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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

Table 1
HAdV detection by conventional methods and AB PCR in the 840 respiratory samples.

DFA	Culture (MRC5 et HUH7)	AB PCR	Type determination by HAdV Consensus®	Co-detection with other viruses: 10 (16%)
19 (2.2%)	40 (4.7%)	61 (7.2%)[a]	35 Ad sp B1 20 Ad sp C 1 Ad sp E 1 Ad sp F 4 ND	3 RSV 4 HRhV 1 MIC 1 MIA 1 hMPV

[a] 3 were co-detected with HBoV.

395 still available samples after other tests carried out on these samples.

2.7. Statistical analysis

Qualitative variables were described as percentages and quantitative variables as means and standard deviation. Viral loads were log transformed before analysis. Qualitative and quantitative variables were compared by Chi-square test or Student *t*-test, respectively. The Epi-Info 6 program (v. 6.04 dfr) and Excel were used for statistical analysis and a *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Sensitivity, reproducibility, and specificity of the duplex real-time AB PCR assay for the detection and quantification of HAdV and HBoV

The detection limits of the AB PCR were reproducibly 10 copies/mL and 2.5 copies/mL in 50% of the samples for HAdV and HBoV, respectively. For both viruses, the correlation coefficient was repeatedly >0.997 and the slopes were −3.5. The amplification efficiency, calculated as $[10^{(-1/\text{slope})} - 1] \times 100$, was 93%. Tests performed to exclude a potential preferential amplification of either HAdV or HBoV showed that low amounts of one of the viruses (HAdV and HBoV detected by the duplex real-time AB PCR) would be detected in the presence of high amounts of the other virus. No preferential amplification was observed; 10 copies/mL of HAdV or HBoV were detected at the attempted cycle threshold (Ct). The sensitivity of the AB PCR for the detection of HBoV in respiratory samples was compared with the sensitivity of the qualitative HBoV PCR assay as described in Section 2.5. The results are (i) in the first nasal aspirate, HBoV was detected by the qualitative assay until 1:10 dilution and by the AB PCR assay the end-point dilution was 1:1000; (ii) in the second nasal aspirate, HBoV was detected only in the non-diluted DNA (1:1) by the qualitative assay and by the AB PCR assay the end-point dilution was 1:100.

Concerning the reproducibility of the AB PCR, the coefficient of variation of the mean cycle threshold (Ct) values within runs ranged from 0.2% to 1.5% for HBoV and from 0.23% to 1.6% for HAdV. From run to run, the coefficient of variation of the mean Ct values ranged from 0.3% to 2.2% for HBoV and 0.6% to 2.8% for HAdV.

As for specificity, there was no signal when the AB PCR was performed on samples that were positive for any of the following viruses: influenza A, B, and C viruses (IFVA, IFVB, and IFVC); respiratory syncytial viruses (RSV); human metapneumovirus (hMPV); parainfluenza viruses 1, 2, 3, and 4 (PIV 1–4); coronavirus OC43, 229E, and NL63; measles virus; mumps virus; human rhinovirus (hRV) prototype strains type 9 and 31; echovirus 30; human herpes virus 1 (HSV1); varicellae-zoster virus (VZV); cytomegalovirus (CMV); Epstein Barr virus (EBV); human herpes virus 6 (HHV6); parvovirus B19; BK and JC viruses; *Mycoplasma pneumoniae*; *Chlamydia pneumoniae*; *Haemophilus influenzae*; *Streptococcus pneumoniae*; *Enterococcus*; *Streptococcus group A*; *Staphylococcus aureus*; *and Pseudomonas aeruginosa*. In contrast, eight serotypes representative of the seven species of adenovirus were amplified and detected by the adenovirus probe (AdHex2921P).

3.2. Performance of the duplex real-time AB PCR

Of the 840 specimens collected, 345 (41%) were positive for a respiratory virus using conventional methods. The following viruses were identified: 131 (15.5%) IFVA; 110 (13%) RSV; 40 (4.7%) HAdV; 27 (3.2%) hRV; 21 (2.5%) hMPV; 7 (0.8%) HSV; 5 (0.5%) PIV3; 2 (0.2%) PIV1, and 2 (0.2%) IFVC.

Adenoviruses were detected in 40 samples (4.7%) using culture methods; 21 (2.5%) of the samples were positive by cell culture but negative by direct immunofluorescence assay (DFA), and 19 (2.2%) were positive for HAdV using both methods. The AB PCR detected adenoviruses in 61 (7.2%) samples (Table 1); of these 61 samples, 21 (34.4%) were negative by DFA and culture methods. In contrast, all samples which were adenovirus-positive by conventional methods were also positive by AB PCR. The 61 AB PCR adenovirus-positive samples were also tested using Adenovirus Consensus® PCR (Argene, France). All 61 samples were confirmed to be positive, and the type distribution was as follows: 35 HAdV sp B1, 20 HAdV sp C, 1 HAdV sp E, and 1 HAdV sp F, 4 not determined. HBoV was detected in 32 samples (3.8% of the total) using duplex AB PCR (Table 2). Of these 32 positive samples, 15 (46.8%) were negative using the conventional PCR method described by Allander.

Other viruses were co-detected with HAdV in 13 cases: 4 hRV, 3 RSV, 3 HBoV, 1 IFVC virus, 1 IFVA virus, and 1 hMPV. HBoV was co-detected with other viruses in 16 cases: 6 RSV, 4 IFVA, 3 HAdV, 2 hMPV, and 1 PIV3 virus. HAdV and HBoV were co-detected in 3 samples by the AB PCR. HBoV was co-detected more frequently with other viruses than HAdV: 16 (50%) of 32 nasal samples positive for HBoV showed co-infection versus 13 (21%) of 61 nasal samples which were positive for HAdV ($P < 0.05$).

3.3. Quantitative results using AB PCR

Quantitation of viral loads in samples that were HAdV-positive using the AB PCR showed a range from 1 to 6.08 log with a mean of 3.07 log and an SD of 1.5 log (Fig. 1A). For HBoV-positive samples, the viral load ranged from 1 to 7.70 log with a mean of 3.02 log and an SD of 2.50 log (Fig. 2A). To standardize HAdV and HBoV quantitation in the samples, the albumin gene was quantified in parallel with HAdV and HBoV DNA in all 90 positive specimens. As expected, the number of cells in the nasal aspirates differed from one sample to another. In nasal samples which

Table 2
HBoV detection by conventional methods and AB PCR in the 840 respiratory samples.

Conventional PCR	AB PCR	Co-detection with other viruses: 17 (53%)
17 (2%)	32 (3.8%)[a]	6 RSV 4 MIA 2 hMPV 1 PIV3

[a] 3 were co-detected with HAdV.

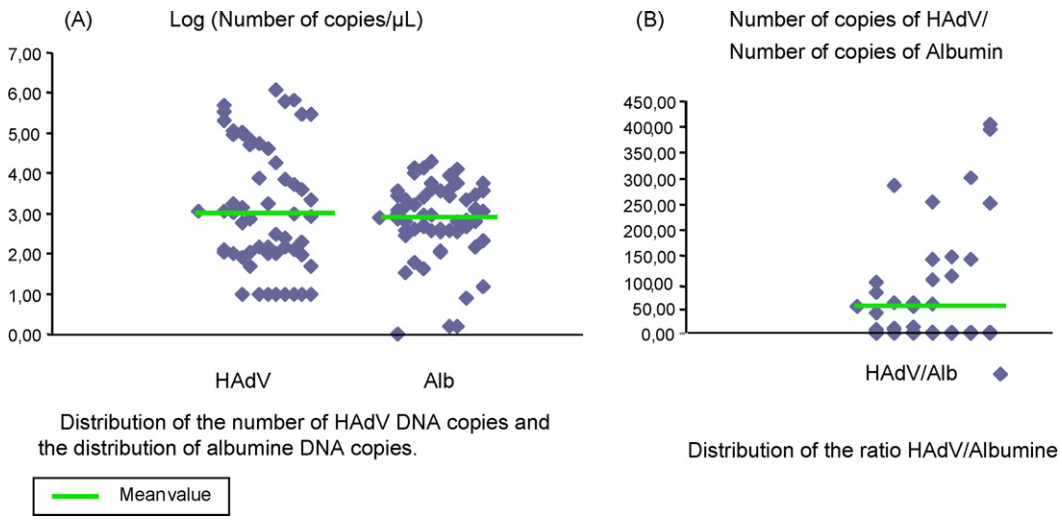

Fig. 1. Quantification of HAdV in positive AB PCR nasal aspirates samples using the real-time duplex ABPCR assay for HAdV and for albumine DNA.

were positive for HAdV, the mean albumin gene copy number was 2.88 log with a range from 0.18 to 4.28 log and an SD of 0.89 log (Fig. 1A); in samples positive for HBoV, the mean copy number was 3.21 log with a range of 1.26–4.15 log and an SD of 0.74 log (Fig. 2A).

The number of copies of the albumin gene is proportional to the number of cells in the sample, and HAdV and HBoV particles are also cell-associated in clinical samples. Thus, results can be expressed as the ratio of the number of copies of HAdV or HBoV to the number of copies of the albumin gene. This allows to compare results obtained from different nasal aspirates and ensures that the number of cells in each nasal sample (and thus the quality of the sampling) is not a limiting factor. The ratio of HAdV copies to albumin DNA copies ranged from 0 to 404.78 with a mean of 53.14 (Fig. 1B) and the ratio of HBoV copies ranged from 0 to 8012.82 (mean ratio: 910). Four samples had a ratio >1000 that indicated a high HBoV DNA level (Fig. 2B). Next, the HAdV DNA and the HBoV DNA were quantitated according to the severity of the disease. A severe disease was defined when low respiratory symptoms were present and a non-severe disease was defined in the presence of high respiratory symptoms only. The HAdV copy number ranged from 1 to 5.53 log with a mean of 3.06 log and a SD of 1.38 log in the severe-disease group. In the non-severe disease group the HAdV copy number ranged from 1 to 6.08 log with a mean of 3.07 log and a SD of 1.65 log. The mean number of HAdV copies was comparable in the two groups, whether the disease was severe or not (3.06 log versus 3.07 log). The ratio of HAdV copies to albumin DNA copies ranged from 0 to 404.78 with a mean of 62.82 in the patients with severe disease, and from 0 to 103.57 with a mean of 26.52 in the patients with non-severe disease. There was no statistical difference between these two groups. The HBoV copy number ranged from 1 to 7.7 log with a mean of 3.99 log and a SD of 2.95 log in the severe disease group and from 1 to 4.3 log with a mean of 1.75 log and a SD of 1.01 log in the non-severe disease group. The mean number of the HBoV DNA copies was higher in the severe-disease group than in the non-severe disease group (3.99 log versus 1.75 log) and the difference was significant statistically ($P = 0.006$). The ratio of HBoV copies to albumin DNA copies ranged from 0 to 8012.82 with a mean of 1883.15 in the patients with severe disease, and from 0 to 87.33 with a mean of 6.35 in the patients with non-severe disease. The difference between the two HBoV-positive groups, severe and non-severe, remain statistically significant after standardiza-

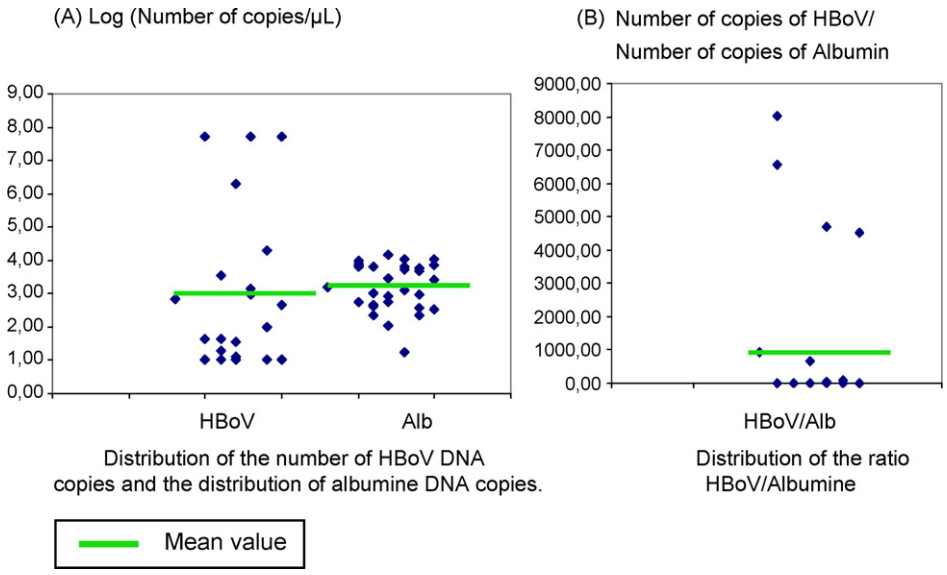

Fig. 2. Quantification of HBoV in positive AB PCR nasal aspirates samples using the real-time duplex ABPCR assay for HBoV and for albumine DNA.

tion of the viral load according to the number of cells in the sample ($P = 0.02$).

For one 6-month old female patient two nasal aspirates were available at a 3-day interval. The viral load of HBoV in the first nasal aspirate was 7.7 log and was dramatically decreased 3 days later. The viral load in the second nasal aspirate reached 3.32 log. This patient was hospitalized in the pediatrics department for feeding difficulties and weight loss in an underlying context of bronchiolitis diagnosed one week before. A first nasal aspirate was sampled at admission and virus detection by conventional methods was negative. Three days later when the second nasal aspirate was performed the symptoms improved. This patient was not included in the statistical analysis because it was an isolated case.

HAdV and HBoV were co-detected in 3 cases. The HAdV viral load was high in one case (5.48 log) and mild in 2 cases (2.18 and 2.04 log). Low level of HBoV viral load was detected in these 3 cases (<1 log). Low respiratory symptoms (severe disease) were present in one case and high respiratory symptoms (non-severe disease) were present in the other 2 cases.

3.4. Results of the internally controlled assay

The research of PCR inhibitors and extraction efficiency was conducted on 100 negative samples for the detection of HBoV and HAdV by the duplex AB PCR. All these samples were tested positive for the detection of the T4 phage. The Ct varied between 30.30 and 33.76 and the Ct for the reference control (water extract with T4 phage) was 30.89. The conclusion is that no PCR inhibitors were present in these samples.

3.5. Clinical evaluation of patients

The clinical and epidemiological characteristics of the HAdV- and HBoV-infected patients are summarized in Table 3. The age distribution of HAdV- or HBoV-infected patients was as follows: 0–2 years (53 patients, 58.8%); 2–5 years (23 patients, 25.5%); 5–16 years (13 patients, 14.4%); and 16–24 years (1 patient, 1.1%). This age distribution was different from the age distribution of the sampled patients. Of the HAdV- and HBoV-positive patients, 69% were under 3 years old. Clinical data were available for 85 of the 90 HAdV- and HBoV-positive patients; data were unavailable for 3 of the 61 HAdV-positive patients and 2 of the 32 HBoV-positive patients. The ages were similar in the two groups; the proportion of males was higher in the HAdV group (1.14 versus 0.81), but the difference was not significant.

Upper respiratory tract infections were more common in the HAdV-infected patients than in the HBoV-infected patients: 63.7% versus 33.3% ($P < 0.05$). In contrast, lower respiratory tract infections were more frequently reported in the HBoV-infected patients than in the HAdV-infected patients: 50% versus 27% ($P < 0.05$). Respiratory distress and the exacerbation of asthma were more frequently reported in the HBoV-positive patients than in the HAdV-positive patients (23.3% versus 13.8% for respiratory distress, and 13.3% versus 8.6% for the exacerbation of asthma, respectively). However, the differences were not significant statistically. Moreover, 7 of the 16 patients in the HAdV-positive group suffering from lower respiratory tract infections were co-infected with other respiratory viruses: respiratory syncytial virus in 3 cases, HBoV in 2 cases, hMPV in 1 case, and rhinovirus in 1 case. Similarly, 10 of the 15 patients suffering from lower respiratory tract infections in the HBoV-positive group were co-infected with other viruses: RSV in 4 cases, hMPV in 2 cases, PIV3 in 1 case, rhinovirus in 1 case, and HAdV in 2 cases. Fever was reported more frequently in the HAdV group than in the HBoV group: 82% versus 40% ($P < 0.001$). Some patients had gastroenteritis, and these patients were more frequently HAdV-positive than HBoV-positive: 27.5% versus 16.6% (NS). Detection of rotavirus in stools was reported in 1 patient in the HAdV-positive group and in 4 patients in the HBoV-positive group (data not shown). Evidence of nosocomial acquisition was found in 1 HAdV-positive, 5-month old girl who had been hospitalized in the pediatric department for malnutrition. The nasal aspirate and stool samples taken 33 days after her admission were HAdV-positive, even though she had not left the hospital.

Because HBoV and HAdV were co-detected in numerous samples, a distinct clinical evaluation of mono-infected patients was conducted and the clinical and epidemiological characteristics of these patients are presented in Table 4. Bronchitis, exacerbation of asthma and respiratory distress were reported more frequently in the HBoV-positive patients than in the HAdV-positive patients (9.5% versus 18.1% for bronchitis, 4.7% versus 27.2% for exacerbation of asthma, and 7.1% versus 27.2% for respiratory distress). The difference was statistically significant only in case of exacerbation of asthma ($P < 0.05$).

4. Discussion

This study had two objectives. The first objective was to develop a real-time duplex PCR method for the detection and quantitation of HAdV and HBoV, both of which are DNA viruses that are detected routinely in nasal aspirates from patients with respiratory symptoms. The second objective was to evaluate the clinical and epidemiological characteristics of the HAdV- and HBoV-infected patients in our sampled population. There was no cross-reactivity with other respiratory agents associated with respiratory infections, and the analytical sensitivity was good, i.e. 10 copies/mL for these two viruses. Thus, the duplex real-time AB PCR is a convenient and useful alternative method for detecting HAdV and HBoV.

Others have also developed conventional or real-time PCR techniques which detect HBoV DNA in respiratory samples (Arnold et al., 2006; Choi et al., 2006; Foulongne et al., 2006; Neske et al., 2007; Simon et al., 2007). Using these methods, the prevalence of HBoV-positive samples from patients with respiratory symptoms is usually about 2–18%; using the AB PCR, the prevalence of HBoV infection in our patients was 3.8%. The differences in detection frequency of HBoV DNA in different studies may be explained by patient characteristics or by seasonal and geographical variations. Moreover, the PCR methods would have to be standardized to be compared directly. In the present study, the sensitivity of the duplex AB PCR was greater than that of the conventional PCR for detecting HBoV, with the AB PCR detecting 15 more HBoV-positive nasal samples than the conventional PCR.

To exclude false positives due to cross-contamination, two complementary studies were conducted. In the first study, a second round of DNA extraction was carried out using 10 nasal aspirate samples that had sufficient volume remaining. All 10 samples were confirmed as positive using a second real-time PCR method carried out in a different laboratory of virology (St. Vincent de Paul Hospital, Paris). In the second study, the AB PCR and the conventional PCR were used to test serial dilutions of two DNA samples extracted from positive nasal aspirates. The AB PCR had 10× greater sensitivity, i.e. it was able to detect virus in a reaction using 10-fold less DNA than was needed for a positive result using conventional PCR. Thus, differences in results using the AB PCR versus the conventional PCR are likely due to samples with low viral loads. In addition, the results of tests by the conventional PCR are not followed by nested PCR or probe hybridization, which also can explain the lower sensitivity of the conventional PCR.

Direct immunofluorescence assay (DFA, Imagen™, Oxoid, France) is a rapid and useful method for the clinical diagnosis of HAdV respiratory infections (Vabret et al., 2004). However, due to its low sensitivity, samples which are negative for HAdV using direct immunofluorescence assay require further testing after iso-

Table 3
Epidemiological and clinical characteristics of the HAdV- and HBoV-infected study patients.

Characteristics	HAdV group ($n = 57$)	HBoV group ($n = 30$)	*P*-Value
Sex ratio M/F	1.14	0.81	–
Age, mean (range)	3.3 (9 days to 24 years)	2.1 (2 mois to 14 years)	–
Fever (>37.5 °C)	48 (84%)	12(50%)	0.00002
Temperature, mean (range)	38.8 °C (36.7–40.4)	38.5 °C (36.7–40.2)	–
Upper respiratory tract illness	37 (64.9%)	10 (33.3%)	0.004
Rhinitis	39 (68.4%)	15 (53.5%)	NS
Pharyngitis	36 (63.1%)	14 (46.6%)	NS
Conjunctivitis	10 (17.5%)	6 (20%)	NS
Otitis	9 (15.7%)	6 (20%)	NS
Lower respiratory tract illness	16 (28%)[a]	15 (50%)[b]	0.042
Bronchitis	8 (14%)	5 (16.6%)	NS
Bronchiolitis	9 (15.7%)	5 (16.6%)	NS
Pneumonia	1 (1.75%)	1 (3.3%)	NS
Exacerbation of asthma	5 (8.7%)	4 (13.3%)	NS
Respiratory distress	8 (14%)	7 (23.3%)	NS
Gastroenteritis	16 (28%)	5 (16.6%)	NS
Vomiting	17 (29.8%)	5 (16.6%)	NS
Diarrhoea	22 (38.5%)	5 (16.6%)	NS

Qualitative variables are reported as *n* (%) otherwise indicated.

[a] Out of the 16 LRTI, in 7 cases HAdV was co-detected with other viruses: RSV in 3 cases; HBoV in 2 cases; hMPV in 1 case and HRhV in 1 case.

[b] Out of the 15 LRTI, in 10 cases HBoV was co-detected with other viruses: RSV in 4 cases, hMPV in 2 cases, PIV3 in 1 case, rhinovirus in 1 case and AdV in 2 cases.

lation in cell culture. Isolation of HAdV in cell culture is lengthy, requiring 2–15 days, which may delay correct management of the patient.

Several other real-time PCR assays have been developed for detection of HAdV (Claas et al., 2005; Damen et al., 2008; Gu et al., 2003; Heim et al., 2003; Mitchell et al., 2003). Gu et al. described a real-time PCR method using six different primer sets to detect all HAdV species. Heim et al. (2003) reported a broadly reactive HAdV assay that used quantitative real-time PCR with a consensus primer and probe. Claas et al. (2005) developed an internally controlled real-time PCR assay to detect and quantify HAdV serotypes involved in disseminated adenovirus disease. Damen et al. (2008) described recently an internally controlled HAdV real-time PCR method that detects all known HAdV serotypes using a mix of degenerate primers. A newly developed method, such as the Luminex xTAG respiratory virus panel, allows detection of 20 respiratory viruses. A test like this can save money and time, as well as increase the diagnosis rate. However, there are some limitations in terms of virus quantitation and detection of low viral loads (Pabbaraju et al., 2008).

In the present study, the diagnosis rate using the AB PCR assay on clinical samples was 10.7%: 3.4% for HBoV detection, 6.9% for HAdV detection and 0.3% dual detection HBoV–HAdV. This is significantly higher than the diagnosis rate using conventional techniques. Our samples were collected during two winter months, January and February 2007, because our aim was not to document year-round changes in prevalence. The duplex AB PCR assay was also able to detect mixed HAdV/HBoV infections; this was one of our aims, even though dual infections were rare in this study (3/90) and the clinical implications of the co-infections remain unclear.

Concerning the quantitative results in the present study, the amount of HAdV in the 61 positive nasal samples did not seem related to the severity of the acute respiratory tract disease ($P = 0.17$). Based on the HAdV/albumin no statistically significant difference was found between patients with severe symptoms versus those with mild symptoms. That is, the severity of the disease did not appear to be linked to HAdV viral load. Conversely, the amounts of HBoV in the 30 positive nasal samples were related to the severity of the symptoms. The difference between the two HBoV groups, severe and non-severe, was statistically significant 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 ($P = 0.02$). Nevertheless, the isolated case of the patient that was sampled twice suggested that the HBoV viral load was linked to the

Table 4
Epidemiological and clinical characteristics of the HAdV and HBoV mono-infected patients.

Characteristics	HAdV group ($n = 42$[a])	HBoV group ($n = 11$[b])	*P*-Value
Sex ratio M/F	1.04	0.85	–
Age, mean (range)	4.03 (9 days to 24 years)	1.26 (15 days to 3 years)	–
Fever (>37.5 °C)	37 (86%)	10 (90.9%)	NS
Temperature, mean (range)	39.1 °C (36.7–40.4)	38.5 °C (36.7–40.2)	–
Upper respiratory tract illness	33 (78.5%)	7 (63.6%)	NS
Rhinitis	28 (66.6%)	5 (53.5%)	NS
Pharyngitis	31 (73.8%)	6 (45.4%)	NS
Conjunctivitis	7 (16.6%)	0 (0%)	NS
Otitis	9 (21.4%)	3 (27.2%)	NS
Lower respiratory tract illness	9 (21.4%)	4 (36.3%)	NS
Bronchitis	4 (9.5%)	2 (18.1%)	NS
Bronchiolitis	4 (9.5%)	1 (9%)	NS
Pneumonia	1 (2.3%)	1 (9%)	NS
Exacerbation of asthma	2 (4.7%)	3 (27.2%)	$P < 0.05$
Respiratory distress	3 (7.1%)	3 (27.2%)	NS
Gastroenteritis	11 (26.1%)	3 (27.2%)	NS
Vomiting	13 (30.9%)	3 (27.2%)	NS
Diarrhoea	15 (35.7%)	4 (36.3%)	NS

[a] The number of HAdV mono-infected patients was 45. Only 42 medical files were consulted (3 were missing).

[b] The number of HBoV mono-infected patients was 13. Only 11 medical files were consulted (2 were missing).

stage of the infection. In this study, the quantitative results were standardized according to the number of cells in the nasal samples. Nevertheless, factors other than viral replication could play a role in symptom severity, as inflammatory response or genetic susceptibility of the patients.

The absence in this assay of an internal control for the PCR inhibitions and extraction efficiency for all negative samples is a limitation of the study. The detection of PCR inhibitor consists in a supplementary PCR assay increasing the costs of the research. Nevertheless, the detection of PCR inhibitors is an important step in routine tests. Other studies on nasal aspiration samples show a very low percentage of inhibitors. The techniques described by Coiras et al. included an internal control supplied with Promega© Kit and no amplification inhibitors were detected during clinical evaluation (Coiras et al., 2003, 2004). Syrmis et al. used an endogenous human retrovirus (ERV-3) as an internal control and found that only 5 out of 396 samples tested were not amplificated (Syrmis et al., 2004). Dingle et al created a stable internal control based on a modified RNA fragment of hepatitis Delta (Dingle et al., 2004). Among the 324 respiratory samples tested, only two cases of inhibition were detected. It is possible that the dilution of samples in the transport medium overcomes the effect of inhibitors (Syrmis et al., 2004).

The limitations of the clinical evaluation in this study are the small number of patients included, missing clinical data due to the retrospective examination of the medical file and the shortness of the study.

In conclusion, a sensitive and specific duplex real-time PCR assay was developed for the detection of HAdV and HBoV DNA in respiratory samples. Further studies are needed to confirm the results concerning the link between the viral load and the severity of the disease.

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