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Effect of genomic drift of influenza PCR tests

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ABSTRACT

Background: Nucleic acid amplification assays have become the method of choice for influenza (Flu) testing due to superior accuracy and faster turnaround time. Although assays are designed to detect highly conserved genomic targets, mutations can influence test sensitivity. Most of the circulating viruses in the United States during the 2014–2015 season were associated with significant genetic drift; however, the effect on testing was unknown.

Objectives and study design: We compared the performance of Prodesse ProFlu+/ProFAST+ (PFlu/PFAST), FilmArray Respiratory Panel (RP), cobas® Influenza A/B test (cIAB), and Xpert® Flu (Xpt) in a retrospective analysis of consecutive nasopharyngeal specimens received for a two-week period during the winter of 2015. Furthermore, limits of detection (LOD) were determined with six isolates of Flu.

Results: Of the 275 specimens, 63 were positive for FluA by PFAST, 60 were positive by RP, 58 were positive by cIAB and 52 were positive by Xpt. Only a subset of 135 specimens was tested by PFlu, of which 32 were positive. The sensitivity/specificity for PFAST, RP, cIAB, Xpt and PFlu was 100/99.1%, 96.7/99.5%, 91.8/99.1%, 85.2%/100%, and 75.6%/98.9%, respectively. LOD analyses demonstrated assay performance variations were strain associated. Specifically, PFlu’s and cIAB’s LODs were higher with A/Texas/50/2012-like and A/Switzerland/9715293/2013-like strains, while Xpt’s highest LOD was with the Swiss strain.

Conclusions: Strain-associated assay performance variation is known to occur with other Flu test methods; hence, it is not surprising that such variation would be observed with molecular tests. Careful monitoring and reporting for strain-associated variations are warranted for all test methods.

1. Background

Flu A and B are some of the most important human pathogens, infecting hundreds of millions of people annually with 250,000–500,000 deaths worldwide [1]. Rapid identification is important for both therapeutic and infection control purposes. Traditional rapid diagnostics, such as immunoassays, produce quick results and are simple to perform; but, have sub-optimal sensitivity [2–5]. Nucleic acid amplification assays (NAAT), which are relatively rapid and have greatly enhanced sensitivity, are becoming the method of choice [6].

NAATs are designed to detect highly conserved genomic targets, generally in the matrix protein (MP) gene [7]; however, mutations can influence test sensitivity. Indeed, Flu viruses mutate and evolve rapidly and the continual antigenic drift is associated with annual epidemics and the need for annual assessments for the viral vaccine strains. These continual changes have also been associated with annual changes in the sensitivity of many rapid immunoassays [2,8], as well as cell culture in terms of cell-line permissiveness [9].

Although the mutation rates of the HA and NA genes are usually higher than those of the internal proteins [10], there have been periods where the mutation rates in the MP gene rivaled that of the surface proteins [11]. Indeed, problematic M gene mutations affecting the performance of commercial assays have been observed with A(H1N1)pdm09 viruses [12–14]. More recently, mutations in the MP gene of H3N2 strains have been reported in Taiwan and Belgium [15,16].

In 2015, the majority of circulating FluA in the United States (USA) was associated with significant genetic drift, loss of vaccine protection and reduced ability to culture in many cell lines [17]. The effect of this drift had on molecular testing warranted investigation.
2. Objectives

To assess the performance of a variety of molecular assay with strains of FluA associated with genetic drift in early 2015.

3. Study design

3.1. Clinical samples

Clinical specimens were initially comprised of 275 consecutive NP specimens in M4RT viral transport media (VTM, Remel, Lenexa, KS) received for the detection of respiratory viruses during a 2-week period in the winter of 2015 (age range 22d–93yr, median 25yr, 45% pediatic cases). Specimens, stored at −80 °C, after original testing by PFlu/PFAST (49%, Hologic/Gen-Probe, San Diego, CA) or by RP (51%, BioFire Diagnostics Inc., Salt Lake City, UT), were thawed for testing by ciAB and RP or PFAST, and returned to −80 °C. Samples were thawed again for testing by Xpt. Additional archived Flu positive samples were analyzed to assess assay performance against other strains of virus. Additional samples included 50 samples from 2009 positive for FluA, of which 4 were seasonal H1N1 (sH1), 4 were seasonal H3N2 (sH3) and 42 were pdm09 H1N1 (09H1N1); 10 09H1N1 from the 2013 to 2014 season; and 40 FluB from spring 2014 or spring 2015. Samples equivalent for FluA with RP were considered to be positive by that test method [18]. Specimens were discarded as not positive if they were positive by two or more assays. Viral nucleic acid concentrations determinations were based on quantified control viral RNA (Hologic/Gen-Probe) using either PFlu or PFAST, depending on amplification efficiency.

3.2. Viruses

Flu isolates A/California/7/2009(H1N1)pdm09-like (H1N1), A/Perth/16/2009(H3N2)-like (Perth), A/Texas/50/2012(H3N2)-like (Texas) and A/Massachusetts/2/2012-like (FluB) were obtained through the New York State Department of Health’s Wadsworth Center Proficiency Testing program (NYS PT). H3N2 isolates A/Switzerland/9715293/2013-like (Swiss) and A/Indiana/09/2012 (H3N2v)-like were gifts from Jennifer LaPlante, NYS Wadsworth Center (NYS Coll) and Judith Lovchik, Indiana State Department of Health (IS), respectively. Viral stocks were serially diluted 1:10 in VTM for LOD analyses. Nucleic acid concentrations were determined in the same manner as above.

3.3. Commercial-real-time PCR assays for influenza detection

PFlu detects FluA, FluB and RSV and PFAST differentiation of 09H1N1, sH3, and sH1. Briefly, viral RNA was extracted from 0.2 ml of specimen in VTM, along with a universal internal control, using easyMAG extractors (bioMerieux, Durham, NC) with 50 μl elution. RNA extract, 5 μl, was amplified on SmartCyclers (Cepheid, Carlsbad, CA) per package insert. Data analysis was performed using the SmartCycler® Dx software Version 3.0.

RP is a closed system which integrates extraction and multiplex amplification for the detection of adenovirus, coronaviruses 229E, HKU1, NL63 and OC43, FluA (with subtyping for hemagglutinin genes sH1, 09H1N1 and sH3), FluB, human metapneumovirus, parainfluenza virus, types 1–4, respiratory syncytial virus, human rhinovirus/enterovirus, Bordetella pertussis, Chlamydia pneumoniae, and Mycoplasma pneumoniae on FilmArray instruments. Results are reported as detected or not detected for each target, or invalid. In addition, a result of equivocal for FluA occurred when only one of two FluA specific targets was amplified.

ciAB (Roche Diagnostics, Indianapolis, IN) is a 20-min point-of-care real-time PCR performed on a cobas® Liat Instrument. The test uses 0.2 ml of specimen in VTM. Results may be detected, not detected, or indeterminate for FluA or B, or assay invalid. Xpt (Cepheid) is a 90-min automated PCR for detection of FluA, 09H1N1 and FluB, performed on GeneXpert® Instrument. Approximately 0.3 ml of specimen in VTM was added to a single-use disposable GeneXpert cartridge. Result of detected, not detected for FluA, FluB, 09H1N1 or invalid (SPC failure) or error (PCC or other instrument errors). Invalid and errored samples by any method were retested to obtain a valid result.

3.4. Sequencing and alignment

Viral RNA was extracted from the clinical specimens using the easyMAG and a 405 bp fragment of the M1 gene was amplified for Sanger sequencing by the Center for Functional Genomics Core Facility at the University at Albany. Because the Texas isolate would not amplify with the standard WHO primers, an alternate forward primer (MP-F69, TGT AAA ACG AGC AGT TTC CRT CRG GCC CCC TC) was used with the WHO MP-R473 [19]. One patient sample was sequenced by Hologic. Sequence alignments were performed using MEGA version 7 (The Biodiges Institute, Tempe, AZ).

3.5. Statistical analysis

Probit analyses for the limit of detection with a 95% probability of detection were performed using SPSS version 8.0 (IBM, Armonk, NY). Sensitivities, specificities, positive and negative predictive values and confidence intervals (CI) were determined using Microsoft Excel 2016 (Redmond, WA) [20].

4. Results

4.1. Assay performance with consecutive clinical samples from 2015

The evaluation involved a retrospective analysis of 275 consecutive nasopharyngeal specimens received into the laboratory for the detection of respiratory viruses between 1/26/15 and 2/9/15, incidence of Flu = 22%, exclusively A/H3N2. Originally, 135 of these specimens were tested by PFlu/PFAST and 140 were tested by RP. PFlu had detected 22%, exclusively A/H3N2. Originally, 135 of these specimens were tested by PFlu/PFAST and 140 were tested by RP. PFlu had demonstrated suboptimal performance with the circulating strain of A/H3N2 (sensitivity of 76%) and was excluded from further testing, as the initial intent of the study was to evaluate the performance of ciAB. All specimens were analyzed by ciAB and Xpt, while RP and PFAST were performed as needed depending on original clinical testing. No specimens were reproducibly problematic by any method, however, three (1%) specimens were initially invalid with ciAB and six (2%) specimens produced instrument errors on the Xpert. As per Cepheid, the instrument errors on the Xpert may be related to the VTM.

Sixty-one specimens were true positive (TP) for FluA, i.e., positive by two or more tests (Table 1). All TP cases were positive for FluA/H3

| Table 1 | Assay Performance with Clinical Specimens Collected Between 1/26/15 and 2/9/15 (total = 275) |
|----------|--------------------------------------------------|
| Virus    | PFAST | RP | ciAB | Xpt |
| sH3N2    | 50    | +  | +   | +   |
| sH3N2    | 5     | +  | +   | +   |
| sH3N2    | 3     | +  | +   | +   |
| sH3N2    | 1     | +  | +   | +   |
| sH3N2    | 1     | +  | +   | +   |
| −        | 2     | a  | −   | −   |
| −        | 1     | a  | −   | −   |
| −        | 2     | a  | −   | −   |
| 209      | −     | −  | −   | −   |

a 2 were positive for FluA with RP but not typed.
b Equivocal for FluA with RP.

60 specimens were true positive (TP) for FluA, i.e., positive by two or more tests (Table 1). All TP cases were positive for FluA/H3

60 specimens were true positive (TP) for FluA, i.e., positive by two or more tests (Table 1). All TP cases were positive for FluA/H3
by PFAST, as well as two additional samples. There were two false negative (FN) and one false positive (FP) result with RP. In addition, two TP samples were not typed with RP and two samples were equivocal for FluA, one TP and one FP. FluA was detected by cIAB in 56 TP and 2 FP samples and by Xpt in 52 TP samples only. Only 135 of the specimens were tested by Pflu, of which 41 were TP, ten were FN and 2 FP samples and by Xpt in 52 TP samples only. Only 135 of the specimens were tested by Pflu, of which 41 were TP, ten were FN and one was FP.

The performance characteristics of all assays are presented in Table 2. The sensitivity/speciﬁcity for, PFAST, RP, cIAB, Xpt and PFlu for the circulating strain(s) of A/H3N2 were 100/99%, 97/100%, 92/99%, 85/100%, and 76/99%, respectively. The positive predictive value for all assays was very good during this period.

4.2. Assay performance with archived Flu positive clinical samples from previous years

Additional archived Flu positive samples were analyzed to assess assay performance against other strains of virus. The 60 FluA samples were analyzed with all five assays, while PFAST was excluded from the analysis of the 40 FluB samples, as appropriate. Of the 52 09H1N1 positive specimens, one was initially invalid with RP and another three produced instrument errors with Xpt, two of which were resolved with repeat testing. The sample with repeated errors was excluded from the analysis of Xpt. Pflu, PFAST and cIAB detected all 52 samples positive for pdm09 H1N1 (Table 3), whereas RP was only positive for 49 (94%). In addition, six of the RP positive specimens were equivocal for FluA. Of the 51 09H1N1 positive samples with valid results on Xpt, 49 (96%) were positive by this system. All 51 09H1N1 positive samples with valid results on Xpt, 49 (96%) were positive by this system. All 51 09H1N1 positive samples with valid results on Xpt, 49 (96%) were positive by this system. All 51 09H1N1 positive samples with valid results on Xpt, 49 (96%) were positive by this system. All 51 09H1N1 positive samples with valid results on Xpt, 49 (96%) were positive by this system.

To demonstrate that assay performance variations were strain associated, we performed LOD on all methods with six strain of Flu. In general, the LODs were highly variable between virus strains and between assays (Table 5). PFlu and cIAB had lower LODs, as compared to RP and Xpt, for H1N1, Perth and H3N2v, but inferior LODs for Texas. In fact, the highest concentration of this virus available (6.28 log copies/ml) was not detected by PFlu. Conversely, PFAST demonstrated lowest LOD for the same strain. Interestingly, inconsistent results were observed across a broad range of dilutions of the H3N2v strain with RP, with equivocal calls being common, suggesting one of the two FluA targets is more effective that the other for this strain. For the Swiss strain, PFAST and RP LODs were superior to cIAB and Xpert, while Pflu’s LOD fell mid-range. PFlu, cIAB and Xpt all performed well with FluB.

Table 4
Sensitivity with archived Flu positive clinical samples from previous years.

| Virus | FluA (sH1, sH3, 09H1) | FluB |
|-------|---------------------|------|
| PFAST | Not applicable      | 100% |
| RP    | 95.0% (89.5-100%)   | 100% |
| cIAB  | 100%                | 100% |
| Xpt   | 95.0% (89.5-100%)   | 100% |
| Pflu  | 96.7% (89.5-100%)   | 100% |

Table 5
Limit of Detection (estimated log copies/ml).

| Virus        | Strain (source) | PFlu | PFAST | RP  | cIAB | Xpt |
|--------------|-----------------|------|-------|-----|------|-----|
| 09H1N1       | A/California/07/2009-Like (NYS-PT) | 3.91 | 3.77 | 4.01 | 3.77 | 4.30 |
| sH3N2        | A/Perth/16/2009-Like (NYS-PT) | 3.51 | 5.23 | 4.42 | 5.23 | 5.23 |
| sH3N2        | A/Texas/50/2012-Like (NYS-PT) | > 6.28 | 3.13 | 4.09 | 5.20 | 4.63 |
| sH3N2        | A/Switzerland/9715293/2013-Like (NYS-CoV) | 4.77 | 3.69 | 5.22 | 5.02 | 5.53 |
| sH3N2        | A/Indiana/09/2012-Like (IS) | 3.48 | 3.43 | 3.47 | 3.48 | 4.92 |
| FluB         | B/Massachusetts/02/2012-Like (NYS-PT) | 3.95 | na | 5.29 | 3.31 | 3.62 |

a not analyzed.
4.4. Viral load (VL) associated performance with clinical specimens

The sensitivities for the commercial assays were highly variable with our sample population. The box and whisker plots in Fig. 1 demonstrate differences in VL for false negative (FN) samples vs the true positive samples for the 3 assays with 5 or more FN results. The assays’ LOD for both the Texas and Swiss isolates are illustrated with dashed lines. The highest VLs were seen with the PFlu FN samples, with 90% lower than the assay’s LOD with Texas strain, but only 50% lower than the LOD for the Swiss strain. The next highest VLs were seen with the Xpt FN samples, but in this case 89% had virus titers lower than the assay’s LOD with Swiss strain and 56% had virus titers lower than the LOD for the Texas strain. All cFLA FN samples had titers below the LODs for both strains of virus. Of the two FN RP samples, both were above the LOD for both strains. There were no FN PFAST samples.

4.5. Nucleotide sequence alignments

MP genes sequences of various historical isolates of FluA, viruses analyzed in the LOD studies and 10 clinical specimens with suboptimal performance with PFlu were aligned (Table 6). Although this illustrated region is considered highly conserved and includes the WHO recommended target region, sequence divergence has been observed. Hologic indicated that the weak positive or false negative FluA results observed by our lab laboratory were due to a H3N2 variant containing 4 point mutations in the PFlu probe binding region with no mutations in the primer binding regions. The exact target regions used by the commercial tests investigated are proprietary, they indicated the general target region of most commercial assays are somewhat similar to the WHO Aw-1 target region [7] (Table 6).

5. Discussion

Prior to the 2014–2015 influenza season, mutations in the matrix gene affecting the performance of molecular tests, had only been observed with isolated cases of 09H1N1 [12-14]. For A/Minnesota/JB1/2013(H1N1)pdm09, false negative PFlu results were due to two point mutations in the assay’s probe binding region [12,14]. Since the probe and primer binding regions are proprietary, it is difficult to speculate which mutations affect probe binding. Based on divergence from historical sequences one would have expected more mutations in the Minnesota 09H1N1 strain than in our strain. Interestingly, Overmeire et al. [16] demonstrated that a C163T point mutation was pivotal in the loss of activity for the probe in a CE-marked assay from Qiagen. Indeed, the C163T mutation was seen in our viruses while a C162T mutation was observed with A/Minnesota/JB1/2013(H1N1)pdm09.

Divergence within the MP gene of H3N2 viruses was first reported in 2013 Taiwanese viruses, with C153T, C163T, and G189T mutations affecting the WHO recommended target region [15]. It has been reported these MP mutations correlated with clade 3C.2a viruses in Europe [16]. We have demonstrated that the same is true in the USA [21]. What is unique to the USA is that 3C.3b viruses also carry this MP gene pattern; while the MP gene in European clade 3C.3b viruses are similar to A/Newcastle/22/2014 [21].

World-wide, the majority of viruses collected from during the 2014–2015 season were clades 3C.2 and 3C.3, with 3C.2a viruses predominating in many regions [22,23]. Originally, it was reported that 32.5% of the USA isolates between September 28–December 6, 2014 were A/Texas/50/2012-like (clade 3C.1) and most of the remaining were antigenically similar to A/Switzerland/9715293/2013 (clade 3C.3a) [17]. However, it appears early in the season in the USA, similar rates of clades 3C.2a, 3C.3, and 3C.3a co-circulated; but 3C.2a viruses quickly predominated, representing more than 70% of the virus population by January 2015 [24].

Interestingly, MP sequences obtained from the Texas-like strain we obtained was different from the published prototype sequences and similar to MP sequence of our clinical isolates. However, this was not surprising given that A/Texas/50/2012 is a clade 3C.1 virus and in 2014 the MP gene from USA 3C.1 viruses evolved to be similar to 3C.2a viruses [21].

The 2014–2015 respiratory season is the first-time reports of false negative results or reduced sensitivity with commercial PCR tests have been associated with an entire clade(s) of viruses. Our lab was fortunate to have identified the problem early in October 2014, with routine interrogation amplification curves which allowed for the recognition of three cases with abnormal curves (Supple Fig. 1). Thus, all samples in our lab with requests for PFlu testing, were tested by both PFlu and PFAST. Hologic did modify the PFlu package insert to indicate limitations with our virus, but did little else to warn other customers as they falsely believed the phenomenon was restricted to our region. On the other hand, Cepheid did recognize issues and has made changes to their currently available assays to enable extended Flu and RSV Coverage.

Competing interests

The authors’ institution received several research grants from Abbott Molecular, BD, BioMerieux, and Roche Diagnostics. Dr. Stellrecht received reimbursement of travel expenses for attending meetings and conferences from Abbott Molecular, Quidel and Roche Diagnostics.

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Ethical approval

Obtained from Albany Medical Center.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2017.05.016.

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Table 6

Nucleotide sequence alignments between the WHO target region in M1 gene of isolates of influenza viruses. The A/Moscow/10/1999(H3N2) strain was used as a reference, homologous of other viruses are indicated by dashed lines and divergent sequences are indicated. The WHO Aw-1 primers and probe sequences are illustrated with underlining and boldface font, respectively [7].