Contribution of Viruses, *Chlamydia spp*. and *Mycoplasma pneumoniae* to Acute Respiratory Infections in Iranian Children

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Summary

The study reports the frequency and clinical presentation of respiratory syncytial virus (RSV), human metapneumovirus, influenza (Inf V), parainfluenza, adenovirus (Adv), *Chlamydia spp*. and *Mycoplasma pneumoniae* in children with acute respiratory infections (ARI) in Rasht, Iran. Nasopharyngeal aspirates and swabs were collected from 261 children in 2003 and 2004. Pathogens were detected using polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR), confirmed with sequence analysis. Ninety-three pathogens were detected in 83 children. RSV was present in 39 (15%), Adv in 37 (14%), Inf A in 11 (4%), *C. trachomatis* in 4 (2%) and *M. pneumoniae*, in 2 (1%) children. Neither parainfluenza nor metapneumovirus were detected. RSV, Inf A and *C. trachomatis* were more frequent in children with lower respiratory infections. Adv presented more frequently as upper respiratory infection. All pathogens, except *M. pneumoniae*, were detected in children with severe pneumonia. Viruses play a significant role in Iranian children with community-acquired ARI.

Keywords: Acute respiratory infections, children, Iran, viruses, *Chlamydia spp*., *Mycoplasma pneumoniae*

Introduction

Acute respiratory infections (ARI) are the leading cause of death related to infection in children, mostly resulting from pneumonia and bronchiolitis [1]. In addition, these are a major cause of childhood morbidity, with increased utilization of health services, hospital admissions and drug prescriptions. In developing countries, viruses represent a considerable proportion of the pathogens associated with ARI, varying from 9% to over 60% across studies [2]. Although this wide range is largely due to geographical variations, study design and laboratory methods, most studies report that respiratory syncytial virus (RSV) is the most frequent pathogen followed by other viruses such as parainfluenza (PIV), influenza (Inf V), adenovirus (Adv) and human metapneumovirus (HMPV) [2, 3].

Most studies in developing countries, however, focus on a small number of pathogens, and information on the relative contribution of each pathogen to ARI is incomplete. We report here, a study to simultaneously describe the relative incidence of a large number of respiratory pathogens (RSV, HMPV, Inf V, PIV, Adv, *Chlamydia spp*. and *Mycoplasma pneumoniae*) in children with ARI presenting to two hospitals in Rasht, north Iran. The study also describes for the first time the clinical and epidemiological characteristics of the pathogens from this region, which could be useful for the development of preventive approaches to ARI in the community.

Acknowledgements

M.N. is supported by the Iranian Ministry of Health and Medical Education.

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Materials and Methods

Time and location

This was a prospective cross-sectional study conducted from November 2003 to March 2004, based

doi:10.1093/tropej/fml089 Advance Access Published on 7 February 2007

on 17-Shahrivar and Rasoul-e-Akram hospitals. 17-Shahrivar is a paediatric reference university hospital with 200 beds and Rasoul-e-Akram is a general regional referral hospital located in Rasht, Guilan province in northern Iran. These hospitals serve as curative and referral centres for both urban and rural populations of the province.

Patients

All the children <5 years of age attending the out-patient clinics and those admitted to the hospital wards from Saturday to Thursday with a clinical diagnosis of ARI were enrolled in the study, irrespective of the severity of their illness. An ARI was defined as the presence of respiratory signs and symptoms of <7 days duration and followed the World Health Organisation diagnostic protocol, which is based on the respiratory rate and the presence of cough and chest indrawing [4]. Oxygen saturations (pO_2) were measured in all the patients admitted to the wards with a pulse oximeter (Nonin Medical, Inc. MPL, MN, USA, model 8500) and before initiation of oxygen therapy. Children were classified into having no/moderate ($pO_2 \geq 94\%$) or severe ($pO_2 < 94\%$) hypoxia. A standardized questionnaire containing clinical, sociodemographic, therapeutic and outcome data was used to collect information from the parents.

Samples and laboratory methods

Nasopharyngeal aspirates (NPA) or swabs (Medical Wire & Equipment Co. Ltd., Corsham, Wilts, UK) were collected from all children using sterile mucus extractors for NPA. The specimens were transported in an icebox and stored at −80°C until processed in the Department of Medical Microbiology, University of Liverpool, UK.

DNA and RNA of all samples were extracted separately using the QIAamp® DNA and RNeasy Mini Kits (Qiagen Ltd., Crawley, West Sussex, UK). Extracted RNA was processed using reverse transcription-polymerase chain reaction (RT-PCR) for RSV, HMPV, Inf A and B and PIV 1–4. PCR assays were also used to amplify the DNA of Adv, *Chlamydia spp*. and *M. pneumoniae* [5]. A duplex RT-PCR for RSV nucleocapsid (N) gene and HMPV matrix (M) gene was used with RSV-N1 and N2 primers for amplifying the N gene between nucleotides 858 and 1135 to give a 278-bp product as described by Cane and Pringle [6] and the HMPV-MF1 and MR1 primers for amplification of the M gene between nucleotides 212 and 331 to make a 120-bp product as described by Greensill *et al*. [7]. Each RSV/HMPV RT-PCR contained 10 µl of RNA with 2.5 units of Amplitaq gold, 20 units of RNase Inhibitor and 2.5 units of MuLV Reverse Transcriptase (Applied Biosystem, Warrington, UK) added to 3 mM $MgCl_2$, 1× PCR buffer, 5 mM dithiothreitol (dTT), 0.4 mM deoxynucleoside triphosphates (dNTPs), 0.4 mM of primer mixed, containing equal volumes of all primers at a concentration of 20 µM in a 50 µl reaction. RT-PCR thermal cycling conditions were 50°C for 30 min, followed by 1 cycle of 94°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and 1 cycle of 72°C for 10 min in a Perkin-Elmer 2400 model thermal cycler (Warrington, Cheshire, UK). Ten microlitres of each PCR product was separated by electrophoresis on 2% agarose gels with Tris-borate buffer. RSV positive PCR products were analysed for N gene typing by digestion with restriction endonucleases, namely *Hind*III *Pst*I, *Bgl*II, *Hae*III and *Rsa*I (Roche Diagnostics GmbH, Roche Applied Science, Nonnenwald, Penzberg, Germany) and typed into NP1 to NP6 genotypes on the base of their digestion profiles [8].

A multiplex influenza RT-PCR was used to identify influenza A and B using the primers Inf-As, Inf-Aas, Inf-Bs and Inf-Bas and a multiplex RT-PCR was utilized for the detection of para-influenza virus types 1–4 using the primers PIV-1s, PIV-1as, PIV-2s, PIV-2as, PIV-3s, PIV-3as, PIV-4s and PIV-4as [9]. A nested PCR was used to amplify the adenovirus *hexon* gene to give a 330-bp product using the primers ADH-01 and ADH-02 for a primary PCR, which was subsequently integrated with the primers ADH-11 and ADH-12 for secondary reaction. The 16S ribosomal RNA genes of *M. pneumoniae* and *Chlamydia spp*. were detected using the primers MYCOP-01 and MYCOP-02 to give a 322-bp product; and primers CHLAM-01 and CHLAM-02 to provide a 609-bp product, respectively [5]. All *Chlamydia spp*. amplicons were subjected to sequencing of both strains by Lark Technologies (Surrey, UK).

Ethical considerations

Ethical approval for the study was obtained from the Research Ethics Committees of the Liverpool School of Tropical Medicine, UK and Guilan University of Medical Sciences, Iran; informed consent was obtained from all the parents.

Results

Frequency of pathogens

A total of 261 specimens were collected from 122 hospitalized children and 139 out-patients. Hospitalized children had a median (interquartile range) age of 9 (3–22) months and were younger than the out-patients with a median of 22 (11–39) months ($p < 0.001$). Overall, 93 respiratory pathogens were detected in 83 (32%) children. RSV was the most frequent pathogen, detected in 39 (15%) cases, followed by Adv in 37 (14%), Inf V in 11 (4%),

Chlamydia spp. in 4 (2%) and *M. pneumoniae* in 2 (1%). Neither PIV nor HMPV were detected.

Pathogens subgroups and sequencing

Thirty-five (90%) of the RSV were subgroup A-NP4, three subgroup A-NP2 and one subgroup B-NP3. All the Inf V viruses belonged to group A. Four amplicons of *Chlamydia spp*. were sequenced and found to be *C. trachomatis* and two randomly selected amplicons of Adv were sequenced as Adv type 3, which is the type most often associated with ARI and pneumonia in young children.

Co-infections

Co-infections with more than one pathogen were identified in eight children. Most of these co-infections involved the presence of Adv with other pathogens, including two co-infections each with RSV, Inf A and *C. trachomatis*. Similarly Adv was recovered in two children with triple infections, together with RSV and *C. trachomatis* in one child and with Inf A and *M. pneumoniae* in another. Males predominated in all infections as shown in the Table 1.

Seasonality

The proportion of specimens from which respiratory pathogens were detected increased from 10 (25%) out of 40 samples in November to a peak of 30 (45%) out of 67 samples in January, as shown in Fig. 1 (chi-square for trend, $P = 0.06$). RSV and Adv were detected most frequently in January and February and very few cases were detected in November and March, whereas 9 of the 11 children with Inf A were detected in December.

Pathogens by location

The ratio of admission to ambulatory management varied with each pathogen, as all children with *C. trachomatis*, 33 (85%) of the 39 children with RSV, 9 (82%) of the 11 with Inf A, but only 18 (49%) of the 37 with Adv were hospitalized (chi-square for trend, $p < 0.01$), suggesting that some pathogens resulted in more severe ARI than others. Children with RSV and Inf A had slightly longer hospitalizations with a mean (standard deviation, SD) hospitalization stay of 6 (2) and 5 (2) days, respectively, than children with Adv and *C. trachomatis* who had similar means (SD) of 4 (2) days.

TABLE 1
Clinical characteristics of children with ARI by respiratory pathogens

Pathogens Variable/number	RSV $N = 39$	Adenovirus $N = 37$	Influenza A $N = 11$	*C. trachomatis* $N = 4$	*M. pneumoniae* $N = 2$
Hospitalized cases, number (%)	33 (85)	18 (49)	9 (82)	4 (100)	1 (50)
Male (%)	23 (59)	23 (62)	8 (73)	3 (75)	1 (50)
Median (IQR) age (months)	5 (3–13)	22 (12–45)*	19 (8–28)*	25 (4–52)	29 (28–30)
Mean (SD) respiratory rate	35 (8)	26 (8)*	32 (6)	33 (14)	25 (8)
Presence of *N* (%)					
Cough	39 (100)	36 (97)	11 (100)	4 (100)	2 (100)
Fever	24 (62)	32 (87)*	11 (100)*	4 (100)	2 (100)
Tachypnoea	28 (72)	10 (27)*	5 (46)	2 (50)	1 (50)
Dyspnoea	27 (69)	11 (30)*	8 (73)	2 (50)	1 (50)
Crackles	22 (56)	10 (27)*	8 (73)	1 (25)	1 (50)
Nasal congestion	13 (33)	24 (65)*	8 (73)	3 (75)	1 (50)
Chest indrawing	8 (21)	6 (16)	4 (36)	2 (50)	1 (50)
Hoarseness	3 (8)	17 (46)*	0	1 (25)	1 (50)
Otitis media	3 (8)	10 (27)*	1 (9)	0	0
Conjunctivitis	2 (5)	1 (3)	2 (18)	0	0
Sore throat	2 (5)	19 (51)*	2 (18)	0	1 (50)
Pallor	2 (5)	2 (5)	0	0	0
Unconsciousness	1 (3)	1 (3)	0	0	0
Wheeze	11 (28)	13 (35)	2 (18)	1 (25)	1 (50)
Skin rash	0	1 (3)	1 (9)	0	0
$pO_2 < 94\%$[a]	8 (24)	7 (39)	2 (22)	2 (50)	0
Chest X ray taken	31 (79)	18 (49)	8 (73)	4 (100)	1 (50)
Hyperinfiltration	20 (51)	8 (22)	5 (45)	2 (50)	0
Consolidation	16 (41)	6 (16)	3 (27)	1 (25)	0
Mean (SD) hospital stay (day)	6 (2)	4 (2)	5 (2)	4 (2)	6
Mixed infection with adenovirus[b]	2		2	2	

[a] pO_2 were obtained only for hospitalized children.
[b] In addition, one adr infection with RSV plus *C.trachomatis* nand one with Inf A and *M. pneumoniae*.
* $p<0.05$ when compared with RSV.

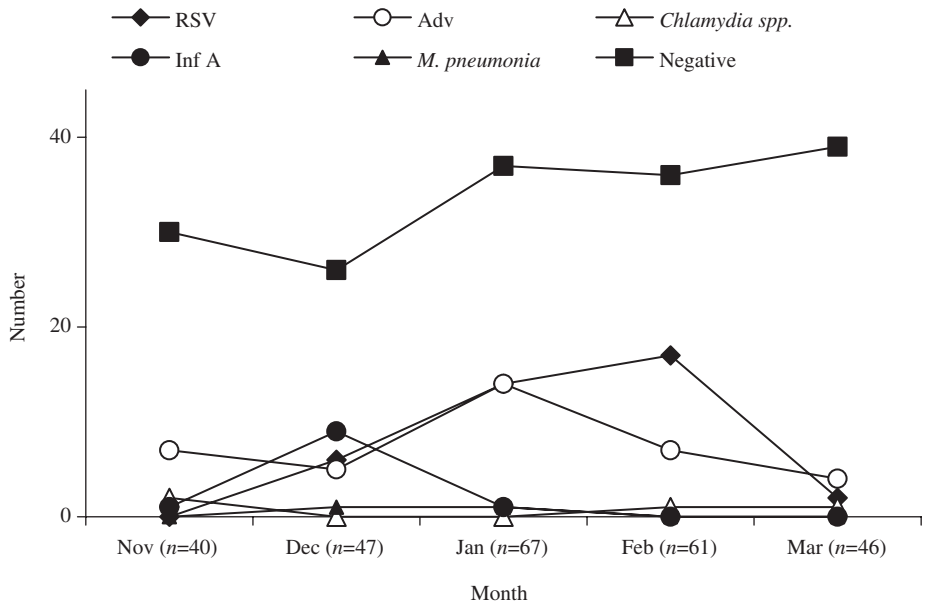

FIG. 1. Proportion of respiratory pathogens identified by month.

Age distribution

The age distribution of the children by pathogen is shown in Fig. 2. RSV infected mostly infants and 20 (51%) of the 39 children with RSV were <6 months with only five (13%) cases being ≥2 years old. In contrast, 18 (49%) of the 37 children with Adv and two children each with *C. trachomatis* (50%) and *M. pneumoniae* (100%) were ≥2 years old ($p < 0.05$ for Adv and *M. pneumoniae*), while patients with Inf A were identified across all ages.

Clinical characteristics

The clinical characteristics of the children are described in Table 1. pO_2 concentrations <94% were observed in 8 (24%) out of 33 children admitted with RSV, 7 (39%) out of 18 with Adv, 2 each out of the 4 children with *C. trachomatis* (50%) and 11 with Inf A (22%). Although a lower proportion of children with Adv were admitted (18/37), a high proportion of those admitted had hypoxia. A high proportion of patients infected with RSV, Inf A or *C. trachomatis* had signs and symptoms of acute lower respiratory infections presenting mostly with tachypnoea, dyspnoea, crackles and chest indrawings. In contrast, patients with Adv had clinical signs of predominantly upper respiratory infections including nasal congestion, hoarseness, sore throat and otitis media. A similar proportion of wheezing was observed in children with RSV, Adv and the rest of the pathogens, confirming that clinical presentation does not facilitate an aetiological diagnosis.

Discussion

Respiratory viruses are responsible for a high proportion of ARI worldwide and among these RSV is the leading pathogen in developing countries [2]. This study confirms that RSV is the virus most frequently associated with ARI in Iranian children and, similar to reports from this region, RSV groups A and B co-circulate in the same season with a predominance of group A [10–13]. Adv and Inf A were also identified in a relatively high proportion of infections (14 and 4%), which is similar to reports from other developing countries [2, 13]. Many of the children with Adv, however, were co-infected with other pathogens, mostly with RSV, Inf A and *C. trachomatis*. It is therefore difficult to determine which of these pathogens were implicated in the pathogenesis of the disease or were simply incidental findings. As children with these co-infections did not have more severe clinical symptoms than those with single Adv infections, it is possible that the latter had no pathogenic role.

Our study did not detect any HMPV, which could be due to the short duration of the study. It has been shown in other studies that HMPV seasonality does not always coincide with other viruses such as RSV [14]. This seasonal variation has also been reported from a study in the Yemen, where the HMPV incidence increased after the peak incidence for RSV [10]. HMPV therefore did not have a significant contribution during the 'bronchiolitis' season in our study which was mostly associated with other pathogens.

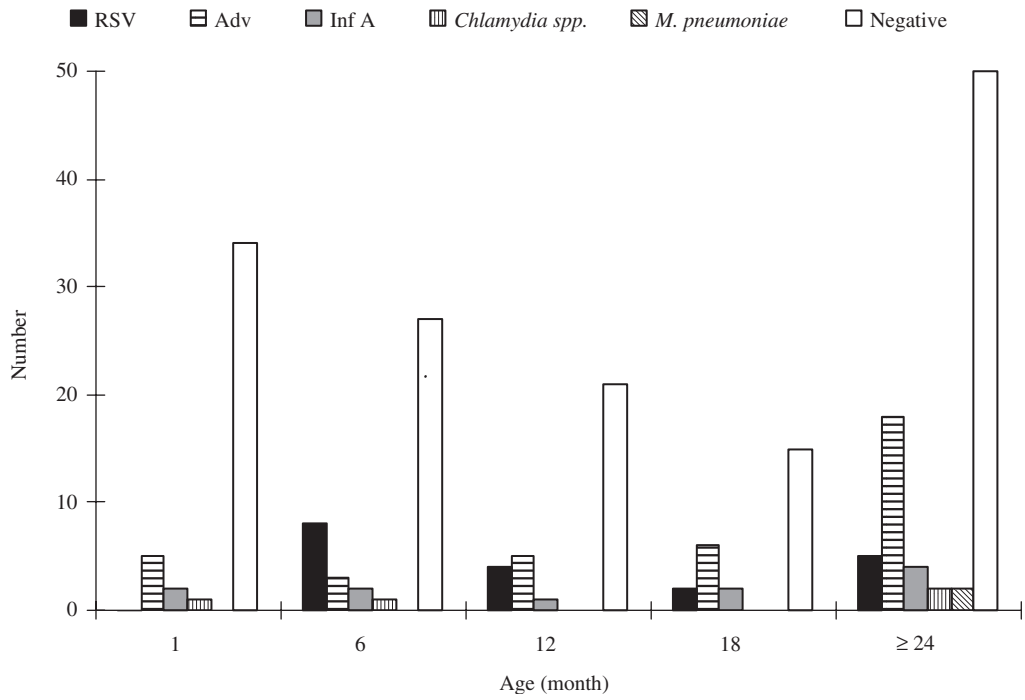

FIG. 2. Age distribution of children by respiratory pathogens.

We detected an overrepresentation of RSV and Inf A in hospitalized children, suggesting that infection with these viruses resulted in more severe ARI than with other pathogens such as Adv, which mostly occurred in older children. Despite the short duration of the survey, we detected a peak of infections with Adv in January, while RSV had a peak in February, reflecting the high frequency of respiratory viruses during the winter season.

The high frequency of co-infections, however, could be explained by the high overall frequency of the viruses in the study. Interestingly, we did not find evidence of a worsening clinical presentation with multiple infections.

Children with RSV and Inf A and *C. trachomatis* infections mostly presented with lower respiratory tract infections, while Adv was mostly associated with upper ARI. Despite these differences, however, it was not possible to differentiate children with a specific pathogen solely on the basis of their clinical signs and symptoms.

This study therefore confirms that viruses play a significant role in ARI in Iranian children and describes for the first time that RSV, Adv and Inf A are frequent pathogens in children with community-acquired ARI. This information can help improve our understanding of the epidemiology of ARI for the development of preventive and control strategies in the community.

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