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Editorial note:

This is the inauguration issue of **Topical Update – The Hong Kong College of Pathologists** published by the Education Committee of the Hong Kong College of Pathologists. It is envisaged that this publication will provide a platform for the presentation and discussion of current topics in pathology and related disciplines, with the aim of updating practical knowledge and guiding best practices. Any feedback and suggestions could be directed to Dr. Janice Lo (e-mail: janicelo@dh.gov.hk) of the Education Committee, the Hong Kong College of Pathologists. Opinions expressed are those of the authors or named individuals, and are not necessarily those of the Hong Kong College of Pathologists. Happy reading !

Laboratory diagnosis of human disease caused by H5N1 influenza virus

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Avian influenza A subtype H5N1 is endemic in poultry across south-east Asia and continues to cause zoonotic disease in humans. So far, transmission of virus from avian to humans appears very inefficient and sustained transmission from human-to-human has not occurred. However, with the continued opportunity for human exposure over an ever increasing geographic range, it is possible (though not inevitable) that H5N1 virus may acquire the ability to transmit efficiently from human-to-human, leading to a pandemic.

Human disease caused by H5N1 influenza virus typically presents either as a rapidly progressing viral pneumonia, often with evidence of marked lymphopenia, leucopenia and mild to moderate liver dysfunction. Some patients also have evidence of diarrhea and other gastro-intestinal manifestations. The disease may progress to acute respiratory distress syndrome (ARDS), multiple organ dysfunction and death (1-5). However, in the individual patient, it is not possible to make a reliable diagnosis of avian influenza H5N1 purely on clinical grounds. Furthermore, some patients may manifest a milder course of the disease presenting merely as a self-limited influenza-like illness. Virological diagnosis is therefore essential.

Whom to test?

Although sporadic cases among wild birds and smuggled or backyard chickens have been detected, H5N1 virus is not presently active among poultry populations in Hong Kong. Thus, patients who require consideration for testing for H5N1 virus are those with history of recent travel to areas where the virus is endemic with poultry exposure; those within Hong Kong exposed to H5N1 virus through occupation (e.g. laboratory staff working with infectious H5N1 virus); close unprotected contact with sick or dead birds; unexplained clusters of pneumonia and those in direct contact with known cases of human H5N1 disease.

In areas where H5N1 is endemic, a severe progressive viral pneumonia in otherwise healthy young adults or children beyond the period of infancy should raise suspicion of avian influenza. Cluster of disease within families is an additional cause for heightened suspicion. It should be noted that up to 30% of cases of avian influenza H5N1 in endemic regions do not have an obvious exposure history to sick poultry (5).

What clinical specimens need to be tested?

Respiratory specimens are required for virus detection and paired serum specimens are useful for a serological confirmation of H5N1 infection. Nasopharyngeal aspirates (NPA), nasopharyngeal swabs, throat and nose swabs are all useful respiratory specimens for detecting avian influenza H5N1. Endo-tracheal aspirates, broncho-alveolar lavage or lung biopsy, when available are excellent specimens for diagnosis of avian influenza H5N1. Nasopharyngeal aspirates were successfully used for H5N1 diagnosis in Hong Kong during the avian influenza outbreak in 1997. In addition, it provides the ideal specimen for rapid (4-6 hours) diagnosis of many other respiratory virus infections (e.g. conventional human influenza A or B, adenovirus, parainfluenza virus), thereby helping to exclude a diagnosis of avian influenza. Such an alternative diagnosis can be rapidly established on NPA specimens but not on swab specimens. Throat and nose swabs (rather than NPA) have been more generally used in recent human cases in Vietnam, Thailand and Indonesia and there is no good recent comparative data on whether swabs or aspirates are the superior clinical specimen for diagnosis of H5N1 disease. Available data comparing throat swabs with nose swabs tested in parallel appears to suggest that a throat swab is superior to nasal swabs. The nose and throat swab may be placed in the same transport medium bottle. If nasopharyngeal secretions are present, for reasons outline above, it is best that a NPA is collected in addition to a throat and nose swab. Appropriate personal protection (mask, eye cover) should be used when collecting such respiratory specimens.

Virus RNA has been detected in faeces and in serum but viral load and diagnostic yield appears to be lower than that found in respiratory specimens. Thus, while these specimens may be collected for investigation, the primary diagnostic specimen should be a respiratory specimen (5).

Autopsy specimens are critical in confirming or excluding avian H5N1 influenza disease. If a full autopsy is not possible, a paramortem biopsy using the Tru-Cut needle is an alternative option.

Once collected, specimens for virus detection should be kept at 4^{o}C until they are sent to the laboratory. They should NOT be frozen at -20^{o}C. If a long delay (>3-4 days) is anticipated before being sent to the virology laboratory, the specimen should be frozen at -80^{o}C.

Demonstrating a serological response to H5N1 virus in paired sera provide a retrospective confirmation of H5N1 infection. Seroconversion by micro-neutralization is generally detectable 14 days after onset of illness (6).

Laboratory tests

Options for detecting influenza A viruses in clinical specimens include a) virus culture, b) virus antigen detection, or c) detection of viral nucleic acid by RT-PCR methods (7). Isolation of H5N1 viruses in culture can be done by inoculation of embryonated eggs or of Madin Darby Canine Kidney (MDCK) cells. Growth of human influenza A viruses requires the addition of exogenous trypsin (2 μg/ml), but H5N1 virus is a

"highly pathogenic avian influenza virus" and virus growth is independent of exogenous trypsin supplements. Viral culture may take 2-6 days but the availability of a virus isolate allows full genome sequencing and opportunity for antigenic characterization. Genetic sequencing of the virus will provide evidence of genetic reassortment or antiviral resistance and clues to possible changes of the virus that may reflect greater adaptation to human transmission.

Viral antigen detection may be carried out by immunofluorescence or enzyme immunoassay (EIA) methods. The EIA based methods are simple and convenient in use and may in theory be applicable as point-of-care tests. Presently, such tests are directed at conserved viral antigens (e.g. virus nucleoprotein, matrix protein) and detect all subtypes of influenza A viruses, whether of human or avian origin. Therefore these tests will not differentiate human virus subtypes H3N2 or H1N1 from avian influenza H5N1. A positive result will require additional tests (e.g. RT-PCR or culture) for differentiation of virus subtype (e.g. H5 vs. H3 or H1). Besides, current viral antigen detection tests, while being sensitive for the detection of human influenza viruses, appear to have low clinical sensitivity for the diagnosis of avian influenza H5N1. A negative result does not exclude H5N1 disease. Thus overall, presently commercially available antigen detection tests have limited clinical utility for diagnosis of H5N1 disease in humans.

RT-PCR tests can be targeted at genes that are relatively conserved across all influenza A viruses (e.g. matrix gene) or to the haemagglutinin or neuraminidase genes which are subtype specific. In practice a panel of such RT-PCR assays (generic influenza A detection plus subtype specific H5 detection) are used to investigate suspected human H5N1 disease. Including the time taken for viral RNA extraction and for amplicon detection, the turn-round time of conventional RT-PCR assays are 6-8 hours (or overnight). However, real time PCR methods can shorten this time interval to around 4-6 hours while providing increased sensitivity and possibility of quantitation of the viral target gene (7).

The clinical sensitivity of tests for detection of avian influenza H5N1 in specimens collected from the upper respiratory tract appears to be lower than commonly observed in patients with conventional human influenza A disease. This lower clinical sensitivity is not explained by a reduced analytical sensitivity. Thus, the reason for the lower clinical sensitivity is likely to be due to the presence of lower levels of H5N1 virus in the clinical specimens collected. This may be due to poor specimen collection and transport. Alternatively, it is possible that there are differences in tissue tropism of the avian flu H5N1 virus, which may involve the upper respiratory tract less that conventional human influenza A. In any event, these observations point to the need for extra care and effort at specimen collection and in laboratory testing when attempting a diagnosis of avian influenza H5N1.

In practice, the microbiologist must take into account the specimen quality, stage of disease, clinical condition and epidemiological exposure to decide on the management and infection control strategy, especially for a negative laboratory result in the context of clinical suspicion, in view of the limited resources such as isolation rooms. Additional investigations may be considered on a case by case basis.

As for serological diagnosis, apart from the micro-neutralization test, which is known to be the most sensitive method, single radial haemolysis and Western blot could be used as supplementary tests.

Quality assurance

With continued evolution of the H5N1 virus, mismatch with PCR primers and probes may occur and this should be taken into consideration when designing test protocols. It is essential that laboratories use only test protocols that have been evaluated against a number of H5N1 strains. Optimization of test methods may be necessary if different equipment is used. Training of personnel, appropriate design of facility and maintenance of equipment are other factors that may affect test results. Participation in external quality assessment programmes should be considered.

Laboratory safety

WHO recommends that procedures that involve virus replication (virus isolation, micro-neutralization tests) should be carried out in biosafety level (BSL)-3 containment. However, procedures that do not involve amplification of infectious virus by culture can be carried out at BSL-2 containment. All H5N1 virus isolates and specimens tested positive for H5N1 virus should be stored in an appropriate containment facility. Inventory of specimens, viruses and genetic materials should be kept and updated regularly.

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