Seasonality and prevalence of respiratory pathogens detected by multiplex PCR at a tertiary care medical center

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Abstract Respiratory tract infections (RTIs) are a leading cause of mortality and morbidity. Seasonality has been reported for many viruses, including influenza virus, respiratory syncytial virus (RSV), and the recently described human metapneumovirus (hMPV). We hypothesize that the availability of rapid, multiplex PCR diagnostics will provide better clinical care and new insights into the etiology and clinical spectrum of RTIs. We conducted a retrospective analysis of the incidence of respiratory pathogens at a 500-bed adult and 154-bed pediatric hospital tertiary care center. A total of 939 specimens from patients with an age range of 5 days to 91 years (median, 2 years) were tested by a multiplex respiratory pathogen PCR from November 14, 2011 to November 13, 2012. Sixty-five percent of specimens were positive for at least one pathogen. As the age of the patient increased, the positivity rate for the PCR decreased proportionately. Rhinoviruses/enteroviruses (Rhino/Entero) were the most prevalent (34.3%) followed by RSV (19.2%) and hMPV (6.2%). Twelve percent of the positive samples were positive for multiple analytes, with Rhino/Entero and RSV being the most common combination. The peak months were September and May for Rhino/Entero infections, January for RSV and February for coronavirus. hMPV peaked 2 months after RSV, as has been observed recently in other studies. Multiplex PCR provides rapid diagnostic information that can be used to make knowledgeable clinical decisions and potentially reduce the use of antibiotics. Active respiratory PCR surveillance could also predict seasonal respiratory epidemics to allow for adequate planning of additional infection control measures.

Introduction

Acute respiratory tract illnesses are the most common illness in all age groups and are an important cause of hospitalization and mortality, especially in the winter months. For children less than 5 years of age, respiratory tract infections are the second leading cause of death [23]. Major causes of bronchiolitis and lower respiratory tract illnesses in children include respiratory syncytial virus (RSV), parainfluenza viruses, influenza virus, and human metapneumovirus (hMPV).

Understanding seasonal variations in these infections may allow optimal planning and utilization of resources in emergency departments, hospitals and clinics. Past epidemiologic studies used routine diagnostic methods such as culture for the detection of viral and bacterial respiratory pathogens [7, 38]. However, viral culture requires 3 to 5 days to detect most agents and detects rhinovirus or coronaviruses poorly or not at all. Multiplex reverse transcriptase PCR has been shown to be more sensitive than standard respiratory virus culture, bacterial culture, and antigen detection methods [5, 8, 18, 27]. Moreover, PCR is much faster. For the recently characterized hMPV, RT-PCR-based techniques are generally the method of choice for detection [35]. No antigen detection methods are commercially available for hMPV.

Expanded multiplex PCR panels now allow the detection of up to 14 different viruses along with influenza A subtyping and the detection of three common bacterial respiratory pathogens, *Mycoplasma pneumoniae, Chlamydia pneumoniae* and *Bordetella pertussis* in one hour.
The high sensitivity and the expanded capability of these tests, therefore, may affect our understanding of the epidemiology of respiratory tract infections. This study explores the seasonality and prevalence of respiratory viral pathogens at a tertiary care medical center using the multiplex PCR respiratory pathogen panel.

Materials and methods

Study participants

Testing took place from November 14, 2011 to November 13, 2012 on nasopharyngeal specimens (NPS) originally sent to Georgia Health Sciences University (GHSU; its name was recently changed to Georgia Regents University) clinical microbiology laboratory (Augusta, GA) from the 500-bed adult and 154-bed pediatric hospital at GHSU for respiratory pathogen PCR assay by the FilmArray Respiratory Panel (RP) (BioFire Diagnostics, Inc., Salt Lake City, UT). NPS specimens were obtained from patients with symptoms of a respiratory infection, collected from the patients using standard technique, and placed in viral transport medium (Remel MicroTest M4RT Viral Transport tube). Specimens were tested as soon as possible after collection. The project was approved by the institutional review board of our institution; informed consent for the project was waived. Demographic data, such as initial symptoms, chief complaint, age, gender, and secondary diagnosis were obtained for each specimen tested.

FilmArray RP assay

The FilmArray assay was performed according to the manufacturer’s instructions. In brief, 1 mL of purified water included in the kit was injected into the FilmArray pouch to rehydrate the reagents. Then, 300 l of the viral transport medium that had contained the NPS specimen was mixed with 500 l of sample buffer and injected into the sample port of the pouch. The pouch was then placed into the FilmArray instrument, and a respiratory PCR panel program was started. The first stage of the program consists of a multiplexed PCR, followed by an individual nested second-stage real-time PCR contained within a microarray chip. The FilmArray RP includes two internal controls: an RNA process control and controls for every step inside the pouch. Results are analyzed using melting curve data. The organism/viruses detected by the FilmArray included adenovirus, influenza A virus (FluA), influenza B virus (FluB), parainfluenza virus 1 (Para 1), parainfluenza virus 2 (Para 2), parainfluenza virus 3 (Para 3), parainfluenza virus 4 (Para 4), respiratory syncytial virus (RSV), coronavirus 229E (CoronaV 229E), CoronaV NL63, CoronaV HKU1, CoronaV OC43, human metapneumovirus (hMPV), Bordetella pertussis, Chlamydia pneumoniae and Mycoplasma pneumoniae. Due to genetic similarity between the human rhinoviruses and enteroviruses, a positive result with PCR primers to these viruses was listed as Rhino/Entero. Rhinovirus A, B and C and enterovirus A, B and C are detected in the assay. Both RSV subtypes A and B are detected in the assay, though not specifically assayed. The FluA viruses could also be subtyped as FluA/H1, FluA/H3 or FluA-2009 if present.

Statistics

A two-sample Student’s t-test between proportions was performed to determine whether there was a significant difference between the viruses with respect to the percentage of initial clinical symptoms in both single virus infections and mixed infections [6]. Statistical analysis was performed using the software package StatPac for Windows (Pepin, WI).

Results

Analysis of positivity rates and prevalence within age groups

Specimens from a total of 939 patients were analyzed by PCR. The age range of the patients was 5 days to 91 years of age (median, 2 years). The male:female ratio was 1.42. Rhino- and enteroviruses were the most prevalent (34.3 %), followed by RSV (19.2 %) and hMPV (6.2 %) (Table 1). The data were divided according to age groups. The largest age group in our study was children ≤2 years of age, followed by the age group 3-21 years of age (Table 1). The older age groups, 22-49, and ≥50 years were small compared to the first two groups, with 24 and 52 patients in each group, respectively.

The highest rate of PCR positivity was observed in children ≤2 years of age, with a rate of 71.6 % (Table 1), followed by 62.5 % for the second-youngest age group, ages 3-21. The positivity rates for the 22-49 and ≥50 age groups were much lower, at 33.3 % and 21.2 %, respectively.

The prevalence of specific viruses and bacteria also differed between age groups (Table 1). Most viruses and bacteria showed the highest prevalence in children ≤2, with the exception of Flu A, for which the highest number of infections was in the 3-21 age group (Table 1). Rhino/Entero viruses were the most prevalent viruses in all age groups except in the 22-49 age group, where there were equal numbers of Rhino/Entero cases and Para 3 cases.
although there were only three cases in each group. No positive results were detected for *B. pertussis* during the study period.

All hMPV cases were detected in the 2-21 and 3-21 age groups, except for two cases. One case was in a 50-year-old female day-care worker admitted for asthma exacerbation, and the second was a 58-year-old female with an underlying hematologic malignancy.

All RSV cases were also detected in the 2-21 age group, except for two cases. One case of RSV was detected in a 33-year-old male who also tested positive for Rhino/Entero, and a second case was detected in a 59-year-old male. The patient that tested positive for both Rhino/Entero and RSV was the only patient above the age of 18 who tested positive for more than one virus.

Comparison of initial symptoms/diagnoses with PCR diagnosis

The major initial clinical symptoms/diagnoses of the patients included respiratory distress, asthma/wheezing, pneumonia, fever or bronchiolitis. The percentages of the major initial symptoms/diagnoses for the more prevalent PCR results are presented in Fig. 1.

When compared to RSV, patients with hMPV showed significantly less respiratory distress (10.9 %, \( p < 0.05 \)) as a presenting symptom, but more fever (28.2 %, \( p < 0.05 \)). There were no significant differences in the percentages of initial presentations with pneumonia, asthma/wheezing or bronchiolitis between RSV and hMPV.

When single RSV or hMPV infections were compared to single rhino/enterovirus infections, there was a significantly higher percentage that presented with pneumonia (18.4 %, \( p < 0.001 \) or 21.7 %, \( p < 0.05 \), respectively) or bronchiolitis (9.5 %, \( p < 0.001 \); 6.3 %, \( p < 0.05 \)). Mixed co-infections with Rhino/Entero and RSV or hMPV showed a significant lower percentage that presented with pneumonia (3.0 %, \( p < 0.05 \), but not with bronchiolitis (6.5 %).

There was a significant increase in the number of patients that presented with respiratory distress with a mixed coronavirus and RSV or hMPV co-infection (61.5 %, \( p < 0.01 \)). There were only 13 cases for

| Analyte           | \( \leq 2 \) years | 3-21 years | 22-49 years | \( \geq 50 \) years | All ages |
|-------------------|-------------------|------------|-------------|-------------------|----------|
|                   | # Pos (n=570)     | # Pos (n=293) | # Pos (n=24) | # Pos (n=52)     | # Pos (n=939) |
| Rhino/Entero      | 196 34.4 %        | 117 40.8 %  | 3 12.5 %    | 6 11.5 %         | 322 34.3 % |
| RSV               | 148 26.0 %        | 30 10.2 %   | 1 4.2 %     | 1 1.9 %          | 180 19.2 % |
| hMPV              | 42 7.4 %          | 12 4.1 %    | 0 0 %       | 2 3.8 %          | 58 6.2 %   |
| Para 1            | 6 1.1 %           | 2 0.7 %     | 0 0 %       | 0 0 %            | 8 0.9 %    |
| Para 2            | 3 0.5 %           | 4 1.4 %     | 0 0 %       | 0 0 %            | 7 0.7 %    |
| Para 3            | 34 6.0 %          | 7 2.4 %     | 3 12.5 %    | 0 0 %            | 44 4.7 %   |
| Para 4            | 1 0.2 %           | 0 0 %       | 0 0 %       | 0 0 %            | 1 0.1 %    |
| Parainfluenza total | 44 7.7 %         | 13 4.4 %    | 3 12.5 %    | 0 0 %            | 60 6.4 %   |
| CoronaV 229E      | 9 1.6 %           | 4 1.4 %     | 0 0 %       | 2 3.8 %          | 15 1.6 %   |
| CoronaV HKU1      | 14 2.5 %          | 2 0.7 %     | 1 4.2 %     | 0 0 %            | 17 1.8 %   |
| CoronaV NL63      | 5 0.9 %           | 2 0.7 %     | 0 0 %       | 0 0 %            | 7 0.7 %    |
| Coronavirus total | 34 6.0 %          | 9 3.1 %     | 1 4.2 %     | 2 3.8 %          | 46 4.9 %   |
| Adenovirus        | 6 1.1 %           | 5 1.7 %     | 0 0 %       | 0 0 %            | 11 1.2 %   |
| Influenza A total | 2 0.4 %           | 14 4.8 %    | 0 0 %       | 0 0 %            | 16 1.7 %   |
| FluA/H1           | 0 0 %             | 0 0 %       | 0 0 %       | 0 0 %            | 0 0 %      |
| FluA/H3           | 2 0.4 %           | 8 2.7 %     | 0 0 %       | 0 0 %            | 10 1.1 %   |
| FluA/2009 H1      | 0 0 %             | 6 2.0 %     | 0 0 %       | 0 0 %            | 6 0.6 %    |
| Influenza B       | 0 0 %             | 0 0 %       | 1 4.2 %     | 0 0 %            | 1 0.1 %    |
| C. pneumoniae     | 2 0.4 %           | 0 0 %       | 0 0 %       | 0 0 %            | 2 0.2 %    |
| M. pneumoniae     | 1 0.2 %           | 2 0.7 %     | 0 0 %       | 0 0 %            | 3 0.3 %    |
| Total positive:   | 408 71.6 %        | 183 62.5 %  | 8 33.3 %    | 11 21.2 %        | 610 65.0 % |
comparison, however. There were only two co-infections with both RSV and hMPV, one as part of a quadruple infection with two coronaviruses and one RSV and hMPV co-infection. The patient with the quadruple infection was admitted to pediatric intensive care with severe respiratory distress. The coinfection RSV and hMPV was an outpatient case seen for asthma exacerbation.

All three cases of M. pneumoniae involved co-infections (Table 2). Two of the cases were co-infected with Rhino/Entero, and the third was a triple infection with adenovirus and Rhino/Entero. With all three cases, the initial symptom was asthma/wheezing. One of the two C. pneumoniae cases was a co-infection with Rhino/Entero with an initial symptom of respiratory distress. The pure infection with C. pneumoniae had an initial diagnosis of pneumonia.

Analysis of multi-analyte-positive samples

Sixty-five percent (65 %) of all specimens were positive for at least one viral or bacterial organism. Of the 610 positive specimens, 12 % (73/610) were positive for more than one analyte (Table 2). The majority (72/73) of multi-analyte-positive samples were from patients ≤ 18 years of age. The most common viruses detected in multi-analyte-positive specimens were Rhino/Entero and RSV, which comprised 88 % of all multi-analyte-positive samples (64/73). Individually, Rhino/Entero and RSV were detected in all multi-analyte-positive samples, 75 % and 53 % of the time, respectively (Fig. 2). There were four triple-positive samples and two quadruple-positive samples (Table 2).

Additional viruses found in multi-analyte positive samples included hMPV, Para 3 and CoronaV (HKU1, NL63, OC43). The majority of the hMPV multi-analyte-positive samples were in conjunction with Rhino/Entero. There were only two samples in which hMPV was detected together with CoronaV (HKU1) or CoronaV (HKU1, NL63) and RSV. Also, the majority of the Para 3 multi-analyte-positive samples were also positive for Rhino/Entero. There were only two Para 3 positives that were positive for RSV.

Seasonal prevalence of respiratory pathogens from November 14, 2011 to November 13, 2012

The prevalence of Rhino/Entero remained relatively high throughout the year, with several incidence peaks, the highest occurring in August/September, with two minor peaks in May and December (Fig. 3). A distinct peak in RSV cases occurred in January, followed by a peak in total coronavirus cases in February, and a peak in hMPV cases in March. A distinct peak for Para 3 cases was observed in May.

The eight cases of Para 1 and seven cases of Para 2 all occurred in the fall, in September-November (data not shown). The one case of Para 4 was detected in November. The eleven cases of adenovirus occurred both in the spring and the fall months. Four cases of FluA type H1-2009 were detected February-April 2012. Two cases of FluA type H3 were detected March 2012, and eight cases were detected November 2012. One case of Flu B was detected February
2012. No seasonal variation was noted for the eleven adenovirus cases. All three *M. pneumoniae* cases occurred in September, and the two *C. pneumoniae* cases occurred in January and November.

**Discussion**

In our study, 939 specimens were analyzed over the course of a year using a respiratory pathogen multiplex PCR that yielded a positivity rate of 65% with multiple analytes detected in 12% of specimens, especially in children. As the age of the patient increased, the positivity rate for the PCR decreased proportionately. Other multiplex respiratory PCR studies have reported lower positivity rates for adults [9, 11]. Studies have shown that older adult patients shed lower titers of viruses, which demands the use of a highly sensitive methodology such as RT-PCR over
conventional culture or DFA [36]. It is possible that the multiplex PCR assays now available may still not be sensitive enough to detect the lower titers of virus shed by older adult patients. It is also possible that adult patients may have other bacterial infections causing the respiratory infections that are not detected by the PCR, such as Legionella or Streptococcus pneumoniae.

We noted a seasonal variation of the number of viral infections. Rhino/Enterovirus was the most prevalent viral infection, with demonstrated peaks in the number of cases in May and September. The September peak appears to correspond to the start of school sessions. RSV was the second most prevalent virus, with a very large number of cases in January, followed two months later by an increased number of cases of hMPV.

RSV and hMPV were the second- and third-most common causes of respiratory infections in our study. The majority of the RSV and hMPV infections in our study occurred in children. Recently, in a study of over 3,000 children less than 5 years of age, the burden of hMPV was determined to be 6% in hospitalized children and 7% of children in both outpatient clinics and emergency departments [14]. We also detected a similar positivity rate of 6.2%.

RSV and hMPV PCR-positive cases were statistically much more likely than Rhino/Enterovirus PCR-positive infections to initially present with pneumonia or bronchiolitis in our study. Huguenin et al. similarly showed that Rhinovirus was the most common finding in the respiratory tract in their study, at 38.0%, and was the predominant virus regardless of season [11]. Although the rhinoviruses are the most prevalent of all the seasonal viruses, peaks of rhinovirus illness are well documented in September, after school starts, and again in the spring [24, 28, 39].

Rhino/Enterovirus was the predominant virus for all age groups and was superseded only by RSV from December to March. Brittain-Long et al. similarly observed that rhinovirus was the most common finding in the respiratory tract in their study, at 38.0%, and was the predominant virus regardless of season [11]. Although the rhinoviruses are the most prevalent of all the seasonal viruses, peaks of rhinovirus illness are well documented in September, after school starts, and again in the spring [24, 28, 39].

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RSV and hMPV PCR-positive cases were statistically much more likely than Rhino/Enterovirus PCR-positive infections to initially present with pneumonia or bronchiolitis in our study. Huguenin et al. similarly showed that RSV was associated with more-severe disease in infants hospitalized for bronchiolitis [16]. RSV and hMPV are also known to cause severe respiratory infections in the elderly [17]. Some of these infections did occur in adults ≥ 50 in our study [13, 25]. Epidemiologic peaks for hMPV occurring 1 to 2 months later than that observed for RSV epidemics have been described in a number of studies [3, 4, 15, 20, 34]. Our study certainly supports this seasonality.

The parainfluenza viruses tend to have different patterns of seasonal variation based on type. Para 3, which can be predominant in spring in temperate climates, increased in the month of May [22]. Both Para 1 and Para 2 cases in our study were seen only in the fall months in our study, the typical season for these viruses [24]. The epidemiology of Para 4 is largely unknown. The number of coronavirus cases peaked in February in our study, indicating a winter seasonality. There is some evidence, however, that the seasonality of the coronaviruses may be dependent on geographic location [32].

There was an unusually low prevalence of influenza A virus (1.7%) for the 2011-2012 season in our study. In a review of the 2011-2012 winter influenza season in the northern hemisphere by the WHO, the influenza season started unusually late in most of North America, the latest in nearly 30 years in the U.S. [1]. The season was remarkably mild in the U.S., where influenza A (H3N2) was the predominant virus circulating. Transmission remained low until the end of the year.

No B. pertussis cases were detected in our hospital setting. The state of Georgia has one of the lowest incidences of reported B. pertussis in the entire U.S., with an incidence of 2.7 per 100,000 [12].

Multiple respiratory pathogens were detected in 12% of the respiratory specimens in our study; 75% of the time, the second analyte was Rhino/Enterovirus. Recently, Rand et al. [28] detected 15.9% co-infections in a study of 200 patients by multiplex respiratory PCR. Other studies have reported slightly lower rates of multiple-analyte-positive specimens of around 10% and 8.7% [9, 21].

The significance of these multi-analyte-positive specimens is not clear, especially in conjunction with Rhino/Enterovirus. It is likely that many of the dual-positive results with RSV and Rhino/Enterovirus samples are due to viral co-infection with a previous Rhino/Enterovirus infection. There was a significant increase in the number of patients who presented with respiratory distress (61.5%) with a mixed coronavirus and RSV or hMPV co-infection. It is possible that these may have represented true co-infections with coronavirus rather than viral shedding. However, the significance of this observation is limited by having only 13 patients in this group.

Some studies that have concluded that more-severe disease occurs in infants with bronchiolitis with co-infections with RSV and hMPV, of which we detected only two cases, one as part of a quadruple infection and one mixed infection with RSV and hMPV [29, 33]. The quadruple infection was a severe respiratory case requiring intensive care.

A possible limitation of the study is that the subjects are biased toward the younger age groups, with only a modest representation of adults. However, the population reflects current physicians’ choice of using PCR for the diagnosis of respiratory symptoms. Young children may seek healthcare earlier in the course of disease, and the reasons for testing in young children may be different in adults compared to children and young adults.

Multiplex respiratory PCR has been used in some cases to reduce nosocomial infections during specific seasonal
respiratory epidemics. For example, some investigators have advocated performing multiplex respiratory PCR on all symptomatic infants during the RSV season, recommending that the RSV-positive patients be cohorted and placed on gown and glove precautions, noting a reduction in nosocomial infection in newborn nurseries after putting such practices into place [19, 37]. Ideally, active respiratory PCR surveillance could help predict seasonal respiratory epidemics so that additional infection control measures can be instituted.

A distinct advantage of the rapidity of diagnosis with the multiplex PCR is the ability to quickly and specifically treat the respiratory pathogen and facilitate therapy. Some of the pathogens detected in the multiplex PCR have specific therapies [2]. The multiplex respiratory PCR detects three bacterial pathogens that can all be treated with specific antibiotics, including *C. pneumoniae, M. pneumoniae*, and *B. pertussis*. Adamanatanes and neuraminidase inhibitors are available for the influenza viruses. Intravenous polyclonal immune globulin enriched against RSV and palivizumab, a monoclonal antibody against the RSV fusion glycoprotein, have been recommended for high-risk infants. Ribavirin has been also been approved for use with RSV and has had some apparent success in cases of infection with hMPV, adenovirus and parainfluenza viruses. Most conventional immunoglobulin preparations seem to contain sufficient amounts of neutralizing titers against hMPV.

In conclusion, molecular testing methods have significantly expanded our ability to diagnose respiratory infections because of their rapidity, sensitivity, and potential for the simultaneous detection of 15 or more respiratory pathogens. The use of a rapid and accurate diagnostic test enhances clinical decision making and safely promotes implementation of cost-effective treatment strategies, including limiting the unnecessary use of antibiotics.

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Conflict of interest We declare that we have no conflicts of interest with respect to this study.

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