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Epidemiology of respiratory syncytial virus infections in Chennai, south India

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1. Introduction

Respiratory syncytial virus (RSV) is a major cause of mortality and morbidity among children[1] and the elderly.[2] It causes mild upper respiratory tract infections to severe life threatening lower respiratory tract infections like bronchiolitis and pneumonia. Based on variations in the glycoprotein gene, RSV exists as two major antigenic subgroups – RSV A and RSV B. RSV A is known to cause infections of greater severity than RSV B.[3] Depending upon the climatic conditions and region, one subtype dominates the other.

The epidemiology of RSV shows various patterns. In tropical regions, RSV outbreaks occur in the rainy months,[4] while in temperate countries it occurs during the winter months.[5] Epidemiological studies are crucial to understanding regional pattern of RSV infections. Meteorological factors such as temperature, humidity and rainfall influence the severity of RSV disease in infants.[6]

Diagnosis of RSV infections is accomplished by several methods including viral culture, serology, antigen detection tests and nucleic acid amplification tests (NAATs). Viral culture is said to be the gold standard; however it is cumbersome, requires expertise, and has a long turnaround time. NAATs are now being used widely. Conventional PCR and nested PCR are considered to be simple and economic for any laboratory setting. However the diagnostic accuracy of these techniques is discrepant.

Hence this study was intended to determine the prevalence of RSV in respiratory specimens from children and adults. The incidence of RSV was correlated with factors such as gender, clinical presentation and meteorological conditions like monthly mean maximum and minimum temperature, average relative humidity and total monthly rainfall to understand its epidemiology. We also assessed the performance of nested RT-PCR in comparison with real time RT-PCR in the detection of RSV A and B to analyse the application of nRT-PCR in resource limited settings for surveillance and diagnosis.

2. Materials and methods

2.1. Collection of specimen and transportation

We conducted a prospective pilot study over a 1 year period from April 2016–April 2017. A total of one hundred and forty six nasal swabs were collected from patients (majorly children) with symptoms of acute respiratory infection (ARI). This study was approved by the institutional human ethical committee. Informed consent was obtained from the parents of all the children and individual participants included in the study. Nasal swabs were collected using sterile flocked nylon swabs, by placing them 2–3 cm deep inside the nostril and rotating 2–3 times. The swabs were immediately placed in sterile viral transport medium (HiViral transport Kit, Himedia, Mumbai) and transported to laboratory on ice. An aliquot of the sample was stored at −80 °C for further investigations. Demographic and clinical details such as age, sex, clinical symptoms, clinical diagnosis and duration of symptoms were collected using well-structured questionnaire.

2.2. RNA extraction and reverse transcription

RNA was extracted from these samples using NucleoSpin RNA virus kit (Macherey-Nagel, Germany) according to manufacturer's instructions. The extracted RNA was immediately subjected to reverse transcription and the remaining RNA was stored at −80 °C. The reaction mixture for reverse transcription consisted of 4 µl of 5X M-MuLV reverse transcriptase buffers, 1 µl of 0.4 µg/µl random hexamer, 2 µl of 10 mM dNTPs, 40U of M-MuLV reverse transcriptase, 8U of RNase inhibitor, 5 µl of RNA template. The volume was made up to 20 µl with RNase free water. The reaction was carried out for one cycle at 37 °C for one hour followed by 93 °C for 5 min cDNA was stored at −20 °C until use.

2.3. Detection of subtypes of RSV by real time RT-PCR

Real time RT-PCR was applied to all the samples using Taqman probes specific for RSV A and B (Table 1). Simplex real time RT-PCR was performed using our optimized cycling parameters. Each 10 µl

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Table 1
Details of primers and probes used.

Nested RT-PCR		References
I round primers		
RSV-A-OF2	CAGCTCCGTTATCACATCTCTAGGAGCC	[7]
RSV-A-OR2	TGGGTTGTCTATGAGCAGATAKKAAACCA	
RSV-B-OF2	CGGGCCAGAAGAGAAGCACCACAGTA	
RSV-B-OR2	TGATCCTTCTTTGATGTTGGTGGTGC	
II round primers		
RSVA-IF2	TGACCCATTAGTGTTCCCCTCTGAT GAAT	
RSVA-IR2	CTTCTGGCCTTRCAGTATARGAG CAGT	
RSV-B-IF1	GTCGCATCTCCAACATTGRAAC	
RSV-B-IR1	TGGTGCATAGAGGTGATGTGTG	
Real time RT-PCR		[8]
RSVA		
RS–F1 Forward primer	AACAGATGTAAGCAGCTCCGTTATC	
RS–F2 Reverse primer	CGATTTTTATTGGATGCTGTACATTT	
RS–F3 Probe	TGCCATAGCATGACACAATGGCTCCT	
RSVB		[9]
Sense	AACAGACATAAGCAGCTCAGTAATT	
antisense	CGATTTTTGTTGGATGCAGTGCATTT	
Probe	CTAGGAGCTATAGTGTCATGCTATGGT	This study

reaction mixture contained 5 µl of 5X FastStart Universal Probe Master (Rox), 5 pmol each of forward and reverse primers, 2 pmol of probe, 2 µl of cDNA and 2.4 µl of nuclease free water. The cycling conditions were 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min followed by 72° for 10 s. An annealing temperature of 56 °C for 1 min was used for RSV B. Positive and negative controls were included in each run. The amplified nucleic acids were detected with the StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The samples were interpreted as positive and negative based on their threshold cycles (C_t value).

2.4. Nested RT-PCR

Multiplex nested RT-PCR (nRT-PCR) was also performed using primer pairs targeting the fusion gene of RSV A and RSV B (Table 1). In the first round, the PCR reaction mixture consisted of 2 µl of 10X Taq buffer, 0.5 µl of 10 mM dNTPs, 1U of Taq polymerase, 5 pmol of each primer (2 sets of forward and reverse primers specific for RSV A and RSV B) and 2 µl of cDNA. The volume was adjusted to 20 µl using sterile nuclease free water. 1 µl of the first round product was used as the template in the second round. The reaction mixture for the second round was same as that of first round. The first round reaction was set up at an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s and a final extension of 10 min at 72 °C. The cycling parameters were same for second round PCR except that 20 cycles were used instead of 35 cycles. Positive and negative controls were included in each run. PCR products were analyzed by electrophoresis with 1.5% agarose gel in 1X Tris- Borate- Ethylenediaminetetra acetic acid (TBE) buffer. PCR products were visualized using the Gel documentation system.

2.5. Statistical analysis

Data comparison based on gender and clinical presentation was analyzed using chi-square test. Descriptive statistics was used for continuous variables. The correlation between meteorological factors and incidence of RSV was assessed using Pearson's correlation coefficient and p-values less than 0.05 were considered as significant. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and negative likelihood ratio (NLR) were calculated to determine the diagnostic performance of nested RT-PCR. The data obtained from both the tests were analyzed by McNemar's test. Data analysis was done using online statistical software, QuickCalcs (GraphPad Software).

Table 2
Demographic details and clinical presentations of RSV positive and negative patients.

Characteristics	Total (n = 146)	RSV positives (n = 32) (22%)	RSV negatives (n = 114) (78%)
Gender (n) (%)			
Male	75 (51.4)	16 (50)	59 (51.8)
Female	71 (48.6)	16 (50)	55 (48.2)
Age group (n) (%)			
≤1 year	32 (22)	3 (9)	29 (25)
1–2 years	37 (25)	7 (22)	30 (26)
2–3 years	25 (17)	6 (19)	19 (17)
3–5 years	26 (18)	6 (19)	20 (18)
5–7 years	12 (8)	1 (3)	11 (10)
7–15 years	3 (2)	1 (3)	2 (1)
15–25 years	6 (4)	6 (19)	0 (0)
> 25 years	5 (3)	2 (6)	3 (3)
Duration of symptoms[a]	5.4 ± 3.6	4.5 ± 2.5	5.7 ± 3.7
Clinical symptoms (n) (%)			
Fever	85 (58)	22 (69)	63 (55)
cough	122 (84)	27 (84)	95 (83)
Rhinorrhea	107 (73)	26 (81)	81 (71)
Myalgia	11 (8)	3 (9)	8 (7)
Nasal congestion	80 (55)	23 (72)*	57 (50)
Sore throat	14 (10)	7 (22)**	7 (6)
Wheezing	10 (7)	2 (6)	8 (7)

Chi-square test was used to analyse the data.
*$p = 0.043$.
**$p = 0.014$.
[a] Duration of symptoms is represented as mean ± SD.

3. Results

3.1. Prevalence of RSV

From April 2016 to April 2017, one hundred and forty six patients with acute respiratory infection were recruited in the study. RSV A was identified in 27/146 (18.4%) samples and RSV B was detected in 5/146 (3.4%) samples by real time RT-PCR. In all, 32/146 (22%) samples were positive for RSV.

3.2. Clinical presentation of RSV in patients

The major clinical symptoms observed in RSV positive patients were cough (27/32, 84%), followed by rhinorrhea and nasal congestion (23/32, 71.8%) (Table 2). Statistically significant association was found between nasal congestion and presence of RSV ($p < 0.05$). One patient had a co-infection with influenza B virus (Data not shown). A male: female ratio of 1:1 was observed among the patients with RSV. Twenty two of the thirty two positive patients were less than 5 years old (Table 2).

3.3. Correlation between incidence of RSV and meteorological factors

The incidence of RSV was highest during the months of November to February (Fig. 1). The total number of RSV positives correlated with mean monthly maximum temperature, mean monthly minimum temperature (Fig. 2) and average relative humidity (Fig. 3). There was no statistically significant association between RSV incidence and total rainfall (Fig. 3) (Table 3).

3.4. Comparison between real time RT-PCR and nRT-PCR

Of the 27 RSV A detected by real time RT-PCR, only two samples

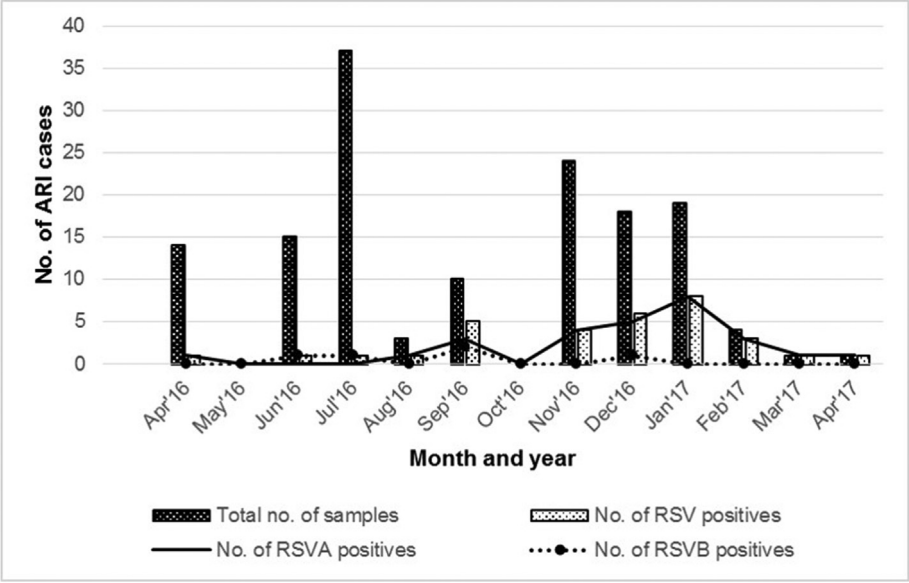

Fig. 1. Seasonal varisations in the incidence of RSV infections.

were positive for RSV A by nRT-PCR. However, all the five RSV B positives were also detected by nRT-PCR. Totally real time RT-PCR detected thirty two positives, while nRT-PCR detected only seven out of 146 samples (4.7%, $p < 0.0001$). The overall agreement between real time RT-PCR and nRT-PCR was found to be 21.8% (95% confidence interval (C.I.): 14%–28.6%). With respect to real time RT-PCR, the overall sensitivity, specificity, NPV, PPV and Negative likelihood ratio of nRT-PCR were found to be 21.9%, 100%, 82.01%. 100% and 0.78 respectively (Table 4).

4. Discussion

RSV is one of the most common viral etiologies of lower respiratory tract infections among infants, young children and the elderly. The prevalence rate of RSV was 22% in our study, which was in agreement with previous reports in our country. Bharaj et al.[10] and Singh et al.[11] reported 20.3% and 21.3% respectively. In contrast, lower prevalence rates of 8.7%, 18.61% and 14.5% were documented in Kolkata,[12] Pune[13] and Chennai[14] respectively. Varying prevalence rates were documented in different parts of the world.[15–18] The difference in epidemiology of RSV in the world could be due to variations in climatic conditions, environmental factors and severity of its epidemics.

RSV infection has a wide range of clinical manifestations and in our study, cough was found to be the predominant symptom followed by nasal congestion and rhinorrhea. Similar findings have been documented in China[19] and Rochester.[20] Others studies have reported fever and rhinorrhea as the frequently occurring symptoms.[10,21]

With regards to gender, male sex has been reported as a risk factor for RSV infections.[22] However, both the sexes were equally infected with RSV in our study. Similar observations were made by Fall et al.[21] and Koetz et al.[23] Zahlan et al.[18] and Liu et al.[24] documented male dominance in their study.

RSV infection is known to occur in seasonal outbreaks peaking during the winter in temperate climates and rainy season in warmer climates. Likewise this study confirmed the seasonality of RSV infection with increased positivity in September and November to February.

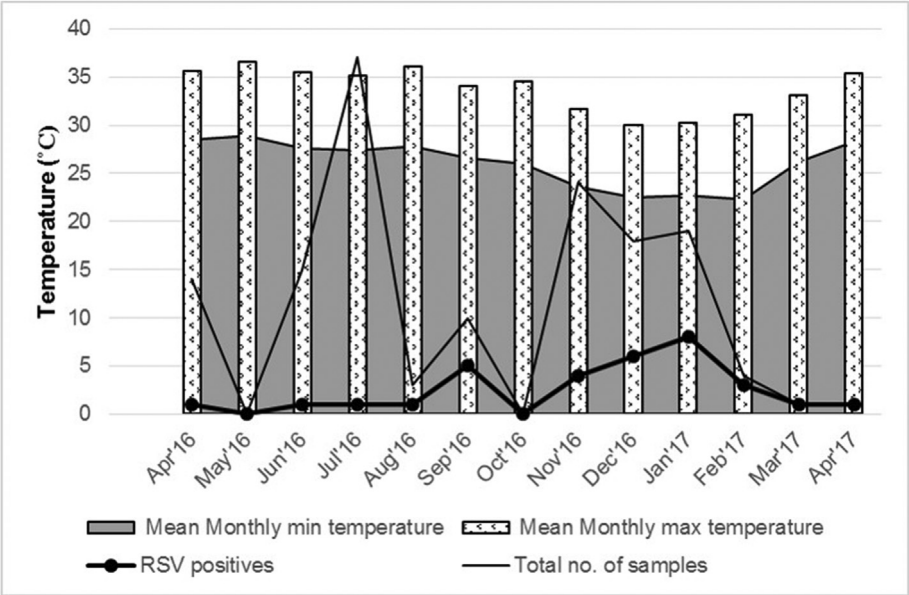

Fig. 2. Incidence of RSV in relation to temperature.

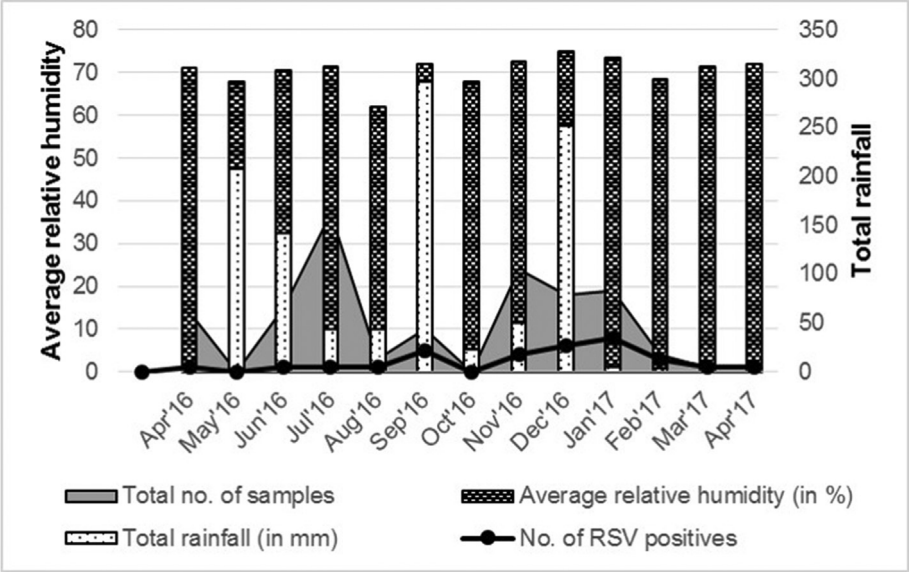

Fig. 3. Incidence of RSV in relation to average relative humidity and total rainfall.

Table 3
Correlation between incidence of RSV and meteorological factors.

	Mean monthly maximum temperature	Mean monthly minimum temperature	Total rainfall	Average relative humidity
Correlation coefficient	0.822	0.761	0.261	0.574
Statistical significance	$p < 0.001$	$p < 0.05$	NS[a]	$p < 0.05$

[a] Not significant.

Similar patterns were reported by Parveen et al.[25] and Choudhary et al.[13] In other countries, varying seasonal distribution of RSV were reported. In Sweden, RSV detections followed a seasonal pattern, with peaks occurring in early February and lasting around 10 weeks.[26] Fall et al. reported that the RSV season peaked between July and November in Senegal.[21] In Central England, RSV peaked from end of November to January, similar to our study.[27]

Of the various meteorological factors which affect the respiratory tract, air temperature and humidity have the most marked impact. Meteorological factors lower the local resistance of the human body to infection, thereby affecting the respiratory system.[28] Local resistance may deteriorate in several ways, for example, low humidity leads to loss of water vapor from the body cells, further paving way to microfissures in the nasal mucosa during cold weather. Moreover cold depresses the ciliary movements in the respiratory tract, consequently increasing vulnerability to infections.[28] A significant positive correlation was found between average relative humidity, mean monthly maximum and minimum temperatures and incidence of RSV in our study, even though the highest number of cases may not always coincide with the lowest temperature. In contrast, several authors have reported a negative correlation between temperature and incidence of RSV,[11,12] while Passos et al.[5] found a positive correlation between them.

The results of the real time RT-PCR assay presented here indicated its high performance compared to nRT-PCR. nRT-PCR was shown to detect significantly more numbers of RSV than tissue culture in a previous study.[18] In India, studies by Nandhini et al.[29] and Agrawal et al.[12] reported that performance of real time RT-PCR in detecting RSV was greater than that of conventional RT-PCR, supporting the findings of our study.

5. Conclusion

The results obtained in the present study indicate high prevalence of RSV among children less than 5 years of age. The seasonality and climatic patterns of RSV infections closely resembled that in other parts of the country. Comparative analysis of real time RT-PCR and nRT-PCR further confirmed the increased sensitivity, specificity and rapidity of real time RT-PCR. The results demonstrate the significance of epidemiological surveillance of respiratory infections caused by RSV in South India. However, further studies with larger sample size are required to elucidate the absolute prevalence of RSV and associated risk factors.

Conflicts of interest

None of the authors have any conflicts of interest.

Table 4
Comparison of performance of nRT-PCR vs. Real time RT-PCR for the detection of respiratory syncytial virus.

Real time RT-PCR	nRT-PCR negative	nRT-PCR positive	Sensitivity % (95% CI)[a]	Specificity % (95% CI)	NLR[b] % (95% CI)	NPV[c] % (95% CI)	PPV[d] % (95% CI)
Negative	114	0	21.88% (9.28%–39.97%)	100% (96.82%–100%)	0.78 (0.65–0.94	82.01% (7.15%–84.56%)	100%
Positive	25	7					
n = 146	139	7					

[a] CI- Confidence interval.
[b] NLR- Negative likelihood ratio.
[c] NPV- Negative predictive value.
[d] PPV- Positive predictive value.

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