Advance Publication by J-STAGE

Japanese Journal of Infectious Diseases

Comparative analysis of epidemiology, clinical features and cytokine response of Respiratory Syncytial and Human Metapneumovirus infected children with acute lower respiratory infections.

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Received: March 4, 2021. Accepted: June 3, 2021.

Published online: June 30, 2021.

DOI:10.7883/yoken.JJID.2021.151

Advance Publication articles have been accepted by JJID but have not been copyedited or formatted for publication.

Title page

Title: Comparative analysis of epidemiology, clinical features and cytokine response of Respiratory Syncytial and Human Metapneumovirus infected children with acute lower respiratory infections.

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Running head: RSV vs hMPV: experience from north India

Word count: Abstract - 195; text – 2353

Tables – 3; Figures – 1; References – 34

Keywords: acute respiratory infections; molecular diagnosis of viral pneumonia; IFN- γ ; MMP-9; epidemiology

Title: Comparative analysis of epidemiology, clinical features and cytokine response of Respiratory Syncytial and Human Metapneumovirus infected children with acute lower respiratory infections.

Summary

Both Human Respiratory Syncytial virus (RSV) and Human Metapneumovirus (hMPV) cause immune-mediated under-five acute respiratory infections (ARI), but differences in their disease pathogenesis, if any, are not well-known. This study was undertaken to analyze the epidemio-clinico-immunological features of RSV and hMPV infections. Naso-pharyngeal aspirates from children (aged two months to five years) with ARI presenting to our tertiary care center between December 2013 to March 2016 were subjected to real-time polymerase chain reaction for detection of RSV and hMPV. Positive samples were analyzed for co-infections and levels of cytokines. Of 349 naso-pharyngeal aspirates, RSV was detected in 40.68% (142/349), hMPV in 6.59% (23/349) and both in 1.4% (5/349). Co-infections were common, rhinovirus being the commonest co-offender. The demographical and clinical parameters of RSV- and hMPV-infected children were comparable. MMP-9/TIMP-1 ratio was significantly higher in RSV-mediated ARI and IFN- γ in hMPV-mediated ARI. Both RSV and hMPV are common among north Indian children with ARI and coinfections are not uncommon. Their clinical features being non-discriminatory, molecular diagnosis should be utilized to ascertain their individual epidemiology. The differences in their immune-pathogenesis (MMP-9/TIMP-1 ratio in RSV and IFN- γ in hMPV) could serve as useful tools for developing newer drugs.

TEXT

Introduction

Acute respiratory infections (ARI) in children present a major healthcare burden worldwide, contributing to significant healthcare visits, morbidity and mortality. Globally, about 13 million children under 5 years of age die annually, one-third of which are attributed to ARI.(1) Viruses constitute the most important etiology of ARI, chiefly Human Respiratory Syncytial virus (RSV), Influenza A, Rhinovirus, Human Metapneumovirus (hMPV) and Adenovirus. Most episodes of ARIs are self-limiting, however, complications are seen with the severe acute respiratory infection (SARI) requiring hospitalization and intensive care.

Global estimates suggest that RSV contributes a significant under-five healthcare burden where 31.8 million new episodes and 3.2 million admissions were reported(2). Among developing countries, India had the highest number of RSV-associated ARI with 7 million episodes in 2015(2). A recent review of 110 studies across the world concluded that hMPV constituted 1-86% of all under-five ARIs with marked differences among different countries.(3) Among Asian children <5 years old, incidence varied from 2.4% in Vietnam to 86% in Sri Lanka; hMPV constituted 25% of all ARI cases in India.(3) Such geographical differences in the incidence of ARI associated with RSV and hMPV necessitates studying epidemiological patterns associated with them.

The disease pathogenesis of both RSV and hMPV is largely immune-mediated. While the perturbation of Th2/Th1 balance may lead to bronchiolitis, it is the Th2 predominance which is associated with disease severity and mortality in RSV and hMPV infections(4). IL-33 mediates Th2-biased response leading to RSV-related asthma in later life (5), however, its role in hMPV is limited to a single study involving seven patients (6). Matrix metalloproteinases (MMP-9) and tissue inhibitor of metalloproteinases (TIMP-1) have also recently been

associated with airway inflammation in severe viral lower respiratory tract infections (7). The imbalance between MMP-9 and TIMP-1 is considered as a correlating factor for RSV-associated disease severity (8) thus necessitating the determination of the individual levels of MMP-9, TIMP-1 and the ratio thereof. There is no data on their role in hMPV-associated ARI. Other than a pilot study involving 15 RSV-infected children from southern India and evaluating only IL-4 and IFN γ (9), there is no Indian study evaluating the levels of different cytokines in patients of RSV or hMPV. Further, to the best of our knowledge, literature reports a single study wherein a comparison between cytokine levels among RSV patients viz-a-viz hMPV patients was attempted, that too analyzing only IL-4, IFN γ , IL-1b and IL-6 on a cohort of 102 Korean children (10).

The present study was, therefore, aimed at estimating the burden of RSV and hMPV-associated ARI among children from Chandigarh and neighboring states of North India and to compare their disease pathogenesis on the basis of clinical features and cytokine analysis.

Materials and methods

Study design: It was a prospective study on children visiting the Advanced Pediatric Center of Postgraduate Institute of Medical Education and Research, Chandigarh, India for ARI between December 2013 to March 2016. Children aged more than 2 months to less than 5 years of age suffering from acute lower respiratory tract infections (bronchiolitis, pneumonia, wheezing illness) and attending the Pediatric emergency and ICU were included in the study. Children with bronchial asthma, pneumonia of non-infectious causes such as aspiration pneumonia or HIV positive were excluded. Written consent was obtained from the guardian of the children. The study was approved by Institute Ethics committee vide no. NK/1232/PhD/20418.

Clinical assessment: Disease severity was assessed according to WHO Classification as ALRTI, severe ALRTI and very severe ALRTI in children from >2 months to 5 years of age (11).

Other relevant clinical details (time interval between onset and admission, history of breast feeding, prematurity, birth weight), demographic details, anthropometric data, co-morbid conditions, other signs and symptoms (auscultation findings, oxygen de-saturation etc.), radiological endpoints, treatment course (requirement of oxygen therapy, beta-agonists, adrenaline, antibiotics), bacterial co-infections, duration of hospital stay, duration of ICU stay and mortality were also recorded and analyzed with the laboratory findings.

Sample collection and processing: Nasopharyngeal aspirate (NPA) was collected from each patient. The child was made to lie down on his back or sit comfortably on mother's lap. 1-2 ml of sterile normal saline was instilled into one or both of the nostrils. The older children were instructed to hold their breath. For younger children, instillation of saline was timed with the expiration phase of breathing or crying. An age-appropriate disposable catheter connected to a mucus extractor was advanced along the floor of the nose until resistance was met on abutting the nasopharynx. The catheter was withdrawn, while applying gentle suction. The aspirated sample was considered adequate if at least 0.5 mL of volume was collected. After collection, samples were transferred into 3ml of viral transport media (Hi-media) properly labelled and immediately transported to the department of Virology, PGIMER maintaining the cold chain~ (4°C). The dilution factor of the specimen was calculated by subtracting the total weight of mucus extractor containing the NPA sample and VTM from the mean weight of the mucus extractor containing only VTM. The actual concentrations of the analyte cytokines were calculated by multiplying with the dilution factor of each sample. The samples were briefly sonicated (10 μ m amplitude for 30 sec) to liquefy the mucus while keeping the sample in ice. After sonication, samples were centrifuged at 5000rpm for 10 minutes at 4°C. The supernatant was aliquoted in three parts and stored at -70°C. The aliquots to be used for the cytokine level

determination, were mixed with protease inhibitor cocktail (Sigma Aldrich, St Louis, USA) and stored in -70°C . Taking all biosafety measures, 140 μl of respiratory sample was aseptically transferred in a 1.5 ml labelled micro- centrifuge tube (MCT) (containing 560 μl AVL Buffer and 5.6 μl carrier RNA per sample) to decontaminate the sample. Viral RNA was extracted using Viral RNA Extraction Mini kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions and stored at -80°C .

Detection of RSV and hMPV viral genomes and co-infections: Reverse transcriptase polymerase chain reactions (RT-PCR) were set up for the detection of RSV (12) and hMPV (13) genome in respiratory samples as per previously described method. Samples positive for RSV and hMPV by RT-PCR targeting N gene were subjected for viral load estimation by Real Time PCR using CDC recommended protocol.(14) Samples in which RSV or hMPV was detected were subjected to respiratory virus panel evaluation for determination of co-infections using FTD Respiratory pathogens 21 kit using manufacturer's instructions (Fast Track Diagnostics, Silema, Malta).

Determining levels of analyte cytokines: level of the cytokines (IL-4, IL-17, IFN- γ , IL-10, IL-17A and TNF α) in respiratory samples from patients infected by RSV and/or hMPV was determined using BD Cytometric Bead Array Human Th1/Th2/Th17 kit (BD Biosciences, San Jose, USA), following manufacturer's instruction. Level of IL-33, MMP-9 and TIMP-1 was done using specific Quantikine ELISA (R & D Systems, San Diego, CA, USA) for each.

Statistical analysis: The statistical analysis was carried out using Statistical Package for Social Sciences (SPSS, version 22.0). All quantitative variables were estimated using measures of central location (mean, median) and measures of dispersion (standard deviation, standard error of mean and inter-quartile range). Qualitative or categorical variables were described as frequencies and proportions. Proportions were compared using Chi square test and quantitative

variables were compared using Mann Whitney U test. A p value of <0.05 was taken as statistically significant.

Results

1. RSV and hMPV positivity by reverse transcriptase PCR and their seasonality:

Out of 349 nasopharyngeal aspirates, RSV was detected in 40.68% (142/349) and hMPV in 6.59% (23/349) samples. There were 1.4% (5/349) samples in which both RSV and hMPV were detected.

The circulation pattern of RSV and hMPV was similar during the study period. The upsurge of cases of ARI and virus positivity was seen in the winter months for both the viruses during the study period. (Figure 1)

2. Co-infections among RSV- and hMPV-positive samples (Table 1)

Among RSV-positive patients, 53% (52/98) of the infections were mono-infection. Rhinovirus was the most common virus (n=19) present along with RSV in ALRTI patients followed by Human Influenza A virus (n=11) and Human Bocavirus (n=9). The RSV and hMPV co-infections were seen in 1.45% (5/349) patients.

Among hMPV-positive patients, 50% (11/22) of the infections were mono-infection. Among co-infections RSV was the most common virus (n=5) present along with hMPV in ALRTI patients followed by Rhinovirus (n=4) and Human Bocavirus (n=3).

3. Comparison between baseline demography, health history, anthropometric data and clinical history among RSV and hMPV positive patients

Five samples were co-infected with both RSV and hMPV, so they were excluded from the comparative analysis between RSV and hMPV positive patients. Among the recruited patients 94.8% (331/349) were hospitalized, out of which 25.67% (85/331) required management in intensive care unit while 74.32% (246/331) were managed in the pediatric emergency ward. Among the very severe ALRTI patients, 16.6% (17/102) had the requirement for intermittent positive pressure respiration (IPPR) through mechanical ventilation because of the poor air entry. The history, clinical features and demography of both RSV and hMPV were almost similar without any significant differences (Table 2).

4. Comparison between cytokine levels in respiratory samples from RSV and hMPV associated ALRTI patients

The local cytokine level of $\text{TNF}\alpha$ (477.81 ± 11 vs 3557.92 ± 3007) and IL-10 (129 ± 13.58 vs 212 ± 94.36) was higher in hMPV infected patients but without any statistical significance (Table 3). hMPV-associated ALRTI children showed a significantly higher secretion of IFN- γ than those of RSV (48.10 ± 8.74 vs 97.7 ± 49.17 ; $p = 0.034$). MMP-9/TIMP-1 ratio was significantly higher in RSV-mediated ALRTI children than hMPV infected patients (39.90 ± 17.2 vs 2.2 ± 1.7).

Discussion

RSV was detected in 40.68% (142/349) under-five ARI cases in the present study. This was higher than the reported rate from other parts of the country; Rajala *et al* (15) from central India (New Delhi) reported RSV in 22.5% (44/200), Agarwal *et al* (16) from eastern India (Kolkata) in 10.29% (177/1720) and Kini *et al* (17) from southern India (Bengaluru) in 24.5% (94/383). The hMPV detection rate by RTPCR varies from 5% to 35% among hospital-based studies and 5% to 15% among community-based studies (18) whereas in current study hMPV

was detected in 6.6% (23/349) under-five children with ARLTI. In comparison to 3% (21/662) positivity of hMPV reported among children with ARI from central India (New Delhi) between 2005-2007(19) and that of 5.11% (118/2309) reported from eastern India between 2006-2009 (20). The higher positivity could be explained by selective recruitment of patients with bronchiolitis and clinically assessed viral pneumonia depending upon the radio-diagnostic features and clinical findings and due to the geographical variations in disease prevalence. Such geographical variation was observed in USA with significant higher prevalence in northern hemisphere than southern hemisphere (10.0% vs 3.0%; $P = 0.001$) (21).

A seasonality towards winter preponderance was observed for both RSV and hMPV in the present study, i.e. from December to March. This was in accordance to previous studies from northern India where upsurge of RSV infections overlapped with lowering temperatures (22) and from eastern India where RSV activity was highest during November to February (23). Global seasonality of RSV documented distinct periods of RSV activity, with waves of RSV experienced from September to December in Northern Hemisphere and from March to June in Southern Hemisphere (23). Similar information on seasonality of RSV from low- and middle-income countries is lacking due to lack of proper surveillance. A pilot study under the aegis of Global Influenza Surveillance and Response System reported maximum RSV-positivity during rainy season (August-September) in India (24). Only a handful of studies have documented seasonality of hMPV in India; Study reported highest number of hMPV infections during rainy season in eastern and southern India (25), (26).

Co-infection with both RSV and hMPV was observed in 5 samples. Co-infection with other respiratory viruses were also screened in representative RSV (n=98) and hMPV (n=22) positive samples with FTD respiratory 21 pathogen kit where 53% (52/98) of the RSV infections were mono-infection, 34% cases had dual infection and the remaining 13% had three or more pathogens in the respiratory samples; co-infections being less common with hMPV

which may be due to small sample size. Rhinovirus, adenovirus, influenza A and corona viruses are associated with upper respiratory tract infections and are frequently associated with prolonged shedding hence their exact pathogenic potential in acute lower respiratory tract infections remains to be enigmatic (27). Conventionally pdmH1N1 infection is less likely to be seen in association with other respiratory viruses(28), however in 4 cases of RSV and pdmH1N1 co-infections was detected in the present study. In context of rhinoviruses, R.M. Greer *et al*(29) hypothesized that double-stranded RNA (dsRNA) intermediates formed during rhinovirus replication trigger IFN-stimulated gene cascade which induces an antiviral state in the host designed to protect from co-infections (30) whereas in the present study Rhinovirus infections were most frequently detected as a part of co-infections with both RSV (n=19) and hMPV (n=4) infected patients. Similar finding was the observations during contemporary SARS-CoV2 pandemic (31).

The levels of IFN- γ was found to be significantly lower in RSV-infected patients (48.10+8.7) than hMPV-infected patients (97.7+49.17) in the present study. Supposedly the suppression of host's IFN- γ production by NS1 and NS2 proteins of RSV (32) through suppressor of cytokine signaling (SOCS) proteins and IFN-stimulated gene-15(ISG15) might be playing a role(33). With the growing evidence of protective effect of IFN- γ , its role as an antiviral agent for treatment of RSV-mediated ALRTI may be explored. The MMP9:TIMP1 ratio was significantly higher in RSV-infected patients than hMPV-infected patients. This was in accordance to the study by Kong *et al* (8) wherein pulmonary MMP9 activity was found to be associated with disease severity in RSV-mediated ARI. In hMPV-related infections, there is a single case report where elevated levels of MMP9 in serum were presumed to be related to acute encephalopathy in a 3-year old child (34). Other than that, the role of MMP-9 and its inactivator has not been explored in hMPV-associated ARI.

RSV and hMPV are important etiological agents of under-five ALRTI in northern India; their prevalence being higher than that reported at other places of the country. The demographical and clinical profile of RSV-infected children was similar to those of hMPV. Unlike hMPV infection, RSV infection was associated with decreased levels of IFN- γ and increased MMP9:TIMP1 ratio.

Acknowledgement: The author acknowledges The Indian Council Of Medical Research, New Delhi for partially supporting the present study.

Funding: Indian Council of Medical Research.

Conflict of interest: None.

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Figure legend

Figure 1. Seasonality pattern of RSV and hMPV from Dec 2013 to April 2016.

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Tables

Table 1. Co-infections detected in patients with RSV and hMPV infections.		
Co-infections with RSV		No. of cases (n)
Dual Infections (N=33)	RSV+ Rhino	11
	RSV+FLU A	7
	RSV+hMPV	5
	RSV+ Boca virus	3
	RSV+Cor 63	2
	RSV+ pdm H1N1	1
	RSV+Adenovirus	1
	RSV+ <i>M. pneumoniae</i>	1
	RSV+ Coronavirus HKU	1
	RSV+ Cor 229	1
Multiple infections (N=13)	RSV+ Rhinovirus+BOV	3
	RSV+ Flu A+ pdmH1N1	2
	RSV+FluA+Rhinovirus	1
	RSV+FluB+Cor43	1

	RSV+Rhino+Cor229	1
	RSV+Rhino+cor63	1
	RSV+BOV+HPIV4	1
	RSV+BOV+pdmH1N1	1
	RSV+Rhinovirus+BOV+HPIV3	1
	RSV+Rhinovirus+FluA+Enterovirus	1
Co-infections with hMPV		Number of cases
Dual Infections (N=9)	hMPV+ RSV	5
	hMPV+Rhinovirus	2
	hMPV + Bocavirus	1
	hMPV+Adenovirus	1
Multiple infections (N=2)	RSV+ Rhinovirus+BOV	2

Table 2. Comparative analysis of demography, health history, anthropometric data and clinical history (radiological findings, clinical investigations and prophylaxis) among children infected with RSV (n=137) and hMPV (n=18).			
	RSV cases	hMPV cases	p value
Sex : Male	89/137(65%)	15/18 (83.3%)	0.119
Female	48/137 (35%)	3/18(16.7%)	
Age (months)[Mean±SD]	6.67±5.652	6.94±5.2	0.846
Age group: A) ≥2-6 months	95/137 (69.3%)	11/18 (61.1%)	0.480
B) >6months-5 years	42/137 (30.7%)	7/18 (38.9%)	
Prematurity	17/116 (14.65%)	5/14(35.71%)	0.120
Low birth weight (<2.5kg)	32/110 (29.09%)	7/14 (50%)	0.284
Breast feeding	106/137 (77.4%)	15/18 (83.3%)	0.566
Hospital stay (days) [Mean±SD]	6.47±4.6(n=133)	7.27±3.4 (n=14)	0.575
Site of management:			
A) Emergency ward+ emergency OPD	93/137 (67.9%)	11/18 (61.1%)	0.565
B) ICU	44/137 (32.1%)	7/18 (38.9%)	
Duration of ICU stay (days) [Mean±SD]	3.66±2.25(n=44)	3.60±2.88 (n=7)	0.959

Presence of co-morbidities	41/137 (29.9%)	3/18 (16.7%)	0.241
Co-morbidities:			
A) Congenital heart disease	11/137 (8%)	1/18 (5.6%)	0.712
B) Hematological malignancy	13/137 (9.5%)	1/18 (5.6%)	0.584
C) Vitamin D deficiency	9/137 (6.5%)	Nil	0.263
Severity:			
A) ALRTI	52/137(38%)	8/18 (44%)	0.701
B) Severe ALRTI	35/137 (25.5%)	3/18 (16.7%)	
C) Very Severe ALRTI	50/137 (36.5%)	7/18 (38.9%)	
Triage classification			
A) Resuscitation	105/137 (76.6%)	13/18 (72.2%)	
B) Emergent	19/137 (13.9%)	3/18 (16.7%)	0.918
C) Urgent	13/137 (9.5%)	2/18 (11.1%)	
Requirement of mechanical ventilation	9/137 (6.6%)	3/18 (16.7%)	0.132
Auscultation findings:			
A) Crepitations			
B) Wheeze			0.891

C) Crepitations+ wheeze	40/137 (29.1%) 32/137 (23.35%) 65/137(47.44%)	4/18 (22.22%) 6/18 (33.33%) 8/18 (44.44%)	
Treatments			
A) 40% FiO2 supplement	131/137(95.6%)	16/18 (88.89%)	0.234
B) Beta agonists	46/137(33.6%)	6/18 (33.33%)	0.984
C) Adrenaline	99/137(72.3%)	16/18 (88.9%)	0.130
X ray findings			
A) Hyperinflation	56/137 (40.87%)	8/18 (44.44%)	0.330
B) Patchy infiltrates	33/137 (24.08%)	5/18 (27.77%)	
C) b/l infiltrates	11/137(8.02%)	1/18 (5.6%)	
D) Consolidation	22/137 (16.05%)	1/18 (5.6%)	
E) Hyperinflation+ consolidation	6/137 (4.37%)	1/18 (5.6%)	
F) Hyperinflation+ infiltrates	9/137 (6.56%)	2/18 (11.1%)	

Table 3. Comparative analysis of concentrations of cytokines in RSV & hMPV infected patients

Cytokine levels	RSV cases	hMPV cases	<i>p</i> value
	Mean±SEM (n)	Mean±SEM (n)	

	[range]	[range]	
IL-17A (pg/ml)	88.6 $\pm$ 7.6 (n=137) [0.3 to 592.75]	66.7 $\pm$ 13.48 (n=18) [0.02 to 205.9]	0.16
IFN- γ (pg/ml)	48.1 $\pm$ 8.7 (n=137) [2.26 to 726.27]	97.7 $\pm$ 49.1 (n=18) [1.24 to 906.23]	0.034
TNF α (pg/ml)	477.8 $\pm$ 113.8 (n=137) [5.84 to 11857.02]	3557.9 $\pm$ 3007 (n=18) [10.4 to 7902.8]	0.902
IL-6 (pg/ml)	8980.9 $\pm$ 785.3 (n=137) [390.76 to 59135.4]	10833.2 $\pm$ 4339.4 (n=18) [19.4 to 67403.1]	0.075
IL-10 (pg/ml)	129 $\pm$ 13.5 (n=137) [7.86 to 778.37]	212 $\pm$ 94.3 (n=18) [5.16 to 1375.4]	0.336
IL-33 (pg/ml)	349.2 $\pm$ 48.6 (n=104) [0.2 to 2939.9]	166.8 $\pm$ 58.6 (n=18) [0.2 to 1223.1]	0.293
MMP-9 (ng/ml)	1325.8 $\pm$ 180 (n=104) [0.13 to 8153.4]	566.3 $\pm$ 306.9 (n=18) [0.13 to 2070.18]	0.101
TIMP-1 (ng/ml)	542.4 $\pm$ 89.2 (n=104) [1.1 to 3981.6]	716.8 $\pm$ 240.2 (n=18) [20.1 to 3981.6]	0.293

MMP-9/TIMP-1 ratio*	39.90±17.2 (n=104) [0.01 to 1641.25]	2.2±1.7 (n=18) [0.01 to 14.1]	0.036
IFN-γ/IL-10 ratio*	0.96±0.1 (n=137) [0.02 to 13.13]	2.89±1.1 (n=18) [0.04 to 16.35]	0.095
<p>For the comparative analysis of cytokines Mann Whitney test was used.</p> <p>Statistical significance was considered at p<0.05</p> <p>*The ratio of MMP/TIMP-1 for each child was calculated by dividing the value of MMP by TIMP-1 for that child. Thereafter, mean of all these ratio was calculated. Similar for IFN-γ/IL-10 ratio.</p>

Accepted Manuscript