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Evaluation of monoclonal antibodies that detect conserved proteins from Respiratory Syncytial Virus, Metapneumovirus and Adenovirus in human samples

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ABSTRACT

Human Respiratory Syncytial Virus (hRSV), human Metapneumovirus (hMPV) and Adenovirus (ADV), are three of the most prevalent viruses responsible for pneumonia and bronchiolitis in children and elderly worldwide, accounting for a high number of hospitalizations annually. Diagnosis of these viruses is required to take clinical actions that allow an appropriate patient management. Thereby, new strategies to design fast diagnostic methods are highly required. In the present work, six monoclonal antibodies (mAbs, two for each virus) specific for conserved proteins from hRSV, hMPV and ADV were generated and evaluated through different immunological techniques, based on detection of purified protein, viral particles and human samples. In vitro evaluation of these antibodies showed higher specificity and sensitivity than commercial antibodies tested in this study. These antibodies were used to design a sandwich ELISA tests that allowed the detection of hRSV, hMPV, and ADV in human nasopharyngeal swabs. We observed that hRSV and ADV were detected with sensitivity and specificity equivalent to a current Direct Fluorescence Assay (DFA) methodology. However, hMPV was detected with more sensitivity than DFA. Our data suggest that these new mAbs can efficiently identify infected samples and discriminate from patients infected with other respiratory pathogens.

1. Introduction

Respiratory tract infections are defined as the set of infections that affect the airways, including the oropharinx, bronchi and lungs (Antibiotic Expert Group, 2010). These infections are globally considered as a serious public health problem affecting all age people; however higher morbidity and mortality rates are more common in children, particularly when they evolve to its more severe manifestations: bronchiolitis and pneumonia (Lozano et al., 2012).

Pneumonia is the leading cause of mortality in children worldwide, even more than diarrhea, malaria and acquired immune deficiency syndrome together. Pneumonia accounts for about 17% of deaths of children under four years old (UNICEF Committing to Child Survival, 2013; Kahn, 2006). Laboratory diagnosis to identify the etiological agent causing a respiratory tract disease is applied to less than half infected patients and this problem becomes more complex in cases of lower respiratory tract infections in children (Ruiz et al., 1999; Zambon et al., 2001; Ampofo et al., 2008). Low respiratory tract damage may result from direct viral agent infection in children and adults, acting as a predisposition factor to bacterial pneumonia (Levin et al., 2010;

Abbreviations: hRSV, Human Respiratory Syncytial Virus; hMPV, human Metapneumovirus; ADV, Adenovirus; mAbs, monoclonal antibodies; ELISA, Enzime-Linked ImmunoSorbent Assay; DFA, direct immunofluorescence; RT-PCR, Reverse Transcriptase Polymerase Chain Reaction; IPTG, Isopropyl-β-D-1-thiogalactopyranoside

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Samransamruajkit et al., 2008; Louie et al., 2005). Among viral agents that can trigger pneumonia, especially in children younger than 2 years of age, highlights Influenza virus, Parainfluenza, hRSV, hMPV and ADV (Bradley et al., 2011; Girardi et al., 2001).

hRSV is an enveloped non-segmented negative sense RNA virus with a genome of 15.3 kb length (Collins et al., 2001; Rudan et al., 2013). hRSV is considered the most important cause of viral pneumonia and bronchiolitis, especially in infants under two years old (Nair et al., 2010). Serological studies indicate that between 70% and 100% of children are exposed at least once to hRSV at the age of 1 and 2 years old, respectively. Worldwide, it is estimated that hRSV causes annually close to 34 million respiratory infections, 3.4 million hospitalizations and nearly 200 thousand deaths in children below the age five (Nair et al., 2010). Importantly, the infection caused by this virus do not promote long-lasting immune memory (González et al., 2016; Lay et al., 2015; Céspedes et al., 2014; González et al., 2008), therefore it annually causes severe outbreaks and the generation of vaccines has not been possible to date.

hMPV is an enveloped virus that belongs to the recently described *Pneumoviridae* family (Afonso et al., 2016). HMPV remained unknown until fifteen years ago, probably due to the difficult isolation in cell cultures, reduced diagnosis methodologies and the fact that it shares with hRSV not only genetic and morphological similarities, but also it has a similar infectivity and spectrum of disease (García et al., 2004; Boivin et al., 2007; Williams et al., 2010). HMPV has been strongly associated with bronchiolitis and pneumonia, being an important cause of hospitalizations in children under 2 years old and the cause of high morbidity in elderly and immunocompromised patients (Heikkinen et al., 2008). Similar to hRSV, hMPV is also able to prevent an appropriate immune host response (Céspedes et al., 2013; Palavecino et al., 2015; Céspedes et al., 2016; Lay et al., 2015, 2016).

ADV is a stable and solid non-enveloped virus. ADV genome consists in double stranded DNA with about 34 to 36 kb size. At present, sixty types and seven species (human ADV A–G) have been described, and most of them are associated to several clinical syndromes (Zhang et al., 2012). Similar to hRSV and hMPV, ADV represents an important viral agent in respiratory tract infections, oscillating in severity from mild self-limited infections, to severe pneumonia and bronchial syndromes, requiring in severe cases the admission to intensive care units. In fact, some novel types of ADV strains have been associated with lethal diseases (Zhang et al., 2012, Adrian et al., 1986).

Diagnosis of these respiratory viruses is important to take prompt clinical actions, especially in children under 1 year of age. Knowing the etiological agent of respiratory tract diseases allow a better decision regarding the management of patients, which can positively influence in their evolution. For this reason, it would be relevant to perform diagnosis at primary health services and not only at emergency rooms and hospitals, where the patients already present a more severe symptomatology. Current and available diagnostic methods include the analysis of nasopharyngeal swab by Direct Fluorescence Assay (DFA) tests and molecular strategies as Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), multiplex RT-PCR and FilmArray® Respiratory Panel (Pretorius et al., 2012; Gharabaghi et al., 2011; Couturier et al., 2013). The selection of the most suitable technique depends of the purpose of the test. For instant, viral detection by molecular techniques achieves excellent sensitivity, but require sophisticated equipment and infrastructure. Therefore, these are more appropriate for large hospitals and clinical laboratories. In primary health care facilities, in which specialized human resources and dedicated infrastructure to run molecular techniques is not always available (specially in developing countries), it would be more suitable the use of rapid screening tests. Diagnostic methods based on protein detection have shown an acceptable clinical value and they are less sophisticated and affordable for the majority of the population than molecular techniques, but their sensitivity and specificity values still are far to be comparable to PCR. For this purpose, availability of more sensitive and efficient monoclonal antibodies (mAbs) useful to implement diverse platforms of immunodiganosis is required to improve viral respiratory detection. Based on this premise, the goal of this study was to characterize new mAbs generated to detect conserved proteins from three viruses of public health importance (hRSV, hMPV, ADV), which allows the identification of all serotypes, also guaranteeing that there is no variation of the antigen as is the case of surface proteins, which are the most used antigens for the generation of antibodies currently available in the market.

2. Materials and methods

2.1. Cell lines and viral strains

Monkey kidney LLC-MK2, Human laryngeal carcinoma Epidermoid cell line 2 (HEp-2) and human lung adenocarcinoma epithelial A549 cell lines (American Type Culture Collection), were grown in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin/ml, and 100 µg of streptomycin/ml and non-essential amino acids (all culture reagents were obtained from Gibco™, Life Technologies, Carlsbad, CA). A titer of 1×10^6 plaque-forming unit per milliliter (pfu/ml) of each virus was used to infect the cell lines mentioned above. hMPV strain CZ0107, serogroup A1, was kindly provided by the Laboratory of Infectology and Virology of the Hospital Clínico de la Pontificia Universidad Católica de Chile, while hRSV strain 13018-8 serogroup A2 and a clinical isolate of ADV were kindly provided by the Instituto de Salud Pública de Chile. LLC-MK2 cells, HEp-2 cells and A549 cells were infected with hMPV, hRSV and ADV respectively. Infections were performed in OptiMEM-I at 37 °C, 5% CO_2 and infections with hMPV were performed as previously described and media growth was supplemented 5 µg/ml of trypsin (Tollefson et al., 2010). After 12 h of incubation, culture media was replaced with fresh OptiMEM-I and incubated for another 48 h, as previously described (Gomez et al., 2013). Next, supernatant from infected LLC-MK2 cells, HEp-2 cells and A549 cells were distributed in aliquots and stored at −80 °C.

2.2. Generation of purified hMPV, hRSV and ADV antigens

The generation of purified proteins used in the production of monoclonal antibodies was performed using the expression vectors pET15b-M-hMPV, pET15b-P-hRSV and pET15b-pIII-ADV and the M-hMPV, P-hRSV and pIII-ADV proteins were over-expressed using *Escherichia coli* BL21 strain, as previous described (Gomez et al., 2013). Single colonies from transformed BL21 strains were selected by ampicillin resistance and sub-cultured for screening studies by PCR, using specific primers. Confirmed strain transformed with the plasmid were grown into 8–10 L of Luria Bertani broth (LB) supplemented with 100 µg/ml ampicillin up to DO_{600} 0.6. For protein induction, 0.5 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG, Winkler Ltd.) was added and cultures were incubated for 3 h at 37 °C. Cell mass were collected by centrifugation at 4600*g* for 5 min and lysed using a lysis buffer that contained 20 mM sodium phosphate, pH 7.5; 500 mM NaCl, and 20 mM Imidazole. In the case of bacteria transformed with pET15b-pIII-ADV, the lysis buffer contained 20 mM sodium phosphate, pH 7.5; 500 mM NaCl, 8 M Urea, plus lysozyme (1 mg/ml final concentration). Lysis buffer was supplemented with proteases inhibitors cocktail (Life Science Roche Biochemical) and lysis mixture was incubated for 30 min at 4 °C. Lysed bacteria were sonicated 10 cycles of 1 min, at a maximum intensity on ice. Lysates were centrifuged for 30 min at 20,000*g* and the cleared supernatants were incubated for 45 min in stirring at 4 °C, in a Ni-NTA column (Invitrogen, Life Technologies, Carlsbad, CA), for His-tagged protein purification. The His-tagged proteins were purified by Imidazole elution and collected in a 1ml aliquot (in the case of M and P proteins). Recombinant pIII protein was purified using denaturant buffer with 8M urea at pH 4.5 and collected in 1 ml aliquots, subsequently 7 µl of NaOH 2M was added per aliquot. The clear protein

content was measured at 280 nm absorbance. Finally, the protein preparation was analyzed by SDS-PAGE and stained with Coomasie Blue.

2.3. Generation of anti M-hMPV, anti P-hRSV and anti pIII-ADV monoclonal antibodies

MAbs production was outsourced to GrupoBios S.A. (Santiago, Chile). Balb/c mice were immunized with the purified proteins described above and the production of specific hybridomas was performed following standard protocols. The hybridomas selected for this study were clones 3G8/C11 and 7G4/A12, which produced anti M-hMPV antibodies, clones 2E6/D2 and 6H5/H1 that produced anti P-hRSV antibodies and clones 6F11 and 7E11 that produced anti pIII-ADV antibodies. Patents have been filled in Chile and other countries for anti M-hMPV, anti P-hRSV and anti pIII-ADV mAbs.

2.4. Clinical samples

Two different approaches were performed to evaluate mAbs in clinical samples. For the first one, clinical samples from anonymous infants with respiratory tract disease were provided by the Laboratory of Infectology and Virology (LIV), of the Hospital Clínico de la Pontificia Universidad Católica de Chile. Samples were obtained by nasopharyngeal swab with Universal Transport Medium UTM™ (COPAN catalog #360C) and were processed by DFA at the LIV. A total number of 70 clinical samples (20 diagnosed as positive for hMPV, 20 diagnosed as positive for hRSV, 20 diagnosed as positive for ADV and 10 controls samples that were negative for hRSV, hMPV or ADV) were initially analyzed by DFA with D3 Ultra™ 8 DFA Kit (Diagnostic Hybrids, Athens, OH) (Table 1). Next, the leftover fraction of each sample was stored at −20 °C. Once the experiments started, samples were thawed once and assessed by ELISA. The samples were treated in an anonymous way. The Scientific Ethics Committee (CEC-MEDUC) from the Pontificia Universidad Católica de Chile approved the study (Approval number 15-332).

For the second approach, children with respiratory tract disease consulting at Hospital Clínico of the Pontificia Universidad Católica de Chile, were exclusively recruited for purposes of this study. Samples were collected as mentioned above and analyzed simultaneously by DFA at LIV and ELISA. It is important to mention that a fraction of the sample was separated for extraction of RNA, which was done to detect the virus by RT-qPCR in case of discordances. These samples were obtained upon the sign of an informed consent by parents of children between 0 and 2 years-old, as described above. This study was approved by the Scientific Ethics Committee (CEC-MEDUC) from the Pontificia Universidad Católica de Chile (Approval number 13-294).

2.5. ELISA assays

For viral proteins detection, mAbs generated in this study were used in an indirect ELISA using commercial antibodies as control of the protein detection. Selection of the commercial antibodies used to compare to the results obtained with the antibodies against P-hRSV and M-hMPV generated in this study was based on two criteria: first, that they were monoclonal and second, that were specific for the same proteins than the mAbs generated in this study. In the case of ADV, no commercial mAbs against pIII protein is currently available. Corning 96-well plates were activated whit 50 ng per well of M-hMPV, P-hRSV or pIII-ADV purified recombinant proteins, for 12–18 h at 4 °C, in 50 µl of PBS. For detection of viral particles, ELISA plates were activated for 1 h at 37 °C with 50 µl of 1×10^6 pfu/ml of hMPV, hRSV or ADV. Viral particles were previously inactivated by exposure to UV radiation (302 nm) for 30 min, using a 15-W lamp transiluminator, then were boiled for 5 min at 85 °C. Activated plates were blocked by the addition of 10% FBS (Sigma-Aldrich) in PBS for 2 h at 37 °C and then washed twice with 0.05% Tween-20 in PBS for 3 min each wash. Each mAb generated in this study were diluted in 10% FBS/PBS at 3.4 µg/ml concentration and incubated 1 h at 37 °C. Commercial antibody used for hRSV detection (Abcam, #ab94965), also was used to final concentration 3.4 µg/ml. Commercial anti M-hMPV antibody (Millipore, #MAB8510) was used at 13.6 µg/ml final concentration. Then, plates were washed and incubated with anti-mouse IgG-HRP (1 mg/ml) (Invitrogen, Life Technologies #62-6520) at 1:2000 dilution in 10% FBS diluted in PBS for 1 h at room temperature (RT), in darkness. Finally, plates were washed again, and 3,3',5, 5'-Tetramethylbenzidine (TMB, 100 mg/ml, Sigma-Aldrich) was used as a colorimetric substrate. The enzymatic reaction was stopped with 2N H_2SO_4, and optical density was measured at 450 nm.

Sandwich ELISA was used to evaluate clinical samples. Plates were coated with 3.4 µg/ml of capture antibodies in PBS for 1 h at 37 °C. Capture antibodies were: anti M-hMPV 3G8/C11, anti P-hRSV 2E6/D2, and anti pIII-ADV 7E11. Then, plates were blocked with 10% FBS/PBS for 2 h at 37 °C and subsequently washed twice with 0.05% Tween-20 diluted in PBS. Plates were incubated with clinical samples overnight at 4 °C (50 µl per well). Clinical samples were previously treated with 100 µl of RIPA Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS and Protease inhibitor cocktail) for 15 min at 4 °C. Then, samples were centrifuged at 2000 rpm for 10 min at 4 °C and the supernatants were recovered to be tested by ELISA. After incubation with each specimen, plates were washed and detection antibodies were added diluted 1:2000 in blocking solution for 1 h at RT, in darkness. Detection antibodies used in this study were anti M-hMPV 7G4/A12, anti P-hRSV 6H5/H1 and anti pIII-ADV 6F11. All secondary antibodies were previously conjugated with HRP, following manufacture instructions from Lightning-Link™ HRP Conjugation Kit (Innova Biosciences, USA, #701-0003). Finally, plates were washed and 3,3', 5, 5'-Tetramethyl-benzidine (TMB, 100 mg/ml, Sigma-Aldrich) was used as a colorimetric substrate. Enzymatic reaction was stopped with 2M H_2SO_4, and optical density at 450 nm was determined. Statistical analysis was performed in GraphPad Prism v5.0 software. A clinical sample was considered positive if the absorbance value was higher at least two times the mean value obtained for the negative control (Table 1).

2.6. Dot-blot assays

To immobilize viral particles in a solid matrix, 20 µg of lysates of LLC-MK2, HEp-2 and A549 cells infected with hMPV, hRSV and ADV, respectively, were spotted onto a nitrocellulose membrane (Thermo Scientific). As positive controls M-hMPV, P-hRSV or pIII-ADV purified proteins were also immobilized at different amounts. Dots containing 1 µg Bovine Serum Albumin (BSA) were used as negative control. Membranes with spots were air-dried for 15 min and subsequently blocked with 5% BSA diluted in 0.05% Tween-20 in PBS, for 1 h at RT. Next, membranes were incubated for 1 h at RT with 3.4 µg/ml of mAbs anti M-hMPV, anti P-hRSV, or anti pIII-ADV in blocking solution. After incubation, membranes were washed with 0.05% Tween-20 in PBS (three times) and incubated for 1 h at RT with a 1:2000 anti-mouse anti IgG-HRP (1 mg/ml) (Invitrogen, Life Technologies), diluted in 5% BSA with 0.05% Tween-20 in PBS. Finally, membranes were washed with 0.05% Tween-20 in PBS (twice), once with PBS and incubated with the enhanced chemiluminescence Western blot detection system (ECL, Amersham, Uppsala, Sweden).

2.7. Immunofluorescence assay

LLC-MK2, HEp-2 and A549 cells were cultured in 24- well plates with 12-mm covers (2×10^5 cells/well), and infected with 1×10^6 pfu/ml of hMPV, hRSV or ADV, respectively. After 48 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min at RT. Cells were permeabilized with 0.2% saponin diluted in 2% BSA-PBS for 30 min at RT. Then, cells were incubated for 1 h at RT with antibodies

anti M-hMPV 3G8/C11 and 7G4/A12 (hMPV), anti P-hRSV 2E6/D2 and 6H5/H1, or anti pIII-ADV 7E11 and 6F11, at final concentration of 3.4 µg/ml. Commercial mouse monoclonal anti M-hMPV antibody (Millipore, #MAB8510) was used at final concentration of 13.6 µg/ml; commercial mouse monoclonal anti P-hRSV antibody (Abcam, #94965) was used at final concentration of 3.4 µg/ml, and commercial rabbit polyclonal anti-ADV antibody (Abcam, #ab1039,) was used at final concentration of 3.4 µg/ml. Then, fixed cells were incubated with IgG FITC-conjugated anti-mouse antibody (0.5 mg/ml) (BD Pharmingen) at a 1:200 dilution (final concentration 2.5 mg/ml) for 1 h at RT, in darkness. ADV infected cells that were tested with commercial rabbit polyclonal anti-ADV antibody (Abcam, nºab1039) at 3.4 µg/ml were incubated with an FITC conjugated anti-rabbit antibody (0.5 mg/ml) at a 1:2000 dilution (ThermoFisher). Finally the cells were washed with PBS and nuclei were stained with TOPRO-3 iodide 642/661 (Invitrogen, #T3605), diluted 1:10,000, for 15 min in darkness. Coverslips were mounted and examined using an Epifluorescence microscope Olympus BX51 (Center Valley, PA) and the pictures were analyzed in INFINITY Software V5.10.

2.8. Determination of viral RNA by RT-qPCR

Viral RNA from nasopharyngeal swabs was purified using TRIzol LS reagent (Invitrogen), following the manufacturer's instructions. Briefly, total RNA was isolated using TRIzol LS reagent (Life Technologies) and 2 µg RNA was reverse transcribed to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, 1708891), in a thermocycler (Applied Biosystems). The virus detection was made using TaqMan™ Fast Advanced Master Mix (Applied Biosystems, 4444556) and 6FAM (5'label)-AAGTGTATTACAGAAGTTTG-MGBNFQ (3' label) probe for hMPV (TaqhMPV_Fw01 5'-GATGGACATACCAAAAATCGCTAGA-3' and TaqhMPV_Rv01 5'-GCCTAATGCTTTGCCATACTCA-3'), 6FAM-(5'label)-TATGGTGCAGGGCAAG-MGBNFQ (3' label) probe for hRSV (forward 5'-TTTTGCTGGATTGTTTATGAATGC-3' and reverse 5'-GACCCCCCAC CGTAACAT-3') and 6FAM-TGCACCAGACCCGGGCTCAGGTACTCC adenoprobe with the forward Adenoquant (AQ1) 5'-GCCACGGTGGGG TTTCTAAACTT-3' and reverse Adenoquant (AQ2) 5'-GCCCCAGTGGT CTTACATGCACATC-3' primer for ADV (Esposito et al., 2016). Amplification was performed with the following cycling conditions for hMPV and hRSV probes: one cycle of 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 min and 58 °C for 10 s and a final cycle of 68 °C for 10 s. Amplification was performed with the following cycling conditions for ADV probe: one cycle of 50 °C for 2 min, one cycle of 95 °C for 8 min, followed by 50 cycles of 95 °C for 15 s and 59 °C for 1 min (Esposito et al., 2016), and a final cycle of 68 °C for 10 s. Abundance of each target cDNA was determined by the relative quantification or comparative CT ($2-\Delta\Delta ct$) method. Normalization versus endogenous control RNA was performed on samples using the TaqMan probe of human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control assays (Applied Biosystems). All samples were analyzed at least by duplicate in an Applied Biosystems Step-One Plus thermocycler.

2.9. Statistical analysis

Statistical analysis was performed in GraphPad Prism v6.0 software. Data are means (± SEM) of at least three independent experiments. $*P < .05$; $**P < .01$; $***P < .001$; ns: no significant difference, for the Fig. 1–5 by one-way ANOVA and posteriori Dunnett's multiple comparisons test or Tukey's multiple comparisons test. In the Fig. 6, the data shown are median and $*P < .05$; $**P < .01$; $***P < .001$, treatment versus control or infected patients versus healthy patients or patients with other virus; by non-parametric student test and Mann Whitney post-test.

3. Results

3.1. Anti M-hMPV, anti P-hRSV and anti pIII-ADV mAbs can detect purified M-hMPV, P-hRSV and pIII-ADV proteins by ELISA

As described in materials and methods, six new mAbs were generated in this study: anti M-hMPV 3G8/C11 and 7G4/A12, anti P-hRSV 2E6/D2 and 6H5/H1, and anti pIII-ADV 2E6/D2 and 6H5/H1. To evaluate the specificity of these mAbs, indirect ELISA was performed. To develop the assay, wells were activated with 1×10^6 pfu/ml of each virus (hMPV, hRSV or ADV) or with 50 ng of purified recombinant viral proteins (M-hMPV, P-hRSV or pIII-ADV) obtained as previously described (materials and methods). MAbs anti-hRSV, hMPV or ADV were used at a 3.4 µg/ml concentration and commercial antibodies were used as recommended by the suppliers (Materials and Methods). MAbs generated to detect the M protein of hMPV could bind hMPV and purified M-hMPV protein (Fig. 1A, left and middle panels), reaching an OD_{450} value significantly higher as compared to controls. The commercial anti M-hMPV antibody was also able to detect hMPV, but the OD_{450} value obtained for this antibody was lower than antibodies 3G8/C11 and 7G4/A12, despite the same amount (3.4 µg/ml) was used in the assay (Fig. 3A, right panel). Further, the commercial anti-M-hMPV antibody showed reduced capacity to detect M-hMPV protein, as compared to the results obtained for 3G8/C11 and 7G4/A12 antibodies (Fig. 1A right panel). For hRSV, the anti P-hRSV mAbs generated in this study and the commercial anti P-hRSV antibody could detect both the recombinant protein and viral particles with equivalent efficiencies (Fig. 1B). Finally, among the two anti pIII-ADV mAbs of this study, anti pIII-ADV antibody 7E11 showed higher sensitivity to detect purified pIII-ADV protein, as compared to anti pIII-ADV antibody 6F11. However, both antibodies showed similar performance to detect ADV (Fig. 1C). No commercial antibodies against ADV pIII protein were commercially available to include in this study, and anti-ADV mAbs that detect different proteins are not recommended for ELISA. In summary, mAbs anti M-hMPV, anti P-hRSV and anti pIII-ADV specifically detected purified proteins and antigens from the viral particles. In addition, control assays were performed to determine that antibodies bind exclusively to the antigen and to rule out unspecific binding to the plates or to the BSA protein. The results show that none of the antibodies used in this study bind non-specifically to the plates or unspecific proteins (Fig. S1).

3.2. Anti M-hMPV, anti P-hRSV and anti pIII-ADV mAbs can detect viral particles with high sensitivity by ELISA

Indirect ELISA tests were performed to determine the lower limit of detection of each mAbs generated in this study. In this assay, 96-well plates were activated with serial dilutions of purified M-hMPV, P-hRSV or pIII-ADV proteins, ranging from 50 to 0.04 ng. The mAbs of this study and the commercial anti P-hRSV antibody were all used at a concentration of 3.4 µg/ml, while commercial anti M-hMPV antibody was used at recommended concentration of 13.6 µg/ml. Currently, anti pIII-ADV commercial antibodies are not available to perform assays to compare with the anti-ADV mAbs generated in this study (Figure S2). Similarly, plates with serial dilution of inactivated hMPV, hRSV or ADV were prepared, ranging from 1×10^6 pfu/ml to 1×10^3 pfu/ml, to detect the minimal amount of viruses that is detected by these mAbs (Fig. 2). Both anti M-hMPV mAbs of this study were able to detect a large number of dilutions of the recombinant protein, displaying a great sensitivity (Fig. S2A). Sensitivity evaluation of the commercial antibody anti-M was not performed because it was observed, after several trials, that this antibody is not able to recognize the purified M-hMPV protein used in this study. For purified P-hRSV protein, both mAbs generated in this study and the commercial anti P-hRSV mAb, could detect nearly all the protein dilutions tested (Fig. S2B). However, in other assays, commercial anti P-hRSV was used as capture antibody in detection of the

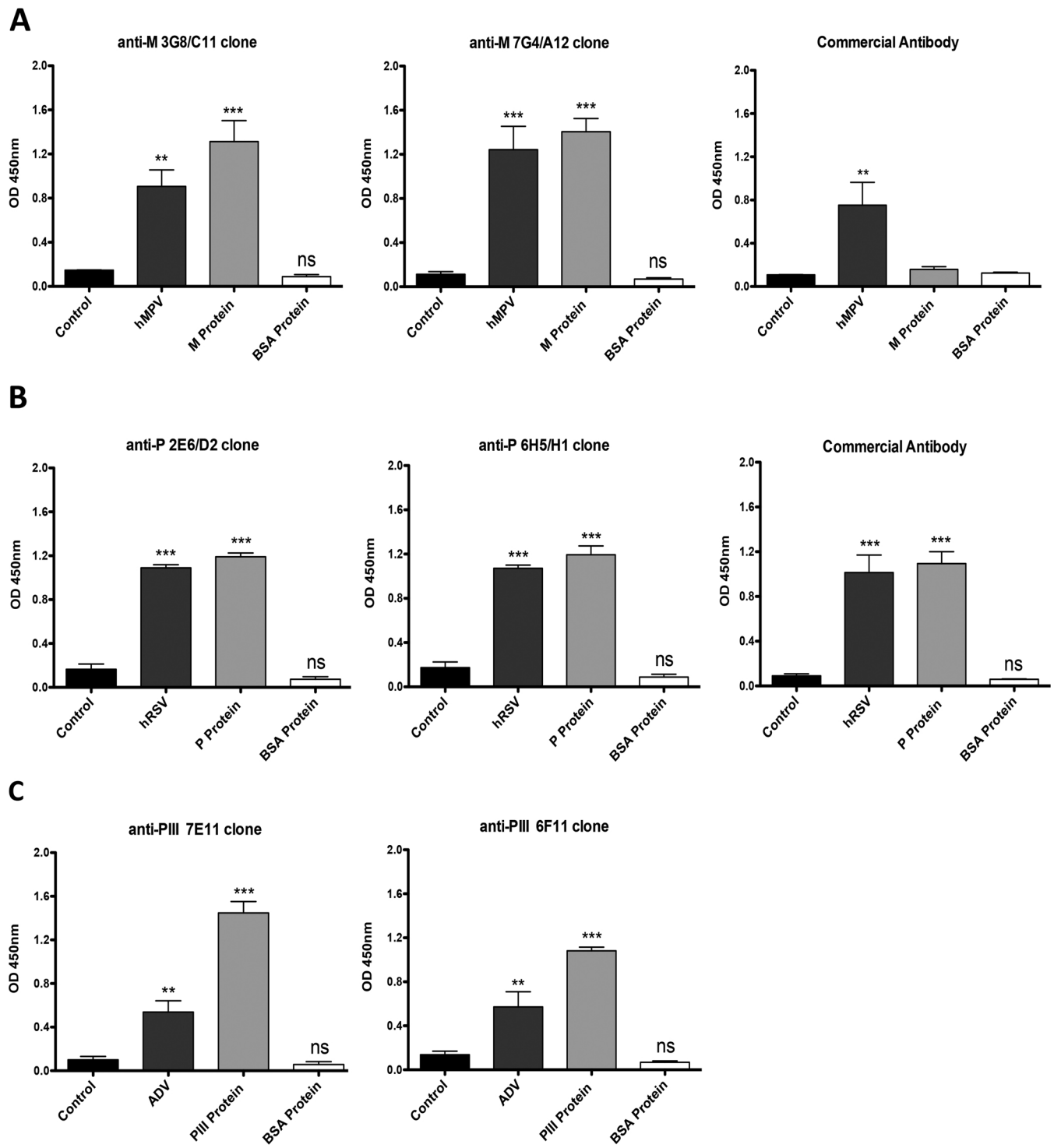

Fig. 1. Indirect ELISA for the detection of hMPV, hRSV and ADV antigens by different monoclonal antibodies. Wells were coated with different antigens: 50 ng of M protein, 1×10^6 pfu/ml of hMPV and 50 ng of BSA protein (A); 50 ng P protein, 1×10^6 pfu/ml of hRSV and 50 ng of BSA protein (B) and 50 ng of pIII protein, 1×10^6 pfu/ml of ADV and 50 ng of BSA protein (C), per duplicate. Wells without antigen and wells with other viruses were included in each assay as negative controls. Data shown in graphs are optical density results obtained with (A) anti-M 3G8/C11 and 7G4/A12 clones at 3,4 μg/ml, and commercial antibody (Millipore) anti-M at 13.6 μg/ml. B) anti-P 2E6/D2 and 6H5/H1 clones at 3,4 μg/ml, and commercial antibody (Abcam) anti-P hRSV at 3,4 μg/ml. C) anti-pIII 7E11 and 6F11 clones at 3,4 μg/ml. Data are mean (± SEM) of at least three independent experiments with $^{**}P < .01$ and $^{***}P < .001$ significance versus control using one-way ANOVA and a posteriori Dunnett's multiple comparisons test. "ns" means no significant difference.

viral protein in clinical samples and compared with the new mAbs. The results showed that the commercial mAb was less sensitive to detect P-hRSV in clinical samples when used as capture antibody (data not shown). For pIII-ADV, both antibodies could detect up to 25 ng of purified protein (Fig. S2C). In terms of sensitivity, all monoclonal antibodies were able to detect hMPV, hRSV or ADV viral particles respectively, and the results were statistically significant until detection of approximately 1×10^3 pfu/ml, excepting for anti M-hMPV 3G8 clone, which showed a decreased sensitivity in comparison with the other antibodies, with a limit of detection of 31,250 viral particles, however being more sensitive than commercial antibodies (Fig. 2). Finally, serial dilutions of each mAb were performed to determine the

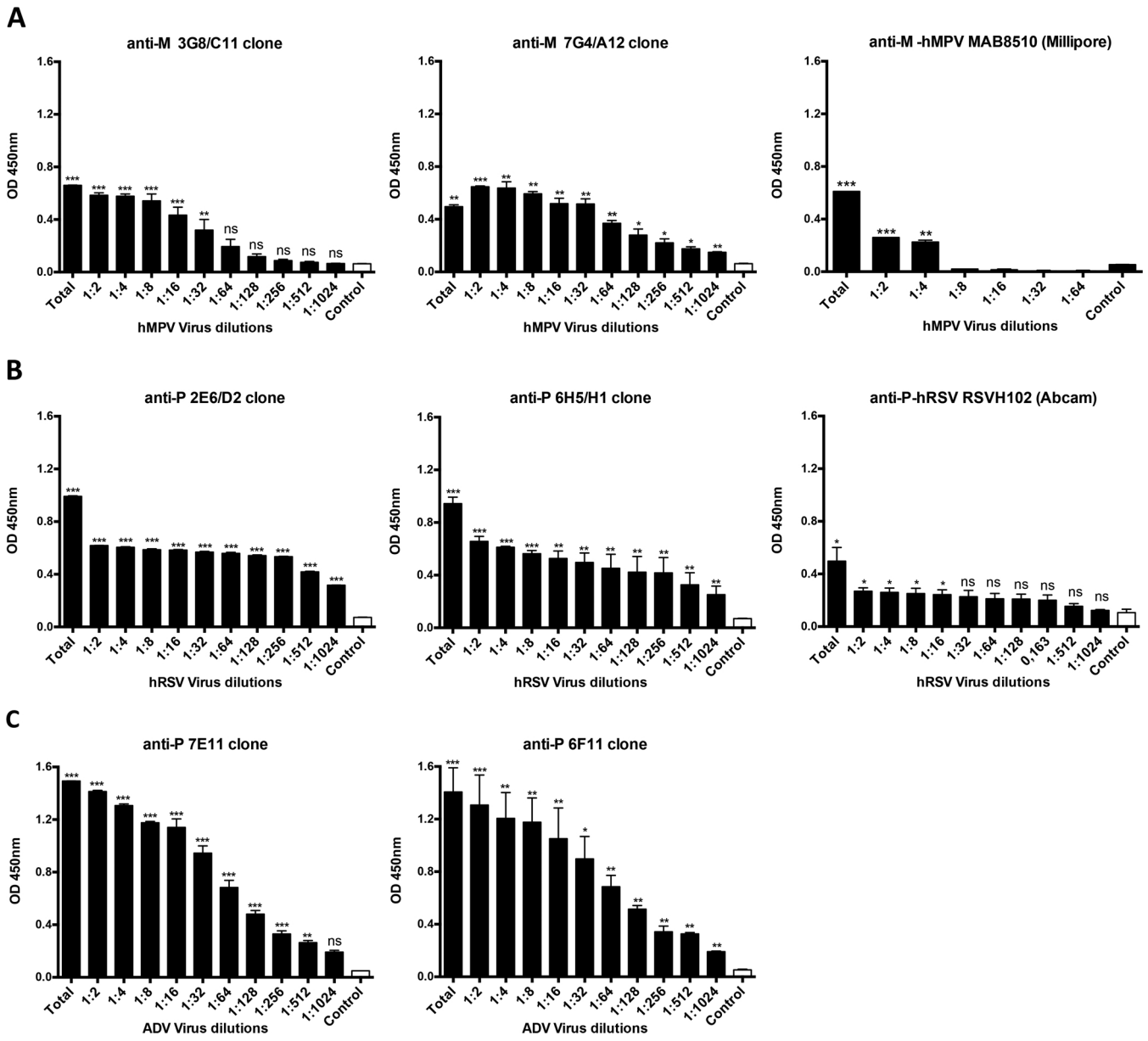

Fig. 2. Serial dilutions assays for the evaluation of sensitivity of the mAbs in detection of hMPV, hRSV and ADV. Wells were coated with dilution range from total virus (1×10^6 pfu/ml) to 1:1024 of hMPV (A), hRSV (B) and ADV (C) virus. Wells without antigen were included as negative controls. Graphs show optical density at 450 nm. A) Anti-hMPV mAbs Anti-M 3G8/C11 and 7G4/A12 clones tested at 3.4 μg/ml; commercial antibody tested at 13.6 μg/ml. B) Anti-hRSV mAbs anti-P 2E6/D2, 6H5/H1 and commercial antibody were tested at 3.4 μg/ml. C) Anti-ADV mAbs anti-pIII 7E11 and 6F11 clones were tested at 3.4 μg/ml. Data are mean (± SEM) of at least three independent experiments with $^*P < 0.05$; $^{**}P < .01$ and $^{***}P < .001$ significance versus control using one-way ANOVA and a posteriori Dunnett's multiple comparisons test.

minimal concentration needed of each antibody to detect 50 ng of protein. According to results, only 0.15 ng are needed of each antibody to detect 50 ng of their specific protein (Fig. 3).

3.3. Anti M-hMPV, anti P-hRSV and anti pIII-ADV mAbs detect viruses in infected cells by indirect immunofluorescence

Indirect immunofluorescence was performed to determine MAbs ability to recognize M-hMPV, P-hRSV or pIII-ADV proteins on infected cells. LLC-MK2, HEp-2 and A549 cells were infected with hMPV, hRSV and ADV, respectively, for two days and evaluated for cytopathic effect. Commercial anti M-hMPV and anti P-hRSV mAbs were used as positive controls to confirm cell infection by hMPV and hRSV (Materials and Methods). Data show that anti M-hMPV mAbs 3G8/C11 and 7G4/A12 could detect hMPV-infected cells with similar reactivity than commercial anti M-hMPV antibody (Fig. 4A). Anti P-hRSV mAbs were also able to detect hRSV-infected cells with equivalent reactivity than commercial anti P-hRSV mAb (Fig. 4B). Data shown for anti pIII mAbs, also demonstrates that 7E11 and 6F11 mAbs can recognize ADV infected cells (Fig. 4C). In this assay, a commercial rabbit polyclonal anti-ADV mAb was used to test its reactivity, but it was not able to detect ADV infected cells by immunofluorescense. Further, in this assay none of the mAbs tested recognized uninfected cells. According to these results, mAbs generated in this work may be used in indirect immunofluorescence and thereby are suitable for the detection of hMPV, hRSV or ADV-infected cells from clinical samples.

3.4. Detection of hMPV, hRSV and ADV antigens by dot blot using anti M-hMPV, anti P-hRSV and anti pIII-ADV mAbs

The ability of the mAbs to detect viral proteins by dot-blot assays was carried out by adding purified viral proteins or protein extracts

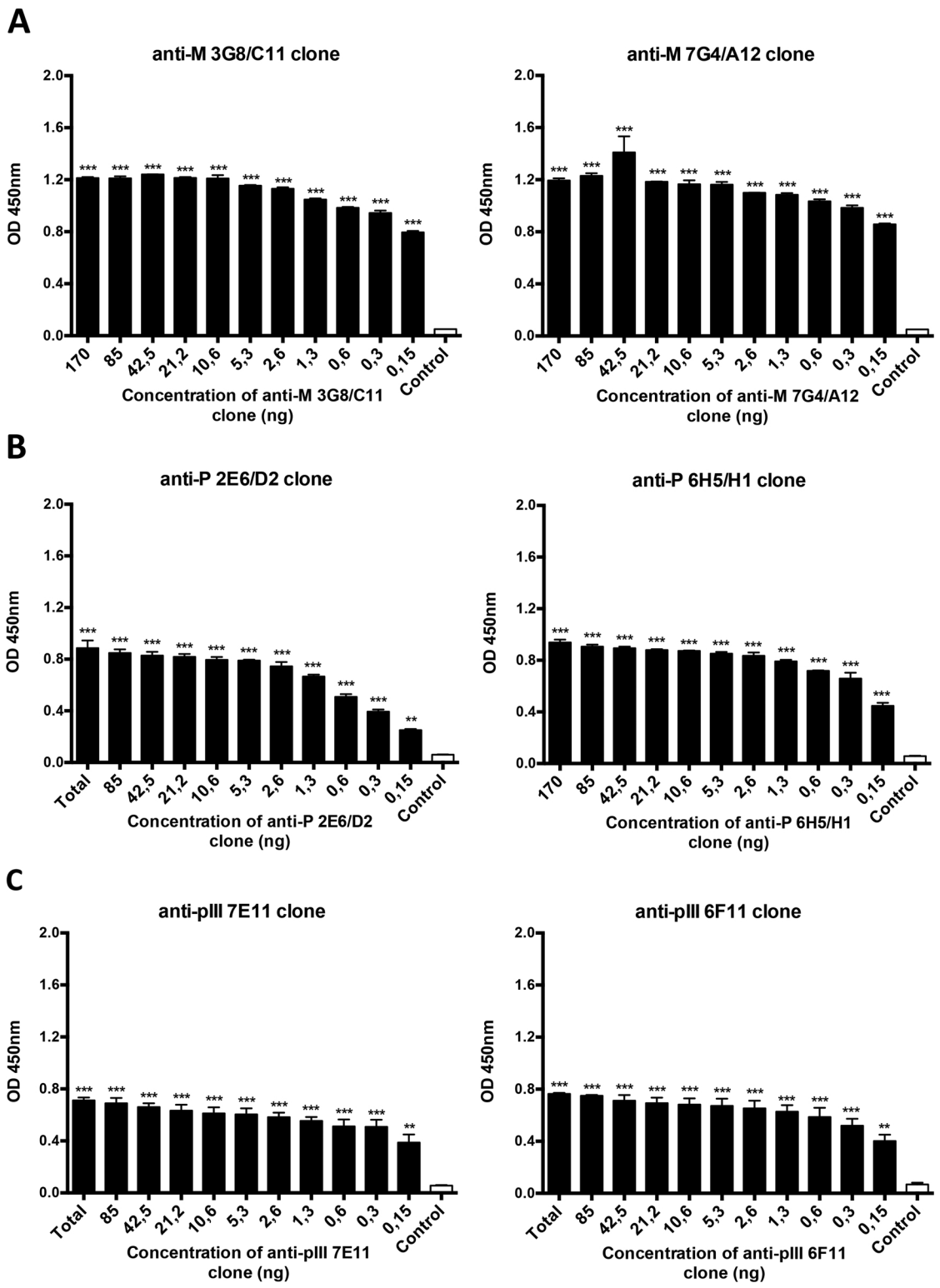

Fig. 3. Serial dilutions assays to evaluate efficiency of mAbs at detection of hMPV, hRSV and ADV antigens. Wells were coated with 50 ng of M protein in (A), P protein (B) and pIII protein (C). Wells without antigen were included as negative controls. Graphs show optical density at 450 nm. A) Anti M-hMPV antibodies 3G8/C11 and 7G4/A12. B) Anti P-hRSV antibodies 2E6/D2 and 6H5/H1. C) Anti pIII-ADV antibodies 7E11 and 6F11. All monoclonal antibodies were added in concentration ranged from 170 to 0.15 ng per well. Data are mean ± SEM of at least two independent experiments with $^{*}P < .05$ and $^{***}P < .001$ significance versus control using one-way ANOVA and a posteriori Dunnett's multiple comparisons test.

from hMPV, hRSV and ADV infected cells onto a nitrocellulose membrane. Anti M-hMPV mAbs 3G8/C11 and 7G4/A12 could recognize specifically M-hMPV protein and hMPV infected cells (Fig. 5A). Furthermore, these mAbs could detect concentrations as low as 50 ng of purified M-hMPV protein by this technique. In addition, the results suggest that anti-M clones are highly specific, because they do not recognize negative controls. Anti P-hRSV mAbs 2E6/D2 and 6H5/H1 were also able to detect purified P-hRSV protein and hRSV infected HEp-2 cells (Fig. 5B). Specifically, using these mAbs at a concentration equal to 3.4 µg/ml it was possible to detect 20 µg/ml of total protein

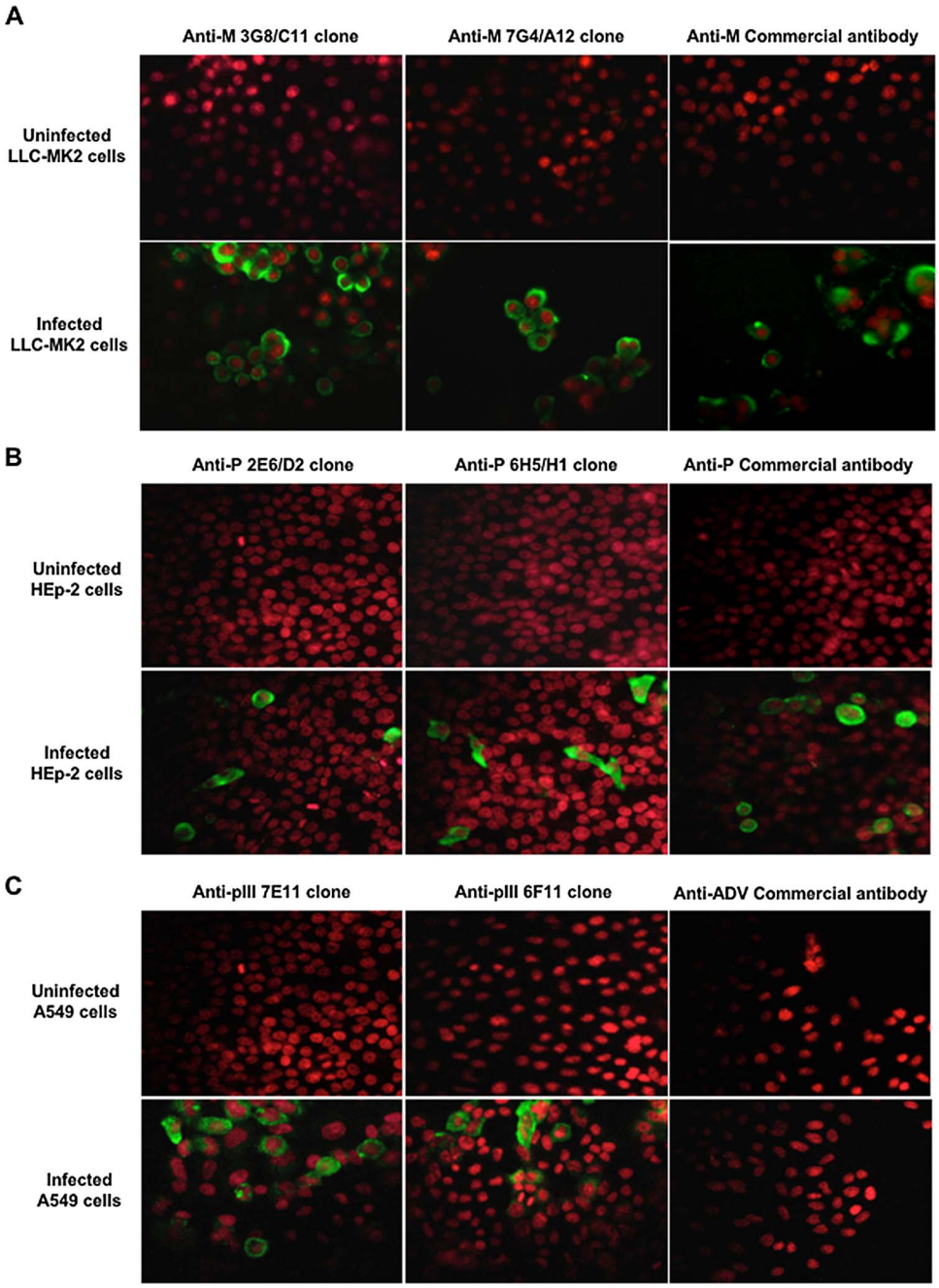

Fig. 4. Detection of hMPV, hRSV and ADV antigens in infected LLC-MK2, HEp-2 and A549 cells by immunofluorescence. LLC-MK2 (A), HEp-2 (B) and A549 (C) cells were infected with hMPV, hRSV or ADV respectively for 48 h and analyzed by immunofluorescence. A) Detection of hMPV infected cells using anti M-hMPV antibody 3G8/C11 (3.4 μg/ml), anti M-hMPV antibody 7G4/A12 (3.4 μg/ml), and commercial anti M-hMPV antibody from Millipore (13.6 μg/ml). B) Detection of hRSV infected cells using anti P-hRSV antibody 2E6/D2 (3.4 μg/ml), anti P-hRSV antibody 6H5/H1 (3.4 μg/ml) and commercial anti P-hRSV antibody from abcam (3.4 μg/ml). C) Detection of ADV infected cells using anti pIII-ADV antibody 7E11 (3.4 μg/ml), anti pIII-ADV antibody 6F11 (3.4 μg/ml) and commercial polyclonal rabbit antibody (0.34 μg/ml). Images were taken at 40× magnification and are representative of two independent experiments. We included nuclear staining in red color by using TO-PRO-3 iodide 642/661. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from hRSV infected cells and even lower concentrations of purified P-hRSV protein. Like anti M-hMPV mAbs, anti P-hRSV mAbs did not bind to any of the negative controls used in this assay. In the case of the anti pIII-ADV mAbs 7E11 and 6F11, the results showed that these mAbs were also able to recognize the purified pIII-ADV protein or A549 cells infected with ADV. No signal was observed for any of the negative controls used in this assay (Fig. 5C). These results confirm the high specificity of the anti M-hMPV, anti P-hRSV and anti pIII-ADV mAbs not only in the detection of purified antigens, but also in infected cells.

3.5. Anti M-hMPV, P-hRSV and pIII-ADV mAbs can detect viral proteins in clinical samples

Sandwich ELISA was performed to evaluate the efficiency of the anti M-hMPV, anti P-hRSV and anti pIII-ADV mAbs in clinical samples. Nasopharyngeal aspirates used in this assay were previously diagnosed with hMPV, hRSV or ADV using the standard immunofluorescence technique. In addition, 10 control samples that were classified previously as negative for hMPV, hRSV and ADV infection were included. To evaluate the capacity of the mAbs of this study to detect viral proteins in these samples, a customized sandwich ELISA test was designed, using anti M-hMPV mAb 3G8/C11, anti P-hRSV mAb 2E6/D2, and anti pIII-ADV mAbs 7E11 as capture antibodies. Anti M-hMPV mAb 7G4/A12, anti P-hRSV mAb 6H5/H1 and anti pIII-ADV mAb 6F11 were conjugated with HRP and used as detection antibodies. For the combination of anti M-hMPV mAbs 3G8/C11 − 7G4/A12, from 20 patients previously diagnosed with hMPV, 18 resulted positive using the ELISA technique (OD_{450} 0.314 ± 0.253) (Fig. 6A), equivalent to a sensitivity of 90% (Table 1). For the combination of anti P-hRSV mAbs 2E6/D2 and 6H5/H1, from 20 patients previously diagnosed with hRSV, 17 resulted positive using the ELISA test (OD_{450} 0.695 ± 0.479) (Fig. 6B), with a sensitivity equal to 85% (Table 1). Using the set anti pIII-ADV

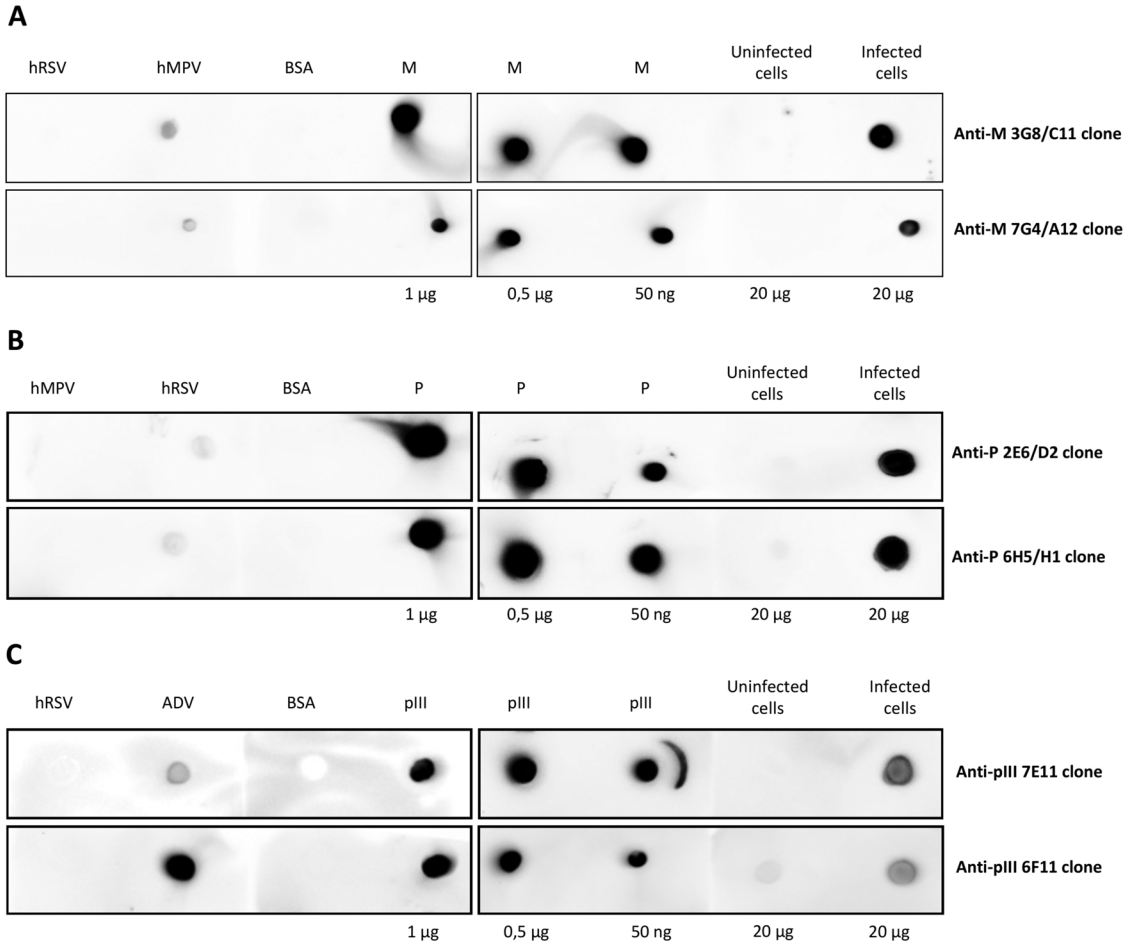

Fig. 5. Dot-blot assays confirm monoclonal antibody specificity for hMPV, hRSV and ADV antigens. Dot blots for anti-M (3G8/C11; 7G4/A12 clones) of hMPV are shown in A, anti-P (2E6/D2; 6H5/H1 clones) of hRSV in B and anti-pIII (7E11; 6F11 clones) of ADV in C. The effective recognition of antigens by each antibody is reflected by the spot intensity, which appears with variability according to the different antibody concentrations tested. The film on the right corresponds to the negative and positive controls including 1µg of hMPV M protein (A), hRSV P protein (B) or ADV pIII protein (C). The film on the left corresponds to other concentrations of M, P and pIII proteins and detection of the hMPV, hRSV and ADV antigen in infected and uninfected LLC-MK2, HEp-2 and A549 cells (20 µg, A, B and C), respectively. The image shown below is representative for two independent experiments.

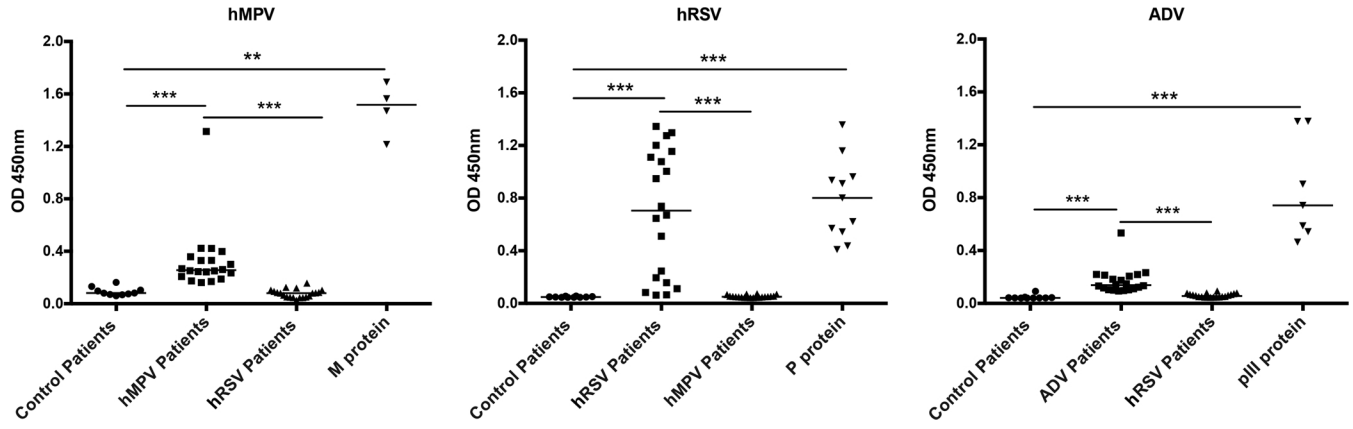

Fig. 6. Sandwich ELISA for detection of hMPV, hRSV and ADV in clinical samples, using as positive control proteins (M, P and pIII) and as a negative controls samples from patients infeceted with other non-related viruses. A) OD obtained for samples from 20 patients infected with hMPV that were tested using the anti M-hMPV antibody 3G8/C11 as capture mAb and anti M-hMPV antibody 7G4/A12 as detection mAb (HRP labeled). B) OD obtained for samples from 20 patients infected with hRSV that were tested using the anti P-hRSV antibody 2E6/D2 as capture mAb and anti P-hRSV antibody 6H5/H1 as detection mAb (HRP labeled). C) OD obtained for samples from 20 patients infected with ADV that were tested using the anti pIII-ADV antibody 7E11 as capture mAb and anti pIII-ADV antibody 6F11 as detection mAb (HRP labeled). In each experiment, samples obtained from 10 patients infected with other viruses were included. Capture monoclonal antibodies were used in 1:700 (3.4 µg/ml) dilution and detection monoclonal antibodies in 1:2.000 (1 mg/ml) dilution. Data are mean (± SEM) of at least three independent experiments with $^{**}P < .01$ and $^{***}P < .001$ significance hMPV, hRSV and ADV patients versus healthy patients or patients infected with other viruses, using non-parametric student test and Mann Whitney test post. "ns" means no significant difference. OD: Optical Densities.

mAbs 7E11/6F11, from 20 patients previously diagnosed with ADV, 17 resulted positive (OD_{450} 0.169 ± 0.098), with sensitivity equal to 85% (Table 1). Importantly, none of the mAbs combinations produced false positive results (Fig. 6 and Table 1). It is worth mentioning that samples used in this assay were freeze-stored before being tested by the assay designed in this study, therefore it is possible that viral proteins in these samples were reduced due to degradation after thawing. Notwithstanding, the customized sandwich ELISA test designed in this study could detect more than 85% of the samples previously diagnosed as positive for hMPV, hRSV and hADV by inmunofluorescence. These results show that the new mAbs described in this study can detect hMPV, hRSV and ADV antigens from clinical samples.

For the second approach, 37 patients younger than 2 years old with respiratory symptoms were recruited. From these patients, 16 were females and 21 males, 23 outpatients, 5 from the emergency service, 7 from hospitalization service and 2 from the intensive care unit. DFA analyses and ELISA assays were performed in parallel. The two clones obtained for each protein (M-hMPV, P-hRSV, pIII-ADV), were used to perform a sandwich ELISA. Results were compared with "gold standard" technique (DFA). Specificity and sensitivity parameters were calculated according to the following formula: Specificity (%): (TN/TN + FP) x 100; Sensitivity (%): (TP/TP + FN) x 100. Results obtained for this part of the study were analyzed at the end of the patient's recruitment and were expressed in sensitivity and specificity terms, compared with DFA as the standard method for this study. The results obtained by ELISA for anti P-hRSV antibodies showed 86% of specificity and 88% of sensitivity. For anti pIII-ADV, none of the samples were positive neither by DFA nor by ELISA; resulting in a 100% of specificity. However, we observed a significant change both in sensitivity and specificity for anti M-hMPV antibodies, when compared with the first results obtained with the 70 first samples (Table 1). Anti M-hMPV antibodies showed a 50% of sensitivity and a 69% of specificity. Considering the discrepancy of results obtained for anti M-hMPV antibodies in comparison with DFA, an additional assay was performed with all the clinical samples, where results obtained from DFA and ELISA were individually compared with a qPCR analysis, as and additional "gold standard". Results showed that, when compared to qPCR, anti M-hMPV antibodies used in the ELISA platform showed less specificity than DFA (92% vs. 100%), but higher sensitivity than DFA (83% vs. 17%) (Table 2). This result indicates that anti-M hMPV mAbs, when used in ELISA, are more sensitive than DFA. We also observed that when compared with qPCR, anti P-hRSV antibodies in ELISA showed more specificity than antibodies used in DFA (94% vs. 86%) (Table 3). Finally, qPCR analysis for ADV showed there is no difference in terms of specificity between DFA and anti-ADV antibodies in the ELISA platform (Table 4). On the other hand, according to the analysis of the results by qPCR, there was just one clinical sample positive for ADV, and not positive data for ELISA nor DFA. For this reason, there are no "True positives" data in these assays, indicating that there is not possible to calculate the sensitivity of the tests. These results are represented as N.A. in Table 4.

4. Discussion

Among the assortment of viral pathogens that cause acute respiratory infections, viral agents such as hRSV, hMPV, Rhinovirus, Influenza virus and ADV have shown to be the most prevalent, especially in children under five years old (Lozano et al., 2012; Ruiz et al., 1999; Louie et al., 2005; Bradley et al., 2011, Jacobs et al., 2013). Since there is no broad effective treatments or prevention procedures to reduce the infections caused by these viruses, many efforts have been made to improve diagnosis strategies. The goal of this work was to generate and characterize new mAbs to detect three of the most important respiratory viruses: hRSV, hMPV and ADV, to design novel tests that could be easier to implement in basic clinic laboratories and more affordable for general population. In this study, mAbs that recognized

Table 1
Detection of hMPV, hRSV and ADV in clinical samples with monoclonal antibodies in ELISA platform.

	hMPV patients	Positive analysis	Negative analysis	hRSV patients	Positive analysis	Negative analysis	Control patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Anti-M hMPV	20 (0.314 ± 0.253)	18	2	20 (0.078 ± 0.033)	0	20	10 (0.097 ± 0.032)	0	10	100	90
	hRSV patients	Positive analysis	Negative analysis	hMPV patients	Positive analysis	Negative analysis	Control patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Anti-P hRSV	20 (0.695 ± 0.479)	17	3	20 (0.055 ± 0.021)	0	20	10 (0.055 ± 0.021)	0	10	100	85
	ADV patients	Positive analysis	Negative analysis	hRSV patients	Positive analysis	Negative analysis	Control patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Anti-pIII ADV	20 (0.169 ± 0.098)	17	3	20 (0.061 ± 0.014)	0	20	10 (0.047 ± 0.016)	0	10	100	85

The two clones obtained por each viral proteins were used to perform a sandwich ELISA. Results were compared with data obtained from "gold standard" technique (DFA). Positive analysis: patients diagnosed as positive for DFA and ELISA. Negative analysis: patients diagnosed as positive for DFA that were negative by ELISA. Clinical samples from control patients were used as specificity controls. Clinical samples caracterized as positive for hMPV, were used in assays with anti-hRSV mAbs to evaluate specificity. Likewise, hRSV positive samples were used to evaluate anti-M and anti-pIII specificity. In parenthesis mean ± SD (Standard Deviation).

Table 2
Comparison of hMPV detection in clinical samples between monoclonal antibodies, DFA and qPCR, by blinded study.

	Reference diagnosis: DFA				
	mAbs-hMPV: 37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: ELISA	Positive analysis Negative analysis	1 1	11 24	69	50
	Reference diagnosis: qPCR				
	mAbs-hMPV: 37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: ELISA	Positive analysis Negative analysis	10 2	2 23	92	83
	Reference diagnosis: qPCR				
	mAbs-hMPV: 37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: DFA	Positive analysis Negative analysis	2 10	0 25	100	17

Table 3
Comparison of hRSV detection in clinical samples between monoclonal antibodies, DFA and qPCR, by blinded study.

	Reference diagnosis: DFA				
	mAbs-hRSV:37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: ELISA	Positive analysis Negative analysis	14 2	3 18	86	88
	Reference diagnosis: qPCR				
	mAbs-hRSV: 37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: ELISA	Positive analysis Negative analysis	16 3	1 17	94	84
	Reference diagnosis: qPCR				
	mAbs-hRSV: 37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: DFA	Positive analysis Negative analysis	14 2	3 18	86	88

Table 4
Comparison of ADV detection in clinical samples between monoclonal antibodies, DFA and qPCR, by blinded study.

	Reference diagnosis: DFA				
	mAbs-ADV: 37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: ELISA	Positive analysis Negative analysis	0 0	0 37	100	N.A
	Reference diagnosis: qPCR				
	mAbs-ADV: 37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: ELISA	Positive analysis Negative analysis	0 1	0 36	100	N.A
	Reference diagnosis: qPCR				
	mAbs-ADV: 37 patients	Positive analysis	Negative analysis	Specificity (%)	Sensitivity (%)
Diagnostic test: DFA	Positive analysis Negative analysis	0 1	0 36	100	N.A

N.A: not apply.

conserved proteins of hMPV, hRSV and ADV were obtained and characterized, using different laboratory methods. The results obtained here demonstrate that these immune reagents not only could be used in different techniques for research, but also for diagnosis. New methodologies for rapid point-of-care tests, easy to implement, based on immunodiagnostics are under constant development. Therefore, it is important to generate mAb that detect conserved proteins of respiratory viruses that can be used in diverse platforms. At baseline, characterizations of mAbs were performed in terms of specificity and sensitivity, evaluating their ability to recognize low concentrations of their specific antigens. As a result, all the mAbs described in this study could recognize only the specific antigen of the virus for which they were produced. Anti M-hMPV antibodies 3G8/C11 and 7G4/A12 were not only able to detect specifically the M-hMPV protein, but also were able to detect low quantities of viral particles and small amounts of purified protein (below 1 ng). In contrast, commercial anti M-hMPV mAb (Millipore) used in this study as a positive control, showed lower sensitivity than the new mAbs described here in the detection of serial viral dilutions. Further, recombinant M-hMPV protein used in this assay was not recognized by this commercial mAb. This observation might be due to specific sequences recognized by Millipore mAb, that probably have some differences in the recombinant protein generated in our laboratory. For instance, it is possible that the his-tag included in the recombinant protein generated in our laboratory is masking some epitope recognized by the commercial anti M-hMPV mAb. Nevertheless, the mAbs generated in this study, using this recombinant M-hMPV protein, could recognize total virus and infected cells, with more sensitivity than the commercially anti M-hMPV mAb. MAbs. 2E6/D2 and 6H5/H1, which recognize P-hRSV protein, showed similar features than the commercially available anti P-hRSV mAb, in terms to sensitivity and specificity. All anti P-hRSV mAbs (new ones and commercially available) could detect specifically protein and total virus, and detected lower dilutions of the hRSV and P-hRSV proteins. Remarkably, it was demonstrated that only 150 pg of each clone against hRSV, hMPV or ADV, are capable to detect less than 50 ng of their specific protein, accounting for the high efficiency of the antibodies to recognize their antigen, which make them widely competent to get into the market of mAbs. The sensitivity evaluation was performed using methods usually implemented in clinical laboratories. The results obtained by immunofluorescence and Dot Blot not only corroborate the data obtained by ELISA, but also account for the versatility of these antibodies through the different options available for detection of viral proteins.

It is also worth mentioning that there is no cross-reaction between P or M antigen of hRSV and hMPV, which can be observed in Fig. 1, where each graph contains a negative control which corresponds to another virus, e.g. hRSV lysate was used as control in the assays with anti-M antibodies of hMPV (Fig. 1A “control”) and hMPV viral lysate was used as control for the anti-P antibodies of hRSV (Fig. 1B “control”). In addition, alignments of the nucleotide sequences and the amino acid sequences of several serotypes of each virus were performed, e.g. both serotypes of hRSV were analyzed against M hMPV protein and vice versa, and the analysis resulted in less than 50% similarity among sequences.

During the further characterization of the mAb described in this study, we established a protocol of detection of each virus in biological samples where the two mAbs generated for each viral protein complement each other in a sandwich ELISA based technique. The best combination was determined to favor the sensitivity in the detection of viral antigen in biological samples. The results obtained by the sandwich ELISA test designed in this study were compared with previous data obtained from direct immunofluorescence, showing 85–90% concordance (Table 1). Later, a head-to-head assay to evaluate viral detection in clinical samples was performed by ELISA with the mAbs described here and DFA performed by LIV, simultaneously. Results obtained for anti pIII-ADV and anti P-hRSV, showed similar sensitivity and specificity than the first clinical approach. However, sensitivity and specificity of anti-M hMPV antibodies decreased in comparison with the first approach. Suspecting that some problems due to sample volume might affect anti-M hMPV capability of detection, a qPCR was performed for hMPV in the clinical samples. qPCR detected 12 hMPV samples from the 37 analyzed, and in this scenario, surprisingly the mAbs generated in this study detected more hMPV than DFA, given that anti M-hMPV mAbs detected 10 samples positive for hMPV and DFA only detected 2 samples. This result might be explained by the fact that anti M-hMPV antibodies are directed towards a conserved viral protein, whereas it is possible that DFA antibodies are directed against proteins of strains that have a low circulation rate in Chile. It is important to mention that comparison with other antibodies already used in diagnostic methods as DFA was not performed, because the information of the characteristics for the antibodies included in these tests is not disclosed by the companies that provides these methods to LIV. However, the assays mentioned above for viral detection in human samples gives an approximation of mAbs performance against antibodies in DFA.

The present study shows that all the mAbs produced in our laboratory could recognize their respective specific antigens with high sensitivity and specificity. Also, they show high sensitivity as compared to the commercial antibodies used in this study. These data suggest that the new mAbs can efficiently discriminate patients infected with hMPV, hRSV and ADV from healthy patients or patients infected with other viruses.

The mAbs described in this study were generated against highly conserved viral proteins, and therefore they would be able to detect several strains of circulating viruses. However, further studies need to be performed to corroborate this statement. The mAbs described in this study can provide an early and general diagnostic, enabling rapid decisions regarding patient management. Further, considering our findings, it could be suggested an application of the antibodies described here in immunotherapy or viral infections prevention, expanding the skyline of the device. However, the study of these applications was not considered in this work. Future studies are required to get a deeper insight of these antibodies and their potential application in areas other than the diagnostic field.

5. Conclusions

This study describes new monoclonal antibodies to detect the most prevalent viruses that cause severe respiratory tract infections. These antibodies target conserved proteins and are efficient at detecting hRSV, hMPV and ADV by diverse techniques. These antibodies could be used to generate simple immunodiagnostic methodologies that could be used in developing countries, were the infrastructure and equipment to use molecular techniques to detect respiratory tract infections are limited. The mAbs described here are appropriate to generate new diagnostic alternatives to improve detection in primary care health services and help to monitor and control the disease caused by these etiological agents.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.01.011.

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