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Development of duplex real-time PCR for detection of two DNA respiratory viruses

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A B S T R A C T

A method was developed for the detection and quantitation of HAdV (human adenovirus) and HBoV (human bocavirus) based on a duplex real-time PCR, the AB PCR, using a Smartcycler instrument. A control real-time PCR was carried out on albumin DNA to standardise the non-homogenous respiratory samples. No cross-reactivity was observed with viruses or bacteria that could be found in the respiratory tract. The diagnosis rate using the AB PCR on clinical samples was 10.7%: 3.4% for HBoV detection, 6.9% for HAdV detection and 0.3% double detection HBoV–HAdV. The clinical and epidemiological characteristics of the HAdV- and HBoV-infected patients were evaluated. In the HAdV-positive group and the HBoV-positive group the samples were classified according to the severity of the disease. The HAdV viral load did not appear to be linked to the severity of the disease. Conversely, the difference between the two HBoV groups, severe and non-severe, was significant statistically when the comparison was based on the viral load ($P = 0.006$) or after adjustment of the viral load to the number of cells in the samples ($P = 0.02$).

1. Introduction

Adenoviruses (HAdV) are common infectious agents that cause sporadic or epidemic respiratory infections (Mitchell et al., 2003). To date, 51 different HAdV serotypes divided into six species (A to F) have been identified (Benkö, 1999; De Jong et al., 1999; Xu et al., 2000). Adenovirus serotypes 1, 2, 5, and 6 from species C, Ad serotypes 3 and 7 from species B1, and Ad serotype 4 from species E are most frequently isolated from patients with respiratory tract diseases (Benkö, 1999; Vabret et al., 2004). According to a WHO (World Health Organization) study, ~20% of HAdV respiratory infections are caused by HAdV7 (Erdman et al., 2002). Immunofluorescence assays, enzyme-linked immunosorbent assays (ELISA), and viral isolation techniques are used routinely to detect HAdV associated with respiratory infections. However, HAdV can take several days to grow in cell culture, are susceptible to specimen toxicity, and can be out-competed by bacteria or fungi. Diagnosis by virus isolation is not rapid enough, while direct antigen detection by immunofluorescence lacks sensitivity. In contrast, molecular identification by PCR can be both rapid and sensitive. Most PCR assays detect sequences in the hexon gene (Allard et al., 1990; Hierholzer et al., 1993; Morris et al., 1996; Raty et al., 1999), whereas other PCR assays use sequences in the VA RNA gene (Kidd et al., 1996; Vabret et al., 2004).

The human bocavirus (HBoV) has been isolated from respiratory samples by large scale molecular virus screening (Allander et al., 2005). The incidence of HBoV detection in the patients with respiratory tract infections varies from 1% to 19%, depending on the study design (Allander, 2008; Arden et al., 2006; Arnold et al., 2006; Ma et al., 2006; Manning et al., 2006; Qu et al., 2007; Sloots et al., 2006; Smuts and Hardie, 2006; Weissbrich et al., 2006). Because HAdV and HBoV are both DNA viruses which are associated with respiratory infections, it would be convenient to have an assay to detect both viruses in the same DNA sample. Towards this end, a duplex real-time PCR assay was developed and validated for rapid screening of HAdV and HBoV in respiratory samples.

2. Materials and methods

2.1. Patients and samples

Respiratory samples ($n = 842$) were collected from patients hospitalized with respiratory symptoms at the University Hospital of Caen and at five Regional Hospitals (Argentan, Avranches, Cherbourg, Flers, and Vire) during January and February 2007. The patients ranged from 1 day old to 99.4-year-old (average age

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21.27; median age 3.53). The age distribution of the sample population was as follows: 361 patients (43%) were <2 years old, 96 patients (11%) were 2–5 years old, 116 patients (14%) were 5–20 years old, 159 patients (18%) were 20–70 years old, and 110 patients (13%) were 70–99 years old. A portion of each respiratory sample was immediately processed using conventional virus detection methods (see below), and the remainder of the sample was frozen at −80 °C and tested retrospectively by the duplex real-time adenovirus–bocavirus PCR (AB PCR) assay.

For all patients who were positive for adenovirus or/and bocavirus by the AB PCR, a retrospective examination of the medical record was conducted and the respiratory diseases were classified as follows: upper respiratory tract illness, lower respiratory tract illness without wheezing, bronchiolitis, or exacerbation of asthma. Cases associated with gastroenteritis were also recorded.

2.2. Conventional virus detection methods

Conventional methods were used to assess the accuracy of the AB PCR technique. For HAdV, a direct immunofluorescence assay (DFA, Imagen™, Oxoid, France) and cell culture isolation using MRC5 and HuH7 cells were used to detect respiratory viruses, including HAdV, as previously described (Freymuth et al., 2005). The PCR Adenovirus Consensus® assay (Argene, France) was used to determine HAdV species and to confirm all adenovirus-positive samples detected by direct immunofluorescence assay (DFA), cell culture, or the AB PCR. The qualitative PCR method previously described by Allander et al. (2005) was used routinely to detect HBoV.

2.3. Real-time quantitative AB PCR assays

Nucleic acids were extracted from 200 μL of nasal sample using MagAttract® DNA Mini M48 Kit (Qiagen, Courtaboeuf, France) with the Biorobot M48 (Qiagen) and eluted with 100 μL of Tris elution buffer (TE) according to the manufacturer's protocol. DNA extracts were stored at −80 °C until use.

The primers and probes for quantitative real-time AB PCR were designed taking into account the variability of adenoviruses, the reported sequences of HBoV, and the compatibility of these primers in the same reaction system using the Primer3 program (v. 0.4.0). The primers and the probe specific for HAdV were selected to target the hexon gene, while those specific for HBoV targeted the NS gene. The names and sequences of the primers and probes were as follows: AdHex2840F (5′-GACATGACCTTTGAGGTGGATCCCATGGA-3′), AdHex2946R (5′-GCGGAGAAGGGCGTGCGCAGGTA-3′) and AdHex2921P (5′-6-FAM CACCAGCCGCACCGCGGCGTCATCGA BHQ1-3′) for HAdV; BocaVSJ (5′-TCATAGTTCGTCTGAGCTAGG-3′), BocaVASK (5′-ACCATAGAACTGAGCACAGGA-3′) and BocaP (5′-Hex-CACTGCCATA TTATAGTTGGGGGAG BHQ1-3′) for HBoV.

DNA extract (5 μL) was added to 20 μL PCR mixture containing 0.5 μL (10 μM) of each primer, 0.2 μL of each 20 μM Taqman probe, 5 μL 10× Qiagen buffer, 0.8 μL of 10 mM dNTP, 3.5 μL of 25 mM $MgCl_2$ and 5 units of HotStart Taq DNA polymerase. The reaction consisted of a 15-min activation step for HotStart Taq DNA polymerase at 95 °C, followed by 45 cycles of amplification that included 15 s at 95 °C, 30 s at 60 °C, and 10 s at 72 °C. Fluorescence data were collected during the 72 °C step. The reactions, data acquisition, and analyses were performed using the Smart Cycler instrument (IL France).

Two plasmids were constructed. The HAdV plasmid contained the nucleotide sequences of the HAdV7 (prototype strain ATCC VR7) hexon partial gene (2944 nt) which was amplified using the primers Ad7Hex31F (5′-AT GGCCACCCCATCGATGATG-3′) and Ad7Hex2975R (5′-TTATGTGGTGGCGTTGCCG GC-3′). The HBoV plasmid contained the NS partial gene (1187 nt) amplified using the primers BocaVSK (5′-GCCGGCAGACATATTGGATTC-3′) and BocaVASI (5′-GTCTAGCAAGTTTAGCATAAG-3′). A wild HBoV strain was selected arbitrarily, sequenced, and used to construct the "bocavirus plasmid." Plasmid construction was carried out using the TOPO^{XL} TA Cloning® Kit, following the manufacturer's recommendations (Invitrogen, France).

2.4. PCR assay for the albumin gene

The albumin gene was quantified in DNA extracts from all nasal samples that were positive for HAdV or HBoV. The real-time PCR was performed using a Smart Cycler instrument according to a method described previously (Laurendeau et al., 1999). A dilution series of human genomic DNA was used as a standard (Human Genomic DNA, Roche, France). Results were expressed as the number of cells per 200 μL of respiratory sample, taking into account that one cell contains two copies of the albumin gene.

2.5. AB PCR sensitivity, reproducibility, and specificity

The analytical sensitivity of the quantitative duplex AB PCR assay was assessed using serial dilutions of HAdV and HBoV plasmids. The 10-fold serial dilutions ranging from 10 to 10^8 copies/mL were tested in duplicate. The mean cycle threshold (Ct) values plotted against the copy number established an external standard curve. To exclude the potential preferential amplification of either HAdV or HBoV by the duplex real-time AB PCR, a wide range of dilutions of HBoV and HAdV in the same sample was tested. The 10-fold serial dilutions of HBoV plasmid ranging from 10 to 10^8 copies/mL were tested in the presence of 10^8 copies/mL dilution of HAdV plasmid and the 10-fold dilutions of HAdV plasmid ranging from 10 to 10^8 copies/mL were tested in the presence of 10^8 copies/mL dilution of the HBoV plasmid. For HBoV, the sensitivity of the AB PCR assay and the qualitative assay (Allander) were also compared for respiratory samples. The DNA obtained from two distinct nasal aspirates was diluted to determine the end-point dilution value for each assay. The extracted DNA was diluted from 1/10 to 1/10,000, and each dilution was tested in parallel using both the qualitative and quantitative assays. To evaluate the intra-assay reproducibility, three replicates of four nasal aspirates (two positive for HAdV and 2 positive for HBoV) were subjected independently to DNA extraction and real-time PCR in the same experiment. In order to estimate of the inter-assay reproducibility, DNA was extracted and amplified from three nasal aspirates in duplicate, in two distinct experiments.

Assay specificity was demonstrated using infectious agents other than HAdV and HBoV which are also commonly found in the respiratory tract. The capacity of the AB PCR to detect most HAdV serotypes was also tested using the following representative HAdV prototype strains: HAdV 12 (spA), HAdV 3 and HAdV 7 (spB1), HAdV 11 (spB2), HAdV 1 (spC), HAdV 8 (spD), HAdV 4 (spE), HAdV 40 (spF).

2.6. Internally control assay

The internally controlled assay for the PCR inhibition and extraction efficiency used in our laboratory was kindly provided by the virology department of the University Hospital of Marseille, France (Pr. Xavier de Lamballerie)-manuscript submitted. The assay consists in adding a T4 phage virus to the clinical samples and to the reference sample (water) in order to perform a real-time PCR for the detection of the T4 phage using the same extraction procedure, the same real-time amplification procedure and the same Taq DNA polymerase. Research of PCR inhibitors and extraction efficiency was conducted on 100 negative samples for the detection of HBoV and HAdV by the duplex real-time AB PCR out of