

The Challenges Associated With the Development of a Multiplex-PCR Influenza Subtyping Panel

Stephanie L. Jeffrey, MS
stephanie.jeffrey@diatherix.com
Donna Hockman, MS
Leslie Malone, MS, MB (ASCP)^{CM}
Elena Grigorenko, PhD
Diatherix Laboratories, Huntsville, AL

Abstract

In 2014-2015 the United States experienced a High Path Avian Influenza (HPAI) outbreak. This outbreak, primarily H5N2 and H5N8 subtypes, affected 211 commercial flocks and 21 backyard flocks with an estimated loss of 49,700,000 birds. Simultaneously, Asia was dealing with three HPAI outbreaks that crossed species to human: H3N2 23,286 cases (1.5% mortality), H5N1 700+ cases (60% mortality), and H7N9 667 cases (34% mortality). The increasing number of outbreaks and associated virulence suggests that the trend of mutation from avian strains spreading to humans is increasing. The development of a multiplex PCR panel for the detection and subtyping of HPAI strains would provide a tool to assist in the containment and prevention of widespread influenza occurrences.

Introduction

In April 2009, Influenza A H1N1pdm09 overwhelmed health agencies across the US with a rapidly spreading outbreak. The large number of people affected by H1N1pdm09 caused the CDC to terminate incident reporting for all but hospitalizations and deaths confirmed to be H1N1pdm09.¹ In response, Diatherix Laboratories developed one of the first assays for H1N1pdm09, and was the first CLIA certified laboratory granted an Emergency Use Authorization (EUA) from the FDA for H1N1-09 during the 2009 pandemic.² The initial goal of this project was to develop a confirmatory influenza subtyping assay for HPAI in preparation for future outbreaks. Current HPAI testing is performed using ELISA or endpoint PCR for a single strain, with most assays targeting only the hemagglutinin protein, making them presumptive. TEM-PCRTM allows for multiple targets to be included in a single assay with greater sensitivity and specificity. Additionally, a panel inclusive of the current HPAI strains would allow one test to be run to identify the HPAI strain from a single sample. There were several challenges encountered during the development of the panel that will need to be overcome in order to complete the development of a TEM-PCRTM HPAI subtyping panel.

Materials & Methods

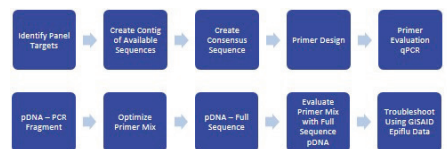


Figure 1. Project Workflow. HPAI targets are determined through identification of emerging influenza strains utilizing information available from WHO, NIH, CDC, and GISAID. Primers are designed following guidelines for TEM-PCRTM and consensi created from available sequences. Primers are evaluated for functionality using qPCR and optimal candidates are used to create a primer mix. Plasmid DNA designed from the PCR fragments is used for the development and optimization of the primer mix, viral RNA is used where available.



Figure 2. TEM-PCRTM Scheme. Low concentration nested gene-specific primers are (Fo – forward out; Fi – forward in; Ri – reverse in; and Ro – reverse out) designed to enrich the targets during the initial PCR cycles. Later in the procedure, a pair of universal SuperPrimers (Fs and Rs) is used to amplify all targets. The Rs primer is labeled with biotin for subsequent detection.

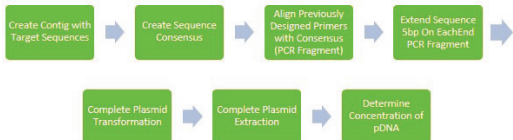


Figure 3. Plasmid DNA Design. Gene-specific primers are aligned to a consensus of all available sequences for the desired target creating a PCR fragment. An additional 50p is added to both the 5' and 3' ends of the PCR fragment, the resulting sequence is the plasmid DNA sequence. The plasmid is then transformed and extracted and the subsequent pDNA evaluated for concentration via Nanodrop Spectrophotometer (ThermoFisher), and tested for functionality via qPCR.

Materials & Methods (continued)

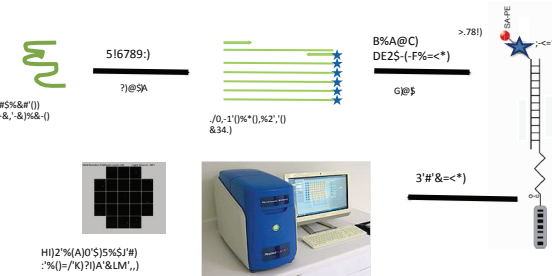


Figure 4. Detection Using Barcoded Magnetic Beads. Extracted nucleic acids are used in TEM-PCRTM with the resulting PCR product being used for detection. Barcoded magnetic "beads" that have been coupled to detection oligos (De) bind to the appropriate biotin labeled PCR products enabling the reader to both detect and identify what is present in the sample. (Applied BioCode, Santa Fe, CA)

Table 1. Influenza Panel Targets. The HPAI targets selected reflect CDC recommendations and current and emerging strains.

Target	Consensus	Target
456532789	456532789	456532789
4<6<	4<6<	4<6<
4>6<	4>6<	4>6<
4<6<	4<6<	4<6<
4>6<	4>6<	4>6<
4E69<	4E69<	4E69<
4E69<	4E69<	4E69<

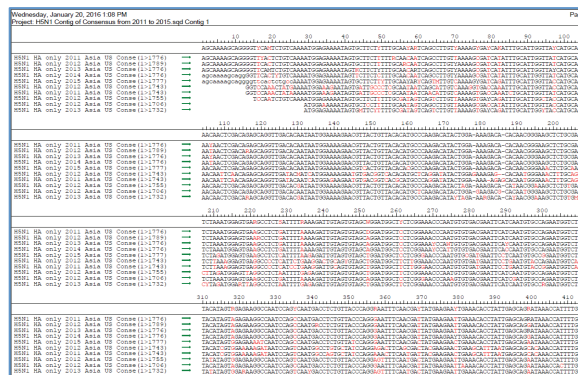


Figure 5. Construction of consensi using H5N1. All available strains for each year were compiled to create a consensus for the specific year. The consensus were then compiled into a single contig to create a consensus of consensus or consensi.

Results

Target	Consensus	Target
456532789	456532789	456532789
4<6<	4<6<	4<6<
4>6<	4>6<	4>6<
4<6<	4<6<	4<6<
4>6<	4>6<	4>6<
4E69<	4E69<	4E69<
4E69<	4E69<	4E69<

Figure 6. TEM-PCRTM Primer Mix Development and Testing Using pDNA at 1e3 and 1e2 copies/μL. All targets were detected at both 1e3 and 1e2 with MFIs easily discernable from background.

Results (continued)

Target	Consensus	Target
456532789	456532789	456532789
4<6<	4<6<	4<6<
4>6<	4>6<	4>6<
4<6<	4<6<	4<6<
4>6<	4>6<	4>6<
4E69<	4E69<	4E69<
4E69<	4E69<	4E69<

Figure 7. TEM-PCRTM Primer Mix Development and Testing Using RNA. Primer mixes were tested with RNA provided by the World Health Organization Pandemic Influenza Preparedness Framework. It was not possible to test H5N8 or H7N9 US due to the lack of virus and/or nucleic acid. Full sequence synthetic nucleic acid (pDNA) were used to further evaluate all targets.

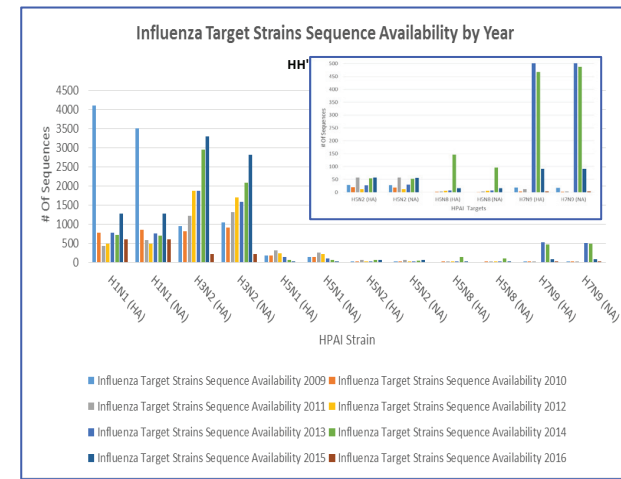


Figure 8. A comparison of the number of sequences deposited in public databases for each of the HPAI targets for years 2009 through 2016. The availability of sequences deposited in public databases varies by HPAI strain. More common strains have a larger number of sequences. Fewer sequences are available for more current strains/years. **The insert is a detailed view of HPAI Strains with fewer sequences available.

Table 2. The number of sequences deposited in public databases for each of the HPAI targets for years 2009 through 2016.

Target	Consensus	Target
456532789	456532789	456532789
4<6<	4<6<	4<6<
4>6<	4>6<	4>6<
4<6<	4<6<	4<6<
4>6<	4>6<	4>6<
4E69<	4E69<	4E69<
4E69<	4E69<	4E69<

Results (continued)

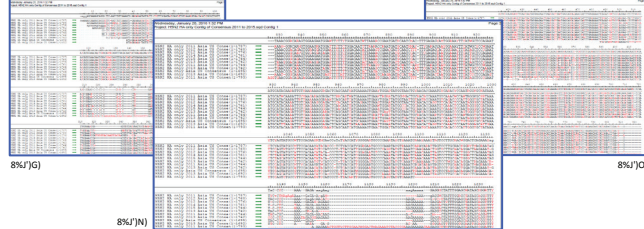


Figure 9. Antigenic Drift and Mutation of H5N2 Hemagglutinin. The above consensi of the H5 hemagglutinin component of Influenza A H5N2 is an example of the SNPs and mutations that occurred from 2009 to 2016. Additionally, there are also deletions and insertions that occur over time and as the influenza virus moves from one continent to another.

Table 3. The Number of Sequences Used to Complete the H5N2 HA Consensi. The GISAID Epiflu database was used to compile all available H5N2 HA sequences for years 2011 to 2015. DNASTar Lasergene software was used to create a contig for each year and a consensi for all years.

Contig	2011	2012	2013	2014	2015
Contig 1	25	27	7	36	3
Contig 2	3	12	19	4	36
Contig 3	35	2			

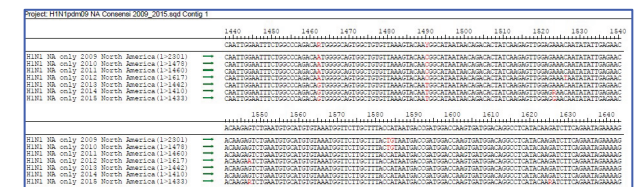


Figure 10. Antigenic Drift and Mutation of H1N1pdm09 NA. H1N1pdm09 also shows a number of mutations over the last 7 years. This example shows the variability of a region within the H1N1pdm09 neuraminidase.

Discussion

The frequency in HPAI mutations indicates an increased possibility of transmission of the avian influenza strains to humans. TEM-PCRTM as a molecular diagnostic tool can be used for subtyping of HPAI. Thus, the molecular test for detection of continual and well-documented antigenic drift among HPAI may be more suitable as a Laboratory Developed Test allowing for the addition or modification of primer sequences in order to adapt to seasonal changes in circulating influenza virus types.

Conclusions

There are several challenges associated with the development of accurate and inclusive molecular tests for HPAI subtyping:

- Continual antigenic drift of influenza viruses
- Delay in current sequence information in publicly available databases
- Lack of commercially available strains (virus or RNA)
- Biosafety level 3 requirements for panel development
- Frequent review required to address continuous antigenic drift
- Panel is more suitable to an LDT than an FDA approved test

Acknowledgements

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• BPL-Inactivated Influenza A Virus, A/Georgia/New Jersey/1355/1998(H5N2)-PR8-IBCC-4
• Influenza A Virus, A/Vietnam/1199/2004(H5N1)
• Influenza A Virus, A/California/07/2009 (H1N1)pdm09 Antiviral Resistance (AVR) - Reference Virus M2: S31N NA: wild type (wt)
• Genomic RNA from Influenza A Virus, A/Vietnam/1203/2004(H5N1)-PR8-IBCC-RG/GLP

References

¹Centers for Disease Control. Flu-related hospitalizations and deaths in the United States from April 2009 – January 30, 2010. Available at http://www.cdc.gov/h1n1flu/hosp_deaths_ahdr.htm
²Koon K, Sanders CM, Green J, Malone L, White H, Delaney Z, Miller R, Lu S, Han J. Co-detection of Pandemic (H1N1) 2009 Virus and Other Respiratory Pathogens. Emerging Infectious Diseases www.cdc.gov/eid Vol 16, No. 12, December 2010.