Relationship between GII.3 norovirus infections and blood group antigens in young children in Tunisia

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Abstract

Noroviruses (NoVs) constitute a major cause of gastroenteritis in Tunisia. One hundred and fourteen matched saliva and stool samples were collected from children (n=114) suffering from acute gastroenteritis at the hospital of Monastir during the winter season 2011–2012. For 98 of 114 children, blood samples were collected for secretor genotyping. NoVs were associated with 36.8% (n=42/114) of the gastroenteritis cases. The GII.3 genotype was the most common (69% of all NoVs). For patients who were phenotyped (n=114) for human blood group antigens (HBGAs), the secretor and non-secretor phenotypes represented 79% and 21%, respectively. Of the NoV infections, 83% were detected in all ABO groups. Five GII.3 isolates, one GII.1 isolate and one GII.7 isolate were detected in Lewis-positive non-secretors, confirmed by genotyping of the FUT2 gene. Even though our data showed that GII.3 NoVs could infect non-secretors, no binding was observed with saliva and GII.3 baculovirus-expressed virus-like particles from the same symptomatic non-secretor individual. This suggests that other factors might also participate in NoV attachment in children and newborns.

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Introduction

Each year, millions of people suffer from acute gastroenteritis (AGE) worldwide. Rotavirus and norovirus (NoV) are the main causative agents of gastroenteritis in all age groups. Group A rotavirus is the leading cause and human NoVs are the second leading cause of AGE in children aged <5 years. NoVs belong to one of the four genera constituting the Caliciviridae family, and are divided into six genogroups, but only genogroup (GI), GII and GIV infect humans. There are eight and 17 genotypes within GI and GII, respectively, according to VP1 coding sequence analysis [1]. Among NoVs, GII genotype 4 (GIi.4) is globally the most predominant, and can be classified into subtypes or variants [2], which follow on one after the other as a global epidemic strain every 2 or 3 years [3].

The human blood group antigens (HBGAs) are involved in NoV attachment to intestinal cells [4]. Expression of the HBGAs (A, B, H and Lewis antigens) in saliva and on the surface of intestinal cells is driven by the FUT2 (A, B and H antigens) and FUT3 (Lewis antigens) genes, which encode type 2 and type 3 fucosyltransferases, respectively [5]. Lewis antigen expression is also partly dependent on the ABO phenotype, as the FUT3 enzyme elongates H type 1 antigen, which is controlled by the active FUT2 enzyme. Approximately 20% of the Caucasian
population are homozygous for a recessive point mutation of the FUT2 gene, which leads to the absence of A, B and H antigen expression, also called the non-secretor phenotype. There is also a Lewis-negative phenotype due to various mutations of the FUT3 gene, as reviewed previously [6], but the percentage of genotype and allele mutations varies considerably with ethnicity [7,8].

Genetic differences may thus play a role in susceptibility to NoV infection in humans, as non-secretor individuals are not susceptible or are less susceptible than secretors to infection [9]. Indeed, HBGA ligands participate in NoV attachment at the surface of intestinal cells via amino acid residues that directly interact with the sugar moiety [4,10,11]. NoV infection mainly occurs in secretor individuals, among whom blood group O individuals are more likely to be infected by any NoV genotype [9,12–15]. NoV infection, mainly with GII.3 and GII.4, has also been reported in non-secretors, although the number of infected individuals was lower [12,13,16,17]. Nonetheless, susceptibility to NoV infection seems to be much more complex. Recent data have shown that adult non-secretor individuals infected with CagA Helicobacter pylori might become more susceptible because of abnormal expression of fucosylated motifs at the surface of intestinal cells, while it is absent from saliva [18]. Additionally, it has been shown that enteric bacteria from unfiltered stools, such as H antigen-expressing Enterobacter cloacae, promote in vitro infection of B-cells by human NoV [19].

A recent epidemiological survey of children in the Monastir region (Tunisia) allowed us to determine whether NoV paediatric infections were linked to secretor status and HBGAs. The observations regarding NoVs were further supported by the analysis of saliva from patients and the use of baculovirus-expressed synthetic particles from a strain that was isolated during the study in a non-secretor patient.

Materials and methods

Study design

One hundred and fourteen individual stool and saliva samples were collected from children aged <6 years who consulted for AGE at the Children’s Hospital of Monastir (Tunisia) from January 2011 to August 2012. At the onset of the disease, blood samples were also collected from 98 of these 114 patients. The study was approved by the Ethics Committee of the Fattouma Bourguiba Public Hospital at Monastir (Tunisia) (committee decision of 9 May 2013), and informed consent was obtained from the parents of the 114 children. Blood and saliva samples were taken in the presence of the parents.

NoV detection and typing

Viruses were screened for and characterized at the National Reference Centre for Enteric Viruses in Dijon, France. For each patient, 800 μL of a 10% stool suspension was prepared in phosphate-buffered saline (PBS) before extraction of nucleic acids with a Nuclisens Easy MAG system (bioMérieux, Marcy l’Etoile, France), according to the manufacturer’s instructions. RNA was eluted in a final volume of 110 μL.

Clinical specimens were first screened by real-time RT-PCR with Taqman technology. Five microlitres of RNA was used for the real-time RT-PCR assay, which was performed with an ABI Prism 7500 sequence detection system (Life Technologies, Saint Aubin, France), according to the manufacturer’s instructions. For GI NoVs, the jJIV1F/jJIV1R primer pair and jJIV1P and RING-1b probes were used [20]. For GII NoVs, the QNIF2d and COG2R primers were combined with the QNIF2 probe as described previously [21]. Samples giving Ct values of >40 were considered to be negative. The samples that were positive by real-time RT-PCR were then genotyped for the polymerase region with the JV12/JV13 primer pair for the polymerase [22]. For the capsid region, the G1SKF/G1SKR and G2SKF/G2SKR primer pairs were used for the amplification of GI and GII NoVs, respectively [23]. Following sequencing of the PCR products, the NoV genotypes were determined by alignments with reference sequences from GenBank by the use of MEGAS software [24].

Cloning of the GII.3 isolate, and production and purification of baculovirus-expressed virus-like particles (VLPs)

A GII.3 NoV isolate (SW4 strain) from a non-secretor Lewis-positive individual from the study was further characterized and cloned into a baculovirus system to produce VLPs for the saliva binding assay. The FW1/RT5 (Table S1) primer pair was used to amplify open reading frame (ORF)2, with the same PCR reagents and amplification conditions as described previously [25]. PCR products were cloned into the pGEM-T easy vector to generate a pGII.3-pGEM construct, which was later sequenced with primers FW1–FW5 and RT1–RT5 (Table S1). pGII.3-pGEM was used as the template to amplify ORF2 with the forward primer 5′-GAAGATCTATGAAGATGGCGTCGAATGACGC-3′, which contains a BglII site at the 5′ end, (underlined), and RT5 as the reverse primer, which overlaps with ORF2 and ORF3 at the 3′ and 5′ ends, respectively. Amplified ORF2 with the BglII site was gel-purified and cloned into pGEM-T easy. One positive clone was digested with BglII and NotI and purified from gel before being cloned into transfer vector pVL1392 (BD Bioscience, Le Pont de Claix, France), which was entirely sequenced to determine the presence of any inadvertent mutations. The recombinant baculovirus was obtained by co-transfection of the pVL vector and the
linearized baculovirus genome into Sf9 cells, according to the manufacturer’s recommendations (BD Bioscience). Following transfection, 20 clones were selected after plaque assay by ELISA with an in-house rabbit polyclonal antibody raised against GI.3. The recombinant baculovirus showing the highest optical density by ELISA was selected, and was cloned a second time by plaque assay. Thereafter, the inoculum of the selected recombinant baculovirus was amplified and titrated by infecting Sf9 at a multiplicity of infection of 0.1. The Sf9-produced inoculum was then used to infect Hi-5 cells (Life Technologies) at a multiplicity of infection of 5 with serum-free Express Five medium (Life Technologies). The cells were incubated for 6 days at 27°C prior to concentration and purification of the recombinant VLPs from infected cell lysates, as described previously [26,27].

Saliva typing for carbohydrates
Saliva samples were screened for the presence of A, B and O blood group and Lewis antigens. Saliva was boiled for 10 min, and then centrifuged at 3000 g for 2 min and then at 10 000 g for 10 min to pellet debris. The saliva samples were diluted 1000-fold in carbonate/bicarbonate buffer at pH 9.6, and incubated overnight at 37°C on an ELISA plate. On the following day, the plates were washed three times with PBS (pH 7.4) containing 0.05% Tween 20% (PBS-T). Non-specific sites were blocked with 4% skimmed milk–PBS for 2 h at 37°C, and then washed three times with PBS-T. A 4% skimmed milk–PBS solution was also used for the dilution of primary and secondary antibodies in a final volume of 100 μL per well. Anti-A and anti-B mouse IgG antibodies (Diagast, Loos, France) were diluted 5000-fold, and anti-H-specific IgM (Thermo Fisher Scientific, Villebon sur Yvette, France) was diluted 1000-fold. Supernatants from anti-Lewis a (clone 7LE) and anti-Lewis b (clone 2-25LE) hybridoma supernatants were diluted 1000-fold. 7LE and 2-25LE supernatants were kindly provided by J. Bara. The primary antibodies were incubated for 1 h (A, B and O blood group antigens) and 1.5 h (Lewis antigens) at 37°C. After three washes with PBS-T, 5000-fold-diluted peroxidase-conjugated anti-mouse antibodies (Vector/ABCYS, Paris, France) were incubated for 30 min at 37°C for the detection of anti-A-specific, anti-B-specific and Lewis-specific IgG. For the detection of anti-H-specific IgM, a 1000-fold dilution of peroxidase-conjugated goat anti-mouse IgM antibodies (Southern Biotech/Clinsciences, Nanterre, France) was incubated for 45 min at 37°C. The plates were then washed four times with PBS-T. Peroxidase activity was detected with 100 μL of 3,3′,5,5′-tetramethyl benzidine (KPL/Eurobio, Courtaboeuf, France). The reaction was stopped after 10 min of incubation at room temperature with 50 μL of 2 M HCl prior to reading of the absorbance at 450 nm.

Genotyping for the FUT2 phenotypes
The FUT2 genotype was determined in the individuals from whom we had obtained blood samples (n = 98). DNA was extracted from a 50-μL sample of whole blood with the QIAamp Blood DNA extraction kit (Qiagen, Les Ulis, France), according to the manufacturer’s recommendations. The FUT2 genotypes were determined by partial sequencing of the FUT2 gene as described previously [28].

Saliva binding assay with VLPs
For GI.3 VLP binding, a preliminary assay showed that only a weak binding signal was obtained with 1000-fold-diluted saliva, and 100-fold-diluted saliva was therefore used for the assay. Five hundred nanograms of purified GI.3 VLPs was used for the assay. Bound VLPs were detected by the use of rabbit polyclonal GI.3-specific serum for 1 h at 37°C. Peroxidase-conjugated anti-rabbit antibodies were incubated for 30 min at 37°C. Peroxidase activity was detected with 3,3′,5,5′-tetramethyl benzidine. The reaction was stopped after 20 min of incubation at room temperature with 2 M HCl prior to reading of the absorbance at 450 nm. Values below an optical density of 0.1 were arbitrarily considered to be negative.

Statistical analysis
StataCorp statistical software (StataCorp LP, College Station, TX, USA) was used for the statistical analyses. To determine whether NoV infected children at different ages, univariate analysis was performed with the Pearson chi-square test and Fisher’s exact test. To determine whether NoV and GI.3 NoV infections were preferentially associated with ABO and Lewis antigens or secretor and non-secretor genotypes, multivariate analysis was conducted. Because of the small sample size, logistic regression models with a robust variance estimator (Huber/White/sandwich) or exact logistic regression models were used [29,30]. Variables with a significance level of p <0.2 were entered into the multivariate model. The final threshold for significance was set at p <0.05.

Results

Epidemiology and clinical features
One hundred and fourteen stool samples collected between January 2011 and July 2012 were screened for the presence of NoV. Eighty per cent of the samples were collected during the cold season from September 2011 to March 2012 (Table S2). Apart from one child aged 6 years, all of the children treated at the paediatric hospital were aged <24 months, and 80% were aged ≤12 months. On average, there were six diarrhoeal episodes per day at the peak of the disease. After diarrhoea, the
most common symptoms were fever (n = 79), vomiting (n = 56, mean of 4 episodes/day), and dehydration (n = 20). Dehydration was mild (n = 8) or moderate (n = 12), with an average severity score between 4 and 5. No cases of severe dehydration were reported during the course of the study. NoVs were detected in 42 specimens. Thirty-five of the 42 NoV-positive samples were collected during the cold season (September 2011 to March 2012). Other NoV-positive samples were collected in January 2011 (n = 3) and March 2011 (n = 3). It is of note that one sample from August 2012 was also positive. The average age of infants infected with NoV was 7.8 months. Eighty percent of the infections occurred in infants aged <10 months. Forty-two clinical specimens were positive for NoVs (Table 1). GII.3 was predominant, accounting for 69% of NoV cases. GII.3 isolates were detected in children aged between 2 and 14 months. The 29 GII.3 isolates belonged to the GII.21pol genotype in the polymerase region. Five GII.4 isolates were detected, and belonged to 2010 (n = 2) and 2012 (n = 3) variants (n = 3). GII.7 isolates were detected in two clinical specimens, and were genotyped as GII.21pol in the polymerase region.

**TABLE 2. Distribution of ABO blood groups, Lewis antigens and secretor status among the patients from the cohort**

<table>
<thead>
<tr>
<th>Lewisa</th>
<th>O</th>
<th>A</th>
<th>B</th>
<th>ABb</th>
<th>Non-secretors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype (n = 114)</td>
<td>37</td>
<td>1</td>
<td>26</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Genotype (n = 98)</td>
<td>Se&quot;/Se&quot;</td>
<td>6</td>
<td>1</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Se&quot;/Se</td>
<td>25</td>
<td>0</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Se&quot;/Se</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

aIndividuals were positive for the presence of A and B antigens.
bSaliva samples were phenotyped for the presence of Lewis a and b antigens.

A hundred and fourteen saliva samples corresponding to the 114 faecal specimens were phenotyped by ELISA for the presence of ABO HBGAs and Lewis antigens (Table 2). A, B and H blood group antigens were detected in 78.9% of saliva samples (n = 90). For 24 samples, the presence of ABH blood group antigens was below the detection threshold. Among the 90 ABO-positive samples, 38 were O, 27 were A, 19 were B, and six were strongly positive for A and B, which characterizes the AB group. The Lewis antigen was detected in 109 individuals. Thirty-seven, 26 and 16 saliva samples were also positive for the Lewis antigen in O, A and B individuals, respectively. The six AB individuals were all positive for the Lewis antigen. For five individuals of the O (n = 1), A (n = 1) and B (n = 3) types, representing 4.4% of the cohort, the Lewis antigen was not detected in the saliva samples. The strong reactivity of these 24 samples against the Lewis a-specific monoclonal antibody rather than against a monoclonal antibody raised against the Lewis b antigen suggested that the 24 individuals had a non-secretor phenotype (Fig. S1). To confirm this hypothesis, the FUT2 secretor phenotype was determined by genotyping the FUT2 gene by sequencing and partial sequencing of the FUT2 gene for 98 patients, including 22 of 24 individuals for whom saliva samples were Lewis a positive and ABO negative. We found that these 22 individuals, representing 22.45% of the study population, had a non-secretor genotype and possessed the mutation G428A. None of the samples showed the A385T mutation. For the other patients with positive saliva test results for either A, B or H antigens, 76 blood samples were available. Genotyping confirmed that they all had a secretor genotype. Among these 76 secretors, 23 (23.47% of the study population) were homozygous (Se"/Se") for the FUT2 gene, whereas 53 (54.08% of the study population) were heterozygous (Se"/Se). The age distribution for the HBGA phenotype is shown in Fig. S2.

**TABLE 1. Distribution of norovirus genotypes according to age group**

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>GII.1</th>
<th>GII.3</th>
<th>GII.4</th>
<th>GII.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2–4</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4–6</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6–8</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8–10</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10–12</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12–14</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>14–16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16–18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18–20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20–22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>22–24</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>&gt;24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>29</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

aVariant 2012 GII.4.
bVariant 2010 GII.4.
Presence of NoVs and the FUT2 phenotype

To determine the relationship between the secretor status and the presence of NoV, paired faecal samples and blood samples, in addition to saliva samples, were analysed in 35 NoV-infected patients. The NoVs infected homozygous and heterozygous secretor individuals equally. However, for GII.3 NoV, the majority of the infections occurred in heterozygous secretor individuals (18 of the 29 infections; Table 3).

Distribution of HBGAs in NoV-infected patients

NoVs were detected in secretor and non-secretor patients (Table 4), and all but two of these patients were Lewis positive. The NoVs infected homozygous and heterozygous secretor individuals equally. However, for GII.3 NoV, the majority of the infections occurred in heterozygous secretor individuals (18 of the 29 infections; Table 3).

GII.3 SW4 strain binding profile of saliva

GII.3 NoV was detected in five stool samples from Lewis-positive non-secretor patients. To further characterize NoVs that infected non-secretor patients, the GII.3 SW4 strain was selected from a Lewis-positive non-secretor patient for whom we had characterized stool, saliva and blood samples. The complete nucleic acid sequence of the SW4 ORF2 (Genbank KM056394) showed 98% nucleic acid identity with the GII.3 ‘693/425’ paediatric strain from Australia (Genbank number KC464328) [31]. The VLPs were purified to >90% homogeneity following production and concentration from insect cells. The origin was confirmed with NoV-specific polyclonal serum (Fig. S3).

GII.3 SW4 VLPs were assayed on the 114 saliva samples collected (Fig. 1), and were found to bind to A, B and AB Lewis-positive saliva. The magnitude of the binding varied greatly from person to person. No binding was observed for the Lewis-negative O, A and B saliva (n = 5). Residual binding to no binding was observed for O and non-secretor saliva. For the patient who provided samples from which the GII.3 VLPs were produced, no binding was observed with 100-fold-diluted and ten-fold-diluted saliva (data not shown), whereas a strong response for the presence of the Lewis a antigen was obtained with 1000-fold-diluted saliva.

Discussion

A recent report from Tunisia showed that NoV affected 7.7% and 10.5% of non-hospitalized and hospitalized patients, respectively, between 2007 and 2010 [32]. GII NoVs were predominant over GI NoVs. Among GII NoVs, the GII.3 genotype became predominant during the winter of 2009–2010.

Our data confirm that GII.3 NoVs mostly circulate in the paediatric population, as reviewed previously [33]. One hypothesis to explain why GII.3 NoVs were associated with paediatric cases is that the intestinal tract is mostly immature during the first years of life, and might provide a favourable environment for opportunistic infection by GII.3 NoVs.

As in previous reports, we observed that NoV infection mostly occurred in secretor individuals [12,13,34]. We observed that GII.3 and GII.7 isolates also affected Lewis-positive non-secretor individuals, as reported previously [12,17,35], whereas NoV infection has never been reported in Lewis-negative non-secretor individuals. This suggests that the
Lewis antigen might play an essential role in NoV recognition by intestinal cells in non-secretor individuals. Nonetheless, VLPs from GII.3 NoV isolated from a chronically infected patient, and called the Chron1 strain, could not bind a panel of non-secretor saliva samples [36]. Our study allowed us to characterize and to clone a GII.3 NoV strain isolated from a symptomatic non-secretor Lewis-positive patient. Our first objective was to determine whether this strain isolated from this non-secretor individual could bind the patient’s own saliva and saliva from other non-secretors. As the VLPs did not bind non-secretor saliva, including the saliva of the patient from whom the isolate came, we formulated several hypotheses. We first hypothesized that there was no binding because HBGAs were absent from the saliva sample. This was very unlikely, as we observed strong binding to saliva for GII.4 VLPs (Fig. S4), and a marked signal was observed by ELISA for the presence of Lewis a antigen during phenotyping (Fig. S1). The second hypothesis was that the absence of binding could be due to an artefact. Indeed, it is possible that no binding was observed because the VLPs were not structurally sound. However, as the GII.3 VLPs did bind with A and B saliva from secretor individuals, we concluded that the GII.3 VLP structure was correct. It is of note that we successfully used SW4 GII.3 VLPs in binding experiments with duodenum tissue from secretor individuals (Fig. S5), again demonstrating that the VLP conformation was correct. Binding for GII3 VLPs, however, was markedly lower than that for GII.4 VLPs, as described previously [36,37] (Fig. S4). We then hypothesized that the presence of HBGAs in saliva did not reflect the ABO status at the intestinal level. This is very unlikely, because HBGAs are present in the intestine at birth, and persist for the first 2 years of life [38]. Moreover, Lewis, A and H antigen expression is complete in saliva at birth for term infants, whereas it is delayed in red blood cells [39]. Finally, in the event that the Lewis antigen does not participate in the attachment of NoV for non-secretor individuals, we hypothesized that non-HBGA ligands or precursor carbohydrates could constitute an alternative attachment pathway for NoVs during the first months of life.

Our data suggested that the HBGAs present in the saliva from young non-secretor individuals did not reflect the binding

![Saliva binding assays for the GII.3 SW strain.](image)

**FIG. 1.** Saliva binding assays for the GII.3 SW strain. The value for each sample is represented by a bar on the graph. Binding values for patients infected with GII.3 and non-GII.3 noroviruses are indicated by red and blue bars, respectively. An arrow indicates the binding value of the patient for whom the GII.3 isolate found in the stool specimens was used for baculovirus-expressed virus-like particle production. The binding experiments were performed in duplicate for each sample, and the mean values are given on the graph (optical density (OD)450 nm; ordinate). The Lewis status is indicated at the top of the graph by minus (absence of Lewis antigen) and plus (presence of Lewis antigen) signs. The non-secretor and human blood group antigens are also indicated on the abscissa, and are separated by vertical dashed lines. The thick horizontal dashed line indicates the threshold below which the binding assay was arbitrarily considered to be negative.
capacity of NoV at the surface of intestinal cells. Other types of ligand might play a role during NoV infection. A recent study provided evidence of this, by showing that previous infections with CagA-positive \textit{H. pylori} might interfere with HBGA expression [18]. Infection with \textit{H. pylori} is, however, very unlikely in the first years of life. Nonetheless, immature carbohydrate-like type 1 chain precursors and non-HBGA ligands might contribute to the increased susceptibility of a large proportion of children to NoV infections. Recent findings have also suggested that certain bacteria (e.g. \textit{E. cloacae}) from the microbiota might participate in NoV infection [19]. These findings support the notion that genetic and microbiological factors are at play during NoV infection. Synergistic and antagonistic effects remain to be determined in future studies.

**Transparency declaration**

The authors declare that they have no conflicts of interest.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.cmi.2015.05.015.

**References**


