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## Research paper

## Occurrence of norovirus infection in an asymptomatic population in Indonesia



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## ABSTRACT

35  
 Norovirus (NoV) is a major cause of nonbacterial acute gastroenteritis worldwide in all age groups, and asymptomatic individuals may contribute to NoV transmission as a reservoir. Nonetheless, little information is available regarding asymptomatic NoV infection in Indonesia. We performed an epidemiological analysis of NoV infection among asymptomatic healthy volunteers in the city of Surabaya, Indonesia (population ~2.75 million). A total of 512 stool samples from 18 individuals (age range 20–42 years) collected from July 2015 to June 2016 were examined. The detection of NoV and the genotype classification were carried out by a reverse transcription-polymerase chain reaction (RT-PCR) direct sequencing method. NoV was detected in 14 of the 512 stool samples (2.7%), with 7 individuals (38.9%) having at least 1 positive stool sample. All 14 of the NoV strains detected belonged to genogroup GII. The phylogenetic analysis indicated that 10 strains (71.4%) were grouped with GII.2, 2 (14.3%) were GII.17, 1 was GII.4 Sydney 2012, and 1 was GII.1. The circulation of GII.Pg/GII.1 and GII.Pe/GII.4 Sydney 2012 recombinant variants was detected among an asymptomatic population in Surabaya, Indonesia. Of the 7 positive individuals, 2 were repeatedly infected with the same strain and heterogenous strains. Taken together, our results suggest that the excretion of NoV from healthy individuals is one of the sources of NoV outbreak.

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1. Introduction

Norovirus (NoV) is considered a leading cause of both sporadic cases and outbreaks of nonbacterial gastroenteritis in all age groups (Bon et al., 2005; Khamrin et al., 2016; Zheng et al., 2010), and is responsible for 1.45 million deaths worldwide every year (Ahmed et al., 2014). Moreover, diarrhea, the most common symptom of gastroenteritis, remains a major cause of morbidity and mortality in all age groups in Southeast Asia. Diarrhea is the third leading cause of overall morbidity and the leading cause of infant mortality in Indonesia (Agtini et al., 2005). However, whether NoV is a major cause of diarrhea in Indonesia or, more generally, Southeast Asia, remains uncertain due to the dearth of epidemiological data.

NoV is a positive-sense, single-stranded, non-enveloped RNA virus that belongs to the family *Caliciviridae* (Jiang et al., 1993). The NoVs

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 are classified into 7 genogroups (GI to GVII) based on the complete capsid protein VP1 sequences. The GI and GII genogroups are further classified into 9 and 22 genotypes, respectively (Vinjé, 2015).

NoV is highly contagious and can be transmitted in various modes including contact with contagious individuals, contaminated environments, and the consumption of contaminated foods and even ice (Parashar et al., 1998). The personal hygiene practices of infected food handlers are considered to be the most important contributing factor in the spread of foodborne diseases (Koopmans and Duizer, 2004).

Several studies have shown that asymptomatic NoV infections are relatively common within different populations (García et al., 2006). Data from Western countries and Japan have demonstrated that NoVs are responsible for 5%–30% of NoV asymptomatic infection in adults (Centers for Disease Control and Prevention, 2011; Ozawa et al., 2007), and that asymptomatic persons can spread the virus although their viral

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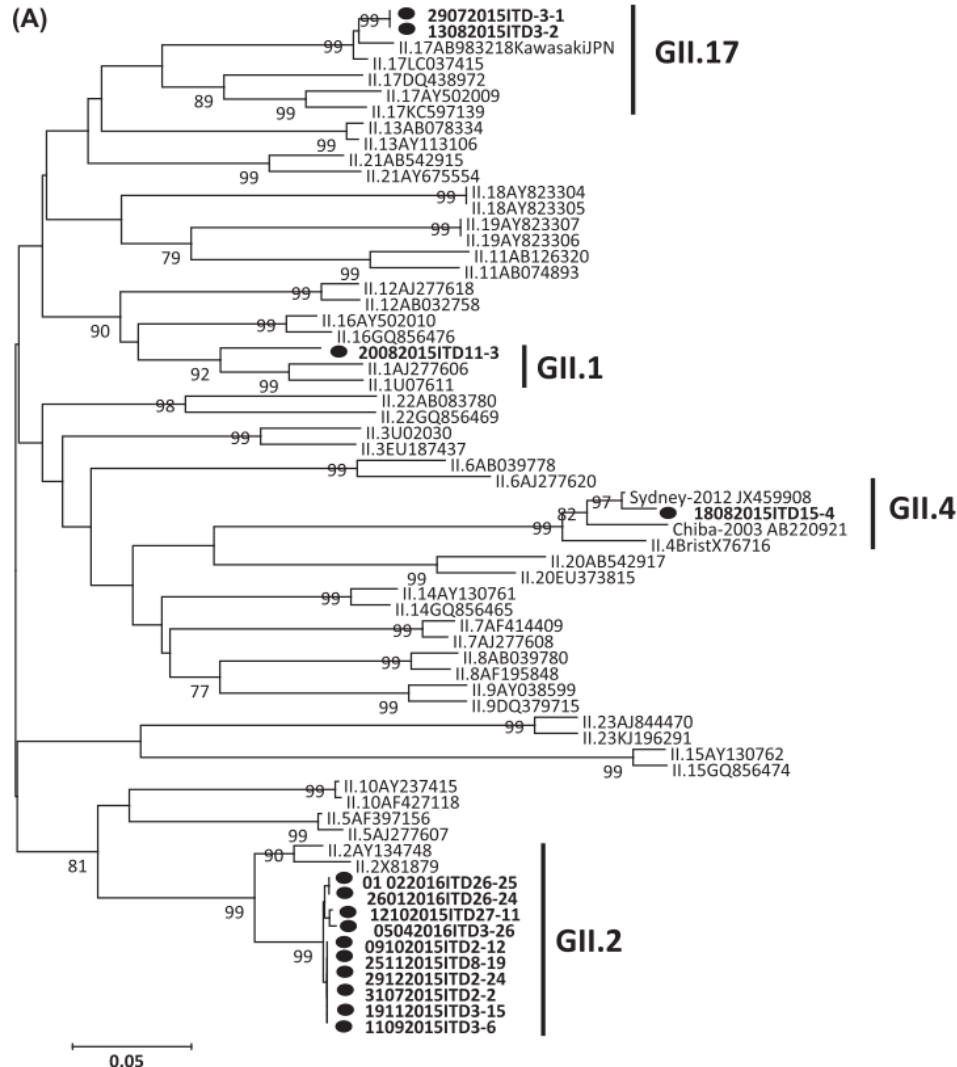
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**Fig. 2.** The phylogenetic tree constructed based on partial sequences of the capsid gene (A) and the polymerase gene (B) of NoV isolated in asymptomatic Indonesian adults. The tree was constructed using the neighbor-joining method incorporated in MEGA 4.0 software. Bootstrap values (> 70) are shown at the branch nodes. The NoV strains detected in the present study are shown in bold font. The nucleotide sequences of the 14 strains were deposited in the GenBank database under accession nos. LC259504 to LC259512, LC259514–LC259517, MF668936, MF668937.

requested to provide their weekly stool samples.

## 2.2. Sample collection

We conducted a 1-year longitudinal prospective study from July 2015 to June 2016. The inclusion criterion was the absence of any gastroenteritis symptoms within the month preceding enrollment. A total of 18 individuals (age range: 20–42 years; 6 males and 12 females) were enrolled in this study, and were prospectively asked to provide their stool samples on a weekly basis for up to one year, provided they continued to experience no symptoms of gastroenteritis (diarrhea, vomiting, weakness, fever). Some participants, however, were unable to provide weekly stool samples consistently for the entire sampling period; the data for these individuals was not included in the analysis. Demographic data (age, sex), source of water, traditional toilet use and educational background were recorded at the enrolment. All of the stool samples were collected at the participant's home, stored in

provided containers, delivered immediately from their homes to specified, air-conditioned sites at the research institution, and immediately stored in a deep freezer at  $-80\text{ }^{\circ}\text{C}$  until tested. A total of 512 stool samples were collected from 18 participants. The study protocol was reviewed and approved by the Ethics Committees of Airlangga University in Indonesia and the Kobe University Graduate School of Medicine in Japan, and all participants signed an informed consent. Asymptomatic infection was defined as the presence of NoV in the stool without any clinical symptoms (diarrhea, vomiting, weakness, fever).

## 2.3. RT-PCR using a commercial kit

All 512 samples collected were examined for the presence of NoV using a One-step RT-PCR Norovirus G1 & G2 Detection Kit ver. 2 (Shimadzu, Kyoto, Japan). A 10% (w/v) stool suspension of each sample in distilled water was prepared by centrifuging at  $21,130 \times g$  for 10 min. The supernatant (1  $\mu\text{l}$ ) from the 10% stool suspension was

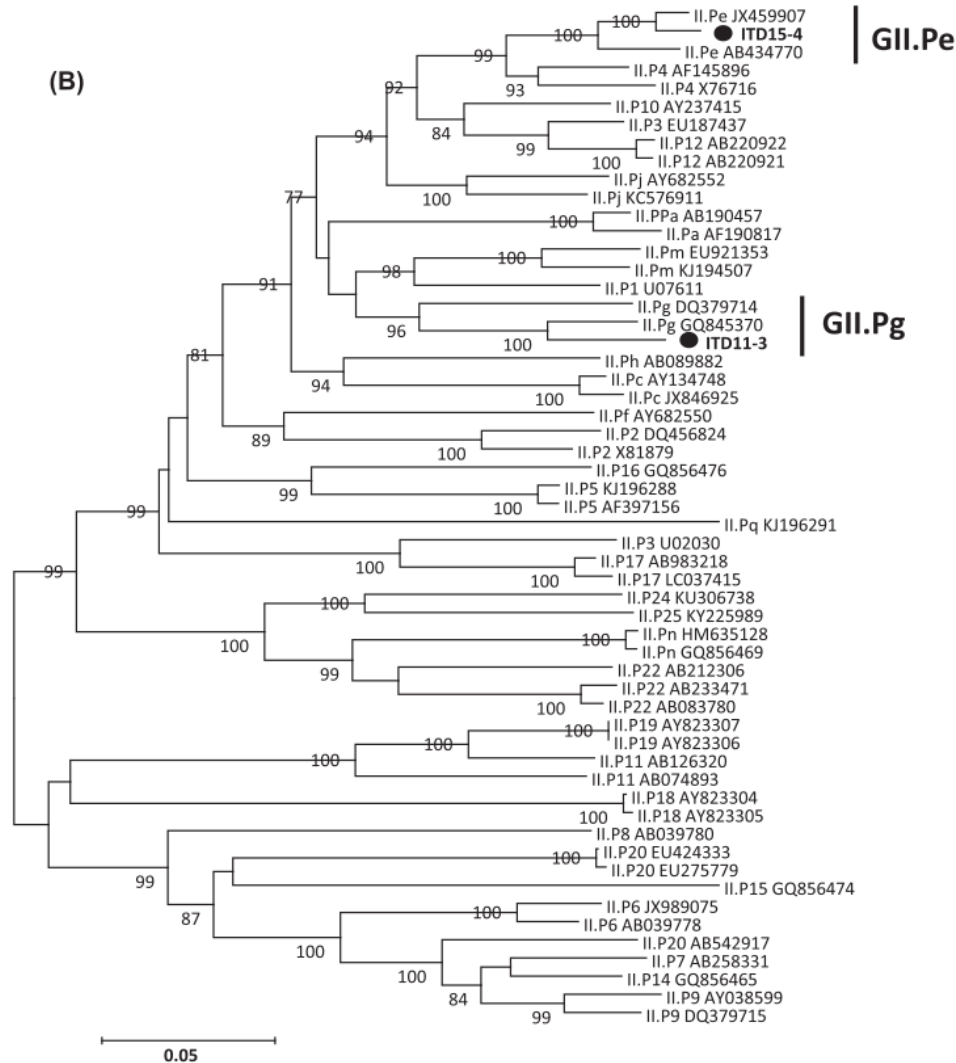


Fig. 2. (continued)

mixed with 19  $\mu$ l of Sample Treatment Reagent from a One-step RT-PCR Norovirus G1 & G2 Detection Kit ver. 2 (Shimadzu, Kyoto, Japan), treated at 90  $^{\circ}$ C for 5 min and stored on ice until further processing. The RT-PCR was carried out according to the manufacturer's instructions. The amplified product specific to the target had an expected size of 85 or 98 base pairs (bp) for G1 or G2, and the amplified product specific to the internal control had an expected size of 251 bp. The supernatant was stored at  $-20^{\circ}$ C until further processing.

#### 2.4. Conventional RT-PCR

Sixteen samples that were determined to be positive using the One-step RT-PCR Norovirus G1 & G2 Detection Kit ver. 2 were then prepared for conventional RT-PCR to confirm NoV RNA and sequencing. Viral RNA was extracted from 140  $\mu$ l of supernatant with a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was eluted with 60  $\mu$ l of RNase- and DNase-free water and stored at  $-80^{\circ}$ C.

We used the previously published primer pair of G1SKF/R and G1F1/R1 and the published primer pair of G2SKF/R and G2F1/R1 for

the detection of NoV GI and GII in the capsid gene, respectively (Kojima et al., 2002). In order to investigate the presence of recombination events of NoV GII, primers were newly designed in the polymerase gene for 1st PCR (ITD15-4158S-PCR1S: 5'-AGGTGCRCTTAAAGATGAG-3'/ITD15-5376A-PCR1A: 5'-CTCTCCACCAGGGCCTTGAC-3') and for 2nd PCR (ITD15-4321S-PCR2S: 5'-GTTGGTATGAACATGAATG-3'/ITD15-5268A-PCR2A: 5'-AACCTCATTGTTGACCTCTG-3'). The RT reaction was performed at 65  $^{\circ}$ C for 3 min, followed by 40  $^{\circ}$ C for 1 h using Superscript III reverse transcriptase (Invitrogen, New York, NY) and random primers (Takara Bio, Kyoto, Japan) for the capsid gene and a newly designed primer (ITD15-5415A-RT: 5'-ATAGTATTTACCTGGAGCG-3') for the polymerase gene. We found that 14 samples were positive for NoV GII by RT-PCR, while neither GI nor GII NoV was detected in two samples in the capsid gene. Out of these 14 samples, the polymerase genes from 2 samples were successfully sequenced. ITD11-3 was also confirmed by next-generation sequencing (NGS) using a MiSeq sequencer (Illumina, San Diego, CA). The amplified PCR products were stored at  $-20^{\circ}$ C.



## 2.5. Sequencing and phylogenetic analyses

The sequences of the 14 NoV amplicons in the capsid gene and 2 amplicons in the polymerase gene were determined directly from the PCR product with a BigDye terminator cycle sequencing kit using an Applied Biosystems 3500XL Genetic Analyzer (Applied Biosystems, Foster, CA). Phylogenetic analyses were performed based on approx. 270-bp nucleic acid sequences of the amplified NoV capsid region and 922-bp sequences of the polymerase region. Reference sequences were retrieved from the DNA Data Bank of the Japan/European Molecular Biology Laboratory/GenBank database. Alignments were performed using CLUSTAL X software, and phylogenetic trees were constructed by the neighbor-joining method. To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times using Molecular Evolutionary Genetic Analysis (MEGA4) software (<http://www.megasoftware.net>).

Finally, the capsid regions of 14 strains and the polymerase regions of 2 strains from the NoV-positive samples in this study were successfully sequenced.

## 2.6. Determination of the NoV RNA viral load

The viral load of 14 NoV samples in the capsid region was assessed by real-time PCR using an ABI 7300 real-time PCR system (Applied Biosystems). Twenty-five microliter of the reaction mixture contained 2.5  $\mu$ l of cDNA, 12.5  $\mu$ l of TaqMan Universal PCR Master Mix (Applied Biosystems), 100 pmol forward (COG2F, ALPF) and 100 pmol reverse (COG2R) primers, and 4 pmol of RING2AL-TP probe for NoV GII detection.

The PCR amplification was performed under the following conditions: incubation at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, and then 45 cycles of amplification with denaturation at 95 °C for 15 s and annealing and extension at 56 °C for 1 min. The quantitation of NoV GII was achieved based on the standard curve generated from the NoV GII standard plasmid provided by Japan's National Institute of Infectious Disease (NIID), Tokyo. The standard curve was established by using a 10-fold dilution series ( $1 \times 10^8$  to  $1 \times 10^0$  copies) (Khamrin et al., 2016; Kageyama et al., 2003).

## 2.7. Statistical analysis

Statistical analysis was performed by Fisher's exact test for categorical variables using SPSS software. The following parameters were compared between the NoV-positive and -negative groups: sex, use of western style toilets, and educational background (higher education or not).

## 3. Results

### 3.1. NoV-positive population

During the study period, 18 asymptomatic participants were enrolled, and 512 samples were collected among them. NoV was detected in 14 stool samples from 7 participants. The NoV-positive individuals are shown in Table 1. No significant differences were found in the relation between NoV positivity and sex ( $P = 0.36$ ), western toilet use ( $P = 0.36$ ), or educational background ( $P = 0.64$ ) of the participants. All of them drank bottled or boiled water.

### 3.2. Prevalence and characterization of NoVs

The overall NoV detection rate was 2.7%, with 14 of 512 samples being found positive. A total of 7 of the 18 subjects (38.9%) had at least 1 NoV-positive stool during the study period. NoV prevalence was highest in July, followed in order by April, August, and November, while the remaining months were associated with relatively low levels

of infection. No infection was found in March, May and June (Fig. 1). Among the 7 subjects with NoV-positive samples, 2 subjects (ITD-2 and ITD-3) (28.6%) had reinfection with the same strain and heterologous strains (Table 1). Two other subjects had two consecutive positive samples with the same strains (ITD3-1 and ITD3-2; ITD26-24 and ITD26-25). When we calculated the incidence, we considered these samples as a single infection. The intervals of each reinfection were approx. 1–4 months (Table 1).

Four subjects (ITD8, ITD11, ITD15 and ITD27) had a single NoV-positive sample. Our data demonstrated that NoV-asymptomatic infection was most prevalent in July (13.3%), but there was no distinct seasonal peak in this study (Fig. 1). The viral loads of the NoV-positive samples were mostly low, except for the subjects ITD11-3 ( $1 \times 10^5$  copies/g), ITD2-12 ( $1 \times 10^3$  copies/g), ITD3-6 ( $1 \times 10^4$  copies/g) and ITD3-15 ( $1 \times 10^2$  copies/g) (Table 1).

### 3.3. Phylogenetic analysis of the NoV strains

Phylogenetic analysis of the 14 NoV strains was performed by amplification of the capsid regions, which had an average sequence length of 270 nucleotides (nt), allowing successful genotyping. All 14 strains originated from healthy subjects, and all belonged to genogroup II (GII). Ten (71.4%) of the GII strains were assigned to GII.2, 2 (14.3%) to GII.17, 1 to GII.4 Sydney 2012, and 1 to GII.1 (Fig. 2A).

The phylogenetic analyses of the 2 NoV strains were performed by amplification of the polymerase regions, which had an average sequence length of 922 nucleotides (nt), allowing successful genotyping. The polymerase region of one strain (ITD11-3) was assigned to GII.Pg and the capsid was assigned to GII.1. The polymerase region of the other strain (ITD15-4) was assigned to GII.Pe and the capsid was assigned to GII.4 Sydney 2012 (Fig. 2).

## 4. Discussion

Our present findings demonstrated that NoVs were commonly detected in stool samples collected throughout the year from asymptomatic individuals in Surabaya, Indonesia. Of the 18 healthy individuals examined in non-outbreak settings throughout the year, 38.9% showed the presence of NoVs. In the only previous study of NoV infections in Indonesia, NoV was not found in the control subjects (D. Subekti et al., 2002b). The prevalence of asymptomatic NoV infection in our present investigation was higher than those reported in non-outbreak settings: 11.9% of a series of food handlers in Japan (Okabayashi et al., 2008), and 1.02%–2.3% in food handlers in Korea (Jeong et al., 2013; Koo et al., 2016) had asymptomatic NoV infection. However, a study performed in a small community in southwestern Cameroon found high asymptomatic NoV prevalences of 27% in adults and 30.8% in children (Ayukekbong et al., 2014a). NoV infection is less prevalent among food handlers than among other workers, because food handlers tend to be well educated on hygiene practices and to maintain their health in good condition in order to avoid transmitting infectious diseases to others. In addition, one of the causes of the high prevalence of NoV infection in this study or in the community in Cameroon in the previous report may have been that diarrhea is endemic in both Indonesia and Cameroon (Gorham et al., 2017).

NoV infections are considered common in the winter season in temperate regions (Dey et al., 2010; Glass et al., 2009), while in tropical regions they are relatively common in the rainy season (Ayukekbong et al., 2014b). Our findings of a major peak of NoV infection in July (dry season) were not consistent with the data previously reported, suggesting that climate does not play a major role in NoV prevalence, at least in the Indonesian population in this study.

In the present study, 2 of the 7 asymptomatic subjects (ITD-2 and ITD-3) were reinfected with NoVs. We considered that the reinfections of these 2 asymptomatic individuals proceeded as follows. Subject ITD-2 shed NoVs three times with the same strain, genogroup II genotype 2

(GII.2). Subject ITD-3 shed NoVs four times with the same strain (GII.2 and GII.17) or heterologous strains (GII.2) within a year. The intervals (considered to be the immunity periods) between infections varied from approx. 1 to 4 months, which was shorter than the ranges of 6 months to 2 years (Simmons et al., 2013) and approx. 2 months to 2 years (Parrino et al., 1977) previously reported for symptomatic cases.

Considering that our population of subjects was limited to asymptomatic individuals, it is possible that antibodies against NoV may not achieve virus neutralization. Additionally, asymptomatic infections may result from acquired immunity that is protective against diseases but does not block infection (Lopman et al., 2014). Immunity to NoV lasts for at least 1 year after symptomatic infection, demonstrating that symptomatic infections strongly stimulate immune responses (Camona-Vicente et al., 2016). We speculate that the asymptomatic subjects in our present study were re-infected less than a year. However, the exact duration of the immune protection remains uncertain, since the study was carried out only for one year.

The interval of NoV excretions between the first and the second infection was 15 days for the subject ITD-3 and 6 days for the subject ITD-26, whereas a previous study demonstrated that adults infected with NoV excreted NoV in their stool samples for up to 22 days (Rockx et al., 2002).

In the present study, all NoV strains turned out to be genotype GII, which is consistent with reported genotypes among the patients with gastroenteritis. Several genotypes that belong to GII were detected in our subjects. Of these, GII.2 appeared to be the dominant genotype. We also identified the following in this study: GII.17 Kawasaki, which is prevalent in East Asian countries such as Japan, China and Korea (Koo et al., 2016; Wang et al., 2016), GII.4 Sydney 2012, which has been the most prevalent in worldwide outbreaks (Eden et al., 2014), and GII.1, which was found to be prevalent in Hawaii and the United Kingdom (Bon et al., 2005).

Genetic recombination frequently occurs in RNA viruses, and several recombinant NoV strains have been described (Hardy et al., 1997). The GII.4 Sydney 2012 recombinant detected in this study has been globally prevalent (Mans et al., 2014). The other GII.17/GII.1 recombinant has been associated with foodborne outbreaks of gastroenteritis in Europe (Mathijs et al., 2011; Vega and Vinjé, 2011). The present study provides evidence suggesting that the NoV strain detected in our asymptomatic population in Indonesia was caused by the recombination of previously reported polymerase and capsid genotypes.

Here we observed that the presence of NoV in stools was associated with asymptomatic infection in Surabaya, Indonesia. As has been described previously, there are three possible explanations for such a finding: (1) long-term shedding from a previous symptomatic episode, (2) true asymptomatic infection, and (3) transient passage of ingested non-replicating virus through the gut (Ahmed et al., 2014; Lopman et al., 2014). The stool samples examined in the present study were collected from physically healthy subjects who had manifested no symptoms for one month before the samples were collected. Therefore, the first possibility is quite unlikely in this study, and no information is available to consider the third possibility.

Some of the asymptomatic individuals in our investigation showed a relatively high viral load, but these viral loads were still lower than those of symptomatic patients. This result is well consistent with the notion that symptomatic individuals have higher viral RNA titers in stool than asymptomatic individuals (Atmar et al., 2008; Kirby et al., 2014).

To the best of our knowledge, this is the first study to profile the characteristics of asymptomatic NoV infection and its genogroup/genotype in Surabaya, Indonesia. Further studies will be needed to determine whether asymptomatic infections cause NoV transmission leading to sporadic cases and outbreaks. Analyses of larger populations will also be necessary to test our findings. Moreover, it is obvious that the reinforcement of hygiene practices must be emphasized regardless of the cause of diarrhea.

In conclusion, our findings demonstrated that asymptomatic NoV infections are common in Surabaya, Indonesia, and some of the asymptomatic individuals shed large amounts of viruses similarly to symptomatic cases, suggesting that the excretion of NoV from healthy individuals is one of the sources of NoV outbreaks.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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