Konya bölgesinde toplum kökenli pnömoni için hastaneye yatırılan çocuk hastalarda solunumsal viral enfeksiyon etkenlerinin multipleks gerçek zamanlı-PCR ile tanımlanması

Identification of respiratory viral infection agents by multiplex real-time PCR among children hospitalized for community-acquired pneumonia in Konya province

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ÖZET

Amaç: Bu çalışmanın amacı; toplum kökenli pnömoni tanısı ile hastaneye yatırılan çocuklarda viral pnömoni etkenlerinin moleküler tanı teknikleri kullanılarak tanımlanmasını değerlendirmektir.

Yöntem: Çalışmaya, Konya bölgesindeki viral pnömoni etkenlerini tanımlamak amacı ile toplum kökenli pnömoni nedeni ile hastaneye yatırılan 64 çocuk dahil edilmiştir. Çalışma, Ekim 2009 ve Aralık 2010 tarihleri arasında gerçekleştirilmiştir. Yaşları bir ay ile sekiz yaş arasında olan ve toplum kökenli pnömoni olarak tanı konulan çocuk hastalardan nazo-faringeal sürüntü örnekleri alınmıştır. Hastalara hastaneye yatışları sırasında respiratuvar sinsityal virüs (RSV), adenovirus, influenza virüs ve paranifluenza virüsler gibi viral pnömoni etkenlerinin varlıklarını tepit etmek için multipleks gerçek zamanlı polimeraz zincir reaksiyonu (PZR) testi yapılmıştır.

Bulgular: Toplam 64 hastanın 22 (%34,4)'si viral enfeksiyonlar, beş (%7,7)'si bakteriyel enfeksiyonlar ve iki (%3,1)'i hem viral hem de bakteriyel enfeksiyonlar

ABSTRACT

Objective: Aim of this study was to evaluate the identification of viral pneumonia agents among children who were diagnosed and hospitalized for community-acquired pneumonia (CAP) by using molecular diagnostic techniques.

Method: Sixty four children hospitalized for community-acquired pneumonia were included in the study in order to identify viral pneumonia agents in the province of Konya. The study was carried out in between October 2009 and December 2010. Nasopharyngeal smears were obtained from children patients aged 1 month to 8 years who were diagnosed as community-acquired pneumonia. Multiplex real time-Polymerase Chain Reaction (PCR) was performed for detection of existence of viral pneumonia agents such as respiratory syncytial virus (RSV), adenovirus, influenza and parainfluenza viruses on admission.

Results: Of all the 64 patients, 22 (34.4%) were positive for viral infections, 5 (7.7 %) were positive for bacterial infections, and 2 (3.1%) were positive for both

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için pozitif olarak bulunmuştur. 20 (%31,2) vakada sadece viral patojen ve üç (%4,6) hastada sadece bakteriyel enfeksiyon belirlenmiştir. RSV (%23,4) ve adenovirüs (%4,6) en sık viral patojenler olarak tespit edilirken, influenza A virüsü (%3,1), parainfluenza tip 1, 2, 3 virüsleri (%3,1) enfeksiyon nedeni diğer virüsler olarak belirlenmiştir. İnfluenza B virüsü vakaların hiçbirinde tespit edilmemiştir. 37 (%57,9) hastada muhtemel hiçbir etken belirlenememiştir. Çalışma süresince ölüm olmamıştır.

Sonuç: Bu çalışmada, hastaneye yatırılan bir ay ve sekiz yaş arasında çocuklarda toplum kökenli pnömoninin en sık etkeninin virüsler olduğu belirlenmiştir. Virüslerin etiyolojik tanılarının geliştirilmesi, tedavilerde özellikle gereksiz antibiyotik kullanılması ve gereksiz izolasyon uygulamaları yapılmasını önlemede yararlı olacaktır. Hastaneye yatırılan çocuk hastalarda toplum kökenli pnömoninin etiyolojisinde virüslerin rollerini açıklamaya odaklanan daha fazla ve yeni çalışmaların gerçekleştirilmesi bu hasta grubunun tedavisinde doğru politikaların belirlenmesine katkı sağlayacaktır.

Anahtar Sözcükler: Çocuklar, toplum kökenli pnömoni, viral etiyoloji, multipleks gerçek zamanlı-PZR.

viral and bacterial infections . A sole viral infection was identified in 20 (31.2%) and a sole bacterial infection in 3 (4.6%) cases. Influenza A virus (3.1%), parainfluenza type 1, 2, 3 viruses (3.1%), respiratory syncytial virus (23.4%) and adenovirus (4.6%) were found as causative viruses. Influenza B virus was not detected in any of cases. No possible etiologic agent was found in 37 cases (57.9%). Respiratory syncytial virus (23.4%) and adenovirus (4.6%) were the most commonly detected viral pathogens. There was no mortality during the study.

Conclusion: This study showed that the viruses commonly detected as the causative agents of community-acquired pneumonia among the hospitalized children aged between 1 month and 8 years. Improving the etiological diagnosis of viral infections may definitely contribute to avoid unnecessary therapy, particularly antibiotics and allow for preventive isolation of infected patients. Performing more and new studies focusing on defining the role of viruses at the etiology of community-acquired pneumonia among hospitalized children will help establishment of true policies on the treatment of these patients.

Key Words: Children, community-acquired pneumonia, viral etiology, multiplex real time-PCR.

INTRODUCTION

In most developing countries, community-acquired pneumonia (CAP) is the leading cause of hospitalization for young children. Although the documented reduction of mortality with the development of antimicrobial therapy, CAP management guidelines, and effective vaccines, 50 to 90% of these infections is caused by viruses in children younger than 5 years of age (1-3).

Viral pathogens are increasingly recognized as playing a major role in the etiology of lower respiratory tract infections (LRTIs), and are considered the predominant pathogens in CAP in preschool children (4).

Respiratory syncytial virus (RSV) is the most commonly identified virus in this setting, with detection rates in infants with bronchiolitis of up to 100% during seasonal winter epidemics. Other respiratory viruses, including influenza virus types A and B, and parainfluenza virus types 1-3 and adenoviruses, are also important causes of LRTIs and hospital admission during epidemics or periods of increased prevalence in the community (3).

As these respiratory viral pathogens cause very similar clinical symptoms, differential diagnosis of the pathogens is required in appropriate sample. Monospecific PCR assays require separate

amplification of each target and are therefore expensive and resource intensive. For clinical diagnosis, multiplex PCR has a significant advantage, as it permits simultaneous amplification of several viruses in a single reaction mixture, facilitating cost-effective diagnosis (5-9). Multiplex real-time PCR method was found to be more sensitive than cell culture on a range of different respiratory samples. The specificity of the real-time PCR was reported to be as high as 93% and the sensitivity as 100% (10). Therefore, in our study we used multiplex real-time PCR method for diagnosis and differentiation of different viral agents.

Detailed information on the etiology of CAP is required for the formulation of treatment recommendations and the introduction of preventive measures. Evaluation of mixed infections and the relative importance of each potential pathogen may also contribute to improved understanding of the etiopathogenesis of CAP (11,12).

We conducted prospective study to investigate the incidence of viral pneumonia in children aged a month to eight years who were hospitalized for CAP. We also investigated the contribution of different viruses including respiratory syncytial virus, adenovirus, influenza and parainfluenza viruses by multiplex RealTime-PCR.

MATERIAL AND METHOD

Study design

The study was a descriptive study. We evaluated the identification of viral pneumonia among children who were diagnosed and hospitalized for CAP. Patients aged 1 month to 8 years old diagnosed as CAP in absence of underlying chronic illnesses by inclusion criteria were recruited into the study. Written consents were obtained from their legal guardians and parents. A patient was enrolled in the study if she/he met the following criteria (13). Fever with body temperature >37.8 °C, respiratory rate more than average per age by WHO criteria, abnormal chest x-ray together with signs of respiratory distress. Children were excluded if they were currently on antibiotic therapy or were admitted to hospital for more than 48 hours. Upon enrollment, demographic characteristics and baseline clinical data were recorded. Pulmonary auscultation findings of each patient were recorded with detailed physical examination.

Study population

From December 2008 to January 2009, 64 children aged 1 month to 8 years (39 girls and 25 boys, median age: 12 months age) who were diagnosed as CAP and were hospitalized at Department of Pediatrics, Konya Training and Research Hospital, Konya, Central Anatolia were included in the study.

Radiology

A senior radiologist, unaware of clinical and laboratory findings, reviewed all chest radiographs. The radiologist assigned standardized and mutually exclusive diagnoses including unequivocally normal chest radiography, with consolidation, with interstitial infiltration, with peribronchitis, with hiler/mediastinal lymphadenopathy and hyperinflation.

Microbiology

Nasopharyngeal smears were taken with swabs and transported to molecular diagnosis unit within specific liquid transport system UTM-RT transport medium (Copan, Italy). A nasopharyngeal sample was kept under -70°C until virologic tests (n = 64) were done. Existence and genotyping of viruses (Influenzae A and B viruses, parainfluenza virus type 1, 2 and 3, respiratory syncytial virus (RSV) and adenovirus) causing viral CAP were investigated with RT-PCR followed by reverse-hybridization method using sequence-specific oligonucleotide probes (SSOP) with three steps. First, RNA isolation were performed with isolation kits RTP-DNA/RNA Virus Mini Kit (Invitek, Germany) and then in-vitro amplification was performed via specific biotin labeled primers

by using thermal cycler (Bioer XP Cycler, Japan) and finally the amplification products were selectively hybridized to the nitrocellulose test strips (GenID GmbH, CAPvir Straßberg, Germany) which contain sequence specific oligonucleotide probes with Auto-LiPA instrument (Tecan ProfiBlot T48, Austria). Each strip contained five specific probes for genotyping of the most common causative agent of the viral CAP. The virologic studies were carried out at the Department of Microbiology, Konya Training and Research Hospital.

Serum procalcitonin (PCT) levels were measured with Elecsys BRAHMS PCT reactive (Roche Diagnostics GmbH, Mannheim, Germany). Assays were performed with Cobas e 601 immunoassay device (Roche Diagnostics GmbH, Mannheim, Germany) by using electrochemiluminescence immunoassay method (ECLIA).

Serum C-reactive protein (CRP) levels were measured with CardioPhase hsCRP reactive (Siemens, Healthcare Diagnostics Products, GmbH Marburg, Germany) by using BN II Nephelometry Device (Siemens, Healthcare Diagnostics Products, GmbH Marburg, Germany).

Erythrocyte sedimentation rate (ESR) measurements were performed by using fully automated ESR assay device (Diesse Ves Cube 200, Diesse Diagnostica Senese SpA, Italy).

Complete blood count (CBC) assays were performed with fully automated CBC device (Cell-Dyne 3700, Abbott Diagnostics Division, Abbott Laboratories, Abbott Park IL, USA).

Blood cultures were obtained via BD Bactec Peds Plus/F vials before initiation of parenteral antibiotic therapy among all patients and incubated in automated blood culture system (Bactec 9120 BD, Becton Dickinson and Company, Sparks MD, USA). Isolated strains were also identified by using automated bacteria identification system (Phoenix 100 Becton Dickinson and Company, Sparks MD, USA).

Statistical analysis

SPSS software (version 15.0) was used for statistical analysis. Descriptive data were presented as mean ± SD. Categorical data were analyzed with the chi-square test or Fischer's exact test. Results were compared using the Kruskal-Wallis test. $p<0.05$ was considered statistically significant.

RESULTS

The study included 25 (39%) boys and 39 (61%) girls. The mean age of the patients was 2.04±2.06 years (range, 1 month-96 months, median, 1.6 years). In 27 of 64 (42.1%) patients, causal agent of infection was obtained with nasopharyngeal smear and blood culture. Viral infection was detected in 22 (34.4%) and bacterial infection in five (7.7%) pneumonia cases. A mixed viral-bacterial infection (RSV in nasopharyngeal smear+*Staphylococcus aureus* in blood culture, adenovirus in nasopharyngeal smear+ *Streptococcus pneumoniae* in blood culture) was seen in two (3.1%) patients. No possible etiologic agent was found in 37 cases (57.9%). A sole viral infection was identified in 20 (31.2%) and a sole bacterial infection in 3 (4.6%) cases. Influenza A virus (3.1%), parainfluenza type 1, 2, 3 viruses (3.1%), respiratory syncytial virus (23.4%) and adenovirus (4.6%) were found as causative viruses. Influenza B virus was not detected in any of cases.

Only five patients had a positive blood culture (*S.aureus, S.pneumoniae* and *Listeria monocytogenes*), while ten other blood cultures were contaminated by *Staphylococcus hominis, Staphylococcus epidermidis, Staphylococcus saprophiticus* and *Staphylococcus capitis*. Table 1 shows viral and bacterial agents causing CAP in hospitalized children.

The mean body temperature on admission was 38.2 ±0.4 ^{o}C. Considering the pulmonary auscultation findings of the patients, crackles, rhonchi and wheezing were found in 58 (90.6%), 39 (60.9%) and eight (12.5%) children, respectively. The mean leukocyte count was 11563±4668 cell/µl.

Table 1. Viral and bacterial agents in children who were hospitalized for CAP

Pathogen	Number of patients (%)
Viral and bacterial agents	**27 (42.1)**
Viruses	**22 (34.4)**
Influenza A virus	2 (3.1)
Influenza B virus	0
Parainfluenza Type 1, 2, 3 viruses	2 (3.1)
Respiratory syncytial virus	15 (23.4)
Adenovirus	3 (4.6)
Bacteria	**5 (7.7)**
Staphylococcus aureus	2 (3.1)
Streptococcus pneumoniae	2 (3.1)
Listeria monocytogenes	1 (1.5)
Unidentified cases	**37 (57.9)**
Total	**64 (100)**

The viral etiology of community-acquired pneumonia according to different age groups was shown in Table 2.

RSV had the highest rate among detected viruses in all age groups. The rates of RSV and adenovirus were equal among 24-59 months of age group. Influenza A virus was detected only in 2-11 months of age group. Parainfluenza 1-3 were found in 2-11 and 24-59 months of age group. Adenovirus was the most common viral agent among 12-23 and 24-59 months of age group.

Table 2. The viral etiology of community-acquired pneumonia according to age

Age (mo)	Patient No	Viral Etiology (%)
<2	4	1 (25)
2-11	22	9 (40.9)
12-23	13	4 (30.7)
24-59	16	5 (31.2)
>59	9	3 (33.3)
Total	**64**	**22 (34.4)**

The numbers in parentheses show percentages

There was a statistically significant difference between 2-11 months (18.86± 11.62) and 12-23 months of age group (31.23 ±18.24) with regard to ESR values (p=0.028). There was statistically significant difference between 24-59 months (0.22±0.52) and >59 months of age group (0.44 ±0.45) with regard to PCT values (p=0.016).

Chest x-rays were interpreted and grouped as normal, with consolidation, with intercystitial infiltration, with peribronchitis, with hiler/mediastinal lymphadenopathy and hyperinflation (Table 3). There was no statistically significant difference according to different viral etiologic agents with regard to chest x-ray findings (p=0.681). There was no statistically significant difference between viral and bacterial pneumonia groups with regard to chest x-ray findings (p=0.613).

There was no statistically significant difference between different age groups according to ages with regard to auscultatory and radiologic findings (p=0.337). The average length of hospitalization was at 5.0±1.9 days. None of the children required assisted ventilation or died.

Table 3. Chest x-ray findings of viral, bacterial etiology and unidentified groups

Chest X-Ray Findings	Viral Etiology	Bacterial Etiology	Unidentified Etiology
Normal	13	2	26
Consolidation	2	1	4
Intercystitial infiltration	5	1	2
Peribronchitis	0	0	1
Hiler/mediastinal lymphadenopathy	1	0	0
Hyperinflation	1	1	4
Total	**22**	**5**	**37**

DISCUSSION

Molecular diagnostic techniques were used in this study to thoroughly examine the etiology of CAP in hospitalized children who were 1 month to 8 years old. By means of these methods, infection with seven viruses was investigated, and the presence of viral infection was identified in 34.4% of the patients. Bacterial infection was detected in five (7.7%) of 64 patients. These results may corroborate previously reported etiological rates; in previous studies, the rate has been reported as 43% to 85% (14-20).

Viruses have been most commonly associated with CAP diagnosed in infants and younger children (12). However, recent evidence suggests that, when sensitive detection methods are used, the prevalence of viral infections in older children with CAP is higher than previously thought (14). In our study, approximately one-third of the patients were found to be infected with RSV, adenovirus, influenza virus or parainfluenza 1-3 viruses. Cilla et al. (21) had investigated 14 respiratory viruses in children aged less than three years old with CAP using molecular or immunochromatographic techniques and/or viral culture. In their study, at least one virus had been detected in 66.9% of the episodes.

Juvén et al. (14) reported a very high rate (62%) of viral etiology in pediatric CAP. In their study, rhinovirus, detected by PCR, accounted for a large proportion of viral pneumonia. In our study, rhinovirus was not investigated.

RSV is accounted for an estimated 13.3, and 0.4 RSV-associated hospitalizations per 1000 children who were younger than one year, one year of age, and in children 2 to 5 years of age, respectively (21). Children who were detected with RSV illness in our study were young than what Cilla et al. (21) determined in their study; 6.6% of the children were younger than two months, and nearly 46.6% were younger than one year.

RSV infections may be attributed to in all age groups of our study population. A high prevalence of RSV infection (5-11.5%) has also been found in this age group in previous studies (4, 14, 21, 22). The most frequent virus in our study was RSV, a result consistent with those of other large series of CAP in children less than three years old.

The second most frequent virus was adenovirus (4.6%), an important finding of this study. This number is close to that was previously reported in a study by Samransamruajkit et al. (23) in which adenovirus was detected in 6.6% of pneumonia patients.

Another finding of this study was the equal frequency of parainfluenza and influenza A viruses (3.1%). Estimated parainfluenza-associated hospitalization rates in the New Vaccine Surveillance Network study were 3.2, 1.5, and 0.4 per 1000 children younger than one year, one year, and 2 to <5 years of age, respectively (24). In our study, 50% of parainfluenza 1-3-associated hospitalizations occurred in children between 2 to11 months of age group and the rest occurred in 24-59 months of age group.

The influenza-associated hospitalization rates are reported to be 1.7, 0.5, and 0.2 per 1000 children younger than one year, one year of age, and in children between two to five years of age, respectively (24). In our study, all the influenza A-associated hospitalizations occurred in children younger than one year.

In a previous study, in 27% of the episodes of childhood CAP multiple viral infections were detected. Infection severity was more in multiple viral infections than single viral infection due to the greater frequency of hospitalization (21). However, we did not detect viral co-infection in any patient.

In our study population, mixed infections were uncommon (3.1%), although in previous studies mixed infections were reported as high as 23% of pneumonia cases in children (25). These findings are in accordance with recent studies suggesting nearly all kind of viral pathogens (six out of seven except

influenza B) often contribute to the pathogenesis of pneumonia (14, 16, 18).

This study, like others that investigated viral pneumonia, has three major limitations: First of all, documentation of infection in the upper respiratory tract does not necessarily prove the etiological agent of pneumonia. However, in 37 of 64 cases no etiological agent was detected. The RT-PCR method is an extremely powerful and useful tool; by the way, if we had been included other viral agents including rhinovirus, human metapneumovirus, and human bocavirus detected by RT-PCR, numbers of diagnosed cases might be higher.

Second, month-to-month variations in the prevalence of different pathogens may affect their association with CAP. Third, we studied only hospitalized children. A study on outpatients might have given different results.

Despite several limitations, overall this study highlights the important role of viruses in causing CAP in children younger than 3 years old.

In conclusion, our study demonstrated the etiological contribution of viral agents in CAP. Improving the etiological diagnosis of viral infections may avoid unnecessary therapy, particularly antibiotics and allow for preventive isolation of infected patients. Our study suggests that the viral infections are commonly found among children aged between 1 month to 8 years who were diagnosed and hospitalized for CAP. We recommend performing more comprehensive studies in children to define the role of viruses among children in CAP.

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