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RESEARCH ARTICLE/KLİNİK ÇALIŞMA

FLORA 2022;27(1):28-36 • doi: 10.5578/flora.20229903

Investigation of Viral and Atypical Pathogens in Patients with Pneumonia Who Need Intensive Care Unit

Yoğun Bakım Gerektiren Pnömonili Hastalarda Viral ve Atipik Patojenlerin Araştırılması

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Cite this article as: *Doğan A, Ersoy Çınar Y, Otlu B, Kuzucu Ç. Investigation of viral and atypical pathogens in patients with pneumonia who need intensive care unit. FLORA 2022;27(1):28-36.*

*This project was supplied by Inonu University Scientific Research Projects Coordination Unit with the grant number 2016/39.
**This paper was presented as an oral presentation in the International 5th Intensive Care Infections Symposium, 9-12 January, 2020, Trabzon-Turkey.

ABSTRACT

Introduction: *Viral pathogens have been reported increasingly in pneumonia patients. There are few studies in Turkey on viral and atypical bacterial etiology in adult patients with community-acquired pneumonia (CAP) or hospital-acquired pneumonia (HAP). In this study, it was aimed to determine atypical and viral pathogens in patients with pneumonia requiring ICU and to research clinical progression.*

Materials and Methods: *Adult patients admitted to adult ICUs between November 2016-October 2017 with either CAP or HAP diagnosis were included prospectively. Viral pathogens and also atypical bacterial pathogens were investigated with the in-house multiplex polymerase chain reaction method.*

Results: *Two hundred patients were enrolled to the study, of whom 63 had CAP (31.5%) and 137 had HAP (68.5%). Viral agents were identified in 31 (15.5%) patients in total, 11 (17.5%) in CAP and 20 (14.6%) in HAP. The most identified viral etiologic agents were rhinovirus, influenza A, and coronavirus HKU. Eight patients (4%) had Mycoplasma pneumoniae. All patients were negative for Legionella pneumoniae and Chlamydophila pneumoniae. Mortality rates were 16.7% for cases with a viral etiology only, 29.2% for cases with bacterial pathogens only, and 23.5% for cases with mixed agents identified.*

Conclusion: *Viral pathogens and M. pneumoniae should be remembered in the etiology of severe pneumonia patients.*

Key Words: *Atypical pneumonia; Viral pneumonia; Community-acquired pneumonia; Hospital-acquired pneumonia; Intensive care*

Received/Geliş Tarihi: 03/11/2021 - Accepted/Kabul Ediliş Tarihi: 14/12/2021

Available Online Date: 24.03.2022

ÖZ

Yoğun Bakım Gerektiren Pnömonili Hastalarda Viral ve Atipik Patojenlerin Araştırılması

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Giriş: *Pnömonili hastalarda viral patojenler giderek daha fazla rapor edilmektedir. Türkiye'de toplum kökenli pnömoni (TKP) veya hastane kökenli pnömoni (HKP) olan erişkin hastalarda viral ve atipik bakteriyel etiyoloji ile ilgili az sayıda çalışma bulunmaktadır. Bu çalışmada yoğun bakım ünitesi (YBÜ) gerektiren pnömonili hastalarda atipik ve viral patojenleri belirlemek ve klinik progresyonu araştırmak amaçlanmıştır.*

Materyal ve Metod: *Kasım 2016-Ekim 2017 tarihleri arasında erişkin yoğun bakım ünitelerine TKP veya HKP tanısı ile başvuran erişkin hastalar prospektif olarak dahil edildi. In-house multipleks polimeraz zincir reaksiyonu yöntemiyle viral patojenler ve ayrıca atipik bakteriyel patojenler araştırıldı.*

Bulgular: *Çalışma periyoduna alınan 200 hastadan, 63 olguda TKP (%31.5) ve 137 olguda HKP (%68.5) vardı. TKP'de 11 (%17.5) ve HKP'de 20 (%14.6) olmak üzere toplam 31 (%15.5) hastada viral ajan tespit edildi. En çok tanımlanan viral etiyolojik ajanlar rinovirus, influenza A ve koronavirüs HKU idi. Sekiz hastada (%4) Mycoplasma pneumoniae vardı. Tüm hastalarda Legionella pneumoniae ve Chlamydophilia pneumoniae negatifti. Mortalite oranları tek viral etiyolojiye sahip olgularda %16.7, yalnızca bakteriyel patojenlerin saptandığı olgularda %29.2 ve karışık etken saptanan olgularda %23.5 idi.*

Sonuç: *Ciddi pnömoni kliniği olan hastaların etiyolojisinde viral patojenler ve M. pneumoniae akılda tutulmalıdır.*

Anahtar Kelimeler: *Atipik pnömoni; Viral pnömoni; Toplum kökenli pnömoni; Hastaneden edinilmiş pnömoni; Yoğun bakım*

INTRODUCTION

Pneumonia is a common cause of infection-related deaths. Both hospital-acquired pneumonia (HAP) and community-acquired pneumonia (CAP) cause significant morbidity and mortality. Pneumonia etiologic agents display differences according to whether they are CAP or HAP. The most common agents for CAP are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and atypical agents, while aerobic Gram-negative bacteria are responsible for HAP[1]. Atypical bacterial pneumonia agents such as *Chlamydophila pneumoniae*, *Legionella pneumophilia*, and *Mycoplasma pneumoniae* are important since they are responsible for 15% of CAP, difficult to diagnose and beta-lactam group antibiotics have no effect. *L. pneumophilia* comes to the fore among CAP and HAP agents for causing severe clinical course. It is responsible for 8% of pneumonia cases requiring hospital admission and is reported to be the 3rd or 4th most common cause among pneumonia cases requiring intensive care unit (ICU) admission[2]. In recent years, development in molecular microbiology techniques has caused increasing importance of viral pneumonia sourced from hospital or community. Studies have reported viruses are included at varying rates of 2-35% in pneumonia etiology[1,3]. However, there are few studies in Turkey on viral and atypical bacterial etiology in adult patients[4].

In this study, it was aimed to determine the atypical and viral pneumonia pathogens in pneumonia cases requiring intensive care and to research the clinical progression.

MATERIALS and METHODS

This study was conducted in six adult ICUs (one neurosurgical ICU, two anesthesiology and reanimation ICUs, two internal medicine ICUs, and one stroke unit) with 66 beds in a tertiary university hospital in Turkey. The hospital bed capacity was 1100. Prospectively, the patients admitted to the ICUs between November 1,

2016 and October 30, 2017, with a diagnosis of pneumonia or who developed pneumonia in the hospital units or ICUs were included in the study. Patients aged 18 years and older who required intensive care admission with CAP diagnosis or HAP diagnosis based on clinical, radiological imaging, and laboratory findings were included in the study. Patients under the age of 18 years, with hematological malignancy, organ transplant patients, neutropenic patients, and HIV positives were excluded from the study.

CAP diagnosis was placed according to the diagnostic criteria of the "Turkish Thoracic Society Diagnosis and Treatment Consensus Report for Adults Acquiring Pneumonia in the Community 2009" and "Infectious Diseases Society of America/American Thoracic Society Consensus Guidelines on the Management of Community-Acquired Pneumonia in Adults 2007"[5,6]. HAP diagnosis was made according to the nosocomial PNU 1 criterion in line with the suggestions of CDC[7]. Patients' clinical and laboratory data were recorded. Patients' outcomes were followed up daily for at least 14 days. Mortality within 14 days was considered mortal progression.

Deep tracheal aspirate or sputum samples were taken from all patients and inoculated in sheep blood agar and eosin-methylene blue agar for standard bacterial culture and in "Buffered Charcoal yeast extract (BCYE) agar (Lab 195, Legionella Isolation Medium, Lab-M, A Neogen Company) intending to identify *L. pneumophila*. The viral pneumonia agents as Respiratory syncytial virus (RSV), human metapneumovirus (HMPV), rhinovirus (RV), enterovirus (EV), human parechovirus (HPeV), parainfluenza virus (PIV) 1, 2, 3, 4, influenza A (InfA), influenza B (InfB), INFA H1N1, human bocavirus (HBoV), adenovirus (AV), coronavirus (CoV) 43, 63, 229, HKU, and the atypical bacterial pneumonia agents of *M. pneumoniae*, *L. pneumophila*, and *C. pneumoniae* were researched using the in-house multiplex PCR method in lower respiratory tract samples. Additionally, nasopharynx swab samples (NPS) were collected and studied using PCR.

All samples for DNA/RNA extraction were stored in a deep freeze at -80°C until studied. DNA isolation from samples was performed using a nucleic acid isolation device (Qiasymphony, Qiagen, Germany). The 50-microliter amplification mixture [25 l 2X SyBr Master Mix, 2 l of every primer (10 pmol/l] was added to 5 l extraction products with amplification performed in a thermocycler device (RotorGene Q, Hilden, Germany). Amplification conditions were initial denaturation for 10 min at 94°C, then 40 cycles of 94°C/1 min denaturation, 57°C/1 min binding, and 72°C/1.5 min extension. Melting curve analysis was performed to image the amplification products. Base sequence analysis was performed using a DNA purification kit to confirm positive amplification products and obtain positive controls. A Big Dye Terminator 3.1 (Applied Biosystems) kit and ABI PRISIM 310 (Applied Biosystems) automatic base sequence device was used with sequence analysis of amplified gene regions with PCR. The obtained base sequences had BLAST analysis performed to identify bacterial species. Specific DNA/RNA primer sequences researched by the In-House Multiplex PCR method are demonstrated in Table 1.

With the aim of bacterial and viral DNA/RNA isolation, the DNA/RNA extraction kit with PCR, SYBR Green PCR Kit, and primer standard were used; and to proliferate *L. pneumophila* species in culture BCYE growth supplement, not including L-cysteine, BCYE growth supplement including L-cysteine, *L. pneumophilia* GVPC supplement with added antibiotic and BCYE *L. pneumophilia* media were prepared in the laboratory.

Ethics Committee Permission

An application was made to the İnönü University Clinical Research Ethics Committee. The study was planned with the decision of the board dated 8/6/2016 and numbered 2016/116. At the same time, approval was obtained from the heads of departments of the ICUs where the study was planned. Daily visits were made to the relevant intensive care units to determine patients to be included in the study.

Statistical Analysis

Statistical analysis was performed by loading the data taken from the patient forms into the "IBM SPSS Statistics 22" program. Continuous and intermittent quantitative variables in

Table 1. The specific DNA/RNA primer sequence used for an in-house multiplex PCR method

	Primer sequences 5'-3'	Amplicon size (bp)
M. pneumoniae	GTTTGCTGCTAACGAGTACGAG GTAATCATCGTCTGACTGCC	360
L. pneumophilia	CAATGGCTGCAACCGATGC GGGATAACTTGTGAAACCTG	487
C. pneumoniae	CGGCTAGAAATCAATTATAAGACTG GGTGTGTTTCTAATACCTGTCC	283
RSV	GGAACAAGTTGTTGAGGTTTATGAATATGC TTCTGCTGTCAAGTCTAGTACACTGTAGT	139
HMPV	AACCGTGTACTAAGTGATGCACTC CATTGTTTGACCGGCCCCATAA	212
RV	GGTGTGAAGACTCGCATGTGCT CCAAAGTAGTCGGTTCCGCTTCTGA	277
EV	GGCCCCTGAATGCGGCTAATCC GCGATTGTCACCATAAGCAGTCA	151
PIV	GCTAAATACTGTCTTMAHTGGAGAT GTAAGGATCACCWACATADAWTGTA	114
InfA	RGGCCCCCTCAAAGCCGA ACTGGGCACGGTGAGYGT	160
InfB	GGG ATA TAC GTA ATG TGT TGT GCA CTG CCT GCT GTA CAC TT	489
HBoV	GACCTCTGTAAGTACTATTAC CTCTGTGTTGACTGAATACAG	354

the data were summarized as median (min-max), and qualitative variables were given as a number (percentage). The Shapiro-Wilk test was used to investigate whether continuous and intermittent quantitative variables had a normal distribution. Mann-Whitney U test was used to investigate the statistically significant differences between the group variable categories in terms of continuous and intermittent quantitative variables. Qualitative variable categories and group variable categories were investigated for statistically significant correlations with Pearson Chi-square, Yates corrected Chi-square and Fisher's exact Chi-square tests. $P< 0.05$ was accepted as the level of statistical significance. Based on estimated pneumonia incidence in intensive care of 0.10, alternative incidence 0.17, type 1 patient (alpha) 0.05, and type 2 patient (beta) 0.01, power analysis calculated at least 200 individuals were required.

RESULTS

In the study period, 249 patients were admitted to the ICUs for different causes. Two hundred patients were enrolled in the study according to CAP or HAP criteria. Mean age of the cases consisting of 126 males (63%) and 74 females (37%) was 62.8 ± 18.09 years, of whom 63 had CAP (31.5%) and 137 had HAP (68.5%). There were no statistically significant differences between viral, bacterial, and mix infectious etiology pneumonia patients for symptom incidence, mean age, CRP, APACHE II score, PaO_2/FiO_2 ratio, and prognosis ($p> 0.05$), except tachypnea ($p= 0.048$). A comparison of the pneumonia patient groups is given in Table 2. During diagnosis, 135 (67.5%) patients were intubated and 65 (32.5%) were not.

When the comorbid status was investigated, 90 cases (45%) had central nervous system (CNS) pathologies, 52 cases (26%) had cardiovascular pathologies (CVD), 33 cases (16.5%) had pulmonary pathologies, 20 cases (10%) had trauma, 14 cases (7%) had malignancy (non-hematological and solid organ malignancy), and lower incidences of endocrine and renal pathologies were identified.

Table 2. Comparison of the variables in the pneumonia groups according to etiologic microorganisms

Variables (n)	Total (200)	Viral only (18)	Bacterial only (65)	Atypical bacterial only (4)	Mix infection (17)	No microorganism (96)	p
Age, years (mean)	62.8	59.6	62.6	61.7	57.6	64.5	>0.05
Male/female	126/74	11/7	41/24	3/1	6/7	61/35	>0.05
Fever (%)	65 (32.5)	5 (27.8)	23 (35.4)	3 (75)	5 (29.4)	29 (30.2)	>0.05
Dyspnea (%)	122 (61.0)	13 (72.2)	40 (61.5)	4 (100)	12 (70.5)	53 (55.2)	>0.05
Tachypnea (%)	112 (56.0)	12 (66.7)	40 (61.5)	4 (100)	12 (70.5)	44 (45.8)	<0.05
Purulent sputum or tracheal secretion (%)	191 (95.5)	17 (94.4)	60 (92.3)	4 (100)	17 (100)	93 (96.9)	>0.05
APACHE II score, mean	16.9	12.8	17.2	16.3	20.2	16.9	>0.05
Blood WBC, mean	14.1	16.6	13.6	13.9	13.1	14.2	>0.05
Serum CRP, mean	10.6	14.1	10.1	6.0	12.0	10.3	>0.05
PaO_2/FiO_2, mean	261	282	249	280	230	270	>0.05
14 days mortality, (%)	54 (27)	3 (16.6)	19 (29.2)	1 (25)	4 (23.5)	27 (28.1)	>0.05

Among 200 cases studied using the PCR test, 31 cases (15.5%) had a viral agent. Totally, eight cases (4%) had *M. pneumoniae*. The most frequently identified viral pathogens were RV, InfA, and CoV HKU, with CoV 43, PIV 2, HMPV, and HBoV identified less often. The distribution of viral etiologic agents is shown in Table 3. When CAP and HAP groups were compared, there were no significant differences observed in terms of viral agents (Table 3). All patients were negative for *L. pneumophilia* and *C. pneumoniae* on PCR.

Bacterial pathogens were identified in 81 cases (40.5%) as a single pathogen, and 17 (8.5%) had two or more pneumonia agents. The distribution of isolated pathogens from bacterial cultures is demonstrated in Table 4. *P. aeruginosa* was isolated from two patients in the CAP group. These patients had hospitalization history in the hospital within one month; however, did not meet the HAP criteria. One patient in the CAP group had *A. baumannii* in the tracheal aspirate culture. This patient was admitted from the emergency department with a history of fainting in the garden bed. This *A. baumannii* isolate was very sensitive to most antibiotics.

L. pneumophilia was not detected in any patients with the BCYE media. No bacteria or viruses have been isolated in 96 patients.

Eighteen cases only had viral pathogens and none of these patients had any bacteria. For CAP patients, 13 cases had viral agents, five of them were mixed with bacteria and four patients had *M. pneumoniae*. For HAP cases, 18 had viral agents, eight of them were viral and bacterial mixed etiology. Four patients had *M. pneumoniae* in the HAP group, 3 of them mixed with bacteria and one case (0.5%) had both Parainfluenza and *M. pneumoniae*. There were no statistically significant differences identified in the viral agent incidences between CAP and HAP patient groups.

Fifty-four patients (27%) died in the pneumonia cases included in the study. Forty-three (31.8%) cases had mortal progression in HAP, and the greatest fatality rate was observed in the HAP group producing *A. baumannii*. Fatal progress occurred in 11 (17.5%) patients in the CAP group. Of the 31 cases identified positive for virus PCR, 6 (18.7%) had mortal progression. Three patients died in the group in whom only viral pathogens were isolated.

Table 3. Distribution of viral and atypical microorganisms identified with PCR and mortality outcomes

PCR results	Total, n (%)	CAP(63), n (%)	HAP (137), n (%)	p	Mortality (54), n (%)
Negative	161 (80.5)	47 (74.6)	114 (83.2)	>0.05	46 (23)
M. pneumoniae	8 (4)	4 (6.3)	4 (2.2)	>0.05	2 (1)
Influenza A Influenza B	5 (2.5) 2 (1)	2 (3.2) 0	3 (2.2) 2 (1.5)	>0.05	2 (1) 0
Rhinovirus	10 (5)	2 (3.2)	8 (5.8)	>0.05	3 (1.5)
Coronavirus HKU	4 (2)	2 (3.2)	2 (1.5)	>0.05	0
Coronavirus 43	1 (0.5)	1 (1.6)	0	>0.05	0
Parainfluenza virus 1 Parainfluenza virus 2	2 (1) 1 (0.5)	0 0	2 (1.5) 1 (0.7)	>0.05	0 0
Human metapneumovirus A/B	1 (0.5)	1 (1.6)	0	>0.05	1 (0.5)
Human bocavirus	1 (0.5)	1 (1.6)	0	>0.05	0
Respiratory syncytial virus A/B	2 (1)	2 (3.2)	0	>0.05	0
Enterovirus	1 (0.5)	0	1 (0.7)	>0.05	0
Adenovirus	2 (1)	1 (1.6)	1 (0.7)	>0.05	0
Coronavirus 63, 229, Human parechovirus parainfluenza virus 3, 4	0 (0)	0	0	-	0
Total viral pathogens	31 (15.5)	11 (17.5)	20 (14.6)	-	6 (3)

Table 4. Distribution of isolated bacterial pathogens from microbiological cultures

Microbiological culture	CAP	HAP	Total
No bacterial growth	43	70	118
Acinetobacter baumanii	1	22	24
Pseudomonas aeruginosa	2	7	9
Klebsiella pneumoniae	1	8	9
Staphylococcus aureus	4	9 (3*)	13
Streptococcus pneumoniae	1	0	1
Escherichia coli	3	5	8
Other gram-negative bacteria	3	4	5
Mix bacterial infection	5	12	17
Total	63	137	200

CAP: Community-acquired pneumonia, HAP: Hospital-acquired pneumonia.
*MRSA: Methicillin-resistant *S. aureus*.

DISCUSSION

Pneumonia is responsible for a significant portion of serious causes of morbidity and mortality in ICUs. Bacterial pathogens are more researched and understood due to their easy identification in culture, but data on the frequency of viral and atypical bacterial infectious agents that are difficult to produce in culture media are limited[8]. Epidemiologic information about the pathogens in pneumonia for the community and the nosocomial source is very few in our region. In this study, both HAP and CAP patients were included to understand the frequency of

these pathogens and clinical course in severe pneumonia cases.

The range of respiratory viruses in patients admitted to the ICU has been reported in a range of 16-49%[8-10]. We demonstrated viral pathogens in 15.5% of the cases in ICUs. In our study, the rate of viral pneumonia may be low because it included the summer season when viral infections were less common.

Viral agents have been reported at a rate of 32-34% in HAP cases. While Inf A and HRV are the most common causes of viral pneumonia, these are followed by RSV, PIV, and BV. Forty-seven percent of the HAP patients were immunocompromised[9,11]. In our study, the PCR method identified a viral agent in 31 cases (15.5%) in total and in 20 (14.6) with HAP. The most frequently identified viral agents were HRV (5%), CoV HKU (2%) and InfA (2.5%). The reason for less viral pneumonia may be explained with the exclusion of the hematologic and solid organ malignancies and transplant patients in our study.

The viral pneumonia agent of the influenza virus has a high mortality risk for immunosuppressed patients, patients over 65 years, and pregnant cases. The influenza (H1N1) virus was first reported in 2009 and rapidly spread around the world. In Turkey, an H1N1 epidemic was experienced, especially in ICUs in our country[12-24]. In our study, despite cases from both the summer and winter periods, 5 cases (2.5%) with INFA and 2 cases (1%) with INFB were identified. These rates are lower compared to other studies in Turkey. The low identification of the influenza virus in our study may be explained by the study not encompassing any epidemic period and including cases admitted during the summer period.

In a retrospective 4-year postmortem study, respiratory viruses have been detected in 20% of the adults in Turkey. The most common viruses in adults were HRV and Inf A. Viral etiology in adults has been reported less than in children[15]. Our results are similar with the studies from our country.

In the advanced age group, pneumonia is observed more frequently in those with other underlying diseases such as diabetes, chronic obstructive pulmonary diseases (COPD), chronic liver disease that progress more severely[16,17]. In our study, when comorbid diseases were investigated, the most common sub-diseases were CNS diseases (45%), COPD (16.5%), and KV diseases (26%). We thought that the reason for the high rate of CNS disease in our study might be because two of the six intensive care units where the study was conducted were Neurology Stroke Unit and Neurosurgical ICU.

Fatality rates in viral pneumonia patients in ICU have been reported 26-35% in different studies[11,18]. In our study, 14-day mortality was detected in 16.6% of viral pneumonia patients. Mortality has been reported between 10-13% in some studies in our country. We found that the mortality rate in all patients was 27%, while it was 17.5% for CAP cases and 31.4% for HAP cases. Compared with the literature, higher mortality rate, which was the main finding of our study, is considered to be due to most cases comprising HAP cases and patients in the study being chosen from patients who needed intensive care admission. There was no statistically significant difference identified between patients with bacterial, viral, mixed, or no agent identified pneumoniae in terms of mortality. In addition, four cases were identified to have CoV HKU and one case had CoV 43 in this study. Despite the new coronavirus, SARS-CoV-2, none of the five cases with CoV identified were fatal.

Patients with a preliminary diagnosis of pneumonia are seen to have common symptoms of fever, shivering, shaking, pleuritic chest pain and mucopurulent sputum accompanied by cough[19]. Studies have shown that some cases (especially the elderly) may not have a cough, increased sputum, or leukocytosis, and nearly 30% of patients may not have a fever[12,13,20]. In our study, 95.5% of the cases had purulent sputum, 61% had dyspnea, 56% had tachypnea, and 32.5% had a fever. While there was no significant difference between the groups in terms of other symptoms, tachypnea was found to be significantly higher in the viral pneumonia group.

With the increasing use of PCR recently, identification of *Legionella*, *Chlamydophila*, and

Mycoplasma in addition to viral pathogens has ensured great benefit, though there is no definite information about whether these agents are colonization or infection[19,20]. Some studies carried out to identify atypical agents in CAP cases in our country have identified 0-28.6% rates for *C. pneumoniae*, 1.3-25% rates for *M. pneumoniae*, and 0-12% rates of *L. pneumophila* [13,21-23]. In our study, only 4% (eight cases) of all cases had *M. pneumoniae*, while no cases had *C. pneumoniae* or *L. pneumophilia*. We thought that antibacterial usage before admission may have affected the identification of these microorganisms; however, most of the patients were in the HAP group. *L. pneumoniae* was not detected in both groups by culture and PCR method, which suggests that *Legionella* and *C. pneumoniae* may be uncommon pathogens in our region and hospital. However, there is a need for multicenter studies.

In pneumonia cases, high WBC, CRP and PCT values are usually observed[11,24]. In our study, cases with one viral agent identified had WBC and CRP values of 16.08 ± 5.3 and 14.2 ± 11.8, (mean ± SD), respectively. Statistical significance could not be obtained for laboratory parameters investigated for differentiating bacterial and viral pneumonia. This situation is considered due to the small number of cases with viral pneumonia included in the study and the inclusion of severe cases requiring intensive care.

CONCLUSION

This study demonstrated epidemiologic data of viral and atypical bacterial etiologic pathogens in CAP and HAP patients who need ICU admission. The most frequently identified viral agents in patients with community-acquired pneumonia were RV and CoV, with influenza in the third place. For HAP, the most common viral agents were RV and influenza, so viral agents should be considered in the etiology of HAP. None of the patients in this study were identified to have *L. pneumoniae* and *C. pneumoniae* and few cases were identified to have *M. pneumoniae* showing that atypical pneumonia agents are not common in our region. Increasingly effective use of molecular methods over time will benefit the management and prognosis of patients with viral and atypical bacterial pneumonia and provide benefit in terms of rational use of antibiotics. There is a need for multicenter, prospective studies including larger number of cases.

ACKNOWLEDGEMENT

All the authors thank to Nafia Canan Gürsoy, Ph.D., from the Department of Medical Microbiology, for help on microbiological analyses in this study.

ETHICS COMMITTEE APPROVAL

The ethical approval for this study was obtained from Clinical Research Ethics Committee (Decision no: 2016/116, Date: 08.06.2016).

CONFLICT of INTEREST

None of the authors had conflict of interest.

AUTHORSHIP CONTRIBUTIONS

Concept and Design: All of authors

Data Collection or Processing: AD, BO, ÇK

Analysis/Interpretation: AD, YEÇ

Literature Search: YEÇ

Writing: AD

Final Approval: All of authors

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