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Two sensitive and specific RT-PCR assays were standardised for testing the presence of human metapneumovirus. A total of 300 nasopharyngeal aspirates collected from infants suffering from bronchiolitis since October 2000 to June 2003 and shown previously as negative to common respiratory viruses were examined. Matrix and polymerase viral genes, which show a low rate of variation, were chosen to design amplification assays to ensure that any genotype of the human metapneumovirus could be detected. A RT-PCR followed by a reverse line blotting hybridisation was developed for viral polymerase gene. For the matrix gene, after the RT-PCR assay, a subsequent nested-PCR was carried out. Both assays had similar sensitivity, equivalent to 0.1 TCID\textsubscript{50} of human metapneumovirus strain NL/1/99 which was used as the positive control. The human metapneumovirus was present in 16.6% of the specimens studied. The approaches described below are not only a robust method for rapid diagnosis of the human metapneumovirus, but also to establish an etiological surveillance tool for epidemiological studies. Based on the results obtained, human metapneumovirus infections in Madrid followed a seasonal pattern, with most of the infections occurring between February and April.

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Keywords: RT-heminested-PCR; Reverse line blotting hybridisation; Human metapneumovirus; Bronchiolitis; Respiratory viruses

1. Introduction

Respiratory infections are the most common diseases in the world being the origin of considerable morbidity and mortality specially in infants and elderly (Monto, 2002). Viruses causing acute respiratory infections include influenza viruses, respiratory syncytial virus, parainfluenza viruses, adenoviruses, coronavirus, enteroviruses and rhinoviruses (Mackie, 2003). Among these, respiratory syncytial virus is a major pathogen of the lower respiratory tract infections in infants and young children, causing bronchiolitis and pneumonia with an important rate of hospitalizations (McCarthy and Hall, 2003).

Another recently discovered paramyxovirus, named human metapneumovirus, has also been associated with respiratory disease in children (van den Hoogen et al., 2001). It causes infections similar to those produced by respiratory syncytial virus, with symptoms ranging from wheeze and fever to severe cough, bronchiolitis or even pneumonia. Assisted ventilation is required in the most severe cases (Boivin et al., 2003; Esper et al., 2003). Since it was discovered in 2001 in The Netherlands, the virus has also been detected in specimens from adults, elderly and immunocompromised patients suffering from acute respiratory infections (Boivin et al., 2002; Pelletier et al., 2002).

Studies in different countries indicate the worldwide circulation of the human metapneumovirus in respiratory specimens from North (Peret et al., 2002) and South America (Galiano et al., 2004); China (Peiris et al., 2003a); Israel (Wolf et al., 2003); Australia (Nissen et al., 2002); The Netherlands
2003; Vicente et al., 2003; Xepapadaki et al., 2004). Two main human metapneumovirus genetic lineages have been described and cocirculate during different years in several geographical areas (Biachessi et al., 2003; Viazov et al., 2003; van den Hoogen et al., 2001) and other European countries (Maggi et al., 2003; Nicholson et al., 2003; Viazov et al., 2004).

The role of the human metapneumovirus in the paediatric acute respiratory infections is frequently deceived due to the difficulty to detect this virus by traditional diagnostic methods such as isolation in cell culture, immunofluorescence assay or enzyme immunoassay. Isolation in cell cultures is inefficient and monoclonal antibodies for immunofluorescence assay or enzyme immunoassay are not easily available and further testing is required to ensure reliability. Consequently, specific and sensitive molecular diagnostic methods are essential to achieve the accurate detection of human metapneumovirus in clinical specimens. This diagnosis may help to the clinical management of patients and may establish the role of this pathogen in the respiratory infection and its epidemiological behaviour.

In the present study, the convenience of using two RT-PCR based assays, designed in two different highly conserved genes (van den Hoogen et al., 2002), which code for matrix and the polymerase viral proteins have been evaluated. These genes were selected to ensure the detection of human metapneumovirus independently of its genetic variation. The assays, a RT-heminested-PCR and a RT-PCR followed by a reverse line blotting hybridisation assay, had high specificity and sensitivity. Their inclusion as routine diagnosis methods can reduce the number of negative results. The presence of human metapneumovirus was evaluated in 300 respiratory secretions collected from children with respiratory disease from October 2000 to June 2003 in Madrid (Spain).

2. Clinical specimens

2.1. Clinical specimens

A total of 747 nasopharyngeal aspirates from young infants and children presenting with acute respiratory infections were collected from October 2000 to June 2003 at the Hospital Severo Ochoa Pediatric Unit (Madrid, Spain). Isolation in cell culture, indirect immunofluorescence assays and/or multiplex RT-nested-PCR (Coiras et al., 2003) were attempted with every sample in order to detect influenza viruses, respiratory syncytial virus, parainfluenza viruses, adenoviruses or enteroviruses. The presence of human metapneumovirus was checked in 300 aspirates, which were proven to be negative for the main respiratory viruses. All specimens were collected in 3 ml of virus transport medium (MEM, Gibco-BRL, Life Technologies, Paisley, Scotland; penicillin 200 U/ml, and streptomycin 200 μg/ml, BioWhittaker, MA; mycostatin 200 U/ml Sigma; bovine serum albumin 0.25%, Merck, Darmstadt, Germany). These specimens were analysed at the Respiratory Virus Laboratory, in the National Centre for Microbiology (ISCIII, Madrid, Spain) to test for the presence of respiratory viruses. The specimens were frozen and stored at −70°C until testing.

2.2. Virus isolation

Rhesus monkey kidney (LLC-MK2) cell line was maintained in DMEM (BioWhittaker, Belgium) supplemented with 5% foetal bovine serum and 1% penicillin-streptomycin (ICN Biomedicals, Ohio). Human metapneumovirus strain NL/1/99 was inoculated in LLC-MK2 in the presence of 5 μg/ml of trypsin in order to prepare positive controls for the amplification assays.

2.3. Nucleic acid extraction

Total nucleic acids were extracted from 200 μl aliquots from nasopharyngeal aspirates and also from the supernatant of infected cell cultures that were used as positive controls. Extraction was made using the guanidinium thiocyanate method described by Casas et al. (1995). Negative controls, consisting on RNAse free sterile water were also submitted to the same extraction process. Lysis buffer including 100 molecules of a cloned amplified product of the RNA control supplied by the Promega RT-PCR system kit (Promega), was used as internal control of the extraction step and the following PCR process (Coiras et al., 2003).

2.4. Primer design for the matrix gene detection

Specific primer pairs were designed to amplify highly conserved regions of the human metapneumovirus gene that codes for matrix (M) protein. All the human metapneumovirus sequences available in GeneBank database were used to perform computer-assisted alignments using Macaw 2.0.5 program (Multiple Alignment Construction and Analysis Workbench, NCBI, Bethesda, MD). These primers were evaluated to ensure they fitted essential criteria for optimal PCR primers (Dieffenbach et al., 1993). The G+C content, melting temperatures and lengths of the primers chosen, were analysed by using PrimerSelect v3.04a (DNAstar Inc., Madison, Wisconsin).

Primers used to amplify a part of the M gene by a RT-heminested-PCR assay were MIS (5′-GAGTCTACACAGTAGACAC-3′) and MIA (5′-TTGTYTCCITTGRTGCTCCA-3′) for RT-PCR and MIS and M2A (5′-TTCTTGCATCAYTYTRCTKATGCT-3′) for the subsequent heminested amplification reaction.

2.5. Primer and probe design for the polymerase gene detection

A conserved fragment of the polymerase gene was amplified using L6 and L7 oligonucleotides (van den Hoogen et al., 2003). L6 oligonucleotide was biotin-labelled at 5′ end to
permit the detection of PCR product by chemiluminescence. The specific probe (5'-CTGTTAATATCCCAACCCA-3'), designed to be used in the reverse line blotting hybridisation with the PCR product, was 5' end amino labelled.

2.6. RT-heminested-PCR assay for matrix gene detection

Reverse transcription PCR amplification for M gene detection was carried out in a single tube, using a commercial kit (Qiagen OneStep RT-PCR Kit, Qiagen, California). To each reaction tube was dispensed a mixture which contained 10 μl of buffer II: 25 mM MgCl₂, 0.1 mM dNTPs, 0.1 μM of specific primers MIS and MIA, 0.1 μM of specific primers for internal control amplification, 2 μl of QIAGEN OneStep RT-PCR Enzyme Mix, according to manufacturer’s instructions, and 5 μl of nucleic acid extracted for a final volume of 50 μl. Thermal cycling conditions were as followed: for the RT process an initial cycle of 45 °C for 45 min, and 95 °C for 5 min; cycling conditions for the PCR were 45 cycles of 95 °C for 30 s, 53 °C for 2 min, 68 °C for 30 s and finally 10 min incubation at 68 °C for 10 min.

The heminested-PCR reaction contained 5 μl of buffer II 5× (Applied Biosystems, New Jersey), 2 mM MgCl₂ (Applied Biosystems), 0.2 mM of each dATP, dGTP, dCTP and dTTP (Amersham Pharmacia Biotech, Piscataway, New Jersey), 2 μM of specific primers MIS and M2A, 0.2 μM specific primers for internal control amplification and 2.5 U Taq DNA polymerase (AmpliTaq DNA polymerase, Applied Biosystems). A total of 2 μl from the first reaction products was added for a final volume of 50 μl. Before heminested PCR, samples were heated to 95 °C for 5 min. Cycling conditions were 35 cycles: 95 °C for 1 min, 68 °C for 1 min, and 95 °C for 30 s; annealing temperatures, thermocycling parameters, concentration of each reaction component and probe hybridisation, both temperature and concentration, were standardised by experimenting with PCR amplification products or positive controls (Kowk and Higuchi, 1989).

2.7. RT-PCR followed by RLB hybridisation assay for polymerase gene detection

The RT-PCR assay for L gene amplification was carried out in a single tube using the Qiagen OneStep RT-PCR Kit (Qiagen) as above described except for dNTPs final concentration, which was 0.2 mM. Ten picomole of each specific oligonucleotide was included to obtain a 170 bp sized amplified product. Antisense primer was labelled with biotin at 5' end to get a biotin-labelled PCR amplification product.

Thermal cycling conditions were the following: an initial cycle of 42 °C for 45 min, and 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 52 °C for 1 min, 68 °C for 30 s and a final incubation of 68 °C for 7 min.

The reverse line blotting hybridisation procedure was performed according to Coiras et al. (2005). Briefly, the carboxyl groups of a Biodine C blotting membrane (Pall Biosupport) were activated with EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Pall Gelman Laboratory, Michigan) and the membrane was placed in a MN45 miniblottter (Biometra, Germany). The specific probe for human metapneumovirus L gene, diluted to a final concentration of 0.8 pmol/μl in 500 mM NaHCO₃ (pH 8.4), was covalently linked to the activated membrane. The remaining active esters on the membrane were hydrolysed by incubation with 0.1N NaOH. The membrane was then washed with 2× SSPE-0.1% SDS and placed in a MN45 miniblottter rotated 90 °C from the previous position. The slots were filled with 40 μl of denatured biotin-labelled PCR products diluted with 2× SSPE-0.5% SDS. The samples were incubated for 2 h at 48 °C. After washing, the membrane was treated with streptavidin-peroxidase conjugate (Roche, Indiana). Finally, the membrane was washed with 2× SSPE and the hybridised PCR products were detected by chemiluminescence using ECL detection reagents (Amersham Pharmacia Biotech, England) and visualised by exposure to a light sensitive film (Hyperfilm ECL; Amersham).

2.8. Sequence analyses for confirmation of the results

Positive specimens for human metapneumovirus M and L genes were purified with QIAquick PCR purification kit (Qiagen). Sequenced were obtained by an automatic DNA sequencer (ABI Prism 3700; Applied Biosystems) using Big Dye Terminator Cycle Sequencing kit Version 3.1 (Applied Biosystems). Nucleotide sequences obtained were aligned with sequences published previously using Macaw 2.0.5 program.

3. Results

The primers designed for the RT-PCR based assays amplify specifically partial sequences of the human metapneumovirus genes which encode for the matrix and the polymerase viral proteins. Primer concentrations and annealing temperatures, thermocycling parameters, concentration of each reaction component and probe hybridisation, both temperature and concentration, were standardised by experimentation. After optimizing the assay conditions, both assays showed concordant results with serial 10-fold dilutions of the human metapneumovirus strain NL/1/99 used as positive control (Fig. 1). The efficient limit of detection was 0.1 TCID₅₀ on each assay. Specificity of the method was assayed by using RNA extracted from different respiratory viruses (influenza A, B and C, respiratory syncytial virus A and B, parainfluenza 1–3, adenoviruses and enteroviruses).
The percentage of human metapneumovirus infections in every season is presented in brackets.

Table 1
Number of human metapneumovirus infections detected in the nasopharyngeal aspirates studied during three consecutive seasons

|          | 2000–2001 | 2001–2002 | 2002–2003 | Total |
|----------|-----------|-----------|-----------|-------|
| Number of samples analysed | 100        | 93         | 107        | 300   |
| Number of positives to HMPV | 14 (14.00%) | 26 (27.9%) | 10 (9.3%)  | 50 (16.6%) |

The percentage of human metapneumovirus infections in every season is presented in brackets.

4. Discussion

Acute respiratory infection comprises a wide range of clinical pictures and is caused by a heterogeneous group of viruses. Thus, there is a need for specific detection of the virus involved in each particular case, including those discovered more recently such as human metapneumovirus, human coronavirus NL63 (van der Hoek et al., 2004) or SARS coronavirus (Dowen et al., 2003). As in the case of other new viruses, the human metapneumovirus is specially difficult to grow in cell cultures. For this reason, molecular amplification methods are a valuable tool to detect the viral RNA present in the respiratory secretions (Kahn, 2003).

Two highly specific and sensitive RT-PCR based assays for the detection of human metapneumovirus RNA in nasopharyngeal aspirates were designed and can be performed rapidly and easily. In order to identify genetic variants of the main human metapneumovirus lineages, the design of amplification assays was focused on fragments of two different genes highly conserved. The use of two alternative RT-PCR assays presents the advantage of confirming the positive results in two independent genes and it also guarantees a decrease in the number of false positive results. The RT-heminested-PCR for the matrix gene is easy to perform and do not need high technology requirements. The reverse line blotting protocol for the viral polymerase gene is a safe procedure since non-radioactive reactives are used for labelling the probe and has the advantage of being suitable for large-scale epidemiological screening. The usage of the probe increases the specificity of the assay. In our experience, both methods were reliable and reproducible. Contamination with amplified products is prevented by careful separation of each mixture preparation, extraction, addition of the sample RNA, amplification and postamplification areas. Different sets of laboratory coats, pipettes and filtered pipette tips are used in each different area.

The assays were validated by examining 300 nasopharyngeal aspirates from a complete cohort of 747 collected from infant population, less than 2 years old, suffering from acute respiratory infections such as bronchiolitis, pneumonia...
Table 2

| Season Type of respiratory viruses | 2000–2001 | 2001–2002 | 2002–2003 | Totals |
|-----------------------------------|-----------|-----------|-----------|--------|
| ADV                               | 37 (4.95%)| 6 (0.8%)  | 20 (3.2%) | 53 (6.8%)|
| EV                                | 6 (0.8%)  | 24 (3.2%) | 10 (1.4%) | 30 (4.0%)|
| IV                                | 26 (3.5%) | 4 (0.5%)  | 0 (0.0%)  | 30 (4.0%)|
| HMPV                              | 14 (1.8%) | 5 (0.6%)  | 5 (0.7%)  | 24 (3.1%)|
| PIV                               | 1 (0.1%)  | 4 (0.5%)  | 0 (0.0%)  | 5 (0.6%) |
| RSV                               | 86 (11.2%)| 67 (8.7%) | 97 (13.9%)| 250 (33.5%)|
| Negative                          | 162 (21.3%)| 148 (19.3%)| 65 (9.3%) | 375 (49.0%)|
| Total                             | 277       | 266       | 204       | 747     |

The total percentage of each virus infection is presented in brackets: ADV, adenovirus; EV, enterovirus; IV, influenza virus; HMPV, human metapneumovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; negative, specimens in which no respiratory virus was found.

On the other hand, the human metapneumovirus was detected in the present study as a unique pathogen because all 300 samples tested were found negative previously for the presence of the most common respiratory viruses. This type of samples had also been tested in other studies (Boivin et al., 2002; Freymouth et al., 2003; Peret et al., 2002). However, when co-infections were studied, some of the researchers concluded that they rarely occurred (Stockton et al., 2002; Vicente et al., 2003). Co-infections would be more numerous if the diagnosis techniques used for common respiratory viruses, mainly cellular cultures and indirect immunofluorescence assays, were as sensitive as the PCR assays are at the
moment. Further studies are necessary to obtain a more real analysis of the respiratory virus infections.

Although this is a retrospective study, some epidemiological conclusions may be drawn. Human metapneumovirus positive specimens were collected mainly during the late winter and early spring months. Some positive cases were detected during the rest of the year. In contrast to other studies, no alternative years of high and low incidence were observed (Maggi et al., 2003). The peak of virus detection was found between February and April on the 3 years period from 2000 to 2003. Besides, the onset of respiratory syncytial virus disease was determined between December and January, just before the peak of human metapneumovirus detection, during the same seasons (data not shown). These results confirm that human metapneumovirus circulates primarily during the late winter and early spring, at the end of the circulation of respiratory syncytial virus (Falsey et al., 2003; Maggi et al., 2003; von Linstow et al., 2004). However, positive specimens for human metapneumovirus were also found during the late spring and summer months. Peiris et al. (2003b) reported human metapneumovirus activity in these months too. Nevertheless, few data related to summer months are available, since most of the studies only examined samples collected during winter season. In relation to the age of the children studied, most of the human metapneumovirus infections affected children between 6 and 12 months of age (data not shown), in agreement with previous data (van den Hoogen et al., 2003). Further studies will be needed to determine the real incidence of human metapneumovirus in community-acquired acute respiratory infections in young children in Spain.

In conclusion, the methods developed in this study and the results obtained permit the detection of human metapneumovirus, indicating its important role as a pathogen causing bronchiolitis in young infants. Diagnosis of human metapneumovirus is essential to reduce the number of respiratory infections in which the etiologic agent is not recognized. It should be noted that human metapneumovirus was detected in 16.6% of the subgroup with negative results out of the total 747 nasopharyngeal aspirates, the use of these specific molecular assays has increased at least in 6.7% the number of specimens in which a viral agent could have been determined. The results obtained indicate that human metapneumovirus should be taken into account in the differential diagnosis of acute respiratory infections, mainly to distinguish it from respiratory syncytial virus infections. The RT-PCR based assays described above are specific and sensitive for a rapid and reliable diagnosis of human metapneumovirus in nasopharyngeal aspirates.

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