Survival rate of H5N1 highly pathogenic avian influenza viruses at different temperatures

M. R. Paek,* Y. J. Lee,*[1] H. Yoon,† H. M. Kang,* M. C. Kim,* J. G. Choi,* O. M. Jeong,* J. S. Kwon,* O. K. Moon,† S. J. Lee,† and J. H. Kwon*

*Avian Disease Division, and †Veterinary Epidemiology Division, National Veterinary Research and Quarantine Service, 173 Jungangro, Anyang, Gyeonggi, 430-757, South Korea

ABSTRACT The survival rate of Korean H5N1 highly pathogenic avian influenza (HPAI) viruses was investigated at different temperatures under the laboratory conditions. The estimated survival days for a starting viral concentration of $10^{6.5}$ 50% egg infectious dose/0.1 mL were 930, 1,042, and 3,213 d at 4°C; 226, 232, and 293 d at 20°C; and 51, 55, and 58 d at 30°C for A/chicken/Korea/ES/03, A/chicken/Korea/IS/06, and A/chicken/Korea/Gimje/08 (Gimje/08) viruses, respectively. The stability of the Gimje/08 virus was statistically significant compared with the other 2 viruses except for the data between Gimje/08 and A/chicken/Korea/IS/06 virus at 30°C. This result indicated that the survival rate of 3 Korean HPAI viruses is different at various temperatures, which might have partially influenced the large scale of HPAI outbreak in Korea in 2008.

Key words: H5N1, highly pathogenic avian influenza, temperature, survival rate

2010 Poultry Science 89:1647–1650
doi:10.3382/ps.2010-00800

INTRODUCTION

Avian influenza viruses are classified in the family *Orthomyxoviridae,* genus *Influenzavirus A*. Avian influenza viruses are relatively unstable in the environment and have lipid envelopes readily inactivated by physical factors, organic solvents, and detergents (Swayne and Halvorson, 2003). In the field, however, influenza viruses are protected by organic material such as nasal secretions or feces, which increase the resistance to physical and chemical inactivation (Swayne and Halvorson, 2003).

The survival of the influenza viruses outside the host is significantly affected by several environmental parameters, such as RH, UV radiation, salinity, and temperature (Brown et al., 2007, 2009; Weber and Stilianakis, 2008; Shahid et al., 2009; Zuk et al., 2009). The lower winter temperature has been considered as the main factor behind the seasonality of influenza prevalence (Lowen et al., 2007). However, the survival rate of the influenza virus at different temperatures varied between individual viruses (Brown et al., 2007). Although the outbreaks of 2003-2004 and 2006-2007 occurred in winter, the 2008 outbreak of highly pathogenic avian influenza (**HPAI**) in Korea occurred in the spring and was at a greater scale compared with previous outbreaks (Lee et al., 2005, 2008; Kim et al., 2010). There was the possibility that the temperature stability of individual viruses might have influenced the season and scale of outbreaks. Therefore, in this study, we investigated the survival rate of HPAI viruses at different temperatures using representative Korean H5N1 HPAI viruses from 3 previous outbreaks in 2003-2004, 2006-2007, and 2008 for the evaluation of the temperature stability of individual HPAI viruses.

MATERIALS AND METHODS

Viruses

Three representative Korean H5N1 HPAI viruses were used in this study: A/chicken/Korea/ES/03 (H5N1) (**ES/03**), A/chicken/Korea/IS/06 (H5N1) (**IS/06**), and A/chicken/Korea/Gimje/08 (H5N1) (**Gimje/08**), which were identified by the National Veterinary Research and Quarantine Service of South Korea. Viruses were propagated in 9- to 11-d-old specific-pathogen-free chicken eggs.

Infectivity Assays

For the infectivity assay, each virus sample (infected allantoic fluid) was diluted with PBS to $10^{6.5}$ 50% egg infectious dose (**EID_{50}**)/0.1 mL and aliquoted into 100

Received March 25, 2010.
Accepted May 15, 2010.
[1]Corresponding author: leeyj700@korea.kr

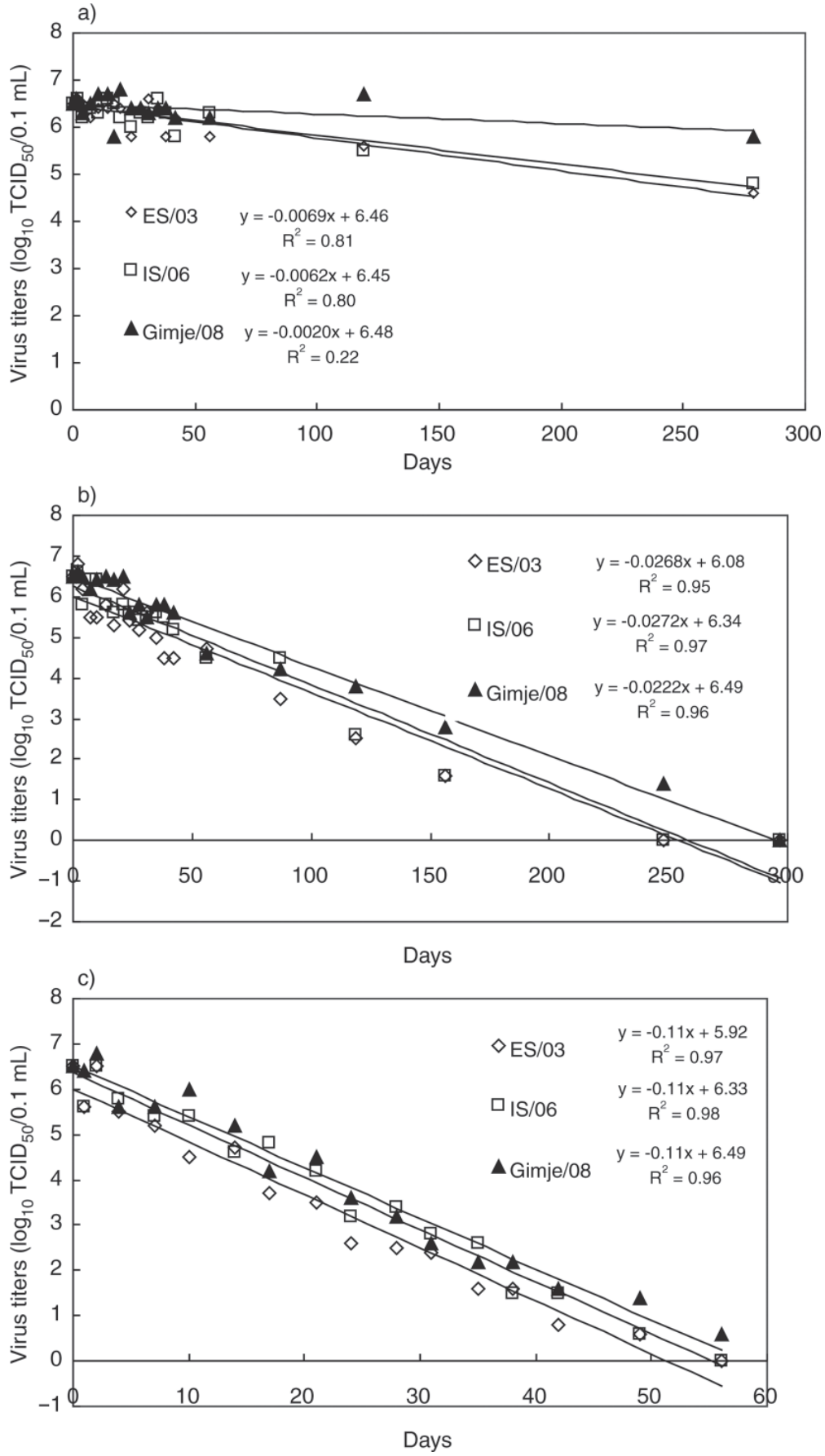

Figure 1. Linear regression models for the survival rate of 3 highly pathogenic avian influenza (HPAI) viruses at (a) 4°C and (b) 20°C. The symbols represent each HPAI viruses [◊ = A/chicken/Korea/ES/03 (ES/03); □ = A/chicken/Korea/IS/06 (IS/06); ▲ = A/chicken/Korea/Gimje/08 (Gimje/08)]. $TCID_{50}$ = 50% tissue culture infectious dose.

Table 1. Survival pattern of 3 different H5N1 highly pathogenic avian influenza viruses isolated in Korea according to days poststorage (dps), estimated using linear regression model

	Regression model[2]				Time (d)	
Virus[1]	Slope	Intercept	R^2	*P*-value[3]	90% reduction[4]	Survival days[5]
4°C						
ES/03	−0.0069	6.46	0.81	0.02	139	930
IS/06	−0.0069	6.45	0.80	0.03	153	1,042
Gimje/08	−0.0020	6.48	0.22	—	485	3,213
20°C						
ES/03	−0.0268	6.08	0.95	$<10^{-3}$	23	226
IS/06	−0.0272	6.34	0.97	0.002	31	232
Gimje/08	−0.0222	6.49	0.96	—	45	293
30°C						
ES/03	−0.1144	5.92	0.97	$<10^{-3}$	4	51
IS/06	−0.1144	6.33	0.98	0.05	7	55
Gimje/08	−0.1112	6.49	0.96	—	9	58

[1]ES/03 = A/chicken/Korea/ES/03; IS/06 = A/chicken/Korea/IS/06; Gimje/08 = A/chicken/Korea/Gimje/08.

[2]Linear regression model, virus titer = (dps) slope + intercept.

[3]Estimated with least squares means in a GLM. Pairwise comparisons were done between ES/03, IS/06 versus Gimje/08, respectively, for the 3 temperatures (4, 20, and 30°C).

[4]Days to reduce the starting viral concentration by 90% (1 $\log_{10}$).

[5]Estimated survival days for a starting viral concentration of $10^{6.5}$ 50% egg infective dose/0.1 mL.

vials and stored at 4, 20, and 30°C in a water bath. Virus infectivity was quantified via microtiter endpoint titration using primary chicken embryo fibroblast cells as described previously (Brown et al., 2007). The viruses of each temperature were tested at 3- or 4-d intervals until 42 d poststorage (**dps**). After 42 d, different temperature groups were sampled as follows: the viruses of 4°C were tested at 56, 119, and 279 dps; the viruses of 20°C were tested at 56, 87, 119, 156, 248, and 297 dps; and the viruses of 30°C were tested at 49 and 56 dps. Each virus sample was diluted 10-fold with minimum essential medium and inoculated with 5 replicates in chicken embryo fibroblast cells in a 96-well microplate. Plates were incubated at 37°C under 5% CO_2 for 96 h. Examination for cytopathic effects was performed with an inverted microscope.

Statistical Analysis

To assess the effect of days poststorage on virus titer, linear regression analysis was performed. A total of 9 different models were estimated according to temperature and viruses. Goodness of fit for these regression models was assessed with deviance, Pearson χ^2, and coefficient of determination (R^2). Predictions from the regression model and the observed values of virus titers were plotted on a $\log_{10}$ scale according to days poststorage.

Distributions of titers for each virus were compared using the least squares method in a GLM. The model was adjusted for days poststorage. Pairwise comparisons were done between ES/03, IS/06 versus Gimje/08, respectively, for the 3 temperature (4, 20, and 30°C). Statistical analysis was performed with PAWS 17.0 (SPSS Korea, Seoul, South Korea).

RESULTS AND DISCUSSION

Using linear regression models, we approximated the survival days of 3 HPAI viruses at different temperatures (Table 1; Figure 1). We estimated the 90% reduction days of viral titers and the remaining days of viral infectivity with linear regression models. The estimated days of 90% reduction titers from initial viral concentration (from $10^{6.5}$ to $10^{5.5}$ EID_{50}/0.1 mL; 1 $\log_{10}$) were 139, 153, and 485 d at 4°C; 23, 31, and 45 d at 20°C; and 4, 7, and 9 d at 30°C for ES/03, IS/06, and Gimje/08, respectively. The estimated survival days for a starting viral concentration of $10^{6.5}$ EID_{50}/0.1 mL were 930, 1,042, and 3,213 d for ES/03, IS/06, and Gimje/08, respectively, at 4°C. The estimated survival days were 226, 232, and 293 d at 20°C and 51, 55, and 58 d at 30°C with ES/03, IS/06, and Gimje/08, respectively. Coefficients of determination (R^2) values for these linear models ranged from 0.80 to 0.98 with the exception of the Gimje/08 virus at 4°C (Table 1), which indicates that individual models accounted for 80 to 98% of observed variation in data. Except for the data of the Gimje/08 virus at 4°C, all of the other data had statistically significant ($P < 0.001$) relationships between virus titers and survival days at each temperature via GLM. All Korean HPAI viruses were more stable at lower temperatures, as described in previous reports (Stallknecht et al., 1990a,b; Brown et al., 2007, 2009; Weber and Stilianakis, 2008; Shahid et al., 2009; Zuk et al., 2009; Wanaratana et al., 2010).

The stability of influenza viruses at specific temperatures was initially reported through the finding that influenza viruses retain infectivity in fecal material in nonchlorinated water for at least 30 d at 4°C and for 7 d at 20°C (Webster et al., 1978). The estimated survival days of our study were much longer than previously re-

ported (Stallknecht et al., 1990a,b; Brown et al., 2007). It appears that the results by Webster et al. (1978) were based on fecal material instead of infected allantoic fluid, which may account for the variation in results. Moreover, we eliminated other environmental factors such as humidity, UV radiation, and salinity under the laboratory conditions. Therefore, in field environmental conditions, the survival days can be reduced by other physical and chemical factors or can be extended by protecting organic materials.

One of the purposes of this study was to investigate whether the Gimje/08 virus was more stable in higher temperature compared with other HPAI viruses because the 2008 HPAI outbreaks in Korea occurred in the spring, unlike the previous 2 outbreaks, which occurred in the winter. Our study showed that although reduction of the survival of the virus was largely dependent on increasing temperature, the survival rate was variable between individual HPAI viruses. To examine the influence of individual viruses on virus titers and survival days, the Gimje/08 virus was compared with ES/03 and IS/06 viruses using pairwise comparison (Table 1). In our study, with the exception of the data between Gimje/08 and IS/06 virus at 30°C, the stability of the Gimje/08 virus compared with the other 2 virus was statistically significant ($P < 0.05$). This was consistent with previous reports that the survival of influenza virus was significantly influenced by temperature and was also variable depending on the virus (Stallknecht et al., 1990a,b; Brown et al., 2007). Although the main reasons behind this enormous outbreak include the concentration of poultry farms in a small area and involvement of the live bird market as described by Kim et al. (2010), our results suggested that the temperature stability feature of the Gimje/08 virus might have influenced the large-scale epidemic of HPAI in the spring season of 2008.

ACKNOWLEDGMENTS

This research was supported by the National Veterinary Research and Quarantine Service of the Ministry of Agriculture and Forestry of South Korea (project C-AD15-2006-14-01).

REFERENCES

Brown, J. D., G. Goekjian, R. Poulson, S. Valeika, and D. E. Stallknecht. 2009. Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. Vet. Microbiol. 136:20–26.

Brown, J. D., D. E. Swayne, R. J. Cooper, R. E. Burns, and D. E. Stallknecht. 2007. Persistence of H5 and H7 avian influenza viruses in water. Avian Dis. 51:285–289.

Kim, H. R., C. K. Park, Y. J. Lee, G. H. Woo, K. K. Lee, J. K. Oem, S. H. Kim, Y. H. Jean, Y. C. Bae, S. S. Yoon, I. S. Roh, O. M. Jeong, H. Y. Kim, J. S. Choi, J. W. Byun, Y. K. Song, J. H. Kwon, and Y. S. Joo. 2010. An outbreak of highly pathogenic H5N1 avian influenza in Korea, 2008. Vet. Microbiol. 141:362–366.

Lee, C. W., D. L. Suarez, T. M. Tumpey, H. W. Sung, Y. K. Kwon, Y. J. Lee, J. G. Choi, S. J. Joh, M. C. Kim, E. K. Lee, J. M. Park, X. Lu, J. M. Katz, E. Spackman, D. Swayne, and J. H. Kim. 2005. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. J. Virol. 79:3692–3702.

Lee, Y. J., Y. K. Choi, Y. J. Kim, M. S. Song, O. M. Jeong, W. Jeong, S. J. Joh, K. S. Choi, M. Her, M. C. Kim, A. Kim, M. J. Kim, E. H. Lee, T. G. Oh, H. J. Moon, D. W. You, J. H. Kim, M. H. Sung, H. Poo, J. H. Kwon, and C. J. Kim. 2008. Highly pathogenic avian influenza virus (H5N1) in domestic poultry and relationship with migratory birds, South Korea. Emerg. Infect. Dis. 14:487–490.

Lowen, A. C., S. Mubareka, J. Steel, and P. Palese. 2007. Influenza virus transmission is dependent on relative humidity and temperature. PLoS Pathog. 3:1470–1476.

Shahid, M. A., M. Abubakar, S. Hameed, and S. Hassan. 2009. Avian influenza virus (H5N1); effects of physico-chemical factors on its survival. Virol. J. 6:38–43.

Stallknecht, D. E., M. T. Kearney, S. M. Shane, and P. J. Zwank. 1990a. Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. Avian Dis. 34:412–418.

Stallknecht, D. E., S. M. Shane, M. T. Kearney, and P. J. Zwank. 1990b. Persistence of avian influenza viruses in water. Avian Dis. 34:406–411.

Swayne, D. E., and D. A. Halvorson. 2003. Influenza. Pages 135–160 in Diseases of Poultry. 11th ed. Y. M. Saif, ed. Iowa State University Press, Ames.

Wanaratana, S., R. Tantilertcharoen, J. Sasipreeyajan, and S. Pakpinyo. 2010. The inactivation of avian influenza virus subtype H5N1 isolated from chickens in Thailand by chemical and physical treatments. Vet. Microbiol. 140:43–48.

Weber, T. P., and N. I. Stilianakis. 2008. A note on the inactivation of influenza A viruses by solar radiation, relative humidity and temperature. Photochem. Photobiol. 84:1601–1602.

Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. G. Murti. 1978. Intestinal influenza: Replication and characterization of influenza viruses in ducks. Virology 84:268–278.

Zuk, T., F. Rakowski, and J. P. Radomski. 2009. Probabilistic model of influenza virus transmissibility at various temperature and humidity conditions. Comput. Biol. Chem. 33:339–343.