Journal of Virological Methods 167 (2010) 125–131

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

# Validation of a method for preparing influenza H5N1 simulated samples

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*Article history:*
Received 24 November 2009
Received in revised form 12 March 2010
Accepted 22 March 2010
Available online 1 April 2010

*Keywords:*
Novel influenza A virus
In vitro diagnostics
Simulated samples
Avian influenza H5N1

A B S T R A C T

Avian influenza virus type A subtype H5N1 and potentially other novel influenza A viruses continue to pose a concern with mutation into a form easily transmitted between humans. The ability to rapidly detect and characterize influenza viruses, and distinguish seasonal and novel influenza A viruses such as H5N1, remains important to minimize morbidity and mortality in humans. As with other rare and emerging viral pathogens, clinical specimens from persons with H5N1 infections are extremely rare. Consequently, development of standardized methods and accepted criteria are necessary for both ensuring the validity of available diagnostic methods and for assessing the potential of new diagnostic tests that can detect and differentiate H5N1 and other novel influenza A viruses. Additionally, genotypic and antigenic evolution of H5N1 poses a challenge with maintaining updated reference virus strains. In this report, a method for preparing simulated samples using defined procedures and carefully selected H5N1 virus strains is described, and the reliability for using these samples in an evaluation protocol with a laboratory test for differentiating H5N1 virus from other influenza A viruses is evaluated.



## 1. Introduction

Since 1997, influenza A/H5N1 viruses have evolved into multiple genetic clades [World Health Organization (WHO) Global Influenza Program Surveillance Network, 2005] that have infected humans in 15 countries [(WHO) Global Alert and Response], 4 March 2010. Though human H5N1 cases are rare, the ongoing exposure of humans in countries with endemic disease in poultry and wild birds poses a continued threat to human health, with concern that mutations could enable transmission between humans (Peiris et al., 2007).

In 2007, a WHO expert consultation meeting addressed considerations and gaps related to H5N1 diagnostic capacity worldwide, with an observation that "re-constructed spiked clinical samples" could be important for evaluating new tests especially when patient clinical specimens are a scarce resource and likely to be compromised during storage. The meeting summary further recommended implementation of procedures for evaluating new tests using simulated samples in lieu of clinical specimens from infected patients, and acceptance of data from such evaluations by regulatory authorities (Chung et al., 2007).

Standardized methods and approaches to construct and assemble simulated samples have not been published or recognized. In the United States, an interagency government working group for pandemic influenza diagnostics comprised of representatives from the Centers for Disease Control and Prevention (CDC), Department of Defense, Food and Drug Administration, Department of Health and Human Services, and National Institute of Allergy and Infectious Diseases, initiated two efforts: (a) to validate and document a method for preparing simulated samples using established procedures and (b) to demonstrate feasibility for using these samples to assess diagnostic test systems for detecting and differentiating H5N1 virus. The working group recommended preparing samples in a uniform human respiratory epithelial cell background at a level typical for human respiratory specimens (approximately $10^5$ cells/mL) (Class et al., 2007) with low-passage viruses representing the four H5N1 clades that have infected humans since 2004, with virus levels roughly correlating with the range reported in specimens from infected persons (approximately 0.5–6 log 10 $TCID_{50}$/mL) (Calfee et al., 1999; Döller et al., 1992; Kaiser et al., 1999; Ng et al., 2005; Ryan-Poirier et al., 1992; Snyder et al., 1986). Additionally, the process for preparing samples should be reproducible within the laboratory, and prepared samples should be characterized fully by viral titer, cellular levels, and for virus identity. Possible alternatives for consideration as background cellular matrix include residual pooled human or animal respiratory spec-

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doi:10.1016/j.jviromet.2010.03.022

imens and laboratory cell lines including human A549 cells (type 2 pneumocytes), a cell line permissive to infection by H5N1 viruses.

Evaluating test systems with H5N1 viruses representing genetic clades associated with human infections is confounded not only by the scarcity of clinical specimens but also the availability of isolated, publicly documented viruses. Biosafety certifications, regulatory requirements, and in some cases international sharing and ownership issues restrict access and may limit the number of strains that are available to test developers and researchers. This last issue in particular may necessitate turning to genetic precursors representing viruses that have infected humans. Use of laboratory-derived genetic reassortants with limited hemagglutinin (HA) and neuraminidase (NA) genes placed into an attenuated background virus addresses some of the safety issues, however their availability and use is also encumbered by regulatory and ownership issues as well as the significant time and effort associated with the demonstration of attenuated pathogenicity. Additionally, these reassortants cannot be used exclusively for assessing test performance as many technical designs include detection targets other than HA.

The objectives of this work were to prepare simulated H5N1-infected samples consisting of A549 cells spiked with varying amounts of wild-type H5N1 influenza viruses, and to assess feasibility for use as alternative samples to generate supportive data for evaluating H5N1 laboratory diagnostic test systems. Seasonal influenza viruses were prepared identically and used in a similar testing protocol as controls, and for comparison with reported performance. To validate the methods for preparing simulated samples containing H5N1 influenza viruses, procedures were documented and sample integrity validated by cell culture infectivity levels, genotypic identity, and reproducibility. To demonstrate feasibility for a protocol to assess diagnostic test systems, an FDA-cleared laboratory test system evaluated previously with clinical specimens for the detection and differentiation of H5N1 influenza viruses and seasonal viruses was used.

## 2. Materials and methods

### 2.1. Viruses

Eight ($n = 8$) different wild-type H5N1 viruses with representatives of recent H5N1 virus clades that have infected humans in different geographical regions were used: six wild-type viruses from clades 1.0, 2.1, 2.2, and 2.3, and two reverse genetics reassortant viruses (Table 1). Six ($n = 6$) recent seasonal influenza viruses (Table 1) were also used: recent A/H1N1, A/H3N2 along with two influenza B viruses. All viruses (Table 1) were provided by the CDC as low-passage stocks and stored at −80 °C for up to one year, or in the vapor phase of a liquid nitrogen cryopreservation tank over longer periods. Permits necessary for the importation and work with highly pathogenic avian influenza H5N1 viruses were acquired in accordance with federal, state, and local laws. When necessary for a particular virus, a Materials Transfer Agreement (MTA) was obtained and signed by both parties, and all terms of the MTA were strictly followed.

### 2.2. Bio-containment facilities

A United States Department of Agriculture (USDA)-approved biosafety level 3-enhanced (BSL3+) containment facility was used for in vitro experiments and the cultivation of H5N1 viruses. Work with seasonal influenza viruses was conducted in BSL2 facilities, and work with reverse genetics reassortant H5N1 viruses (Table 1) followed BSL2-enhanced requirements in a USDA-approved biosafety level 2-enhanced (BSL2+) containment facility.

**Table 1**
List of H5N1 and seasonal influenza viruses.

| Virus category | Type | Subtype or clade | Strain |
|---|---|---|---|
| Seasonal influenza viruses | A | H1N1 | A/New Caledonia/20/1999 |
| | | H1N1 | A/Hawaii/15/2001 |
| | | H3N2 | A/New York/55/2004 |
| | | H3N2 | A/Wisconsin/67/2005 |
| | B | (NA) | B/Florida/07/2004 (Yanagata/16/88-like) |
| | | | B/Ohio/01/2005 (Victoria/2/87-like) |
| H5N1 reassortant viruses | A | Clade 1.0 | A/Vietnam/1203/2004xPR8 |
| | | Clade 2.3 | A/Anhui/01/2005xPR8 RG-5 |
| Wild-type H5N1 Viruses | A | Clade 1.0 | A/Chicken/Yunnan/1251/03 |
| | | Clade 2.1 | A/Duck/Hunan/795/02 |
| | | Clade 2.2 | A/Chicken/Korea/IS/06 |
| | | Clade 2.3 | A/scaly breasted munia/Hong Kong/45/2007 |
| | | | A/Japanese white-eye/Hong Kong/1038/2006 |
| | | | A/common magpie/Hong Kong/645/2006 |

NA: not applicable.

### 2.3. Cultivation of cell lines

Madin–Darby canine kidney (MDCK) cells were obtained at passage 73 were used until passage 77 (Diagnostic Hybrids, Inc., Athens, OH). The cells were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-Alanyl-L-Glutamine (GlutaMAX™, Invitrogen Corp., Carlsbad, CA), bicarbonate, antibiotics (PSN; penicillin/streptomycin/neomycin) (Invitrogen Corp.), and gamma-irradiated fetal bovine serum (HyClone, subsidiary of Thermo Fisher Scientific, Inc., Pittsburgh, PA), at 37 °C and 5% $CO_2$. Cells were observed daily to ensure normal morphology and assess confluency.

The hypotriploid human cell line A549 [ATCC, catalogue CCL-185, Manassas, VA] was obtained at passage 93 and used until passage 97. The A549 cells were cultured as monolayers at 37 °C and 5% $CO_2$ in F12K medium (Invitrogen Corp.) supplemented with 10% gamma-irradiated FBS and PSN. The cells were grown and maintained between $6 \times 10^3$ and $6 \times 10^4$ cells/cm$^2$ with subcultivation and medium renewal two to three times per week. Cells were observed daily to ensure normal morphology and to approximate confluency. Cells were harvested for use in sample preparation when ~90% confluent.

Both MDCK and A549 cells were negative for mycoplasma DNA by testing with a PCR-based Mycoplasma Detection kit (Takara Bio, USA, Thermo Fisher).

### 2.4. Embryonating (embryonated) chicken eggs

Specific pathogen free (SPF) *Chicken anemia virus* (CAV)-free embryonating chicken eggs (ECE) [Charles River Laboratories, Wilmington, MA] were used. Prior to inoculation with virus, the ECE were incubated with automatic rotation at 39 °C, and candled to cull dead or otherwise defective eggs as recommended in the World Health Organization Manual on Animal Influenza Diagnosis and Surveillance (2002).

### 2.5. Virus propagation in MDCK cells and ECE

H5N1 viruses were propagated at 37 °C in the chorioallantoic cavity (CAC) of 9–11-day-old ECE following standard methods (Szretter et al., 2006). Briefly, for each ECE, the embryo and CAC