Original article

Detection of rhinovirus-associated asthma exacerbations using reverse transcriptase-polymerase chain reaction in Egyptian children

Background: *Acute exacerbations of asthma are the leading cause of emergency department visits in pediatric patients. The development of sensitive diagnostic polymerase chain reaction (PCR) based techniques permitted demonstration of an already clinically suspected association between common viral respiratory infections and asthma exacerbations. Respiratory viruses have been identified in 80–85% of exacerbations in school-aged children, with human rhinoviruses (HRVs) being the most frequently detected. A recently identified HRV genotype, HRV-C, is circulating worldwide and is an important cause of febrile wheeze and asthmatic exacerbations in children requiring hospitalization.* ***Objectives:*** *This study aimed to detect HRV- induced asthma exacerbations (including the new HRV-C genotype) among a group of Egyptian children.* ***Methods:*** *This cross-sectional study was conducted on 31 asthmatic children in exacerbations in the period from September 2014 till October 2015. Patients were recruited from the emergency department and chest clinic, Children's hospital, Ain Shams University. Sputum (for children ≥7years) and nasopharyngeal aspirates (for infants and children<7years) were collected for one-step, real-time pan Rhinovirus reverse transcription polymerase chain reaction (RT-PCR) assay. One step RT-PCR was done to detect Rhinovirus C among positive cases.* ***Results:*** *This study included 31 asthmatic children in exacerbations. They were 15 males (48.4%) and 16 females (51.6%). Their ages ranged from 7 months to 12 years with a mean and SD of (4.47 ± 3.15) years. Eight (25.8%) of the studied patients showed positive Rhinovirus RT-PCR test and 4 (50%) of the HRV positive patients were of the Rhinovirus C genotype (12.9% of the total population). HRV positive patients showed higher percentage of positive family history of bronchial asthma (p=0.002), higher mean values of respiratory rate (p=0.001) and temperature (p=0.001), but lower mean value of oxygen saturation (p =0.011). There were statistically significant differences regarding the exacerbation severity (p=0.024) and outcome (p=0.048) between HRV positive and negative patients.* ***Conclusion:*** *HRVs are important triggers of asthma exacerbations among Egyptian children. The newly described HRV-C genotype accounts for a significant proportion of HRV- associated asthma exacerbations. Further studies on a larger scale are needed for HRV-C and other possibly undiscovered HRV genotypes.*

Keywords: Rhinovirus, Rhinovirus C genotype, asthma exacerbation, real time RT-PCR.

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INTRODUCTION

Acute asthma exacerbation is a cause of strong concern among children and parents and represents a challenge for pediatric healthcare providers[1]. Studies reported the issue of "virus-induced exacerbation in asthma and chronic obstructive pulmonary disease" and evidence of viral infection is found in 80% to 90% of childhood wheezing episodes[2,3]. More than 200 different types of viruses, such as human rhinovirus (HRV), human metapneumovirus (HMPV), respiratory syncytial virus (RSV), and human parainfluenza virus (HPIV) are known to cause acute respiratory illness[4]. However, among these causative viruses, HRV is now recognized to have a major impact on asthma pathogenesis[5,6]. Rhinovirus (genus Enterovirus, family Picornaviridae), a virus of considerable heterogeneity comprising over 100 serotypes, is the main cause of common cold and is consistently associated with sudden-onset asthma attacks in children and adults[7]. Typically, HRVs are segregated into two groups, HRV-A and HRV-B, although a new potentially more virulent and

exacerbation-relevant group (HRV-C) has recently been identified[8]. HRVs are predominant during the spring and autumn. They have been incriminated for the peak in pediatric asthma exacerbations at this time of year termed the September epidemic[9].

The definitive diagnosis of respiratory viral infections is complicated by the lack of commercial availability of a rapid and cost-effective laboratory test to confirm the presence of viral respiratory infections[10]. The gold standard laboratory requirement for the culture confirmation of viral infection was difficult to apply, had low sensitivity, and resulted in underdetection of viral infections[11]. The rhinovirus and coronavirus were difficult to culture, and convalescent serology was often not available. The recent increasing use of PCR amplification methods in studies of respiratory viruses (in particular the rhinovirus) has provided new information on viral epidemiology and has contributed to the understanding of the role of viruses in exacerbations of asthma[12]. Furthermore, the use of induced sputum samples, obtained non-invasively, facilitates viral detection, because induced sputum (RT-PCR) has been found to be a more sensitive and rapid diagnostic method than conventional serology and immunofluorescence[13].

This study aimed to detect HRV-induced asthma exacerbations (including the new HRV-C genotype) in a group of Egyptian children.

METHODS

This cross-sectional study was conducted on 31 asthmatic children in exacerbations in the period from September 2014 till October 2015. Patients were recruited from the emergency department and chest clinic, Children's hospital, Ain Shams University. An informed consent was obtained from the parents or caregivers before enrollment. The study protocol was approved by the ethics committee of the Pediatric department, Ain Shams University.

Detailed history was taken stressing on residence, history of atopy (atopic dermatitis and allergic rhinitis) and family history of asthma. Patients with comorbid illnesses or those suffering from any other broncho-pulmonary diseases were excluded from the study. The age of the studied patients was categorized according to Eunice Kennedy Shriver National Institute of Child Health and Human Development in the United States (2011).

Full clinical examination was done and the severity of exacerbations was classified according to GINA, 2015 into mild- moderate, severe and life threatening. The criteria for exacerbation severity were based on symptoms, parameters of physical examination and oxygen saturations.

RT-PCR test was done to detect HRV in sputum samples (children ≥7years) and nasopharyngeal aspirates (infants and children<7years) and according to the results, the studied population were classified into HRV negative and positive groups.

Sample preparation

Each sample was mixed in the mucous collector with 2 ml of virus transport media and votexed and transferred to 2 tubes each 1.5 ml, one tube used for RNA extraction and the second was frozen as back up. Total sample RNA extraction was done using QIAgen viral RNA Kit® (QIAgen, Germany). The preparation of kit reagents and RNA extraction were done according to the manufacturer's instruction.

One-Step, Real-Time PCR Assay

In this one-step, real-time PCR assay, reverse transcription and PCR amplification were performed in the same reaction tube. The total reaction volume was 50 µl, and the reaction mixture contained 0.9 µmol/L (each) of the primers, 0.15 µmol/L of the probe, TaqMan One Step PCR Master Mix Reagents kit (Applied Biosystems) components, and 5 µl of extracted sample. The primers and TaqMan probe used had the following sequence[14]: primer Pic-1 (positions 377 to 393), 5'-TCCTCCGGCCCCTGAAT-3' (melting temperature of 59.9°C); Pic-3 (positions 496 to 474), 5'-GAAACACGGACACCCAAAGTAGT-3' (melting temperature of 58.1°C); and probe Pic-5 (positions 395 to 410), Fam 5'-YGGCTAACCYWAACCC-3' (melting temperature of 68.8°C to 76.8°C). All sample lysates and controls, were tested in duplicate reactions. The thermal cycling program consisted of 48°C for 30 minutes, 95°C for 10 minutes, and 45 two-step cycles of 95°C for 15 seconds and 60°C for 1 minute, and was conducted by using the Step One Sequence Detection System (Applied Biosystems). Each run included the testing of the positive and negative extraction control lysates, Tris-EDTA buffer in four reactions (no template controls), and diethylpyrocarbonate-treated water (QIAgen, Germany) in duplicate reactions for each set of five specimen lysates (negative reagent controls). The no template controls and negative reagent controls were used to detect any nonspecific fluorescent signal or carry-over contamination. Run acceptability required obtaining the expected results from each control. The total run time for this protocol was 2.5 hours. Samples were considered

positive if the amplification plots (i.e., change in normalized reporter signal [delta Rn] versus PCR cycle number) from duplicate reactions showed definite exponential increase in fluorescent signal (Figure 1).

Rhinovirus C specific conventional RT-PCR

One step RT-PCR was used to detect Rhinovirus C among positive cases detected by pan-Rhino real time PCR. Two primers were used FC and RC which are specific for rhino viruses C[15]. The Test was done using QIAgen one step RT-PCR kit (QIAgen, Germany) in 50 µl volumes as described by the manufacturer. The cycling parameters were initial RT step at 50ºC for 30 minutes followed by hot start enzyme activation at 95ºC for 15 minutes followed by 40 cycles each of 94 ºC for 45 seconds, annealing at 55ºC for 45 seconds and extension at 72ºC for 1 minute. PCR product was detected by Gel electrophoresis with specific product at 330 base pairs (Figure 2).

Statistical analysis

Data was analyzed using the Statistical Program for Social Science (SPSS) version 18.0. Quantitative data was expressed as mean± standard deviation (SD). Qualitative data was expressed as frequency and percentage. Chi-square (X^2) test was used to compare the proportions between two qualitative parameters. Independent-samples t-test was used to compare between two means. P-value of <0.05 was considered significant.

RESULTS

This study included 31 asthmatic children. They were 15 males (48.4%) and 16 females (51.6%) presented with acute asthma exacerbations. Their ages ranged from 7 months to 12 years with a mean and SD of (4.47 ± 3.15) years. Eight (25.8%) of the total population showed positive Rhinovirus RT-PCR. Four (12.9%) of the total population (50% of the RV positive patients) were of the Rhinovirus C group.

Analysis of demographic data of the studied population showed no statistically significant differences between HRV negative and positive groups as regards to age, sex, seasonal distribution and history of atopy but HRV positive patients showed a statistically significant higher percentage of positive family history of bronchial asthma when compared to the HRV negative patients (Table 1).

All HRV positive patients required hospital admission, 7(87.5%) in the ward and one (12.5%) died in the pediatric intensive care (PICU), while 15(65.2%) of the HRV negative group required admission in the ward and no one was admitted in the PICU. There was a statistically significant difference between both groups as regards to the outcome (X^2= 6.073, p=0.048).

Characteristics of HRV-C positive patients: Of the total population, 4 had RV-C infection (2 toddlers and 2 preschoolers). All had life-threatening exacerbations. One was complicated by acute pneumonia and died in PICU. All cases were detected in winter and all had a positive family history of bronchial asthma. However, statistical comparison between the HRV-C positive patients and those with other types was non-applicable due to small sample size.

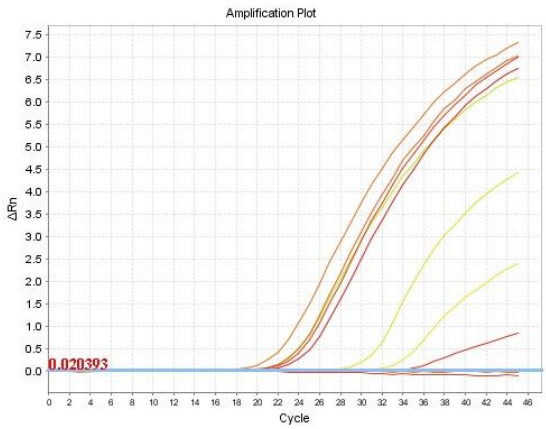

Figure 1. Real time Pan Rhinovirus RT-PCR

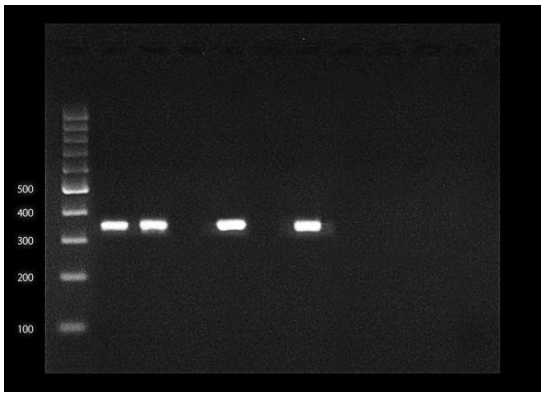

Figure 2. Specific Rhinovirus C conventional PCR

Table 1. Comparison between HRV negative and positive groups as regards to Demographic data

			Pan Rhinovirus Real time PCR		Chi-square test	
		No.	Negative(n=23)	Positive(n=8)	X^2	P-value
Age group (years)	Infant	7	5 (21.7%)	2 (25%)	1.037	0.792
	Toddler	6	5 (21.7%)	1 (12.5%)		
	school age	10	8 (34.8%)	2 (25%)		
	Preschool	8	5 (21.7%)	3 (37.5%)		
	Mean ± SD		4.78 ± 3.42	3.60 ± 2.17	0.908	0.372
	Range		0.6- 12	0.9- 6		
Gender	Female	16	12 (52.2%)	4 (50%)	0.011	0.916
	Male	15	11 (47.8%)	4 (50%)		
Seasonal distribution	Early fall	4	3 (13%)	1 (12.5%)	0.002	0.968
	Winter	27	20 (87%)	7 (87.5%)		
History of atopy	Allergic dermatitis	4	2 (8.7%)	2 (25%)	3.394	0.183
	Allergic rhinitis	6	6 (26.1%)	-		
	Negative	21	15 (65.2%)	6 (75%)		
Family history of Bronchial asthma	Negative	18	17 (73.9%)	1 (12.5%)	9.194	0.002*
	Positive	13	6 (26.1%)	7 (87.5%)		

Table 2. Comparison between HRV negative and positive groups as regards to the respiratory manifestations

		Pan Rhinovirus Real time RT-PCR		Chi-square test	
		Negative (n=23)	Positive (n=8)	X^2	P-value
Cough	Positive	21(91.3%)	8(100%)	0.744	0.389
	Negative	2 (8.7%)	-		
Dyspnea	At rest	9 (39.1%)	7 (87.5%)	5.56	0.018*
	On exertion	14 (60.9%)	1 (12.5%)		
Alertness	Agitated	12 (52.2%)	8 (100%)	5.930	0.015*
	Calm	11 (47.8%)	-		
Wheeze on Auscultation	Loud inspiration and expiration	1 (4.3%)	7 (87.5%)	21.953	0.000*
	Loud in expiration only	16 (69.6%)	-		
	Moderate	6 (26.1%)	1 (12.5%)		

DISCUSSION

Respiratory viruses are the most common triggers of exacerbations, accounting for approximately 85% of cases, and HRV represents the great majority of viruses detected[16]. The presentation of children with infection-induced wheezing is influenced by age, previous respiratory infections, genetic background, and sensitization to aeroallergens, environmental influences and interactions between these factors[17].

The advent of molecular viral diagnostics has expanded our understanding of the epidemiology of respiratory illnesses in infancy by improved detection rates for known viruses, and surprisingly, has led to the discovery of previously unrecognized respiratory viruses. These new diagnostic techniques have been particularly helpful in understanding the role of HRV, which are particularly difficult to grow in tissue culture, in acute illness and asthma exacerbations[18].

With this background, this study aimed to detect HRV- induced asthma exacerbations in a group of Egyptian children and to determine the presence of HRV- C genotype among them.

This cross-sectional study was conducted on 31 asthmatic children in exacerbations in the period from September 2014 till October 2015. Patients were recruited from the emergency department and chest clinic, Children's hospital, Ain Shams University. They were 15 males (48.4%) and 16 females (51.6%). Their ages ranged from 7 months to 12 years with a mean and SD of (4.47 ± 3.15) years.

It was estimated that approximately two-thirds of infections associated with asthma exacerbations were caused by HRV[19]. In this study, eight (25.8%) out of 31 patients showed positive Rhinovirus RT-PCR test. Four out of 31 (12.9%) were of the Rhinovirus C genotype (50% of HRV positive cases). In agreement with this study, *Lau et al.*[20] detected HRVs in 178 (29.7%) of 600 NPAs taken from children. Of them, HRV-C was found in 78(13%) of patients (43.8% of HRVs). *Venarske et al.*[21] conducted a cohort study of 101 asthmatic patients during 1999–2003, enrolled at hospital admission for an asthma exacerbation and found that HRV was detected by RT-PCR in 21% of hospitalized patients over a 4-year period. Furthermore, *Louie et al.*[22] studied 43 children admitted to the intensive care unit due to asthma exacerbation and detected HRV in 21(48.8%) of them. Also, *Lau et al.*[8] found HRV-C among 21 (10.3%) of 203 NPAs from children hospitalized for acute respiratory illness. Moreover, *Arden et al.*[23] detected HRV in 41(52.6%) of 78 NPAs of children with asthma exacerbation not requiring admission to hospital. They found that HRV-Cs were the viruses most frequently detected as single virus detection in 23(29.5%) of the total population (56.1% of HRVs).

Although rhinoviruses are the most perennial of the respiratory viruses, with transmission in all months of the year, peaks of RV illness have been well –documented in September, after school opens and again in the spring[24]. In this study, most (87.1%) of the total population were collected in winter with no significant difference between both groups. This can be attributed to other respiratory viruses that could have been involved among the HRV negative group such as Respiratory syncytial virus (RSV) and influenza. RSV and influenza have been associated with increased asthma exacerbations during winter months[25]. Moreover, *Arden et al.*[26] found that HRV detections were made in every season while, *Piotrowska et al.*[27] reported that HRV-C induced asthma exacerbation can happen all over the year, without peaks in the spring and fall. Also, *Lau et al.*[20] found that a diverse set of HRV-C genotypes was circulating throughout the year. This variation underscores the importance of studies over multiple years and seasons to understand fully the geographical and seasonal rhinovirus epidemiology and its role in asthma exacerbation[28].

A positive family is a known risk factor for asthma development, and it has recently been found that infants of mothers with asthma are more likely to have severe respiratory tract infections with HRV[29]. In this study, HRV positive patients showed a statistically significant higher percentage of positive family history of bronchial asthma when compared to HRV negative patients. In accordance with this study, the Tennessee Children's Respiratory Initiative reported that having a mother with atopic asthma significantly increased the risk of the infant experiencing HRV infection compared with RSV infection. Interestingly, maternal asthma increased the severity of infant HRV infection but not that of RSV infection. Thus, compared with infants with RSV infection, infants with HRV infections are more likely to have an atopic predisposition reflected by positive family history. These findings suggest that there is likely an underlying genetic basis for risk of and response to respiratory infections[30].

The relative importance and disease severity associated with the two-pre-existing species, HRV-A and HRV-B and the newly described species, HRV-C are not well understood[20]. Recent reports have implicated HRVs in more severe upper and lower respiratory tract infections, especially in children[31]. In this study, HRV positive patients showed statistically significant higher percentage of dyspnea, agitation, distress and desaturations. Comparatively, they showed statistically significant higher percentage of life-threatening exacerbations and thus hospitalization and no one experienced mild to moderate degree of exacerbation although, the higher proportion of life-threating cases may be an overestimate as this is a tertiary hospital- based study to which the severe and life threatening cases are referred to. In agreement with our results, *Venarske et al* [21] found that HRV detection was strongly associated with hospitalization for asthma and HRV positive patients were more severe than HRV negative patients.

Unfortunately, in other studies that described the detection of HRV-A and HRV-B in addition to the new genotypes, the results were often limited by either the small number of samples with positive results or a lack of clinical and epidemiological data[32]. Further clinical studies that involve more patients with HRV infection are required to investigate whether certain HRV species or strains may be more virulent than others[20]. In this study, All HRV-C patients were hospitalized for life-threatening exacerbations. Unfortunately, no systematic study has been carried out to examine the epidemiology and clinical features of HRV-C, compared with those of HRV-A and HRV-B as most of the performed studies only focused on the

molecular identification of HRV-C from clinical samples[20].

Finally, the population's characteristics and our diagnostic methods may explain the slight variations to other investigators as we included only children with a previous diagnosis of asthma and excluded patients with comorbid illnesses, patients on systemic steroids and those suffering from other broncho-pulmonary diseases such as bronchiectasis and pneumonia, while other studies of hospitalized children enrolled all children with wheezing including bronchiolitis and pneumonia.

Limitations of this work were the absence of detection of other viruses precipitating exacerbations of bronchial asthma for comparison and the relatively small sample size.

CONCLUSION

HRVs are important triggers of asthma exacerbations among Egyptian children. The newly described HRV-C genotype accounts for a significant proportion of HRV- associated asthma exacerbations. Further studies on a larger scale are needed for HRV-C and other possibly undiscovered HRV genotypes that may help future attempts to produce a protective pan-serotype human Rhinovirus vaccine with possible cross-serotype protection that will guard against life-threatening asthma exacerbations.

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