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Development and Validation of a Complete Workflow Solution for SIV Testing

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ABSTRACT

Swine influenza virus (SIV) is a highly contagious viral infection of pigs, resulting in significant economic losses in the swine industry and posing a significant threat to human health through zoonotic transmission. SIV subtypes are defined by the surface glycoproteins: hemagglutinin and neuraminidase, with H1N1, H3N2, and H1N2 representing the predominant subtypes in swine.

We have validated an SIV testing workflow consisting of high throughput nucleic acid purification, SIV detection, and SIV subtyping from porcine nasal swab samples. SIV can be detected using the USDA-licensed VetMAX™-Gold SIV Detection Kit, a single-tube one-step real-time RT-PCR kit for the rapid and accurate screening for influenza A. The assay targets three independent regions of the SIV genome to dramatically limit the number of false-negatives due to mutation of the viral genome. The VetMAX-Gold SIV Detection assay also incorporates multiple degenerate primers and probes designed to detect all known strains of SIV. It is multiplexed with an internal positive control (IPC) to monitor for nucleic acid recovery and PCR inhibition. Laboratories wishing to obtain more information about SIV-positive samples can utilize the VetMAX·Gold SIV Subtyping Kit to further characterize their samples and confirm positive results. The VetMAX-Gold SIV Subtyping Kit is a pair of single-well real-time RT-PCR assays to detect and differentiate the H1, H3, N1 and N2 alleles.

We validated the screening and workflow by testing >100 SIV-positive and >100 SIV-negative porcine nasal swab field samples and virus isolates originating from diverse geographic regions in the US with the screening and subtyping kits. The SIV status and subtype of each sample was confirmed prior to the start of the study with Virus Isolation (VI) and/or whole genome sequencing. Collaborator laboratories purified the viral nucleic acid using the MagMAX™-96 Viral RNA Isolation Kit (AM1836) and MagMAX Express-96 Magnetic Particle Processor. Extracted nucleic acid (8uL) was tested with the VetMAX-Gold SIV Detection Kit and VetMAXTM-Gold SIV Subtyping Kit on the AB 7500-Fast Real-Time PCR System.

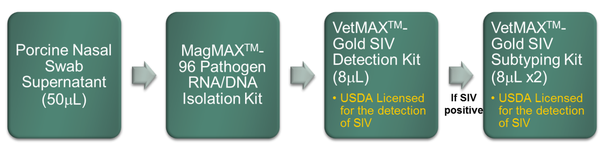
Results of validation testing were used to determine diagnostic sensitivity and specificity for each kit. Detection with the VetMAX-Gold SIV Detection Kit resulted in calculated diagnostic sensitivity and specificity values of 98.4% and 99.1%, respectively. The VetMAXTM-Gold SIV Subtyping Kit produced >97% sensitivity and specificity for identifying the SIV subtype from nasal swab samples. This study indicates that RNA isolated from diagnostic porcine nasal swab samples, tested with the VetMAX-Gold SIV Subtyping Kit in conjunction with the VetMAX-Gold SIV Detection Kit, provides an economical and rapid solution for SIV screening and subtype identification.

INTRODUCTION

Swine influenza virus (SIV) subtypes are defined by the surface glycoproteins: hemagglutinin and neuraminidase, with H1N1, H3N2, and H1N2 representing the predominant subtypes in swine.

We have validated an SIV subtyping workflow consisting of high throughput nucleic acid purification, SIV detection, and SIV subtyping from porcine nasal swab samples. The VetMAX™-Gold SIV Subtyping Kit is a pair of single-well real-time RT-PCR assays to detect and differentiate the H1, H3, N1 and N2 alleles. The H1 and H3 assays are multiplexed into a single reaction with the H3 probe labeled with FAM™ and the H1 probe labeled with VIC®. The N1 and N2 assays are multiplexed into a single reaction with the N2 probe labeled with FAM™ and the N1 probe labeled with VIC®. All fluorescent probes in the kit are modified with a 3' non-fluorescent quencher, Eclipse™ Q. When performed in conjunction with the VetMAX-Gold SIV Detection Kit to screen samples for SIV as well as monitor sample isolation and inhibition, the VetMAX™-Gold SIV Subtyping Kit provides a robust method for subtyping the predominant SIV subtypes in swine (Figure 1).

Figure 1. SIV Detection Workflow



MATERIALS AND METHODS

The sensitivity and specificity of the SIV subtyping workflow was evaluated with 169 SIV-positive and 150 SIV-negative porcine nasal swab field samples and virus isolates originating from diverse geographic regions in the US. The SIV status and subtype of each sample was confirmed prior to the start of the study with virus isolation (VI) as well as with whole-genome sequencing or qRT-PCR. The characterized positive samples processed in this study consisted of the H1N1, H3N2, H1N2, and H3N1 genotypes. Of the 169 characterized positive samples processed in this study 42% were of the H1N1 genotype, 47% were of the H3N2 genotype, 10% were of the H1N2 genotype, and 0.5% were of the H3N1 genotype. These values roughly correspond to the typical distribution of genotype prevalence described in the literature (1,2), with the majority of samples representing strains circulating within the swine population in the last five years (Table 1).

SIV Status	Subtype	SIV Screening #	SIV Subtyping #
SIV-positive	H1N1	71	76
	H3N2	80	17
	H1N2	17	26
	H3N1	1	2
SIV-negative	N/A	150	105

Table 1. VetMAX™-Gold SIV Detection Kit and VetMAX™-Gold SIV Subtyping Kit validation tested numerous field samples in separate studies. Samples were from diverse geographic origins (>10 unique US states) and were well characterized by either virus isolation or sequencing to determine true SIV status.

The collaborator laboratory purified the viral nucleic acid from 50µL porcine nasal swab supernatant using the MagMAX™-96 Pathogen RNA/DNA Kit (Life Tech #4462359) and MagMAX™ Express-96 Deep Well Magnetic Particle Processor. 20,000 copies of Xeno™ RNA were spiked into each nucleic acid isolation to serve as an extraction control and to monitor for the presence of PCR inhibitors. Two negative extraction controls (PBS) per extraction plate were processed through the testing workflow to monitor for contamination of the sample preparation reagents. 8µL of extracted RNA was tested with the VetMAX™-Gold SIV Subtyping Kit to classify the hemagglutinin and neuraminidase alleles. Samples were tested in the Applied Biosystems 7500 Fast Real-Time PCR System. The sensitivity, specificity, and positive/negative predictive values were calculated for the SIV subtyping workflow.

To test kit robustness across diverse testing conditions, SIV nucleic acid was purified using the MagMAX™-96 Viral RNA Isolation Kit (AM1836) and MagMAX™-Express Instruments. A panel of twenty (20) blinded samples (a mix of SIV positive and negative) were tested in duplicate by three different laboratories with two lots of kits on two different days (Figure 2).

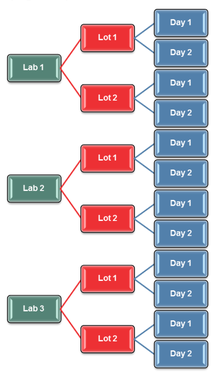


Figure 2. Repeatability and Reproducibility (R&R) Study Set-up. A panel of 20 blinded samples (mix of positive and negative of various subtypes) were tested in duplicate by three laboratories with two kit lots on two consecutive days.

RESULTS

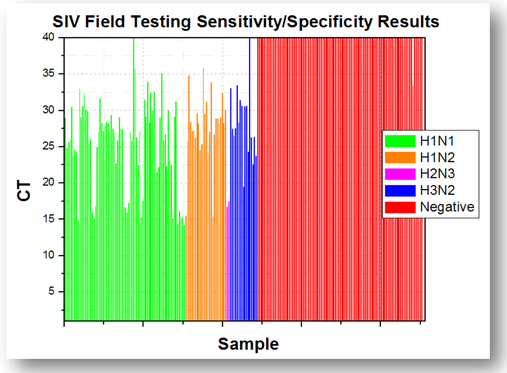


Figure 3: **VetMAX™-Gold SIV Detection Kit sensitivity and specificity results.** Results of field testing of 121 SIV-positive and 105 SIV-negative nasal swab samples of the major SIV subtypes resulted in 98.4% sensitivity and 99.1% specificity when compared to Virus Isolation. Discrepant positive samples were very low titer samples amplifying near the assay's limit of detection, which resulted in inconsistent detection upon re-testing.

Call	Sample #
Final Call: True Positive	166
Final Call: False Negative	3

Table 2. SIV-Positive Field Sample Results for the VetMAX-Gold SIV Subtyping Kit . The results of testing were a calculated diagnostic sensitivity of 98.2%. Discrepant negative samples were very low titer samples amplifying near the assay's limit of detection, which resulted in inconsistent or absent detection upon re-testing.

Call	Sample #
Final Call: True Negative	150
Final Call: False Positive	0

Table 3. SIV-Negative Field Sample Results for the VetMAX·Gold SIV Subtyping Kit . The results of testing were a calculated diagnostic sensitivity of 100%.

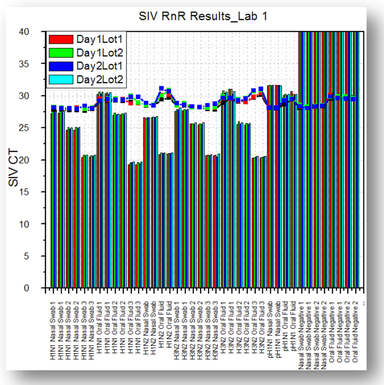


Figure 4. VetMAXTM-Gold SIV Detection Kit Precision. The figure shows representative results from one of the labs in the R&R study. Testing showed highly consistent detection of SIV across multiple lots, days, and replicates. There was 100% agreement for all test results across all laboratories, days, and kit lots.

SIV Subtype	True Positive Samples	Test Positive Result	Test Suspect Result	% Agreement
H1	240	240	0	100%
H3	144	144	0	100%
N1	144	145	2	99.3%
N2	240	241	3	99.6%

Table 4. VetMAX™-Gold SIV Subtyping Kit R&R Results for Positive Samples. There was >99% agreement in diagnostic calls across all testing conditions for positive samples.

SIV Subtype	True Negative Samples	Test Negative Result	% Agreement
H1	240	240	100%
H3	336	336	100%
N1	336	333	99.1%
N2	240	236	98.3%

Table 5. VetMAX™-Gold SIV Subtyping Kit R&R Results for Negative Samples. There was >98% agreement in diagnostic calls across all testing conditions for negative samples.

CONCLUSIONS

The VetMAX·Gold SIV Detection Kit and VetMAX-Gold SIV Subtyping Kit showed highly repeatable and reproducible results across multiple labs, kit lots, and testing days. The VetMAX·Gold SIV Detection Kit demonstrated diagnostic sensitivity and specificity was 98.4% and 99.1% respectively. The VetMAX·Gold SIV Subtyping Kit demonstrated diagnostic sensitivity and specificity of 98.2% and 100% respectively. Results of testing lead to successful USDA licensing of both kits for the detection of SIV from porcine nasal swabs.

ACKNOWLEDGEMENTS

Thank you to our collaborators for participating in this study:
Amy Vincent and Michelle Harland from USDA- ARS/NADC
Alfonso Clavijo and Megan Schroeder from Texas Veterinary Medical Diagnostic Laboratory
Richard D. Oberst and Lalitha Peddireddi KSU Veterinary Diagnostic Laboratory
Dr. Sabrina Swenson and Leo Koster from USDA-NVSL
Dr. Marie Culhane from the University of Minnesota Veterinary Diagnostic Lab
Dr. Sharon Stewart from Murphy Brown Laboratory

TRADEMARKS/LICENSING

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