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Absolute Humidity Influences the Seasonal Persistence and Infectivity of Human Norovirus

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Norovirus (NoV) is one of the main causative agents of acute gastroenteritis worldwide. In temperate climates, outbreaks peak during the winter season. The mechanism by which climatic factors influence the occurrence of NoV outbreaks is unknown. We hypothesized that humidity is linked to NoV seasonality. Human NoV is not cultivatable, so we used cultivatable murine norovirus (MNV) as a surrogate to study its persistence when exposed to various levels of relative humidity (RH) from low (10% RH) to saturated (100% RH) conditions at 9 and 25°C. In addition, we conducted similar experiments with virus-like particles (VLPs) from the predominant GII-4 norovirus and studied changes in binding patterns to A, B, and O group carbohydrates that might reflect capsid alterations. The responses of MNV and VLP to humidity were somewhat similar, with 10 and 100% RH exhibiting a strong conserving effect for both models, whereas 50% RH was detrimental for MNV infectivity and VLP binding capacity. The data analysis suggested that absolute humidity (AH) rather than RH is the critical factor for keeping NoV infectious, with an AH below 0.007 kg water/kg air being favorable to NoV survival. Retrospective surveys of the meteorological data in Paris for the last 14 years showed that AH average values have almost always been below 0.007 kg water/kg air during the winter (i.e., 0.0046 ± 0.0014 kg water/kg air), and this finding supports the fact that low AH provides an ideal condition for NoV persistence and transmission during cold months.

Acute gastroenteritis affects millions of people every year, worldwide. Most cases of gastroenteritis are viral infections, and norovirus (NoV) is the most common causative agent of the disease accounting for >90% of nonbacterial cases of gastroenteritis (1). In addition to person-to-person transmission of NoV (2), other factors also contribute to the spread of NoV throughout the population. These include the high infectivity of viral particles, the long-term shedding of infectious particles by infected individuals, short-term immunity, and NoV resistance in the environment (2, 3). The majority of NoV-related gastroenteritis cases occur in winter, as is the case for illnesses due to respiratory viruses (e.g., influenza virus) and other enteric viruses (e.g., rotavirus). Many studies have investigated the reasons for the winter peak of influenza virus outbreaks. Early on, a correlation was found between aerosols, relative humidity (RH), and influenza virus infectivity in a mouse model. It was shown that influenza virus was the most infectious at the lowest and highest RH levels, while infectivity and mortality in mice were diminished at intermediate RH levels (4, 5).

This observation was confirmed in a recent study, which showed that influenza virus transmission in guinea pigs was reduced in medium-range RH and high RH at room temperature (6). Retrospective analysis of published data showed that temperature-independent absolute humidity (AH) rather than temperature-dependent RH correlated with winter seasonality (7). This observation was later confirmed by epidemiological data from the United States: statistical models that took account of AH fit the seasonal cycle of the influenza virus (8). Moreover, the higher survival rate of infectious particles at low and high humidity levels has been extensively described in aerosol studies, suggesting that the seasonality observed with the influenza virus might be partly explained by AH (9–11). Studies on the survival of enteric viruses, such as rotaviruses on nonporous surfaces, also showed that viral particles could remain infectious in feces for a long period of time under low- and high-RH conditions, whereas medium-range RH had a deleterious effect on virus survival. Besides the influence of humidity, as the temperature decreased, a protective effect was observed without modifying RH trends (12–14). Several studies on other enteric viruses, such as hepatitis A virus and poliovirus, showed some discrepancies about RH parameters (15–18). However, RH ranging between 40 and 70% usually has a deleterious effect on viral infectivity, while infectious particles were best preserved at low and high RH (19).

The lack of a cell culture for human NoV has hampered investigations of its persistence and inactivation in environment studies. Therefore, caliciviruses, such as feline calicivirus (FCV), and more recently Tulane virus and murine norovirus (MNV) have been used as surrogates to study human NoV (20, 21). It has been found that FCV remained infectious after 7 days of incubation when immobilized on inert surfaces (22). An RH ranging between 30 and 70% was found to affect virus survival in aerosols (23). Few studies have been conducted on MNV to investigate its persistence and survival in the environment and the influence of physical parameters such as humidity and temperature. It has been shown

Received 28 May 2014 **Accepted** 8 September 2014
Published ahead of print 12 September 2014
Editor: K. E. Wommack
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01871-14.

doi:10.1128/AEM.01871-14

that infectivity was better preserved since the virus remained infectious for over a week at 4°C than at room temperature. MNV survival was somewhat similar to FCV survival in that both viruses show a faster decrease in the infectious titer when viral particles were dried (i.e., 75 to 85% RH) on stainless steel coupons than in wet conditions (24). Of note, stool material or foodstuffs have a protective effect on MNV infectivity whatever the humidity and thus also play a role in virus survival (25). The relationship between MNV infectivity and RH suggests that MNV is best preserved at a low RH (16).

For NoVs, though viral and host characteristics could explain part of the NoV epidemiology, the seasonal mechanisms of NoV infection remain unclear. That being said, retrospective analyses of epidemiological data suggest that cold, dry weather along with herd immunity might explain the high number of NoV gastroenteritis cases during winter (26, 27). In addition, previous studies showed that human NoV infections could occur via fomites or aerosols (i.e., vomitus), as reviewed previously (28), and the presence of the NoV genome could be detected for up to 7 days in stool samples stored at room temperature (22). The NoV genome could be recovered from stainless steel, Formica, and ceramic surfaces for long periods of time (29, 30). Unfortunately, the presence of the viral genome does not necessarily correlate with the presence of infectious particles (31–33), and the correlation between genome detection and infectivity is not known for human NoVs. To date, the mechanisms by which environmental parameters could influence human NoV transmission especially during the winter season are still largely unknown. Our objective was to determine the role of humidity parameters in NoV infectivity and to identify the mechanisms by which infectious particles become inactive. We first explored the role of humidity in the survival of MNV, which was used as a surrogate for human NoV. We then studied human NoV resistance to humidity by using virus-like particles (VLPs) that mimic native NoV particles.

MATERIALS AND METHODS

Murine NoV and baculovirus-expressed VLPs. The murine NoV CW1 strain was provided by Herbert W. Virgin (Washington University, St. Louis, MO). Virus propagation, plaque assay, and real-time reverse transcription-PCR (RT-PCR) were performed as described previously (34, 35). An MNV positive control and resuspended MNV particles, which had been recovered from the drying experiment, were frozen in liquid nitrogen and stored at −20°C prior to titration without loss of infectivity during storage (see Fig. S1 in the supplemental material). VLPs from the GII.4 Cairo4 strain, which belongs to the Osaka variants, were used for the binding assay since they have the capacity to bind saliva from secretor and nonsecretor individuals (36, 37). VLPs were produced and purified as described previously (38). The protein content for the VLP preparation was determined using the BCA assay kit (Pierce, Brebieres, France). VLP quality was checked by electron microscopy (EM) after negative staining and SDS-PAGE using NuPAGE gel (Life Technologies, Saint Aubin, France).

Controlled-atmosphere chamber. Sealed boxes containing saturated saline solution were used as the controlled-atmosphere chamber as described previously (39). Salts used for the experiments were lithium chloride, magnesium chloride, magnesium nitrate, and potassium chloride, for equilibration at 10, 35, 55, and 85% RH, respectively. Bidistilled water was used for saturated conditions (i.e., 100% RH). Small electric fans were incorporated in the chamber to speed up equilibration of the chamber and the drying process for the virus suspension. Temperature was recorded throughout the experiment by a USB thermometer/hygrometer (IMH, Rochford, United Kingdom). All of the experiments were carried out in the dark at 25°C ± 1°C.

MNV and VLP drying and RH experiment. Glass coupons were spotted with 20 μl of either MNV clarified cell lysate at $10^{6.2}$ PFU/ml (representing $10^{4.5}$ PFU per assay) or 20 μg of purified GII-4 VLP at 1 mg/ml and immediately placed in a controlled-atmosphere chamber. For each condition, the experiments were performed in triplicate unless otherwise stated. VLP and MNV preparations were first dried for 1 h at 10% RH, unless otherwise stated, at room temperature and were kept at 10% RH or were rapidly transferred to chambers that had been equilibrated at other RHs (35, 55, 85, and 100% RH).

After exposure to different RHs, the glass coupons with MNV were immersed in 3 ml of TGEB buffer (1% beef extract, 50 mM glycine, 100 mM Tris [pH 7.2]) that was agitated at 25°C for 15 min to fully recover the viral particles. The viral suspension was immediately frozen in liquid nitrogen and placed at −80°C until further use. For each time point, 100 μl of the viral suspension was used for MNV titration. One milliliter of Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) was added to 100 μl of the assay. The inoculum was then serially diluted at 2-fold dilution in DMEM–10% FBS prior to cell inoculation. The cells were infected with 1 ml of each dilution, followed by incubation for 1 h at 37°C. A plaque assay was then performed as described previously (35) with a detection threshold of 10 PFU/assay (data not shown). In addition, RNA was extracted from 100 μl of the assay and used for real-time RT-PCR as described previously (34).

For human NoV VLP, a similar protocol to that described for MNV was followed except that 20 μg of VLP suspended (20 μl) in TNC buffer (10 mM Tris, 140 mM NaCl, 10 mM $CaCl_2$ [pH 7.4]) were spotted onto the glass coupons. GII.4 VLPs were maintained at RH ranging from 10 to 100% for 1 week at 25°C after 1 h drying at 10% RH. VLPs that had been dried for 1 h at 10% RH prior to resuspension were used as the positive control (10% RH-1 h) for statistical analysis. Dried material was resuspended in 30 μl of TNC buffer. The protein content was determined for each assay using a BCA protein assay (Pierce, Rockport, IL) prior to freezing in liquid nitrogen until further use. Each sample was used for the SDS-PAGE analysis, EM observation, and saliva binding assay.

Saliva binding assay. For the binding assay, we used a subset of saliva samples, the use of which had previously been approved in previous studies (authorization number BRD02/2-P from the Nantes University Hospital Review Board) (37). The saliva binding assay was performed as described previously (37, 40). Briefly, 1,000-fold-diluted saliva from ABO and nonsecretor individuals was coated overnight on Immulon ELISA plates (Nunc, Roskilde, Denmark). Nonspecific binding sites were quenched with 4% blotto prior to incubating the VLPs. Resuspended VLPs were 2-fold serially diluted in phosphate-buffered saline (PBS) to concentrations ranging from of 250 to 2 ng/well and were incubated for 2 h at 37°C. Attached VLPs were then detected with an in-house conformational GII.4-specific monoclonal antibody (MAb) as described previously (37) (see Fig. S2 in the supplemental material). Each assay was performed in duplicate.

Protein analysis in SDS-PAGE and EM. VLPs that had been dried for 1 week were resuspended in TNC buffer pH 7.4 and examined by EM. Protein analysis was done by SDS-PAGE. One microgram of resuspended VLPs was resolved in a 10 to 20% Tris-glycine (Tris-Gly) polyacrylamide gel in denaturing conditions with morpholinepropanesulfonic acid buffer (Life Technologies) prior to transfer onto 0.45-μm nitrocellulose (Bio-Rad, Marnes-la-Coquette, France). VP1 protein was detected using an in-house GII.4-specific MAb that recognizes the linear epitope (see Fig. S2 in the supplemental material). Bound antibodies were detected with alkaline phosphatase-conjugated antibody. Alkaline phosphatase activity was detected using NBT-BCIP (Roche, Boulogne-Billancourt, France) for 10 min at room temperature. The reaction was stopped with 50 mM EDTA. For electron microscopy, 10 μl of the VLPs was incubated for 5 min at room temperature on a carbon-coated grid before staining with phosphotungstic acid for 1 min. Negatively stained VLPs were observed on a Hi-

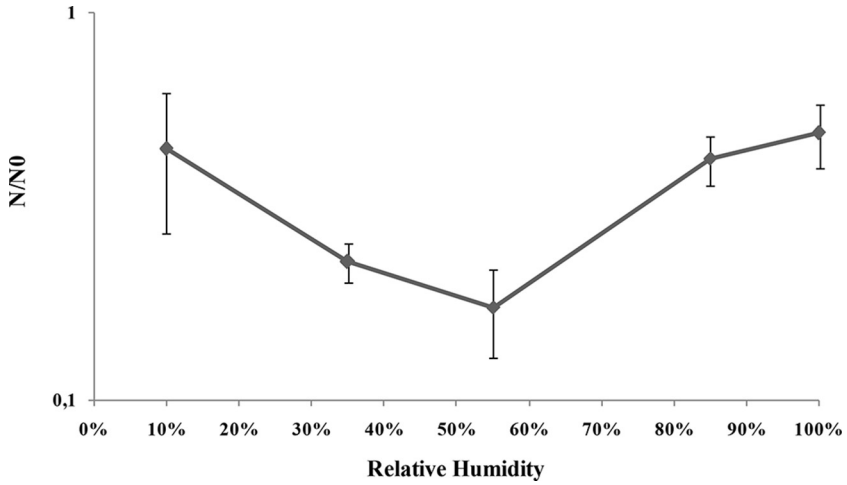

FIG 1 Effect of the RH level on MNV survival during the evaporation step. Droplets (20 μl) were spotted onto glass coupons and directly placed in various RH environments for 1 h for drying. For each experimental condition, *N* represents the mean titer by plaque assay of three independent experiments ($n = 3$). *N0* represents the mean titer of the MNV inoculum before the drying process. The *N/N0* ratio is given as a percentage and is indicated on the ordinate for this figure and the following figures. Error bars represent the standard deviations of at least three repeated experiments.

tachi 7500 transmission electron microscope equipped with an AMT camera driven by AMT software (AMT, Danvers, MA).

Statistical analysis. The statistical analyses were performed with Stata statistical software (Stata Corp., College Station, TX). A Kruskal-Wallis test was used to study the effect of low temperature on MNV infectivity. To study the effect of RH on the binding capacity of VLP to A, B, O, and nonsecretor saliva, fractional polynomials were used to model the VLP-ligand interaction by linear regression.

Meteorological data. To correlate our results with the weather conditions of a temperate country, we used meteorological data from Parc Montsouris meteorological Station in Paris from the Météo France station network. The station is located in a city park. The urban area around the park has not changed for the last 20 years and therefore had no modifying effect on temperature and humidity measurements over time. The minimum and maximum daily temperatures and RH levels for a 14-year period spanning 1 January 2000 through 31 December 2013 ($n = 5{,}113$ days) were plotted on a separate graph for each season (winter from 21 December through 20 March, spring from 21 March through 20 June, summer from 21 June through 20 September, and Autumn from 21 September through 20 December in accordance with Météo France guidelines). The AH was calculated from the RH by using the following equations (41):

$$\mathrm{AH} = \frac{0.622\, p'_\theta\, \mathrm{RH}}{10{,}132{,}500 - p'_\theta\, \mathrm{RH}}$$

AH is expressed in kilograms of water per kilogram of dry air (kg water/kg air). p'_θ is the saturation vapor pressure in pascals at temperature θ (°C). p'_θ was calculated from the following equation:

$$p'_\theta = \exp\left(23.3265 - \frac{3{,}802.7}{\theta + 273.18} - \left(\frac{472.68}{\theta + 273.18}\right)^2\right)$$

For the survey, the extreme (i.e., minimum and maximum) daily AH levels were plotted against the corresponding temperature for each day. The graph was generated using Excel software from Microsoft.

RESULTS

Effects of drying and RH on MNV infectivity. In a preliminary experiment, we observed that a large volume of inoculum (i.e., 100 to 500 μl) markedly extended the time required for complete dehydration of the sample and that this prolonged drying period led to complete inactivation of the MNV at 25°C. Therefore, the inoculum size for each experiment was kept low (e.g., 20 μl) to allow fast equilibration of the sample with the chamber atmosphere. In the next experiment, the glass coupons with MNV suspensions were directly placed in atmosphere control chambers set at various RHs for 1 h (Fig. 1).

The time for complete dehydration of the droplets (time for the disappearance of visible moisture) was RH dependent: the lower the RH, the faster the drying process. MNV inactivation was the highest at 55% RH and the lowest at 10 and 100% RH (44.7% reduction titer). Our data suggested that the removal of water was not responsible for MNV inactivation, but the speed of evaporation was probably involved in the process. Infectious MNV was detected at 85% RH, at which droplets were noticed even after 1 h of drying. We thus concluded that the drying process was not complete and that RH equilibrium between the viral phase and the atmosphere had not been reached. Therefore, infectious MNV present at 85% RH probably came from virus in suspension (i.e., droplets).

Preliminary drying kinetic experiment was performed by weight loss measurements and showed that complete drying was achieved in less than 1 h (data not shown). Moreover, 20 μl of inoculum was completely evaporated within 30 min at 25°C and 10% RH with minor effects on the virus titer (*N/N0* = 44.5% of the initial virus titer; Fig. 1). To avoid artifacts as mentioned above, which are caused by the drying process, the inoculum was first dried at 10% RH and then placed at an RH ranging between 10 and 100% for the subsequent experiments.

The infectivity of MNV was determined after incubation for 1, 3, 6, 12, and 20 h (Fig. 2). The highest recovery of infectious material was obtained for 10% RH. For 55 and 85% RH, a sharp decrease was observed after 6 h of incubation, and no infectious MNVs were detected after 12 h.

We then pursued the experiment by incubating MNV for 2 weeks in a chamber that was maintained at either 10 or 35% RH at 25°C. A viral suspension was used as the control and was stored in the dark for the same period of time. Infectious particles were still detected after 2 weeks of incubation in both conditions (5.41 ± 0.2 $\log_{10}$ PFU/ml at 10% RH; 3.28 ± 0.17 $\log_{10}$ PFU/ml at 30% RH and 3.31 ± 0.23 $\log_{10}$ PFU/ml for the control). The experiment

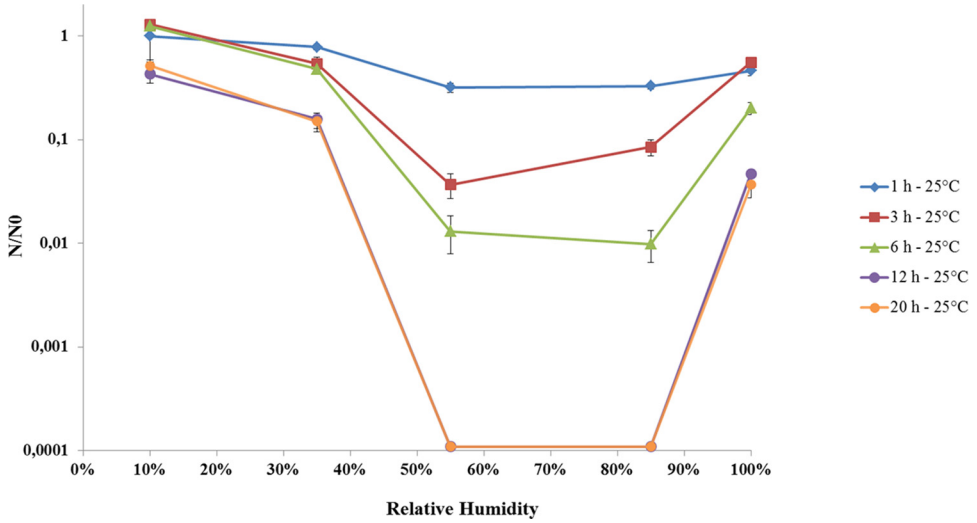

FIG 2 Effect of RH on MNV survival during the equilibrium phase. All of the coupons were dried for 1 h at room temperature in a chamber with 10% RH before being placed in RHs ranging from 10 to 100% (ordinate). Incubation times at room temperature are given in hours and are indicated on the right side of the graph. For each experimental condition, *N* represents the mean titer by plaque assay of three independent experiments ($n = 3$). *N0* represents the mean titer of the MNV inoculum, which had been drier for 1 h at 10% RH prior to resuspension. Error bars represent the standard deviations of at least three individual experiments. Each drying condition is color coded, and the coding system is indicated on the right side of the graph.

was pursued for 3 months at 10% RH and 25°C. Infectious MNV particles were still present after 3 months with almost no decrease in the infectious titer (5.22 ± 0.18 $\log_{10}$ PFU/ml − the initial titer 6.2 $\log_{10}$ PFU/ml). The data showed that low RH had a conservative effect on MNV infectivity, especially at 10% RH.

MNV survival at low temperature. The first experiments showed that low RHs were more suitable to MNV survival. Nevertheless, because RH is temperature dependent, our next objective was to determine whether a lower temperature could affect virus survival. MNV suspensions were first dried for 1 h at 10% RH and 25°C and placed in chambers for 20 h at 9°C (Table 1). The data showed that the MNV titer was always higher at 9°C than at 25°C ($P = 0.0069$, Kruskal-Wallis) (Table 1). Moreover, we found that virus survival after 20 h at 55 and 85% RH at 9°C was significantly higher than that at 25°C. These data demonstrated that a low temperature was favorable for MNV survival.

Viral genome detection. To determine whether humidity could induce genome degradation, we performed MNV-specific real-time RT-PCR analysis on inoculum kept for 1 and 20 h for all RHs (Fig. 3). Viral genomes were almost entirely recovered for all RHs after 1 h of incubation. A reduction in the number of viral genome copies was observed after 20 h of incubation. We observed 0.6- and 0.8-$\log_{10}$ reductions for 10 and 35% RH, respectively. The decrease in genome copies was the highest for 55 and 85% RHs with a 1.4-$\log_{10}$ reduction, while no infectious particles were detected at these RHs. The loss of infectious material while viral genomic RNA was still present suggested that viral inactivation was essentially due to capsid alteration rather than genomic RNA degradation. RNA degradation might then occur after capsid degradation.

Relationship between MNV survival and humidity. At 9°C, AH never exceeded 0.007 kg water/kg air for up to 100% RH and

TABLE 1 Relationship between relative humidity, absolute humidity, and MNV survival[a]

RH (%)	9°C		25°C	
	AH (kg water/kg air)	Mean % survival (*N*/*N0*) ± SD	AH (kg water/kg air)	Mean % survival (*N*/*N0*) ± SD
10	0.0007	63.9 ± 33.9	0.002	51.4 ± 7.0
35	0.002	55.6 ± 15.6	0.007	14.9 ± 3.1
55	0.004	24.3 ± 6.3	0.011	ND
85	0.006	56.4 ± 4.4	0.017	ND
100 (humid fraction)	0.007	59.5 ± 15.4	0.020	3.7 ± 0.9

[a] RH, relative humidity; AH, absolute humidity; *N*, mean titer by plaque assay from three independent experiments under the temperature and relative humidity conditions specified in the table; *N0*, mean titer of MNV following drying for 1 h at 10% RH/25°C; ND, not detected.

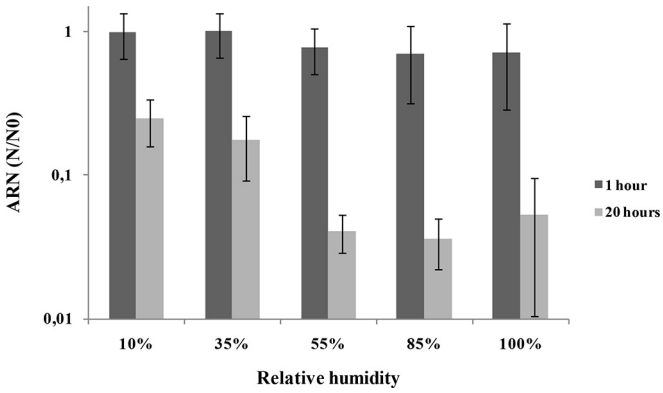

FIG 3 Persistence of the MNV genome under various RH levels. Bars—1 h (dark gray bar) and 20 h (light gray bar)—are defined on the right side of the graph. The ratio between the number of MNV genome copies (*N*), which was determined by real-time RT-PCR, and the *N0* (the number of recovered genome copies after 1 h of drying at 10% RH) is indicated as a percentage (ordinate). Error bars represent the standard deviations of three repeated experiments ($n = 3$).

infectious MNV was observed for all RH (Table 1). At 25°C, AH ranged between 0.002 and 0.02 kg water/kg air. Infectious MNV was detected in atmospheres containing 0.002 (10% RH) and 0.007 (35% RH) kg water/kg air. Inversely, no infectious MNV was detected with 0.011 and 0.017 kg water/kg air corresponding to 55 and 85% RH, respectively. Our data suggested that an AH below 0.007 kg water/kg air probably provides ideal conditions for the survival of infectious MNV particles, whereas an AH above 0.01 kg water/kg air is detrimental to NoV persistence. However, at 100% RH and 25°C, corresponding to 0.02 kg water/kg air, infectious particles were still observed, likely due to the presence of viruses in residual liquid suspension. These data suggest that NoV survival was AH dependent when the atmosphere was not entirely saturated (i.e., 100% RH) and that low AH values were favorable to MNV survival.

Saliva binding assay of human NoV VLPs. Our observations for MNV suggested that the humidity of the air acted primarily on the capsid protein, while the nucleic acid of the viral genome was better preserved. We investigated whether a similar mechanism might alter the binding capacity of the capsid of human NoV VLPs toward human blood group antigens (HBGAs) when exposed to various RH conditions (Fig. 4). The binding capacity of VLPs at different RHs was first compared to those of control VLPs stored at −80°C (control) throughout the experiment and diluted to the same concentration. Binding with saliva from secretor and nonsecretor individuals was significantly higher for the control than for the VLPs dried at all RHs at 25°C ($P \leq 0.0001$), suggesting that maintaining VLPs at an RH ranging between 10 and 85% reduced the binding capacity of NoV VLPs to that observed with VLPs in suspension.

Binding to secretor saliva from A, B, O, and nonsecretor individuals was best preserved at 10% RH. VLP binding to saliva samples from secretor individuals was also observed for 85 and 100% RH, although it was lower than that observed for 10% RH. For 35 and 55% RH, VLP binding to saliva was totally abolished ($0.0001 \leq P \leq 0.02$) for secretor and nonsecretor individuals.

For O and B saliva, similar binding profiles were observed. There was no statistical difference in binding for VLPs that had been stored at 10% RH for 1 h and 1 week. Binding was reduced at 100% RH and was significantly lower at 85% RH ($P = 0.001$). For A saliva, binding for VLP that had been stored for 1 h or 1 week at 10% RH was similar. VLP binding was significantly lower for 85% RH ($P = 0.001$) and 100% RH ($P = 0.031$). VLP binding to nonsecretor saliva was the most labile to drying and storage. Binding to nonsecretor saliva was only observed for 10% RH, although it was markedly reduced ($P = 0.002$). Residual binding was observed for 85% RH and 100% RH ($P = 0.021$ and 0.018, respectively).

Overall, the data showed that the binding properties of human NoV VLPs could be influenced by humidity in the air.

EM of human NoV VLPs. We first performed an SDS-PAGE analysis of the protein content corresponding to each RH. A doublet corresponding to VP1 protein was observed as described previously (38). Specific staining of the VP1 protein after blotting showed no degradation of the VP1 protein (Fig. 5A).

Resuspended VLPs were observed by EM to determine the shape of the VLPs (Fig. 5B). Unscathed VLPs were observed for 10% RH, while a few scathed VLPs in the presence of a large amount of aggregated materials were observed for RHs at 35 and 55%, which was consistent with the reduced binding capacity of VLPs at these RHs.

Nevertheless, we noticed that binding was still observed at 85% RH, even though only amorphous material was observed. This binding might be due to disaggregation of the VLPs into VP1 dimers. For 100% RH, well-structured VLPs were observed, although the binding capacity during the saliva binding assay was only partially preserved, suggesting that inactivation mechanisms beyond particle disassembly were possible. Overall, the EM showed that the capsid structure could be affected by RH ranging from 35 to 85% and that viral particles were best preserved in a very dry atmosphere (e.g., 10%).

Meteorological data. The daily AH and temperature for the last 14 years recorded at Montsouris Park (Paris, France) are plotted for each season (Fig. 6). The vertical line indicates the limit of 0.007 kg water/kg air, and the percentage of data lower than this value is indicated for each season. This limit represents the critical value below which the virus showed properties of high persistence (>2 weeks for MNV). Regarding the mean AH, autumn and spring had a similar intermediate patterns with 0.0066 ± 0.0019 kg water/kg air and 0.0063 ± 0.0020 kg water/kg air, respectively, and 65.3 and 68.9% of the data below 0.0070 kg water/kg air, respectively. The summer and winter seasons showed a markedly different distribution with 0.0089 ± 0.0017 kg water/kg air and 0.0045 ± 0.0014 kg water/kg air on average, respectively. Only 20.3% of the data were below the limit value of 0.007 kg water/kg air for the summer, whereas 96.3% of data were below 0.007 kg water/kg air during the winter.

DISCUSSION

NoV gastroenteritis is often called gastric flu when compared to the marked winter seasonality observed for influenza since epidemiological data show a winter peak for infection. NoV outbreaks are often reported indoors, where multiple modes of transmission through direct contact between people or food or via contaminated surfaces occur (1, 3). In some cases, prolonged outbreaks of gastroenteritis have been related to NoV persistence in semiclosed environments (e.g., airplanes, restaurants, and concert halls), and the risk associated with airborne transmission (e.g., vomit or toilet flushing) and the deposit of viral particles on various surfaces has been increasingly described in the literature (42–44). It is nevertheless difficult to determine the contribution of environmental factors to NoV transmission (1). Our study sought to identify the conditions that foster NoV persistence and that, as a result, might enhance the risk of environmentally mediated transmission by focusing on NoV seasonality.

We used cultivatable MNV as a surrogate and human NoV VLPs, which mimic the binding properties of native human NoV particles. We acknowledge that MNV and human NoV VLPs are not ideal surrogates of human NoVs. However, MNV is a useful tool in the absence of cell culture for human NoV because it is cultivatable and it belongs to the same genus as human NoVs (28). It should be noted that MNV has often been used as a substitute in virucidal assays to assess by extrapolation human NoV resistance to antiseptics or survival in water (34, 45). Because MNV and human NoV attachment factors are different, we used human NoV-derived VLPs to study NoV binding properties after long-term storage in a controlled atmosphere to complete our infectivity assay with MNV. Of note, VLPs have recently been used successfully as an NoV substitute for environmental studies (46).

For the first experiments, we observed that the drying step was critical for virus survival. The evaporation process should be kept

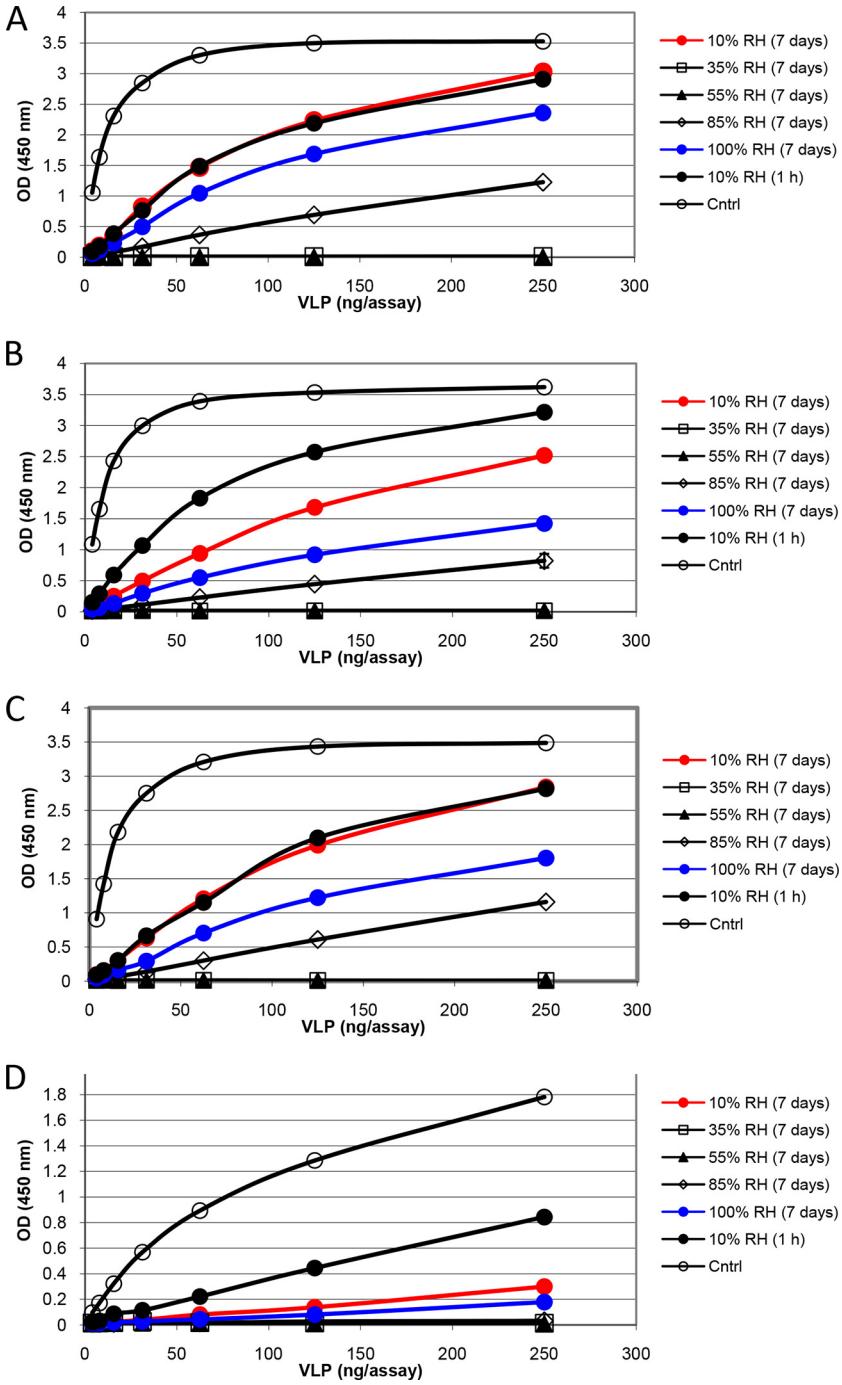

FIG 4 Saliva binding assays of the GII.4 Osaka variant (Cairo4 strain). VLPs had been dried for 1 week under different conditions at room temperature and then resuspended in PBS at 500 ng/μl. Twofold serial dilutions of resuspended VLPs were tested on 1,000-fold-diluted saliva samples from individuals that were typed for secretor (A to C) and nonsecretor (D) phenotypes. Saliva samples from O (A), A (B), B (C), and nonsecretor (D) individuals were characterized for the presence of the ABO and Lewis antigens, as documented previously (61). For the positive control, purified VLPs were diluted in PBS and stored at −80°C for 1 week prior to being used for the binding assay. The binding experiments were performed in duplicate for each sample, and the mean values are given on the graph (optical density at 450 nm [OD_{450}], ordinate). Each RH is color coded, and the coding system is indicated on the right side of the graph with the incubation period indicated in parentheses.

as short as possible with the lowest RH. Regarding MNV persistence at 10 to 35% RH and 25°C, most of the MNV particles remained infectious for weeks to months. With increasing RH, we observed a decrease in the virus titer as noted previously (16). Similar observations have been made for FCV (23), rotavirus (12, 14, 47), influenza virus (9, 10), and coronavirus (48). The marked impact of humidity parameters on the fate of viral integrity is largely documented in the literature and confirmed in our study

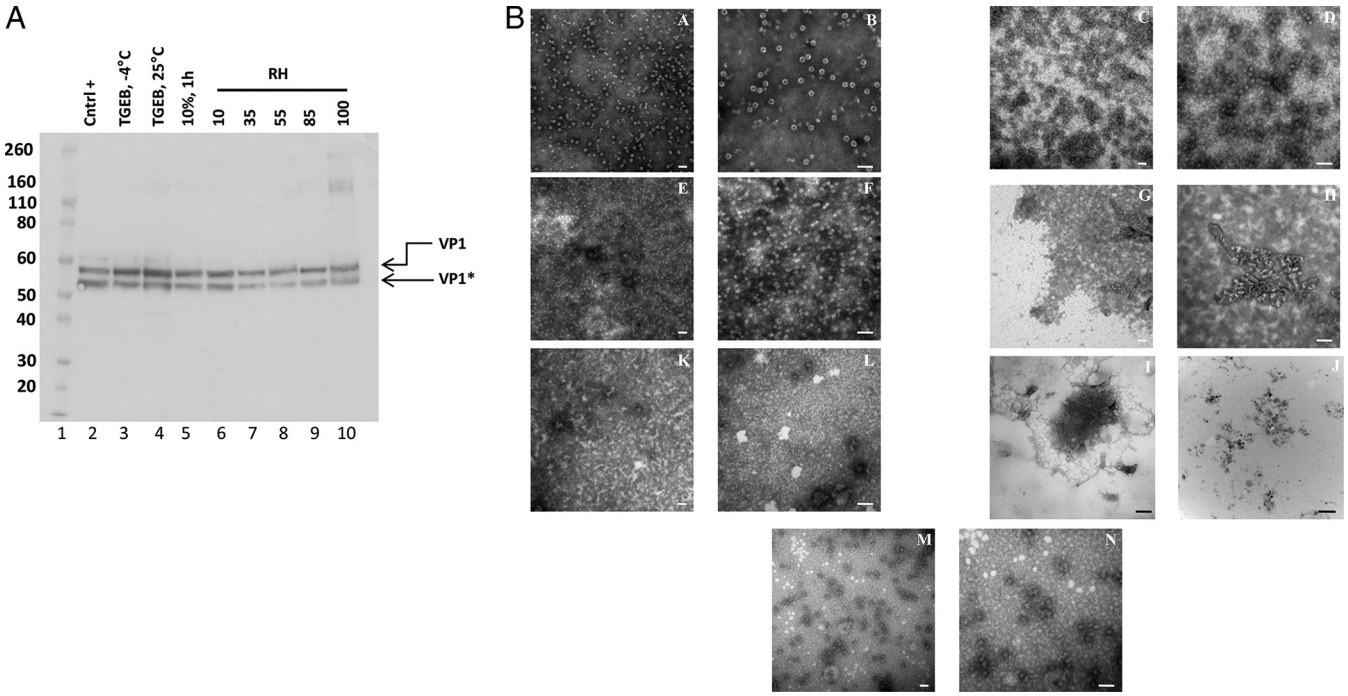

FIG 5 (A) SDS-PAGE of the VLP from the saliva binding assay. One aliquot of 1 μg each of the VLPs that had been resuspended in buffer after 1 week of drying under different RHs was resolved under denaturing conditions by SDS-PAGE using NuPAGE bis-Tris gel. VLPs were diluted in TGEB buffer and directly resolved by SDS-PAGE (lane 2) or stored at −40°C (lane 3) or 25°C (lane 4). For the drying experiments, VLP were dried under 10% RH at 25°C for 1 h (lane 5) and 1 week (lane 6). For 35 to 100% RH, VLP were first dried for 1 h at 10% RH and then immediately placed in RHs that are indicated above the gel (lanes 7 through 10). Resolved proteins were then transferred onto a nitrocellulose membrane prior to immunoblotting. VP1 protein was labeled with in-house specific MAbs which were detected with anti-mouse alkaline phosphatase-labeled antibody. Alkaline phosphatase activity from the mouse-specific alkaline phosphatase-labeled secondary antibody was detected with NBT-BCIP solution. The complete (VP1) and truncated (VP1*) capsid proteins for GII.4 VLPs, indicated by arrows, have been described (38). Lanes corresponding to RH levels and controls are indicated above the gel. The molecular mass (in kilodaltons) (lane 1) is indicated on the left side of the gel. (B) Electron micrographs of VLPs after storage at 25°C under various RH levels. VLPs were dried for 1 h at 10% RH before being placed at the various RH levels. Subpanels: A and B, control, C and D, drying at 10% RH for 1 h; E and F, 1 week at 10% RH; G and H, 1 week at 35% RH; I and J, 1 week at 55% RH; K and L, 1 week at 85% RH; M and N, 1 week at 100% RH. Preparations were observed at ×80,000 and ×150,000 magnification. Scale bars correspond to 100 nm, except for panel J (2 μm).

for MNV and human NoV VLPs. However, NoV persistence on fomites is often discussed without considering the RH.

Our experiments showed that MNV can be fully inactivated in 12 h at 55 and 85% RH (Fig. 2), while viral RNA was still detectable by real-time RT-PCR (Fig. 3). These data suggest that humidity primarily acts on capsid structure. Because the capsid protects genomic RNA from the environment, we assumed that capsid degradation led to virus inactivation and was very probably followed by degradation of unprotected viral RNA. These results were later confirmed by the VLP analysis. VLP binding to HBGAs was RH dependent. Despite the fact that viral RNA is absent from VLPs, our data demonstrated that the capsid structure was well preserved. The presence of VLPs correlated with strong binding properties at extreme RH levels (i.e., 10 and 100% RH), as was the case for the MNV infectivity assay. At 25°C, 55% RH was detrimental to MNV infectivity and VLP binding. However, residual VLP binding was observed at 85% RH, while no infectious MNV particles were observed. That being said, VLP binding might be due to the presence of a functional VP1 dimer, corresponding to the amorphous materials observed by EM in the absence of complete VLP. One could then hypothesize that 85% RH at 25°C would also induce major changes in native human NoV. We finally observed that MNV was still infectious at 35% RH and 25°C, while VLP binding was totally abolished. This discrepancy might be explained by minute structural changes, possibly induced by the absence of genomic RNA in human NoV VLPs. Capsid alteration might be due to various concomitant physical and chemical events, such as VLP dissociation and/or chemical modifications of amino acids due to the Maillard reaction or oxidization, which are well known to destabilize protein structure, especially in the mid-range RH levels. These mechanisms could lead to protein destabilization and subsequently to denaturation. Inversely, dehydration, down to low water content levels, generally enhances protein preservation during storage (49–51).

At the molecular level, it has been demonstrated that amino acid variation in the P1 and P2 domains might change the binding profile of GII.4 NoV (52, 53). Our data clearly showed that variations in the level of RH might also influence NoV binding patterns to HBGA ligands. Therefore, RH variations probably influence NoV binding capacity, and the size of the population that could become infected will thus change. In addition, binding to nonsecretor saliva was the most labile even at low RH, which suggests that GII.4 infection of nonsecretor individuals mostly occurs through direct person-to-person transmission. Further experiments will be required to determine whether the properties of genogroup I NoV and other genotypes with regard to RH are similar to those of GII.4 NoV.

Since the discovery of NoV, there have been countless reports

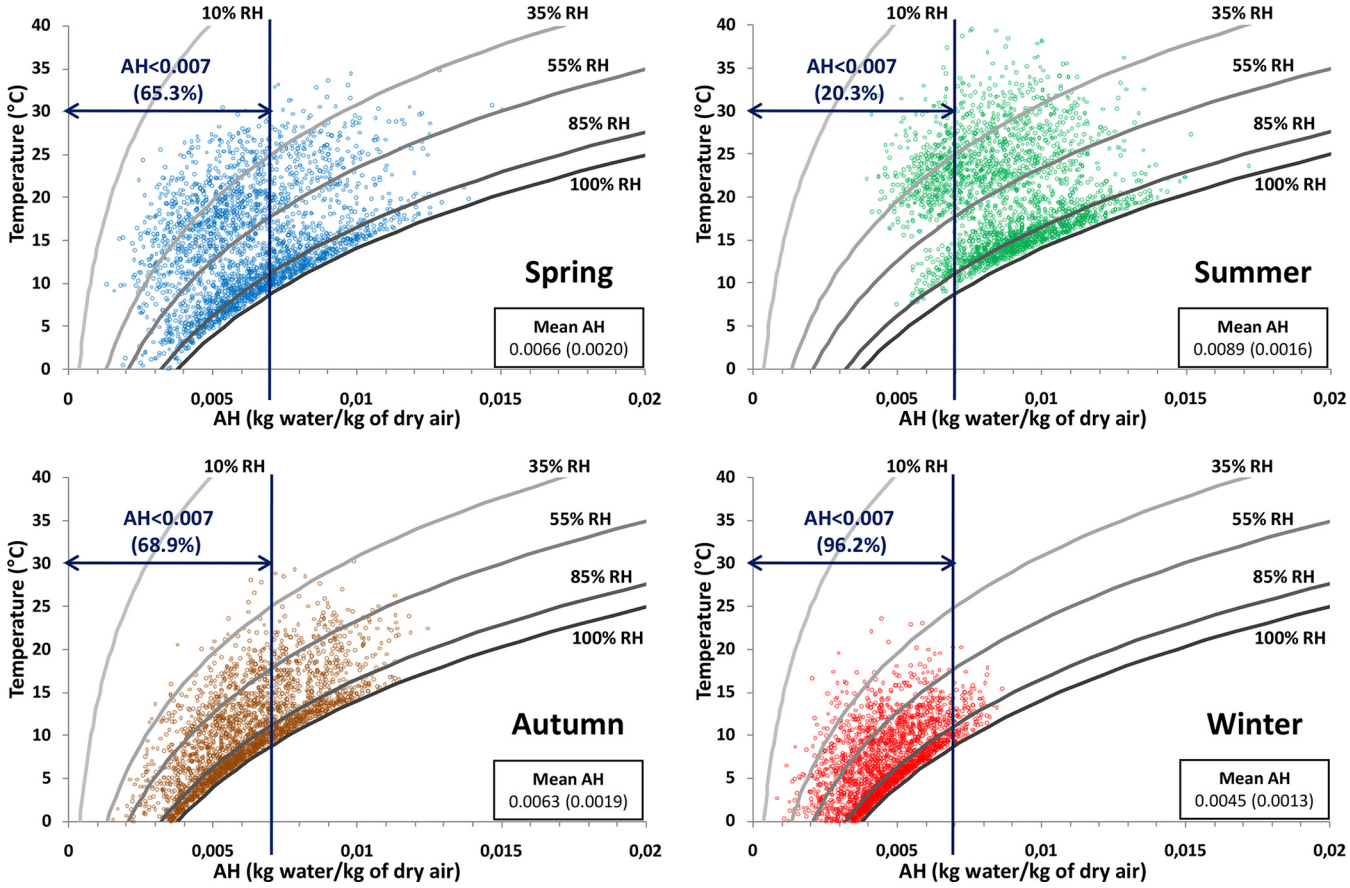

FIG 6 Distribution of daily temperature and AH in Montsouris Park, Paris, France, between 2000 and 2013. Temperature (expressed in °C) and AH (expressed in kg water/kg air) are indicated on the ordinate and abscissa, respectively. Minimal and maximal daily temperatures and corresponding AH are indicated by dots on the graph. Each dot corresponds to 1 day. Th RH is indicated by a curve on the graph and is plotted against temperature and AH. A vertical line is drawn across the graph for AH corresponding to 0.007 kg water/kg air. The percentage of days for the 14-year survey for which AH was below 0.007 kg water/kg air is indicated on the graph for each season: spring, blue dots; summer, green dots; autumn, brown dots; and winter, red dots.

showing that NoV-related gastroenteritis mostly occurred during the winter season in temperate countries (28, 54). A review of epidemiological and meteorological surveys suggested that outdoor temperature, dry conditions, and NoV outbreaks are to some degree related to NoV transmission (27).

Recently, a number of studies have highlighted the potential use of AH rather than RH to explain the seasonality of some infections. The transmission of influenza virus, for example, has been related to AH rather than RH and temperature factors in experimental (6, 7) and epidemiological studies (8, 55). As mentioned above, most NoV outbreaks are reported in closed or semiclosed environments. Because people spend most of their time indoors (even more so during the cold season), outdoor conditions may be a poor indicator of human exposure to humidity parameters (56). Unlike RH, AH parameters show a high correlation ($R^2 = 0.96$) between indoor and outdoor environments all year round (56).

MNV remained infectious at all RHs at 9°C, while infectious particles were only detected at 10 and 35% RH at 25°C (Table 1). A 10 to 100% RH at 9°C and 10 and 35% RH at 25°C correspond to AHs ranging from 0.0007 to 0.007 kg water/kg air. Therefore, we hypothesize that an AH below 0.007 kg water/kg air has a strong preservative effect on viral particles whatever the temperature considered. Inversely, our data suggest that AH from 0.007 to 0.010 kg water/kg air is detrimental for NoV infectivity and persistence for temperature ranging from 10 to 25°C and RHs ranging from 35 to 85%. A completely water-saturated atmosphere (i.e., 100% RH) is comparable to a liquid suspension in which human NoV and MNV remain infectious for a long period of time, as shown earlier (45, 57).

We may then hypothesize that winter conditions and especially low AH (96.3% of the day with AH $<$ 0.007 kg water/kg air, mean AH = 0.0045 kg water/kg air in winter) corresponding to a temperate climate such as in Paris provide the ideal conditions for keeping human NoV infectious whether indoors or outside. The mid seasons, spring and autumn, provide intermediate AH conditions, whereas summer is characterized by a substantial rise in both temperature and AH (20.3% of the day with AH $<$ 0.007 and 0.009 kg water/kg air on average), which could greatly affect NoV persistence and transmission from the environment.

A recent report suggested that small seasonal shifts in influenza virus transmission (i.e., contact rates and survival of pathogen) combined with the population's susceptibility to infection (drift in viral antigens, low herd immunity) could generate a large seasonal incidence because of amplification by dynamic resonance (58, 59). For NoVs, it has been reported that herd immunity very

probably plays a major role in the emergence of new GII.4 variants. Our present data support a similar scenario for the noroviruses: low winter AH provides favorable conditions for NoV transmission and thus increases the prevalence by dynamic resonance, as observed regularly for the emergence of new NoV variants, and as exemplified by the rapid global spread of the recent 2006b GII.4 NoV variant (60).

Even though NoV and influenza virus are structurally different, both viruses show striking similarities in terms of seasonality, variant changes, and the preponderant role of immunity. The role of AH in the seasonality of influenza virus has recently been documented, and the impact of AH parameters on NoV viability may also contribute to the long-lasting pathogenicity of NoV particles present in the environment. AH may better reflect the higher persistence of infectious particles at low temperatures and low humidity and should therefore be used in epidemiological studies of NoVs that try to relate humidity to epidemiological data. Animal models (i.e., mouse, pig, or calf) may be of some use for the study of NoV transmission by using NoV surrogates, as previously described for influenza virus (6).

The accurate measurement of environmental parameters such as AH (i.e., calculated from RH and temperature parameters) and temperature during NoV outbreaks will bring new insights into the epidemiology and environmental transmission of NoV. By taking humidity into account, it should be possible to better define risks associated with NoV infection.

ACKNOWLEDGMENTS

This study was funded by a grant from the French National Agency for Research (Agence Nationale de la Recherche, project SPICECLEAN). This study was partly supported by the National Reference Center for Enteric Viruses, the Public Hospital of Dijon, and the University of Burgundy.

CW1 MNV strain was kindly provided by Christiane Wobus and Herbert Virgin (Washington University, St. Louis, MO). Saliva samples were kindly provided by Jacques Le Pendu (INSERM 892/CNRS 6299, Institut de Recherche en Santé de l'Université de Nantes, Nantes, France). We thank Météo France (Paris, France) for providing meteorological data and Yves Richard from the University of Burgundy for administrative and technical support. We thank Kim Y. Green (National Institute for Allergy and Infectious Diseases, Bethesda, MD) for scientific comments on the manuscript. We thank Pia Villa (Mario Negri Institute, Milan, Italy) for providing the RAW cells. We thank Jeannine Lherminier (DImaCell, INRA, Dijon, France) and Philip Bastable (Public Hospital of Dijon, Dijon, France) for technical support with the EM and for editorial assistance, respectively.

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