

# Ring test evaluation of the detection of influenza A virus in swine oral fluids by real-time reverse-transcription polymerase chain reaction and virus isolation

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## Abstract

The probability of detecting influenza A virus (IAV) in oral fluid (OF) specimens was calculated for each of 13 assays based on real-time reverse-transcription polymerase chain reaction (rRT-PCR) and 7 assays based on virus isolation (VI). The OF specimens were inoculated with H1N1 or H3N2 IAV and serially diluted 10-fold ( $10^{-1}$  to  $10^{-8}$ ). Eight participating laboratories received 180 randomized OF samples (10 replicates  $\times$  8 dilutions  $\times$  2 IAV subtypes plus 20 IAV-negative samples) and performed the rRT-PCR and VI procedure(s) of their choice. Analysis of the results with a mixed-effect logistic-regression model identified dilution and assay as variables significant ( $P < 0.0001$ ) for IAV detection in OF by rRT-PCR or VI. Virus subtype was not significant for IAV detection by either rRT-PCR ( $P = 0.457$ ) or VI ( $P = 0.101$ ). For rRT-PCR the cycle threshold (Ct) values increased consistently with dilution but varied widely. Therefore, it was not possible to predict VI success on the basis of Ct values. The success of VI was inversely related to the dilution of the sample; the assay was generally unsuccessful at lower virus concentrations. Successful swine health monitoring and disease surveillance require assays with consistent performance, but significant differences in reproducibility were observed among the assays evaluated.

## Résumé

La probabilité de détecter le virus de l'influenza A (VIA) dans des échantillons de fluide oral (FO) a été calculée pour chacune des 13 épreuves basées sur une réaction d'amplification en chaîne en temps réel utilisant la polymérase réverse (rRT-PCR) et 7 épreuves basées sur l'isolement viral (IV). Les échantillons de FO ont été inoculés avec du VIA H1N1 ou H3N2 et dilués en série par facteur de 10 ( $10^{-1}$  à  $10^{-8}$ ). Huit laboratoires participants ont reçu 180 échantillons randomisés de FO (10 répliquats  $\times$  8 dilutions  $\times$  2 sous-types de VIA plus 20 échantillons témoins négatifs sans VIA) et ont réalisé la méthode de rRT-PCR et d'IV de leur choix. L'analyse des résultats à l'aide d'un modèle de régression logistique pour les effets mélangés a identifié la dilution et l'épreuve comme étant des variables significatives ( $P < 0,0001$ ) pour la détection de VIA dans du FO par rRT-PCR ou IV. Le sous-type de virus n'était pas significatif pour la détection de VIA soit par rRT-PCR ( $P = 0,457$ ) ou par IV ( $P = 0,101$ ). Pour les épreuves rRT-PCR les valeurs seuils de cycle (Ct) augmentaient de manière constante avec la dilution mais variaient énormément. Ainsi, il n'était pas possible de prédire le succès de l'IV sur la base des valeurs de Ct. Le succès de l'IV était inversement relié à la dilution de l'échantillon; l'épreuve était généralement négative aux faibles concentrations de virus. Pour avoir du succès dans la surveillance des maladies et de la santé des porcs il est nécessaire d'avoir des épreuves avec des performances constantes, mais des différences significatives dans la reproductibilité ont été observées parmi les épreuves évaluées.

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## Introduction

Influenza A virus (IAV) causes illness, death, and economic losses at all stages of pig production, particularly when infection occurs concurrently with infection by other respiratory pathogens such as Porcine reproductive and respiratory syndrome virus (PRRSV) (1). In addition, IAV is zoonotic and moves relatively easily between pigs and humans (1). There have been well-recognized pig-to-human transmission events, such as outbreaks at county fairs in Indiana (2) and Ohio (3), but human-to-pig transmission of IAV is now also well-documented (4). In the United States influenzavirus infections in humans and swine are monitored by the Centers for Disease Control and Prevention (5) and the United States Department of Agriculture (USDA) (6). This effort tracks the regional distribution and genetic changes of viral subtypes circulating in human and swine populations. In pigs, current antemortem surveillance is based on individual animal sampling (nasal swabs), although the short duration of shedding in nasal secretions greatly limits the probability of virus isolation (VI) or detection by real-time reverse-transcription polymerase chain reaction (rRT-PCR) after day 6 and day 8 of infection, respectively (7). This window of detection is reduced in vaccinated animals to day 4 and day 5 for VI and rRT-PCR, respectively (7). As an alternative to testing individual pig samples, assays of pen-based oral fluid (OF) specimens by rRT-PCR may be used to detect IAV for at least 14 d after inoculation in unvaccinated animals and at least 7 d in vaccinated animals (7). For ease of sampling and improved rates of IAV detection in swine populations, OF assays are gaining favor in surveillance programs (David Pyburn, USDA-APHIS, 2010 personal communication). Regardless of specimen, successful swine health monitoring and disease surveillance depend on reliable diagnostic assays. Various VI procedures, as well as commercial and in-house IAV rRT-PCR assays, are in place in veterinary diagnostic laboratories, but no direct comparisons of assay performance have been done on swine specimens. Although proficiency panels are useful in test validation and harmonization (8–10), they also serve to assess competence, supplement laboratory quality-control measures, and improve uniformity in assay performance (9,11–13). In follow-up performance studies in which identical protocols, reagents, and/or training were provided, greater assay reproducibility and repeatability were observed (14–16). Ring tests are proficiency tests coordinated among multiple collaborating laboratories (17). Inclusion of sufficient numbers of replicates and dilutions allows for statistical analysis of assay reproducibility (between laboratory) and repeatability (within laboratory) and facilitates process improvement. The objectives of this study were to evaluate IAV OF assays, determine the correlation between rRT-PCR results and the probability of successful VI, and identify the most sensitive rRT-PCR and VI protocols using a ring-test design.

## Materials and methods

### Experimental design

The probability of detecting IAV in swine OF samples was calculated for each of 13 rRT-PCR and 7 VI assays done at 8 laboratories. Swine OF was inoculated with H1N1 or H3N2 IAV and diluted

10-fold ( $10^{-1}$  to  $10^{-8}$ ) serially. The complete sample set consisted of 180 randomized samples (10 replicates of each subtype at each dilution and 20 IAV-negative OF samples). A mixed-effect repeated-measures logistic-regression model (Proc GLIMMIX, version 9.3; SAS Institute, Cary, North Carolina, USA) was used to determine the association between the detection of IAV and the variables of interest (IAV subtype, dilution, assay, and interactions).

### Ring-test samples

The study was conducted with the use of 4 sows in isolation at the Iowa State University Livestock Infectious Disease Isolation Facility, Ames, Iowa. The protocol for the animal procedures was approved by the university's Institutional Animal Care and Use Committee. The sows were clinically healthy, but to verify their health status before OF collection a serum sample was tested at the Iowa State University Veterinary Diagnostic Laboratory for evidence of infection with PRRS (IDEXX PRRS X3 Ab Test; IDEXX Laboratories, Westbrook, Maine, USA) (18), *Mycoplasma hyopneumoniae* (IDEXX M. hyo. Ab Test; IDEXX Laboratories) and IAV (IDEXX AI Multi-Screen Ab Test; IDEXX Laboratories) with established laboratory procedures (19). Oral fluid was collected by allowing the animals to chew on, and thus saturate, 5/8-inch 3-strand twisted 100% cotton rope (Web Rigging Supply, Lake Barrington, Illinois, USA) suspended in the pen. The wet portion of the rope was inserted into a resealable plastic bag and severed from the dry portion. The bag containing the wet rope was then passed through a wringer (Dyna-Jet Products, Overland Park, Kansas, USA), and the OF that pooled in the bottom of the bag was decanted into tubes. At the end of each daily collection the OF was centrifuged (at  $13\,000 \times g$ ) for 10 min and stored in 50-mL tubes at  $-80^{\circ}\text{C}$ . The collection process was repeated for 28 d, until a total of 5.4 L was accumulated (20).

To prepare ring-test samples, OF was thawed, aggregated in a sterile 6-L flask, and mixed on a magnetic stir plate for 1 h in a biosafety cabinet. Negative-control samples were generated by dispensing 2.6-mL aliquots of aggregated OF into 3-mL cryovials. Sufficient aliquots were dispensed to supply 20 negative samples per sample set. Thereafter, the aggregated OF was split into 2 flasks for the creation of subtype-specific IAV stock solutions by adding either A/Swine/Ohio/511445/2007  $\gamma$  H1N1 virus (kindly provided by Dr. Amy Vincent, USDA National Animal Disease Center, Ames, Iowa) or A/Swine/Illinois/02907/2009 cluster IV H3N2 virus (kindly provided by Dr. Marie Culhane, University of Minnesota, St. Paul, Minnesota, USA) to 1 of the 2 flasks. Previously the viruses had been propagated on Madin-Darby canine kidney (MDCK) cells to a concentration of  $1 \times 10^{6.5}$  median tissue culture infectious dose ( $\text{TCID}_{50}$ ) per milliliter. From these virus stock solutions, 10-fold dilutions ( $10^{-1}$  to  $10^{-8}$ ) of H1N1 and H3N2 were created and dispensed as 2.6-mL sample aliquots into 3-mL cryovials. Ten replicates per dilution of each viral subtype were created for each sample set. Thus, 1 sample set (180 samples) included 20 negative samples and 160 IAV-inoculated samples (10 replicates of each IAV subtype at each dilution). Finally, 1 mL of each stock solution was retained for back titration.

Eight sample sets were prepared for distribution to participating laboratories. Each aliquot was identified by set number and a random sample number between 1 and 180. The sample sets were sorted

**Table I. Number of samples of swine oral fluid (OF) positive for influenza A virus (IAV) by real-time reverse-transcription polymerase chain reaction (rRT-PCR) and mean cycle threshold (Ct) value by dilution<sup>a</sup> (assays listed in descending order of performance)**

Assay	Lab	Neg. <sup>b</sup>	Dilution, number of samples, and mean Ct value															
			Swine OF with H1N1 <sup>c</sup>								Swine OF with H3N2 <sup>d</sup>							
			10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>
PCR 1	G	1	10	10	10	10	10	10	5	1	10	10	10	10	10	6	1	0
		36.3	16.6	19.9	23.2	26.5	29.9	33.3	35.4	36.1	19.7	22.9	26.1	29.5	32.6	35.7	36.2	—
PCR 2	A	1	10	10	10	10	10	10	2	1	10	10	10	10	10	5	2	1
		39.2	19.9	23.5	27.0	30.4	33.8	37.3	39.6	39.5	19.7	22.9	26.1	29.4	37.3	39.0	39.0	39.8
PCR 3	G	2	10	10	10	10	10	10	4	1	10	10	10	10	10	3	1	0
		37.7	18.7	22.1	25.4	28.6	32.0	35.6	36.9	38.5	21.7	25.2	28.4	31.6	34.7	36.0	36.7	—
PCR 4	G	0	10	10	10	10	10	10	7	0	10	10	10	10	10	6	0	0
		—	18.6	21.8	25.1	28.6	32.2	35.4	37.3	—	21.6	24.6	28.3	31.7	35.0	37.6	—	—
PCR 5	D	1	10	10	10	10	10	10	1	1	10	10	10	10	10	0	1	0
		34.2	18.1	21.6	25.0	28.2	31.0	35.4	36.0	35.4	21.3	25.0	28.1	31.2	33.1	—	33.3	—
PCR 6	H	2	10	10	10	10	10	3	0	0	10	10	10	10	10	—	3	1
		37.5	21.6	25.1	28.8	31.8	35.6	41.5	—	—	21.0	24.5	27.8	31.3	35.3	37.0	36.2	36.9
PCR 7	D	1	10	10	10	10	10	5	0	1	10	10	10	10	10	0	1	0
		36.9	20.5	23.9	27.7	31.0	34.8	37.7	—	37.5	22.1	26.0	29.5	33.0	35.8	—	37.3	—
PCR 8	E	1	10	10	10	10	8	1	1	2	10	10	10	9	6	5	2	0
		39.1	23.1	26.7	29.7	34.4	38.2	35.6	36.4	35.4	23.4	27.7	29.9	34.6	36.7	35.7	38.4	—
PCR 9	G	1	10	10	10	10	10	6	0	1	10	10	10	10	9	1	0	1
		36.0	20.8	24.8	28.2	31.2	35.0	37.7	—	38.8	23.8	27.3	30.6	33.8	36.4	38.8	—	37.2
PCR 10	F	2	10	10	10	10	3	1	0	0	10	10	10	10	9	0	2	1
		40.2	24.7	28.2	31.1	34.1	41.1	37.8	—	—	23.5	27.1	30.4	34.0	37.4	—	40.5	39.9
PCR 11	C	1	10	10	10	9	5	1	0	0	10	10	10	10	9	4	0	0
		39.0	26.3	29.5	32.8	36.4	39.2	42.3	—	—	24.1	27.7	31.2	34.7	38.0	38.6	—	—
PCR 12	B	1	10	10	10	10	10	1	1	0	10	10	9	6	0	0	0	0
		33.7	22.3	26.2	29.5	32.6	36.1	38.6	38.1	—	25.8	29.3	32.9	35.9	—	—	—	—
PCR 13	E	0	10	10	10	8	2	0	0	2	10	9	10	9	5	1	1	0
		—	22.8	26.8	29.2	34.0	36.9	—	—	33.6	22.2	25.4	28.5	32.2	34.7	33.6	38.5	—

<sup>a</sup> Results based on a sample set composed of 20 negative controls and 10 samples at each dilution (10<sup>-1</sup> to 10<sup>-8</sup>) for each IAV subtype.

<sup>b</sup> Detection represents false-positive results.

<sup>c</sup> For undiluted fluid the concentration [median tissue culture infective dose per milliliter (TCID<sub>50</sub>/mL)] of the  $\gamma$  H1N1 virus A/Swine/Ohio/511445/2007 was estimated at 1 × 10<sup>7.5</sup>/mL.

<sup>d</sup> For undiluted fluid the concentration of the cluster IV H3N2 virus A/Swine/Illinois/02907/2009 was estimated at 1 × 10<sup>6.6</sup>/mL.

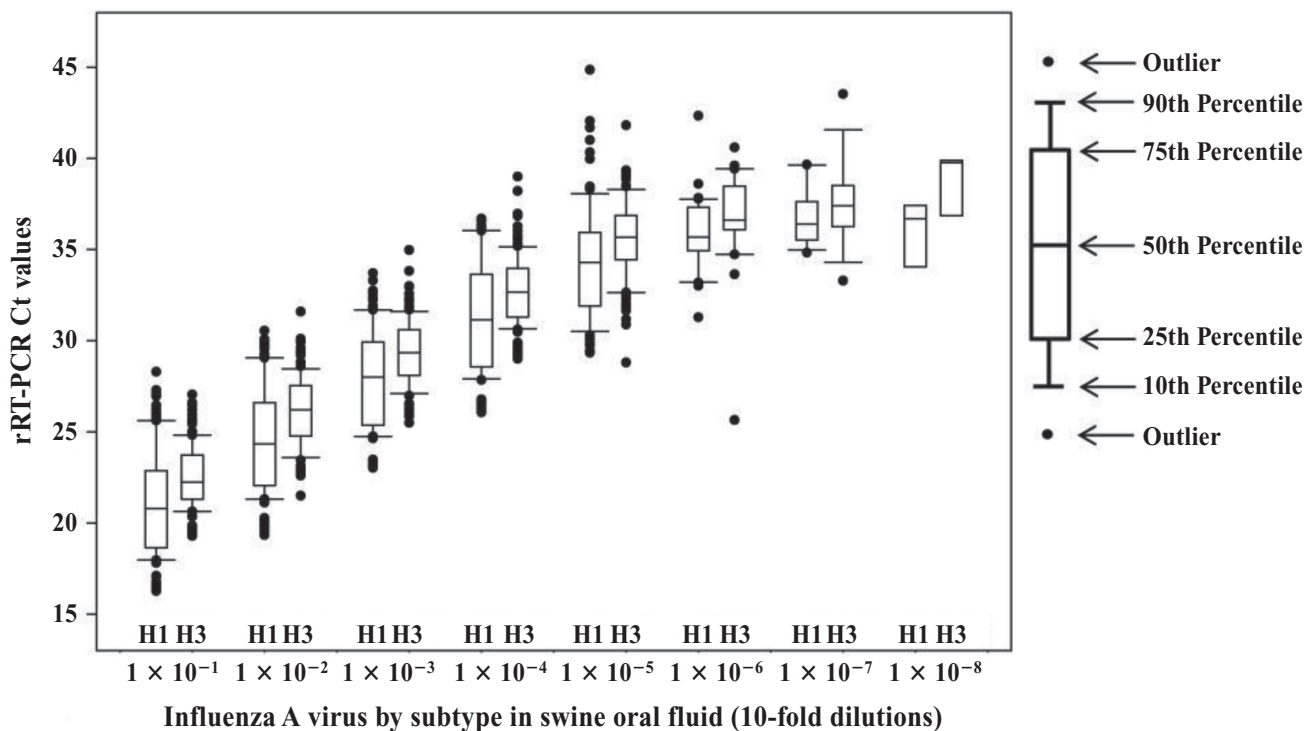
by random number, stored at -80°C, and then shipped overnight on dry ice to the 8 laboratories. Temperature indicators (WarmMark Time-Temp Tags; ShockWatch, Graham, Texas, USA) were placed in each box (13 × 13 cm) of cryovials (60 samples) to detect exposure of the contents to temperatures of -18°C or higher during the shipment process. The laboratories confirmed that the samples were still frozen and that the boxes contained dry ice at the time of arrival.

Samples were immediately returned to -80°C until tested at the recipient laboratory. All laboratories invited to participate had prior experience with molecular techniques for the detection of IAV in swine specimens including nasal swabs, tissue, and oral fluid. All invited laboratories agreed to participate. Each laboratory was at liberty to perform the IAV rRT-PCR and VI procedure(s) of their choice. Among the 8 laboratories, 5 tested the samples using 1 rRT-PCR procedure, 2 laboratories conducted 2 PCR procedures, and 1 laboratory conducted 4 PCR procedures (PCR assays 1 to 13). Six laboratories conducted VI, one using 2 different methods (VI assays 1 to 7). Testing results were reported by sample number as binary (Yes/No)

outcomes, along with cycle threshold (Ct) values for the rRT-PCR-positive samples. Positive or negative rRT-PCR status was determined by each laboratory independently and according to previously established cut-off values for the particular assay, protocol, and equipment used.

### Data analysis

The Proc GLIMMIX mixed-effect repeated-measures logistic-regression model was used to analyze the binary responses: detection of IAV in OF by rRT-PCR (Yes/No) and VI (Yes/No). The explanatory variables of interest include dilution (10<sup>-1</sup> through 10<sup>-8</sup>), virus subtype (H1N1 or H3N2), assay (PCR 1 to 13 or VI 1 to 7), and relevant interactions, with sample as a random effect. Fixed effects were considered significant at an  $\alpha$ -value = 0.05. The degree of variability among all aspects of the assay protocols precluded statistical analysis of the individual components. Pairwise differences between assays were assessed with the use of Tukey's *t*-tests. The probability of detecting IAV in known-positive OF samples by dilution was



**Figure 1.** Distribution of cycle threshold (Ct) values from real-time reverse-transcription polymerase chain reaction (rRT-PCR) assay of influenza A virus (IAV) in serially diluted swine oral fluid (OF) obtained from all virus-positive results from each laboratory, by virus subtype (H1N1 or H3N2) and dilution.

estimated for rRT-PCR and VI tests from the mixed-effect repeated-measures logistic-regression model. This model was also used to estimate the detection dose 50 ( $DD_{50}$ ) for each assay; that is, the inoculum dilution at which there was a 50% probability of detection.

## Results

All sow serum was negative for evidence of exposure to PRRSV, *M. hyopneumoniae*, and IAV. Back titration of the virus stock solutions estimated the initial concentrations at  $1 \times 10^{7.5}$  TCID<sub>50</sub>/mL and  $1 \times 10^{6.57}$  TCID<sub>50</sub>/mL for the H1N1 and H3N2 viruses, respectively.

Table I shows the rRT-PCR test results by assay. Of the 13 assays 11 reported at least 1 false-positive result among the 20 negative samples. All the assays detected virus in all the H1N1-inoculated samples through the  $10^{-3}$  dilution, after which detection decreased by assay and dilution. For the H3N2-inoculated samples all the assays detected virus in 10 of 10 samples at the  $10^{-1}$  dilution, and 12 of the 13 assays detected virus in 10 of 10 samples at the  $10^{-2}$  and  $10^{-3}$  dilutions, after which detection decreased by assay and dilution. The mean Ct values by dilution varied widely among the assays, although the values increased consistently within each assay as the dilution increased (Figure 1).

Table II shows the VI results by assay. Successful isolation was inversely related to sample dilution. One assay reported a single false-positive result among the 20 negative samples. Virus was detected in all H1N1- and H3N2-inoculated samples at the  $10^{-1}$  dilution in 6 of the 7 assays; the probability of VI decreased by assay and dilution thereafter.

Analysis of the data in the mixed-effect logistic-regression model identified the variables significant to IAV detection in OF by rRT-PCR or VI as dilution ( $P < 0.0001$ ) and assay ( $P < 0.0001$ ). Virus subtype was not significant to IAV detection by rRT-PCR ( $P = 0.457$ ) or VI ( $P = 0.101$ ). The interaction between dilution and subtype was also not significant for detection by either rRT-PCR or VI ( $P = 0.757$  and  $P = 0.066$ , respectively). Therefore, virus subtype was subsumed into a single variable for subsequent analyses.

The probability (Pr) of IAV detection over dilution was calculated with the following equations.

$$\text{Equation 1: } \lambda = \text{Logit}(P(X)) = \alpha + \beta_1 X_1 + \beta_2 X_2$$

Where:

$\alpha$  = intercept

$\beta_1$  = regression coefficient for concentration

$\beta_2$  = regression coefficients for assay procedure

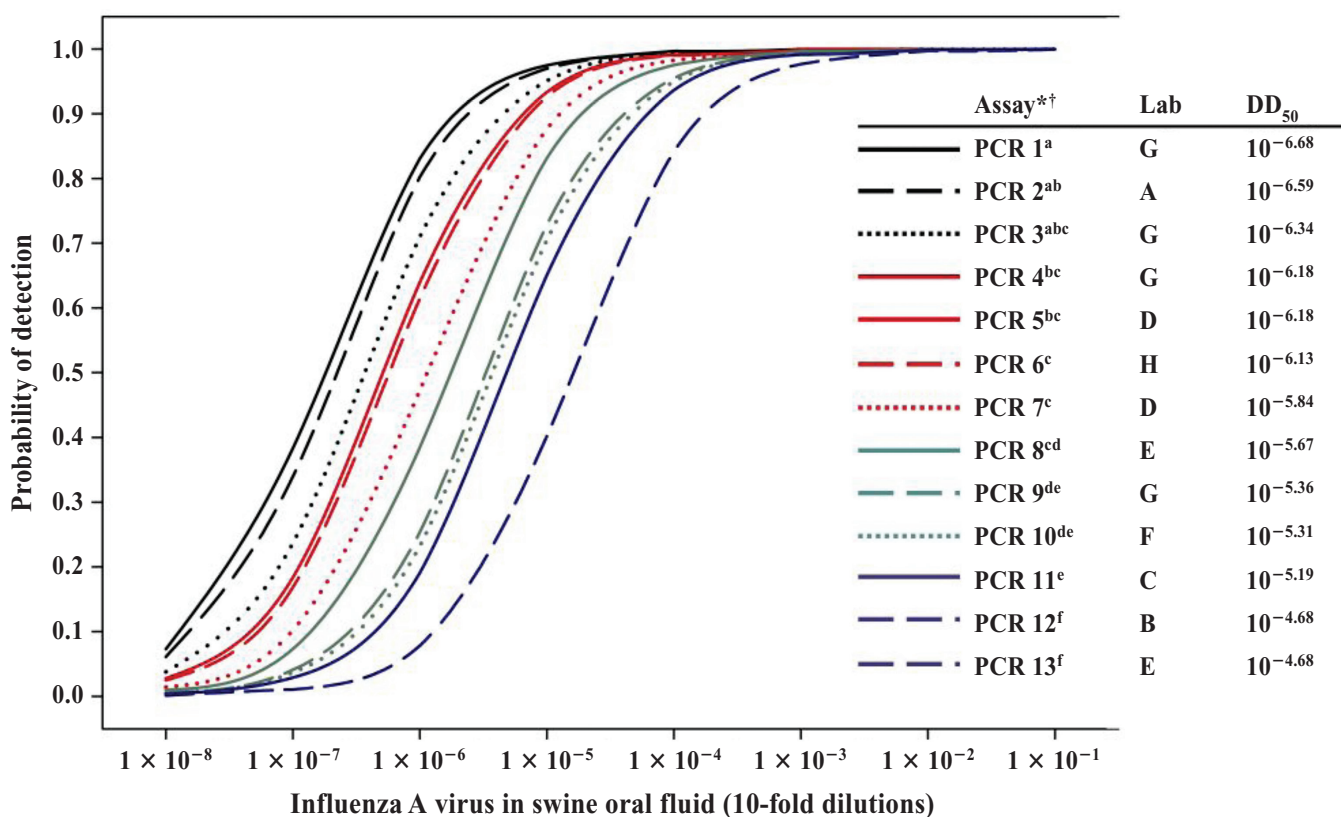
$$\text{Equation 2: Probability (Pr)} = e^{(\lambda)} / (1 + e^{(\lambda)})$$

The probability of detecting IAV by rRT-PCR as a function of dilution is shown in Figure 2. Notably, there was a  $10^2$  range in  $DD_{50}$  between the lowest-performing and highest-performing assays:  $1 \times 10^{-4.68}$  versus  $1 \times 10^{-6.68}$ . Pairwise comparisons among the 13 assays identified significantly different levels of detection performance; assays 1, 2, and 3 exhibited the highest performance and were statistically equivalent. The protocols for these 3 assays are given in the Appendix.

**Table II. Number of samples from which IAV was isolated, by dilution (assays listed in descending order of performance)**

Assay	Lab <sup>a</sup>	Neg.	Dilution and number of samples															
			Swine OF with H1N1								Swine OF with H3N2							
			10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>
VI 1	E	0	10	10	10	8	3	1	1	0	10	10	10	5	1	2	0	0
VI 2	F	0	10	10	9	9	2	1	0	0	10	9	10	9	1	0	0	0
VI 3	B	0	10	10	10	6	1	0	0	0	10	10	9	4	0	0	0	0
VI 4	H	0	10	10	9	2	0	0	0	0	10	10	10	2	0	0	0	0
VI 5	C	1	10	9	1	0	0	0	0	0	10	10	3	2	0	0	0	0
VI 6	D	0	10	4	1	0	0	0	0	0	10	7	1	1	0	0	0	0
VI 7	C	0	9	4	1	0	0	0	0	0	8	6	1	0	0	0	0	0

<sup>a</sup> Not all of the 8 participating laboratories conducted virus isolation studies. All other footnotes as for Table I.

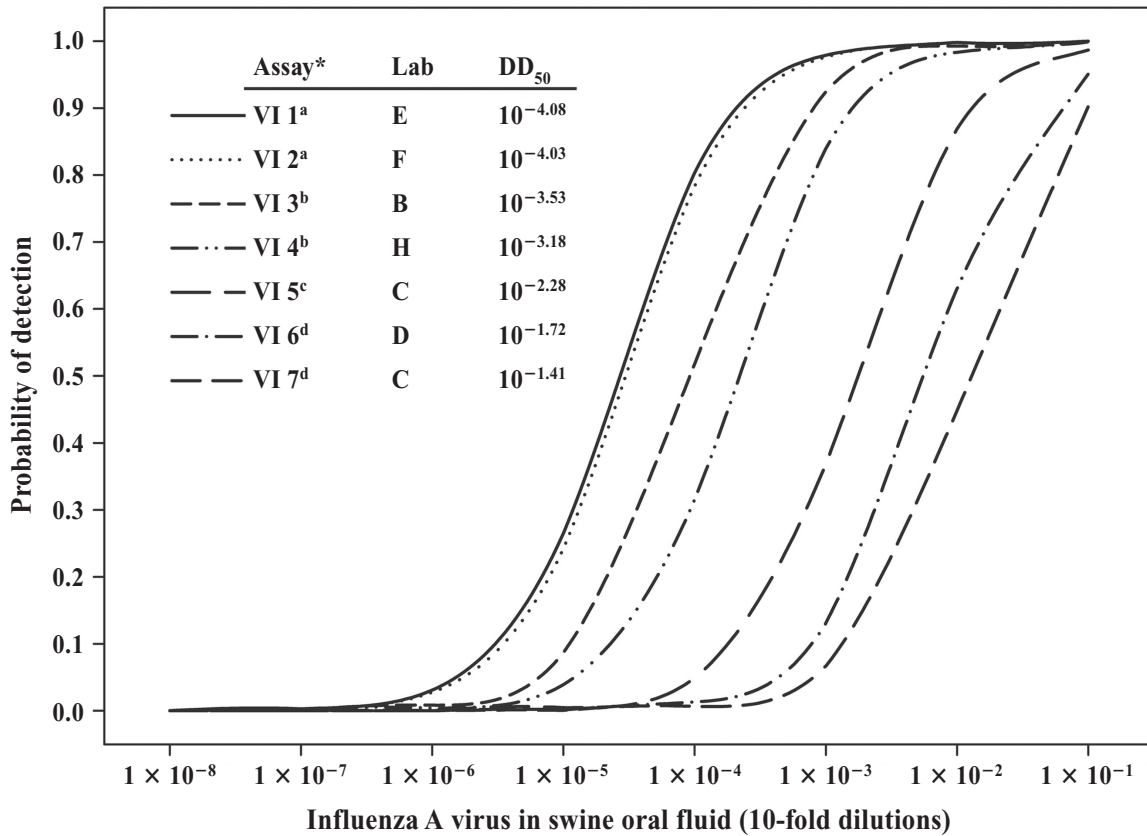


**Figure 2. Probability of detecting IAV in swine OF by rRT-PCR according to combined H1N1 and H3N2 test results. Differences between superscripts reflect significant differences ( $P < 0.05$ ) in assay performance between the 13 assays. Duplicate line patterns of the same color indicate identical probability curves. DD<sub>50</sub> — detection dose 50; that is, the inoculum dilution at which there was a 50% probability of detection.**

The probability of isolating IAV from OF as a function of dilution is shown in Figure 3. Significantly different levels of performance were identified among the VI assays, with a 10<sup>2.67</sup> range in DD<sub>50</sub> between the lowest-performing and highest-performing assays: 1 × 10<sup>-1.41</sup> versus 1 × 10<sup>-4.08</sup>. Virus isolation was generally unsuccessful at dilutions greater than 10<sup>-4</sup>. Pairwise comparisons among the 7 assays identified significantly different levels of performance; assays 1 and 2 showed the highest performance and were statisti-

cally equivalent. The protocols for these 2 assays are given in the Appendix.

An evaluation of the relationship between the rRT-PCR Ct values and VI positivity determined that the probability of a positive rRT-PCR result had no association with the probability of successful VI within the same laboratory except for the dilutions with the highest concentrations of virus. This absence of an association resulted from the variability in both PCR Ct values and VI performance within laboratories.



**Figure 3. Probability of detecting IAV in swine OF by virus isolation according to combined H1N1 and H3N2 test results. Differences between superscripts reflect significant differences ( $P < 0.05$ ) in assay performance between the 7 assays.**

## Discussion

The use of OF specimens for the detection of IAV infections in swine is a relatively recent innovation (7,21,22) but is compatible with previous reports on the use of such specimens in human and veterinary diagnostics (23). In particular, pen-based OF specimens provide the advantage of a higher probability of disease detection in populations compared with samples collected from individual animals (7,22,24,25). For example, Romagosa et al (22) reported that the probability of detecting IAV in swine OF by rRT-PCR was 69% when the prevalence of infection in a pen was 9% and 99% when the prevalence was 10% or greater.

The primary aim of this study was to evaluate the performance of rRT-PCR and VI assays for the detection of IAV in swine OF specimens. Interlaboratory assessment of molecular diagnostic assay performance is challenging because procedures are often nonuniform among veterinary diagnostic laboratories and because the technology continues to evolve at a rapid pace. In this study, performance was assessed by analyzing and ranking 13 rRT-PCR and 7 VI assays using results from identical sets of samples tested at 8 laboratories. The analysis identified marked differences in rRT-PCR and VI performance and a general lack of reproducibility among the participating laboratories. These results were compatible with the results of previous ring-test studies, in which high variability was observed among diagnostic laboratories performing similar tests (26–30).

Although a recent publication compared MDCK cells with embryonated eggs for isolation of IAV (31), no studies comparing IAV VI ring tests were found in the literature. Thus, there are no reports with which to compare the VI DD<sub>50</sub> estimates (10<sup>-1.41</sup> to 10<sup>-4.08</sup>) in this study. However, comparison of these VI DD<sub>50</sub> estimates with the DD<sub>50</sub> estimates for the rRT-PCRs in this study (10<sup>-4.68</sup> to 10<sup>-6.68</sup>) confirms that PCR is a more analytically sensitive test. Although the mean Ct values (Figure 1) and the number of rRT-PCR-positive samples (Table I) correlated with the concentration of IAV in OF, the range of Ct values within dilutions demonstrated a substantial lack of precision. As a consequence, although samples with a higher virus concentration had a higher probability of virus detection by both rRT-PCR and VI, it was not possible to predict VI success on the basis of the Ct values.

A second aim of this study was to identify specific assay or laboratory factors associated with IAV detection in swine OF specimens. Achieving this aim is inherently difficult because of the number of potential sources of variability: the assay (the procedure, reagents, and equipment), the technician(s) performing the test, sample mishandling at any point in the chain of custody, and random errors introduced at any point in the process. Identifying factors affecting assay performance and reproducibility is further compromised by the fact that many sources of variability are difficult or impossible to document. In the case of molecular diagnostics, many of the components that potentially affect performance are proprietary and not

available for analysis. Although the analysis in this study disclosed a large range in detection limits for both rRT-PCR and VI assays (a greater than 100-fold difference in the  $DD_{50}$  values), the variability in equipment, conditions, reagents, and protocols precluded statistical analysis that might have identified specific variables positively (or negatively) associated with detection. Furthermore, some laboratories participating in the study declined to provide complete protocols on the grounds of protecting intellectual property.

A possible weakness of the study was the uniformity of the OF specimens; that is, we used OF from 4 sows housed under experimental conditions rather than field samples. However, this approach assured uniformity among samples and made it possible to generate the large number of samples ( $n = 1980$ ) required for statistical analysis. In addition, there is no documented difference between experimental and field OF samples in terms of assay performance.

Assays based on PCR play an ever-larger role in routine diagnostics because their diagnostic and analytic sensitivities are considered superior to those of VI, and they also provide for high throughput. However, issues with PCR reproducibility and repeatability were recognized soon after the method was introduced (32). To assure minimum standards of PCR performance for high-consequence human pathogens, the European Union implemented proficiency testing of laboratories handling specimens from humans (32). Subsequent developments in PCR technology (e.g., single-tube reactions and robotic automation) have resulted in improvements that have been documented in proficiency testing over the last decade (9). Still, significant differences in assay performance remain, particularly in samples with lower target concentrations, as seen in this study and others (11,26,33,34). Both routine diagnostics and programmatic surveillance require tests that provide accurate, consistent performance. Achieving this goal will require a collaborative approach that starts with more stringent requirements for validation of molecular assays and includes continuous, rigorous quality-control measures, such as proficiency panel and ring-trial assessments.

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## Appendix. Optimal procedures for the diagnosis of influenza A virus (IAV) infection

### Real-time reverse-transcription polymerase chain reaction (rRT-PCR) assay

*Procedure 1* — Oral fluid (OF) specimens (200  $\mu$ L) were centrifuged at 10 000  $\times$  g for 30 s. Supernatant (140  $\mu$ L) was extracted, purified, and processed with the QIAamp Viral Mini QIAcube kit (Qiagen, Valencia, California, USA) according to the manufacturer's recommendations. An inhibition control (Tetracore, Rockville, Maryland, USA) was incorporated into the extraction process and used as an extraction and PCR-inhibition control for each sample. The final elution volume of the extracted sample was 60  $\mu$ L. Extracted samples were stored at 4°C and tested within a day.

The rRT-PCR assay was done with commercial reagents and a dry master mix (Universal Influenza A Matrix MPX 2.0; Tetracore) prepared according to the manufacturer's recommendations. For each reaction 20  $\mu$ L of rehydrated master mix and 5  $\mu$ L of sample were loaded into 1 well of a 96-well plate. The reactions were run with the following cycling conditions: 50°C for 30 min (stage 1), then 95°C for 2 min (stage 2), followed by 45 cycles of 95°C for 15 s, 52°C for 15 s, and 60°C for 33 s (stage 3). The thermocycler (7500 Fast Real-Time PCR System; Applied Biosystems, Carlsbad, California, USA) was run in "standard" mode, and fluorescence data were collected during the 60°C step in the FAM and CY5 channels. The baselines were set automatically and the thresholds set manually for each channel. A sample was considered positive for IAV if it yielded a FAM cycle threshold (Ct) value of less than 37. A sample was re-extracted and retested if it yielded a FAM Ct of 37 or more or a negative CY5 Ct, indicative of PCR inhibitors in the reaction.

*Procedure 2* — Lysates were prepared by adding OF specimens (300  $\mu$ L) to the lysis/binding solution in a commercial kit (MagMAX Pathogen RNA/DNA Kit; Applied Biosystems) and preclarified by centrifugation at 16 000  $\times$  g for 2 min. Nucleic acid from the OF samples was extracted and purified from the entire lysate according to the manufacturer's recommendations with the same kit and the MagMAX Express-96 Magnetic Particle Processor (Applied Biosystems). The final elution volume of the extracted sample was 90  $\mu$ L. The extracted samples were stored at 4°C and tested within a day.

The rRT-PCR assay was done with commercial reagents (Swine Influenza Virus RNA Test Kit and VetMAX-Gold SIV Detection Kit; Applied Biosystems), and an internal control (Xeno RNA Control; Applied Biosystems) was used as an extraction and PCR-inhibition control for each sample. The rRT-PCR reaction was run with the following cycling conditions: 48°C for 10 min (stage 1), then 95°C for 10 min (stage 2), followed by 40 cycles of 95°C for 15 s and 60°C for 45 s (stage 3). The thermocycler (7500 Fast Real-Time PCR System) was run in "standard" mode, and fluorescence data were collected during the 60°C annealing/extension stage. Analysis was done with the control-based threshold setting, with thresholds for swine influenzavirus RNA set at 5% of the positive control  $\Delta$ Rn at cycle 40. A sample was considered positive for IAV if it yielded a Ct value of less than 38.

*Procedure 3* — Before extraction, OF specimens (280  $\mu$ L) were centrifuged at 10 000  $\times$  g for 30 s. Supernatant (175  $\mu$ L) was extracted



and purified with the MagMax 96 Viral RNA Isolation Kit (Applied Biosystems), with exceptions, according to the manufacturer's recommendations, and processed with the Qiagen BioSprint 96, according to the manufacturer's recommendations, and the program AM\_1836\_DW50\_v2 of the KingFisher H 96 Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Exceptions to the extraction procedure were as follows: preparation of the lysis/binding solution [237  $\mu$ L of lysis/binding solution concentrate (without isopropanol) and 3  $\mu$ L of carrier RNA per reaction]; the lysis step [sample supernatant was added to the lysis/binding solution with 4  $\mu$ L of inhibition control (QIACube), then mixed and clarified by centrifugation at 14 000  $\times g$  for 3 min]; and preparation of the lysis/binding plate (85  $\mu$ L of bead mix, 65  $\mu$ L of 100% isopropanol, and 115  $\mu$ L of lysate added in order to each well). The remaining reagents were prepared according to the manufacturer's recommendations. The final elution volume of the extracted sample was 75  $\mu$ L. The extracted samples were stored at 4°C and tested within a day.

The rRT-PCR assay was done with commercial reagents and a dry master mix (Universal Influenza A Matrix MPX 2.0) prepared according to the manufacturer's recommendations. For each reaction 20  $\mu$ L of rehydrated master mix and 5  $\mu$ L of sample were loaded into 1 well of a 96-well plate (MicroAmp Optical 96-Well Reaction Plate; Applied Biosystems). The reactions were run with the following cycling conditions: 50°C for 30 min (stage 1), then 95°C for 2 min (stage 2), followed by 45 cycles of 95°C for 15 s, 52°C for 15 s, and 60°C for 33 s (stage 3). The thermocycler (7500 Fast Real-Time PCR System) was run in "standard" mode, and fluorescence data were collected during the 60°C step in the FAM and CY5 channels. The baselines were set automatically and the thresholds set manually for each channel. A sample was considered positive for IAV if it yielded a FAM Ct value of less than 37. A sample was re-extracted and retested if it yielded a FAM Ct of 37 or more or a negative CY5 Ct, indicative of PCR inhibitors in the reaction.

#### **Virus isolation assay**

*Procedure 1* — The following protocol is fully described elsewhere (1). Briefly, confluent monolayers of Madin–Darby canine kidney (MDCK) cells were prepared in flasks 25 cm<sup>2</sup> (Corning, Corning, New York, USA). The cell culture medium was removed, and the cell monolayers were washed 3 times with Minimum Essential Medium (MEM), pH 7.2, containing Earl's salts, 3X antibiotic solution (penicillin, 25 IU/mL; streptomycin, 75  $\mu$ g/mL; gentamicin, 75  $\mu$ g/mL;

and amphotericin B, 3  $\mu$ g/mL), and 5  $\mu$ g/mL of trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (MEM/3X/Tr; Worthington Biochemical, Lakewood, New Jersey, USA). Next, 2 mL of the OF sample was placed in each flask and incubated at 37°C for 60 min, after which the inoculum was removed. The cell monolayers were rinsed 3 times with MEM/3X/Tr and then incubated for 5 to 7 d with 8 to 10 mL of MEM/3X/Tr. The cell cultures were examined for the appearance of cytopathic effect (CPE) daily. If CPE was present, the culture was tested by indirect fluorescent antibody (IFA) detection or rRT-PCR. Cells with no CPE were subjected to 1 freeze–thaw cycle (at –80°C and 37°C) and reinoculated onto fresh MDCK cells. Contaminated cell-culture fluids were filtered (at 0.45  $\mu$ m) (Millipore; Billerica, Massachusetts, USA) and reinoculated onto fresh MDCK cells. Samples were considered IAV-negative if no CPE or IFA was detected after the 2nd passage in cell culture.

*Procedure 2* — The following protocol is described in detail elsewhere (2). Briefly, confluent monolayers of MDCK cells were prepared in flasks 25 cm<sup>2</sup>. The cell culture medium was removed, and the cell monolayers were washed once with Hanks' balanced salt solution (Gibco, Grand Island, New York, USA). Next, 0.5 mL of the OF sample was placed in each flask and incubated at 37°C for 1 h, after which the inoculum was replaced with MEM plus Earl's Salts and L-glutamine (Gibco), 2  $\mu$ g/mL of sterile filtered trypsin from porcine pancreas (MP Biomedicals, Santa Ana, California, USA), and antibiotics (25 IU of penicillin and 25  $\mu$ g of streptomycin). The cell cultures were incubated at 37°C in 5% CO<sub>2</sub> and evaluated for the appearance of CPE daily for 5 to 7 d. If CPE was present, the culture was tested by IFA detection. Cells with no CPE were subjected to 1 freeze–thaw cycle (at –80°C and 37°C) and reinoculated onto fresh MDCK cells. Contaminated cell-culture fluids were filtered (at 0.22  $\mu$ m) (Acrodisc; Pall Corporation, Port Washington, New York, USA) and reinoculated onto fresh MDCK cells or else the original sample was retested. Samples were considered IAV-negative if no CPE or IFA was detected after the 2nd passage in cell culture.

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