Viral etiology of respiratory infections in children in southwestern Saudi Arabia using multiplex reverse-transcriptase polymerase chain reaction

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ABSTRACT

الأهداف: فحص 15 فيروس من فيروسات الجهاز التنفسي في الأطفال الذين يعانون من التهاب الجهاز التنفسي باستخدام اختبار تفاعل سلسلة انزيم البلمرة المتعدد (multiplex RT–PCR)، وتحليل المظاهر السريرية والوبائية لهذه الفيروسات.

الطريقة: شملت الدراسة على 135 طفل أقل من 5 سنوات من العمر مصابين بالتهاب الجهاز التنفسي خلال الفترة بين أكتوبر 2012م ويوليو 2013م. وتم تسجيل البيانات الاجتماعية والديموغرافية والسريرية ونتائج المختبر باستخدام استبيان موحد. تم جمع مسحتين من البلعوم لكل طفل: مسحة للفحص الميكروبي والأخرى لفحص 15 فيروس من فيروسات الجهاز التنفسي باستخدام اختبار تفاعل سلسلة انزيم البلمرة المتعدد (multiplex RT–PCR).

النتائج: كانت مسببات التهابات الجهاز التنفسي هي العدوى بفيروس واحد في 76 من المرضى، عدوى فيروسية بأكثر من فيروس في 9 من المرضى، العدوى الفيروسية والبكتيرية المختلطة في 15 حالة. تم عزل فيروس (hRSV) في 33 مريض، وفيروسات (hRhV) في 22 حالة، فيروس (hADV) في 19 مريض، فيروس (hMPV) في 13 مريض، وفيروس الإنفلونزا (hIFV) في 10 حالات، وفيروس الكورونا (hCoV) في 4 حالات، وفيروس (hBoV) في حالة واحدة.

الخاتمة: كانت فيروسات (hRSV) و (hRhV) و (hADV) هي الأكثر شيوعاً، بينما فيروسات أخرى مثل (hCoV OC43) و (hCoV NL63) و (hMPV) قد تلعب دوراً هاماً في التهاب الجهاز التنفسي في الأطفال. يعتبر اختبار (multiplex RT–PCR) مفيداً وهاماً للكشف المبكر عن ظهور فيروسات الجهاز التنفسي وخاصة في الفترة الأخيرة ومع ظهور فيروس (MERS–CoV).

Objectives: To investigate 15 respiratory viruses in children with acute respiratory tract infections (ARTIs) using multiplex reverse-transcriptase polymerase chain reaction (RT-PCR), and to analyze the clinical and epidemiological features of these viruses.

Methods: In a cross-sectional study, 135 children, ≤5 years of age who presented with ARTIs in Najran Maternity and Children Hospital, Najran, Saudi Arabia between October 2012 and July 2013 were included. The clinical and sociodemographic data, and the laboratory results were recorded using a standardized questionnaire. Two nasopharyngeal swabs were collected from each child: one for bacteriological examination, and the second for viral detection using multiplex RT-PCR.

Results: A single viral pathogen was detected in 76 patients, viral coinfections in 9, and mixed viral and bacterial pathogens in 15. Respiratory syncytial virus was isolated in 33 patients, human rhinovirus (hRV) in 22, adenovirus (AdV) in 19, human metapneumovirus in 13, influenza virus in 10, parainfluenza virus in 7, human corona virus (hCoV) in 4, and human bocavirus in one.

Conclusion: Respiratory syncytial virus, hRV, and AdV were the most frequent viruses, accounting for more than two-thirds of the cases. Other viruses, such as MPV, hCoV NL63, and hCoV OC43, may play a role in pediatric ARTIs. Of significance is the potential use of multiplex RT-PCR to provide epidemiological and virological data for early detection of the emergence of novel respiratory viruses in the era of the Middle East respiratory syndrome coronavirus.

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Acute respiratory tract infections (ARTI's) are a leading cause of infectious disease-related morbidity and mortality among children under 5 years of age worldwide, particularly in the developing countries.[1] The ARTI's can be caused by bacteria and fungi, but viral infections are the most common causes. In developing countries, viruses represent a considerable proportion of the pathogens associated with ARTI's, varying from 40-90% across studies.[2-4] The most frequently implicated viruses among children are respiratory syncytial virus (RSV), influenza A and B viruses (IAV and IBV), parainfluenza viruses (PIVs), adenoviruses (AdV), human rhinoviruses (hRV), and human enteroviruses (hEV). Several recently discovered viruses, such as human metapneumovirus (hMPV), human bocavirus (hBoV), and the human coronaviruses (hCoVs) (NL63 and OC43), have been identified as potential respiratory pathogens. In addition, 2 novel human polyomaviruses (Washington University virus [WU] and Karolinska Institute virus [KI]) have been detected in patients with respiratory infections.[5-7] Lower respiratory tract infections (LRTI's) are the most severe forms of infection and cause of approximately 1.9 million annual deaths among children,[8] while upper respiratory tract infections (URTI's) are the most frequent presentation of respiratory infections. An estimated 50% of all illnesses in all age groups, and approximately 75% of illnesses in young children are viral URTI's.[9] Proper diagnosis of viral ARTI's has been shown to reduce the misuse of antibiotics and shorten the length of hospital stay. However, current challenges in detecting respiratory viruses include inconsistent clinical manifestations of viral RTI's and variable sensitivity and specificity of available diagnostic assays. The traditional diagnostic methods for the respiratory viruses including cell culture and direct immunofluorescence assays are time consuming and technically demanding.[10] Several types of molecular biological methods, including reverse transcription PCR (RT-PCR), PCR-hybridization, and real time PCR, have been introduced as more rapid and sensitive detection methods for respiratory infections.[3,5,11-13] Most studies in Saudi Arabia focused on a small number of viral pathogens, and information on the relative contribution of each pathogen to clinical forms of ARTI's is incomplete.[14-16] Hence, the aims of our study were to investigate the distribution of 15 respiratory viruses in children with ARTI's using multiplex RT-PCR, and to evaluate the presenting clinical, demographic, and epidemiological features of these different viral infections.

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Methods. This study was conducted in Najran Maternity and Children Hospital, a 200-bed, tertiary-care hospital in Najran, southwestern Saudi Arabia between October 2012 and July 2013. Included in the study were children ≤5 years of age who presented with signs and symptoms of upper and/or lower respiratory tract infection seen in the pediatric emergency room, or admitted to the pediatric ward, or the pediatric intensive care unit (PICU). Written consent was obtained from their parents. Inclusion criteria were as follows: a temperature of ≥38^{0}C, and at least one of the following: rhinitis, pharyngitis, cough, earache, hoarseness of voice, rhonchi, crepitations, or wheezy chest. The diagnosis for each patient was performed by the attending pediatrician based on the World Health Organization (WHO) standard clinical criteria.[17] The clinical and socio-demographic data and routine laboratory tests results were recorded using a standardized questionnaire, including the age, gender, underlying medical conditions, signs and symptoms at presentation, findings on physical examination, laboratory findings (complete blood count [CBC], C-reactive protein [CRP], and bacterial cultures), and definitive clinical diagnosis.

Sampling. Two nasopharyngeal swaps were collected for each patient under strict infection control guidance using rayon-budded swabs with 2 ml of virus transport medium (Virocult, Wiltshire, UK); one for bacteriological examination and the other for respiratory virus detection using RT-PCR multiplex. The specimens were transported in an icebox to the Microbiology Department, College of Medicine, Najran University for further processing.

Bacteriological examination. Each nasopharyngeal sample from each case was inoculated on blood and MacConkey agar media (Difco Laboratories, Detroit, MI, USA). The plates were incubated at 37^{0}C for 24-48 hours. Suspected colonies were identified as pathogenic organisms using standard laboratory procedures according to the biochemical and serological techniques recommended by the WHO.[18]

Multiplex RT-PCR analysis. Viral nucleic acids were extracted from the nasopharyngeal samples using QIAamp viral mini kit (Qiagen, Maryland, USA)

according to the manufacturers' instructions. The total RNAs extracted from the clinical samples were used for the synthesis of the first-strand cDNAs (Revert AidKit, Fermentas, Canada) according to the manufacturer's instructions. The sample cDNAs were tested in a 3-tube reaction following the protocol supplied by the manufacturer (Qiagen, Maryland, USA). Tube-1 contained primers set A targeting AdV, hCoV 229E/NL63, and PIV1-3. Tube-2 contained primers set B targeting hCoV OC43/HKU1, hRV A/B/C, RSV A/B, and IAV. Tube-3 contained primers set C targeting hBoV 1/2/3/4, IBV, hMPV, PIV4, and hEV. Positive controls included a mixture of all 15 virus clones. The negative control contained only double distilled water. The PCR amplification was performed using 10 µl of Seeplex RV master mix (Seegene, Seoul, Korea), 4 µl of 5X multiplex primer sets, 3 µl of 8-MOP solution, and 3 µl of newly synthesized first-strand cDNA (1 µg/3 µl). The Seeplex RV contained A, B, and C sets of primers designed from highly conserved regions of genetic sequences for the 15 respiratory viruses. An initial pre-PCR step of 94°C for 15 minutes was performed in a thermocycler 9600 (Perkin-Elmer, Ohio, USA), followed by a total of 35 PCR cycles under the following conditions: 94°C for 30 sec, 60°C for 1.5 min, and 72°C for 1.5 min. The final cycle was followed by an extension step at 72°C for 10 min. The amplified PCR products were separated on 2% agarose gel and stained with ethidium bromide. The type of respiratory virus was identified by comparison with the reference band size provided by the manufacturer.

Statistical analysis. Values were expressed as percentages for discrete variables, or as mean and standard deviation for continuous variables. Clinical characteristics and laboratory variables were compared using the chi-square or the Student t-test or Fisher's exact test, as appropriate. Statistical significance was defined as a *p*-value less than 0.05. All analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 15.0 (SPSS Inc., Chicago, IL, USA).

Results. A total of 135 patients were included in the study period. The median age of patients was 12 months, and the number of males was 80 (63.3%). A single viral pathogen was detected in 76 (56.3%); viral co-infections in 9 (6.7%); and mixed viral and bacterial pathogen in 15 (11.1%) patients. The specific pathogens were RSV in 33 patients, hRV in 22, AdV in 19, hMPV in 13, IFV in 10, PIV's in 7, hCoV in 4, and hBoV in one) (**Table 1**). Children were divided into 5 age-groups: ≤12 months (n=73; 54.1%), 13-24 months (n=32; 23.7%), 25-36 months (n=9; 6.7%), 37-48 months (n=9; 6.7%), and 49-60 months (n=12; 8.9%). The infection rate in children ≤12-month age was significantly higher than in the other age groups. Interestingly, we observed a significantly larger number

Table 1 - Distribution of viral etiologic agents and co-infections.

Respiratory viruses	Types of infection			Total (%)
	Single viral	Viral coinfection	Mixed viral and bacterial*	
RSV	26 (23.9)	5 (4.6)	2 (1.8)	**33 (30.3)**
RSV A	26 (23.9)	3 (2.8)	1 (0.9)	**30 (27.5)**
RSV B	0 (0)	2 (1.8)	1 (0.9)	**3 (2.8)**
hRV	16 (14.7)	4 (3.7)	2 (1.8)	**22 (20.2)**
AdV	12 (11.0)	0 (0)	7 (6.4)	**19 (17.4)**
hMPV	6 (5.5)	7 (4.6)	0 (0)	**13 (11.9)**
IV	7 (6.4)	0 (0)	3 (2.8)	**10 (9.2)**
IAV	6 (5.5)	0 (0)	2 (1.8)	**8 (7.4)**
IBV	1 (0.9)	0 (0)	1 (0.9)	**2 (1.8)**
PIV	6 (5.5)	0 (0)	1 (0.9)	**7 (6.4)**
PIV-1	5 (4.6)	0 (0)	0 (0)	**5 (4.6)**
PIV-3	1 (0.9)	0 (0)	1 (0.9)	**2 (1.8)**
hCoV	2 (1.8)	2 (1.8)	0 (0)	**4 (3.7)**
hCoV NL63	1 (0.9)	1 (0.9)	0 (0)	**2 (1.8)**
hCoV OC43	1 (0.9)	1 (0.9)	0 (0)	**2 (1.8)**
hBoV	1 (0.9)	0 (0)	0 (0)	**1 (0.9)**

Data are expressed as number and percentage (%)
*mixed viral and bacterial infection: *Staphylococcus aureus* (*S. Aureus*) with respiratory syncytial virus (RSV) in 2 patients, *Staphylococcus aureus* with adenoviruses (AdV) in 2 patients, *S. Aureus* with human rhinoviruses (hRV) and AdV in 2 patients, *S. Aureus* with IV and parainfluenza viruses (PIVs), in one patient, *Staphylococcus pneumoniae* with AdV and IV in 2 patients and *Staphylococcus pneumoniae* with AdV in one patient. IAV and IBV - influenza A and B viruses, hEV - human enteroviruses, hMPV - human metapneumovirus, several recently discovered viruses, such as human metapneumovirus, hBoV - human bocavirus, hCoVs - human coronaviruses (NL63 and OC43), RSV - respiratory syncytial virus

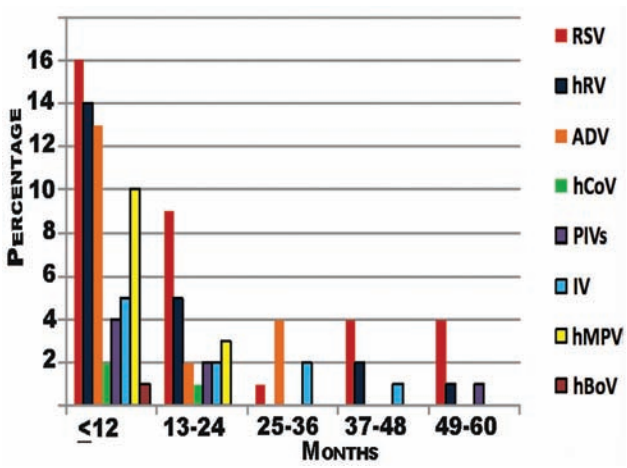

Figure 1 - The distribution of viral agents according to age among the studied patients.

of positive cases for RSV infection in patients aged <24 months (25/34 versus 9/34, p=0.01). Moreover, hMPV and hCoV infections were detected only in children <24 months of age, p=0.001 (**Figure 1**).

Demographic data and clinical presentation of patients were compared to general pathogens identified (**Table 2**) and the individual viral pathogens (**Table 3**). Generally, there were no significant differences in the clinical presentation, as well as, the gender of patients for the various pathogens isolated. However, the median age of children with hMPV was significantly higher than that of children infected with the other viruses (p=0.036). Wheeze was more frequently associated with viral infections (p=0.005) mainly RSV (p=0.01).

Table 2 - Demographic and clinical presentations of the studied patients.

Presentation	Microbial isolation					*P*-value
	None (n=22)	Virus (n=85)	Bacteria (n=13)	Bacteria and viruses (n=15)	Total (n=135)	
Age (months) median	14	10.5	6.5	7	**12**	0.186
Gender (males)	12 (54.5)	54 (63.5)	9 (69.2)	8 (53.3)	**83 (61.5)**	0.498
Smoking among parents	17 (77.3)	63 (74.1)	10 (76.9)	11 (73.3)	**101 (74.8)**	0.594
Number of siblings (>4)	9 (40.9)	39 (45.9)	5 (38.5)	8 (53.3)	**61 (45.2)**	0.476
Temperature median	39	39	39	39	**39**	0.724
Tachypnea	7 (31.8)	31 (36.5)	4 (30.8)	1 (6.7)	**43 (31.9)**	0.124
Wheeze	9 (40.9)	62 (72.9)	5 (38.5)	10 (66.7)	**86 (63.7)**	0.005
Chest in drawing	10 (45.5)	48 (56.5)	4 (30.8)	8 (53.3)	**70 (51.9)**	0.769
Difficulty in breathing	6 (27.3)	33 (38.8)	4 (30.8)	5 (33.3)	**48 (35.6)**	0.521
Use of accessory muscles	10 (45.5)	53 (62.4)	4 (30.8)	8 (53.3)	**75 (55.6)**	0.666
Hoarseness of voice	3 (13.6)	6 (7.1)	0 (0)	1 (6.7)	**10 (7.4)**	0.133
Diarrhea	11 (50.0)	35 (41.2)	1 (7.7)	6 (40.0)	**53 (39.3)**	0.122
URTI	11 (50.0)	28 (32.9)	7 (53.8)	6 (40.0)	**52 (38.5)**	0.485
Bronchiolitis	8 (36.4)	40 (47.1)	4 (30.8)	4 (26.7)	**56 (41.5)**	0.747
Pneumonia	3 (13.6)	17 (20.0)	1 (7.7)	6 (40.0)	**27 (20.0)**	0.404
TLC Median	9800	11000	12400	11200	**11200**	0.167
CRP	6 (27.3)	28 (32.9)	3 (23.1)	4 (26.7)	**41 (30.4)**	0.914

URTI - upper respiratory tract infections, TLC - total leucocytic count median, CRP - C-reactive protein

Table 3 - Demographic and clinical presentations of the studied patients infected with individual respiratory viruses.

Presentation	Respiratory viruses								*P*-value
	hRV (n=22)	RSV (n=33)	AdV (n=19)	hCoV (n=4)	PIVs (n=7)	IVs (n=10)	hMPV (n=13)	hBoV (n=1)	
Age (months) median	8	15	13	15	15	8	4.5	10	0.036
Gender (males)	14 (63.6)	23 (69.7)	11 (57.9)	3 (75)	4 (57.1)	6 (60)	4 (30.8)	0 (0)	0.506
Smoking among parents	18 (81.8)	24 (72.7)	13 (68.4)	3 (75)	5 (71.4)	10 (100)	9 (69.2)	0 (0)	0.743
No of siblings (>4)	10 (45.5)	18 (54.5)	10 (52.6)	2 (50)	4 (57.1)	6 (60)	4 (30.8)	0 0)	0.702
Temperature median	39	39	38.5	39	38.5	39	39	39	0.756
Tachypnea	7 (31.8)	15 (45.5)	2 (10.5)	2 (50)	2 (28.6)	1 (10)	4 (30.8)	0 0	0.296
Wheeze	11 (50.0)	31 (93.9)	6 (31.6)	2 (50)	5 (71.4)	4 (40)	6 (46.2)	1 (100)	0.010
Chest indrawing	8 (36.4)	25 (75.8)	3 (15.8)	2 (50)	2 (28.6)	4 (40)	6 (46.2)	1 (100)	0.274
Difficulty in breathing	6 (27.3)	16 (48.5)	3 (15.8)	1 (25)	2 (28.6)	1 (10)	11 (84.6)	0 (0)	0.249
Use of accessory muscles	8 (36.4)	26 (78.8)	6 (31.6)	1 (25)	2 (28.6)	4 (40)	11 (84.6)	1 (100)	0.102
Hoarseness of voice	1 (4.5)	3 (9.1)	3 (15.8)	0 (0)	0 (0)	0 (0)	2 (15.4)	0 (0)	0.289
Diarrhea	12 (54.5)	10 (30.3)	11 (57.9)	0 (0)	3 (42.9)	6 (60)	4 (30.8)	0 (0)	0.902
URTI	17 (77.3)	1 (3.0)	16 (84.2)	0 (0)	1 (14.3)	6 (60)	0	0 (0)	0.122
Bronchiolitis	4 (18.2)	13 (39.4)	3 (15.8)	2 (50)	5 (71.4)	4 (40)	11 (84.6)	1 (100)	0.077
Pneumonia	1 (4.5)	20 (60.6)	0 (0)	1 (25)	1 (14.3)	0 (0)	2 (15.4)	0 (0)	0.370
TLC median	9800	12800	10800	12000	9900	9900	7800	7400	0.861
CRP	4 (18.2)	18 (54.5)	5 (26.3)	0 (0)	2 (28.6)	0 0 (0)	4 (30.8)	0 (0)	0.843

URTI - upper respiratory tract infections, TLC - total leucocytic count median, CRP - C-reactive protein, hRV - human rhinoviruses, RSV - respiratory syncytial virus, AdV - adenoviruses, hCoVs - human coronaviruses, PIV - parainfluenza virus, IV - influenza virus, hMPV - human metapneumovirus, hBoV - human bocavirus

Discussion. Viral infection is a major cause of respiratory illness among children. The frequency of respiratory virus detection in 135 children with ARTI's, in this study, was 80.7%. This finding is in support of previous studies, which reported viral detection rates of 47-95% in children.[2-5,19] Possible explanations for the wide differences in detection rates in the literature include heterogeneity in study populations, differences in presenting respiratory symptoms, number of respiratory pathogens tested, method used for detection and genetic variability between populations.[2,20] Overall, in 6.7% of the patients, viral co-infections were found. Previous studies using RT PCR techniques reported viral co-infection rates of 5-10%, with RSV, hRV, PIVs, and hMPV being the most commonly implicated viruses in cases of mixed infections.[12,19-21] In this study, the high detection rate of RSV with hMPV (4/9; 44.4%) and hRV with hCoV (2/9, 22.2%) in viral co-infections was an interesting finding. Huijskens et al[3] found that in 67% of the hRV-positive patients and 42% of the RSV cases, hRV and RSV were co-detected with another virus.

It has been reported that the unique characteristic of RSV facilitates infection with a second respiratory virus.[22,23] Previous studies reported that hRV in URTI's could serve as a clinical illness promotion factor, functioning additively or synergistically in the pathogenesis of lower respiratory syndromes such as bronchiolitis.[9,23,24] However, identification of 2 or more viruses in a patient may be due to prolonged viral shedding or asymptomatic persistence of viruses.[5] Further, work would be needed to clarify this situation. Quantitative identification of the viral genome may help in explaining co-infections.

As expected, most of the respiratory pathogens in this study were detected in children <1 year. The higher detection rate of respiratory pathogens among infants and young children has been ascribed to a higher infection rate, lower viral clearance rate due to underdeveloped immune system, and higher load of the infectious agent associated with living conditions such as crowding.[2,3,20] Furthermore, the parents of younger children may seek healthcare earlier in the course of disease due to parental anxiety. Our results confirm that RSV and hRV play a key role in RTI's in children; RSV was the most frequent respiratory virus detected, followed by hRV. The predominance of RSV in this study is in accordance with the assertion that this virus is the single most frequent lower respiratory tract pathogen in infants and young children in Saudi Arabia and worldwide.[4,5,15,20] It would be important for local pediatricians to use antibiotics cautiously when children are hospitalized with ARTI's. The hRV has been known to be responsible for URTI's and some LRTI's in children.[19,24] Our findings showed that the presence of hRV in all age groups. Most hRVs (17/22; 77.3%) were associated with URTI's and 22.7% of hRV were detected in bronchiolitis (4 cases), or pneumonia (one case).

Adenovirus was reported to be responsible for 5-10% of ARTI's in children, and has been largely associated with bronchiolitis obliterans and acute wheezing episodes.[25-27] In our study, ADVs were found in 17.4% of all patients, being the third most frequent viral pathogen, and most ADVs (16/19; 84.2%) were associated with URTI's. The hMPV is one of the causes of upper and lower RTI's, especially in preschool and older children. In our study, hMPV was the fourth most frequent viral pathogen accounting for 11.9% of all cases. In a previous Saudi study,[14] hMPV was identified in 8.3% of 489 children with ARTI's. In contrast to some studies,[12,26,28] all hMPV-positive cases in this study were below 2 years of age and almost half of the cases (7/13; 54%) were co-infected with other viruses. The detection rates for IVs and PIVs in our study were comparable with other studies, reporting rates of 0.8-12.6% for IV's and 2.8-19.4% for PIV's.[3,5,13,19,20,23,26]

In our study, hCoVs were detected in 3.7% of all patients including 2 cases with bronchiolitis and 2 cases with pneumonia. In a previous Saudi study,[14] hCoVNL63 type was detected in 2.8% of 489 children.[14] However, in our study 2 hCoV types (hCoV NL63 and hCoV OC43) were reported. In September 2012, a novel human coronavirus, called the Middle East respiratory syndrome coronavirus (MERS-CoV), was first identified in samples obtained from a Saudi Arabian businessman who died from acute respiratory failure.[29] As of May 2013, a total of 49 confirmed cases of MERS-CoV infection with 26 deaths have been reported to the WHO, including 37 in Saudi Arabia with 21 deaths.[30] These figures highlight the characteristic distribution of hCoVs emerging types circulating in Saudi Arabia.

The similar clinical presentations of patients infected by various respiratory viruses and some bacterial pathogens make etiological diagnoses difficult when decisions are based only on clinical presentation.[3-5,12] In our study, except the strong association between RSV infection and chest wheeze, the clinical manifestations of other viral infections were largely nonspecific with fever and cough as the main symptoms.

One limitation of this study is the inability to describe the seasonal distribution of respiratory viruses due to the short duration of the study. Further, molecular-based

studies of longer surveillance duration are necessary to elucidate the seasonal patterns and disease burden associated with respiratory viral pathogens.

In conclusion, our study provided background information concerning the respiratory viral etiology in Najran, southwestern Saudi Arabia. The RSV, hRV and AdV were the most frequent pathogens, accounting for over than two-thirds of cases with ARTI's. However, other emerging viruses as hMPV, hCoV NL63 and hCoV OC43 seem to play an important role in pediatric LRTI's. Moreover, the use of multiplex RT-PCR is needed to provide not only epidemiological and virological data, but also the opportunity to understand the emergence of novel respiratory viral pathogens ahead of time.

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