Shedding of norovirus in symptomatic and asymptomatic infections

P. F. M. TEUNIS1,2 *, F. H. A. SUKHRIE3,4, H. VENNEMA3, J. BOGERMAN4,5, M. F. C. BEERSMA4 AND M. P. G. KOOPMANS4

1 CIb/RIVM, Epidemiology and Surveillance Unit, Bilthoven, The Netherlands
2 Rollins School of Public Health/Emory University, Hubert Department of Global Health, Atlanta, GA, USA
3 CIb/RIVM, Laboratory for Infectious Diseases and Perinatal Screening, Bilthoven, The Netherlands
4 Erasmus Medical Centre, Department of Virology, Rotterdam, The Netherlands
5 Municipal Health Service, Rotterdam-Rijnmond, The Netherlands

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SUMMARY
Norovirus is the most frequent cause of acute infectious gastroenteritis and it is difficult to control in crowded environments like hospitals and nursing homes. Transmission depends on oral intake of virus deposited in the environment by infectious subjects. Data from volunteer studies indicate that virus concentrations in stool are highly variable, but systematic studies of the time-course of shedding and its individual variation are lacking. This paper quantifies norovirus shedding in a large population of 102 subjects, including asymptomatic shedders, and uses a longitudinal model to generalize shedding patterns. Enhanced surveillance for studies of transmission of norovirus in hospital outbreaks has yielded a considerable number of faecal samples from symptomatic and asymptomatic shedders, both from patients and staff. Norovirus concentrations were determined by real-time PCR. A quantitative dynamic model was fitted to the shedding data, in a multilevel Bayesian framework, to study the time-course of shedding and its variation. The results indicate that shedding in asymptomatic subjects is similar to that in symptomatic infections, both showing considerable variation in peak levels (average $10^5$–$10^9$ /g faeces) as well as duration of virus shedding (average 8–60 days). Patients appear to shed higher numbers of virus than staff, for slightly longer durations, but the differences are too small to be significant. Given equal shedding, the greater contribution of symptomatic cases to transmission must be caused by their higher efficiency in spreading these viruses. The results of this study will be helpful for risk studies that need to quantify the deposition of virus in the environment.

Key words: Asymptomatic viral infections, gastrointestinal infections, Norwalk agent and related viruses, virology (human) and epidemiology, virus infection.

INTRODUCTION
Viral gastroenteritis is an important concern for public health and a major cost factor for society. Hospitals and nursing homes are affected in particular [1, 2]. The epidemic potential of norovirus is striking [3, 4] and has been partly attributed to its high infectivity [5]. There is no clinical treatment for norovirus disease, or a vaccine that protects against infection,
although clinical trials are ongoing [6]. Hygiene measures and quarantining of cases are therefore currently the only option for containing an outbreak [1, 4]. Norovirus is transmitted via the faecal–oral route: primary foodborne or waterborne cases may shed virus into their environment. Virus deposited on surfaces can then cause outbreaks [7, 8] and virus can be transferred by hand contact [9] followed by ingestion [10]. In order to better understand how much virus is deposited in the environment, we have studied virus shedding in infected subjects.

Data from human challenge studies have shown that there may be considerable heterogeneity in the numbers of viruses that are shed by infected subjects [11, 12]. Generalization of the results from these studies to nosocomial outbreaks raises uncertainty because of small study sizes and the use of experimental infections, with a Norwalk virus (GI.I) or Snow Mountain virus (GII.2) inoculum in healthy volunteers [13].

The present studies extends these experimental findings with outbreak data, involving GII.4 norovirus, a common cause in recent outbreaks [3, 14, 15]. Moreover, in contrast to these experimental studies, the time-course of virus shedding was analysed with a multilevel longitudinal model, incorporating individual variation and measurement error.

In a study of norovirus transmission during outbreaks in a hospital and nursing homes [16, 17], enhanced tracking of cases provided data from patients with symptoms of acute norovirus infection as well as any known contacts. Data from respondents from this transmission study were used for the present study of faecal shedding.

MATERIALS AND METHODS
Data
Four distinct outbreaks of gastroenteritis caused by norovirus GII.4 were detected by prospective monitoring during the winters of 2009–2011, in a tertiary-care hospital and three nursing homes. Upon detection of outbreaks of two or more PCR-confirmed cases in the same ward, data were collected from as many patients and healthcare workers as possible, with or without symptoms of norovirus gastroenteritis [17]. Faecal samples were collected from all subjects who gave consent and these were followed up weekly, until they tested negative for norovirus.

Virus concentrations were measured by quantitative (real-time) RT–PCR. Details about sample collection and preparation, and assay performance have been published [18]. All Ct readings were done once, not in duplicate. To avoid inhibition on the target RNA, yeast rRNA was added that can react competitively with the RNase. For quality control a dilution series in yeast rRNA was included until $10^{-15}$.

Four NoV GII.4 outbreaks studied in the winters of 2009–2011 were selected for further analysis, yielding a total of 230 faecal samples in 102 (out of a total of 125) subjects. Of these, 47 were patients (32 with symptoms, 15 asymptomatic) and 54 healthcare workers (38 with symptoms, 16 asymptomatic). For one symptomatic case the patient/staff status was missing. There were also 23 subjects (six patients and 17 healthcare workers) with symptoms of gastroenteritis who did not consent to collection of samples. Details are shown in Table 1.

Model for the time-course of virus shedding
A realistic model of the time-course of virus shedding must include an initial increase in virus concentration, followed by a decrease to undetectable levels. For virus produced at an infection site and transported by bowel movement, the observed virus concentration in faeces can be written as

$$C(t|\alpha, \beta) = C_0 e^{-\alpha t} (1 - e^{-(\beta - \alpha)t}) \quad (\beta > \alpha).$$  \hspace{1cm} (1)

At onset of shedding ($t = 0$) the virus concentration $C(t)$ increases from 0 [with rate $C_0(\beta - \alpha)$], reaches a maximum and decreases to zero (with rate $\alpha$). More details, as well as a tentative biological mechanism, are given in the Supplementary online Appendix, section A.

Assuming the parameters ($C_0$, $\alpha$, $\beta$) vary among individual infected subjects, this model can be applied in a Bayesian hierarchical framework [19]. This results in individual estimates of the time-course of virus shedding, with the variation among these individuals characterized by (joint) probability distributions of the parameters of the regression model [equation (1)]. All models were run in JAGS (v. 3.3.0) [20] using rjags (v. 3.3-10) [21] in R (v. 3.0.1) [22]. Due to censoring tests of model fit using the DIC cannot be used, but in the Supplementary online Appendix the reader can check that the model accurately fits the observed $C_t$ data. Supplementary Figure A1b shows residuals for all observed $C_t$ values in all subjects.
### Table 1. Statistics of the sampled outbreak populations: numbers of infected patients and staff sampled for norovirus shedding, by symptoms, and their ages (mean age in years and 95% range), and collected numbers of samples (no. of observations) per individual

<table>
<thead>
<tr>
<th>Out-break Setting</th>
<th>Symptomatic</th>
<th>Asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Age (years) mean (95% range)</td>
</tr>
<tr>
<td>1 H Patients</td>
<td>5</td>
<td>62.2 (50.6–73.6)</td>
</tr>
<tr>
<td>11 Staff</td>
<td>5</td>
<td>34.8 (20.9–54.9)</td>
</tr>
<tr>
<td>2 NH Residents</td>
<td>8</td>
<td>85.9 (76.0–92.4)</td>
</tr>
<tr>
<td>11 Staff</td>
<td>11</td>
<td>40.0 (18.2–59.6)</td>
</tr>
<tr>
<td>3 NH Residents</td>
<td>8</td>
<td>80.5 (71.3–93.1)</td>
</tr>
<tr>
<td>9 Staff</td>
<td>43.8 (23.4–54.9)</td>
<td>2</td>
</tr>
<tr>
<td>4 NH Residents</td>
<td>11</td>
<td>88.0 (81.4–93.6)</td>
</tr>
<tr>
<td>7 Staff</td>
<td>52.1 (47.6–61.3)</td>
<td>1</td>
</tr>
</tbody>
</table>

H, Hospital; NH, nursing home.

*Non-compliant, symptomatic subjects who were approached for inclusion but did not give consent.

†The total number of subjects add up to 124 and not 125. In outbreak 1 there was one symptomatic case for whom status (patient or staff) was missing.

The onset of shedding \( t = 0 \) in equation (1) cannot be observed. For symptomatic subjects the onset of symptoms may be assumed to occur shortly after the onset of shedding [17]. For asymptomatic subjects an estimate of the onset of shedding may be found that provides the best agreement of the observed virus titres with the time-course of equation (1). This has been achieved by expressing time of onset of shedding relative to the first faecal sample that was collected, and treating the delay between onset and first sample as a parameter that can be estimated. Technical details of this procedure are given in the online Appendix, section B. For the symptomatic subjects the estimates of shedding onset agreed well with the observed symptom onsets, as shown in the online appendix of [17].

#### Calibration curve

To obtain a titration curve a standard suspension was made of RNA prepared as run-off transcript from cloned fragments (GII.4 Bristol region B, junction ORF I and II). The number of genome copies in an undiluted PCR sample of that suspension can be calculated from the RNA concentration measured by Nano Drop (ND-1000 Isogen Life Science, USA) as follows: an equivalent volume of 0.33 μl (2/15 × 2.5μl) of the RNA solution contains 2279 ng/μl RNA, with 1684 nucleotides \((MW \approx 1684 \times 320 + 159 = 539.04 \text{ kDa})\). The concentration of genome copies in undiluted suspension is then

\[
2279 \times \frac{10^{-9}}{10^{-6}} \times \frac{0.33 \times 10^{-6}}{539.04 \times 10^{3}} \times N_{Av}
\]

\[
\approx 8.487 \times 10^{11} \text{ genome copies,}
\]

where \(N_{Av}\) is Avogadro’s number \((6.02 214 \times 10^{23})\). Tenfold serial dilutions of this standard suspension were used for obtaining a calibration series of \(C_t\) values.

A calibration curve, obtained by linear regression, allows translation of observed \(C_t\) values in faecal samples into numbers of genome copies of NoV (GII.4). In the regression procedure, \(C_t\) values of 40 were considered censored \((C_t \geq 40)\). Supplementary Figure A1c shows the regression curve with (95%) prediction intervals. A Monte Carlo sample of the estimated parameters of this regression model was used to translate \(C_t\) estimates into virus concentrations, incorporating measurement error due to translation.

#### RESULTS

Faecal samples containing norovirus were obtained from 71 symptomatic and 31 asymptomatic subjects. Numbers of patients/staff and their age ranges are given in Table 1.

In Figure 1 observed virus concentrations are compared with predicted levels of virus shedding as a function of time, in symptomatic and asymptomatic subjects. Details of the longitudinal model and its biological basis, as well as details of the statistical
framework, are given in the Supplementary online Appendix; fitted responses for all individuals may be obtained from the corresponding author as a separate document.

For ease of interpretation, instead of rates of increase and decrease, the time from onset of shedding to peak levels and the duration of shedding (time from onset of shedding to decline to \( C_t = 40 \), considered the diagnostic detection limit) can be calculated (Fig. 2b, c). In addition, the peak levels of virus shed (Fig. 2a) and the area under the shedding curve can be calculated (Fig. 2d).

There is considerable variation, in the averages of peak concentration (\( 10^5 - 10^9 \) genome copies/g faeces),...
time to peak (<0·5 to >5 days from onset of shedding),
duration of shedding (8–60 days) and area under the
shedding curve (10^6–10^10 genome copies/g faeces).

There is an indication that patients, in particular
when they have symptoms, may shed higher levels of
norovirus, for somewhat longer periods (Fig. 2).
However, it should be noted that Figure 2 shows the
variation in individual means of predicted character-
istics: when uncertainty is taken into account the
differences are insignifi
ant (P levels for a non-
parametric test for differences in any of these para-
eters may be found in Supplementary Table A2).
The posterior distributions of these characteristics
also do not indicate that symptomatic and asympto-
matic subjects have different shedding characteristics.
There is, however, considerable uncertainty, as illu-
strated in the individual estimates in Figure 3.

In Figure 3 individual estimates are grouped by age,
illustrating that none of the four characteristics of the
time-course of shedding (peak levels, time to peak,
duration of shedding and area under the shedding
curve) appears to depend on age of the infected sub-
jects (Fig. 3).

Estimated time to peak and durations of shedding
show positive correlation (Pearson correlation
coefficients of 0·873 and 0·973 for symptomatic and
asymptomatic subjects, respectively), as do peak levels
and area under the shedding curve (correlation coeffi
cient 0·960). For additional details see Supplementary
Table A1.

**DISCUSSION**

Norovirus is transmitted via the faecal–oral route: its
high infectivity and persistence in the environ-
ment allow the virus to exploit various pathways.
Norovirus present on fomites is considered to be im-
portant for transmission [10]. Such virus can be de-
posited by infected subjects, via contaminated hands
[9]. As a potential driver for the numbers of viruses
that are deposited by infected subjects, the concen-
tration of virus shed in faeces is of interest. Two stu-
dies of virus shed by volunteers challenged with
Norwalk virus (GI.I) showed considerable variation
in the numbers of viruses in stools of infected subjects
[11, 12]. Another study of nosocomial norovirus in-
fected in aged-care facilities showed a range of
concentrations and durations of shedding [23].

The present study comprises a larger number of
subjects than previous studies, including some with
asymptomatic infections, sampled in the course of a study on nosocomial transmission of norovirus in The Netherlands [17]. In addition, the data were analysed with a longitudinal model, accounting for individual variation in response parameters, as well as measurement error in the quantitative PCR assay. Using these enhanced analysis methods, patterns and quantities of shedding in infected symptomatic and asymptomatic subjects are markedly similar. The variation in peak levels in individual subjects is large, with the minimum as low as a few hundreds of virus genomes/g, and the maximum (95th percentile of the predicted concentration) >10^{10} virus genomes/g faeces. The peak shedding levels are in the same range as those reported by [23], and slightly lower than those reported by [11]. It remains unknown whether the difference may be attributed to the different genotype (GI.I) used in the latter study.

The rates of increase and, more importantly, decrease of virus shedding are also variable, but to a lesser extent. In infected subjects shedding had dropped to low levels (<10^3 viruses/g) by day 60 post-infection. As symptoms usually do not last longer than 2 weeks the contribution of such long-lasting shedding to transmission may remain small [17].

In a study in hospitalized patients more serious symptoms (longer duration) were associated with higher virus concentrations [24]. In the study reported here such an association was not apparent, in fact, symptomatic subjects seem to shed norovirus in similar numbers as asymptomatic subjects (Fig. 3a, d), while both categories show considerable variation (mean peak levels of virus/g stool vary over a range of ~4 log_{10} units).

One may be tempted to interpret the numbers of viruses shed as an indicator for the intensity of infection, with higher numbers corresponding to more infected cells in the intestinal mucosa. If that is true, the variation in numbers shed may not have great clinical significance because shedding appears equally intense in symptomatic and asymptomatic infections. Patients, either symptomatic or asymptomatic, seem to shed higher levels of norovirus than staff, for a longer period. However, the difference is small and insignificant against the large variability in responses (Supplementary Table A2).

Also of note is that a transmission study in the same cohort has shown that symptomatic cases (staff and even more so patients) are more likely to transmit the virus than asymptomatic shedders [17]. As asymptomatic shedders appear to produce equal numbers of viruses as symptomatic cases, this difference in transmission must be attributed to enhanced environmental seeding of the virus, associated with vomiting and diarrhoea. Increased environmental seeding of symptomatic cases may be associated with their producing greater amounts of faeces than asymptptomatically infected subjects, and/or a higher frequency of defecation, or diarrhoea may result in a higher probability of contamination. It is important to note that the present study does not capture any shedding of norovirus in vomit. Vomiting has also been documented as a means of depositing norovirus in the environment [25] and norovirus has been detected in the oral cavities of infected subjects [26]. If faecal shedding indicates virus replication in the intestinal tract, it must be assumed that virus is then shed orally as well.

It is likely that only a fraction of the shed virus genomes is in infectious virus particles, as has been observed for other viruses [27–29]. Assuming that the fraction of infectious virus does not depend on the level of shedding (the peak concentration, or area under the curve), the large variation in peak concentration and/or area under the shedding curve may imply that different infected subjects may shed very different numbers of infectious noroviruses into their environment, and consequently they may have considerably different infectiousness to their contacts.

The present study is part of a greater project aimed at quantifying nosocomial transmission of norovirus in hospital and care facilities. It will be interesting to use the individual shedding patterns as a proxy for the infectiousness of each subject, allowing adjustment of the contribution of any individual to transmission according to the numbers of viruses they shed at any time following infection [4, 30, 17]. Such an individual-based approach may considerably improve the faithfulness of tracking of infections during an outbreak [31]. Direct use of the estimated virus concentrations to predict risk of transmission from individual infectious subjects seems attractive. It must, however, be realized that at present little is known quantitatively about the role of human behaviour both in spreading shed virus in the environment and in picking up that virus from contaminated fomites.

CONCLUSIONS

During norovirus infection, high numbers of virus are shed in faeces. Numbers of viruses in faeces increase rapidly, reaching peak levels within a few days following onset of infection, followed by a slow decline for
several weeks. It may take more than 2 months until faecal concentrations of virus reach undetectable levels. Both peak levels and duration of shedding show considerable individual variation that is not different between symptomatic and asymptomatic infections.

SUPPLEMENTARY MATERIAL
For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S095026881400274X.

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DECLARATION OF INTEREST
None.

REFERENCES

