Applied and Environmental Microbiology

Effects of Temperature, Relative Humidity, Absolute Humidity, and Evaporation Potential on Survival of Airborne Gumboro Vaccine Virus

Yang Zhao, Andre J. A. Aarnink, Remco Dijkman, Teun Fabri, Mart C. M. de Jong and Peter W. G. Groot Koerkamp

Appl. Environ. Microbiol. **2012, 78(4):1048. DOI: 10.1128/AEM.06477-11.**
Published Ahead of Print 9 December 2011.

AEM
Journals.ASM.org

Effects of Temperature, Relative Humidity, Absolute Humidity, and Evaporation Potential on Survival of Airborne Gumboro Vaccine Virus

Yang Zhao,[a,c,d] Andre J. A. Aarnink,[a] Remco Dijkman,[b] Teun Fabri,[b] Mart C. M. de Jong,[c] and Peter W. G. Groot Koerkamp[a,d]

Wageningen UR Livestock Research, Lelystad, the Netherlands[a]; Animal Health Service (GD), Deventer, the Netherlands[b]; Quantitative Veterinary Epidemiology, Wageningen University, Wageningen, the Netherlands[c]; and Farm Technology Group, Wageningen University, Wageningen, the Netherlands[d]

Survival of airborne virus influences the extent of disease transmission via air. How environmental factors affect viral survival is not fully understood. We investigated the survival of a vaccine strain of Gumboro virus which was aerosolized at three temperatures (10°C, 20°C, and 30°C) and two relative humidities (RHs) (40% and 70%). The response of viral survival to four metrics (temperature, RH, absolute humidity [AH], and evaporation potential [EP]) was examined. The results show a biphasic viral survival at 10°C and 20°C, i.e., a rapid initial inactivation in a short period (2.3 min) during and after aerosolization, followed by a slow secondary inactivation during a 20-min period after aerosolization. The initial decays of aerosolized virus at 10°C (1.68 to 3.03 ln % min^{-1}) and 20°C (3.05 to 3.62 ln % min^{-1}) were significantly lower than those at 30°C (5.67 to 5.96 ln % min^{-1}). The secondary decays at 10°C (0.03 to 0.09 ln % min^{-1}) tended to be higher than those at 20°C (−0.01 to 0.01 ln % min^{-1}). The initial viral survival responded to temperature and RH and potentially to EP; the secondary viral survival responded to temperature and potentially to RH. In both phases, survival of the virus was not significantly affected by AH. These findings suggest that long-distance transmission of airborne virus is more likely to occur at 20°C than at 10°C or 30°C and that current Gumboro vaccination by wet aerosolization in poultry industry is not very effective due to the fast initial decay.

Epidemics of viral diseases in livestock production have triggered research on airborne transmission as a possible mechanism for spreading disease between farms (11, 24). However, this mechanism is still not well understood, because many parameters relating to the fate of viruses in the processes of airborne transmission have not been extensively investigated. The survival of airborne viruses, one of the most important parameters, determines the impact of airborne transmission and infection in the recipient animals (27).

Among the significant factors affecting the survival of viruses during transportation in ambient air are temperature and humidity. The effect of temperature and humidity on viral survival has been frequently investigated since the 1950s. Initially, the metric used most often for air moisture was relative humidity (RH), which is the ratio between the actual water vapor pressure (VP) of the air and the water VP of saturated air at a certain temperature (1, 2, 16, 17). Later, absolute humidity (AH), i.e., the actual water content of the air, was introduced and was found to constrain the survival of some species of airborne virus more significantly than the RH (23, 31). The effect of humidity on microbial survival can also be analyzed with other metrics for air moisture, such as evaporation potential (EP), which is the numeric difference between the actual water VP in the air and the water VP in saturated air. The question is which metric is the best predictor of viral survival, and the answer might depend on the species of virus. Gumboro virus, known as the infectious agent of infectious bursal disease (IBD) in poultry, is a nonenveloped double-stranded RNA (dsRNA) virus (13) that may lead to immunosuppression and mortality in young chickens (36, 37) and cause huge economic losses (21). This virus was recently detected in the air of a broiler room (our unpublished data), suggesting a potential risk of disease transmission through air. However, information on the viral survival at different temperatures and humidities is limited.

To study microbial survival, suspensions containing microorganisms can be aerosolized into an enclosed air space and the aerosolized microorganisms collected with bioaerosol samplers at different moments (10, 15). The levels of viable microorganisms collected at these moments are compared and expressed in several measures of survival, such as decay rate, survival rate, or half-life (7, 25, 34). During aerosolization and the intervals between air-sampling moments, the bioaerosols inevitably deposit and/or impact onto surfaces. This physical elimination of bioaerosols from the air is not ascribable to the biological response of microorganisms and should therefore be excluded when calculating the microbial survival rate. The physical elimination can be determined by aerosolizing microbial suspension together with a tracer. The fluorescent compound uranine (also known as fluorescein sodium) is a popular tracer due to its advantages in use: it is harmless to many microorganisms (44), and it is detectable at very low concentrations. The disadvantage of using uranine is that an extra infectivity test is necessary beforehand, to ensure that microbial survival is not affected by adding uranine. An alternative tracer is the genetic materials (DNA or RNA) of microorganisms, which can be quantified with the PCR technique. Because the genetic materials originate from microorganisms themselves, no additional tracer is required and infectivity control is not necessary. However, the suitability of using genetic materials quantified by the PCR technique as microbial tracer has seldom been investi-

Received 7 August 2011 **Accepted** 3 November 2011
Published ahead of print 9 December 2011
Address correspondence to Andre J. A. Aarnink, andre.aarnink@wur.nl.

doi:10.1128/AEM.06477-11

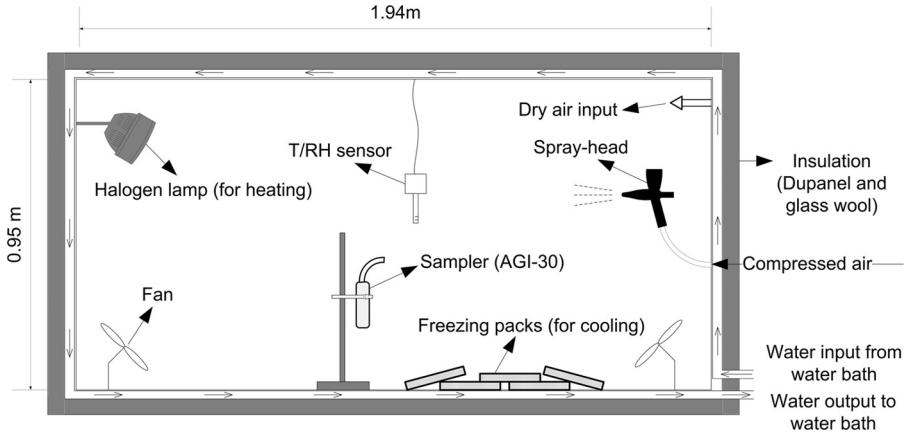

FIG 1 Drawing of the isolator system. Halogen lamp (for heating) and freezing packs (for cooling) were used when the measured temperature did not fully fit the required temperature.

gated. A drawback of the PCR technique is that the genetic material might also be degraded when exposed to environmental and sampling stresses (38, 39). For instance, the viral genetic material degrades at high temperature (8, 26) and under UV radiation (35).

The objective of this study was to investigate the survival of Gumboro virus aerosolized in an isolator at three temperatures (10°C, 20°C, and 30°C) and two RHs (40% and 70%). The climate metrics, i.e., temperature, RH, AH, and EP, were evaluated with respect to their suitability for predicting the survival of airborne virus. In addition, viral RNA quantified with the PCR technique was used to indicate the physical elimination of bioaerosols, and its efficacy as a tracer was compared with that of uranine. For safety reasons, a vaccine strain was used instead of the virulent Gumboro virus.

MATERIALS AND METHODS

Viral suspension for aerosolization. Gumboro vaccine virus (L258577; Gallivac IBD) was purchased from Merial, Velserbroek, Netherlands. One vial contained approximately 7 $\log_{10}$ 50% egg infective doses (EID_{50}s). One viral suspension was made by diluting virus from two vials in 5 ml Hanks' balanced salt solution (HBSS; Gibco, Breda, Netherlands). One liter of HBSS contains 8,000 mg NaCl, 1,000 mg D-glucose, 400 mg KCl, 350 mg $NaHCO_3$, 60 mg KH_2PO_4, 48 mg Na_2HPO_4, and 10 mg phenol red. In a previous study (Y. Zhao, A. J. A. Aarnink, W. Wang, T. Fabri, P. W. G. Groot Koerkamp, and M. C. M. de Jong, submitted for publication), it was shown that 0.1% uranine addition did not harm Gumboro vaccine virus infectivity. Therefore, in the present study, 0.1% uranine was added to all viral suspensions.

Quantification of infective virus. Virus survival in a liquid sample was quantified by performing an egg embryonic death test to determine the level of virus that remained infective. The liquid sample was first decimal diluted (10^{-1}, 10^{-2}. . .). A volume of 0.5 ml of each decimal dilution was injected into the allantoic cavities of five 9-day-old specific-pathogen-free (SPF) egg embryos. The inoculated eggs were incubated at 37°C for 7 days, and the viral concentration was calculated from the death of the embryos and specific abnormalities of the living embryos by using the method of Spearman and Karber (16a, 33). The Spearman-Karber method obtains an estimate of a weighted average of the midpoints between successive $\log_{10}$ viral concentrations (14). The uncertainty of this estimation is from 0.03 to 0.1 $\log_{10}$ EID_{50} ml^{-1} (14). The final viral concentrations were approximately 6 to 7 $\log_{10}$ EID_{50} ml^{-1} in the viral suspensions for aerosolization and 0.5 to 3 $\log_{10}$ EID_{50} ml^{-1} in liquid air samples. The airborne viral concentrations, ($\log_{10}$) EID_{50} m^{-3}, were obtained by dividing the level of collected virus in the liquid samples by the air volumes drawn in through the bioaerosol samplers.

Quantification of viral RNA by PCR technique. Viral RNA for real-time reverse transcription-PCR (RT-PCR) amplification was extracted from 0.1 ml of sample with the MagMAX express system (Applied Biosystems by Life Technologies Corporation, Carlsbad, CA) using the MagMAX viral RNA isolation kit (AM1836), following the protocols recommended by the manufacturer. The primers and probe were developed from the VP2 sequence of the Gumboro vaccine virus (3). Their sequences were as follows: forward primer IBDV-gal-F1, 5′-TGA TGG GAC AAC GGT AAT CAC C-3′; reverse primer IBDV-gal-R1, 5′-AGG TCA CTA TCT CCA GTT TGA TGG-3′; and probe IBDV-gal-P1, 6-carboxy-fluorescein (6-FAM)-CCG CAA ACA ATG GGC TGA CGA CCG-BHQ1. PCR was performed on the Applied Biosystems 7500 real-time PCR system using the AgPath-ID one-step RT-PCR reagents (Applied Biosystems by Life Technologies Corporation, Carlsbad, CA) and the primers and probe. Viral RNA amplification was a reverse transcription at 45°C for 30 min, followed by enzyme activation at 95°C for 15 min and then 45 cycles of denaturation at 95°C for 20 s and a combined annealing/extension step at 60°C for 60 s.

Sensitivity of the PCR was determined using a 10-fold dilution series of the Gumboro vaccine virus containing 2.5×10^6 EID_{50} ml^{-1} as was determined by titration using the egg embryonic death test. Serial dilutions ranging from 2.5×10^{-3} EID_{50} ml^{-1} to 2.5×10^6 EID_{50} ml^{-1} were prepared in triplicate in HBSS. Viral RNA in serial dilutions was extracted and subjected to PCR, and the standard curve was constructed using the dilution steps which were positive in the PCR. The PCR efficiency was expressed as $10^{(-1/\text{slope})}$.

Uranine analysis. Uranine (CAS#518-47-8) was purchased from Fisher Scientific, Landsmeer, Netherlands. The uranine was analyzed with a fluorescence detector (HP 1046 A; Hewlett-Packard) that had a detection limit of 0.002 μg ml^{-1}. The excitation and emission wavelengths for uranine were chosen as 494 nm and 521 nm, respectively. A series of reference uranine solutions (of known concentration) were measured with the detector before analyzing the samples, and a standard curve was made which showed the relationship between uranine concentration and fluorescence intensity. The uranine concentrations in samples were determined by comparing their fluorescence intensities with the standard curve.

Isolator and insulation. A stainless steel isolator (Beyer and Eggelaar, Utrecht, Netherlands) of 1.38 m^3 (1.95 m × 0.75 m × 0.95 m) was used (Fig. 1). HEPA filters were installed at the air inlet and outlet of the isolator. To reduce heat transfer, the isolator was insulated by Dupanel boards and glass wool. A 50-m-long plastic tube was entwined between the glass

of the isolator and the insulation materials, and water from a water bath was circulated through it. The temperature of the circulating water could be adjusted from 0 to 100°C as required. This heating and insulation system maintained the air inside the isolator at the required temperature during the experiment.

Aerosolization. A Walther Pilot spray head (Walther Spritz- und Lackiersysteme, Wuppertal, Germany) connected to an air compressor (Mecha Concorde type 7SAX, 1001; SACIM, Verona, Italy) was used to aerosolize 5 ml of viral suspension each time. The duration of aerosolization was about 20 s, with an air pressure of 2×10^5 Pa. The aerosol size distribution of the spray head was characterized by laser diffraction (Mastersizer-S long bed; Malvern Instruments, Malvern, United Kingdom); the volume median diameter of the sprayed aerosol near the spray head was approximately 10 μm. When aerosols were expelled away from the spray head, the aerosols shrank into small ones due to evaporation. A spectrum of the aerosol sizes, measured by a particle counter (Grimm 1.109; Grimm Aerosol Technik GmbH and Co. KG, Ainring, Germany), in a 20-min experimental run can be found in our previous studies (44, 45). Two small fans were used to uniform the viral aerosols.

Temperature and humidity. Survival of aerosolized Gumboro vaccine virus was investigated at three temperatures (10°C, 20°C, and 30°C) and two RHs (40% and 70%). A combination of temperature and humidity level was called a treatment; all combinations were tested, and each treatment was repeated four times. So, in total there were 24 aerosolization events.

When the measured temperature did not exactly match with the required value it was adjusted by a halogen lamp (for heating) or by freeze packs (for cooling). The required RH was achieved by spraying a certain amount of HBSS medium into the dry air inside the isolator. The amounts that needed to be sprayed were calculated with the Vaisala humidity calculator (version 2.1; Vaisala, Vantaa, Finland). All these adjustments were done before aerosolization. The temperature and RH values were recorded by a sensor (HygroClip2; Acin Instrumenten, Rijswijk, Netherlands) every 6 s. The actual mean temperature values in all treatments during the experiments deviated by less than 1°C from the levels required, and the RH values fell within 5% of the levels required.

Water VP (kPa) was used as the measure of AH; water VP deficit (VPD) (kPa) was used as the measure of EP. VPD was calculated as the difference between actual VP in the air and the VP in the saturated air. VP and VPD were calculated from the measurements of actual temperature and RH. To check the significance of temperature and humidity on viral survival, the viral survival (both initial and secondary survival; see "Survival rate of aerosolized virus" below) was regressed over the above-mentioned metrics.

Bioaerosol sampler and sampling. The all-glass impinger (AGI-30) was used to sample aerosolized virus. In this experiment, the AGI-30 was filled with 20 ml of HBSS containing 0.005% silicone antifoam (85390; Sigma-Aldrich Inc., Netherlands) as the collection medium. Two identical AGI-30 samplers were placed in the middle of the isolator before the experiment started. One of the AGI-30 samplers took samples immediately after aerosolization, and the other took samples 20 min after aerosolization. Each sampling lasted 2 min with a sampling flow rate of 12.5 liter min^{-1}. To clean the air inside the isolator, the isolator was ventilated at 70 m^3 h^{-1} for 2 h between two aerosolization events. This ventilation scheme has been proven to be able to effectively reduce residue bioaerosols to an undetectable level (19, 20).

Survival rate of aerosolized virus. Previous studies have shown that microorganisms are inactivated at two different rates (2, 30). In the first few seconds or minutes after aerosolization, the inactivation rate is high; this is followed by a lower inactivation rate. Accordingly, in our experiment, the survival of Gumboro vaccine virus at each temperature and RH level was expressed in two phases: initial survival and secondary survival. The initial survival rate (S_0) was determined using equation 1 by comparing the virus/tracer ratio (EID_{50} μg^{-1}) in an air sample collected immediately after aerosolization to the ratio in the viral suspension (with either uranine or the RNA analyzed by PCR as the tracer):

$$S_0 = \frac{C_{virus,\ sample} / C_{tracer,\ sample}}{C_{virus,suspension} / C_{tracer,\ suspension}} \times 100\% \quad (1)$$

where $C_{virus,\ sample}$ is the concentration of virus in air sample collected immediately after aerosolization (EID_{50} ml^{-1}), $C_{tracer,sample}$ is the concentration of tracer in air sample collected immediately after aerosolization (μg ml^{-1} [uranine] or $TCID_{50}$ ml^{-1} [RNA]), $C_{virus,suspension}$ is the concentration of virus in viral suspension (EID_{50} ml^{-1}), and $C_{tracer,suspension}$ is the concentration of tracer in viral suspension (μg ml^{-1} [uranine] or $TCID_{50}$ ml^{-1} [RNA]).

The survival rate of virus at 20 min after aerosolization (S_{20}) was calculated in the same way as S_0, i.e., by dividing the virus/tracer ratio in the air sample at 20 min by the ratio in the viral suspension. Then, the secondary survival (S_{0-20}) was determined by equation 2:

$$S_{0-20} = S_{20} / S_0 \times 100\% \quad (2)$$

For many microorganisms, the slope of the natural logarithm survival rate (ln %) over time generally fits nicely to a linear plot and has been termed the "decay constant" (9). In our study, the initial decay constant (k_0) and secondary decay constant (k_{0-20}) were determined from S_0 and S_{0-20} (equation 3). As noted above, the aerosolization of 5 ml of suspension lasted for approximately 0.3 min (20 s), and the first sampling immediately after aerosolization lasted 2 min; therefore, the time denominator for k_0 was set to 1.2 min (half of 2.3 min). The time denominator for k_{0-20} was 20 min.

$$k_i = (\ln 100 - \ln S_i) / t \quad (3)$$

where k_i is the decay constant (i is "0" or "0–20"), S_i is the survival rate (i is "0" or "0–20"), and t is the time denominator ($t = 1.2$ min [when i is "0"] or 20 min [when i is "0–20"]).

RESULTS

The average threshold cycle (C_T) values obtained from the PCR on the dilution series of the Gumboro vaccine virus were plotted against the $\log_{10}$ of the dilution, and the linear equation for the PCR was $y = 3.68x + 10.64$. Using the slope from the linear equation, a PCR efficiency of 87% was obtained and the quantitative range was at least 6 $\log_{10}$ dilution steps with coefficients of regression exceeding 0.99. The detection limit of the PCR using 0.1 ml as the input for viral RNA extraction is the PCR equivalent of 25 EID_{50} ml^{-1} (L258577; Gallivac IBD).

Table 1 shows the virus survival rates and corresponding decay constants, which were corrected either for RNA with PCR techniques or for uranine to exclude physical deposition of aerosolized virus, at different temperatures and RHs. There was a pronounced decrease of initial survival at all the temperatures and RHs. The decrease was especially striking at 30°C: only 0.1 to 0.2% of the aerosolized virus remained infective. At 10°C and 20°C, the secondary survival of virus was 7.4 to 76.4% when corrected for RNA and 23.9 to 114.9% when corrected for uranine. The concentration of viable virus at 20 min after aerosolization at 30°C was below the detection limit of the bioaerosol samplers, i.e., 3.3 $\log_{10}$ EID_{50} m^{-3}; therefore, S_{0-20} was not calculated.

The initial decay constants at 30°C were significantly higher than those at 10°C and 20°C. The initial decay constant at 10°C/70% was significantly lower than that at 20°C/40%; however, neither of these decay constants differed from the initial decay constants at 10°C/40% and 20°C/70%. The secondary decay constants were significantly lower than the initial decay constants ($P < 0.001$). The secondary decay constants at 10°C were significantly higher than those at 20°C.

TABLE 1 Survival rates and decay constants of airborne Gumboro vaccine virus at different temperatures and RHs (physical elimination of virus from air was corrected either for RNA or for uranine)

Treatment	S variable[d]	Survival rate (±SE, %)		k variable[e]	Decay constant (±SE, ln % min^{-1})[c]	
		RNA	Uranine		RNA	Uranine
10°C/40%	S_0	6.0 ± 1.9	4.0 ± 1.8	k_0	2.55*† ± 0.39	3.03*† ± 0.48
10°C/70%	S_0	19.7 ± 5.0	14.0 ± 2.3	k_0	1.42† ± 0.18	1.68† ± 0.16
20°C/40%	S_0	2.2 ± 0.5	1.6 ± 0.5	k_0	3.23* ± 0.19	3.62* ± 0.35
20°C/70%	S_0	6.0 ± 2.6	3.8 ± 1.6	k_0	2.65*† ± 0.46	3.05*† ± 0.47
30°C/40%	S_0	0.1 ± 0.0	0.1 ± 0.0	k_0	5.61‡ ± 0.27	5.96‡ ± 0.32
30°C/70%	S_0	0.2 ± 0.1	0.1 ± 0.0	k_0	5.34‡ ± 0.37	5.67‡ ± 0.22
10°C/40%	S_{0-20}	18.0 ± 6.0	58.0 ± 16.4	k_{0-20}[a]	0.10¶§ ± 0.02	0.03¶§ ± 0.02
10°C/70%	S_{0-20}	7.4 ± 2.3	23.9 ± 10.8	k_{0-20}	0.14¶ ± 0.02	0.09¶ ± 0.03
20°C/40%	S_{0-20}[a]	76.4 ± 9.4	114.9 ± 8.2	k_{0-20}[a]	0.01‖ ± 0.01	−0.01§ ± 0.00
20°C/70%	S_{0-20}	50.2 ± 18.8	97.7 ± 24.2	k_{0-20}	0.05§‖ ± 0.03	0.01§ ± 0.01
30°C/40%	S_{0-20}[b]	—	—	k_{0-20}[b]	—	—
30°C/70%	S_{0-20}[b]	—	—	k_{0-20}[b]	—	—

[a] There is a significant difference between the survival (or decay) corrected for the RNA quantified with the PCR technique and that corrected for uranine.
[b] —, no airborne virus could be detected at 20 min after aerosolization; therefore, the S_{0-20} and k_{0-20} values were not calculated.
[c] Means of k_0 in one column lacking a common symbol (*, †, ‡) are significantly different ($P < 0.05$). Means of k_{0-20} in one column lacking a common symbol (¶, §, ‖) are significantly different ($P < 0.05$).
[d] S_0 and S_{0-20} represent initial viral survival and secondary viral survival, respectively.
[e] K_0 and K_{0-20} represent initial and secondary decay constants, respectively.

In most cases, the survival rates corrected for the two tracers were not significantly different. This was also true for the decay constant. Three exceptions were noticed when using the two tracers to correct S_{0-20} and k_{0-20} at 20°C/40% and k_{0-20} at 10°C/40%. In these situations, when the tracer was RNA rather than uranine the calculated survival rate was lower and the calculated decay constant was higher. To understand the preservation of viral RNA in the air, the pooled RNA preservation in all treatments was calculated using uranine as the tracer with equation 1 (by replacing infectious virus data with RNA data). A one-sample t test shows that the RNA preservation, 105.5% ± 9.5%, was not significantly different from 100% ($P = 0.147$), which means that viral RNA was well preserved under the tested climate conditions.

The response of viral survival to temperature, RH, VP, and VPD is shown in Fig. 2, both as data points and as the results of linear regression analysis. Because the spot plots showed an obvious nonlinear relationship between the survival rate (%) and these environmental metrics, the natural logarithm survival rate (ln %) was used as the dependent variable. The initial survival of aerosolized Gumboro vaccine virus was inversely related to temperature, VP, and VPD (Fig. 2a, c, and g). In contrast, no relationship was found between RH and viral survival ($P = 0.537$). As no virus remained infective at 20 min after aerosolization at 30°C (Table 2), the secondary survival of virus at this temperature was excluded from linear regression analysis (Fig. 2b, d, f, and h). The secondary survival was positively related to temperature ($P = 0.007$) and VPD ($P = 0.006$) but was not related to RH ($P = 0.236$) and VP ($P = 0.275$).

Table 2 shows the multiple linear regression of viral survival rate on the climate metrics. The viral initial survival was negatively related to temperature and positively related to RH ($P = 0.035$). VP was not related to initial viral survival ($P = 0.413$), but there was a tendency for a relationship between VPD and initial survival ($P = 0.085$). Temperature (standardized coefficient = −0.89) was more important than RH for the initial viral survival (standardized coefficient = 0.23). The results also show that the secondary survival was significantly correlated with temperature and that there was a tendency for a relationship with RH ($P = 0.075$).

DISCUSSION

Previous studies have reported biphasic survival kinetics for many species of aerosolized viruses, including Newcastle disease virus (32), influenza virus (30), and infectious bovine rhinotracheitis virus (32). Biphasic kinetics were also noticed in this study of aerosolized Gumboro vaccine virus.

The first phase was a rapid initial loss of infectivity during a 20-s aerosolization of the 5-ml viral suspension and the 2-min air sampling. The decay of virus was faster at the highest temperature (30°C) than at the lower temperatures (10°C and 20°C). In this phase, it is unlikely that the inactivation of virus was caused by sampling stress, because it has been reported that the AGI-30 imposes negligible stress on airborne Gumboro vaccine virus (Zhao et al., submitted). Therefore, the inactivation was probably due to the stress of the sudden changes of temperature and humidity, caused by evaporation of water from the droplets, and the shear force stress that deagglomerated the big droplets into small aerosols during aerosolization (22, 28, 44, 45). With the setup we used, it is not possible to precisely distinguish the percentages of virus inactivated by each of the two stresses; however, the loss of infectivity due to shear force stress seemed less important because the sizes of aerosols containing virus (cutoff diameter was 10 μm) generated by the spray head were much larger than the virus itself (0.06 to 0.09 μm) (44, 45). A more precise discrimination of the effects of the two starting stresses on viral survival could be achieved by passing the aerosolized virus through a volume of saturated air at the same temperature as the viral suspension before the viral aerosols enter the isolator. By doing this, the inactivation of virus by the shear force can be determined from the loss of viral survival in the saturated air.

Our finding of a rapid initial loss of viral infectivity especially at high temperature suggests that the current method of vaccinating poultry by wet aerosolization might not be effective. Other vaccination methods should be investigated, e.g., the dry aerosolization of vaccine virus (5, 6).

Decay of viral infectivity was much slower during the second phase than that during the first phase. We found that the second-

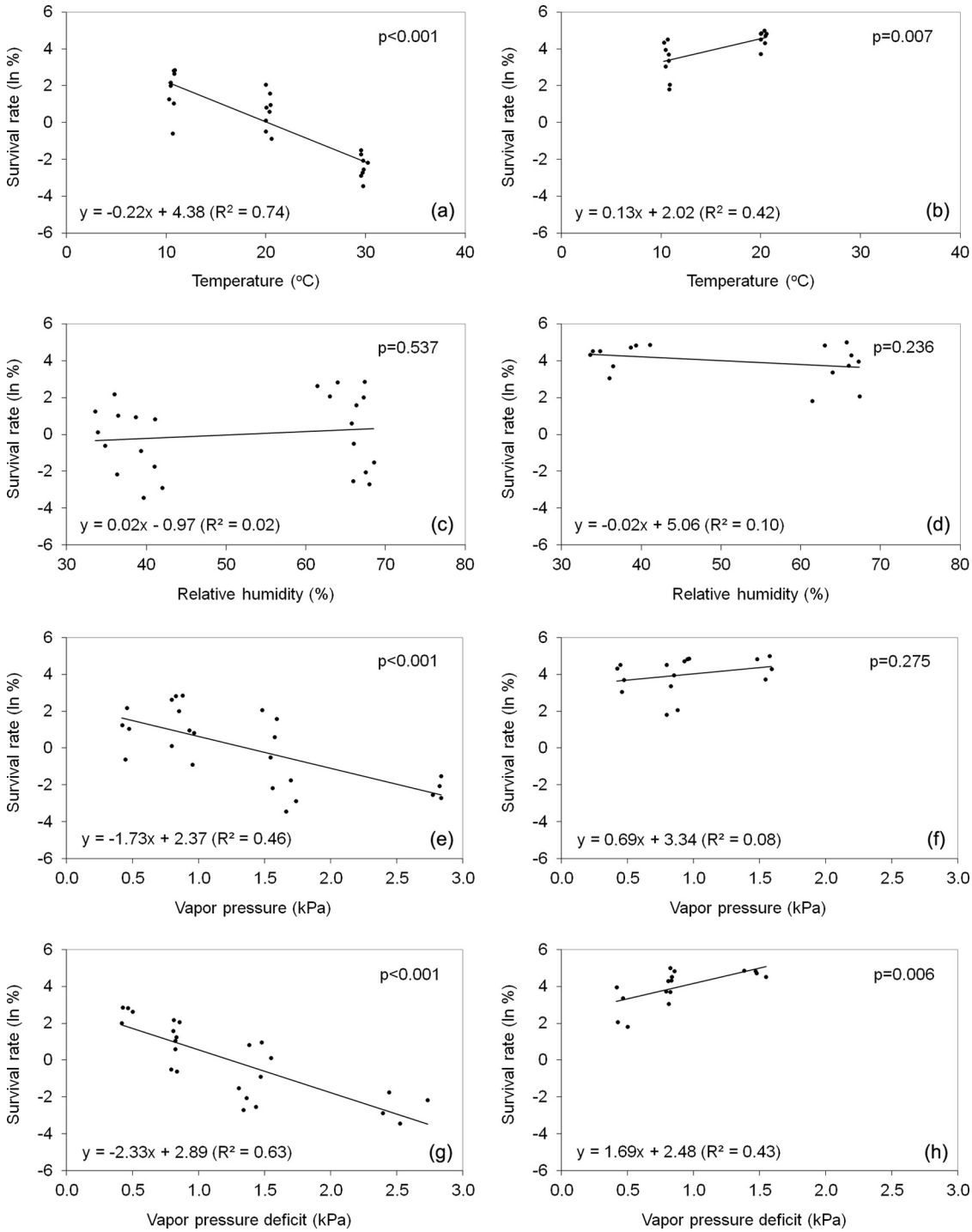

FIG 2 Natural logarithm initial survival rate, $\ln S_0$ (a, c, e, and g), and logarithm secondary survival rate of Gumboro vaccine virus, $\ln S_{0-20}$ (b, d, f, and h), plotted against temperature, RH, vapor pressure, and vapor pressure deficit. Regression formula, regression lines, and regression results are shown in each plot. No infective virus was detected at 30°C at 20 min after all aerosolization events; thus, the regression of $\ln S_{0-20}$ was done with data on 10°C and 20°C only.

ary decay constants at 10°C (0.03 to 0.09 ln % min^{-1}) tended to be higher than those at 20°C (−0.01 to 0.01 ln % min^{-1}). Some of the survival rates at 20°C were calculated to be higher than 100%, probably due to variations in virus quantification analyses. At 30°C, no infective virus was detected in the air sampled 20 min after aerosolization. The secondary survival at this temperature was not calculated, because it was not clear whether the failure in viral detection was caused by inactivation of all virus or by the airborne viral concentration being below the detection limit of the assay method and the sampler. However, the range of the secondary survival at 30°C could be estimated. The average concentration of virus immediately after aerosolization was 3.5 $\log_{10}$ EID_{50}

TABLE 2 Results of the multiple linear regression of $\ln S_0$ and $\ln S_{0-20}$ of airborne Gumboro vaccine virus on temperature and humidity metrics

Dependent	Factor	Standardized coefficient	Significance	Adjusted R^2
$\ln S_0$	Temp	−0.89	<0.001	0.77
	RH	0.23	0.035	
$\ln S_0$	Temp	−1.01	<0.001	0.73
	VP	0.17	0.413	
$\ln S_0$	Temp	−0.62	0.002	0.76
	VPD	−0.31	0.085	
$\ln S_{0-20}$	Temp	0.67	0.003	0.48
	RH	−0.36	0.075	
$\ln S_{0-20}$	Temp	0.96	0.006	0.42
	VP	−0.42	0.171	
$\ln S_{0-20}$	Temp	0.37	0.187	0.43
	VPD	0.40	0.159	

m^{-3}. Assuming the viral concentration at 20 min after aerosolization was in the range from 0 to the detection limit, i.e., 3.3 $\log_{10}$ EID_{50} m^{-3} (Zhao et al., submitted), then the range of the secondary survival of virus at 30°C was from 0 (all virus was inactivated 20 min after aerosolization) to 63.1% (viral concentration equaled the detection limit 20 min after aerosolization). Secondary survival at 10°C (23.9 to 58.0%) and 30°C (0 to 63.1%) was lower than the secondary survival at 20°C (97.7 to 114.9%). This suggests that 20°C is the optimal temperature for the long-distance airborne transmission of Gumboro vaccine virus.

In terms of the airborne transmission of virus, the initial survival data at 20°C and 30°C obtained in this study are more informative for the practical situation, because the viruses are shed by chickens and become airborne inside poultry houses, where the temperature is generally controlled at 20°C (for laying hens) or higher (for broilers). The secondary survival is more meaningful when long-distance airborne transmission between farms is of concern.

Our analysis revealed that temperature was more influential than humidity on the survival of airborne Gumboro vaccine virus. The effects of different humidity metrics on viral survival varied when different analysis methods were applied. For instance, AH had a significant relationship with the initial viral survival as an individual variable (Fig. 2e), but this relationship was no longer significant after temperature had been introduced into the model (Table 2). This is because of the confounding relationship between AH and temperature (AH is calculated from temperature and RH). A large part of the AH effect on viral survival actually was attributable to the effect of temperature. Our results also show that temperature and RH together gave the best prediction of initial (77%) and secondary (48%) viral survival and that there was a tendency ($P = 0.085$) for EP to affect the initial survival (Table 2).

Temperature affects both the capsid protein coating and the RNA of nonenveloped viruses (8, 26). It has been suggested that the stabilities of capsid protein coating and RNA vary with temperature independently of each other, so inactivation of virus at a particular temperature occurs through whichever component is the least stable at that temperature; additionally, the RNA of some viral species, e.g., poliovirus, has been shown to be more susceptible at temperatures of <44°C, whereas protein coating is more susceptible at temperatures of >44°C (8). The temperatures used in our study were much lower than 44°C. We infer that the inactivation of airborne Gumboro vaccine virus was mainly due to the protein coating being damaged at temperatures below 30°C, because during the experiment the viral RNA was quite stable (Table 1). The probable reasons for the differences in inactivation between poliovirus and Gumboro vaccine virus are the unique characteristics of the individual virus species or the different living microcosms for virus (poliovirus in liquid medium, but Gumboro vaccine virus in air). The more likely mechanism of inactivation of Gumboro vaccine virus probably starts with the removal, deformation, or denaturation of a critical site in the protein coating, causing release of viral RNA, which subsequently decays (29). The exact mechanism of the humidity effect on the survival of aerosolized virus is not yet fully understood, but it seems that humidity might affect the virus differently in the two successive phases. In the first phase, i.e., immediately after aerosolization, water in the viral aerosols evaporates into the ambient air, resulting in water moving out of the wet aerosols and in a decrease in temperature of the aerosol (evaporative cooling). The extent of water movement greatly depends on the deficit of aerial water content, i.e., RH and EP (4, 18), and has been suspected to be an important reason for intervention of the natural structure of protein coating (40–42). In the latter phase, there is less water movement, and the response of the virus to humidity is probably due to the slower reaction between water and viral constituents (43).

No extra tracer compound is needed in survival studies if viral genetic material is used to indicate physical elimination of viral aerosols. However, the prerequisite is that the genetic material is stable under all the conditions tested. Hermann et al. (12) reported that porcine reproductive and respiratory syndrome virus (PRRSV) RNA did not decay under their experimental conditions and therefore was a promising tracer for measuring physical loss. In our study, the survival and decay rates of Gumboro vaccine virus corrected either for viral RNA or for uranine were not significantly different in most cases (17 out of 20). Moreover, the pooled preservation of RNA (105.5% ± 9.5%) was not significantly different from 100%. This finding suggests that the decay of the viral RNA was negligible under these conditions and that RNA is a potential tracer. In some situations, i.e., secondary decay at 20°C/40% and 10°C/40%, the virus decay was higher when corrected for RNA than when corrected for uranine. This finding suggests that the viral RNA was prone to be destroyed at the lower temperatures (8), at the lower RH, and in the later airborne phase (29).

Conclusion. This study investigated the response of airborne Gumboro vaccine virus's survival to temperature and humidity. We conclude the following. (i) The initial viral survival (0 to 2.3 min after aerosolization) was affected by temperature and RH and potentially by EP; the secondary viral survival (2.3 to 20 min after aerosolization) was affected by temperature and potentially by RH. However, viral survival was not significantly affected by AH in both phases. (ii) The initial viral survival was much worse than the secondary survival at 10°C and 20°C and at 40% and 70% RH. (iii) The fact that the best secondary viral survival was found at 20°C suggests that long-distance transmission of airborne virus is more likely to occur at 20°C than at 10°C or 30°C. (iv) Current Gumboro vaccination by wet aerosolization in poultry industry is not very effective due to fast initial decay of the aerosolized vaccine virus.

ACKNOWLEDGMENTS

This work was supported by the AI-FES budget from the Dutch Ministry of Economic Affairs.

We thank Joy Burrough for advising on the English.

REFERENCES

1. **Akers TG, Bond S, Goldberg LJ.** 1966. Effect of temperature and relative humidity on survival of airborne Columbia SK group viruses. Appl. Environ. Microbiol. **14**:361–364.
2. **Benbough JE.** 1969. The effect of relative humidity on the survival of airborne Semliki forest virus. J. Gen. Virol. **4**:473–477.
3. **Bolink G, Dijkman R.** 2010. Development of a quantitative real time PCR (Q-PCR) for detection of infectious bursal disease virus (IBDV)—Gallivac vaccine strain project 5082061. Animal Health Service (GD), Deventer, the Netherlands.
4. **Chow LC, Chung JN.** 1983. Evaporation of water into a laminar stream of air and superheated steam. Int. J. Heat Mass Transf. **26**:373–380.
5. **Corbanie EA, et al.** 2007. Spray drying of an attenuated live Newcastle disease vaccine virus intended for respiratory mass vaccination of poultry. Vaccine **25**:8306–8317.
6. **Corbanie EA, Vervaet C, van Eck JHH, Remon JP, Landman WJM.** 2008. Vaccination of broiler chickens with dispersed dry powder vaccines as an alternative for liquid spray and aerosol vaccination. Vaccine **26**: 4469–4476.
7. **de Jong JC, Harmsen M, Trouwborst T.** 1975. Factors in the inactivation of encephalomyocarditis virus in aerosols. Infect. Immun. **12**:29–35.
8. **Dimmock NJ.** 1967. Differences between the thermal inactivation of picornaviruses at "high" and "low" temperatures. Virology **31**:338–353.
9. **Dunklin EW, Puck TT.** 1948. The lethal effect of relative humidity on air-borne bacteria. J. Exp. Med. **87**:87–101.
10. **Gannon BW, Hayes CM, Roe JM.** 2007. Survival rate of airborne Mycobacterium bovis. Res. Vet. Sci. **82**:169–172.
11. **Gloster J, et al.** 2003. Airborne transmission of foot-and-mouth disease virus from Burnside Farm, Heddon-on-the-Wall, Northumberland, during the 2001 epidemic in the United Kingdom. Vet. Rec. **152**:525–533.
12. **Hermann J, et al.** 2007. Effect of temperature and relative humidity on the stability of infectious porcine reproductive and respiratory syndrome virus in aerosols. Vet. Res. **38**:81–93.
13. **Hirai K, Shimakura S.** 1974. Structure of infectious bursal disease virus. J. Virol. **14**:957–964.
14. **Hoekstra JA.** 1991. Estimation of the LC50, a review. Environmetrics **2**:139–152.
15. **Ijaz MK, Karim YG, Sattar SA, Johnson-Lussenburg CM.** 1987. Development of methods to study the survival of airborne viruses. J. Virol. Methods **18**:87–106.
16. **Ijaz MK, Sattar SA, Johnson-Lussenburg CM, Springthorpe VS, Nair RC.** 1985. Effect of relative humidity, atmospheric temperature, and suspending medium on the airborne survival of human rotavirus. Can. J. Microbiol. **31**:681–685.

16a. **Karber G.** 1931. Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. Arch. Exp. Path. Pharmak. **162**:480–483.

17. **Karim YG, Ijaz MK, Sattar SA, Johnson-Lussenburg CM.** 1985. Effect of relative humidity on the airborne survival of rhinovirus-14. Can. J. Microbiol. **31**:1058–1061.
18. **Kincaid DC, Longley TS.** 1989. A water droplet evaporation and temperature model. Trans. ASAE **32**:457–463.
19. **Landman WJM, Corbanie EA, Feberwee A, van Eck JHH.** 2004. Aerosolization of Mycoplasma synoviae compared with Mycoplasma gallisepticum and Enterococcus faecalis. Avian Pathol. **33**:210–215.
20. **Landman WJM, van Eck JHH.** 2001. Aerosolization of Newcastle disease vaccine virus and Enterococcus faecalis. Avian Dis. **45**:684–687.
21. **Lasher HN, Shane SM.** 1994. Infectious bursal disease. Worlds Poult. Sci. J. **50**:133–166.
22. **Marthi B, Fieland VP, Walter M, Seidler RJ.** 1990. Survival of bacteria during aerosolization. Appl. Environ. Microbiol. **56**:3463–3467.
23. **McDevitt J, Rudnick S.** 2010. Role of absolute humidity in the inactivation of influenza viruses on stainless steel surfaces at elevated temperatures. Appl. Environ. Microbiol. **76**:3943–3947.
24. **Mikkelsen T, et al.** 2003. Investigation of airborne foot-and-mouth disease virus transmission during low-wind conditions in the early phase of the UK 2001 epidemic. Atmos. Chem. Phys. **3**:2101–2110.
25. **Moe K, Harper GJ.** 1983. The effect of relative humidity and temperature on the survival of bovine rotavirus in aerosol. Arch. Virol. **76**:211–216.
26. **Norman A, Veomett RC.** 1960. Heat inactivation of poliovirus ribonucleic acid. Virology **12**:136–139.
27. **Otake S, Dee S, Corzo C, Oliveira S, Deen J.** 2010. Long distance airborne transport of infectious PRRSV and *Mycoplasma hyopneumoniae* from a swine population infected with multiple viral variants. Vet. Microbiol. **145**:198–208.
28. **Reponen T, Willeke K, Ulevicius V, Grinshpun SA, Donnelly J.** 1997. Techniques for dispersion of microorganisms into air. Aerosol Sci. Technol. **27**:405–421.
29. **Salo RJ, Cliver DO.** 1976. Effect of acid pH, salts, and temperature on the infectivity and physical integrity of enteroviruses. Arch. Virol. **52**:269–282.
30. **Schaffer FL, Soergel ME, Straube DC.** 1976. Survival of airborne influenza virus: effects of propagating host, relative humidity, and composition of spray fluids. Arch. Virol. **51**:263–273.
31. **Shaman J, Kohn M.** 2009. Absolute humidity modulates influenza survival, transmission, and seasonality. Proc. Natl. Acad. Sci. U. S. A. **106**: 3243–3248.
32. **Songer JR.** 1967. Influence of relative humidity on the survival of some airborne viruses. Appl. Environ. Microbiol. **15**:35–42.
33. **Spearman C.** 1908. The method of right and wrong cases (constant stimuli) without Gauss's formulae. Br. J. Psychol. **2**:227–242.
34. **Stephenson EH, Larson EW, Dominik JW.** 1984. Effect of environmental factors on aerosol-induced Lassa virus infection. J. Med. Virol. **14**:295–303.
35. **Tseng CC, Li CS.** 2005. Inactivation of virus-containing aerosols by ultraviolet germicidal irradiation. Aerosol Sci. Technol. **39**:1136–1142.
36. **van den Berg TP.** 2000. Acute infectious bursal disease in poultry: a review. Avian Pathol. **29**:175–194.
37. **van den Berg TP, Gonze M, Meulemans G.** 1991. Acute infectious bursal disease in poultry: isolation and characterisation of a highly virulent strain. Avian Pathol. **20**:133–143.
38. **Verreault D, Moineau S, Duchaine C.** 2008. Methods for sampling of airborne viruses. Microbiol. Mol. Biol. Rev. **72**:413–444.
39. **Wang Z, Reponen T, Grinshpun SA, Gorny RL, Willeke K.** 2001. Effect of sampling time and air humidity on the bioefficiency of filter samplers for bioaerosol collection. J. Aerosol Sci. **32**:661–674.
40. **Ward RL, Ashley CS.** 1977. Inactivation of enteric viruses in wastewater sludge through dewatering by evaporation. Appl. Environ. Microbiol. **34**: 564–570.
41. **Webb SJ.** 1960. Factors affecting the viability of airborne bacteria. II. The effect of chemical additives on the behavior of airborne cells. Can. J. Microbiol. **6**:71–87.
42. **Webb SJ.** 1960. Factors affecting the viability of airborne bacteria. III. The role of bonded water and protein structure in the death of airborne cells. Can. J. Microbiol. **6**:89–105.
43. **Yeager JG, O'Brien RT.** 1979. Structural changes associated with poliovirus inactivation in soil. Appl. Environ. Microbiol. **38**:702.
44. **Zhao Y, et al.** 2011. Investigation of the efficiencies of bioaerosol samplers for collecting aerosolized bacteria using a fluorescent tracer. I. Effects of non-sampling processes on bacterial culturability. Aerosol Sci. Technol. **45**:423–431.
45. **Zhao Y, et al.** 2011. Investigation of the efficiencies of bioaerosol samplers for collecting aerosolized bacteria using a fluorescent tracer. II. Sampling efficiency, and half-life time. Aerosol Sci. Technol. **45**:432–442.