Review

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Received 10 April 2007
Accepted 29 June 2007

Establishment of a UK National Influenza H5 Laboratory Network

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Avian (H5N1) influenza continues to pose a significant threat to human health, although it remains a zoonotic infection. Sensitive and robust surveillance measures are required to detect any evidence that the virus has acquired the ability to transmit between humans and emerge as the next pandemic strain. An integral part of the pandemic planning response in the UK was the creation in 2005 of the UK National H5 Laboratory Network, capable of rapidly and accurately identifying potential human H5N1 infections in all regions of the UK, and the Republic of Ireland. This review details the challenges that designing molecular detection methods for a rapidly evolving virus present, and the strategic decisions and choices required to ensure successful establishment of a functional national laboratory network, providing round the clock testing for H5N1. Laboratory partnerships have delivered improved real-time one-step multiplex PCR methodologies to ensure streamlined testing capable of not only detecting H5 but also a differential diagnosis of seasonal influenza A/B. A range of fully validated real-time PCR H5 confirmatory assays have been developed to run in parallel with a universal first-screening assay. Regular proficiency panels together with weekly surveillance runs, intermittent on-call testing for suspect cases of avian flu in returning travellers, and several outbreaks of avian influenza outbreaks in poultry that have occurred since 2005 in the UK have fully tested the network and the current diagnostic strategies for avian influenza. The network has clearly demonstrated its capability of delivering a confirmed H5N1 diagnosis within 3–4 h of receipt of a sample, an essential prerequisite for administration of the appropriate antiviral therapy, effective clinical management, disease containment and implementation of infection control measures. A functional network is an important means of enhancing laboratory capability and building diagnostic capacity for a newly emerging pandemic of influenza, and is an essential part of pandemic preparedness.

Introduction

Given the serious threat to public health, early and accurate diagnosis of H5N1 infection is critical for effective clinical management, disease containment and infection control measures. Since re-emerging in 2003, avian H5N1 has maintained a steady global advance from the epicentre in South-East Asia, infecting wild birds and poultry, spreading into Europe and parts of Africa. Viral evolution has led to significant genetic variation among H5N1 viruses, associated with increased virulence and an expanded mammalian host-range. Numerous lineages, HA clades and subclades have been described, with recent isolates clustering into two distinct clades: clade 1 and the more diverse clade 2. Moreover, the ability of H5N1 to directly transmit from infected birds to humans following close contact, although a rare event, has been repeatedly demonstrated in more than 270 cases with an

approximate case fatality of 50–60 % (http://www.who.int/csr/disease/avian_influenza/en/). Although H5N1 remains an avian virus not yet adapted to transmission between humans, there is concern that small genetic changes may significantly alter the pandemic potential of this virus, allowing it to emerge as the next influenza pandemic strain. Highly sensitive, specific and rapid tests for H5N1 detection are therefore essential to control the threat to human health posed by this zoonotic infection. In view of the genetic diversity, molecular assays capable of detecting a wide range of circulating H5N1 variants are essential.

Pandemic planning and H5N1 threats in the UK

The detection of H5N1 in an imported finch in a quarantine centre in October 2005, and in a dead

Whooper swan washed up in Scotland in March 2006, avian influenza (H7N3) outbreaks in two poultry farms in Norfolk in April 2006, and more recently the H5N1 outbreak in a turkey farm in Suffolk in February 2007, emphasize the clear and present danger of H5N1 to animals and wildlife in western Europe, despite the distance from South-East Asia. Human exposure to H5N1 may occur not only in returning travellers potentially exposed in distant regions, but also as a consequence of exposure to virus in wildlife or poultry in the UK. The ability to detect and confirm rare zoonotic infections is usually the preserve of a reference laboratory. However, the threat posed by H5N1 and consideration of strategies to mitigate a pandemic (Ferguson *et al.*, 2006) suggest that at least one counter measure that can be considered is the use of antivirals to contain an epidemic at source. This requires the ability to detect and accurately diagnose infection at or close to the source/outbreak with minimum delay, a tactic consistent with global experience during the SARS epidemic in 2003, underlining the desirability of decentralised specialist testing, at least to a regional level.

UK regional diagnostic capability

Development of robust and rapid regional diagnostic capability for detection of H5N1 is part of national pandemic planning activity. Delivery of a seamless, integrated national diagnostic service, capable of delivering a reliable result for either a returning traveller with an influenza-like illness or exposed poultry worker, requires coordination of scientific, public health and logistical activities through a functional laboratory network. Diagnosis of H5N1 influenza can be achieved by a variety of techniques, the most reliable and sensitive of which are nucleic acid amplification techniques applied to clinical respiratory samples (de Jong *et al.*, 2006). Such tests target the most highly variable gene of the H5N1 influenza virus genome, the viral HA gene. However, as with any test used in a very low incidence setting (such as with rare zoonotic infections), the predictive value of a single positive test is <70%. It is essential to ensure that there is a confirmatory strategy to improve the certainty of diagnosis. In view of the frequency of returning-traveller queries (over 100 000 UK nationals return every week from South-East Asia), it was recognized that H5 testing needed to be based in regional laboratories, close to the patients, with the diagnostic service available 24/7 to deliver a confirmed result in a timely fashion, 3–4 h following receipt of a sample.

H5 assay development

The last few years has witnessed some exciting new developments relating to rapid H5 diagnosis. A number of real-time RT-PCR assays exist for the detection of avian influenza viruses, including assays that can detect all influenza A subtypes with an internal control (Di Trani *et al.*, 2006; Das *et al.*, 2006), a triplex assay that can specifically detect H5, N1 and all influenza A subtypes

(Payungporn *et al.*, 2006) and a monoplex assay that specifically detects H5 only (Ng *et al.*, 2005; Chen *et al.*, 2007). Evaluating which of the assays are suitable for primary screening and which might be better used for confirmation, ensuring that such assays are both sensitive and specific for the detection of H5 in relevant clinical material and are fully validated for clinical use, is a tall order. Within the UK, prior to the establishment of the National Influenza H5 Laboratory Network in 2005, significant progress had been made in some regional laboratories towards implementing multiplex real-time PCR methods for respiratory virus screening (Coyle *et al.*, 2004; Moore *et al.*, 2004; Templeton *et al.*, 2004; Gunson *et al.*, 2005), but H5 diagnosis was provided centrally at the Influenza Reference Laboratory in London and the Regional Virology Laboratory in Glasgow. Access to panels of influenza virus subtypes and different strains of H5 influenza to ensure subtype specificity and sensitivity of detection for relevant circulating strains is essential for the validation of assays that will be used to decide important/high profile public health measures. Clinical material from human H5 cases is globally highly restricted, since such material is in short supply and generally located outside western Europe. Assay validation involves comprehensive testing of reference influenza H1–H16 isolates, recently circulating H5 viruses from all H5 HA clades, related respiratory pathogens and clinical material containing human seasonal influenza. Preparation and analysis of simulated clinical material spiked with relevant concentrations of different influenza H5 (clade 1, 2 and 3) and other virus strains is an important part of assay validation, but is generally the preserve of specialist reference laboratories. However, measuring assay performance for different platforms (TaqMan, LightCycler, Rotor-Gene, Smart Cycler etc.) and fine-tuning technical/confirmatory strategies suitable for use in diagnostic settings require input from laboratories providing front-line diagnostic services in order to develop a coherent national strategy.

H5 assay validation

Creation of laboratory partnerships to overcome difficulties in developing tests suitable for a wide variety of settings is essential to ensure that robust and reliable assays are implemented. In the UK, collaborations between national reference and regional virology laboratories has led to the development of several national standard operating procedures (SOPs) for molecular detection of H5 (Ellis *et al.*, 2007; M. D. Curran, J. S. Ellis and M. C. Zambon, unpublished results), and a suite of confirmatory assays targeting different regions of the H5 HA using different technical approaches and platforms. Pyrosequencing, melting point analysis (fluorescence resonance energy transfer), restriction digestion of the screening assay RT-PCR amplicon (RFLP), an agarose gel-based RT-PCR with a post-amplification restriction enzyme digestion step (PCR-RFLP) and sequencing have been comprehensively validated on appropriate platforms to distinguish amplification of a

circulating H5 strain from the assay control material (Ellis *et al.*, 2007). This allows regional laboratories some choice in assay and confirmation strategies to provide a more streamlined work flow alongside other molecular diagnostic work performed in the same laboratories. Efficient diagnostic testing requires multiplex assays that can detect all influenza A subtypes (H1–H16) and influenza B, as well as specifically identify the presence of H5, with an internal control in a single reaction. A quadriplex real-time assay, which was developed for the network, fulfils these criteria, and allows simultaneous differential diagnosis (flu A or flu B) and the whole process of nucleic acid extraction, reverse transcription and amplification to be monitored, providing quality assurance and thereby minimizing the possibility of issuing a false-negative result (M. D. Curran, J. S. Ellis and M. C. Zambon, unpublished results).

Establishing a network

Whilst the requirement for a national/regional H5 laboratory network was evident, the practicalities of establishing it required substantial coordination. Choice of laboratories for inclusion in a laboratory network was based on:

- geographical coverage of the UK
- location in major urban centres
- existing laboratory facilities and expertise
- existing molecular diagnostic service capability
- core of molecular biology expertise
- ability to scale up capacity in the event of a pandemic.

Initial choice of laboratories and assays was followed by a comprehensive training course demonstrating selected assay protocols, to ensure that key laboratory personnel would be able to implement appropriate assays in their own laboratories. Participants returned to their laboratories with knowledge of the diagnostic and confirmatory assays. Regional laboratories aimed to provide a molecular-based diagnostic service, as well as a surveillance service, for influenza A, B and the avian H5N1 virus. A single assay was initially adopted as a front-line H5 screening test (Ellis *et al.*, 2007), providing the necessary national standardization and adaptability to other real-time platforms. While consensus existed for a confirmatory strategy using different assays, the development of a second real-time confirmatory assay, capable of distinguishing a control from wild-type H5N1 and run in parallel with the diagnostic assay, streamlined testing and made a 24/7 'test and confirm' service an achievable objective. To facilitate low-level surveillance and maintain proficiency, weekly runs using both assay formats performed on 10–20 routine clinical respiratory specimens by all laboratories providing an H5 service were undertaken.

Network support activities

Supporting activities for the UK National Influenza H5 Laboratory Network include:

- monthly teleconferences
- a secure website
- e-mail network
- reliable courier services
- provision of CE-marked (complies with the essential requirements of applicable European Union directives) H5 control material
- proficiency panel exercises.

A secure H5 laboratory network website has proved extremely useful for facilitating information deposition and exchange, identifying problems with methodologies, providing technical and information updates, clinical guidelines and newly released SOPs, and allowing laboratories to see and provide supporting evidence and data to substantiate issues raised during regular monthly teleconferences. Monthly teleconferences have been pivotal to the success, and ongoing development and testing strategy of the network. As part of the network support by the reference laboratory, regular sequence review of newly deposited H5 sequences is undertaken, and new H5N1 isolates from around the world are analysed to ensure that no modifications are necessary as a consequence of sequence diversity occurring in the target sites, and that all approved diagnostic assays used by the network are effective. Centrally provided CE-marked inactivated H5N3 virus control material, which is suitably easy to distinguish from currently circulating H5 strains, is used as positive control material throughout the network.

Annual proficiency testing

Annual proficiency testing has been introduced to ensure that participating laboratories are accredited for H5 diagnostic testing, and demonstrate that regional laboratories in the National Influenza H5 Laboratory Network can provide a quality-assured diagnostic service for H5 detection. The first H5 proficiency-testing panel of simulated clinical samples containing high and low titre Vietnam H5N1 viruses (inactivated), and influenza A and B viruses, was distributed in October 2005 simultaneously to 19 laboratories in the UK and 1 laboratory in the Republic of Ireland, timed to arrive at each laboratory at the same time. The results of proficiency testing demonstrated an overall excellent performance by the laboratories. All participating laboratories had succeeded in establishing H5 assays. The high completion rate (18/19) and overall performance showed that laboratories had been able to implement H5 specific assays in a 3–4 month period, irrespective of local problems. The following conclusions were possible following the first proficiency exercise:

- all laboratories using H5 RT-PCR assays could have results available in 6–8 h after receipt of a sample
- clear confirmation strategy is essential for accurate H5 diagnosis. The major errors that occurred were in laboratories that used single assays without confirmatory strategies. This underlined the necessity for a comprehensive testing strategy for low-volume tests

in non-specialist laboratories to ensure a very high predictive value in the event of a positive test result

- generic molecular influenza A/B diagnosis and subtype analysis in the regional laboratories can be used to enhance national influenza surveillance programmes.

Proficiency testing has continued, with a second proficiency panel in November 2006. This was designed to test whether laboratories could reliably detect virus strains representative of all circulating H5 lineages at clinically relevant concentrations. The panel contained 12 samples, including all 3 major H5 HA clades, as well as a number of other influenza A subtypes (H1, H3 and H7) and influenza B. All UK regional laboratories included in this exercise demonstrated the capability to detect and confirm diverse H5 strains, and deliver H5-screening results in a very timely way, which was substantially improved from the previous exercise, and suitable for dissemination in the event of a suspect case of avian influenza. Timed proficiency-panel exercises are an important and useful way of assessing laboratory service provision, testing technical decision making in complex algorithms and clinical interpretation, as is required in high-profile outbreak situations where the time to result delivery is calculated in hours, not days. Results of proficiency panels provide objective evidence of improving proficiency and familiarity with assays, and performance in these assays provides a means of deciding on service delivery capability for the National Influenza H5 Laboratory Network.

Network performance

The number of suspect H5N1 cases in the UK fulfilling the clinical algorithm for exposure and illness presentation (http://www.hpa.org.uk/infections/topics_az/influenza/avian/algorithm.htm, http://www.hpa.org.uk/infections/topics_az/influenza/avian/documents/J3poultrymanagement080207.pdf) between 2005–2006 has been limited (approximately 1–2 per month), although presentation of possible cases has occurred throughout the UK. The outbreak of avian H7N3 influenza (low pathogenicity) in a poultry flock in Norfolk in April 2006, and its subsequent identification in two other free-range poultry farms close by, provided an opportunity to examine the national strategy for avian influenza. Suspect H7 human cases arising during this episode were screened at the local regional laboratory and specimens fast-tracked to the National Reference Laboratory for confirmation and further analysis. On the 27th April 2006, a poultry worker presented with conjunctivitis, and testing within 4 h identified influenza A in all specimens (eyes, nose and throat). This was subsequently confirmed as H7N3. Samples from a further 17 suspect human cases, all displaying conjunctivitis or other respiratory symptoms, were screened using the quadriplex assay and found to be negative for influenza A. Despite only one human case in this outbreak, the outbreak proved extremely informative, and highlighted strengths

and minor weaknesses in the regional testing strategy, suggesting attention to certain aspects of logistics planning.

More recently, an outbreak of H5N1 in turkeys in a farm in Suffolk, England, at the beginning of February 2007 again triggered outbreak-response testing in two different regions of the UK. Of six cases presenting with influenza-like symptoms, none were found to contain the highly pathogenic H5N1 subtype, but three did test positive for influenza A, confirmed to be the circulating influenza A H3N2 virus. Fifty to sixty per cent of suspect H5N1 cases in travellers returning to the UK have been cases of circulating seasonal influenza A H1 or H3 or influenza B viruses, emphasizing the need to test for seasonal influenza in parallel with H5 testing (J. S. Ellis & M. C. Zambon, unpublished results), in the event of returning-traveller illness or outbreak investigations.

Network development and role of commercial assays

Commercial assays for diagnosis of H5 have not played a significant role in the last few years in the provision of H5 diagnostic capability in the UK. This has partly been due to the difficulty for companies that prepare kits for human diagnosis in accessing strains and sequences from newly emerging H5 viruses to achieve CE marking required on kits for human clinical use. Several companies have begun to find ways around these difficulties, often by working in partnerships with laboratories in countries in South-East Asia affected by H5N1. It is likely that the next few years will see the availability of different commercial kits for use in diverse laboratory settings. One role of the National Influenza H5 Laboratory Network will be to evaluate such kits and determine their suitability for different laboratory environments. Several kits are available that are based on the technique of isothermal amplification of viral RNA. For example, a rapid and sensitive CE-marked test, based on a novel amplification method (Imai *et al.*, 2006, 2007), is now available (<http://loopamp.eiken.co.jp/e>). While nucleic acid extraction is needed for this test, there is no necessity for a real-time PCR instrument or thermal cycler since amplification is isothermal (63 °C) and amplification can be visualized by eye, or using a UV lamp. Such a test would be extremely useful in a simple front-line clinical laboratory lacking complex equipment close to an outbreak/incident site. Alternatively, an example of a more complex methodology is a recent CE-marked multiplex diagnostic test that permits the detection of 16 different respiratory viruses in a single test, including avian H5 influenza, seasonal influenza H1 or H3 viruses and SARS virus (<http://www.idtag.tmbioscience.com>), and requires moderately complex equipment. Such tests have the potential to significantly streamline molecular diagnostics across microbiology disciplines, but may not be suitable for front-line laboratories with simple equipment and few staff.

Conclusion

The creation of a national laboratory network for influenza H5 diagnosis ensures a rapid testing response to suspect H5N1 clinical cases can be mounted anywhere in the UK and Republic of Ireland. Ongoing influenza A surveillance using molecular methods within the network provides enhanced capability within the UK for detection of newly emerging influenza strains in humans. Assessing the suitability of commercial tests and matching them to appropriate laboratory environments is part of national and regional pandemic preparedness, and an important role for an influenza laboratory network, which also needs to adapt and evolve in response to the formidable challenge offered by the virus it is attempting to track. The supporting structures within the network provide a generic capability and capacity to permit the network to adapt quickly to any new emerging infectious pathogen, and provide necessary national molecular diagnostic capability in the absence of commercially available reagents.

Acknowledgements

The authors would like to thank all participants in the UK National Influenza H5 Laboratory Network. National SOPs are available upon request from the authors.

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