

Influenza



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Influenza

8. Influenza

8.1 Epidemiological and virological monitoring of human influenza viruses in India

LR Yeolekar, staff of NIV influenza group & staff of all Regional Influenza Centers

The following projects under the 'Multi-site epidemiological and virological monitoring of human influenza virus' jointly funded by ICMR-CDC have been initiated.

1. Influenza surveillance in and around Pune city by ICMR Regional center.
2. Antigenic and genetic analyses of influenza isolates and compilation of national data: ICMR Referral center.

8.1.1 Activities of ICMR Regional Centers

Under the multi-site monitoring project five regional centers have been established in India. Each regional center is responsible for collection of samples from cases suffering with acute respiratory infection, conduct virus isolation and identify the circulating strains of influenza in their region. NIV is responsible for influenza surveillance in and around Pune.

Influenza surveillance in Pune city

During the course of surveillance between April 2005 to March 06, a total of 477 respiratory specimens were collected from two hospitals and five dispensaries located in different areas of Pune. Nineteen samples of the above yielded influenza virus isolates, 7 were identified as A(H₁N₁), 6 as A(H₃N₂) and 6 as type B Influenza. Increased influenza activity was from months of June to October and a small peak in March 2006. Influenza A(H₁N₁) was reintroduced in the community after a gap of five years with marked increase in activity in the month of June, July and August 2005. Antigenically A(H₁N₁), A(H₃N₂) and type B were identified as similar to A/New Caledonia/20/99, A/Wyoming/3/03 and B/Sichuan/379/99 from June to October and A/Wyoming/3/03 and B/Hong Kong/330/01 in the March peak.

8.1.2 Influenza surveillance during pilgrimage of Pandharpur

Lord Vitthal of Pandharpur is one of the most revered deities of Maharashtra. Every year, nearly a million devotees walk to Pandharpur. A total of 42 respiratory specimens were collected on 19th July 2005, the last day of the pilgrimage, from patients with respiratory symptoms visiting the government OPD. Majority of the samples were from adults. Nine isolates were obtained 7 A(H₁N₁) and 2 of A(H₃N₂) subtypes and these strains were antigenically similar to the strains isolated from Pune during the same period.

8.1.3 Virus isolation in embryonated eggs

Influenza viruses are known to grow in embryonated eggs. However the presently prevailing strains of influenza virus do not grow to high titres in embryonated eggs. To reconfirm the susceptibility of eggs to these strains, virus isolation was attempted in this system. A total of 11 samples that yielded virus in MDCK cells and 27 negative samples were inoculated in eggs by the allantoic route. The studies clearly indicated that isolation of human influenza viruses in eggs is not a sensitive method.

8.2 ICMR Referral center activity

Under the multi-site monitoring project all the five regional centers sent virus isolates to the ICMR Referral center at NIV. This center is responsible for reconfirming the antigenic analysis of the strains received, conduct genetic analysis, dispatch representative isolates to the WHO Collaborating center at CDC, Atlanta and compile national data. NIV also provides technical support to the regional centers whenever required.

8.2.1 Clinical samples received from other regional centers for isolation

A total of 80 clinical samples received from the Regional center at Dibrugarh were subjected to isolation. Six isolates were obtained, 5 A(H₁N₁) and 1 type B. These samples had been collected in the month of July-August 2005 and antigenically similar to A/New Caledonia/20/99 and B/Shanghai/361/2002. One hundred samples received from regional center at Kolkata (collected in the month of October), yielded 2 isolates which were confirmed as A(H₃N₂) and type B respectively. These were antigenically similar to A/Wyoming/03/03 and B/Hong Kong/330/01.

8.2.2 Analysis of National data

A total of 153 virus isolates were received at NIV from the five regional centers, which were tested in HI test, 32 isolates did not grow to high titers. Influenza typing and sub-typing of these strains was confirmed using RT-PCR. Representative strains were genetically analysed by sequencing of the approximately 1.1 kb of the HA gene covering the HA1 of all the three subtypes. This data was then analyzed with respect to seasonality, age distribution, antigenicity and genetic variability

8.2.2.1 Seasonality

Seasonal pattern of influenza viruses circulating in different regions of India

Influenza activity was observed round the year. Peak activity in New Delhi coincided with low temperatures whereas peak activity in Pune and Chennai was recorded during monsoon rains.

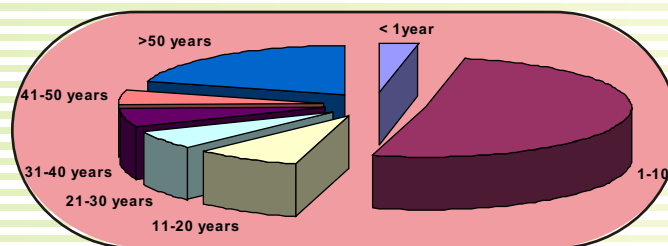


Fig. 1 : Age distribution

Influenza virus strains were isolated from all age groups with maximum number of virus isolation from children between the age group 1-10 years followed by adults >50 years of age.

Antigenic analysis

Antigenic strains of influenza A and B circulating in India

Only one antigenic variant of A(H₁N₁), 5 of A(H₃N₂) and 5 of type B were circulating in Indian community. Strains of A(H₁N₁) circulating in India were similar to A/New Caledonia/20/99, which is the vaccine recommended strain for the period 2004-2005, 2005-2006 and 2006-2007. Three H₃N₂ strains recommended for vaccine in the years 04-05, 05-06 and 06-07 are circulating in India. Both the lineages of type B were co-circulating in 2005.

Genetic analysis

LR Yeolekar, VAPotdar, AAJoshi, SR Waregaonkar, M Joglekar.

Phylogenetic analysis of HA1 domain of HA gene of influenza A virus isolates received from regional centers as well as those isolated at NIV, Pune was carried out. The results of which are depicted as follows.

Table 1: Percentage nucleotid & Aminoacid identity of Indian A (H₁N₁) KA (H₃N₂) with vaccine recommended H₁N₁ & H₃N₂ strain for the years 04-05, 05-06, 06-07

Virus Type No. of nt sequenced		Homology b/w Indian strains		Remark
		nt level	aa level	
A(H1N1)	1000	96.95-98.8%	97.25-98.47%	Genetically similar to A/New Caledonia/20/99 recommended vaccine strain for the years 04-05, 05-06 and 06-07
A(H3N2)	979	95.44-99.79%	92.44-99.31%	Genetically similar to A/Wyoming /3/03 and A/Wisconsin/67/05 recommended vaccine strains for years 05-06, 06-07

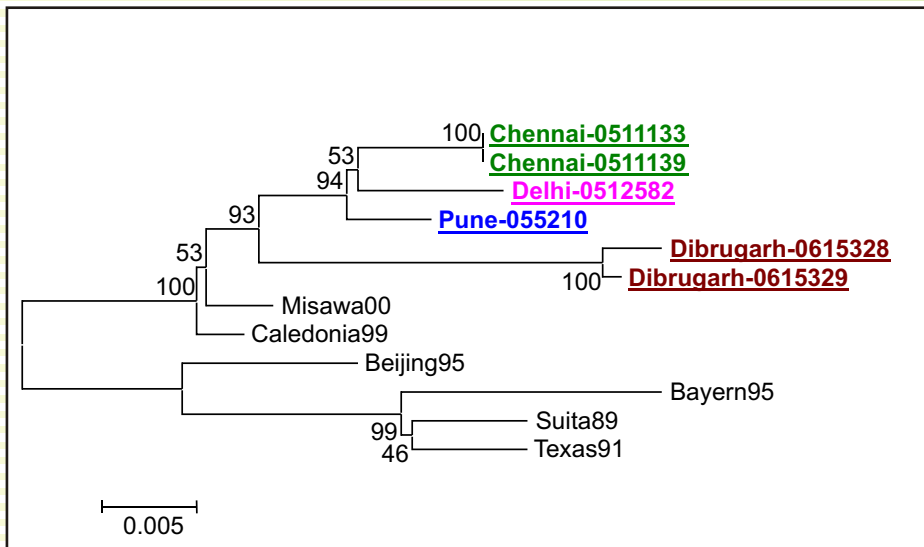


Fig. 2 : Phylogenetic analysis of A(H1 N1) HA1 gene

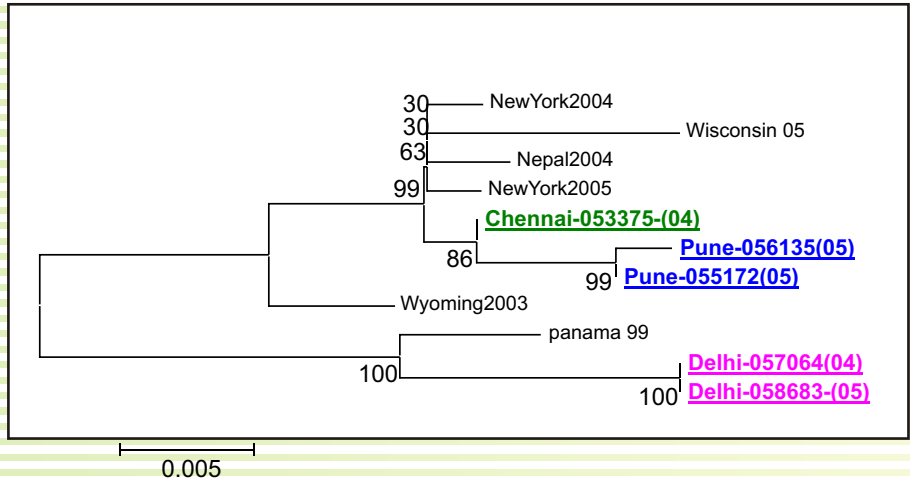


Fig. 3 : Phylogenetic analysis of A(H3N2) HA1 gene

Type B

Sequence analysis of 1024 nucleotide of the HA1 gene of 3 type B strains from Pune indicated 98.24% homology between the two strains of the Victoria lineage and 89.82% homology with the strain of Yamagata lineage at nucleotide level and 99.12% homology and 90.59% at amino acid level respectively. The Yamagata lineage strain from Pune showed 97.94% homology at amino acid level with B/Shanghai the recommended influenza type B vaccine component for the years 04-05 and 05-06 influenza seasons

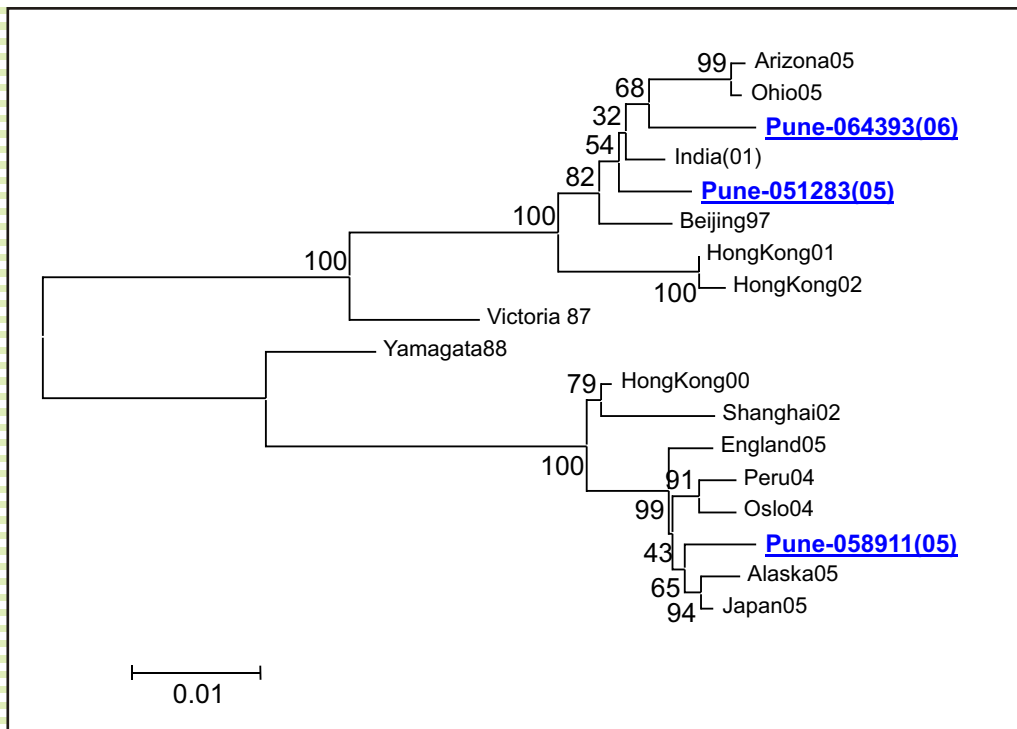


Fig. 4 : Phylogenetic analysis of type B, HA1 gene

8.3 Other studies on diagnosis and antiviral sensitivity

8.3.1 Detection of amantadine sensitivity of influenza A virus isolates

A study to check naturally emerging amantadine resistant influenza A virus strains in Pune has been undertaken. A total of 22 Influenza A virus strains isolated from 2000-2004, that were previously screened for resistance in MDCK cells and found to be sensitive were taken for the study. RT-PCR and nucleotide sequencing was carried out to confirm the amantadine sensitivity of these strains.

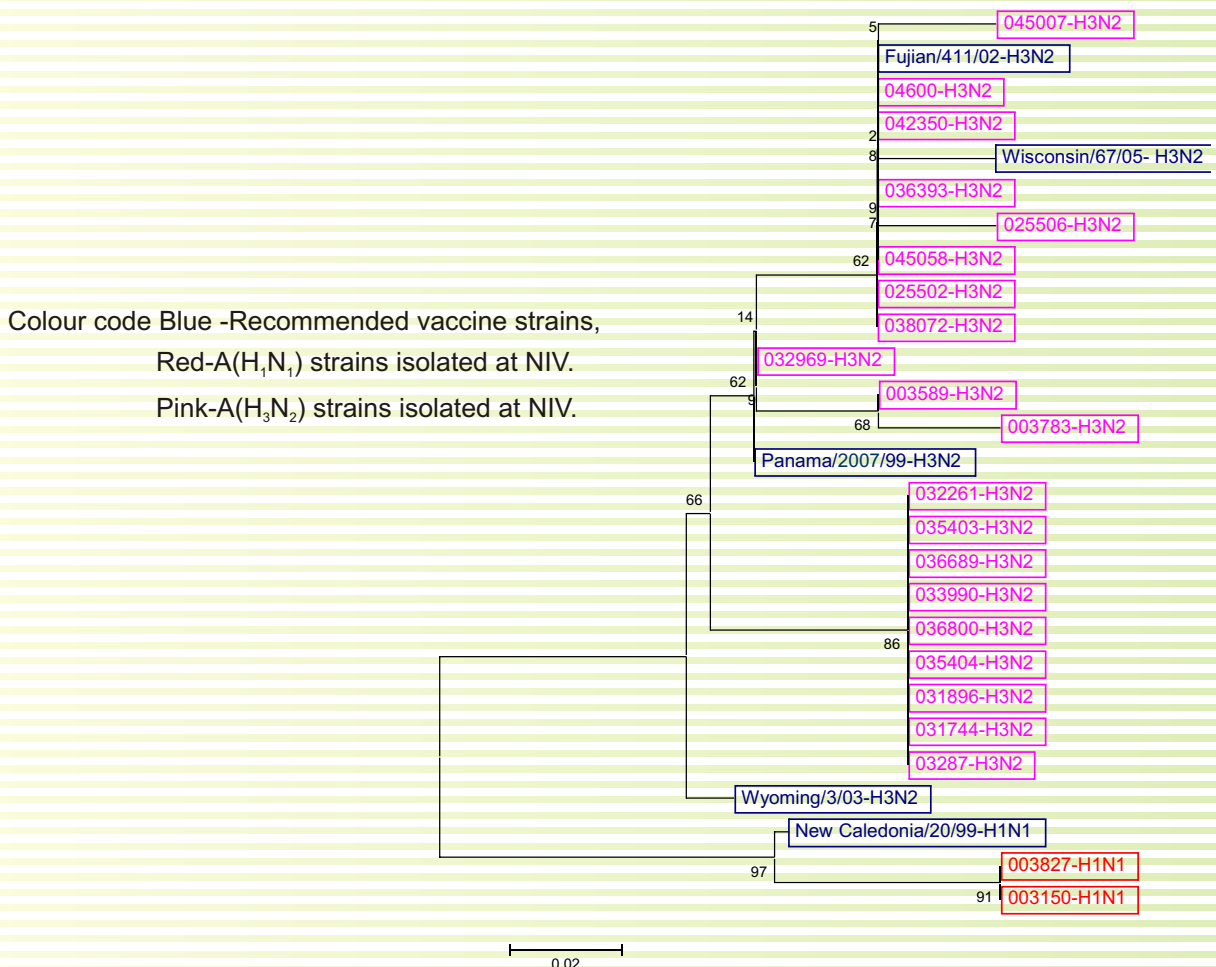


Fig. 5 : Phylogenetic tree based on deduced amino acid sequences of 22 strains of Influenza type A isolated from 2000-2004.

Amantadine interacts with the M2 protein of influenza and single mutation in amino acids 26, 27, 30, 31 and 34 are known to induce resistance. Specific primers to the highly conserved region of M2 gene covering the region encoding for aa 26-34 for influenza A were synthesized to give an amplicon of 270 bp following sequencing of RT-PCR products. The amino acid sequence of previously in vitro tested amantadine sensitive strains was confirmed. No substitution at any of these positions was detected in the 22 strains undertaken for the study, confirming them to be amantadine sensitive. Phylogenetic analysis of the 48 aa sequences indicated clustering of A(H₁N₁) and A(H₃N₂) strains with 91.70-100 % homology within the sub-types and 81.2085.40% between sub-types.

8.3.2 Monoclonal antibodies to influenza A virus

SD Pawar, LR Yeolekar, RG Damle, BL Rao.

Preparation of monoclonal antibodies (MAbs) to influenza A(H₃N₂) has been reported before. These MAbs were used for epitope analysis and year wise strain analysis of influenza A viruses. Also attempts to use these MAbs for antigen capture ELISA were made and compared with virus isolation and antigen capture using WHO Mabs.

Epitope mapping of influenza A(H₃N₂) NP using anti-NP MAbs by performing ELISA additivity test

Epitope mapping was done to determine if these MAbs are reactive with the same epitope or different epitopes on the influenza A(H₃N₂) NP. Epitope mapping was done by performing an additivity index (AI) ELISA. Epitope mapping was done in the following steps;

- Determination of saturation concentration of MAbs in an indirect ELISA;
- Additivity ELISA;
- To generate epitope map using computer programme “Epitope” developed at NIV, Pune.

Six MAbs from three hybrids [E6/3F1, D10/3F1, G9/6A11, B10/6A11, E11/4F11 and H6/4F11] were used in the study. The criterion for the selection of MAbs in epitope mapping was their reactivity in IF tests. Monoclonal antibodies were grouped into three groups, based on their reactivity in IF tests. Representative MAbs (from three hybrids) from the three groups defined in IF test viz. MAbs reacting with influenza A(H₁N₁), A(H₃N₂) and A(H₃N₂) subtypes [E6/3F1, B10/6A11, E11/4F11], MAbs reacting with only A(H₃N₂) strain [D10/3F1, G9/6A11] and MAb not reacting in IF tests [H6/4F11] were selected for epitope mapping.

An additivity index ELISA test was employed for epitope mapping, which is based on an estimation of the number of antigenic sites simultaneously available to a pair of antibodies on an antigen.

Determination of saturation points of MAbs in an indirect ELISA

Each well of the ELISA was coated with 50 ng of purified egg-grown A(H₃N₂) virus. A low antigen concentration was chosen so that saturation of antigen did not require high concentration of MAbs. The saturation points of all the six MAbs were determined in an indirect ELISA and saturation curve for each MAb was plotted. The saturation concentrations of the MAbs ranged from 1:400 to 1:1000.

Additivity index ELISA

Monoclonal antibodies were added separately & together at saturation concentrations to the coated antigen on polystyrene ELISA wells, to test whether two MAbs could bind simultaneously to the virus. After determination of saturation points of all the six MAbs, two MAbs were mixed with each other in such a way that all the possible combinations of MAbs were made. Both the MAbs were diluted in such a way that they achieve their saturation points after mixing. For each pair of MAbs, the additivity index was calculated with the equation, $AI = 100\{[2A_3 - A_1 + A_2] - 1\}$, where A₁, A₂ & A₃ are OD values at 490 nm in an indirect ELISA for the first MAb alone, the second MAb alone & two MAbs together respectively.

The epitope model of the NP was drawn by computer programme “Epitope” developed at NIV that uses AI values to graphically represent relationships between epitopes. Additivity index values were given as input in the programme & the output was the overlapping circles showing map of epitope defined by MAbs. Overlapping circles represent epitopes in close proximity.

The AI values of these six MAbs are shown in the table. A low AI value (<20) indicates that the pair of tested MAbs recognizes the same antigenic site & a high AI value (>40) indicates simultaneous binding of two MAbs recognizing distinct epitopes.

Table 2 : Additivity indices of six influenza A Mabs.

MAb	E6/3F1	D10/3F1	G9/6A11	B10/6A11	E11/4F11	H6/4F11
E6/3F1	-	2	53	58	76	76
D10/3F1		-	55	60	74	81
G9/6A11			-	3	64	74
B10/6A11				-	81	77
E11/4F11					-	8
H6/4F11						-

“Epitope programme” algorithm:

Additivity index values were given as input values in “Epitope programme” developed in NIV, Pune.

The results of epitope mapping indicated that

- The six MAbs from three hybrids delineated three non-overlapping distinct epitopes on NP of influenza A(H₃N₂) virus;
- Two MAbs from each hybrid reacted with the similar epitope.

Year wise analysis of reactivity of A(H₁N₁) & A(H₃N₂) strains

Influenza type A(H₁N₁) & A(H₃N₂) strains isolated from Pune, India spanning a period of 28 years were analyzed to observe variation in the strains with respect to the year of virus isolation. Year-wise analysis of nine A(H₁N₁) strains and 29 A(H₃N₂) strains was performed with E6/3F1, B9/6A11, E11/4F11 and WHO pool A MAbs. The percent reactivity of the strains with MAbs in AC-ELISA was calculated.

The reactivity of $\geq 100\%$ was considered similar to that obtained with the homologous strain, against which MAbs were prepared. Year wise analysis showed variation in the three epitopes of NP of human A(H₁N₁) and most of the human A(H₃N₂) strains during circulation in the community. These epitopes on the strains were predominant in different years without showing any definite pattern.

Rapid detection of influenza A virus directly from respiratory specimens

A total number of 238 respiratory specimens were directly tested in AC-ELISA for influenza diagnosis from respiratory specimens. All these specimens were also processed in MDCK cell culture and 18 of these specimens yielded influenza A(H₃N₂) virus isolates.

When AC-ELISA tests developed using E6/3F1, G9/6A11 and WHO pool A MAbs were used for the rapid detection of influenza A virus antigens from respiratory specimens, sensitivity of the tests ranged from 38.8 % to 88.88%, while specificity of the tests were 100%. Higher sensitivity of AC-ELISA using WHO pool A MAbs might be due to the pool of IgG and IgM MAbs. The results of evaluation of AC-ELISA tests are shown in the tables.

Other monoclonal antibodies B9/6A11 and E11/4F11 also detected respiratory specimens. As limited number of known positive respiratory specimens were available, it was not possible to evaluate AC-ELISA using all these MAbs.

These in-house AC-ELISA tests will be useful in influenza surveillance for rapid and specific diagnosis; to detect sudden influenza outbreaks, These tests would enable cost-effective screening of large number of specimens and selection of positive specimens for influenza virus isolation and strain analysis. Although, respiratory specimens positive only for A(H₃N₂) virus were available for evaluation, the AC-ELISA will also be useful for detection of novel influenza subtypes from respiratory specimens as NIV and WHO pool A MAbs are influenza type A specific.

Table 3 : Antigen capture ELISA results using E6/3F1 NIV-MAb.

AC-ELISA E6/3F1 NIV-MAb	Virus isolation & HAI identification (Gold standard)		Total
	Positive	Negative	
Positive	10	0	10
Negative	08	220	228
Total	18	220	238

Sensitivity: 55.55 %, Specificity: 100 %, PPV: 100%, NPV: 96.4%.

Table 4 : Antigen capture ELISA results using G9/6A11-NIV-MAb.

AC-ELISA G9/6A11 NIV-MAb	Virus isolation & HAI identification (Gold standard)		Total
	Positive	Negative	
Positive	07	0	07
Negative	11	220	231
Total	18	220	238

Sensitivity: 38.8 %, Specificity: 100 %, PPV: 100%, NPV: 95.2%.

Table 5 : Antigen capture ELISA results using WHO pool A MAbs.

AC-ELISA WHO pool A MAb	Virus isolation & HAI identification (Gold standard)		Total
	Positive	Negative	
Positive	16	0	16
Negative	02	220	222
Total	18	220	238

Sensitivity: 88.88 %, Specificity: 100 %, PPV: 100%, NPV: 99.0%.

Note: The results were recorded by calculating P/N ratio. Positive to negative ratio ≥ 2 was considered as a positive ELISA result.

8.3.3 Antigenic comparison among strains of influenza type B victoria lineage in Pune.

Influenza type B Victoria lineage related strains have circulated in Pune in the year 2002. Reemergence of influenza type B Victoria like strains was observed in 2005. Antigenic cross reactivity among the strains of Victoria lineage of year 2002 (NIV # 021492) and 2005 (NIV # 05774) was studied using HI and NT tests.

Polyclonal sera were raised against both the strains 021492 and 05774 cross reacted to the same extent in HI tests with the titre in the range of 1: 40- 1: 80 with both the homologous and heterologous strains. However, antibodies raised against strain 021492 showed high neutralizing titres (1:3200 - 1:6400) with homologous strain but low titers (1: 100 - 1: 200) heterologous strains. Antibodies raised against strain 05774 showed similar neutralizing titers (1:100 - 1:400) with both the homologous and heterologous strains.

It appears that though strains of both the years appeared antigenically similar in HI test showed variability in NT test.

8.3.4 Effect of glutaraldehyde and para-formaldehyde inactivation on immunofluorescence test.

LR Yeolekar, MR Khude.

IF test of cells from nasopharyngeal aspirates is conducted for rapid detection of different respiratory viruses. The effect of glutaraldehyde and paraformaldehyde was tested on influenza virus infected cells. MDCK cells infected with influenza A virus were treated with different concentrations of the two inactivating agents at room temperature for 60 minutes. Cells were then probed with virus specific MAbs in an indirect IF test using anti-mouse FITC conjugate and observed under fluorescent microscope.

Pretreatment with glutaraldehyde lead to change in fluorescence color from bright apple green to bright yellow. Since non-specific fluorescence often appears bright yellow this change in color due to glutaraldehyde could interfere in interpretation of results. Paraformaldehyde at a concentration of 3% did not have a visible effect on the fluorescence and hence appears to be a good virus inactivating agent for pretreatment of cells before IF test.

8.3.5 Screening of clinical samples under electron microscope

LR Yeolekar, PB Kulkarni, RG Damle

Fourteen coded original nasopharyngeal aspirates positive in IF test were screened under electron microscope for direct visualization of virus particles. Virus like particles were detected in only 3 positive samples (Influenza B, RSV and PIV) of the 14 screened.

8.4 Epidemiology of respiratory syncytial virus (Flu 05-2)

LR Yeolekar, RG Damle, M Pore

8.4.1 Detection of HRSV from TS/NS specimens from the year 2004 by NIV ELISA and comparison with RT-PCR.

Out of 133 NS/TS specimens tested, 10 specimens showed P/N= 2.0 hence were positive for HRSV infection. These 10 specimens were also PCR positive, as they gave bands of 1+ to 4+ intensity in either of PCR-A or PCR-B. Additionally two NIV ELISA borderline positive specimens showing P/N ratio 1.91 and 1.98 also showed visible band only in PCR-B after gel electrophoresis. This indicates higher sensitivity of the NIV ELISA. Out of 121 NIV ELISA negative specimens only 13 were tested in RT-PCR. These specimens and other negative controls were also negative in RT-PCR. This indicates the high specificity of the NIV ELISA.

Table 6 : TComparison of NIV ELISA positive results with PCR

Sr. No.	NIV No.	P/N in ELISA	PCR-A		PCR-B			
			+/-	Band intensity	Neat		1: 5 diluted	
					+/-	Band intensity	+/-	Band intensity
1	04 11056	2.06	-ve		+ve	1+	+ve	1+
2	04 5767	2.22	+ve	2+	+ve	4+	+ve	4+
3	04 2757	2.55	-ve		-ve	-	+ve	1+
4	04 2293	2.59	-ve		+ve	2+	+ve	2+
5	04 2162	3.07	-ve		-ve	-	+ve	3+
6	04 2337	3.12	-ve		+ve	2+	+ve	2+
7	04 6211	4.06	+ve	3+	+ve	4+	+ve	4+
8	04 3057	4.98	-ve		+ve	2+	+ve	4+
9	04 10341	4.98	-ve		+ve	2+	+ve	4+
10	04 5773	5.58	-ve		+ve	4+	+ve	4+

+ve: Positive for HRSV infection. ve: Negative for HRSV infection

Table 7 : Comparison of NIV ELISA borderline positive results with PCR

Sr. No.	NIV No.	P/N	PCR-A Band intensity	PCR-B Band intensity		
				Neat	1: 5 diluted	
1	04 3019	1.91	Negative	-ve	+ve	3+
2	04 3192	1.98	Negative	-ve	+ve	4+

+ve: Positive for HRSV infection

-ve : Negative for HRSV infection

8.5 Genotyping of HRSV strains

LR Yeolekar, RG Damle, S Pardeshi.

Standardization of genotyping PCR was done using BS-C-1 stock of nine strains of HRSV isolated during the year 2002 and one strain isolated during the year 2004. Primers from the hypervariable region of the G gene of HRSV were synthesized. Using this procedure sequencing of the second hypervariable region (270 nucleotides) of 9 isolates from 2002 and one from 2004 was done. All the strains were located in the same cluster GA5. This cluster also contained two strains one each isolated during the year 1993 and 1995. Cluster GA1 contains two reference strains and was very close to prototype A2 strain. GA2 cluster consisted of two 1995 isolates and two reference strains and GA3 cluster consisted of one Indian isolate each from the year 1991, 1994, 1995 and two reference strains. GA4 consisted of only one reference strain, whereas GA6 and GA7 consisted of two reference strains each.

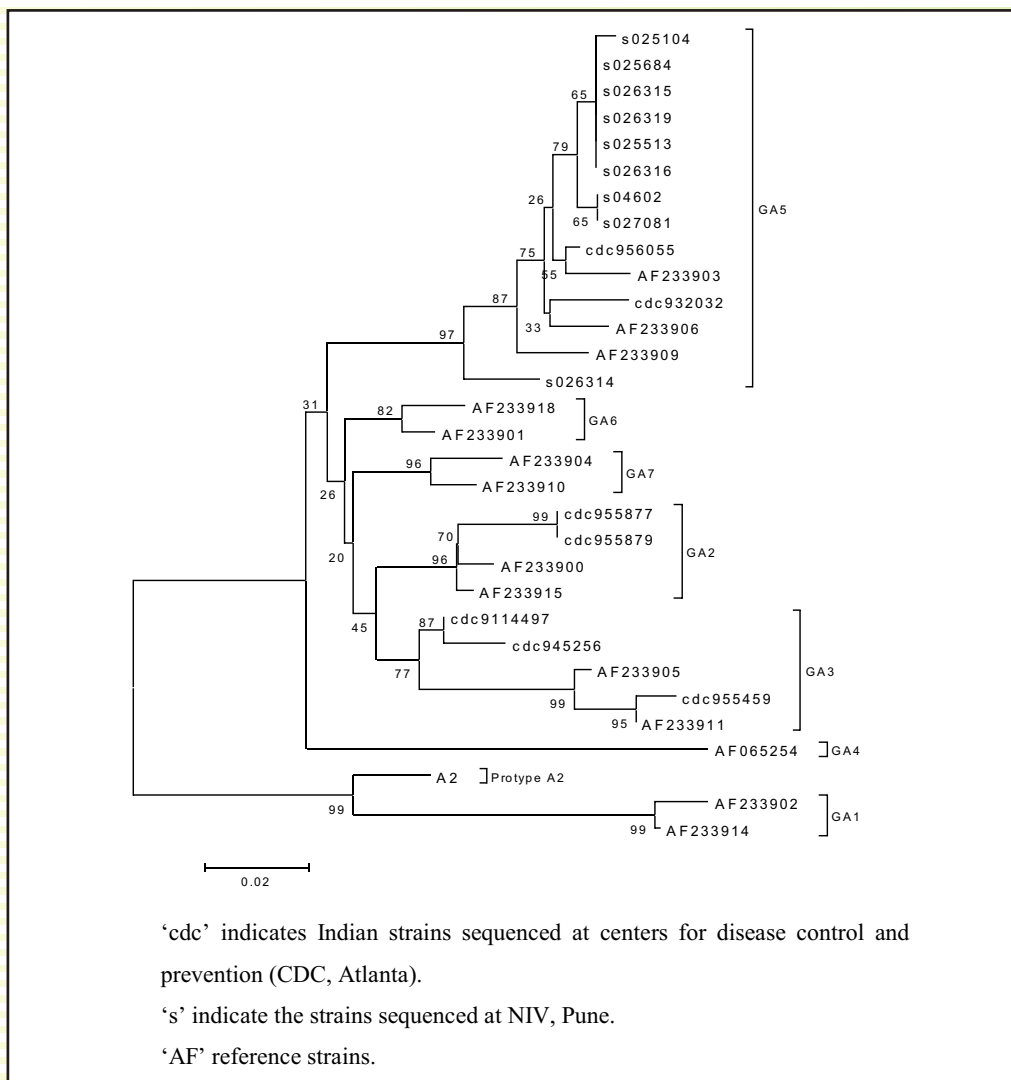


Figure 6 : Phylogenetic tree of HRSV strains.

Amino acid alignment of the G protein for group A HRSV.

The amino acid sequences of the 270 nt region of C terminal third of G glycoproteins was deduced. The predicted amino acid sequences of the isolates in the different genotypes were compared to prototype strain A2 for group A virus. The differences in the amino acid sequences correspond to replacement of the amino acid/or change in position of stop codon. In the analyzed amino acid sequence, accumulation of the changes was observed mainly in last 57 amino acids as compared to initial 30 amino acids of the deduced G glycoprotein. The prototype strain A2 strain consisted of 87 amino acids for this region whereas isolates from the year 2002 and 2004 consisted of 86 amino acids.

The data was further analyzed with respect to % identity to all other members in the same phylogenetic clusters as well as in two different clusters. The average p-distances is the proportion of the differences; i.e. the number of pair-wise nucleotide or amino acid differences divided by the total number of the nucleotide or amino acid in the sequenced region calculated with MEGA3.1.

Nucleotide percent (%) identity within and between the groups

The nucleotide % identity determined between and within the genotypes is shown in table 15 and table 16. The average nucleotide % identity within the groups ranged from 96.31-98.18% and average nucleotide % identity between the groups ranged from 78.54- 94.80%.

Amino acid % identity within and between the groups

The amino acid % identity determined between and within the genotypes The average amino acid % identity within the genotypes ranged from 86.22-98.83% and average amino acid % identity between the genotypes ranged from 65.65- 90.87%.

8.6 Animal influenza surveillance

LR Yeolekar, VA Potdar, SR Waregaonkar, AA Joshi

8.6.1. Detection and analysis of H5 antigen in BPL inactivated virus stocks

Single primer pair H5-1 & H5-2 was used to amplify WHO reference Ag of H5 which produce amplicon of 340 bp of +4 intensity. Amplicon was further subjected to TA cloning to obtain transcribed RNA, which can be further used as positive control. Transcribed RNA obtained after TA cloning was used for standardization of RT-PCR using WHO recommended and indigenously developed primer pairs. 1:10 diluted Transcribed RNA was further serially diluted and using different primers sensitivity for nested PCR was checked. Nested PCR showed +4 band up to 1:1000 dilution.

8.6.2 Detection H7 antigen in BPL inactivated virus stocks

Different primer combinations were used to amplify H7 WHO reference antigen and results confirmed by sequencing of PCR product.