ORIGINAL ARTICLE

Performance testing of two new one-step real time PCR assays for detection of human influenza and avian influenza viruses isolated in humans and Respiratory Syncytial Virus

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Key words

Diagnosis of respiratory infection • Influenza drift • Avian influenza

Summary

Introduction and Methods. *Two real time one-step RT-PCR assays were developed for simultaneous detection and typing of influenza A and B viruses and detection of Respiratory Syncytial Virus (RSV). As regard influenza, primers were designed to amplify specific sequences of gene M of A/H1N1, A/H3N2, A/H5N1, A/H7N7 and A/H9N2 viruses and of gene NP of type B viruses belonging both Yamagata and Victoria lineage. Specificity, analytical and clinical sensitivity, dynamic range, linearity of the new assays were evaluated.*

Results. *Dynamic ranges for Influenza A and B, and RSV, were at least five logs and linearity was conserved. In order to evaluate the specificity, 80 nasopharyngeal swabs resulting Influenza and RSV negative by multiplex nested PCR and cell culture, were tested and 79 resulted negative. The detection limits for influenza A and B, calculated by 95% probit, was 0,008 and 0,09 PFU, respectively, resulting more sensible than nested PCR. A total of 75 specimens (10 A/H1N1, 3 A/H1N2, 8 A/H3N2 Johannesburg/94-like, 10 A/H3N2 Panama/2007/99-like, 10 A/H3N2 Fuijian/411/02-like, 2 A/H5N1, 2 A/H7N7 and 2 A/H9N2, 15 B/Yamagata-like and 13 B/Victoria-like) collected between 1994 and 2004 or received by WHO Influenza Centre, London, were chosen as representative of the circulating strains and tested. All samples resulted positive although one B/Victoria sample was not clear typed. Thirty swabs nested RT-PCR positive for RSV collected during the four seasons, were also analysed by real-time PCR, resulting positive. To evaluate the performance of the new assay on fresh material, 250 specimens, collected during the 2004/05 seasons, were tested by nested-PCR, cell culture and real-time PCR.*

Discussion and Conclusion. *The new assays provide accurate and sensitive diagnosis of influenza and RSV infection and they represent a sensitive tool for virological surveillance and management of patient with ILI.*

Introduction

The influenza A and B viruses as well as Respiratory Syncytial Virus (RSV) are prominent among viral causes of respiratory illness. A rapid and sensitive assay for the detection of Influenza virus and RSV, in clinical samples from subjects with influenza-like illness (ILI), bronchiolitis or pneumonia is a fundamental tool for various reasons, concerning the epidemiological and virological surveillance as well as the management of the patient. First of all, intensive influenza surveillance efforts are continuously conducted to monitor for the emergence of pandemic strains and to monitor antigenic drift so that an effective vaccine can be produced annually. This epidemiological and virological surveillance, coordinated by WHO, requires a sensitive procedure not only to detect the virus, but also to effect isolation by cell culture. Secondly, the contribution of RSV to respiratory illness in adults has not been fully elucidated and the availability of a rapid and inexpensive assay may contribute to gathering information concerning the epidemiology of this virus. Finally, a rapid diagnostic test would allow a specific diagnosis to be made at an early stage of the disease and thus help the clinician in making therapeutic decisions and to prevent nosocomial infections. The availability of improved drug treatment with neuraminadase inhibitors, for influenza viruses, has emphasized the importance of an efficient laboratory test. Early diagnosis of RSV could be useful, although controversy exists regarding ribavirin and immunoglobulin therapy [1]. Current methods used in the diagnosis of influenza and RSV infection include cell culture isolation, direct antigen detection and serology. The major limitations of these techniques are prolonged time to completion, subjective evaluation, low sensitivity and low specificity. The introduction of nucleic acid amplification techniques has made sensitive diagnosis of influenza and RSV feasible and real time approach has made it rapid [2-5].

Aim of the present report is to describe the evaluation of the performance in terms of dynamic range, linearity, specificity, analytical and clinical sensitivity, and the suitability of a new real time PCR for typing influenza viruses and detection of RSV. The new assay was designed to detect (i) influenza A, subtype H1N1 and H3N2, including the new A/H3N2/Hong

Kong/1143/02-like variant, appeared during the 2002-03 season, (ii) avian influenza A virus recently infecting humans (H5N1, H9N2, H7N7) and (iii) the two lineages of influenza B (Victoria/2/87 and Yamagata/16/88 lineages), that co-circulate since 2001/02 season [6-8].

Methods

Real time PCR

Primer design

Primers were designed from conserved regions of genes codifying the Matrix Protein, the Nucleoprotein and Fusion protein of Influenza virus type A, B and RSV, respectively. Sequences of influenza A, influenza B and RSV isolates were selected with FASTA software [9] and multi-aligned with CLUSTALW software [10]. Multi-alignments were used to select highly conserved regions using MEGA package, version 1.01 of the Pennsylvania State University (PA, USA). Primers were designed manually to obtain homogeneous melting temperatures calculated with AnnHyb software [11 12]. Primers and probes sequences for influenza A, influenza B and RSV were as follow: FLUAV_FOR 5'- ACA AgA CCA ATC CTg TCA CCT CT-3'; FLUAV_REV 5'- ggC ATT TTg gAC AAA gCg TCT AC-3'; FLUAV_TM 5'-FAM- CAg TCC TCg CTC ACT ggg CAC ggT(p)-BHQ1-3'; FLUBV_FOR 5'- CCA gTg ggA CAA CCA Ga-3'; FLUBV_REV 5'- TgC TCT TTC Cgg ggA Tg-3'; FLUB_TM 5'-JOE- ATC ATC AgA CCA gCA ACC CTT gCC (p)- BHQ1-3'; RSVA_FOR 5'- CCA TAT ATT gAA CAA CCC AAA AgC ATC 3'; RSVA_REV 5'- TgT ACC TCT gTA CTC TCC CAT TAT 3'; RSVA_TM 5'-FAM- Agg CCA gCA gCA TTg CCT AAT ACT ACA(p)- BHQ1-3'; RSVB_FOR 5'- ACC ATA TAT TgA ACA ATC CAA AAg CAT C 3'; RSVB_REV 5'- TgT ACC TCT ATA CTC TCC CAT TAT gC 3'; RSVB_TM 5'-FAM – ACC TgC TgC ATT gCC TAg gAC CAC(p)- BHQ1-3'.

Procedure

Viral RNA was extracted with QIA techniques, according to the manufacturer's instructions (RNeasy Minikit, Qiagen, Valencia, CA, USA). Real Time PCR was performed using Fast Set Inf A/Inf B and Fast Set RSV A/B (Arrow Diagnostics, Genoa). The reaction mixture consisted of 2x reaction mix (Invitrogen, Carlsbad, CA, USA), PCR additive I, Flu mix (primer 250 nM, TaqMan probe 75 nM) or RSV mix (primer 300 nM, TaqMan probe 100 nM) respectively, SuperScript III RT/Platinum Taq mix, RNase OUT and 10 μl of template with a total volume of 50 μl. The PCR thermal profile consisted of initial RT step of 15 min at 50 °C followed by 2 min at 95 °C and 35 cycles of 15 sec at 95 °C and 60 sec at 62 °C or 60 sec at 60 °C for Influenza A, B and RSV respectively.

With regard to influenza type A and B multiple fluorescent signals were obtained with detectors corresponding to FAM and JOE respectively. RSV signal was acquired on FAM. Real Time Measurements were taken and a threshold cycle (Ct) value for each sample was calculated determining the point at which the fluorescence exceeded a threshold limit of 0,01.
Amplification, detection and data analysis were performed with Rotor-Gene 3000 (Corbett Research, Australia).

Multiplex nested PCR

Procedure

Viral RNA was extracted with QIA techniques, according to the manufacturer's instructions (RNeasy Minikit, Qiagen, Valencia, CA, USA). RT was performed using random primers (RT-kit plus, Amplimedical, Buttigliera Alta, Torino, Italy). The first amplification was carried out by adding previously synthesized cDNA and DNA polymerase to the pre-aliquoted mix, that contained the 3 pairs of outer primers, KCl, Tris-HCl, Triton X-100; MgCl2, dNTPs (Influenza/RSV Multiplex, Amplimedical, Buttigliera Alta, Torino, Italy). The second amplification was performed using the pre-aliquoted mix, containing the 3 pairs of inner primers. Biochemical optimisation of the amplification condition was performed and a final condition of 2 mM MgCl2, 50 mM KCl and pH 8.8 was found to be optimal for maximum yield of the specific product for each primer set. PCR was performed on a 5332 Thermocycler (Eppendorf, Hamburg, Germany) as follows: heat inactivation of the RT at 95 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, for the first amplification; 30 cycles, using the same parameters, for the second amplification. The influenza A and B and the RSV primer set amplified specific products of the expected sizes, 169, 309 and 218 bp, respectively, which could be easily distinguished by agarose gel electrophoresis. RNA extraction control, containing phage MS2 RNA, was added to all samples before extraction, while primers for phage amplification were added after RT (RECK, RNA easy check, Amplimedical, Buttigliera Alta,Torino, Italy). Positive (Influenza A, Influenza B and RSV plasmids) and negative controls, included in the kit were added to each run.

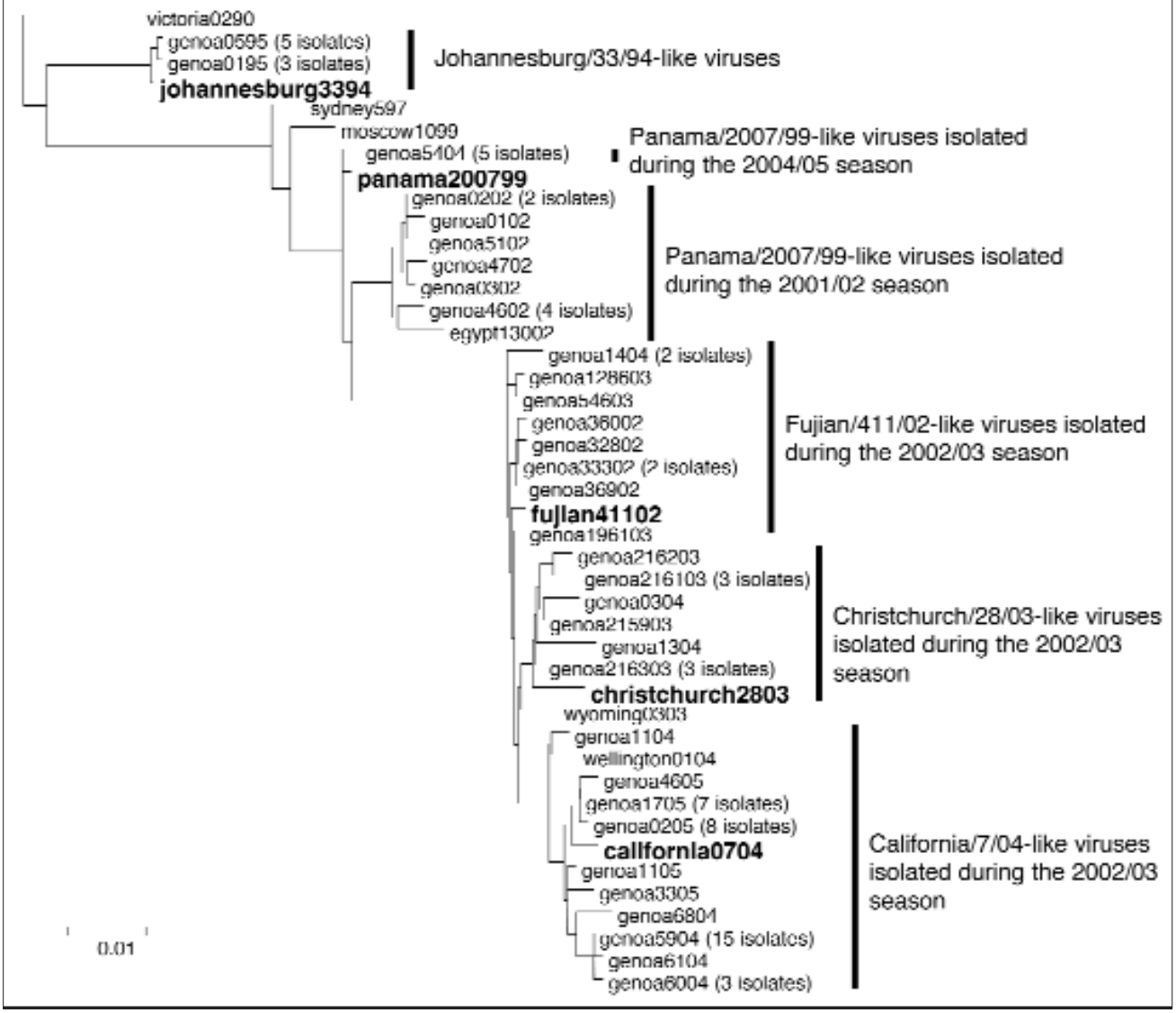
Prevention of PCR contamination

To avoid contamination of reaction tubes or carry-over with previously amplified product from other specimens and controls, extraction of RNA, preparation of reagents, first step PCR, nested PCR and amplicon detection were performed in safety cabinets located in separated areas of laboratory. Each cabinet was equipped with an independent batch of reagents, micropipette sets, sterile tubes and filtered tips.

Cell culture

Conventional viral culture was performed inoculating 0.3 ml of each specimen into Madin-Darby Canine

Fig. 1. Phylogenetic tree of strains circulating during last decade (1995-2004).



Kidney (MDCK) and HEp-2 cells for influenza and RSV isolation, respectively. Virus detection was performed by hemagglutination test and indirect immunofluorescence (Mouse anti-Influenza A/HA blend and Mouse anti-Influenza B/NP blend monoclonal antibody, Mouse anti-RSV fusion protein 1b monoclonal antibody and Mouse anti-RSV fusion protein 1c monoclonal antibody, Chemicon, CA) for influenza and RSV identification after isolation, respectively [13].

Antigenic and molecular characterization

The antigenic characterization of isolates was carried out by the haemagglutination inhibition (HI) test using ferret post-infection sera at the WHO Influenza Centre, London, UK. The molecular characterization of the most representative isolates in the HI test was carried out by sequence analysis of the globular head region of HA protein (HA1 subunit) as described elsewhere [6].

Design of the evaluation

Virological surveillance and influenza epidemiological picture

Epidemiological and virological surveillance of influenza in Italy is performed by a national network, coordinated by the Inter-University Centre for Research on Influenza (CIRI) and the National Influenza Centre, National Health Council, with the collaboration of Regional Health Authorities. The sentinel network comprises paediatricians and practitioners, who collaborate with hospital physicians and community officers in the collection of specimens, in order to integrate the epidemiological and clinical surveillance with the virological surveillance. During the last decade (1995-2004), antigenic and molecular characterization of the circulating influenza strains revealed the presence of all 3 types/subtypes; A/H1N1 virus was substantially stable during that period (New Caledonia/20/99-like variant). As far as concerns influenza virus type A/H3N2 four

antigenic drifts were observed: a novel A/H3N2 antigenic variant (Sydney/5/97 and the subsequent variant Panama/2007/99) appeared during the 1997/98 season and replaced strains that were previously predominant (Johannesburg/33/94); during the 2002/03 and 2004/05 seasons, two new A/H3N2 variants (Fujian/411/02 and California/7/04) appeared on the epidemiological scene [14]. The 2002/03 season was also characterized by isolation of recombinant A/H1N2 in three children. The phylogenetic tree is shown in Figure 1.

As far as concerns influenza virus type B, Yamagata/16/88-like strains prevailed until the 2001-02 season when Victoria/2/87 lineage re-appeared after a decade. A co-circulation of strains belonging to Victoria/2/87 and Yamagata/16/88 lineages was observed during the 2002/03, 2003/04 and 2004/05 seasons.

Retrospective study

(i) *Specificity*. In order to evaluate the specificity of the assay, 50 nasopharyngeal swabs, collected from patients with ILI resulting negative for Influenza and RSV by nested RT-PCR and cell culture, were tested. Those included specimens positive for Adenovirus (No = 3), Mumps virus (No = 3), Parainfluenza viruses (No = 9). Furthermore, to evaluate influenza-RSV cross-reactivity, 15 nasopharyngeal specimens nested-PCR and cell culture positive for Influenza (5 A/H1N1, 5 A/H3N2 and 5 B) and RSV were tested for RSV and Influenza, respectively.

(ii) *Analytical sensitivity*. The analytical sensitivity of the test under investigation was determined testing serial dilution of Influenza A and B and RSV cell culture-grown virus. The titration of viral suspension was performed by plaque reduction assay [13 15].

(iii) *Clinical sensitivity*. Out of 410 specimens uniplex nested RT-PCR and culture positive for influenza, 85 were selected as representative of the circulating strains over the last decade. In particular, 10 specimens were A/H1N1/New Caledonia/20/99-like, 3 were A/H1N2 recombinants, 8 A/H3N2/Johannesburg/33/94-like, 10 A/H3N2/Panama/2007/99-like, 10 A/H3N2/Fujian/411/02-like, 10 A/H3N2/Christchurch/28/03-like, 13 were type B strains belonging to Victoria/2/87 lineage and 15 were type B strains belonging to Yamagata/16/88 lineage. Furthermore, to determine the clinical sensitivity of Real Time PCR in detecting avian influenza viruses isolated in humans, 6 specimens culture positive for A/H5N2, A/H7N7 and A/H9N2 were tested. The specimens were kindly supplied by Alan Hay, WHO Collaborating Centre for Reference and Research on Influenza, London, UK.

Sixteen and 14 specimens, collected during the last four seasons and resulted nested RT-PCR positive for RSV A and B, respectively, were also analysed by real-time PCR.

Prospective study

Between 1 October 2004 and 31 January 2005, 250 specimens were collected from patients with influenza-like illness as above described and stored into three equal fractions: one was inoculated into the MDCK and HEp-2 cell line for isolation of influenza and RSV viruses, one was used for nested RT-PCR and one for real-time PCR.

Results

Retrospective study

Specificity

Out of 50 respiratory samples negative for influenza and RSV included in specificity evaluation, 49 (98%) resulted negative by both real-time one step RT-PCR assays. One of the samples (2%) tested showed a fluorescence increase in influenza test and was re-tested using real-time PCR and nested PCR resulting negative by both assays (Tab. I). Probably, the fluorescence increase was due to an aberrant signal caused by a fluorescence spike. No influenza-RSV cross-reactivity was observed with samples from a cross-reactivity panel as all specimens nested-PCR and culture positive for influenza and RSV resulted negative when tested by real time RT-PCR specific for detection of RSV and influenza, respectively.

Analytical sensitivity

The endpoint dilutions of 1:10,000 PFU for influenza B and 1:100,000 PFU for influenza A and RSV viruses resulted positive in every run of the real time PCR, with the point at which the fluorescence crosses the threshold (Ct) mean of 31.6 ± 1.9 (95% I.C. 27-36.3), 33.2 ± 0.3 (95% I.C. 32.4-34), and 34.1 ± 0.7 (95% I.C. 32.4-35.8) respectively. The A/H1N1 dilutions corresponding to 0.13 PFU resulted positive in 1 out of 6 runs (Tab. II).

Clinical sensitivity

All influenza subtypes and variant representative of the circulating strains in Italy between 1995 and 2004 tested by influenza real-time PCR assay, as well as both RSV A and B isolated during the last four seasons and tested by RSV real-time PCR test, resulted positive. The 6 avian strains all were detected by the new assay. No signal above background was detected in these two assays with the samples from cross-reactivity panel. This primer set and TaqMan probe led to specific detection of influenza (type A and B) and RSV (Tab. I).

Prospective study

As shown in Table III, with regard to influenza A, 37 out of 250 samples tested resulted positive by real time PCR, in concordance with both nested PCR and cell culture; 9 samples resulted positive by nested PCR and negative by cell culture, resulted positive by real time PCR. As regard as influenza B, 6 samples were positi-

Tab. I. Specificity and clinical sensitivity evaluation.

Evaluation	Sample	N.	Influenza Real-time PCR		RSV Real-time PCR	
			Pos (%)	Neg (%)	Pos (%)	Neg (%)
Specificity	Influenza and RSV nested PCR neg.	50	1 (2)	49 (98)	0	50 (100)
	RSV nested PCR pos	15	0	15 (100)	-	-
	Influenza nested PCR pos	15	-	-	0	15 (100)
Clinical Sensitivity	10 A/H1N1/ New Cal./20/99-like	10	10 (100)	0	-	-
	A/H1N2	3	3 (100)	0	-	-
	A/H3N2/Johannesburg/94-like	8	8 (100)	0	-	-
	A/H3N2/ Panama/2007/99-like	10	10 (100)	0	-	-
	A/H3N2/ Fuijian/411/02-like	10	10 (100)	0	-	-
	A/H3N2/ Christchurch/28/03-like	10	10 (100)		-	-
	A/H5N1	2	2 (100)	0	-	-
	A/H7N7	2	2 (100)	0	-	-
	A/H9N2	2	2 (100)	0	-	-
	B/Yamagata-like	15	15 (100)	0	-	-
	B/Victoria-like	13	13 (100)	0	-	-
	RSV A	16	-		16 (100)	0
	RSV B	14	-		14 (100)	0

ve by real time PCR, in concordance with both nested PCR and cell culture and all sample negative by nested PCR and cell culture also resulted negative by real time PCR. As far as RSV, 18 swabs were positive by nested PCR, cell culture and real time PCR and 5 resulted positive in real time PCR, in concordance with nested PCR. Three and 5 out of 175 negative by both nested PCR and cell culture were positive by real time PCR for influenza A and RSV, respectively.

Discussion

Rapid detection of Influenza viruses is becoming of the utmost importance, especially with the advent of specific antiviral drugs for influenza viruses A and B and the emergence of highly pathogenic viruses like avian influenza A/H5N1. The efficacy of antiviral drugs largely depends by the early administration that should be given within 36-48 hours since the onset of symptoms. Considering the emergence of respiratory disease caused by highly pathogenic avian influenza virus A/H5N1 in South-East Asia and the confirmation of human cases in Europe, Africa and Mid-east Asia, a rapid sensitive diagnosis plays a fundamental role in early war-

Tab. II. Analytical sensitivity:obtained in analysis of serial dilution of influenza A, influenza B and RSV cell culture-grown virus.

Virus	Dilution	Virus Concentration (PFU)	Ct Mean ± s.d.	Ct 95% I.C.	Ct CV%
A/H3N2 Influenza	1:1	100	17.5 ± 0.5	16.7-18.3	2.9
	1:10	10	20.5 ± 1.2	18.5-22.3	5.9
	1:100	1	24.6 ± 0.6	23.7-25.5	2.4
	1:1,000	0.1	27.5 ± 0.7	26.3-28.5	2.5
	1:10,000	0.01	30.4 ± 1.2	28.5-32.3	3.9
	1:100,000	0.001	33.2 ± 0.3	32.4-34	0.9
B Influenza	1:1	100	20.7 ± 0.9	19.3-22	4.3
	1:10	10	23.3 ± 1.1	21.5-25.1	4.3
	1:100	1	26.9 ± 1.3	24.9-28.9	4.8
	1:1,000	0.1	29.3 ± 0.9	27.9-30.6	3.1
	1:10,000	0.01	31.6 ± 1.9	27-36.3	6
	1:100,000	0.001	-		
RSV	1:1	100	19.9 ± 0.6	18.9-20.8	3
	1:10	10	23.5 ± 1	22-25.1	4.3
	1:100	1	26.7 ± 1.2	24.8-28.5	4.5
	1:1,000	0.1	29.8 ± 1.8	26.9-36.7	6
	1:10,000	0.01	32.3 ± 2	29.1-35.5	6.2
	1:100,000	0.001	34.1 ± 0.7	32.4-35.8	2.1

Tab. III. Results obtained in analysis of 250 specimens collected during the 2004/05 season.

Influenza A		Influenza B		RSV		N.	Real time PCR		
Nested PCR	Cell cult.	Nested PCR	Cell cult.	Nested PCR	Cell cult.		Influ A Pos	Influ B Pos	RSV Pos
Pos	Pos	Neg	Neg	Neg	Neg	37*	37	0	0
Pos	Neg	Neg	Neg	Neg	Neg	9	9	0	0
Neg	Neg	Pos	Pos	Neg	Neg	6	0	6	0
Neg	Neg	Pos	Neg	Neg	Neg	0	0	0	0
Neg	Neg	Neg	Neg	Pos	Pos	18	0	0	18
Neg	Neg	Neg	Neg	Pos	Neg	5	0	0	5
Neg	Neg	Neg	Neg	Neg	Neg	175°	3	0	5
						250			

* 1 A/H1N1/New Caledonia-like, 5 A/H3N2/Panama-like, 31 A/H3N2/California/7/04-like; °3 A/H3N2 and 5 RSV

ning of surveillance system. Amplification techniques are particularly beneficial to survey difficult-to-culture organisms, such as RSV. Laboratory methods as cells culture are labor-intensive and time consuming. PCR based tests are considered the gold standard for detection of respiratory viruses [16-18] even if the application of a PCR panel to respiratory specimens has the disadvantage of requiring the use of different tubes for each sample thus increasing the risk of cross contamination and the presence of a multiple primer sets may affect the specificity and sensitivity of the assays. The real time PCR approach overcomes these limitations. In this study is reported the evaluation of two real-time one step RT-PCR assays as sensitive methods for detection of respiratory viruses using a close-tube system that minimizes the risk of contamination and a single step procedure easy and rapid to perform. The findings showed that our assays are highly specific and sensitive for detection of all tested influenza A and B virus strains, belonging to all subtypes and lineages within subtypes, and RSV type A and B.

As regard as clinical evaluation, these two real-time PCR detected all viral culture- and multiplex-nested PCR positive clinical samples. The data of prospective study highlighted that the real time PCR was more sensitive than both cell culture and nested-PCR, showing that some positive samples could be missed by routine methods. The analytical sensitivity resulted from analysis of a dilution series of a cell culture-grown virus showed that the sensitivity was increased by up to a factor of 100 with respect to multiplex for influenza A and B and RSV viruses. These results are extremely important in routine diagnostics studies particularly when the amount of virus RNA in specimens is low.

In conclusion, this two new real-time PCR showed to be specific and sensitive in rapid detection of influenza and RSV. They could represent an important tool for human influenza surveillance due to both human and avian influenza virus; moreover they are not affected by differences in the circulating strain so that also drifted viruses could be detected such as viruses belonging to subtypes other than A/H3N2 or A/H1N1, such as A/H5N1, A/H7N7 and A/H9N2.

A rapid detection of RSV is necessary since RSV is the most common viral pathogen for lower respiratory tract infection among infants and young children and an important agent for respiratory disease in the elderly and in transplant patients.

References

[1] Falsey AR, Walsh EE. *Respiratory syncytial virus infection in adults*. Clin Microbiol Rev 2000;13:371-84.

[2] Paton AW, Paton JC, Lawrence AJ, Goldwater PN, Harris RJ. *Rapid detection of respiratory syncytial virus in nasopharyngeal aspirates by reverse transcription and polymerase chain reaction amplification*. J Clin Microbiol 1992;30:901-4.

[3] Claas EC, van Milaan AJ, Sprenger MJ, Ruiten-Stuiver M, Arron GI, Rothbarth PH, et al. *Prospective application of reverse transcriptase polymerase chain reaction for diagnosing influenza infections in respiratory samples from a children's hospital*. J Clin Microbiol 1993;31:2218-21.

[4] Herrmann B, Larsson C, Zweygberg BW. *Simultaneous detection and typing of influenza viruses A and B by a nested reverse transcription-PCR: comparison to virus isolation and antigen detection by immunofluorescence and optical immunoassay (FLU OIA)*. J Clin Microbiol 2001;39:134-8.

[5] Zambon M, Hays J, Webster A, Newman R, Keene O. *Diagnosis of influenza in the community: relationship of clinical diagnosis to confirmed virological, serologic, or molecular detection of influenza*. Arch Intern Med 2001;161:2116-22.

[6] Ansaldi F, D'Agaro P, De Florentiis D, Puzelli S, Lin YP, Gregory V, et al. *Molecular characterization of influenza B viruses circulating in northern Italy during the 2001-2002 epidemic season*. J Med Virol 2003;70:463-9.

[7] http://www.cdc.gov/flu/

[8] http://www.who.int/csr/disease/influenza/

[9] Pearson WR. *Flexible sequence similarity searching with the FASTA3 program package*. Methods Mol Biol 2000;132:185-219.

[10] Thompson JD, Higgins DG, Gibson TJ. *CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice*. Nucleic Acids Res 1994;22:4673-80.

[11] Allawi HT, Santa Lucia J Jr. *Thermodynamics and NMR of internal G.T mismatches in DNA*. Biochemistry 1997;36:10581-94.

[12] Rozen S, Skaletsky H. *Primer3 on the WWW for general users and for biologist programmers*. Methods Mol Biol 2000;132:365-86.

[13] Manuguerra JC, Hannoun C. *Influenza and other viral respiratory diseases. Surveillance and laboratory diagnosis*. Paris: Institut Pasteur 1999.

[14] Ansaldi F, Icardi G, Gasparini R, Campello C, Puzelli S, Bella A, et al. *New A/H3N2 influenza variant: a small genetic evolution but a heavy burden on the Italian population during the 2004-2005 season*. J Clin Microbiol 2005;43:3027-9.

[15] Ansaldi F, Bacilieri S, Amicizia D, Valle L, Banfi F, et al. *Antigenic characterisation of influenza B virus with a new microneutralisation assay: comparison to haemagglutination and sequence analysis*. J Med Virol 2004;74:141-6.

[16] Magnard C, Valette M, Aymard M, Lina B. *Comparison of two nested PCR, cell culture and antigen detection for the diagnosis of upper respiratory tract infections due to influenza viruses*. J Med Virol 1999;59:215-20.

[17] Grondhal B, Puppe W, Hoppe A, Kuhne I, Weigl JAI, Schmitt HJ. *Rapid identification of nine micro-organisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study*. J Clin Microbiol 1999;37:1-7.

[18] Weinberg A, Zamora MR, Li S, Torres F, Hodges TN. *The value of polymerase chain reaction for the diagnosis of viral respiratory tract infections in lung transplant recipients*. J Clin Virol 2002;25:171-5.

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