Evaluation of the U.S. Department of Agriculture’s Egg Pasteurization Processes on the Inactivation of High-Pathogenicity Avian Influenza Virus and Velogenic Newcastle Disease Virus in Processed Egg Products

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MS 12-369: Received 21 August 2012/Accepted 27 November 2012

ABSTRACT

Globally, 230,662 metric tons of liquid egg products are marketed each year. The presence of highly pathogenic avian influenza (HPAI) or Newcastle disease in an exporting country can legitimately inhibit trade in eggs and processed egg products; development and validation of pasteurization parameters are essential for safe trade to continue. The HPAI virus (HPAIV) A/chicken/Pennsylvania/1370/1983 (H5N2) and velogenic Newcastle disease virus (vNDV) AMPV-1/chicken/California/S01212676/2002 were inoculated into five egg products and heat treated at various times and temperatures to determine thermal inactivation rates to effect a 5-log viral reduction. For HPAIV and vNDV, the pasteurization processes for fortified, sugared, plain, and salted egg yolk, and homogenized whole egg (HPAIV only) products resulted in >5-log reductions in virus at the lower temperature–longer times of U.S. Department of Agriculture (USDA)–approved Salmonella pasteurization processes. In addition, a >5-log reduction of HPAIV was also demonstrated for the five products at the higher temperatures–shorter times of USDA-approved pasteurization processes, whereas the vNDV virus was adequately inactivated in only fortified and plain egg yolk products. For the salted and sugared egg yolk products, an additional 0.65 and 1.6 min of treatment, respectively, at 63.3°C was necessary to inactivate 5 log of vNDV. Egg substitute with fat does not have standard USDA pasteurization criteria, but the D_{59}-value was 0.75 min, adequate to inactivate 5 log of vNDV in <4 min.

Highly pathogenic avian influenza (HPAI) and virulent Newcastle disease (vND) in poultry are notifiable diseases to the World Organization for Animal Health. The World Trade Organization uses the World Organization for Animal Health health standards for guidance in safe trade of animals and animal products, with HPAI and vND being legitimate, non-tariff trade barriers.

Experimentally, the HPAI virus (HPAIV) has been detected in the breast and thigh meat, blood, bones, and eggs of infected chickens (15). The increase in the global trade in eggs and egg products along with the increased incidence of H5N1 HPAI outbreaks in Southeast Asia, Africa, and Eastern Europe, and the endemic nature of vND in many developing countries, have raised concerns for potential risks of importation of processed egg products from affected countries. Both diseases are caused by heat-labile, single-stranded, negative-sense RNA viruses, i.e., HPAIV and the vND virus (vNDV). HPAIV and vNDV replicate in many internal organs including the reproductive tract, causing systemic disease with near 100% mortality in unvaccinated poultry.

In the United States, liquid eggs are pasteurized to assure safety for human consumption. Previous egg pasteurization research demonstrated 4- to 7.5-log reductions of Salmonella in various egg products (9, 11, 12). The U.S. Food and Drug Administration’s recommendation is that the pasteurization process must render the product free of Salmonella. Furthermore, pasteurization for in-shell eggs requires specified parameters to inactivate 5 log of Salmonella (6, 8). The U.S. Department of Agriculture (USDA) recommends pasteurization as follows:

(i) Fortified egg yolk: 61.1°C for 6.2 min or 62.2°C for 3.5 min
(ii) 10% sugared or salted egg yolk: 62.2°C for 6.2 min or 63.3°C for 3.5 min
(iii) Plain egg yolk: 60°C for 6.2 min or 61.1°C for 3.5 min
(iv) Homogenized whole eggs: 60°C for 3.5 min

There is no USDA Food Safety and Inspection Service standard for egg substitute. The standard for imitation egg could be used (56.7°C for 4.6 min), or depending on the content of egg white, egg white without pH adjustment pasteurization standard (57.7°C for 6.3 min) may also be used (7, 8). Kinetic data have been limited for the thermal
inactivation requirements and thermal resistance of HPAIV and vNDV in various commercial egg products, with D-values being available for homogenized whole egg, liquid egg white, and dried egg white (16, 19). Such data are necessary to ensure that the pasteurization processes for egg products are effective in inactivation of these viruses. Therefore, the objective of this study was to develop survival curves for both HPAIV and vNDV, from which the inactivation rate and change in thermal resistance (D- and z-values) were generated and used to validate the times and temperatures used in the standard USDA pasteurization process for Salmonella to be effective for inactivation of HPAIV and vNDV in egg yolk products. The performance standard in this study was based on the maximum titer of H5N2 HPAIV in an egg laid by an infected chicken (16), i.e., 5 log.

MATERIALS AND METHODS

Experimental design. HPAIV A/chicken/Pennsylvania/1370/83 (H5N2) (HPAIV/PA/83), and vNDV APMV-1/chicken/California/S01212676/2002 (vNDV/CA/2002) were inoculated into fortified, sugared, salted, and plain egg yolk products (Michael Foods, Minnetonka, MN). In addition, HPAIV was artificially inoculated in homogenized whole eggs (Southeast Poultry Research Laboratory, Athens, GA) and egg substitute with fat (Egg Beaters with Cheese and Chives, ConAgra, Omaha, NE) at 6-log mean tissue culture infective doses (TCID\textsubscript{50}) per ml, and then heat treated at various time-temperature parameters (Table 1). Survival curves were generated to calculate D- and z-values.

Virus inoculum preparation. HPAIV/PA/83 and vNDV/CA/2002 were propagated by inoculation of 10-day-old specific-pathogen-free embryonating White Leghorn chicken eggs (Southeast Poultry Research Laboratory). Titration of the virus stock was completed with tissue culture standard methods (17). In brief, 10-fold serial dilutions of infected allantoic fluid were prepared in brain heart infusion broth (BD, Sparks, MD) with antibiotics (200 μg/ml gentamicin, 2,000 U/ml penicillin, and 4 mg/ml amphotericin-B; Sigma Chemical Co., St Louis, MO), and then 0.1 ml of each dilution was inoculated onto tissue culture and expressed as a TCID\textsubscript{50} value (13).

Sample preparation. Samples of sugared, fortified, plain, and salted egg yolk, homogenized whole eggs, and egg substitute with fat (8) were artificially inoculated with a 1:10 dilution of virus, resulting in 6 log TCID\textsubscript{50}/ml. Ninety-microliter aliquots of the inoculated homogenized egg products were transferred into 200-μl thin-walled thermocycler polypropylene tubes (ThermoWell, Corning, Inc., Lowell, MA) and maintained at 4°C. The homogenized egg substitute with fat was filtered through cheese cloth prior to inoculation.

Heat inactivation. Samples were equilibrated at 25°C for 2 min and then heated to the desired temperature. On reaching the desired temperature, samples at the zero time point were removed, and the timing of inactivation began. The samples were heated at 57, 58, 59, 60, 61 or 61.1, 62.2, and 63.3°C for 0 to 20 min in a PCR thermocycler (GeneAmp PCR System 9700, Perkin Elmer, Boston, MA). Then, the samples were immediately cooled to 4°C in a cooling block (Diversified Biotech, Dedham, MA). Samples were serially diluted in Dulbecco’s minimum essential medium (Gibco, Invitrogen, Carlsbad, CA) with 1× antibiotics-antimycotic (Hyclone, Thermo Scientific, Suwanee, GA) for inoculation into cell culture. Each experiment was replicated three times. Previously, the thermocycler and cooling block temperature precision, and ramp-up (27 to 30 s) and cool-down (30 to 75 s) times were confirmed with thermocouples for validation (15, 16).

Table 1. Comparison of the lethality for the pasteurization process for high-pathogenicity avian influenza (H5N2) (HPAI, A/PA/83) and velogenic Newcastle Disease virus (vNDV, vNDV/CA/2002) in egg products against the standard USDA pasteurization process for 5-log reductions in Salmonella

<table>
<thead>
<tr>
<th>Product</th>
<th>Standard USDA pasteurization process for Salmonella</th>
<th>HPAIV</th>
<th>vNDV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temp (°C) Time (min) D-value (min) Process lethality (D)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified egg yolk</td>
<td>61.1 6.2</td>
<td>0.23</td>
<td>27 1.1</td>
</tr>
<tr>
<td>62.2 3.5</td>
<td>0.14</td>
<td>25 0.53</td>
<td>6.6</td>
</tr>
<tr>
<td>62.2 6.2</td>
<td>0.05 \textsuperscript{b}</td>
<td>124 \textsuperscript{b}</td>
<td>1.1</td>
</tr>
<tr>
<td>63.3 3.5</td>
<td>0.02 \textsuperscript{b}</td>
<td>175 \textsuperscript{b}</td>
<td>1</td>
</tr>
<tr>
<td>Plain egg yolk</td>
<td>60 6.2</td>
<td>0.06 \textsuperscript{b}</td>
<td>103 \textsuperscript{b}</td>
</tr>
<tr>
<td>61.1 3.5</td>
<td>0.03 \textsuperscript{b}</td>
<td>117 \textsuperscript{b}</td>
<td>0.5 \textsuperscript{b}</td>
</tr>
<tr>
<td>10% salted egg yolk</td>
<td>60 6.2</td>
<td>0.06 \textsuperscript{b}</td>
<td>103 \textsuperscript{b}</td>
</tr>
<tr>
<td>62.2 3.5</td>
<td>0.04 \textsuperscript{b}</td>
<td>81.5 \textsuperscript{b}</td>
<td>0.87</td>
</tr>
<tr>
<td>Egg substitute (with fat)</td>
<td>56.7 \textsuperscript{c}</td>
<td>4.6</td>
<td>5.6</td>
</tr>
<tr>
<td>57.7 \textsuperscript{c}</td>
<td>6.3</td>
<td>2.3 \textsuperscript{b}</td>
<td>2.7 \textsuperscript{b}</td>
</tr>
<tr>
<td>59/\textsuperscript{c}</td>
<td>4.0</td>
<td>0.75</td>
<td>5.3</td>
</tr>
<tr>
<td>Homogenized whole egg</td>
<td>60</td>
<td>3.5</td>
<td>0.56</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Process lethality, log reduction for virus at specified pasteurization temperatures and times.

\textsuperscript{b} Estimate was made based on the z-value equation.

\textsuperscript{c} Imitation egg without fat pasteurization standard.

\textsuperscript{d} ND, not done.

\textsuperscript{e} Liquid egg white pasteurization standard.

\textsuperscript{f} Proposed pasteurization process.
saline, inoculated with virus, and incubated for 1 h. After incubation, medium was added to the inoculated monolayer and incubated at 37°C with 5% CO₂-humidified atmosphere for 4 days. The viruses were titered with an hemagglutination assay. In brief, 100-μl aliquots of supernatant from the inoculated chicken embryo fibroblast or Madin-Darby canine kidney monolayer cells were transferred to microtiter plates, and serial log dilutions were accomplished by the successive transfer of 50 μl to wells containing 50 μl of the diluent (1:2), which was then overlaid with 0.5% red blood cells. After 30 min of incubation, the presence or absence of agglutination was recorded. Virus titer was determined by calculation of the TCID₅₀ value (13).

**Statistical analysis.** Survival curves were generated by plotting the TCID₅₀ log value as a function of the heating times at a specified temperature by using Excel 2003 (Microsoft, Inc., Seattle, WA). The statistical distribution of the curves was examined, and linear, exponential, and polynomial curves were generated. Goodness-of-fit tests were conducted to determine if linear and nonlinear functions best fit the data. There was no specific nonlinear function that fit the data at the various temperatures; therefore, linear regression was selected. The D-value, or time (in minutes) to inactivate the viral load by 90%, was calculated with the inverse slope value of the most linear portion of the regression plot. In those cases where the inactivation curves had a rapid exponential decline followed by tailing, the “endpoint” data were used to calculate the D-value. The (D₁, D₂) spline model was also tested and found to be inadequate for these data (10). The z-value is the change in temperature required to decrease the D-value by 1 log or 10-fold, and it was determined with Excel 2003 by computing the regression of the mean log D-values versus heating temperatures. z-Values were the absolute value of the inverse slope. The process lethality corresponds to the time-temperature combination necessary to reduce the concentration of the target organism to an acceptable level and was expressed as a given number of D-values (5). In short, the log reduction or lethality process equals Dreference divided by Dexperimental.

**RESULTS**

The thermal death rate (D) for HPAIV/PA/83 inoculated into fortified egg product ranged from 29 min for D₅₃ to 0.14 min for D₆₂.₂. For the standard pasteurization temperatures of fortified egg products, the D₆₁.₁ and D₆₂.₂ were <0.3 min, so a 5-log viral reduction would result in 1.15 and 0.7 min, respectively. The lethality of the pasteurization process for HPAIV in fortified egg yolk, (61.1°C for 6.2 min and 62.2°C for 3.5 min) was at least fivefold higher than this study’s pasteurization baseline of a 5-log pathogen reduction (Fig. 1A and Table 1).

The D for HPAIV/PA/83 inoculated into 10% sugared egg yolk product ranged from the 18 min for D₅₃ to 0.22 min for D₆₀. At the standard pasteurization temperature, the D₆₂.₂ and D₆₃.₃ were estimated to be ≤0.05 min, with 5-log viral reductions occurring at 0.25 and 0.1 min, respectively. The lethality of the pasteurization process (62.2°C for 6.2 min and 63.3°C for 3.5 min) was estimated at >20-fold higher than the 5-log pathogen reduction needed (Fig. 1B and Table 1). In both fortified and sugared egg yolk, HPAI/PA/83 survived low-temperature (53°C to 57°C) heating, showing a slow, linear decline, with <3-log reductions of the virus after 10 min of exposure. At temperature higher than 59°C, there was rapid exponential decline in survival (Fig. 1A and 1B).

The D for HPAIV/PA/83 inoculated into plain egg yolk product ranged from the 2.54 min for D₅₅ to 0.14 min for D₉₀. The D₆₀ and D₆₁.₁ were estimated to be ≤0.06 min, and the resulting 5-log viral reduction occurred at 0.3 and 0.15 min, respectively. Viral reduction at standard USDA pasteurization times was 20-fold higher than the 5-log viral reduction baseline for this pasteurization study (Fig. 1C).

The D for HPAIV/PA/83 inoculated into 10% salted egg yolk product ranged from 1.2 min for D₅₂ to 0.37 min for D₅₉. Standard pasteurization temperatures for D₆₂.₂ and D₆₃.₃ were estimated to be ≤0.06 min, and 5-log viral reductions in 0.30 and 0.20 min, respectively. The log reductions due to the pasteurization process (62.2°C for 6.2 min and 63.3°C for 3.5 min) were estimated at >16-fold higher than the 5-log pasteurization lethality (Fig. 1D). At lower heating temperatures (52 to 55°C), the inactivation was biphasic, suggesting heat resistance at those temperatures.

The D for HPAIV/PA/83 inoculated into egg substitute product with fat ranged from 5.7 min for D₅₀ to 0.53 min for D₆₀. Since there was no pasteurization standard for egg substitute with fat, the thermal inactivation was compared with imitation egg (56.7°C for 4.6 min) and liquid egg white without pH adjustment (57.7°C for 6.3 min) pasteurization processes. The D₆₆.₇ and D₅₇.₇ were estimated to be 5.6 and 2.3 min, respectively, and a 5-log viral reduction in 28 and 11.5 min, respectively. The viral log reductions due to the pasteurization process (56.7°C for 4.6 min and 57.7°C for 6.3 min) for HPAIV/PA/83 in egg substitute product was poor, <3-D reduction (Fig. 1E). However, a D₅₀ was estimated to be 0.75 min and would be effective at inactivating 5 log within 4.0 min.

The D for HPAIV/PA/83 inoculated into homogenized whole egg product ranged from 12.5 min for D₅₅ to 0.2 min for D₆₁. The D₆₀ and D₆₁.₁ were estimated to be <0.6 min, with 5-log viral reductions at 2.8 and 1 min, respectively. The lethality of the pasteurization process at 60°C for 3.5 min was >1 log higher than the 5-log viral reduction baseline of this pasteurization study (Fig. 1F).

The thermal death rates (D) for the vNDV/CA/2002 virus were 4 to 50 times longer than all corresponding temperatures for the HPAI/PA/83 virus, indicating greater thermal resistance (Figs. 1 and 2 and Table 1). However, a 5-log reduction of vNDV/CA/2002 was achievable with the standard USDA Salmonella pasteurization process for fortified egg yolk at 61.1 and 62.2°C, 10% sugared egg yolk at 62.2°C, plain egg yolk at 60 and 61.1°C, and 10% salted egg yolk at 62.2°C (Fig. 2A to 2D and Table 1). Yet, a 5-log reduction of vNDV/CA/2002 was not achieved with the standard USDA pasteurization process for 10% sugared yolk or 10% salted yolk at 63.3°C (Fig. 2B and 2D and Table 1). In sugared egg product, the survival curve for 57 and 58°C demonstrated thermal resistance, with a biphasic rate of inactivation and a <4-log reduction after 15 min of heating.

**DISCUSSION**

In egg products, the pasteurization performance criterion is the total elimination of Salmonella from the product.
In this study, our aim was to determine the adequacy of the USDA egg product pasteurization process for Salmonella inactivation to also inactivate 5 log of HPAIV/PA/83 and vNDV/CA/2002. The pasteurization process for fortified, sugared, plain, and salted egg yolk products challenged with HPAIV/PA/83 and vNDV/CA/2002, and for homogenized whole eggs with HPAIV/PA/83, resulted in 5-log reductions in virus at the lower temperatures–longer times.

FIGURE 1. Heat inactivation curves for HPAIV/PA/83 (H5N2) in (A) fortified egg yolk, (B) sugared egg yolk, (C) plain egg yolk, (D) salted egg yolk, (E) egg substitute product with fat, and (F) homogenized whole egg. Samples were heat treated at 53 to 63.3°C. Values (±) are the log-transformed numbers of the tissue infectious dose (TCID_{50}) per ml of product, shown as the mean of three independent replications: (A) initial titer of 6.3 log TCID_{50}/ml; (B) initial titer of 6.0 log TCID_{50}/ml; (C) initial titer of 6.1 log TCID_{50}/ml; (D) initial titer of 5.7 log TCID_{50}/ml; (E) initial titer of 6.1 log TCID_{50}/ml; and (F) initial titer of 6.3 log TCID_{50}/ml.

In this study, our aim was to determine the adequacy of the USDA egg product pasteurization process for Salmonella inactivation to also inactivate 5 log of HPAIV/PA/83 and vNDV/CA/2002. The pasteurization process for fortified, sugared, plain, and salted egg yolk products challenged with HPAIV/PA/83 and vNDV/CA/2002, and for homogenized whole eggs with HPAIV/PA/83, resulted in >5-log reductions in virus at the lower temperatures—longer times.
of USDA-approved Salmonella pasteurization processes. In addition, a >5-log reduction of HPAIV/PA/83 virus was also demonstrated for fortified, sugared, plain, and salted egg yolk. Samples were heat treated at 53 to 63.3°C. Values (●) are the log-transformed numbers of TCID<sub>50</sub> per ml of product, shown as the mean of three independent replications: (A) initial titer of 6.8 log TCID<sub>50</sub>/ml, (B) initial titer of 7.1 log TCID<sub>50</sub>/ml, (C) initial titer of 6.8 log TCID<sub>50</sub>/ml, and (D) initial titer of 8.2 log TCID<sub>50</sub>/ml.

Inadequate inactivating 5 log of vNDV (5). In the egg industry, much higher temperatures and longer times than the USDA standards are used for egg substitute products to attain adequate shelf life for the products. Pasteurization temperature and treatment time are adjusted based on the specific product formulation, such as the addition of cheese or other non-egg ingredients (14).

When evaluating the potential risk of HPAIV and vNDV in imported pasteurized egg products, two criteria must be assessed: (i) thermal inactivation parameters for each virus in specific egg products and (ii) potential quantity of virus in infected or contaminated eggs. The data from this study only addressed the thermal inactivation parameters, generating D- and z-values. For the quantity of virus in eggs, the study assumed a 5-log reduction in virus titer as a beginning point, which corresponded to the maximum titer of H5N2 HPAIV in an egg laid by an infected chicken in a previous study (16), and would be analogous to the regulatory standard for 5-log reductions of Salmonella spp. in egg products. However, a more accurate assessment would take into account the actual titers of HPAIV and
vNDV reported in eggs laid by infected hens from additional experimental studies.

In the United States, vNDV was classified as an agent that spreads rapidly and poses a severe risk to animal health, human health, and animal products in the 2002 National Preparedness and Bioterrorism Act (20). However, there have been few reports of NDV being transmitted via infected eggs, and no quantification of the virus was recorded in eggs laid by infected hens. The instance of infection occurred in embryonating eggs from a breeder farm was confirmed experimentally (2–4).

However, field data have shown that HPAIV/PA/83 (H5N2) was detected in 57% of eggs laid by infected chickens in a 1983 outbreak in Northeastern United States (1). A recent experimental study with three replicate trials reported 53% of eggs laid by hens infected with H5N2 HPAIV were infected with HPAIV. Samples from a single infected egg had maximum virus titers of $10^{4.8}$ and $10^{5.1}$ 50% egg infective dose (EID$_{50}$) per ml for yolk and albumen, respectively (17). When birds become infected with HPAIV, the egg production drops to 0%, and the mortality rate reaches 100% within 4 days. Because multiple eggs are pooled and blended, the mean titers of infected eggs (yolk $= 10^{2.17 \pm 0.19}$ and albumen $= 10^{3.01 \pm 0.23}$) would be a more appropriate estimation of the potential titer of virus in the blended egg product; therefore, the 5-log assumption used in our study would provide a >100-fold margin of safety. Furthermore, vaccination as a mitigation strategy is known to reduce the number of virus-contaminated eggs (23% decrease) and to reduce the quantity of virus in contaminated eggs (for yolk to $10^{1.5 \pm 0.4}$, for albumin to $10^{1.38 \pm 0.23}$ EID$_{50}$/ml) by the time the last infected eggs are laid 4 days postchallenge. Furthermore, because not all the eggs in a flock are infected, this creates an additional dilution effect, further reducing the virus titers and validating the safety margin of this study’s 5-log reduction performance baseline. Additional modeling is needed to determine the risk of HPAI virus and vNDV in eggs laid by infected hens and the appropriate standard of inactivation needed to make potentially infected egg products safe for trade.

ACKNOWLEDGMENTS

The authors thank the American Egg Board for their financial support, and Hershell Ball of Michael foods for supplying the egg products for this study. Thanks also go to Kira Moresco for her technical support.

REFERENCES