Egyptian Journal of Medical Microbiology Volume 29 / No.3 / July 2020 129-136 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Rapid Detection of Viral Pneumonia in Children with Primary Immunodeficiency

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Key words:
Primary immunodeficiency,viral pneumonia, multiplex PCR

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ABSTRACT

Background: *In children with primary immunodeficiency (PID), the most common types of infection are respiratory tract infections, among which viral pneumonia representing the majority. Early diagnosis and treatment can prevent or diminish the respiratory complications. Multiplex polymerase chain reaction (Multiplex PCR) can permit amplification of a lot of viruses all together in a single reaction mixture.* ***Objective:*** *Determining different viruses causing pneumonia in children with PID by multiplex PCR.* ***Methodology:*** *A cross-sectional study included 33 nasopharyngeal swabs collected from children suffering from PID with clinical respiratory signs of pneumonia. For each collected sample, nucleic acid extraction and multiplex PCR were done using primers specific for influenza A, influenza B, adenovirus, respiratory syncytial virus (RSV), cytomegalovirus (CMV), parainfluenza virus 1 (PIV1), parainfluenza virus 2 (PIV2), parainfluenza virus 3 (PIV3) and human coronavirus (HCoV-NL63).* ***Results:*** *Viruses were detected in 11 nasopharyngeal samples (11/33, 33.33%). Viral pneumonia was mostly found in children with severe combined immune deficiency (5/11; 45.45%), followed by children with common variable immunodeficiency (3/11; 27.27%) and children with hyper Ig E syndrome, hyper Ig M syndrome and selective Ig A deficiency (1/11; 9.09%) for each. RSV and CMV were the most common detected viruses; each virus was detected in 3 cases. Influenza virus A was detected in 2 cases and adenovirus, PIV1 and PIV3 each was detected in one case. Influenza virus B, PIV2 and HCoV-NL63 were not detected in any sample. No viruses were detected in the nasopharyngeal swabs taken from children with chronic granulomatous disease. No mixed viral infection was detected in any sample.* ***Conclusion:*** *Severe viral pneumonia is frequent in the children with PID. Multiplex PCR has a major benefit, as it allows simultaneous amplification of several viruses in a single reaction mixture with cost-effective diagnosis.*

INTRODUCTION

Primary immunodeficiencies (PIDs) are hereditary disorders with one or some immune system components are diminished, absent, or of inappropriate function[1]. Primary immunodeficiency disorders can be divided into 2 large groups: disorders caused by defects in the innate immune system such as Toll-like receptors (TLRs), natural killer (NK) cells, phagocytes and interferons (IFNs) and other disorders caused by defects in the adaptive immune system such as lymphocyte-derived cellular immunity and antibody response [2].

T cells chiefly eradicate the infecting virus, while antibody is mainly responsible for protection against recurrence of viruses. Consequently, viral infections are more considerable in patients with deficiency of T-cell immune response. Both the extent of T- cell response and the character of the invading virus will determine the severity of disease[3]. Also, it was reported that reduced formation of type I and type III interferons was associated with severe viral infection specially with influenza virus[4]. So, severe viral infections could result from poor lymphocyte response especially $CD8^+$ T-cell response and also deficient innate immune response[2].

Children with PIDs are susceptible to severe viral infections particularly respiratory ones. Children under 5 years, with PIDs have six to eight respiratory infections every year. The winter months, daycare attendance, exposure to smokers, and atopy may increase these infections considerably[5]. Viral infections are possibly the characteristic feature and could be considered the most important morbidity for these children. Children with a frequent or severe viral pneumonia are often referred for an immunologic assessment. Now, newborn screening for detection of severe combined immune deficiency (SCID) are routinely done, but this is not present worldwide[3].

DOI: https://doi.org/10.51429/EJMM29317

In most cases of PIDs, bacterial super-infection often occurs on the top of viral pneumonia particularly with prolonged viral infections. So, it is common to see mixed infections with both viruses and bacteria. In children with antibody defects, pneumonia may falls into this type[3].

Children with PID are susceptible to the common respiratory viruses causing pneumonia in immunocompetent population. RSV and influenza virus are classically widespread during the respiratory season[6]. In addition, corona virus and metapneumovirus are known to cause viral pneumonia in children with PID[5].

Early diagnosis of viral pneumonia and proper treatment can prevent or at least minimize the respiratory complication of PIDs[1]. All respiratory pathogens cause very similar clinical symptoms. So, differential diagnosis of the pathogens is required in a single sample. Monospecific PCR separately amplify each target but with costly and rigorous supply. On the other hand, multiplex PCR has a considerable benefit, because it allows simultaneous amplification of several viruses in a single reaction mixture, with cost-effective diagnosis. Then, these multiplex PCR assays distinguish the target according to PCR fragment size by electrophoresis or hybridization with specific probes[7].

This study aimed at determining incidence of viral pneumonia among children suffering from PID and determining causative viruses in these children by multiplex PCR.

METHODOLOGY

Study design

This study was performed as a cross-sectional study from June 2017 to May 2019, after the approval of Institutional Review Board of the Faculty of Medicine, Mansoura University, code number: R/18.09.275. The study included 33 respiratory samples (nasopharyngeal swabs) that were collected from 33 children suffering from PID with clinical respiratory signs of pneumonia. These children were classified according to the International Union of Immunological Societies, Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity[8]. These children were either admitted or coming to the outpatient clinic of Mansoura University Children Hospital. Nasopharyngeal swabs were taken from the patients before initiation of treatment. These samples were taken by entering the nose with a swab and moving it two or three times on both tonsil medials and behind the uvula. The taken samples were carried to the laboratory using the eSwab Liquid Amies transport medium (Copan, Italia). The samples were kept at -20°C until they were worked on.

Exclusion criteria

Children under treatment with immunosuppressive drugs or infected with HIV or children with immunodeficiency secondary to any disease such as severe malnutrition, nephrotic syndrome or protein losing enteropathy were excluded from this study.

Nucleic acid isolation

Isolation procedures were done using a commercial viral DNA/RNA extraction kit (Viral Gene Spin, Boca Scientific Inc, USA). From each sample, 150 µl was taken and vortexed. Then, the extraction process was carried out according to the manufacture instructions.

cDNA synthesis

cDNA was achieved using the reverse transcription kit (Revert Aid First Strand cDNA synthesis kit, Fermantas, USA) according to the manufacture instructions.

Multiplex PCR

Table 1 illustrates the used primers sequences and the size of each amplified product (Promega, Madison, WI, USA). The PCR reaction contained 1.5 U of Takara Taq (Takara Bio, Dalian, China), 2.5 µl of 10× PCR buffer, 2.0 mM $MgCl_2$, 250 µM dNTPs, 25 pM of each primer, 2 µl DNA and nuclease-free water was added to a total volume of 25 µl. The amplification conditions of each PCR were as follows: predenaturation step for 5 min at 94°C, 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. Water was used instead of nucleic acid as a negative control[9]. The multiplex PCR was performed with DNA thermal cycler (Bio- Rad Laboratories Inc., Hercules, CA, USA).

Table 1: Respiratory viruses primers used for PCR assay (from GenBank database)[11-14]

Primer	Sequence (5' to 3')	Size of amplified products
Influenza A	F: AGGYWCTYATGGARTGGCTAAAG R: GCAGTCCYCGCTCASTGGGC	105 bp
Influenza B	F: GGAGAAGGCAAAGCAGAACTAGC R: CCATTCCATTCATTGTTTTTGCTG	503 bp
RSV	F: CTGTCATCCAGCAAATACAC R: ACCATAGGCATTCATAAACAATC	683 bp
PIV1	F: TCTGGCGGAGGAGCAATTATACCTGG R: ATCTGCATCATCTGTCACACTCGGGC	84 bp
PIV2	F: GATGACACTCCAGTACCTCTTG R: GATTACTCATAGCTGCAGAAGG	197 bp
PIV3	F: GATCCACTGTGTCACCGCTCAATACC R: CTGAGTGGATATTTGGAAGTGACCTGG	266 bp
Adenovirus	F: GCCGCAGTGGTCTTACATGCACATC R: CAGCACGCCGCGGATGTCAAAGT	308 bp
CMV	F: CAAGCGGCCTCTGATAACCAAGC R: CTCTTCCTCTGGGGCAACTTCCTC	370 bp
HCoV-NL63	F: ACACAGCTGAATCTTAAGTATGC R: TGGGATTATCCCAAATGTGA	251 bp

RSV: respiratory syncytial virus, PIV: parainfluenza virus, HCoV: human coronavirus, CMV: cytomegalovirus, F: forward primer, R: reverse primer, bp: base pair.

Agarose gel electrophoresis

Electrophoretic separation of the PCR product (10 µl) was done for 30 min at 130 to 160 mA on 2% agarose gels and UV illumination visualized the PCR product[10]. Ø X174 *Hae III* digested (Thermo Fisher Scientific, USA) was used as a marker to determine the fragment lengths (figure 1).

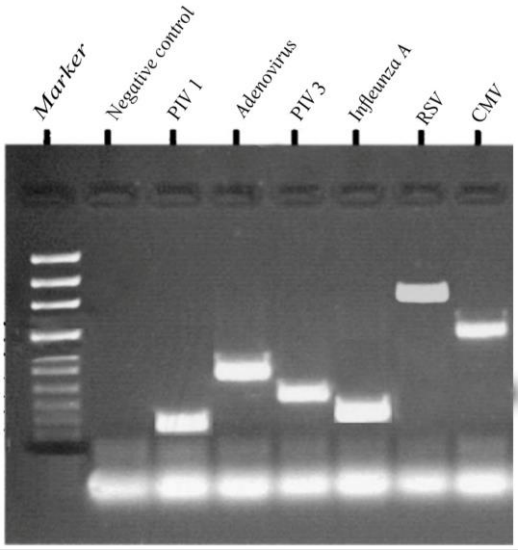

Fig. 1: Multiplex-PCR products on agarose gel. The sizes of the DNA fragments of the marker (Ø X174 *Hae III* digested) are as follows: 1,353 bp, 1,078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 271bp, 234 bp,194 bp,118 bp, 72 bp). The expected product lengths of each virus are given in the text.

Statistical Analysis

The data were analyzed using Package for Social Sciences 17.0 for Windows" (SPSS-17) software (SPSS Inc., Chicago, IL, USA). Data were expressed as median, range for numerical data; frequencies, percents for categorical data.

RESULTS

Clinical and demographical data of children with PID and pneumonia

Thirty three children with PID had clinical respiratory signs of pneumonia during the studied period. In this study, 8 children had severe combined immunodeficiency, 7 children had common variable immunodeficiency, 6 children had selective IgA deficiency, 4 children had hyper IgE syndrome, 3 children had hyper IgM syndrome and 5 children had chronic granulomatous disease. The study population comprised 22 boys and 11 girls with a male to female ratio of 2:1, and age ranged from 3 to 122 months (table 2).

Table 2: Clinical and demographical data of children with PID and pneumonia

Category	No of cases	Disorder	Age by months Median (Range)	Sex Boys/Girls
Combined B, T	8	Severe combined immune deficiency	12 (9-35)	6/2
	4	Hyper IgE syndrome	18 (16-36)	3/1
	3	Hyper IgM syndrome	52 (52-122)	2/1
Antibody deficiency	7	Common variable immunodeficiency	60 (30-120)	4/3
	6	Selective IgA deficiency	22 (12-84)	4/2
Phagocytic immunodeficiency	5	Chronic granulomatous disease	18 (12-22)	3/2
Total	33		22 (9-122)	22/11

Clinical and demographical data of children with PID and viral pneumonia

Clinical data of children with PID and viral pneumonia are listed in Table 3. Viruses were detected in nasopharyngeal samples of 11 children (11/33, 33.3%). Viral pneumonia was mostly found in children with severe combined immunodeficiency (5/11), and children with common variable immunodeficiency (3/11). Viral pneumonia was also detected in 3 children each had selective IgA deficiency, hyper IgM syndrome or hyper IgE syndrome (figure 2). RSV and CMV were the most common detected viruses; each virus was detected in 3 cases. Influenza virus A was detected in 2 cases and adenovirus, PIV1 and PIV3 each was detected in one case (figure 3). Influenza virus B, PIV2 and HCoV-NL63 were not detected in any sample. No viruses were detected in the nasopharyngeal swabs taken from children with chronic granulomatous disease. No mixed viral infections were detected in any sample.

Table 3. Clinical and demographical data of children with PID and viral pneumonia

Patient	Underlying PID	Age (months)/Sex	Virus isolated
P1	Severe combined immune deficiency	14/boy	RSV
P2		36/boy	Adenovirus
P3		16/girl	PIV 3
P4		10/boy	CMV
P5		9/boy	RSV
P6	Hyper Ig E syndrome	36/boy	Influenza A
P7	Hyper Ig M syndrome	52/boy	CMV
P8	Common variable immunodeficiency	30/girl	RSV
P9		43/boy	Influenza A
P10		60/boy	CMV
P11	Selective IgA deficiency	26/boy	PIV 1

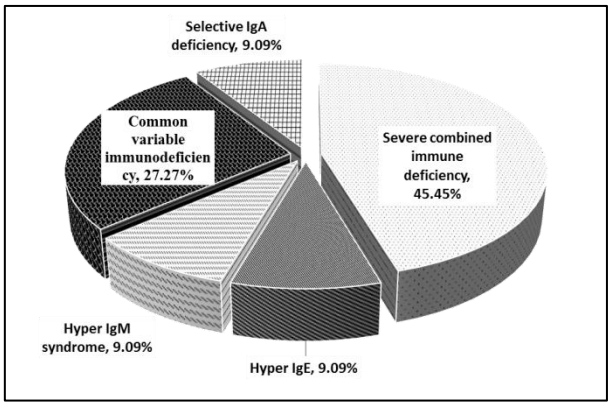

Fig. 2: Distribution of viral pneumonia in children with PID

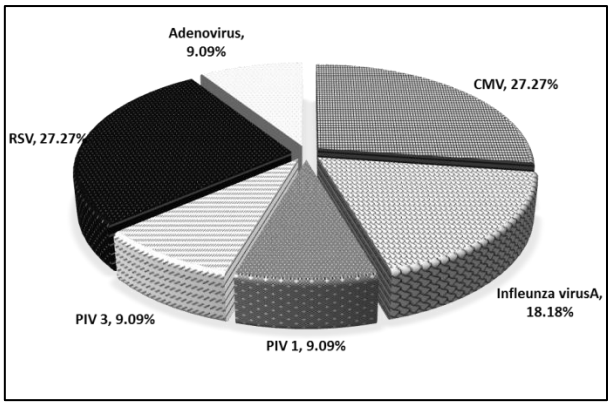

Fig. 3: Frequency of viruses causing pneumonia in children with PID

DISCUSSION

Science of primary immunodeficiency represents an interesting branch of the present medicine and clinical immunology. Respiratory symptoms are characteristically the earliest presentations in children with different PIDs[1]. In a retrospective study including 64 children with PID from Egypt, recurrent pneumonia represented the most frequent manifestation[15]. Also, in a study in Egypt, pneumonia was one of the most

common manifestation with 20% mortality rate in children with PID[16]. Another study done in Kuwait found that the most common presentation in PID children was viremia (28.8%) followed by pneumonia (28.2%) and skin infections (17.6%)[17].

In the present study, combined B, T cells deficiency represented the most isolated cases with pneumonia (15/33; 45.45%) followed by antibody deficiencies (13/33; 39.39%). Reda et al[15] observed that frequency of combined T and B cells immunodeficiency was the most frequent in their cohort. In contrast, two studies done in both Egypt[16] and USA[18] registered that antibody deficiencies were more frequent than combined immune deficiencies.

In our research, children with primary immunodeficiency and pneumonia showed male to female ratio of 2:1. Similar result was observed by Hussien and coauthors[16], but Joshi and coauthors[19] in Minnesota found that the majority was females in their study. Also, the children age ranged from 9 to 122 months with median age of 22 months in our research. Different studies found that the respiratory infections are expressed almost early on life, while, the non-infectious complications frequently become visible in the adolescent and adult age throughout the course of PID[1]. Again, in our research, the age of children with antibody deficiency and pneumonia was 12 months or above. Similarly, it was reported that the clinical pictures in humoral deficiency usually occur after the first 6 months of life (after the fading of mother's IgG)[1]. But, another study reported that lung infections in this category could begin earlier[20].

In this study, we took nasopharyngeal swabs from children with PID as respiratory samples to diagnose viral pneumonia. It was found that nasopharyngeal swab is the favorite method of sampling for diagnosis of viral pneumonia in PERCH (Pneumonia Etiology Research for Child Health; a multisite case-control study of pneumonia etiology in the developing world)[21].

In the present work, multiplex-PCR was selected as a method to detect viruses in the nasopharyngeal swabs taken from children with PID. Confirmation of a viral infection by means of conventional techniques such as viral cultures, direct immunofluorescence tests, and rapid antigen tests are either difficult or unreliable in many cases. Direct immunofluorescence tests and rapid antigen tests give results within short time, but in many cases they are deficient in sensitivity and specificity and are not available for all viruses[22]. Oosterheert et al[23] reported that the confirmatory rate of the lower respiratory tract infection increased from 21% to 43% if PCR is used for detection of respiratory pathogens, rather than conventional methods.

Clinically, a large number of pathogens may cause respiratory tract infections and there are not specific clinical symptoms for each pathogen. In a study of 20 or more possible pathogens, using of monoplex-PCR for diagnosis of viral pneumonia took too prolonged time, but multiplex PCR allowed testing the pathogens all together in a single analysis. So multiplex PCR was time saving, since it analyzed the different pathogens simultaneously[24].

As RSV infection in children is highly suspected and the rapid RSV test frequently gives a negative result, PCR can therefore be done. In addition, because RSV co-infection with other respiratory viruses is frequent and can increase the severity of pneumonia specially in children with PID, multiplex-PCR should be carried out in such patients[25].

As regard influenza viruses, early identification of influenza viruses by PCR is useful because treatment with neuraminidase inhibitors becomes then the drug of choice. Furthermore, the use of multiplex-PCR helps in the detection of mixed infections or superinfection[26].

Moreover, since infections with adenoviruses, parainfluenza viruses, or RSV frequently cause a severe course in immunocompromised children, therefore, rapid technique such as multiplex-PCR should be used. So, it is recommended to use multiplex-PCR in immunocompromised children especially in the first months of life to be aware of the clinical consequences in such case[24].

In the present work, viruses were detected in the nasopharyngeal samples of 11 children (11/33). So, viral pneumonia here represented 33.33% of the total cases with pneumonia. In children, respiratory tract infections are the most common types of infection, amongst viral infections represent the majority[27]. Furthermore, it was found that viral infections in immunocompetent children are usually asymptomatic or cause mild clinical manifestations. These children have effective immune responses that either clear the virus from the body or, maintain the virus in a dormant state in case of viruses that have latency. For children with PID, viral infections may cause life-threatening infection. Such cases often have infections with low virulent widespread viruses and these infections may be severe, persistent, recurrent, or refractory to therapy[2].

The present research found that viral pneumonia was mostly detected in children with severe combined immunodeficiency (5/11; 45.45%) and the viral pneumonia in this category of PID was caused by RSV (2cases), CMV, adenovirus and PIV3. Combined T- and B-cell immunodeficiency frequently takes extremely severe form of immunological defects, that necessitates rapid identification and proper management. Cases with combined T-and B-cell immunodeficiency are liable to infection by intracellular pathogens[1]. In SCID, the most common site of infection is the respiratory tract and the most commonly involved pathogens are RSV, CMV, adenovirus, PIV type 3. Paramyxoviruses and adenoviruses are frequent infecting viruses in these patients with considerable bad effect on bone marrow transplant outcome[28]. Another study found that 21% of

SCID had a pulmonary infection before transplant with PIV which was the most frequent one followed by RSV, rhinovirus, and influenza[29]. Also, Szczawińska-Popłonyk et al[30] found that a viral pneumonia was the earliest finding of SCID in all the children studied and this infection was caused by cytomegalovirus (CMV) and coronavirus in them. So, rapid diagnosis and aggressive treatment with virostatics, immunoglobulins and corticosteroids may decrease viral reproduction, lung injury with pulmonary function improvement[31].

Influenza A virus was detected in one case with hyper IgE syndrome in our study (1/11; 9.09%). Hyper-IgE syndrome (Job's syndrome) is a combined immunodeficiency. There are at least 3 types of hyper-IgE syndromes (HIES). Mutation in signal transducer and activator of transcription (STAT3) results in autosomal dominant form; AD-HIES. While mutation in gene for tyrosine kinase 2 (*TYK2)* or gene for dedicator of cytokinesis 8 (*DOCK8)* results in the other two autosomal recessive forms; AR-HIES. Pneumonias occur frequently in all the three types of HIES[32]. Mutations in the dedicator of cytokinesis-8 gene (DOCK8) were found to account for the majority of patients with AR-HIES. Absence of DOCK8 results in failure of dendritic cells migration to lymph nodes and defective CD4+ T-cell priming[33]. In B cells, DOCK8 is an adaptor protein that encourages B cell proliferation and immunoglobulin production. Deficiency of DOCK8 inhibits long-term memory of B cells as well as of virus specific CD8+ T cells, which could explain the susceptibility to persistent viral infections[34].

In our work, CMV was detected in the nasopharyngeal swab of a boy suffering from hyper IgM syndrome (1/11; 9.09%). Patients with hyper IgM syndrome (HIGM) syndrome have an inability to switch from the production of IgM to IgG, IgA or IgE antibodies. Therefore, children with hyper IgM syndrome have diminished IgG and IgA levels while the level of IgM is normal or high. The previous antibodies perform an essential role in combating infections. As a rule, B-lymphocytes are able to make IgM by themselves, but they need the help from T-lymphocytes to switch from IgM to IgG, IgA or IgE. Genetic defect of this T-lymphocytes and B-lymphocytes interaction results in HIGM syndrome. A deficiency of CD40 ligand protein that is found on the surface of activated T-lymphocytes leads to the most common form of HIGM syndrome. Normally, CD40 ligand binds to CD40 protein on B-lymphocytes. CD40 ligand is encoded by a gene on the X-chromosome. Therefore, HIGM is inherited as an X-linked recessive trait. Children with HIGM syndrome always have increased susceptibility to infection including recurrent pneumonia. The viral infections are frequent and severe. Pneumonia in HIGM syndrome is frequently caused by herpes viruses[35].

As regard children with humoral immunodeficiency in our study, viral pneumonia was detected in 3 cases with common variable immunodeficiency (3/11; 27.27%) and one with selective IgA deficiency (1/11; 9.09%). The viruses detected were RSV, CMV, influenza A virus and PIV1. Humoral immunodeficiencies represent clinically significant group of inherited immune deficiency. The most frequent and characteristic manifestation of primary antibody deficiencies is recurrent pneumonia. The pneumonia in children with humoral deficiencies is typically caused by encapsulated bacteria such as *Streptococcus pneumonia*, *Haemophilus influenzae*, and *Staphylococcus*[36]. These children have also the increased susceptibility to severe and prolonged viral pneumonia with rhinovirus, herpes simplex virus, and cytomegalovirus[37].

In the present work, no viruses were detected in the nasopharyngeal swabs taken from children with chronic granulomatous disease. Chronic granulomatous disease is the most commonly encountered phagocyte immunodeficiency, which result from a disorder of the NADPH oxidase system, making the phagocyte unable to generate superoxide, leading to the defective killing of microbes. Repeated respiratory infections with bacterial and fungal pathogens, as well as the formation of granulomas in tissue were found in cases with chronic granulomatous disease[38]. However susceptibility to frequent viral infections was not reported[39,40].

CONCLUSION

In children with PID, viral pneumonia is a common type of infection. So, the use of multiplex-PCR is recommended in these children for rapid diagnosis and accurate treatment. This can diminish lung damage and improve the respiratory function outcome.

Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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