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# Occult respiratory viral infections in coronial autopsies: a pilot project

## Key Messages

1. The presence of clinically undiagnosed and untreated RNA respiratory viral infections in autopsies performed under the Hong Kong coronial system in 2003 was investigated.
2. There was a relatively low incidence (11/130, 8.4%) of polymerase chain reaction-positive cases with respiratory syncytial virus and NL63 accounting for four and seven cases, respectively. Immunohistochemical staining did not show any evidence of viral antigen.
3. Untreated viral infections may contribute to a minority of sudden respiratory deaths in Hong Kong, but more attention should be focused on the role of newly discovered agents such as NL63 on population mortality.
4. Occult viral infections appear to play a minor role compared to bacterial disease.

## Introduction

Respiratory viral infections in Hong Kong have a different seasonality from those in temperate regions of the world, and in the past few years well-controlled studies have investigated the degree to which influenza and respiratory syncytial virus (RSV) contribute to morbidity and mortality in Hong Kong.<sup>1,2</sup> The opportunity for accurate disease modelling is enhanced because of the centralised records of the Hong Kong Census and Statistics Department and the major role of the Hospital Authority (HA)—90% of all admissions are managed by the HA. The influenza surveillance programme of the Department of Health showed that influenza accounted for 4942 cases of influenza in 2004.<sup>3</sup> At Queen Mary Hospital other viruses such as adenovirus and parainfluenza accounted for fewer infections than influenza or RSV.<sup>1</sup> Detection of patients with no microbiological cause for respiratory tract infections led to the discovery of novel infectious agents. Three of the four new viral agents discovered over the past 3 years were coronaviruses and the remaining one was a parainfluenza virus, known as human metapneumovirus (HMPV). Polymerase chain reaction (PCR) amplification of common or consensus regions in viral genomes contributed to this discovery by enabling wider detection of viral genes followed by more specific amplification and sequencing.

The Forensic Pathology Service (FPS) works closely with the Hong Kong Police and provides professional input on medico-legal aspects of criminal cases. In 2000, there were 33 933 registered deaths in Hong Kong, and the impact of influenza on the death rate has also been reported.<sup>2</sup> In the same year, 5400 post mortems (ie 16% of all deaths) were performed by the FPS to determine the cause of death (many of these were violent deaths eg traffic accidents, murder, suicide). In sudden unexplained deaths, if the lungs showed histological evidence of pneumonia, chest infection or pneumonia was rendered as the cause of death. Further investigation to determine whether the agent was viral or bacterial was usually not undertaken. The newer viral infectious agents for respiratory tract were identified through a number of samples from the respiratory tract; in the past it was mainly from nasopharyngeal aspirates. The autopsy materials from the FPS provide an untapped source of data, as the patients who died suddenly had not been treated with antiviral agents.

## Methods

This study was conducted from December 2005 to December 2006.

### Sample selection

The causes of death were obtained from all autopsies performed in 2003 by pathologists at the three public mortuaries in Hong Kong; all deaths attributable to either chest infection or pneumonia were recorded. We aimed to determine if there were any undocumented cases of fatal severe acute respiratory syndrome (SARS) during February to June 2003.

There were a total of 6879 deaths attributable to natural causes and 1293 were due to diseases of the respiratory system. As autopsies were not performed (ie 'waived') in 810 patients, there were 1293 cases from which material was available. The age and sex distributions of the samples are shown in Table 1. We excluded patients with malignancy, and in over half of records examined

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**Table 1. Age and sex distribution of 1293 samples**

Sex	Age-group (years)								Unknown	Sub-total
	0-9	10-19	20-29	30-39	40-49	50-59	60-69	≥70		
Male	8	4	12	11	42	61	124	608	1	871
Female	-	2	6	15	22	15	30	332	-	422

**Table 2. Analysis of cases from three public mortuaries\***

Mortuary	Total No. of cases	RSVA PCR positive	RSVB PCR positive	FluA PCR positive	NL63 PCR positive
Wan Chai	32	3	0	0	4
Tai Wai	49	0	0	0	0
Kwai Chung	49	1	0	0	3
<b>Total</b>	<b>130</b>	<b>4</b>	<b>0</b>	<b>0</b>	<b>7</b>

\* RSV denotes respiratory syncytial virus, and PCR polymerase chain reaction

there was insufficient fixed lung material present for DNA or RNA extraction.

The paraffin tissue was obtained and haematoxylin and eosin staining was performed to determine which of the sites in the lung are suitable for further examination. The presence or absence of viral inclusions was recorded.

#### ***Polymerase chain reaction amplification***

The first step was to determine if PCR amplification methods used on non-tissue samples in the clinical laboratory could be applied to tissue sections. Initial results on previously confirmed influenza cases were negative. So we modified existing protocols and adapted them for formalin-fixed tissues. We then confirmed the positive cases using immunohistochemistry.

#### ***Immunohistochemistry***

From an analysis of the records of the three public mortuaries (Tai Wai, Wan Chai and Kwai Chung), we identified 130 cases where the cause of death was recorded as chest infection and tissue blocks were available for further examination. Histological examination of the sections confirmed the presence of acute pneumonia, bronchopneumonia, chronic obstructive lung disease, tuberculosis or acute pulmonary oedema. Only one case showed an interstitial infiltrate suggestive of viral infection (03-378F). In no cases were intranuclear or intracytoplasmic viral inclusions seen.

### **Results**

#### ***Influenza***

We used control positive cases of H5N1 proven autopsy material and found that protocols developed for the clinical virology laboratory did not result in amplification of any PCR product (data not shown). When we used a protocol developed by the United States Armed Forces Institute of Pathology,<sup>5</sup> it required some modification. In particular, we found that the proteinase K digestion prior to phenol-chloroform-isoamyl alcohol extraction was more effective than using a commercially available purification kit. We

then found that 3x10 µm consecutive sections per sample were just as effective as 10x10 µm consecutive sections for a positive finding and later modified this to 6x6 µm sections. Based on data obtained from previous studies, a product size of less than 120 (for influenza) resulted in a more positive band than when a larger amplicon (404 bp) was selected.<sup>4</sup> The detection of RSV and NL63 proved less problematic than that for influenza. We then analysed the cases from the three public mortuaries (Table 2). No positive SARS cases were identified in that year.

#### ***Immunohistochemical analysis***

The PCR positive cases of RSV and NL63 were then analysed by both immunohistochemistry and sequencing. Immunohistochemistry identified no positive cells in any of the samples, though control samples were positive. Sequencing showed homology with published RSV and NL63 sequences, indicating that the results were not artefacts (data not shown). We therefore concluded that the positive findings were more indicative of past rather than recent infection (as published previously).<sup>5</sup>

### **Discussion**

This pilot study aimed to optimise the diagnosis of viral infection of archival tissue fixed in formalin; for over a century formalin has been the fixative of choice for tissue samples. Fixation of tissue is necessary because as soon as death occurs or tissue is removed from the body in a vital state, it undergoes a degenerative process called autolysis. The fixation of tissue samples in formaldehyde leads to extensive cross-linking of all tissue components. This preserves tissue and prevents autolysis. Recently, the safety of formalin has come under scrutiny; in the European Union it is classified as a Class I carcinogen. Alternatives such as Glyo-Fixx, STF-Streck, Omnifix, Histochoice, and Histofix have been proposed, but none of these has met with much approval for morphological diagnosis by pathologists. From a research perspective, the main drawback with formalin fixation is its limited ability to extract DNA and RNA from this fixed tissue. Only 0.1% contamination of an extraction

reagent by formalin may inhibit the ability of the reagent to extract mRNA.

For molecular diagnosis of a viral infection, it is best not to use any form of fixative, because RNA can readily be extracted from fresh tissue using either a combined guanidinium thiocyanate acid–phenol chloroform procedure or a guanidinium thiocyanate–caesium chloride gradient. Commercially available RNA extraction methods are available (RNeasy [Qiagen], Trizol [Invitrogen] and ToTally RNA [Ambion]), but in PCR amplification of fresh samples their success rate is varied, possibly due to the steps of the individual techniques and the affinity of viral RNA for the spin column. These solutions do not fix the tissues in the same way as formalin does, so they are not useful for morphological analysis or long-term storage at room temperature. It is very difficult to interpret morphological analysis from tissues placed in this reagent.

Fixation in alcohol is superior to formalin for the preservation of RNA, but results in poor morphology. Although immunohistochemistry is not altered, it is conceivable that in alcohol-fixed tissue some antibodies will not react as the antibodies are developed for use in formalin-fixed material and small molecules (eg peptides) may be solubilised and lost in alcohol-based fixatives. Carnoy's fixative is superior to formalin and ethanol for RNA extraction, but is not widely used in routine practice. Bouin's fixative should be avoided as it causes damage to DNA and RNA. If ethanol fixation is used, 70% seems to be the ideal concentration though it should only be used for small samples.

Our findings confirmed that in analysing fatal lung conditions, there was a problem with the extraction and amplification of RNA which hampered the process, as protocols used by clinical virology laboratories for non-formalin-fixed specimens may be unsuitable for autopsy specimens. Protocols developed for the extraction of RNA for influenza virus needed to be modified in order to enhance the successful amplification of RNA. Our results indicated that our protocols were just as effective as the commercially available kits for RNA extraction.<sup>4</sup>

From our retrospective analysis of cases dying in the community that had autopsies, there was a low incidence of occult respiratory RNA viral infections as determined by PCR. As optimisation of the previously published techniques required much more effort than anticipated, we were unable to analyse the novel respiratory agents (such as HMPV or CoV-HKU1) apart from NL63 within the time frame. However, this will be addressed in future experiments. No cases of undocumented SARS were identified in 2003.

There are a number of limitations to this investigation. First, tissue sampling is dependent on the pathologist's

expertise. Given that only one tissue block from each lobe of the lung is sampled, only a small area of the lung is assessed. Second, as many respiratory viruses are RNA in type and the standard fixative is formalin, the extraction and detection of high quality nucleotides suitable for PCR amplification remains a concern. Hence, any positive findings most likely represent the lower range of the real situation. Third, it appears that given the relatively low number of documented viral infections over a calendar year, having routine viral culture or analysis of tissue samples from patients with chest infection is probably not warranted. However, given that 7/130 (5.3%) of mortuary cases (most of whom were elderly) were coronavirus NL63 PCR-positive, further investigation into the nature and pathogenesis of this newly discovered entity seems warranted. Such studies should have included seasonal changes, existing co-morbidities and cellular tropism, which were beyond the scope of this investigation.

## Conclusions

This was a pilot project to test the feasibility of a retrospective analysis of formalin-fixed tissues and demonstrated that it was a less-than-optimal method for detecting viral infections. Conventional protocols in use by diagnostic laboratories for clinical specimens should be amended and focus in particular on smaller PCR products. A newly discovered coronavirus (NL-63) is present in 5% of autopsy cases and further investigation into this novel agent is warranted. If prospective studies are to be performed, tissue should be fixed in ethanol in addition to formalin. At this stage there appears to be little justification for routinely sending autopsy specimens for viral culture.

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