ABSTRACT

Acute Respiratory Tract Infections (ARTIs) are the major cause of morbidity and mortality in young children, geriatric population and in immunocompromised patients caused by various strains of Influenza virus, Respiratory syncytial virus (RSV), Parainfluenza viruses (PIVs), Adenovirus (AdV) and Rhinovirus (RV). The seasonality of Influenza and RSV involved in this process remain unexplored with respect to mechanism of action at molecular level evincing very few epidemiological data. Although there are antiviral drugs available, there is an increasing chance of developing resistance to those available drugs. This has necessitated a hunch for a biocompatible, cheap, reliable, effective biomaterial having broad spectrum antiviral activity for containment process.

A surveillance study from 2012 to 2015 was conducted on all age group from the outpatients attending five tertiary care Government hospitals in and around Chennai and investigated at Department of virology, King Institute of Preventive Medicine and Research, Chennai, Tamilnadu. Samples (throat swab / nasal swab (n = 1960)) were screened for the presence of Influenza Viruses and RSV using Real-time and conventional RT-PCR respectively and the positive ones were stored at -80°C for further experimental work. Further, genetic analysis was performed and compared with the vaccine strain to detect any mutation responsible for the development of resistance pattern. In vitro anti-Influenza activity of crude extract of Adathoda vasica (L.) Nees was attempted after subjecting it to preliminary phytochemical analysis and cytotoxic studies using MDCK cells.

A total of 1960 samples were screened during the study period which showed 406 (20.7%) positive cases of respiratory viruses (Influenza virus and RSV) and 9 (0.45%) cases of co-infections. Among the screened samples, 190 (9.7%) were positive for influenza virus comprising 3.7%, 4.4% and 1.6% influenza type A/H3N2, A (H1N1)pdm09 and IVB type. In the same way, 11% of the total samples screened accounted for RSV positive cases comprising 9.2% and 1.8% attributing RSV A and RSV B with RSV A as predominant type.
Influenza viruses were found to be predominant in the preschool children in the age group of 2-5 years (13.8%) followed by adults > 18 years (11.4%), 6-8 years (8.9%) and 0-1 years (6.5%). Males (53.1%) were significantly affected than females (46.84%) with its high positivity rate during September to December (rainy season). During the study period, overall Influenza virus detection rate by virus culture was 53.7% when compared with PCR. Individual virus isolation rate was high in Influenza B (67.7%) and low in Influenza A (H1N1) pdm09 (48.3%) in MDCK cell line. Over all RSV detection rate by PCR was 60.2%. RSV B isolation rate was slightly high (63%) when compared with RSV A (60%) in HEp-2 cell line.

Antigenic characterization using HAI detected all strains of influenza as follows: Influenza A (H1N1) pdm09 (n=42), Influenza A/H3N2 (n=39), Influenza B/ Victoria (n=15) and Influenza B/ Yamagata (n=6). Similarly, of all 21 Influenza B virus isolates, 71.4% attributed to the B/Victoria lineage and were characterized as B/Brisbane/60/2008 and 28.5% were identified as Influenza B/Wisconsin/1/2010 related to B/Yamagata lineage.

The RSV panel showed 51.85% and 48.15% positive for male and female respectively with its high positivity observed from August to December. RSV positivity was found to be high in children in the age group 0-1 year (12.9 %) followed by 2-5 years (11.6 %), 6-18 years (10.8 %) and > 18 years (7.7 %). Statistically significant difference in clinical symptoms was observed between RSV and Influenza virus (p value <0.0001). In the study, Influenza and RSV positive cases were at a higher side when the specimen was collected within 3 to 4 days from the onset of illness.

The phylogenetic analysis on partial HA1 gene sequences of Chennai Influenza A (H1N1) pdm09 strains demonstrated the genetic variants of prototype vaccine strain Influenza A/California/07/2009 and showed 98.9-99.8% similarity. Analysis of partial amino acid sequences of HA1 gene (from aa 100 to516) revealed the presence of minor mutations in antigenic epitope of HA1 domain ( Sa, Ca1, Ca2, and Sb) which induce immune response and are also responsible for binding to the host cell sialic acid receptors.

Molecular analysis of Influenza A(H3N2) strains on HA diversity suggest that all strains circulating in Chennai were related more closely to the prototype strain,
Influenza A/Victoria/361/2011 than the earlier year’s vaccine strains; A/Perth/16/2009. In the HA1 domain, four substitutions; S198A, T212A, V223I, and N312S were identified that were found to be stable in all the studied strains as well as 2012-13 vaccine strain (A/Perth/16/2009) when compared with 2011-2012 vaccine strain (A/Victoria/361/2011).

It is noteworthy that there were antigenic variations in IBV in the HA gene likely in the the 120- loop (HA1 116–137), 150-loop (HA1 141–150), 160-loop (HA1 162– 167) and in the 190-helix (HA1 194–202). But the study revealed that there is no alteration at amino acid 129 with slight amino acid substitution at amino acid 131 and 137 residues of HA1 regional, Yamagata lineage strains had a deletion in the amino acid position 178, whereas Victoria lineage strains had asparagine at aa 178 of HA1 domain. B/Victoria like strains had isoleucine at position 190, instead of valine.

Phylogenetic analysis of Chennai isolates revealed that all RSV-A strains clustered with strains of the novel ON1 genotype (NA1 group). RSV-B genotype BA was the predominant genotype co-circulating with RSV-A in Chennai, South India from 2012 to 2015. Five sequences from present study clustered in cluster B within BA9 genotype, and one sequences in genotype BA12. Specific amino acid substitutions for BA genotype was identified among Chennai RSV-B viruses including; K218T, K223T, S247P, T270I, V271A and two strains (KY419506, KY419507) showed H287Y mutations.

Investigation on the antiviral resistant markers viz. M2 protein of Influenza A (H1N1) pdm09 revealed S31N and I43T mutations which conferred amantadine resistance. No such mutations/ substitutions were found in the amino acid leucine at 26, valine at residue 27, alanine at residue 30 and glycine at residue 34. But all the isolates from Chennai had Histidine (H) at 274 amino acid position which indicated that these strains were sensitive towards Oseltamivir.

The Analytical sensitivity was determined by Multiplex PCR and the detectable RNA was optimized as of 0.1 TCID50 for RSV, Influenza A, Rhino virus and 1 TCID50 for hMPV, PIV1, PIV2, PIV3 and Influenza B RNA. Further, it was validated by comparing with Real-time PCR data showing 94.2% (for Rhino virus) to 100% sensitivity (remaining viruses) and 100% specificity for all viruses.
The crude extracts were subjected to qualitative phytochemical analysis. In ethanolic extract contains Steroid, Tannin, Saponin, Coumarins, Alkaloids, Diterpenes, Phenol, Phlobatannin, Cardiac glycosides and Flavonoids were as present. Whereas aqueous extract contain Tannin, Saponin, Alkaloids, Amino acids and Flavonoids. Anti-Influenza (at TCID<sub>50</sub> concentration 10<sup>-5</sup> dilution) activity was determined to be 50µg/ml (inhibiting 80% virus titre) for ethanolic extract and 60µg /ml for aqueous extract (inhibiting 100% virus) within 72 hrs and compared with the control. Further, HA method was adopted to determine the inhibitory action on virus replication using both the extracts. In simultaneous assay, aqueous extract shows 80% viral inhibition was observed at a concentration of 50µg/ml, whereas 100% viral inhibition was observed in ethanol extract at concentration of 60µg/ml. In post treatment assay, aqueous extract did not show any inhibition whereas the ethanol extract showed 100% viral inhibition at concentration of 60µg/ml.

In conclusion, this study investigated the epidemiological pattern of RSV and Influenza infection in patients with Influenza like illness (ILI) in Chennai highlighting RSV infection as predominant compared to Influenza infection with relatively less co-infection. The isolates were subjected to HAI for antigenic characterization and gene sequencing for confirming its lineage. This study finds that Influenza A (H1N1) pdm09 viruses circulating in Chennai during study period December 2014 – December 2015 were resistant to Amantadine and sensitive to Oseltamivir due to S31N and I43T mutations. Adathoda vasica (L.) Nees seems to be a promising antiviral candidate for the development of third generation anti influenza drugs, thus challenging the neuraminidase drug resistant viruses in an attempt to safeguard human health and the global economy. However, there should be a continuous monitoring / surveillance of the respiratory viruses in all age groups, at all seasons and throughout the country for containing the emergence of drug resistant viruses circulating in India.