharmaceutical Co., Ltd., Japan), Enterobacteriaceae enrichment mannitol broth (Nihon Pharmaceutical Co., Ltd.), or enterobacteria enrichment broth-Mossel (Nihon Pharmaceutical Co., Ltd.) was added to each well. After overnight incubation at 37°C under microaerobic conditions, the enriched cells were cultured further by streaking onto an agar plate containing the same medium, and several colonies were selected randomly. The selected colonies were cultured again by streaking onto the same agar plates, and a single colony was selected. The selected cells were cultured in the same culture broth, collected by centrifugation, and suspended in PBS. Tenfold dilution series were prepared with PBS, and the OD_{600} values of the diluted cell suspensions were measured using a spectrophotometer. The presence of HBGA-like substances on the cell surface in the diluted cell suspensions were detected on the basis of their aggregation activities with anti-blood group A, B, or O(H) antibody-coated beads from a blood typing kit (ABO sphia; Kamakura Techno-Science Inc., Japan). The names of the antibodies used in the blood typing kit were not disclosed, but it could detect at least type 1 and type 2 HBGAs because human blood, saliva, and sperm could be used as samples. The minimum OD_{600} in the tenfold dilution series that gave a

positive result was determined for each isolated strain. The isolated bacterial strains were identified by 16S rRNA gene sequencing by targeting its 1466 nucleotides using the primers 27F, 530F, 907R, and 1492R (18).

Assay for HBGA binding activity of NoVLPs. NoVLP genotypes GI.7 (AB758449), GII.3 (AB758450), GII.4 (AB668028), and GII.6 (AB758451) were prepared according to a published method (19). These genotypes were selected because GII.3, GII.4, and GII.6 are the most prevalent GII noroviruses (20-22), while GI.7 is also detected frequently in epidemiological studies (23). The HBGA binding profile of each genotype (Table 1) was tested as follow. Fifty microliters of NoVLP suspension were added to each well of an ELISA plate and kept at 4°C for 24 h to coat the well. Triplicate wells were used for each sample. The wells were washed two times with PBS and blocked with 5% bovine serum albumin (BSA) in PBS. After incubation at RT for 2 h, the wells were washed two times with PBS, and 50 μL of biotin-conjugated type 1 A- (GLT 01-084, GlycoTech, MD, USA), B- (GLT 01-085, GlycoTech), H- (GLT 01-037, GlycoTech), or Lewis b-carbohydrate (GLT 01-042, GlycoTech) (diluted to 1:10 with PBS containing 5% BSA; Eve Bio-Science Co., Ltd., Japan) were added to the wells. Plates were incubated at RT for 2 h. The wells were washed two times with PBS and 50 μL of horseradish peroxidase (HRP)-conjugated streptavidin (518-59941, Thermo Scientific Japan) diluted to 1:10 with PBS containing 5% BSA was inoculated into each well. After incubation at RT for 1 h, the wells were washed four times with PBS, and the bound HRP-conjugated streptavidin was measured by coloring with o-phenylenediamine dihydrochloride (P-7288; Sigma-Aldrich, Japan) and H_2O_2 in citrate-phosphate buffer for 30 min. The coloring reaction was stopped with 2 M H_2SO_4. The absorbance at 490 nm was measured using a plate reader (ARVO MX; Perkin Elmer, Japan).

Assay to measure the binding activity of NoVLP to bacterial cells. NoVLP binding to isolated bacterial cells was investigated by ELISA and TEM. The isolated bacterial strain *Enterobacter* sp. SENG-6 was incubated in Luria–Bertani medium at 37°C for 18 h, and the number of cells suspended in PBS (pH 6.5) was adjusted to 10^9 cells/mL. *Staphylococcus epidermidis* (ATCC 35984), an HBGA-negative strain, was incubated in Reasoner's 2A medium at 37°C for 18 h, and the cell number was adjusted to 10^9 cells/mL. One milliliter of the cell suspension was centrifuged at 3000 ×*g* for 5 min, re-suspended with 400 μL of NoVLPs (GI.7, GII.3, GII.4, or GII.6) in PBS ($10^{9–10}$ particles/mL), and incubated at RT for 1 h. NoVLPs suspended in PBS (without bacterial cells) were used as controls. The mixture was filtered using a mixed cellulose acetate membrane filter (0.20-μm pore size and 13-mm diameter; 13CP020AN; Advantec, Toyo Roshi Kaisha Ltd., Japan) to recover unbound NoVLPs. The recovered NoVLPs were detected using ELISA as follows. Fifty microliters of NoVLP suspension were added to each well of an ELISA plate and kept at RT for 1 h to coat the well. Triplicate wells were used for each sample. The wells were then washed two times with PBS and blocked with 5% BSA in PBS. After incubation at RT for 2 h, the wells were washed two times with PBS, and 50 μL of anti-NoVLP rabbit antibody (diluted to 1:100 with PBS containing 5% BSA; Eve Bio-Science Co., Ltd., Japan) was added to the wells (the anti-NoVLP rabbit antibodies were purified using protein A from the antisera of rabbits immunized separately with each genotype). The plates were incubated at RT for 1 h. The wells were washed two times with PBS, and 50 μL of HRP-conjugated anti-rabbit IgG antibody (SAB-300; Stressgen Bioreagents, Belgium) diluted to 1:500 with PBS containing 5% BSA was inoculated into each well. After incubation at RT for 1 h, the wells were washed four times with PBS, and the bound HRP-conjugated antibodies were measured as described previously.

154 TEM imaging of NoVLP binding to bacterial cells was performed as follows.
155 *Enterobacter* sp. SENG-6 was incubated at 37°C for 18 h, as described previously, and the number
156 of cells suspended in M9 broth was adjusted to 10^9 cells/mL. One milliliter of the cell suspension
157 was centrifuged at 3000 ×*g* for 5 min, and the resulting cell pellet was re-suspended in M9 broth (a
158 washing step). The washing step was repeated two times. The cell suspension (10^9 cells/mL) was
159 centrifuged at 3000 ×*g* for 5 min, resuspended with 100 μL of GII.6 NoVLPs in PBS ($10^{11–12}$
160 particles/mL), and incubated at 4°C for 1 h. The washing step with M9 broth was repeated two
161 times, and the washed cells were placed on collodion membrane-coated copper grids, negatively
162 stained with 1% phosphotungstic acid, and observed using a transmission electron microscope
163 (JEM-1400; JEOL Ltd., Japan).
164 ***Immuno-TEM observation for localizing HBGA-like substances***. The localizations of
165 HBGA-like substances were analyzed by immuno-TEM. *Enterobacter* sp. SENG-6 was incubated
166 at 37°C for 18 h, as described previously. Two milliliters of cell suspension were centrifuged at
167 3000 ×*g* for 5 min, re-suspended in 2 mL of PBS containing 4% paraformaldehyde and 0.1%
168 glutaraldehyde, and incubated at 4°C for 2 h. The fixed cells were collected by centrifugation at
169 12,000 ×*g* for 5 min. The resulting cell pellet was washed by soaking in 2 mL of PBS at 4°C for 10
170 min. This washing step was repeated two times. The cell pellet was dehydrated by soaking in 2 mL
171 of increasing concentrations of ethanol (70, 80, 90, and 95%) and then mixed gently using a rotator
172 at 4°C for 15 min. The following dehydration and infiltration reactions were also conducted on a
173 rotator at 4°C. For complete dehydration, the pellet was soaked in 2 mL of 100% ethanol for 20 min.
174 This final dehydration step was repeated two times. Next, the cell pellet was infiltrated with 2 mL
175 of LR white resin (medium grade; London Resin Company Ltd., England) and 100% ethanol (1:2)

mixture for 12 h, followed by 2 mL of LR white resin and 100% ethanol (2:1) mixture for 12 h. The solution was replaced with 2 mL of pure LR white resin for 1 h, followed by another 2 mL of pure LR white resin for 12 h. The cell pellet in the pure LR white resin were polymerized using an ultraviolet polymerizer (TUV-100; Dosaka EM Co., Ltd., Japan) at 4°C for 3 days. Ultrathin sections (70-nm thick) were prepared from the embedded cell pellet using a diamond knife on an ultramicrotome (RMC MTXL; Boeckeler Instruments Inc., USA) and then placed on nickel grids. The ultrathin sections on grids were washed by floating the grid upside-down on a drop of PBS at RT for 1 min. The following incubations were also conducted by floating the grid upside-down on drops of reagent at RT. The sections were blocked with 4% BSA in PBS for 5 min and incubated with anti-blood group A, B, or O(H) mouse monoclonal antibody (sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc.) diluted to 1:30 with PBS containing 1% BSA for 2 h, followed by washing with PBS (six washes, 1 min each). In a competitive adsorption test, an ultrathin section was incubated with 100 μg/mL of *Soybean* agglutinin prior to incubation with anti-blood group A antibody (sc-69951, Santa Cruz Biotechnology Inc.). The bound primary antibodies were localized by incubating the sections on anti-mouse IgM antibody gold conjugate (10 nm, ab39613; Abcam, Japan) diluted to 1:10 with PBS containing 1% BSA for 1 h, followed by washing with PBS (six washes, 1 min each). The sections were fixed with 2% glutaraldehyde in PBS for 15 min, followed by washing with deionized distilled water (DDW; seven washes, 1 min each). Finally, the sections were stained with 5% uranyl acetate, followed by washing with 50% ethanol for 1 min and DDW (four washes, 1 min each). Stained sections were observed by TEM. A grid was also prepared without the incubation with primary antibodies, which was used as a control.

Assay of NoVLP binding to HBGA-like substances in bacterial polymeric substances.

198 Based on a hypothesis that HBGA-like substances were polysaccharides in EPS or LPS, we
199 extracted EPS and LPS from *Enterobacter* sp. SENG-6. Bacterial EPS was also extracted from *S.*
200 *epidermidis* ATCC 35984. EPS was extracted according to Liu and Fang (24) with some
201 modifications. This procedure was used because Pan et al. found that the application of this method
202 to algae–bacteria biofilm produced a higher yield of carbohydrate in EPS, whereas the protein yield
203 was lower than that with other extraction methods (25). The bacterial strains were incubated at
204 37°C for 18–20 h in 400 mL of each medium, as described previously. The cell suspension ($10^{10–11}$
205 cells/mL) was transferred to 2 × 225-mL centrifuge tubes and centrifuged at 3000 ×*g* for 5 min. The
206 resulting cell pellet was re-suspended in 40 mL of PBS using a vortex mixer for 2 min to displace
207 EPS from the cells. No chemicals were used in this first step of EPS recovery. Therefore, the
208 destruction of bacterial cells and the recovery of cellular proteins and genomic DNA components
209 were minimized. Eighty milliliters of the cell suspension were transferred to 2 × 50-mL centrifuge
210 tubes and centrifuged at 9000 ×*g* for 5 min. The supernatant containing EPS was collected and
211 filtered using a mixed cellulose ester membrane filter (0.20-μm pore size and 25-mm diameter;
212 25AS020AS; Advantec, Toyo Roshi Kaisha Ltd.) to remove bacterial cells. Eighty milliliters of the
213 filtrate were purified with mixed cellulose dialysis tubing (MWCO of 3500–5000 Da; 131 204;
214 Spectrum Laboratories Inc., USA) in DDW at 4°C for 24 h. The purified solution was lyophilized at
215 −50°C for 48 h, and the resulting pellet was dissolved in 4 mL of DDW.
216 The remaining bacterial cells in the EPS extract from *Enterobacter* sp. SENG-6 were used
217 to extract SOM, which is mainly composed of LPS according to Takaara et al. (26). The cell pellet
218 was lyophilized using a vacuum freeze dryer, and the lyophilized cells were suspended in 4 mL of
219 autoclaved distilled water at 65°C. Next, 4 mL of 90% phenol (65°C) was added. After vigorous

stirring with a vortex mixer, the samples were stirred for 15 min at 65°C and left for 15 min on ice. The cooled samples were centrifuged at 4000 ×*g* for 30 min at 2°C. Components of the cell-associated hydrophilic substances, which comprised mainly LPS and cellular RNA (27), were fractionated in the aqueous phase. The aqueous phase (4 mL) was then mixed with 4 mL of 90% phenol at 65°C. The samples were cooled on ice and centrifuged at 4000 ×*g* for 30 min at 2°C. The aqueous phase was collected and treated with 100 mg/mL RNase A (Sigma-Aldrich) for 6 h at 37°C to eliminate RNA components. Next, RNase A molecules were removed by phenol chloroform precipitation, and organic substances in the supernatant were purified further by ethanol precipitation. The degradation of RNA molecules by the RNase A treatment was confirmed visibly by agarose gel (1.5%) electrophoresis, ethidium bromide staining, and UV light excitation. The pellet collected after ethanol precipitation was suspended in DDW and stored as an SOM fraction at −80°C until further analysis. In addition to SOM, LPS of *Enterobacter* sp. SENG-6 was extracted separately using a LPS Extraction Kit (17141; Cosmo Bio Co., Ltd., Tokyo, Japan). The endotoxin units of SOM and LPS were quantified using an EndoLISA Endotoxin Detection Kit (609033; J.K. International, Tokyo, Japan) to confirm the presence of lipid A in the extracted bacterial substances.

NoVLP binding to the extracted EPS, SOM, and LPS was analyzed by ELISA. Fifty microliters of NoVLP GII.6 suspension in PBS ($10^{9–10}$ particles/mL) was added to each well of an ELISA plate and maintained at RT for 1 h to coat the well. Triplicate wells were used for each sample. The wells were washed two times with PBS and blocked with 5% BSA in PBS. After incubation at RT for 2 h, the wells were washed two times with PBS and 50 μL of the extracted EPS, SOM, or LPS was applied to the wells. After incubation at RT for 1 h, the wells were washed two times with PBS and 50 μL of anti-blood group A, B, or O(H) mouse monoclonal antibodies

(sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc., USA) diluted to 1:100 with PBS containing 5% BSA was applied to the wells. After incubation at RT for 1 h, the wells were washed two times with PBS and 50 μL of HRP-conjugated anti-mouse IgM antibody (A90-101P; Bethyl Laboratories, USA) diluted to 1:500 with PBS containing 5% BSA was inoculated into each well. After incubation at RT for 1 h, the wells were washed four times with PBS and the bound HRP-conjugated antibodies were measured as described previously. Wells without addition of EPS were used as controls.

In order to prove the specific binding of NoVLPs to the bacterial EPS, GI.1 NoVLPs (Norwalk virus, strain 8fIIa, GenBank accession number: M87661) and its mutant strain (W375A), kindly provided by Dr. Mary K. Estes, Baylor College of Medicine, were tested against the EPS extracted from *Enterobacter* sp. SENG-6. The mutant strain (W375A) does not recognize A and O(H) antigens (28). Furthermore, the extracted EPS treated with α-N-acetyl-galactosaminidase, which specifically catalyzes the hydrolysis of α-linked D-N-acetyl-galactosamine residues from oligosaccharides, was also used in ELISA for detecting A antigen. The enzyme treatment was performed as follows. One hundred micro-litters of reaction mixture contained 88 μL of extracted EPS, 10 μL of 10X G7 Reaction Buffer, 1 μL of α-N-acetyl-galactosaminidase to yield a final concentration of 0.2 U/mL (New England Biolabs, Inc., Japan), and 1 μL of 10 mg/mL BSA. One micro-liter of DDW was added instead of α-N-acetyl-galactosaminidase to control EPS. The tubes were incubated at 37 °C for 24 hr.

Specific interaction between NoVLPs and HBGA-like substances. The QCM method was used to evaluate the specific interaction between GII.6 NoVLPs and HBGA-like substances in the extracted EPS. The principle of QCM is the same as an electric scale where the frequency of an

264 energized quartz crystal changes relative to the amount of substances accumulated on the quartz
265 crystal surface. Therefore, the specific interaction between ligand and receptor molecules is
266 detected when the ligand molecules are inoculated to the quartz crystal surface where the receptor
267 molecules are immobilized. Fifty microliters of NoVLP suspension in PBS at a concentration of 20
268 μg total protein/mL was placed on a sensor chip and incubated at RT for 1 h. The sensor chip was
269 blocked with 0.1% BSA in PBS. After incubation at RT for 1 h, the chip was washed with DDW
270 and installed in the reactor of a QCM instrument (Single-Q0500; BioLab; AsOne Co., Ltd., Japan).
271 The reactor was filled with 500 μL of PBS and stirred at 300 rpm during measurements. When the
272 frequency of the chip stabilized, 3-μL aliquot of 1 mg TOC/mL of the extracted EPS in PBS was
273 inoculated into the reactor consecutively to produce a total of 24 μL, and the change in the
274 frequency of the chip (ΔF) was recorded. This adsorption experiment was performed three times,
275 and the adsorption isotherms were obtained. As controls, adsorption isotherms were obtained
276 without NoVLPs immobilized on the sensor chip. The difference between the ΔF values of the test
277 and control conditions (ΔΔF) was used to calculate the equilibrium binding constant. The molecular
278 weight of the HBGA-like EPS from *Enterobacter* sp. SENG-6 was estimated to calculate the
279 apparent equilibrium binding constant of the HBGA-like EPS from *Enterobacter* sp. SENG-6 and
280 GII.6 NoVLP according to the Langmuir adsorption isotherm. In brief, the extracted EPS was
281 fractionated using an ultrafiltration device (Nanosep Centrifugal Device; MWCO of 300 kDa; Pall
282 Corporation, USA), and the filtrate was collected. The amount of A-like substances in the filtrate
283 was measured by ELISA using anti-blood group A antibody, as described above.

284 ***Nucleotide sequence accession number.*** The GenBank/EMBL/DDBJ accession number
285 for the partial 16S rRNA gene sequence of *Enterobacter* sp. SENG-6 is AB758448. The accession

numbers for the capsid genes of the HuNoV genotypes of GI.7, GII.3, GII.4, and GII.6 are AB758449, AB758450, AB668028, and AB758451, respectively.

Results

Isolation of human enteric bacteria bearing HBGA-like substances. We screened blood group-active enteric bacteria from human feces using a biopanning technique with anti-HBGA antibodies. Of 35 isolated strains, 21 screened bacterial strains had blood group activity with A, B, or O(H) (Table 2). The phylogenetic relationships of the 16S rRNA gene sequences of the 21 isolated strains and related enteric bacteria are shown in Fig. 1. These enteric bacteria were gram-negative and were genetically related to *Enterobacter cloacae* (ATCC 13047^T), *Shigella flexneri* (ATCC 29903^T), or *Escherichia fergusonii* (ATCC 35469^T). From these, we isolated a bacterium (designated strain SENG-6) that was closely related to *Enterobacter cloacae* (ATCC 13047^T) with a 16S rRNA gene sequence similarity of 99.9%. Strain SENG-6 had A, B, and O(H) blood group activities. Twelve of 15 strains that were closely related to *S. flexneri* (ATCC 29903^T), sharing 99.3–99.9% of its 16S rRNA gene sequence, had both A and O(H) blood group activities (e.g., NMCA1-7 and NMC20B1-4), whereas two other strains (MURA1-1 and MUR18B1-4) with similarity of 99.5 and 99.6%, respectively, had only A blood group activity, while NMC20B1-6 with a similarity of 99.5% had only O(H) blood activity. All 5 strains that were closely related to *E. fergusonii* (ATCC 35469^T), sharing 99.4–99.8% of its 16S rRNA gene sequence, had both A and O(H) blood group activities.

Strains SENG-6 and NMC18B1-6 produced HBGA-positive results at the minimum optical density (OD_{600}) of 0.068 and 0.058, respectively (Table 2), which showed that these strains

carried a high abundance of HBGA-like substances. SENG-6 possessed A, B, and O(H) blood group activities; therefore, this strain was used in further experiments. *Staphylococcus epidermidis* (ATCC 35984) was used as the HBGA-negative strain because it had an HBGA-negative result, even at an OD_{600} of 1.357 (Table 2).

NoVLP binding to isolated bacterial cells. The blood group activity test suggested that HBGA-like substances were present in the bacterial outer components, including LPS of gram-negative bacteria and/or EPS, including the capsule. The binding of NoVLP genotypes GI.7, GII.3, GII.4, and GII.6 to *Enterobacter* sp. SENG-6 and *S. epidermidis* ATCC 35984 were tested. Each NoVLP genotype was mixed with each strain, and unbound NoVLPs were detected by ELISA (Fig. 2). Each NoVLP genotype was also mixed with PBS (without bacterial cells), which was used as a negative control. All NoVLP genotypes tested bound significantly to *Enterobacter* sp. SENG-6 ($p < 0.01$). While GII.4 NoVLP bound to *S. epidermidis* ATCC 35984 (Fig. 2A), it was previously shown that this genotype recognizes a broad range of ligands other than HBGAs, such as those containing sialic acid (29-31).

The direct visualization with TEM confirmed that NoVLP genotypes GI.7 and GII.6 bound to *Enterobacter* sp. SENG-6 (Fig. 3A and 3B). The majority of these NoVLPs were observed to bind mainly to the EPS of *Enterobacter* sp. SENG-6 (Fig. 3A and 3B), whereas a few NoVLPs were observed in the region adjacent to bacterial cells. However, it was not determined by this technique whether NoVLP bound to LPS of *Enterobacter* sp. SENG-6 because most LPS components were covered by EPS, which would hamper the interaction between NoVLPs and LPS.

Localization of HBGA-like substances in bacterial cell components. Ultrathin sections of *Enterobacter* sp. SENG-6 were examined by immuno-TEM to determine the localization of

HBGA-like substances. *Enterobacter* sp. SENG-6 was labeled with gold nanoparticles carrying anti-mouse IgM after reacting with anti-blood group A, B, or O(H) antibody (used as the primary antibody). A number of gold nanoparticles were observed in the extracellular spaces of *Enterobacter* sp. SENG-6 (Fig. 3C), which had reacted with anti-A antibody. The scattered occurrence of A-like substances outside bacterial cells suggests that these antigens were present at the terminals of each extracellular polymer, although this needs to be confirmed by the structural analysis of EPS. Fig. 3D shows that the number of gold nanoparticles in the ultrathin section was reduced dramatically by pretreatment of the ultrathin section with *Soybean* agglutinin, which is a lectin specific to N-acetylgalactosamine. This showed that anti-A antibodies labeled with gold nanoparticles in the ultrathin section in Fig. 3C specifically bound to A-like substances that contained N-acetylgalactosamine. In contrast, few B- and O(H)-like substances were observed in *Enterobacter* sp. SENG-6 (Fig. 3E and 3F, respectively), although the B and O(H) blood group activities of this bacterial strain were confirmed using the blood group typing kit (Table 2). The failure to detect B- and O(H)-like substances in the immuno-TEM images may be attributable to a subtle difference in the recognition of the antibodies used in the blood group typing kit and immuno-TEM or insufficient amounts of B- and O(H)-like substances in immuno-TEM, although it is difficult to compare the relative amounts of each HBGA-like substance. Gold nanoparticles did not accumulate at the edges of the cell surfaces (Fig. 3C), which showed that only limited amounts of HBGA-like substances were present in LPS of *Enterobacter* sp. SENG-6, even if blood type-active substances were present.

NoVLP binding to HBGA-like substances in bacterial EPS, SOM, and LPS. TEM observations showed that NoVLPs were captured mainly in the bacterial EPS where the A-like

substances were present, whereas LPS had none or a low level of specific binding sites for NoVLPs (Fig. 3). To further elucidate the localization of HBGA-like substances, EPS, SOM, and LPS were extracted separately from *Enterobacter* sp. SENG-6 and tested to detect HBGA-like substances by ELISA using GII.6 NoVLP. GII.6 is one of the best genotypes for the binding assay because this genotype was shown to bind to A, B, and O(H) antigens (Table 1). The bacterial EPS were also extracted from *S. epidermidis* ATCC 35984 and used in the same ELISA. SOM and LPS of *S. epidermidis* ATCC 35984 were not extracted because *S. epidermidis* ATCC 35984 is gram-positive.

Fig. 4A shows that A- and B-like substances were detected in the extracted EPS of *Enterobacter* sp. SENG-6, which were trapped by GII.6 NoVLP immobilized in ELISA. B-like substances were not detected in immuno-TEM (Fig. 3E), but the extraction and concentration of EPS allowed them to be detected by ELISA. However, no significant signals for A-, B-, and O(H)-like substances were detected in SOM and LPS of *Enterobacter* sp. SENG-6. Likewise, no signals for A-, B-, and O(H)-like substances were detected in EPS of *S. epidermidis* ATCC 35984 (Fig. 4A). *Enterobacter* sp. SENG-6 was positive for all A, B, and O(H) blood group antigens (Table 2), whereas O(H)-like substances were not detected by ELISA, which may be caused by the difference in the recognition of anti-O(H) antibodies used in the blood typing kit and ELISA.

The binding between GII.6 NoVLP and EPS of *Enterobacter* sp. SENG-6 containing A-like substances is shown clearly in Fig. 4A, although it is not clear whether the A-like substances in EPS of *Enterobacter* sp. SENG-6 are the main binding sites for GII.6 NoVLP. To test the function of A-like substances in EPS as the binding site for NoVLP, we performed ELISA using the NoVLPs of GI.1 wild-type (8fIIa) and mutant (W375A) strains. The GI.1 wild-type strain (8fIIa) can recognize A antigen, whereas the mutant strain (W375A) has lost its ability for binding to A

antigen because of a point mutation from tryptophan to alanine at the 375th position (28). Fig. 4B shows that A-like substances in EPS of *Enterobacter* sp. SENG-6 were detected when NoVLP of the GI.1 wild-type strain (8fIIa) was immobilized in ELISA, but they were not detected when NoVLP of the GI.1 mutant strain (W375A) was immobilized. Furthermore, the enzymatic treatment of EPS with N-acetylgalactosaminidase, which can release the terminal N-acetylgalactosamine from A antigen, weakened the interaction between EPS and NoVLP of the GI.1 wild-type strain (8fIIa) (Fig. 4B). These results show that A-like substances in EPS of *Enterobacter* sp. SENG-6 are the binding sites of NoVLPs. The A antigen has also been found in HBGA-like substances in the digestive tissues of oysters (32), and the critical role of the terminal N-acetylgalactosamine residue in the strain-dependent accumulation of HuNoVs in oysters has been discussed previously (29, 30). Bacterial A-like substances can also affect the genotype-dependent fate of HuNoVs in the environment because each HuNoVs genotype has its own HBGA binding profile (12).

The adsorption isotherms of the extracted EPS were determined for GII.6 NoVLP, which was immobilized on a sensor chip (Fig. 5A and 5B). The ΔF values for EPS of *Enterobacter* sp. SENG-6 with GII.6 NoVLP were higher than those under the NoVLP-negative condition, and this difference was statistically significant according to a Mann–Whitney U test performed for each EPS concentration (Fig. 5A). In contrast, the ΔF values for the EPS extracted from *S. epidermidis* ATCC 35984 were not different from those under the NoVLP-negative condition (Fig. 5B). These results indicated that the EPS extracted from *Enterobacter* sp. SENG-6 can interact specifically with NoVLP. In order to estimate the molecular weight of HBGA-positive EPS from *Enterobacter* sp. SENG-6, the amount of A-like substances in the EPS extracted from *Enterobacter* sp. SENG-6 was determined before and after ultrafiltration with a molecular weight cut off (MWCO) of 300 kDa. As

a result, the amount of A-like substances was significantly reduced by ultrafiltration (Fig. 5C), which suggested that the majority of A-like substances existed in EPS with a molecular weight >300 kDa. If we assume that A-like antigens dominated EPS and that EPS carrying the HBGA-like substances had a molecular weight of 300 kDa, the reciprocal plots of ΔΔF (the difference between ΔF with NoVLPs and that without NoVLPs in Fig. 5A) and EPS concentration is obtained (Fig. 5D), which gives the apparent equilibrium binding constant of 3.1×10^6 M^{-1} according to the Langmuir adsorption isotherm. Since the molecular weight of HBGA-positive EPS is expected to be larger than 300kDa (Fig. 5C), the apparent equilibrium binding constant for the HBGA-positive EPS of *Enterobacter* sp. SENG-6 and GII.6 NoVLP must be $>3.1 \times 10^6$ M^{-1}.

Discussion

Blood group activities of human enteric bacteria. The presence of blood group-active enteric bacteria was reported many years ago. Springer et al. tested 282 gram-negative bacterial strains from genera such as *Escherichia*, *Salmonella*, and *Klebsiella*, which were isolated from clinical specimens, and approximately 50% of the strains inhibited anti-blood group A, B, and/or O(H) agglutinins. Of the tested strains belonging to *Escherichia*, some strains had a single aggregation activity (A, B, or O(H)), whereas others had multiple aggregation activities (ABO(H), AO(H), AB, or BO(H)) (17). In the present study, 21 blood group-active strains were isolated from human feces using a biopanning technique with anti-HBGA antibodies (Table 2). The isolated bacterial strains must represent only a small fraction of the actual population of HBGA-positive bacteria, because we only analyzed culturable strains in a fecal sample from a single individual under microaerobic conditions in this study, while it is recognized that the majority of enteric bacteria in human feces

418 are likely to be dead, viable but not culturable (33), or obligate anaerobes.

419 The blood group activities of various widely distributed gram-negative bacteria were
420 reported initially by Springer et al. (17), as described above, and these blood group activities of
421 enteric bacteria were attributed to the presence of HBGA-like "O antigen" in LPS. The structural
422 basis of the HBGA-like O antigen was clarified recently. For example, Anderson et al. showed that
423 the O-antigen polysaccharide of *E. coli* O86 contained a B-type trisaccharide where alpha-L-fucose
424 and beta-D-galactose formed part of the O-antigen backbone, while alpha-D-galactose was
425 conjugated to beta-D-galactose (34). *Salmonella milwaukee* O:43 (group U) also has human blood
426 group B activity with the O-polysaccharide (35). Therefore, we initially postulated that HBGA-like
427 substances were present in LPS of the isolated strains. However, immuno-TEM imaging (Fig. 3)
428 and ELISA (Fig. 4) clearly showed that HBGA-like substances were localized mainly in the
429 bacterial EPS. Only *Klebsiella* sp. was suggested to have blood group active substances in its
430 capsular polysaccharide, although this was not proved (17). This is the first study to show that
431 human enteric bacteria possess HBGA-like substances in their EPS and that they can capture
432 HuNoVLPs.

433 ***Interaction between bacterial substances and NoVLPs.*** Four genotypes of NoVLP (GI.7,
434 GII.3, GII.4 and GII.6) were used in the binding assay to HBGA-positive (*Enterobacter* sp.
435 SENG-6) and HBGA-negative (*S. epidermidis* ATCC35984) cells, and only GII.4 bound to both
436 strains (Fig. 2A). It has been shown that NoV GII.4 may bind to multiple carbohydrate moieties,
437 including alpha-D-Gal, alpha-D-Man/alpha-D-Glc and alpha-L-Fuc, in addition to GalNAc,
438 GlcNAc and sialic acid (30). Although extracellular polymeric substances of planktonic *S.*
439 *epidermidis* cells have not been well analyzed so far, the broad binding capacity of NoV GII.4 is

likely to contribute to the binding to HBGA-negative cells of *S. epidermidis* ATCC35984 observed in the present study. This binding property of NoV GII.4 did not allow us to use this genotype in the following binding assays indicated in Fig. 3 to 5, because it was necessary to analyze the specific interaction between bacterial HBGAs and NoVLPs. In that context, NoV GII.6 was available for those binding assays in the present study, because this genotype can recognize all HBGAs tested (Table 1) but seemed not to have a binding spectrum as broad as GII.4, judged by the binding assay shown in Fig. 2. Further analysis on the binding spectrum of multiple genotypes of HuNoVs other than GII.4 may provide a clue for better understanding the genotype-specific lifestyle of HuNoVs.

Significance of the blood group-active enteric bacteria: a hypothesis. The binding is likely to affect at least two aspects of the ecology of HuNoVs, particularly the fate of HuNoVs in their hosts after HuNoVs enter the human intestine and encounter large numbers of enteric bacteria. In this regard, there is increasing understanding of the roles played by the intestinal microbiota in viral infections. Kane et al. showed that bacterial LPS plays a key role in the infection of mouse mammary tumor virus (MMTV) using antibiotic-treated and germ-free mice (36). MMTV-bound bacterial LPS was recognized by Toll-like receptor 4, and it affected the induction of the inhibitory cytokine interleukin-10. However, this enhanced viral titers and MMTV overcame the immune response. Kuss et al. found that antibiotic-treated mice were less susceptible to poliovirus infection and demonstrated that exposure to bacteria or their N-acetylglucosamine-containing surface polysaccharides, including LPS and peptidoglycan, enhanced the infectivity of poliovirus (37). These results suggest that antibiotic-mediated microbiota depletion reduces virus infections and that viruses may exploit intestinal microbes for replication and transmission. Therefore, it is likely that the commensal microbiota in the human intestine will also affect the likelihood of infection with

462 human enteric viruses, including HuNoVs, in the intestine. A higher level of blood group-active
463 EPS in the mucosa of the intestine (jejunum and ileum) probably provides HuNoVs with a greater
464 opportunity for infecting intestinal cells because norovirus particles may be retained in the intestine
465 provided that norovirus-binding bacteria are present, although it is also possible that bacterial
466 HBGA will be an inhibitor of HuNoV binding to human HBGA on intestinal cells. The abundance
467 of blood group-active EPS could also affect rotavirus infection, another major gastroenteritis virus,
468 because spike protein VP8 of human rotavirus recognizes HBGAs (38, 39). Thus, enteric virus
469 infections may be characterized on the basis of the bacterial community composition of the human
470 intestine, which varies considerably with age, lifestyle, and season. Understanding the role of the
471 intestinal microbiota in viral transmission and pathogenesis may lead to the development of new
472 antiviral strategies, although the role of bacterial HBGAs in the infections of intestinal mucosal
473 cells with HuNoVs remains largely speculative at this moment.
474 Another important implication concerning HBGA-positive bacterial EPS for the HuNoVs
475 life cycle is the transport of viral particles from acute gastroenteritis patients to aquatic
476 environments. Enteric viruses are known to be associated mainly with particles in environmental
477 waters (40). Various environmental materials can capture human enteric viruses, including activated
478 sludge (41), estuarine sediment (41, 42), river sediment (43), and soils (41, 44). da Silva et al.
479 showed that the GI and GII noroviruses found in wastewater samples were associated mainly with
480 particles measuring <180 μm (45). Sano et al. demonstrated that indigenous noroviruses were not
481 present in the permeates of mixed liquor and treated wastewater filtered using a microfiltration
482 membrane (pore size, 0.1 μm) (46). Gentry et al. showed that an unexpectedly large number of
483 HuNoVs were present in the fraction collected using 63- and 200-μm-mesh plankton nets, which

484 suggested the binding of HuNoV particles to phytoplankton and zooplankton (47). Viral particles
485 associated with environmental materials are physically more stable than freely moving viral
486 particles (48). Viruses associated with organic or inorganic particles are protected from inactivation
487 by chlorine (49), UV (40), and salinity (osmotic shock) (50) because these exogenous stresses are
488 impeded by the entities that capture the viral particles. Furthermore, enteric viruses associated with
489 environmental suspended solids may be transported in a different manner from freely moving
490 virions (51, 52). Thus, HuNoV particles associated with HBGA-positive EPS that is attached to or
491 disengaged from enteric bacterial cells must have different fates from free viral particles. Since the
492 proteolytic virucidal activity of some bacterial species has been reported (53), the association of
493 HuNoV particles with HBGA-positive EPS detached from bacterial cells may have an advantage in
494 terms of its environmental persistency. These hypotheses about the impact of bacterial HBGAs on
495 the environmental persistency of HuNoVs largely remain to be proved, and are challenging issues
496 in further studies.

497 ***Avidity of the blood group-active bacterial EPS with HuNoV particles.*** Thus, the effect
498 of the blood group-active bacterial EPS on the fate of HuNoVs is totally dependent on the binding
499 avidity of EPS to HuNoV particles. If we assume that all the components in the extracted EPS are
500 blood group-active and that they have a molecular weight >300 kDa (Fig. 5C), we can estimate the
501 apparent equilibrium binding constant according to the Langmuir adsorption isotherm as $>3.1 \times 10^6$
502 M^{-1}. This value is comparable with that obtained for the interactions between ß-galactose specific
503 lectins and lactose-carrying glycopolystylenes, which have binding constants of 10^6 to 10^7 M^{-1} (54).
504 The interactions between enteric viruses and environmental adsorbents were previously shown to
505 have no binding specificity (41-45, 47), whereas HuNoV binding to bacterial HBGAs is regarded as

specific, as illustrated by human HBGAs and HuNoVs (12). In addition to enthalpy-driven adsorption via an array of hydrogen binding routes (28), the entropy effect caused by multiple binding sites also contributes to the increased binding avidity, although structural analyses of bacterial EPS are essential to obtain an insight into the true value of the binding constant. The specific binding capacity of blood group-active enteric bacteria with HuNoVs provides a new scientific platform for understanding the interactions between two types of microbes that were previously regarded as biologically unrelated.

Conclusions

Enterobacter sp. SENG-6, an isolated strain from a fecal sample of a healthy person, exhibited blood group activity. We observed A-like substances in its extracellular region. EPS extracted from *Enterobacter* sp. SENG-6 captured norovirus particles, specifically via A-like substances, with an avidity comparable to those between multi-glycoconjugate polymers and lectins. Further studies of human enteric bacteria capable of binding to HuNoVs are warranted to gain new insights into the biological role of HBGA-positive bacterial EPS in the transmission and infection of HuNoVs in the environment, and are likely to provide a clue for the development of novel prevention strategies.

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686 **Figure Legends**

687 **FIG. 1.** Phylogenetic relationships of 16S rRNA gene sequences of isolated bacterial strains and
688 related enteric bacteria. The phylogenetic tree was constructed by the neighbor-joining method with
689 1,000 bootstrap replicates using ClustalX (version 2.1). Bold type indicates the bacterial strains
690 isolated in this study, and *Salmonella enterica* serovar Typhimurium SL1344 was used as an
691 outgroup. The scale bar represents the number of substitutions per site.

692 **FIG. 2.** NoVLP binding to bacterial cells. (A) *Enterobacter* sp. SENG-6 and *Staphylococcus*
693 *epidermidis* (ATCC 35984) were mixed with each NoVLP genotype (GI.7, GII.3, GII.4, and GII.6)
694 in PBS. Unbound NoVLPs were recovered by filtration and detected by ELISA. Suspended
695 NoVLPs in PBS that were not mixed with bacterial cells were filtered and used as controls. The
696 results were expressed as the absorbance relative to the control; therefore, values statistically <1
697 (dashed line) indicated the significant binding of NoVLPs to bacterial cells. The error bars represent
698 the standard deviations of triplicate independent measurements. ** $p < 0.01$ (*t*-test). (B) GI.7
699 NoVLPs. (C) GII.3 NoVLPs. (D) GII.4 NoVLPs. (E) GII.6 NoVLPs. Black dots in the viewing
700 field in (B)–(E) are avidin-conjugated gold nanoparticles (average diameter = 5nm), which were
701 added to estimate the diameter of NoVLPs.

702 **FIG. 3.** Binding of NoVLPs to bacterial cells and localization of HBGA-like substances of
703 *Enterobacter* sp. SENG-6. *Enterobacter* sp. SENG-6 was reacted with GI.7 (A) or GII.6 (B)
704 NoVLPs in PBS. NoVLPs are indicated by arrows in (A) and (B). For the localization of
705 HBGA-like substance, ultrathin sections of *Enterobacter* sp. SENG-6 were labeled with
706 immuno-gold nanoparticles after reacting with anti-blood group A, B, and O(H) antibodies (primary
707 antibodies) and observed by TEM. Ultrathin sections without primary antibodies were used as

controls. (C) A-like substances. (D) A-like substances after pretreatment with *Soybean* agglutinin, a lectin specific to N-acetylgalactosamine. (E) B-like substances. (F) O(H)-like substances. (G) Primary antibody-negative control.

FIG. 4. Detection of HBGA-like substances in extracted bacterial polymers. (A) The wells on an ELISA plate were coated with GII.6 NoVLPs, and EPS, SOM, and LPS extracted from *Enterobacter* sp. SENG-6, and EPS extracted from *Staphylococcus epidermidis* (ATCC 35984) were inoculated into the wells. We used ELISA to measure A-, B-, and O(H)-like substances in the EPS, SOM, or LPS which bound to NoVLPs. A well without the incubation of bacterial polymeric substances was used as a control. (B) The wells were coated with GI.1 NoVLPs (8fIIa or W375A), and the EPS extracted from *Enterobacter* sp. SENG-6 or the EPS treated with α-N-acetyl-galactosaminidase was reacted. A-like substances which bound to the NoVLPs were detected by ELISA. All results in (A) and (B) were expressed as the absorbance relative to the control; therefore, values statistically >1 (dashed line) indicated the presence of A-, B-, or O(H)-like substances. The error bars represent the standard deviations of triplicate independent measurements. * $p < 0.05$; ** $p < 0.01$ (*t*-test).

FIG. 5. Binding avidity between NoVLPs and bacterial EPS. Adsorption isotherm between GII.6 NoVLPs and EPS from *Enterobacter* sp. SENG-6 (A) and *Staphylococcus epidermidis* (ATCC 35984) (B), measured using the QCM method. GII.6 NoVLPs were immobilized on a sensor chip and various amounts of extracted EPS were inoculated. The change in frequency of the sensor chip was measured, which depended on the amount of EPS bound to GII.6 NoVLPs. The error bars represent the standard deviations of triplicate independent measurements. Black dots (diamonds in A and triangles in B) are ΔF values with NoVLPs, while white dots are those without NoVLPs. (C)

730 Detection of A-like substances in EPS extracted from *Enterobacter* sp. SENG-6 before and after
731 ultrafiltration. The wells of an ELISA plate were coated with GII.6 NoVLPs, and the wells were
732 inoculated with EPS extracted from *Enterobacter* sp. SENG-6 before or after ultrafiltration with a
733 MWCO of 300 kDa. A well without EPS was used as a control. The results were expressed as the
734 absorbance relative to the control. The error bars represent the standard deviations. $*p < 0.05$ (*t*-test).
735 (D) Reciprocal plots of ΔΔF and the concentration of EPS. ΔΔF is the difference between the ΔF
736 value with NoVLPs and that without NoVLPs.
737
738

739 Table 1. Binding profiles of norovirus-like particles prepared in this study

Genotype (GenBank accession number)	Histo-blood group antigens			
	A (Type 1)	B (Type 1)	H (Type 1)	Lewis b (Type 1)
GI.7 (AB758449)	+	−	−	−
GII.3 (AB758450)	+	−	−	−
GII.4 (AB668028)	+	+	+	+
GII.6 (AB758451)	+	+	+	+

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741

742 Table 2. Blood group activities of isolated and reference bacterial strains used in this study

Strain	Growth medium	Blood group activities			Min. OD_{600} with HBGA positive	Genetically similar strain based on 16S rRNA gene analysis (strain, similarity)
		A	B	O(H)		
SENG-6	BGLB[b]	+	+	+	0.068	*Enterobacter cloacae* (ATCC 13047^T, 99.9%)
NMCA1-7	BGLB	+	−	+	0.920	*Shigella flexneri* (ATCC 29903^T, 99.6%)
NMCA1-8	BGLB	+	−	+	0.082	*Escherichia fergusonii* (ATCC 35469^T, 99.8%)
NMC18B1-3	BGLB	+	−	+	0.091	*Escherichia fergusonii* (ATCC 35469^T, 99.4%)
NMC18B1-6	BGLB	+	−	+	0.058	*Escherichia fergusonii* (ATCC 35469^T, 99.5%)
NMC20B1-4	BGLB	+	−	+	0.821	*Shigella flexneri* (ATCC 29903^T, 99.5%)
NMC20B1-6	BGLB	−	−	+	0.302	*Shigella flexneri* (ATCC 29903^T, 99.5%)
KKA1-1	EC[c]	+	−	+	0.465	*Escherichia fergusonii* (ATCC 35469^T, 99.8%)
KKB1-1	EC	+	−	+	0.502	*Escherichia fergusonii* (ATCC 35469^T, 99.5%)
TZWH1-1	EEM[d]	+	−	+	0.502	*Shigella flexneri* (ATCC 29903^T, 99.6%)
TZWH1-2	EEM	+	−	+	0.089	*Shigella flexneri* (ATCC 29903^T, 99.6%)
TZWH2-2	EEM	+	−	+	0.515	*Shigella flexneri* (ATCC 29903^T, 99.3%)
TZWH2-3	EEM	+	−	+	0.570	*Shigella flexneri* (ATCC 29903^T, 99.6%)
TZWH2-4	EEM	+	−	+	0.568	*Shigella flexneri* (ATCC 29903^T, 99.4%)
TZWA1-2	EEM	+	−	+	0.532	*Shigella flexneri* (ATCC 29903^T, 99.6%)
TZWA1-3	EEM	+	−	+	0.504	*Shigella flexneri* (ATCC 29903^T, 99.6%)
TZWA1-4	EEM	+	−	+	0.257	*Shigella flexneri* (ATCC 29903^T, 99.4%)
MURA1-1	Mossel[e]	+	−	−	0.407	*Shigella flexneri* (ATCC 29903^T, 99.5%)
MURA1-6	Mossel	+	−	+	0.428	*Shigella flexneri* (ATCC 29903^T, 99.9%)
MUR18B1-4	Mossel	+	−	−	0.367	*Shigella flexneri* (ATCC 29903^T, 99.6%)
MUR20B1-4	Mossel	+	−	+	0.263	*Shigella flexneri* (ATCC 29903^T, 99.7%)
ATCC 35984[a]	R2A[f]	−	−	−	−	−

743 a, *Staphylococcus epidermidis* ATCC35984 was used as the HBGA-negative strain, and it gave an
744 HBGA-negative result, even at an OD_{600} of 1.357; b, 2% Brilliant green bile broth; c, *Escherichia*
745 *coli* broth; d, Enterobacteriaceae enrichment mannitol broth; e, Enterobacteria enrichment
746 broth-Mossel; f, Reasoner's 2A.