Short Communication

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Performance of direct immunofluorescence assay for the detection of human metapneumovirus under clinical laboratory settings

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ABSTRACT

Introduction: Human metapneumovirus (hMPV) is an emergent human respiratory pathogen. This study aimed to evaluate the performance of direct immunofluorescence (DIF) to detect hMPV in a clinical laboratory setting. **Methods:** Nasopharyngeal aspirate samples (448) of children and adults with respiratory illness were used to detect hMPV by using DIF and real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays. **Results:** In all, 36 (8%) samples were positive by DIF and 94 (21%) were positive by qRT-PCR. Direct immunofluorescence specificity was 99% and sensitivity was 38%. **Conclusions:** DIF is not very sensitive under clinical laboratory settings.

Keywords: Human metapneumovirus. Direct immunofluorescence. qRT-PCR.

Acute respiratory infections are serious public health problems worldwide due to their global distribution, ease of spread in the community, and high morbidity, especially in pediatric patients. These infections might be caused by a wide variety of viruses such as parainfluenza, influenza, adenovirus, coronavirus, human respiratory syncytial virus, bocavirus, and human metapneumovirus (hMPV)[1].

The hMPV belongs to the family Paramyxoviridae, subfamily Pneumovirinae, and genus *Metapneumovirus*. It is a segmented, enveloped, and pleomorphic virus with a negative-sense ribonucleic acid (RNA) genome of 13,000 nucleotides. It has been associated with acute respiratory infections in children under five years as well as in immunocompromised adults and elderly individuals[1]. The most common clinical manifestations include pneumonia, bronchiolitis, and bronchitis[2].

This virus was primarily characterized in the Netherlands in 2001[2]. Subsequently, its frequency was found to be 1.5% to 41% in respiratory diseases according to the geographic region and population group across different studies[3]. In Brazil, virus frequency rates of 5.6% were reported in Campinas, State of São Paulo[4]; 6.4%, in Curitiba, State of Paraná[5]; and 14.5%, in Porto Alegre, State of Rio Grande do Sul[6].

Laboratorial diagnosis was based on viral cell culture after the discovery of hMPV. Subsequently, reverse transcription-polymerase chain reaction (RT-PCR) was shown to be efficient and more sensitive method to detect hMPV and is presently considered the gold standard assay[7]. However, efforts have also been directed to develop rapid antigen tests for nasopharyngeal samples by using hMPV-specific monoclonal antibodies. Direct immunofluorescence (DIF) assays involving the use of these antibodies have become a standard in some countries, because it allows rapid detection of viral proteins from clinical samples, with acceptable performance[2]. This study aimed to evaluate the performance of the DIF assay for the detection of hMPV in an epidemiological surveillance laboratory setting between 2009 and 2011.

Nasopharyngeal aspirate (NPA) samples were collected from patients with respiratory influenza-like illness (mostly with severe acute respiratory syndrome) from different cities of the State of Rio Grande do Sul (RS) and sent to a public laboratory [*Instituto de Pesquisas Biológicas-Laboratório Central de Saúde Pública do Rio Grande do Sul* (IPB-LACEN-RS)] for influenza A H1N1pdm09 virus detection between 2009 and 2011. Of more than 2,000 NPA samples, 448 (91 from adults and children over five years and 357 from children up to five years) were selected for this study. All the samples were negative

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for influenza A virus (tested by **r**eal time reverse transcription-PCR), influenza B virus, parainfluenza virus (types 1, 2, and 3), adenovirus, and human respiratory syncytial virus (the last four viruses tested using specific indirect immunofluorescence assays).

The hMPV was detected using the reagent Millipore® CAT.5091, approved by the Food and Drug Administration (FDA) for research use only. Nasopharyngeal aspirate samples were smeared and fixed with acetone on a microscopic slide. Next, 25µL of the monoclonal antibody (anti-IgG hMPV) was added to the sample. The slides were incubated at 37°C in a humid chamber for 15 min, washed by immersion in phosphate buffer, and dried with air. The slides were mounted with buffered glycerol and analyzed using a Leica microscope equipped with a fluorescence lamp (HBO100W®).

Samples of nucleic acids were obtained using commercial Pure-Link Viral ribonucleic acid/deoxyribonucleic acid (RNA/DNA) Mini Kit (Invitrogen-Life Technologies, CA, USA) and/or RNA Mini Kit (Qiagen, CA, USA). Each sample was submitted to real-time RT-PCR by using oligonucleotides (primers and probe) specific to hMPV as previously described[8]. All reactions were performed on a Step-One Plus thermocycler (Applied Biosystems, CA, USA). Positive and negative controls were used in all assays.

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) program. A p value of <0.05 was considered significant as per the chi-squared test (χ^2). The agreement between the techniques was measured according to accuracy and Kappa index (where values below 0.4 indicate low; between 0.4 and 0.6, good; and above 0.6, strong correlation). Clinical sensitivity and specificity as well as positive and negative predictive values were obtained from a contingency table showing the frequency distribution of the positive and negative results obtained with both the assays.

Between 2009 and 2011, 448 samples were evaluated using DIF and qRT-PCR assays. In all, 357 (80%) NPA samples were collected from children up to five years and 91 (20%) from adults and children over five years. Further, 98 (22%) samples were hMPV positive by DIF and/or qRT-PCR. In a year-based stratified analysis, 36 (18%) samples of the 208 samples were positive in 2009; 10 (9%) of 124 in 2010; and 52 (37%) of 142 in 2011. Direct immunofluorescence assay detected 36 (8%) positive samples, 34 (9%) of 357 samples from children up to five years, and two (2%) of 91 samples from the remaining patients, whereas qRT-PCR detected 94 (21%) positive samples, 86 (25%) of 357 samples from children up to five years and 8 (9%) of 91 samples from the remaining patients. In addition, 36 (8%) of the samples were positive in the two assays, four (0.9%) were detected only by DIF assay, 58 (13%) were positive strictly by qRT-PCR, and the remaining 350 (78%) were negative in both the assays.

The overall performance of the DIF assay is shown in **Table 1**. Analytical specificity was very high (99%), considering the global analysis of all the samples or even in the separate evaluation of the two studied population groups (children and adults). Similar results have been reported previously[9] [10] [11]. In contrast, analytical sensitivity was low: 38% for all patient samples; 39.5%, for children; and 25%, for adults. Previous studies have also not shown very good sensitivities for DIF assays[9] [10]. Only one study showed a relatively high (73.9%) sensitivity value after enrichment of the viral load with cell culture[8]. In all, 386 samples (302 from children up to five years and 84 from the remaining patients) showed agreement between the DIF and qRT-PCR results with a Kappa index of 0.47, suggesting a good correlation. However, this correlation was noted only for the children when the two groups of patients were separately analyzed.

Human metapneumovirus detection relies on the analysis of viral antigens and nucleic acids in clinical samples. Real-time RT-PCR has been commonly used for the detection of hMPV, but DIF offers an easy alternative and enables rapid diagnosis. In the present study, DIF was found to be rapid and simple, requiring relatively little hands-on time in a clinical laboratory setting. However, it had poor performance. Many factors are related to this situation in a clinical laboratory setting. First, the monoclonal antibody anti-hMPV needs to be specific to circulating strains to prevent background staining[9] [10]. The reagents used in the present study were developed for North American and European viral strains. Brazilian hMPV could present some specific antigenic characteristics as previously shown[12] [13]. In addition to these technical characteristics, factors associated with sample collection and technical

TABLE 1 - Performance of the direct immunofluorescence assay compared to the gold standard qRT-PCR.

Age group		Cases (n)				Sensitivity %	Specificity %	Accuracy %	PPV %	NPV %	χ^2	Kappa
		RT-PCR (+)		RT-PCR (-)								
	Total	DIF (+)	DIF (-)	DIF (+)	DIF (-)							
Children	357	34	52	3	268	39.5	99.0	85.0	92.0	84.0	<0.05*	0,47**
Adults	91	2	6	1	82	25.0	99.0	92.0	67.0	93.0	0.121	0.33***
Total	**448**	**36**	**58**	**4**	**350**	**38.0**	**99.0**	**86.0**	**90.0**	**86.0**	**0.123**	**0.47*** **

qRT-PCR: real time quantitative reverse transcription-polymerase chain reaction; **RT-PCR:** reverse transcription-polymerase chain reaction; **PPV:** positive predictive value; **NPV:** negative predictive value; **DIF:** direct immunofluorescence. **χ^2:** Chi-squared test. *p <0.05 was considered statistically significant. **Good correlation; ***Weak correlation.

procedures in the laboratory should always be considered. Direct immunofluorescence assays also depend on the experience of laboratory technicians, and some studies have restrictions in using this method for large-scale analysis[14].

Acute respiratory viral disease is the leading cause of hospitalization in children, and hMPV is one of the most important pathogens related to such a disease. Clinically, these infections cannot be differentiated due to the symptom similarities, even with bacterial respiratory diseases. Despite the viral etiological origin, antibiotics are often prescribed to patients for the treatment of such diseases, exacerbating antibiotic abuse[15]. A large proportion of co-morbidity with other viruses and bacteria is also observed, resulting in severe outcomes and long periods of hospitalization. In this sense, accurate hMPV identification is important to prevent unnecessary use of antibiotics and to define the proper treatment of complications in patients with co-morbidities. Further studies are necessary to detect the occurrence of hMPV co-infection with other respiratory viruses (influenza A, influenza B, parainfluenza, adenovirus, and human respiratory syncytial) considering the severity of clinical symptoms.

The development of DIF assay to virus detection was important in the field for the rapid diagnosis of respiratory tract viral infections. DIF enables the direct evaluation of cell specimens, providing rapid results with low cost and a subsequent early medical management. However, DIF use should be restricted to a first-line approach, and a confirmatory test is necessary for the hMPV diagnosis. Further, DIF assay showed limited performance in a public health laboratory. Quantitative RT-PCR is more consistent and suitable for the detection of hMPV.

Ethical considerations

This study was approved by the Ethics Committee of the *Fundação Estadual de Produção e Pesquisa em Saúde do Estado do Rio Grande do Sul* (FEPPS/RS), Protocol 11/2010.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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