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MULTIPLEX PCR IN DIAGNOSING RESPIRATORY TRACT INFECTIONS IN HOSPITALIZED CHILDREN

Gorica Popova[1, 2], Tatjana Jakjovska[1, 3], Ivana Arnaudova-Danevska[1, 3], Katerina Boskovska[1, 3], Olga Smilevska Spasovska[1]

[1] Institute for Respiratory Diseases in Children-Kozle, Skopje, RN Macedonia

[2] Faculty of Medical Sciences, Goce Delcev University, Stip, RN Macedonia

[3] Faculty of Medicine, Ss. Cyril and Methodius University in Skopje, RN Macedonia

Corresponding author: Gorica Popova, Tel. +38975265407, E-mail: gorica.popova@yahoo.com

ABSTRACT

Objectives: To elaborate the utility of multiplex quantitative polymerase chain reaction (multiplex qPCR) for the accurate diagnosis of severe respiratory tract infections (RTIs) in hospitalized children.

Methods: In two separate periods during 2022, 76 respiratory specimens (combined throat/nasopharyngeal swabs) were submitted for multiplex qPCR regarding 26 respiratory pathogens. The specimens were obtained from children with severe RTIs hospitalized in the Institute for Respiratory Diseases in Children, Skopje.

Results: Multiplex qPCR detected at least one respiratory pathogen in all examined specimens (76/76), with 83% (63/76) rate of co-infections. Considering that positive results are only the ones with Ct value below 28, the rates of detected pathogens and co-infections decrease to 75% and 22%, respectively. The most commonly detected pathogens during the spring period were Parainfluenza type 3 (PIV3) followed by Adenovirus (AdV) and Respiratory syncytial virus type B (RSVB) with frequency rate of 23%, 19% and 19%, respectively. During the autumn period, the most common were RSVB and *Streptococcus pneumoniae* with frequency rate of 31% and 17%, respectively.

Conclusion: Multiplex qPCR is a powerful tool for diagnosing RTIs. Semi-quantification of the viral load by reporting Ct values added higher level of evidence for accurate diagnosis. Seasonal detection of the examined viruses was notable with higher prevalence of PIV3 in spring and RSVB in autumn period.

Keywords: Multiplex qPCR, Respiratory tract infections, children

INTRODUCTION

Respiratory tract infections (RTIs) are a significant cause of morbidity and mortality worldwide, especially in young children and elderly. The majority of RTIs are caused by respiratory viruses, followed by bacterial pathogens, and sometimes, mixed bacterial-viral co-infections can occur [1]. Even though the majority of RTIs are caused by respiratory viruses, the burden of the antimicrobial prescriptions is evident especially in developing countries, and one of the reasons for this is probably the lack of rapid and accurate laboratory tests for respiratory pathogens [2,3]. On the other hand, viral respiratory infections sheared many similar clinical features, so testing should be encouraged also to prevent unnecessary prescriptions of antivirals in "similar looking" non-influenza cases, where neuraminidase inhibitors would be ineffective [4]. In the past decades, a great advantage in diagnosing viral infections is achieved by the

development of molecular diagnostic technologies based on the principle of nucleic acids amplification. Advanced molecular technologies such as multiplex quantitative polymerase chain reaction (multiplex qPCR), further enhance the microbiological diagnostic capacity enabling simultaneous detection and quantification of multiple pathogens in a single tube [5]. Created in this manner, the panels often detect multiple pathogens which can cause confusion and prevent their correct interpretation, especially if the results are reported only as positive or negative. The reason for this is the high sensitivity of the method which can detect residuals of non-infectious RNA or DNA of the responsible pathogens beyond the acute phase of the disease and infectivity of the patient. This is the reason why according to the hierarchical framework for the causality of the detected respiratory pathogens, PCR binary positive/negative results represent the weakest level of evidence [6]. In this context, the aim of this work was to elaborate on the use of multiplex qPCR not only as a method for qualitative detection of the target pathogens, but also for their semi-quantification by issuing cycle threshold (Ct) values that would add a higher level of evidence and lead to accurate diagnosis of respiratory infections.

MATERIAL AND METHODS

The study population comprised hospitalized children with severe acute respiratory tract infections in the Institute for Respiratory Diseases in Children, Skopje. The study was done in two separate periods – the first period was from April 12 to May 03, 2022, and the second period from October 05 to December 02, 2022. Out of 76 patients involved in the study, 36 were included in the first period and 40 in the second period. Only one specimen (combined throat/nasopharyngeal swab) was obtained per patient. The patients were sampled in a period of one to three days after their admission to hospital. Sampling was done for the Reverse Transcription quantitative multiplex PCR (RT-qPCR) regarding 26 respiratory pathogens.

Diagnostic test

Multiplex-qPCR was performed in the certified microbiological laboratory at the Institute for Respiratory Diseases in Children, Skopje. Nucleic acid extraction, multiplex-qPCR and results interpretation were performed according to the instructions of the manufacturer. Briefly, RNA/DNA was isolated and purified using the STARMag 96 ProPrep extraction kit (Seegene, South Korea) on an automatic nucleic acid extractor SEEPREP 32 (Seegene South Korea). Amplification was performed in a four-tube assay using Allplex Respiratory full panel Seegene Inc**.,** consisting of:

Respiratory Panel 1 - One-step RT-PCR for detection of: Influenza A virus, Influenza B virus, Human respiratory syncytial virus A, Human respiratory syncytial virus B, and Influenza A virus subtypes (Human influenza A virus subtype H1, H3 и H1pdm09).

Respiratory Panel 2 - One-step RT-PCR for detection of: Human adenovirus, Human metapneumovirus, Human enterovirus, Human parainfluenza virus 1, Human parainfluenza virus 2, Human parainfluenza virus 3, Human parainfluenza virus 4.

Respiratory Panel 3 - One-step RT-PCR for detection of: Human bocavirus 1/2/3/4, Human rhinovirus A/B/C, Human coronavirus 229E, Human coronavirus NL63 and Human coronavirus OC43.

Respiratory Panel 4 - Multiplex real-time PCR for detection of: *Chlamydiophila pneumoniae, Mycoplasma pneumoniae, Legionella pneumophila, Bordetella pertussis, Bordetella parapertusis, Streptococcus pneumoniae, Haemophilus influenzae.*

Amplification and detection were performed on a CFX-96 real-time thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The conditions consisted of 1 cycle of 20 min. at 50°C, 15 min. at 95°C and followed by 45 cycles of 10 s at 95°C, 1 min at 60°C, 10 s at 72°C. The results were interpreted with Seegene Viewer data analysis software, in which the threshold Cycle (Ct) was automatically determined, and the positive result was defined as amplification of the targeted genes within the cut-off values < 42.

Additional calculation was performed by defining positive detection within the cut-off values < 28.

Statistical analysis

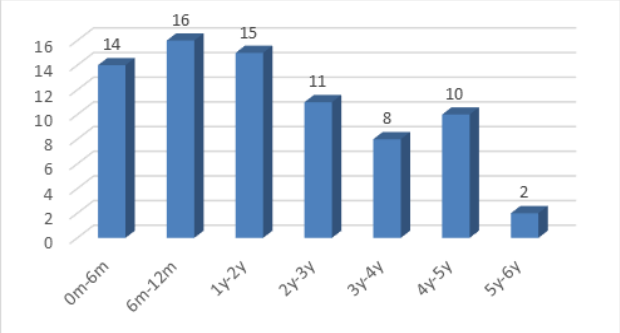
Ct values of all positive detections of target pathogens were used to establish a database for

descriptive statistical analysis. The comparison of counting data was performed by χ^2 test, and $p < 0.05$ was used to indicate significant differences.

RESULTS

In this study, out of 76 included patients, 42 (55.3%) were male and 34 (44.7%) female. Age distribution of the patients enrolled is given in Figure1.

Figure 1. *Age distribution of the patients enrolled*



In the summary calculation of both periods, multiplex PCR detected at least one respiratory pathogen in all examined specimens 76/76 (100%), with 63/76 (82.9%) rate of co-infections. Single detection was observed in 13 specimens (17.1%), dual detections were observed in 21 specimens (27.6%), triple in 28 (36.8%) quadruple in 7 (9.2%), five detections in 5 specimens (6.6%), six detections in 1 specimen (1.3%) and seven detections in 1 specimen (1.3%).

Considering that positive results were only if the Ct value was below 28, the rate of detected pathogens decreased from 100% (76/76) to 75% (57/76). The rate of detected co-infections decreased from 82.9% (63/76) to 22.4% (17/76) which was statistically significant χ^2 (1, N=76) = 46, $p < 0.001$. Single detection was observed in 40 patients (52.6%), dual detections were observed in 14 patients (18.4%), three detections in two patients (2.6%) and five detected targets were observed in one patient (1.3%) (Table1).

Defining positivity within the cut off < 28, during the spring period, of all positive detections the most prevalent were Parainfluenza virus 3 (PIV3) followed by Adenovirus (AdV) and Respiratory syncytial virus type B (RSVB) with frequency rate of 22.6%, 19.3% and 19.3% respectively (Figure 2). During the autumn period, of all positive detections, the most prevalent were RSVB and Streptococcus pneumoniae (SP) with frequency rate of 31% and 17% respectively (Figure 3).

The reduction of individual target positivity depending on its Ct value is shown in Figure 4. The highest decrease in positivity was seen in Human bocavirus (HBoV), Haemophilus influenzae (HI), Human rhinovirus (HRV), SP and AdV with 92.3%, 80.5%, 66.7%, 66.7% and 57.7% respectively (Figure 4), which was of statistical significance ($p < 0.001$).

Table 1. *Number of pathogens detected per sample with positivity defined within Ct < 28*

One pathogen detected n=40	PIV3 n=6 (24.38); (15.81); (22.74); (26.25); (20.05); (17.92); RSVB n=12 (25.98); (25.34); (27.08); (25.64); (18.06); (24.57); (27.56); (22.74); (24.08); (26.11); (25.71); (27.13); AdV n=7 (23.75); (26.7); (20.22); (25.41); (17.4); (14.19); (19.51); hMPV n=3 (26.94); (22.98); (27.11); HRV n=4 (24.43); (26.2); (27.64); (23.35); SP n=2 (27.79); (24.93); OC43 n=2 (17.0); (23.83); HEV n=1 (27.77); NL63 n=1 (23.93); BPP n=1 (23.55); HBoV n=1 (17.42);
Two pathogens detected n=14	PIV3 (22.73) + SP (26.75); RSVB (23.86) + HI (27.74); AdV (25.55) + HI (26.45); RSVB (16.24) + SP (24.53); RSVB (25.15) + SP (25.25); RSVB (23.16) + HI (23.31); RSVA (27.21) + HI (22.73); AdV (22.54) + SP (24.22); HEV (26.16) + SP (26.69); hMPV (25.76) + HI (27.94); AdV (16.88) + HI (27.4); RSVB (21.83) + HRV (26.12); RSVB (20.93) + HRV (25.31); RSVB (27.34) + HRV (26.12);

Three pathogens detected n=2	RSVA (23.43) + SP (26.28) + HI (27.09); RSVB (25.25) + SP (27.32) + HI (27.87);
Five pathogens detected n=1	RSVB (19.61) + AdV (27.31) + HRV (25.41) + SP (23.95) + HI (27.13);

*Each target is accompanied with its Ct value given in numbers in parentheses

n - number of samples

Haemophilus influenzae (HI), Respiratory syncytial virus B (RSVB), Streptococcus pneumoniae (SP), Human adenovirus (AdV), Human metapneumovirus (hMPV), Parainfluenza virus 3 (PIV3), Human rhinovirus A/B/C (HRV), Respiratory syncytial virus A (RSVA), Human bocavirus1/2/3/4 (HBoV), Human enterovirus (HEV), Parainfluenza virus 1 (PIV1), Bordetella parapertusis (BPP), Parainfluenza virus 4 (PIV4), Human coronavirus NL63 (NL 63), Human coronavirus OC43 (OC43)

Figure 2. *Targets detected during the spring period with positivity defined within the cut-off < 28*

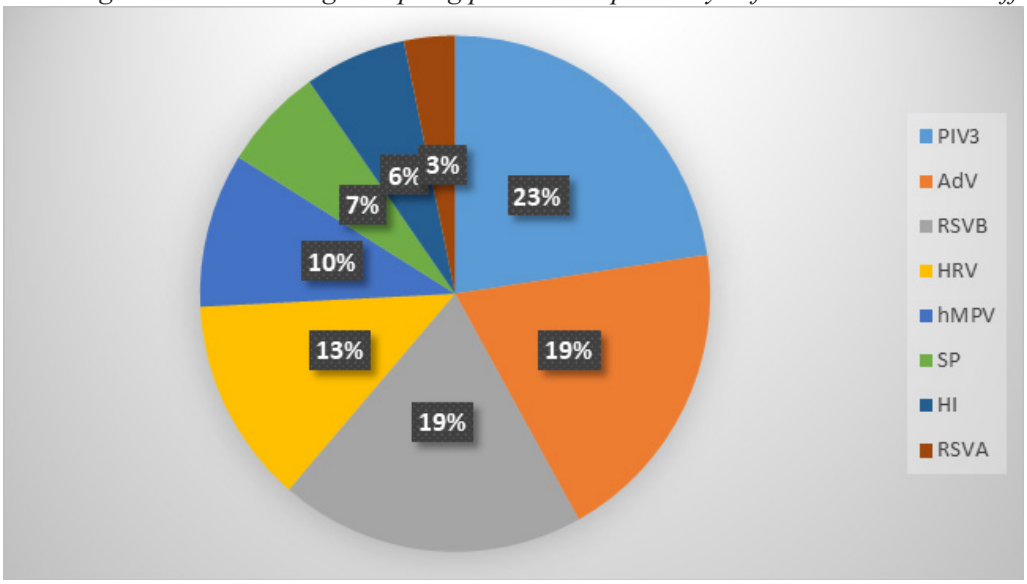


Figure 3. *Targets detected during the autumn period with positivity defined within the cut-off <28*

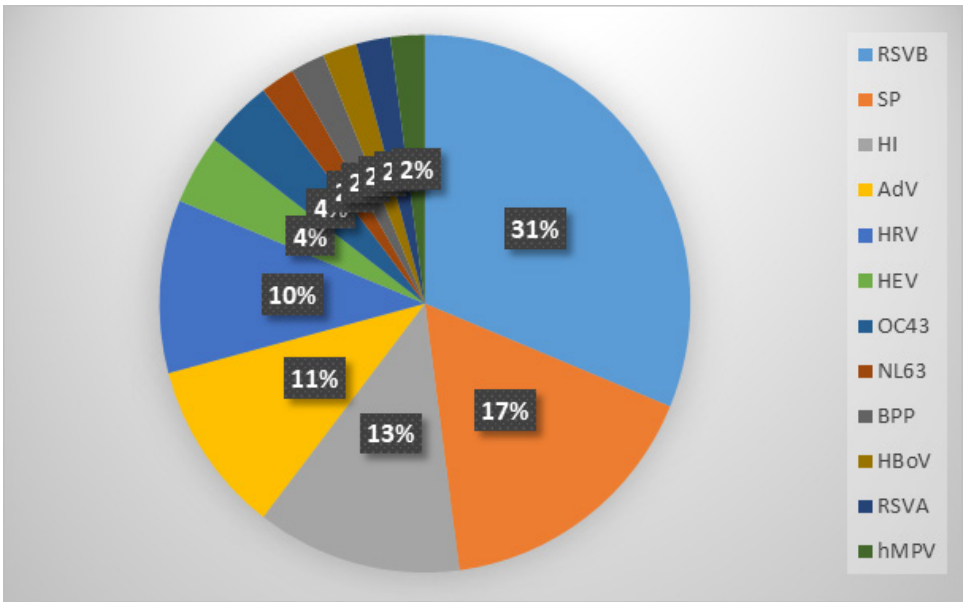
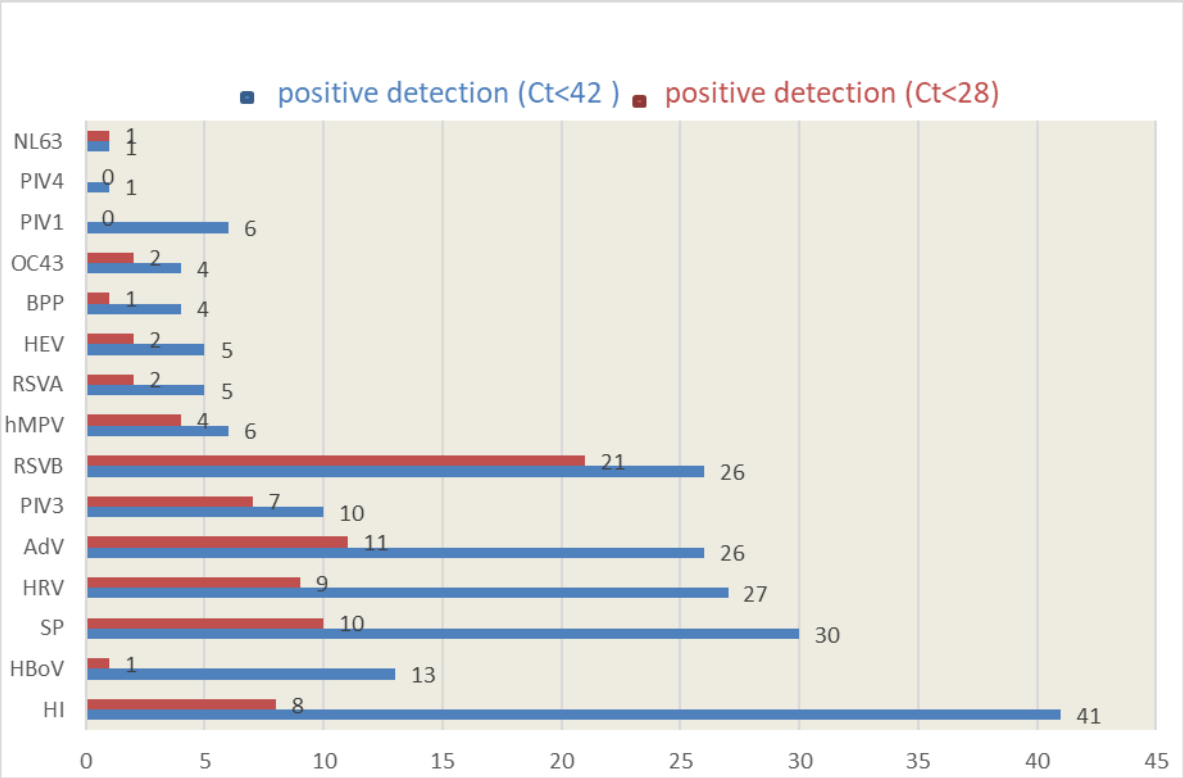


Figure 4. *Differences between the targets detected with different positivity criteria*



Blue series represent the number of positive targets within the Ct cut-off < 42, while the red series represent positive targets within the Ct cut-off < 28.

DISCUSSION

In this study, Multiplex qPCR was used for detection of respiratory pathogens among children hospitalized due to the severity of the respiratory infections. Multiplex qPCR was carried out by the panel designed to detect broad spectrum of targets (26 respiratory pathogens). So, taking this into account, together with the small age of the study group (59% were under the age of two, Figure 1) and the reporting of the results in a binary manner (positive/negative), the high positivity of tested samples is not very surprising. At least one pathogen was detected in all examined specimens with co-infection rate of 83%. Using the binary manner of reporting, Kholyet et al. [7] reported similar results with overall 89.9% positivity and co-infection rate of 46.9%, using a panel designed for 16 viral targets, whereas Bierbaum et al. [8] reported 82% positivity among group of children younger than 2 years, using panel for 15 targets. The deference in positivity between age groups is also described in the study of Yang et al. [9]. Similar results are shown in the study by Zimmerman et al. [10], where co-infections were reported more commonly than single infections in children in comparison with older adults (≥65 years; $p = 0.01$). The reason for that was thought to be the higher rate of acute respiratory infections in young children and because of the short period between infections it was assumed that they overlap.

However, reporting the results in semi-quantitative manner brings higher level of evidence about the causality of detected respiratory pathogens. In this context, during the SARS-CoV-2 pandemic, the increasing body of evidence demonstrated a close relationship between the viral load (e.g., copy number and cycle thresholds - Ct) and the probability of culturing replication-competent virus, defining cut-off as 10^5 copies/ml as threshold value for the absence of cytopathogenic effect (CPE) [11, 12, 13, 14]. The working group of the Nationwide Harmonization Effort for Semi-Quantitative Reporting of SARS-CoV-2 PCR Test Results in Belgium [15] defined

a correlation between the Ct values and the RNA copies/ml, and the 17 PCR assays that had met the inclusion criteria, and the mean Ct values which correspond to the 10^5 RNA copies/ml were in the range of 21.9 to 27.4. The same working group mentioned that from their work on cell cultures the highest Ct value that resulted in CPE was 28.5. In the work of Marot et al. [16] no isolate was recovered from the cell lines when the viral load was below 5.83 log10 cp/ml (i.e., Ct > 28).

Based on the data from the literature, we decided to make an additional calculation defining the positive detection within the cut-off < 28. When this criterion was applied, the overall positivity fell from 100% to 75%. The multiple-detection fell from 83% to 22%. Under these conditions, only one pathogen was detected in most of the examined specimens 40 (53%), which significantly differs compared to the previous calculation 13 (17%). By defining the positivity within the cut-off < 28 in the co-pathogens group, the majority of 76% (13/17) was a combination of viral and bacterial detection (Table 1). Dual viral targets (RSVB + HRV) were detected in three samples. One sample was with five detections of which three were viral (RSVB+AdV+HRV+SP+HI), with remarkably highest concentration of RSVB with Ct=19.6 (Table 1). Overall, when defining positivity only with Ct < 28, multiple-viral detection was found to be very low, only in four patients out of 76 (5.3%).

The detected respiratory pathogens with high Ct values refer to the non-infective traces of viral RNA or DNA detectible after the acute phase of the infection. In this work, the highest rate of such detection was observed for HBoV, HI, HRV, SP and AdV in the rate of 92.3%, 80.5%, 66.7%, 66.7% and 57.7% respectively (Figure 4).These findings correlate with the data from the literature regarding high detection of HRV, AdV, HBoV in healthy children, probably reflecting the previous infections and the ability of these pathogens to persist in a concentration detectable by PCR beyond the active phase of the infection [7, 17]. Besides the high rate of detection of HBoV with Ct > 28 and the controversy of its clinical importance, in one patient aged 7 months manifested with bronchiolitis, HBoV was detected with Ct=17.42 (Table 1) which implies a high viral load. This finding correlates with the data published by Allander et al, about the connection of high HBoV load of the respiratory tract and the acute wheezing in children [18]. The high detection of HI and SP with Ct > 28 probably reflects high bacterial colonization of the upper respiratory tract in young children [19, 20].

The most commonly detected pathogen with Ct < 28 during the spring period was PIV3 with 23% (Figure 2). During the autumn period, the most detected target with Ct < 28 was RSVB with 31% (Figure3). The hMPV was detected in a total of six specimens, but in four of them with remarkable viral loud (Ct < 28). These findings correlate with the data published by Rhedin et al. showing that the nucleic acids of RSV, hMPV, and parainfluenza virus can be confirmed almost exclusively in symptomatic children and very rarely in asymptomatic children [21]. In our study this finding did not correlate with respect to PIV1 which was detected in six specimens and in all detections the Ct values were > 28 (Figure 4).

Even though the second part of the study was performed during the influenza season, we had no detection of Influenza RNA in our study group. The reason for that is probably the fact that in the period examined (October 05-December 02, 2022) the epidemiologic results obtained from the influenza virological surveillance were below the 10% positivity limit. According to these data, North Macedonia had a sporadic prevalence and low intensity of influenza virus activity in this period [22]. With the results from multiplex qPCR, clinicians were alerted that anti-influenza therapy was not indicated, although there were influenza-like symptoms in the studied patients during this period.

CONCLUSION

Multiplex qPCR is a powerful tool in diagnosing respiratory tract infections. Reporting results in semi-quantitative manner instead of in binary manner (positive/negative) adds additional value to the interpretation of the positive results allowing clinicians to make informed and correct therapeutic and epidemiological decisions. Sometimes these broad-spectrum panels can reveal an unexpected respiratory pathogen as was the case with HBoV in this study. The tendency for seasonal occurrence of certain viruses was determined by a higher prevalence of PIV3 in spring and RSVB in autumn period.

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Резиме

УПОТРЕБАТА НА МУЛТИПЛЕКС ПВР ВО ДИЈАГНОЗА НА РЕСПИРАТОРНИТЕ ИНФЕКЦИИ КАЈ ХОСПИТАЛИЗИРАНИ ПЕДИЈАТРИСКИ ПАЦИЕНТИ

Горица Попова[1, 2], Татјана Јаќовска[1, 3], Ивана Арнаудова-Даневска[1, 3], Катерина Бошковска[1, 3], Олга Смилевска Спасовска[1]

[1] Институт за белодробни заболувања кај децата-Козле, Скопје, РС Македонија

[2] Факултет за Медицински науки, Универзитет „Гоце Делчев“, Штип, РС Македонија

[3] Медицински факултет, Универзитет „Св. Кирил и Методиј“, Скопје, РС Македонија

Цел: Да се утврди вредноста на мултиплекс полимераза верижна реакција (ПВР) во поставувањето на етиолошката дијагноза на респираторни инфекции кај хоспитализирани педијатриски пациенти.

Метод: Во два одвоени периоди во текот на 2022 година, кај 76 хоспитализирани пациенти беше реализирана мултиплекс ПВР за детекција на 26 респираторни патогени (19 вируси и 7 бактерии).

Резултати: Со мултиплекс ПВР беше детектиран барем еден патоген во сите испитувани примероци (76/76), при што кај 83 % (63/76) беа детектирани копатогени. Дефинирајќи ги позитивнте резултати само со Ct < 28, позитивната детекција падна на 75 %, а детекцијата на копатогени на 22 %. Во текот на пролетниот период најчесто детектирани патогени беа Parainfluenza type 3 (PIV3) со 23 %, Adevovirus (AdV) со 19 % и Respiratory syncytial virus type B (RSVB) со 19 %, додека во текот на есенскиот период RSVB со 31 % и *Streptococcus pneumoniae* со 17%.

Заклучок: Мултиплекс ПВР е моќно орудие во дијагнозата на респираторните инфекции. Семиквантитативното одговарање преку Ct-вредностите овозможува попрецизна дијагноза на акутните инфекции. Евидентно е сезонско детектирање на вирусите со поголема преваленца на PIV3 во текот на пролетниот период и на RSVB во текот на есенскиот период.

Клучни зборови: респираторни инфекции, мултиплекс ПВР, педијатрија