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Genetic Characterization and the Development of Multiplex PCR for Common Respiratory viruses, in Chennai during September 2013 to January 2014

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Abstract : Totally 200 samples were collected during the period of September 2013 to January 2014, from different Hospitals (Private and Government Hospitals) in Chennai. All the samples were subjected to two tubes multiplex PCR. First tube will detect RSV and FLU; second tube will detect Para influenza viruses 1-4 and HmPV. Positive results were obtained and confirmed with gel electrophoresis, where RSV showed the highest positivity followed by FLU A and B.Co-infections were observed with RSV+INFA, RSV+PIV 2 and RSV+PIV 3.The RT-PCR products of the viruses were sequenced using the ABI PRISM Big Dye Terminator V3.1cycle kit and the sequence was documented in NCBI. To conclude, a simplified multiplex PCR for the detection of seven respiratory viruses in samples from patients with ALRI was developed. This assay was found to be more sensitive, less time consuming and economical than virus isolation. Multiplex PCR format allowed the detection of co infections which cannot be done using monoplex PCR or culture as shown in the present study.
Key words : HmPV,RSV,RSV+PIV 2 RSV+PIV 3 and PCR.

Introduction

Acute respiratory infections are estimated to cause approximately 1.9 million childhood deaths annually[1]. The contribution of acute respiratory infections to overall childhood mortality ranges from <5% in the developed countries to 25% in some developing countries. Bacterial infections in general have a higher case fatality than acute viral infections, but viruses are far more common causes of acute respiratory infection. Overall they contribute to at least one-third of the deaths caused by acute respiratory infection in the developing world[2]

The respiratory tract can be divided into upper and lower parts, with the boundary at the lower end of the larynx. Viral infections confined to the upper part (upper respiratory tract infection, URTI) are rarely life-threatening, with the exception of croup. They can be uncomfortable but do not usually call the individual's future into question. These infections do not automatically spread to the lower respiratory tract, but where the lower respiratory tract is involved the process is extensive and rarely confined to one lobe or even one lung. This contrasts with pneumococcal pneumonia, which is typically confined to one lobe of one lung.

The most common manifestations of a lower respiratory tract infection (LRTI) are bronchiolitis (in infants) or an atypical pneumonia. Even when an LRTI occurs, the upper tract is also usually involved and the causative virus can usually be recovered from it. There are no clear-cut differences between the clinical

presentations of any viruses in the respiratory tract. For example, although Respiratory Syncytial Virus (RSV) is the most common cause of bronchiolitis worldwide, this clinical condition may also be caused by parainfluenza viruses, influenza viruses, adenoviruses or rhinoviruses. Consequently, it must not be assumed that two patients with similar clinical illnesses would have been infected by the same virus. This is particularly so in babies and young children. Conversely, the same virus may cause a range of clinical manifestations in different patients[3]

Materials Method

Sample collection criteria

Inclusion Criteria:

Influenza Like Illness (ILI): a person presenting with sudden onset of fever >38°C or history of sudden onset of fever in the recent past, cough, sore throat.Severe Acute Respiratory Illness (SARI): a person presenting with sudden onset of fever >38°C or history of sudden onset of fever in the recent past, cough, sore throat, and/or rhinorrhea and in addition having breathlessness or difficulty in breathing and clinically suspected pneumonia (in children).

Exclusion criteria

If consent not given.In seriously ill, and patients with terminal disease who are unable to give samples.

Sample collection

200 Throat and nasal swabs were collected from patients suffering from Influenza like illness cases.

Methodology for Sample Collection

Between September 2013 to January 2014 , nasopharyngeal aspirates (NPAs) were collected from patients with ALRI as given in Appendix 1.

RNA extraction

RNA was extracted from 140 µl of each sample, using a commercial reagent (QIAamp viral RNA mini kit®, Qiagen).Whenever possible, the extracts were tested immediately after extraction. If this was not possible, they were divided into aliquots and kept frozen at −80°C. Each aliquot was used only once to avoid the loss of viral genomic material during repetitive freezing and thawing (Refer Appendix 2).

Methodology for RNA Extraction (QIAamp Total Viral RNA Mini kit (Qiagen)

Viral RNA in throat / Nasal swab was extracted using a QIAamp Viral RNA Mini kit (Qiagen), in accordance with the manufacturer's instructions.

560 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube.140 µl throat swab specimen was added to the buffer AVL–carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 sec and incubated at room temperature (25°C) for 10 min.The tubes were briefly centrifuged to remove drops from the inside of the lid.560 µl of ethanol (100%) was added to the sample, and mixed by pulse-vortexing for 15 sec. After mixing, the tubes were briefly centrifuged to remove drops from inside the lid.630 µl of the solution from the above step was carefully added to the QIAampMini column (in a 2 ml collection tube) without wetting the rim. The cap closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate discarded.tep 6 was repeated.To the QIAamp Mini column 500µl of Buffer AW1 was added and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded.500 µl of Buffer AW2 was added to the QIAamp Mini column and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini column was kept in a new 2 ml collection tube and the old collection tube with the filtrate discarded centrifuged at full speed for 1 min.TheQIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube, the old collection tube containing the filtrate discarded.To the QIAamp Mini column 60 µl of Buffer AVE was added and equilibrated

to room temperature and incubated at room temperature for 1 min. centrifuged at 6000 x g (8000 rpm) for 1 min.Viral RNA is stable for up to one year when stored at –20°C until used for cDNA synthesis.

RT- PCR

All seven sets of primers when combined led to impairing and nonspecific amplification. After trying different combinations, it was observed that RSV, Influenza A and B viruses in one set (TUBE 1) and PIV1–3 and hMPV in another set (TUBE 2) gave specific amplification for each virus (Refer Appendix 3).

Methodology for Multiplex Conventional RT-PCR (AmbionAgPath-IDTM RT-PCR kit)

25µl Master mix contain Buffer, Enzyme, Forward primers, Reverse primes, Nuclease free water (AmbionAgPath-IDTM RT-PCR kit) and 5 µl of Template (RNA) was taken into the reaction mix. Each component (Reagents) maintain in proper concentration

Reaction assay mixtures were made as a cocktail and dispensed in to the PCR tubes. Water and extracted nucleic acid positive template controls were then added to the appropriate test reactions and controls.

Sensitivity and specificity of Multiplex PCR

The sensitivity of detection by two tube multiplex PCR was 0.1TCID50 for RSV, Influenza A and 1TCID50 for hMPV, PIV1, PIV2, PIV3 and Influenza B. There were no non-specific amplification products against RNA from heterologous sources.

Product analysis by Agarose Gel Electrophoresis

The PCR amplified products were analyzed on a 1.5% agarose gel with intercalating ethidium bromide dye. 10 µl of the amplified product was mixed with 1 µl of 10X loading dye and loaded into the wells along with 1 µl of molecular weight marker (100-1000 bp ladder). The electrophoresis was run at 100 volts in 0.5X TBE buffer. The gel was visualized under UV transilluminator and the product was compared with molecular weight marker.

Methodology for sequencing

PCR products were subjected to electrophoresis on 2% agarose gel in TBE buffer. Amplicons were purified using QIA quick PCR purification kit (QIAGEN) and subjected to cycle sequencing using ABI PRISM Big Dye Terminator V3.1 cycle sequencing kit. Post cycle sequencing purification was done using dye ex 2.0 spin kit (Qiagen) and eluted DNA was dried in a vacuum centrifuge, resuspended in 20µl of hi-diformamide (ABI) and denatured at 94°C for 2 minutes and quenched on ice. Sequencing was performed on ABI PRISM 310 sequence alignment and phylogenetic analysis were done using mega version 6.

Results

In the present study 200 samples were collected during the period from September 2013 to January 2014 and investigated for the Human Acute Lower Respiratory Viral Infection (ALRI) in Chennai.

Table: 5 and Graph: 1 show, From September 2013 - January 2014, 200 ALRI samples were collected from all over Govt and private Hospitals in Chennai. Out of 200 samples 83 (41.5%) were detected for Respiratory viruses by Multiplex PCR. December month shows the highest positivity rate.Out of 83 positive samples, higher positivity of RSV was found **(21.5%)** throughout the monsoon season. The month of December showed higher number of positives with FLU **(15.5%)** to have higher positivity followed by RSV. Positivity of FLU started from the month of November gradually reducing towards the month of January.

Multiplex PCR detected respiratory viruses in 83 (41.5%) of 200 samples of which RSV was detected in 40 (20%), PIV3 in 4 (2%), PIV2 in 2 (1%), hMPV in 3 (1.5%) and influenza A in 17 (8.5%) and influenza B in 14 (7%) samples. Co-infections were observed.From the above graph, it is observed that fever is the most observed symptom among the positive cases, followed by nasal discharge, cough, sore throat and breathlessness.

The development of multiplex PCR for the detection of respiratory viruses as a rapid, sensitive and time saving technique has not gained priority in India even though ~0.5 million children die each year in this country due to ALRI each year, accounting for one fourth of the 1.9 million global ALRI deaths. Among all the major ALRI causing viruses namely RSV, PIVs and influenza viruses A and B, the presence of RSV has been documented to be the most commonly identified pathogen followed by PIV3[4].In the present study, we standardized and evaluated a two-tube multiplex PCR assay devoid of any further confirmatory steps. The sensitivity of our multiplex PCR assay was similar or better than previously described mPCR assays for these viruses[5].We did not make direct comparisons of the performance of the different assays in our laboratory.In the present study we could culture majority of the viruses detected by mPCR with the exception of RSV which is known to be highly thermolabile. The detection rate of viruses was similar to detection rate reported earlier[6]. A higher proportion of males were found to have infection with respiratory viruses as compared to females as reported earlier[7].RSV was most commonly identified viral pathogen similar to viral identifications by mPCR[8]

The detection rate of co-infections was similar to previously reported multiplex PCR studies [9]. It was observed that higher percentage of children with mixed infections had severe and very severe ALRI as compared to ALRI. Previous studies have shown that co-infection with different respiratory viruses might lead to a more severe disease or multiple viruses have been detected from patients with severe disease as described by.

ALRI caused by RSV was more common in younger children as reported earlier [7]. RSV and HmPV were associated with bronchiolitis. PIVs and Influenza viruses were associated with pneumonia similar to previous findings[10]. However, the number of all the viral detections except RSV was too few to comment on the association of the virus with bronchiolitis or pneumonia.In the present study, RSV was detected during the fall season similar to previously described studies [11]. The rest of the virus identifications were few and their seasonality cannot be commented upon.

Table 1: Master Mix for 1st Tube Multiplex Conventional RT-PCR

SNO	Reagent	Volume of reagent added per reaction (1X)
1	2X RT-PCR Buffer	12.5 µl
2	Enzyme Mix (RT and Taq polymerase) 2 IU/ul	1.0 µl
3	Forward primer for INF A (10 Pmol/ µl)	0.5 µl
4	Forward primer for INF B (10 Pmol/ µl)	0.5 µl
5	Forward primer for RSV (10 Pmol/ µl)	0.5 µl
6	Reverse primer for INF A (10 Pmol/ µl)	0.5 µl
7	Reverse primer for INF B (10 Pmol/ µl)	0.5 µl
8	Reverse primer for RSV (10 Pmol/ µl)	0.5 µl
9	Nuclease free water	3.5 µl
	Total	**20 µl**

Table 2: Master Mix for 2nd Tube Multiplex Conventional RT-PCR

SNO	Reagent	Volume of reagent added per reaction (1X)
1	2X RT-PCR Buffer	12.5 µl
2	Enzyme Mix (RT and Taq polymerase) 2 IU/µl	1.0 µl
3	Forward primer for PIV1 (10 Pmol/ µl)	0.5 µl
4	Forward primer for PIV2 (10 Pmol/ µl)	0.5 µl
5	Forward primer for PIV3 (10 Pmol/ µl)	0.5 µl
6	Forward primer for hMPV (10 Pmol/ µl)	0.5 µl

7	Reverse primer for PIV1 (10 Pmol/ µl)	0.5 µl
8	Reverse primer for PIV2 (10 Pmol/ µl)	0.5 µl
9	Reverse primer for PIV3 (10 Pmol/ µl)	0.5 µl
10	Reverse primer for hMPV (10 Pmol/ µl)	0.5 µl
11	Nuclease free Water	2.5 µl
	Total	**20 µl**

Table 3: Master Mix for Nested PCR for RSV sub-typing

SNO	Reagent	Volume of 1X
1	2X RT-PCR Buffer	12.5 µl
2	Enzyme Mix (RT and Taq polymerase) 2 IU/ul	1.0 µl
3	Forward primer for RSV A (10 Pmol/ µl)	0.5 µl
4	Reverse primer for RSV A(10 Pmol/ µl)	0.5 µl
5	Forward primer for RSV B (10 Pmol/ µl)	0.5 µl
6	Reverse primer for RSV B(10 Pmol/ µl)	0.5 µl
7	Nuclease free water	7.5 µl
8	Template (Pan RSV PCR product)	1.5 µl
	Total	**20 µl**

Table: 4 Primers used for this study Y, W, R, S are wobbles for C/T, A/T, A/G and G/C

Target Gene	Primer Name	Sequence (5' - 3')	Amplicon size
1.RSV N Gene	P-RSVNF	CTGTCATCCAGCAAATACAC	**683bp**
	P-RSVNR	ACCATAGGCATTCATAAACAATC	
RSV N gene (nested primers)	RSVAF	AAGCAAATGGAGTGGATGTAACAAC	**260bp**
	RSVAR	CTCCTAATCACAGCTGTAAGACCCA	
	RSVBF	CAAACTATGTGGTATGCTATTAATCA	**328bp**
	RSVBR	ACACAGTATTATCATCCCACAGTC	
2.Influenza A matrix gene	INFA-F	AGGYWCTYATGGARTGGCTAAAG	**105bp**
	INFA-R	GCAGTCCYCGCTCASTGGGC	
	INFB-F	GGAGAAGGCAAAGCAGAACTAGC	**503bp**
	INFB-R	CCATTCCATTCATTGTTTTTGCTG	
3.PIV1 N gene	PIV1NF	TCTGGCGGAGGAGCAATTATACCTGG	**84bp**
	PIV1NR	ATCTGCATCATCTGTCACACTCGGGC	
4.PIV2 N gene	PIV2NF	GATGACACTCCAGTACCTCTTG	**197bp**
	PIV2NR	GATTACTCATAGCTGCAGAAGG	
5.PIV3 N gene	PIV3NF	GATCCACTGTGTCACCGCTCAATACC	**266bp**
	PIV3NR	CTGAGTGGATATTTGGAAGTGACCTGG	
6.hMPV N gene	hMPVNF	AAGCATGCTATATTAAAAGAGTCTCA	**440bp**
	hMPVNR	ATTATGGGTGTGTCTGGTGCTGA	

Table 5: Month wise Distribution of Total no of samples and Total no of positive samples

Month / Year	Total No of Samples	No of Positive Samples
September (2013)	13	7
October (2013)	17	9
November (2013)	66	25
December (2013)	75	32
January (2014)	29	10
Total	**200**	**83 (41.5%)**

Table 6: Month wise distribution of respiratory viruses

Month / Year	No of Positives	RSV	FLU	PARA FLU	Hmpv
September (2013)	7	5	-	1	1
October (2013)	9	6	-	2	1
November (2013)	25	12	11	1	1
December (2013)	32	15	16	1	
January (2014)	10	5	4	1	-
Total	**83 (41.5%)**	**43 (21.5%)**	**31 (15.5%)**	**6 (3%)**	**3 (1.5%)**

Table 7: Gender wise distribution of Respiratory samples

Total No of Samples	Male (%)	Female (%)
200	92 (46%)	108 (54%)

Out of 200 samples, the number of female samples received were higher (54%) compared to the male samples (46%)

Table 8: Month wise positivity with different age groups

Month / Year	Less than 1 year	1-5 years	6-12 Years	13-18 years	Above 18 Years
September (2013)	3	3	-	-	1
October (2013)	2	4	1	1	1
November (2013)	8	10	1	1	5
December (2013)	10	11	2	5	4
January (2014)	3	2	2	1	2
Total	**26 (13%)**	**30 (15%)**	**6 (3%)**	**8 (4%)**	**13 (6.5%)**

Out of the 83 positive samples, 1-5 paediatric age group patients showed highest positive (15%) followed by paediatric patients less than 1 year (13%).

Table 9: Virus identifications with ALRI patients detected positive for viral infections and Co infections by multiplex PCR

SNO	Viruses detected by mPCR	Number of positive specimens
1	Respiratory Syncytial Virus (RSV)	40
2	Influenza A (INFA)	16
3	Influenza B (INFB)	14
4	Para Influenza-1 (PIV1)	0
5	Para Influenza-2 (PIV2)	1
6	Para Influenza-3 (PIV3)	3
7	Human Metapneumo Virus (hMPV)	3
8	RSV+PIV3	1
9	RSV+PIV2	1
10	RSV+INFA	1

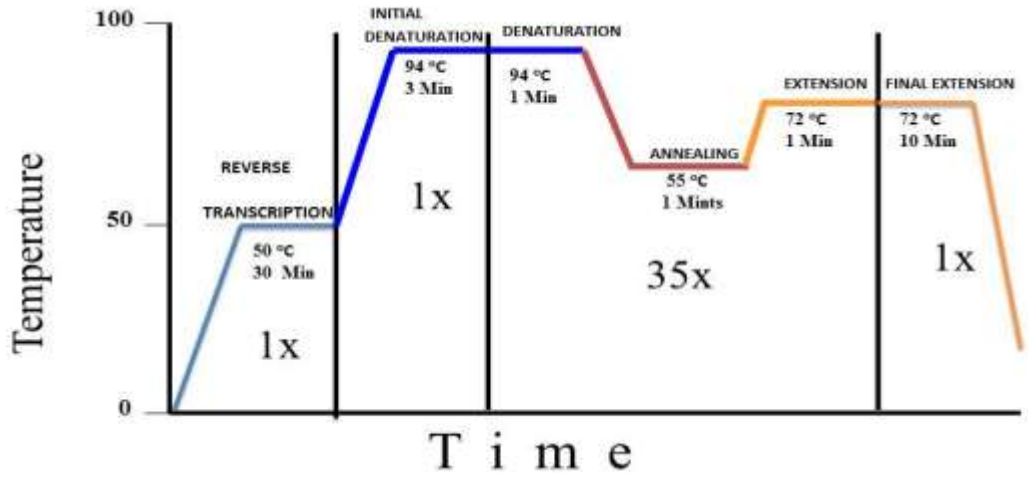


Figure 1: Cyclic Conditions for 1st Tube Multiplex Conventional RT-PCR

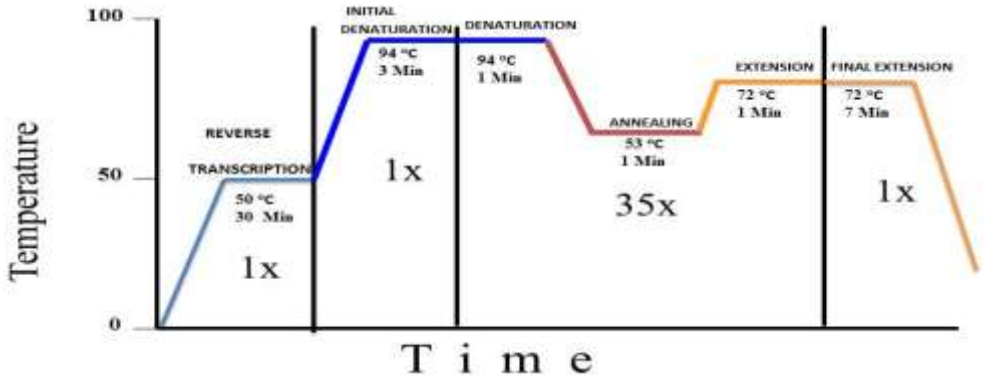


Figure 2: Cyclic Conditions for 2nd Tube Multiplex Conventional RT-PCR

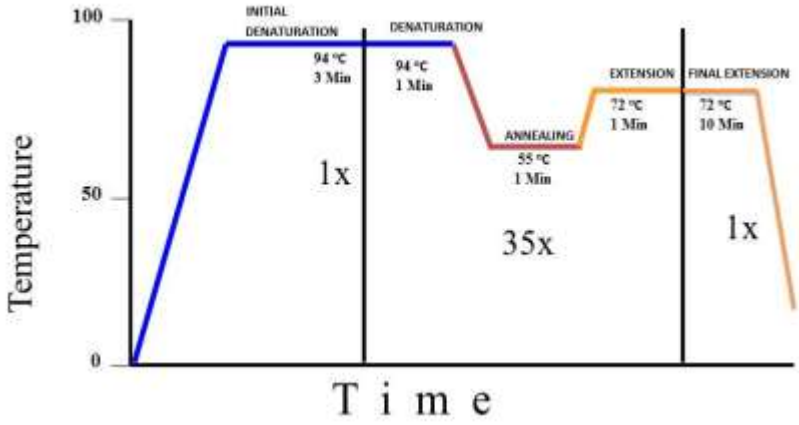


Figure 3: Cyclic conditions for Nested PCR

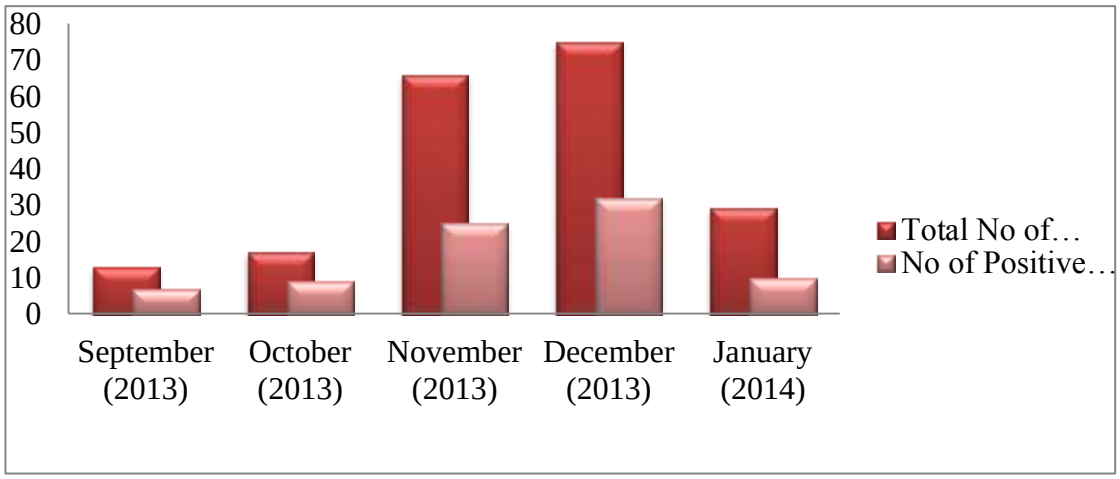


Figure 4: Month wise Distribution of Total no of samples and Total no of positive samples

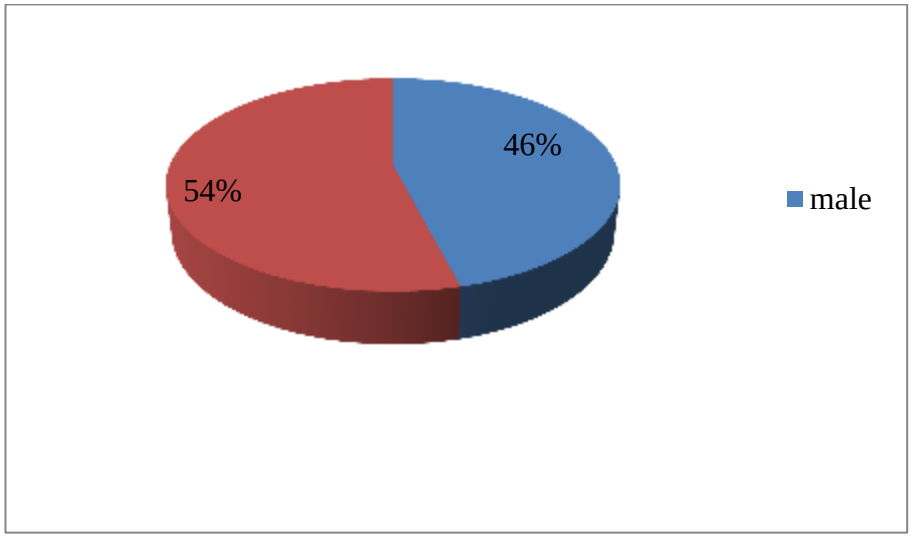


Figure 5: Gender wise distribution of Respiratory samples

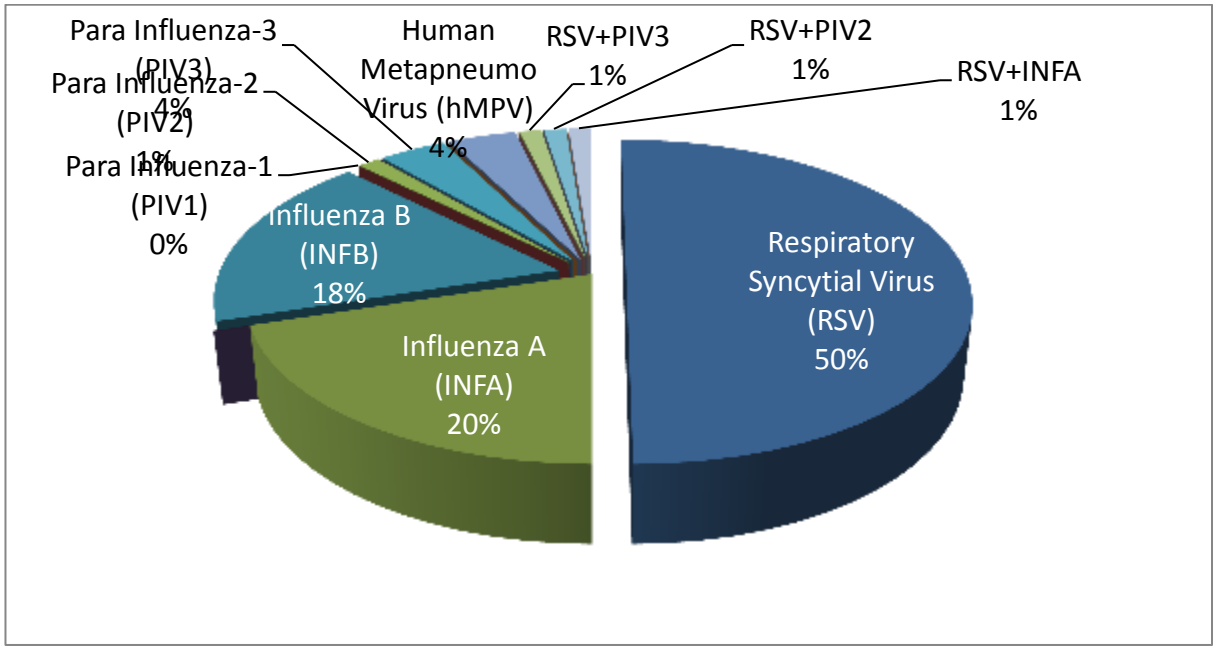


Figure 6: Virus identifications with ALRI patients detected positive for viral infections and Co infections by multiplex PCR as 1.5% for RSV+PIV3, RSV+PIV2, RSV+INFA.

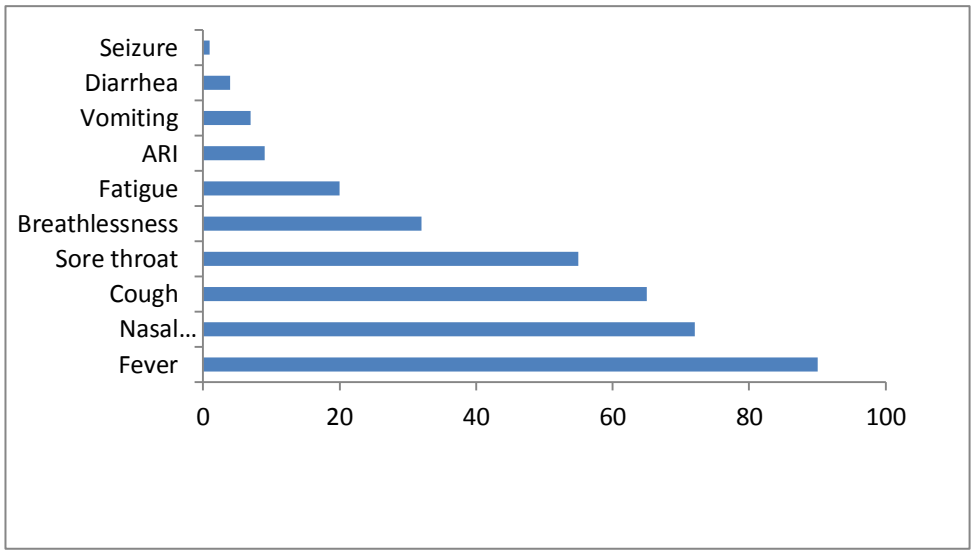


Figure 7: Result identification in positive samples according to symptoms

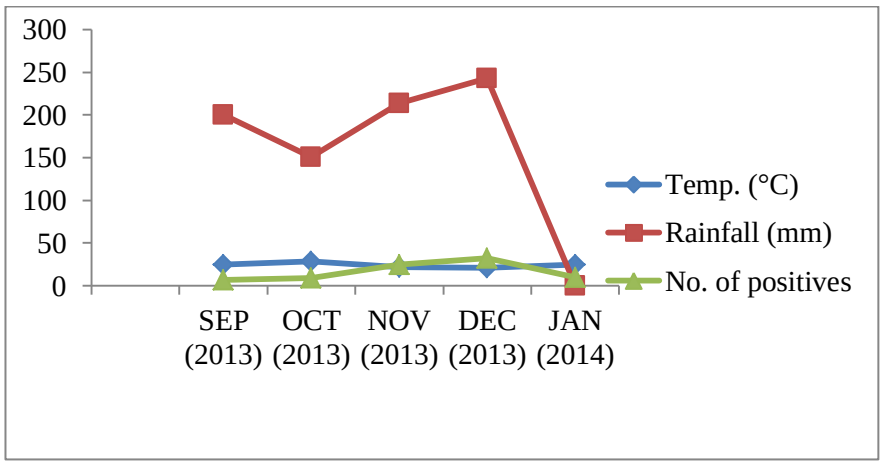
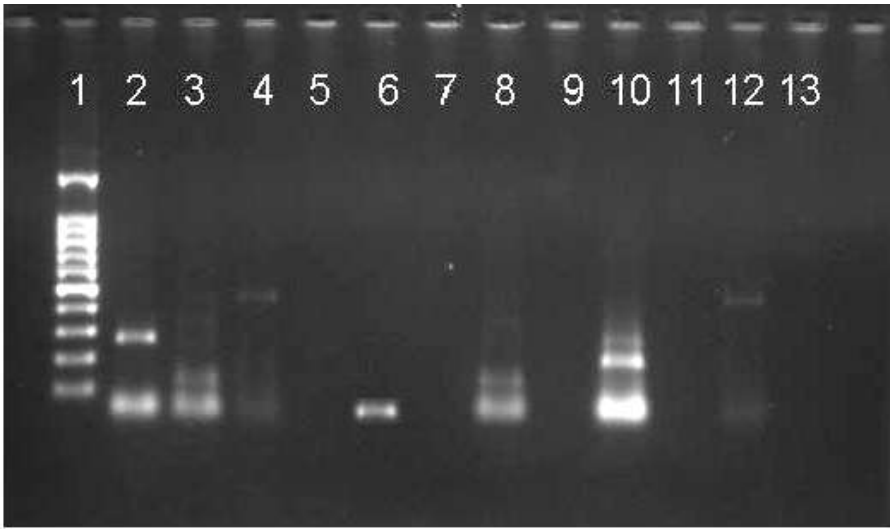


Figure 8: Meteorological Data Vs Virus positive

Lane 1 : molecular marker (100bp) , Lane 2, 4 : Pan RSV - 683bp, Lane 3 : Flu B - 503bp , Lane 5 : Flu A -105bp, Lane 7,9,11 : negative controls (Flu A, B, Pan RSV) ,Lane 8,10,12 : positive controls (Flu A, B, Pan RSV)

Figure 9: Gel documentation result for tube-1 Multiplex PCR



Lane 1: molecular marker (100bp) , Lane 2: Para 3 - 266bp,Lane 3 : Para 2 - 197bp ,Lane 4 : HmPV - 440bp, Lane 5,7,9,11 : negative controls (Para 1,2,3, HmPV), Lane 6,8,10,12: positive controls (Para 1,2,3, HmPV).

Figure 10: Gel documentation result for tube-2 Multiplex PCR

Lane 1 : molecular marker (100bp), Lane 2,3 : RSV A - 260bp Lane 4,5 : Pan RSV - 683bp, Lane 6, : RSV B - 328bp Lane 8,10,12 : negative controls(Pan RSV, RSV A, RSV B)Lane 9,11,13 : positive controls(Pan RSV, RSV A, RSV B).

Figure11: Gel documentation result of Nested Multiplex PCR for RSV sub typing

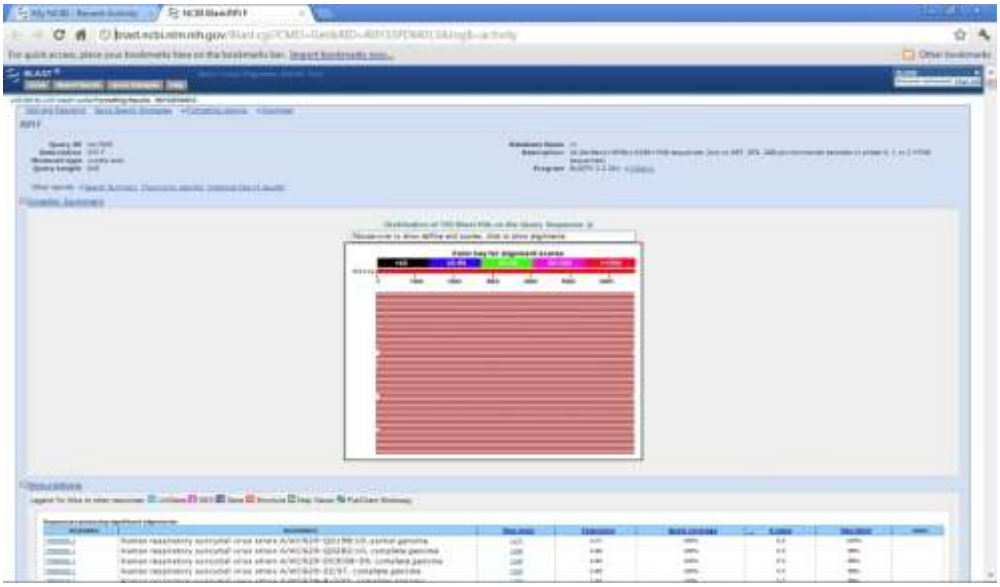


Figure 12: Blast alignment for (rsv a) sequences obtained

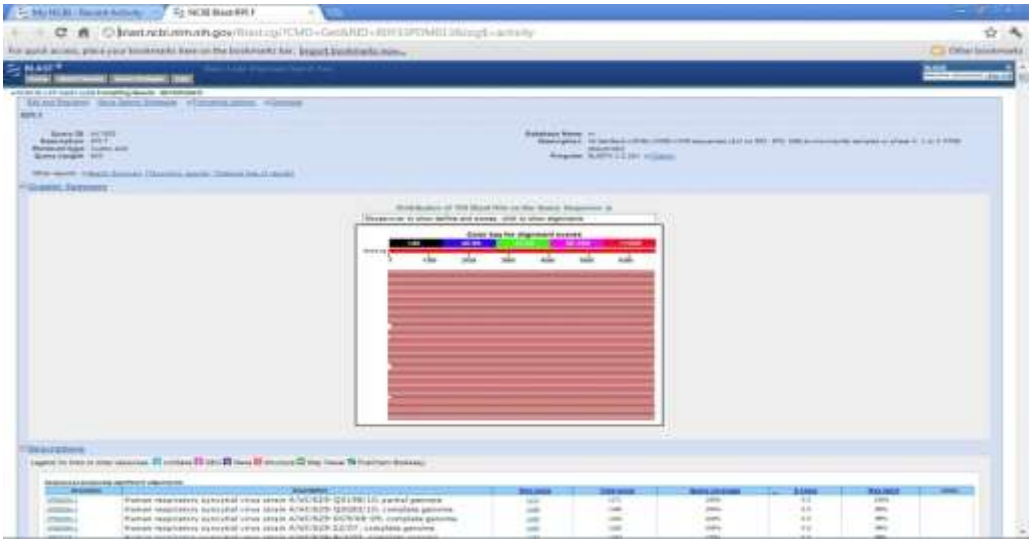


Figure 13: blast alignment for (rsv b) sequences obtained

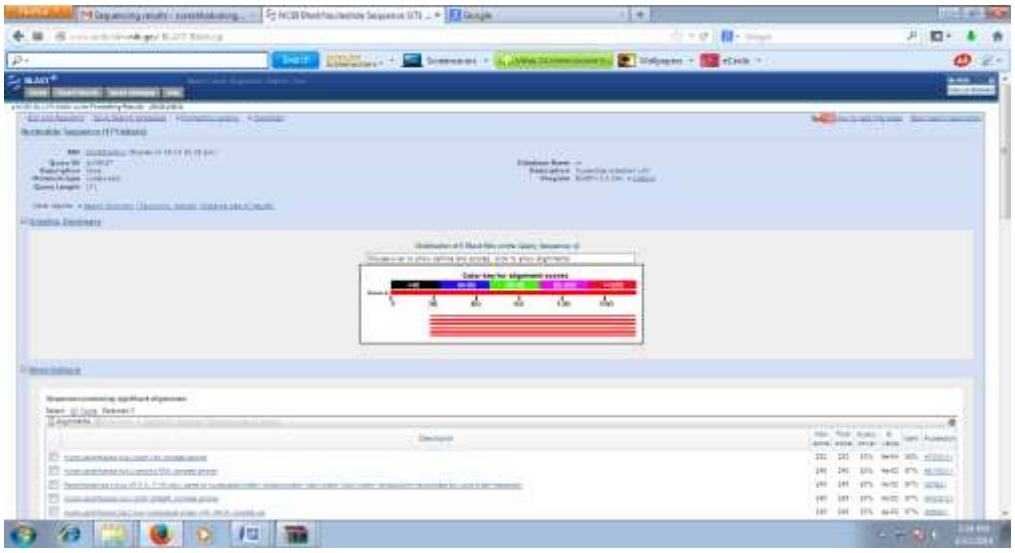


Figure 14: blast alignment for (piv 2) sequences obtained

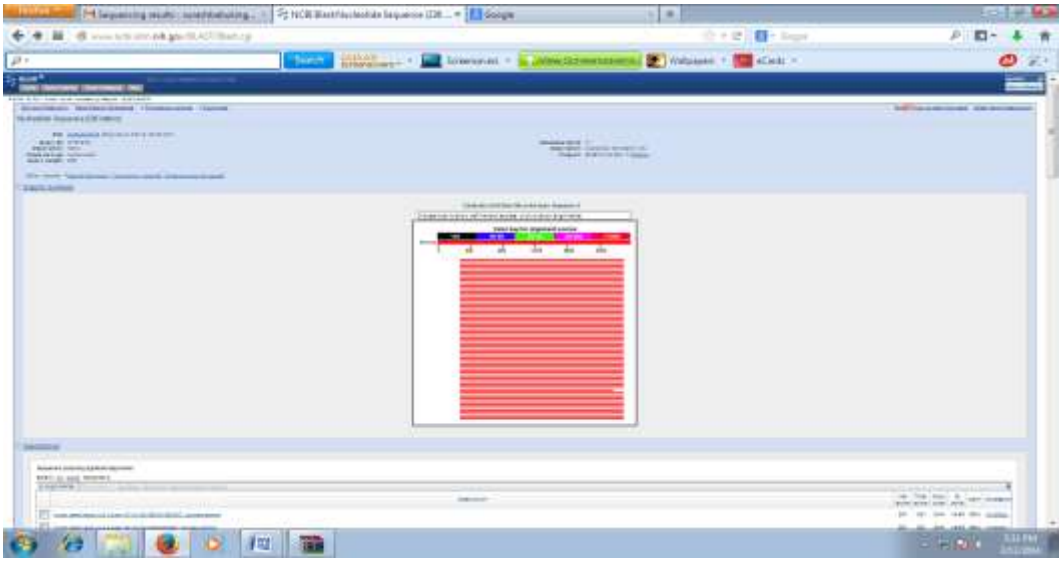


Figure 15: blast alignment for (piv 3) sequences obtained

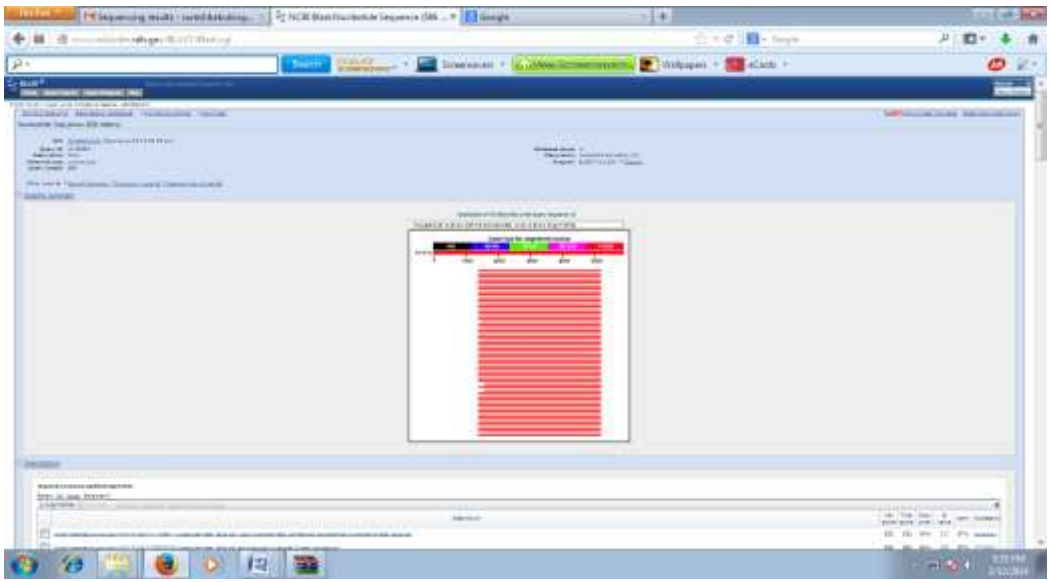


Figure 16: blast alignment for (hmpv) sequences obtained

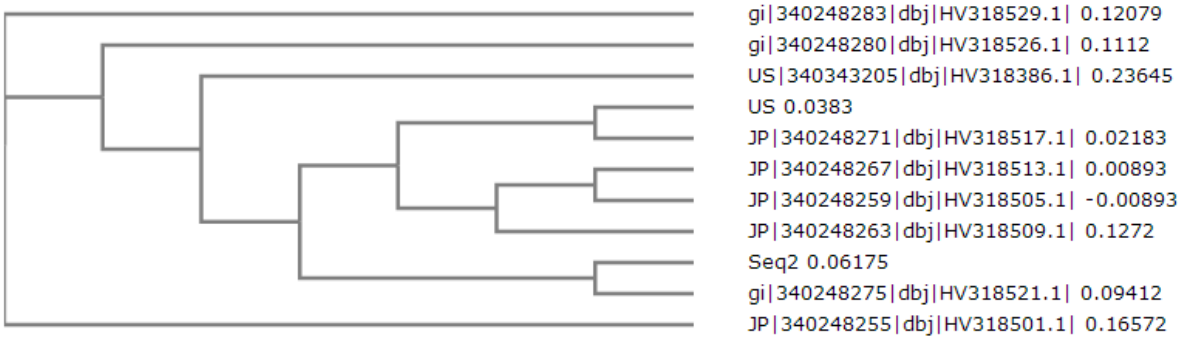


Figure 17: Phylogenetic tree for Respiratory Syncytial Virus

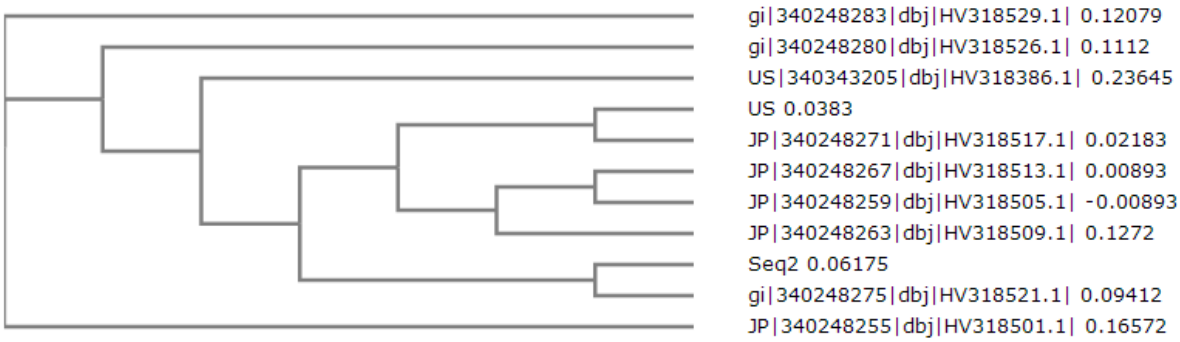


Figure 18: Phylogenetic tree for Human Metapneumo Virus

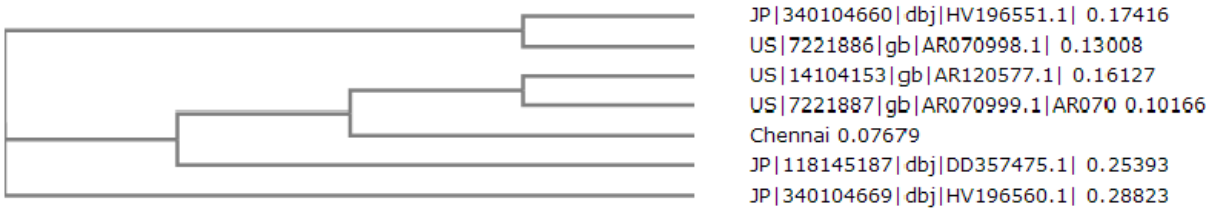


Figure 19: Phylogenetic tree for Parainfluenza virus 2

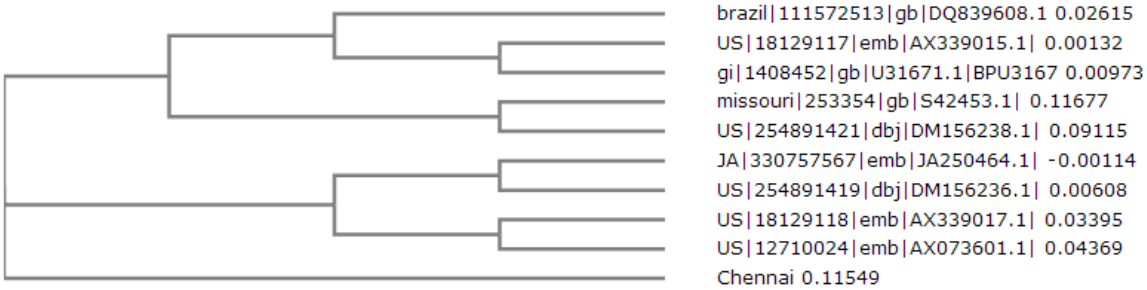


Figure 20: Phylogenetic tree for Parainfluenza virus3

Conclusion

To conclude, a simplified multiplex PCR for the detection of seven respiratory viruses in samples from patients with ALRI was developed. This assay was found to be more sensitive, less time consuming and economical than virus isolation. Multiplex PCR format allowed the detection of co infections which cannot be done using monoplex PCR or culture as shown in the present study.

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