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Next-Generation Sequencing Confirmation of Real-Time RT-PCR False Positive Influenza-A Virus Detection in Waterfowl and Swine Swab Samples

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Abstract

Surveillance for the Influenza A virus is the most important method used to monitor poultry and other animal species for the presence of the Influenza A viruses. Waterfowl and swine swab samples that tested positive for the Influenza A virus by real-time RT-PCR (rRT-PCR) but tested negative for the virus by virus isolation were further investigated in our recent studies using next-generation sequencing (NGS). A total of seven such pooled swab samples (four swine oral-pharyngeal (OPH) swab pools and three wild duck OPH and cloacal swab pools) were tested for influenza A virus by whole genome sequencing using NGS with the Illumina MiSeq system. Our sequencing results confirmed that none of these rRT-PCR positive samples (Ct-values of 22 to 28) contained Influenza A virus contigs; instead, all the samples contained multiple non-target contig sequences mismatched to the rRT-PCR primer and probe sequences, which led to the positive rRT-PCR results. These genome sequence findings provide scientific evidence that the rRT-PCR false positive results were caused by non-target contigs and not by target viral RNA. Additionally, these samples tested negative for the influenza A virus by conventional RT-PCR (cRT-PCR), which suggests cRT-PCR can serve as an alternative approach for identifying rRT-PCR false positive results.

Keywords: Influenza A virus; Surveillance; Real-time RT-PCR false positive; Genome sequencing; Virus isolation

Introduction

Influenza A virus is a genus of the Orthomyxoviridae family of viruses and causes influenza in birds and some mammals [1-3]. Many wild birds and waterfowl are thought to be natural hosts of Influenza A viruses, also known as avian influenza viruses (AIVs), without necessarily exhibiting signs of infection [4-6]. The type of Influenza A virus that infects swine is named swine influenza virus (SIV) [7,8]. Influenza A viruses are negative-sense, single-stranded, segmented RNA viruses. The Influenza A subtypes are classified according to the type of hemagglutinin and neuraminidase surface proteins on the virus, designated H and N, respectively. So far, 18 distinct H antigens (H1 to H18) and 11 distinct N antigens (N1 to N11) have been identified [9]. H17 was isolated from fruit bats in 2012, and H18N11 was discovered in a Peruvian bat in 2013 [10,11].

The surveillance and rapid diagnosis of Influenza A viruses are essential strategies in the prevention and control of outbreaks [12,13]. Although the classic laboratory methods for virus isolation (VI) are used worldwide as standard assays, emerging novel technologies have been rapidly developed and applied to support influenza surveillance and diagnosis since outbreaks of H5N1 highly pathogenic avian influenza (HPAI) occurred in southeast Asia in early 2000 [14-18]. The newly developed virus detection assays consist primarily of monoclonal antibody-based antigen capture assays and various molecular assays, including conventional reverse transcription polymerase chain reaction (cRT-PCR), multiplex RT-PCR (mRT-PCR), real-time RT-PCR (rRT-PCR), real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP), duplex rRT-PCR (drRT-PCR), and rapid PCR-based molecular pathotyping of the AIV H5 and H7 subtypes. These newly developed techniques provide urgently needed alternatives for rapid diagnosis during AIV outbreaks [19-31].

The advanced molecular assay of real-time PCR (rPCR), also known as rRT-PCR, has completely revolutionized the detection of DNA and RNA in the life sciences and molecular biology over the last decade [27,32]. The rPCR technology allows the detection of PCR amplification and the measurement of the reaction kinetics during the early phases of the reaction, thus providing a distinct advantage over cPCR detection [33-35]. Because rPCR detects the accumulation of amplicons as the reaction progresses, the data are acquired during the exponential phase of the PCR reaction. By contrast, cPCR methods use agarose gels or other post-PCR methods to detect PCR amplification at the final phase or the end-point of the PCR reaction; these methods are time consuming and may not be as precise as rPCR. The exponential phase, or real-time, is commonly considered to be the optimal point for data analysis, and rPCR is considered to be the easiest method for quantifying detected DNA and RNA. Theoretically, there is a quantitative relationship between the amount of DNA in the starting target sample and the amount of PCR product at any given cycle number [34,35].

Despite these advantages, rPCR has some shortcomings, including easy cross-contamination, the need for expensive equipment and a high cost per test. Molecular assays can detect viral RNA or DNA from both live and dead viruses therefore, samples that are positive for virus detection by molecular assays but negative by VI are commonly interpreted as being positive for viral RNA but not for live viruses

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[27,36-40]. However, we have recently conducted genome sequencing studies of samples that tested positive by rRT-PCR but not by VI and confirmed that the positive rRT-PCR results acquired in our research studies were actually false positives. In this report, we describe our diagnostic investigation using next-generation sequencing (NGS) and provide genome sequence evidence that identifies rRT-PCR false positives for Influenza A virus detection in wild duck and swine swab samples.

Materials and Methods

Ethics statement

All animal procedures were performed in accordance with the regulations of the Pennsylvania state university (PSU) animal welfare and ethics guidelines (http://www.research.psu.edu/training/sari/teaching-support/animal-welfare-1) and approved by PSU Institutional animal care and use committees (IACUC). The swab sample collection, virus propagation and isolation test were carried out in our avian virology lab. All experimental protocols were approved by PSU institutional biosafety committee.

Duck and swine swabs

For Influenza A virus surveillance during our previous research studies, duck OPH and cloacal swabs were obtained from wild ducks in the northwest region of Pennsylvania, and swine OPH swabs were obtained from farm-raised pigs in the source of Pennsylvania. A total number of 50 wild duck swab pools obtained in three collections and 56 pig OPH swabs obtained in one collection were used in this study.

rRT-PCR and VI

All swab samples were processed for the detection of the Influenza A virus by one-step rRT-PCR assay using influenza A virus matrix gene primers and probe per USDA-NVSL AI rRT-PCR protocol (NVSL-SOP-DVL-AV, SOP-AV-0001, NVSL, Ames, IA, USA, 2013). QIAGEN RNeasy (Spin columns) Mini Kit (250) (Cat No./ID: 74106) was used for viral RNA extraction. QIAGEN OneStep RT-PCR Kit (100) (Cat No./ID: 210212) was used for the RT-PCR reaction. ABI 7500 fast real-time PCR machine was used for the assay performance. One AIV reference strain H5N2 (A/H5N2/chicken/PA/7659/1985) and one AIV H11N9 (A/H11N9/duck/PA/02099/2012) positive duck OPH swab pool of surveillance case stored in our lab were used for RNA extraction positive controls. The extracted H5N2 and H11N9 RNAs and also AIV matrix RNA obtained from USDA-NVSL were used for rRT-PCR positive controls. Wild duck OPH-CS swabs and farm-raised pig OPH swabs previously tested negative were used for RNA extraction and rRT-PCR negative controls. Swab samples that tested positive for the Influenza A virus by the rRT-PCR were repeated one or two times to confirm the positive rRT-PCR results, and then were subjected to VI using 9 to 11 day old specific-pathogen-free (SPF) embryonating chicken eggs (ECE) [41].

cRT-PCR

First, the rRT-PCR products (10 µl of PCR product mixed with 2 µl of loading dye/sample) were loaded onto an agarose gel (1.0% to 1.2%) and electrophoresed to test for the presence of a true PCR band in each sample. Second, if the rRT-PCR products contained PCR bands, the same RNAs that tested positive by rRT-PCR were used for cRT-PCR with the Influenza A group primers designed in our previous studies [25].

NGS

Wild duck and swine swab samples that resulted in strong positive signals by rRT-PCR (Ct-values <30) but that tested negative by VI and cRT-PCR, were used for whole genome sequencing by NGS with

Serial No.	Animal ID and Type of swabs	rRT-PCR Ct-Value	cRT-PCR gel band	Virus Isolation
1	Duck/OPH-CS/31477-3	30.36	-	-
2	Duck/OPH-CS/31477-4*	22.45	-	-
3	Duck/OPH-CS/31477-5*	28.35	-	-
4	Duck/OPH-CS/31968-4	22.45	-	-
5	Duck/OPH-CS/31968-11	27.32	-	-
6	Duck/OPH-CS/31968-13	29.78	-	-
7	Duck/OPH-CS/32646-7*	26.72	-	-
8	Duck/OPH-CS/32646-9	31.45	-	-
9	Duck/OPH-CS/32646-10	33.46	-	-
10	Swine /OPH/Y045-Y046	33.83	-	-
11	Swine /OPH/Y047-Y048*	24.67	-	-
12	Swine /OPH/Y049-Y050	29.42	-	-
13	Swine /OPH/Y051-Y052*	22.74	-	-
14	Swine /OPH/Y053-Y054*	26.34	-	-
15	Swine /OPH/Y055-Y056*	22.53	-	-
16	Swine /OPH/YR23-YR24	32.32	-	-
AIV (+) duck swab	Duck/OPH/H11N9 (+)* (A/H11N9/duck/PA/02099/2012)	30.55	+	+
AIV (+) H5N2 virus	AIV H5N2 (10^3EID$_{50}$/ml)* (A/H5N2/chicken/PA/7659/1985)	30.33	+	+
Negative duck swabs	Negative wild duck OPH-CS swabs for RNA extraction control	0	-	-
Negative Swine swabs	Negative pig OPH swabs for RNA extraction negative control	0	-	-
* Samples selected for next-generation sequencing (NGS)				

Table 1: A summary of the results of Influenza A virus detection for swine oral-pharyngeal (OPH) swab and duck OPH and cloacal swab (CS) samples by real-time RT-PCR (rRT-PCR), conventional RT-PCR (cRT-PCR) and virus isolation.

Type of swabs and animal ID	rRT-PCR Ct-value	NGS total reads	Assembled contigs	Unused reads	Influenza A virus contigs	Contigs mismatched to primers and probe		
						3'FP	Pro	5'RP
Duck/OPH-CS/31477-4	22.45	2,001,391	1,174	222,143	0	5	3	4
Duck/OPH-CS/31477-5	28.35	1,874,558	245	345,61	0	2	2	3
Duck/OPH-CS/32646-7	26.72	1,271,086	309	85,805	0	2	4	6
Swine /OPH/Y047-Y048	24.67	1,379,134	1,174	162,903	0	2	5	9
Swine /OPH/Y051-Y052	22.74	1,979,241	1,802	341,676	0	5	4	8
Swine /OPH/Y053-Y054	26.34	1,441,454	1,071	208,290	0	5	6	8
Swine /OPH/Y055-Y056	22.53	1,855,902	1,255	368,201	0	8	3	5
AIV H5N2, 10^3EID_{50}/ml (A/H5N2/chicken/PA/7659/1985)	30.33	1,158,024	1,398	325,748	8	2	1	3
Duck/OPH/H11N9 (+) (A/H11N9/duck/PA/02099/2012)	30.55	1,578,432	190	628,782	8	1	1	2
Negative SPF chicken swabs	0	1,707,525	259	81,517	0	0	0	0
Note: FP=Forward Primer; RP=Reverse Primer; Pro=Probe								

Table 2: Summary of the results of Influenza A virus detection by next-generation sequencing (NGS) of swine and duck swab samples. All the swabs tested positive for the influenza A virus matrix gene by real-time RT-PCR (rRT-PCR) but negative by conventional gel RT-PCR (cRT-PCR) and virus isolation (VI).

the Illumina MiSeq system [42]. Viral RNAs that were extracted from duck and swine swab samples for rRT-PCR and cRT-PCR were used for NGS. Procedures for sequencing, viral genome assembly, obtaining 5' and 3' termini, and sequence analyses were as previously described [43].

Results

Influenza A virus detection in wild duck and swine swabs by rRT-PCR, cRT-PCR and VI

Nine wild duck oral-pharyngeal (OPH) and cloacal swab pooled samples (one pool per duck) and seven swine OPH swab pooled samples (one pool per two pigs) tested positive for the influenza A virus by rRT-PCR but tested negative by both cRT-PCR and VI (Table 1).

rRT-PCR result validation

To validate the rRT-PCR results, first, the rRT-PCR products were analyzed by gel electrophoresis, and clear PCR bands were obtained at approximately 100 bp; and second, the Ct-values were analyzed using the ABI real-time PCR program, which confirmed that the Ct-values were technically valid (Figure 1). These results indicated that the Ct-values of these swab samples represented true reactions that occurred during the rRT-PCR assay.

Influenza A virus detection by NGS

Four swine swab pooled and three wild duck swab pooled samples that showed strong positive results (Ct-values of 22-28) for the Influenza A virus matrix gene by rRT-PCR were selected for whole genome sequencing using NGS with the Illumina MiSeq System. Our sequencing results revealed that none of the samples contained Influenza A virus contigs; instead, they all contained mismatched contigs of multiple non-specific sequences that partially matched to the rRT-PCR primer and probe sequences (Table 2). The two AIV positive samples H5N2 and H11N9 yielded 1.1 million and 1.5 millions of NGS total reads, respectively and each of the swab test samples yielded about 1.2 to 2.0 millions of NGS total reads (Table 2), which were sufficient number of MiSeq reads per sample to detect viral sequences. Furthermore, the mismatched contigs were derived from host cells and other microorganisms contained in the swab samples and thus led to false-positive rRT-PCR reactions (Tables 3 and 4). These genome sequence findings provide scientific evidence that the rRT-PCR false positives were caused by non-specific contig sequences and not by the target viral RNA. The whole genomes of the two positive control samples (an H11N9 positive duck OPH swab pool and H5N2 AIV) were sequenced successfully and matched correctly to their subtypes (Tables 2 and 5).

Discussion

In the present study, we have investigated three duck and four swine swab pooled samples that tested positive for the influenza A virus by rRT-PCR but tested negative by VI and cRT-PCR. We processed these swab samples for genome sequencing analysis by NGS, and we confirmed that the rRT-PCR positive results were false positive reactions induced by background, non-target RNAs contained in the swab samples. The NGS data identified the non-target RNAs and their segments of identical bases that match to corresponding segments of the rRT-PCR primers and probe. Furthermore, the multiple non-target contigs, or RNA segments, in each swab sample were able to make a perfect match, similar to the target virus contigs, with the whole primer and probe sequences, thus resulting in true PCR reactions that represented false positives. This is a novel methodology of obtaining genome sequence evidence to confirm false positive results generated by rRT-PCR. Therefore, the common interpretation of positive rRT-PCR results for target viral RNA accompanied by negative VI results – that this represents the presence of non-viable viruses-could be wrong.

As we know that the USDA AI rRT-PCR assay used in this study was well validated to be 100% specificity for domestic poultry samples. However, this rRT-PCR assay or other rRT-PCR assays could be subject to non-specific reactions when they were used to test wildlife samples, because wildlife samples contain much complicated microorganisms or exotic/unknown molecular substances which may interference the rRT-PCR reactions. Thus, it is not surprising we obtained false rRT-PCR positive results of the wild duck and swine swab samples in this study, and similar results of other published studies as discussed below.

A published study of AIV detection in wild birds reported that 97 of the 137 birds (70.8%) tested positive for AIV by rRT-PCR using cloacal and/or OPH swabs, but only nine of the 137 birds (6.6%) tested positive for AIV by VI. Thus, we know that at least 88 (97 to 9), or 90.72%, of the rRT-PCR-positive samples failed in the AIV isolation; these samples should not be misinterpreted as being positive for AIV RNA, because they could in reality be rRT-PCR false positives. Another excellent report of AIV surveillance in wild birds in Georgia (conducted between 2009 and 2011 and published by Lewis et al. in

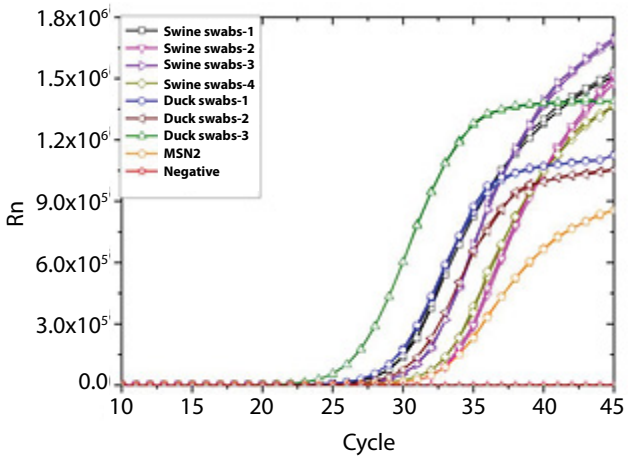

Figure 1: The Ct-value curves of the swine and duck swab samples that tested positive for the influenza A virus matrix gene by real-time RT-PCR (rRT-PCR).

Detected contig ID	Matched contig sequence bases by next-generation sequencing								Average coverage	Contig origin	GenBank Accession#
	(1) Duck/OPH-CS/31477-4, Ct=22.45										
3' F-Primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig0525	AGA	TGA	GTC	TTC	TA				3.49	Bacteria	DQ207481
Contig0209			C	TTC	TAA	CCG	TGG	T	11.08	Bacteria	CP010368
Contig0295	AGA	TGA	GTC	T					19.51	rRNA	NR_076702
Contig0205	GA	TGA	GTC	TT					23.16	rRNA	AF202181
Contig0026		TGA	GTC	TTC	T				295.83	Bacteria	CP007549
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA				
Contig0226			CCC	CTC	AAA	G			107.14	Duck	KC466567
Contig0183			C	CTC	AAA	GCC			19.76	rRNA	NR_076625
Contig0080				TC	AAA	GCC	GA		211.84	rRNA	CP007619
5' R-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig0334		AA	AAC	ATC	TTC				7.99	Unknown	
Contig0139		A	AAC	ATC	TTC	AAG	T		10.66	Bacteria	AP014630
Contig0312			AAC	ATC	TTC	A			9.65	Unknown	
Contig0269		AAA	AAC	ATC	T				19.66	rRNA	AY245110
	(2) Duck/OPH-CS/31477-5, Ct=28.35										
3' F-Primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig0004	G	ATG	AGT	CTT	CT				159.22	Phage	KC352403
Contig0073	G	ATG	AGT	CTT					2.22	rRNA	JN935869
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA				
Contig0135	CA	GCC	CCC	CTC	AAA				81.39	Bacteria	CP007410
Contig0006			CCC	CTC	AAA	G			834.82	Duck	KJ833587
5' R-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig0198			AC	ATC	TTC	AA			4.35	Duck	XM_5027579
Contig0023			AC	ATC	TTC	AA			36.79	Unknown	
Contig002			C	ATC	TTC	AAG			7.85	Bacteria	CP007766
	(3) Duck/OPH-CS/32646-7, Ct=26.72										
3' F-Primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig0282	AGA	TGA	GTC	TT					2.84	rRNA	NR_103040
Contig0055		TGA	GTC	TTC	T				86.81	Bacteria	CP007549
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA				

Contig0296			CCC	CTC	AAA	GCC			8.45	Duck	XM_5020670
Contig0132	TCA	GGC	CCC	CTC	AAA				182.02	rRNA	CP006649
Contig0127		GGC	CCC	CTC	A				51.39	Bacteria	CP008921
Contig0017			CCC	CTC	AAA	G			815.69	Duck	KJ833587
5' R-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig0309			C	ATC	TTC	AAG			1.86	Duck	XM_5017337
Contig0271	C	AAA	AAC	ATC					3.39	Duck	KC466567
Contig0193					TC	AAG	TCT	CT	18.91	*rRNA*	HQ402849
Contig0077			AC	ATC	TTC	AA			1145.11	rRNA	GU323337
Contig0067			AC	ATC	TTC	AA			547.63	rRNA	AB303970
Contig0045			AC	ATC	TTC	AA			410.67	Bacteria	CP010307

Table 3: The results of Influenza A virus detection by next-generation sequencing (NGS) of duck swab samples (pool of oral-pharyngeal (OPH) swabs and cloacal swabs per duck). All of the duck swab pools tested positive for the Influenza A virus by real-time RT-PCR (rRT-PCR) but negative by conventional gel RT-PCR (cRT-PCR) and virus isolation.

PLoS One) described detailed studies and the results of tests using first rRT-PCR and subsequently VI for rRT-PCR-positive samples to detect AIV in fresh faecal, cloacal or tracheal samples from various waterfowl species [40]. Only 23 out of 84 rRT-PCR-positive swab samples (27%) were positive for AIV isolation. Of the 23 VI-positive samples, 20 showed strong rRT-PCR results (Ct-values of 16.69 to 29.99), and the remaining three were weakly positive (Ct-values of 30.00 to 33.96). Of the 60 samples that tested negative by VI but positive by rRT-PCR, 27 showed strong rRT-PCR positive results (Ct-values of 17.89 to 29.99), and 33 showed weak positive results (Ct-values of 30.00 to 39.38). The authors' declaration that "We were generally successful in isolating virus from M RRT-PCR positive samples with a CT-value of under 30 is applicable to the 23 VI-positive samples (20 with Ct-values under 30 and 3 with Ct-values above 30) but cannot explain the 27 VI-negative samples with strong rRT-PCR positive results (Ct-values under 30). Furthermore, among the 23 AIV isolates, 11 were obtained from duck samples and 12 were obtained from gull samples; thus, we cannot agree with the following assumption made by the author: "However we also note that we appeared somewhat more unsuccessful isolating virus from low CT-value samples if they were taken from gulls rather than ducks. This might indicate that some influenza A viruses in gulls do not optimally replicate to high titers in our current culture and isolation system". Interpretations such as these are speculative and not based on scientific evidence. Another report of AIV surveillance in wild birds reported that 332 virus isolates were recovered from 992 rRT-PCR-positive samples, representing an overall recovery rate of 33.5% (332/992) and a VI failure rate of 66.5% [37]. In fact, the majority of the rRT-PCR-positive samples that failed in VI appear to be either true negatives or rRT-PCR false positives. Our research findings using NGS for the identification of rRT-PCR false positive results in the present study provide a new approach and interpretation based on scientific evidence for the confirmation of rRT-PCR false positive results.

The rRT-PCR product amplified by the primers and probe for the influenza A virus M gene is only 95 bp long, as determined by the difference between the forward 3'-25+ primer (5-25) and the reverse 5'-124 primer (76-99), which is appropriate for the rapid collection of amplicon signals during rRT-PCR reactions, but which may be easily subject to cross-reaction with non-target RNAs [27]. In contrast, the duck and swine swab samples in our report tested negative for the influenza A virus by cRT-PCR, indicating the cRT-PCR reactions were not affected by the non-target RNA sequences in the samples, or some related factors we need to investigate. Nonetheless, cRT-PCR appears having an advantage over rRT-PCR in its ability to eliminate such false positive results and thus could be used as an alternative approach for confirming rRT-PCR false positive results in swab samples.

On the other hand, influenza surveillance field samples that tested positive by rRT-PCR and negative by VI can be due to dead virus and/or extremely low virus titers in the samples or a new field variant strain that is not feasible in VI. Although these possibilities are not easy to confirm or rule out in general, alternatively, testing additional collections of the same source samples are more practical in monitoring influenza virus true status.

The confirmation of the rRT-PCR false positive results for influenza A virus detection in this study indicates that it is important and necessary to maintain VI as the "gold standard" for influenza A virus surveillance, despite the availability of advanced molecular assays. It is especially important to use VI to acquire accurate results when conducting Influenza, A virus surveillance in field samples obtained from complicated background sources, when a high rate of non-specific RNA cross contamination is likely, or from clinically healthy animals. Conversely, it is clear that the influenza A virus rRT-PCR assay is more suitable and accurate for the testing of laboratory samples or relatively clean swab (e.g. tracheal swab) samples than in the testing of dirty samples (e.g. cloacal or faecal swabs) or complicated source samples of wildlife species. In fact, most field samples, especially wildlife samples, contain complicated background substances including a large number of unidentified host mRNAs or mutated viral RNAs that can easily produce false positive results. The wild duck and swine swab pooled samples used in the present study are good examples of animal swabs that are not suitable to be called truly positive for Influenza A based only on rRT-PCR testing. Additionally, the classic VI technique remains useful for detecting unexpected, unknown and unforeseen viruses, as well as for identifying entirely new viral agents; therefore, VI remains the "gold standard" and may continue to do so in the future.

NGS is a powerful high-throughput sequencing methodology that generates millions of sequencing reads and can directly sequence viral RNAs. NGS methodologies have revolutionized the detection of emerging variants and novel or unknown viruses that are beyond the capabilities of the VI method. As the most powerful genome

Detected contig ID	Matched contig sequence bases by next-generation sequencing								Average coverage	Contig origin	GenBank Accession#
	(1) Swine /OPH/Y055-Y056, Ct=22.53										
3' F-primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig1217			GTC	TTC	TAA	C			2.61	Bacteria	CP001650
Contig0903	AGA	TGA	GTC	T					4.44	Bacteria	CP002696
Contig0796	GA	TGA	GTC	TT					3.32	rRNA	KC249997
Contig0792	GA	TGA	GTC	TT					9.52	rRNA	JQ311014
Contig0301		TGA	GTC	TTC	T				4.61	Unknown	
Contig0253	GA	TGA	GTC	TT					23.55	rRNA	AF202181
Contig0182			C	TTC	TAA	CCG			441.35	Bacteria	CP000764
Contig0027		TGA	GTC	TTC	T				138.03	Bacteria	LM997153
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA				
Contig1037				TC	AAA	GCC	GA		2.51	Swine	KJ746666
Contig0800	TCA	GGC	CCC	C					16.43	rRNA	NR076498
Contig0258				TC	AAA	GCC	GA		150.77	rRNA	NR121978
5' R-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig1061		A	AAC	ATC	TTC	AA			11.07	Unknown	
Contig1218				TC	TTC	AAG	TCT		2.47	Unknown	
Contig0147		A	AAC	ATC	TTC	A			1686.85	rRNA	FJ498886
Contig0798	TGC	AAA	AAC	ATC	T				4.72	Unknown	
Contig0765				ATC	TTC	AAG	TGT	C	42.29	rRNA	AJ542473
Contig1107		A	AAC	ATC	TTC				2.90	Bacteria	CP003040
Contig1002			C	ATC	TTC	AAG			2.45	Bacteria	AP012044
Contig0835	C	AAA	AAC	ATC					3.56	Unknown	
Contig0830			AAC	ATC	TTC	A			3.67	Unknown	
Contig0356		A	AAC	ATC	TTC				5.05	rRNA	NR_076582
Contig0353	C	AAA	AAC	ATC					37.34	Swine	KF569218
Contig0267			AC	ATC	TTC	AA			186.58	rRNA	NR_121978
Contig0108			AAC	ATC	TTC	A			6.54	Unknown	
	(2) Swine /OPH/Y053-Y054, Ct=26.34										
3' F-Primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig0035		A	GTC	TTC	TAA	CCG			760.61	rRNA	NR_121989
Contig0892	AGA	TGA	GTC	T					3.64	Bacteria	CP002696
Contig0409	GA	TGA	GTC	TT					14.53	rRNA	JQ034423
Contig0097		TGA	GTC	TTC	T				152.61	Trematoda	LL266921
Contig0092			C	TTC	TAA	CCG			420.54	Bacteria	CP005586
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA				
Contig0013	TCA	GGC	CCC	CT					145.3	rRNA	JQ337010
Contig0923			CC	CTC	AAA	GCC	GA		3.08	rRNA	NR_103206
Contig0764	TCA	GGC	CCC	C					21.39	rRNA	GQ877606
Contig0590				TC	AAA	GCC	GA		97.68	Bacteria	CP010896
Contig0477			C	CTC	AAA	GCC			10.55	Bacteria	KC246835
Contig0012			CCC	CTC	AAA	G			9173.2	rRNA	NR_076885
5' R-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig0913	TGC	AAA	AAC	ATC	TTC	AA			1.59	Bacteria	CP007208
Contig0518		A	AAC	ATC	TTC	AA			45.94	Bacteria	AM295250
Contig0827	C	AAA	AAC	ATC					8.77	Swine	JN601068
Contig0778			AAC	ATC	TTC	A			2.11	Unknown	
Contig0707			AAC	ATC	TTC	A			2.06	Unknown	
Contig0185			AC	ATC	TTC	AA			174.61	rRNA	GU926746
Contig0044		A	AAC	ATC	TTC				156.42	rRNA	EU778531
Contig0008				TC	TTC	AAG	TC		168.3	rRNA	NR_076878
	(3) Swine/OPH/Y051-Y052, Ct=22.74										
3' F-Primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig0810	GA	TGA	GTC	TT					27.62	rRNA	AY305327
Contig0851		A	GTC	TTC	TAA	C			4.99	Por.Para.Flu	JX857409
Contig0650	GA	TGA	GTC	TT					3.58	rRNA	AF202181
Contig0138		TGA	GTC	TTC	T				149.69	Trematoda	LL266921
Contig0111			C	TTC	TAA	CCG			1224.61	rRNA	FO681347
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA		1328.37	rRNA	EU066039
Contig0301	TCA	GAC	CCC	CTC	AAA						

Contig1113	TCA	GGC	CCC	CTC	AA				37.16	rRNA	NR_076498
Contig0619	TCA	GGC	CCC	C					445.77	rRNA	NR_076128
Contig0172	A	GGC	CCC	CTC					443.03	rRNA	HM545299
5' R-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig0885		A	AAC	ATC	TTC	AA			17.59	Bacteria	FR695868
Contig0827	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG	2.25	Unknown	
Contig0900			AAC	ATC	TTC	A			14.96	Unknown	
Contig0843		AAA	AAC	ATC	T				42.68	rRNA	JX515400
Contig0723			C	ATC	TTC	AAG			57.35	rRNA	X67761
Contig0694	C	AAA	AAC	ATC					20.85	rRNA	4V19_A
Contig0161				ATC	TTC	AAG	TGT	C	2733.7	rRNA	NR_076295
Contig1167			AC	ATC	TTC	AA			288.84	Unknown	
	(4) Swine/OPH/Y047-Y048, Ct=24.67										
3' F-Primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig231				C	TAA	CCG	AGG		233.37	Bacteria	CP002631
Contig80		TGA	GTC	TTC	T				227.46	Bacteria	CP004084
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA				
Contig0716	TCA	GGC	CCC	CT					24.47	rRNA	JX635010
Contig0505	TCA	GGC	CCC	CTC	AAA				41.85	rRNA	JX638985
Contig0592				TC	AAA	GCC	GA		87.03	Bacteria	AM181176
Contig0545				TC	AAA	GCC	GA		66.60	Bacteria	EU063768
Contig0359				TC	AAA	GCC	GA		257.15	rRNA	GQ876410
5' R-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig0763		A	AAC	ATC	TTC	AA			15.2	Bacteria	FJ872373
Contig1110			C	ATC	TTC	AAG	TCT	CTG	2.62	Unknown	
Contig0776				ATC	TTC	AAG	TGT	C	52.36	rRNA	NR_076295
Contig0535	TGC	AAA	AAC	A					77.42	Bacteria	FN668375
Contig0418	TGC	AAA	AAC	A					132.61	rRNA	NR_121991
Contig0283		A	AAC	ATC	TTC				531.11	rRNA	EU778531
Contig0249			C	ATC	TTC	AAG			63.01	Bacteria	CP006809
Contig0205			C	ATC	TTC	AAG			201.79	Bacteria	CP006772
Contig0060			AC	ATC	TTC	AA			289.82	rRNA	NR_121995

Table 4: The results of Influenza A virus detection by next-generation sequencing (NGS) for swine swabs (two pig oral-pharyngeal (OPH) swabs pooled for each swab sample for the NGS test). Each of these swine swabs individually tested positive for the Influenza A virus by real-time RT-PCR (rRT-PCR) but negative by conventional gel RT-PCR (cRT-PCR) and virus isolation.

Detected contig ID	**Matched contig sequence bases by next-generation sequencing**								**Average coverage**	**Contig origin**	**GenBank Accession#**
	AIV H5N2 (10^3 EID_{50}/ml), Ct=30.33										
3' F-Primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig0269	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG	576.51	Influenza A	KP674444
Contig0987					AA	CCG	AGG	TC	3.37	Chicken	BX931775
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA				
Contig0269	TCA	GGC	CCC	CTC	AAA	GCC	GA		576.51	Influenza A	KP674444
5' R-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig0269	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG	576.51	Influenza A	KP674444
Contig0691				TC	TTC	AAG	TC		6.46	Unknown	
Contig0563					C	AAG	TCT	CTG		Chicken	XR_214244
	H11N9 positive duck OPH swabs, Ct=30.55										
3' F-Primer	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG			
Contig086	AGA	TGA	GTC	TTC	TAA	CCG	AGG	TCG	17.7	Influenza A	CY149605
Probe	TCA	GGC	CCC	CTC	AAA	GCC	GA				
Contig086	TCA	GGC	CCC	CTC	AAA	GCC	GA		17.7	Influenza A	CY149605
Contig147			C	CTC	AAA	GCC	GA		7.08	Unknown	
3' F-Primer	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG			
Contig086	TGC	AAA	AAC	ATC	TTC	AAG	TCT	CTG	17.7	Influenza A	CY149605

Table 5: The results of Influenza A virus detection by next-generation sequencing (NGS) for two positive control Influenza A viruses of the low pathogenic avian influenza virus (LPAIV) H5N2 subtype (A/chicken/PA/7659/1985, GenBank Accession No. KP674444-KP674451) and H11N9 subtype (A/duck/PA/02099/2012, GenBank Accession No. KR870234-KR870241), propagated in SPF embryonating chicken eggs.

sequencing technology, NGS overcomes the limitations of VI and rRT-PCR, as described here, by identifying every sequenceable RNA in each sample and providing direct genome sequence evidence for false positive rRT-PCR reactions caused by mismatching of non-target RNAs to the rRT-PCR primers and probe. In the analysis of the rRT-PCR results from seven swine and nine duck swab pooled samples, the Ct-values obtained indicated high "viral RNA" concentrations in these swab samples. However, when seven of the swab samples with strong rRT-PCR positive results (Ct-values of 22-28) were selected for NGS analysis, none of them were found to contain influenza A virus-related contigs. Conversely, the positive control H5N2 virus (A/chicken/PA/7659/1985, 10^3EID_{50}/ml) and the H11N9 positive duck OPH swabs (A/H11N9/duck/PA/02099/2012), both of which yielded a weak positive rRT-PCR result (Ct-value of 30), were fully sequenced and found to contain eight influenza A virus contigs. These NGS results provide the first genome sequence evidence for false positive rRT-PCR results in the detection of influenza A virus in wild duck and swine swab samples.

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References

1. Couch RB (1996) Orthomyxoviruses (Chapter 58). In: Baron S (Ed.) Medical Microbiology (4thedn.), Galveston (TX): University of Texas Medical Branch at Galveston.
2. Baigent SJ, McCauley JW (2003) Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. BioEssays 25: 657-671.
3. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56: 152-179.
4. Rutz C, Dalessi S, Baumer A, Kestenholz M, Engels M, et al. (2007) Avian influenza: wildbird monitoring in Switzerland between 2003-2006. Schweiz Arch Tierh 149: 501-509.
5. Haider N, Sturm-Ramirez K, Khan SU, Rahman MZ, Sarkar S, et al. (2015) Unusually High Mortality in Waterfowl Caused by Highly Pathogenic Avian Influenza A(H5N1) in Bangladesh. Transbound Emerg Dis.
6. Astorga RJ, Leon L, Cubero MJ, Arenas A, Maldonado A, et al. (1994) Avian influenza in wild waterfowl and shorebirds in the Donana National Park: Serological survey using the enzyme-linked immunosorbent assay. Avian pathol 23: 339-344.
7. Du N, Yang XX, Wang M, Lan Y, Yang L, et al. (2009) Review on the etiological property of the swine influenza virus. Chinese J Virol 25: 39-47.
8. Dunlap MK (1948) Human and swine influenza; A review. Vet Clin N Am 29: 782.
9. Wahlgren J (2011) Influenza A viruses: An ecology review. Infect Ecol Epidemiol.
10. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, et al. (2012) A distinct lineage of influenza A virus from bats. PNAS 109: 4269-4274.
11. Tong S, Zhu X, Li Y, Shi M, Zhang J, et al. (2013) New world bats harbor diverse influenza A viruses. Plos Pathog 9: e1003657.
12. Suarez DL, Das A, Ellis E (2007) Review of rapid molecular diagnostic tools for avian influenza virus. Avian Dis 51: 201-208.
13. Peacey M, Hall RJ, Bocacao J, Huang QS (2009) Diagnostic assay recommended by the world health organization for swine origin influenza a (H1N1) virus cross-reacts with H5N1 influenza virus. J Clin Microbiol 47: 3789-3790.
14. Cappucci DT Jr, Johnson DC, Brugh M, Smith TM, Jackson CF, et al. (1985) Isolation of avian influenza virus (subtype H5N2) from chicken eggs during a natural outbreak. Avian Dis 29: 1195-1200.
15. Edwards S (2006) OIE laboratory standards for avian influenza. Devel Biologicals 124: 159-162.
16. Zowalaty El, Abin ME, Chander M, Redig Y, Goyal SM, et al. (2011) Improved method for the isolation and sub-typing of avian influenza viruses from oropharyngeal samples of ducks. Avian Dis 55: 439-442.
17. Ferguson NM, Cummings DA, Cauchemez S, Fraser C, Riley S, et al. (2005) Strategies for containing an emerging influenza pandemic in Southeast Asia. Nature 437: 209-214.
18. Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus AD, et al. (2006) Global patterns of influenza A virus in wild birds. Science 312: 384-388.
19. Zhang A, Jin M, Liu FF, Guo X, Hu Q, et al. (2006) Development and evaluation of a DAS-ELISA for rapid detection of avian influenza viruses. Avian Dis 50: 325-330.
20. Lu H (2003) A longitudinal study of a novel dot-enzyme-linked immunosorbent assay for detection of avian influenza virus. Avian Dis 47: 361-369.
21. Koskinen JO, Vainionpaa R, Meltola NJ, Soukka J, Hanninen PE, et al. (2007) Rapid method for detection of influenza A and B virus antigens by use of a two-photon excitation assay technique and dry-chemistry reagents. J Clin Microbiol 45: 3581-3588.
22. Ho HT, Qian HL, He F, Meng T, Szyporta M, et al. (2009) Rapid detection of H5N1 subtype influenza viruses by antigen capture enzyme-linked immunosorbent assay using H5-and N1-specific monoclonal antibodies. Clin Vaccine Immunol 16: 726-732.
23. Starick E, Romer-Oberdorfer A, Werner O (2000) Type- and subtype-specific RT-PCR assays for avian influenza A viruses (AIV). J Vet Med B Infect Dis Vet Public Health 47: 295-301.
24. Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK (2001) Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J Virol Methods 97: 13-22.
25. Lu H, Xie Z, Liu J, Lin L (2013) Studies on Multiplex RT-PCR for Detection of Avian Influenza Virus Type A Group and Specific H5 and H7 Subtypes. J Veter Sci Med 2: 5.
26. Chaharaein B, Omar A, Aini I, Yusoff K, Hassan S (2009) Detection of H5, H7 and H9 subtypes of avian influenza viruses by multiplex reverse transcription-polymerase chain reaction. Microbiol Res 164: 174-179.
27. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, et al. (2002) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 40: 3256-3260.
28. Hoffmann B, Harder T, Starick E, Depner K, Werner O, et al. (2007) Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR. J Clin Microbiol 45: 600-603.
29. Soheili Z and Samiei S (2005) Real time PCR: principles and application. Hepat Mon 5: 83-87.
30. Imai M, Ninomiya A, Minekawa H, Notomi T, Ishizaki T, et al. (2006) Development of H5-RT-LAMP (loop-mediated isothermal amplification) system for rapid diagnosis of H5 avian influenza virus infection. Vaccine 24: 6679-6682.
31. Teifke JP, Klopfleisch R, Globig A, Starick E, Hoffmann B, et al. (2007) Pathology of natural infections by H5N1 highly pathogenic avian influenza virus in mute (Cygnus olor) and whooper (Cygnus cygnus) swans. Vet Patho 44: 137-143.
32. Niesters HG (2001) Quantitation of viral load using real-time amplification techniques. Methods 25: 419-429.
33. Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, et al. (2006) The real-time polymerase chain reaction. Mol Aspects of Med 27: 95-125.
34. Bar T, Kubista M, Tichopad A (2011)Validation of kinetics similarity in qPCR. Nucleic Acids Res 40: 1395-1406.
35. Hatt JK, Löffler FE (2012) Quantitative real-time PCR (qPCR) detection chemistries affect enumeration of the Dehalococcoides 16s rRNA gene in groundwater. J Microbiol Meth 88: 263-270.
36. Klein D (2002) Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 8: 257-260.
37. Munster VJ, Baas C, Lexmond P, Waldenström J, Wallensten A, et al. (2007) Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLos Pathog 3: e61.

38. Jindal N, de Abin M, Primus AE, Raju S, Chander Y, et al. (2010) Comparison of cloacal and oropharyngeal samples for the detection of avian influenza virus in wild birds. Avian Dis 54: 115-119.

39. Hulsager CK, Breum SØ, Trebbien AR, Handberg AK, Therkildsen OR, et al. (2012) Surveillance for avian influenza viruses in wild birds in Denmark and Greenland, 2007-10. Avian Dis 56: 992-998.

40. Lewis NS, Javakhishvili Z, Russell CA, Machablishvili A, Lexmond P, et al. (2013) Avian influenza virus surveillance in wild birds in Georgia: 2009-2011. Plos one 8: e58534.

41. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, et al. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme J 6: 1621-1624.

42. Lu H, Xie Z, Liu J, Lin L (2013) Studies on multiplex RT-PCR for detection of avian influenza virus type A group and specific H5 and H7 subtypes. Avens Publishing Group, J Vet Sci Med.

43. Tang Y, Lu H, Sebastian A, Yeh YT, Praul CA, et al. (2015) Genomic characterization of a turkey reovirus field strain by Next-Generation Sequencing. Infect Genet Evol 32: 313-321.