AVIAN DISEASES 54:725–728, 2010

Research Note—

Avian Influenza Virus H9N2 Survival at Different Temperatures and pHs

I. Davidson,[A] S. Nagar, R. Haddas, M. Ben-Shabat, N. Golender, E. Lapin, A. Altory, L. Simanov, I. Ribshtein, A. Panshin, and S. Perk

Division of Avian and Fish Diseases, Kimron Veterinary Institute, P.O. Box 12, 50250, Israel

Received 27 April 2009; Accepted and published ahead of print 17 October 2009

SUMMARY. The H9N2 avian influenza virus (AIV) subtype has become endemic in Israel since its introduction in 2000. The disease has been economically damaging to the commercial poultry industry, in part because of the synergistic pathology of coinfection with other viral and/or bacterial pathogens. Avian influenza virus viability in the environment depends on the cumulative effects of chemical and physical factors, such as humidity, temperature, pH, salinity, and organic compounds, as well as differences in the virus itself. We sought to analyze the viability of AIV H9N2 strains at three temperatures (37, 20, and 4 C) and at 2 pHs (5.0 and 7.0). Our findings indicated that at 37 C AIV H9N2 isolate 1525 (subgroup IV) survived for a period of time 18 times shorter at 20 C, and 70 times shorter period at 4 C, as measured by a decrease in titer. In addition, the virus was sensitive to a lower pH (pH 5.0) with no detectable virus after 1 wk incubation at 20 C as compared to virus at pH 7.0, which was viable for at least 3 wk at that temperature. The temperature sensitivity of the virus corresponds to the occurrence of H9N2 outbreaks during the winter, and lower pH can greatly affect the viability of the virus.

RESUMEN. *Nota de Investigación*—Viabilidad del virus H9N2 de influenza aviar a diferentes temperaturas y pH.

El subtipo H9N2 del virus de influenza aviar se ha convertido endémico en Israel desde su introducción en el año 2000. La enfermedad ha causado daños a la industria avícola comercial, en parte debido a la patología sinergística de coinfección con otros patógenos virales y/o bacterianos. La viabilidad del virus de influenza aviar en el ambiente depende de los efectos acumulativos de factores físicos y químicos tales como: humedad, temperatura, pH, salinidad y compuestos orgánicos, así como características del propio virus. Se analizó la viabilidad de las cepas H9N2 del virus de influenza aviar a tres temperaturas (37 C, 20 C y 4C) y a dos niveles de pH (5.0 y 7.0). Los hallazgos indicaron que a 37C el aislamiento 1525 del virus de influenza aviar H9N2 (subgrupo IV), sobrevivió por un tiempo 18 veces menor que a 20 C y 70 veces menor que a 4C, según se determinó por descensos en el titulo. Adicionalmente, el virus mostró ser mas sensible a un pH menor (pH 5) sin niveles de virus detectables después de una semana de incubación a 20C en comparación con un virus incubado a pH 7.0, el cual mostró viabilidad por al menos tres semanas a la misma temperatura. La sensibilidad a la temperatura del virus corresponde con la presentación de brotes de H9N2 en el invierno y a un pH menor puede afectar significativamente la viabilidad del virus.

Key words: avian influenza virus, strain H9N2, temperature stability, pH stability

Abbreviations: AF = allantoic fluids; AIV = avian influenza viruses; EID = egg infective dose; HA = hemagglutinating activity; LP = low pathogenic; PBS = phosphate-buffered saline

Avian influenza viruses (AIV), in general, and the low pathogenic (LP) H9N2 strains, in particular, possess a multifaceted importance for poultry. The H9N2 AIV continues to have a major economic impact in commercial flocks in Israel as well as other countries in the region. The complex environmental conditions and coinfecting pathogens contribute to the severity of infection with LP-AIV H9N2 virus strains. The LP-AIV H9N2 virus strains have been spreading in Israel since the year 2000, and almost 1000 isolates have been isolated. Extensive genetic characterization studies revealed that these H9N2 virus strains are changing continuously; the phylogenetic analysis of their external and internal genes revealed the existence of four subgroups (2). The AIV H9N2 isolates that were isolated during the last year provide genetic evidence that a fifth H9N2 subgroup has emerged in Israel.

In contrast to the numerous studies performed on the genetic characterization of H9N2 viruses, much less scientific effort has been dedicated to virus spread and survival in the environment. The presence of AIV in the environment depends on the cumulative effects of physical and chemical factors, such as 1) persistence in dry, humid, or liquid environments; 2) whether the virus is mixed with organic compounds; 3) the pH; 4) salinity; and 5) temperature of the environment. The AIV strain, virus titer, and time after dissemination are also important factors in the AIV prevalence.

Stallknecht *et al.* (6) showed the importance of low temperatures on the persistence of five AIV strains (H3N8, H4N6, H6N2, H12N5, and H10N7). The viruses contained in allantoic fluids (AF) were diluted in distilled water and incubated at 17 and 28 C. At the lower temperature of 17 C AIV viability was increased, with live virus being detected at 207 days compared to 102 days for incubation at 28 C. Three viruses (H6N2, H4N6, and H10N7) were further examined for their viability in more complex physical conditions, including temperature, pH, and salinity (5), and a strong interactive effect was observed between pH and salinity. A recent study reported findings regarding 12 AIV isolates from wild-bird origin, and showed that the viruses were more stable at slightly basic pH (7.4–8.2), low temperatures (<17 C) and fresh to brackish salinities (0–20,000 ppm) (1). When incubated in AF or in the presence of manure, which destabilizes the viability of the virus, the temperature was critical with the H7N2 strain tested (3). Unexpectedly, AIV strain H7N2 was not affected by brief exposure to pHs in the range of 5.0–12.0.

[A]Corresponding author. E-mail: davidsoni@int.gov.il

Table 1. AIV H9N2 virus concentration determination for use in the study.

H9N2 subgroup	Virus isolate	EID_{50} of original AF	EID_{50} of multiplied AF	EID_{50} of AF used for death time	Day of last embryo death
II	965	$10^{-9.3}$	$10^{-9.2}$	$10^{-8.2}$	2 or 3
III	1567	$10^{-12.1}$	$10^{-9.2}$	$10^{-8.0}$	5
IV	1525	$10^{-11.0}$	$10^{-10.3}$	$10^{-8.0}$	7

As previous studies suggest, various AIV strains vary in their stability to various environmental conditions. This study evaluated the properties of recent AIV H9N2 Israeli isolates to gain appropriate information for risk assessments and AIV control in poultry. The influence of temperature to represent climates (winter, summer, spring, and autumn) in conjunction with moderate pHs (5.0 and 7.0) were assessed on the viability of three AIV isolates belonging to H9N2 genetic subgroups II, III, and IV.

MATERIALS AND METHODS

Viruses. The H9N2 AI isolates have been obtained from commercial poultry flocks since their first detection in 2000. Based on phylogenetic analyses, the Israeli H9N2 isolates have been classified into four main subgroups, which have appeared sequentially in the country since 2000. Although virus subtype H9N2, subgroup 1, included only two isolates from May 2000, subgroup II included isolates from December 2001 to April 2003, subgroup III included isolates from April 2003 to March 2007, and subgroup IV included isolates from December 2006. The four subgroups differ from each other in amino acid sequences by 2%–11%. Three virus strains, belonging to subgroups II, III, and IV of H9N2 AI, were studied:

Subgroup II: A/ty Givat Haim/**965**/17/03/02
Subgroup III: A/ty/Shadmot Dvora/**1567**/06/01/04
Subgroup IV: A/ck/Gshor/**1525**/10/12/06

Preparation of the AIV isolates for viability assessment. AIV seed stocks were propagated by inoculation in 9–10-day-old specific-pathogen–free (SPF; SPAFAS, N. Franklin, CT) embryonated eggs and stored at −80 C (4,7). The virus titers were first determined in the original AF, and then tenfold dilutions in sterile phosphate-buffered saline (PBS) were used for inoculation in order to calculate the egg infective dose (EID_{50}) by the method of Reed and Munch, as described

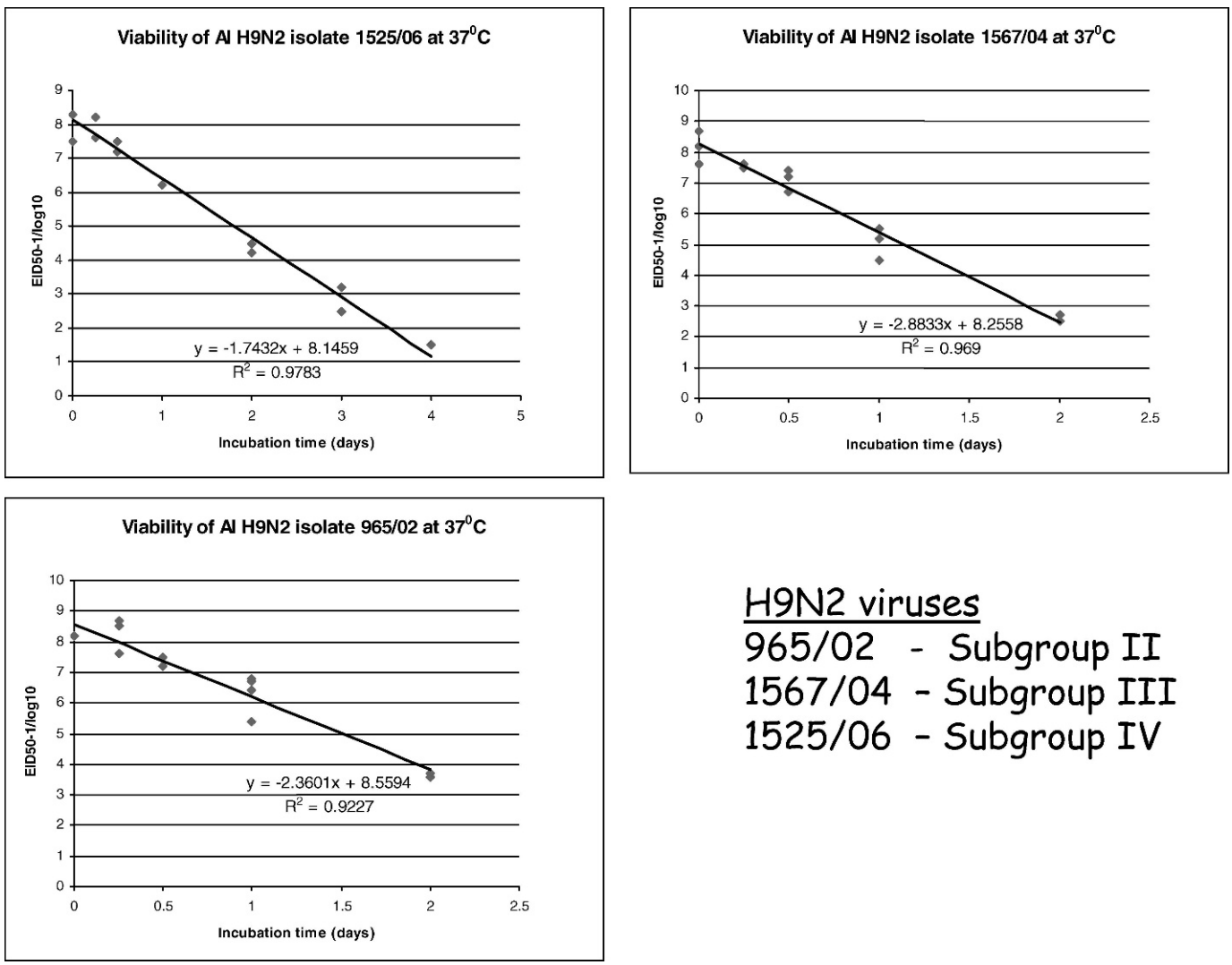

Fig. 1. Viability of AIV H9N2 strains 965/02, 1567/04, and 1525/06 at 37 C.

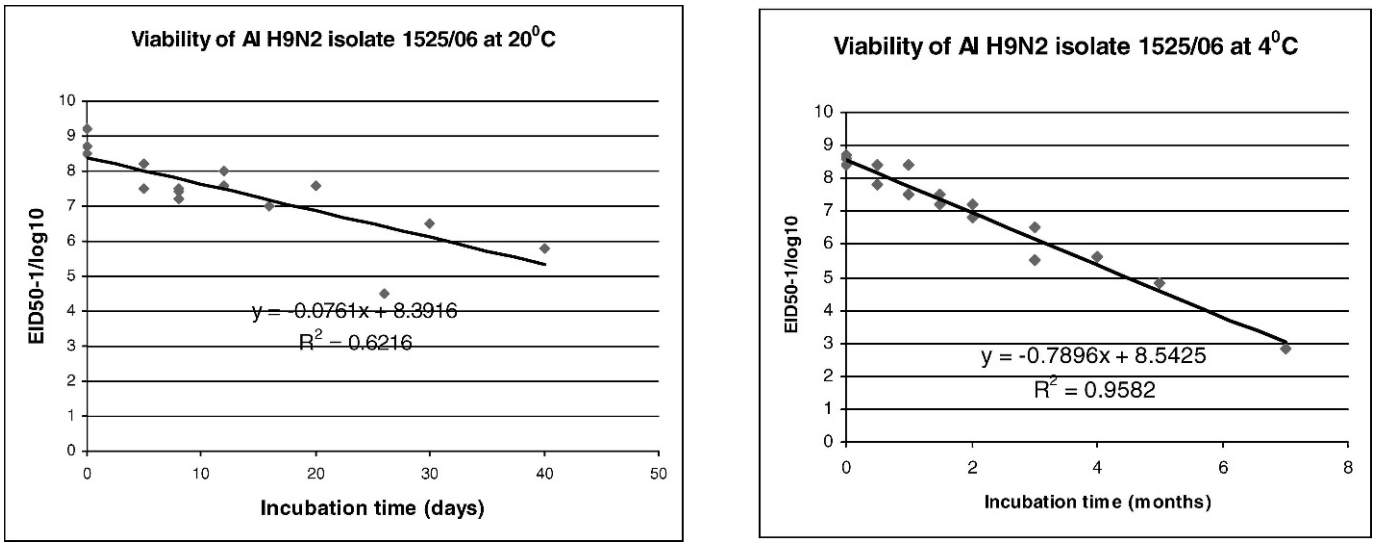

Fig. 2. Viability of AIV H9N2 strain 1525/06 at 20 and 4 C.

in Villegas (8). As a large amount of AF was needed for the study, a further amplification was performed. Accordingly, the titer of the three viruses in AF was adjusted to about $10^{-8.0}$ EID_{50} and then the final virus titer was confirmed in embryonating chicken eggs (Table 1).

Determination of EID_{50}. At each time point a sample was quantified for infectivity and lethality in embryonating chicken eggs and the virus titer was expressed as embryo infectious dose per milliliter (EID_{50}) (8). Briefly, the inoculated eggs were inspected daily for embryo mortality for 6 days postinoculation. Embryo death during the first 24 hr was considered nonspecific. The eggs were chilled at 4 C on day 6 of incubation and the AF were harvested and analyzed for hemagglutinating activity (HA).

Exposure of AIV to various temperatures. AIV in allantoic fluid that was adjusted to $\sim 10^{8.0}$ EID_{50}, was distributed in 0.5-ml aliquots. The number of aliquots was determined according to the sample times at each temperature, and considering the analysis of three replicates at each data point. The incubation was performed in PCR cyclers that were adjusted to the three temperatures, 4, 20, and 37 C. At the appropriate incubation time the three tubes were stored at −80 C until use. Each inoculation was performed by using a new tube.

Exposure of AIV to pH 5.0 and 7.0. The AIV H9N2 strain 1525/06 was tested for its viability at pH 5.0 and 7.0 and incubated at two different temperatures, 4 and 20 C, as above. The AF was diluted 1:10 to obtain a virus EID_{50}/ml of $10^{-7.6}$. The dilution was made in buffer solutions pH 7.0 and 5.0 (Fluka, Buchs, Switzerland; Cat. Nos. 82571 and 82567, respectively). Further dilutions were made in PBS. The tubes were incubated at 4 and 20 C for 3 wk. The virus presence and EID_{50} was determined at 0, 1, 2, and 3 wk of incubation. At these points the pHs of the solutions were also determined and found to be stable. The viability of the chick embryos in the presence of the pH solutions was determined and found unaffected.

RESULTS AND DISCUSSION

Viability of AIV H9N2 strains at three temperatures—37, 20, and 4 C. The general perception on the higher stability of several AIV strains at lower temperatures motivated us to determine systematically the sensitivity of the highly prevalent and economically important AIV H9N2 strains at the three temperatures that typify the Israeli climate, the summer (37 C), spring and autumn (20 C), and winter (4 C). The experimental model of AIV incubation in AF enabled the accurate evaluation of the temperature factor only. Figs. 1, 2 show the viability of the AIVs in embryonating chicken eggs, expressed as the EID_{50}/ml at various times of incubation in the AF. The linear regression equation and its statistical significance, in the form of the correlation coefficient (R^2) of the linear relationship provided the quantitative evaluation. From the three viruses that were studied at 37 C (Fig. 1), representing the three subgroups of AIV H9N2, only the isolate 1525/06 was selected for further study. Although no significant differences were noted between isolates 965/02, 1567/04, and 1525/06, isolate 1525/06 was selected because it was the most recent. Fig. 2 shows the viability of isolate 1525/06 at 20 and at 4 C.

The significant influence of lower temperatures on the H9N2 isolates could be illustrated from the experiment time scale, ranging from days up to 7 mo. Table 2 presents the calculated time for the complete decay of the H9N2 viabilities at the three temperatures. Although at 37 C the complete decay time of the three isolates ranges between 3 and 5 days, the decay time of isolate 1525/06 at 20 C it is almost 18 times higher than it was and at 37 C, and almost 65 times higher at 4 C than it was and at 37 C . In summary,

Table 2. The viability decay time of AIV H9N2 isolates at three temperatures.

Temperature of incubation	Virus	Correlation coefficient, R^2	Decay time (days)
37 C	965/02	0.9227	3.62
37 C	1567/04	0.969	2.86
37 C	1525/06	0.9783	4.67
20 C	1525/06	0.6216	85.29
4 C	1525/06	0.9582	327.6

Table 3. Viability (%) of AIV H9N2 1525/06 isolate at pH 5.0 and 7.0 and at 4 and 20 C.

Incubation time (weeks)	pH 7.0		pH 5.0	
	4 C	20 C	4 C	20 C
0	100		100	
1	100	100	100	0
2	60	100	80	0
3	100	100	0	0

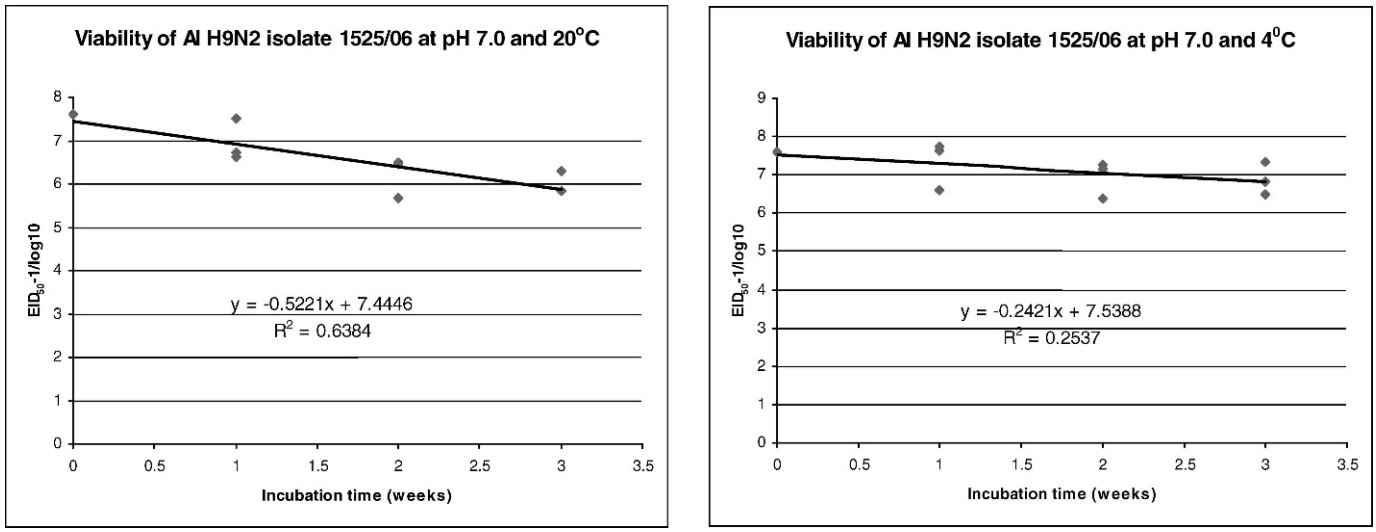

Fig. 3. Viability of AIV H9N2 strain 1525/06 at pH 7.0 and incubation at 20 and 4 C.

it seems that the AIV H9N2 resistance to the winter cold (4 C) is greater than in the autumn and spring (20 C) and much greater than in the summer (37 C).

Viability of H9N2 strain 1525/06 at pHs 5.0 and 7.0 at 20 and 4 C. Table 3 shows the viability of the 1525/06 isolate at pH 5.0 and 7.0 and at 4 and 20 C. To evaluate the presence of virus, the AF was inoculated into embryonating chicken eggs as undiluted sample (Table 3). It seems that incubation at pH 5.0 has a much greater affect on virus viability than at pH 7.0, as at 4 C the virus does not survive beyond the second week. When incubated at 20 C the viruses that are incubated at pH 5.0 do not survive even for 7 days. Moreover, even immediately after adjusting the pH of the AF to 5.0, at the experiment initiation time, week 0, the original virus EID_{50} of $10^{8.0}$ decreases to $10^{4.7}$. However, at pH 7.0 the viability at 4 and at 20 C was relatively stable, as shown in Fig. 3 and Table 4.

In conclusion, we demonstrated the high sensitivity of the AIV H9N2 strains to slightly lowered pHs and higher temperatures. This information can potentially be useful to develop effective control measures to eradicate the virus.

Table 4. The viability decay time of AIV H9N2 isolate 1525/06 at pH 7.0.

Temperature of incubation	Virus	Correlation coefficient -R^2	Decay time (days)
20 C	1525/06	0.6384	99.70
4 C	1525/06	0.2537	218.0

REFERENCES

1. Brown, J. D., G. Goekjian, R. Poulson, S. Valeika, and D. E. Stallknecht. Avian influenza in water: infectivity is dependent on pH, salinity and temperature. Vet. Microbiol. 136:20–26. 2008.

2. Golander, N., A. Panshin, C. Banet-Noach, S. Nagar, S. Pokamunski, M. Pirak, Y. Tendler, I. Davidson, M. C. Garcia, and S. Perk. Genetic characterization of avian influenza viruses isolated in Israel during 2000–2006. Virus Genes 37:289–297. 2008.

3. Lu, H., A. E. Castro, K. Pennick, J. Liu, Q. Yang, P. Dunn, D. Weinstock, and D. Henzler. Survival of avian influenza virus H7N2 in SPF chickens and their environment. Avian Dis. 47:1015–1021. 2003.

4. Senne, A. Virus propagation in embryonated eggs. In: Isolation and identification of avian pathogens, 4th ed. D. E. Sawyne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 235–240. 1998.

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

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

7. Swayne, D. E., D. A. Senne, and C. W. Beard. Avian influenza. In: Isolation and identification of avian pathogens, 4th ed. D. E. Sawyne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 150–155. 1998.

8. Villegas, P. Titration of biological suspensions. In: Isolation and identification of avian pathogens, 4th ed. D. E. Sawyne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 248–253. 1998.

ACKNOWLEDGMENT

This study was supported by the FLURESIST project SSPE-CT-2006-044311 of EU-FP6.