

ENVIRONMENT, WELL-BEING, AND BEHAVIOR

Tenacity of low-pathogenic avian influenza viruses in different types of poultry litter

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ABSTRACT To determine the risk of infection associated with exposure to low-pathogenic avian influenza (AI) virus-contaminated poultry litter, the tenacity of low pathogenic A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/Ohio/313053/04(H3N2) was evaluated. Viral stocks were incubated with poultry litter from commercial flocks at 25°C. Three types of poultry litter, wood shavings, shavings plus gypsum, and shavings plus peanut hulls, from commercial broiler flocks were used. The 3 low-pathogenic avian influenza viruses retained infectivity for one day in wood shavings and shavings plus peanut hulls litter types, whereas in wood shavings plus gypsum, litter viruses remained infective for up

to 3 d. In contrast to the survivability in litter, all the viruses maintained infectivity in water for 4 d at titers of log₁₀4.5. The infectivity of A/Ck/CA/431/00(H6N2) shed by experimentally infected layers, broilers, and turkeys was retained for one day, independently of the type of litter. In commercial production where a high density of birds are housed, the viral load shed by an infected flock will be significantly higher than the viral load shed 3 d postinfection obtained under the experimental conditions used in this study. Therefore proper management and disposal of poultry by products, such as windrow composting of litter and the composting of carcasses during an AI outbreak should be implemented.

Key words: infectivity, avian influenza, poultry litter

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INTRODUCTION

Avian influenza (AI) viruses (*influenza A virus*, *Orthomyxoviridae*; Lamb, 2001) can infect a wide variety of animals, from wild birds to mammals. Wild ducks and shorebirds are the natural host and reservoir of this diverse group of viruses. Avian influenza viruses are classified on the antigenic profiles of the surface glycoproteins; these include 16 hemagglutinin (H1 through H16) and 9 neuraminidase (N1 through N9) subtypes, for a total of 144 possible different influenza virus subtype combinations (Lamb, 2001).

Although poultry is not the natural host for AI viruses, introduction to poultry occurs frequently (Alexander, 2000) and can result in 2 distinct types of AI infections. The low-pathogenic (LPAI) virus infection is characterized by a mild respiratory disease with low morbidity and no mortality and the highly pathogenic (HPAI) virus infection is characterized by severe respiratory disease accompanied with high morbidity and

mortality (Spickler et al., 2008). The mechanisms by which AI viruses are transmitted, the extent, route, duration of viral shedding, and tenacity outside of the host are extremely variable and depend on the viral strain, species of bird infected, and a variety of environmental factors (Alexander et al., 1986; Makarova et al., 2003; Alexander, 2007; Yee et al., 2009; Pillai et al., 2010). In poultry, respiratory tract replication and aerosol transmission of the virus are the most important factors in the spread of the disease within a flock.

However, poor management during movement of poultry, poultry by-products, and poultry waste will promote mechanical transmission and consequently the spread of avian influenza viruses (McQuiston et al., 2005; Swayne and Halvorson, 2008).

It has been established that composting of poultry wastes contributes to the rapid inactivation of LPAI and Newcastle disease viruses (Kinde et al., 2004; Guan et al., 2009); for example, composting of carcasses has been effectively used for emergency disposal during LPAI outbreaks (Akey, 2003; Wilkinson, 2007). Among poultry wastes, litter is the most significant in volume and value and its disposal and management is vital in the control of infectious diseases (Coufal et al., 2006; Bernhart et al., 2010). To assess the risk of mechanical

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spreading of LPAI viruses via the movement of non-composted litter, the infectivity of one LPAI virus in layer litter was evaluated (Lu et al., 2003). The objective of this study was to expand the *in vitro* assessment initiated by Lu et al. (2003) by testing the tenacity of 3 LPAI viruses in commercial broiler litter started from wood shavings alone, combined with peanut hulls, or combined with gypsum and to determine the infectivity of A/Ck/CA/431/00(H6N2) in litter contaminated by experimentally infected layers, broilers, and turkeys.

MATERIALS AND METHODS

Viruses

Three viruses were used in this study, the poultry-adapted A/Ck/CA/431/00(H6N2) originally isolated from a layer flock in California between 2000 and 2001 (Webby et al., 2002), A/Mallard/MN/355779/00(H5N2) originally isolated from a cloacal swab sample of a wild mallard (Costa et al., 2010), and A/turkey/Ohio/313053/04(H3N2) originally isolated from a 34-wk-old turkey breeder flock in Ohio (Tang et al., 2005). Viruses were propagated in 10-d-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) as previously described (Swayne et al., 2008b); second ECE passage of each virus was used to mix with used broiler litter samples and to infect layers, broilers, and turkeys.

Infectivity of A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/Ohio/313053/04(H3N2) in Different Types of Broiler Litter

Different used broiler litters of pine shavings (shavings), pine shavings plus gypsum (gypsum), or pine shavings plus peanut hulls (peanut hulls) were obtained from AI virus-negative local commercial flocks. Three samples from each litter type (0.5 g) were placed in 5-mL snap cap tubes in triplicates and mixed by vortex for 5 min with 1 mL of H6N2, H5N2, or H3N2 infected allantoic fluid at a titer of $10^{7.5}$ EID₅₀/mL followed by moderate shaking for 15 min to ensure the virus was spread homogeneously and absorbed by the litter. As a negative control, 3 samples from each litter type (0.5 g) were mixed with uninfected allantoic fluid as indicated above. As a positive control, distilled water aliquots (900 μ L) were mixed with 100 μ L of H6N2, H5N2, and H3N2 ($10^{7.5}$ EID₅₀/mL) in triplicates. All tubes (36) were incubated at 25°C. Aliquots from each tube were collected and tested for virus isolation at 0, 1, 2, 3, and 4 d post mixing. At the predetermined time point, 2 mL of brain-heart infusion (BHI) media (Sigma-Aldrich, St. Louis, MO) with antibiotics (10,000 U/L of penicillin-G potassium, 250 g/L of gentamycin sulfate, 25 mg/L of amphotericin B (Fisher Scientific), 500 g/L of kanamycin (Sigma-Aldrich), and 1 g/L of streptomycin sulfate (Sigma-Aldrich) was added to each tube,

mixed by vortex for 30 min, centrifuged at $7,000 \times g$ for 5 min and filtered through a 0.45- μ m syringe filter (Whatman), filtrates were stored at -80°C until inoculation in embryos to determine viral titers.

Experimental Infection of Layers, Broilers, and Turkeys with A/Ck/CA/431/00(H6N2). As approved by the animal use committee of the University of Georgia (AUP A2009 10-032-Y3-A0), the infectivity of A/Ck/CA/431/00(H6N2) shed by layers into different types of used litter (shavings, gypsum, peanut hulls) was evaluated as follows: eighteen 3-wk-old layers were inoculated intranasally with A/Ck/CA/431/00(H6N2) virus at a $10^{7.2}$ embryo infectious dose (EID)₅₀ per bird in a volume of 200 μ L, as negative controls, 6 layers were inoculated intranasally with 200 μ L of noninfected allantoic fluid. Infected layers, were distributed into 3 stainless-steel, polycarbonated, negative pressure, HEPA filtered, isolator units 6 layers per isolation unit and 6 noninfected layers were housed in a fourth isolation unit. Before placing infected and noninfected chickens, litter beds of used broiler litter obtained from AI-negative local commercial broiler flocks were spread on the floor of the 4 isolation units (4 pounds per unit). Used litter started from shavings was spread on the floor of 2 isolation units. One unit housed 6 H6N2-infected layers and the second unit housed 6 noninfected layers. Used litter started from gypsum was spread on the floor of a third isolation unit, and used litter started from peanut hulls was spread on the floor of the fourth isolation unit, both isolation units housed infected layers. To determine if layers were properly infected 3 d postinfection (PI), 3 pools of cloacal swabs (2 swabs per pool) were collected from each treatment group and placed in BHI media with antibiotic and tubes were briefly vortex and stored at -80°C for virus titration. In addition, at 3 d postexposure of litter to infected layers, approximately 300 g of litter was separated from isolation units housing infected and noninfected layers. Three litter samples of 100 g each were placed in 3 open plastic containers. Containers were then returned to the isolation units and placed away of direct contact with chickens to preserve the litter viral loads as when collected at 3 d postexposure while keeping it in the same environment as the chickens. At 0, 0.5, 1, 1.5, and 2 d postplacement, 10 g of litter was collected from each plastic container placed in triplicates per isolation unit. Litter samples (10 g each) were resuspended in 30 mL of BHI with antibiotics and mixed by vortex for 30 min, centrifuged at $7,000 \times g$ for 5 min, and filtered through a 0.45- μ m syringe filter (Whatman), filtrates were stored at -80°C until inoculation in embryos to determine viral titers.

A second experiment was performed to determine the infectivity of A/Ck/CA/431/00(H6N2) in used broiler litter started from shavings shed by broilers and turkeys. One-day-old commercial broilers and turkeys were obtained from a local producer; at 3 wk of age, 12 broilers and 12 turkeys were distributed into 4 isolation units (6 broilers/unit; 6 turkeys/unit). Six broilers and 6 tur-

keys were inoculated with A/Ck/CA/431/00(H6N2) virus, while the remaining broilers and turkeys were inoculated with noninfected allantoic fluid as described above. Before challenge, each unit was prepared with a bedding of used broiler litter starter from shavings as described above. At 3 d PI, 3 pools of cloacal swabs were collected, and litter from units housing infected and noninfected birds was collected and divided into 3 open plastic containers that were placed back in each of the units and remained inside the isolation unit until the end of the experiment. At 0, 1, 1.5, and 2 d post-placement, 10 g of litter was collected from each of the 3 containers placed per unit and resuspended in 30 mL of BHI with antibiotics and processed as described above for virus isolation and titration. All birds were kept in the isolation units for 2 wk, monitored daily after inoculation with LPAI viruses, fed a standard diet with water ad libitum, and were euthanized by neck dislocation.

Virus Isolation and Titration

Virus isolation and titrations on each of the collected and processed samples were performed in 9- to 11-d-old SPF ECE. Three embryos were inoculated in the allantois with 200 μ L of undiluted 10^{-1} to 10^{-7} serial dilutions of the processed litter samples. After inoculation, eggs were incubated for 4 d and candled daily to monitor mortality rates. On the fourth day, surviving embryos were stored at 4°C for 3 to 6 h. The allantoic fluid was collected from each embryo and tested for hemagglutinin (HA) activity (Swayne et al., 2008; Thayer, 2008). Samples were considered positive if allantoic fluid had an HA titer higher than 2. The presence of AIV in HA-positive samples was confirmed by hemagglutination inhibition (HI) assay. Viral titers were determined using Reed and Muench (1938), and expressed as EID₅₀. Mean titer per treatment was estimated from 3 independently collected samples.

HI Assay

To confirm that isolated virus was of the expected serotype, chicken hyperimmune sera against chicken H6N2, duck H5N2, and turkey H3N2 isolates was produced and used to type by HI-positive allantoic fluid samples. Hyperimmune sera against A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/Ohio/313053/04(H3N2) were produced in chickens. Briefly, 3 groups of 7-d-old SPF layer chickens were immunized intravenously with $10^{7.5}$ EID₅₀/mL of each virus. Three weeks after the first immunization, chickens were boosted intravenously with similar doses of each virus. Fifteen days after the second immunization, sera were collected and used in the HI assay following protocols previously described (Swayne et al., 2008; Thayer, 2008). Hemagglutinin inhibition antibody titers on sera from infected layers, broilers, and turkeys with HI titers $\geq 4 \log_2$ were considered positive (Thayer, 2008).

Statistical Analysis

Linear regression model with robust standard errors was used to estimate the magnitude of the decrease in log₁₀ titer (tenacity) over time. The slopes were compared between treatments (litter types) or within treatments (virus subtypes) by evaluating the significance of treatment by day interactions. Bonferroni's procedure was used to limit the type I error probability to 0.05 overall comparisons (PASW statistics 18, SPSS, Chicago, IL).

RESULTS

Infectivity of A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/Ohio/313053/04(H3N2) Viral Stocks in Broiler Litter

Decrease of H6N2 (Figure 1a), H5N2 (Figure 1b), and H3N2 (Figure 1c) titers during a 4-d period, when in contact with the 3 types of used broiler litter are shown in Figure 1. Viral titers for all 3 viral subtypes were detected for a 2-d period in the wood shavings and wood shavings plus peanut hulls treatments and for a 3-d period in the wood shavings plus gypsum litter treatment. No viral titers were detected after 4 d in contact with the 3 broiler litter treatments. Viral titers in distilled water remained at 4 log₁₀ EID₅₀ 4 d postinoculation (Figure 1).

Linear regression models were constructed for the 3 virus subtypes to estimate the magnitude of viral titer decrease over time among viral subtypes within a litter treatment (data not shown). There were no differences in the slopes among viral subtypes within one litter treatment from 0 to 2 d (Table 1). Linear regression models were also constructed to estimate titer decrease over time (0 to 2 d) for a single subtype in contact with different litter treatments (Figure 1). Overall *P*-values for the slope differences of H6N2, H5N2, and H3N2 linear regression models were 0.013, 0.014, and <0.001, respectively (Table 1). Differences in slopes were detected for H5N2 virus in wood shavings as compared with H5N2 virus in wood shaving plus gypsum (*P* = 0.011), indicating faster decrease in titers for the shavings treatment but not for the shavings plus peanut hulls treatment. Differences in slopes were detected for H3N2 in wood shavings (*P* = 0.011) and wood shaving plus peanut hulls (*P* < 0.001) as compared with wood shavings plus gypsum, indicating a faster H3N2 titer decrease on these 2 later treatments. No differences in slopes were detected for H6N2 in wood shavings, wood shavings plus peanut hulls, and wood shaving plus gypsum treatments (Table 1).

H6N2 Cloaca Viral Titers

At d 3 PI, average viral titers in cloacal swab pools from H6N2-infected layers ranged from 4.33 to 4.42

\log_{10} , whereas H6N2 averaged cloaca swabs viral titers of 4.5 and 2.28 \log_{10} , estimated for broilers and turkeys, respectively.

H6N2 HI Antibody Titers

At 11 d PI, H6N2-infected layers, broilers, and turkeys showed HI titers ranging from 4 \log_2 to 8 \log_2 , confirming infection. All negative-control birds remained sero-negative.

Infectivity of H6N2 Virus Shed By Layers, Broilers, and Turkeys

Ten grams of litter contaminated with fecal droppings from H6N2-infected layers, were collected at 0 (3 d PI), 0.5, 1, 1.5, and 2 d, whereas collection of litter from infected broilers and turkeys was performed at 0 (3 d PI), 1, 1.5, and 2 d. In litter contaminated by infected layers, virus was detected at 0, 0.5, and 1 d postplacement but not at 1.5 and 2 d. In litter contaminated by infected broilers and turkeys, virus was detected at 0 and 1 d postplacement but not at 1.5 and 2 d. At 0 h postplacement, average H6N2 titers shed by layers in shavings, peanut hull, and gypsum litter were 1.83 and 1.50 \log_{10} EID₅₀, whereas in shavings contaminated by infected broilers and turkeys, an average H6N2 titer of 1.46 \log_{10} EID₅₀ was detected. At 1 d postplacement, the average titers found in all litter treatments was 0.5 \log_{10} EID₅₀ (Figure 2). The magnitude of H6N2 titer decreased over time in different litter treatments and R² values were estimated (Table 2). Linear regression analysis for H6N2 titer decrease from 0, 0.5, to 1 d postplacement among litter types contaminated by layers showed no differences among the slopes. Although only 2 time points were analyzed for H6N2 titer decrease (from 0 to 1 d postplacement), no slope differences were found in shavings contaminated by infected broilers and turkeys (Table 2).

DISCUSSION

The objective of this study was to expand the in vitro assessment initiated by Lu et al. (2003) by testing the tenacity of 3 LPAI viruses in used commercial broiler litter started from wood shavings, wood shavings combined with peanut hulls, or combined with gypsum. Compared with the Lu et al. (2003) study where LPAI A/CK/PA/3779-2/97(H7N2) remained infective in manure from commercial layers for more than 2 d at ambient temperatures of 15° to 20°C and for one day at 30° to 37°C, in this study, LPAI viruses H6N2, H5N2, and H3N2 retained infectivity for one day in used broiler litter originated from shavings or shavings plus peanut hulls and for 3 d in litter originated from shavings plus gypsum at 25°C. Due to the high hydrophobicity of the gypsum component, the allantoic fluid was not completely absorbed and consequently the virus was

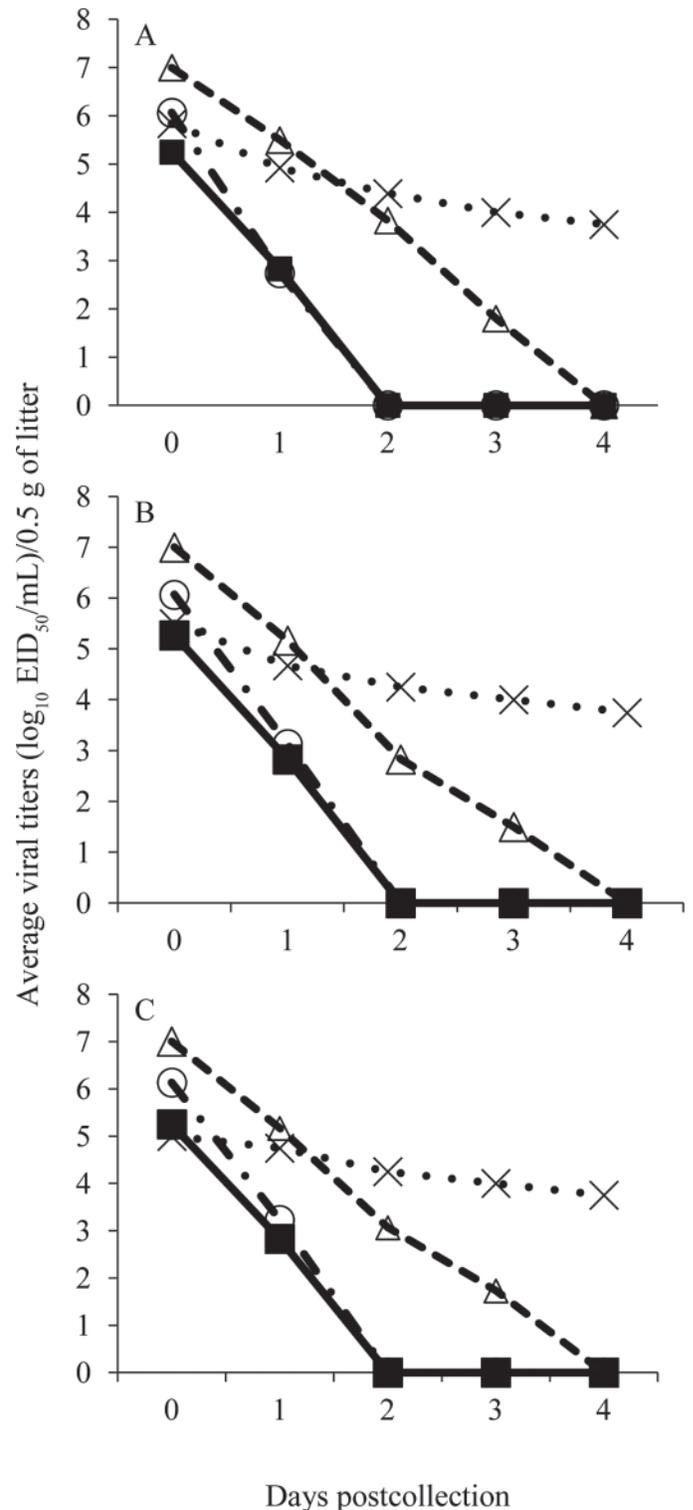


Figure 1. In vitro evaluation of viral titers of low-pathogenic avian influenza viruses when in contact with used litter. Average viral titers of H6N2 (A), H5N2 (B), and H3N2 (C) (\log_{10} EID₅₀) at 0 to 4 d post-contact with different types of used litter: Δ shavings plus gypsum, \circ shavings plus peanut hulls, \blacksquare shavings, and \times water.

not as effectively inactivated as when mixed with the shavings and shavings plus peanut hulls treatments. Therefore, the extended infectivity of the LPAI viruses in the shavings plus gypsum litter was anticipated.

Experimentally, LPAI viruses have been shown to persist for prolonged periods of time in distilled water

Table 1. Linear regression models for H6N2, H5N2, and H3N2 virus infectivity when in contact with different types of used broiler litter over a period of 2 d

Treatment	H6N2		H5N2		H3N2		P-value ¹
	Equation	R ²	Equation	R ²	Equation	R ²	
Wood shavings	$y = 5.97 - 3.03x^A$	0.968	$y = 6.19 - 3.07x^A$	0.991	$y = 6.10 - 3.03x^A$	0.993	0.382
Wood shavings plus gypsum	$y = 7.17 - 1.77x^A$	0.989	$y = 6.88 - 1.74x^B$	0.992	$y = 6.83 - 1.77x^B$	0.986	0.563
Wood shavings plus peanut hulls	$y = 5.32 - 2.63x^A$	0.982	$y = 5.32 - 2.63x^B$	0.982	$y = 5.33 - 2.63x^A$	0.994	0.951
P-value ²	0.013		0.014		<0.001		

^{A,B}Within columns, slopes with a superscript in common are not significantly different when using the Bonferroni procedure to limit the type I error probability to 5% over all comparisons.

¹Comparison of viruses within a treatment.

²Comparison of treatments within a virus.

(Stallknecht et al., 1990; Brown et al., 2009). In this study, LPAI virus infectivity was maintained in distilled water for 4 d at titers of log₁₀4.5. Therefore, virus infectivity in distilled water was used as a control to compare virus survivability in litter. In addition, virus infectivity in water has been shown to be dependent on temperature, pH, and salinity (Webster et al., 1978; Stallknecht et al., 1990; Brown et al., 2009); a pH range of 7.4 to 8.2 was determined to be the optimal range for stability of LPAI viruses in water (Brown et al., 2009). The pH of shavings, shavings plus gypsum, and shavings plus peanut hulls after adding allantoic fluid ranged from 8.0 to 8.4, which was within the range required for prolonged virus persistence in water; however, a rapid loss of infectivity of LPAI was observed in litter. As previously reported, allantoic fluid may promote bacterial growth at 25°C, resulting in decreased viral titers due to viral inactivation by bacterial metabolites and removal of virions by adherence to bacteria (Nazir et

al., 2010). Overall, the magnitude of titer decrease for individual viral subtypes over a 2-d period yield significantly higher slopes for shavings plus gypsum and shavings plus peanut hulls litters contaminated with H5N2 and for shavings plus gypsum litter contaminated with H3N2. While no significant slope differences were estimated for H6N2 in either litter treatment.

In contrast to the in vitro experiment, where the infectivity of the H6N2 virus was prolonged from 2 d, the H6N2 shed by experimentally infected birds remained infective for only one day, and neither the type of litter nor the source of fecal contamination had an effect in the virus infectivity.

Several factors may have contributed to accelerate the inactivation of the H6N2 virus shed by birds. Infected birds had a limited period of 3 d postinfection to shed virus before the litter was collected and placed in containers away from the chickens. Mainly because the starting titers were much lower than those of the in vitro experiment, regression models for the in vivo experiment showed low correlations for viral titer reductions over time, influencing the precision of measurements and consequently limiting the range of measurements acquired.

Another factor that may have accelerated inactivation was the decrease in moisture content observed during a 2-d period from 31% to 7.7% in shavings and shavings plus peanut hulls and from 19% to 8% in shavings plus gypsum litter.

In general, although low humidity and limited fecal contamination of the litter may have contributed to the accelerated inactivation of the viruses, LPAI viruses retained infectivity for one day. These results further emphasize the importance of cleaning and disinfection of chicken houses and proper handling of poultry by-products, such as the composting of carcasses and windrow composting of litter during AI outbreaks.

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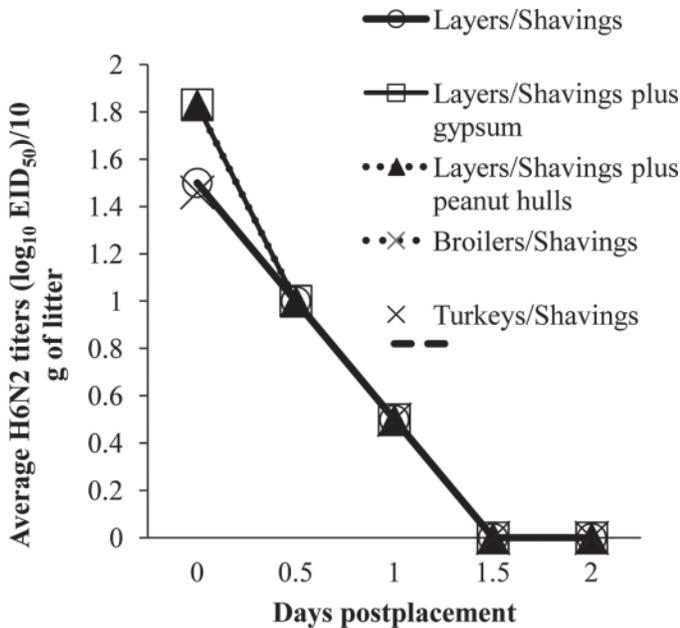


Figure 2. In vivo evaluation of A/Ck/CA/431/00(H6N2) infectivity shed by layers, broilers, and turkeys in different types of used litter. Average viral titers (log₁₀ EID₅₀) in different types of used litter measured 0 to 2 d postplacement (3 to 5 d postexposure to infected birds).

Table 2. Linear regression models for H6N2 virus tenacity shed by layers, turkeys, and broilers on different types of used litter for a period of 1.5 d

Treatment	Equation ¹	R ²
Layers/Shavings ²	$y = -1.2x + 1.7$	0.380
Layers/Shavings and peanut hulls ²	$y = -1.2x + 1.7$	0.490
Layers/Shavings and gypsum ²	$y = -1.0x + 1.5$	0.560
Broilers ³ /Shavings	$y = -1.0x + 1.5$	0.700
Turkeys ³ /Shavings	$y = -1.0x + 1.5$	0.700

¹ $y = \log_{10}EID_{50}$; x = persistence in days.

²Comparison of litter type contaminated by layers, $P = 0.904$.

³Comparison of bird type with contamination of shavings-based litter.

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