Updated Recommendations for Heat Inactivation of High Pathogenicity Avian Influenza Virus in Dried Egg White for Import/Export Purposes

Colleen Thomas and David E. Swayne
USDA-ARS Southeast Poultry Research Laboratory, Athens, GA 30605

High pathogenicity avian influenza viruses (HPAIV) cause severe systemic disease with high mortality in chickens. Isolation of HPAIV from the internal contents of chicken eggs has been reported, and this is cause for concern because HPAIV can be spread by movement of poultry products during marketing and trade activity. This study presents thermal inactivation data for the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) in dried egg white with a moisture content (7.5%) similar to that found in commercially available spray-dried egg white products. The 95% upper confidence limits for D-values calculated from linear regression of the survival curves at 54.4ºC, 60.0ºC, 65.5ºC, and 71.1ºC were 475.4, 192.2, 141.0, and 50.1 minutes, respectively. The line equation y=[(0.05494)(ºC)]+5.5693 (RMSE = 0.0711) was obtained by linear regression of experimental D-values versus temperature. Conservative predictions based on the thermal inactivation data suggest that standard industry pasteurization protocols would be very effective for HPAIV inactivation in dried egg white. For example, these calculations predict that a 7-log reduction would take only 2.6 days at 54.4ºC.

INTRODUCTION

High pathogenicity avian influenza virus (HPAIV) strains cause severe disease with high mortality in chickens and related gallinaceous poultry (*14*). In chickens, the initial replication site for HPAIV is the respiratory or intestinal tract which is followed by systemic spread of the virus. During the 1983-1984 outbreak of high pathogenicity avian influenza in the northeastern U.S., HPAIV was isolated from albumen, yolk, and the shell surface of eggs obtained from affected flocks in Pennsylvania (*4*). In one experimental study, HPAIV titers as high as 4.9 log EID_{50} (mean embryo infective dose) per ml of egg product were found in eggs laid by infected hens (*13*).

The presence of HPAIV in eggs is cause for concern because the virus can spread to susceptible poultry via the movement of infected poultry products. Due to the high economic cost of controlling HPAI in poultry, the World Organization for Animal Health (Office International des Epizooties; OIE), the intergovernmental organization that establishes health standards for international trade of animals and animal products, recommends that poultry products from HPAIV-affected countries, zones, or compartments be treated to inactivate HPAIV prior to export (*10*). The demonstration of heat inactivation of avian influenza viruses in poultry products suggests that thermal processing could be an effective treatment for many products (*8*, *12*, *13*, *17*, *18*).

A previous study performed in our laboratory reported D-values for the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) in various egg products (*13*). Using this data, calculations were done to determine whether U.S. industry standards for egg product pasteurization, which were developed to inactivate contaminating *Salmonella* (*6*), are also sufficient for HPAIV inactivation. The calculations predicted that 15 days would be required to completely inactivate high titers of HPAIV in dried egg white at 54.4ºC, rather than the 7-10 days specified by the industry standard. However, the moisture content of the freeze-dried egg white prepared for the previous study was not controlled and was probably much lower than that in commercially available spray-dried egg white products. USDA regulations state that the moisture content of spray-dried egg white must be greater than 6.0% for adequate destruction of *Salmonella*, and commercially available spray-dried egg white products typically have 6.5-8.0% moisture to provide adequate pathogen kill with an additional margin of safety (*5*).

For this study, HPAIV-contaminated freeze-dried egg white with an average moisture content of 7.5% was prepared and used for thermal inactivation experiments. A mathematical model for HPAIV inactivation in dried egg white was derived from survival curve data and used to predict the log reductions of HPAIV expected in dried egg white pasteurized according to standard industry protocols.

RESULTS

Survival curves and D-values for PA/83 HPAIV in dried egg white. Figure 2 shows survival curves for PA/83 HPAIV in dried egg white at 54.4ºC, 60.0ºC, 65.5ºC, and 71.1ºC. The coefficients of determination (R^2) and the D-values calculated from each survival curve are shown in Table 1. A linear model provided a fair-to-good fit for the survival curves, with R^2 values of 0.90 or higher for all temperatures except for 65.5ºC. As shown in the 65.5ºC graph (Fig. 2), the 4 hour time point had an unusually large standard deviation. Similar results were obtained for a second set of triplicate samples. This variability probably accounts for the relatively low R^2 value for the 65.5ºC curve. For each survival curve, the last data points include at least one sample in which PA/83 HPAIV was not detected: 1/3 samples for 54.4ºC, 2/3 samples for 60ºC, 3/3 samples for 65.5ºC, and 2/3 samples for 71.1ºC. Negative samples were graphed as 1.7 log EID_{50}/g, which is just below the detection limit of the assay (1.8 log EID_{50}/g). PA/83 HPAIV was not detected in additional samples incubated at 54.4ºC for 2 days (3 samples) or 3 days (9 samples).

Regression line equation and the z-value. Figure 3 shows a linear regression plot of log D-value versus temperature for PA/83 in dried egg white. The line equation and RMSE for the linear model are shown in the figure legend. A z-value of 18.2ºC was calculated from the graph, with a 95% upper confidence limit of 23.0ºC.

DISCUSSION

HPAIV has been isolated in eggs collected during natural outbreaks and experimental infection studies. During the 1983-1984 outbreak of H5N2 HPAI in the Northeastern U.S., HPAIV was isolated from the internal contents of 22% of the chicken eggs sampled from flocks in Pennsylvania 1-18 days after clinical signs typical of HPAIV appeared, but the quantity of HPAIV in the samples was not determined (*4*). One report in the literature describes the isolation of 4.6 to 6.2 log EID_{50}/ml H5N1 HPAIV from the internal contents of Japanese quail eggs collected during a 2003-2004 H5N1 HPAI outbreak in Thailand (*11*), demonstrating that HPAIV may be present in eggs from other susceptible avian species.

In studies with experimentally infected chickens, H5N2 HPAIV was not detected in eggs laid 1-2 DPI, but was isolated from 88% of the eggs laid 3-4 DPI (*2*). Some of these eggs had virus titers greater than 4 log EID_{50}/ml (*2*), with a maximum titer of 4.9 log EID_{50}/ml (*13*). Liquid egg white is 87.81% water and the density of liquid egg white is 1.035 g/ml (*6*). Therefore, 1 ml of liquid egg white would be expected to yield about 0.136 g of dried product with 6.5 to 8.0% moisture. If no loss in virus titer occurred during the drying process, liquid egg white with 4.9 log EID_{50}/ml HPAIV would yield dried egg white with 5.8 log EID_{50}/g HPAIV. The dried egg white samples used in this study had 5.6 to 6.3 log EID_{50}/g HPAIV and could therefore be considered “worst case” samples. Based on the conservative D-value shown in Table 1, pasteurization at 54.4ºC for 7-10 days would result in a 19.1 to 27.3 log reduction of HPAIV titer. A 7-log reduction, which would reduce an HPAIV titer of 6 log EID_{50}/g to one EID_{50}/0.1 g, would take only 2.6 days.

In contrast, a previous study done in our laboratory suggested that pasteurization at 54.4ºC for 7-10 days would not be sufficient for inactivating high titers of HPAIV in dried egg white (*13*). The same PA/83 HPAIV strain was used for both studies, but the dried egg white used in the previous study had a titer of 7.3 log EID_{50}/g (*13,* J. Beck, unpublished data).

Our previous studies of HPAIV inactivation in chicken meat showed that HPAIV inactivation rates at 57ºC were similar in meat samples with HPAIV titers ranging from 3.7 to 8.0 log EID_{50}/g (*17*, *18*). Therefore, differences in HPAIV titer are unlikely to account for the large differences in HPAIV inactivation rates measured during the dried egg white studies. Likewise, the results cannot be explained by differences in thermal inactivation methods. For the previous study, dried egg white samples were heated in thin-walled PCR tubes. The thin-walled tubes probably provided more efficient heat transfer to the sample compared to the glass vials used in this study. If anything, this would result in faster rather than slower HPAIV inactivation rates. We conclude that the differences are most likely due to the moisture content of the dried egg white.

Moisture content is known to affect the thermal inactivation of pathogens in a variety of food products, including *Salmonella* in dried egg white (*7*). For this study, 1.5 ml aliquots of liquid egg white (138 ml total) were freeze-dried for 7 hours and 45 minutes, resulting in a product with 6.5 to 8.0% moisture. The moisture content of the freeze-dried egg white prepared for the previous study was not controlled or measured. However, given that 80 microliter aliquots (approximately 10 ml total) were freeze-dried for 7 hours (*3*), we would expect the moisture content to be quite low and not comparable to that found in commercially available spray-dried egg white products. We conclude that the moisture content of dried egg white affects PA/83 HPAIV inactivation. We predict that pasteurization guidelines already in place for *Salmonella* inactivation will effectively inactivate high titers of PA/83 HPAIV in commercially available spray-dried egg white products with a large margin of safety.

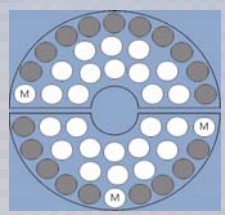

FIGURE 1. Serum vials containing liquid egg white with PA/83 HPAIV (gray circles) or no virus (white circles) placed in trays for freeze drying. “M” indicates samples for moisture analysis.

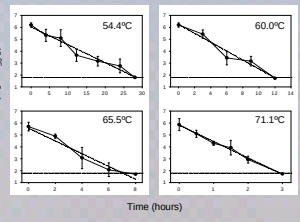

FIGURE 2. Survival curves for PA/83 HPAIV in dried egg white. Each data point represents the average titer of at least three samples, and the error bars indicate standard deviations. The detection limit of the assay is 1.8 log EID_{50}/g.

Temp (ºC)	D-value (min)[a]	R^2[b]	95% PI upper limit for D-value (hours)[c]
54.4	400.6	0.90	8.80
60.0	160.7	0.93	4.33
65.5	109.4	0.83	2.16
71.1	43.7	0.95	1.06

TABLE 1. D-values for PA/83 HPAIV in dried egg white.
[a] Experimental D-values calculated from the inactivation curves shown in Figure 2.
[b] Coefficient of determination.
[c] Upper limit of the 95% prediction interval for the D-value, calculated from the regression line equation + 2RMSE (Fig. 3). RMSE, root mean square error.

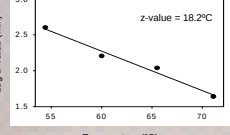

FIGURE 3. Linear regression plot of log D-value (in minutes) versus temperature (in ºC) for PA/83 HPAIV in dried egg white. Line equation: $y = [(-0.05494)\ (ºC)] + 5.5693$. RMSE (root mean square error) = 0.0711. $R^2 = 0.98$.

MATERIALS AND METHODS

Virus strain and preparation of virus-infected material. Working stocks of the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) were prepared by propagation in embryonating chicken eggs (*15*). All work with PA/83 HPAIV and with PA/83 HPAIV-infected materials was performed in USDA-certified Biosafety Level 3 Enhanced (BSL-3E) facilities.

Preparation of samples for freeze-drying. Liquid egg white was prepared by mixing 0.2 g spray-dried egg white (Michael Foods, Wakefield, NE) per ml of sterile deionized and distilled water. After mixing, approximately 200 ml of liquid egg white were filtered through sterile cheesecloth to remove any dried egg white that did not go into solution. For protocol development trials, no virus was added to the liquid egg white. For experiments with HPAIV, 1 ml of 8.1 log EID_{50}/ml PA/83 HPAIV stock was added to 100 ml liquid egg white, and no virus was added to the remainder. Sterile 5 ml serum vials (22 mm diameter, 40 mm height) were placed in drying trays, and 1.5 ml of liquid egg white was dispensed into each vial according to the pattern shown in Fig. 1. Stoppers were placed in the vials in the “up” position used for freeze-drying. The vial trays were placed in sealed bags and frozen at -80ºC for a maximum of 3 days before freeze-drying.

Freeze-drying liquid egg white containing HPAIV. Using samples with no virus added, the drying time and sample configuration required for reducing the moisture content of liquid egg white to 6.5-8.0% was determined empirically. The batches used for 54.4, 60, 65.5, and 71.1ºC had HPAIV titers of 6.2, 6.2, 5.7, and 5.9 log EID_{50}/g, respectively.

Thermal inactivation procedure. Sample vials were completely submerged in a water bath (Model 280 Precision Water Bath, Thermo Scientific, Waltham, MA) set to the target temperature. Water temperature and negative control sample temperature were simultaneously monitored with a data logger thermometer (Omega, Stamford, CT). The t=0 time point was set when the sample temperature was within 0.5ºC of the water temperature (*9*). Instead of adjusting equations to account for heating lag time, the time required for samples to reach t = 0 at each temperature was determined before starting the thermal inactivation experiments. Because the heating lag time ranged from 5-10 min regardless of temperature, t = 0 was set at 10 min for all temperatures. Therefore, t = 0 samples were removed from the water bath after a 10 min incubation, and timing for other samples started 10 min after the samples were placed in the water bath (e.g. t = 30 min samples were placed in the water bath for a total of 40 min). Samples were immediately chilled on ice after removal from the water bath. Unless otherwise noted, triplicate samples were analyzed for each data point. The average HPAIV titers at t = 0 were 6.2, 6.2, 5.7, and 5.9 log EID_{50}/g for 54.4, 60, 65.5, and 71.1ºC had HPAIV titers of 6.2, 6.2, 5.7, and 5.9 log EID_{50}/g, respectively.

Virus isolation and titration. 1.5 ml of 4ºC sterile phosphate-buffered saline was added to each sample vial. The samples were placed on ice for 30-60 minutes and allowed to dissolve, and then were gently mixed by swirling and pipetting. A 1:10 dilution series in brain-heart infusion medium (Becton Dickinson, Sparks, MD) was prepared for each sample, and 1.5 ml of each dilution was inoculated into 9- to 11-day-old embryonating chicken eggs (*15*). Amnioallantoic fluid was harvested from eggs after a 2-3 day incubation and tested for hemagglutination activity (*16*). The 50% endpoints were calculated by the Reed and Muench method (*19*). The detection limit of the assay was 1.8 log EID_{50}/g.

Statistics and graphs. Statistical operations were performed with Sigma Stat version 2.03 (1992-1997, SPSS, Chicago, IL). Graphs were prepared with Sigma Plot version 6.00 (2000, SPSS). Experimental D-values were calculated from linear regression of virus titer versus time at the given temperature (D-value = -1/slope). The upper limit of the 95% confidence interval for each experimental D-value was calculated from the following equation: $b_1 + t(s_{b_1})$ where b_1 is the slope coefficient, t is obtained from a t-test critical values table (two-tailed test, $\alpha = 0.05$), and s_{b_1} is the standard error of the slope coefficient. The coefficients of determination (R^2) for the D-value graphs describe all of the data points rather than only averages. The z-value was calculated from linear regression of log D-values (minutes) versus the temperature in ºC (z-value = -1/slope). The upper limit of the 95% confidence interval for the z-value was calculated as described for the D-values, except that the following generic equation was used to calculate the upper limit of the 95% confidence interval for the slope coefficient: $b_1 + 2(s_{b_1})$. For each D-value calculated from the z-value graph regression line equation, the upper limit of the 95% prediction interval for the D-value was calculated as follows: $y + 2(\text{RMSE})$ where y is the predicted log D-value (minutes) and RMSE is the root mean square error, or the standard error of the y estimate.

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