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H5N2 vaccine viruses on Russian and US live attenuated influenza virus backbones demonstrate similar infectivity, immunogenicity and protection in ferrets

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Abstract

The continued detection of zoonotic influenza infections, most notably due to the avian influenza A H5N1 and H7N9 subtypes, underscores the need for pandemic preparedness. Decades of experience with live attenuated influenza vaccines (LAIVs) for the control of seasonal influenza support the safety and effectiveness of this vaccine platform. All LAIV candidates are derived from one of two licensed master donor viruses (MDVs), cold-adapted (*ca*) A/Ann Arbor/6/60 or *ca* A/Leningrad/134/17/57. A number of LAIV candidates targeting avian H5 influenza viruses derived with each MDV have been evaluated in humans, but have differed in their infectivity and immunogenicity. To understand these differences, we generated four H5N2 candidate pandemic LAIVs (pLAIVs) derived from either MDV and compared their biological characteristics *in vitro* and *in vivo*. We demonstrate that all candidate pLAIVs, regardless of gene constellation and derivation, were comparable with respect to infectivity, immunogenicity, and protection from challenge in the ferret model of influenza. These observations suggest that differences in clinical performance of H5 pLAIVs may be due to factors other than inherent biological properties of the two MDVs.

Keywords

avian influenza; Leningrad; Ann Arbor; LAIV; pandemic

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CONFLICT OF INTEREST STATEMENT

Hong Jin and Zhongying Chen are employees of MedImmune. The Icahn School of Medicine at Mount Sinai has filed patent applications regarding influenza virus vaccines. No other authors have potential conflicts to report.

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INTRODUCTION

Circulation of seasonal influenza viruses is responsible for an estimated 226,000 excess hospitalizations and 36,000 excess deaths per year in the United States alone (1). Additionally, several subtypes of avian influenza viruses have caused human illness; of these, H5N1 viruses are particularly notable with respect to incidence, morbidity and mortality, and evidence of increasing genetic diversification (2). Vaccination remains a critical element of influenza prevention and control strategies. To this end, the development of candidate vaccines for influenza viruses with pandemic potential has been the subject of substantial international effort (3).

The live attenuated influenza vaccine (LAIV) platform is particularly attractive for the development of a pandemic influenza vaccine. In well-controlled efficacy trials in children, despite induction of lower titers of serum antibody, trivalent seasonal LAIV offered superior protection against matched and antigenically drifted influenza viruses compared to trivalent inactivated vaccine (4–9). LAIVs can elicit diverse and robust T cell responses (10, 11), a feature that is associated with increased breadth of protection (5, 9, 12) and is particularly relevant given the difficulty in predicting which strain may emerge as a pandemic threat. Robust T-cell responses and mucosal immunity elicited by LAIVs are also associated with protection from severe disease (13–19). The potential advantages of this platform are recognized by its inclusion in the World Health Organization's pandemic preparedness plans (20, 21).

LAIVs are derived from genetically stable, attenuated influenza A and B “master donor viruses” (MDV; MDV-A and MDV-B). Two types of MDVs have been independently generated and licensed in Russia and the United States. These MDVs are used as backbones for LAIVs bearing the surface antigens of wild-type (*wt*) viruses and are generated by either classical reassortment or reverse genetics. The internal protein genes of the MDV confer the properties of attenuation (*att*), cold adaptation (*ca*) and temperature sensitivity (*ts*), that limit LAIV replication to the upper respiratory tract in humans (4).

A growing number of LAIV candidates for potential pandemic influenza strains (pLAIVs) based on each MDV, A/Ann Arbor/6/60 cold-adapted (*ca*) H2N2; (AA/6/60) or A/Leningrad/134/17/57 (*ca* H2N2; Len/17/57), have been evaluated in phase I clinical trials (4, 22, 23). The development of a robustly immunogenic pLAIV candidate for H5N1 avian influenza viruses has been challenging. An H5N2 pLAIV derived on the Len/17/57 MDV backbone was infectious and immunogenic in 50% of participants, although antibody titers were modest compared to those elicited by seasonal influenza vaccination (23). In contrast, an H5N1 pLAIV formulated with the AA/6/60 MDV was shed in a minority of participants and was not immunogenic in healthy adults (24). However, both H5 pLAIVs induced long lasting B cell memory responses, as demonstrated by a rapid and robust recall response following boost with inactivated H5 vaccine (25, 26).

It has been speculated that the Len/17/57-based viruses are more infectious and immunogenic than the AA/6/60-based vaccine viruses but the basis for the observed differences in viral replication and antibody response between the AA/6/60- and Len/17/57-

derived H5 LAIVs is not known. To directly compare characteristics of H5 pLAIV formulated with each MDV, we generated reassortant viruses by reverse genetics. All vaccine viruses bore surface antigens from the same low pathogenic avian influenza (LPAI) virus, but varied in internal gene composition to represent the formulations of H5 pLAIV candidates that have been evaluated in clinical studies. We compared the biological characteristics of these viruses *in vitro* and evaluated replication, immunogenicity, and protection from challenge with a heterologous highly pathogenic H5N1 avian influenza (HPAI) virus in the ferret model. Ferrets are naturally susceptible to most influenza A viruses, generate robust antibody responses to influenza antigens, and demonstrate clinical signs of infection (27) and are therefore a valuable model for pre-clinical evaluation of influenza vaccine candidates.

METHODS

Cells and viruses

293T and Madin-Darby canine kidney (MDCK) cells were maintained as described previously (28). Primary human nasal epithelial cells (hNEC; PromoCell) and primary human bronchial epithelial cells (NHBE; Lonza) were seeded onto membrane supports (6.5 mm Transwell; Corning, Inc.) coated with collagen type I (Corning, Inc) and maintained following manufacturer's recommendations. After monolayers reached confluence, an air liquid interface (ALI) was established and cells were differentiated for four weeks before infection.

All viruses in this study were propagated in embryonated chicken eggs (Charles River SPAFAS). Viral cDNA of the HA and NA of *wt* A/duck/Potsdam/1402-6/1986 (A/dk/Potsdam; H5N2 LPAI) were generated by RT-PCR following isolation of viral RNA. The unmodified hemagglutinin (HA) and neuraminidase (NA) coding sequences were cloned into pAD3000, a derivative of pHW2000 (28). Reassortant attenuated vaccine viruses were generated using an eight plasmid reverse genetics system as previously described, by co-transfection of 293T and MDCK cells (29). Briefly, the two 6:2 reassortant vaccine viruses were rescued with the six internal protein genes of either Len/17/57 or AA/6/60; the 7:1 vaccine viruses were rescued with only the H5 HA paired with the N2 segment from either MDV (Table S1). The 50% tissue culture infectious dose (TCID₅₀) was determined as previously described (14) and titer was calculated by the Reed-Muench method (30). Sequences of pLAIV stock viruses were verified by Sanger sequencing (BigDye v3.1, ABI).

In vitro analysis of H5N2 pLAIVs

The *ts* phenotype of the recombinant and *wt* A/dk/Potsdam viruses was evaluated by plaque assay, as described previously (29), with duplicate plates incubated at the permissive temperature (33°C) or the restrictive temperature (39°C). Results were independently verified by TCID₅₀ assay on MDCK cells. Growth curves were generated by inoculating MDCK cells, NHBE cells, and hNEC cells at a multiplicity of infection (MOI) of 0.01, as described previously (31, 32). Supernatant was sampled at serial time points. The amount of infectious virus in each sample was determined by TCID₅₀ assay in MDCK cells at 33°C.

Ferret studies

All ferret experiments were approved by and conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee at the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH). All study procedures and experiments were carried out in biosafety level 3 facilities in accordance with the Select Agent guidelines of the NIH, Centers for Disease Control and Prevention (CDC), and the U.S. Department of Agriculture (USDA). Adult (24 week old) outbred male ferrets (*Mustela putorius furo*; Triple F Farms) that were seronegative for circulating H1N1 and H3N2 viruses by hemagglutination inhibition (HAI) assay were housed in individual cages.

Prior to vaccination, a subcutaneous transponder was implanted to monitor temperature and to facilitate animal identification. Body weight, temperature, and activity level were assessed daily for 6 days following vaccination as well as after challenge. Ferret activity level was scored daily to assess disease-related changes in behavior, as previously described (33). Ferrets that exhibited ataxia or hind limb paralysis were humanely euthanized within 12 hours of first observation of neurological signs.

Six groups of four ferrets each were lightly sedated with isoflurane and vaccinated with a single dose of either one of the four H5N2 LAIVs or infected with *wt A/dk/Potsdam*, by intranasal inoculation of 10^7 TCID₅₀ of virus in a volume of 1 mL. Mock-vaccinated ferrets were inoculated with an equivalent volume of Leibovitz's L-15 media (Lonza). Nasal washes were obtained 1, 3, and 5 days post-immunization to evaluate the replication of each candidate LAIV and the *wt A/dk/Potsdam* virus.

Four weeks following immunization, all ferrets (n=24) were challenged intranasally with 10^6 TCID₅₀ of *wt A/Vietnam/1203/2004* (H5N1 HPAI) in a volume of 1 mL. Nasal washes were collected at 3 and 5 days post-challenge and all ferrets were euthanized on day 5 post-challenge for titration of virus from tissue homogenates of lungs and nasal turbinates (NT), as described previously (29). To collect nasal washes, ferrets were anesthetized by intramuscular injection of a ketamine hydrochloride cocktail (ketamine (25 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg)). Sterile PBS in a volume of 1 mL was instilled into the nose. A sneeze response was elicited by stimulation of the nostrils. The expelled secretions were collected in a petri dish, transferred to cryovials, and stored at -80°C until titration.

Serologic Assays

Baseline serum samples were collected prior to and four weeks following vaccination. Antibody titers were assessed by HAI assay using horse erythrocytes (Lampire Biological Laboratories) and by micro-neutralization assay (MN) against *wt A/dk/Potsdam* or *wt A/Vietnam/1203/2004*, as described previously (29).

Statistical analysis

Comparison of virus titers *in vitro* was analyzed by two-way ANOVA with Tukey's correction for multiple comparisons. All other comparisons were performed by Kruskal-Wallis test with Dunn's correction for multiple comparisons. All analysis was performed in

GraphPad Prism v7 (La Jolla, CA). A comparison was considered to be statistically significant when $p < 0.05$.

RESULTS

We evaluated growth characteristics of each virus in MDCK cells at the restrictive temperature (39°C) compared to the permissive temperature (33°C). The infectivity of each pLAIV was highly (>100-fold) restricted at 39°C, consistent with the known shut-off temperatures of the two MDVs (34, 35). In contrast, the infectivity of the *wt* A/dk/Potsdam virus was slightly increased at 39°C (Figure 1A). These findings were independently verified by TCID₅₀ assay (Figure 1B).

The *in vitro* growth kinetics of all 4 pLAIVs in MDCK cells were similar. One candidate pLAIV (7:1 Len/17/57) replicated to lower titers than the others at 24 hpi. However, peak titers were comparable among the pLAIVs (Figure 2A). The *wt* A/dk/Potsdam replicated to a lower titer than all pLAIVs in MDCK cultures. Differentiated primary airway epithelial cells are more sensitive to differences in growth kinetics among influenza viruses than MDCK cultures (31, 32). We therefore evaluated growth kinetics in hNEC and NHBE cells. In NHBE cells (Figure 2B), 6:2 Len/17/57 and 7:1 AA/6/60 replicated to modest titers, but replication of 6:2 AA/6/60 and 7:1 Len/17/57 were minimal. In contrast, hNECs were not permissive to the replication of the pLAIVs or *wt* A/dk/Potsdam (Figure 2C).

We compared *in vivo* infectivity of the H5N2 pLAIVs in ferrets. All four candidates were infectious and the amounts of infectious virus shed by ferrets in each vaccine group were comparable (Figure 3A). Vaccination was not associated with weight change, temperature elevation or behavioral changes in the ferrets (Figure 3B–D). However, vaccination with 6:2 AA/6/60 was associated with modest (>5%) weight loss in two of four ferrets; one recovered baseline weight within five days and the other did not continue to lose weight (Figure 3B).

Infection with *wt* A/dk/Potsdam virus elicited robust homologous antibody titers. Antibody titers were modest in ferrets vaccinated with H5N2 pLAIVs; no differences in immunogenicity could be detected among the four pLAIV candidates (Table 1). Furthermore, we evaluated the ability of serum antibodies elicited by immunization or infection to cross-neutralize A/Vietnam/1203/2004 (H5N1), a heterologous HPAI virus. None of the candidate pLAIVs elicited cross-neutralizing antibody while neutralizing antibody was detected in 1 of 4 ferrets infected with *wt* A/dk/Potsdam virus.

To probe the ability of the four candidate pLAIVs to protect against heterologous challenge, ferrets were challenged with a lethal dose of A/Vietnam/1203/2004. We evaluated protection after a single dose of LAIV to permit the detection of differences in protection conferred by the different pLAIVs because we previously demonstrated that two doses of pLAIV induced complete protection from pulmonary replication of challenge virus (36). Mock-vaccinated ferrets rapidly developed clinical signs associated with H5N1 infection, including weight loss and transient temperature elevation (Figure 4A, B). All mock-vaccinated ferrets exhibited severe lethargy followed by the appearance of neurological signs, including poor balance and ataxia and accompanied by a decrease in body temperature (Figure 4C). In

contrast, all vaccinated ferrets (with one exception) were protected from weight loss. Although vaccinated ferrets demonstrated some lethargy and concomitant decrease in body temperature following challenge, temperature and behavioral patterns returned to baseline by 5 days post-challenge. Differences in weight loss, temperature changes, and behavioral changes were not detected among the vaccine groups. Neurological signs were not observed in vaccinated ferrets.

Virological evidence for protection in pLAIV-vaccinated ferrets was consistent with clinical observations. At day 3 post-challenge, approximately 10^4 TCID₅₀/ml of infectious virus was detected in nasal wash samples from mock-vaccinated ferrets. In contrast, titers recovered from nasal wash samples of all vaccinated ferrets were significantly reduced compared to mock-vaccinated ferrets (Figure 5A). In mock-vaccinated ferrets, high virus titers were also detected in the nasal turbinates (Figure 5B) and the lungs (Figure 5C) at day 5, whereas vaccinated ferrets were nearly universally protected from challenge virus replication. Virus was detected at low or modest titers in the tissues of three ferrets vaccinated with either 6:2 Len/17/57 or 7:1 Len/17/57; only one of these ferrets demonstrated clinical signs of influenza virus infection.

DISCUSSION

To be effective, LAIVs must achieve a balance between safety and sufficient replication to elicit a protective immune response. Clinical data from several phase I studies of candidate H5 pLAIVs suggest that those derived on the Len/17/57 MDV backbone may be more infectious and immunogenic in humans than those derived on the AA/6/60 MDV backbone. However, a comparison of isogenic pLAIVs has not been possible because H5 pLAIV candidates from Russia and the United States bore distinct surface antigens. This is significant because differences in infectivity and immunogenicity have been observed even among distinct H5 pLAIVs derived on the same MDV (23, 24). Therefore, we performed a direct comparison of otherwise identical candidate H5N2 pLAIVs bearing internal protein genes derived from either AA/6/60 or Len/17/57 MDV. We prepared 6:2 and 7:1 reassortant viruses to represent the standard formulations of pLAIV candidates previously generated using the AA/6/60 and Len/17/57 MDV backbones, respectively. All viruses were generated by reverse genetics and all expressed surface antigens derived from same LPAI (H5N2) virus.

Previous *in vitro* studies suggested that AA/6/60 may be more attenuated and that the replication of LAIVs on the AA/6/60 backbone was more highly restricted at 39°C than Len/17-based viruses (37). In contrast, we found that all four H5N2 pLAIV candidates demonstrated comparable levels of restriction at 39°C when evaluated by two independent assays. This may be attributable to properties conferred by (human) H3 versus (avian) H5 HA, or it may be a reflection of gene constellation, as the previous study compared an H3N2 6:2 LAIV derived on the AA/6/60 backbone to a 5:3 LAIV derived on the Len/17/57 backbone.

Consistent with the results of the *ts* phenotype assays, we did not detect any substantial differences in the growth kinetics of the four H5N2 pLAIVs in MDCK cells. Interestingly,

primary human airway cell cultures were less permissive to replication. The experiments in human airway cells were inconclusive as we could only detect replication of two LAIVs, one derived from each MDV. All H5N2 pLAIVs replicated to higher titers in MDCK cells than the *wt* H5N2 virus; this is likely because avian influenza viruses are adapted to replicate well at the higher temperature of their (avian) hosts and are restricted at temperatures below 39°C (38). The ability of some H5N2 pLAIVs, but not the *wt* H5N2 virus, to replicate in human airway cultures may be explained by contributions of the internal protein gene segments of the pLAIVs.

All viruses were infectious in ferrets. Nasal wash titers collected during the week following vaccination were comparable across groups, suggesting that the *in vivo* infectivity of the four H5N2 candidate LAIVs was similar. Consistent with these observations, all candidate pLAIVs elicited serum neutralizing antibody responses, with no clear differences between groups. Pandemic vaccines are licensed based on safety and immunogenicity in humans because efficacy and effectiveness data cannot be obtained. This can be problematic for evaluation of pLAIVs because HAI antibody titer, the primary immunogenicity criterion evaluated for licensing of seasonal influenza vaccines (39) is not a robust correlate of protection in clinical or pre-clinical studies of pLAIVs (25, 40–44).

Therefore, we challenged vaccinated ferrets with a highly pathogenic H5N1 virus. Notably, vaccination with each H5N2 pLAIV was associated with significant virological protection, despite the absence of A/Vietnam/1203/2004 -specific neutralizing antibodies prior to challenge. One ferret that had been vaccinated with 6:2 Len/17/57 demonstrated weight loss (>10%) without overt clinical disease; this is consistent with previous reports of weight loss in vaccinated ferrets exhibiting virological protection following challenge with H5N1 HPAI viruses (44, 45). Overall, no single candidate pLAIV was clearly superior in providing protection from morbidity (weight loss, activity, temperature) or challenge virus replication in the respiratory tract. In fact, challenge virus titers in the lungs were either below the limit of detection or just above the detection threshold in all vaccinated ferrets at day 5 post-challenge.

Although clinical observations have led many to speculate that the replication of AA/6/60-derived LAIVs are more restricted than those derived on Len/17, our data suggest that viruses derived from each “master donor virus” replicate similarly *in vitro* and in ferrets. As findings in ferrets may not accurately reflect findings in humans, the continued evaluation of pLAIV candidates bearing the surface antigens of additional avian influenza viruses will be critical for identifying determinants of LAIV-mediated immunogenicity. Indeed, our observations are supported by a previous study demonstrating that the shedding and immunogenicity of a pair of Russian and American H3N2 LAIVs were remarkably similar in healthy adults, despite differences in *in vitro* phenotype (37). Observed differences in shedding and immunogenicity in phase I studies of H5 pLAIVs may therefore be attributable to differences in dose and derivation of Russian and American pLAIV candidates, differences in study populations, or differences in the assays by which viral replication and immunogenicity were evaluated (4, 23, 24, 46).

In summary, we report that, when produced by similar methods and carrying identical surface antigens, H5N2 pLAIVs derived on a backbone of either AA/6/60 or Len/17/57 MDV offer comparable immunogenicity and protection in ferrets. These findings have promising public health implications. Numerous clinical trials have supported the safety of candidate pLAIVs derived from both MDVs. The work presented here further suggests that the two MDV backbones are comparable and that pLAIVs formulated with either MDV have a potential role in pandemic preparedness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Two master donor viruses (MDVs) are licensed for the generation of seasonal live attenuated influenza vaccines (LAIVs) and both have been used as backbones to generate candidate vaccines for avian influenza viruses belonging to the H5 subtype
- To directly compare the biological properties of LAIVs derived on these MDVs, four distinct H5N2 pandemic LAIV (pLAIV) candidates were generated under identical conditions
- All viruses replicated and induced similar neutralizing antibody responses in ferrets
- All viruses offered comparable protection against challenge with a highly pathogenic H5N1 influenza virus, suggesting that both MDVs are appropriate for the generation of candidate pLAIVs

Figure 1. Relative infectivity of pLAIVs derived on AA/6/60 or Len/17/57 and *wt* A/dk/Potsdam virus (H5N2 LPAI)

The infectivity of pLAIVs and the *wt* parental virus were evaluated by (A) plaque assay and by (B) TCID₅₀ assay, with duplicate plates assayed at either the permissive temperature (33°C) or the restrictive temperature (39°C). Results are representative of two independent experiments. Dashed lines indicate the limit of detection for each assay.

* $p < 0.05$, 33°C vs. 39°C for each virus;

† $p < 0.05$, each pLAIV vs. *wt*, 39°C

Figure 2. *In vitro* growth kinetics of pLAIVs derived on AA/6/60 or Len/17/57 and wt A/dk/Potsdam (H5N2 LPAI)

Growth kinetics were evaluated by inoculation at moi = 0.01 of (A) MDCK cells, (B) NHBE cells, or (C) hNEC at the permissive temperature (33°C).

Each experiment was performed in triplicate; wells were sampled at the indicated time points. Infectious virus was quantified on MDCK cells at 33°C. Symbols represent the mean titer ± standard deviation at each time point.

Dashed lines indicate the limit of detection for each assay.

A **B**

Figure 3. Clinical signs in ferrets following infection with *wt* A/dk/Potsdam (H5N2) or related pLAIVs

Groups of four ferrets were inoculated intranasally with 10^7 TCID₅₀ of the indicated pLAIVs or with *wt* A/dk/Potsdam virus. An additional four ferrets were mock-vaccinated. (A) Nasal washes were collected at the indicated time points (days post-infection; dpi) and virus was titrated by TCID₅₀ assay. (B) Ferrets were weighed daily to track changes in body weight up to five days following vaccination. Each line represents an individual ferret. (C) Temperature and (D) activity level were monitored daily.

Briefly, changes in activity level were scored as follows: (0) ferret initiated playful behavior, (1) ferret did not initiate play, but responded to play overtures, (2) ferret was alert but did not

respond to play overtures, or (3) ferret was not alert or playful. Ferrets were scored by the same individual at all time points.

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Figure 4. Clinical signs in ferrets immunized with influenza A/dk/Potsdam (H5N2) *wt* virus or related pLAIVs following challenge with heterologous H5N1 influenza virus

Groups of four ferrets that had been mock-vaccinated, infected with *wt* A/dk/Potsdam (H5N2 LPAI), or vaccinated with A/dk/Potsdam pLAIVs were challenged with 10^6 TCID₅₀ of A/Vietnam/1203/2004 (H5N1 HPAI). (A) Body weight, (B) temperature, and (C) activity were monitored daily (days post-infection; dpi). Each line represents an individual ferret. The cross symbol in panel (A) indicates that a ferret was humanely euthanized due to substantial weight loss associated with influenza virus infection on 4 dpi. For this reason, no clinical values are shown for this ferret at the 5 dpi time point.

Briefly, changes in activity level were scored as follows: (0) ferret initiated playful behavior, (1) ferret did not initiate play, but responded to play overtures, (2) ferret was alert but did not respond to play overtures, or (3) ferret was not alert or playful. Ferrets were scored by the same individual at all time points.

Figure 5. Protective efficacy of influenza A/dk/Potsdam (H5N2) *wt* virus and related pLAIVs in ferrets challenged with heterologous H5N1 influenza virus

Groups of four ferrets that had been previously mock-vaccinated, infected with *wt* A/dk/Potsdam (H5N2 LPAI), or vaccinated with A/dk/Potsdam pLAIVs were challenged with 10^6 TCID₅₀ of A/Vietnam/1203/2004 (H5N1 HPAI). To evaluate protection from virus replication in the respiratory tissues, infectious virus was titrated by TCID₅₀ assay from (A) nasal washes collected 3 days post-infection, (B) nasal tissues collected 5 days post-infection, and (C) lung tissues collected 5 days post-infection. Each symbol represents an individual ferret and the horizontal line represents the group mean.

* $p < 0.05$, vs. mock; differences among pLAIVs were not significant

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Table 1
Immunogenicity [GMT (95% CI)] of influenza A/dk/Potsdam (H5N2) *wt* virus and related pLAIVs in ferrets

Groups of 4 ferrets were inoculated intranasally with 10^7 TCID₅₀ of the indicated vaccines or with *wt* virus. Sera were collected 28 days after immunization. Antibody titers by hemagglutination inhibition assay (HAI) are shown against the homologous virus (*wt* A/dk/Potsdam; H5N2 LPAI). Antibody titers by micro-neutralization assay (MN) are shown against the homologous virus and a heterologous avian virus (A/Vietnam/1203/2004; H5N1 HPAI). The data shown are representative of two independent experiments. The limit of detection for the heterologous MN assay and for HAI were 1:5. GMT: geometric mean titer; CI: confidence interval

Immunogen	Serum antibody response in ferrets vaccinated with the indicated viruses		
	HAI	MN	
	<i>A/dk/Potsdam</i>	<i>A/dk/Potsdam</i>	<i>A/Vietnam/1203/2004</i>
A/dk/Potsdam <i>wt</i>	95 (55, 165)	640 (373, 1099)	5 (4, 7)
6:2 AA/6/60	10 (3, 36)	202 (120, 339)	<5
6:2 Len/17/57	7 (2, 21)	116 (74, 184)	<5
7:1 AA/6/60	14 (2, 117)	208 (75, 577)	<5
7:1 Len/17/57	14 (5, 43)	196 (77, 496)	<5