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Clinical Accuracy of a PLEX-ID Flu Device for Simultaneous Detection and Identification of Influenza Viruses A and B

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Respiratory tract infections caused by influenza A and B viruses often present nonspecifically, and a rapid, high-throughput laboratory technique that can identify influenza viruses is clinically and epidemiologically desirable. The PLEX-ID Flu assay (Abbott Molecular Inc., Des Plaines, IL) incorporates multilocus PCR and electrospray ionization-mass spectrometry to detect and differentiate influenza A 2009 H1N1 (H1N1-p), seasonal H1N1 (H1N1-s), influenza A H3N2, and influenza B viruses in nasopharyngeal swab (NPS) specimens. The clinical performance characteristics of the PLEX-ID Flu assay in symptomatic patients were determined in this multicenter trial. A total of 2,617 prospectively and retrospectively collected NPS specimens from patients with influenza-like illness between February 2008 and 28 May 2010 were eligible for inclusion in the study. Each specimen was tested in parallel by the PLEX-ID Flu assay and by the Prodesse ProFLU+ assay (Prodesse Inc., Madison, WI), to detect influenza A and B viruses. Specimens testing positive for influenza A virus by ProFLU+ were subtyped as H1N1-p, H1N1-s, or H3N2 by using the ProFAST+ assay (Gen-Probe Prodesse Inc.). The reproducibility of the PLEX-ID Flu assay ranged from 98.3 to 100.0%, as determined by testing a nine-specimen panel at three clinical sites on each of 5 days. Positive percent agreements (PPAs) and negative percent agreements (NPAs) of the PLEX-ID Flu assay were 94.5% and 99.0% for influenza A virus and 96.0% and 99.9% for influenza B virus, respectively. For the influenza A virus subtyping characterization, the PLEX-ID Flu assay had PPAs and NPAs of 98.3% and 97.5% for H1N1-p, 88.6% and 100.0% for H1N1-s, and 98.0% and 99.9% for H3N2, respectively. The overall agreements between the PLEX-ID and Prodesse ProFLU+/ProFAST+ assays were 97.1 to 100.0%. Bidirectional Sanger sequencing analysis revealed that 87.5% of 96 discrepant results between the PLEX-ID Flu and ProFLU+/ProFAST+ assays were found upon influenza A virus detection and H1N1-p subtyping. The PLEX-ID Flu assay demonstrated a high level of accuracy for the simultaneous detection and identification of influenza A and B viruses in patient specimens, providing a new laboratory tool for the rapid diagnosis and management of influenza A and B virus infections.

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Influenza (Flu) is a contagious respiratory illness caused by influenza viruses that are associated with significant morbidity and mortality. These infections are a major economic burden in terms of health care costs and lost income and productivity of workers (1). Antiviral treatment is available and effective for influenza if the disease is identified early in the course of illness (2–4). Treatment within the first 4 days of illness can have a positive impact on viral clearance and the disease course (5, 6); therefore, rapid and accurate laboratory diagnosis is particularly important in both the inpatient and outpatient settings. In addition, knowledge of the etiological agent of these infections can result in significant improvements in patient management by permitting the judicious use of antiviral agents in an era where antiviral resistance is continuing to increase.

The introduction of highly sensitive and rapid molecular assays for respiratory virus detection has prompted many laboratories to discontinue culture or rapid antigen-based techniques for influenza virus testing (7–10). Previous studies have shown the superior sensitivity of molecular diagnostic assays compared to other assays for the detection of respiratory viruses (11–14). During the 2009 H1N1 influenza A virus (H1N1-p) pandemic, the subtyping of influenza virus strains became important for monitoring the spread of the outbreak and managing high-risk patients. The emergence of this pandemic strain prompted several manufacturers to develop laboratory test systems to detect and subtype influenza viruses. The Food and Drug Administration (FDA) issued emergency use authorizations for several nucleic acid amplification-based assays for the diagnosis of H1N1-p infection in patients with signs and symptoms of respiratory infections and for differentiating seasonal H1N1 (H1N1-s), H3N2, and Flu B viruses (http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm). Some of these devices have been evaluated in clinical trials and have obtained regular FDA clearance (15–19).

The PLEX-ID Flu assay (Abbott Molecular Inc., Des Plaines, IL) is a qualitative nucleic acid *in vitro* diagnostic test intended for the detection and identification of H1N1-p, H1N1-s, H3N2, and Flu B viruses in nasopharyngeal swab (NPS) specimens from symptomatic patients. The PLEX-ID Flu assay is intended for use with the PLEX-ID system as an aid in the diagnosis of influenza virus infection in conjunction with clinical and epidemiological information. The assay incorporates broad-range multilocus re-

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verse transcription-PCR (RT-PCR) and electrospray ionization-mass spectrometry (ESI-MS) and has the potential to rapidly detect and semiquantify different pathogens simultaneously (20). To date, RT-PCR/EMI-MS has been used to detect and characterize a variety of bacterial and viral pathogens causing respiratory tract infections (21–27).

For this investigational study, the objectives were to validate the performance of the PLEX-ID Flu assay on the investigational PLEX-ID system for the qualitative determination of H1N1-p and for the qualitative detection and differentiation of other influenza viruses (H1N1-s, H3N2, and Flu B viruses) in NPS specimens from subjects presenting with influenza-like illness during the 2009-2010 Flu season. A summary of these clinical trial data served as the basis for the PLEX-ID Flu assay premarket submissions to regulatory agencies.

(This study was presented in part at the 28th Annual Clinical Virology Symposium, Daytona Beach, FL, 22 to 25 April 2012.)

MATERIALS AND METHODS

Study design. A multicenter trial was designed to determine the clinical performance of the PLEX-ID Flu assay for the qualitative detection and subtyping of H1N1-p, H1N1-s, H3N2, and Flu B viruses in NPS specimens from subjects presenting with Flu-like illness. A prospective collection study was conducted at four enrollment sites, including BioMed Supply Inc., Carlsbad, CA; Johns Hopkins University, Baltimore, MD; ProMedDx, LLC, Norton, MA; and Vanderbilt University Medical Center, Nashville, TN, from December 2009 through May 2010. Additional NPS specimens retrospectively collected during February 2008, January to March 2009, and September 2009 to June 2010 were provided by the Vanderbilt University Medical Center, Discovery Life Science, Los Osos, CA; the NorthShore University HealthSystem Research Institute, Evanston, IL; and the Centers for Disease Control and Prevention, Atlanta, GA. Specimen testing was done at AthoGen, Irvine, CA; Vanderbilt University Medical Center; and Johns Hopkins University. Comparator testing was done at Prodesse Inc., Waukesha, WI. Bidirectional Sanger sequencing was done at SeqWright Inc., Houston, TX. The study was approved by the local institutional review boards (IRBs) at each investigational site.

The reproducibility of the PLEX-ID Flu assay was defined by using a nine-member panel including a negative sample and spiked positive samples at two levels (low positive near the assay limit of detection [LOD] and approximately 2 to 3 times the LOD) for influenza B virus and the three influenza A virus subtypes detected by the assay. Panels were coded, randomized, and shipped to the three testing laboratories. Each NPS specimen was tested in parallel by using the PLEX-ID Flu assay and a predicate method, the Prodesse ProFLU+ assay (Gen-Probe Prodesse Inc., Madison, WI), to detect influenza A and B viruses (28). Specimens testing positive for influenza A virus by ProFLU+ were subtyped as H1N1-p, H1N1-s, or H3N2 by using the ProFAST+ assay (15) (Gen-Probe Prodesse Inc.). Discrepant results between the PLEX-ID Flu and the Prodesse ProFLU+/ProFAST+ assays were resolved by bidirectional Sanger sequencing of the Flu A virus hemagglutinin (HA) gene. Only results from the Prodesse ProFLU+/ProFAST+ assays (comparator method) were used to determine the performance of the PLEX-ID Flu assay.

Patient recruitment and specimen collection. All of the inclusion and exclusion criteria were met in order for the patient to be enrolled in the prospective component of the study. The following inclusion criteria were used: (i) a NPS specimen was obtained as part of a routine laboratory evaluation for respiratory virus infection and respiratory virus nucleic acid amplification testing and/or viral culture was ordered by a physician, (ii) a sufficient volume of a NPS specimen was available (≥2.0 ml), (iii) informed consent was provided if required by the corresponding IRB, and (iv) the date of onset of Flu-like symptoms was within the past 4 days. Exclusion criteria included (i) patients previously enrolled in this study, (ii) patients for whom obtaining a NPS swab specimen was contraindicated, and (iii) patients with a medical condition that prevented a NPS specimen type from being obtained. Case report forms (CRFs) were utilized to document the completion of collection and testing procedures. The clinical monitor reviewed the data and CRFs to ensure compliance with the study-directed procedures and to monitor the progress of the study. A minimum of three NPS aliquots were prepared for testing with the PLEX-ID Flu assay, the Prodesse ProFLU+/ProFAST+ assays, as well as SeqWright bidirectional Sanger sequencing analysis. All aliquots were frozen and stored at −70°C prior to testing with PLEX-ID Flu and Prodesse ProFLU+/ProFAST+ at the collection sites or shipped to other laboratories on dry ice for testing.

PLEX-ID Flu assay. The PLEX-ID system is composed of front-end processing instrumentation and the PLEX-ID analyzer. Sample, reagent, and processing information is tracked by a workstation networked to the analyzer. Nucleic acids were extracted from NPS specimens by using the PLEX-ID SP instrument and the PLEX-ID viral RNA isolation kit (list 05N80). Total nucleic acids were bound to magnetic beads, and non-nucleic-acid sample material was washed away during multiple wash steps. Purified nucleic acids were then released from the magnetic beads with an elution buffer. The PLEX-ID FH liquid handler distributes sample eluates. The PLEX-ID Flu amplification reagent kit (list 05N21) contains all PCR amplification reagents. PCR amplification, amplification product desalting, and mass spectrometry analysis were performed as previously described (21, 24). A software algorithm converted the mass information to base composition using the exact masses of each of the bases that comprise DNA and the known information about the primer sets present in each well. The PLEX-ID analyzer identified the influenza virus species and subtype by comparing the base composition signature of the observed products to a database that links the base composition signature to influenza virus species and subtype identity (21, 24).

Prodesse Flu assays. The Prodesse ProFLU+ and ProFAST+ real-time PCR assays were used as validation references and were performed according to the manufacturer's instructions (15, 28). The ProFLU+ assay has manufacturer-disclaimed sensitivities and specificities of 100.0% and 92.6% for Flu A virus and 97.8% and 98.6% for Flu B virus, respectively. The ProFAST+ assay has manufacturer-disclaimed sensitivities and specificities of 94.4 to 100.0% and 99.0 to 99.36% for H1N1-s, 95.4 to 100.0% and 100.0% for H1N1-p, and 100.0% and 99.6 to 100.0% for H3N2, respectively. Extractions were performed with either the MagNA Pure LC System/MagNA Pure Total Nucleic Acid Isolation kit (Roche) or NucliSENS easyMAG system/Automated Magnetic Extraction reagents (bioMérieux Inc., Durham, NC). SmartCycler instrumentation (Cepheid, Sunnyvale, CA) was used for amplification. Samples positive for Flu A virus by ProFLU+ were then tested by using the ProFAST+ assay to identify the virus subtype.

Bidirectional Sanger sequencing analysis. Blind bidirectional Sanger sequencing analysis was performed to resolve discrepant results between the PLEX-ID Flu assay and the Prodesse ProFLU+/ProFAST+ assays (SeqWright Inc., Houston, TX); at least 200 bp of the Flu A virus HA gene was queried. RT-PCR was by performed using the SuperScript III One-Step RT-PCR system with Platinum *Taq* (catalog number 12574018; Invitrogen). DNA cycle sequencing was performed on the RT-PCR products by using standard BigDye Terminator v3.1 chemistry in conjunction with proprietary primers. Sequencing reactions were purified by using Sephadex. Sequence delineation and base calling were performed by using an ABI model 3730xl automated fluorescent DNA sequencer.

Independent primer pairs were designed for each of the three categories to be discriminated, including seasonal H1N1, 2009 pandemic H1N1, and seasonal H3N2 viruses. Primer pair CP1 (5′-CCA GAA ATA GCC AAR AGA CC-3′)/CP2 (5′-AAC CAT CYA CCA TYC CAG TCC-3′) was used to amplify and sequence seasonal H1N1 virus-positive samples. These primers amplify a region from positions 685 to 1093 of the reference sequence under GenBank accession number JQ714075. Primer pair CP3 (5′-AGG GAG AAT GAA CTA TTA CTG G-3′)/CP4 (5′-AAA TAG GCC

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TABLE 1 Demographics and clinical features of patients recruited prospectively

Parameter	Value for prospectively collected specimens				P value[c]
	Site 1 (n = 58)	Site 2 (n = 177)	Site 3 (n = 560)	Site 4 (n = 288)	
No. (%) of patients of gender[a]					<0.001
Female	33 (56.9)	92 (52.0)	346 (61.8)	132 (46.0)	
Male	25 (43.1)	85 (48.0)	214 (38.2)	156 (54.0)	
Mean age of patients (yr) ± SD[b]	28.9 ± 14.8	33.0 ± 15.1	37.3 ± 14.2	4.6 ± 10.3	<0.001
Mean no. of days after onset of symptoms ± SD	2.2 ± 1.17	2.2 ± 0.95	2.0 ± 1.03	2.0 ± 1.12	0.1263
No. (%) of patients on antiviral treatment					<0.001
Yes	1 (1.7)	12 (6.8)	2 (0.4)	7 (2.4)	
No	3 (98.1)	164 (92.7)	557 (99.5)	275 (95.5)	
Unknown	54 (5.2)	1 (0.6)	1 (0.2)	6 (2.1)	
No. (%) of patients with influenza vaccination					<0.001
Yes	14 (24.1)	67 (37.9)	138 (24.6)	43 (14.9)	
No	41 (70.7)	105 (59.3)	420 (75.0)	94 (32.6)	
Unknown	3 (5.2)	5 (2.8)	2 (0.4)	151 (52.4)	

[a] Site 4 had 1 subject of an unknown gender.
[b] Site 1 had 1 subject whose age was not documented.
[c] *P* values represent site comparisons. Continuous variables were analyzed by analysis of variance (ANOVA), with site being the main effect. Discrete variables were analyzed by a Cochran-Mantel-Haenszel test for general associations.

TCT AGA TTG-3′) was used to amplify and sequence 2009 H1N1 virus-positive samples. These primers amplify a region from positions 723 to 1041 of the reference sequence under GenBank accession number HM189312.1. Primer pair CP5 (5′-ATA AGC ATY TAT TGG ACA ATA G-3′)/CP6 (5′-TCC CTC CCA ACC ATT TTC TAT G-3′) was used to amplify and sequence H3N2 virus-positive samples. These primers amplify a region from positions 755 to 1102 of the reference sequence under GenBank accession number CY114558.1. The amplified and purified RT-PCR products were used as the templates for DNA cycle sequencing using BigDye Terminator v3.1 chemistry (Applied Biosystems).

The quality of sequencing data generated was measured by using a Phred score program. Sequences were considered valid if at least 200 bp from a single read had a Phred score of 20 or higher and the overlapping consensus sequence had an average Phred score of 40 or higher. The sequence data were analyzed by BLAST against a SeqWright in-house database. Sequence data from the amplified RT-PCR products should match the correct reference sequence of virus of the expected subtype in the BLAST analysis with an E value from the BLAST analysis of e^{-5} (10^{-5}) or lower and should match FDA sequence acceptance criteria.

Statistical analysis. Agreements and 95% confidence intervals (CIs) of the reproducibility rates between the three testing sites were calculated based on the score method for individual sites and all sites combined. The acceptable criterion for the point estimate of the reproducibility rate was ≥95.0% for all testing sites combined. The results of the PLEX-ID Flu assay were compared to the results of the Prodesse ProFLU+/ProFAST+ assay for the calculation of positive percent agreement (PPA) (sensitivity), negative percent agreement (NPA) (specificity), and overall agreement as well as their 95% CIs. A minimum of 114 specimens with positive results was set to have a statistical power of 80% to detect the difference between a null-hypothesis proportion, π_0, of 0.800 and an alternative proportion, π_A, of 0.900, based on a score test with a nominal two-sided significance level of 0.050. Statistical Analysis Software (SAS), version 8.2, on a UNIX operating system was used to analyze the data, and a *P* value of ≤0.05 was considered statistically significant.

RESULTS

A total of 2,665 specimens were selected for the study. Among them, 1,087 were specimens prospectively collected from December 2009 to May 2010, 89 were preselected positive banked specimens collected during February 2008 and January to March 2009, and 1,489 were leftover archived specimens collected from September 2009 through June 2010. Forty-eight (1.8%) specimens were excluded due to missing/duplicated results (n = 38) or results that could not be resolved by the comparator method when the sample was negative for all three influenza A virus subtype markers and the internal control (n = 10). A total of 2,617 NPS specimens were included in the analysis. The demographic and clinical features of the patients recruited prospectively are listed in Table 1. Both adults and children were recruited in sites 1 to 3, while children were dominantly recruited in site 4. No significant differences in days until the onset of symptoms between the four sites were observed. Demographic data and clinical features of illness were not recorded for the retrospectively collected specimens.

The reproducibility of the PLEX-ID Flu assay was evaluated at 3 clinical sites. Samples were tested in replicates of 4, with 1 replicate each of the positive and negative controls per batch. Two batches were run per day per site. Each site had two operators, and a different operator tested each batch. A total of 319 results obtained during 5 days of testing were analyzed (120 results for each of six panel members and 199 results for each of three panel members). An overall agreement of 98.3 to 100.0% was observed between the three sites (Table 2). A slightly lower agreement (98.3%) was noticed for low-positive H1N1-p and H1N1-s specimens. The acceptance criterion for overall site reproducibility was met.

In comparison to the Prodesse ProFLU+ and ProFAST+ assays, the positive percent agreement (PPA) and negative percent agreement (NPA) of the PLEX-ID Flu assay were 94.5% (95% CI, 92.6 to 95.9%) and 99.0% (95% CI, 98.4 to 99.4%) for Flu A virus and 96.0% (95% CI, 86.5 to 98.9%) and 99.9% (95% CI, 99.7 to 100.0%) for Flu B virus, respectively. For the subtype characterization, the PLEX-ID Flu assay had PPAs and NPAs of 98.3% (95% CI, 96.9 to 99.1%) and 97.5% (95% CI, 96.8 to 98.1) for

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TABLE 2 Reproducibility of the PLEX-ID Flu assay

Panel member description	No. of samples tested (% agreement)			Total no. of samples tested at all sites (% agreement) (95% CI)
	Site 1	Site 2	Site 3	
Negative ($n = 40$)	40 (100.0)	40 (100.0)	40 (100.0)	120 (100.0) (96.9, 100.0)
Flu A H1N1-p, low positive ($n = 40$)	40 (100.0)	39 (100.0)[a]	40 (95.0)[b]	117 (98.3) (94.1, 99.5)
Flu A H1N1-p, moderate positive ($n = 40$)	40 (100.0)	40 (100.0)	40 (100.0)	120 (100.0) (96.9, 100.0)
Flu A H1N1-s, low positive ($n = 40$)	40 (97.5)[c]	40 (100.0)	40 (97.5)[c]	118 (98.3) (94.1, 99.5)
Flu A H1N1-s, moderate positive ($n = 40$)	40 (100.0)	40 (100.0)	40 (100.0)	120 (100.0) (96.9, 100.0)
Flu A H3N2, low positive ($n = 40$)	40 (100.0)	40 (100.0)	39[a] (100.0)	119 (100.0) (96.9, 100.0)
Flu A H3N2, moderate positive ($n = 40$)	40 (97.5)[d]	40 (100.0)	40 (100.0)	119 (99.2) (95.4, 99.9)
Flu B, low positive ($n = 40$)	40 (100.0)	39 (100.0)[a]	40 (97.5)[e]	118 (99.2) (95.4, 99.9)
Flu B, moderate positive ($n = 40$)	40 (100.0)	40 (100.0)	39 (97.5)	119 (99.2) (95.4, 99.9)

[a] One missing replicate due to instrument error, which was excluded from the calculation.
[b] Reported "not detected" in 2 separate runs.
[c] One reported "H3N2" in site 1 but "not detected" in site 3.
[d] One reported "H3N2 and H1N1-s."
[e] One reported "H3N2."

H1N1-p, 88.6% (95% CI, 74.0 to 95.5%) and 100.0% (95% CI, 99.9 to 100.0%) for H1N1-s, and 98.0% (95% CI, 89.7 to 99.7%) and 99.9% (95% CI, 99.7 to 100.0%) for H3N2, respectively. The overall agreement between the PLEX-ID Flu assay and Prodesse ProFLU+/ProFAST+ assays for Flu A and Flu A virus subtype characterization was 97.1 to 99.9% (Table 3). There were no statistically significant differences in PPA and NPA values between adults and children for the detection of different viruses and subtypes (data not shown).

There were 96 specimens identified as being discrepant between the ProFLU+ and ProFAST+, ProFLU+ and PLEX-ID Flu, and ProFAST+ and PLEX-ID Flu assays. Bidirectional Sanger sequencing was used to test 93 of the specimens. There were 40 positive and 19 negative specimens with discrepant results for Flu A virus by the ProFLU+ assay. Of the 40 ProFLU+-positive/PLEX-ID Flu-negative specimens, 20 were negative, 3 were unresolved, and 17 were positive by bidirectional Sanger sequencing. Of the 19 ProFLU+-negative/PLEX-ID Flu-positive specimens, 15 were positive and 1 was negative by bidirectional Sanger sequencing, and 3 could not be tested. A large number of discrepant results were seen between the PLEX-ID Flu and ProFLU+/ProFAST+ assays for the H1N1-p subtype. Of the 50 negative ProFLU+/ProFAST+ specimens, 44 specimens were positive, 2 were negative, and 1 was unresolved by bidirectional Sanger sequencing. Three specimens could not be tested. Of the 10 positive ProFAST+ specimens, 8 were positive and 2 were negative by bidirectional Sanger sequencing (Table 3). These data demonstrate the high level of accuracy of the PLEX-ID Flu assay for the simultaneous detection and identification of influenza A and B viruses in NPS specimens.

DISCUSSION

Influenza viruses are continually evolving through site-specific mutations and segment reassortment. This evolutionary plasticity occasionally allows "novel" influenza viruses to move from animal hosts to humans, potentially causing destructive pandemics. Most currently used rapid molecular diagnostics for influenza virus detection use analyte-specific reagents (PCR primers and antibodies, etc.) to detect and identify well-characterized, circulating strains of influenza virus and are therefore generally incapable of detecting novel influenza viruses. The PLEX-ID system uses deliberately nonspecific primers that amplify all known variants (all H/N sub-

TABLE 3 Performance of the PLEX-ID Flu assay in comparison to Prodesse assays

Influenza virus subtype	Total no. of specimens	No. of specimens with test result				PPA (%) (95% CI)	NPA (%) (95% CI)	Overall agreement (%) (95% CI)
		Prodesse$^+$ PLEX-ID$^+$	Prodesse$^+$ PLEX-ID$^-$	Prodesse$^-$ PLEX-ID$^-$	Prodesse$^-$ PLEX-ID$^+$			
Flu A	2,617	684	40[a]	1,874	19[b]	94.5 (92.6, 95.9)	99.0 (98.4, 99.4)	97.7 (97.1, 98.2)
H1N1-p	2,615	570	10[c]	1,985	50[d]	98.3 (96.9, 99.1)	97.5 (96.8, 98.1)	97.7 (97.1, 98.2)
H1N1-s	2,615	31	4[e]	2,580	0	88.6 (74.0, 95.5)	100.0 (99.9, 100.0)	99.8 (99.6, 99.9)
H3N2	2,615	50	1[f]	2,562	2[g]	98.0 (89.7, 99.7)	99.9 (99.7, 100.0)	99.9 (99.7, 100.0)
Flu B	2,617	48	2[h]	2,564	3[h]	96.0 (86.5, 98.9)	99.9 (99.7, 100.0)	99.8 (99.6, 99.9)

[a] Seventeen specimens were positive for a Flu A virus subtype, and 20 were negative by bidirectional sequencing. Three samples were unresolved, since the sequence data failed the quality criteria.
[b] Fifteen specimens were positive and one was negative for Flu A virus by bidirectional sequence analysis. Among the 15 positive ones, 13 were H1N1-p and 2 were H3N2 by bidirectional sequencing. Three samples had an insufficient sample volume for further analysis.
[c] Eight samples were positive and two were negative for H1N1-p by bidirectional sequencing.
[d] Forty-four samples were positive and two were negative for H1N1-p by bidirectional sequencing. One sample was unresolved since the sequence data failed the quality criteria, and three had insufficient sample volumes.
[e] Negative for H1N1-s by bidirectional sequencing.
[f] Negative for H3N2 by bidirectional sequencing.
[g] Positive for H3N2 by bidirectional sequencing.
[h] Bidirectional sequencing was not performed for discrepant Flu B virus results.

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types) of influenza virus, including human, other mammalian, and avian influenza viruses, and is therefore likely to generate analyzable amplicons from any novel influenza virus that might emerge in humans (21, 24, 27). The advantage of mass spectrometry as an amplicon characterization method is that it does not require the sequence of the amplicon to be known, as is required when probe-based methods are employed. The mass spectrometer simply weighs the amplicons present, without prejudice, and reports their base composition. The reported base composition for the sample is compared to a curated database of calculated base compositions from reference sequences of known influenza viruses, and the identification is made. This method provided the first molecular identification of the 2009/H1N1 pandemic influenza virus (24, 29). A universal Flu virus diagnostic device that can detect previously unrecognized strains in addition to all known strains therefore has potential utility to provide prompt identifications in the setting of pandemics that alter the landscape of human influenza virus infection.

The PLEX-ID Flu assay, by incorporating the use of multilocus PCR and electrospray ionization-mass spectrometry, has been reported to be a rapid and powerful technique for the detection, identification, and characterization of influenza viruses for both clinical diagnosis and epidemiological investigations (21, 24, 27, 29–32). The clinical trail data demonstrated that the PLEX-ID Flu assay is able to correctly detect and differentiate influenza A 2009 H1N1 (H1N1-p), seasonal H1N1 (H1N1-s), influenza A H3N2, and influenza B viruses in nasopharyngeal swab (NPS) specimens with a reproducibility ranging from 98.3 to 100.0%. In comparison to the FDA-cleared Prodesse ProFLU+ and ProFAST+ assays, the PLEX-ID Flu assay results yielded positive and negative agreement rates of between 88.6% and 100.0% for influenza A and B virus detection as well as for the discrimination of seasonal H1N1, pandemic (2009) H1N1, and seasonal H3N2 virus subtypes. The overall agreement rates between the PLEX-ID Flu assay and Prodesse ProFLU+/ProFAST+ assays were 97.1 to 99.9%, with some discrepant results involving Flu A virus detection and pandemic (2009) H1N1 virus subtyping. The PLEX-ID Flu assay demonstrated a high level of accuracy for the simultaneous detection and subtype identification of influenza A and B viruses in patient specimens, providing another laboratory tool for the rapid diagnosis and management of influenza virus infections.

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