Rapid typing, subtyping and RNA quantification of influenza virus type A strains in respiratory secretions

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SUMMARY

During the winter-spring season 2006-2007, 38 influenza virus strains were identified in patients admitted to hospital with an acute respiratory tract infection. Infections were diagnosed in parallel by direct fluorescent antibody (DFA) staining using type-specific monoclonal antibodies and real-time reverse transcription (RT)-PCR targeting the gene M (nt 25-124). In addition, virus strains were isolated in MDCK cells. Overall, 37 influenza virus strains were type A, and one type B. Of these, 35 (80.4%) were detected and typed by real-time RT-PCR, 34 (80.1%) by DFA, and 27 (71.0%) by virus isolation. Subtyping of 37 influenza virus A strains by RT-PCR and DFA gave the following results: 4/6 H1 strains were correctly subtyped by both methods, while of the 29 H3 strains subtyped by RT-PCR 7 were missed by DFA. Thus, the overall concordance of the two subtyping methods was 28/37 (75.7%). Viral RNA quantification by real-time PCR showed that when respiratory secretion collection was done within 5 days after the onset of symptoms, viral load was greater than $1x10^6$ RNA copies/ml. In conclusion, typing and subtyping of influenza virus type A strains may benefit from both MAbs and RT-PCR, while viral RNA quantification may provide an indication of symptom onset.

KEY WORDS: Influenza virus A, Typing and subtyping, RNA quantification, Respiratory secretions, Acute respiratory tract infections

INTRODUCTION

Following the emergence of the risk for transmission of avian influenza H5N1 to human beings in 1997 (Claas *et al.*, 1998; Subbarao *et al.*, 1998), it immediately became evident that medical virology laboratories needed methods for rapid detection, isolation, typing and subtyping of influenza virus type A strains. Prior to and in parallel, veterinary virology reference laboratories were massively and critically involved in subtyping influenza virus type A strains detected in birds, both wild and domestic, for the earliest possible detection of the transmission of highly pathogenic viral variants to domestic poultry. This epidemiological situation opened the door to a strengthened collaboration between medical and veterinary virologists and new collaborative research programmes have started. As a result, veterinary and medical surveillance of influenza virus type A strain circulation is currently performed in Italy.

In this study, we detected, typed and subtyped influenza virus A strains recovered from respirato-

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ry secretions of patients admitted to hospital during the winter-spring season 2006-2007 by using, besides virus isolation, two different approaches for rapid viral diagnosis, i.e. monoclonal antibodies (MAbs) and real-time reverse transcription (RT)-PCR. In addition, influenza virus A viral load in respiratory secretions was determined by real-time RT-PCR to define its etiologic role in the current episode of acute respiratory tract infection (ARTI) under diagnosis. In this respect, coinfections by different respiratory viruses are often encountered in patients with acute respiratory infections.

MATERIALS AND METHODS

Patient and respiratory samples
During the winter-spring season 2006-2007, all subjects admitted to hospital for an ARTI episode or staying in the hospital because of an underlying disease but affected by an episode of ARTI, were subjected to nasopharyngeal aspiration (NPA) or, during bronchoscopy, broncoalveolar lavage (BAL) sampling as close as possible to symptom onset. In a few patients, a second, and less frequently, a third respiratory sample was taken towards resolution of respiratory symptoms.

Respiratory sample processing
NPA and BAL samples were processed and divided into four aliquots as follows: one aliquot was used for molecular assays, the second for direct or indirect fluorescent antibody (DFA or IFA) staining of respiratory cells from NPA or BAL, the third for virus isolation following inoculation of short-term (shell-vial cultures, SVC) and long-term cultures, and the fourth was frozen in multiple aliquots as a back-up. Specimens were examined for influenza viruses A and B, parainfluenza viruses types 1-4, human respiratory syncytial viruses A and B, human adenoviruses, human metapneumoviruses A and B, human coronaviruses 229E and HKU-1 by DFA and short-term virus isolation in SVC using MAbs (Sarasini *et al.*, 2006). In addition, all four human coronaviruses (229E, NL63, OC43 and HKU-1), and human rhinoviruses were detected by RT-PCR (Gerna *et al.*, 2006a, 2007a), while influenza virus A as well as human respiratory syncytial viruses (A and B), human metapneumoviruses (A and B)

and human bocavirus were quantified by real-time RT-PCR (Gerna *et al.*, 2007b).

Cell cultures
Clinical samples were routinely inoculated onto shell-vials of mixed mink lung (Mv1Lu) and A549 epithelial cells (Mix-SVCs) for rapid influenza virus detection and typing as well as for rapid detection of other respiratory viruses (Huang and Turchek, 2000). SVCs were fixed and stained with MAbs 48h after inoculation for rapid virus detection following SVC centrifugation. In the present study, influenza virus type A strains detected by DFA/IFA in respiratory cells from nasopharyngeal secretions and identified in SVC 48h after inoculation were considered as a single group in the analysis of results provided by MAbs in comparison with results provided by RT-PCR on clinical samples and with results given by conventional virus isolation.

DFA/IFA staining of respiratory cells and cell cultures
DFA staining of respiratory cells and cultured cells from SVCs for conventional respiratory viruses was applied using commercially available virus-specific MAbs, as reported (Sarasini *et al.*, 2006; Gerna *et al.*, 2006a). In addition, in-house developed MAbs to human coronaviruses 229E and HKU-1 (Gerna *et al.*, 2006a, 2007a) and to human metapneumoviruses A and B (Percivalle *et al.*, 2005; Gerna *et al.*, 2006b) were added to the commercial MAb panel and routinely used for IFA staining. As for influenza virus A detection and typing, pan-Flu A MAbs (a pool of three MAbs-P8E11, P8D11, and P9H3), developed in collaboration with Dr. Ilaria Capua's laboratory (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padua, Italy) and reactive with the NP protein (the gene 5 product) of all human and avian influenza viruses A so far tested by both Western blot and ELISA, were routinely assayed in parallel with the commercial Flu A MAb (Chemicon). Typing was done in parallel by IFA on respiratory cells, SVC (after 48h) and MDCK cells (after 7 days). Subtyping of influenza virus A strains was done by IFA using commercial MAbs directed against influenza A H1N1 (Chemicon MAB8256) and influenza A H3N2 (Chemicon MAB8254). In addition, typing and subtyping of influenza A virus strains was done

by IFA on virus strains isolated in MDCK cells seven days after inoculation using the same set of MAbs.

RT-PCR for influenza virus A strain typing and subtyping
Influenza virus A detection and typing was performed using a real-time RT-PCR protocol as follows (Table 1). Viral nucleic acid was extracted from 200 μl of NPA or BAL samples using the automatic extraction kit Nuclisens® easy MAG™ (BioMèrieux, Lyon, France), and eluted in 50 μl. RT reaction was done in a final volume of 15μl (10 μl of reaction mix plus 5μl extract) using the

forward primer specific for the target gene M (nt 25-nt 124) of influenza virus A, as previously reported (Spackman *et al.*, 2002) and the enzyme SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the following thermal profile: 30 min at 42°C and 15 min at 70°C. The reaction product was then amplified by real-time PCR in a final volume of 50μl reaction mixture [25μl TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems International, Foster City, CA), 0.2μM of each primer and 0.2 μM of the FAM-labeled probe]. Amplification was performed in 96-well microplates, using the ABIPRISM® 7000 Sequence

TABLE 1 - *List of primers used for identification, typing and subtyping of influenza virus strains detected and isolated from patients admitted to hospital during the winter-spring season 2006-2007.*

Influenza virus A type and subtype	PCR method	Target gene (nt-nt)	Thermal profile	Cycle no.	Primer pair	Reference
Panflu A	Real-time RT-PCR	M (25-124)	<95°C/15" 60°C/1'	40	Forward: 5'-agatgagtcttctaaccgaggtcg-3' Reverse: 5'-tgcaaaaacatcttcaagtctctg-3' Probe FAM-tcaggcccccctcaaagccga-TAMRA	Spackman E. et al., J Clin Microbiol 2002; 40: 3256-60
Panflu A	RT-PCR	NP (8-541)	94°C/45" 52°C/1'30" 72°C/2'30"	40	Forward: 5'-gcagggtagataatcactcac-3' Reverse: 5'-gagagcacattctgggatcc-3'	ISS[a]
Influenza A H1	RT-PCR	HA (44-1058)	94°C/1' 60°C/1' 72°C/1' 94°C/1' 52°C/1' 72°C/1'	10[b] 40	Forward: 5'-cagatgcagacacaatatgt-3' Reverse: 5'-aaaccggcaatggctccaaa-3'	Stockton J. et al., J Clin Microbiol 1998; 36: 2990-5
Influenza A H1	RT-PCR	HA (303-1117)	94°C/45" 52°C/1'30" 72°C/2'30"	40	Forward: 5'gaatcatggtcctacattgtagaaa-3' Reverse: 5'-atcattccagtccatcccccttcaat-3'	ISS[a]
Influenza A H3	RT-PCR	HA (250-588)	94°C/45" 52°C/1'30" 72°C/2'30"	40	Forward: 5'-ccttgatggagaaaactgcacac-3' Reverse: 5'-tgtttggcatagtcacgttca-3'	ISS[a]
Influenza A H3	RT-PCR	HA (291-897)	94°C/1' 60°C/1' 72°C/1' 94°C/1' 52°C/1' 72°C/1'	10[b] 40	Forward: 5'- ctgaacgtgactatgccaaac -3' Reverse: 5'-gcttccatttggagtgatgcat-3	in-house
Influenza A H5	RT-PCR	HA (894-1245)	94°C/1' 60°C/1' 72°C/1' 94°C/1' 52°C/1' 72°C/1'	10[b] 40	Forward: 5'-actccaatggggcgataaa-3' Reverse: 5'-caacggcctcaaactgagtgt-3'	Poddar S.K., J Virol Methods 2002; 99: 63-70
Influenza A H7	RT-PCR	HA (405-1113)	94°C/1' 60°C/1' 72°C/1' 94°C/1' 52°C/1' 72°C/1'	10[b] 40	Forward: 5'-ggagacaatgggattcacct-3' Reverse: 5'-gccataccacccatcaatca-3'	in-house
Influenza A H9	RT-PCR	HA (585-1112)	94°C/1' 60°C/1' 72°C/1' 94°C/1' 52°C/1' 72°C/1'	10[b] 40	Forward: 5'-cgacacagaacagacagact-3' Reverse: 5'-ccaactccctgatcgtttga-3'	in-house
Influenza B	RT-PCR	HA (154-1053)	94°C/1' 60°C/1' 72°C/1' 94°C/1' 52°C/1' 72°C/1'	10[b] 40	Forward: 5'-gtgactggtgtgataccact-3' Reverse: 5'-tgttttcacccatattgggc-3'	Stockton J. et al., J Clin Microbiol 1998; 36: 2990-5

[a]ISS, Istituto Superiore di Sanità; [b]Annealing temperature decreasing by 1°C/cycle

Detection System (Applied Biosystem) according to the following thermal profile: 2 min at 50°C, and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 60 s at 60°C. External DNA standards were obtained from 10^6 through 10^1 DNA copies following cloning of the selected region of gene M of a reference influenza virus A strain by the TA-Cloning kit (Invitrogen). These standards allowed the construction of a calibration curve, from which the amount of virus present in the clinical sample was extrapolated.

As for influenza A H1 and H3 subtyping, a set of primers reported in Table 1 were used for RT-PCR. For H5, H7, and H9 avian strains, primer pairs were used as reported in Table 1. RT-PCR assays for conventional respiratory viruses were developed to detect at least ten input plasmid copies, as previously reported (Sarasini *et al.*, 2006). In addition, hCoVs 229E, NL63, OC43, and HKU-1 were detected by RT-PCR as reported elsewhere (Gerna *et al.*, 2006a, 2007a).

Statistical analysis

The diagnostic value (sensitivity, specificity, positive and negative predictive values) of DFA/IFA (MAbs) and conventional virus isolation was calculated using RT-PCR as a gold standard method. The comparison of influenza virus A load in clinical samples from some patients at different times after onset of symptoms was done by using the Mann-Whitney U test for unpaired data.

RESULTS

Influenza virus A detection and typing

As shown in Table 2, during the winter-spring season 2006-2007, 38 influenza virus strains were detected in respiratory samples from patients ad-

mitted to hospital or staying in hospital with an episode of ARTI. Overall, 37 were type A, and one type B. Of these, 35 (80.4%) were detected and typed by real-time RT-PCR, 34 (80.1%) by DFA (including strains detected directly in cells from respiratory samples and those detected in short-term SVCs), and 27 (71.0%) by conventional virus isolation. Three strains were missed by real-time RT-PCR, four by DFA (all PCR-positive), and 11 (9 positive by real-time RT-PCR, and 8 positive by DFA) by conventional virus isolation. The unique type B strain was detected by all three procedures (RT-PCR, MAbs, and conventional virus isolation). No influenza virus strain was detected by conventional virus isolation alone. On the other hand, conventional virus isolation was able to detect 24/30 (80.0%) influenza A strains positive by both DFA and real-time RT-PCR. Of the seven samples detected by either real-time RT-PCR or DFA, only 2 (28.6%) were positive by culture.

Typing of influenza virus A isolates in respiratory cells, SVC, and MDCK cells by DFA is reported in Figure 1 A-C. Comparative growth of a recent influenza virus A isolate in SVC of mixed Mv1Lu and A549 cells, and separate Mv1Lu and A549 cells, as detected with a MAb to influenza A NP, is reported in Figure 1 J-L, showing comparable efficiency in level of viral replication in both cell systems.

Thus, using real-time RT-PCR as the reference method for influenza virus A detection and typing, diagnostic values of DFA (including use of MAbs with both respiratory cells from clinical samples and SVCs) and conventional virus isolation in MDCK cells, on the total number of 602 respiratory samples examined during the winter-spring season 2006-2007, were as follows: sensitivities 89% and 74%, specificities 99% and 100%, positive predictive values 91% and 96%, and neg-

TABLE 2 - *Diagnostic value of DFA, real-time RT-PCR, and conventional virus isolation in detecting influenza viruses in 38 patients during the winter season 2006-2007.*

Conventional virus isolation	Influenza A				Influenza B	Total (%)
	$DFA^+ PCR^+$	$DFA^- PCR^+$	$DFA^+ PCR^-$	$DFA^- PCR^-$	$DFA^+ PCR^+$	
Positive	24 (63)	1 (0.3)	1 (0.3)	0	1 (0.3)	27 (71)
Negative	6 (16)	3 (0.8)	2 (0.5)	0	0	11 (29)
Total	30 (79)	4 (1.1)	3 (0.8)	0	1 (0.3)	38

ative predictive values 99% and 98%, respectively. In terms of turnaround times, real-time RT-PCR results were provided 24-48 h after sample collection, DFA results were obtained on the same day of sample collection for direct staining of res-
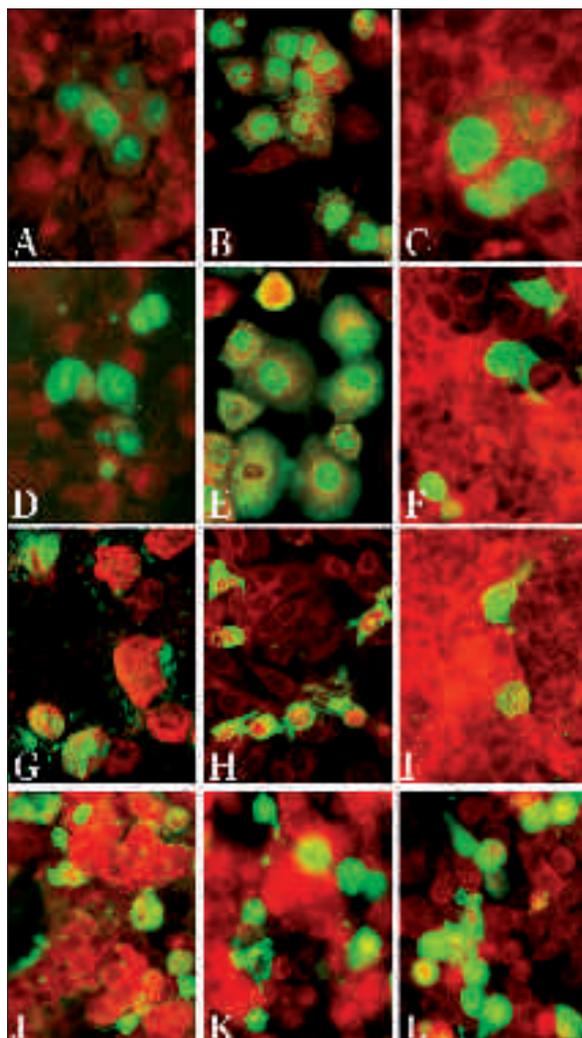

FIGURE 1 - *DFA/IFA staining of respiratory cells from NPAs showing cells positive for (A) influenza virus A (MAb anti-NP of influenza A), (D) H1 from a H1N1 strain (MAb anti-H1), and (G) H3 from a H3N2 strain (MAb anti-H3). In addition, shell-vial cultures (SVC) of mixed mink lung and A549 epithelial cells stained for (B) influenza A virus, (E) H1, and (H) H3 with specific MAbs, are shown. The same influenza A strains inoculated onto mixed SVCs were inoculated onto MDCK cells, in which the number of infected (stained) cells was very poor for (C) influenza A, (F) H1, and (I) H3. Finally, SVCs were found to be highly susceptible to influenza virus A infection, both as (J) mixed cultures, and as separate (K) mink lung and (L) A549 epithelial cells.*

piratory cells or within 48h after SVC inoculation and cultured cell MAb staining, while results of conventional virus isolation required seven days before MAb staining of cultured MDCK cells, and a possible further passage. Thus, in this study, the true comparison was between the molecular (real-time RT-PCR) and the immunological (MAb) method for rapid detection of influenza virus A strains, while conventional virus isolation was aimed at recovery of virus strains for further characterization.

Subtyping of influenza A strains by RT-PCR and MAbs

Subtyping of 37 influenza virus A hemagglutinin (H) strains by RT-PCR and IFA gave the following results (Table 3). On the whole, 4/6 H1 strains were correctly subtyped by both methods, while, of the remaining two H1 strains, one was correctly subtyped by RT-PCR, and the other one by IFA. Thus, one H1 strain was missed by each method. In addition, of the 29 H3 strains tested by RT-PCR, seven were missed by IFA. Finally, subtyping of two strains was missed by both methods. Overall, of five H1 strains typed by RT-PCR, four (80%) were typed also by IFA, and of 29 H3 strains typed by RT-PCR, 22 (75.9%) were typed by IFA. The overall concordance of the two subtyping methods was 28/37 (75.7%). Subtyping of H1 and H3 viral isolates by IFA using MAbs is reported in Figure 1 D-F and G-I.

Influenza virus A RNA quantification in respiratory samples

Quantitative findings of RNA present in respiratory secretions from patients with influenza virus A infection are reported only for those patients in whom the date of onset of respiratory symptoms was available (Table 4). In this respect, the median viral load detected in respiratory samples from nine immunocompetent individuals with acute influenza virus A infection within five days after onset of symptoms was 1.7×10^7 RNA copies/ml (range 2.5×10^6-2.0×10^8). After a median time of seven (6-14) days, the median viral load in respiratory secretions dropped (p=0.001) to 1.5×10^5 (2.5×10^2-8.0×10^5). In lung transplant recipients, the median viral load was 1.6×10^8 (1.3×10^6-3.4×10^8) within five days after onset of symptoms. In a single patient, viral load dropped to 7.1×10^5 after six days and to 5.0×10^1 after 13

TABLE 3 - *Subtyping of 37 influenza virus type A strains by RT-PCR and monoclonal antibodies.*

Influenza A subtyping by MAbs	No. of influenza A strains subtyped by RT-PCR			
	H1	H3	Not subtyped	Total
H1	4	0	1	5
H3	0	22	0	22
Not subtyped	1	7	2	10
Total	5	29	3	37

TABLE 4 - *Median influenza virus A load in respiratory secretions from three groups of patients.*

Patient group	Age range	Influenza virus A load	
		Median	Range
Immunocompetent children	1 mo-4 yrs		
Early (≤5 d)[a] sampling (n=9)		5.0×10^6	$2.5 \times 10^6 - 2.0 \times 10^8$
Late (>5 d)[a] sampling (n=5)		1.7×10^5	$2.5 \times 10^2 - 8.0 \times 10^5$
Young HSCT[b] recipients (n=7)	3-17 yrs	5.0×10^6	$1.0 \times 10^6 - 6.9 \times 10^7$
Lung transplant recipients (n=5)	14-75 yrs	1.6×10^8	$1.3 \times 10^6 - 3.4 \times 10^8$

[a]Days after onset of symptoms; [b]HSCT, hematopoietic stem cell transplant.

days. In pediatric hematopoietic stem cell transplant recipients, the median viral load in respiratory secretions was 5.0×10^6 ($1.0 \times 10^6 - 6.9 \times 10^8$) RNA copies/ml within five days after onset, dropping to 7.9×10^5 ($1.3 \times 10^4 - 4.5 \times 10^6$) copies/ml (p=0.1) 12 (6-15) days after onset of symptoms. Thus, in this group of patients viral load appeared to decline at a slower rate than in the other groups of patients.

In conclusion, viral RNA quantification by real-time RT-PCR showed that, whenever respiratory secretions were collected within five days after onset of symptoms (fever, upper or lower RTI symptoms), viral load in NPA or BAL was greater than 1.0×10^6 RNA copies/ml. This threshold value did not seem to be different between H1N1 and H3N2 influenza virus type A strains. For three type A strains collected ≥20 days after onset of symptoms during the course of the disease, viral RNA could not be quantified. These three patients included a 6-year-old child, a 3-year-old HSCT recipient, and one adult lung transplant recipient. In all three patients, influenza virus A was detected in ≤5 cells of Mix-SVC inoculated with respiratory secretions collected late after onset of symptoms. Subtyping was not possible in any of these three samples.

Coinfections

In the group of 38 influenza virus A strains recovered from the same number of patients, coinfections by other respiratory viruses were detected in five patients as follows: four were detected in infants and young children, and two were caused by human parainfluenzavirus types 1 and 3, one by human respiratory syncytial virus, and one by human bocavirus, while the fifth coinfection was caused by human coronavirus OC43 in an adult lung transplant recipient. However, while in the four young coinfected patients, influenza virus A load was $>1.0 \times 10^6$ RNA copies/ml and, thus, was highly likely to be the virus actually causing the ARTI episode (coinfecting viruses provided an indication of a previous infection or the first indication of a new infection), in the adult patient a very low influenza virus A load indicated that hCoV OC43 was the respiratory virus responsible for the current ARTI.

DISCUSSION

Surveillance of acute respiratory virus infections caused by influenza virus A within human (and animal) communities has become a major goal of diagnostic (human and veterinary) virology laboratories. This requirement has become particularly demanding following the numerous reports of avian influenza spread, and particularly of avian influenza H5N1 to human beings. In this respect, there is an urgent need for diagnostic laboratories operating within hospitals to be able to detect, type and subtype influenza virus A strains affecting patients in multiple geographic areas. Along this line, it appears mandatory that rapid methods for detection, typing, and subtyping of influenza virus A strains be developed to meet requirements for an adequate surveillance programme.

Our study was developed using the two major lines of reagents and methods now available for virus detection, i.e. immunological method using MAbs, and molecular method using RT-PCR. It is known that virus isolation is conventionally considered the gold standard for influenza virus diagnosis. However, it must be taken into account that not all cell culture systems are susceptible to influenza viruses and primary monkey kidney cell cultures from either rhesus or African green monkeys are not readily available. In this respect, their use has been replaced by that of established cell lines, such as MDCK, Mv1Lu, and LLC-MK2. MDCK cells are especially useful for isolation of influenza B virus, while Mv1Lu have been reported to be more sensitive than MDCK for rapid detection of influenza virus in culture (Reina *et al.*, 1997; Huang and Turchek, 2000). Since influenza virus isolates generally do not cause a cytopathic effect in cell cultures, virus identification may be accomplished by immunofluorescence using MAbs. Thus, virus isolation requires 5-7 days to complete.

In order to speed up diagnostic procedures and adopt adequate preventive epidemiological measures and, when required, timely therapeutic intervention with antiviral drugs, DFA using MAbs has become more and more popular either applied directly to respiratory cells from NPAs or BAL samples or to cell monolayers from SVCs, following 24-48 h incubation at 33°C after centrifugation of clinical samples onto cell monolayers (Dunn *et al.*, 2003). DFA staining with MAbs both directly (respiratory cell smears) and indirectly (SVC cells) permits a dual approach to the rapid immunological diagnostic procedure. For this reason, we considered data provided by NPA smears and SVC as a single result.

The other diagnostic approach used in this study, i.e. the molecular approach using RT-PCR, is progressively replacing virus isolation as a standard reference method (Petric *et al.*, 2006). Most RT-PCR assays for influenza viruses A and B use primers complementary to the conserved gene 7 and may identify all influenza virus strains detected to date (Fouchier *et al.*, 2000). RT-PCR is commonly considered to be more sensitive than virus isolation and DFA (Herrmann *et al.*, 2001; Templeton *et al.*, 2004). In our study, the sensitivities of DFA and virus isolation were 89% and 74%, respectively, with respect to RT-PCR. However, we believe that all samples positive by DFA only, were true positives. This conclusion was based on the finding that the great majority of samples (24/30, 80%) were positive for influenza virus A by all three procedures. Discrepant results were all relevant to samples containing small amounts of virus. Few virus particles or viral components may not be detected by RT-PCR because of RNA degradation. Respiratory cell smears for DFA staining may not contain an adequate number of respiratory cells carrying viral antigens. Finally, cell cultures used for virus isolation may not be susceptible to viral infection, or an insufficient number of viable virus particles may be present in clinical samples. In conclusion, we believe that epidemiological surveys may use both immunological and molecular assays to achieve maximal sensitivity, while for clinical studies either one of the two diagnostic approaches may be routinely employed.

As for subtyping of influenza virus A strains, RT-PCR using primers targeting gene 4 (hemagglutinin) is the gold standard for identification of H subtypes. However, MAbs specific for H1 and H3 are commercially available at this time, and were used in comparison with RT-PCR. Results showed that, while sensitivity in subtyping H1 strains was comparable, MAbs were unable to type 7/29 (24.1%) H3 strains typed by RT-PCR. Thus, although both primers and MAbs must be continuously updated according to the emergence of

new viral variants, RT-PCR remains the gold standard for H subtyping.

Viral RNA quantification by real-time RT-PCR revealed a correlation between viral load in respiratory secretions and clinical symptoms. Reduction in viral load was associated with progressive disappearance of respiratory symptoms both in immunocompetent and immunocompromised patients. However, in a stem cell transplant recipient, a trend towards a delayed time to viral disappearance in respiratory secretions was observed. Monitoring respiratory viral infections in these patients may be useful for both patient isolation and therapeutic interventions. The reported association of NPA RNA load and respiratory symptoms was already described by our group for other respiratory viruses, such as respiratory syncytial virus (Campanini *et al.*, 2007) and human metapneumovirus (Gerna *et al.,* 2007b). Similarly, in case of coinfection by two or more respiratory viruses, it has already been suggested that virus quantification may be the only tool available for the identification of the virus actually responsible for the current ARTI episode. Results of the present study indicate that the same approach appears useful also for defining the role of influenza virus A during coinfection of both immunocompetent and immunocompromised patients.

In conclusion, our study shows the following:

1. detection and typing of influenza virus A in clinical samples may achieve good results by using either the immunological or the molecular approach. However, best results may be provided by their simultaneous use;
2. real-time RT-PCR appears to be the best method for H subtyping of influenza virus A strains;
3. viral RNA quantification may provide useful indications on the role of influenza virus A in the current ARTI episode.

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