Comparison of Influenza Virus Detection Methods

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In this study, we provide a cross-sectional comparison of existing influenza virus detection
methods in terms of their sensitivity, sample volume required, and process time needed for
an assay. Virological techniques, immunological techniques, and real-time polymerase chain
reaction were examined. An identical lot of influenza virus stock was used for the experiments.
The result gives an index for the evaluation of the performance abilities of virus detection
systems.

1. Introduction

The fields of clinical medicine and preventive epidemiology call for simple and high-
performance techniques for the sensitive detection and early identification of pathogens, such
as the influenza virus. Influenza is an infectious disease that can be global in scope in annual
outbreaks. Children ages 5 years or younger, the elderly aged 65 years or older, pregnant
women, and patients with underlying diseases of the respiratory, metabolic, and immune
systems are at heightened risk of increased severity of infection, according to the World Health
Organization, with outbreaks profoundly affecting economic activities.¹

The influenza virus is a negative-sense RNA virus of the Orthomyxoviridae family with an
RNA genome of eight or seven segments.² It is further classified into variable strains on the
basis of the viral surface antigens hemagglutinin (HA) and neuraminidase (NA). The virus has
been isolated from humans, other mammals, and birds, and the host range is determined by the
combination of HAs and NAs.² Eighteen HAs and 11 NAs have been identified so far.³ An
antigenic shift occurs when different influenza virus strains recombine their genomes, resulting
in a new strain having a mixture of HAs and NAs of original strains.² This occasionally
triggers outbreaks by producing a strain that has never infected humans. A well-known
example is the 1968 influenza pandemic caused by the emergence of the H3HA subtype.⁴
Moreover, highly pathogenic viruses that have arisen from an avian influenza virus such as
the H5N1 subtype have been threatening human public health.² It is thus important that
surveillance of the influenza virus is achieved precisely to prevent the pandemics.

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Methods of influenza virus detection include virological techniques, such as hemagglutination and plaque assays,\(^\text{5}\) immunological techniques, such as immunochromatography,\(^\text{6}\) and enzyme-linked immunosorbent assay (ELISA),\(^\text{7}\) and nucleic acid amplification techniques, such as real-time polymerase chain reaction (RT-PCR)\(^\text{8}\) and loop-mediated isothermal amplification.\(^\text{9,10}\) In addition, new methods have been developed on the basis of these detection strategies.\(^\text{11,12}\) However, to the best of our knowledge, there is no index for the comparison of the detection abilities of these methods. Such an index would be important for selecting an appropriate method matching for a purpose and evaluating the merits and demerits of a newly emerging technology.

In this research, we carried out a cross-sectional comparison of influenza virus detection methods to determine the index of sensitivity, sample volume required, and process time required for an assay using an identical lot of influenza virus stock.

### 2. Materials and Methods

#### 2.1 Virus

Frozen aliquots of the influenza A virus strain A/Panama/2007/99 (H3N2), propagated from a purified plaque, were used in the experiments. In this study, dilution factor is used as an index for comparing sensitivity. Here, the dilution factor means the dilution ratio of the virus suspension used for each assay from the stock solution. Dilution with reagent solutions is not included in the calculation of the dilution factor in an assay in which reagents are mixed in a virus suspension. What is important is the original concentration of the sample, not the concentration in the final detection system, which depends on the volume of reagents added. If we compare the performance using the virus concentration after adding reagents, an assay taking into consideration the reagents added will result in the evaluation showing higher sensitivity, and the performance will be overestimated. This is the reason why the dilution factor as the index for comparing sensitivity does not include the dilution rate with reagent solutions.

#### 2.2 Hemagglutination assay

Hemagglutination titers were determined by hemagglutination assay. The principle of the hemagglutination assay is shown in Fig. 1. Twofold serial dilutions of a virus stock were made in 25 μl of phosphate-buffered saline (PBS) in 96-well U-bottom plates. Fifty microliters of 0.5% (v/v) chicken red blood cells (Nippon Bio-test Laboratories, Japan) in PBS was added to each well. The plates were kept at 4 °C for 1 h and the hemagglutination patterns were read. Hemagglutination titers were determined from the last dilution showing complete hemagglutination.
2.3 Plaque assay

The infectivity of the virus was determined by plaque assay. The principle of the plaque assay is shown in Fig. 2. The virus was serially diluted 10-fold in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, and 100 µg/ml streptomycin (Thermo Fisher Scientific). Madin–Darby canine kidney cell monolayers maintained in 6-well tissue culture plates were inoculated with 400 µl of the virus dilutions and incubated at 37 °C for 1 h with rocking every 15 min. The inoculum was removed and 2 ml of serum-free medium (KBM220, Kohjin Bio, Japan) containing 0.8% SeaPlaque agarose (50101; Lonza, USA) and 1.25 µg/ml acetylated trypsin (Sigma-Aldrich, USA) was added to each well to be solidified. The plates were incubated for 3 days at 37 °C in 5% CO₂ and plaques were counted. Infectivity was calculated as plaque forming units (pfu) per ml. Assays were performed in three independent experiments.

2.4 Chromatographic rapid diagnostic test (RDT)

The ESPLINE Influenza A & B-N (Fujirebio, Japan) chromatographic influenza RDT was used. This test is currently commercially available in Japan. The sensitivity of the kit to another influenza virus isolate was previously reported. The detection principle is based on an antigen–antibody reaction with monoclonal antibody(ies) specific to nucleoprotein (NP), which is one of the most abundant proteins in influenza virions. Twenty microliters of each 10-fold serial virus dilution in PBS was mixed with 200 µl of sample preparation buffer. Twenty microliters of the mixtures was subsequently dispensed on the immunoassay cassettes. Results were interpreted on the basis of observations with the naked eye after 15 min incubation at room temperature. This assay was repeated independently three times.
2.5 Sandwich ELISA

A commercially available sandwich ELISA kit (Influenza A virus Nucleoprotein Antigen ELISA Kit, DEIA-CL036; Creative Diagnostics, USA) was used for NP antigen detection in accordance with the manufacturer's instruction. Briefly, the virus was serially diluted 10-fold in PBS and 200 µl of each dilution was mixed with 50 µl of the sample preparation reagent. One hundred microliters of each mixture including 80 µl of virus dilution was added to the antibody-coated plate and incubated for 30 min at room temperature. The influenza A virus detection antibody was dispensed with an equal volume of a sample mixture and incubated for 45 min. After washing the plate six times with the wash buffer, the sample was reacted with 100 µl of chromogen for 10 min for color development, and the reaction was stopped by adding 100 µl of stop solution. The optical density (OD) at 450 nm was measured using an iMark microplate reader (Bio-Rad, USA). The cutoff value was determined by multiplying the negative control value by 2, where the negative control was provided with the kit. Assays were carried out in triplicate independently.

2.6 One-step RT-PCR

One-step RT-PCR was employed to detect influenza virus RNA. Total RNA was prepared from 140 µl of the virus stock of serially diluted 10-fold in PBS using a QIAamp® Viral RNA Mini Kit (Qiagen, USA) in accordance with the manufacturer’s instructions. Total RNA was eluted with 60 µl of buffer AVE included in the kit. RT-PCR was performed using a QuantiTect® Probe RT-PCR Kit (Qiagen) in accordance with the manufacturer’s instructions. The PCR mixture consisted of 10 µl of 2× QuantiTect Probe PCR Master Mix, 0.2 µl of QuantiTect RT Mix, 0.8 µl of 10 µM forward primer, 0.8 µl of 10 µM reverse primer, 0.4 µl of 5 µM probe, 3.8 µl of water, and 4 µl of the RNA template, which is equivalent to 9.3 µl of virus dilutions. Thermal cycling conditions and the primer design were described previously.\(^8\) Thermal cycling was performed as follows: 30 min at 50 °C to activate reverse transcriptase, followed by initial denaturation for 15 min at 95 °C with subsequent 45 cycles of amplification (denaturation at 94 °C for 15 s and annealing as well as an extension at 60 °C for 60 s) using
MyGo Mini (IT-IS Life Science Ltd., Ireland). The following probe and primers were used in the experiment: MP-96-75ProbeAs (5’-FAM-ATYTCGGCTTTGAGGGGGCCTG-MGB-3’), MP-39-67For (5’-CCMAGGTCGAACGTAYGTCTCTCTATC-3’), and MP-183-153Rev (5’-TGACAGRATYGGTCTTTGCTTTTAGCCAYTCCA-3’). The assay was performed in triplicate.

3. Results

3.1 Stock virus characteristics

To determine the stock virus characteristics used in this study, we performed two classical virological assays: the hemagglutination and plaque assays. These two assays revealed that the hemagglutination titer of the virus stock was 256 (Fig. 3) and the concentration of infectious virus particles was $4.03 \times 10^7$ pfu/ml. The amount of the virus suspension used in the hemagglutination assay was 25 μl per well and the time required to detect the virus (observing aggregation) was ~2 h, which included 1 h of virus incubation. As the hemagglutination titer was 256, the detection limit (the detectable highest dilution factor) was between $10^2$ and $10^3$. In the plaque assay, the amount of the virus suspension per assay was 400 μl. The plaque assay detects infectious virus particles. Since the virus stock concentration was $4.03 \times 10^7$ pfu/ml, approximately 16 infectious virus particles were contained in 400 μl of the sample diluted $10^6$-fold. The detectable highest dilution factor of the plaque assay was about $10^7$.

3.2 Immunological detection assays based on antigen–antibody interaction

Figure 4 shows a photograph of the virus detection result obtained using the RDT, ESPLINE Influenza A & B-N. A positive band was observed on the test line in the testing of samples diluted 10- and $10^2$-fold. In contrast, it was difficult to discern positive bands in samples diluted $10^3$-fold or greater. This result means that the detectable highest dilution factor of the RDT kit was between $10^2$ and $10^3$.

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Fig. 3. (Color online) Titration of the virus stock by the hemagglutination assay. The dilution factors of the virus stock used in the assay (a), (b), (c), (d), (e), (f), (g), (h), (i), (j), and (k) were 1, 2, $2^2$, $2^3$, $2^4$, $2^5$, $2^6$, $2^7$, $2^8$, $2^9$, and $2^{10}$, respectively. Negative control (PBS) was applied instead of the virus (l).
Figure 5 shows the result of the NP antigen detection using the commercially available ELISA kit. Each data point expresses the average of three independent experiments. Each error bar indicates ±3 standard deviations. The cutoff value was determined in accordance with the manufacturer’s instruction using the negative control provided with the kit. The detectable highest dilution factor of the NP capture ELISA was between $10^3$ and $10^4$. The total incubation time of the NP capture ELISA was 85 min, and all the assays took approximately 2–3 h.

3.3 Real-time RT-PCR assays

Figure 6 depicts cycle threshold (Ct) values against the original virus stock dilution factor in the RT-PCR analysis using the MyGo Mini. Each value expresses the average of triplicate data and each error bar indicates the standard deviation. The detectable highest dilution factor of the method was $10^7$–$10^8$. The PCR analysis took approximately 3 h for the detection, which included 30 min for viral RNA extraction and 100 min for amplification.

4. Discussion

Table 1 summarizes the detection results. In the table, ‘target’ indicates the substances detected by each method. ‘Sample volume’ is the amount of the virus sample used for the sample preparation at the beginning of each assay, which means that the volumes of reagents used in the assays are excluded from the calculation. The numbers in parentheses are the amount of virus samples actually introduced into the assays. ‘Process time’ is the time required for virus detection by each method. The number in parentheses for the hemagglutination assay and ELISA indicates the time required for the incubation processes, and that for the RT-PCR indicates the time required for the amplification. ‘Normalized sensitivity’ gives an exponent of the detectable highest dilution factor of each assay normalized by the value of the hemagglutination assay.

The virological virus titration methods included the hemagglutination and plaque assays. The hemagglutination assay is widely used for the quantitative evaluation of influenza virus. As shown in Table 1, the hemagglutination assay displayed a low sensitivity and was not suitable for the detection of a trace amount of the virus. However, since chicken red blood
cells react with all the viral surface antigens and the measurement result reflects the number of viral particles in the sample and can be obtained without the need of a special device or a measurement kit, the hemagglutination assay is considered to be suitable for use as a standard of the sensitivity of immunoassay or PCR, which evaluates protein or RNA originating from virus particles.

Fig. 5. Result of the NP antigen detection using the commercially available ELISA kit. ‘PC’ and ‘NC’ indicate the positive and negative controls included in the kit, respectively.

Fig. 6. Ct values against the dilution factor of the virus stock observed in the RT-PCR analysis. ‘ND’ indicates that substantial amplification was not observed.

Table 1
Summary of results of comparison of the influenza virus detection methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Sample volume (µl)</th>
<th>Process time</th>
<th>Detectable highest dilution factor</th>
<th>Normalized sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemagglutination assay</td>
<td>Virus particles</td>
<td>25</td>
<td>2 h (1 h)</td>
<td>$10^2$–$10^3$</td>
<td>0</td>
</tr>
<tr>
<td>Plaque assay</td>
<td>Infectious particles</td>
<td>400</td>
<td>3 days</td>
<td>$\sim10^3$</td>
<td>4</td>
</tr>
<tr>
<td>Chromatographic RDTs</td>
<td>Protein (NP)</td>
<td>20 (1.8)</td>
<td>15 min</td>
<td>$10^2$–$10^3$</td>
<td>0</td>
</tr>
<tr>
<td>ELISA</td>
<td>Protein (NP)</td>
<td>200 (80)</td>
<td>2–3 h (85 min)</td>
<td>$10^3$–$10^4$</td>
<td>1</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>RNA (M1 gene)</td>
<td>140 (9.3)</td>
<td>3 h (100 min)</td>
<td>$10^2$–$10^8$</td>
<td>5</td>
</tr>
</tbody>
</table>
We determined the stock virus infectious titer as $4.03 \times 10^7$ pfu/ml by the plaque assay. Among the assays we assessed, only the plaque method targets infectious viruses. The other approaches cannot determine virus infectivity. The plaque assay is highly sensitive and can detect only infectious viruses, so it is possible to evaluate the number of viruses that may harm humans. However, since it takes days to obtain the results, it is not suitable as a virus sensor.

The RDT provided detection in 15 min from a 20 µl sample. The highest dilution for detection was $10^2$–$10^3$. In the case of using the commercially available ELISA kit targeting NP, a sensitivity one digit higher than that of the RDT was obtained with a measurement time of 2–3 h. The measurement time required for the RDT is short and the assay method is simple, but its low sensitivity is a problem. The ELISA kit is highly sensitive, highly quantifiable, and excellent as a virus concentration evaluation tool. However, the time required for measurement is long, and the technical level of the measurer has a large effect on the measurement result.

The detection sensitivity of immunosensors based on the antigen–antibody reaction is sometimes expressed as pfu, as compared with the detection results obtained by the plaque assay. However, as described above, since the plaque assay is a tool for evaluating the number of infectious virus particles and the immunosensors are used for evaluating the concentration of the target protein, it is not appropriate to evaluate the sensitivity of the immunosensors using pfu. If the sensitivity of the immunosensor is to be evaluated, it is considered better to compare it with hemagglutination titer, which reflects the virus particle number.

In the RT-PCR using a conventional thermal cycler, a 140 µl specimen volume was applied. The amplification step took 100 min and the highest dilution for detection was $10^7$–$10^8$. Recently, it has been reported that a microfluidic RT-PCR device yielded a similar sensitivity to the conventional thermal cycler RT-PCR device, where the time required for the amplification was approximately one-tenth of that of the conventional thermal cycler RT-PCR.\(^{15,16}\) The sensitivity of the RT-PCR is higher than that of the plaque assay, because the RT-PCR detects RNA not only in infectious viruses but also in noninfectious ones. The sensitivity of the RT-PCR is considered to be sufficient to detect viruses in the environment and the RT-PCR is applied as a sensor for the prevention of infections. On the other hand, viruses that are likely to infect humans are only infectious virus particles. The detection of even trace amounts of viral RNA or viral proteins is no guarantee of a danger of infection due to an infectious virus. Therefore, for preventing infection, a method that can enumerate infectious viruses, such as the plaque assay, is suitable. Several techniques for evaluating virus infectivity based on PCR have been reported.\(^{17–19}\) In the future, it will be necessary to develop infectious virus sensors that can replace the plaque assay.

In this study, we compared various virus detection methods with different detection principles and targets. As mentioned above, the hemagglutination assay, which provides a value that correlates with the number of virus particles, can be used as an evaluation standard of immunoassays and RT-PCR that detect viral proteins and viral RNAs, respectively. However, depending on the collection method and/or preservation state of the virus, the virus particles or RNA may be damaged, and the protein may be degraded. If mutation occurs in the RNA targeted by PCR or the RNA is not present, the virus can be detected by immunoassay, but not by PCR. Thus, the values obtained by these methods do not necessarily correlate with each other.
5. Conclusions

This is the first study directly comparing the volumes, times, and sensitivities between different assays using an identical virus stock. These parameters significantly varied as expected. Although smaller amount, shorter time, higher sensitivity, and accuracy are desired and pursued in an assay, it is obvious that every method has both merits and demerits in different points. This clearly indicates that we need to change the methods of testing the influenza virus depending on the situation. We hope that this study will be a helpful for choosing a virus detection method in future examinations.

References