Pandemic Preparedness in Hawaii: A Multicenter Verification of Real-Time RT-PCR for the Direct Detection of Influenza Virus Types A and B

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SYNOPSIS

Objective. We integrated multicenter, real-time (RTi) reverse transcription polymerase chain reaction (RT-PCR) screening into a statewide laboratory algorithm for influenza surveillance and response.

Methods. Each of three sites developed its own testing strategy and was challenged with one randomized and blinded panel of 50 specimens previously tested for respiratory viruses. Following testing, each participating laboratory reported its results to the Hawaii State Department of Health, State Laboratories Division for evaluation and possible discrepant analysis.

Results. Two of three laboratories reported a 100% sensitivity and specificity, resulting in a 100% positive predictive value and a 100% negative predictive value (NPV) for influenza type A. The third laboratory showed a 71% sensitivity for influenza type A (83% NPV) with 100% specificity. All three laboratories were 100% sensitive and specific for the detection of influenza type B. Discrepant analysis indicated that the lack of sensitivity experienced by the third laboratory may have been due to the analyte-specific reagent probe used by that laboratory. Use of a newer version of the product with a secondary panel of 20 specimens resulted in a sensitivity and specificity of 100%.

Conclusions. All three laboratories successfully verified their ability to conduct clinical testing for influenza using diverse nucleic acid extraction and RTi RT-PCR platforms. Successful completion of the verification by all collaborating laboratories paved the way for the integration of those facilities into a statewide laboratory algorithm for influenza surveillance and response.

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More than 200,000 people are hospitalized from influenza complications, and about 36,000 people die from influenza in the United States each year.[1] Influenza infection is primarily transmitted person to person by respiratory droplets, and symptoms are typically observed after incubation of one to four days. Symptoms commonly include fever, sore throat, dry cough, headache, myalgia, malaise, and/or anorexia.[2,3]

Historically, influenza testing was limited to virology laboratories, often associated with health departments, and used primarily for retrospective surveillance and vaccination development. However, advances in nucleic acid amplification and detection platforms, especially real-time (RTi) reverse transcription polymerase chain reaction (RT-PCR), have made these systems more available and useful to clinical laboratories. This technology offers the fastest turnaround time and the most sensitive detection and monitoring laboratory test for influenza. Testing performed by hospital laboratories would help individual patients by providing high-quality diagnostic testing in a clinically relevant time frame for both diagnosis and administration of antiviral therapy.[4–8] Incorporating the local private laboratories into the statewide testing algorithm (Figure 1) has the distinct advantages of both improving the timeliness of influenza test results and increasing the testing capacity of the state during abnormally high influenza endemicity. Early detection offers the potential to prevent and contain an epidemic or pandemic.[9–11]

The Hawaii State Department of Health (HDOH) recently established a five-year memorandum of agreement with three local, private, clinical laboratories to integrate them into the statewide influenza testing algorithm by establishing RTi RT-PCR influenza testing in these facilities. Laboratory verification is the important quality-management step that confirms or determines test performance characteristics before the test or system is used for patient testing.[12] Validation consists of the related, but ongoing, quality-assurance processes that ensure test performance continues to perform satisfactorily over time. Participants considered this multicenter verification a critical component in the process of establishing the cooperative influenza screening, surveillance, and response program. This article describes the performance of the collaborating laboratories in the use of RTi RT-PCR for influenza types A and B, and discusses the benefits of a novel public-private partnership in pandemic preparedness.
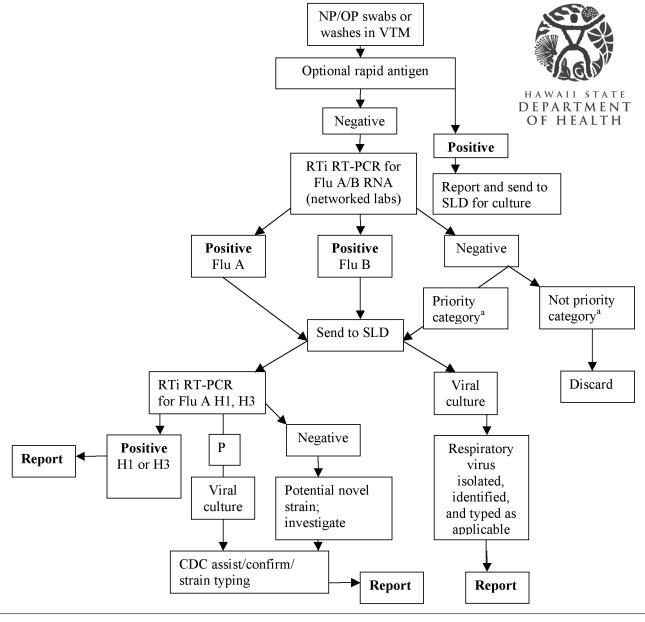
METHODS

Memoranda of agreement
HDOH outlined expectations for all parties and provided some start-up funding for participating laboratories. The state public health laboratory (PHL) served as coordinator and provided technical assistance. Planning was facilitated by statewide coordination meetings, which provided a forum for technical consultations, electronic reporting, consensus-building, logistical considerations, and verification strategies. Considerable discussion was devoted to the advantages of RTi RT-PCR, which enabled much higher quality rapid influenza testing than antigen-based assays.[13–15]

Laboratories were responsible for acquiring and implementing manual or automated nucleic acid extraction systems and RTi RT-PCR platforms, which included training, competency assessments, verification, equipment maintenance, and consumables procurement. Additionally, each laboratory was required to maintain a constant 1,000-clinical-specimen capacity to accommodate statewide surge capacity. The HDOH retained access to both the data, via electronic reporting, and the specimens, which were submitted to the State Laboratories Division (SLD) for culture, subtyping, and archiving. Specimen submission and SLD testing were guided by patient risk categories. For example, in addition to influenza PCR-positive specimens, PCR-negative specimens were submitted to SLD if the patient exhibited acute respiratory disease of unknown etiology, traveled outside the United States within 10 days prior to illness onset, or was epidemiologically linked to a respiratory disease outbreak as determined by HDOH disease investigators. Although the institutions had primary responsibility for ongoing validation, typically through accreditation by the College of American Pathologists or Centers for Medicare & Medicaid Services, submission of both positive and negative specimens to SLD for additional analysis further reduced the chance of test problems going undetected.

Verification panels
SLD prepared randomized, blinded verification panels of 50 specimens. Panels consisted of viral transport medium (VTM) that had been inoculated either with patient respiratory specimen or spiked with virus from cell culture. All patient identifiers were removed before assembling the panels. Twenty VTMs positive for influenza A were used; however, only subtype H1 was available, due to seasonal variation during which the H1 subtype dominated.[16] Influenza B-positive VTM also was not readily available, so 0.1 milliliter (ml) of supernatant from influenza B-positive cell culture was

**Figure 1. Hawaii statewide influenza testing algorithm used in the
Hawaii State Department of Health study to integrate multicenter,
RTi RT-PCR screening into a statewide laboratory algorithm for influenza surveillance and response**

[a]Priority categories include: hospitalized, acute respiratory disease of unknown etiology, X-ray-confirmed pneumonia, travel outside the U.S. within 10 days prior to illness onset, and outbreak associated.

RTi RT-PCR = real-time reverse transcription polymerase chain reaction

NP/OP = nasopharyngeal/oropharyngeal

VTM = viral transport medium

Flu A = influenza type A

Flu B = influenza type B

RNA = ribonucleic acid

SLD = State Laboratories Division

CDC = Centers for Disease Control and Prevention

diluted 1:10 in VTM. This spiked master VTM was tested to ensure the cycle threshold (Ct) value was realistic and aliquoted into 200 microliter (µL) volumes to serve as influenza B-positive test specimens. Twenty VTMs that were negative for influenza were also selected from the specimen archives. The 20 influenza A, 10 influenza B, and 20 negative samples were aliquoted into 200 µL volumes and placed in 2.0 ml CryoVials® (Sarstedt AG & Co., Nümbrecht, Germany). A total of four identical, randomized, blinded verification panels were prepared.

A secondary panel was prepared in the event that discrepant analysis required further testing. This smaller panel was to be used for discrepant analysis and was not intended to be a second process verification. It comprised a set of 20 samples: five influenza A (four H1 and one H3), five influenza B, and 10 negatives. The H3 specimen was a spiked VTM prepared in the same manner that the aforementioned influenza B samples were prepared. The second panel was randomized as described for the first panel.

Quality control
SLD analysts who were blinded to the key tested both the primary and the secondary verification panels, which ensured analysis yielded expected results and that no contamination had occurred during the panel preparation. It also was important to establish baseline

Ct values, especially for the spiked samples. Specimens were extracted using a QIAamp viral ribonucleic acid (RNA) mini-kit (cat #52906; Qiagen, Valencia, California) with a specimen processing control bead from the influenza A/B analyte-specific reagent (cat #ASRFLU-150N-040; Cepheid, Sunnyvale, California). RTi RT-PCR was performed using Superscript™ III Platinum One-Step Quantitative RT-PCR System master mix (cat #11732-088, Invitrogen, Life Technologies, Carlsbad, California) on a SmartCycler® II (Cepheid) amplification platform.

Multicenter verification
Participating laboratories designed their individual testing strategies utilizing various nucleic acid extraction methods, PCR amplification reagents, and RTi RT-PCR platforms (Figure 2). Reagent and test platform decisions were site-specific and based on the appropriateness of the extraction method and RTi RT-PCR platform for the particular site. Procurement of all reagents and equipment was the responsibility of each collaborating laboratory test site.

Sites entered results into a spreadsheet and submitted the data to SLD for evaluation. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each site.[17] One site (laboratory 3) required discrepant analysis and secondary panel testing.

Figure 2. Equipment and reagents selected by three private laboratory test sites to conduct clinical testing for influenza in the Hawaii State Department of Health study to integrate multicenter, RTi RT-PCR screening into a statewide laboratory algorithm for influenza surveillance and response

Lab site identification	Nucleic acid extraction kit	Nucleic acid extraction platform	Real-time amplification	Real-time detection platform
1	MagNA pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science;[a] cat #03038505)	MagNA pure LC (Roche Applied Science[a])	Flu A/B ASR (Cepheid;[b] cat #ASRFLU-150N-040) and QIAGEN®[c] OneStep RT-PCR Kit (cat #210212)	SmartCycler® II (Cepheid[b])
2	NucliSENS® easyMAG® consumables (Biomerieux)[d]	NucliSENS® easyMAG® (Biomerieux)[d]	Flu A/B ASR (Cepheid;[b] cat #ASRFLU-150N-040) and QIAGEN®[c] OneStep RT-PCR Kit (cat #210212)	SmartCycler® II (Cepheid[b])
3	MagNA pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science;[a] cat #03038505)	MagNA pure LC (Roche Applied Science)[a]	ProFlu-1™ Real Time Assay and ProFlu-Plus™ Real Time Assay (Prodesse, Inc.;[e] cat #H44VK77)[f]	Rotor-Gene™ 6000 (Corbett Life Science, QIAGEN®[c])

[a]Indianapolis, Indiana

[b]Sunnyvale, California

[c]Valencia, California

[d]Durham, North Carolina

[e]Waukesha, Wisconsin

[f]ProFlu-Plus™ Real Time Assay (Prodesse, Inc.; cat #H44VK77) replaced ProFlu-1™, which was used for panel #2. ProFlu-1 is no longer available.

RTi RT-PCR = real-time reverse transcription polymerase chain reaction

RESULTS

The results for SLD quality-control testing and all three sites, including Ct values, mean, and standard deviation, are listed in the Table and Figure 3. The SLD Ct value for all influenza B specimens was the value from the spiked master VTM prior to aliquoting into 200 µL volumes. Consequently, the SLD Ct values for influenza B were identical. Results from laboratories 1 and 2 were the same as the key, so the sensitivity [true positive (TP)/(TP+false negative [FN]) × 100], specificity [true negative (TN)/(false positive [FP]+TN ×

100], PPV [TP/(TP+FP) × 100], and NPV [TN/(TN+FP) × 100] were 100% for both influenza type A: [20/(20+0) × 100], [30/(30+0) × 100], [20/(20+0) × 100], [30/(30+0) × 100]; and type B: [10/(10+0) × 100], [40/(40+0) × 100], [10/(10+0) × 100], [40/(40+0) × 100], respectively.

The results from laboratory 3 indicated a problem with the detection of influenza type A. Only 12 (60%) of the 20 type A specimens (Table) were positive. Although the specificity [30/(30+0) × 100] and PPV [20/(20+0) × 100] for influenza type A were 100%,

Table. Ct values, mean, and SD for specimens testing positive for influenza types A and B at three private laboratory test sites in the Hawaii State Department of Health study to integrate multicenter, RTi RT-PCR screening into a statewide laboratory algorithm for influenza surveillance and response

Sample	SLD key	SLD (Ct)	Lab 1 (Ct)	Lab 2 (Ct)	Lab 3 (Ct)	Mean (Labs 1–3)	SD
1	Flu A	35.5	35.5	37.8	0.0[a]	36.7	1.6
3	Flu A	27.0	26.5	29.1	31.1	28.9	2.3
6	Flu A	28.7	27.8	30.1	34.7	30.9	3.5
8	Flu A	32.6	32.1	34.4	0.0[a]	33.3	1.6
10	Flu A	27.9	27.9	30.3	32.2	30.1	2.2
14	Flu A	31.2	30.8	33.6	0.0[a]	32.2	2.0
20	Flu A	21.5	20.4	23.4	24.6	22.8	2.2
21	Flu A	31.1	30.9	33.1	0.0[a]	32.0	1.6
22	Flu A	24.8	25.4	27.8	27.3	26.8	1.3
26	Flu A	30.2	31.4	33.3	0.0[a]	32.4	1.3
27	Flu A	29.3	29.1	31.4	35.9	32.1	3.5
29	Flu A	23.4	23.8	23.8	28.2	25.3	2.5
30	Flu A	29.8	29.5	29.3	0.0[a]	29.4	0.1
35	Flu A	25.4	25.1	24.8	30.8	26.9	3.4
41	Flu A	27.0	26.2	26.4	34.6	29.1	4.8
43	Flu A	31.9	31.6	31.3	0.0[a]	31.5	0.2
49	Flu A	27.8	27.3	27.1	29.3	27.9	1.2
51	Flu A	33.5	32.6	32.9	0.0[a]	32.8	0.2
52	Flu A	27.6	27.9	27.5	31.4	28.9	2.1
55	Flu A	32.2	31.3	31.5	34.9	32.6	2.0
2	Flu B	20.9	20.8	23.7	21.8	22.1	1.5
11	Flu B	20.9	20.7	23.6	21.8	22.0	1.5
18	Flu B	20.9	27.2	31.0	30.4	29.5	2.0
23	Flu B	20.9	20.5	24.0	23.7	22.7	1.9
28	Flu B	20.9	20.1	23.9	22.7	22.2	1.9
31	Flu B	20.9	20.6	20.8	23.4	21.6	1.6
39	Flu B	20.9	20.1	20.7	22.8	21.2	1.4
44	Flu B	20.9	21.1	20.9	22.5	21.5	0.9
45	Flu B	20.9	20.5	20.8	21.9	21.1	0.7
48	Flu B	20.9	24.2	24.9	25.7	24.9	0.8
n=20 other	Negative	0.0	0.0	0.0	0.0	NA	NA

[a]Not included in calculations

Ct = cycle threshold

SD = standard deviation

RTi RT-PCR = real-time reverse transcription polymerase chain reaction

SLD = State Laboratories Division

Flu A = influenza type A

Flu B = influenza type B

NA = not applicable

the sensitivity $[20/(20+8) \times 100]$ was 71% with an NPV $[40/(40+8) \times 100]$ of 83%. The sensitivity, specificity, PPV, and NPV were 100% at laboratory 3 for influenza type B: $[10/(10+0) \times 100]$, $[40/(40+0) \times 100]$, $[10/(10+0) \times 100]$, $[40/(40+0) \times 100]$, respectively. After discrepant analysis revealed the problem was the PCR reagent, the laboratory acquired a new generation of reagent[18] and tested a secondary panel. Test results for the secondary panel demonstrated 100% sensitivity $[5/(5+0) \times 100]$, specificity $[15/(15+0) \times 100]$, PPV $[5/(5+0) \times 100]$, and NPV $[15/(15+0) \times 100]$ for influenza types A and B.

Ct values among all three sites were very comparable, showing little inter-laboratory variation (Table, Figure 3).
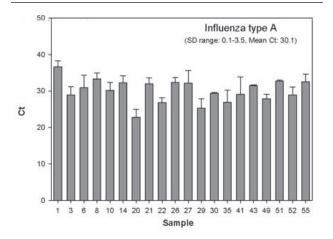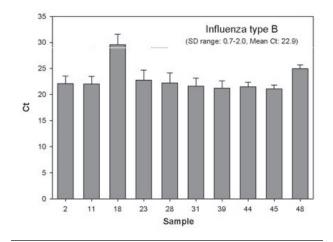
DISCUSSION

This evaluation intended to verify that the partner laboratories' testing systems were yielding the correct results; it was not an evaluation of specific products or a substitute for institutional quality management. The three collaborating sites independently developed processes that yielded excellent test performance for RTi RT-PCR detection of influenza. All sites demonstrated 100% sensitivity, specificity, PPV, and NPV following discrepant analysis. These testing data, along with fairly consistent Ct values, indicated a high-quality testing outcome could be expected regardless of site.

Rapid and timely testing can offer providers relevant diagnostic information that can directly influence their decision to utilize antiviral therapy.[4–8] Expansion of influenza testing from a health department-directed, surveillance-only task to decentralized diagnostic testing not only improved day-to-day patient care, but also greatly increased statewide capacity from a single state government laboratory to a total of four laboratories for pandemic preparedness. Furthermore, this collaboration mitigated the inherent risks associated with having all influenza testing for an isolated state dependent on one facility. A single laboratory facility would be inadequate during a pandemic and would expose the state to vulnerabilities if significant damages were sustained from a natural disaster.

Logistical advantages included increased stocks of consumables and less risk of expiration with multiple laboratories rotating stock, both of which are critical to an island state. Testing efficiency was realized because private laboratories agreed to submit nucleic acid extracts on RTi RT-PCR-positive specimens; therefore, the PHL could perform influenza A subtyping without having to perform another extraction. Furthermore, private laboratories continued to send unextracted

Figure 3. Graphic depiction of Ct values, mean, and SD range for specimens testing positive for influenza types A and B at three private laboratory test sites in the Hawaii State Department of Health study to integrate multicenter, RTi RT-PCR screening into a statewide laboratory algorithm for influenza surveillance and response

Ct = cycle threshold

SD = standard deviation

RTi RT-PCR = real-time reverse transcription polymerase chain reaction

specimens in VTM, which was required for cultivation and necessary for Hawaii's year-round contribution to national influenza surveillance, antiviral resistance detection, and annual vaccine development. Hawaii's travel-centric location in the Pacific provides isolates that are often distinct from mainland isolates. Through this laboratory partnership, the private laboratories assisted the HDOH in monitoring viral respiratory illness not caused by influenza (Figure 1).

The community laboratories enjoyed distinct benefits

as a result of their participation. The effort incorporated molecular platforms for influenza detection into the clinical laboratories and provided the foundation for further expansion of the technology into their diagnostic repertoire. It also brought enhanced collaboration, exemplified by agreements to standardize some electronic data elements such as Health Level Seven messages, which will enable data exchange that will reduce both keystrokes and errors. These benefits clearly extend beyond influenza testing and have strengthened laboratory networks in Hawaii.

Multicenter verifications simplify discrepant analysis, which is a distinct advantage over single-laboratory verifications. Several experienced laboratories testing blinded specimens in a collaborative verification can bring more clarity to complex processes. Proficiency testing, one of the cornerstones of quality laboratory management, is another good example of the effectiveness of multi-laboratory evaluations.[19,20] A European report indicated excellent performance of the Prodesse ProFlu-1™ Real Time Assay;[21] however, a multisite study in Hawaii identified the product as insensitive for the detection of locally circulating influenza type A subtypes.[22] Community laboratory collaboration and experience, in conjunction with objective discrepant analysis, verified that the next-generation ProFlu-Plus™ Real Time Assay, which was Food and Drug Administration-approved in 2008,[18] had resolved sensitivity problems. In the present study, the difficulties encountered by laboratory 3 were quickly narrowed from the many variables in the testing process to a locally established limitation of the ProFlu-1 Real Time Assay. This discrepancy was easily resolved at laboratory 3 using a smaller secondary panel.

CONCLUSIONS

Public and private laboratory collaboration can optimize routine testing capacity and provide a foundation for effective response to public health emergencies. Mutual support agreements, such as the memoranda of agreement described in this article, outline the preparation, planning, and response framework critical to an effective crisis-response relationship.[23] The present agreement between private laboratories and the HDOH was an unprecedented, mutually beneficial partnership. This innovative collaboration provides a unique opportunity to maximize limited resources.

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