The use of PCR in the surveillance and diagnosis of influenza

Report of the 4th meeting of the WHO working group on polymerase chain reaction protocols for detecting subtype influenza A viruses
Geneva, Switzerland 14–15 June 2011

The polymerase chain reaction (PCR) assay is a rapid and sensitive method for detecting the genetic material of influenza viruses, and is now the first-choice laboratory test for influenza infection in both humans and animals. Since its initial application for detecting A(H5N1) viruses, the use of PCR has expanded to cover other influenza types, subtypes and lineages, and the assay is increasingly used for routine seasonal influenza surveillance and diagnosis.

The WHO working group on polymerase chain reaction protocols for detecting subtype influenza A viruses (hereafter "the PCR working group") was established in 2007 to serve as an expert technical group to provide guidance to WHO on the use of PCR in the context of the WHO Global Influenza Surveillance and Response System (GISRS). At its fourth meeting held on 14–15 June 2011, the PCR working group reviewed:

- developments since the previous PCR working group meeting
- the role of PCR in virological surveillance and diagnostics
- the updating of PCR protocols
- H5 protocols
- PCR quality-assurance activities.

Following consideration of the future of PCR within the GISRS and the use of sequencing, discussion then centred on the role, objectives and operational aspects of the PCR working group.

Participants included representatives from WHO Collaborating Centres (WHOCCs) for Reference and Research on Influenza, WHO H5 Reference Laboratories, Essential Regulatory Laboratories (ERLs), National Influenza Centres (NICs) and the World Organisation for Animal Health–United Nations Food and Agriculture Organisation Network of Expertise on Animal Influenza (OFFLU).

Developments since the previous PCR working group meeting

A number of updated PCR protocols and kits are now available or are being finalized. These include an updated protocol for the A(H1N1) 2009 pandemic virus and new PCR kits developed by the WHOCC Atlanta, United States Centers for Disease Control and Prevention (CDC) along with associated laboratory-support and performance-evaluation initiatives and the revised Influenza Reagent Resource (IRR) web site.[1] In addition, the validation of H5 primers against recent viruses is ongoing with a need for updated protocols in this area.

The WHO manual for the laboratory diagnosis and virological surveillance of influenza has now been published and is available on the WHO web site.[2] As updated PCR protocols become available they will also be posted on the WHO web site and linked to the manual. The previously identified need for the WHO External Quality Assessment Project (EQAP)[3] to incorporate an assessment of the proficiency of RNA extraction has been addressed and further discussion is now needed on the optimum composition and scope of future panels.

[1] www.influenzareagentresource.org/
[2] www.who.int/csr/disease/influenza/manual_diagnosis_surveillance_influenza/en/
[3] www.who.int/entity/csr/disease/avian_influenza/guidelines/eqa_project/en/index.html

The requirement for suitably equipped NICs to conduct virus isolation continues to be emphasized to avoid the entire burden falling on WHOCCs. Although PCR (both real-time and conventional) is increasingly the method of choice for influenza surveillance, this should not distract from the crucial role of virus isolation. It is the antigenic characteristics of emerging viruses that determine the need to make changes to vaccine virus recommendations, while virus phenotypic determinations provide comprehensive antiviral resistance monitoring. The importance of promptly shipping unsubtypable influenza A viruses to a WHOCC was also reiterated. Updated WHO guidance is now available on selecting clinical specimens for virus isolation and on shipping specimens and virus isolates to WHOCCs.[4]

The role of PCR in virological surveillance and diagnostics

There is a need to distinguish between the use of PCR for virological surveillance and its role in diagnostic activities. Despite issues such as false positives (caused by contamination or the non-specific hydrolysis of primers) and false negatives (caused by factors such as poor sample quality, inefficient extraction of nucleic acids or the presence of reaction inhibitors) PCR is increasingly the first-choice assay for both activities. It is a rapid, sensitive and specific assay (applicable at a low bio-containment level) for detecting A(H1N1) 2009, A(H3N2) and influenza B viruses, as well as viruses with pandemic potential including A(H5N1) and A(H9N2).

National-level presentations highlighted the central and expanding role of PCR in the work flow of NICs as they tracked national influenza trends. It was reported that PCR results were now a primary criterion used to determine which viruses to culture in accordance with recent WHO guidance on how best to meet the needs of the GISRS while addressing local needs. The widespread use of PCR testing has led to significant improvements in the quality of surveillance and diagnostic data, in the capacity of laboratories to support national and regional activities, and in the ability of national authorities to respond quickly to emerging situations. There remains a pressing need to develop testing strategies that include PCR and other methods to best meet public health demands at national, regional and global levels.

PCR has unparalleled advantages in helping to meet the surveillance aim of rapidly detecting the emergence of new influenza viruses, as illustrated during the A(H1N1) 2009 pandemic. For example, the updated CDC protocol (2009)[5] for the detection and characterization of the A(H1N1) 2009 pandemic virus was the basis for diagnostic kits developed and distributed by the Chinese Center for Disease Control and Prevention. The use of these kits clearly revealed the spread of the pandemic to all parts of China in the first half of 2010. In addition, PCR diagnosis during the period 2005–2010 identified 31 human cases of infection with the A(H5N1) virus in mainland China, with no new cases found since June 2010. The National Influenza Surveillance Network (NISN) of China expanded rapidly following the onset of the A(H1N1) 2009 pandemic and PCR testing has become a key approach in national virological surveillance activities.

Due to variations in laboratory capacity and sample quality, PCR testing needs to be robust in order to detect circulating viruses during influenza seasons. For example, in the WHO

[4] www.who.int/csr/disease/influenza/influenzanetwork/2010_12_06_clinical_specimens_for_virus_isolation_and_virus_for_shipment_from_nic_to_who_collaborating_center.pdf

[5] www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html

Western Pacific Region there is wide diversity in the geographical and socioeconomic characteristics of countries. However, despite variations in the infrastructure, operational approaches and technical sophistication of NICs in the Region, almost all laboratories have or soon will have real-time PCR, in many cases backed up by conventional PCR. Where resources are limited, issues such as staff turnover, staff training and equipment maintenance can be challenging, and sample quality can vary due to sampling procedures and transport issues. Despite recent initiatives in the Region such as the development of subregional networks of countries and the provision of freezers, shipping issues remain, especially where countries are isolated. Lessons are being learnt to further improve the transportation of clinical specimens, virus isolates and reagents.

The role of NICs in supporting national PCR testing is determined by their specific circumstances, capabilities and public health responsibilities. NIC capabilities in areas such as PCR protocol design, adaptation and validation may also rely upon national capacities in these areas. It was felt that, wherever possible, advanced NIC capabilities should be advocated for by the GISRS in order to maximize their global contribution. In terms of responsibilities, some NICs support a national network of laboratories, for example in the use of recommended protocols, provision of kits, reagents and controls, and proficiency testing. Such initiatives can be complicated by a lack of direct NIC authority to instruct national network laboratories. In some settings this has led to increases in the numbers of specimens forwarded to NICs in transport media unsuitable for subsequent virus isolation, while the quality of PCR-based subtyping at the sub-national level can be highly variable.

Virological surveillance needs which are insufficiently addressed at present include the need for molecular testing to provide information comparable to that obtained from virus isolation and characterization (for example, the subtype of influenza A viruses and the lineage of influenza B viruses). The GISRS needs a routine assay to generate more-timely data on influenza B lineages. In Norway, protocol validation for influenza B lineage differentiation as part of a duplex real-time RT-PCR testing strategy has been undertaken by the NIC with good results, and at least one conventional RT-PCR protocol for influenza B developed by the WHOCC Beijing in China has proven to be very useful. Both these methods can be made broadly available.

Developing parallel or multiplex PCR approaches to include the differentiation of influenza B lineages, and eventually to incorporate the surveillance of respiratory viruses other than influenza, could potentially increase efficiency and promote sustainability. In China, the process of establishing PCR surveillance of other viral infections is already under way, while in Thailand funding pressures after the A(H1N1) 2009 pandemic are driving a move towards the expanded use of PCR surveillance to cover other pathogens. The potential for increased automation of PCR techniques was also noted. However, such benefits must be considered alongside disadvantages such as cost, the need to maintain standards and the increasing trend for laboratories to prioritize the technique (particularly real-time PCR) over more traditional approaches such as virus isolation.

There is also a need to reliably detect genuine cases of co-infection with more than one influenza virus. In some cases, false indications of co-infection have been attributed to the use of live-attenuated influenza vaccine (LAIV) in some parts of the world. Conversely, in other cases a single infection indicated by PCR has subsequently been found to be a genuine co-infection following virus isolation and further testing.

Genetic sequencing should continue to be supported as a vital extension of PCR testing. Sequencing remains key to correlating amino acid substitutions with antigenic variation, accurate virus subtyping, detection of co-infection and antiviral susceptibility screening. WHO held a sequencing workshop in Singapore and a guidance document prepared on behalf of the European Centre for Disease Prevention and Control (ECDC) will be modified and added to the WHO web site. During discussion it was noted that the quality of sequence-related data on public databases varies, but most participants are now submitting to the GISAID public-access database which is developing stringent curation algorithms. It was agreed that developing guidance on next-generation sequencing techniques was unnecessary given the stage of development and the analytical requirements associated with the large volume of data generated. The use of PCR technologies by NICs to replace sequencing in the monitoring of antiviral susceptibility cannot be recommended at present as there are a number of significant technical difficulties.

Updating PCR protocols

To maintain the required sensitivity and accuracy of PCR tests in detecting ever-evolving influenza viruses, PCR primers and protocols must be updated in a timely manner. During 2011, the current WHO 2009 protocols[6] will be updated and streamlined to better reflect the priorities for influenza surveillance following the A(H1N1) 2009 pandemic. Specific adjustments include the provision of protocols for the detection of former seasonal A(H1N1) viruses, and of A(H3N2), A(H5N1) and type B influenza viruses.[7] PCR protocols for H7 and H9 detection now under development will be considered for future inclusion.

Discussions on the optimal range and number of protocols centred on issues specific to each virus type, subtype or lineage. It was felt that the virological surveillance of seasonal H1, H3 and B viruses, as well as highly pathogenic avian H5 viruses, remains the minimum requirement for NICs. It is likely that the updating of the WHO protocols in addition to the availability of protocols from other sources would result in sufficient coverage of these particular viruses. As the number of different assays increase, the level of demands and associated complications will also increase. It was reported in one country that a focus on H1 surveillance had distorted national influenza surveillance with adverse affects for example on H3 surveillance. This raises the issue of testing for subtypes at the sub-national level, where routine H3 and H5 testing may strengthen surveillance but will increase complexity.

In the United States, high-level discussions have been held on developing the most appropriate combination of PCR resources for the surveillance of viruses with pandemic potential. Canine H3, and avian H7 and H9 viruses are all under consideration for inclusion in a representative panel of reference materials. In the case of H7, one CDC protocol and one protocol developed by the Netherlands NIC exist but the former at least is likely to be out of date and specific to viruses in North America. Large variability within the subtype prevents the development of a universal protocol. The endemic nature of H9 in some countries, the recent detection of avian H9 infections caused by a different sub-clade, and the emergence of human cases of H9 infection in Bangladesh support the development of additional assays. However, ensuring the broad reactivity of individual reagents is problematic, and the value of

[6] www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf
[7] The in-house real-time and conventional PCR duplexes for influenza B lineage viruses reported above by the NIC Norway and WHOCC Beijing respectively will be considered for inclusion.

attempting to pre-empt emerging H9 viruses needs to be demonstrated before the required resources can be justified. At present, the CDC will only provide current H9 assay kits to centres reporting increased H9 infections in humans.

Generally, there appears to be no current epidemiological urgency to develop PCR protocols for H7 and H9. The small number of human cases of infection with H9 appears to be limited to one country and there have been no recent human cases of infection with H7. It was felt that the existing system of immediately forwarding unsubtypable viruses (as clinical specimens and/or virus isolates) to WHOCCs would detect human cases of infection with either subtype.

The successful development of H9 reagents is likely to depend upon effective surveillance in animals and cooperation with animal-sector agencies. The tracking of H9 by OFFLU is currently limited as the subtype typically has only limited economic and public health impact and is not included in the list of notifiable animal influenzas. However, recent events in Bangladesh have demonstrated the capacity of H9 viruses to infect humans.

A view was expressed that there needs to be an even broader approach taken given the possibility of the emergence of other reassortants involving H1 and H3 viruses. The recent A(H1N1) 2009 pandemic highlights the unpredictability of emerging threats, and the addition of a universal protocol for H2 viruses may be prudent due to their proven pandemic potential in 1957. Once again, influenza surveillance among animals with a focus on detecting reassortants in swine will be a key factor with animal-sector agencies taking the lead role on this issue.

Despite the advantages of reducing the overall number of WHO and other protocols, there is also value in maintaining a range of different methods covering both conventional and real-time PCR assays for seasonal and H5 influenza. This will provide “back-ups” for the critical analysis of specific types and subtypes, and help to address the varying compatibilities of different protocols for use in multiplexing approaches.

The WHOCC Atlanta continues to provide protocols, reagents and training in support of real-time PCR for influenza. Of the two current CDC protocols, one provides generic procedures for all CDC real-time influenza PCR assays while the other is specific to the detection of the A(H1N1) 2009 virus. In the past, CDC has noted that once the protocol sequences are published online there have been cases of non-validated commercial kits appearing on the market, some claiming CDC approval. It is not known if the publication of sequences by the WHO GISRS is associated with the same issue. During 2011, CDC intends to discontinue two existing positive control kits and to make available a Pooled Influenza Positive Control (PIPC) kit containing four inactivated human influenza viruses (A/H1, A/H12009pdm, A/H3 and B) and cultured human cells. Enzyme kits and RNA extraction kits will also be made available. Reagent ordering will continue to be coordinated through the CDC Influenza Reagent Resource (IRR) for approved and registered laboratories.

To strengthen harmonization and support efforts, CDC will also expand the provision of specific procedures for nucleic acid extraction and amplification, and provide support for the setting up, analysis and interpretation of assays. Through a registration web site scheduled to launch in 2011, CDC will provide information and support for multiple procedures and methods on a range of platforms. This will also allow for coordinated communication with registered laboratories, timely notification of assay updates and the sharing of platform-

specific procedures for assay setup. As the web site develops it is intended that public health and research laboratories will be able to selectively access only those resources most relevant to their needs. The possibility of providing a link to the web site through the GISRS EZCollab communication platform was raised. Follow-up actions in this area will be taken.

H5 protocols

The emergence of multiple A(H5N1) genetic groups and their continuous mutation makes the review, update and validation of H5 primers and protocols complex but crucial. Current CDC procedures for H5 remain the same but there is evidence of regional differences in circulating clades and sub-clades. To help address this, a redesigned reverse primer has been developed by CDC allowing a second kit to be offered in addition to the existing set. Although it is likely that one set will eventually fall out of use, the current sets are considered to work well. One problem identified has been the development of commercial H5 (and multiple-pathogen) assay kits that perform suboptimally. As well as efforts to determine which kits are used by individual laboratories as part of quality-assurance activities, promoting the use of centralized resources such as the IRR would help to ensure consistency.

Despite its lower sensitivity, conventional PCR still has great utility as it is less expensive to perform than real-time PCR and larger products are generally made (suitable for sequence analysis) employing primers located in highly conserved regions of the HA gene. Conventional PCR may therefore pick up evolving H5 strains with mutations that reduce the sensitivity of real-time PCR assays.

Comprehensive surveillance of H5 infection in both animals and humans remains problematic, with indications of mismatches between H5N1 primers and circulating clade 2.2 (and to a lesser extent 2.3.2) viruses. In some settings, animal surveillance is limited as infections outside the formal commercial sector often go unreported. Nevertheless, the absence of significant numbers of human cases suggests that sustained human-to-human transmission is not occurring, and recent H5 human fatalities have all involved contact with sick birds. Where human cases have been detected, samples are to be sent for characterization in time for the September 2011 vaccine composition meeting. Antigenic data indicate that H5 is drifting sufficiently to support the updating of the vaccine virus candidate panel currently recommended by WHO.

PCR quality-assurance activities

Acknowledgement was given to the WHO H5 Reference Laboratory and National Influenza Centre based in the Virology Division, Centre for Health Protection in China, Hong Kong Special Administrative Region for its ongoing efforts in coordinating the WHO EQAP.[8] Since its establishment in 2007, the EQAP has been used to monitor and improve the quality of virological testing by GISRS laboratories and other national influenza reference laboratories, and to confidentially identify any weaknesses. Since 2007, the number of participating laboratories has risen from 64 to 160 covering all six WHO regions. Obtaining good results in the EQAP is a WHO criterion for accepting positive PCR test results for H5 infection in humans from national reference laboratories. In 2010 the scheme was accredited

[8] www.who.int/entity/csr/disease/avian_influenza/guidelines/eqa_project/en/index.html

in accordance with ISO 17043 and the twice-yearly test panels extended to include the detection of influenza B viruses. In the first half of 2011 (panel 9) two inactivated viruses (H1 and H3) were added to the simulated RNA specimens to provide a test of RNA-extraction procedures. Dispatching inactivated viruses presents no additional difficulties other than the need to ensure sufficient inactivation, and thorough steps were taken in this respect.

Careful consideration continues to be given to panel composition, and greater efforts are being made to verify the content and homogeneity of samples whilst ensuring their stability during distribution. As part of the shipping of panel 9, temperature monitors were selectively used and significant variations found in the conditions to which panels were exposed during transit, sometimes due to detention of shipments at customs for prolonged periods. In general, inactivated viruses appear to be more stable than the simulated RNA specimens. Other problems include the lack of PCR capacity and/or reagents in some settings, delays in obtaining import permits and varying customs requirements.

Problems associated with the use of EQAP panel 9 included inconsistent technical performance, cross-reaction during H5 detection, inappropriate use of positive controls, laboratory contamination, misinterpretation of results, mismatching of primers and probes, and transcriptional errors. At present there is likely to be great variation in the precise protocols, extraction kits and other methodologies in use at NICs with consequences for the consistency and reliability of results.

In 2010 the associated annual questionnaire-based survey covered the issues of personnel; quality management; design, equipment and consumables; pre-analytical procedures; analytical procedures; post-analytical procedures; reporting and record keeping; and safety. Areas identified as needing improvement include an inability to evaluate reagents and/or assays, and a lack of auditing programmes. It was noted that laboratories with 100% correct answers for EQAP panels were significantly more likely to meet the quality parameters outlined in the survey. Laboratories have indicated that some survey questions are vaguely worded and lengthy, and consideration is being given to ways of improving the clarity and conciseness of the questionnaire.

Discussion centred on the best range of virus types, subtypes and H5 sub-clades to be covered in future panels. Concern was raised that some GISRS laboratories cannot be completely certain of their H5 results as not all circulating H5 viruses are currently available for assay validation and other purposes. It is hoped that the recent successful conclusion of initiatives such as the Open-Ended Working Group of Member States on Pandemic Influenza Preparedness (OEWG/PIP): Sharing of Influenza Viruses and Access to Vaccines and Other Benefits[9] will lead to improvements in this area.

The issue of how best to enhance performance through training was raised and opinions invited on the optimal frequency of assessment (currently twice-yearly). It was generally agreed that without follow-up support to poorly performing laboratories, the utility of the EQAP and other quality-assurance activities is severely constrained. The WHOCC Melbourne reported that it responds to training needs highlighted by the WHO Regional Office for the Western Pacific. It was felt that the training in PCR techniques for influenza diagnosis currently provided by a range of WHOCCs, H5 Reference Laboratories and suitably equipped NICs could be better coordinated through WHO to improve efficiency and

[9] http://apps.who.int/gb/pip/e/E_Pip_oewg.html

avoid duplication of efforts. It was also felt that although the accreditation requirements of diagnostic laboratories may not directly apply to national surveillance and reference laboratories, some form of accreditation of the latter is likely to become increasingly important. In the case of NICs, the WHO Regional Office for Europe sends out annual letters to governments highlighting the importance of their role in public health. In all WHO regions, recognition of an NIC by the GISRS is itself a form of accreditation.

CDC has conducted laboratory quality assessments based upon the provision of simulated respiratory specimens containing cultured human epithelial cells with or without inactivated influenza viruses, along with fictional clinical case histories. In 2008, 52 laboratories submitted their results electronically together with related information on the testing parameters and commercial kits used. It was found that 50 laboratories were using a version of the CDC real-time PCR protocol, with only 14 of these using current (2007) CDC protocol recommendations. In addition, most laboratories were not following protocol recommendations with adverse effects on testing performance. It is clear that simply posting updated protocols is insufficient to ensure their widespread use and correct implementation.

The upcoming voluntary CDC Performance Evaluation Program (PEP) for the molecular testing/diagnosis of influenza has been designed to provide enhanced support to public health laboratories in the United States as part of meeting a regulatory requirement for successful twice-yearly performance testing. Pilot deployment is scheduled for late 2011 with the electronic submission of results and information on the processes and equipment used. Assessment of the performance of individual laboratories will be confidential with a strong emphasis placed on helping laboratories to improve rather than on appraisal or regulation. Prior to launch, CDC will continue to fine-tune the electronic submission forms, data-collection methods and shipping procedures.

The initiative will also allow for the monitoring of CDC reagent performance and provide empirical evidence of success in monitoring laboratory performance and planning appropriate follow-up support and training. The provision of validated reagents is now under way and future assessments will evaluate the effect of this and allow for the fine-tuning of the CDC support web site and associated database. The input of results and associated specific platform data by laboratories may eventually allow for an evaluation of the performance of different commercial kits. It is intended that the initiative will also cover the laboratory monitoring of antiviral susceptibility and the conducting of genetic sequencing. An international version of the initiative is at the pilot phase and if successful will be offered to laboratories worldwide on a request basis.

The results of the 2011 World Organisation for Animal Health (OIE) proficiency panel were reported. This compulsory panel is designed to standardize diagnostic testing for avian influenza viruses (H5 and H7) and for the A(H1N1) 2009 pandemic virus by international reference laboratories working in the animal sector. The panel also allowed an assessment to be made of the utility of different primers and probes in detecting viruses from various regions. Overall performance by the 45 participating laboratories was good particularly in the conducting of generic PCR testing for the presence of avian influenza viruses, and determining their degree of pathogenicity. Related OFFLU activities reported included the development of coupled PCR-pyrosequencing assays, and an electronic “microarray” for detecting and subtyping influenza viruses.

As quality-assurance activities are enhanced, an increasing range of resources will become available to laboratories. Efficient coordination of these resources will be needed to avoid overburdening laboratories and prevent unnecessary duplication. In light of the different objectives of different molecular testing activities, clear descriptions of their precise purpose must be provided. For example, for surveillance purposes increasing the sensitivity of detection may not be a priority and may lead to an increase in the number of unsubtypable specimens forwarded to WHOCCs. An alternative approach would perhaps focus upon WHOCCs helping laboratories to determine their sensitivity levels through the use of highly standardized controls, as is done in the molecular testing of other infectious agents such as HIV.

Future direction of the PCR working group

There was broad agreement that the PCR working group provides a valuable forum for GISRS and partner agencies and laboratories. Originally established to provide guidance on H5 protocols, the scope of the group has increased and it is now viewed as an efficient way of highlighting updated protocols, and addressing quality-assurance and other key issues such as the coordination of training activities and assessing the potential utility of PCR techniques in areas such as antiviral susceptibility monitoring.

In addition, the PCR working group provides a direct communication channel for participants that supports and supplements existing channels of communication. This was felt to have great benefit as national authorities seek to develop and refine their surveillance activities and to place their findings in the broader global context. This support could be further enhanced by improving the linking of the GISRS EZCollab platform to other key web-based resources for conducting PCR and updating protocols. Through initiatives such as the WHO EQAP it should also be possible to identify and incorporate a broader range of providers of key resources.

It was generally agreed that the group should continue to convene annually to keep up with developments in this area. It was also suggested that a more concerted and coordinated approach to implementing the recommendations made at each meeting would be beneficial, for example in areas such as the improved coordination of PCR training provision by WHOCCs and other GISRS laboratories.