HHS Public Access

Author manuscript

J Wildl Dis. Author manuscript; available in PMC 2024 September 04.

Published in final edited form as:

J Wildl Dis. 2013 January ; 49(1): 180–185. doi:10.7589/2011-11-322.

Infectivity of Avian Influenza Virus-Positive Field Samples for Mallards: What Do Our Diagnostic Results Mean?

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Abstract

Most surveillance programs for avian influenza (AI) virus in wild birds utilize molecular tests such as real-time reverse transcription-PCR (RRT-PCR) or virus isolation (VI) in embryonating chicken eggs. To provide insight into the relationship between positive diagnostic test results and infectivity for an avian host, we challenged Mallards (*Anas platyrhynchos*) with Mallard-derived cloacal swab field samples found positive by VI or RRT-PCR. Six of 11 samples that were both RRT-PCR positive and VI positive infected Mallards. Sample infectivity for Mallards appeared to be dependent on concentration of infectious virus in the sample; five of the six samples that replicated in Mallards had a measurable virus titer, whereas four of the five samples that did not infect Mallards had titers below the limit of detection ($10^{0.9}$ median embryo infectious dose/0.2 mL). None of seven samples that were RRT-PCR positive and VI negative infected Mallards. These results indicate that embryonating chicken eggs are a sensitive diagnostic tool for detecting Mallards excreting infectious AI virus at a high enough concentration to infect another Mallard; however, not all cloacal swab field samples that are positive by VI or RRT-PCR are infective to another Mallard. Additionally, our results indicate that Mallards are susceptible to Mallard-origin AI viruses that have not been propagated in embryonating chicken eggs and that some of these virus strains can infect birds at titers that are lower than those typically used in experimental challenge studies. These data highlight a need to examine the effects of using egg-propagated AI viruses in experimental trials.

Keywords

Avian influenza virus; Ct value; diagnostic; infectivity; Mallard; PCR; virus isolation

Diagnostic tests used to detect avian influenza (AI) virus infection in waterfowl vary among laboratories, but most often include virus isolation (VI) in embryonating chicken eggs, molecular tests such as real-time reverse-transcription PCR (RRT-PCR), or a combination of the two. Differences in sensitivity between VI and RRT-PCR are well established (Munster

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et al., 2009); however, both have been used to provide estimates of the prevalence of infection in wild populations. Although surveillance data based on molecular or classic egg-based diagnostic tests have provided the foundation for defining the patterns of AI virus infection in wild bird populations, basic knowledge gaps relating to diagnostics limit our ability to fully interpret surveillance results and use existing field data to address broader questions on viral transmission. For example, it is not known to what extent positive surveillance results, generated by RRT-PCR or VI, reflect samples that are infectious for a wild bird host. Conversely, it is not known how many wild bird samples containing infectious virus are missed because the viruses are not sufficiently adapted for replication in chicken eggs. To provide insight into these unknowns, we used cloacal swab field samples collected from Mallards (*Anas platyrhynchos*) to examine the relationship between diagnostic test results (RRT-PCR and VI in embryonating chicken eggs) and infectivity in juvenile Mallards.

Cloacal swab samples used in this study were collected from Mallards at premigrational staging areas in Minnesota, USA during 2007 to 2010. After collection, samples were transported to the University of Georgia and preserved at −80 C. Cloacal swab samples were thawed once, and simultaneously tested by VI in embryonating chicken eggs and RRT-PCR, and the remaining volume of cloacal swab media (approximately 0.9 mL) was returned to −80 C. On the basis of the RRT-PCR and VI results, 19 of these cloacal swab samples were selected for inclusion in this study to represent various combinations of diagnostic results; in the following scheme we use a plus sign (+) to indicate positive and a minus sign (−) to indicate negative: RRT-PCR−/VI− (n=1); RRT-PCR+/VI− (n=4); RRT-PCR+/VI+ (n=14). Samples were chosen using only the laboratory identification number, without knowledge of viral subtype. At the time the samples were selected, the infectious viral titers of the field samples were unknown, but were assumed to be lower than concentrations that are typically used in experimental challenge studies. Consequently, proportionally more RRT-PCR+/VI+ samples were selected to increase the likelihood that samples infectious for inoculated Mallards would be included in the study.

The 19 cloacal swab samples were thawed a second time and the inoculum was prepared by diluting the residual cloacal swab medium in brain heart infusion (BHI) medium to a final volume of 2.5 mL. For clarity, throughout this manuscript, the term “cloacal swab field sample” will refer to the original sample collected from Mallards in Minnesota, whereas “inoculum” will refer to the sample after it was thawed a second time and diluted to a final volume of 2.5 mL. After dilution, each of the inoculum samples was simultaneously tested by 1) VI in embryonating chicken eggs, 2) virus titration in embryonating chicken eggs, 3) RRT-PCR, and 4) experimental inoculation of juvenile Mallards. General techniques for VI and virus titration were performed following standard published procedures (Swayne et al., 2008). Virus isolations were performed by injecting 0.2 mL of the inoculum into the allantoic sac of five 10-day-old embryonating chicken eggs. Inoculum samples were considered VI+ if there was evidence of AI virus replication in one or more of the five injected eggs. For virus titration, the inoculum samples were serially diluted, 10-fold, in BHI medium and 0.1 mL was inoculated into five 10-day-old embryonating chicken eggs per dilution. Virus titers were calculated using the method described by Reed and Muench (1938). For RRT-PCR, viral RNA was extracted from the inoculum samples using the

QIAamp® Viral RNA Mini Kit (QIAGEN, Valencia, California, USA), and the samples were tested for influenza A virus nucleic acid using a protocol targeting the internal matrix gene (Spackman and Suarez, 2008).

For the infectivity trials, 1-day-old, captive-bred Mallards were purchased from a commercial breeder (McMurray Hatchery, Webster City, Iowa, USA) and raised at the University of Georgia (UGA) until 4 wk of age. At 4 wk, ducks were randomly separated into 19 groups (five birds/group), and each group was challenged with inocula prepared from the samples listed in Table 1. Individual ducks were inoculated with 0.2 mL of the inoculum (matching the volume injected into each egg for VI), which was split evenly between the intranasal and intratracheal routes. Blood was collected from each Mallard on 0 and 14 days postinoculation (DPI) for serologic testing using a commercial blocking enzyme-linked immunosorbent assay (bELISA; Flock-Chek AI MultiS-Screen antibody test kit, IDEXX Laboratories, Westbrook, Maine, USA). Oropharyngeal and cloacal swabs were collected from each Mallard on 0, 2, and 4 DPI and placed in separate vials containing 2.0 mL of BHI medium with antimicrobial drugs (250 μg/mL gentamicin, 500 μg/mL kanamycin, 1,000 μg/mL streptomycin, 1,000 units/mL penicillin G, and 25 μg/mL amphotericin B) for VI in embryonating chickens eggs (Swayne et al., 2008). The timing of these sample collections was based on data from prior experimental challenge studies with low-pathogenic (LP) AI viruses in age-matched Mallards (Costa et al., 2010; Brown, pers. comm.), which indicate that peak levels of shedding occur during this period and residual inoculum is unlikely to be detected in oropharyngeal swabs collected at 2 DPI. Individual Mallards were considered to be infected if any postinoculation oropharyngeal or cloacal swabs were VI+ or postinoculation serum was positive on the bELISA. The trial was terminated at 14 DPI and all birds were euthanized by CO_2 inhalation. General animal care was provided and experimental sampling was performed according to an animal care and use protocol approved by the Institutional Animal Care and Use Committee at UGA.

On the basis of VI and serology, respectively, none of the Mallards used in the challenge study was shedding virus or had antibodies to AI virus at the time of inoculation. The following information is summarized in Table 1: 1) RRT-PCR and VI results for the cloacal swab field samples, 2) RRT-PCR, VI, and virus titration results for the 19 inoculum samples, and 3) pre- and postinoculation VI and serology results for Mallards experimentally challenged with the 19 inoculum samples. Of the 14 cloacal swab field samples originally RRT-PCR+/VI+, all 14 inoculum samples were RRT-PCR+ but only 11 were VI+. The inability to reisolate virus from three of these inoculum samples presumably relates to the combined effects of a second freeze–thaw event and dilution of the cloacal swab field sample in BHI medium. Consistent with a prior study (Munster et al., 2009), virus was isolated more frequently from RRT-PCR+ inoculum samples with higher viral loads, as evidenced by lower cycle threshold (Ct) values; RRT-PCR+/VI+ samples (n=11) had a mean Ct value of 28.90 (range: 26.16–32.88) compared with RRT-PCR+/VI− samples (n=7) that had a mean Ct value of 35.00 (range: 29.07–39.28). It was not our primary intent to evaluate the relationship between RRT-PCR and VI or virus titration results for the inoculum samples. Consequently, using these data to draw conclusions concerning relationships between molecular and egg-based diagnostic tests may be inappropriate, as

the two freeze–thaw events and the dilution step could significantly alter inoculum sample infectivity with minimal change to Ct values.

Six of the 11 (54.5%) inoculum samples that were RRT-PCR+/VI+ infected Mallards on the basis of postinoculation viral shedding or seroconversion (Table 1). As all viruses were originally isolated from Mallards, the failure of five RRT-PCR+/VI+ inoculum samples to infect Mallards does not likely relate to host adaptation. Rather, the failure to infect Mallards appeared to be associated with the virus titer of the inoculum samples; five of six (83%) RRT-PCR+/VI+ inoculum samples that replicated in Mallards had titers that ranged from $10^{0.9}$ to $10^{3.8}$ median embryo infectious doses (EID_{50})/0.2 mL (the volume of a single bird inoculum), whereas only one of five (20%) RRT-PCR+/VI+ inoculum samples with a titer below the limit of detection ($<10^{0.9}$ EID_{50}/0.2 mL) infected Mallards. Although the inability to infect Mallards with inoculum samples that replicated in embryonating chicken eggs was associated with virus titer of the sample, this factor alone did not fully explain the results, as inoculum samples A/Mallard/MN/AI09–2434/09 had a titer of $10_{1.9}$ EID_{50}/0.2 mL and did not infect any Mallards, whereas A/Mallard/MN/Sg-00570/08 had a titer below the detectable limit of the test but infected all five challenged Mallards. Such exceptions suggest that the sensitivity of Mallards to LP AI virus infection (i.e., differences in infectious dose) varies between AI viral strains, as has been reported for both domestic poultry and waterfowl (Swayne and Slemons, 2008).

Consistent with the results described above, none of the seven inoculum samples that were RRT-PCR+/VI− infected Mallards. This is a common diagnostic result in wild-bird AI surveillance, which is difficult to interpret, particularly if the sample has a high Ct value (>35). As none of the RRT-PCR+/VI− inoculum samples replicated in eggs or inoculated Mallards, the inability to isolate virus using classic egg-based diagnostic tests does not necessarily appear to reflect a lack of virus adaptation. It is important to note that our experimental protocol involved two freeze–thaw events, and a loss of infectivity associated with freeze–thaws has been previously demonstrated with AI viruses (Stallknecht et al., 2010). Although this is an experimental artifact, it is one that is relevant to wild-bird AI surveillance programs. Most AI surveillance testing protocols involve at least one freeze–thaw of diagnostic samples and, if the viral load in the sample is low, as evident by higher Ct values (>35), poor success in isolating virus may relate to inactivation during sample processing.

Regarding diagnostic interpretation, our data suggest that if cloacal swab field samples are collected, handled, and stored appropriately, few Mallards excreting infectious virus in the feces at a concentration transmissible to another Mallard would be missed with VI in embryonating chicken eggs. However, not all RRT-PCR+ or VI+ field samples can infect the host of origin, in this case Mallards. Finally, four of the six inoculum samples that infected Mallards had viral titers below $10^{1.5}$ EID_{50}/0.2 mL, which are significantly lower than the concentrations typically used in experimental challenge studies in ducks (4 to 6 $\log_{10}$ EID_{50}) and lower than previously reported infectious doses for most AI virus strains in Pekin ducks (Swayne and Slemons, 2008). These results indicate that Mallards are highly susceptible to infection with non-egg-passaged Mallard-origin LP AI viruses, as would be expected for a species widely considered a global reservoir for this virus. The difference between the

results of this study and previously reported infectious doses for AI viruses in Mallards may relate to egg adaptation. Nearly all previous experimental challenge studies with AI virus in waterfowl, including those with H5N1 highly pathogenic AI viruses, have utilized viral stock propagated in embryonating chicken eggs (Brown et al., 2011). This is a variable that, to our knowledge, has not been considered in the interpretation of experimental results or in the extrapolation of observations from experimental challenge studies to natural conditions. Our study demonstrates the ability to infect Mallards with non-egg-propagated AI viruses. Additionally, the results suggest that further research is necessary to evaluate potential biases associated with using egg-propagated viruses in experimental challenge studies, and our novel study design provides a potential template for how trials examining this topic could be conducted.

Acknowledgments

Funding for this work was provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract HHSN266200700007C, and the National Science Foundation under grant DEB-0917853. The opinions expressed herein are those of the authors and do not necessarily reflect the views of any of the funding agencies.

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TABLE 1.

Summary of testing results for 19 cloacal swab field samples and inoculum samples included in this study. Cloacal swab field samples refers to the samples that were collected from Mallards (*Anas platyrhynchos*) in Minnesota, frozen at −80 C, thawed, and tested by virus isolation (VI) and real-time reverse-transcription PCR (RRT-PCR). Inoculum samples refer to the cloacal swab field samples after they were refrozen at −80 C and thawed one additional time and the residual cloacal swab medium was diluted to 2.5 mL.

					Inoculum inoculation testing						
					Mallards						
						Postinoculation VI					
	Cloacal swab field samples testing		Inoculum concentration testing			2 DPI[c]		4 DPI			
Sample identification	VI	RRT-PCR (Ct value[a])	Titer ($log_{10}EID_{50}$[b])	RRT-PCR (Ct value)	VI (Eggs)	OP	Cl	OP	Cl	14 DPI serology	Mallard infection summary based on VI and serology
A/Mallard/MN/AI10-2106/10 (H4N6)	+	29.93	3.8	27.58	5/5	4/5	5/5	2/5	5/5	5/5	5/5
A/Mallard/MN/AI09-3094/09 (H6N1)	+	28.65	2.3	32.88	5/5	1/5	1/5	0/5	2/5	1/5	2/5
A/Mallard/MN/AI09-2434/09 (H3N8)	+	26.16	1.9	26.16	5/5	0/5	0/5	0/5	0/5	0/5	0/5
A/Mallard/MN/Sg-00929/08 (H3N8)	+	22.37	1.5	29.11	5/5	5/5	5/5	5/5	5/5	5/5	5/5
A/Mallard/MN/AI09-2684/09 (H6N1)	+	29.25	0.9	28.48	5/5	5/5	5/5	3/5	5/5	5/5	5/5
A/Mallard/MN/AI09-3100/09 (H6N3)	+	28.65	0.9	27.91	3/5	4/5	5/5	2/5	5/5	5/5	5/5
A/Mallard/MN/Sg-00680/08 (H8N4)	+	22.89	<0.9[d]	29.85	4/5	0/5	0/5	0/5	0/5	0/5	0/5
A/Mallard/MN/Sg-00570/08 (H8N4)	+	22.92	<0.9	30.41	1/5	2/5	3/5	0/5	5/5	5/5	5/5
A/Mallard/MN/AI09-2855/09 (H4N8)	+	30.19	<0.9	29.66	2/5	0/5	0/5	0/5	0/5	0/5	0/5
A/Mallard/MN/AI09-2564/09 (H3N8)	+	29.71	<0.9	27.84	5/5	0/5	0/5	0/5	0/5	0/5	0/5
A/Mallard/MN/AI10-2050/10 (H11N9)	+	29.16	<0.9	28.07	5/5	0/5	0/5	0/5	0/5	0/5	0/5
A/Mallard/MN/Sg-00684/08 (H8N8)	+	26.08	-[e]	34.31	0/5	0/5	0/5	0/5	0/5	0/5	0/5
A/Mallard/MN/Sg-00927/08 (H6N1)	+	29.91	-	39.28	0/5	0/5	0/5	0/5	0/5	0/5	0/5
A/Mallard/MN/Sg-00932/08 (H6N1)	+	25.96	-	35.57	0/5	0/5	0/5	0/5	0/5	0/5	0/5
AI07-5395	−	28.45	-	29.07	0/5	0/5	0/5	0/5	0/5	0/5	0/5
AI07-4041	−	33.85	-	34.83	0/5	0/5	0/5	0/5	0/5	0/5	0/5
AI07-2844	−	34.98	-	35.64	0/5	0/5	0/5	0/5	0/5	0/5	0/5
AI07-3907	−	35.42	-	36.28	0/5	0/5	0/5	0/5	0/5	0/5	0/5
AI08-3605	−	-	-	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a] Ct = cycle threshold.

[b] Titer in 0.2 mL inoculated into embryonating chicken eggs and Mallards.

[c] DPI = day postinoculation.

[d] Virus isolation positive, but below the limit of detection with viral titration.

[e] [−] = negative result.