Evaluation of a Field-Deployable Insulated Isothermal Polymerase Chain Reaction Nucleic Acid Analyzer for Influenza A Virus Detection at Swine Exhibitions

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Abstract

Rapid detection of influenza A virus (IAV) at swine exhibitions, where zoonotic transmission has occurred, can allow exhibition officials to quickly implement mitigation strategies and reduce public health risk. While laboratory diagnostic methods using PCR exist, pen-side detection of IAV can reduce lag time between sample collection and results. Portable insulated isothermal PCR (RT-iiPCR) has been used for point-of-care pathogen detection in veterinary medicine. This study compared laboratory methods of real-time reverse transcription PCR (rRT-PCR) to RT-iiPCR to determine the potential effectiveness of RT-iiPCR for detection of IAV in swine in the field. Two methods of extraction (magnetic bead and spin-column) and the two PCR platforms were used in a crossover study design to detect IAV in nasal wipes of 150 individual swine from one exhibition. Magnetic bead extraction is considered the laboratory gold standard while spin-column purification is considered the field-deployable method. IAV RNA was detected in 17 samples using Mag/rRT-PCR (reference assay) and 16 samples using Mag/RT-iiPCR (Sensitivity-S 76.5%), whereas only 14 samples using Spin/rRT-PCR (S 88.2%) and 12 samples using Spin/RT-iiPCR (field method) (S 58.8%) were positive, demonstrating a reduction in detection of viral RNA using column purification. There is moderate agreement (Cohen's kappa=0.6575) between Mag/rRT-PCR and Spin/RT-iiPCR. There is good agreement between both PCR assays when using the same method of extraction (Mag: Cohen's kappa=0.8203, Spin: Cohen's kappa=0.7642). RT-iiPCR requires testing of 10 more samples than the rRT-PCR to detect disease at the 95% confidence level in a population of 300 animals with a disease prevalence of 20%. In conclusion, although there is some reduction in sensitivity, RT-iiPCR used in conjunction with spin-column purification is an acceptable method of IAV in swine detection at exhibitions where it may help reduce lag time and allow for rapid control of an IAV outbreak.

Keywords: influenza A virus, RT-iiPCR, swine, point-of-care systems, sensitivity and specificity, real-time polymerase chain reaction

Introduction

INFLUENZA A VIRUS (IAV) is a single-stranded, segmented RNA virus that infects various species from wild waterfowl to humans (Webster et al. 1992). IAV causes clinical and subclinical respiratory disease in swine and is endemic in swine populations (Yoon et al. 2014). With the susceptibility of swine hosts to various genetic lineages of IAVs, swine are considered mixing vessels of IAV that facilitate bidirectional, interspecies transmission (Brown 2000). Swine exhibition environments are conducive to the rapid spread of IAVs in

swine, where subclinical infection makes recognition of the presence of IAV difficult (Bowman et al. 2012a).

Cases of variant IAVs (IAVs that typically circulate in swine found in humans) with association to swine exhibitions have occurred due to the extensive interaction between swine and humans during such exhibitions (Bowman et al. 2012b, 2014, Wong et al. 2012, Bowman et al. 2017). As evidenced by the most recent swine-lineage H1N1 IAV pandemic in 2009, pandemic potential of variant IAVs exists, although human-to-human transmission has been limited (Jhung et al. 2011). As of the end of 2017, 468 variant IAV cases have occurred since

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2005, with the majority of these cases having association to swine exhibitions (CDC 2017). Therefore, early detection and containment of IAVs in swine at exhibitions is imperative for the protection of animal and public health.

Many regulatory and surveillance agencies use methods of nucleic acid extraction and PCR to detect IAV in swine that require large, immobile equipment that is often recognized for its ease of use (*i.e.*, automatic runs and large sample processing volume), but is only found in the laboratory, introducing lag time between sample collection and diagnostic results. Point-of-care detection is often necessary in cases where time is of the essence and laboratory equipment is not quickly or easily accessible.

The portable POKKITTM Nucleic Acid Analyzer (GeneReach Biotechnology Corporation, Taichung City, Taiwan) uses insulated isothermal PCR (RT-iiPCR) technology for rapid point-of-care detection of various pathogens (Tsai et al. 2012). When used with the POKKIT Swine Influenza A Reagent Set (GeneReach Biotechnology Corporation), RT-iiPCR can detect IAV nucleic acids in swine nasal secretions in the field and without the need for stationary laboratory equipment, reducing lag time.

With rapid and reliable detection of IAV at swine exhibitions, RT-iiPCR could give exhibition officials the ability to detect and contain an IAV outbreak before it presents a risk to public health. The current study aimed to validate the use of RT-iiPCR for the detection of IAV in exhibition swine when compared with laboratory methods of real-time reverse transcription PCR (rRT-PCR).

Materials and Methods

Laboratory testing

Nasal wipe samples from 150 individual swine were collected at an Ohio, USA swine exhibition as part of an ongoing active IAV surveillance program in exhibition pigs as previously described (Edwards et al. 2014). After storage at -80°C for 1 year, all 150 samples were tested for the presence

of IAV by nucleic acid extraction and PCR. Nucleic acid extraction was performed by magnetic bead extraction and spin-column extraction in parallel on all samples. Magnetic bead extraction (Mag) was performed using a laboratory-modified protocol for 100 μL sample using the Mag-Bind Viral DNA/RNA 96 Kit (Omega Bio-tek, Inc., Norcross, GA) in conjunction with a MagMAXTM Express-96 Magnetic Particle Processor (Applied Biosystems, Foster City, CA) as previously described (Bliss et al. 2016). Spin-column extraction (Spin) was executed using the E.Z.N.A.[®] Viral RNA Kit (Omega Bio-tek, Inc.) according to the manufacturer's instructions. Both methods of extraction were performed on the same freeze-thaw cycle for all samples and all RNA was eluted into 50 μL of elution buffer (regardless of extraction method).

Upon completion of both extraction methods, all nucleic acids underwent rRT-PCR and RT-iiPCR in parallel. The rRT-PCR was performed using the VetMAXTM Gold SIV Detection Kit (multiple gene detection assay) (Applied Biosystems) as per the manufacturer's instructions in conjunction with 7500 Fast Real-time PCR System (Applied Biosystems). The rRT-PCR requires the addition of 8 μL of eluted RNA into the reaction.

The RT-iiPCR was completed using the POKKIT Swine Influenza A Reagent Set (single gene detection assay) in conjunction with the POKKIT Nucleic Acid Analyzer. The RT-iiPCR was done as per the manufacturer's instructions with the exception of the addition of 100 μM M-124 Swine Influenza Virus reverse primer (5'-TGCAAAGACACTTTC CAGTCTCTG-3') (Integrated DNA Technologies, Stokie, IL) to buffer B in a 1:100 dilution to broaden the target of the assay and facilitate the detection of known matrix genes currently present in IAV strains (Sponseller et al. 2010). RT-iiPCR requires the addition of 5 μL of eluted RNA into the reaction.

A crossover analysis study design of the two methods of extraction and two PCR platforms was used: Mag/rRT-PCR, Mag/RT-iiPCR, Spin/rRT-PCR, and Spin/RT-iiPCR (Fig. 1). The crossover analysis was used to compare laboratory and field methods while also assessing discrepancies due to

FIG. 1. Laboratory work flow. A crossover analysis study design was used to detect influenza A virus in 150 swine nasal wipe samples from a swine exhibition. All samples were tested by two methods of nucleic acid extraction, magnetic bead (Mag) and spin-column (Spin) extractions, and two PCR platforms, rRT-PCR and RT-iiPCR. Each sample was tested by four methods (Mag/rRT-PCR, Mag/RT-iiPCR, Spin/rRT-PCR, and Spin/RT-iiPCR) resulting in a total of 600 PCRs. Mag/rRT-PCR is the reference assay (*shaded gray*). RT-iiPCR, insulated isothermal PCR; rRT-PCR, real-time reverse transcription PCR.

differences in extractions and PCR platforms. Mag/rRT-PCR is the in-house laboratory gold standard for testing swine nasal wipes for the presence of IAV nucleic acids and is considered the reference assay. Spin/RT-iiPCR is considered the field-deployable, point-of-care method. Mag/RT-iiPCR and Spin/rRT-PCR were used to evaluate discrepancies in RNA extraction methods.

The VetMAX Gold SIV Detection Kit uses a cycle threshold (Ct) value of 38 for positive samples. The POKKIT Swine Influenza A Reagent Set defaults to “+” for a positive sample, “-” for a negative sample, or “?” for an undetermined result based on the POKKIT Nucleic Acid Analyzer’s built-in algorithm.

Statistical analysis

Using Mag/rRT-PCR as the reference assay, sensitivity and specificity were calculated for each testing method. The differences in sensitivities and specificities were averaged across each method of nucleic acid extraction and each PCR platform to evaluate any differences in sensitivity and specificity between Mag/rRT-PCR and Spin/RT-iiPCR. Cohen’s kappa was used to determine the level of agreement between Mag/rRT-PCR and Spin/RT-iiPCR. Freedom of disease sample size calculations were conducted to determine the utility and effectiveness of Spin/RT-iiPCR in a surveillance study design when compared with Mag/rRT-PCR.

Results

Laboratory testing

Of the 150 swine nasal wipe samples, IAV genetic material was detected in 17 samples using Mag/rRT-PCR; 16 samples using Mag/RT-iiPCR; 14 samples using Spin/rRT-PCR; and

12 samples using Spin/RT-iiPCR (Table 1). Nine samples had discrepant results and all of the samples that were negative by Spin/RT-iiPCR had a Ct value >34.5 by the reference assay (Table 2). RT-iiPCR found IAV nucleic acid in one sample that rRT-PCR did not (Table 2).

Statistical analysis

The determined sensitivities were as follows: 76.5% for Mag/RT-iiPCR, 88.2% for Spin/rRT-PCR, and 58.8% for Spin/RT-iiPCR; the specificities for Mag/RT-iiPCR, Spin/rRT-PCR, Spin/RT-iiPCR were 99.2%, 99.2%, and 98.5%, respectively (Table 1). Between Mag/rRT-PCR and Spin/RT-iiPCR, there is moderate agreement with a Cohen’s kappa of 0.6575, 95% CI 0.4247–0.8134. There was good agreement between PCR assays when the same nucleic acid extraction method is used (Mag: Cohen’s kappa=0.8203, 95% CI 0.6169–0.9219, Spin: Cohen’s kappa=0.7642, 95% CI 0.5389–0.8893).

Of the 41.2% reduction in sensitivity between rRT-PCR and RT-iiPCR, 14.75% was attributed to discrepancies in extraction methods and 26.45% was due to the RT-iiPCR platform. Of the 1.5% reduction in specificity, 0.75% was attributed to spin-column extraction, whereas 0.75% was due to the RT-iiPCR platform. In a typical population of 300 exhibition swine with an IAV prevalence of 20%, Mag/rRT-PCR requires a sample size of 15 individuals to detect disease at a confidence level of 95%. Spin/RT-iiPCR requires a sample size of 25 under the same parameters.

Discussion

This study demonstrates that RT-iiPCR performed by the portable POKKIT Nucleic Acid Analyzer used in conjunction with spin-column extraction, which can be performed in the field, is an acceptable point-of-care detection method when

TABLE 1. SENSITIVITY AND SPECIFICITY OF INFLUENZA A VIRUS RNA DETECTION IN CLINICAL SAMPLES USING TWO RNA PREPARATION METHODS AND TWO POLYMERASE CHAIN REACTION PLATFORMS

	Number of IAV positive samples (n=150)	2 × 2 Tables		Sensitivity (%)	95% CI	Specificity (%)	95% CI
		Test assay	Referent				
			+ -				
			+ -				
			a b				
			c d				
Mag/RT-iiPCR	14	13	1	76.5	50.1–93.2	99.2	95.9–100.0
		4	132				
Spin/rRT-PCR	16	15	1	88.2	63.6–98.5	99.2	95.9–100.0
		2	132				
Spin/RT-iiPCR	12	10	2	58.8	32.9–81.6	98.5	94.7–99.8
		7	131				
Mag/rRT-PCR	17	Referent		Referent		Referent	

Swine nasal wipe samples were tested in a crossover analysis study design of magnetic bead (Mag) and spin-column (Spin) extractions and rRT-PCR performed on the laboratory thermocycler and RT-iiPCR performed on the portable POKKIT™ device; four methods of detection were used (Mag/rRT-PCR, Mag/RT-iiPCR, Spin/rRT-PCR, and Spin/RT-iiPCR). An IAV-positive sample is defined by the Mag/rRT-PCR (reference assay) for comparison. A total of 150 samples were tested with 17 (14.16%) defined as positive with the Mag/rRT-PCR cycle threshold cutoff of <38.

CI, confidence interval; IAV, influenza A virus; RT-iiPCR, insulated isothermal PCR; rRT-PCR, real-time reverse transcription PCR.

TABLE 2. DISCREPANCIES BETWEEN METHODS OF INFLUENZA A VIRUS DETECTION

<i>Mag/ rRT-PCR</i>	<i>Mag/ RT-iiPCR</i>	<i>Spin/ rRT-PCR</i>	<i>Spin/ RT-iiPCR</i>
37.2	—	37.3	—
—	—	31.0	+
36.2	+	35.9	—
36.0	?	35.9	—
34.9	+	36.3	—
36.5	—	—	—
37.7	—	—	—
34.5	+	35.3	—
—	+	—	+

Nasal wipe samples of 150 individual swine from one exhibition were tested four ways in a crossover analysis study design using two methods of nucleic acid extraction, magnetic bead (Mag) and spin-column (Spin) extractions, and two polymerase chain reaction (PCR) platforms, real-time reverse transcription PCR (rRT-PCR) and insulated isothermal PCR (RT-iiPCR). Positive and negative determinations are shown for the nine samples where there was disagreement between the methods of detection. A number indicates the positive cycle threshold value of the sample by rRT-PCR; “+” indicates a positive sample by RT-iiPCR; “—” indicates a negative sample by both methods of PCR; “?” indicates an undetermined result by both methods of PCR. Overall, a moderate level of agreement was reached between Mag/rRT-PCR (reference assay) and Spin/RT-iiPCR (field-deployable assay) (Cohen’s kappa = 0.658, 95% CI 0.425–0.813). All samples that are negative by the field-deployable method but positive by the reference assay have a cycle threshold value >34.5.

compared with the in-house, gold standard laboratory testing method and may provide quick, reliable results for detecting and mitigating IAV outbreaks at swine exhibitions. Early, point-of-care detection of IAV-infected swine at exhibitions could reduce viral transmission and decrease the risk to animal and human health (Bliss et al. 2016). Various rapid (15 min) influenza antigen detection tests exist and can be employed at swine exhibitions, but have a lower sensitivity when compared with classical laboratory techniques of RNA extraction and PCR, which can detect IAV at the molecular level (Brussel et al. 2013).

The sensitivity of rapid influenza detection tests is often lessened during early and late infection (when viral shedding is lowest) and is only increased by testing multiple pigs where an outbreak may be evident (Duquette et al. 2013). In a swine exhibition setting, each individual pig that is infected with IAV might be at various time points in the course of their infection, making the rapid detection tests less sensitive at the individual level. Environmental conditions, such as extreme heat or extreme cold, can also often have a negative effect on rapid influenza detection tests, sometimes rendering them inoperable. Where rapid flu detection tests may fail to detect active IAV infection in individual swine in the field, PCR is a more reliable method of detection (Brussel et al. 2013).

Although it should not entirely replace the more sensitive laboratory methods of magnetic bead extraction and rRT-PCR for detection of IAV, RT-iiPCR performed with the POKKIT Nucleic Acid Analyzer can omit the need for an accessible laboratory stocked with large, stationary equipment that is typically required for PCR. However, it should be noted that Spin/RT-iiPCR would still require an electric power source and smaller, portable laboratory equipment, such as pipettes and a centrifuge, unlike the antigen detection tests, which may prove difficult in some field work.

As nucleic acid extraction is required for the detection of IAV by PCR, spin-column extraction can be efficiently performed in the field without fixed laboratory equipment and easily done on individual samples, whereas magnetic bead extraction is often performed on multiple samples at once and requires stationary laboratory machinery and equipment for efficiency. However, spin-column extraction entails multiple pipetting and transfer steps that may introduce an excess of human error and contamination during the procedure. Spin-column extraction is also often critiqued for its small surface area for nucleic acid capture, reducing its efficiency.

If a point-of-care magnetic bead extraction method can be employed, such as a portable magnetic bead processor or a magnetic bead stand, and the need for the less reliable spin-column purification can be eliminated, the reduction in sensitivity and specificity due to the spin-column extraction could potentially be reduced and the sensitivity of the RT-iiPCR could be increased to that of the Mag/RT-iiPCR method (76.5%).

Although discrepancies between the reference assay and the field-deployable method exist and could be attributed to differences in extraction method, the concentration of viral nucleic acid added and gene detection differences between assays could also be contributing to these discrepancies. The objective of this study was to do a side-by-side comparison of the detection/purification platforms following all manufacturers’ protocols and recommendations with the understanding that the rRT-PCR assay uses $1.6 \times$ as much eluted RNA per reaction as the RT-iiPCR and that the assays differ in gene detection, both of which may have affected and contributed to the decreased sensitivity of the RT-iiPCR assay, particularly at low copy numbers (i.e., Ct >35–38).

This study assessed samples that were taken as part of an active surveillance program; samples were taken randomly, regardless of clinical signs, and the low number of positive samples is a limitation of this methodology. However, with a larger sample size requirement, a limit of running a maximum of eight samples at a time, and a lower sensitivity, RT-iiPCR may not be the method of choice in an active surveillance study design where mass number of samples need to be run efficiently and sensitivity should be high to avoid false negatives. With high specificity, RT-iiPCR may be most effective for detecting and diagnosing IAV in swine at exhibitions during an outbreak situation, where clinical signs are apparent. To properly control an outbreak, specificity should be high to avoid false positives, and lag time between sampling and results should be reduced, allowing for quick decision making and mitigation.

Implementation of RT-iiPCR at swine exhibitions for IAV detection would reduce lag time between sampling and obtaining results by eliminating travel and laboratory processing time, giving veterinarians and animal health officials the ability to make rapid decisions and quickly implement mitigation strategies. However, due to the slight reduction in sensitivity, RT-iiPCR may be more useful for commercial herd use, where disease detection is often done at the herd level (testing of multiple individuals in the herd) as compared with the individual level used at swine exhibitions. Despite this, the use of RT-iiPCR may aid in the quick containment of an IAV outbreak at a swine exhibition and, in turn, potentially reduce the risk to animal and public health.

Conclusions

With continued evidence of zoonotic transmission of IAV at swine exhibitions, rapid detection of IAV infection in swine is necessary for preventing and controlling outbreaks. In summary, this study supports the use of RT-iiPCR in conjunction with a field-deployable magnetic bead extraction method at swine exhibitions for the detection of IAV in individual pigs during an outbreak. Although it should not replace detection through traditional laboratory methods, spin-column extraction and RT-iiPCR is an acceptable method of rapid, point-of-care detection of IAV at swine exhibitions that uses the reliable technology of PCR.

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Author Disclosure Statement

No conflicting financial interests exist.

References

- Bliss N, Nelson SW, Nolting JM, Bowman AS. Prevalence of influenza A virus in exhibition swine during arrival at agricultural fairs. *Zoonoses Public Health* 2016; 63:477–485.
- Bowman AS, Nelson SW, Page SL, Nolting JM, et al. Swine-to-human transmission of influenza A(H3N2) virus at agricultural fairs, Ohio, USA, 2012. *Emerg Infect Dis* 2014; 20:1472–1480.
- Bowman AS, Nolting JM, Nelson SW, Slemons RD. Subclinical influenza virus A infections in pigs exhibited at agricultural fairs, Ohio, USA, 2009–2011. *Emerg Infect Dis* 2012a; 18:1945–1950.
- Bowman AS, Sreevatsan S, Killian ML, Page SL, et al. Molecular evidence for interspecies transmission of H3N2pM/H3N2v influenza A viruses at an Ohio agricultural fair, July 2012. *Emerg Microbes Infect* 2012b; 1:e33.
- Bowman AS, Walia RR, Nolting JM, Vincent AL, et al. Influenza A(H3N2) virus in swine at agricultural fairs and transmission to humans, Michigan and Ohio, USA, 2016. *Emerg Infect Dis* 2017; 23:1551–1555.
- Brown IH. The epidemiology and evolution of influenza viruses in pigs. *Vet Microbiol* 2000; 74:29–46.
- Brussel S, Risser J, Galina-Pantoja L. Swine influenza surveillance using FluDETECT test kit. *Am Assoc Swine Veterinarians* 2013:263–264.
- Centers for Disease Control and Prevention (CDC). *Reported Infections with Variant Influenza Viruses in the United States Since 2005*. Centers for Disease Control and Prevention, 2017.
- Duquette A, Ertl J, Garmer M, Ramirez VN, et al. Evaluation of Pfizer Flu DETECT antigen test kit for the detection of laboratory isolates and experimentally infected swine with influenza A virus. *Am Assoc Swine Veterinarians* 2013:77–78.
- Edwards JL, Nelson SW, Workman JD, Slemons RD, et al. Utility of snout wipe samples for influenza A virus surveillance in exhibition swine populations. *Influenza Other Respir Viruses* 2014; 8:574–579.
- Jhung MA, Sverdlow D, Olsen SJ, Jernigan D, et al. Epidemiology of 2009 pandemic influenza A (H1N1) in the United States. *Clin Infect Dis* 2011; 52 Suppl 1:S13–S26.
- Sponseller BA, Strait E, Jergens A, Trujillo J, et al. Influenza A pandemic (H1N1) 2009 virus infection in domestic cat. *Emerg Infect Dis* 2010; 16:534–537.
- Tsai YL, Wang HT, Chang HF, Tsai CF, et al. Development of TaqMan probe-based insulated isothermal PCR (RT-iiPCR) for sensitive and specific on-site pathogen detection. *PLoS One* 2012; 7:e45278.
- Webster RG, Bean WJ, Gorman OT, Chambers TM, et al. Evolution and ecology of influenza A viruses. *Microbiol Rev* 1992; 56:152–179.
- Wong KK, Greenbaum A, Moll ME, Lando J, et al. Outbreak of influenza A (H3N2) variant virus infection among attendees of an agricultural fair, Pennsylvania, USA, 2011. *Emerg Infect Dis* 2012; 18:1937–1944.
- Yoon SW, Webby RJ, Webster RG. Evolution and ecology of influenza A viruses. *Curr Top Microbiol Immunol* 2014; 385:359–375.

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