Preventive Veterinary Medicine 145 (2017) 73–77

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Thermal Inactivation of avian influenza virus in poultry litter as a method to decontaminate poultry houses

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ARTICLE INFO

Article history:
Received 24 April 2017
Received in revised form 27 June 2017
Accepted 28 June 2017

Keywords:
Cleaning and disinfection
Virus heat inactivation
Avian influenza virus
Poultry litter
Animal disease outbreak

ABSTRACT

Removal of contaminated material from a poultry house during recovery from an avian influenza virus (AIV) outbreak is costly and labor intensive. Because AIV is not environmentally stable, heating poultry houses may provide an alternative disinfection method. The objective was to determine the time necessary to inactivate AIV in poultry litter at temperatures achievable in a poultry house. Low pathogenic (LP) AIV inactivation was evaluated between 10.0°–48.9 °C, at ~5.5 °C intervals and highly pathogenic (HP) AIV inactivation was evaluated between 10.0°–43.3 °C, at ~11 °C intervals. Samples were collected at numerous time points for each temperature. Virus isolation in embryonating chicken eggs was conducted to determine if viable virus was present. Each sample was also tested by real-time RT-PCR. Low pathogenicity AIV was inactivated at 1 day at 26.7 °C or above. At 10.0, 15.6 and 21.1 °C, inactivation times increased to 2–5 days. Highly pathogenic AIV followed a similar trend; the virus was inactivated after 1 day at 43.3 °C and 32.2 °C, and required 2 and 5 days for inactivation at 21.1 °C and 10.0 °C respectively. While low pathogenicity AIV appeared to be inactivated at a lower temperature than high pathogenicity AIV, this was not due to any difference in the strains, but due to fewer temperature points being evaluated for high pathogenicity. Endpoints for detection by real-time RT-PCR were not found even weeks after the virus was inactivated. This provides a guideline for the time required, at specific temperatures to inactivate AIV in poultry litter and likely on surfaces within the house. Heat treatment will provide an added level of safety to personnel and against further spread by eliminating infectious virus prior to cleaning a house.

1. Introduction

Avian influenza virus (AIV) is among the most economically important viruses that affects poultry worldwide and is potentially zoonotic (Swayne et al., 2013). Outbreaks of AIV have severe economic consequences for the poultry industry. In response to an outbreak of highly pathogenic (HP) AIV, and often to outbreaks of the H5 and H7 subtypes of low pathogenic (LP) AIV, infected birds are quickly depopulated. Unless the carcasses are disposed of by in-house composting they are removed immediately, then the house must be cleaned and disinfected. Removal of all organic material from the house is recommended, which includes the poultry litter (USDA, 2016b). Removing contaminated litter from the house for disposal prior to decontamination is costly and carries some risk of spreading the virus to other poultry flocks and exposing personnel. Litter cannot be decontaminated by chemical methods because of the high organic load (Stringfellow et al., 2009), but AIV is susceptible to heat inactivation.

Most data on the thermal stability of AIV are based on cooking conditions (Swayne and Beck, 2004; Isbarn et al., 2007; Thomas et al., 2008) and composting temperatures (e.g. >56 °C [133 °F]) (Senne et al., 1994; Guan et al., 2009; Elving et al., 2012) or at lower temperatures on hard surfaces (Guan et al., 2016), in manure (Chumpolbanchorn et al., 2006) and in water (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2009). Currently in the US, heat treatment at 37.8–48.9 °C (100–120 °F) for 7 days with 3 consecutive days at the maximum temperature, may be used to help decontaminate surfaces within affected poultry houses (USDA, 2016a). However, there are inadequate data available for AIV inactivation at lower temperatures such as those that can be achieved

Abbreviations: AIV, avian influenza virus; Ct, cycle threshold; ECE, embryonating chicken eggs; EID, egg infectious dose; HM, high moisture; HP, highly pathogenic; LM, low moisture; LP, low pathogenic; rRT-PCR, real-time reverse transcription polymerase chain reaction.

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http://dx.doi.org/10.1016/j.prevetmed.2017.06.012

in a poultry house, and for a mixed organic material like litter which is comprised of wood, manure and decaying feathers,

The objective of this study was to determine the time necessary to inactivate AIV across a range of temperatures to simulate different maximal achievable temperatures for different styles of poultry houses in different weather conditions. To simulate different litter conditions within a house, both high moisture (HM) and low moisture (LM) litter were tested. Finally, while all evidence suggests that LPAIV and HPAIV have the same thermal stability HPAIV was tested at a subset of temperatures to confirm this assumption.

A secondary objective was to determine if there was any correlation between heat treatment and detection of AIV by the rRT-PCR test, and compare these results to virus isolation in ECEs. Regardless of cleaning method, farms are still routinely tested for the absence of virus before the farm can be restocked. Currently, virus isolation is used because rRT-PCR can detect inactivated virus (Suarez et al., 2003). Although rRT-PCR can't be used to differentiate live from inactivated virus, if samples are negative by rRT-PCR it could provide a rapid and more cost effective method for screening saving substantial time and money.

2. Materials and methods

2.1. Viruses

Both HP and LP AIV isolates were selected for their ability to grow to high titers in embryonating chicken eggs (ECEs) in order to maximize the sensitivity of detection from the litter in downstream applications. The LPAIV strain utilized was rgA/gyrfalcon/WA/41088/2014x PR8 H5N1, which is a reverse genetics generated strain with the hemagglutinin gene from A/gyrfalcon/WA/41088/2014 H5N8 that was engineered to be LP, with the other 7 gene segments from A/Puerto Rico/8/1934 H1N1, a common laboratory influenza A strain which replicates to high titers in ECE. The HPAIV isolate selected was A/turkey/Italy/4580/1999 H7N1 (Banks et al., 2001), and was obtained from the Southeast Poultry Research Laboratory, USDA-ARS repository. Each isolate was titrated according to standard procedures in ECE (Spackman and Killian, 2014). The titer of the LPAIV isolate used was 8.4 $\log_{10}$ 50% egg infectious dose per ml (EID_{50}/ml), and the titer of the HPAIV isolate used was 8.8 $\log_{10}$ EID_{50}/1 ml. The viruses were added to the litter undiluted to simulate the highest viral loads in the litter possible.

2.2. Litter

Used litter, consisting of kiln dried, medium flake, mixed wood shavings was obtained from Southeast Poultry Research Laboratory specific pathogen free (SPF) chicken flocks. High moisture litter was collected from under the drinkers and LM litter was collected from dryer areas of the house. Litter pH for both LM and HM litter was determined to be 7.0–7.5. Moisture content was measured using soil moisture probes (EC-5 Small Soil Moisture Sensors; Decagon, Pullman, WA). Low moisture litter had a mean moisture level of 0.025 cubic meters of water per cubic meter of litter (m^3/m^3) (standard deviation = ± 0.053 m^3/m^3), which did not change during the course of any treatment. The HM litter had an average moisture content of 0.409 m^3/m^3 (standard deviation = ± 0.110 m^3/m^3) and decreased an average of 0.040 m^3/m^3 during treatment.

2.3. Experimental design

Litter treatment groups are shown in Table 1. Three replicates of each moisture level were run at each temperature. Samples were collected at a minimum of 24hr intervals at which time one vial was removed, sealed, and immediately stored at −80 °C. Sample collection times varied by temperature and were based on the expected endpoint (Table 1).

Individual 5 ml plastic vials were filled with 1.5–2.0 g of LM or HM litter, and then 0.1 ml of titrated virus was added to each vial. The vials were used to prevent lateral diffusion of the virus through the litter so the change in virus titer per gram could be accurately measured. Each vial served as an individual time-point sample at each temperature. All litter samples in vials were pre-heated to ambient temperatures before the virus was added. Individual litter vials were placed in 1 L canisters that had been filled with the same litter as the vials and when a sample vial was removed, an empty 5 ml vial was added back to the litter canister to maintain the density. An untreated sample of litter to which virus was added (time 0 sample) was collected at the time the virus was added to all the litter sample vials for the same temperature treatment, and was immediately stored at −80 °C until it was processed for virus detection. Individual vials and 1L canisters were not sealed so the litter could evaporate naturally (Supplemental Fig. S1). However, all material was sealed in a secondary container to contain the virus. Temperatures were maintained in an environmental chamber.

Changes in moisture content were monitored using data loggers with soil moisture probes (EM5B Analog Data Loggers and EC-5 Small Soil Moisture Sensors; Decagon, Pullman, WA). As a secondary measure of moisture change, canisters were weighed before and after each temperature treatment. A separate 5 ml vial of litter with no virus added was included in each 1 L canister and was weighed before and after each treatment, to confirm that moisture loss in the 5 ml vials was equivalent to the 1 L canister. A data logging thermometer with a probe were placed at a depth of 2–5 cm into the center of the litter in each canister (SD200 3-Channel Temperature Data logger; ExTech, Nashua, NH) to verify the temperature of the litter.

2.4. Extraction of virus from litter

The procedure for extracting virus from the litter was optimized for virus recovery prior to processing samples. It was determined that storage at −80 °C with one total freeze-thaw cycle did not decrease virus titers. Each sample was processed and titrated individually as follows: brain heart infusion (BHI) broth (3.5 ml for LM litter and 3.0 ml for HM litter) was added to each vial, and the material was then mixed by vortexing and incubated at ambient temperatures for 10 min. Samples were homogenized with a pestle by hand to minimize heat generation, then centrifuged at 2900 × *g* for 10 min, the supernatant was collected and centrifuged again at 1900 × *g* for 10 min. The supernatant from the second centrifugation step was used for virus isolation and RNA extraction.

2.5. Virus isolation and titration

The supernatant was treated with antibiotics (final concentration: Penicillin G 1000 IU/ml, Streptomycin 200 μg/ml, Gentamicin 100 μg/ml, Kanamycin 65 μg/ml, Amphotericin B 2 μg/ml) for 1 h at ambient temperature which was necessary for the antibiotics to kill any contaminating bacteria. Each virus isolation was set-up as a titration to maximize sensitivity and to quantify virus. Titrations were conducted in ECEs by standard methods (Spackman and Killian, 2014). Briefly, 5 ECEs were inoculated with 0.1 ml of each dilution by the chorioallantoic sac route using undiluted material through a dilution of 10^{-5} in BHI broth. Standard hemagglutination assay was used to test the fluid from each egg for virus replication (Killian, 2014). Titers were calculated with the Reed-Meunch method (Reed and Muench, 1938). The endpoint was defined as the first time point where all three replicates were negative. Controls collected at time 0 had an average titer of 4.29 ± 0.78 $\log_{10}$