

Characterization of circulating respiratory viruses in the Riyadh region of Saudi Arabia by real-time multiplex PCR

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Respiratory viruses are common in causing acute respiratory illnesses with significant annual morbidity and mortality. Detection of these viruses is critical to guiding appropriate patient management and infection control. To date, many viral infections are under-reported or misdiagnosed due to the limitations of current techniques available for routine respiratory viruses testing. Thus, we aimed to characterize circulating human respiratory viruses causing acute respiratory disease and hospitalization using real-time multiplex PCR. Of the 129 samples tested for human respiratory viral infections, 96 (74.42%) samples were positive for at least one virus, whilst the remaining 33 (25.58%) samples were negative for all. Among the positives, 60 (62.5%) cases were infected with a single respiratory virus, compared with 36 (37.5%) cases infected with multiple respiratory viruses. Throughout the study period, the most prevalent viruses detected in both single- or multiple-infections were human rhinovirus (HRV) with 42 (43.75%) positive cases followed by human metapneumo virus, respiratory syncytial virus B, and influenza A virus infections with 20 (20.83%), 17 (17.71%), and 14 (14.58%) positive cases each, respectively. In summary, 45.7% of all hospitalized cases were infected with a single respiratory virus, and HRV was characterized as the dominant virus causing acute respiratory illness in Riyadh. Continued monitoring of circulating respiratory viruses in the region is necessary to understand their epidemiology.

Key words: Respiratory viruses, multiplex real-time PCR, Riyadh.

Respiratory viruses are known to cause respiratory diseases in both humans and animals throughout the year¹⁻². Over 24 viruses and subtypes have been classified to date including influenza virus (IFV) type A and B, parainfluenza virus (PIV) type 1, 2, 3, and 4, respiratory syncytial

virus (RSV) type A and B, human metapneumovirus (HMPV), adenovirus (ADV), coronavirus (COV), human rhinovirus (HRV), human enterovirus (HEV), human bocavirus (HBOV). Some of these viruses have been reported to infect humans of different age groups³⁻⁵. Viruses such as HRV and ADV tend to cause mild upper respiratory symptoms. On the other hand, IFV, PIV, and RSV tend to cause severe lower respiratory illnesses with significant annual morbidity and mortality⁶. Detection of these viruses is critical to patient management, infection control and epidemiological

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studies. Current virological techniques used for the detection of respiratory viruses include tissue culture, direct immunofluorescence assay (DIFA), rapid detection tests, and polymerase chain reaction (PCR)⁷⁻⁸. These methods differ in their sensitivity and specificity, and are mostly designed to identify the major respiratory viruses such as RSV, IFV, PIV, ADV and HEV. For example, several studies from the Riyadh region which used either DIFA, tissue culture or both to detect respiratory viruses in young children admitted to hospital, were only able to detect five respiratory viruses: RSV, ADV, IFV, HEV, and PIV.⁹⁻¹² To date, many viral infections are under-reported or misdiagnosed due to variability in the sensitivity of kits available for the detection and screening of respiratory viruses. In addition, limited information is available about respiratory viruses currently circulating in the Riyadh region, and whether infections are caused by single or multiple respiratory viruses. Thus, in this study, multiplex PCR was used, to determine the most prevalent respiratory viruses causing acute lower respiratory illnesses in Riyadh during the November–March period.

MATERIAL AND METHODS

Patients study group and sample collection

This study was conducted between November 2013 and March 2014 at the King Khalid University Hospital (KKUH), Riyadh. A total of 129 nasopharyngeal aspirate or swab samples were collected in viral transport media (remel MicroTest™ M4RT® transport) from patients hospitalized for symptoms of acute lower respiratory illness. These included 82 males and 47 females, aged between 1 and 83 years. Samples were received by the Virology laboratory at KKUH, and analyzed by real-time multiplex PCR as described below. This study was approved by, and performed according to the guidelines of The KKUH and College of Medicine Institutional Review Board (IRB) committee.

Nucleic acid extraction and reverse transcription

Total viral nucleic acid was extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Indianapolis, IN). According to the protocol, 400 µL of each sample was used for extraction, and eluted (with elution buffer) in a final volume of 100 µL. During the final

step, 10 µL of internal control was added to each extract.

Viral RNA was then reverse transcribed into cDNA using the cDNA Synthesis Premix (V1.1) kit (Seegene), according to the manufacturers' instruction. Briefly, each 8 µL of extracted nucleic acid (with internal control) was mixed with 10 µL of premix master mix and 2 µL of random hexamer. Reactions were performed on the thermocycler Primus 96 plus (MWGAG BIOTECH) as follows: 1 cycle at 25°C for 5 mins followed by 1 cycle at 37°C for 1 hour, and a final cycle at 95°C for 2 mins.

Qualitative real-time PCR

The amplification and detection of respiratory viruses was performed using the Anyplex™ II RV16 Detection kit (Seegene) on the CFX96 real-time PCR system (BIO-RAD). The RV16 kit allows for the simultaneous detection of 16 respiratory viruses including IFV type A and B, PIV type 1, 2, 3, and 4, RSV type A and B, HMPV, HRV, ADV, HEV, HBOV, COV types NL63, 229E, and OC43 via two different primer sets (A and B). Briefly, for each tested sample, 8 µL of cDNA was mixed with 5 µL of 4X RV16 set A primer, 5 µL of 4X Anyplex PCR master mix and 2 µL RNase-free water. In a separate reaction, another 8 µL of the same sample was mixed with 5 µL of 4X RV16 set B primer, 5 µL of 4X Anyplex PCR master mix and 2 µL RNase-free water. Reactions were performed on the CFX96 real-time PCR system, following the conditions recommended in the Anyplex RV16 protocol.

Statistical analysis

Data was collected and entered into Microsoft Office Excel for ease of handling and statistical analysis. The percentage and the mean with standard deviation were used where applicable. The Mann-Whitney test, Fisher's Exact test, and Z-test of proportion drawn from two samples were applied for comparisons to determine the p value. The significance level was established at $p < 0.05$.

RESULTS

During the period November 2013 to March 2014, 129 samples were screened for respiratory viral infections. Ninety six (74.42%) of these were positive for at least one respiratory virus, and 33 (25.58%) were negative for all (Fig. 1). Among the ninety six positives, a single infection

Table 1. The distribution of the respiratory viruses detected throughout the study period

Virus	Age [mean \pm SD (range)] year	Nov,13	Dec,13	Jan,14	Feb,14	Mar,14	Total
IFVA	16.4 \pm 23.4(1–75)	8	2	1	–	3	14
IFVB	58 \pm 24 (41–75)	2	–	–	–	–	2
PIV1	5.25 \pm 4.5 (5–11)	1	3	–	–	–	4
PIV2	1	–	–	–	–	1	1
PIV3	1 \pm 0 (1–1)	–	2	1	–	–	3
PIV4	4 \pm 3.6 (1–8)	1	1	–	1	–	3
RSVA	8.5 \pm 16.4 (1–42)	2	2	2	–	–	6
RSVB	3.6 \pm 3 (1–12)	4	6	1	6	–	17
HMPV	3.5 \pm 2.8 (1–11)	1	4	8	5	2	20
HRV	6 \pm 11.3 (1–66)	19	17	2	2	2	42
ADV	3.9 \pm 3.5 (1–12)	1	2	–	4	3	10
HEV	4.1 \pm 4 (1–12)	5	4	2	–	–	11
HBOV	3.7 \pm 2.5 (1–8)	3	2	1	1	–	7
COVNL63	8	1	–	–	–	–	1
COV229E	1	1	–	–	–	–	1
COVOC43	6.2 \pm 5.1 (1–12)	1	–	1	–	4	6

IFV: influenza virus; PIV: parainfluenza virus; RSV: respiratory syncytial virus; HMPV: Human metapneumovirus; HRV: human rhinovirus; ADV: adenovirus; HEV: human enterovirus; HBOV: human bocavirus; COV: coronavirus.

was detected in 60 (62.5%) cases, while the remaining 36 (37.5%) cases were classified as multiple infections (Fig. 1). The rate of single infections was more than twofold higher, 70% (42 cases, $p=0.0094$) in the period (between November and December in comparison with the period between January and March, 30% (18 cases) (Fig. 1). However, no significant differences were observed with the rate of multiple infections (Fig. 1). Throughout the study period, HRV was the most prevalent virus (42 cases, 43.75%) in both single and multiple infections. This was followed by

HMPV, RSVB, and IFVA with 20 (20.83%), 17 (17.71%), and 14 (14.58%) positive cases each, respectively (Fig. 2). Table 1 shows the distribution of the respiratory viruses detected by month during the study period. Between November and December 2013, the most prevalent virus detected was HRV, with 36 (37.5%) positive cases followed by RSVB, IFVA, and HEV infections representing 10 (10.42%), 10 (10.42%), and 9 (9.37%) positive cases each, respectively (Table 1). Interestingly this pattern changed between January and March 2014, with the highest infections observed by

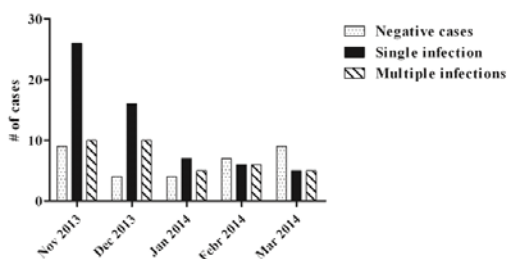


Fig. 1. Seasonality of single and multiple respiratory viral infections in Riyadh. The bar graph shows the positive and the negative samples tested. White, dotted bars represent negative cases. Black bars represent positive single respiratory viral infections. Striped bars represent positive multiple respiratory viral infections.

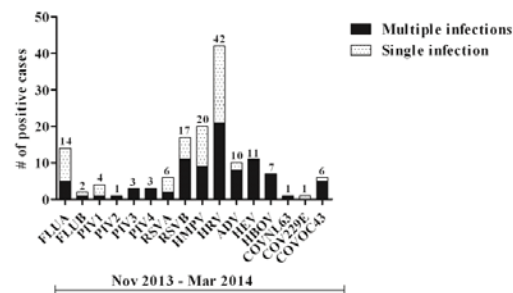


Fig. 2. Circulating respiratory viruses detected in Riyadh during the study period. The bar graph shows positive samples detected by the multiplex-PCR as indicated with numbers. White, dotted portions of the bars represent the occurrence of the virus as a single infection. Black portions of the bars represent the occurrence of the virus as multiple infections.

HMPV accounting for 15 (15.62%) positive cases followed by RSVB, ADV and HRV, with 7 (7.29%), 7 (7.29%), and 6 (6.25%) positive cases each, respectively (Table 1). All cases of HEV ($p=0.03$) and HBOV ($p=0.026$) throughout the study period were detected only as multiple infections (Fig. 2). Furthermore, most of the respiratory viral infections were observed in young children (4.80 ± 7.72 years), with the exception of IFV, which was more frequent in adults and the elderly (21.63 ± 26.76 years, $p=0.032$) (Table 1).

DISCUSSION

This is the first report within the Riyadh region using sensitive real-time multiplex PCR to monitor winter respiratory viruses causing acute respiratory illness. During the study period, most infections were caused by HRV followed by HMPV and RSVB. Real-time multiplex PCR has a relatively high sensitivity and specificity compared with traditional techniques such as DIFA, rapid tests, or tissue culture. Studies from the same region have been unable to report infections caused by HRV and HMPV due to the limitation of the analytical techniques used. Jamjoom and colleagues (1993) in their study of children admitted to a Riyadh hospital observed that 69 (54%) out of 127 nasopharyngeal aspirate samples were positive for RSV by DIFA. Among those positives, only a few cases could be cultured and produced two positive isolates.¹² Two other reports of young children analyzed over three and six year periods, revealed that only five viruses were positively identified by DIFA: RSV (79–95% prevalence), PIV3 (1.2–8%), IFVA (3–6%), IFVB (3%), and ADV (2–3%).^{10–11} The limitation of tissue culture for the analysis of respiratory viruses was shown by Al-Hajjar and colleagues.⁹ Their study of viral infections in infants and young children over a three-year period showed that 256 samples were positive by tissue culture, but again only five viruses could be isolated: RSV (28.5%), ADV (27.3%), IFV (23.8%), HEV (15.2%), and PIV (2.3%).⁹ Recently, two studies from the Mecca region using real-time multiplex PCR to detect respiratory viruses demonstrated that HRV was the most predominant circulating virus causing respiratory symptoms in pilgrims, and healthcare workers serving pilgrims during the Hajj period.^{13–14} Other similar studies

conducted in Europe, America and Asia also observed HRV to be one of the most frequent viruses causing respiratory symptoms.^{15–17} Thus, the selection of sensitive and specific methods for the detection and correct identification of respiratory viruses is critical to the accurate reporting of the causative pathogen. It is well known that HRV usually cause mild respiratory symptoms such as the common cold. However, our report shows that HRV infection may also cause acute respiratory symptoms, often requiring hospitalization. This data is consistent with other studies from Europe and Asia that have shown HRV to be capable of causing severe respiratory illnesses in both children and adults.^{18–20}

In the current study, we were able to detect sixteen respiratory viruses in the study population. However, variations were observed in the seasonal distribution patterns and rates of infection of these viruses. Seasonal distributions of HRV, IFV, RSV, HEV, and HBOV were observed between November and December 2013, whilst other viruses such as HMPV, COV, and ADV were observed between January and March of 2014. Between November and December 2013, the highest rates of infection were due to HRV followed by RSVB, IFVA, and HEV. This changed slightly between January and March 2014, with most infections being caused by HMPV followed by RSVB, ADV, and HRV. One report from a Riyadh region similarly observed peak seasonal distribution of IFVA between November and December, which was replaced by RSV as the most prevalent virus between January and February.⁹ Another study conducted in France showed peak seasonality of IFVA between November and December, while the seasonality of RSV and HMPV was greatest between December and February²¹.

In this study, the rate of single infections was higher than that of multiple infections. This has been observed in other studies.^{15, 22–23} However, these studies did not specify the seasonality of single- versus multiple-viral infections. We observed single infections to be greater than twofold higher in the period (between November and December) compared with (January to March). It has been proposed that environmental factors, such as humidity and temperature influences, significantly affect the infectivity rate of respiratory viruses. One report in a guinea pig model

demonstrated that the infectivity rate of IFV correlated inversely with increased humidity and temperature.²⁴ Thus, it is possible that in winter, the cold weather preserves more viruses in the environment for a longer period, and therefore more viruses are available to be acquired.

Our data suggests that most respiratory viruses affected young children (< 10 years old), with the exception of IFV which appeared to affect more adults and the elderly. Other studies have also similarly reported increased rates of IFV infection in elderly people²⁵⁻²⁶. Finally, of the 37 (27.41%) specimens that were negative for all viral infections (although corresponding patients displayed symptoms of acute respiratory infection), we cannot exclude the presence of respiratory pathogens not detected by our multiplex PCR. In these cases, further investigations are necessary.

The lack of sensitivity of virological techniques such as tissue culture and DIFA in characterizing respiratory viruses such as HRV and HMPV have led to under estimation of their prevalence globally. This article analyzed viral infections in patients hospitalized for acute respiratory illnesses in the Riyadh region between November 2013 and March 2014. Viral infections were not monitored during the whole year as acute cases requiring hospitalization outside the November to March period are rare. Additionally, respiratory specimens were not available for non-hospitalized outpatients for comparison. Nevertheless, the use of sensitive real-time multiplex PCR in this study enabled the characterization of HRV as the etiological agent responsible for 45.7% of all hospitalized cases. Infections were more often caused by a single respiratory virus, predominantly HRV. We recommend the use of more sensitive techniques such as real-time multiplex PCR for reporting respiratory viral infections, especially in patients with low immunity and chronic respiratory disease (e.g. asthmatics) to avoid misdiagnosis. Identification of the exact causative agents could help guide patient management and infection control.

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