Clinical specimens from paediatric acute gastroenteritis patients were examined for evidence of norovirus (NoV) infection as well as their secretor status. Secretors express ABH histo blood group antigens (HBGAs) on the intestinal epithelium and in secretions (saliva, gastric juice, etc.), whilst non-secretors do not. In cases of NoV infection, the detected virus was genotyped according to the nucleic acid sequence of the capsid N-terminal region. The percentages of non-secretors in all infected patients in major genotypes of NoV (GII.2, GII.3, GII.4 and GII.6) varied from 5.7 to 25%. As two non-secretors were infected with GII.4 NoV clade 2006b, detailed analysis of the main structural protein (capsid protein) was conducted. When the amino acid residues of these specific viruses were compared with four viruses of the same clade obtained from secretors and the outbreak, one virus had two unique amino acids and another had three. However, no common differences were noted between these two viruses. In addition, the predicted HBG-A-binding sites (conserved sites) and putative variant-specific epitopes that were recognized by generated human mAbs (that block carbohydrate binding) were also examined. However, no crucial characteristic amino acid changes in the strains from non-secretors were observed. Nevertheless, these preliminary findings might suggest an alternative route of infection or existence of coincidental molecules.

NoVs in the family *Caliciviridae* are one of the leading viral causes of acute gastroenteritis in the world. HBGAs have been implicated in the susceptibility of individuals to NoV infection (Hutson et al., 2002). These antigens are carried by specific types of carbohydrate chains, of which the following types are common: type 1 (Galβ1–3GlcNAc-) chains expressed on the epithelium and in secretions of the digestive and respiratory tracts; type 2 (Galβ1–4GlcNAc-) chains present on both glycolipids and glycoproteins expressed mainly on erythrocytes and vascular endothelial cells; type 3 (Galβ1–3GlcNAcα-) chains which are typically restricted on glycoproteins; and type 4 (Galβ1–3GlcNAcβ-) chains which are found only on glycolipids (Koda et al., 2001). In humans and animals, ABH antigen expression is more abundant on the epithelial cells of the digestive, respiratory, urinary and reproductive tracts than on erythrocytes (Koda et al., 2001). According to Marroigneau et al. (2002), the virus binds to HBGAs on gastroduodenal epithelial cells of secretors, but not of non-secretors. Secretor-negative individuals are at much lower risk of infection with genotypes GI.1 (Lindesmith et al., 2003), GI.3 (Tan et al., 2008; Liu et al., 2014) and GI.4 (Thorven et al., 2005; Larsson et al., 2006; Kindberg et al., 2007). However, the relationship of secretor status to risk of infection with other genotypes is less clear.

The gene responsible for the secretor phenotype, *FUT2*, encodes 1,2-α-fucosyltransferase (Kelly et al., 1995; Rouquier et al., 1995), which results in an additional fucose molecule at the terminal galactose to form the H antigen. This is the precursor of the ABO HBGAs expressed in body fluids such as saliva and in the intestinal mucosa. Individuals who express ABH antigens are termed secretors (Se individuals), whilst those who have the homozygous non-functional *FUT2* allele and fail to express ABH antigens in secretions such as saliva or on epithelial cells are termed non-secretors (se individuals) (Koda et al., 2001). Approximately 20% of individuals in various populations in the world show the non-secretor phenotype (Koda et al., 2001). For example, the nonsense mutation G428A (Trp143→stop, se428) causes a non-secretor allele in Caucasian and African populations, whereas the missense mutation A385T (Ile129→Phe, se385) is included in a common Se enzyme-deficient allele in Asian populations (Koda et al., 2001).

A homozygous missense mutation at nt 385 is considered as weak secretor status. Kudo et al. (1996) and Henry et al. (1996) examined the fucosyltransferase activity of this missense mutation and compared it with that of the WT gene using acceptor substrate and lysates from Cos cells transfected with the *FUT2* gene. Fucosyltransferase activities of the missense mutation were reportedly as weak as 2–3% (Kudo et al., 1996) or even less than 2% (Henry et al., 1996). In 226 Japanese individuals, the mutations of *FUT2* genes were localized only at nt 357 or at both nt 357 and 385 in the study of Kudo et al. (1996). The same group investigated the *FUT2* gene status as well as the phenotype of secretor status in saliva (ABH antigen detection in saliva by haemagglutination inhibition test) and found that non-secretor status (undetectable ABH antigen) was 100% consistent with homozygous mutations in A385T. Considering their results of abundant expression of ABH antigen on the epithelial cells and in secretions compared with the surface of erythrocytes, the homozygous A385T missense mutation in Japanese individuals is defined as a non-secretor phenotype in this study.

Regarding susceptibility to NoV, Lindesmith et al. (2003) demonstrated the resistance of non-secretors to Norwalk virus infection in a human challenge model. There were several subsequent reports in which non-secretors with the homozygous nonsense mutation (G428A) were resistant to symptomatic NoV GII.4...
infections (Thorven et al., 2005; Larsson et al., 2006; Kindberg et al., 2007). However, another study from the same group reported that one symptomatic patient was a non-secretor, indicating that the nonsense mutation in FUT2 provides strong but not absolute protection (Carlsson et al., 2009).

In this study, the relationship between susceptibility to NoV infection and non-secretor status in Japanese patients with acute gastroenteritis was investigated. In addition, the amino acid sequences of the capsid region (ORF2) of the GII.4 NoVs obtained from non-secretors were compared with those detected from secretors. The amino acid sequences of these viruses were compared with those of the predicted HBGAs (conserved sites) as well as previously reported putative variant-specific epitopes (Lindesmith et al., 2008, 2012, 2013; Allen et al., 2009; Carlsson et al., 2009; Tan et al., 2009; Debbink et al., 2013) to determine the different amino acid changes responsible for the infection of non-secretors.

Japanese clinical specimens (faecal samples and oral epithelial cell suspension in TE/saline buffer) were collected between October 2003 and July 2012 from acute gastroenteritis patients (aged 0–11 years) whose parents signed consent forms in two paediatric clinics. Clinical samples were submitted to the Osaka Prefectural Institute of Public Health for investigation of NoV infection and secretor status. Study design was approved by the ethical committee of the Osaka Prefectural Institute of Public Health.

To determine the ratio of non-secretors in a Japanese population, oral epithelial cell suspensions were collected from 118 Osaka prefectural officers and their families who signed consent forms, and were examined between 2004 and 2011.

Regarding NoV infection screening, an in-house primer set RT-LAMP (reverse transcription loop-mediated isothermal amplification) assay was performed as described by Yoda et al. (2009) using extracted RNA from faecal samples. Reverse transcription (RT)-PCR was conducted in accordance with a standard protocol (Kojima et al., 2002) for NoV-infected samples. Phylogenetic analysis of NoV genotypes was performed based on sequence data of the RT-PCR products using a BLAST search of the DNA Data Bank of Japan (DDBJ) database. Extracted DNA from oral epithelial cells was amplified by using specific primers and the PCR products digested by restriction enzyme to differentiate secretor status (see Supplementary Data, available in the online Supplementary Material).

The 119 paediatric patients confirmed to be infected with major NoV strains (GII.2, GII.3, GII.4 and GII.6) and 118 otherwise healthy Japanese subjects were examined for their secretor status. The relationship between NoV genotype and secretor status, and the secretor status of otherwise healthy Japanese subjects are given in Table 1. The percentages of non-secretors in the NoV-infected group and otherwise healthy group were nearly identical. However, the percentages of non-secretors amongst patients in each genotype varied from 5.7 % in GII.4 to 25.0 % in GII.2. Fisher’s exact test was applied to observe the difference in the ratio of secretors to non-secretors between groups. This ratio between GII.4 and all other genotypes pooled (GII.2, GII.3 and GII.6) was significantly different (odds ratio 0.21 with 95 % confidence interval 0.05–0.94) in a two-tailed test. NoV GII.4 infection was disproportionately observed among secretors. This observation was not found for the other genotypes.

Six NoV strains were selected to obtain whole ORF2 sequences. The backgrounds of the selected strains were: outbreak strain NV2210 (the secretor status of the patient was not known); strains T158, T162 and T169 (obtained from secretors); and strains T164 and T272 (obtained from non-secretors). Complete ORF2 sequences of the RT-PCR products of these six NoV were determined and used for clade categorization. The clade categorization was done using the DDBJ database. Amino acid comparisons were analysed using DNASIS for Mac version 3.4 (Hitachi Solutions).

The selected six GII.4 NoV strains were all categorized into clade 2006b. The amino acid sequences of the GII.4 ORF2 detected from non-secretors (T164 and T272) were compared with those of the other four strains. Two differences in amino acid residues were noted in T164 (at aa 130 and 307), whilst three were noted in T272 (at aa 6, 205 and 393), as shown in Fig. S1(a). Among five differences, aa 130 and 307 of T164 and aa 205 of T272 were unique when compared with 176 GII.4 strains reported by Lindesmith et al. (2008) and three representative clade strains [Den Haag 2006b (DDBJ accession number EF126965), New Orleans 2009 (DDBJ accession number GU445325) and Sydney 2012 (DDBJ accession number JX459907)]. The predicted HBGA-binding sites reported by Shanker et al. (2011) are indicated in Fig. S1(b) for a comparison of

### Table 1. Relationship between secretor status and NoV infection

<table>
<thead>
<tr>
<th>NoV genotype</th>
<th>No. of secretors (Se/Se, Se/se)</th>
<th>No. of non-secretors (se/se, A385T)</th>
<th>Total</th>
<th>Percentage of non-secretors</th>
</tr>
</thead>
<tbody>
<tr>
<td>GII.2</td>
<td>9</td>
<td>3</td>
<td>12</td>
<td>25.0</td>
</tr>
<tr>
<td>GII.3</td>
<td>31</td>
<td>8</td>
<td>39</td>
<td>20.5</td>
</tr>
<tr>
<td>GII.4</td>
<td>33</td>
<td>2</td>
<td>35</td>
<td>5.7</td>
</tr>
<tr>
<td>GII.6</td>
<td>25</td>
<td>8</td>
<td>33</td>
<td>24.2</td>
</tr>
<tr>
<td>Total</td>
<td>98</td>
<td>21</td>
<td>119</td>
<td>17.6</td>
</tr>
<tr>
<td>Distribution of secretors and non-secretors in otherwise healthy group</td>
<td>101</td>
<td>17</td>
<td>118</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Correspondence
the amino acid sequences with the predicted HBGA-binding sites. Den Haag, the same clade (2006b) as the six strains in this study, was used as a representative strain. An amino acid change was observed at aa 393 (G or N) in site 2 of T164 and T272 obtained from non-secretors. However, this change was not characteristic for non-secretors, as the same change was observed in secretor strains (T158, T162) and the other 176 GII.4 strains (Lindesmith et al., 2008).

The predicted GII.4 NoV evolving blockade epitopes, i.e. A-E presented by Lindesmith et al. (2012) and shown in Fig. 5(c), were used for comparison of amino acid sequences with the predicted GII.4 NoV evolving blockade epitopes. The amino acid sequences of the epitope sites of T164 and T272 obtained from non-secretors were compared with those of the other strains from three secretors and one outbreak strain in this study, as well as the 176 GII.4 strains reported by Lindesmith et al. (2008) and three representative clade strains (Den Haag 2006b, New Orleans 2009 and Sydney 2012). The neighbouring amino acid residue next to epitope A at 414 (H) was highly conserved in most of the NoV GII.4 strains, such as the 176 strains as well as the representative three clade strains. The amino acid residue at 414 (P) in T164 and T272 of non-secretors was common and unique, but shared only with T158.

There was a strong correlation between the genotype of GII.4 NoVs and secretor status, in which individuals with a homozygous nonsense mutation at nt 428 (i.e. non-secretors) were found in the majority of non-infected Caucasian populations (Thorven et al., 2005; Larsson et al., 2006; Kindberg et al., 2007; Carlsson et al., 2009). This null allele does not exist in most East Asian populations; the majority of weak secretors in Asian populations have a missense mutation at nt 385. However, this homozygous missense mutation in the Japanese population was defined to indicate non-secretor status in this study.

The relationship between secretor status and NoV GII.3 and/or GII.4 infection in an Asian population was reported with contradictory results, i.e. some reports showed a relationship (Tan et al., 2008; Liu et al., 2014), but others did not (Jin et al., 2013). The results of the present study demonstrated that secretor status affected the susceptibility of Japanese individuals to NoV GII.4 infection, but not to NoV GII.3 infection. The observation of a low distribution of non-secretors relative to all patients amongst GII.4 infection cases was of interest.

Therefore, the main structural protein, i.e. the capsid protein, of GII.4 NoV from two non-secretors was analysed in detail. However, no crucial common single amino acid changes were observed in the HBGA-binding sites nor in the putative variant-specific epitopes of these viruses that were responsible for infection of non-secretors. Our hypothesis is that a combination of the unique amino acid variation of the capsid proteins (unique aa 130, 307 and 414 of T164 and aa 205 and 414 of T272) was an important factor in changing the conformational structure of the HBGA-binding site. However, this hypothesis is difficult to demonstrate, because of various limitations, i.e. no suitable HBGA assay of the recombinant virus-like particle using synthetic glycoconjugate with clear results nor stable NoV cell culture system was available. Nevertheless, our findings might suggest the following possibilities. (1) The HBGA-binding site was involved in infection; however, there might be an alternative route of infection or coincidental molecules might exist, as Jones et al. (2014) described ‘human NoV infection of B cells required the presence of HBGA-expressing enteric bacteria’. (2) The infected non-secretors might have had a massive exposure to GII.4 NoVs, leading to symptomatic infection similar to a few cases of foot-and-mouth disease virus infection in humans (Bauer, 1997). Further study would be expected to elucidate the infection mechanisms of NoV GII.4 in the future.

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Abbreviations: DDBJ, Data Bank of Japan; HBGA, histo blood group antigen; NoV, norovirus; RT, reverse transcription.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of norovirus strains NV22101, T158, T162, T164, T169 and T272 are AB665364, AB663884–AB663887 and AB974408, respectively.

Supplementary data and one supplementary figure are available with the online Supplementary Material.

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