The use of PCR in the surveillance, characterization and diagnosis of influenza

Report of the 7th meeting of the WHO Working Group on Polymerase Chain Reaction (PCR) Protocols for Detecting Influenza Viruses

Geneva, Switzerland, 19–20 June 2014
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Abbreviations and acronyms

CC  collaborating centre (WHO)
CDC  Centers for Disease Control and Prevention
CHP  Centre for Health Protection (Hong Kong SAR)
Ct  cycle threshold
EQA  external quality assurance
EQAP  External Quality Assessment Project
ERLI-Net  European Reference Laboratory Network for Human Influenza
EU  European Union
FAO  Food and Agriculture Organization of the United Nations
GISRS  Global Influenza Surveillance and Response System
GLP  good laboratory practice
GP  general practitioner
HA  haemagglutinin
HE  haemagglutinin-esterase
HPAI  highly pathogenic avian influenza
ILI  influenza-like illness
IRR  Influenza Reagent Resource
LPAI  low-pathogenic avian influenza
MERS CoV  Middle East respiratory syndrome coronavirus
NA  neuraminidase
NDV  Newcastle disease virus
NIC  national influenza centre
NIID  National Institute of Infectious Diseases
NIPH  Norwegian Institute of Public Health
NP  nucleoprotein
NS  non-structural proteins
NSW  New South Wales
OFFLU  OIE/FAO Network of Expertise on Animal Influenza
OIE  World Organisation for Animal Health
PCR  polymerase chain reaction
QA  quality assurance
QC  quality control
RNA  ribonucleic acid
rRT-PCR  real-time RT-PCR
RSV  respiratory syncytial virus
RT-PCR  reverse transcription PCR
SAR  Special Administrative Region (Hong Kong)
USA  United States of America
VCM  WHO Consultation on the Composition of Influenza Virus Vaccines
WHO  World Health Organization
1 Introduction

1.1 Background – PCR and the WHO PCR Working Group
The reverse transcription polymerase chain reaction (RT-PCR) assay is both rapid and sensitive. Increasingly, RT-PCR is the first-choice laboratory test for diagnosing and monitoring influenza virus infections in both humans and animals. Since its initial application in detecting influenza A(H5N1) viruses, the assay has been simplified and applied more broadly to both routine seasonal influenza surveillance and pandemic preparedness activities. Therefore, it is vital that the sensitivity and utility of this assay in detecting evolving viruses be maintained, and that laboratories continue to be supported in their efforts to comply with recommended good operating practices. These are some of the primary roles of the WHO Working Group on PCR Protocols for Detecting Subtype Influenza Viruses (the PCR Working Group), which was established in 2007 “... to provide guidance to WHO on the use of PCR in the Global Influenza Surveillance and Response System (GISRS) for the detection of influenza A viruses”. The group also provides advice to the WHO External Quality Assessment Project (EQAP) and advises on other activities related to the use of PCR technology in GISRS.

1.2 Meetings of the PCR Working Group
Since its inception in 2007, the PCR Working Group has held annual meetings at WHO headquarters in Geneva, Switzerland. The meeting previous to the one reported on in this document was held on 2–3 July 2013. Its objectives were to:

- define the role of PCR in surveillance and diagnostics;
- update PCR protocols;
- obtain input from the World Organisation for Animal Health/Food and Agriculture Organization of the United Nations (OIE/FAO) Network of Expertise on Animal Influenza (OFFLU);
- define the role of sequencing and virus isolation; and
- improve quality assurance (QA) and quality control (QC).

Outputs from the 2013 meeting were as follows:

- protocols on the WHO website for the detection of H5, H9 and seasonal viruses were updated;
- protocols to detect H7N9 and H10N8 viruses were uploaded to the WHO website and shared with the GISRS community; and
- a meeting report was published on the website, and an executive summary was published in the Weekly Epidemiological Record (1).

1 http://www.who.int/influenza/gisrs_laboratory/pcr_working_group/en/

2 http://www.who.int/influenza/gisrs_laboratory/molecular_diagnosis/en/
The objectives of this 2014 meeting were to:

- review the work done since the last meeting;
- discuss new PCR developments, and provide an update on the Influenza Reagent Resource (IRR);
- identify gaps in the PCR protocols currently recommended by WHO, and determine how to develop protocols to fill these gaps;
- identify QC issues and the need to adjust the current EQAP;
- discuss the roles of sequencing and virus isolation;
- discuss the possible addition of protocols for the detection of respiratory syncytial virus (RSV); and
- review and update the functions and operational mechanisms of the PCR Working Group.

The expected outcomes of this meeting were to:

- update PCR protocols and guidance on the Internet;
- determine how EQAP can best meet changing needs;
- develop actions and a way forward for the PCR Working Group; and
- report the outcomes of the meeting via a publication on the WHO website and an executive summary in the *Weekly Epidemiological Record*.

2 Updates from WHO collaborating centres and WHO H5 reference laboratories

Representatives from WHO collaborating centres (CCs) and WHO H5 reference laboratories provided general updates on their activities over the previous year, as outlined below.

WHO CC for Reference and Research on Influenza, London, United Kingdom of Great Britain and Northern Ireland (United Kingdom)

- The importance of a low cycle threshold (Ct) value for successful virus isolation was discussed. It is unlikely that an isolate with a Ct value >30 would be obtained from a sample; hence, national influenza centres (NICs) were asked to consider samples with Ct values <30 as a priority for isolation in the WHO CC.
- Occasionally, NICs submit isolates that test negative for influenza by real-time RT-PCR (rRT-PCR). The CC is working to improve methodologies in these laboratories.
- Computer-based analysis showed that the rRT-PCR protocols in use in European laboratories should work against H7N9 viruses.
- Consideration should be given to cataloguing primer sets because this would facilitate rapid access to primers in the event of a zoonotic outbreak.
- The H3 core primer may need to be redesigned due to the identification of a new mutation.
WHO CC for Reference and Research on Influenza, Melbourne, Australia

- A trend was observed in Yamagata influenza B viruses. In 2013 these viruses were mainly Massachusetts/2/12-like, but in 2014 they were reverting to become Wisconsin/1/10-like.

- Four influenza B Yamagata/Victoria reassortants detected in Singapore, Sri Lanka and Thailand contained Yamagata lineage haemagglutinin (HA) and Victoria lineage neuraminidase (NA) genes. One virus contained a Victoria lineage non-structural proteins (NS) gene, and three contained a Victoria lineage nucleoprotein (NP) gene. The remaining internal genes were of Yamagata lineage.

- A new assay to detect the haemagglutinin-esterase (HE) gene of influenza C viruses – essentially a combination of the HA and NA genes – was developed. The full HE gene sequence was obtained from viruses grown in Madin Darby canine kidney cells.

- A highly pathogenic avian influenza (HPAI) virus of the H7N2 subtype was isolated in poultry in New South Wales (NSW), Australia. The virus had an HA gene that was similar to an HPAI H7N7 virus isolated in NSW in 2012.

- An H7N9 virus was detected in a Chinese tourist in Malaysia in February 2014. The sample had a high Ct value. The HA and NA genes were successfully sequenced by the Malaysian NIC, with help from the CC, and both the HA and NA genes were found to be identical to those found in Chinese H7N9 viruses.

WHO CC for Reference and Research on Influenza, Tokyo, Japan

- The CC published an rRT-PCR protocol to differentiate between the Yamagata and Victoria lineages of influenza B viruses (2).

- Positive control ribonucleic acid (RNA) was designed to differentiate between contamination and positive control RNA in PCR reactions.

- EQAP is currently being established in Japan. Preliminary testing and initial improvements have been completed, and the proficiency of the PCR is currently being assessed. Final improvements in accuracy and sensitivity are planned for 2015.

WHO H5 Reference Laboratory, Centre for Influenza Research, The University of Hong Kong, Hong Kong Special Administrative Region (SAR), China

- Since the previous PCR Working Group meeting, no H5 influenza cases were reported in Hong Kong SAR.

- Computer-based analysis showed that H5 primers validated previously are still effective.

- The primer and probe set specific for human lineage H7N9 viruses were published (3). One nucleotide change was observed in the region specific for the forward primer, so this primer will need to be re-evaluated.

- H9N2/H7N7 reassortants need to be monitored closely. These viruses have a different gene constellation from H7N9, due to continued reassortment after the H7N9 outbreak. Therefore, primer sets may need to be updated to include H9N2 primers.

- PCR methodologies to detect Middle East respiratory syndrome coronavirus (MERS CoV) are planned. Countries in Africa and the Middle East are potential recipients of these primers.
• A primer set was developed and validated to detect H6N1, using a sample from a human case in Taiwan. This probe was specific and did not show cross-reactivity with other viruses, including other H6N1 viruses.

• Three cases of H10N8 were reported in China, with similar characteristics to H6N1. One isolate was obtained. No cases were reported in Hong Kong SAR.

• Surveillance in China revealed the existence of 29 different viral genotypes in swine, most of which contained HA and NA genes from H3N2, and internal genes from (H1N1)pdm09.

WHO CC for Studies on the Ecology of Influenza, St Jude Children’s Research Hospital, Memphis, Tennessee, United States of America (USA)

• No new H5 case reports were received.

• External quality assurance (EQA) panels were distributed to member laboratories as part of the National Institute of Allergy and Infectious Diseases Centers of Excellence for Influenza Research and Surveillance QA/QC programme. Forty-one laboratories participated in at least one panel, and an overall pass rate of >75% was observed.

WHO H5 Reference Laboratory, Centre for Health Protection (CHP), Hong Kong SAR, China

• No new H5 case reports were received.

• Ten cases of H7N9, four of which were fatal, were reported in Hong Kong SAR. All cases were imported from southern China, and underlying conditions were a common factor in the fatal cases.

• The laboratory testing strategy is transitioning from viral culture to molecular detection, which is faster and more sensitive, can detect non-cultivable viruses and increases biosafety.

• In H7N9 cases, nasopharyngeal swabs yield higher Ct values than nasopharyngeal aspirates because the virus replicates more readily in the lower respiratory tract. Therefore, samples should be taken from the lower respiratory tract if possible.

WHO H5 Reference Laboratory, Institut Pasteur, Paris, France

• No H5 case reports were received.

WHO CC for Reference and Research on Influenza, Beijing, China

• Since the 2009 pandemic, the number of laboratories in China that can perform virus isolation or PCR detection (or both) has increased from 62 before 2009 to 395 in 2012–2013.

• In the past influenza season, the viruses that have caused outbreaks in China have shown greater variability than was the case in previous years. Cases in the 2012–2013 season were predominantly caused by (H1N1)pdm09 viruses, whereas outbreaks during the 2013–2014 season were caused by (H1N1)pdm09, H3N2 and influenza B viruses.

• As of June 2014, 448 cases of H7N9 had been confirmed in China (including Hong Kong SAR and Taiwan), 168 of which were fatal. In mainland China the number of H7N9 cases was greater in the 2013–2014 season than in the 2012–2013 season, but the case fatality rates were similar.
EQAP has been conducted with the 110 Chinese national influenza surveillance network laboratories. Results showed that provincial-level network laboratories performed better than county-level network laboratories.

Since the last meeting, considerably more effort has been placed on developing techniques surrounding PCR, including sequencing and deep sequencing.

Viral load in throat swab specimens obtained from H7N9 cases was lower than in (H1N1)pdm09 and H3N2 cases. Sputum or tracheal aspirates showed a higher virus yield, consistent with the greater replication of H7N9 in the lower respiratory tract.

3 PCR-related activities in OFFLU
A representative of OFFLU gave an overview of animal influenza virus infections and PCR-related diagnostics. In contrast to the clinical setting, economics is an important factor in the notification of HPAI virus outbreaks in poultry and in the response to such outbreaks. Direct economic losses due to influenza virus infection include the mortality in poultry caused by HPAI viruses and the reduced productivity in layer hens caused by infection with low-pathogenic avian influenza (LPAI) viruses. Indirect costs, which can also be substantial, occur due to harsh restriction measures (holdings) instituted in response to notifications of an HPAI virus infection. These measures have a significant impact on the area; also, if there are large poultry facilities nearby, the larger supply chain can be affected, impacting an even greater area. Hence, there is trepidation in the industry about reporting; also, despite the fact that LPAI viruses affect the health and productivity of poultry, infections are not notifiable events. Between January 2013 and June 2014, there have been 270 outbreaks of HPAI viruses worldwide, most of which occurred in the Republic of Korea, Mexico, Nepal and Viet Nam. In the same period, there were 104 outbreaks of LPAI viruses, most of which occurred in China and South Africa; however, this number is probably a large underestimation because LPAI virus infections do not elicit overt clinical symptoms and are usually only detected by chance. The mechanisms by which an LPAI virus becomes an HPAI virus are not known, although it would be extremely beneficial to both animal and public health to understand these mechanisms.

Other topics discussed were the:

- discovery of H5N8 reassortant viruses in Republic of Korea, which potentially has a wild bird reservoir;
- recent measures put in place in Italy in response to an H7 HPAI virus outbreak in poultry;
- ongoing outbreaks of H7 HPAI viruses in poultry farms in Mexico;
- results of swine surveillance conducted in Europe – European Union (EU) project ESNIP3 – which revealed new reassortant viruses containing (H1N1)pdm09 genes, such as pH1/N2 (4, 5); and
- changing ecology of influenza virus, such that cats and dogs could potentially become new mixing vessels, based on data in several recent publications (6, 7).

Results were reported of an OFFLU ring trial on “molecular avian influenza virus diagnosis”, conducted in 2013 using samples prepared and shipped by the US Department of Agriculture National Veterinary Services Laboratories, Iowa, USA. Taking part in the trial were nine OIE
There was less variation in Ct values between OIE/FAO laboratories or centres than between regional or national laboratories, and there was a trend whereby laboratories whose assays were most sensitive yielded more false positives. American laboratories obtained more accurate results using American viruses, and Eurasian laboratories obtained more accurate results using Eurasian viruses, highlighting the need for primers applicable to both hemispheres.

A method to detect H7N9 viruses was described; the method was developed with a veterinary perspective, and used both generic and specific primer sets for H7 and N9. A duplex assay to detect H7N9 viruses was also described. A low-density rRT-PCR assay to detect all 16 HA and 9 NA genes has been developed and will be published soon. It was also noted that all reference laboratories must be able to detect all subtypes.

4 Discussion topics

In general discussion, the issue of receiving specimens in the CCs before the February meeting of the WHO Consultation on the Composition of Influenza Virus Vaccines (VCM) was raised. It was suggested that a threshold number of samples could be introduced such that, once that threshold was reached, the laboratory would initiate a shipment of all samples. This could help to prevent a large number of samples being shipped just before the VCM, and thus reduce the pressure on the receiving laboratory.

The issue of shipments not meeting deadlines was discussed. It was recognized that shipment and customs clearance remain the most prominent delaying factors. Failures in timely reporting may be symptomatic of problems in the laboratory, and a careful approach in dealing with this issue is needed.

Political and market-driven issues have resulted in relatively restricted sharing of avian reservoirs of LPAI viruses. This means that only a few laboratories have access to isolates, and can thus perform viral phenotyping and contribute to molecular characterization databases.

4.1 EQAP: Observations on the progress made, issues and future actions

A representative of the Department of Health, Hong Kong SAR, China, presented an update on the global EQAP, which was initiated in 2007 to “improve the global laboratory capacity for influenza diagnosis by the detection of influenza virus type A by PCR”.

EQAP milestones were discussed. The number of material dispatches was reduced from two per year between 2007 and 2011 to one per year from 2012. The EQAP provider has been accredited according to ISO17043 since 2010. The EQAP panels have evolved from the original panels 1–5, which included influenza A H1, H3 and H5 viruses. New panels have been added that included an (H1N1)pdm09 virus (panel 6), Victoria lineage influenza B virus

3 http://www.who.int/influenza/gisrs_laboratory/external_quality_assessment_project/en/
(panel 7), gamma-ray irradiated viruses to test RNA extraction protocols (panel 9), and the addition of Yamagata lineage influenza B virus and the removal of the (H1N1)pdm09 virus (panel 10). All viruses in panel 11 in 2012 were gamma-ray irradiated, and an H9 virus was included. All viruses in panel 12 in 2013 were inactivated using beta-propiolactone, and a component of an (H1N1)pdm09 virus was introduced, for testing susceptibility to NA inhibitors. All viruses in panel 13 in 2014 were inactivated using Triton X-100.

The outcomes of EQAP revealed no common cause related to the reporting of incorrect results; however, the failure to correctly subtype H5 appears to be in part due to mismatches between primer and probe. It was also evident that laboratories that use rRT-PCR assays tend to perform better in terms of sensitivity than laboratories that use conventional PCR. The comparability of the results from EQA panels between years was discussed. Panel composition and preparation methods have varied, making direct comparisons between panels difficult. Also, the introduction of extremely challenging samples in the panels may have introduced bias. Nevertheless, since the inception of EQAP, there has been a trend towards improvement in the performance of participants, evident by 90% of participants consistently reporting correct results for all H5 samples in each panel in recent years.

Good laboratory practice (GLP) surveys were introduced in 2010 and 2012. The results of the 2012 panel showed that most EQAP participants have adopted elements of GLP in the molecular diagnosis of influenza. Further, compared to the 2010 GLP survey, there have been improvements in areas such as staff training, equipment maintenance, laboratory accreditation status, the conduct of internal audits and the establishment of unidirectional workflows for molecular diagnosis. Areas identified as requiring further improvement were regular reagent validation and primer evaluation.

One of the programme challenges discussed was shipping, including fulfilling customs and import requirements, and requests for protocols, additional samples, controls and reagents. Another challenge was RNA fragmentation during the inactivation process. The pros and cons of viral inactivation strategies were then discussed. Gamma irradiation is impractical because it is not available on-site, beta-propiolactone can cause cross-linking that limits amplicon length, and Triton X-100 may affect virion envelope integrity and thus render the viral RNA more vulnerable to degradation. Further work is needed to optimize inactivation procedures to ensure that they do not affect the ability to produce large amplicons (>1000 base pairs) by conventional RT-PCR.

### 4.2 Observations from the European EQA

The WHO Regional Office for Europe, Copenhagen, Denmark, presented the results of an external QA (EQA) conducted by the European Reference Laboratory Network for Human Influenza (ERLI-Net) and WHO Regional Office for Europe, in which 53 NICs from the WHO European Region participated in detection and culture panels, and 35 NICs participated in the antiviral susceptibility panel. In general, molecular detection capabilities were found to be good across the region. In terms of virus isolation, 78% of the laboratories isolated all samples, and the 16 false negatives were from only nine laboratories. Further, 75% of laboratories used antigenic characterization, and a higher than average score was achieved
with genetic characterization. Over the period of the past three EQAs, improvements in culture and strain characterization were also observed. A/Anhui/1/2013 (H7N9) was subtyped by 90% of laboratories, with only five participants reporting false negatives. There was also an improvement in the number of laboratories that could differentiate between influenza B lineages. Overall, a minority of laboratories accounted for the majority of errors. To help solve this problem, targeted support and training will be offered. Future plans by the WHO Regional Office for Europe include an annual molecular EQA and continued collaboration with the ERLI-Net on a 2-yearly culture and antiviral susceptibility EQA.

4.3 CDC initiatives and future plans
A representative of the WHO CC for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, discussed the supply of panels by the CDC to laboratories in both North and South America. Although the CDC does not provide accreditation, panels can be useful in helping laboratories to gain or retain accreditation. These kits contain “challenge” specimens; that is, samples of high Ct value, which are more difficult to obtain results from than regular samples. These samples are useful in identifying issues, particularly in RNA extraction methodologies. An assay for live attenuated influenza vaccine, developed by the CDC, is currently for in-house use only, but US Food and Drug Administration accreditation is being sought, which would allow wider dissemination.

4.4 Discussion on EQA
The value of EQAs and the effort required to conduct them was recognized and appreciated. CCs can provide non-seasonal viruses of, for example, H5N1, for inclusion in future EQAPs.

It was agreed that the need of NICs for virus isolation EQAs should be discussed further. Possible modifications to EQAs were also discussed:

- the inclusion of a specimen with a high viral load to assess adoption of good practices;
- measures against cross-contamination; and
- the inclusion of a high viral load specimen for phenotypic resistance testing in EQAP (this specimen could be provided by the WHO Working Group on Surveillance of Influenza Antiviral Susceptibility).

4.5 Regional perspective
The recent emergence of the H7N9 virus and MERS CoV has emphasized the need for vigilance and investment in pandemic preparedness across the globe. To assess the preparedness and expertise of laboratories in the WHO European Region, a precourse questionnaire was conducted across the region, to which 56 laboratories responded. About 50% of the laboratories used in-house PCR assays; the remaining laboratories used kits from GISRS, although these kits were not necessarily intended for diagnostic applications. Thirty laboratories indicated a need for validation training; of these, 22 required the training in order to improve quality, and the remaining eight did not report a reason.

Expansion of the GISRS network to include RSV surveillance was discussed in the context of an RSV vaccine in the foreseeable future. This expansion needs careful consideration, to
ensure that it does not interfere with the influenza-related activities of GISRS. The network was established to study influenza, and the structure and surveillance approach may need to be re-evaluated for RSV because of differences in the epidemiology of these viruses. For example, the age of the main at-risk group for RSV is 5 years and under – a cohort that may be missed using current influenza surveillance protocols. The timing of the RSV season is also different, peaking just before the start of the influenza season, then continuing. In addition, unlike influenza, there is no need for a seasonal vaccine for RSV. It was suggested that although laboratory infrastructure would not need to be changed, the deliverables and overseers would be different; therefore, some aspects would run in parallel but workflows would “branch off” at some point. There is a need to collect existing data on a global level about transmission and caseload in different regions, and to consult with members of the RSV community for expertise.

The results of an internal questionnaire conducted in European countries showed that 69% of laboratories routinely test for other pathogens, and 33% routinely test cases of influenza-like illness (ILI) for other pathogens. Some 71.4% of countries surveyed were willing to report data collected on other pathogens, and 55% of countries that were already testing for other pathogens had established multiplex PCR protocols. Constraints to implementation include finance, time and trained personnel.

4.6 Country perspective

Influenza molecular surveillance in Norway

The representative of the NIC at the Norwegian Institute of Public Health (NIPH), Oslo, Norway, noted that PCR is now “taking the front seat” in influenza detection over virus isolation and subsequent characterization, making QA of PCR critical. Also, scientific training now focuses more on molecular techniques, meaning that proficiency in fundamental virological techniques can no longer be taken for granted.

The main part of the work of the Norwegian NIC is as a national reference laboratory. About 2000 influenza virus positive samples per year are forwarded from Norwegian laboratories to the NIPH for verification and further identification or characterization. However, most of the influenza virus positive samples identified are not forwarded. To ensure that all relevant human and zoonotic influenza viruses can be detected in Norwegian laboratories, adequate, correct protocols need to be followed, and reagents need to be used for their intended purposes. The assays that can be shared in the public domain are better suited for subnational network implementation than those that cannot be shared beyond the GISRS laboratories.

Influenza molecular surveillance in France

The representative of the H5 Reference Laboratory and NIC at the Institut Pasteur, Paris, France, discussed primary care and hospital surveillance. Primary care surveillance used case definitions of ILI and acute respiratory infections, and a sentinel surveillance system involving 506 general practitioners (GPs) in the GROG and Sentinel networks using nasopharyngeal swabs. In the 2012–2013 season, 3967 specimens were tested, of which 1967 (49.6%) were positive for influenza; and 99% of 842 influenza A viruses detected were subtyped. Hospital surveillance used case definitions of severe acute respiratory illness and
intensive care unit admittance in the RENAL network, which comprised non-sentinel surveillance in 52 hospital laboratories. Numerous sample types were included, such as nasopharyngeal, bronchoalveolar lavage, biopsies and serum. In the 2012–2013 season, 62 859 specimens were collected, of which 7665 were positive for influenza; and 34% of the 3945 influenza A viruses detected were subtyped. Some 8899 samples were positive for RSV. rRT-PCR was used for detection in both sentinel and hospital surveillance systems.

The NIC used an in-house rRT-PCR method to perform A/B typing, H1/H3 subtyping, lineage differentiation between influenza B viruses and discrimination of viruses containing the H275Y mutation. Conventional PCR was used to obtain sequencing data on the HA, NA and M genes, and also for pyrosequencing. Cell culture was used for virus isolation and for haemagglutination tests and haemagglutination inhibition tests. Other respiratory viruses were detected using rRT-PCR.

Hospital laboratories used rRT-PCR to perform A/B typing and H1 subtyping, using a commercial kit for the (H1N1)pdm09 virus. Cell culture was used less frequently, and other respiratory viruses were detected using rRT-PCR.

In the face of an outbreak, the NIC would analyse the first cases and develop relevant rRT-PCR assays. These assays could be shared with hospital laboratories, which would then send positive specimens to the NIC for validation.

The GROG network is threatened financially because it is expensive. However, the rapid detection of influenza virus by the network provides incentive and motivation to the GPs involved. Sample sharing between hospital laboratories and the NIC is limited because of the expenses associated with shipping regulations.

5 Global Influenza Surveillance and Response System

5.1 Review of current PCR protocols for GISRS
A representative of the NIC, Public Health England, London, United Kingdom, provided a summary of the current documents on the web. Two particular documents were discussed, as outlined below.

WHO information for molecular diagnosis of influenza virus – update (9)
This document describes:

- conventional RT-PCR protocols to detect the:
  - M gene of influenza viruses;
  - HA and NA genes of H5N1, seasonal (H1N1)pdm09, H3N2, influenza B viruses and former seasonal H1N1 viruses;

- rRT-PCR protocols to detect the:
  - M gene of influenza A viruses;
  - NS gene of influenza B viruses; and
  - HA and NA genes of H5N1, seasonal (H1N1)pdm09, H3N2 and influenza B (including differentiation of influenza B lineages), and former seasonal H1N1 viruses.
viruses, as well as H7N9 and H10N8 viruses, in addition to sequencing protocols.

There was discussion on additions to this document that may be beneficial, such as a conventional PCR protocol for H7N9 viruses, multiplex PCR protocols, PCR protocols for other subtypes (e.g. H6), additional sequencing protocols, and pyrosequencing protocols for lineage and subtype differentiation.

Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases (10)

This document describes recommendations and protocols for the detection of avian influenza A(H5N1) viruses in specimens from suspected human cases. It provides two conventional RT-PCR protocols for the detection of the influenza A M gene, H5 gene and N1 gene. The M gene protocol has since been updated by the CHP and the WHO CC Tokyo. rRT-PCR protocols are described to detect influenza A M and H5 genes, which were sourced from Hong Kong SAR and the Erasmus MC, Rotterdam, Netherlands. The document also describes a further rRT-PCR protocol from the National Institute of Infectious Diseases (NIID) to detect the influenza A M, H5 and N1 genes. There was discussion about whether this document should be retained, updated or archived because of its age. The status of this document will be investigated.

5.2 Observations from CCs

A representative of the WHO CC NIID, Tokyo, Japan, discussed diagnostic protocols for H7N9 viruses that are used in different laboratories and have been shared with GISRS. The NIID H7 diagnosis kit for RT-PCR and conventional PCR was supplied to 74 local public health institutes and 16 quarantine laboratories in Japan. This kit and positive control RNA from A/Anhui/1/2013 (H7N9) were supplied on request to NICs in Indonesia, Iran, Mongolia, Myanmar and Viet Nam. There was also discussion on the mechanisms that should be in place regarding the modification of a protocol, in relation to how (and how quickly) the information should be made available. It was thought that laboratories should know the contact details in their respective regions for information if needed.

5.3 Future use of PCR in GISRS, and role of WHO and WHO CCs

A representative of the WHO CC for Studies on the Ecology of Influenza, St Jude Children’s Research Hospital, Memphis, Tennessee, USA, facilitated discussions on molecular techniques. Such techniques have become the first line of detection in most instances, with virus isolation being performed less frequently, although isolation is still extremely important for influenza vaccines and other public health concerns. The concern about losing virus isolation skills will be further addressed following the results from the 2014 NIC survey. It was suggested that NICs should be reminded about the importance of isolation, as stated in their terms of reference.

Newcastle disease virus (NDV) has been detected in samples at St Jude; NDV can give a false positive for influenza. The virus can be detected by rRT-PCR with influenza probes. Since St Jude does not receive human samples, it is unlikely that this institution will develop methodology for RSV detection.
There was discussion about the use of glycerol in isolating virus from specimens. At St Jude, 50% glycerol is routinely used, and this method seems to make it easier to isolate virus from samples that have been incorrectly stored, without affecting RNA extraction using the QIAGEN RNeasy kit. It is possible that the glycerol concentration could be reduced to 5% and still be effective; if so, this would further reduce potential interference with downstream processes. There was also a recommendation to store sample tubes in ziplock bags within the secondary container during dry ice shipment, to prevent CO₂ vapour entering improperly closed tubes. If CO₂ enters the tubes, it causes acidification and could thus destroy the infectivity of any influenza viruses present.

There was discussion about the volume of rRT-PCR reactions from a financial viewpoint. Volumes used were 25 µL, 12.5 µL and 10 µL – all of which can be used successfully. To manipulate reaction volumes, it was recommended that laboratories purchase PCR master mixes that allow for the addition of water.

Transportable PCR machines were also discussed, such as those used in the field in the animal sector. Such machines greatly reduce the time needed to obtain a result. There was a concern about the fact that the end user could read and interpret results, which would reduce contact with the laboratory and might decrease reporting or lead to misinterpretation of results (or both).

Multiplex assays were discussed as a potential method for cutting costs, because they allow several targets to be amplified in a single reaction. The discussion emphasized the importance of internal controls and of limiting competition between primer sets by limiting the number of targets in each reaction. It was agreed that using a “mini-multiplex” assay of no more than four targets per reaction would minimize the problem of competition, and that an outcome of this meeting should be the collection of multiplex assays for the detection of influenza viruses. Concerns were raised about fluorophore selection, because most are proprietary, meaning that a monopoly is given to the manufacturer once a fluorophore is validated.

5.4 Role of sequencing in GISRS
Dr Yi-Mo Deng of the WHO CC for Reference and Research on Influenza, Melbourne, Australia, facilitated discussion on sequencing in GISRS. There has been a trend to sequence the whole HA (HA₀) as opposed to just HA₁, because there is now an awareness of mutations in HA₂ and a recognition of the importance of neutralizing antibodies against the stalk region of HA, which is located in HA₂. However, sequencing of HA₀ can change the structure of phylogenetic trees, and therefore the naming of the genetic groups.

There was discussion about the timeliness of receiving specimens from laboratories before the VCM in February. A limited number of European countries have specimens available in time to allow consideration at the meeting, and few submit full-length sequences. Reasons for this include the late shipping of the samples (possibly due to the grouping of samples in one shipment to reduce costs), and an increase in the shipping of clinical specimens, which take longer to analyse than isolations because the virus must be isolated before it can be sequenced. However, despite fluctuations in the number of cases reported, the number of
samples being received just before the VCM is relatively constant. To facilitate preparation for the VCM, laboratories should be encouraged to send sequences in a timely manner, whether or not they are complete, to facilitate tree construction and identification of potentially interesting viruses for further consideration. It was agreed that NICs should try to sequence a greater number of original specimens, then send both the virus and the sequence to the CC, to help with preparations for the VCM.

The potential introduction of a sequencing QA programme was also discussed. The aim of the programme would be to help in assessing and improving the sequencing capability of NICs that are currently conducting Sanger sequencing.

There was discussion of next-generation sequencing, and the potential use of this technology in the GISRS network for detection and diagnostics. This technology is currently being used primarily for research, but has the potential to be broadly applied to detection and diagnostics. Next-generation sequencing yields a large amount of data that require substantial analysis and interpretation. Therefore, difficulties in gaining access to appropriate analysis pipelines and bioinformaticians could be significant bottlenecks. The possibility of sharing pipelines once they have been completed and validated was discussed. The interpretation of data is also an important consideration because of the volume of data generated; also, there were concerns about how quickly equipment becomes obsolescent. Next-generation sequencing does not require the use of specific primers, which means that this approach could:

- be useful in identifying novel influenza strains where specific primers or probes have not been developed or validated (e.g. H10N8); and
- save time and money (compared to the use of specific probes), and facilitate the discovery of unexpected influenza strains.

In summary, next-generation sequencing is a promising technology, but more development is needed before it can be used by the GISRS. Such development may be facilitated by inviting experts to future meetings. It is hoped that, in the next few years, laboratories will become more familiar with this technology, particularly as it has the potential to be more cost and time effective than the methods currently in use.

It was agreed that nothing more was immediately needed for GISRS to facilitate its sequencing activities.

### 5.5 rRT-PCR reagents and situation update

A representative of the WHO CC for Surveillance, Epidemiology and Control of Influenza, CDC, Atlanta, Georgia, USA, provided an update on H3N2v cases in the USA. Cases increased from seven during the 2009–2010 season, to 12 during the 2010–2011 season, to 309 during the 2011–2012 season. This increase was not thought to be due to increased transmissibility of the virus but rather to the high prevalence of these viruses in pigs, and the high human exposure rate at state fairs where these pigs are shown. This was evident in the 2012–2013 season, when there were only 18 cases; this decrease reflected changes introduced at state fairs, including limiting exposure of people to pigs and preventing sick pigs from being shown. A prospective clinical study is now being conducted using swabs collected from ILI
cases, particularly those with swine exposure. This study would also serve to validate the RT-PCR assay that has been developed to distinguish between H3N2v and seasonal H3N2.

Influenza B genotyping panels to distinguish between lineages of B viruses are now available. Also, the influenza A H5 Asian lineage subtyping panel has been updated to improve reactivity for H5a and H5b assays, to detect all clades of HPAI Eurasian lineage H5N1 influenza.

The availability and distribution of CDC kits and pooled influenza positive control material was discussed. These materials can be ordered through the IRR website. The CDC Laboratory Support for Influenza Surveillance SharePoint Site was also discussed. This website provides public health and research laboratories with access to multiple assays, procedures and methods; facilitates coordinated communication with registered laboratories; and provides timely notification of assay updates. There are more than 170 registered users.

Enzyme chemistry options for use with CDC assays were discussed. Invitrogen SuperScript III Platinum One-Step Quantitative RT-PCR System and Quanta qScript One-Step qRT-PCR Kit were recommended. The CDC has an arrangement with Thermo and Quanta to provide fair pricing of these reagents to laboratories performing CDC assays, to prevent price gouging by distributors. BHQ1 (Biosearch Technologies Inc) or ZEN probes (Integrated DNA Technologies) were recommended. There would be legal implications if WHO were to recommend a commercial product; however, the goal should be to facilitate the availability of these reagents as cheaply as possible, particularly to laboratories in poorer regions.

QA of laboratories in the USA has been provided by the CDC in the form of the Molecular Diagnostic Performance Evaluation Panel. The CDC does not certify laboratories; rather, it provides this panel to facilitate assay qualification. The ultimate goal of this approach is to improve the quality of and confidence in results obtained from laboratories that provide seasonal influenza surveillance. Results were encouraging and two QA panels are being planned in 2014. QA panels were also provided for laboratories in Brazil and Chile, to facilitate internal evaluations. A second evaluation with laboratories in the Pan American Health Organization Region is planned.

The shift from culture to molecular techniques in the USA was noted. The CDC now supports two large state laboratories to provide culture facilities, to ensure that the CDC does not become the only culture facility in the country.

5.6 General discussion points

The scope of the GISRS is expanding to include provision of supporting roles and the building of laboratory capabilities, which also extends the reach of the network and yields better data. However, the core role of the PCR Working Group – to “improve the global laboratory
capacity for influenza diagnosis by the detection of influenza virus by PCR’’ – will remain. The group will continue to provide advice and guidance on PCR EQA, and will also evaluate new technologies to determine whether they are mature enough to be included in GISRS. Members will check the validity of their protocols, and inform the group and WHO as soon as possible. It is the responsibility of the PCR Working Group to ensure that the protocols provided on the WHO website are valid for public health purposes.

Annual meetings of the WHO PCR Working Group will continue to be held. Inviting experts from other fields (e.g. bioinformatics, high-throughput sequencing or technology development) would be beneficial for specific discussions.

6 Report and proposed action points
The outcomes of the meeting have been published in an executive summary in the Weekly Epidemiological Record (11).

It is proposed that the PCR Working Group:

- investigate the status of the document Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases (10), and consider additions to the document WHO information for molecular diagnosis of influenza virus (9), such as a conventional PCR protocol for H7N9 viruses, multiplex PCR protocols, PCR protocols for other subtypes (e.g. H6) and pyrosequencing protocols for lineage and subtype differentiation;
- establish a collection of multiplex assay protocols for the detection of influenza viruses;
- ask members to review the validity of their protocols, and to inform the group and WHO of their findings;
- assist laboratories with fair-pricing negotiations with manufacturers of rRT-PCR kits and primers and probes;
- encourage NICs to sequence a greater number of original specimens, and to then send the viruses and the sequences to the CC in a timely manner, to support the preparations for the VCM;
- optimize virus inactivation protocols in order to guarantee inactivation while not affecting the ability to produce large amplicons by conventional RT-PCR;
- ascertain NICs’ need for virus isolation EQAs;
- at a global level, collect data on RSV in relation to transmission and caseloads in different regions, and consult with experts in the RSV field (in particular, about the requirements for standardized RSV surveillance); and
- consult experts on next-generation sequencing and bioinformatics, to determine how this technology could be applied to diagnostics and surveillance, and how best to analyse and interpret data.

http://www.who.int/influenza/gisrs_laboratory/pcr_working_group/en/
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Annex 2: Declarations of interest

The 7th meeting of the WHO Working Group on Polymerase Chain Reaction (PCR) Protocols for Detecting Influenza Viruses (the PCR Working Group), 19–20 June 2014, was organized by the Global Influenza Surveillance and Response System (GISRS) and the Influenza Viruses and Vaccine Support team of WHO, with participation from WHO collaborating centres (CCs) on influenza, WHO H5 reference laboratories, national influenza centres (NICs) and a representative from the veterinary sector.

In accordance with WHO policy, all the PCR Working Group expert members completed the WHO Declaration of Interests for WHO Experts form before being invited to the meeting. These declarations were then evaluated by the WHO Secretariat before the meeting. At the start of the meeting, the interests declared by the expert members were disclosed to all consultation participants. The table below shows the personal current or recent (within the past 4 years) financial or other interests relevant to the subject of work declared by participants.

<table>
<thead>
<tr>
<th>Institution</th>
<th>Representative</th>
<th>Personal interest</th>
</tr>
</thead>
<tbody>
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<td>Dr Janice Yee-Chi Lo</td>
<td>None</td>
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</tbody>
</table>

Based on the WHO assessment of the interest declared by Dr Lindstrom, it was concluded that the interest declared did not present a conflict of interest with the objectives of the technical consultation. In view of this assessment, Dr Lindstrom participated in the meeting as an expert working group member.
References


