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ORIGINAL ARTICLE

Diagnostic capacity of rapid influenza antigen test: Reappraisal with experience from the 2009 H1N1 pandemic

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KEYWORDS

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Background: The rapid influenza antigen test (RIAT) has been questioned because of its poor sensitivity. Clinicians are confused as to what diagnostic help it may provide. RIAT was reappraised by other laboratory confirmatory tests for its diagnostic capacity.

Methods: Records of RIAT, RT-PCR and virus culture, performed for upper respiratory tract samples during the period from July 2009 to January 2010, were reviewed. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of RIAT were evaluated with RT-PCR and virus culture as reference.

Results: With either positive RT-PCR or positive virus culture as confirmation of presence of the virus, the overall sensitivity was 44.2% (235/532) and the overall PPV was 91.1% (235/258). With both negative RT-PCR and negative virus culture as confirmation of absence of the virus, the overall specificity was 98.2% (239/264) and the overall NPV was 92.6% (239/258). The PPV reached 96.2% during peak prevalence of infection and the NPV increased to 91.7% with low prevalence. The sensitivity for seasonal H3N2 was 56% (56/100), significantly better than the 39.6%, (156/394) for 2009 pandemic H1N1. Although RIAT positivity correlated to the viral load in samples, a substantial amount of negative RIAT samples had high viral load, with 16.8% (260/1548) of Ct value less than 36 and 8.8% (136/1548) of Ct value less than 31.

Conclusion: An algorithm is derived for the fast and inexpensive point-of-care laboratory test RIAT for appropriate application in clinical diagnosis of influenza virus infection. In peak seasons, positive RIAT confirms the diagnosis, with PPV over 96%. In low seasons, negative RIAT sufficiently excludes the diagnosis, with NPV over 91%. The sensitivity of RIAT may vary with

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different species of the influenza virus. Negative RIAT is not necessarily equal to low viral load in the upper respiratory tract or low infectivity of the patient.

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Introduction

Influenza virus infection is an important respiratory disease because it is highly contagious and responsible for considerable morbidity and mortality. Certain populations including, but not limited to, the elderly and compromised individuals are at risk for severe complications.^{1–3} Clinical diagnosis of influenza can be difficult because of the nonspecific presentations. Timely diagnosis, with laboratory confirmation if possible, is crucial for appropriate management including distancing and antiviral medication.⁴ The rapid influenza antigen test (RIAT), as a point-of-care test, is frequently employed to confirm clinical suspicion. Unfortunately, the diagnostic capacity of RIAT has been questioned because it has poor sensitivity.^{5,6} RIAT is matchless for the speed with which test results are available, less than 30 minutes, and its cost is relatively low compared with molecular diagnosis with real-time polymerase chain reaction (RT-PCR) or virus culture. During the 2009 H1N1 influenza pandemic, a huge number of people undertook RIAT and other confirmatory laboratory tests for influenza virus infection. These clinical datas in our hospital were reviewed to reappraise the diagnostic capacity of RIAT to provide information on RIAT interpretation for better support of clinical diagnosis.

Materials and methods

Clinical materials

A cross-sectional diagnostic accuracy study was conducted at Chang Gung Memorial Hospital Linkou Medical Center, a 4000-bed tertiary care hospital in northern Taiwan. Electronic medical records of clinical specimens tested for influenza from July 2009 to January 2010 were reviewed. Samples were from out-patients or in-patients suspected to have influenza virus infection. Specimens were collected for QuickVue influenza A+B RIAT (Quindal, San Diego, USA) which is a commercially available lateral-flow immunoassay for detection of influenza A and B virus antigen, RT-PCR, or respiratory virus culture. Only upper respiratory tract specimens (nasal swab or throat swab) were included. Samples of lower respiratory tract specimen or from other sites were excluded. This study was approved by Chang Gung Memorial Hospital Institutional Review Board.

Rapid influenza antigen test

Specimens were sampled by physicians at clinical settings, sent to a laboratory within 30 minutes of being sampled and processed at the laboratory by trained technicians under manufacturer's instructions (QuickVue influenza A+B test).

Patient specimens were placed in a reagent tube for virus nucleoprotein extraction. A test strip containing mouse monoclonal anti-influenza virus A and B antibodies was placed in the reagent tube for detection of virus antigens.

Quantitative reverse transcriptase RT-PCR

The specimens for RT-PCR were stored at 4°C in a virus transport medium and sent to the virology laboratory within 30 minutes of being sampled. Viral RNA was extracted by MagNA PURE Autoextractor with MagNA Pure LC Total Nucleic Isolation Kit (Roche Diagnostics, Germany). Extracted nucleic acid was amplified in ABI 7000/7900 instrument with commercial kit TaqMan one-step RT-PCR mix reagent (Applied Biosystems, Foster City, CA). The total reaction mix volume was 25 µL containing H₂O 1.03 µL, 2× TaqMan Mix 12.5 µL, Flu A forward/reverse primer 2.4 µL, Flu B forward/reverse primer 2.4 µL, Flu A probe 0.5 µL, Flu B probe 0.5 µL, 40 × RNase inhibitor 0.67 µL, and RNA 5 µL. Matrix gene of influenza A virus and hemagglutinin gene of influenza B virus were chosen as the detection target. Probes and primers for influenza A were (InfA Forward) 5' AAG ACC AAT CCT GTC ACC TCT GA 3', (InfA reverse) 5' CAA AGC GTC TAC GCT GCA GTC C 3', and (InfA probe) FAM-5' TTT GTG TTC ACG CTC ACC GT 3'-TAMRA. Probes and primers for influenza B were (InfB Forward) 5' GAG ACA CAA TTG CCT ACC TGC TT 3', (InfB Reverse) 5' TTC TTT TCC CAC CGA ACC AAC 3' and (InfB probe) VIC-5' AGA AGA TGG AGA AGG CAA AGC AGA ACT AG C3'-TAMRA. Cycling conditions were as follows: initial hold at 48°C for 30 minutes and 95°C for 10 minutes followed by 45 cycles at 95°C for 15 seconds and 58°C for 1 minute. Human RNase P gene was detected with RNase P primers and probe for all clinical specimens to ensure acceptable quality and avoid false negative reports. A positive control containing 200 µL influenza A and influenza B isolates was used as external positive control. A sample containing 200 µL polymerase chain reaction (PCR) grade H₂O was used as negative control.

The cycle threshold (Ct) represented the amplification cycle number at which a significant increase in fluorescence signal was detected. Lower Ct values indicate less amplification cycles for signal to be detected and therefore specify more viral nucleic acid in the specimen. Specimens presumed to be positive for influenza A (Ct ≤ 40) were then subtyped for 2009 H1N1 swine influenza A and human seasonal influenza A (H1 and H3). The NP gene of the swine influenza virus was the detection target. The probes and primers are (SW FluA forward) 5' GCA CGG TCA GCA CTT ATY CTR AG 3', (SW FluA reverse) 5' GTG RGC TGG GTT TTC ATT TGG TC 3', and (SW FluA probe) 5' CYA CTG CAA GCC CAT ACA CAC AAG CAG GCA 3'. Amplification was performed in a LightCycler 1.0 (Roche Molecular Biochemicals,

Indianapolis, IN) with total reaction mix volume of 25 μ L containing H₂O 2.33 μ L, 2 \times TaqMan Mix 12.5 μ L, Swine Flu A forward/reverse primer 4 μ L, Swine Flu A probe 0.5 μ L, 40 \times RNase inhibitor 0.67 μ L, and RNA 5 μ L. Cycling conditions were as follows: initial hold at 48°C for 30 minutes and 95°C for 10 minutes followed by 45 cycles at 95°C for 15 seconds and 55°C for 30 seconds.

For the quantitative assay, a reference standard was prepared using the plasmid DNA serial dilution with PCRII-TOPO vector (Invitrogen, San Diego, CA). The lowest limit of RT-PCR for detection of influenza A and influenza B was approximately 100 copies/ μ L and was 10 copies/ μ L for swine H1N1.

Virus culture

Nasal and throat swabs were transported via a virus isolation transport medium. The respiratory specimen was inoculated with a human laryngeal carcinoma cell, human embryonal lung diploid fibroblast cell, human malignant embryonic rhabdomyosarcoma, monkey kidney cell (MK-2), and Madin–Darby canine kidney cell (MDCK) separately. Cells were grown in Eagle's Minimum Essential Medium (Gibco, Grand Island, NY) containing tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (2 μ g/mL) (Sigma, St. Louis, MO) at 5% CO₂ in a 35°C incubator for 14 days. Cytopathic effect (CPE) was checked daily. Specimens with positive CPE were tested with immunofluorescence antibody staining for virus type confirmation. Specimens inoculated in MK-2 and MDCK culture cells without CPE were also tested with commercial immunofluorescence antibody staining (CHEMICON International Inc., Temecula, CA) for seven respiratory viruses.

Statistical analysis

We calculated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the RIAT, with RT-PCR and virus culture as the references. We used an exact binomial method deriving 95% confidence intervals. Fisher's exact tests or Chi-square tests were used to examine the associations in contingency tables when appropriate. A two-tailed *p* value < 0.05 was considered statistically significant. All other analyses were conducted with an IBM Statistical Package for the Social Sciences version 18.0 (SPSS Inc, Somers, NY, USA).

Results

Specimens and laboratory tests

After excluding samples positive for influenza B in any of the three laboratory tests (*n* = 18), a total of 14,072 upper respiratory tract specimens were tested for RIAT, 6661 for RT-PCR and 2519 for respiratory virus culture, during the period from July 2009 to January 2010. The diagnostic capacity of RIAT was examined by RT-PCR or virus culture which was performed less than 3 days apart from RIAT. Then 1787 RIAT results were checked by RT-PCR, 680 by respiratory virus culture, 1835 by either RT-PCR or virus culture, and 374 by both RT-PCR and virus culture.

Sensitivity and specificity

The sensitivity and specificity of RIAT can be evaluated with RT-PCR or viral culture as the standards. They can also be assessed with RT-PCR and viral culture combined together, with either positive RT-PCR or positive virus culture as the presence of infection and with both negative RT-PCR and negative virus culture as the absence of infection. The overall sensitivity ranged from 28.5% to 44.2% and the overall specificity ranged from 90.5% to 98.1%, depending on reference test used (Table 1).

Positive predictive value

PPV is the likelihood for patients with a positive test to have the disease in reality. Because RIAT was a test of very high specificity, from 90.5% to 98.1%, it is not surprising that the PPV of RIAT is also high. RIAT has an overall PPV ranging from 72.5% to 91% depending on which confirmatory laboratory tests were used as the reference. There were 1787 samples of RIAT that could be checked with RT-PCR. Among them, 89.5% (214/239) of the positive results were confirmed by positive RT-PCR. For 680 RIAT samples checked by virus culture, 72.5% (37/51) of the positive RIAT were confirmed with positive culture. Combined together, 1835 RIAT specimens were checked with either RT-PCR or virus culture. As high as 91.1% (235/258) positive RIAT were confirmed by either RT-PCR or virus culture.

The performance of RIAT was even approaching being perfect as the disease prevalence was high. During the month of peak activity of influenza infection in August 2009, the PPVs were 94.1% (96/102), 75.0% (9/12) and 96.2% (100/104), as confirmed by RT-PCR, virus culture, and either RT-PCR or virus culture, respectively. That is, during peak season, influenza virus infection was substantiated for 96.2% of the RIAT positive patients by RT-PCR or virus culture.

However, the accuracy of PPVs decreased in relation to the monthly reduction in disease prevalence. It became as low as only 76.5% (13/17) as verified by RT-PCR or virus culture in December 2009 and January 2010 as the 2009 H1N1 influenza pandemic came to an end. (Table 2, Fig. 1).

Negative predictive values

Because RIAT is a test of low sensitivity, ranging from 28.5% to 44.2%, it has been well known that negative RIAT is not

Table 1 Diagnostic accuracy of RIAT for influenza virus infection

Reference	Total (<i>n</i>)	Sensitivity	Specificity
RT-PCR	1787	43.3% (214/494)	98.1% (1268/1293)
Virus culture	680	28.5% (37/130)	97.5% (536/550)
RT-PCR(+) OR culture (+)	1835	44.2% (235/532)	—
RT-PCR(–) AND culture(–)	374	—	90.5% (239/264)

RIAT = rapid influenza antigen test.

Table 2 Monthly variation of positive predictive values of RIAT

Reference	Aug	Sep	Oct	Nov	Dec/Jan	Overall
RT-PCR	94.1% (96/102)	88.1% (59/67)	90.9% (30/33)	81.8% (27/33)	78.6% (11/14)	89.6% (223/249)
Culture	75.0% (9/12)	75.0% (9/12)	54.5% (6/11)	75% (9/12)	100% (4/4)	72.5% (37/51)
RT-PCR (+) OR culture (+)	96.2% (100/104)	90.1% (64/71)	90.6% (29/32)	85.3% (29/34)	76.5% (13/17)	91.1% (235/258)

RIAT = rapid influenza antigen test.

reliable for exclusion of the diagnosis of influenza infection; that is, the NPV is low. The NPV was 80% (36/45) in our patient population during the peak season of influenza infection. That is, one out of five RIAT negative patients were indeed with the infection, as confirmed later by either RT-PCR or virus culture.

However, NPV will be improved if more patients with no disease are tested. This was shown in our data as the disease prevalence went down. The NPVs were around 90% in succeeding months after the peak in August 2009; NPVs were over 90% at the end of the 2009 H1N1 epidemic (Table 3, Fig. 1).

RIAT has overall NPVs ranging from 81.9% to 92.6%. For 1787 RIAT samples checked by RT-PCR, 81.9% (1268/1548) of the negative RIAT also had negative RT-PCR. For 680 RIAT specimens verified by virus culture, 85.1% (533/626) negative RIAT revealed no culture growth either. For 374 RIAT samples cross checked with both RT-PCR and virus culture, 92.6% (239/258) of the RIAT negatives were neither RT-PCR nor virus culture positive.

Correlation between RIAT and viral load

RIAT detection is supposed to be correlated to the viral load in the samples. As expected, the RIAT positive rate was in reverse proportion to Ct values of RT-PCR (Fig. 2). For RIAT

positive specimens, 82.4% (197/239) had RT-PCR Ct values less than 35 and 71.5% (171/239) had Ct values less than 31 (Table 4). In other words, 82.4% of the RIAT positive samples had a viral load more than 10 copies/ μ L and 71.5% had viral loads more than 100 copies/ μ L.

However, it does not mean that all RIAT negative samples had a negligible amount of virus because 15.6% (241/1548) of RIAT negative samples had Ct values less than 35 and 8.8% (136/1548) had less than 31. These results mean that 15.6% had viral loads more than 10 copies/ μ L and around 10% had viral loads more than 100 copies/ μ L.

Different performance for swine H1N1 and seasonal H3N2

Interestingly, the performance of RIAT for 2009 swine H1N1 influenza and the seasonal strain H3N2 influenza virus cocirculating in that same year is different. Compared with RT-PCR, the sensitivity was 39.6% (156/394) for swine H1N1 and 56% (56/100) for seasonal H3N2 ($p = 0.003$). With virus culture growth as the reference, the sensitivity was 27.8% (35/126) for swine H1N1 and 50% (2/4) for seasonal H3N2 (statistically insignificant). With either positive PCR or positive culture growth as the standard, the sensitivity was 43% (227/528) for swine H1N1 and 57% (57/100) for seasonal H3N2 ($p = 0.01$) (Table 5). RIAT performed differently for two influenza A strains cocirculating in the same time period of year 2009.

Discussion

Influenza virus infection is an important disease with the potential to spread quickly and cause severe illness. As an ever changing agent with frequent genetic drifts and shifts, it has caused many epidemics and pandemics in history and poses a continuous threat to our human society. Clinicians should be ready for precise recognition of influenza among a variety of infections which also cause influenza-like illness. Help from laboratory methods is usually desired.

It is a well-accepted thought that RIAT is a test with poor sensitivity and it is even obsolete in many medical institutions. In the view of statisticians, sensitivity is the percentage of sick people who are correctly identified. As a screening test, RIAT-based diagnosis will miss half of the diseased because of its 50% or so sensitivity. However, people, even some medical professionals, may misunderstand 50% sensitivity as random positivity, and consider that

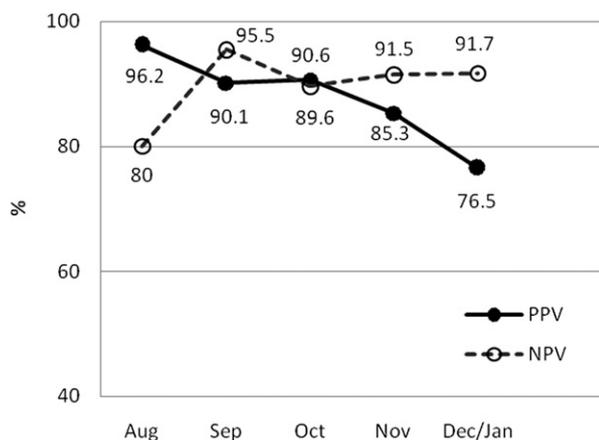


Figure 1. Change of RIAT PPV and NPV with month as confirmed by positive PCR or culture. NPV = negative predictive value; PCR = polymerase chain reaction; PPV = positive predictive value; RIAT = rapid influenza antigen test.

Table 3 Monthly variation of negative predictive values of RIAT

Reference	Aug	Sep	Oct	Nov	Dec/Jan	Overall
RT-PCR	61.0% (191/313)	88.3% (409/463)	84.2% (186/221)	82.1% (206/251)	92.0% (276/300)	81.9% (1268/1548)
Culture	78.5% (62/79)	91.7% (144/157)	81.5% (97/119)	75.4% (98/130)	93.6% (132/141)	85.1% (533/626)
RT-PCR (-) AND culture (-)	80.0% (36/45)	95.5% (84/88)	89.6% (43/48)	91.5% (54/59)	91.7% (22/24)	92.6% (239/258)

RIAT = rapid influenza antigen test.

the chances that RIAT is positive or negative are equal to tossing a coin. It is a misconception. RIAT is a test of high specificity; that is, patients with no disease rarely turned out to be RIAT positive.

Beyond sensitivity and specificity, front-line clinicians are more concerned about the PPVs and NPVs of RIAT when they are contemplating the likelihood of infection with either positive or negative test results. The very high PPV with high disease prevalence and very high NPV with low disease prevalence indicate that RIAT is indeed a valuable test. An algorithm is derived for interpretation and application of this fast and relatively inexpensive point-of-care test for clinical diagnosis of influenza infection. During a high prevalence period, positive RIAT confirms the diagnosis of influenza infection because 96.2% of them can be verified by RT-PCR or virus culture. However, negative RIAT does not preclude measures against influenza infection if clinical suspicion is high. During a low prevalence period, the simple RIAT with negative result almost excludes the possibility of infection. Further study with PCR or virus culture will only be indicated for those few strongly suspected to have the infection. In contrast to a high prevalence period, positive RIAT in low prevalence is not equal to infection. The positive test has to be verified by other laboratory tests.

This algorithm is of crucial practical significance. Diagnosis of influenza infection based on clinical manifestations is only difficult because many other respiratory pathogens also cause influenza-like illness. Laboratory confirmation is indispensable. Taking into account the huge number of

upper respiratory infections in influenza seasons, indiscriminate employment of laboratory tests, especially the molecular method RT-PCR, is costly with formidable consumption of medical resources. With the algorithm derived from our studies, during epidemics RT-PCR or culture may be saved for at least half of the infected patients in whom RIAT are positive. RIAT itself confirms the diagnosis. During peak seasons, RIAT negative patients still have to be managed as with the infection. For some of them, especially those with the potential of evolution into a severe disease, RT-PCR and culture may be applied for definitive diagnosis. Negative RIAT during a low prevalence period also saves the use of PCR and virus culture in the majority of patients because negative RIAT itself excludes the diagnosis.

This algorithm has actually already been laid down in the 2009 Infectious Diseases Society of America guideline for diagnosis and treatment of seasonal influenza infection.⁷ However, it is based on expert opinions only. Our data is the first solid evidence to support this scheme of healthcare logistics.

Although definitive diagnosis is desired by clinicians, it is still crucial to ask whether definitive diagnosis is always indicated. The majority without severe illness can be treated as with the infection even without an ultimate diagnosis. The definitive diagnosis is more important for patients at risk of evolution into severe disease.

It has been reported that RIAT positivity correlates to the viral load in the specimens.^{8,9} Our data also confirmed this association. However, it cannot be assumed that patients with negative RIAT always have a low viral load in their upper respiratory tract. They may have a high load and be very infectious as well. In addition, we and other groups have observed a paradoxical fact that the majority of patients with severe disease had negative RIAT.^{10,11} The

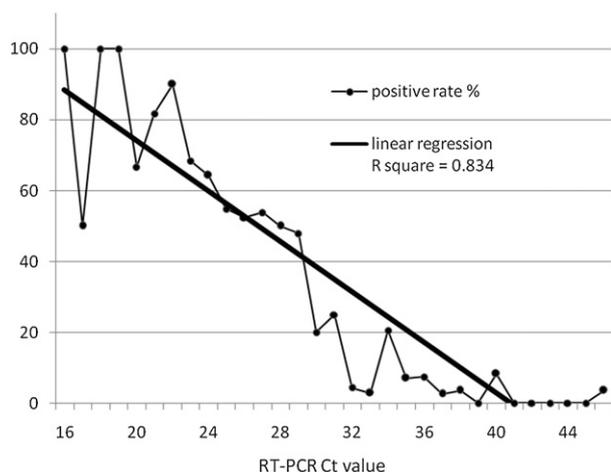


Figure 2. Positive rates of RIAT were in reverse proportion to RT-PCR Ct value. Ct = cycle time; RIAT = rapid influenza antigen test.

Table 4 RIAT and RT-PCR cycle-time values

RIAT	Viral load (copies/ μ L)	Positive (n = 239)	Negative (n = 1548)
Ct <31	100	71.5% (171/239)	8.8% (136/1548)
Ct 31–35	10	10.9% (26/239)	6.8% (105/1548)
Ct 35–40	—	1.6% (4/239)	2.8% (38/1548)
No signal	—	15.9% (38/239)	82.0% (1269/1548)

Ct = cycle time; RIAT = rapid influenza antigen test.

Table 5 Sensitivity of RIAT for swine H1N1 and seasonal H3 influenza in 2009

Reference	Swine H1N1	Seasonal H3	p
RT-PCR	39.6% (156/394)	56.0% (56/100)	0.003
Virus culture	27.8% (35/126)	50.0% (2/4)	NS
RT-PCR or virus culture	43.0% (227/528)	57.0% (57/100)	0.01

NS = not significant; RIAT = rapid influenza antigen test.

reason behind this phenomenon is under study. It is possible that the virus has descended to the lower respiratory tract and that the RIAT in the upper respiratory tract specimen can rarely turn positive.

During the year 2009, two strains of influenza A virus cocirculated. Our data indicated that the performance of RIAT may vary with different strains of virus. The results are similar to what people have reported.^{8,12,13}

There are some limitations of our study. First, this is a retrospective study that reviewed the medical and laboratory database. Specimen quality may vary with the experience and skill of different clinicians who took the nasal or throat swab samples. Second, our results were limited only to the QuickVue test, one of the many RIATs commercially available. Different test kits may give different results.^{6,13–16} Third, virus culture is the confirmatory test with lowest sensitivity. RIAT specificity may be overestimated when virus culture was taken as the standard for comparison.

In conclusion, RIAT is a fast and relatively inexpensive point-of-care test. Its high PPV and specificity has a valuable role in helping clinicians diagnose influenza infection. A positive RIAT result denotes a high amount of viral loads and mandates timely antiviral agents use.

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