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Research Article

Detection of Respiratory Syncytial Virus in Suspected Asthmatic Children by Conventional RT-PCR Assays in Kathmandu

Stuti Khadka[1], Anil Kumar Sah[2], Jeena Amatya[1]

[1] St. Xavier's College, Maitighar, Kathmandu, Nepal
[2] Annapurna Research Center, Maitighar, Kathmandu, Nepal

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CORRESPONDENCE

Anil Kumar Sah; Stuti Khadka

Annapurna Research Center; St. Xavier's College, Maitighar, Kathmandu, Nepal

Email: anilshah4in@gmail.com
stuti.khadka104@gmail.com

0000-0001-5428-8503

Abstract

Respiratory Syncytial Virus (RSV) is the most common causative agent for lower respiratory tract infections in children particularly in infants. At least 50% of children are infected with RSV by the age of 2 to 3 years of age. However, the diagnosis of the RSV infection and its association with risk factors for asthma in asthmatic children is not studied in Nepal. The objectives of this study were to determine the prevalence of RSV in asthmatic children. This work was a hospital based cross-sectional prospective study and conducted from September 2017 to February 2018. Ethical approval was obtained from the Institutional Review Committee (IRC) of Kanti Children's Hospital, Maharajgunj. Suspected asthmatic children visiting the Special Asthma OPD of Kanti Children's Hospital, Maharajgunj were selected with the help of asthma specialist using a set of inclusion criteria for asthma. A total of 32 nasal swab samples were obtained from suspected asthmatic children. Initially, samples were processed for RNA extraction. The extracted RNA was then used for cDNA synthesis followed by PCR using primers for the Nucleocapsid (N) gene of RSV. Out of total 32 samples, 9 (28%) samples were positive for the N gene of RSV. There was no significant association of different variables including age (P=0.187), sex (P=0.264), family history of asthma (P=0.115), passive smoking (P=0.88), birth weight (P=0.954), seasonality (P=0.298) and history of pneumonia (P=0.457) with the RSV infection in this study.

Keywords: Asthma, Nucleocapsid (N) gene, RSV, RT-PCR

Introduction

Asthma is a chronic disease with no exact mechanism of pathology. It is often represented by the episodic shortness of breath characteristically associated with presence of wheezing, chest tightness and cough (Balkissoon, 2008). The Global Burden of Diseases Study (GBD) published in 2019 showed that as many as 334 million people in the world have asthma. In Nepal, 3.6 % of total deaths were due to asthma according to the Nepal burden of disease 2019 (MoHP, 2019). In children, asthma is not diagnosed until the age of 5 years. In children aged 5 years or below, the hyperresponsiveness is often described as presence of wheezing or acute bronchiolitis (Saglani, 2013). The high risk of RSV infection in children can increase the susceptibility to asthma and can also aggravate asthmatic episodes. Respiratory Syncytial Virus belongs to the genus of *paramyxovirus* under the *paramyxoviridae* family of viruses and it has a negative sense single-

stranded RNA genome (Crowe & Williams, 2014). It is an enveloped virus that exists both in filamentous and spherical form (Gower et al., 2005). RSV is responsible for the thousands of illnesses with symptoms of headache, cough, fever, chill and sore throat (Xu et al., 2018). The virus infects 90 % of the children by 2 years of age and has been found to be responsible for the exacerbations when the child is suffering from asthma in the following years (Heymann et al., 1998; Wu & Hartert, 2011). Several studies support that children are prone to the onset of asthma and allergy when sensitized by RSV in early childhood especially in infancy (Wang & Forsyth, 1998). Published data supports that re-infection or re-exposure to RSV may help in the development of asthma (Glezen et al., 1986). However, the underlying mechanisms are still unknown. Genetic and environmental factors interplay have been found to be important in the development of hyperresponsiveness (Han et al., 2011).

Increased level of eosinophils by the immune system of the body due to RSV infection induces the wheezing and asthmatic condition in the children (Kato & Kimura, 2004). Diagnosis of RSV infection by antigen/antibody detection from nasopharyngeal specimens is difficult and having low sensitivity (Falsey et al., 1996). Reverse transcription-PCR (RT-PCR) is a highly sensitive method for diagnosis of viral infection and has been used successfully in children with RSV (Henkel et al., 1997; Stockton et al., 1998). The increased RSV surveillance and treatment are likely to reduce the morbidity and associated disease burden in asthmatic children. Thus, this study was conducted with the aim of the accurate and timely detection of the RSV in children who were diagnosed with asthma.

Materials and Methods

The study was conducted in Kanti Children's Hospital, Maharajgunj, Kathmandu and Annapurna Research Center, Maitighar, Kathmandu from September 2017 to February 2018. Informed consent was taken from the parents or guardians of the participant children. Ethical clearance was obtained on September, 2017 by the IRC of Kanti Children's Hospital, Maharajgunj, Kathmandu. The study population was selected by the asthma specialist based on set inclusion criteria. The inclusion criteria were cold, cough, wheezing and shortness of breath in the children visiting the asthma OPD of the hospital. Data of age, sex, family history of asthma, family smoking (passive smoking), birthweight, seasonality of asthmatic episodes and history of pneumonia were noted.

Sample collection and processing

Sterile synthetic swab tipped swabs with polypropylene shafts were used to collect the specimen from the anterior nares of the selected children. The collected specimen was immediately dipped in the viral transport media (VTM) and transported to the lab on wet ice. The samples were processed immediately or stored at 4°C for RNA extraction to be done within 48 hours.

RNA extraction

The nasal swabs were vortexed in VTM followed by centrifugation. The extracted sample in solution was then used for RNA extraction using the manufacturer's protocol (QIAamp Viral RNA Minikit, Qiagen 2014). Briefly, 25 µl of protease was kept into a 1.5 ml microcentrifuge tube then 200 µl of VTM containing nasal swab. The 200 µl Buffer AL (containing 28 µg/ml of carrier RNA) was added, and then incubated at 56°C for 15 min in a heating block. Then 250 µl of ethanol (96–100%) added to the sample and incubated the lysate for 5 min at room temperature. All the lysate applied onto the QIAamp MinElute column and centrifuged at 8000 rpm for 1 min. Then, the QIAamp MinElute column placed in a clean 2 ml collection tube and discarded the collection tube containing the filtrate. Carefully opened the QIAamp MinElute column, and 500 µl of Buffer AW1 was added and centrifuged at 8000 rpm for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube and discarded the collection tube containing the filtrate. Carefully opened the QIAamp MinElute column, and 500 µl of Buffer AW2 added then centrifuged at 8000 rpm for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube and the collection tube was discarded containing the filtrate. Carefully opened the QIAamp MinElute column and 500 µl of ethanol (96–100%) was added and centrifuged at 8000 rpm for 1 min. The collection tube was discarded containing the filtrate. The QIAamp MinElute column placed in a clean 2 ml collection tube and centrifuged at full speed 14,000 rpm for 3 min to dry the membrane completely. The QIAamp

MinElute column was placed in a clean 1.5 ml microcentrifuge tube and collection tube was discarded with the filtrate. Carefully opened the lid of the QIAamp MinElute column and 70 µl of Buffer AVE were added to the center of the membrane. The lid was closed and incubates at room temperature for 1 min then centrifuged at full speed 14,000rpm for 1 min. RNA presence was confirmed by quantification using Qubit 4 fluorometre. The extracted RNA was stored at -20°C until used.

Conventional RT-PCR assay

The extracted RNA was then used for reverse transcription using manufacturer's protocol Reverse Transcription kit (Promega, USA). Following reverse transcription, briefly the components of the Reverse Transcription Mix kit were thawed on ice, mixed gently, and centrifuged briefly. For each sample RNA extracted, 6 µl of Nuclease free water, 4 µl of GoScript Reaction buffer Random primer, 2 µl of GoScript Enzyme mix and 8 µl of extracted RNA in elution buffer were mixed. After each addition the components were mixed gently. The reverse transcription reactions in the tubes were then incubated in a programmed thermal cycler. The cDNA samples were run for PCR. 12.5 µl of working solution of master mix (Thermofisher, USA), 0.5 µl of 10 pM concentrations of forward and reverse primers for RSV N gene (F: 5'-GGAACAAGTTGTTGA GGTTATGAATATGC-3' R: 5'- TTCTGCTGTCAAGTCT AGTACACTGTAGT-3') (DNA Macrogen), 6 µl of sample cDNA and 5.5 µl of nuclease free water was used for a total volume of 25 µl of one reaction mix. Optimal annealing temperature was determined by gradient PCR and Freymuth et al. (1995) was taken as a reference for overall cycles. PCR settings at Initial denaturation: 94°C; 30 Cycles of denaturation: 93° C for 30 seconds, Annealing: 50° C for 30 seconds and Extension at 72° C for 30 seconds. The PCR products were then detected by running in 2% gel electrophoresis with 100bp of DNA ladder (SolisBiodyne) and visualized in gel-documentation system (Figure 1). The expected band of PCR products was 277bp of N gene of cDNA.

Results and Discussion

RSV N gene was detected in 28% of the total study population (Figure 2). The age group ranged from 7 months to 8 years in the asthmatic children who showed positive results. The mean age of the children was 35.625 months. A majority (66%) of the children who showed positive results were above 24 months of age (Table 1). Also, 7 out of 9 (77.78 %) positive children were males. When family history of asthma was considered, 6 out of 9 (66.67 %) children who had RSV also had a family history of asthma. Out of the total RSV positive children, only 1 had exposure to passive smoking. Only 1 child was found to have low birth weight out of the children who had RSV. In the terms of seasonality, 6 (66.67 %) out of 9 RSV children were found to be having asthmatic conditions in the winter season. Finally, 3 out of 9 children who were positive for RSV had a history of pneumonia. There was no significant association of RSV in the asthmatic children with their age, sex, family history of asthma, passive smoking, birth weight seasonality and history of pneumonia ($p>0.5$). However, this study does show majority of the RSV positive children were males, had family history of asthma and had asthma in winter.

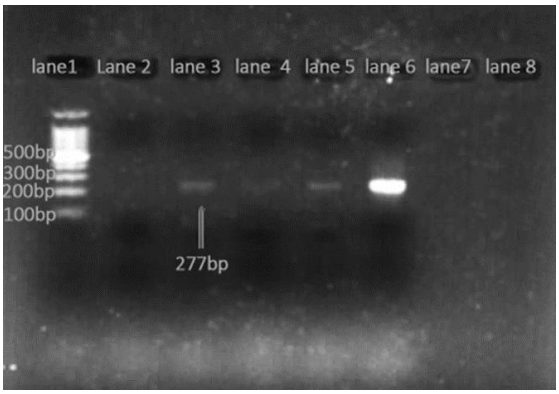

Figure 1: Gel Electrophoresis; Lane 1: 100 bp ladder, lane 2: RSV N gene Negative Control, lane 3; RSV N Gene positive control, lane 4, 7 and 8 negative samples for RSV N gene and lane 5 and 6 positive samples with band appearing at 277 bp.

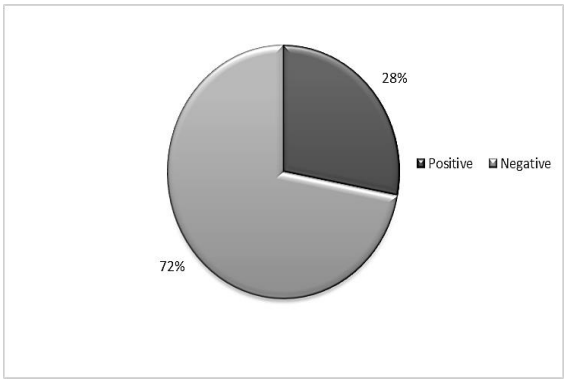

Figure 2: Prevalence of RSV in asthmatic children.

Table 1: Distribution of RSV in children presenting risk factors of asthma.

Age group	n	RSV presence	P-value
<24 months	8	3 (37.5%)	0.187
24 months	7	-	
>24 months	17	6 (35.2%)	
Gender			
Male	20	7 (35.0%)	0.264
Female	12	2 (16.6%)	
Family history of asthma			
Present	11	6 (54.5%)	0.115
Absent	21	3 (14.2%)	
Family smoking			
Present	4	1 (25.0%)	0.88
Absent	28	8 (28.5%)	
Birth weight			
<2500 gm	1	0 (0)	0.954
2500 gm	10	3 (30.0%)	
>2500 gm	21	6 (28.5%)	
Seasonality			
Winter season	28	6 (21.4%)	0.025
Other season	4	3 (75.0%)	
History of pneumonia			
Present	14	3 (21.4%)	0.457
Absent	18	6 (33.3%)	

A total of 32 suspected asthmatic children were included in this study. About 28% of the study population was found to have RSV RNA in their nasal secretion. This showed that a considerable number of children in the asthmatic study population had RSV and there is a possibility that RSV could have triggered the asthma in these children. The findings were similar to the two year prospective study of Finland (Jartti et al., 2004). In that study, 27% of the viruses detected from the wheezing children of 3 months to 16 years of age were found to be RSV. Similarly, 18.9% of the RSV viruses were detected in children with acute asthma by Dziublyk et al. (2013).

No studies were found about the RSV prevalence in the asthmatic children that were conducted in Nepal. However, RSV was sought out in several research among the children with various respiratory infections. The detection rate in this study is higher when compared to the detection rates of RSV in children of Nepal with other pre-existing diseases. Helen Chu and colleagues conducted the research to study the prevalence of RSV burden in the Acute Respiratory Infection in infants of sub-tropical plains of rural southern Nepal. The study found out that RSV was involved in 9% of the study population; 311 out of the total children presenting acute respiratory tract infection had RSV (Chu et al., 2017). Mathisen and colleagues found out 40% i.e., 887 out of 2219 children of Bhaktapur showed viral positive result by PCR test. Among the results, 15% of the viral burden was comprised of RSV (Mathisen et al., 2010). The Majority of children who had RSV in this study were above 24 months of age in contrast to that found in the literature which was mostly below 24 months of age. This could be due to the small sample size where the mean age is already 35.6 months in the study population. Additionally, susceptible children can get reinfection of RSV. Asthmatic episodes are found to be more frequent and exacerbated in boys than girls (Almqvist et al., 2008). This study also followed the same pattern that among the total consecutive asthma children included 62.5% of them were males. Moreover, among the asthma patients for RSV 77% of them were males as well. However, none of the risk factors for asthma had a significant association with presence of RSV. Many studies have shown a significant association between asthma and RSV presence (Aujard & Fauroux, 2002; Bradley et al., 2005; Castro et al., 2006). RSV is considered to be a very important causative agent for respiratory distress in children and the immune response to RSV has shown to be supportive of airway hyper-responsiveness. In Nepal, RSV has been studied in several other respiratory infections such as pneumonia and lower respiratory tract infection (LRTI) (Chu et al., 2016). However, studies for asthma and RSV associations are few to none in Nepal, despite the abundant literature on genetic linkage of these two conditions.

Conclusion

The finding of this study will help early diagnosis and management of the asthma children in low-income country like Nepal. These types of study need to be conducted with large sample size to create clear conclusion on the presence of asthma children in Nepal. Further study needs to perform for the genotyping of RSV.

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