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

Report of the 6th meeting of the WHO Working Group on Polymerase Chain Reaction Protocols for Detecting Subtype Influenza A Viruses
Geneva, Switzerland 2–3 July 2013
The reverse transcription polymerase chain reaction (RT-PCR) assay is both rapid and sensitive, and is increasingly the first-choice laboratory test for diagnosing and monitoring influenza infection in both humans and animals. Since its initial application in detecting A(H5N1) viruses, the assay has been simplified and applied more broadly to both routine seasonal influenza surveillance and pandemic preparedness activities. As a result, it is vital that its sensitivity and utility in detecting evolving viruses is maintained, and that laboratories continue to be supported in their efforts to comply with recommended good operating practices.

The WHO Working Group on Polymerase Chain Reaction Protocols for Detecting Subtype Influenza A Viruses (hereafter “the WHO PCR Working Group”) was established in 2007 to serve as an expert technical group to guide WHO on the use of RT-PCR in the context of the WHO Global Influenza Surveillance and Response System (GISRS). At its sixth meeting held on 2–3 July 2013, the WHO PCR Working Group reviewed:

- developments since the previous WHO PCR Working Group meeting;
- the role of PCR and virus isolation in influenza surveillance, characterization and diagnostics;
- current GISRS PCR protocols;
- PCR assurance and quality-control activities.

Following discussions on the future use of RT-PCR, on the role of sequencing in influenza surveillance, and on the use of PCR in evaluating mixtures of antiviral drug resistant and sensitive viruses, consideration was given to the role, objectives and operational aspects of the WHO PCR Working Group itself.

Participants (Annex 1) included representatives from WHO Collaborating Centres (WHOCCs) 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 WHO PCR Working Group meeting**

Real-time RT-PCR is now the basis of influenza surveillance, characterization and diagnosis in GISRS national laboratories to such an extent that the burden of primary screening placed on WHOCCs has been greatly reduced as most viruses received have already been RT-PCR-subtyped by the forwarding laboratory. Recent exceptions to this were the need for identification by the WHOCC at the National Institute for Medical Research in London of a number of “unsubtypable” viruses provided by some eastern European laboratories. Using materials provided by the WHOCC at the Centre for Disease Control in Atlanta all such viruses were found to be seasonal influenza viruses. WHOCC London also reported on an assay-validation study conducted in April 2013 in response to the occurrence of human cases of H7N9. Laboratories in the WHO European Region with biosafety level (BSL) 3 capacity were sent live virus (A/Anhui/1/2013 in l ml allantoic fluid) in addition to the viral ribonucleic acid (RNA) on dry ice packages sent to all participants. Of 34 packages sent out, 25 reporting forms were returned indicating the successful use of the positive control material. The WHOCC London also approached OFFLU and other groups that could have been expected to have A(H7N9) assays already in place.
To determine whether NICs and other laboratories in the WHO European Region could detect A(H3N2)v and A(H7N9) viruses a questionnaire was also sent out to assess the in-house and/or commercial assays being used. Assays targeting the A(H7N9) haemagglutinin (HA) gene were found to work well – in particular those using the updated CDC protocol. For the neuraminidase (N) gene, most assays were based on the Chinese NIC assay with four laboratories reporting a ten-fold decrease in sensitivity. It was clear that during the H7N9 event a range of assays were rapidly implemented in Europe with countries using a variety of kits on different real-time platforms. In terms of generic influenza A detection, 20 reports from 17 countries indicated that all were targeting the Matrix (M) gene. At a subsequent meeting of European laboratories it was felt that a range of suitable assays are available for the detection of the M, HA and NA genes of A(H7N9) viruses and that these are likely to work across a range of platforms employing different kits. As a result, the WHO European Region appears to be well prepared for A(H7N9) virus detection – and this situation will improve once a WHO/CDC reagent kit is widely distributed.

The importance of validating influenza surveillance was also highlighted by the work of a European molecular task group. Rather than being a rapid-response group, the main function of this initiative is instead to provide recommendations to European countries on the meaningful and accurate validation of assays. It was noted that during the H7N9 event many laboratories in the region developed their own protocols without waiting for WHOCC validation and protocol release – thus highlighting that the central control and validation of assays during an event is unlikely to be realistic.

Highlighted PCR-related activities at the WHOCC Atlanta included the development of testing strategies for seasonal influenza, and testing algorithms for highly pathogenic avian influenza H5 – including updated assays for A(H5N1) virus detection. In addition, attention has been given to the detection of A(H7N9) viruses in Eurasia (and to the use of Vietnamese A(H7N3) viruses to monitor North American H7 lineages), the monitoring of A(H3N2)v influenza in the United States of America, and to the development of assays and strategies for monitoring H6 viruses in Asia. The continually evolving package of CDC RT-PCR resources and performance-evaluation support initiatives includes the revised Influenza Reagent Resource (IRR) web site,1 and the CDC Laboratory Support for Influenza Surveillance (CLSIS) web site. Through this latter “SharePoint” initiative, CDC provides information and support for multiple procedures and methods on a range of platforms. With around 100 registered users the site facilitates the distribution of protocols, assays and procedures and the process of registration is now being optimized, for example through improved categorization of different laboratory types and their requirements.

The emergence of cheaper real-time PCR platforms represents an attractive proposition for low-income laboratories, as do competitively priced enzymes and kits. However, WHOCC Atlanta emphasised that commercially available extraction kits are highly variable in terms of quality and do not always perform as expected. In terms of evaluating probe options, new in-house and other quenching options are available with promising early indications. During the 2009 H1N1 pandemic, the production of quencher was a limiting factor and redundancy in available manufacturing options is needed to ensure large-scale production. Although CDC has also undertaken some evaluation of rapid diagnostic kits to assess their likely clinical

1 www.influenzareagentresource.org/, accessed 7 October 2013
performance, these have only been approved by the United States Food and Drug Administration (FDA) for use in North America and may not be universally relevant.

Given on-going budgetary constraints, efforts to the increase the targeting and monitoring of support are intended to improve the focus and efficiency of CDC efforts in this area, and input from the WHO PCR Working Group concerning current priorities would be welcome.

The GISRS H5 Reference Laboratory at the Centre for Health Protection, China, Hong Kong Special Administrative Region (Hong Kong SAR) reported no cases of human A(H5N1) infection and only one avian case, compared to the 20 avian cases recorded in 2012, suggesting that the number of cases among wild birds is falling. The laboratory conducts molecular testing with a primary focus on severe cases in hospital, clinical and outpatient settings, and covers a number of other respiratory diseases. The H5 testing algorithm in use has been validated to cover several clades and incorporates RT-PCR, virus-isolation and sequencing components as required. In addition, preparedness activities were conducted in relation to H7N9 based upon a positive H7 control provided by the WHOCC Beijing. This allowed for validation of the preparedness assay which was subsequently used to test samples from 48 cases meeting the reporting criteria for H7N9 infection – all of which were found to be negative. An in-house M gene one-step RT-PCR protocol was also evaluated and was found to exhibit greater sensitivity than the current two-step protocol. At present, the laboratory has two parallel strategies for influenza testing – a one-step RT-PCR (streamline testing strategy) and a multiplex PCR in-house assay. For other respiratory viruses the focus is on singleplex due to lowered sensitivity when multiplexing multiple viruses. Efforts are currently under way to assess and streamline testing and/or virus-isolation strategies, and advice from the WHO PCR Working Group would be welcome. The GISRS H5 Reference Laboratory at the Institut Pasteur, Paris also reported on the H5 assays and protocols currently in use as part of its established laboratory system for influenza detection and characterization. In 2013, A(H5N1) isolates from Cambodia had been received.

The School of Public Health, University of Hong Kong continued its on-going surveillance and training activities. Recent efforts have included the provision of reference reagents, primers and protocols for influenza virus detection both during virology and PCR training sessions and upon request. This included the sending out of reagents and/or protocols for the 2009 pandemic H1N1 2009 to around 15 overseas laboratories in Asia, and the preparation and distribution of 2009 pandemic H1N1 2009, avian H5, human H7N9 and influenza A reagents and/or protocols to other countries. Molecular assays for H5, 2009 pandemic H1N1 and influenza A sequence detection were also published. Other recent activities include routinely conducted swine influenza surveillance which indicated that A(H1N1)pdm09 viruses have been reintroduced into pig populations and are undergoing reassortment; evaluation of H5N1 RT-PCR assays using viruses from different clades, which indicated little activity; and the development of a specific assay – based upon published Global Initiative on Sharing Avian Influenza Data (GISAID) sequences – to detect A(H7N9) viruses in advance of the potential re-emergence of human cases. It was reported that H7 and N9 components are still circulating in poultry with one A(H7N9) virus found in a wild bird in Hong Kong SAR. Assays are also being developed and evaluated to detect mutations associated with resistance to neuraminidase inhibitors in H7 viruses.

The WHOCC Beijing reported that during 2013 two human cases of H5 infection had been detected from the same province. Other recent activities included the conducting of the 2013 quality assessment programme for the internal laboratory network, the completion of which
has now been delayed following the H7N9 event. Environmental surveillance for avian influenza viruses is continuing, and in 2012 six positive samples were detected (one A(H3N2); three A(H5N1); and two A(H9N2). Results for 2013 are still being analysed. Both in-house and commercial multiplex RT-PCR assays for the detection of multiple respiratory viruses in samples collected from SARI sentinel sites are in use with the in-house assays requiring further modification to overcome problems of sensitivity. Improvements to the primer design of a real-time RT-PCR assay used for subtyping all HA and NA genes is planned to address issues of subtype cross-reactivity.

The WHOCC Melbourne continues to receive samples primarily from Australasia and South-East Asia. Samples received are typically already subtyped with only limited identification by RT-PCR required in specific circumstances, such as outbreak investigations or suspected mixed-infection samples. Other real-time RT-PCR assays used include those to detect antiviral resistance associated with S31N and H275Y mutations. The 2012–13 influenza season was characterized by a slow start and by detection of a single case of influenza C originating in Fiji.

The importance of swine influenza surveillance was again highlighted with a number of reassortants being detected in pigs. In swine samples collected from Queensland and Western Australia many of the confirmed A(H1N2) mixed subtypes had internal genes from A(H1N1)pdm09 virus, with one case exhibiting a seasonal H1 HA gene. None of the swine viruses present were detected in swine workers. Some H3 viruses detected dated back to the 1970s and 1980s, and a few samples showed mixed infection. HA, NA and full-genome sequences were submitted to GISAID. Preparedness efforts for H7N9 included the designing of in-house real-time RT-PCR primers and probes and sequencing primers for both H7 and N9. Evaluation indicated that the sequencing primers used can detect H7 and N9 genes, with 13 laboratories having requested primers via a dedicated web site link. Following receipt of A/Anhui/1/2013 virus, RNA was also sent to 15 laboratories, protocols shared with GISRS and comparisons made of the Chinese NIC and CDC protocols. The WHOCC Melbourne also continues to provide RT-PCR-related technical support and training to the highly diverse range of NICs and other laboratories in the WHO Western Pacific Region. A regional workshop was held on sequencing and phylogenetic analysis of influenza viruses (involving both wet lab and computer practical experience). For laboratories submitting viruses a feedback process is in place involving a newsletter and individual reports.

The WHOCC Tokyo reported the identification of a 2004 A(H7N7) duck virus with 94% similarity to A/Anhui/1/2013 (A(H7N9)), An in-house assay using primers and probes based on conserved regions in A(H7N9) has demonstrated high sensitivity. In addition, a conventional PCR assay was designed that exhibited similar sensitivity to real-time PCR. A diagnostic kit (enzyme, primers and probes, and manual) and positive control (A/Duck/Fukui/1/2004) was developed and supplied to six NICs in the region and to a range of Japanese public health laboratories. A TaqMan RT-PCR assay for detecting the H275Y mutation was shown to discriminate between 275H (sensitive) and 275Y (resistant) variants and to detect 275H/Y mixed cultures with high sensitivity through the detecting and plotting of the fluorescence values of VIC and FAM probes. Although mixtures consisting of more than 60% resistant variants were phenotypically resistant, there was not always a simple correlation between the phenotypic and resistance characteristics of a mixture. The assay can be performed using clinical isolates in cultured supernatants directly without RNA extraction thus making it simple and rapid.
Efforts to assure the quality of PCR implementation in Japan continued with the completion of the inaugural three-step quality-assurance programme launched in September 2011. In 2013, the programme was extended to all 74 local public health institutes. A detailed indication was given of both panel composition and of summary results for 2011 and 2012, with the results of the 2013 panel now in preparation. Following the preliminary analysis, it was determined that, despite good overall results, there was clear evidence of poor levels of equipment maintenance, deterioration of primers and probes, and cross contamination. All participating laboratories were advised to review their standard operating procedures. It is likely that the issue of cross-contamination in particular will need to be addressed in laboratories worldwide as more-sensitive assays are increasingly used. Early benefits at national level have included increased awareness by the WHOCC Tokyo of the levels of virus detectable in individual laboratories, and an opportunity for core-site laboratories to address a range of associated issues. The continuation of this national programme beyond 2013 and the scope of any future panels are likely to be largely determined by budgetary considerations.

An update from the WHOCC Memphis focused on swine influenza surveillance in North America in the face of reductions in funding. A private contractor is now being used to collect samples from farms. The prevalence of influenza did not appear to vary between the different age groups sampled, with around 8% of approximately 10 000 samples received from several states testing positive. A(H1N1)pdm09, A(H3N2) and A(H1N2) viruses were all found. No genotypic information is yet available. Validations performed on CDC H9 primers and probes indicated good sensitivity and coverage against 12 new and previous isolates obtained during the past 12 years, while assistance was also provided to the GISRS H5 Reference Laboratory, University of Hong Kong in validating H9 protocols. In addition, training courses in PCR and reverse-genetics techniques were provided to a small number of participants. The WHOCC Memphis also provided support to the NIAID Centers of Excellence for Influenza Research and Surveillance (CEIRS) programme in the development and distribution of a quality-assurance panel sent to all sentinel sites. The results of this initiative are still pending. Avian surveillance activities were however scaled down due to budgetary pressures, with a focus placed mainly on known active sites in Bangladesh and Egypt. Although samples from live bird markets indicate on-going H5 and H9 co-infection in birds, and high rates of infection with HPAI, no poultry deaths were recorded, probably because viral levels were below the CFU values (10^4 – 10^5) associated with fatalities. In terms of H7 viruses, several low-pathogenic avian cases were detected in North American shorebirds and waterfowl with none detected abroad. An in-house H7 assay was not developed as the WHO web site H7 protocol seemed to be effective.

The implementation of the 2013 OFFLU Ring Trial for Avian Influenza was then reported on. The goal of this initiative remains to standardize diagnostic testing for avian influenza viruses (including H5 and H7) in all World Organisation for Animal Health (OIE) and Food and Agriculture Organization of the United Nations (FAO) reference laboratories, and to evaluate the proficiency of detection of such viruses across different hemispheres. It is intended that this initiative will also allow for the development of guidelines to harmonize testing and to complement current OIE laboratory guidance. Based upon a combination of molecular and serological testing, the test panel allows for assessment to be made of the utility of different primers, probes and approaches in detecting viruses from various regions. In 2013, the trial was extended to include several regional OFFLU laboratories in Africa. Analysis of the results is under way with reports individualized for each laboratory expected to be available by September 2013 and a summary report scheduled for presentation at the
next OFFLU Technical Working Group Meeting. Direct comparison of the 2012 and 2013 RT results will determine if laboratories have improved their detection capabilities between ring trials.

Lessons learnt during implementation of the initiative include the need for prompt acquisition of import permits by laboratories upon trial notification to help prevent shipping and other delays and thus help ensure the participation of all laboratories. It was also clear that assays (for example for H7) must detect all subtypes if they are to be considered suitable for OFFLU laboratories. Other issues include overcoming the broad range of challenges inherent in sharing viruses between animal health and human health laboratories, including acquiring a better understanding of Pandemic Influenza Preparedness (PIP) Framework processes and their operational exemptions in order to optimize intersectoral collaboration. A feedback opportunity was highlighted as a WHO Working Group has been scheduled to deal with issues of PIP Framework implementation in practice.

The role of RT-PCR and virus isolation in influenza surveillance, characterization and diagnostics

RT-PCR and virus isolation remain the foundations of influenza virological surveillance, virus characterization and diagnosis in a broad range of settings. At the WHOCC Atlanta current activities in support of real-time RT-PCR use include the development of testing strategies for seasonal influenza and of testing algorithms for HPAI H5, including an updated assay developed for A(H5N1) viruses; responding to the Eurasian H7N9 event; and the provision of ongoing technical support through the refined CLSIS web site and the IRR reagent-ordering facility. The currently imposed IRR ordering limits are considered to be realistic and are based upon the testing of 2500 samples a year which most laboratories do not exceed. Although external access to IRR enzymes and extraction kits are limited to laboratories working in countries on a United Nations approved list, CDC procurement activities mean that prices are favourably fixed and the provision of assistance to NICs in setting a fixed price or obtaining manufacturer discounts is feasible. Other recent activities include the monitoring of A(H3N2)v influenza in the USA the development of H6N1 influenza assay design strategies and the continuing implementation of the performance evaluation panel (PEP) initiative. In terms of the overall support offered to NICs there remains an emphasis on surveillance first and then diagnostic activities.

WHOCC Atlanta also evaluates and qualifies real-time RT-PCR platforms and automated extraction platforms available on the market. In addition, new enzymes and probes have been evaluated and added to the list of available approved materials. Intended future developments include strengthening influenza B genotyping (YAM and VIC lineages), and developing approaches for detecting Live attenuated influenza vaccine (LAIV) viruses (types A and B), distinguishing between swine-origin H3 and human H3 infections, and developing assays for the detection of N9, North American avian H7 and Asian avian H9 viruses.

The WHOCC Beijing reported that although the role of RT-PCR has not changed in the past year, its performance at local level needs to be improved. For specimens from ILI sentinel surveillance, laboratories can perform either PCR or virus isolation as the method of detection. For routine surveillance virus isolation alone is often preferred but for unknown or severe cases PCR is the first choice followed by virus isolation. Following PCR diagnosis by
country-level or provincial laboratories clinical specimens are then sent to laboratories with BSL3 virus-isolation facilities for this purpose.

In China, PCR is also the first choice assay for H5 and other subtype detection, including during the recent H7N9 event when probe sets and diagnostic kits (n = > 200 000) were rapidly sent out, including export to Cambodia, and a full genome sequence was completed by 29 March 2013. Ten provinces reported human cases and one province reported animal cases with no human cases. The WHOCC Beijing collaborated with companies to modify and enhance assays, resulting in the production of a hospital-suited H7 positive control and to significantly increased sensitivity of the N9 assay. A single case of human infection with Eurasian avian-like A(H1N1) virus was detected through ILI surveillance. The subject recovered without intervention and appeared to have no obvious risk factors in relation to animal contact. Investigation of the case was prompted by the observed HA titre results which did not accord with the lower levels typically seen with A(H1N1)pdm09 viruses.

Laboratories in the Chinese national network are also encouraged to increase their capacity to perform virus isolation, and this is reflected in the scoring of evaluated laboratory capability. In line with WHO vaccine virus recommendations, the use of eggs is particularly encouraged with around 20 laboratories a year receiving financial support to carry out such work. In the WHO European Region, two training courses involving 22 laboratories and covering cell and egg culture were conducted. As a result, the number of isolates received by the WHOCC London has increased perceptibly – course students were reported to be enthusiastic and the WHOCC London will continue to encourage the forwarding of both isolates and clinical specimens from European NICs. The WHOCC London also noted that H3 virus characteristics are changing with the beneficial consequence that H3 egg-isolation rates are increasing. During 2012, 93% of H3 isolates could be tested using the HAI assay.

At national level, NICs need to robustly detect any influenza A or B viruses and identify common seasonal viruses. In addition, uncommon and unsubtypable viruses need to be promptly recognized and appropriate action taken in accordance with WHO Terms of Reference (ToRs) for NICs. Despite often being faced with limited resources, NICs need to be able to support other laboratories in order to cover national needs in surveillance and response. The typing and subtyping PCR approaches used – as well as the testing algorithms followed – need to fit in with these requirements. WHO guidance on dealing with unsubtypable viruses using PCR now needs to be updated and strengthened to better address issues of data interpretation.

Multiplex PCR also continues to be used to diagnose a broad range of respiratory illnesses in a wide range of settings. There is an increasing need for clear understanding of the objectives and issues involved in simultaneously testing for a range of influenza viruses and for respiratory viruses other than influenza. Issues to be addressed include the interpretation of results and other technical considerations, economic realities and a range of operational and other aspects. These must be addressed before laboratories implement testing programmes based on multiplex or multi-target technologies. The challenges of multiplexing include the complexity of design, which limits the flexibility of assays for any individual component, and the potential for the individual chemical reactions to affect each other. Multi-target approaches which run on the same card but in parallel are also likely to become a focus of developmental interest. In some national settings, for example France, the multiplex detection of a broad range of viruses and bacteria is part of an established programme of laboratory surveillance and diagnostic activities. Seasonal surveillance and emergency surveillance
programmes incorporate the co-circulation of all seasonal viruses, as well as testing for other respiratory viruses.

The applications of genetic sequencing in surveillance include the monitoring of antigenic drift in influenza viruses, monitoring the circulation of influenza B lineage viruses, detecting novel influenza strains with the potential to cause a pandemic, and tracking antiviral drug resistance mutations. It was felt that WHOCCs should maintain a good pool of primers to be able to sequence all novel viruses for both surveillance and diagnosis purposes given that real-time RT-PCR assays are not immediately available for newly emerging viruses.

Concerns surrounding sequencing quality in some NICs could best be addressed by training and follow up. It was suggested that sequencing training should be coupled to demonstrable NIC virus-isolation capacity and that laboratories should be encouraged to send both isolates and sequences to WHOCCs to encourage the retaining of virus-isolation skills in suitably equipped facilities. Areas such as bioinformatics and the use of related tools available on GISAID and allied databases such as Flusurver to analyse sequencing data are also vitally important but expertise is often lacking in GISRS laboratories. Although the timely sharing of sequencing information has improved – mostly through the use of GISAID – strengthened collaboration is required with animal-sector organizations such as OFFLU, for example on the use of established sequencing protocols for avian influenza viruses and facilitating access to existing animal-sector data during outbreak events.

There is a general consensus that the use of sequencing is an inevitable trend – and that helping to develop WHO advice on sequencing is an issue for consideration by this WHO PCR Working Group. For example in the WHO Region of the Americas sequencing is rapidly being adopted despite early resistance, but the need for sequencing information must also be balanced against a number of emerging concerns. It was suggested that any WHO guidance and recommendations in this area should have an integrated quality-control dimension and be linked to skills training to empower NICs and other laboratories to comfortably work with this type of data, including when advising governments. In addition, unless sequence data is tied to virus-isolation requirements, it will be of little use in the influenza system, and would simply represent a costly means of showing the subtype, which even in the case of a novel virus would be of limited benefit if no virus isolate was available.

In all WHO regions, and despite great variability in available capacities, it is clear that PCR is playing a greater role in the detecting of influenza viruses. In the WHO African Region, collaborative projects have been initiated with the United States Agency for International Development (USAID) and CDC to establish or strengthen the PCR capacities of sentinel sites, even in countries with very limited resources. WHO is also working to strengthen virus-isolation capacities and to provide support through initiatives such as WHO FluNet, the WHO Shipping Fund Project and the WHO External Quality Assessment Project for the detection of influenza virus type A by PCR (EQAP). Strong collaboration between WHO and its range of external partners is leading to laboratory capacity strengthening across the Region in an effort to build sustainable national capacity for influenza surveillance. In addition, NICs with advanced capacity are providing limited training and other support regionally while WHOCCs provide advanced training and testing services, including in areas such as sequencing, antiviral-susceptibility testing and antigenic characterization. As in other WHO regions, there is also increasing interest in the use of multiplex assays to detect other respiratory viruses. Major challenges to be addressed include poor procurement procedures, a lack of technical expertise in PCR and virus-isolation techniques, high rates of staff turnover.
and a lack of political will and funding to implement and sustain influenza surveillance programmes.

Improved WHO guidance may now be needed in light of the shift towards molecular testing approaches and the impact this has both on virus-isolation practices and on the criteria for forwarding viruses to a WHOCC. Such trends necessitate careful reflection on the role of classical virology (including virus isolation) and the preserving of capacity in this area. As well as the need to maintain and strengthen surveillance, enough viruses need to be grown to support activities such as vaccine virus strain selection and other activities for which genetic sequencing is insufficient. As molecular testing expands further, guidance to GISRS laboratories on virus isolation and submission will need to be adjusted accordingly.

Proposed action points

- Consideration should be given to coordinating the work of CDC and WHO procurement services as part of identifying potential cost-saving approaches for RT-PCR testing. Centralized negotiations with manufacturers may lead to significant procurement savings through the bulk purchasing of PCR equipment and reagents by GISRS laboratories.

- The current WHO guidance document on the optimal use of RT-PCR and virus isolation should be reviewed by the WHO PCR Working Group and feedback provided on any areas potentially in need of revision and updating. Potential areas for improvement include clarifying the criteria for submitting viruses to WHOCCs.

- The requirement for suitably equipped NICs to conduct virus isolation must continue to be emphasized. Although RT-PCR (both real-time and conventional) is increasingly the method of choice for influenza virological surveillance, this should not distract from the crucial role of virus isolation.

- Consideration to be given to the role of sequencing and to identifying specific topics in this area (for example, diagnostic applications, antiviral-resistance monitoring and genotype surveillance) prior to the next meeting to help guide decisions on the need for complementary expertise in this area.

- WHO to consider ways in which it could better work with WHOCCs and others in the delivery of practical training to NICs and other laboratories in the use of sequencing, and to raise awareness of tools available in this area.

Current GISRS PCR protocols

The conventional and real-time RT-PCR protocols published on the WHO web site continue to be evaluated for further updating and streamlining. Thanks were given to participants for all the updated protocols received to date and a call made for any new updates to be indicated.

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Discussion revolved around potential next steps in ensuring that the protocols provided are representative and presented in an easily accessible format. Despite the advantages of streamlining WHO and other protocols and reducing their overall number, there is also value in maintaining a range of different methods covering both conventional and real-time RT-PCR assays for seasonal and H5 influenza.

In addition, although a single WHO document may remain the best approach, flexibility will be needed. It was felt that an improved table of contents for the document could show more clearly which protocols are available for what sub-types. Such an expanded table of contents could be ordered by subtype, by targets of interest or even by source institutions for individual protocols. In terms of content, the two extremes are to publish only the contact information and/or link for the originating institutes or to assess protocols and publish the best. It was agreed that there were both practical advantages and disadvantages to both these approaches. Further enhancements such as providing the virus strains used to validate protocols or indicating which strains were likely to be picked up by specific protocols were raised for consideration.

Caution was urged in rushing to decide on an optimal approach as the current method works reasonably well. There is also a need to avoid overwhelming laboratories with too much choice. Related issues in updating WHO protocols include determining the selection criteria for inclusion, ensuring some degree of redundancy, and designing a viable system for archiving previous protocols for research publication and other purposes. It was felt that old protocols should remain accessible but that a very clear indication should also be made that these are outdated – with a version-control or time-stamped system put in place wherever links to previous versions were added to a current updated protocol.

Although the publication of a protocol in the broader literature does not equate to “approved”, WHO cannot exclude other good resources and guidance from the WHO PCR Working Group is needed on the extent to which WHO should attempt to evaluate alternative protocols. The highlighting and dissemination of comparative studies in this area (as conducted for example by the WHOCCs London and Melbourne) might be a way forward, though it must be made clear that a degree of subjectivity may be involved. In any case all protocols that are posted on the WHO web site should be updated as required and responsibility for this taken by the protocol source. However there must also be a clear distinction made between the provision of protocols to laboratories and follow-up support which represent two very different activities. It is the responsibility of laboratories to adhere to protocols and to ensure the quality of independently produced primer sets. Similarly the formal accreditation of assays remains the responsibility of WHO Member States with NIC and WHOCC support.

If the virological surveillance of seasonal H1, H3 and B viruses, as well as highly pathogenic avian H5 viruses, is proposed as the minimum requirement for NICs, then the availability and updating of WHO and other protocols and algorithms must sufficiently cover these particular viruses. NIC Norway (Oslo) reported on the on-going modification of an influenza B probe to correct for a recent development in Yamagata virus evolution, and agreed to share the results should the duplex method involved prove to be successful.

RT-PCR protocols for the detection of H7 and H9 now under development will be considered for future inclusion, along with protocols specific for example to A(H3N2)v and A(H7N9)
viruses. Expert validation and assessment of published protocols in this area would help to determine if they should be included in the approved WHO protocol document. Institutions must however be responsible for updating their methods and informing all users. The creation of tables showing the sensitivity and specificity of available assays (for example for H7) would allow laboratories to choose the protocol that best suited their needs.

An enquiry was made concerning the provision by CDC of genetic sequencing information for A(H7N9) virus primers. The WHOCC Atlanta indicated that sequencing data were available on request as this helped to avoid the use of outdated protocols. A query was also raised concerning the resources available for the translation of protocols into languages other than English. Requests have been made for other language versions to the WHOCC Atlanta. It was suggested that one possible approach would be for WHO to decide upon the updates to address and then to initiate translations with regional office support. It was reiterated that any translation would need to be technically correct and that WHO would need to take responsibility for this. The NIC Brazil (Rio de Janeiro) indicated it already performs some degree of quality assessment and checking of received protocol translations.

**Proposed action points**

- Further consideration to be given to updating and improving the usability of RT-PCR protocols across the current range of WHO and other sources. This could include further evaluation of the utility of an upgraded WHO RT-PCR protocol resource, providing, for example, information in areas such as multiplexing and optimum RT-PCR chemistry conditions for globally available and well-performing assays, along with information on the specific viruses used to qualify assays.

- WHOCCs to provide WHO with any comparative studies of currently relevant PCR protocols.

- Institutes requested to check everything that currently appears under their name in the WHO protocol document and to advise WHO of any required changes or additions by no later than 15 August 2013, and to submit any revised annexed protocols at the same time.

- Consideration to be given to adding the CDC CLSIS web site link to the updated WHO protocol document to strengthen the linkage between these complementary resources.

- WHO to continue to work with its regional offices in the translation of protocols into languages other than English, and to ensure their technical correctness.

**PCR assurance and quality-control activities**

The WHO H5 Reference Laboratory and NIC based in the Virology Division, Centre for Health Protection in China, Hong Kong SAR continues to coordinate, update and refine the WHO EQAP. By 2012 (panel 11), participation had increased to over 160 laboratories,

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primarily NICs, in all six WHO regions. Panel 11 also signalled a switch from the previous biannual assessment of performance to one dispatch per year over the period April–June. For 2013 (Panel 12) a different sample-inactivation method was used (from $\gamma$-ray inactivation to $\beta$-propiolactone (BPL) inactivation) and a resistant virus (H275Y) included. Following previous feedback from the WHO PCR Working Group and from laboratories, the panel volume was also increased from 200 µl to 500 µl. EQAP panel 12 results are expected to be made available to WHO in the second half of 2013 with early indications being that the number of responding laboratories and their performance levels were broadly similar to those for panel 11, though with an increase in the accuracy of H9 detection following a move to HA sequencing. In general, laboratories reporting correct results also scored highly in terms of their assessed adherence to good laboratory practice (GLP), highlighting the key importance of regular reviews of laboratory procedures and methods.

Potential next steps include the possibility of including live virus. Despite some concerns over contamination, lack of appropriate BSL and import issues, a significant number of respondents ($n = 46$) to the associated annual GLP questionnaire indicated their interest in such an addition. Consideration must also be given to the subtypes to be included in future panels (including consideration of covering both influenza B lineages), further improving the method of virus inactivation (especially where longer PCR products need to be generated), and further diversifying H5 clade representation.

Identifying the optimum approach for achieving the more-specialized aims of the programme is also becoming a more-pressing issue as the range of tested capabilities increases. At room temperatures panel transport costs are halved giving rise to the possibility of one broad-based room temperature panel (BPL inactivated) for general distribution and another targeted cold-chain panel sent to far fewer laboratories. Although it was felt that enhancements such as the electronic submission of results might lead to efficiency and other savings, any attempt to reduce the current 30-day turnaround and reporting period would be problematic given the realities of sending out panels and receiving results from different laboratories in widely differing regions, and the delays in obtaining permits etc. that can prevent a rapid response.

EQAP implementation also includes the follow-up of laboratories that either did not respond or did not participate. In addition, following the analysis of results, potential problems are identified and suggestions made in the confidential summary report prepared for each laboratory. Laboratories are then invited to review and correct any deficiencies. In parallel, the results are sent by WHO to the relevant regional office, which in some cases will approach individual laboratories.

The voluntary CDC Performance Evaluation Program (PEP) was originally developed to help domestic laboratories in the United States meet their regulatory requirements, and was extended to evaluate the performance of laboratories operating in CDC cooperative agreement (CoAg) countries. The scheme continues to allow for a demonstration that CoAg funding is having an impact, with results appearing to indicate that further funding would be both beneficial and justified. The information already gathered has been used to identify and address technical issues faced both by domestic laboratories and by laboratories in CoAg countries, for example in RNA recovery, real-time RT-PCR testing and platform-specific data analysis.

In terms of follow-up, the rationales for domestic and international laboratories differ. For domestic laboratories there is a possibility of moving to a biannual approach to support
statutory requirements related to the primary goal of assay qualification and laboratory accreditation. Problematic serially failing laboratories have tended to be in a distinct category, with issues primarily of departures from recommended protocols. Follow-up has typically been via state authorities. Currently no CDC respiratory virus panel is used for broader evaluation of domestic laboratories – though harmonization efforts are taking place with other respiratory virus agencies. For international laboratories, incorrect results were associated with issues such as CT interpretation, logistical problems and data capture. Consideration is now being given to the best approach to providing assistance to such laboratories with the two options appearing to be to wait another year and test again or to immediately implement another panel to see if issues have been addressed.

In 2013, the occurrence of human cases of A(H7N9) took precedence over PEP – with the emergency distribution of reagents by CDC and the qualification of assays. However, the verification of positive and negative samples by laboratories was problematic given the absence of positive specimens. CDC therefore produced a panel of positive and negative samples which was sent to 103 laboratories – with 89 results now in. It is clear that a number of laboratories performed poorly against the pass threshold of 100% correct allocation of positives and negatives. Although CDC does not directly collaborate in manufacturer development efforts, and in the case of H7 could only provide inactivated qualified materials, the potential return of H7 influenza cases in the winter would potentially lead to a demand for WHO/CDC validation of diagnostic kits. In any case, the H7N9 event delayed PEP activities which are now intended for rollout in the second half of 2013.

Discussion then turned to the objectives and optimal frequency of quality-assurance activities and the coordination of the various national, regional and international panels now available. Samples included in the EQAP panel should be relevant both to the viruses being detected in the field and to the realities of a range of national testing approaches and policies (for example in the degree to which subtyping is conducted). As the original scope of the EQAP to evaluate H5 detection continues to broaden, and additional clades of H5 are being considered for inclusion, new strategies such as the periodic use of additional special panels as outlined above could be considered. This might better incorporate aspects such as laboratory capacity for antiviral resistance monitoring which may not require annual assessment.

Virus-isolation capacity is also needed but is increasingly a gap in GISRS activities – indicators of capacity and successful implementation need to be defined and accurately measured. At present, the overall picture at laboratory level in terms of virus-isolation capacities, practices and intentions is not known. It was suggested that the upcoming WHO 4th global NIC survey intended for later this year could be adjusted accordingly.

The inclusion of a subtype in the EQAP that will not be identified by the majority of laboratories might also emphasize the importance of dealing with unsubtypable viruses and serve as a reminder to NICs of their ToRs. The inclusion of clinical information in future panels might also help laboratories decide how to test the samples.

As additional and specific quality-assessment panels are run by WHOCCs and other laboratories – nationally regionally or internationally (for example, a virus-isolation EQAP distributed in Europe) – there will be increasing benefit in comparing performances across the whole range of quality-assurance initiatives in which laboratories participate. Such comparisons allow for the possibility of accurately determining the reasons for repeated
under-performance. As requests to CDC and others from WHO Member States for advice in this area are increasing it seems likely that this will become an increasingly important issue.

Discussion was held on how best to support laboratories that consistently underperform in international quality-assurance assessments. WHO headquarters and regional office staff will continue to be informed of laboratory under-performance and will encourage laboratories in need of assistance to obtain help from a WHOCC. The reasons for non-optimal performance in the CDC PEP and/or WHO EQAP initiatives by specific laboratories should become clear, thus allowing for the targeted provision of training and other assistance, for example through local or regional high-performing laboratories.

**Proposed action points**

- In light of stable funding for the WHO EQAP and evolving needs, increased attention should now be given to its intended objectives and to its precise structuring. For example, the core year-on-year components should be identified along with more-flexible areas of need and consideration given to how best to address these.

- Further discussion to be held with WHOCCs and others on how the content and implementation of the WHO EQAP can be optimized in order to achieve its specific capacity-building objectives.

- Consideration to be given to the addition of a section to the EQAP panel results form in which laboratories could indicate how promptly they submit unsubtypable viruses to WHOCCs.

- Consideration could be given to greatly improving the processes for co-ordinating and comparing the results of WHO EQAP, CDC PEP and other national, regional and international quality-assurance efforts.

- Consideration to be given to increase support to laboratories following EQAP and other quality-assurance programme outcomes. This could include asking laboratories which fail the EQAP to follow up with a WHOCC or with the vendor of their equipment for technical support, clarifying the procedures by which WHO regional offices are informed of results, and providing information to WHOCCs on specific aspects of performance requiring attention in poorly performing laboratories.

**Role of the WHO PCR Working Group**

The WHO PCR Working Group continues to act as a valuable forum for GISRS and partner agencies to advise upon the development of WHO guidance for global influenza virological surveillance for public health purposes. As the range of guidance provided by the group continues to expand into broader issues surrounding the use of PCR techniques, there remains an expectation that implementable actions and recommendations to guide WHO will be generated. Any expansion or selected invitations to future meetings – for example, the incorporation of expertise on sequencing or on the use of multiplex approaches – should reflect this. In addition, further strengthening the representation of animal influenza agencies and organizations continues to be a key requirement of improved intersectoral cooperation and collaboration.
Recent years have seen great improvements in areas such as the rapid development and distribution of PCR protocols and reagents, and the performance evaluation of GISRS laboratories. Nevertheless, further guidance is increasingly required not only in identifying the precise role of PCR-based technologies within the GISRS but in determining the public health objectives of a wide range of emerging related technologies, such as next-generation sequencing and the use of multiplex approaches, especially in resource-constrained settings.

It was agreed that the WHO PCR Working Group will continue to meet each year and will work to strengthen its processes for generating guidance and recommendations to WHO based on observed national and regional trends in the use of RT-PCR and associated technologies.
Annex 1

6th WHO Working Group Meeting on PCR Protocols for the Detection of Subtype Influenza A Viruses

WHO Headquarters, Geneva, 2 - 3 July 2013

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