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Influenza A H9N2: Aspects of Laboratory Diagnosis

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Influenza A viruses of subtype H9N2 are now considered to be widespread in poultry (1). They have recently been isolated from pigs originating from southern China (5) and from two children with influenza-like symptoms in Hong Kong (2). Crossing the species barrier to mammals highlights the pandemic potential of H9N2 virus. Recent findings indicate that the H5N1 viruses responsible for the severe human disease that occurred in Hong Kong in 1997 (7) are reassortants that obtained their internal genes from avian H9N2 viruses (3). It is important for pandemic influenza preparedness to have diagnostic options for recognizing H9N2 viruses. Given the genetic heterogeneity of recent nonhuman H9N2 isolates (3), it is important to examine not only human H9N2 isolates but also those from porcine and avian hosts.

The viruses used in this study are listed in Table 1. In the Direct*igenFluA* test (Becton Dickinson, Lockeysville, Md.), the detection limits for human, pig, chicken, and quail H9N2 isolates diluted in virus-free nasopharyngeal aspirates ranged between 5 and 2,000 50% tissue culture infective doses ($TCID_{50}$).

Commercially available reagents for influenza A virus antigen detection and subtyping by immunofluorescence were evaluated with virus-infected cells fixed in acetone. Influenza A virus specific monoclonal antibody (MAb) reagents DAKO (Glostrup, Denmark) IMAGEN influenza A and Respiratory Screen and Bartels (Issaquah, Wash.) influenza A were reactive with human, porcine, chicken, and quail H9N2 isolates.

The influenza virus H3 subtype MAb 8254 (Chemicon, Temecula, Calif.) is known to be reactive with most contemporary human H3N2 variants, while Chemicon H1 MAb 8252 fails to react with some recent H1N1 isolates (4). The H3 MAb cross-reacts only with H4 subtype viruses (Table 1), while the H1 MAb was monospecific for H1.

MAbs HA1-71 and HA2-76 (provided by N. Cox, World Health Organization [WHO] Influenza Center) are used for subtyping influenza A viruses on the basis that the former is H3 specific while the latter reacts with both H3 and H1 subtypes as well as other avian H subtypes, including H9 (9). These MAbs were tested for immunofluorescent reactivity with the viruses listed in Table 1. We confirm that HA1-71 is monospecific for H3. HA2-76 cross-reacts with contemporary H9N2 viruses from different hosts. Our results with HA2-76 are in broad agreement with a previous report (9) with two exceptions. We find that it cross-reacts (i) with an H7 isolate (A/duck/Hong Kong/47/76) and (ii) with H2 subtype viruses A/duck/Hong Kong/319/78 and, weakly, A/Asia/57. Until now, a human virus isolate reactive with HA2-76 and not with HA1-71 would have been presumptively subtyped as H1. However, one should be aware that this reaction profile may also occur with two other subtypes of potential relevance to humans, viz., H9 and H2.

A pool of MAbs to H5N1 virus (CP58, CP62, CP76, 364/1) provided by R. G. Webster, St. Jude Children's Research Hospital, Memphis, Tenn., used for rapid diagnosis of H5N1 influenza (7) had no cross-reaction with any other H subtype.

Comparison of acetone and methanol fixation suggested that acetone gave better fluorescence results with the influenza A type-reactive reagents which recognize the nucleoprotein antigen. In contrast, either fixative was suitable for the subtype-specific reagents (those that react with the hemagglutinin), although methanol was marginally superior.

The hemagglutination inhibition (HAI) test was carried out with the 1998–1999 WHO influenza virus subtyping kit. In addition, R. G. Webster provided H9-specific sera raised in rabbits or chickens. Significant antigenic heterogeneity among H9N2 viruses isolated from different hosts (e.g., quail versus chicken) was seen (Table 2). The WHO H3 subtyping antiserum cross-reacted with some quail H9N2 isolates grown in eggs but not with the same virus grown in MDCK cells. Further, a rabbit hyperimmune H9 antiserum cross-reacted with the WHO reference H3 antigen. Unless differential HAI tests including H9-specific reagents are carried out in parallel, cross-reaction with H3 antiserum may lead to the misdiagnosis of egg-grown H9 isolates as H3. At the time of writing, HAI reagents reactive with the human H9N2 isolates are not widely available.

An H9 subtype-specific RT-PCR was developed. The primer and probe sequences were as follows: for the sense primer, 5′ TTGCACCACACAGAGCACAAT; for the antisense primer, 5′ TGATGTATGCCCCACATGAA; and for the internal probe, 5′ AATGGAATGTGTTACCC. Viral RNA was extracted with the QIAGEN (Hilden, Germany) QIAamp viral RNA kit. Reverse transcriptase PCR (RT-PCR) was carried out as a one-step reaction using GIBCO Superscript RT/Taq with 1.5 mM Mg^{2+}. Reverse transcription for 60 min at 45°C

TABLE 1. Immunofluorescence reaction patterns of MAbs with influenza A viruses of different hemagglutinin subtypes

Virus[a]	Subtype	Reaction with[b]:		
		Chemicon H1 (MAb 8252)	Chemicon H3 (MAb 8254)	H5 MAb pool
A/Beijing-like/98 (human)	H1N1	+	−	−
A/Asia/57 (human)	H2N2	−	−	−
A/duck/Hong Kong/319/78	H2N2	−	−	−
A/Sydney-like/99 (human)[c]	H3N2	−	+	−
A/duck/Hong Kong/477/78	H4N6[d]	−	+	−
A/Hong Kong/486/97 (human)	H5N1	−	−	+
A/goose/Hong Kong/38/79	H6N1	−	−	−
A/duck/Hong Kong/47/76	H7N2	−	−	−
A/turkey/Ontario/6118/68	H8N4	−	−	−
A/Hong Kong/1073/99 (human)	H9N2	−	−	−
A/Hong Kong/1074/99 (human)	H9N2	−	−	−
A/swine/Hong Kong/9/98	H9N2	−	−	−
A/swine/Hong Kong/10/98	N9N2	−	−	−
A/chicken/Hong Kong/G24/97	H9N2	−	−	−
A/chicken/Hong Kong/G9/97	H9N2	−	−	−
A/quail/Hong Kong/G1/97	H9N2	−	−	−
A/duck/Hong Kong/865/80	H10N3	−	−	−

[a] The human H9N2 isolates were provided by W. L. Lim, Government Virus Unit, Department of Health, Hong Kong. Other avian and human influenza A viruses were from the influenza virus repository of the Department of Microbiology, The University of Hong Kong.
[b] +, positive; −, negative.
[c] Eight isolates tested.
[d] Three other isolates gave similar results.

TABLE 2. HAI reaction patterns of H9N2 viruses with various antisera

Antigen preparation	Reciprocal antibody HAI titer of:				
	WHO reference		H9 antiserum		
	H1	H3	Chicken (G1 strain)	Chicken (G9 strain)	Rabbit
H9N2 A/Hong Kong/1073/99 (MDCK cell)	<40	<40	640	80	320
H9N2 A/quail/Hong Kong/G1/97 (allantoic fluid)	80	320	2,560	80	40
H9N2 A/quail/Hong Kong/G1/97 (MDCK cell)	<40	<40	640	<40	320
H9N2 A/chicken/Hong Kong/G9/97 (allantoic fluid)	<40	<40	160	640	640
H9N2 A/chicken/Hong Kong/G9/97 (MDCK cell)	<40	<40	160	320	320
H9N2 A/chicken/Hong Kong/AP2/99 (allantoic fluid)	<40	<40	320	1,280	1,280
H9N2 A/quail/AP22/99 (allantoic fluid)	80	320	1,280	40	80
H9N2 A/quail/AP22/99 (MDCK cell)	<40	<40	2,560	40	80
H9N2 A/quail/AP24/99 (allantoic fluid)	<40	<40	160	40	320
H9N2 A/chukar/AP28/99 (allantoic fluid)	<40	<40	80	80	<40
H1 control antigen (WHO reference)	1,280	<40	<40	<40	<40
H3 control antigen (WHO reference)	<40	640	<40	<40	160

was followed by 3 min at 95°C. PCR amplification (45 cycles of 1 min each at 95, 50, and 72°C) was followed by a final extension at 72°C for 10 min. The 432-bp product was detected by agarose gel electrophoresis and confirmed by Southern hybridization. Once the nucleotide sequence of the hemagglutinin of the human H9N2 virus was available (6), a modified primer set was made by substituting T for C at position 18 of the forward primer and C for T at position 17 of the reverse primer. These were compared with the initial primer set for amplification of human and quail H9N2 viruses. The initial primer set was 3- to 100-fold more sensitive and was used for the studies reported below.

The RT-PCR successfully amplified human ($n = 2$), pig ($n = 2$), and avian ($n = 26$) H9N2 isolates from chicken, quail, pheasant, and chukar in Hong Kong. Compared with cell culture, the sensitivities of RT-PCR (by agarose gel detection) for the human, chicken, quail, and pig H9N2 isolates diluted in virus transport media were 1, 1, 2, and 6 $TCID_{50}$, respectively. When diluted in a pooled negative nasopharyngeal aspirate, sensitivity was reduced to 63 $TCID_{50}$. The RT-PCR would be suitable for subtyping virus isolates (8), and may even be sensitive enough for direct detection of H9N2 virus in clinical specimens as was reported for H5N1 (7). It did not cross-amplify any of the other human or avian influenza virus subtypes listed (Table 1).

There is a need for awareness about the pandemic potential of H9N2 influenza viruses. At present, positive immunofluorescence with either H3 monoclonal antibody or the Chemicon H1-specific MAb excludes H9N2. RT-PCR or a differential HAI test in which an H9-specific antiserum is used in addition to the H3 and H1 antisera provides definitive diagnosis.

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