

Original Research Article

Multiplex Polymerase Chain Reaction for Detection of 16 Respiratory Viruses

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Abstract: The objective of this study is to identify different respiratory viruses in a population of patients hospitalized at Rabat Hospital for severe acute respiratory infection for a period of one year. Over the period: from 12/09/2013 to 09/12/2014, 62 respiratory samples from 59 patients reached the central laboratory of virology. All samples were extracted using QIAGEN columns and analyzed by multiplex PCR. 67.7% of the samples are positive and 32.3% negative. 78.5% from pediatric and 21.5% from adult intensive care. Co infection viral rate was 48.5% in pediatrics and 33.3% in adult intensive care. Rhinovirus is the most frequently isolated in both pediatric and adult intensive care, it is also the most found in the viral co-infections, the association with bocavirus is the most common. In winter, the number of positive samples was the largest compared to other seasons with predominantly of rhinovirus, adenovirus and respiratory syncytial virus A.

Keywords: virological diagnosis, multiplex PCR, respiratory viruses

INTRODUCTION

Severe acute respiratory infections (SARI) are a major cause of morbidity and mortality as well as overuse of antibiotics. Rapid identification of a viral etiology can reduce the prescription of antibiotics, additional examinations, hospitalization time and the risk of nosocomial transmission [1,2]. Traditionally, the diagnosis of respiratory viral infections based on a combination of direct examination and isolation in cell culture. The diversity of the virus involved and the number of different viral strains make the diagnosis long and difficult [3]. In recent years, it has been shown that the use of PCR (polymerase chain reaction) could improve the diagnosis of respiratory viruses by combining sensitivity, specificity and speed [4]. Multiplex PCR has the advantage to allow, from the same sample, the amplification of several different viral genomic sequences [5]. It is particularly interesting to simultaneously search for different viruses involved in SARI because epidemics usually occur concurrently, reflecting the importance of using multiplex PCR.

MATERIALS AND METHODS

This is a retrospective study over a one year period from 12 September 2013 to 12 September 2014, made from 62 samples obtained by nasopharyngeal swab, nasopharyngeal aspiration and bronchoalveolar lavage. Samples derived from 59 patients hospitalized in University Hospital of Rabat for severe acute respiratory infections.

Nasopharyngeal swabs were placed in a special transport medium to prevent desiccation of the sample. All samples were subjected to a first extraction step of the nucleic acids (ribonucleosic acid: RNA, and desoxyribonucleosic acid: DNA) of different virus using the commercial kit "Qiagen QIAamp MinElute". Nucleic acids (DNA / RNA) underwent reverse transcription step (RT) for transforming the DNA into RNA. Then, a multiplex PCR using the commercial kit"

"Anyplex II RV16 Detection V1.1" of Seegene was performed on the DNA and complementary DNA.

This kit uses a bacteriophage "MS2" as an internal control. Before extraction, internal control is added to each sample to verify the whole process from the extraction of nucleic acids to the RT-PCR. The test "Anyplex II RV16 Detection V1.1" allows simultaneous amplification, the qualitative detection and differentiation of target nucleic acids of 16 different respiratory viruses: Influenza A (Flu A) and B (Flu B), A respiratory syncytial virus (RSV A) and B (RSV B), human metapneumovirus (MPV), human adenovirus (ADV), human coronavirus 229 E, NL63, OC43, human rhinovirus (HRV) A, B and C human enterovirus (HEV), human bocavirus (HBoV) 1/2/3/4, and human parainfluenza virus (PIV) 1,2,3,4. This test uses a technology "TOCE" to detect several pathogenic viruses in a single channel of fluorescence based on the melting temperature values for each virus. The detection is done on the equipment real-time PCR BioRad CFX96. For the realization of this test, two Mastermix A and B are prepared. They contain the primers and probes required for amplification of a genome sequence of the 16 viruses mentioned above. The Mastermix A contains primers and probes specific for 8 virus (PIV4, ADV, PIV1, PIV2, PIV3, FluA, flub, HRV), the Mastermix B contains primers and probes specific for the 8 other remaining virus (MPV, HBoV, CoV 229E, NL63 CoV, CoV OC43, MVSr, RSVB, HEV).

In this kit are included 2 positive controls: PC1 and PC2. PC1 contains pathogenic clones of 5 for the panel A (PIV4, ADV, PIV1, PIV2, PIV3) and 5 pathogens of panel B (MPV HBoV, CoV 229E, NL63 CoV, OC43 CoV). PC2 contains clones for 3 pathogens of panel A (FluA, flub, HRV) and 3 pathogens of panel B (MVSr, RSVB, HEV) and also internal control. The negative control consists of water quality molecular biology. In the PCR tubes, we use a symmetric scheme for the PCR plate: a strip for the Mastermix A in position 1 and another link for the Mastermix B at position 7, then it is distributed negative control, the samples and the two positive controls PC1 and PC2. The real-time PCR is then started and the interpretation of the result is based on amplification curves obtained for each virus. A negative result is manifested by the

absence of any amplification curve except that of the internal control, while a positive result is manifested by the presence of the corresponding virus amplification curve. The result obtained will be compared to the curves of the positive controls for each viruses.

RESULTS

Population

Among the 59 patients with SARI and in whom the respiratory sample was taken, we have identified a viral respiratory infection in 41/59 patients (69.5%) including 25/41 (61%) male and 16/41 (39%) female. In 18/59 patients (30.5%) none of respiratory virus has been identified. 32/41 patients (78%) come from the hospital children of Rabat: pediatric infectious and respiratory diseases: 25/41 and 7/41 pediatric intensive care unit. 9/41 (22%) patients come from the service of the adult medical intensive care unit.

Types of samples and the identified virus

58/59 (98.3%) patients had nasopharyngeal swab. 1/59 (1.7%) a bronchoalveolar lavage and 3/58 patients (5.17%) had more of nasopharyngeal swab rhino pharyngeal aspiration. 62 samples are obtained ultimately.

42/62 samples (67.7%) were positive and 20/62 (32.3%) were negative.

One virus was isolated in 51.5% (17/33) in pediatrics and in 66.7% (6/9) in adult intensive care. The combination of two or more viruses is found in 48.5% (16/33) in pediatric and 33.3% in adult intensive care unit.

In pediatrics, rhinovirus was found in 17/33 samples (51.5%). In 11/17 cases (64.7%) it is associated with other viruses. The bocavirus is identified in 12/33 samples (36.3%) mainly associated with other viruses 9/12 (75%). The adenovirus was found in 8/33 samples (24.2%), it is associated with other viruses in 7/8 (87.5%) cases. The respiratory syncytial virus A was found in 4/33 (12%) cases. The influenza virus A, respiratory syncytial virus B, coronavirus 229 E, are each identified only in one sample. The enteroviruses and parainfluenza 1 virus are found in a single sample each associated with rhinovirus (Figure 1 and Table I).

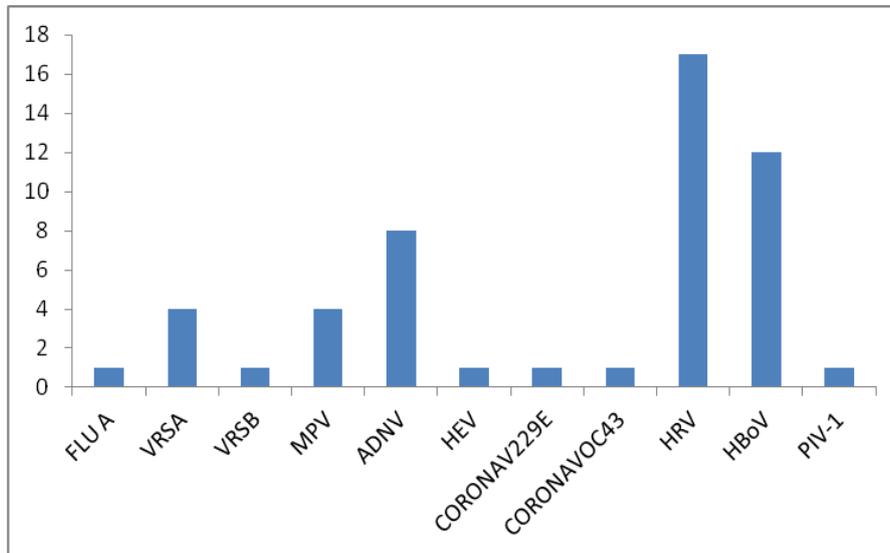


Fig-1: Distribution of Virus Found In Pediatric Unit.

In adult intensive care, rhinovirus is identified in 5/9 samples (55.5%), it is associated in 3/5 cases. The Influenza A virus is identified in 3 / 9 (33.3%) samples, associated in one case to rhinovirus. The bocavirus, and the metapneumovirus, each

identified in a single sample and are both alone. The VRSA, CoV and OC43 are each identified in a single sample and are each associated with rhinovirus (Figure 2 and Table I).

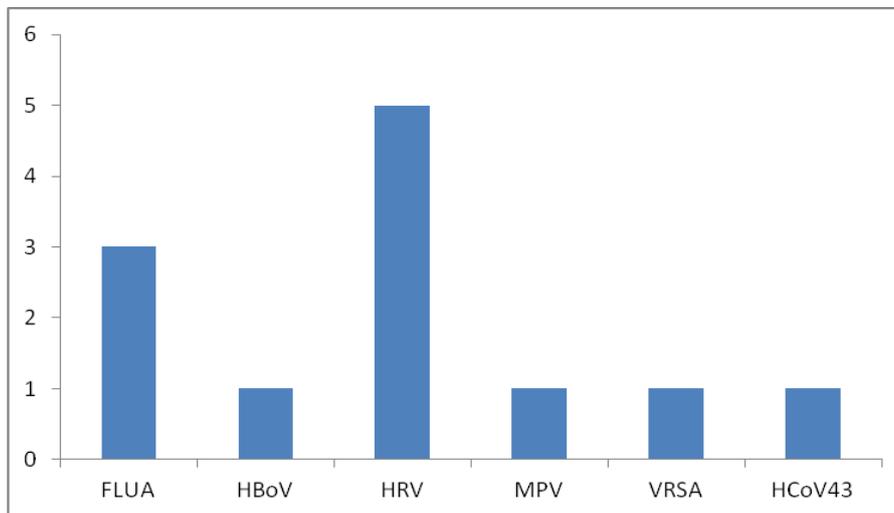


Fig-2: Distribution of Virus Found In Adult Intensive Care

Table-1: Different viral co-infections found

| Samples | Unit | Viral Coinfection Found |
|---------|------|-------------------------|
| S1 | PI | MPV+ HBoV |
| S2 | PUIC | ADV+ HBoV |
| S3 | PI | ADV+ HRV |
| S4 | PI | ADV+ VRSA |
| S5 | PI | ADV+ VRSB |
| S6 | PUIC | PIV-1+ HRV |
| S7 | AUIC | VRSA+ HRV |
| S8 | AUIC | CoV OC43+ HRV |
| S9 | PI | ADV+ VRSA |
| S10 | PI | VRSA+ FLUA+ ADV+ HRV |
| S11 | AUIC | HRV+ FLUA |
| S12 | PI | HRV+ HBoV |
| S13 | PI | HRV+ HBoV |
| S14 | PI | HRV+ HBoV |
| S15 | PUIC | HRV+ MPV+ HBoV |
| S16 | PI | HRV+ MPV+ HBoV |
| S17 | PI | ADV+ HRV |
| S18 | PI | ADV+ HRV+ HBoV |
| S19 | PI | HRV+ HBoV |

AUIC: Adult Unit Intensive Care
 PUIC: Pediatric Unit Intensive Care
 PI: Pediatric Infectious

Seasonal distribution of viruses

In Autumn, the Bocavirus is isolated in 3/3 samples, the adenovirus and the metapneumovirus are isolated each in a single sample. In Winter, the Rhinovirus, the Adenovirus and VRSA are isolated each in 5/18 samples (27.7%). The BRSV virus, the Influenza A and the Bocavirus are isolated each in 2/18 samples (11%). The Metapneumovirus, the Coronavirus 229E, the Coronavirus OC43 and the Parainfluenza 1 viruse are isolated each in a single sample 1/18 (5.5%). In spring, the Rhinovirus is identified in 5/7 samples (71.4%), the Influenza A virus is isolated in 2/7 samples (28.5%). The VRSA, the Metapneumovirus the Adenovirus and the Enterovirus are identified each in a single sample 1/7 (14.2%). In Summer, the Rhinovirus is isolated in 12/14 cases (85.7%), the Bocavirus is identified in 9/14 cases (64.2%), the Metapneumovirus and the Adenovirus are isolated each in 2/14 samples (14.2%) .

DISCUSSION

The major limitation of this study is the small number of cases evaluated in relation to the high number of patients with respiratory infections found in

practice. Indeed, the vast majority of cases do not require hospitalization, which limits our knowledge of the actual situation at the level of the general population.

The identification of respiratory viruses in patients included in the study was made by a new method in Morocco based on a viral molecular analysis (multiplex PCR) performed in the Central Laboratory of Virology of rabat and allowing identifying at the same time of sixteen respiratory viruses. Indeed, the multiplex PCR is a very sensitive technique, which exceeds the other two techniques previously used for viral biological diagnosis: the cell culture and research of viral antigens [6]. It is more sensitive in particular for certain pneumotropic virus (Rhinovirus Bocavirus and some Coronavirus) that can set default other diagnostic techniques [7]. Multiplex PCR allows searching simultaneously several viruses and identifying viral co-infection in the same patient. Its use has allowed us to better understand the different viruses responsible for respiratory tract infections and limit the prescription of antibiotics deemed unnecessary in this case. The viral coinfection rate in our study was 48.5% in pediatrics

and 33.3% in adult intensive care. In the literature, co-infection rates ranges are from 8% to 76%, depending on the series [8-17].

In this study, the rhinovirus is the most frequently isolated in both pediatrics and adult intensive care, it is also found in most of the viral co-infections, the association with the Bocavirus is the most common. After rhinovirus comes adenovirus then MVSR in pediatric while in adult intensive care the virus of Influenza A is positioned after the Rhinovirus. A French study in a pediatric unit shows that respiratory syncytial virus is most often found in the viral co-infections and the association with the adenovirus is the most common [18].

Viral coinfection does not appear as a severity factor according to the study of Brouard and al and the study of A. Martínez-Roig *et al.* [18,19]. However, other studies show that co-infection is a bad prognostic risk factor [15, 20-25]. According to the study of Aberle and al between 2000 and 2004, the airway obstruction was more severe when the RSV had helped co-infection, and the average hospital stay was extended when the rhinovirus was involved [15]. In another study, Grensill and al showed that when co-infection is composed of respiratory syncytial virus and metapneumovirus, the prognosis is worse [23]. In addition, Richard and al who studied coinfection relationship with the admission requirement pediatric intensive care unit (PICU), concluded that the presence of two or more viruses has increased the probability of admission from 2 to 7 times in PICU [25]. But, the main limitation of the multiplex PCR is that it cannot testify the active nature of viral infection especially for the nucleic acids of the rhinovirus that can exist for long periods, which can be positive PCR several weeks after an infection [26]. The development of quantitative techniques in multiplex PCR, could overcome this obstacle [27].

In Winter, the number of positive samples was the largest compared to other seasons with predominance of rhinovirus, adenovirus and the MVSR and the presence of other viruses: RSVB virus, Influenza A, bocavirus, metapneumovirus, coronavirus 229E, coronavirus OC43 and parainfluenza 1. The winter peak of respiratory infections is multifactorial, but exposure to cold causes vasoconstriction of the nasal mucous membranes and upper respiratory tract, decreasing the local defenses and allows latent viral

infections to become Patent [28]. In the spring, the number of positive samples decreased compared to the winter season. In summer, the number of positive samples increased compared to the spring predominantly Rhinovirus and Bocavirus, but the pathogenic role of these viruses remains uncertain since the nucleic acid fragments can persist for several weeks after an actual infection [15].

CONCLUSION

Despite its cost, which is relatively expensive, multiplex PCR is a powerful technique for quick and efficient diagnosis of viral respiratory infections. It allows to highlight a broad range of pneumotropic viruses with time savings and higher sensitivity compared to older traditional techniques. Virological evidence of respiratory infection reduces hospital stay and avoid the deemed ineffective overuse of antibiotics in this case and that could cause serious antibiotic resistance. For better clinical benefit, epidemiological and economic health, it is desirable that this technique is accessible to a greater number of laboratories especially in developing countries. In perspective, broader studies examining the different virological profiles of respiratory infections and the study of viral co-infections would be of interest to achieve.

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