

Efficacy of swine influenza A virus vaccines against an H3N2 virus variant

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Abstract

We compared the efficacy of 3 commercial vaccines against swine influenza A virus (SIV) and an experimental homologous vaccine in young pigs that were subsequently challenged with a variant H3N2 SIV, A/Swine/Colorado/00294/2004, selected from a repository of serologically and genetically characterized H3N2 SIV isolates obtained from recent cases of swine respiratory disease. The experimental vaccine was prepared from the challenge virus. Four groups of 8 pigs each were vaccinated intramuscularly at both 4 and 6 wk of age with commercial or homologous vaccine. Two weeks after the 2nd vaccination, those 32 pigs and 8 nonvaccinated pigs were inoculated with the challenge virus by the deep intranasal route. Another 4 pigs served as nonvaccinated, nonchallenged controls. The serum antibody responses differed markedly between groups. After the 1st vaccination, the recipients of the homologous vaccine had hemagglutination inhibition (HI) titers of 1:640 to 1:2560 against the challenge (homologous) virus. In contrast, even after 2nd vaccination, the commercial-vaccine recipients had low titers or no detectable antibody against the challenge (heterologous) virus. After the 2nd vaccination, all the groups had high titers of antibody to the reference H3N2 virus A/Swine/Texas/4199-2/98. Vaccination reduced clinical signs and lung lesion scores; however, virus was isolated 1 to 5 d after challenge from the nasal swabs of most of the pigs vaccinated with a commercial product but from none of the pigs vaccinated with the experimental product. The efficacy of the commercial vaccines may need to be improved to provide sufficient protection against emerging H3N2 variants.

Résumé

Une étude comparative de l'efficacité de 3 vaccins commerciaux contre le virus de l'influenza porcine A (SIV) et d'un vaccin expérimental homologue a été réalisée chez de jeunes porcs qui ont été soumis à une infection défi avec un variant H3N2 du SIV, A/Swine/Colorado/00294/2004, sélectionné d'une collection d'isolats sérologiquement et génétiquement caractérisés de SIV H3N2 obtenus de cas récents de maladie respiratoire porcine. Le vaccin expérimental a été préparé à partir du virus servant à l'infection. Quatre groupes de 8 porcs chacun ont été vaccinés par voie intramusculaire à l'âge de 4 et 6 sem avec le vaccin commercial ou le vaccin homologue. Deux semaines après la 2^e injection, ces 32 porcs et 8 porcs non-vaccinés ont été inoculés par voie intra-nasale profonde avec le virus. Un groupe additionnel de 4 porcs a servi de témoin non-vacciné, non-infecté. La réponse en anticorps sériques a varié de façon marquée entre les groupes. Après la 1^{ère} vaccination, les animaux ayant reçu le vaccin homologue avaient des titres d'inhibition de l'hémagglutination (HI) variant de 1:640 à 1:2560 dirigés contre le virus (homologue) ayant servi à l'inoculation. À l'opposé, même après la 2^e vaccination, les animaux ayant reçu du vaccin commercial avaient des titres en anticorps non-détectables contre le virus (hétérologue) utilisé pour l'infection. Après la 2^e vaccination, tous les groupes avaient des titres d'anticorps élevés contre le virus de référence H3N2 A/Swine/Texas/4199-2/98. La vaccination a réduit les signes cliniques et le pointage des lésions pulmonaires; toutefois, le virus a été isolé 1 à 5 jours après l'infection défi à partir d'écouillons nasaux de la majorité des porcs vaccinés avec un produit commercial mais d'aucun des porcs vaccinés à l'aide du produit expérimental. L'efficacité des vaccins commerciaux pourrait avoir besoin d'être augmentée afin de fournir une protection suffisante envers les variants émergents de H3N2.

(Traduit par Docteur Serge Messier)

Introduction

Respiratory disease in pigs is frequently caused by infection with *Porcine reproductive and respiratory syndrome virus* (PRRSV), swine influenzavirus (SIV), *Mycoplasma hyopneumoniae*, or *Haemophilus parasuis*. These agents can induce the disease independently, but coinfection with 2 or more agents is common under field conditions (1).

In recent years, SIV appears to be playing an important role in respiratory disease of pigs. Three subtypes of SIV — H1N1, H3N2, and H1N2 — are currently circulating in US swine populations (1–7). Throughout most of the 20th century, H1N1 was exclusively detected (8), but H3N2 and H1N2 have been isolated since 1998 (2,6,9,10). With the detection of these new subtypes, the swine industry has paid greater attention to SIV, and bivalent SIV vaccines have been routinely used on swine farms.

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Table I. Reciprocal hemagglutination inhibition (HI) titers of antibody to various H3N2 strains of swine influenza A virus (SIV) in antiserum from pigs immunized by intramuscular injection of H3N2 field isolates A to E of SIV or commercial SIV vaccines X to Z

| H3N2 strain; phylogenetic cluster | Antiserum ^a or vaccine; HI titer | | | | | | | |
|--------------------------------------------------------|---------------------------------------------|-----|------|------|------|-----|------|-----|
| | A | B | C | D | E | X | Y | Z |
| A/Swine/Colorado/00294/2004 (challenge strain); III | < 40 | 40 | 1280 | 1280 | < 40 | 80 | < 40 | 40 |
| A/Swine/Texas/46710-35/02 (field isolate E); II | < 40 | 640 | < 40 | < 40 | 1280 | 80 | 80 | 80 |
| A/Swine/Texas/4199-2/98 (reference strain); I | < 40 | 40 | 320 | 160 | 40 | 640 | 640 | 640 |

^a Containing antibody to the following SIV strains: A — A/Swine/North Carolina/39615/02; B — A/Swine/Missouri/22585/02; C — A/Swine/North Carolina/5854/02; D — A/Swine/Minnesota/23062/02; E — A/Sw/TX/46710-35/02.

In the United States, H3N2 SIV isolates have been triple-reassortant viruses containing genes of human, swine, and avian lineages. Gene sequence analyses have shown that their hemagglutinin (HA) molecules belong to 1 of 3 phylogenetically distinct human-like HA lineages; thus, H3N2 viruses have been classified into clusters I, II, and III (7,11). In addition, we and others (10,11) have observed serologic diversity. In hemagglutination inhibition (HI) tests, using antiserum against 5 H3N2 viruses and the 3 commercial bivalent SIV vaccines, we classified 97 H3N2 field isolates into 4 serogroups. These results indicate that H3N2 viruses of varying genetic and serologic diversity are circulating in US pig populations.

Reports from veterinary diagnostic laboratories also show an increasing number of H3N2 isolates with low serologic cross-reactivity to reference virus A/Swine/Texas/4199-2/98 antiserum. As well, there have been anecdotal reports from US swine operations of apparent vaccination failure with current commercially available bivalent SIV vaccines. Therefore, we investigated whether 3 commercially available bivalent vaccines provide satisfactory protection against an H3N2 variant of SIV.

Materials and methods

Challenge virus and SIV vaccines

The challenge virus, A/Swine/Colorado/00294/2004, was selected from a bank of SIV H3N2 isolates at the University of Minnesota Veterinary Diagnostic Laboratory. The virus had low serologic cross-reactivity (HI titer \leq 1:80) with antiserum from pigs vaccinated with commercial SIV vaccines (Table I) and was classified as a cluster III virus according to HA gene sequence analysis (Figure 1). It had been isolated from lung tissue collected postmortem from 10-wk-old pigs with clinically severe influenza-induced respiratory disease despite a history of SIV vaccination.

For preparation of an experimental homologous inactivated H3N2 virus vaccine, the challenge virus was grown in Madin-Darby canine kidney (MDCK) cells by routine methods (13); the HA titer was 1:256/0.1 mL at the time of inactivation by the addition of formalin (final concentration 0.1%). The virus was added to an adjuvant mix-

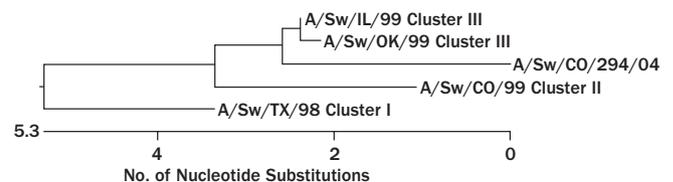


Figure 1. Phylogram, constructed in Megalign (DNASTar, Madison, Wisconsin, USA), demonstrating the inferred genetic relationship of the hemagglutinin genes from the H3N2 challenge strain of swine influenza A virus, A/Swine/Colorado/00294/2004, and 4 H3N2 reference strains. The length of each pair of branches represents the distance between sequence pairs. The units (0 to 5.3) at the bottom of the tree indicate the number of nucleotide substitutions. IL — Illinois; OK — Oklahoma; TX — Texas.

ture of mineral oil (9 parts) and emulsifier (1 part; equal volumes of Span 85 and Tween 85) in a 1:1 ratio and sonicated at 25 W for 2 min. Three bivalent SIV vaccines licensed by the US Department of Agriculture ("X", "Y", and "Z") were purchased from commercial sources. All of the vaccines were stored at 4°C until used.

Experimental design

Forty-four 3-wk-old pigs were purchased from Midwest Research Swine (MRS), Gibbon, Minnesota, USA. All the pigs were free from infection with PRRSV, *M. hyopneumoniae*, and SIV and free from antibody to SIV. The pigs were randomly divided into 5 groups of 8 pigs each (groups 1 to 5) and 1 group of 4 pigs (group 6), ear-tagged, and housed in large pens at the MRS swine farm. Throughout the study, the pigs were handled and cared for according to an approved University of Minnesota Institutional Animal Care and Use Committee protocol.

The pigs in groups 1, 2, and 3 were vaccinated intramuscularly with the commercial bivalent X, Y, and Z SIV vaccines, respectively, according to label directions, at 4 and 6 wk of age. The pigs in group 4 were vaccinated intramuscularly with the experimental homologous vaccine (2-mL dose) at 4 and 6 wk of age. Groups 5 and 6 served as nonvaccinated controls.

Ten days after the 2nd vaccination, the pigs were transported to the isolation units at the University of Minnesota and housed in 6 different rooms, by group. They were acclimated for 4 d and then challenged with A/Sw/CO/294/04. For the challenge, each pig was

Table II. Mean reciprocal HI titers of antibody to the reference (R) and challenge (C) H3N2 viruses after inoculation with a commercial SIV vaccine or the experimental homologous vaccine (H) followed by challenge, after challenge only, or after no treatment

| Pig group | Treatment ^a | Virus tested | No. of days after vaccination; HI titer | | | |
|-----------|------------------------|--------------|-----------------------------------------|-----------------|-----------------|------------------|
| | | | 0 | 14 | 28 | 33 or 34 |
| 1 (n = 8) | Vaccine X | R | < 40 | < 40 | 470.0 (80–1280) | 960.0 (320–1280) |
| | | C | < 40 | < 40 | < 40 | 185.0 (40–160) |
| 2 (n = 8) | Vaccine Y | R | < 40 | 55.0 (< 40–160) | 1160 (320–2560) | 1840 (640–2560) |
| | | C | < 40 | < 40 | 57.5 (< 40–160) | 430.0 (80–1280) |
| 3 (n = 8) | Vaccine Z | R | < 40 | 48.6 (< 40–160) | 290.0 (80–640) | 920.0 (320–2560) |
| | | C | < 40 | < 40 | < 40 | 145.0 (40–320) |
| 4 (n = 8) | Vaccine H | R | < 40 | 72.5 (< 40–160) | 245.0 (40–640) | 340.0 (80–1280) |
| | | C | < 40 | 1280 (640–2560) | 2560 (2560) | 2560 (2560) |
| 5 (n = 8) | Challenge only | R | < 40 | < 40 | < 40 | < 40 |
| | | C | < 40 | < 40 | < 40 | 33.8 (< 40–80) |
| 6 (n = 4) | None | R | < 40 | < 40 | < 40 | < 40 |
| | | C | < 40 | < 40 | < 40 | < 40 |

^a The pigs in groups 1 to 4 were vaccinated on days 0 (at 4 wk of age) and 14 (at 6 wk of age) and challenged on day 28 (at 8 wk of age). The pigs in group 5 were also challenged on day 28.

Table III. Numbers of pigs with clinical signs of coughing (C), nasal discharge (N), and lethargy (L) each day after challenge

| Pig group | No. of days after challenge; no. of pigs with clinical signs | | | | | | | | | | | | | | | Total |
|-----------|--------------------------------------------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-------|
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | |
| | C | N | L | C | N | L | C | N | L | C | N | L | C | N | L | |
| 1 | 0 | 0 | 0 | 1 | 0 | 0 | 4 | 1 | 1 | 2 | 1 | 1 | 2 | 0 | 0 | 13 |
| 2 | 0 | 1 | 0 | 0 | 1 | 1 | 2 | 0 | 2 | 0 | 1 | 1 | 1 | 0 | 1 | 11 |
| 3 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 3 | 2 | 3 | 3 | 0 | 1 | 16 |
| 4 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 8 |
| 5 | 2 | 2 | 0 | 1 | 6 | 0 | 3 | 1 | 1 | 4 | 2 | 2 | 6 | 1 | 1 | 32 |

sedated by an intramuscular injection of tiletamine hydrochloride and zolazepam hydrochloride (Telazol; Fort Dodge Animal Health, Fort Dodge, Iowa, USA) and then inoculated by the deep intranasal route with 1 mL per nostril of the virus at a median tissue culture infective dose per milliliter of 10^{8.5} by means of a syringe adapted with a tight-fitting plastic nasal cannula 5 cm long.

Clinical observation, sampling, and pathological examination

The pigs were observed for clinical signs of lethargy, anorexia, coughing, and nasal discharge every morning for 30 min in each room from 2 d before until 5 d after challenge. In addition, rectal temperature was taken daily and nasal swabs were collected daily during the postchallenge period. Blood samples were collected from all pigs at the times of the 1st and 2nd vaccinations, challenge, and euthanasia.

At 5 or 6 d after challenge, the pigs were euthanized and their lungs examined for gross lesions of pneumonia by 1 examiner blinded as to treatment group. For percentage calculations, we used previously defined weighted proportions of each lobe to the total lung volume (12): the 9 accessory lobes were assigned 5 points each, and the dorsal and ventral caudal lobes were assigned 15 and

12.5 points each, respectively, for a total of 100 points. The percentage of lung consolidation in each lobe was estimated for individual pigs and then a mean lung lesion score calculated for each group. Arbitrary categories of mild (< 10%), moderate (≥ 10 to < 20%), and severe (≥ 20%) consolidation were also used for comparisons. Tracheal swabs were collected for virus isolation. Portions of the nasal turbinates, trachea, and each lung lobe were formalin-fixed, paraffin-embedded, thin-sectioned, and then stained with hematoxylin and eosin for microscopic assessment.

Virus isolation and serology

Immediately after collection, each swab was suspended in 1 mL of phosphate-buffered saline (PBS, pH 7.2) containing antibiotics and vigorously vortexed. The supernatant was collected after centrifugation at 2000 × g for 5 min and used for virus isolation on MDCK cell monolayers (13). Briefly, the monolayers were washed with PBS containing 1 µg/mL of modified trypsin (trypsin treated with tosylphenylalanylchloromethane [TPCK]), inoculated with each supernatant, and incubated for virus absorption. After absorption for 2 h, the samples were removed, and Eagle's minimum essential medium containing 1 µg/mL of modified trypsin and 0.3% bovine serum albumin was added. The samples were then incubated at 37°C.

Table IV. Numbers of pigs for which virus was isolated from nasal swabs after challenge

| Pig group | No. of days after challenge; no. of pigs with virus isolation | | | | | | | Total |
|-----------|------------------------------------------------------------------|---|---|---|---|----------------|----------------|-------|
| | 0 | 1 | 2 | 3 | 4 | 5 ^a | 6 ^a | |
| 1 | — | 8 | 8 | 6 | 6 | 3 | — | 31 |
| 2 | — | 8 | 5 | 6 | 5 | 3 | — | 27 |
| 3 | — | 8 | 8 | 8 | 6 | 4 | — | 34 |
| 4 | — | — | — | — | — | — | — | 0 |
| 5 | — | 8 | 8 | 8 | 8 | 7 | — | 39 |
| 6 | — | — | — | — | — | — | — | 0 |

^a On day 5 after challenge, 4 pigs in each of groups 1 to 5 and 2 pigs in group 6 underwent necropsy. On day 6 after challenge, all remaining pigs underwent necropsy. For at least 1 pig in each of groups 1, 2, 3, and 5 but none of the pigs in groups 4 and 6, virus was isolated from tracheal swabs collected at necropsy.

Each well was observed for cytopathic effect (CPE) daily for 5 d. The supernatants with CPE were tested for hemagglutination with turkey erythrocytes to confirm the presence of SIV. Samples without CPE were passed again on MDCK cell monolayers and observed for another 5 d. The samples with no CPE after the 2nd passage were considered negative for virus.

All serum samples were pretreated with kaolin and turkey erythrocytes as previously described (14) and tested for antibody titers by HI. The HI tests were performed against the challenge virus and the reference H3N2 virus A/Sw/TX/4199-2/98 (14). Briefly, serial 2-fold dilutions of treated serum were made in 96-well round-bottom microtiter plates. Each serum dilution was mixed with 4 HA units of H3N2 virus and incubated at room temperature for 45 min. A 0.5% suspension of turkey erythrocytes was then added. After another 45 min of incubation at room temperature, the wells were read for complete inhibition of hemagglutination.

Statistical analysis

Mean body temperature, results of attempted virus isolation from daily nasal swabs, and mean gross lung lesion score in the 4 vaccinated and the 2 control groups were compared by 1-way analysis of variation. All the data were then analyzed with the use of Statistix 7 software (Analytical Software, Tallahassee, Florida, USA). A *P*-value of < 0.05 was considered to indicate a statistically significant difference between groups.

Results

Antibody response to vaccination

The HI titers of antibody against the challenge and reference H3N2 viruses in the pigs before and after vaccination and after challenge are summarized in Table II. For the reference virus, low titers were detected 2 wk after the 1st vaccination, and the titer increased to 1:80 to 1:2560 by 2 wk after the 2nd vaccination with the 3 commercial vaccines (in groups 1, 2, and 3). The titers were similar in the pigs inoculated with the experimental homologous vaccine (group 4). However, with the challenge virus, the HI antibody titers were

Table V. Gross lung lesions at necropsy (5 or 6 d after challenge)

| Pig group | Gross lung lesions; ^a no. of pigs | | | | Mean lung lesion score ^b (standard deviation) |
|-----------|----------------------------------------------|------|----------|--------|-------------------------------------------------------------|
| | None | Mild | Moderate | Severe | |
| 1 | 2 | 6 | 0 | 0 | 3.54 (3.57) |
| 2 | 5 | 3 | 0 | 0 | 0.73 (1.74) |
| 3 | 5 | 3 | 0 | 0 | 1.72 (2.51) |
| 4 | 5 | 3 | 0 | 0 | 0.28 (0.44) |
| 5 | 0 | 2 | 3 | 3 | 16.92 (10.30) |
| 6 | 4 | 0 | 0 | 0 | 0 |

^a Mild — consolidation < 10%; moderate — consolidation ≥ 10 to < 20%; severe — consolidation ≥ 20%.

^b The mean scores for groups 1, 2, 3, and 4 were significantly less (*P* < 0.0001) than the mean for group 5. The mean score for group 1 was significantly higher (*P* < 0.05) than that for group 4.

negative or low 2 wk after the 2nd vaccination with the 3 commercial vaccines but were high (1:640 to 1:2560) just 2 wk after the 1st vaccination in the pigs inoculated with the homologous vaccine. At the time of euthanasia, all the pigs in groups 1 to 4 had positive HI titers of antibody to the challenge virus. Among the unvaccinated pigs, 3 of the 8 in group 5 had HI titers ≥ 1:40 against the challenge virus 5 to 6 d after challenge, whereas all 4 pigs in group 6, who were not challenged, remained antibody negative throughout the experimental period.

Clinical signs and virus shedding after challenge

Clinical signs of respiratory disease (Table III) were minimal to moderate in all the pigs. On day 1 after challenge, the pigs in groups 1 and 5 were febrile, with a mean rectal temperature of 40.1°C, significantly higher (*P* < 0.05) than on the day of challenge. Although the temperature of the pigs in groups 1 and 3 was more than 40°C on day 2 after challenge, no significant difference in temperature was noted between the groups after challenge. The pigs in group 5 (challenge only) showed typical clinical signs of influenza, including coughing, nasal discharge, and lethargy; the signs were milder in the pigs vaccinated with commercial products and mildest in the pigs vaccinated with the experimental homologous product. No clinical signs of disease were detected among the pigs in group 6 throughout the experiment.

Virus was isolated 1 to 5 d after challenge from nasal swabs of most of the pigs vaccinated with a commercial product and from most of those challenged but not vaccinated, whereas virus was not isolated from any of the pigs vaccinated with the experimental homologous product or from any of the unvaccinated, unchallenged pigs (Table IV). Virus was isolated from tracheal swabs of at least 1 pig in each of groups 1, 2, 3, and 5 but from none of the pigs in groups 4 and 6 at necropsy. The frequency of virus isolation from nasal swabs was not significantly different between groups 1, 2, 3, and 5 (*P* > 0.05).

Gross lung lesions

The numbers of pigs with gross lung lesions at necropsy are summarized in Table V. The mean lung lesion score for the pigs in

group 5 (challenge only) was significantly higher ($P < 0.0001$) than the mean scores for the pigs in each of the vaccinated groups. The mean score for the pigs in group 1 was significantly higher ($P < 0.05$) than the mean for the pigs in group 4.

Microscopic lesions

Epithelial changes and subepithelial inflammation in the nasal turbinates and trachea were minimal to mild in all the groups. The pigs in group 5 (challenge only) had mild to moderate bronchiolar epithelial metaplasia, necrosis, or both, whereas the other pigs in the study had no or minimal bronchiolar epithelial lesions.

Discussion

Vaccination against SIV is now a routine method of respiratory disease control in swine farms because SIV has become 1 of the common causes of respiratory disease in pigs. The vaccines used are mostly bivalent, containing both H1N1 and H3N2 subtypes. Several cross-protection studies with various SIV vaccines in Europe and the United States have shown the vaccines to be effective under experimental conditions (15–20). However, there have been debates on the efficacy of commercial SIV vaccines (7,15), since SIV isolates with antigenic and genetic diversity have been recovered from diseased pigs, and insufficient protection by the vaccines used on swine farms has been reported.

Our study was initiated with a hypothesis that SIV H3N2 variants are increasingly prevalent in swine farms and that the immunity induced by the currently available commercial vaccines may not be sufficient to protect against these variants. As expected, the results with the 3 commercial vaccines were not fully satisfactory. The vaccinated pigs showed relatively mild clinical signs and reduced pneumonic lesions, thereby fulfilling the label claims of the manufacturers. However, daily nasal shedding of the challenge virus was noticeable in the pigs that received the commercial vaccines, and there was no difference in the duration of virus shedding between the pigs that received the commercial vaccines and the nonvaccinated pigs. In contrast, the pigs that received the experimental homologous vaccine had no detectable virus in nasal secretions, high antibody response to the challenge virus, and significantly lower lung lesion scores compared with the pigs that received the commercial vaccines.

The unsatisfactory results obtained with commercial vaccines in this study are believed to be due to heterogeneity of the challenge virus, though this remains unproven. The challenge virus was classified as genetic cluster III, whereas the commercial vaccines contained H3N2 viruses from genetic cluster I. The challenge virus demonstrated low serologic cross-reactivity with the antiserum induced by the commercial vaccines. Differences in vaccine efficacy may also be due to differences in antigen concentration, the adjuvant used, or both factors. However, the improved protection provided by the experimental vaccine is most likely due to its preparation with a virus strain homologous to the challenge virus. Heinen et al (18) reported that homologous protection was excellent when compared with heterologous protection. In addition, Van Reeth et al (15) and Heinen et al (18) have suggested that heterologous protection does not necessarily correlate with serum HI antibody

levels. Although the challenge virus used in this study was genetically and serologically highly divergent from the vaccine viruses, it is representative of the cluster III H3N2 SIV currently circulating in US swine.

Although the commercial SIV vaccines used in this study did prove beneficial in reducing clinical signs and lung lesions, they failed to significantly reduce virus shedding after a challenge. This failure could be critical in swine influenza epidemiology, possibly increasing the risk of infection for susceptible animals and humans and favoring genetic mutation and the generation of virus variants (21). If the current vaccines are insufficiently controlling the spread of H3N2 virus variants, these viruses may continue to change and infect pigs or even reassort with other influenzaviruses that could infect humans and other animals. Therefore, further genetic and serologic evaluations of currently circulating SIV should be done. Upon review of the information obtained, it may become evident that updating and improving commercial SIV vaccines is necessary.

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