INACTIVATION OF AVIAN INFLUENZA VIRUS IN CHICKEN FEED

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BACKGROUND
Highly pathogenic (HP) avian influenza (AI) viruses (AIV) belonging to the H5 or H7 types continue to threaten the world poultry industry and are zoonotic agents with pandemic potential for humans (6). Secondary spread of AI occurs mainly from human-related activities such as movement of personnel, vehicles, equipment, and fomites along with restocking of birds in establishments without adequate biosecurity measures. AIV is relatively sensitive in the environment. Heat, extreme pH values, dryness as well as a variety of disinfectants quickly inactivate the virus. However, AIVs are protected in the presence of organic material which increases resistance to physical and chemical inactivation. For example, H5N1 HPAIV has been shown to survive 4 days in chicken feces maintained at 25-32°C in the shade (1). Thus disinfection of premises, footwear and clothing, vehicles, crates, farm equipment and other materials must be carried out properly to significantly reduce infection persistence in poultry populations. This work is aimed at evaluating the efficacy of the commercially available disinfectants Termin-8® (liquid and powder forms) and Finio® (Anitox, Lawrenceville, U.S.A.) at inactivating AIV in chicken feed.

OBJECTIVES AND ACTIVITIES
1. Evaluate the effectiveness of Termin-8® (liquid) on inactivation of AIV.
2. Determine protective effect of protein on survival of AIV in chicken feed and ground corn.
3. Evaluate the effectiveness of Finio® and Termin-8® (powder) on inactivation of AIV.
4. Evaluate the residual effectiveness of Finio® and Termin-8® (liquid) on inactivation of AIV.

OBJECTIVE 1. Evaluate the effectiveness of Termin-8® (liquid) on inactivation of AIV.

Rationale. Non-pelleted chicken feed could become contaminated with AIV from contaminated ingredients or at other phases during the production processes. Because contaminated chicken feed could play a role on AIV dissemination, we evaluated the efficacy of the commercial disinfectant Termin-8® in liquid form at inactivating AIV in chicken feed.

Materials and procedures

Virus stock. The well-characterized low pathogenic (LP) AIV strain A/turkey/Wisconsin/68 (H5N9) (4,5) was used. The virus was replicated in 10 day-old specific pathogen free (SPF) embryonated chicken eggs (ECE) and virus contained in the amnion-allantoic fluids (AAF) was titered as accepted in SPF ECE (3,7). The virus, with a titer of $10^{8.7}$ EID$_{50}$/ml, was stored at -80°C until use.
Chicken feed. Non-pelleted commercially available layer feed and ground corn were used.

Treatments. Feed was treated with liquid Termin-8 at 2 dose levels (3 kg/ton and 1 kg/ton) by thorough mixing using an automated pressurized mixer.

AIV contamination of treated and untreated feed. AIV (contained in allantoic fluid) was applied once at a dose of $10^5$ EID$_{50}$ per 1 gram of feed and vortexed thoroughly using a conventional laboratory vortex for 30 seconds. AIV contamination began 1 hour after Termin-8 treatment of feed. Trials were performed with 8 replicates; thus 8 tubes/treatment/sampling points containing 1g of feed were inoculated. After inoculation vortexed tubes were stored at room temperature (~22°C) until sampling.

Sample collection. Samples were obtained at 1, 4, 16, and 24 hours by suspending the full content of each tube into tryptose broth (5 ml) containing a commercial antibiotic mix (Corning cellgro® Antibiotic-Antimycotic Solution, Mediatech, Inc.) at a 10X concentration. The material contained in each tube was then centrifuged at 3,000 XG for 10 min and moved to a new tube. This process was repeated twice for clarification of the supernatants. Supernatants of each tube were sterile filtered through a 0.45µm filter and resuspended in a 1:2 dilution of tryptose broth containing antibiotics and maintained refrigerated (approximately 1 h) until inoculation. Two SPF ECE (9 to11 day of incubation) were inoculated via the allantoic route with 0.2 ml of each processed sample. ECE were also inoculated with untreated feed suspension containing AIV as the untreated positive control. Eggs were incubated at 37°C and candled at 8 h intervals. Allantoic fluids were harvested 72 hours post inoculation. AIV RNA was extracted from the allantoic fluid collected from each egg and viral concentration determined by quantitative RT-PCR (qRT-PCR).

AIV viral RNA by qRT-PCR. Inactivation of virus by Termin-8 treatment was determined by comparison of AIV RNA concentration in the allantoic fluids of eggs at each sampling time point in treated and untreated feed using analysis of variance (ANOVA) and a multiple comparisons post-test. In addition, incidence of viral RNA in eggs was recorded and compared by Chi-square.

AIV RNA was extracted using the QIAamp Viral RNA Mini Kit from the allantoic fluids collected from each egg. qRT-PCR to quantitate AIV matrix RNA was performed as described (2).

Results. Viral RNA concentrations determined at 1, 4, 16 and 24 hours are shown in Fig 1. As seen in this figure, treatment with Termin-8 significantly reduced (P<0.05) AIV RNA in samples obtained 1 hour after AIV contamination of the feed. Samples obtained at 4, 16 and 24 hours did not achieve significant differences because untreated samples also showed reduced AIV RNA.

Discussion. AIV inactivation effectiveness of Termin-8 (liquid) was demonstrated in samples collected 1 hour after AIV contamination of the feed. Indeed, Termin-8 treatment significantly reduced (P<0.05) AIV RNA compared to untreated controls. No significant differences were detected between dosages of Termin-8; i.e. 1 and 3 kg/ton work equally well. The inactivating effect of Termin-8 was not apparent at further time points because the virus was inactivated in untreated controls likely by environmental factors (dryness, temperature, etc.). In
the field the presence of organic material, e.g. feces, will likely increase the livability of AIV as reported previously (1).

**OBJECTIVE 2. Determine the protective effect of protein on survival of AIV in chicken feed and ground corn.**

*Rationale.* AIV has been shown to survive for up to 4 days at 22°C and up to 18 days at 4°C in feces (8). Several properties of the environment in fecal material are responsible for increased virus survival but protection by proteins from cell debris is likely the most relevant. In order to resemble natural conditions, instead of clarified virus, we added skimmed milk to the virus suspension at a ratio of 0.002% before contamination of the feed and evaluated AIV survival at room temperature (22°C).

**Materials and procedures**

*Virus stock.* AIV described above was used.

*Chicken feed.* Chicken feed was obtained from a local supplier: a commercially available non-pelleted layer feed and ground corn.

*AIV contamination of chicken feed and sampling.* Stock H5N9 AIV was diluted 1:100 in tryptose broth containing 10% antibiotic/antimycotic solution (Corning 30-004-CL) and 0.002% skimmed milk (Saco Foods, Middleton WI). AIV suspension was applied once at a dose of 10^7.7 EID₅₀ per 1 gram of feed or ground corn and mixed thoroughly using a conventional laboratory vortex for 30 sec. Trials were performed with 5 replicates per sampling point (i.e., 5 tubes/sampling point) per feed type. After inoculation, vortexed tubes were maintained at room temperature (~22°C) until sampling at 2, 19, 24, 48, 96, and 144 hours. Sampling was performed by adding 5ml of tryptose broth with antibiotics to each tube. All tubes were then centrifuged at 4000xg for 15min. 2 ml of supernatant per tube were collected and centrifugation repeated. The supernatants were then sterile-filtered using a 45µm filter (VWR Sterile syringe filter w/0.45um cellulose acetate membrane Cat# 28145-481). 300 µl of filtered supernatant was diluted 1:1 (by adding 300µl) of tryptose broth with antibiotics and maintained in refrigeration until used to inoculate 10 day specific-pathogen-free embryonated chicken eggs. Each sample was inoculated into 4 eggs (100µl per egg). Eggs were harvested 3 days after inoculation. RNA was extracted from the allantoic fluids using the QIAamp Viral RNA Mini Kit (Qiagen, Cat # 52906). qRT-PCR was performed as described (2).

**Results and discussion.* We previously demonstrated that AIV survival in chicken feed maintained at room temperature and humidity is relatively short (see results of Objective 1). Live virus was detectable 1 hour post-inoculation but virus had been inactivated by 4 hours post-inoculation. AIV RNA concentrations determined at 2, 19, 24, 48, 96, and 144 hours are shown in Fig 2. As seen in this figure, adding skimmed milk to the virus suspension significantly increased AIV survivability compared to experiment 1. Live virus was detectable through 24 hours post-inoculation. It was interesting to see that considerably higher concentrations of live virus were detected in commercial feed compared to ground corn. Samples obtained from both feed and ground corn at 2, 4 and 6 days post-inoculation were negative for live AIV.
OBJECTIVE 3. Evaluate the effectiveness of Finio® and Termin-8® (powder) on inactivation of AIV.

Rationale. Because protein (skimmed milk 0.002%) prolongs survival of AIV in chicken feed (see results objective 2), two disinfectants were evaluated for effectiveness at inactivating AIV protected by protein.

Materials and procedures

Virus Stock. Virus stock was diluted 1:100 in tryptose broth containing 10% antibiotic /antimycotic solution (Corning 30-004-CL) and 0.002% skimmed milk (Saco Foods, Middleton WI) before contamination of feed.

Chicken feed. Non-pelleted, commercially available layer feed was used.

Treatments. Feed was treated with Termin-8 powder at 3-kg/ton or with Finio at 2-kg/ton by thorough mixing using an automated pressurized mixer. Untreated feed served as the positive control.

AIV contamination of chicken feed and sampling. AIV contamination was conducted 1 hour after treatment of feed with AIV applied once at a dose of \(10^5\) EID\(_{50}\) per 1 gram of treated or untreated feed and vortexed thoroughly using a conventional laboratory vortex for 30 seconds. Trials were performed with 6 replicates. After inoculation vortexed tubes were stored at room temperature (~22°C) until sampling at 1, 6 and 24 hrs by suspending the full content of each tube into tryptose broth (5 ml) as described above (see objective 1). Two eggs were inoculated with 0.2 ml of each processed sample. Eggs were at 37°C and allantoic fluids harvested 72 hrs post inoculation. AIV RNA was extracted from the allantoic fluid collected from each egg and viral concentration determined by qRT-PCR as described above.

Results and Discussion. Viral RNA concentrations determined at 1, 6 and 24 hrs are shown in Fig 3. As seen in this figure, treatment with Termin-8 (powder) and Finio resulted in AIV inactivation at 1 and 6 hr compared to untreated control feed. Samples obtained from treated feed at 24 hrs did not achieve significant differences because untreated samples also became negative to AIV. As seen in Fig. 3, results obtained at 1 hr indicate that Termin-8 showed a more drastic inactivation of AIV compared to Finio.

OBJECTIVE 4. Evaluate the residual effectiveness of Finio® and Termin-8® (liquid) on inactivation of AIV.

Rationale. In previous experiments AIV contamination of feed was performed around 1 hr after disinfectants were applied to the feed. It was relevant to learn about the residual effect of the disinfectants.

Materials and procedures

Treatments. Feed was treated with Termin-8® liquid at 6 kg/ton or with Finio® at 2 kg/ton 7 days prior to AIV contamination (\(10^5\) EID\(_{50}\) per 1 gram of feed). Six replicates of each treatment performed. Skimmed milk was added to the AIV suspension at 0.002% before contamination of
feed. Samples of AIV contaminated feed were collected at 6 hrs after AIV contamination, and inoculated into ECE. Allantoic fluids were harvested 72 hours after inoculation and AIV RNA determined by quantitative RT-PCR.

**Results and Discussion.** As seen in Fig. 4, both Termin-8 and Finio maintained their capacity to inactivate AIV after 7 days at room temperature. Similar as in objective 3, Termin-8 determined a more drastic inactivation of the virus.

**REFERENCES**

Fig. 1. **Effect of Termin-8® (liquid) on inactivation of avian influenza virus (AIV).** AIV RNA was determined in the allantoic fluid of embryonated chicken eggs inoculated with AIV-contaminated chicken feed. Feed was treated with Termin-8® (T8) liquid at 3 kg/ton or 1 kg/ton 1 hour prior to AIV contamination (10⁵ EID₅₀ per 1 gram of feed) (8 replicates of each treatment). Samples of AIV contaminated feed were collected at 1, 4, 16, and 24 hrs after AIV contamination, suspended in tryptose broth (clarified by centrifugation, treated with antibiotics, and filtered), and inoculated in 2 eggs/sample. Allantoic fluids were harvested 72 hours after inoculation. AIV RNA in allantoic fluids was determined by qRT-PCR. Different letters indicate significant differences (P<0.05).
Fig. 2. **Survival of AIV protected by protein in layer feed and ground corn.** AIV contained in allantoic fluid was added skimmed milk at 0.002% prior to contamination of feed and ground corn. AIV contaminated chicken feed and ground corn were maintained at room temperature (~22°C) and room humidity (~30 - 40%). Samples were collected at 2, 19, 24, 48, 96 and 144 hours, and inoculated into embryonated eggs. Allantoic fluids from inoculated eggs were harvested 72 hours after inoculation and AIV RNA determined by quantitative RT-PCR. Mean and SEM are shown.
Fig. 3. **Effect of Finio® and Termin-8® (powder) on inactivation of AIV.** AIV RNA was determined in the allantoic fluid of embryonated chicken eggs (ECE) inoculated with AIV-contaminated chicken feed. Feed was treated with Termin-8® (T8) powder form at 6 kg/ton or with Finio® at 2 kg/ton (Anitox) 1 hour prior to AIV contamination (10^5 EID_{50} per 1 gram of feed) (six replicates of each treatment). Skimmed milk was added to the AIV suspension at 0.002% before contamination of feed. Samples of AIV contaminated feed were collected at 1, 6, and 24 hours after AIV contamination, and inoculated in ECE. Allantoic fluids were harvested 72 hours after inoculation and AIV RNA determined by quantitative RT-PCR.
Fig. 4 Residual effect of Finio® and Termin-8® (liquid) on inactivation of AIV. AIV RNA was determined in the allantoic fluid of embryonated chicken eggs (ECE) inoculated with AIV-contaminated chicken feed. Feed was treated with Termin-8® (T8) liquid form at 3 kg/ton or with Finio® at 2 kg/ton (Anitox) 7 days prior to AIV contamination ($10^5$ EID$_{50}$ per 1 gram of feed) (six replicates of each treatment). Skimmed milk was added to the AIV suspension at 0.002% before contamination of feed. Samples of AIV contaminated feed were collected at 6 hours after AIV contamination, and inoculated in ECE. Allantoic fluids were harvested 72 hours after inoculation and AIV RNA determined by quantitative RT-PCR.