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A Highly Sensitive Europium Nanoparticle-Based Immunoassay for Detection of Influenza A/B Virus Antigen in Clinical Specimens

Panhe Zhang,[a] Sai Vikram Vemula,[a] Jiangqin Zhao,[a] Bingchen Du,[a] Haleyurgirisetty Mohan,[a] Jikun Liu,[a] Haja Sittana El Mubarak,[b] Marie L. Landry,[c] Indira Hewlett[a]

Laboratory of Molecular Virology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA[a]; Center for Devices and Radiological Health, Food and Drug Administration, Silver Spring, Maryland, USA[b]; Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut, USA[c]

We report the development of a novel europium nanoparticle-based immunoassay (ENIA) for rapid detection of influenza A and influenza B viruses. The ENIA demonstrated sensitivities of 90.7% (147/162) for influenza A viruses and 81.80% (9/11) for influenza B viruses compared to those for an in-house reverse transcription (RT)-PCR assay in testing of influenza-positive clinical samples.

Influenza continues to remain a major public health problem worldwide (1–3). Based on World Health Organization estimates, influenza viruses infect 5 to 15% of the global population annually, resulting in 250,000 to 500,000 deaths (4–6). In the United States alone, influenza viruses infect >50 million people annually, resulting in >200,000 hospitalizations and 30,000 to 50,000 deaths (3, 7). New diagnostic approaches that can rapidly and accurately detect newly emerging viral variants are required for early initiation of antiviral therapy and prophylaxis to control infection during seasonal and pandemic outbreaks. Here, we report the development of a novel europium nanoparticle-based immunoassay (ENIA) for rapid and accurate detection of influenza viruses in humans.

To identify nucleoprotein (NP) antibodies with broad reactivity, we screened 8 influenza A virus NP antibodies (clone numbers 5D8 [Abbiotec, LLC], lnA108 [MyBioSource], 9G8 [Santa Cruz Biotechnology, Inc.], 2F4 and DD9 [BEI Resources], C43 [Abcam], and anti-influenza A NP polyclonal antibodies [ProSci Inc.]) and 9 influenza B virus NP antibodies (clone numbers 1B6/B3 [Abbiotec, LLC], 9D6 [TaKaRa Bio], 2/3 and 3E9 [Santa Cruz Biotechnology, Inc.], 8L390 [MyBioSource], and anti-influenza B NP polyclonal antibodies) in a direct enzyme-linked immunosorbent assay (ELISA) (8, 9). Monoclonal antibodies lnA108 and 5D8 demonstrated broad reactivity against diverse influenza A viruses from subtypes H1, H2, H3, H5, H7, and H9, respectively, while monoclonal antibodies 1B6/B3 and 8L390 demonstrated reactivity against different influenza B viruses tested (data not shown).

Based on the performance in the direct ELISA, antibodies lnA108 and 5D8 (for influenza A virus detection) and 1B6/B3 and 8L390 (for influenza B virus detection) were selected for FluA and FluB ENIAs. ENIAs were performed as previously described (10). Briefly, microtiter plates (Nunc, USA) were coated with anti-influenza A and -influenza B virus NP-specific capture antibodies lnA108 and 8L390 (2 μg/ml) and blocked using StartingBlock T20 buffer (Thermo Scientific, USA). Influenza viruses inactivated in 1% Triton X-100 for 5 min at 37°C were added and incubated for 30 min at 37°C. Biotinylated 5D8 and 1B6/B3 antibodies (0.5 μg/ml) and streptavidin-conjugated europium nanoparticles were added and incubated at 37°C for 30 min. The entire procedure could be completed in <1 h. Optical density measurements were taken using a Victor 3V multilabel plate reader (PerkinElmer, USA).

The specificity of the FluA ENIA was evaluated using 25 influenza A virus strains from subtypes H1, H2, H3, H5, H7, and H9; 10 influenza B virus strains; and 7 human respiratory viruses, respiratory syncytial virus A (RSV-A), RSV-B, herpes simplex virus 1 (HSV-1), HSV-2, adenovirus-1, corona virus, and rhinovirus (viruses were procured from ZeptoMetrix Corporation, and virus concentrations ranged from 10^4 to 10^8 50% tissue culture infective dose [$TCID_{50}$]/ml). The FluA ENIA detected all of the influenza A virus strains tested, with no cross-reactivity observed with influenza B viruses or any of the respiratory viruses. Similarly, The FluB ENIA detected the 10 influenza B virus strains with no cross-reactivity observed with influenza A virus strains and the respiratory viruses.

The analytical sensitivities of the ENIAs were evaluated by testing serially diluted egg or tissue culture-grown pretitrated stocks of reference strains of influenza viruses A/Brisbane/59/07 (H1N1), A/California/07/09 (2009 pandemic H1N1 [pdH1N1]), A/Colorado/14/12 (pdH1N1), A/Victoria/361/11 (H3N2), and B/Brisbane/60/08 (type B). The highest dilution of virus where 19/20 (95%) replicates tested positive was defined as the limit of detection. The ENIAs demonstrated an analytical sensitivity of $1.00 \times 10^{2.4}$ 50% egg infective dose (EID_{50})/ml for A/Brisbane/59/07 (H1N1), $1.00 \times 10^{2.2}$ EID_{50}/ml for A/California/07/09 (pdH1N1), $1.00 \times 10^{1.0}$ $TCID_{50}$/ml for A/Colorado/14/12 (pdH1N1), $1.00 \times 10^{2.0}$ EID_{50}/ml for A/Victoria/361/11 (H3N2), and $1.00 \times 10^{3.0}$ EID_{50}/ml for B/Brisbane/60/08 (type B) (Table 1).

The performance of the ENIAs was compared to that of a commercial photometric influenza A and B NCP antigen capture

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Address correspondence to Sai Vikram Vemula, saivikram.vemula@fda.hhs.gov, or Indira Hewlett, indira.hewlett@fda.hhs.gov.

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TABLE 1 Analytical sensitivity of the ENIA for influenza A and B viruses

Influenza viral strain	Viral type	Limit of detection[a]
A/Brisbane/59/07	A/H1N1	$1.00 \times 10^{2.4}$ EID_{50}/ml
A/California/07/09	A/pdH1N1[b]	$1.00 \times 10^{2.2}$ EID_{50}/ml
A/Colorado/14/2012	A/pdH1N1	$1.00 \times 10^{1.0}$ $TCID_{50}$/ml
A/Victoria/361/2011	A/H3N2	$1.00 \times 10^{2.0}$ EID_{50}/ml
B/Brisbane/60/2008	Type B	$1.00 \times 10^{3.0}$ EID_{50}/ml

[a] EID_{50}, 50% egg infectious dose; $TCID_{50}$, 50% tissue culture infectious dose.
[b] pdH1N1, 2009 H1N1 pandemic virus.

ELISA (Virusys Corporation, USA). The photometric ELISA was performed according to the manufacturer's instructions using serial 2-fold dilutions of Triton X-100-inactivated influenza viruses. The cutoff values for both the ELISA and ENIAs were set at a mean of $+3 \times$ standard deviations of the negative control. The ENIAs showed from 4- to 128-fold higher sensitivity than the photometric ELISA for the various strains of influenza A and influenza B viruses tested (Table 2).

Finally, the clinical performance of the ENIAs was evaluated using a total of 193 nasopharyngeal swab specimens submitted during the 2012-2013 flu season to the Clinical Virology Laboratory, Yale New Haven Hospital, New Haven, CT (institutional review board [IRB] approval RIHSC:13-048B). Patient ages ranged from 20 months to 97 years. The initial detection and subtyping of the clinical samples were done using a direct fluorescent-antibody assay (DFA) (11) and/or a real-time TaqMan PCR assay (12, 13) at Yale New Haven Hospital. Of the 193 samples tested by either the DFA or the real-time TaqMan PCR assay, 162 (83%) were positive for influenza A virus, 11 (5%) were positive for influenza B virus, and 20 (10%) were negative for both influenza A and B viruses. Influenza A virus-positive clinical samples included pandemic 2009 H1N1 and seasonal H3N2 viruses.

Results obtained using the ENIAs were compared to those for an in-house RT-PCR assay (14, 15). The in-house RT-PCR assay was performed as follows. Briefly, viral RNA was extracted from 140 μl of clinical samples using a QIAamp viral RNA minikit (Qiagen, USA) and eluted in 30 μl of water. The viral RNA was then reverse transcribed into cDNA using a SuperScript III first-strand synthesis kit (Invitrogen, USA), and the cDNA was used to amplify the eight genomic segments of influenza A viruses using a set of universal primers: forward, 5′-ACGACGGGCGACAAGCA AAAGCAGG-3′; reverse, 5′-ACGACGGGCGACAAGTAGAAA CAAGG-3′. For influenza B virus detection, the matrix gene segment was amplified using the following primers: forward, 5′-TC GCTGTTTGGAGAC-3′; reverse, 5′-TTTATTTGCTGACATTG ATTAC-3′. The amplification protocol consisted of 1 cycle of 5 min at 94°C; 35 cycles of 30 s at 94°C, 40 s at 50°C, and 2.4 min at 68°C; and 1 cycle of 7 min at 68°C. The amplification products were visualized using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA) and confirmed by sequencing on an Illumina MiSeq next-generation sequencing platform. The in-house RT-PCR assay correctly detected all 173 (100%) influenza-positive samples tested, yielding a specificity of 100%. No cross-reactivity was observed with influenza-negative clinical samples.

The ENIAs demonstrated sensitivities of 90.7% (147/162; 95% confidence interval [CI], 86 to 95%) for influenza A viruses and 81.80% (9/11; 95% CI, 61 to 100%) for influenza B viruses. The specificities for FluA and FluB ENIAs against influenza A and influenza B viruses, respectively, were 100%. Twenty clinical specimens that previously tested negative for influenza A and B viruses showed no reactivity with either FluA or FluB ENIAs. Overall, these results highlight the potential of the ENIA for diagnosis of influenza infections in humans. This approach is cost-effective and easily adaptable to point-of-care formats to facilitate clinical testing and diagnosis in both seasonal and pandemic situations. The improved sensitivity of the influenza antigen ENIA may provide an added benefit for influenza virus testing in clinical settings, given the lower analytical sensitivity of the current licensed antigen-based rapid diagnostic tests (RDTs) (16–18). A meta-analysis of 159 studies involving 26 commercial RDTs reported a pooled assay sensitivity of 62.3% compared to a RT-PCR approach for diagnosis of influenza virus infections (19). Moreover, during the 2009 H1N1 pandemic, published studies using RDTs demonstrated variable sensitivities, ranging from 10% to 70% (20, 21).

TABLE 2 Sensitivity of the ENIA in comparison with that of a photometric ELISA[a]

Influenza virus strain	Virus type	Dilution in: Photometric ELISA	Dilution in: ENIA	Fold change compared to photometric ELISA
A/Brisbane/59/07	A/H1N1	1:2,048	1:32,768	16
A/Solomon Island/03/06	A/H1N1	1:2,048	1:16,384	8
A/swine/Canada/6294/09	A/H1N1	1:512	1:16,384	32
A/Japan/305/57	A/H2N2	1:1,024	1:16,384	16
A/Panama/2007/99	A/H3N2	1:2,048	1:32,768	16
A/Minnesota/10/12	A/H3N2	1:256	1:32,768	128
A/Vietnam/1203/04	A/H5N1	1:2,048	1:32,768	16
A/turkey/Virginia/4529/02	A/H7N2	1:4,096	1:16,384	16
A/ruddy turnstone/NJ/65/85 (H7N3)	A/H7N3	1:4,096	1:16,384	8
A/chicken/Hong Kong/G9/97	A/H9N2	1:4,096	1:16,384	16
B/Victoria/304/06	Type B	1:512	1:2,048	4
B/Panama/45/90	Type B	1:128	1:1,024	8
B/Pennsylvania/7/07	Type B	1:512	1:2,048	4

[a] The sensitivities of the ENIA tests were compared to those of a photometric ELISA, as described in the text.

The ENIA was previously evaluated for diagnosis of HIV-1 and anthrax and demonstrated higher sensitivity than the other antigen-based approaches. The ENIA for HIV-1 demonstrated up to 150-fold higher sensitivity than a colorimetric ELISA (10). Similarly, an ENIA for anthrax toxin demonstrated up to 100-fold higher sensitivity than an ELISA (22). The higher sensitivity of the ENIA can be attributed to the high content of europium in each nanoparticle along with the unique properties of lanthanide chelates to retain the fluorescence signal for long periods of time. Overall, the ENIA-based diagnostic approach holds promise for use in a point-of-care setting for rapid and accurate diagnosis of influenza A and influenza B infections in humans.

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