

Surveillance of influenza virus B circulation in Northern Italy: summer-fall 2008 isolation of three strains and phylogenetic analysis

Giuseppe Gerna¹, Elena Percivalle¹, Antonio Piralla¹, Vanina Rognoni¹, Antonietta Marchi², Fausto Baldanti¹

¹Servizio di Virologia, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

²Dipartimento di Pediatria, Università degli Studi di Pavia, Pavia, Italy

SUMMARY

Influenza virus type B strains were unexpectedly detected and isolated in Italy during summer-fall 2008 from three patients travelling to Italy from Lebanon, Senegal and Uzbekistan. The three summer-fall strains matched to a high degree the hemagglutinin (HA1) of influenza virus type B strains circulating in Italy in the second part (January through April) of the 2007/2008 season, and HA1 of the type B strains included in the 2008/2009 vaccine (B/Yamagata/16/88 lineage). Surveillance of influenza virus circulation in Western countries also during the summer-fall season may help to trace and anticipate the appearance of new influenza virus variants.

KEY WORDS: Influenza virus B, Respiratory viruses, Vaccines, Phylogenetic analysis, Real-time RT-PCR, Hemagglutinin

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Due to the epidemiological characteristics of the influenza virus, its circulation is usually observed only during the winter-spring season, i.e. between November of the preceding year and May of the following year. Starting from the summer-fall season 2008, we monitored all known respiratory viruses in all nasopharyngeal aspirates (NPAs) taken from patients admitted to hospital during the period November 2007-October 2008. According to this surveillance programme, we surprisingly detected influenza B virus in three patients from non-European community countries during the summer-fall 2008. In the last two decades, influenza B virus variants circulating in Italy, as well as in other countries, all belonged to one of the two major lineages: B/Yamagata/16/88 (B/YM), or B/Victoria/2/87 (B/VI) (Rota *et al.*, 1992; Ansaldi *et al.*, 2003; Chi *et al.*, 2003; Puzelli *et al.*, 2004; Pariani *et al.*, 2008).

Corresponding author

Giuseppe Gerna

Servizio di Virologia, Fondazione IRCCS

Policlinico San Matteo

V.le Taramelli, 5 - 27100 Pavia, Italy

E-mail: g.gerna@smatteo.pv.it

The present study sequenced the influenza B virus strains recovered from the three individuals from foreign countries in a portion of the hemagglutinin (HA1) and phylogenetically analyzed to compare their sequences with those of both strains circulating in the same Italian area in 2007-2008, and strains included in vaccines for the 2007-2008 and 2008-2009 influenza seasons. Patients admitted to the University Hospital of the Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo during the period November 1, 2007 - October 31, 2008 with acute respiratory tract infection had respiratory secretions collected and examined for the presence of respiratory viruses.

The collection of nasopharyngeal aspirates (NPAs) was conducted according to a standardized procedure.

NPAs were aliquoted and handled as previously reported (Rovida *et al.*, 2005; Sarasini *et al.*, 2006). Respiratory secretions were tested by immunofluorescence and monoclonal antibodies for influenza virus A and B, human parainfluenza viruses, human respiratory syncytial virus, and human adenoviruses (Millipore, Chemicon

International Inc., Temecula, CA) as well as for human coronaviruses 229E and HKU1 (Gerna *et al.*, 2006a, 2007), and human metapneumovirus (Percivalle *et al.*, 2005; Gerna *et al.*, 2006b) (monoclonal antibodies developed in the laboratory). Specimens positive for influenza were quantified by real-time reverse-transcription PCR (RT-PCR) for influenza virus A (Spackman *et al.*, 2002) and B (Ward *et al.*, 2004). In addition, NPAs were tested by real-time RT-PCR for human coronaviruses (229E, NL63, OC43 and HKU1) (Gerna *et al.*, 2006a, 2007) and human rhinoviruses (Deffernez *et al.*, 2004) and by PCR for human bocavirus (Lu *et al.*, 2006). Influenza A H1 and H3 subtyping was performed by RT-PCR, as previously reported (Percivalle *et al.*, 2008).

In addition, NPAs were routinely inoculated onto shell-vials of mixed mink lung (Mv1Lu) and A549 epithelial cells for rapid (48h) influenza virus and other respiratory virus detection (Huang and Turchek, 2000). As for influenza virus isolation, MDCK cell cultures were routinely inoculated. When virus isolation failed in MDCK cells, NPAs were inoculated onto MDCK cells stably transfected with cDNA of human 2,6-sialyltransferase (SIAT1) expressing higher amounts of 6-linked sialic acids than parent MDCK cells (Matrosovich *et al.*, 2003).

Sequencing of the HA1 gene of influenza B strains was done by a single-step RT-PCR using the enzyme SuperScript™ One-Step RT-PCR and Platinum® Taq (Invitrogen). The HA1 gene (nt 118-879) was amplified using the method described by Stockton *et al.*, 1998. Besides the primer pair used for RT-PCR, two additional in-house designed primers were used as follows: 4F, 5'-TTCGCAACAATGGCTTGG-3', and 12R, 5'-CCAAGCCATTGTTGCGAA-3'. Sequences were then analyzed using the Genetic Analyzer 3100 System (Applied BioSystems). In addition, sequences were cut and checked by using the 4.7 version of the Sequencher Software (Gene Codes Corp., Ann Arbor, MI). Neighbor-joining phylogenetic trees were generated following alignment with the MEGA 4.0 software using the Clustal W method with the kimura 2-parameter correction for multiple base substitutions. In parallel, maximum likelihood trees were generated using the PAUP programme. Both methods generated similar topologies. The bootstrap value was 1000 repetitions.

Overall, 1536 respiratory samples from 917 patients were examined for the presence of respiratory viruses. On the whole, 465 (50.7%) patients and 683 (44.5%) samples were positive for one or more respiratory viruses.

From December 2007 through April 2008, 43 strains of influenza virus were recovered from 42 patients, as follows: 32 influenza B, 6 influenza A (H1N1), 3 influenza A (H3N2), and 2 influenza A (undetermined). In addition, at the beginning of September, influenza B virus was unexpectedly recovered from two patients: an adult from Senegal, and a child from Lebanon. In addition, influenza B virus was recovered at the end of October 2008 from an Italian woman returning to Italy from Uzbekistan. Thus, influenza B virus was detected in three individuals from foreign countries during the summer-fall season, when the virus was not circulating in Italy.

Of the 35 patients infected by influenza virus B, 27 (77.1%) had fever greater than 38°C, 34 (97.1%) had respiratory symptoms, and 18 (51.4%) had systemic symptoms. All cases of influenza virus B occurred between December 2007 and April 2008, and after January 2008, they were predominant or exclusive with respect to influenza A strains.

Influenza virus B was detected in NPA by multiple diagnostic approaches. The direct fluorescent antibody (DFA) technique was positive in 13/35 (37.1%) patients, while the shell vial culture (SVC) was positive in 35/35 (100%) patients and virus isolates were recovered from 32/35 (91.4%) patients. The median viral load in NPAs of the 35 influenza virus B-infected patients was 5.8×10^5 RNA copies/ml (range 1.3×10^3 to 6.6×10^7 copies/ml). It is worth mentioning that 9/32 (28.1%) viral isolates could not be recovered on regular MDCK cells, but were recovered on MDCK cells over-expressing the sialyl- α 2,6-galactose-containing virus receptor.

The HA1 gene was sequenced for all 35 influenza virus B strains. These sequences were used to construct a phylogenetic tree using as prototypes the two influenza virus B strains included in the influenza vaccines 2007/2008 (B/Malaysia/2506/2004, B/VI-derived) and 2008/2009 (B/Florida/4/2006, B/YM-derived). As shown in Figure 1, about half of the influenza B strains (18/35, 51.4%) belonged to the B/VI cluster, while the other half (17/35, 48.6%) belonged to the B/YM cluster.

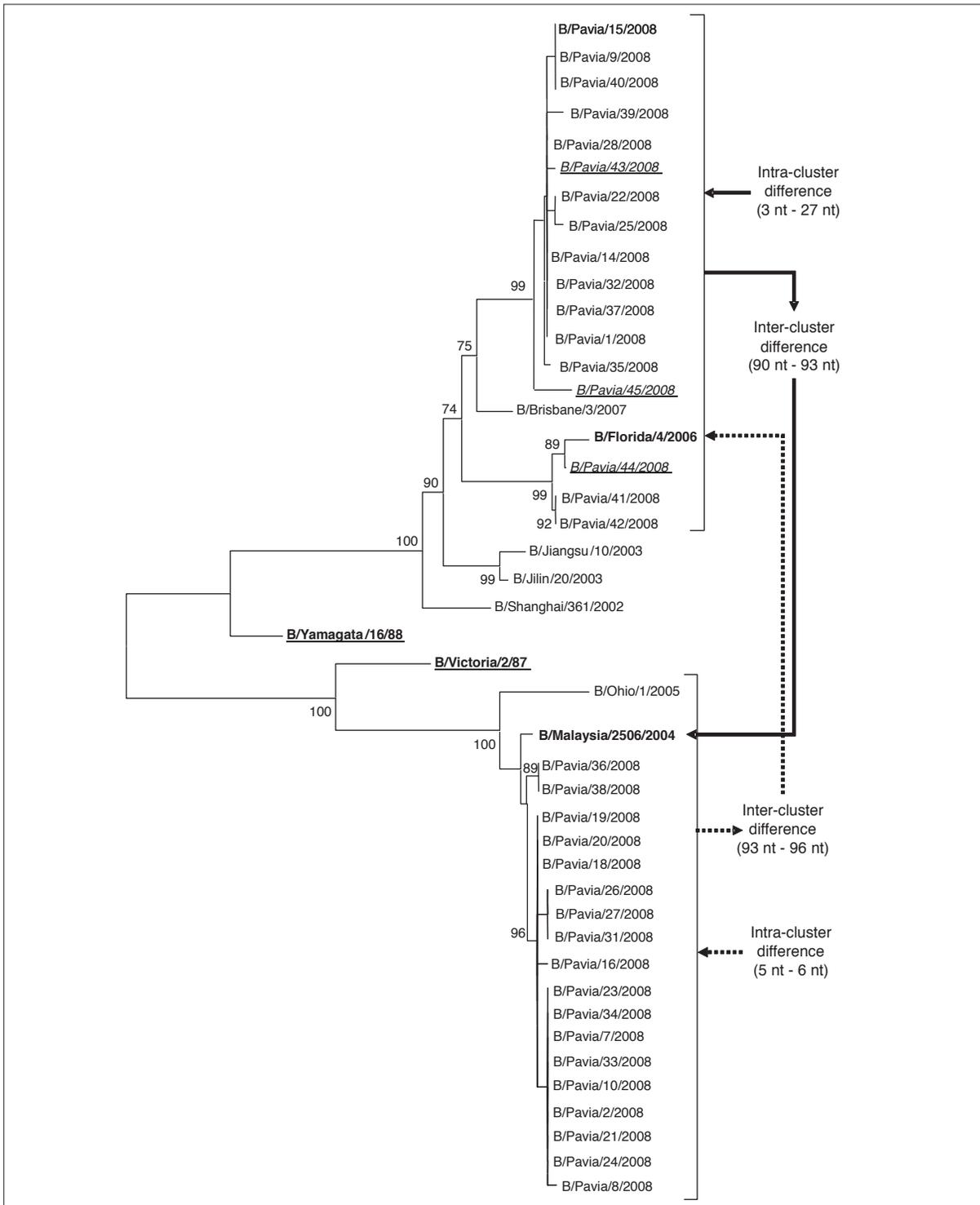


FIGURE 1 - Phylogenetic analysis of the HA1 gene of influenza virus B strains (nt 118-879). The two prototypes from the two major influenza B lineages (B/Yamagata/16/88 and B/Victoria/2/87) as well as the two strains included in the vaccine 2007-2008 (B/Malaysia/2506/2004) and 2008-2009 (B/Florida/4/2006) are in bold. The three underlined strains (in italics) from the B/Yamagata/16/88 lineage were recovered from patients coming to Italy from abroad.

During the 2007-2008 winter-spring season, influenza virus B strains deriving from the B/VI lineage were predominant in the first months (December through February), while strains deriving from the B/YM lineage predominated in the last part of the season. In fact, they were the only influenza strains recovered in April 2008. All three influenza virus B strains recovered during summer-fall 2008 belonged to the B/YM lineage. The HA1 aa sequence of the 17 B/YM strains was very divergent with respect to the influenza virus B vaccine of the 2008-2009 season (B/Florida/04/06). Of these 17 strains, 12 had the same pattern of 8 aa mutations with respect to the vaccine strain (including the strain recovered from the Senegalese patient), while the Lebanese patient (B/Pavia/44/2008) had only three substitutions and the patient from Uzbekistan (B/Pavia/45/2008) had 8 partially different aa substitutions (Fig. 2). In addition, another patient suspected to have had contact with non-Italians (B/Pavia/

39/2008) during the season showed 10 aa substitutions.

In addition, three vaccinated individuals presented with clinically overt influenza B disease caused by B/VI-derived strains. All three vaccinated individuals showed two aa substitutions at position 46 (G→A) and 109 (K→N) within the HA1 gene product with respect to the vaccine influenza virus B strain of the 2007-2008 season (B/Malaysia/2506/04). These two aa changes were present in all 17 strains from the B/VI-lineage, together with an additional change (N→K) at position 165 for 8 strains, and at position 262 for two additional strains (Fig. 2).

The central finding of this investigation was the isolation of influenza virus B strains during late summer-early fall 2008, i.e. in a period when influenza viruses were not circulating in Italy. The three patients came to Italy from other countries, carrying the influenza virus from the relevant country. This finding suggests that surveillance

A					B											
Victoria-lineage strain	HA1 aa position				Yamagata-lineage strain	HA1 aa position										
	46	109	165	262		40	46	52	56	88	150	162	165	196	215	229
B/Malaysia/2506/04	G	K	N	T	B/Florida/04/06	Y	G	K	N	K	S	K	N	D	S	S
B/Pavia/18/2008	A	N	.	.	<u>B/Pavia/44/2008</u>	.	.	.	D	R	.	.	.	N	.	.
B/Pavia/26/2008	A	N	.	.	B/Pavia/42/2008	H	A	.	D	R	.	.	.	N	.	.
B/Pavia/27/2008	A	N	.	.	B/Pavia/41/2008	H	A	.	D	R	.	.	.	N	.	.
<u>B/Pavia/19/2008</u>	A	N	.	.	<u>B/Pavia/45/2008</u>	.	A	R	D	R	I	.	Y	N	.	D
<u>B/Pavia/31/2008</u>	A	N	.	.	B/Pavia/37/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/20/2008	A	N	.	.	B/Pavia/9/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/16/2008	A	N	.	.	B/Pavia/32/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/38/2008	A	N	.	A	B/Pavia/22/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/36/2008	A	N	.	A	<u>B/Pavia/43/2008</u>	H	A	.	D	R	I	.	Y	N	.	D
<u>B/Pavia/7/2008</u>	A	N	K	.	B/Pavia/35/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/2/2008	A	N	K	.	B/Pavia/15/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/10/2008	A	N	K	.	B/Pavia/1/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/8/2008	A	N	K	.	B/Pavia/25/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/21/2008	A	N	K	.	B/Pavia/14/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/24/2008	A	N	K	.	B/Pavia/28/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/33/2008	A	N	K	.	B/Pavia/40/2008	H	A	.	D	R	I	.	Y	N	.	D
B/Pavia/23/2008	A	N	K	.	B/Pavia/39/2008	H	A	.	D	R	I	E	Y	N	F	D
B/Pavia/34/2008	A	N	K	.												

FIGURE 2 - Amino acid differences in HA1 sequences (aa 40-293) of (A) Victoria-lineage and (B) Yamagata-lineage influenza virus B strains recovered during the period November 2007 – October 2008. In (A), virus strains from the three vaccinated individuals are underlined. In (B), the three influenza B virus strains from patients from non-European countries are underlined.

for influenza virus circulation should be extended in the European countries to the summer-fall season, in order to detect early circulation of new influenza virus variants.

The phylogenetic analysis indicated that the three strains recovered during summer-fall were B/YM variants and were close to variants circulating in Italy in the second part of the 2007-2008 season, and to the influenza virus B vaccine strain of the 2008-2009 season.

In the first part of the 2007-2008 influenza season, the B/Malaysia-like (B/VI lineage) and the B/Florida-like strains (B/YM lineage) were equivalent in number, whereas in April only B/Florida-like strains were detected. This epidemiological trend showed that the B/Florida-like strains progressively replaced the B/Malaysia-like strains (included in the 2007-2008 vaccine) during the last part of the season.

In addition, during our surveillance programme, it was found that as many as three patients suffering from influenza virus B disease caused by the B/Malaysia-like strain had been vaccinated in previous months, as already reported (Pariani *et al.*, 2008). Sequencing of the HA1 gene indicated the presence of two aa changes at positions 46 and 109 identical in all three strains. In this respect, crystal structure and selective pressure studies have contributed to the identification of positively selected sites, which appear to be located predominantly within the four major antigenic epitopes of HA1: the 120 loop (HA1 116-137), the 150-loop (HA1 141-150), the 160-loop (HA1 162-167), and the 190 helix (HA1 194-202), and their relevant surrounding regions (Wang *et al.*, 2008; Shen *et al.*, 2009). In general, all sites not adjoining the 150-loop, 160-loop, or the 190-helix epitopes are referred to as the 120-loop region due to their spatial proximity. It might be speculated that the mutations observed at positions 109 and, possibly, 46 of HA1 of the three strains isolated from the three vaccinated patients (with respect to the B/Malaysia/2506/04 vaccine strain) may have been sufficient to originate escape mutants.

A greater degree of variability is well recognized for HA1 of B/Florida-like strains (B/YM variants) as compared to the B/Malaysia-like (B/VI) strain (Ansaldi *et al.*, 2003). In our study, all 17 influenza B strains of the B/YM lineage showed a number of aa substitutions with respect to the vac-

cine B/Florida/04/06 (B/YM) strain, included in the 2008-2009 vaccine. Based on the recent definition of the major epitopes of influenza virus B HA1 (Wang *et al.*, 2008; Shen *et al.*, 2009), it appears evident that multiple epitopes harbour mutations in all field strains isolated, thus suggesting that, if variants of influenza virus B detected in the past season will circulate also in the next season, a large number of patients would not be protected against influenza virus B, even if vaccinated.

Results of the present study document and confirm that HA1 antigenic drift not only occurs in influenza virus A strains, but also in influenza virus B strains, thus lowering the protective efficacy of the vaccine. In this respect, attempts to develop influenza vaccines based on conserved influenza virus proteins, such as protein M, or proteins of the replication complex (PA, PB1, PB2) should be encouraged and investigated in the near future (Heini *et al.*, 2007).

In conclusion, the recovery of influenza virus B strains during the 2008 summer-fall season from patients travelling abroad (outside Europe) should prompt surveillance of influenza virus circulation also in non-European countries during summer and fall. This approach could help in tracking new influenza virus variants.

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REFERENCES

- ANSALDI F., D'AGARO P., DE FLORENTIIS D., PUZELLI S., LIN Y.P., GREGORY V., BENNETT M., DONATELLI I., GASPARINI R., CROVARI P., HAY A., CAMPELLO C. (2003). Molecular characterization of influenza B viruses circulating in Northern Italy during the 2001-2002 epidemic season. *J. Med. Virol.* **70**, 463-469.
- CHI X.S., BOLAR T.V., ZHAO P., RAPPAPORT R., CHENG S.-M. (2003). Cocirculation and evolution of two lineages of influenza B viruses in Europe and Israel in the 2001-2002 season. *J. Clin. Microbiol.* **41**, 5770-5773.

- DEFFERNEZ C., WUNDERLI W., THOMAS Y., YERLY S., PERRIN L., KAISER L. (2004). Amplicon sequencing and improved detection of human rhinovirus in respiratory samples. *J. Clin. Microbiol.* **42**, 3212-3218.
- GERNA G., CAMPANINI G., ROVIDA F., PERCIVALLE E., SARASINI A., MARCHI A., BALDANTI F. (2006a). Genetic variability of human coronavirus OC43-, 229E-, and NL63-like strains and their association with lower respiratory tract infections of hospitalized infants and immunocompromised patients. *J. Med. Virol.* **78**, 938-949.
- GERNA G., PERCIVALLE E., SARASINI A., CAMPANINI G., PIRALLA A., ROVIDA F., GENINI E., MARCHI A., BALDANTI F. (2007). Human respiratory coronavirus HKU1 vs other coronavirus infections in Italian hospitalized patients. *J. Clin. Virol.* **38**, 244-250.
- GERNA G., SARASINI A., PERCIVALLE E., GENINI E., CAMPANINI G., REVELLO M.G. (2006b). Simultaneous detection and typing of human metapneumovirus strains in nasopharyngeal secretions and cell cultures by monoclonal antibodies. *J. Clin. Virol.* **35**, 113-116.
- HEINI A.T., MIOTTO O., SRINIVASAN K.N., KHAN A.M., ZHANG G.L., BRUSIC V., TAN T.W., AUGUST J.T. (2007). Evolutionarily conserved protein sequences of influenza A viruses, avian and human, as vaccine targets. *PLoS ONE*, **2**, e1190.
- HUANG Y.T., TURCHEK B.M. (2000). Mink lung cells and mixed mink lung and A549 cells for rapid detection of influenza virus and other respiratory viruses. *J. Clin. Microbiol.* **38**, 422-423.
- LU X., CHITTAPANITCH M., OLSEN S.J., MACKAY I.M., SLOOTS T.P., FRY A.M., ERDMAN D.D. (2006). Real-time PCR assays for detection of bocavirus in human specimens. *J. Clin. Microbiol.* **44**, 3231-3235.
- MATROSOVICH M., MATROSOVICH T., CARR J., ROBERTS N.A., KLENK H.-D. (2003). Overexpression of the α -2, 6-Sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. *J. Virol.* **77**, 8418-8425.
- PARIANI E., AMENDOLA A., ZAPPA A., BIANCHI S., COLZANI D., ANSELMINI G., ZANETTI A., TANZI E. (2008). Molecular characterization of influenza viruses circulating in Northern Italy during two seasons (2005/2006 and 2006/2007) of low influenza activity. *J. Med. Virol.* **80**, 1984-1991.
- PERCIVALLE E., ROVIDA F., PIRALLA A., ROGNONI V., ZAVATTONI M., BALDANTI F., MARCHI A., GERNA G. (2008). Rapid typing, subtyping and RNA quantification of influenza virus type A strains in respiratory secretions. *New Microbiologica*. **31**, 319-327.
- PERCIVALLE E., SARASINI A., VISAI L., REVELLO M.G., GERNA G. (2005). Rapid detection of human metapneumovirus strains in nasopharyngeal aspirates and shell vial cultures by monoclonal antibodies. *J. Clin. Microbiol.* **43**, 3443-3446.
- PUZELLI S., FREZZA F., FABIANI C., ANSALDI F., CAMPITELLI L., LIN Y.P., GREGORY V., BENNETT M., D'AGARO P., CAMPELLO C., CROVARI P., HAY A., DONATELLI I. (2004). Changes in the hemagglutinins and neuraminidases of human influenza B viruses isolated in Italy during the 2001-02, 2002-03, 2003-04 seasons. *J. Med. Virol.* **74**, 629-640.
- ROTA P.A., HEMPHILL M.L., WHISTLER T., REGNERY H.L., KENDAL A.P. (1992). Antigenic and genetic characterization of the haemagglutinins of recent cocirculating strains of influenza B virus. *J. Gen. Virol.* **73**, 2737-2742.
- ROVIDA F., PERCIVALLE E., ZAVATTONI M., TORSSELLINI M., SARASINI A., CAMPANINI G., PAOLUCCI S., BALDANTI F., REVELLO M.G., GERNA G. (2005). Monoclonal antibodies versus reverse-transcription-PCR for detection of respiratory viruses in a patient population with respiratory tract infections admitted to hospital. *J. Med. Virol.* **75**, 336-347.
- SARASINI A., PERCIVALLE E., ROVIDA F., CAMPANINI G., GENINI E., TORSSELLINI M., PAOLUCCI S., BALDANTI F., MARCHI A., REVELLO M.G., GERNA G. (2006). Detection and pathogenicity of human metapneumovirus respiratory infections in pediatric Italian patients during a winter-spring season. *J. Clin. Virol.* **35**, 59-68.
- SHEN J., KIRK B.D., MA J., WANG Q. (2009). Diversifying selective pressure on influenza B virus hemagglutinin. *J. Med. Virol.* **81**, 114-124.
- SPACKMAN E., SENNE D.A., MYERS T.J., BULAGA L.P., GARBER L.P., PERDUE M.L., LOHMAN K., DAUM L.T., SUAREZ D.L. (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol.* **40**, 3256-3260.
- STOCKTON J., ELLIS J.S., SAVILLE M., CLEWLEY J.P., ZAMBON M.C. (1998). Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. *J. Clin. Microbiol.* **36**, 2990-2995.
- WANG Q., CHENG F., LU M., TIAN X., MA J. (2008). Crystal structure of unliganded influenza B virus hemagglutinin. *J. Virol.* **82**, 3011-3020.
- WARD C.L., DEMPSEY M.H., RING C.J.A., KEMPSON R.E., ZHANG L., GOR D., SNOWDEN B.W., TISDALE M. (2004). Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. *J. Clin. Virol.* **29**, 179-188.