Human adenovirus replication and persistence in hypertrophic adenoids and palatine tonsils in children.

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ABSTRACT

The role of human adenovirus (HAdV) infection in different acute diseases, such as febrile exudative tonsillitis, conjunctivitis, and pharyngoconjunctival fever is well established. However, the relationships, if any, of HAdV persistence and reactivation in the development of chronic adenotonsillar disease is not fully understood. The present paper reports a 3-year cross-sectional hospital-based study aimed at detecting and quantifying HAdV DNA and mRNA of the HAdV hexon gene in adenoid and palatine tonsil (PT) tissues and nasopharyngeal secretions (NPS) from patients with adenotonsillar hypertrophy or recurrent adenotonsillitis. HAdV C, B, and E were detectable in nearly 50% of the patients, with no association with the severity of airway obstruction, nor with the presence of recurrent tonsillitis, sleep apnea or otitis media with effusion. Despite the higher rates of respiratory viral co-infections in patients with HAdV, the presence of other viruses, including DNA and RNA viruses, had no association with HAdV replication or shedding in secretions. Higher HAdV loads in adenoids showed a significant positive correlation with the presence of sleep apnea and the absence of otitis media with effusion. Although this study indicates that a significant proportion (~85%) of individuals with chronic adenotonsillar diseases have persistent non-productive HAdV infection, including those by HAdV C, B, and E, epithelial and subepithelial cells in tonsils seem to be critical for HAdV C production and shedding in NPS in some patients, since viral antigen was detected in these regions by immunohistochemistry in four patients, all of which were also positive for HAdV mRNA detection.

Keywords: Human adenovirus, Chronic Adenotonsillar Disease, Hexon mRNA

INTRODUCTION

Human adenovirus (HAdV) is a non-enveloped icosahedral DNA virus that is highly prevalent in human populations [1]. Since its discovery in the early 1950s [2,3], more than 84 HAdV genotypes, including all the 50 previously characterized serotypes were described. Currently, 7 HAdV species (A – G) have been identified and are classified in the genus *Mastadenovirus* of the family *Adenoviridae* [4].

HAdV can infect a large variety of cell types and tissues in humans, leading to a broad array of diseases, including acute respiratory infections (ARI) [5], febrile exudative tonsillitis [6], acute conjunctivitis [7], cystitis, gastroenteritis [4], and rare cases of encephalitis [8], myocarditis [9], and hepatitis [10]. Although HAdV infections are generally asymptomatic in immunocompetent individuals, acute HAdV diseases have a significant impact on children (especially under 4 years of age), elderly, immunosuppressed individuals, and military recruits [4].

While HAdV can replicate in several cells types *in vitro* and is associated with productive infections in different tissues in humans, several HAdV species present varied tissue specificities. For instance, HAdV C (serotypes 1, 2, 5, and 6) are commonly associated with acute tonsillitis and respiratory diseases, whereas HAdV F (serotypes 40 and 41) and HAdV D (serotypes 8, 19, and 37) are typically associated with gastrointestinal infections and a relatively severe and highly contagious form of epidemic keratoconjunctivitis, respectively [1].

Following the HAdV replication cycle, the viral genome can persist in the nucleus [11,12]. Such a fact is best exemplified by the persistence of HAdV C after primary infections of the respiratory tract, with intermittent viral excretion in nasopharyngeal secretions and feces [13–15].

Numerous studies have shown that lymphocytes of tonsils and adenoids are essential sites of HAdV persistence, namely of the species C [16]. Indeed, seminal studies indicated the ability of HAdV to persist in tonsils and adenoids, since it was possible to recover HAdV from these tissues weeks to months after the establishment of explant cultures [2,17]. More recent studies using tissue cell separation and sorting have revealed that HAdV DNA is present in T lymphocytes of tonsils and adenoids [18]. In addition, several established human lymphocyte cell lines, including a lymphoblastoid cell line derived from a bone marrow transplant recipient with adenovirus pneumonia, may sustain prolonged and non-cytopathic adenovirus infection [19,20].

Although substantial knowledge has been obtained regarding mechanisms associated with viral persistence in human cell lines *in vitro* [21], the strategies of viral persistence and reactivation in human lymphoid tissues *in vivo* have been poorly elucidated. In fact, the cells types involved in the process of viral reactivation *in vivo* and the possible roles that HAdV replication may play in the development of chronic diseases, such as adenotonsillar hypertrophy and recurrent tonsillitis, is not entirely understood. The present cross-sectional study of HAdV replication in adenotonsillar hypertrophy was conducted to help comprehend the association between viral replication in those tissues and shedding in secretions and the development of adenotonsillar hypertrophy and recurrent tonsillitis. Quantification of the HadV genome and the detection of mRNA of the HAdV hexon gene were performed in human adenoids and tonsils, nasopharyngeal secretions (NPS), and peripheral blood (PB) from patients with tonsillar hypertrophy, and were compared to those obtained in samples from control patients.

PATIENTS AND METHODS

Ethics: The present study was conducted according to the principles expressed in the Helsinki Declaration and was approved by the local Research Ethics Committee (#10466/2008). All patients and caregivers signed informed consent and voluntarily agreed to participate in the survey.

Study design: This was a cross-sectional study that evaluated the presence of HAdV in different samples of tissues and secretions from the upper respiratory tract of children with obstructive sleep apnea or recurrent tonsillitis, comparing the results with control patients.

Patients and samples. Fragments of surgically removed adenoids and palatine tonsils, as well as samples of NPS and PB, were obtained from 180 patients (93 males) aged 1 to 18 years (median 5.0 years) who underwent adenotonsillectomy due to obstructive sleep apnea (OSA) or recurrent tonsillitis. Small punch biopsies from tonsillar tissues, NPS, and PB were also obtained from 12 control patients (7 males, median 3.0 years) undergoing cochlear implantation in the absence of chronic adenotonsillitis, without ARI symptoms and with normal nasofibroscopy. All patients enrolled in the study were undergoing treatment at the Otorhinolaryngology Division of the Clinical Hospital of the University of São Paulo Medical School, in the city of Ribeirão Preto, Brazil, from May 2010 to July 2012. Exclusion criteria for both the patient and control groups comprised the presence of ARI symptoms at the time of the surgical procedure and the use of antibiotics within one month prior to surgery. OSA was diagnosed by clinical evaluation, and recurrent tonsillitis using Paradise criteria

[22]. A detailed description of the criteria used for disease classification and the methods employed in clinical sample processing was previously published by our research group [23].

DNA and RNA extraction. Tissue samples, including those from sick and healthy individuals, were maintained in a preservative solution (RNA later – Invitrogen, Carlsbad, CA, USA) at -86°C until nucleic acid extraction. DNA and RNA were extracted from approximately 30.0 mg of adenotonsillar tissue samples using the AllPrep DNA and RNA mini kits (Qiagen, Hilden, Germany), respectively. Total nucleic acids were extracted from 200 μL of NPS and 1.0 mL of PB using the QIAamp MinElute Virus Spin Kit and the QIAamp RNA and DNA blood mini kit, respectively, both from Qiagen GmbH (Qiagen, Hilden, Germany). All nucleic acid extraction procedures were performed according to the manufacturer's instructions.

Detection and quantification of HAdV genomes. HAdV detection was performed by TaqMan real-time PCR (qPCR) following a previously published protocol[23]. Briefly, the final reaction volume (10.0 μL), which contained 50.0 ng of DNA, 10 mM of forward and reverse primers (HAdV-F: 5'-GCCACGGTGGGGTTTCTAAACTT-3'; HAdV-R: 5'-GCCCCAGTGGTCTTACATGCACAT-3'), 5 mM of the probe (HAdV-P: 5'-FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-TAMRA), and 5.0 μL of TaqMan master mix (Applied Biosystems, Foster City, CA, USA), underwent the following cycling parameters: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. All PCR assays were done on a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA, USA), and qPCRs for the β-actin

and RNAseP reference genes were conducted simultaneously in all tissues or secretion samples, respectively [23]. Applicable measures to prevent cross-contamination of the PCR reactions were taken, including sample handling and mix preparations done in separate rooms. In addition, all the qPCR plates included appropriate blanks.

The quantitative PCR (qPCR) for HAdV was targeted to the same region used for viral detection (hexon gene). To quantify the viral genomes, all qPCR assays included a standard curve produced using serial decimal dilutions of a plasmid in which the target DNA sequence of the HAdV hexon gene had been cloned, and the detection limit of the assay was approximately 1 copy of the HAdV hexon gene. A qPCR for HAdV was considered positive when the threshold was reached before the 40th cycle. All HAdV qPCR assays were performed in triplicate, and the results were normalized by amplification of the β-actin or RNAseP gene included in duplicate in all tested batches. With this approach, viral loads were determined as the number of copies of HAdV DNA per g of tissue of mL of NPS or blood

Detection of hexon gene mRNA. Hexon gene mRNA detection was performed by real-time RT-PCR in tissue and NPS samples to ascertain the presence of HAdV replication using the same strategy employed in our previously published human bocavirus study [24]. Briefly, reverse transcription (RT) was performed on 1.0 mg of RNA using 10 pmol of oligo (dT) primer, according to the manufacturer's protocol. PCR was then carried out using 150 ng of cDNA, with 10 mM of each primer (HAdV-F and HAdV-R), 5 mM of the probe (HAdV-P), and 5.0 mL of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA), following the conditions described above. The total RNA extraction product was treated with

DNAse I (Invitrogen, Carlsbad, CA, USA) for 2 hours before the PCR to ensure target-specific amplification. As a negative control, the same RNA extraction product was used for PCR without previous reverse transcription. Samples were considered PCR-positive for HAdV hexon mRNA only when they were also simultaneously negative for the same target using the extracted RNA without previous RT. All samples, including all cDNAs and the RNAs pre-treated with DNAse, were tested by qPCR for β-actin mRNA, following the previously described protocol [24].

HAdV molecular typing. A molecular typing assay based on conventional nested-PCR amplification and sequencing of a hypervariable region contained in the hexon gene was performed to determine which species of HAdV were present in the patients included in this study, following a previously published protocol [25]. Briefly, the first PCR reaction was conducted using a final volume of 50.0 μL containing 100 ng of DNA, 0.2 μM of forward and reverse primers (AdhexF1 - 5'-TICTTTGACATICGIGGIGTICTIGA-3' and AdhexR1 – 5'-CTGTCIACIGCCTGRTTCCACA-3'), 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM of each deoxynucleotide triphosphate, and 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The cycling conditions were: 94ºC for 2 min, followed by 35 cycles of 94ºC for 1 min, 45ºC for 1 min, and 72ºC for 2 min, and final extension at 72ºC for 5 min. For the second PCR (nested reaction), 0.5 μL of the first PCR product was amplified using the same parameters described above, with the following forward and reverse primers, respectively: AdhexF2- 5'-GGYCCYAGYTTYAARCCCTAYTC-3' and AdhexR2 – 5'-GGTTCTGTCICCCAGAGARTCIAGCA-3'. The amplified products were separated on 1% agarose gels, and the nested-PCR products were purified using the QIAquick®

PCR Purification Kit (Qiagen, Chatsworth, CA, USA). Sanger sequencing was performed in both directions using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit Ver. 3.1 and the AdhexF2 and AdhexR2 primers on an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis. A maximum likelihood (ML) phylogenetic tree was inferred using nucleotide sequences from strains of adenoviruses described in this study and representative members of the *Adenoviridae* family. Multiple sequence alignment (MSA) was generated using MAFFT v.7 [26] with manual adjustments. The ML tree was constructed using IQ-TREE version 1.6.8 software with 1,000 ultrafast bootstraps and the best-fit nucleotides model determined by Bayesian Information Criterion, which considered 88 reversible DNA substitution models [27,28]. Statistical support for individual nodes was estimated using the bootstrap value, and the phylogenetic tree was visualized with the FigTree (v.1.4.2) program.

Detection of other respiratory viruses. In this study, the association between the replication of HAdV and the presence of other respiratory viruses in adenotonsillar tissue were analyzed. All samples were tested for the presence of the following respiratory viruses by qPCR, according to previously described procedures [23]: human enterovirus (HEV), human rhinovirus (HRV), human respiratory syncytial virus (HRSV), human metapneumovirus (HMPV), influenza A and B (FLU), human parainfluenza (HPIV), human coronavirus 229E and OC43 (HCoV), and human bocavirus (HBoV).

Immunohistochemistry for HAdV in adenotonsillar tissue. Positive and negative tissues for HAdV by qPCR were tested regarding the presence of HAdV antigen by immunohistochemistry. Fragments of adenoid and PT tissues were fixed for 12 hours in formaldehyde (10%), dehydrated, embedded in paraffin, and subsequently sectioned and placed on microscope slides. Tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. For antigen retrieval, the sections were treated with trypsin (0.05% in distilled water with 0.1% calcium chloride, pH 7.8) at 37°C for 15 minutes. To detect HAdV antigen, the tissue sections were washed in PBS, incubated for 1 h in PBS with BSA and 3% horse serum, and incubated for 2 hours with anti-HAdV mouse monoclonal antibody (MAB8052 - Millipore, Billerica, MA, USA) diluted 1:1000 in PBS/BSA (pH 7.4) with 0.1% of Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at room temperature. The sections were then incubated with biotinylated horse anti-mouse IgG (Vector, Burlingame, CA, USA) diluted 1:2000 in PBS (pH 7.4) for 30 min at room temperature. Detection of biotinylated antibody was carried out with 1:300 Streptavidin-peroxidase Ultrasensitive Polymer (Sigma-Aldrich, St. Louis, MO, USA), and color development was obtained using NovaRed (Vector, Burlingame, CA, USA). The slides were counterstained with hematoxylin and eosin and mounted with Permount (Thermo Fisher Scientific, Waltham, MA, USA). For the positive controls, HAdV-infected Hep-2 cells [Human adenovirus 7 (ATCC® VR-7™)] were suspended in a small volume of human plasma, clotted by treatment with thrombin, then fixed and paraffin-embedded. Equally treated non-infected Hep-2 cells were used as negative controls.

Statistical analysis. The patient groups were compared using the Chi-square and Fisher's Exact tests; viral loads among patient groups were assessed using the Mann-Whitney or unpaired T-test. Comparisons between three or more groups were conducted with one-way ANOVA and the Bonferroni test. All assays were carried out using the GraphPad Prism software version 5.00 for Mac (GraphPad Software, San Diego, CA, USA), and a p-value of ≤ 0.05 was adopted for significance.

RESULTS

Frequency of HAdV. Of the 180 patients with chronic adenotonsillar diseases, 95 (52.8%) had HAdV detected by real-time PCR in adenoids and/or tonsils. In 43 of the 95 patients (45.3%), HAdV was found simultaneously in the tissues and NPS, suggesting that some patients could have productive HAdV infection in the adenoid and/or palatine tonsils. The virus was not identified by real-time PCR in peripheral blood from any of the enrolled patients, indicating lack of viremia, in spite of HAdV detection in the upper airways.

HAdV was detected significantly ($p<0.05$) more often in adenoids (48.9%) than palatine tonsils (27.2%) (Tables 1 and 2), and the frequency of HAdV detection in tonsillar tissues from patients with chronic adenotonsillar disease was not significantly different from that observed in the tissues from the control patients (Tables 1 and 2).

Detection of HAdV in adenoids or palatine tonsils was not significantly associated with any of the specific clinical features, including a degree of nasal obstruction, sleep apnea, otitis media with effusion (OME), and allergy (Tables 1 and 2). Among all the analyzed parameters, the only fact worth mentioning was that the viral co-detections were significantly more frequent ($p=0.002$) in tissues positive for HAdV than in HAdV-negative ones (Table 2).

HAdV viral load. The median HAdV load determined by qPCR in adenoids from patients with chronic adenotonsillar disease was $1.6x10^5$ copies of genome/g (mean $9.4x10^6 \pm 5.1x10^7$ copies/g), while in the control patients, the median HAdV load was $2.6x10^6$ copies/g (mean $3.9x10^7 \pm 6.5x10^7$ copies/g). In the palatine tonsils from

patients with chronic adenotonsillar disease, the median HAdV load was $5.5x10^4$ copies/g (mean $7.0x10^5 \pm 1.8x10^6$ copies/g), whereas, in the control group, the median was $1.8x10^5$ copies/g (mean $1.8x10^5 \pm 3.2x10^4$ copies/g). Regarding the NPS samples, the median HAdV load was $1.4x10^4$ copies/mL (mean $1.2x10^6 \pm 7.2x10^6$ copies/mL) and $3.4x10^4$ copies/mL (mean $3.4x10^4 \pm 4.7x10^4$ copies/mL) in the patients with and without chronic adenotonsillar disease, respectively. The median HAdV load was almost three times higher in the adenoids than the others infection sites, although the difference was not significant (Figure 1A). However, HAdV loads in the adenoids were not uniformly high when compared to the other sampling sites of the same patients (Figure 1B). Differences in HAdV viral loads between patients with chronic adenotonsillar disease and the controls were not significant (Figures 1C – 1E).

In general, the HAdV loads in the adenoids, PTs, and NPS were not significantly different among the patients with and without any of the several clinical features analyzed in the present study (Figure 1C – 1H). Of note, the HAdV viral loads were significantly higher in patients with sleep apnea ($1.4x10^7 \pm 6.7x10^7$ copies/g) than in those without the condition ($2.4x10^6 \pm 7.3x10^6$ copies/g) ($p=0.03$), although lower in patients with OME ($1.6x10^6 \pm 5.6x10^6$ copies/g) than in those without the disease ($1.07x10^7 \pm 5.6x10^7$ copies/g) ($p=0.006$). The HAdV load was also significantly lower in palatine tonsils from patients with OME ($1.6x10^5 \pm 3.1x10^5$ copies/g) than those without the condition ($1.1x10^6 \pm 2.4x10^6$ copies/g) ($p=0.02$), suggesting that higher HAdV load may be a possible protective factor against the development of OME. However, since the number of patients in this group is very small and there are overlap between these 2 populations, including patients with recurrent tonsillitis and adenotonsillar hypertrophy in both groups, any kind of conclusion about this finding is very risky.

HAdV loads between patients with and without simultaneous detection of the virus in other sampling sites were also compared, as well as the influence of co-detection of other respiratory viruses on HAdV loads. The analysis suggested an apparent trend for patients with HAdV in multiple infection sites with higher HAdV loads than individuals with HAdV detection in only one infection site, although the differences were not significant by one-way analysis of variance applying Bonferroni as post-test (Figures 1I – 1K). Therefore, the HAdV loads in adenotonsillar tissues were not significantly associated with the detection of the virus in NPS or with the simultaneous detection of other respiratory viruses (Figures 1L - N and Figure 2).

Productive infections by HAdV. The high frequency (27%) of patients with significant viral loads in the adenotonsillar tissues (>10^6 copies/g tissue), along with the high rate of HAdV detection in NPS (45.3%), is indicative that some of the enrolled patients had productive infection. Thus, to verify the presence of active viral gene expression, suggestive of viral replication in the adenoids and tonsils, we attempted to detect the mRNA of the hexon gene in the tissues by real-time PCR. Hexon gene mRNA was found in 12 (14.1%) of the HAdV-positive adenoids, 4 (8.2%) of the HAdV-positive palatine tonsils, and 2 (4.6%) of the HAdV-positive NPS. Importantly, the presence of HAdV mRNA was correlated with high viral load, mainly in adenoids (Figure 1L – 1N), indicating that the latter appears to be the primary site of HAdV replication in patients with tonsillar hypertrophy.

The presence of HAdV hexon gene mRNA in the adenoid was not associated with age, gender, or any specific clinical feature analyzed in the present study, including the presence of sleep apnea, OME, recurrent tonsillitis or the intensity of airway obstruction (Table 3). Remarkably, mRNA for the HAdV hexon gene was

detected in one of the 3 (33.3%) HAdV-positive adenoid biopsies obtained from control patients without adenotonsillar diseases (Table 4), indicating that the replicative activity of HAdV in tonsils does not necessarily lead to the development of chronic adenotonsillar disease.

HAdV typing by DNA sequencing. To demonstrate which HAdV species can replicate or establish persistence in the adenoids and palatine tonsils of the enrolled patients, the amplification and sequencing of a hypervariable region of the hexon gene were attempted. Of the 95 HAdV-positive patients by real-time PCR, 20 (21%) were positive for HAdV by conventional nested-PCR, with the visualization of DNA products in agarose gels ranging from 688 to 821 bp in size (Figure 3A), always using DNA obtained from adenoid tissue. The palatine and nasopharyngeal secretion samples were not positive by this nested-PCR. Based on the phylogenetic analysis (Figure 3B), 15 isolates (75%) were classified as human mastadenovirus C (13 related with human adenovirus type 1, and the others clustered with human adenovirus type 5 or human adenovirus type 6). Also, four isolates (20%) grouped with human adenovirus type 3 (human mastadenovirus B), and one was classified as human mastadenovirus E, clustering with human adenovirus type 4. Interestingly, viral replication was only detected in human mastadenovirus C-infected adenoids, indicating that this species is able to replicate efficiently in tonsillar tissues. In contrast, the tissues infected with human adenovirus B and E did not show any sign of viral replication, indicating that adenotonsillar tissue can sustain non-productive infections of HAdV B and E.

Immunodetection of HAdV in adenotonsillar tissues. To localize the sites of HAdV replication *in vivo*, histological sections of HAdV PCR-positive and negative tissues were tested by immunohistochemistry using anti-HAdV antibodies (Figure 4). In the presence of the positive control, which consisted of HAdV-infected Hep-2 cells, viral structural proteins were detected in the epithelial layer of adenoids from four patients with HAdV detectable by PCR. Also, HAdV was simultaneously detected in the subepithelial layer and lymphoid parenchyma of a palatine tonsil from one patient. All positive immunohistochemistry patients were also positive for viral mRNA detection by qRT-PCR. Thus, it can be concluded that HAdV C can replicate in epithelial and lymphoid cells from adenoids and palatine tonsils.

DISCUSSION

HAdV is among the leading causative agents of acute respiratory infection in humans [4]. In addition to causing ARI, a previous study by our group showed that HAdV is one of the most frequent respiratory viruses detected in children with chronic adenotonsillar disease in the absence of ARI symptoms [23]. The near 50% detection rate of the HAdV genome reported herein confirms previous findings and agrees with adenoids being preferred sites of HAdV infection when compared with palatine tonsils [16,18].

HAdV has been detected in peripheral blood from patients with HAdV-related tonsillitis in the presence of interleukin-6 production by endothelial cells, fibroblasts or activated T lymphocytes, an essential mechanism for the persistence of fever [6]. In the present study of patients without symptoms of ARI or acute tonsillitis, HAdV was

384 undetectable in PB, suggesting that asymptomatic viremia is not frequent in
385 asymptomatic HAdV carriers.

386 As previously published by our group in a small cohort of patients [23], HAdV
387 was detected more frequently in tonsil tissues where co-detection of other respiratory
388 viruses was present. However, we demonstrated herein that such co-detection is not
389 linked to higher HAdV loads (>10^6 copies/g), nor with the detection of mRNA of the
390 HAdV hexon gene. These findings indicate that the presence of HAdV in tonsils, with
391 or without evidence of structural viral protein production, is not associated with a
392 simultaneous increase in permissiveness of adenotonsillar tissues to other respiratory
393 viruses. Furthermore, this observation infers that HAdV replication is not activated,
394 nor reduced, by the presence of co-infection with other respiratory viruses.

395 Although adenovirus DNA is frequently found in tonsils, adenoids, and
396 intestinal tissues (varying from 30 – 80% of cases), infectious viruses are rarely
397 detected in these tissues, as measured by *in situ* hybridization or co-culture with
398 permissive cells [16,29]. Corroborating these findings, we were able to detect HAdV-
399 specific mRNA (signaling productive infection) in tonsillar tissue from 12 (14.1%)
400 patients, suggesting that the majority of HAdV PCR-positive patients with chronic
401 adenotonsillar disease have a persistent non-productive infection.

402 Among the HAdV-associated respiratory diseases, viruses of the species
403 HAdV B (HAdV-3, -7, -11, -14, -16, -21, -34, -35, -50, -55, and -66), HAdV C
404 (HAdV-1, -2, -5, and-6), and HAdV E (HAdV-4) are frequently described as capable
405 of replicating in the respiratory tract [30]. As expected, we found HAdV-1, -5, and -6
406 (species HAdV C), HAdV-3 (HAdV B) and HAdV-4 (HAdV E) in adenoids obtained
407 from the studied patients. Interestingly, viral mRNA was detected only in adenoids

408 from patients infected with HAdV-1, indicating that HAdV C was able to replicate in
409 the chronically inflamed adenoids analyzed.

410 Recent studies have pointed out the possibility of adenotonsillar tissue to act
411 as a site for DNA respiratory virus production, helping viral spreading between
412 healthy individuals, since HAdV or human bocavirus (HBoV) are frequently
413 undetectable in asymptomatic individuals in adenotonsillectomy follow-ups [31]. In fact,
414 some published data have demonstrated that lymphoid cells from adenoids, palatine
415 tonsils, and intestinal lamina propria are the main sites of HAdV latency in humans,
416 while epithelial cells from these tissues are essential for virus production and
417 shedding in NPS or stools [16,30]. In addition, corroborating with these findings, HAdV
418 antigen was detected in the epithelial layer of adenoids from 4 patients by
419 immunohistochemistry in this study, suggesting that epithelial cells from tonsillar
420 tissue comprise a site of viral proliferation preceding viral dissemination. We also
421 detected HAdV antigen in the lymphoid parenchyma of one patient, indicating that
422 other cells, aside from epithelial cells, can sustain HAdV replication in tonsillar
423 tissue.

424 Persistent infection by HAdV has been associated with chronic airway
425 obstructive diseases in children, such as asthma [32,33]. In those studies, HAdV antigen
426 or genome was found in bronchoalveolar lavage from more than 75% of children with
427 asthma, respectively, by immunohistochemistry [32] or PCR [33]. In the present study, the
428 detection of HAdV was not significantly correlated with chronic adenotonsillar
429 disease, respiratory symptoms or OME, nor with any other detectable disease
430 phenotype.

431 Some clinical studies have associated the detection of respiratory viruses with
432 OME. Viral infections caused by respiratory syncytial virus, influenza virus (types A

433 and B), and adenovirus have been shown to increase the risk of OME, which can be in
434 part attributed to these viral infections facilitating colonization of the nasopharynx by
435 *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *M. catarrhalis* [34,35], and the
436 adhesion of *S. pneumoniae* to epithelial cells of the respiratory tract [36]. In contrast, the
437 development of sleep apnea is partially associated with upper airway obstruction due
438 to enlargement of the palatine tonsils and adenoids, seen significantly more often in
439 obese patients with asymptomatic viral infections, such as those caused by
440 adenoviruses [37,38]. Proinflammatory cytokines released by visceral adipocytes seem to
441 contribute to tonsillar inflammation and the development of sleep apnea [39–41].
442 Although no substantial association of HAdV and the severity of adenotonsillar
443 enlargement was found in this study, a significant correlation was observed regarding
444 HAdV quantities and the presence of sleep apnea or OME.

445 The present study has shown that patients without OME had significantly
446 higher viral loads than individuals with the condition. Some viruses, such as human
447 cytomegalovirus, are known to target dendritic cells, subverting and compromising
448 the host's adaptive immunity by interfering with the cellular transport of major
449 histocompatibility complex molecules [42,43]. Dendritic cells infected with HAdV
450 strongly stimulate T cell proliferation [44], which may result in increased cellular
451 response to other infectious agents, protecting the host from the development of
452 OME. In addition, persistent adenoviral infection, with small HAdV loads, could
453 function as a chronic stimulus for the development of OME.

454 In contrast, patients with sleep apnea exhibited significantly higher HAdV
455 loads than individuals lacking the condition. Cellular and humoral responses are
456 critical for the control of HAdV infection. The recruitment of macrophages and
457 natural killer cells leads to the release of a range of pro-inflammatory cytokines,

458 stimulating both $CD4^+$ and $CD8^+$ T cells, and, consequently, B cell proliferation with
459 humoral antibody response [45]. Thus, in keeping with this idea, it is reasonable to
460 consider that high levels of HAdV may induce the production of pro-inflammatory
461 and vasoactive cytokines, which increase chances of developing apnea.

462 In conclusion, the present study demonstrated that a high proportion of
463 patients with chronic adenotonsillar disease had persistent HAdV infection in the
464 adenoids and tonsils. However, the presence of productive HAdV infection was not
465 associated with the severity of nasal obstruction, recurrent tonsillitis or viral co-
466 infections. The presence of higher HAdV loads in patients with apnea, in parallel with
467 a protective effect against secretory otitis media, indicates that additional studies are
468 required to provide a definitive role for HAdV during chronic adenotonsillar diseases.
469

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471

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476

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FIGURE LEGENDS

Figure 1. Viral load of HAdV in adenoids, palatine tonsils, and nasopharyngeal secretions by qPCR. **A.** HAdV viral loads in ADs, PTs, and NPS from patients with adenotonsillar chronic diseases. **B.** Patterns of HAdV viral loads in patients with simultaneous detection in several sites. The viral loads in the same patient were connected by a straight line. **C.** HAdV viral loads in palatine tonsils in the different clinical conditions. **D.** HAdV viral loads in adenoids in the different clinical conditions. **E.** HAdV viral loads in NPS in the different clinical conditions. **F.** HAdV viral loads in palatine tonsils according to gender and age. **G.** HAdV viral loads in adenoids according to gender and age. **H.** HAdV viral load in NPS according to gender and age. **I.** HAdV viral loads in palatine tonsils from patients with this virus detectable only in this tissue or when the agent was also detectable in other sites, including as adenoids and NPS. **J.** HAdV viral loads in adenoids from patients with this virus detectable only in this tissue or when the agent was also detectable in other sites, such as palatine tonsils and NPS. **K.** HAdV viral loads in NPS from patients with this virus detectable only in this site or when the agent was also detectable in other tissues, including adenoids and palatine tonsils. **L.** Association of HAdV viral loads in palatine tonsils with the presence of viral-coinfection or with the detection of the mRNA of the HAdV hexon gene. **M.** Association of HAdV viral loads in adenoids with the presence of viral-coinfection or with the detection of the mRNA of the HAdV hexon gene. **N.** Association of HAdV viral loads in NPS with the presence of viral-coinfection or with the detection of the mRNA of the HAdV hexon gene. The red line in all graphs represents the median of the viral load in the analyzed condition.

627 **Figure 2.** HAdV viral loads in adenoids (**A**) and palatine tonsils (**B**) from patients
628 with HAdV as a single agent or in dual infection with other respiratory viruses:
629 human bocavirus (HBoV), human rhinovirus (HRV), human enterovirus (HEV),
630 human respiratory syncytial virus (HRSV), and human metapneumovirus (HMPV).
631 Boxes extend from the 25th to 75th percentiles, middle whiskers mark median values,
632 and upper and lower whiskers mark the highest and the lowest values, respectively.
633
634 **Figure 3.** HAdV typing by conventional nested-PCR and DNA sequencing. **(A).**
635 Representative agarose gel electrophoresis denoting the detection of genomic hexon
636 gene sequences after nested-PCR. **(B).** Maximum likelihood phylogeny of strains of
637 adenoviruses identified in this study within representative members of the
638 *Adenoviridae* family**.** The tree was inferred using nucleotide alignments of the partial
639 hexon gene based on TIM2+F+I+G4 of the DNA substitution model. Phylogeny is
640 midpoint rooted. Scale bar indicates evolutionary distance in numbers of substitutions
641 per nucleotide sites. Bootstrap values of 1,000 replicates are shown in primary nodes.
642 The "isolated" adenovirus sequences obtained herein are shown in red. HAdV:
643 Human adenovirus, SAdV: Simian adenovirus. Viral species are indicated by color
644 and letter on the right.
645
646 **Figure 4.** Immunohistochemistry for HAdV of non-infected and infected Hep-2 cells,
647 palatine tonsils and adenoids from patients with adenotonsillar chronic diseases. **A.**
648 Non-infected Hep-2 cells as negative controls counterstained with Hematoxylin and
649 Eosin. **B.** HAdV-infected Hep-2 cells as positive controls counterstained with
650 Hematoxylin and Eosin. **C.** Representative palatine tonsil from a patient without
651 HAdV detectable by qPCR. **D.** Representative palatine tonsil from an HAdV-positive

patient. **E.** Representative adenoid from a patient without HAdV detectable by qPCR. **F.** Representative adenoid from a patient with HAdV detectable by qPCR, illustrating the presence of viral antigens in superficial cells. The positive signal is visible as brown color. The adenoids and palatine tonsils shown here were obtained from the same patient.

Tables

Table 1. Clinical and demographic data of patients with HAdV detected in adenoids.

	Disease Patients Group		Control Patients Group	
	HAdV +	**HAdV -**	**HAdV +**	**HAdV -**
Patients	85 (48.9%)	89 (51.1%)	3 (25.0%)	9 (75.0%)
Males	40 (47.1%)	51 (57.3%)	1 (33.3%)	6 (66.6%)
Age (median of years)	5.0	5.0	3.0	3.0
Viral co-infection*	62 (72.9%)	45 (50.6%)	2 (66.6%)	4 (44.4%)
0-50% nasal obstruction	8 (9.4%)	9 (10.1%)	-	-
50-75% nasal obstruction	36 (42.3%)	41 (46.1%)	-	-
75-100% nasal obstruction	41 (48.3%)	39 (43.8%)	-	-
Sleep apnea	50 (58.8%)	54 (60.6%)	-	-
Otitis media with effusion	12 (14.1%)	18 (20.2%)	-	-
Allergy	27 (31.8%)	21 (23.6%)	-	-

* $p<0.05$

Table 2. Clinical and demographic data of patients with HAdV detected in palatine tonsils.

	Disease Patients Group		Control Patients Group	
	HAdV +	**HAdV -**	**HAdV +**	**HAdV -**
Patients	49 (27.2%)	131 (72.8%)	2 (16.6%)	10 (83.3%)
Males	23 (46.9%)	70 (53.4%)	1 (50.0%)	6 (60.0%)
Age (median of years)	5.0	5.0	3.0	3.0
Viral co-infection*	33 (67.3%)	15 (11.4%)	0 (0.0%)	0 (0.0%)
Recurrent Tonsillitis	29 (59.2%)	84 (46.6%)	-	-
Tonsillar hypertrophy	41 (83.7%)	107 (81.7%)	-	-
Sleep apnea	29 (59.2%)	75 (57.3%)	-	-
Otitis media with effusion	8 (16.3%)	22 (16.8%)	-	-
Allergy	14 (28.6%)	34 (25.9%)	-	-

* $p<0.05$.

Table 3. Clinical and demographic data of patients with replicant and persistent HAdV detected in adenoids.

	Disease Patients Group		Control Patients Group	
	mRNA Hexon +	**mRNA Hexon -**	**mRNA Hexon +**	**mRNA Hexon -**
Patients	12 (14.1%)	73 (85.9%)	1 (33.3%)	2 (66.6%)
Males	6 (50.0%)	34 (46.6%)	0 (0.0%)	1 (50.0%)
Age (median of years)	4.0	6.0	2.0	3.0
Viral co-infection	62 (72.9%)	45 (50.6%)	1 (100%)	1 (50.0%)
Viral load (median copies/g)*	$1.08x10^7$	$4.31x10^4$	$1.1x10^8$	$1.3x10^6$
High viral load ($>10^6$)*	10 (83.3%)	13 (17.8%)	1 (100%)	1 (50.0%)
Detection in several sites	9 (75.0%)	50 (68.5%)	1 (100%)	1 (50%)
Spreading of HAdV in NPA	7 (58.3%)	34 (46.6%)		
0-50% nasal obstruction	1 (8.3%)	7 (9.5%)	-	-
50-75% nasal obstruction	3 (25.0%)	33 (45.2%)	-	-
75-100% nasal obstruction	8 (66.6%)	33 (45.2%)	-	-
Sleep apnea	9 (75.0%)	41 (56.2%)	-	-
Otitis media with effusion	1 (8.3%)	11 (15.1%)	-	-
Allergy	2 (16.6%)	25 (34.2%)	-	-

* $p<0.05$.

671 **Table 4**. Clinical and demographic data of patients with replicant and persistent HAdV detected in
672 palatine tonsils.

	Disease Patients Group		Control Patients Group	
	Replicant HAdV	**Persistent HAdV**	**Replicant HAdV**	**Persistent HAdV**
Patients	4 (8.2%)	45 (91.8%)	0 (0.0%)	2 (100.0%)
Males	2 (50.0%)	21 (42.3%)	-	1 (50.0%)
Age (median of years)	4.0	5.0	-	3.0
Viral co-infection	3 (75.0%)	30 (66.6%)	-	0 (0.0%)
Viral load (median copies/g)	$9.56x10^5$	$4.50x10^4$	-	$1.8x10^4$
High viral load ($>10^6$)*	2 (50.0%)	5 (11.1%)	-	0 (0.0%)
Detection in several sites	4 (100.0%)	37 (82.2%)	-	2 (100%)
Spreading of HAdV in NPA	3 (75.0%)	20 (44.4%)		
Recurrent Tonsillitis	3 (75.0%)	26 (57.7%)	-	-
Tonsillar hypertrophy	4 (100.0%)	37 (82.2%)	-	-
Sleep apnea	4 (100.0%)	25 (55.5%)	-	-
Otitis media with effusion	0 (0.0%)	8 (17.7%)	-	-
Allergy	1 (25.0%)	13 (28.8%)	-	-

673 * $p<0.05$.
674
675

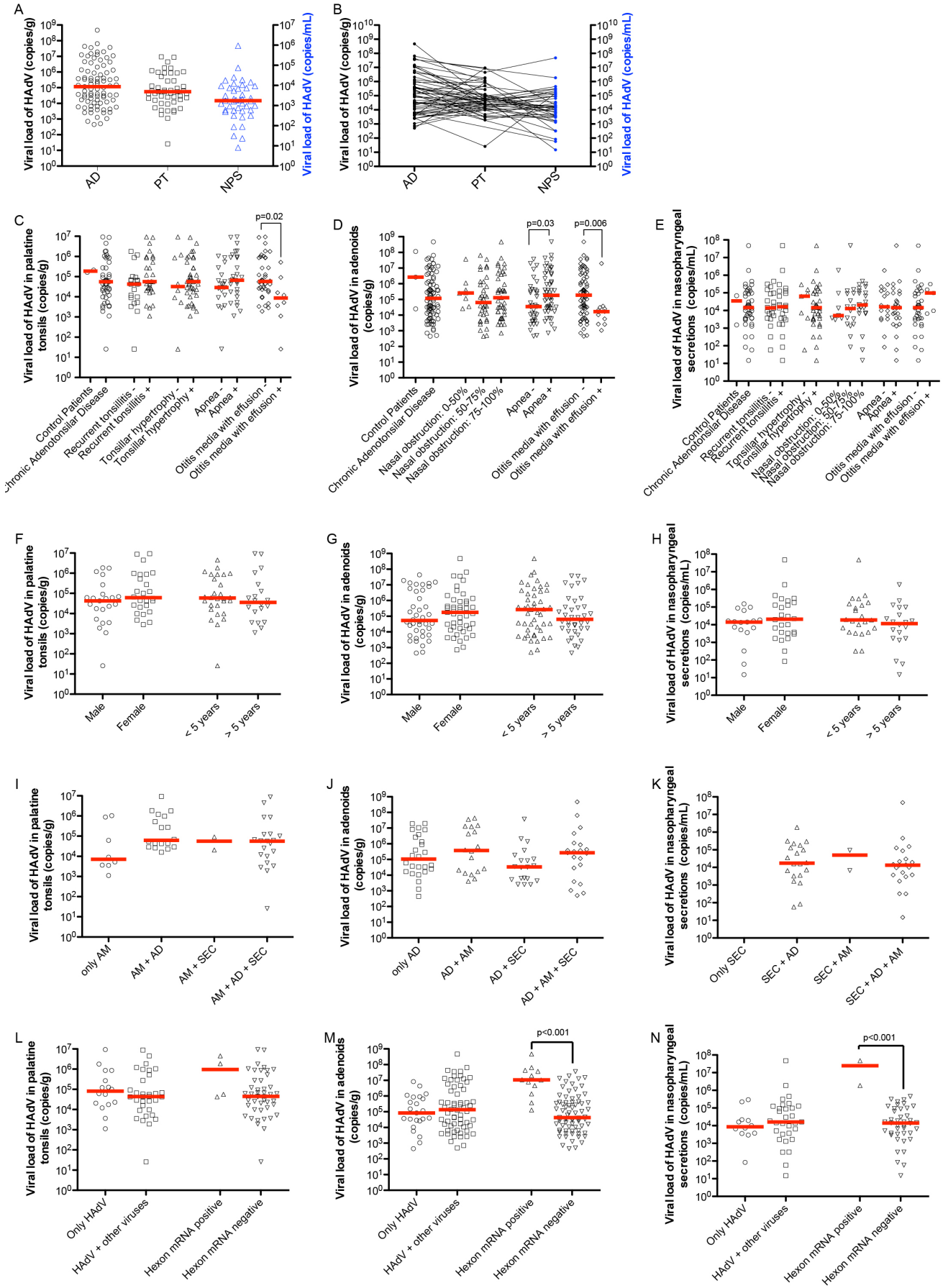
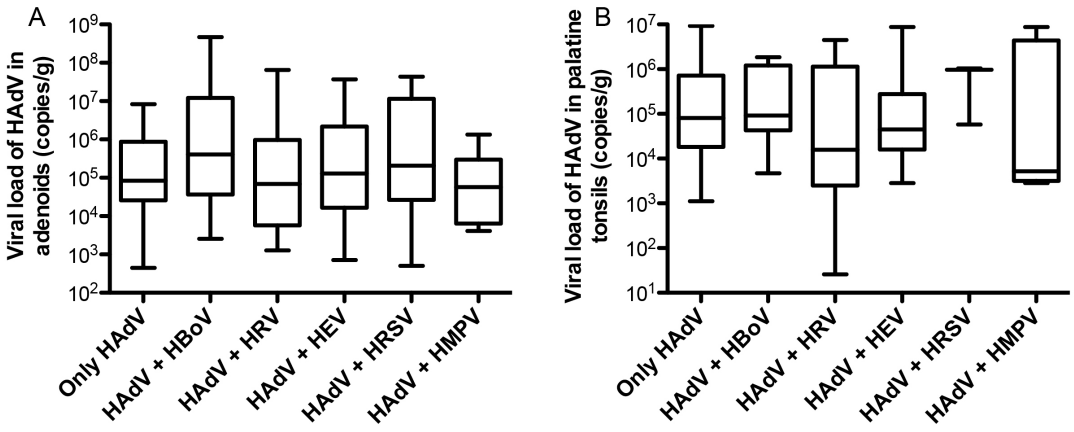
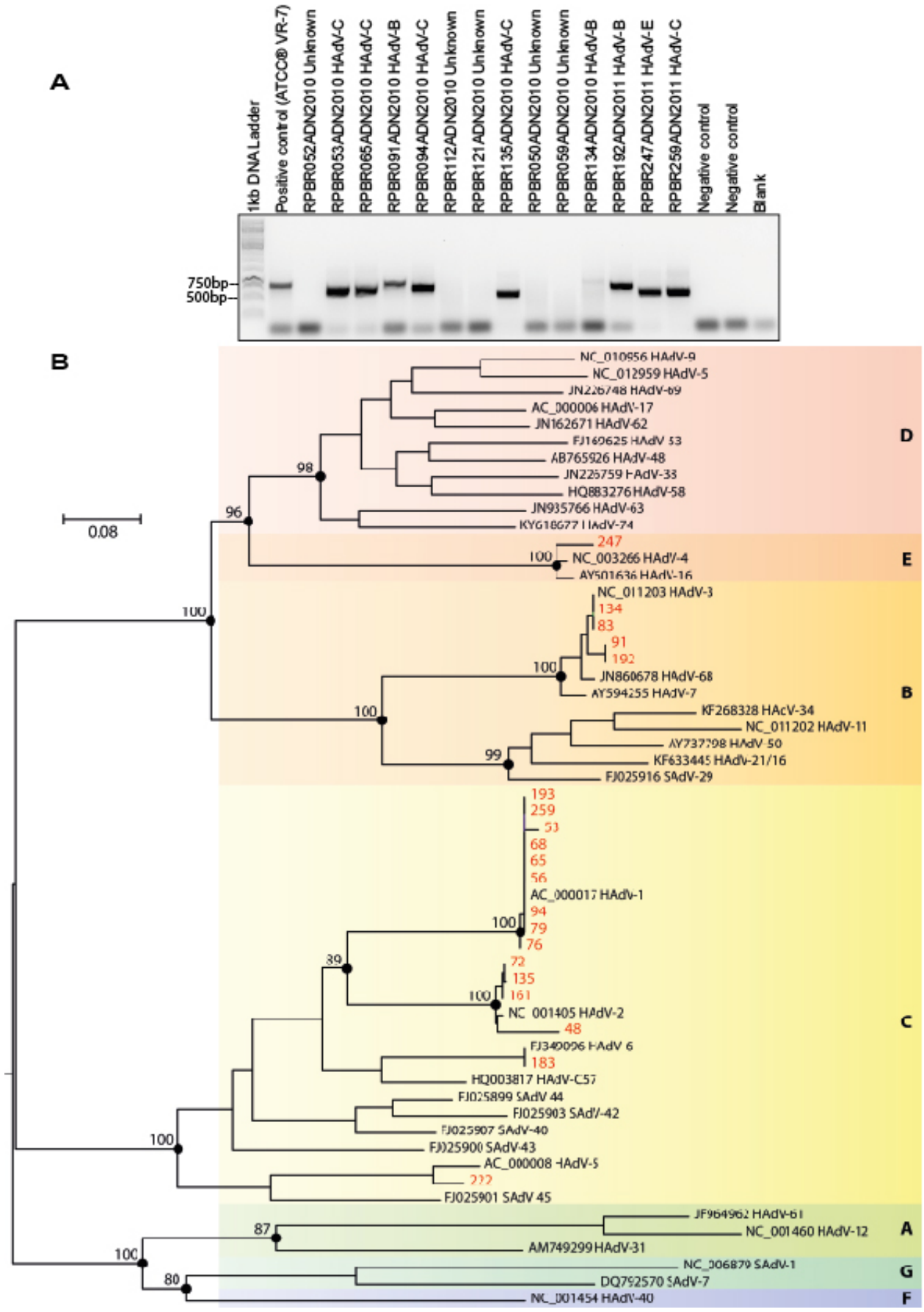

A
Viral load of HAdV (copies/g)
Viral load of HAdV (copies/mL)
AD
PT
NPS
B
Viral load of HAdV (copies/g)
Viral load of HAdV (copies/mL)
AD
PT
NPS
C
Viral load of HAdV in palatine tonsils (copies/g)
p=0.02
Control Patients
Chronic Adenotonsilar Disease
Recurrent tonsillitis -
Recurrent tonsillitis +
Tonsillar hypertrophy -
Tonsillar hypertrophy +
Apnea -
Apnea +
Otitis media with effusion -
Otitis media with effusion +
D
Viral load of HAdV in adenoids (copies/g)
p=0.03
p=0.006
Control Patients
Chronic Adenotonsilar Disease
Nasal obstruction: 0-50%
Nasal obstruction: 50-75%
Nasal obstruction: 75-100%
Apnea -
Apnea +
Otitis media with effusion -
Otitis media with effusion +
E
Viral load of HAdV in nasopharyngeal secretions (copies/mL)
Control Patients
Chronic Adenotonsilar Disease
Recurrent tonsillitis -
Recurrent tonsillitis +
Tonsillar hypertrophy -
Tonsillar hypertrophy +
Nasal obstruction: 0-50%
Nasal obstruction: 50-75%
Nasal obstruction: 75-100%
Apnea -
Apnea +
Otitis media with effusion -
Otitis media with effusion +
F
Viral load of HAdV in palatine tonsils (copies/g)
Male
Female
< 5 years
> 5 years
G
Viral load of HAdV in adenoids (copies/g)
Male
Female
< 5 years
> 5 years
H
Viral load of HAdV in nasopharyngeal secretions (copies/mL)
Male
Female
< 5 years
> 5 years
I
Viral load of HAdV in palatine tonsils (copies/g)
only AM
AM + AD
AM + SEC
AM + AD + SEC
J
Viral load of HAdV in adenoids (copies/g)
only AD
AD + AM
AD + SEC
AD + AM + SEC
K
Viral load of HAdV in nasopharyngeal secretions (copies/mL)
Only SEC
SEC + AD
SEC + AM
SEC + AD + AM
L
Viral load of HAdV in palatine tonsils (copies/g)
Only HAdV
HAdV + other viruses
Hexon mRNA positive
Hexon mRNA negative
M
Viral load of HAdV in adenoids (copies/g)
p<0.001
Only HAdV
HAdV + other viruses
Hexon mRNA positive
Hexon mRNA negative
N
Viral load of HAdV in nasopharyngeal secretions (copies/mL)
p<0.001
Only HAdV
HAdV + other viruses
Hexon mRNA positive
Hexon mRNA negative

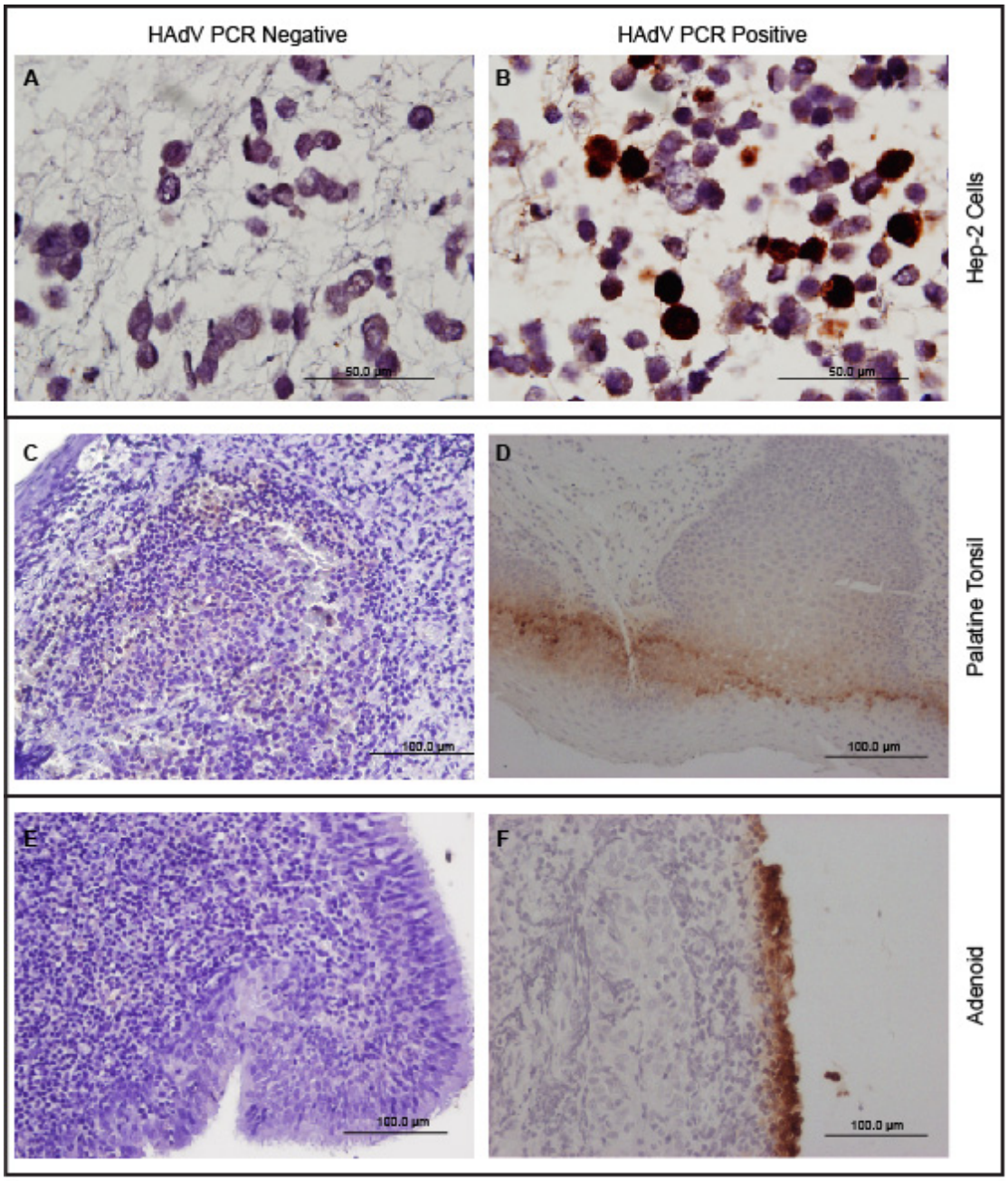
Figure 1.



679
680 **Figure 2.**
681



682
683 **Figure 3.**
684



685
686 **Figure 4.**
687