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Norwalk Virus: How Infectious is It?

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Noroviruses are major agents of viral gastroenteritis worldwide. The infectivity of Norwalk virus, the prototype norovirus, has been studied in susceptible human volunteers. A new variant of the hit theory model of microbial infection was developed to estimate the variation in Norwalk virus infectivity, as well as the degree of virus aggregation, consistent with independent (electron microscopic) observations. Explicit modeling of viral aggregation allows us to express virus infectivity per single infectious unit (particle). Comparison of a primary and a secondary inoculum showed that passage through a human host does not change Norwalk virus infectivity. We estimate the average probability of infection for a single Norwalk virus particle to be close to 0.5, exceeding that reported for any other virus studied to date. Infected subjects had a dose-dependent probability of becoming ill, ranging from 0.1 (at a dose of 10^3 NV genomes) to 0.7 (at 10^8 virus genomes). A norovirus dose response model is important for understanding its transmission and essential for development of a quantitative risk model. Norwalk virus is a valuable model system to study virulence because genetic factors are known for both complete and partial protection; the latter can be quantitatively described as heterogeneity in dose response models. ***J. Med. Virol. 80:1468–1476, 2008.***

KEY WORDS: primary inoculum; secondary inoculum; norovirus; viral gastroenteritis; dose response; virus aggregation

INTRODUCTION

Since they were first recognized as an agent of viral gastroenteritis [Adler and Zickl, 1969], noroviruses have been identified as the cause of many outbreaks [Ciarlet and Estes, 2001; Estes et al., 2006; Kroneman et al., 2006]. In the US alone, noroviruses have been estimated to cause 23,000,000 infections each year, resulting in 50,000 hospitalizations and 310 fatalities [Mead et al., 1999]. The frequent occurrence of these viruses in outbreaks suggests high infectivity: exposure to small numbers of virus particles may be associated with high rates of infection [Glass et al., 2000] and high person-to-person transmission rates [Goodgame, 2007]. For these reasons, there is substantial interest in the development of quantitative risk assessments for noroviruses in water and food, and establishment of a dose response relation for this group of pathogens.

To date, quantitative risk assessment for any enteric virus has been based on the published dose response relation for infection with rotavirus [Ward et al., 1986; Haas et al., 1993]. A commonly mentioned justification for using this organism as a surrogate for any enteric virus is its high infectivity. Ingestion of a small dose consequently implies a high risk of infection, compared to many other pathogens [Teunis et al., 1996]. However, recent studies on the variation in infectivity among different strains of a single pathogen species have demonstrated that genetic relatedness does not necessarily imply similar infectivity. The variations in

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infectivity found in three different strains of *Cryptosporidium parvum* were found to be of the same order of magnitude as, for example, the differences in infectivity among different bacterial species [Teunis et al., 1996, 2002]. Therefore, in the absence of additional information on infection mechanisms and host susceptibility, generalizations about the dose response of new pathogens remain highly speculative.

Although several human challenge studies with Norwalk virus have been published [Johnson et al., 1990; Graham et al., 1994; Okhuysen et al., 1995], establishing the pathogenicity of this virus, the results could not be used for dose response assessment because the dose remained unknown. Titration of infectious virus particles in the inoculum was not possible because culturing these viruses proved to be highly complicated [Straub et al., 2007]. However, the presence of Norwalk virus RNA can be detected by reverse transcription-polymerase chain reaction (RT-PCR), providing an alternative method for Norwalk virus enumeration.

Compared to other dose response studies, there are two complications: differential host susceptibility and aggregation of the viruses in the inoculum. Both of these issues have not been dealt with in previous studies of virus infectivity. We will discuss both problems and show how a modified dose response model can be used to estimate the infectivity of Norwalk virus. In addition, we show that the probability that infected subjects become ill is also dose dependent, increasing with higher virus dose. The results will enable refinement of risk models for a food- or waterborne, category B biothreat, virus and support the development of population models for epidemic transmission.

MATERIALS AND METHODS

Norwalk Virus Challenge Data

Inocula for human challenge were prepared by dilution of a stock suspension of Norwalk virus. A primary inoculum was prepared from the original Norwalk virus isolate in 1971 [Dolin et al., 1971], identified as "8fIIa," and was used in two challenge experiments: one at moderate to high doses, the second experiment exploring the low dose range (combined in top part of Table I).

The "8fIIa" inoculum has been stored in a stock suspension for more than 25 years [Dolin et al., 1972]. This suspension (veal infusion broth with 0.5% bovine serum albumin) contains high concentrations of protein, acting as a "sticky" matrix, resulting in considerable aggregation of suspended virus. Judged by electron microscopy, these aggregates could not be dissociated by sonication (Fig. 3a). Because of this matrix, dilution is also likely to not result in virus dissociation, and the low doses administered in the challenge study represent virus clumps rather than single viruses.

A stool sample from one of the infected subjects in the first experiment was used to prepare a secondary inoculum in 1997, designated "8fIIb," which was then used in a third challenge experiment (bottom part of Table I). The inoculum was prepared from a 20% stool suspension, extracted with Freon and then with 1× phosphate buffered saline (PBS). The first two extractions were titered, combined and filtered through a 0.2 μm Nylon filter. The filtrate was titered and safety-tested for a range of pathogens using standard methods and protocol that was approved by the University of North Carolina School of Medicine Internal Review Board.

TABLE I. Combined Results From Three Norwalk Virus Challenge Studies

Dose		Se^-			Se^+		
μl	Genomes	Tot.	Inf.	Ill.	Tot.	Inf.	Ill.
8fIIa							
1.0×10^{-6}	3.24×10^{1}	2	0	0	8	0	0
1.0×10^{-5}	3.24×10^{2}	2	0	0	9	0	0
1.0×10^{-4}	3.24×10^{3}	6	0	0	9	3	1
1.0×10^{-3}	3.24×10^{4}	1	0	0	3	2	1
1.0×10^{-1}	3.24×10^{5}	2	0	0	8	7	6
1.0×10^{0}	3.24×10^{6}	3	0	0	7	3	1
1.0×10^{1}	3.24×10^{7}	2	0	0	3	2	2
1.0×10^{2}	3.24×10^{8}	4	0	0	6	5	4
		22	0	0	53	22	15
8fIIb							
1.0×10^{0}	6.92×10^{5}	2	0	0	8	3	2
1.0×10^{1}	6.92×10^{6}	4	0	0	18	14	7
3.0×10^{1}	2.08×10^{7}	0	0	0	1	1	NA
		6	0	0	27	18	9(?)

8fIIa: Responses of human volunteers to challenge with different doses of the primary inoculum of norwalk virus (8fIIa isolate)
Separate categories for subjects with positive and negative secretor status (Se^- and Se^+). 8fIIb: responses of human volunteers to challenge with different doses of the secondary inoculum of Norwalk virus (8fIIb isolate). NA, not available.

Titration of the Inocula

Cell culture methods are not available for quantifying infectious Norwalk virus. Therefore the virus stock suspension was titrated by quantitative real time RT-PCR with a NV RNA standard.

Full-length Norwalk virus cDNA, that was cloned into a pSPORT1 vector, was used as a template to prepare the NV RNA standard. NV plasmid was linearized by M1UI enzyme at 37°C overnight, in vitro transcribed with T7 RNA polymerase (Ambion, Inc., Austin, TX), precipitated by lithium chloride, washed with 70% ethanol and suspended in DEPC-treated water. The concentration of the NV RNA standard was determined by measuring the absorbance at 260 nm by spectrophotometer. The number of genome copies per μl RNA standard was calculated using the relation $1OD_{260nm} = 40$ μg/ml, converting to mole concentrations and multiplying by Avogadro's number. This standard of genome equivalent copies was serially diluted and used to quantitate numbers of genome copies of NV inocula used in this study.

Norwalk virus inocula (8fIIa, 8fIIb) were diluted 100- and 1,000-fold with DEPC-treated water. RNA was released by mixing 1 μl of diluted suspension with 8.8 μl of DEPC-treated water and 0.2 μl of RNAse inhibitor (Promega, Madison, WI), heated to 95°C for 5 min followed by chilling on ice for 2 min.

TaqMan real-time RT-PCR was performed by using a Qiagen one-step RT-PCR kit (Qiagen, Hilden, Germany) and a Stratagene Mx3000P (Stratagene, La Jolla, CA) real-time PCR instrument. The reaction mixture was made up to a volume of 25 μl containing 10 μl of heat-released RNA or diluted Norwalk virus RNA standard, 0.4 mM dNTP mixture, 0.4 μM of both sense and antisense primer, 0.4 μM of fluorescent labeled TaqMan probe, 1× Qiagen one-step RT-PCR buffer, 10 U RNase inhibitor, 1 μl Qiagen RT-PCR enzyme mixture of HotStart Taq DNA polymerase and reverse transcriptase. The probe was dual-labeled with 5′-reporter dye FAM (6-carboxy-fluorescein) and 3′-quencher day TAMRA (6-carboxy-tetramethyl-rhodamine). Specific primers for Norwalk virus amplification were sense NVKS1 5′-AC-AGCATGGGACTCAACACA-3′ and antisense primer NVKS2 5′-GGGAAGTACATGGGAAT-CCA-3′ and probe NVKS3 5′(FAM)-TCACCAGAATTG-GCCGAGGTTGT-3′(TAMRA), which are all located in the Norwalk virus RNA polymerase region. The RT-PCR program was started with 32 min at 50°C for reverse transcription followed by 15 min of HotStart Taq DNA polymerase activation, and reverse transcriptase inactivation. Amplifications were carried out for 45 cycles (15 sec at 94°C, 15 sec at 55°C, and 45 sec at 70°C).

The two dilutions (100- and 1,000-fold) of both inocula were tested in triplicate against a calibration series of four dilutions of the Norwalk virus RNA standard. The whole experiment, including calibration series, was repeated three times: in the first experiment 8fIIa was tested, in the second experiment both 8fIIa and 8fIIb, and in the third experiment only 8fIIb was tested (two batches, see details in Table II). Joint regression analysis of the calibration curve with the measurements of the two inocula resulted in single maximum likelihood estimates of 8fIIa and 8fIIb titers. A summary of the results is shown in Table II. These joint maximum likelihood estimates were used to calculate the doses for the analysis of the challenge study data.

Responses to Challenge

Infection was defined as fecal excretion of virus and seroconversion, illness included diarrhea and/or vomiting combined with other symptoms as abdominal pain, myalgia, fatigue, chills and headache more than 8 hr after challenge.

Consistent with our earlier reports [Lindesmith et al., 2003], a percentage of volunteers were resistant to infection regardless of dose (Table I). Although low doses did appear to cause infection in some volunteers, high doses did not infect all exposed subjects, in agreement with earlier studies [Johnson et al., 1990]. As a consequence, the Beta Poisson model of microbial infection did not adequately fit these data (as judged by deviance from a saturated model [Teunis and Havelaar, 2000]).

Previous work has indicated that ABH histoblood group antigens are associated with susceptibility to Norwalk virus infection [Hutson et al., 2002; Lindesmith et al., 2003]. In particular, virus binding to intestinal epithelial cells depends on the secretor phenotype (Se^+). Nonsecretors (Se^-) lack the receptor, and the virus cannot bind to infect their intestinal cells [Lindesmith et al., 2003]. In Se^- subjects, infection has not been found, not even at the highest doses. We therefore restricted the dose response assessment to subjects with the Se^+ phenotype (statistics in Table III).

TABLE II. Likelihood Analysis of Real Time RT-PCR Data

	8fIIa	8fIIb			
Experiment	$\log_{10}\hat{c}$	$\log_{10}\hat{c}$		Deviance	df
1	6.47	NA	NA	21.08	5
2	6.48	5.57	5.70	52.41	7
3	NA	5.92	5.85	19.61	6
Joint	6.51	5.84		99.14	14

Maximum likelihood estimates $\hat{c}$ of the 10-base logarithm of the concentration (genomes/μl) of 8fIIa and 8fIIb from three consecutive experiments separately (two batches of 8fIIb were tested) and outcome of a joint regression model with separate calibration curves but joint titers (also both 8fIIb titers joined). Deviance = −2 times log-likelihood; df = degrees of freedom. Deviance of the joint model compared to the separate ones is not significant: $\chi^2_{5+7+6-14}(99.14 - 21.08 - 52.41 - 19.61) \rightarrow 0.804$ level.

Dose Response Models

The most important basic concept in microbial infectivity is the particulate nature of microbial inocula. A biologically relevant dose of even the most potent toxic chemicals consists of many particles. In contrast, pathogenic microbes affect their hosts at doses consisting of few particles, or even a single organism [Tillett and Lightfoot, 1995; Yang et al., 2000]. Consequently, exposure to microbial pathogens must be treated as a discrete number of organisms.

Dose however, is often expressed as a probable number, calculated by multiplying the titer of the inoculum suspension by the applied volume. The translation of such estimated numbers to actual ingestion of (discrete) numbers of pathogens is necessarily part of any dose response assessment [Teunis and Havelaar, 2000]. At low doses, the probability of not ingesting any infectious organisms at all may be considerable, limiting the infection risk at low doses for any infectious organism.

The Hit Theory Model for Microbial Infection

The hit theory model considers microbial infection as the result of a chain of conditional events: ingestion of one or more organisms from a Poisson-distributed suspension, followed by successful passage through any number of defensive barriers that may be present in the host. Individual organisms are thought to act independently, and any single surviving organism may reach an appropriate host cell and cause infection. Heterogeneity in the probability of individual organisms to achieve infection is modeled as a beta distribution [Furumoto and Mickey, 1967; Haas, 1983; Teunis and Havelaar, 2000].

TABLE III. Maximum Likelihood Estimates For The 8fIIa and 8fIIb Experiments

Infection	$\hat{\alpha}$	$\hat{\beta}$	$\hat{a}$	$\hat{\mu}_a$	Dev.	P
8fIIa (no aggr.)	0.111	32.81	—	—	7.977	0.760
8fIIa (aggr.)	5.35×10^{-3}	2.51×10^{-3}	0.9998	581.9	4.979	0.582
8fIIb	0.631	6.50×10^{5}	—	—	0.246	0.380
8fIIa + 8fIIb	0.040	0.055	0.9997	396.4	9.420	0.692
Illness	$\hat{\eta}$	$\hat{r}$		$\hat{\mu}_a$	Dev.	P
8fIIa (no aggr.)	0.508	0.095		—	4.371	0.373
8fIIa (aggr.)	8.73×10^{-4}	0.095		58.30	4.371	0.373
8fIIb	∞	0		—	0.281	—
8fIIa + 8fIIb	2.55×10^{-3}	0.086		396.4	5.126	0.472

8fIIa: no aggr. and aggr: dose response models without and with virus aggregation applied to susceptible (Se^+) subjects.
8fIIb: dose response model (no aggregation) for Se^+ subjects. 8fIIa + 8fIIb: combined 8fIIa and 8fIIb experiments. Deviance re. binomial supremum (Dev) and corresponding confidence level (p) also shown. Separate models can be compared to pooled models in a likelihood ratio test, by calculating the differences in deviance. Pooled models for infection do not differ significantly from separate models (Infection: $\chi^2_{3+2-3}(9.420 - 4.979 - 0.246) \rightarrow 0.877$ level; Illness: $\chi^2_{2+2-2}(5.126 - 4.371 - 0.281) \rightarrow 0.211$ level).

The probability of infection as a function of the dose can then be written as

$$f(c \cdot V; \alpha, \beta) = 1 - {}_1F_1(\alpha, \alpha + \beta; -c \cdot V) \quad (1)$$

where $c \cdot V$ is the dose: a volume V of an inoculum suspension of concentration c. Parameters α and β characterize infectivity; ${}_1F_1$ is a confluent hypergeometric function.

Virus Aggregation and the Single Hit Model

Microorganisms suspended in water can cluster into aggregates of varying sizes. Viruses especially may be "sticky" in suspension, depending on the ionic strength, pH, and properties of the viral protein coat or envelope. The virus stock used for the present study had been stored for many years in a stabilizing solution, high in protein, that promotes viral aggregation (Fig. 3a). For dose response assessment, we need to take into consideration that the virus may not have been administered as a monodisperse suspension of single virions, but rather as a mixture of aggregates of varying sizes.

When the titration method enumerates particles, and not single virions, aggregation of infectious particles changes the dose response relation in several ways. The apparent dose may be lower than the number of viruses that was actually present. Also, as virus aggregates do not all contain the same number of virions, the inoculum may not be Poisson distributed, as is usually assumed for the single hit model of infection. There may be other effects associated with being part of an aggregate, like protection or facilitation of entry into host cells. These will not be dealt with in this paper because we have no data in these areas.

In the absence of heterogeneity in the interaction between a monodisperse virus and its host, the single hit dose response relation is exponential [Teunis and Havelaar, 2000]. We will characterize an aggregated virus suspension as a Poisson stopped logarithmic series distribution: the distribution of aggregates (particles) is Poisson while the aggregate sizes are logseries distributed with parameter a.

For an aggregated inoculum, in the absence of heterogeneity in the interaction between virus and host, the single hit model is modified

$$P_{inf}(C, V, a, p_m) = 1 - \left(1 + p_m \frac{a}{1-a}\right)^{C \cdot V / \log(1-a)} \quad (2)$$

with C is the concentration of virus aggregates, V the volume of the administered inoculum, and a the parameter of the (logarithmic series) aggregate size distribution, and p_m the single hit infectivity.

Heterogeneity in p_m can be expressed as a Beta distribution (parameters α and β), leading to a dose response relation

$$P_{inf}(C, V, a, \alpha, \beta) = 1 - {}_2F_1\left(\alpha, \frac{-C \cdot V}{\log(1-a)}, \alpha + \beta; \tfrac{-a}{1-a}\right). \quad (3)$$

This is the hit theory dose response model of microbial infection for an inoculum with aggregates of organisms (${}_2F_1$ is a hypergeometric function, details in Supplementary material).

In case the dose is characterized by the total number of viruses present, the dose response relation is

$$P_{inf}(c, V, a, \alpha, \beta) = 1 - {}_2F_1\left(\alpha, \frac{c \cdot V(1-a)}{a}, \alpha + \beta; \frac{-a}{1-a}\right) \quad (4)$$

with c is the concentration of viruses, whether in aggregates or not.

Illness dose Response

More than half of the infected Se^+ volunteers developed symptoms of acute enteric illness. Illness is an important endpoint for risk assessment, especially for disease burden calculations.

As illness is conditional on infection [Teunis et al., 1999], we wanted to study the probability of illness in infected subjects as a function of the applied dose. We used an existing model for illness dose response that is based on the concept of illness hazard during infection [Teunis et al., 1999]. The dose response relation for the conditional probability of illness in infected subjects is

$$h(C, V|\eta, r) = 1 - (1 + \eta CV)^{-r} \quad (5)$$

with parameters η and r (details in Teunis et al. [1999]).

Model Fitting to Experimental Data

In the setting of a clinical challenge study with binary responses, we have a binomial likelihood

$$L(\alpha, \beta) = \prod_i (f(c \cdot V|\theta))^{k_i} (1 - f(c \cdot V|\theta))^{n_i - k_i} \quad (6)$$

where $f(c \cdot V|\theta)$ is the dose response model, with parameter vector θ. The first term in Eq. (6) corresponds to positive (either infected, or symptomatic) subjects (number k_i); the second term represents negative (either uninfected, or asymptomatic) subjects (number $n_i - k_i$).

For the infection dose response model, parameter estimation is improved by transformation of the beta distribution parameters (α, β) to

$$u = \text{logit}(\alpha/(\alpha + \beta)); \quad v = \log(\alpha + \beta) \quad (7)$$

so that we estimate the logit (u) of the mean value of the Beta distribution for the single hit probability p_m (Appendix) and a quantity that is inversely related to its variance (for very large positive values of v the variance tends to zero). As the aggregation parameter a is also defined on (0, 1), it is logit-transformed as well.

For the illness dose response model we used the following parameter transformations

$$w = \log(r\eta); \quad z = \log(r/\eta) \quad (8)$$

Maximum likelihood estimates were obtained by direct numeric optimization of likelihood functions (using Mathematica® v. 6, [Wolfram Research, 2007]).

Monte Carlo uncertainty estimates were obtained by adaptive rejection sampling [Teunis and Havelaar, 2000]. To improve stability, we used non-informative uncorrelated normal (0,10) priors for all transformed parameters and checked whether posterior mode estimates coincided with the maximum likelihood values. Markov chains were constructed with the algorithm of Metropolis and Hastings [Gilks et al., 1996] and implemented in Mathematica® v. 6. To improve the speed of calculations, values for the confluent hypergeometric function were calculated by means of an externally linked application, written in C [Teunis and Havelaar, 2000].

RESULTS

Combined Infection Dose Response for 8fIIa and 8fIIb

Human passage may have changed the infectivity of the secondary (8fIIb) inoculum, compared to its precursor, the 8fIIa isolate. Such a comparison is of interest to other pathogens as well, as passage may result in adaptations that change the infectivity or transmission properties of the pathogen. One must be careful in comparing their dose response relations, however: the 8fIIb inoculum was not aggregated and apparent differences in the shape of the dose response relation may be attributed to differences in aggregation state of the inocula.

This hypothesis may be tested by assuming identical infectivity for the 8fIIa and 8fIIb inocula (shared parameters α and β) but different aggregation states (aggregated 8fIIa and dissociated 8fIIb), and comparing the resulting deviance in a likelihood ratio test to that of the separately fitted models (Table III). The difference is not significant, hence the difference between the two experiments can be explained by the difference in aggregation state of the two inocula. Aggregation is quantified by the parameter a, with corresponding average aggregate size μ_a (also shown in Table III).

Jointly fitted dose response relations for both aggregated (8fIIa) and disperse (8fIIb) virus are shown in Figure 1. The X-axis (dose) shows the expected numbers of viruses, in aggregates or not. Note that in an aggregated suspension the number of particles is

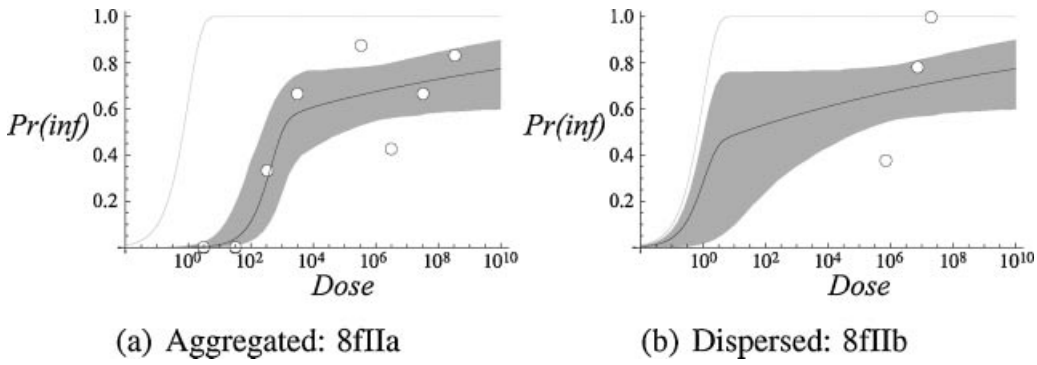

Fig. 1. Infection dose response for 8fIIa (**a**) and 8fIIb (**b**), with and without virus aggregation, respectively. Best (maximum likelihood) curve and 95% (predictive) interval. Also shown: observed fractions (circles) and (gray line) dose response relation for a completely infectious virus ($p_m = 1$).

smaller than the number of viruses. This causes the dose response relation for the aggregated suspension to decrease more rapidly with decreasing dose than it would for the same virus in disaggregated suspension.

The dose response relation for the aggregated NV inoculum in Se^+ subjects has a 50% infectious dose (ID_{50}) of 1,015 genome copies, approximately equivalent to 2.6 (aggregated) particles. The dose response relation for completely disaggregated virus leads to an estimated $ID_{50} = 18$ viruses.

Note that at low doses the dose response relation for infection is very close to the exposure dose response curve for a Poisson inoculum (the gray line in Fig. 1b). This curve represents the theoretical upper limit for the infection dose response relation [Teunis and Havelaar, 2000]. At high doses, the dose response relation for infection levels off to remain below 1 even at very high doses. This may indicate partial immunity, even among susceptible (Se^+) subjects.

Single Hit Infection Probability

The estimated infectivity parameters $(\hat{\alpha}, \hat{\beta})$ define the distribution of the probability of infection per ingested virus particle, p_m. This beta-distributed probability can be interpreted as the probability that any single virus particle will pass through all the barriers of the host defense system and remain infectious [Teunis and Havelaar, 2000]. As both α and β are less than 1, the distribution of p_m has considerable variance (Fig. 2), and the corresponding dose response relation does not reach 1, even at very high doses (Fig. 1a,b). This suggests that a fraction of the exposed population has low susceptibility to infection, while the remainder has high susceptibility. Part of the study population, therefore, still appears to be protected from infection, possibly due to acquired immunity [Lindesmith et al., 2003].

Aggregate Sizes

In addition to an infectivity estimate, fitting the dose response model has also produced an estimate for a, the aggregation parameter. The maximum likelihood estimate of the average aggregate size is 396.4 virus particles (Table III), with uncertainty shown by a Monte Carlo sample of its distribution (Fig. 3b). Note that, to estimate these aggregate sizes, we have only used the infection data from Table I.

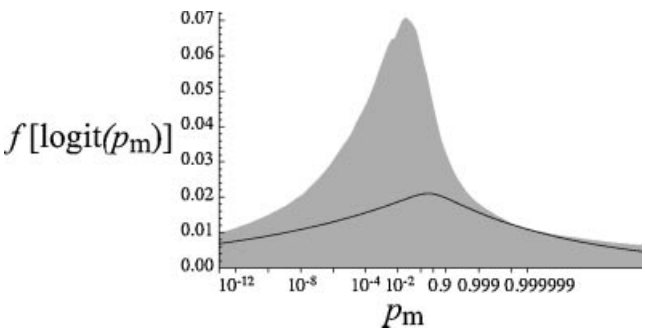

Fig. 2. Single hit probability p_m for the combined 8fIIa and 8fIIb data. Note that p_m has been transformed to a logit scale. Maximum likelihood distribution and 95% predictive intervals are shown.

Although the best estimate of μ_a is as large as 400 virus particles, aggregates of smaller sizes not unlikely. It should be noted that the logseries distribution used for modeling aggregate sizes is highly skewed: at an average aggregate size of 400 the probability of having more than 1,000 viruses in any aggregate is less than 9%. About 60% of the aggregates are smaller than 100 virions. Such aggregate sizes are consistent with directly observed virus aggregates in the 8fIIa inoculum by electron microscopy (Fig. 3a).

Illness dose Response

The conditional probability of illness (among infected subjects) also appears to show dose dependence (Fig. 4). Although the 8fIIb data provide very little information on the shape of the illness dose response relation, they do not contradict the curve obtained from the 8fIIa data (as judged from deviance, Table III). Therefore, the illness dose response parameters $(\hat{\eta}, \hat{r})$ are assumed identical for both inocula. The apparent difference in shape (Fig. 4) is caused by a differently calibrated dose axis. Doses were obtained by dilution of a stock suspension which, in case of the aggregated 8fIIa inoculum, implies diluting of aggregates. The dose for the 8fIIa illness relation is expressed in aggregates instead of single viruses.

DISCUSSION

Noroviruses are a major cause of human gastroenteritis, and they are frequently associated with foodborne and waterborne outbreaks. Regulatory agencies responsible for food and water safety recognize the need for developing quantitative risk assessments for these viruses. To characterize the health hazards associated with noroviruses, dose response information is critical.

This is the first report to document quantitative estimates of Norwalk virus infectivity. Norwalk virus is highly infectious: exposure to minute amounts of virus carries a high risk of infection (near 49% for a single infectious virus particle). Virus inocula made from primary and secondary isolates were shown to have the same infectivity, indicating that prolonged storage and passage through the human host did not enhance or decrease infectivity.

The estimated infectivity of norovirus is similar to that reported for rotavirus [Teunis and Havelaar, 2000]. We should however be cautious about such a comparison. In the rotavirus study [Ward et al., 1986], dosage was titered by a completely different assay method and expressed in focus forming units (ffu). The validity of any comparison of infectivity depends on how many of the ingested virus particles are infectious. The enumeration method for the rotavirus inoculum only counts units capable of infecting cultured host cells. A similar cell culture method does not exist for norovirus.

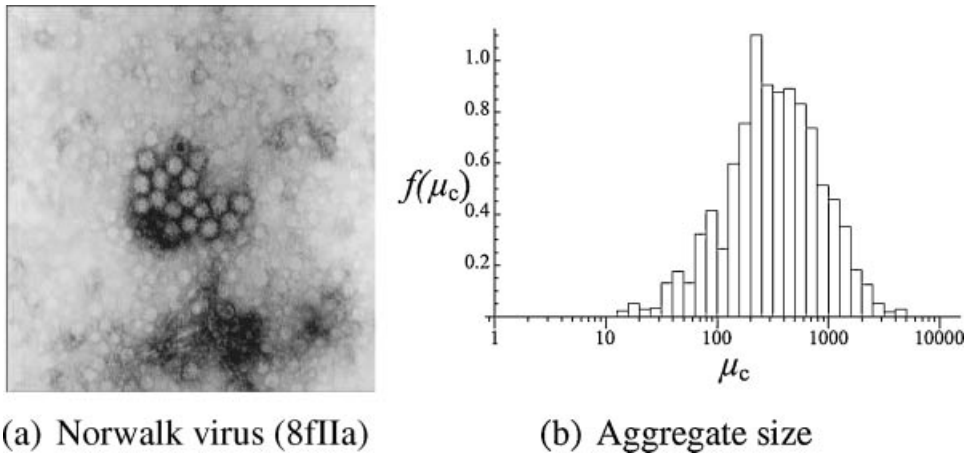

Fig. 3. **a**: Electron micrograph of Norwalk virus inoculum 8fIIa showing aggregation. **b**: Distribution of average aggregate sizes (μ_a) as derived from infection dose response with virus aggregation. Shown here is a Monte Carlo sample from the distribution of μ_a, calculated from the (posterior) distribution of a.

A complicated human norovirus cell culture system has been reported recently [Straub et al., 2007], as yet unconfirmed by other groups, which may or may not be suitable for enumeration of infectious virus.

Detection of noroviruses in environmental samples is based on RT-PCR, as are the titrations of Norwalk virus inocula. If the ratio of total to infectious numbers of viruses is constant, and exposure estimates are based on the same enumeration method as used for the 8fIIa and 8fIIb inocula, the resulting risk estimates may still be unbiased, even though we do not know how many infectious virus particles are actually present. Virus infectivity in cell culture may also differ from infectivity in animal or human hosts [Smith et al., 2006]. However, our best estimate of Norwalk virus infectivity is so close to 1 that we can only conclude that at least half of the virus particles (virions) in both the 8fIIa and 8fIIb inocula were infectious to humans. It should be noted that noroviruses undergo recombination [Bull et al., 2005; Rohayem et al., 2005] and in cells infected with debilitated viruses, recombination may restore their infectivity. If possible, a quantitative comparison of NV enumeration by q-RT-PCR and cell culture should be conducted to examine the relation between genome copy and cell culture infectious units.

A critical factor in this infectivity study was the identification of a genetic marker of innate resistance to Norwalk virus infection. Previous modeling attempts using conventional dose response models failed because of lack of fit due to too much unexplained heterogeneity. Although only secretor-positive volunteers were included in this analysis, the dose response relations still showed weak signs of residual immunity. This may be partly due to some volunteers being heterozygous for the susceptibility allele leading to weak expression of the Se^+ phenotype, some Se^+ subjects with blood group B having a lower risk of infection [Lindesmith et al., 2003; Hutson et al., 2005] because they have fewer NV binding sites [Marionneau et al., 2005], and some subjects with acquired immunity associated with prior episodes of norovirus infection.

Although a specific fraction of the population may be susceptible to Norwalk virus [Lindesmith et al., 2003; Hutson et al., 2005], for other noroviruses this fraction may be different [Lindesmith et al., 2005; Cheetham et al., 2007]. Clearly, this complicates risk assessment: not only do we need to identify similar genetic susceptibility factors for other human pathogens, population studies are also needed to find out how these genetic factors are distributed among the exposed population. If

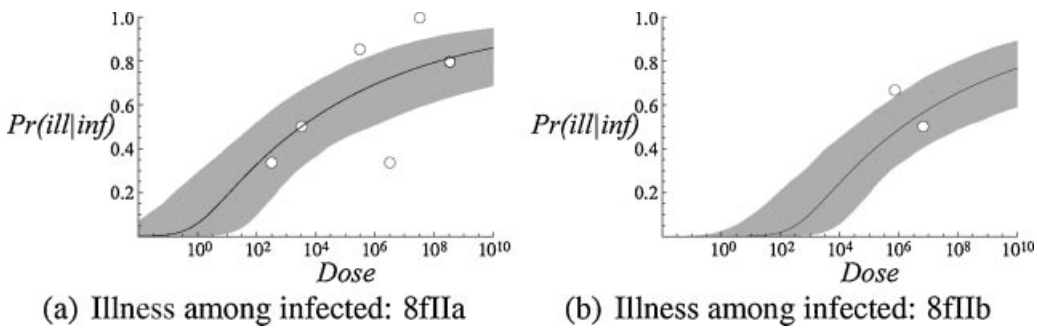

Fig. 4. Dose response relation for illness among infected subjects, both for aggregated (8fIIa) and disaggregated (8fIIb) inocula. Shown are maximum likelihood curves and 95% (predictive) intervals for the illness risk.

we take a prudent (i.e. worst case) approach and assume the whole population is sensitive (in cases where such information is unavailable) we may overestimate the health risks, but possibly not to a great extent as any successful pathogen would have to be capable of infecting a major fraction of its host population in order to survive.

Although it seems plausible that the virus suspension used to prepare the challenge inocula contained aggregated virus, norovirus in an environmental sample may be in a vastly different aggregation state. Therefore, it was useful to explicitly incorporate virus aggregation into the dose response model so that the infectivity of the virus may be expressed in terms of single virus particles. Any exposure assessment for norovirus can then deal with the issue of aggregation and provide a description of the (distribution of) sizes of the viral aggregates so that the numbers of ingested virus particles can be estimated.

Because of aggregation, the "single hit" in our model may actually be caused by a clump and not by a single particle. Whether this clump later dissociates in the intestinal tract before one or more of the viruses in the clump causes infection is irrelevant. A single ingested unit (virion or clump) may contain a varying number of virus particles, and the infectivity of these units will show additional heterogeneity compared to a completely dissociated inoculum. This is analogous to the situation in Cryptosporidium where every oocyst contains four sporozoites that may not all have the same infectivity. The single infectious unit is an oocyst, and differences in the infectivity of individual sporozoites cause heterogeneity in infectivity and influence the parameter estimates and the shape of the dose response relation. Without any variation in infectivity among the pathogens in an inoculum, the single hit dose response relation would be exponential. Compared to this "baseline" relation, heterogeneity can only cause flatter (less steep) dose response relations [Teunis et al., 2002].

Infection with Norwalk virus was associated with a high probability of illness. Out of 22 infected (Se^{+}) subjects, 15 developed acute symptoms of gastroenteritis (68%). Increasing doses led to an increasing probability of becoming ill. Conversely, when a person was infected after exposure to a low dose, the probability of illness was lower than after exposure to a high dose. This means that a high risk event, where heavy contamination occurs, may produce not only many infections but also many cases of illness, making it more likely that the event will be recognized by public health authorities. Low levels of endemic exposure, as may be encountered via contaminated drinking water, for example, may lead to infection but may produce relatively low numbers of illnesses, thereby lowering the recognition of the role of this exposure route.

The methods developed here may allow for more sophisticated modeling of infectivity of other human pathogens. Norwalk virus is a valuable model system to study virulence because of its clear cut (but complex) properties. Genetic factors determining secretor status appear to confer absolute protection and eliminate part of the population as susceptible hosts. Other factors, such as blood type, exhibit considerable polymorphism and result in graded susceptibility that can be conveniently described as heterogeneity in dose response models. The Norwalk virus dose response model may replace the rotavirus model as the generic dose response relation for enteric virus risk assessment: noroviruses are at least as infectious as rotavirus, cause disease in all ages, and the model incorporates differential host susceptibility and virus aggregation.

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