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Short communication

Microassay for measuring thermal inactivation of H5N1 high pathogenicity avian influenza virus in naturally infected chicken meat

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Abstract

A precise, reproducible microassay was developed to measure thermal inactivation of high pathogenicity avian influenza (HPAI) virus in chicken meat. Small pieces of breast or thigh meat (0.05 g) from chickens infected with A/chicken/Pennsylvania/1370/1983 (H5N2) (PA/83) or A/chicken/Korea/ES/2003 (H5N1) (Korea/03) HPAI viruses were tested for inactivation in the heating block of a thermocycler. Korea/03 infected thigh and breast meat had higher virus concentrations ($10^{6.8}$ and $10^{5.6}$ mean embryo infectious doses [EID_{50}]/g, respectively) compared to PA/83 infected thigh and breast meat ($10^{2.8}$ and $10^{2.3}$ EID_{50}/g, respectively). The samples were ran through a ramp-up cycle from 25 to 70 °C, and meat samples were removed and examined for virus infectivity at 30, 40, 50, 60 and 70 °C, and after treatment for 1, 5, 10, 30 and 60 s at 70 °C. The reduction in virus infectivity titers was dependent on virus concentration and no HPAI virus was isolated after 1 s of treatment at 70 °C. A change in coloration from pink-tan to white was associated with a loss in recovery of infectious virus. The microassay provided a predictable and reproducible method to measure thermal inactivation of HPAI virus in chicken meat.
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Keywords: Avian influenza; Chicken; H5N1; Meat; Thermal inactivation

1. Introduction

Infections by high pathogenicity avian influenza (HPAI) viruses in chickens and other gallinaceous birds cause an initial respiratory or intestinal tract infection followed by viremia, and localization and replication in skeletal muscle fibers of the meat (Mo et al., 1997; Perkins and Swayne, 2001; Capua et al., 2003; Swayne and Beck, 2005). This systemic infection and the localization of HPAI viruses in meat provide the basis for World Organization for Animal Health (OIE) recommendations that poultry products not be traded from HPAI infected countries, zones or compartments unless they are treated to inactivate the virus (Office International des Epizooties, 2003). Presence of HPAI in a country zone or compartment is the basis for legitimate non-tariff trade barriers on live poultry and raw poultry products by trading partners. This barrier is for prevention of transmission to both poultry and humans.

Thermal inactivation has been demonstrated in cloacal bursa homogenate for artificially added infectious bursal disease virus (Alexander and Chettle, 1998), ground chicken meat for artificially added Newcastle disease virus (Alexander and Manvell, 2004), and serum and egg products for Newcastle disease and avian influenza viruses (Gough, 1973; King, 1991; Swayne and Beck, 2004). For studies examining artificially added viruses in liquid products, the virus was evenly dispensed within the product by mixing and resulted in a uniformly distributed pathogen. However, with poultry meat, the study used ground meat with artificially added virus followed by mechanical mixing, but the resulting product did not have uniform dispersion of the virus and such a virus was not present within the skeletal muscle fibers. Furthermore, this virology and other studies with *Salmonella* sp., *Listeria monocytogenes* (Murphy et al., 2004) and *Escherichia coli* (Apostolou et al., 2005) used large samples of meat for inactivation which are physically prone to long and uneven heat-up and cool-down times which make accurate determination of inactivation times impractical. For example, in the Newcastle disease virus study (Alexander and Manvell, 2004), higher virus titers were seen in some

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samples after longer periods of treatments than shorter treatment periods. This inconsistent measurement of inactivation could be the result of either virus titer variations in samples from uneven dispersion of the virus or inconsistent heating of the large sample between runs. The development and use of a thermocycler microassay method for measuring inactivation of artificially added avian influenza and Newcastle disease viruses into egg products eliminated inconsistent inactivation times as compared to a large sample volume water bath method (Swayne and Beck, 2004).

The current study was undertaken to develop a precise microassay for determining thermal inactivation of pathogens in meat samples. A HPAI virus model system that produces infection in skeletal muscle fibers of meat was used for development and testing of the microassay system (Swayne and Beck, 2005).

2. Materials and methods

2.1. Virus inoculum

Two highly pathogenic avian influenza (HPAI) viruses were used, A/chicken/Pennsylvania/1370/1983 (H5N2) (PA/83) and A/chicken/Korea/ES/2003 (H5N1) (Korea/03). The virus working stocks were second passage in 10 day-old embryonating chicken eggs via allantoic sac inoculation of the original materials. Amnioallantoic fluid (AAF) was harvested from the eggs at 30–48 h post inoculation and diluted in brain heart infusion medium (BHI) to deliver 10^6 mean embryo lethal dose (ELD_{50}) per bird in 0.1 ml. All work with these viruses or infected material was conducted in a USDA-certified biosafety level 3 agriculture (BSL-3AG) facility.

2.2. Animal experimental design

Six 4-week-old specific-pathogen-free (SPF) White Plymouth Rock (WPR) chickens were inoculated intranasally (IN) with 10^6 mean embryo infectious doses (EID_{50}) of highly pathogenic avian influenza (HPAI) viruses PA/83 or Korea/03 in 0.1 ml volume. The three birds inoculated with PA/83 virus were severely depressed at 4 days post-inoculation (DPI) and were euthanatized (sodium pentobarbital, 100 mg/kg body weight, intravenously). Thigh and breast tissue were collected and stored at −70 °C. The three birds inoculated with Korea/03 were found dead at 2 DPI, and breast and thigh tissue were collected and stored at −70 °C. All chickens were housed in negative pressure HEPA stainless steel isolation cabinets illuminated under continuous light. Feed and water were provided *ad libitum*.

2.3. Laboratory methodology

2.3.1. Thermal inactivation procedure

Breast and thigh meat samples were thawed, minced, dispensed (0.05±0.002 g) into thin-walled 0.2 ml polypropylene thermocycler tubes (Thermowell 6531, Corning Incorporated, Corning, New York, USA) and centrifuged (Daigger Microcentrifuge PX4250, A. Daigger and Company, Vernon Hills, Illinois, USA) at 2100 ×*g* for 10 s to pack the meat in the bottom of the tube. The tubes were held at 4 °C before placing in the test instrument. Triplicates of each sample were tested.

For testing of thermal inactivation of HPAI virus, tubes containing the samples were placed in a tube-holding heating block of the thermocycler with heated lid (iCycler, BioRad Laboratories, Hercules, California, USA) and the heat cycle initiated to 70 °C. The initial block temperature was ambient (25 °C) and single samples were removed upon reaching 30, 40, 50, 60 and 70 °C during the ramp-up period of the heat cycle, along with samples taken after treatment for 1, 5, 10, 30 and 60 s at 70 °C. Upon removal from the block, all samples were immediately chilled to 4 °C in a chilled-block (CoolSafe, Diversified Biotech, Boston, Massachusetts, USA) and processed for virus isolation and titration.

2.3.2. Virological assay

Following thermal treatment, samples were transferred from thermocycler tubes to 1.7 ml polypropylene centrifuge tubes (SafeSeal Microcentrifuge Tubes, PGC Scientifics, Fredrick, Maryland, USA) containing 0.1 ml volume of sterile sand and the meat sample was ground using 0.5 ml pestles (Pellet Pestle, Kimble-Kontes, Vineland, New Jersey, USA). Brain heart infusion media containing 5 μg/ml amphotericin B (A9528, Sigma-Aldrich, St. Louis, Missouri, USA), 100 μg/ml gentamicin (G1397, Sigma) and 1000 units/ml penicillin (P3032, Sigma) were added to obtain a 10% tissue suspension, vortexed and allowed to settle at 4 °C for 15 min. The sample was centrifuged at 8000 ×*g* for 2 min (Eppendorf 5415, Eppendorf, Westbury, New York, USA) and 0.4 ml of supernatant was divided in 0.1 ml aliquots and inoculated into 9–11 day embryonating hen's eggs for virus isolation and titration using standard procedures (Swayne et al., 1998). Fifty percent end points were calculated (Villegas, 1998), and virus titers were reported as $\log_{10}$ EID_{50}/g of tissue. The minimum virus titer detected with a three embryonating hen's egg assay was $10^{2.2}$ EID_{50}/g.

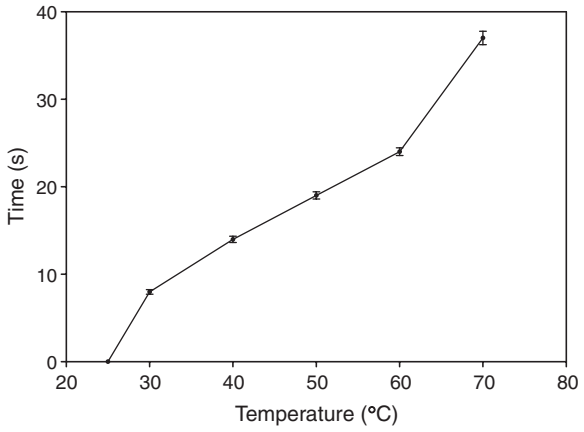

Fig. 1. Reproducibility of the ramp-up temperature curve from ambient (25 °C) to treatment temperature (70 °C) plotted against time in seconds ± standard deviation through 44 test runs.

3. Results and discussion

The reproducibility of the thermal cycle was examined from ambient temperature to 70 °C through 44 ramp-up test runs. Minimal variation was observed between the resulting times to reach each of the target temperatures (i.e. 30, 40, 50, 60 and 70 °C) (Fig. 1). The final treatment temperature of 70 °C was reached in less than 40 s and was maintained through the 60 s treatment period.

The initial quantity of virus in the meat samples varied with the type of meat and inoculating virus. The highest average viral titer ($10^{6.8}$ EID_{50}/g) was present in thigh meat from Korea/03-infected chicken followed by Korea/03-infected breast meat ($10^{5.6}$ EID_{50}/g), PA/83-infected thigh meat ($10^{2.8}$ EID_{50}/g) and PA/83-infected breast meat ($10^{2.3}$ EID_{50}/g) (Fig. 2). A previous study using these two HPAI viruses in chickens demonstrated similar average virus titers in breast and thigh meat for Korea/03 ($10^{7.3}$ EID_{50}/g) and PA/83 ($10^{2.5-3.2}$ EID_{50}/g) HPAI viruses thus indicating the HPAI virus infections were systemic and virus was present within meat samples (Swayne and Beck, 2005). Immunohistochemically, the HPAI virus was visualized in skeletal muscle fibers of the meat samples (Mo et al., 1997; Perkins and Swayne, 2003).

During the heating process, the virus titers of Korea/03 in breast and thigh meat remained unchanged at 30, 40 and 50 °C segment of the ramp-up cycle, but a reduced virus titer was identified beginning with 60 °C. When the meat samples reached 70 °C (0 s), no virus was isolated from the Korea/03-infected breast meat, but 2 of 3 samples of Korea/03-infected thigh meat had low virus titers ($10^{2.3}$ EID_{50}/g). One sample of Korea/03 infected thigh meat had a low titer ($10^{2.3}$ EID_{50}/g) at 1 s for 70 °C. However, no Korea/03 virus was isolated from thigh or breast meat treated at 70 °C for 5, 10, 30 and 60 s. For PA/83-infected meat, the initial virus titers in the meat were lower than with Korea/03 virus and inactivation was seen earlier in the treatment process. In all four virus–meat combinations, all samples had reductions in virus titers as the samples were ramped-up to 70° followed by shorter treatment period at 70 °C before inactivation. In a previous study, a comparable thermocycler microassay demonstrated consistent and reproducible thermal inactivation of artificially added low pathogenicity (LP) and HPAI viruses, and Newcastle disease viruses in liquid egg products using pasteurization temperatures and times (Swayne and Beck, 2004).

The microassay gave predictable and reproducible measurement of HPAI virus thermal inactivation in chicken meat and eliminated the inconsistent virus inactivation reported in a previous chicken meat study using a traditional macroassay (Alexander and Manvell, 2004). In our preliminary development and standardization of the microassay, critical laboratory factors were identified as necessary for producing the accurate methodology. First, the volume of meat in the sample tube must be below the surface of the thermal plate. Use of 0.05 g samples resulted in consistent inactivation of virus which was accompanied by a change from the pink-tan color of the soft, raw product to a uniformly white-colored, firm cooked product within 1 s at 70 °C. By comparison, 0.1–0.2 g samples whose positions within the tubes were level with or above the thermal plate surface had inconsistent virus inactivation and the area of meat above the thermal plate retained the pink-tan coloration of the raw meat even to 60 s at 70 °C. Second, the meat sample required centrifugation to pack the specimen in the bottom of the assay tube in order to eliminate air gaps that provided insulatory effect which resulted in uneven thermal inactivation. Finally, the heated-lid from the thermal cycler must be kept closed throughout the inactivation process and opened only to remove samples for testing.

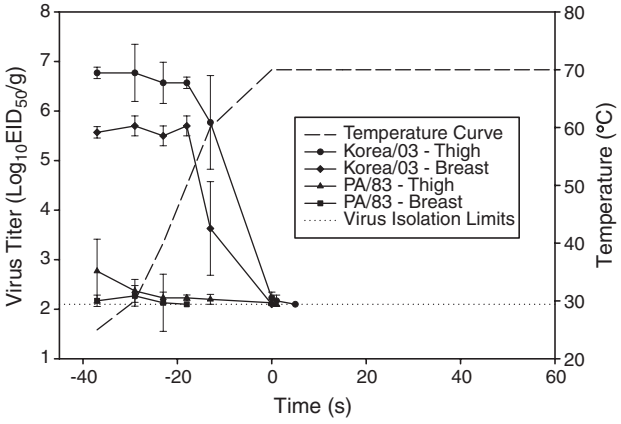

Fig. 2. Decrease in titer (left *y*-axis) of breast and thigh meat samples from chickens naturally infected with two different HPAI viruses (Korea/03 and PA/83) as observed through the thermal ramp-up (25, 30, 40, 50, 60 and 70 °C; −40 to 0 s) and thermal treatment (70 °C; 0, 1, 5, 10, 30, and 60 s) periods (*x*-axis). Each time point represented the mean of three replicates ± standard deviation with the minimal measurable virus titer being $10^{2.2}$ EID_{50}/g tissue. The temperature ramp-up curve is transposed on the graph for comparison of temperature (right *y*-axis) versus time (*x*-axis).

Inactivation of infectious agents is dependent upon concentration of the agent, physical/chemical properties of the contaminated medium, and length of time and temperature of the treatment. Because the physical and chemical properties of AI viruses are similar (Swayne and Halvorson, 2003), strain-to-strain variation is anticipated to have minimal impact on thermal inactivation, but should be examined. In establishing standards for manufacturing cooked poultry meat and other products, multiple time and temperature curves need to be constructed, and D_t (length of time for one $\log_{10}$ reduction in infectious titer at a given temperature) and Z (increase in temperature for one $\log_{10}$ reduction in infectious titer) values calculated. This will allow the future development of international standards for thermal inactivation of AI viruses in poultry products using industrial processes.

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