Protocol Title

Evaluation of novel molecular assays for the detection of influenza virus

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1. PROTOCOL SYNOPSIS

Protocol title	Evaluation of novel molecular assays for the detection of influenza virus
Hypothesis	Our novel molecular assays for the detection of influenza A virus will be non-inferior to current molecular assays published by the World Health Organization and the US Centers for Disease Control and Prevention
Primary objective	To evaluate the sensitivity and specificity of different molecular assays
Clinical specimens	Nasopharyngeal and saliva specimens previously tested for influenza A virus
Study design	We will randomly retrieve archived nasopharyngeal and saliva specimens that were previously tested for influenza A virus using commercially available assays in our laboratory, tested for influenza A virus at the Public Health Laboratory Service Branch in Hong Kong. These specimens will be tested for influenza A virus by 4 different RT-PCR assays as listed below: 1. Our new RT-PCR assay targeting PB2 gene 2. Our new RT-PCR assay targeting NS gene 3. M gene RT-PCR published by the World Health Organization 4. M gene RT-PCR published by the US CDC.
Primary outcome	Results of different RT-PCR assays
Secondary outcome	Cycle threshold (Ct) value of different RT-PCR assays
Data analysis	Sensitivity, specificity, positive predictive value and negative predictive value will be determined

2. BACKGROUND

Seasonal influenza virus causes an estimated 0.3-0.6 million deaths per year. Avian influenza virus H5N1, H7N9 and H5N6 has fatality rate of over 30% [1-3]. Swine influenza viruses from pigs have also infected humans [4].

Molecular assays are now used routinely in the detection of influenza viruses. The M gene is often used as the target for all influenza A viruses because the nucleotide sequence of this gene is relatively conserved among all the influenza A viruses. The World Health Organization and the US Centers for Disease Control and Prevention (CDC) have published protocols for molecular detection of influenza A virus M gene [5, 6].

However, recent studies have shown that mutations in the M gene have led to a reduced sensitivity of RT-PCR assay targeting this gene [7, 8]. Therefore, it is important to use

alternative conserved genes as the target of RT-PCR. In this study, our aim is to evaluate two new RT-PCR assays that are based on PB2 and NS gene segment.

3. STUDY OBJECTIVES

1. To evaluate the sensitivity and specificity of different molecular assays

4. STUDY DESIGN

4.1. Overall study design

We will randomly retrieve archived nasopharyngeal and saliva specimens that were previously tested for influenza A virus using commercially available assays in our laboratory, tested for influenza A virus at the Public Health Laboratory Service Branch in Hong Kong. These specimens will be tested for influenza A virus by 4 different RT-PCR assays as listed below:

1. Our new RT-PCR assay targeting PB2 gene
2. Our new RT-PCR assay targeting NS gene
3. M gene RT-PCR published by the World Health Organization
4. M gene RT-PCR published by the US CDC.

Sensitivity and specificity will be determined as we described previously [9].

4.2. Outcome measurements

4.2.1. Primary outcome measure

1. Results of different RT-PCR assays

4.2.2 Secondary outcome measure:

1. Cycle threshold (Ct) value of different RT-PCR assays

4.3. Confidentiality of data

The specimens will be tested in an anonymous manner**.**

4.4. Archive of data

The investigator will retain all study documentation pertaining to the conduct of the study at the study site for a period of at least 5 years.

4.5. Ethical endorsement

The use of archived clinical specimen for microbiological testing has been approved by the Institutional Review Board (IRB) of the University of Hong Kong and Hospital Authority.

5. SELECTION OF CLINICAL SPECIMENS FOR TESTING

5.1. Inclusion criteria

1. Nasopharyngeal or saliva specimens of patients in Queen Mary Hospital of Hong Kong
2. Tested for influenza A virus using a commercially available assay or by the Public Health Laboratory Services Branch in Hong Kong

5.2 Exclusion criteria

1. Insufficient specimen volume

6. STUDY PROCEDURES

6.1 Nucleic acid extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR) for influenza A virus

Saliva and nasopharyngeal specimens will be subjected to total nucleic acid (TNA) extraction by NucliSENS easyMAG (BioMerieux, Boxtel, Netherlands) as we described previously [10]. Briefly, 250 μL of each specimen will be mixed with lysing buffer. After extraction, the nucleic acids will be recovered using 55 μL of elution buffer.

Monoplex real-time RT-PCR assays for influenza A virus will be performed using QuantiNova Probe RT-PCR Kit (QIAGEN, Hilden, Germany). The reagent mixture (20 μL) will contain 1x QuantiNova Probe RT-PCR Master Mix, 1x QN Probe RT-Mix, 0.8 μM of each forward and reverse primer, 0.2 μM of probe and 5 μL of TNA as the template. The thermal cycling conditions will be 10 min at 45 °C for reverse transcription, 5 min at 95 °C for PCR initial activation, and 50 cycles of 5 s at 95 °C and 30 s at 55 °C. All reactions will be performed using the LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). The primers and probes for the M gene RT-PCR have been published by the WHO and the US CDC [5, 6].

7. STATISTICAL METHODS

7.1 Sample size

We will perform all 4 RT-PCR assays on a total of 320 specimens, including
- 80 nasopharyngeal specimens which tested positive for influenza A by commercially-available molecular assays or by testing performed at the Public Health Laboratory Services Branch in Hong Kong
- 80 nasopharyngeal specimens which tested negative for influenza A by commercially-available molecular assays or by testing performed at the Public Health Laboratory Services Branch in Hong Kong

- 80 saliva specimens which tested positive for influenza A by commercially-available molecular assays
- 80 saliva specimens which tested negative for influenza A by commercially-available molecular assays

The sample size is based on feasibility of the study

7.2 Analysis of the study

Sensitivity, specificity, positive predictive value and negative predictive value will be calculated for

1. All specimens
2. Nasopharyngeal specimens only
3. Saliva specimens only

8. REFERENCES

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