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Prevalence of Epstein–Barr virus in tonsils and adenoids of United Arab Emirates nationals

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ABSTRACT

Objective: Given that Epstein–Barr virus (EBV) often inhabits human tonsils and adenoids, it remains to be distinctively determined its prevalence and in which cell and microenvironment the virus is present.
Methods: To determine the prevalence of EBV in the tonsils and adenoids of the United Arab Emirates (UAE) nationals and to provide a basis for understanding the origin and biology of EBV-infected cells, the immunophenotype of all EBV-infected cells in 46 tonsils and 46 adenoids was determined by EBER in situ hybridization and immunohistochemistry with monoclonal antibodies to T cells (CD3), B cells (CD20), and epithelial cells (cytokeratin AE1/AE3), as well as immunostaining with antibodies to EBV latent membrane protein-1 (LMP-1).
Results: EBV was found in 43% of tonsillectomy specimens and 15% of adenoidectomy specimens. All EBV-infected cells were found to be B lymphocytes. About 90% of the infected B cells are found in the interfollicular regions of tonsils and adenoids and the remaining 10% are found within the follicles. There is no significant association between EBV infection, age ($P = 0.324$) and gender ($P = 0.442$).
Conclusion: EBV is associated with tonsillar hypertrophy and is prevalent in 43% of our cases. EBV is only detected in B lymphocytes and we believe that B lymphocytes are sites of primary infection and latency. In situ hybridization is the gold standard for the detection of EBV in tissue.

1. Introduction

Tonsils and adenoids, the common sites for recurrent inflammation in the pediatric population, are continually exposed to antigens, hence hyperplasia of their lymphoid component accounts for the increase in their size [1]. Some authors have demonstrated a strong relation between viral infection [2–4] and recurrent pharyngotonsillitis. There are many viruses involved in the pathogenesis of pharyngotonsillitis including adenoviruses, parainfluenza viruses, rhinoviruses, herpes simplex viruses, respiratory syncytial viruses, Epstein–Barr viruses (EBV), influenza viruses, coxsackie A viruses, corona viruses, and cytomegaloviruses [2,3,5,6]. In spite of high prevalence of viral infection in adenotonsillar tissue, the methods to detect viruses make this approach difficult in routine practice [2,3,5,6].

Commonly causing infectious mononucleosis, EBV is a member of γ1-herpesvirus and has a genome comprised approximately 172 nucleotide base pairs [7]. EBV has a linear genome which is characterized by a distinctive sequence reiteration. At the termini there are 20 copies of a 500 bp repeated sequence that is complementary and therefore permits circularization of the linear genome to form the EBV episome. EBV episome is the molecular basis for latent infection. It is a circular intracellular form of genome that maintains a persistent relationship within the cell, like an autonomous piece of DNA situated in the chromatin [7,8]. EBV is B-lymphotropic and has the ability to transform memory B-cells into blast cells, with permanent proliferation, leading to tonsillar enlargement [9]. EBV is associated not only with infectious mononucleosis but also with benign diseases, such as oral hairy leukoplakia, and malignancies, such as Hodgkin's lymphoma, non-Hodgkin's lymphomas, nasopharyngeal carcinoma, gastric carcinoma and breast carcinoma. It is recently being associated with autoimmune diseases, such as lupus erythematosus and multiple sclerosis [7,10,11]. For these reasons, our study is designed to identify the magnitude of EBV infection and types and pattern of distribution of EBV-infected cells in tonsils and adenoids among UAE population which will be of great help in future planning to prevent this infection.

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2. Materials and methods

2.1. Review of cases

In total, 46 cases of tonsillectomy with adenoidectomy, which were performed due to tonsillar and adenoidal enlargement, were randomly selected from surgical pathology archive in the department of pathology at Tawam hospital in Al Ain city for the period June 2004 through May 2005. Forty-six paraffin blocks of tonsillectomy specimens and another 46 paraffin blocks of adenoidectomy specimens were available for this study. The available Hematoxylin and eosin (H&E) and immunohistochemical stained sections were reviewed. The age, sex, and clinical presentation were obtained by reviewing all the histopathologic reports and request forms of all cases. For the detection of EBV, two methods were used to increase the specificity and sensitivity of detection, the streptavidin-biotin immunohistochemical immunoperoxidase method to detect Epstein–Barr virus latent membrane antigen type I (EBV-LMP-1) and in situ hybridization (ISH) for EBV encoded RNA (EBER).

2.2. Immunohistochemistry (IHC)

Immunohistochemical (IHC) staining was performed by standard streptavidin–biotin immunoperoxidase technique [12] using the following mouse antihuman monoclonal antibodies (DAKO Cytomation, Glostrup, Denmark); EBV-latent membrane protein-1(LMP1)(clone CS 1–4), CD3 (clone PC3/188A), CD20 (clone L26), cytokeratin (clone AE1/AE3), all diluted to 1:100, and visualized by a commercially available detection kit (DAKO EnVision Plus-HRP, DAKO, Glostrup, Denmark) and 3-3′-diaminobenzidine (DAKO, Glostrup, Denmark) as a chromogen substrate to obtain a brown end-product. Lymph node sections were used as positive controls for CD20, CD3. Skin epidermis was used as a positive control for cytokeratin AE1/AE3. Hodgkin Lymphoma (HL) LMP-1-positive sections were used as positive controls for LMP-1. For negative controls, primary antibody was replaced with normal mouse serum.

2.3. In situ hybridization (ISH)

In situ hybridization (ISH) was performed by standard techniques using a specific oligonucleotide probe (Novocastra–LEBV-K, UK) which hybridizes to EBV encoded RNA (EBER) transcripts concentrated in the nuclei of latently infected cells. With each batch of cases studied, positive and negative control slides were also run. The positive control slide was a known case of EBV positive HL to which a specific EBER oligonucleotide probe was added. The negative control slide was another section of the same case of known EBV positive HL to which a random probe consisting of fluorescein labeled oligonucleotide cocktail was added. In addition, for each case studied two sections were used; the EBER oligonucleotide probe was added to one section, and the random probe was added to the other. Using this random probe as a negative background control alongside the EBV probe contributes to the validation of staining obtained by the EBV probe. If this negative control slide showed significant background staining in a particular case, the slide having the EBER probe was considered non-interpretable and the test was repeated for that particular case.

2.4. Triple staining technique

Five-micrometer sections were stained for EBER using in situ hybridization protocol described earlier. After nuclear visualization, mouse antihuman monoclonal antibodies for CD20 (DAKO Cytomation, Glostrup, Denmark) were added and visualized by a commercially available detection kit (DAKO EnVision Plus-HRP, DAKO, Glostrup, Denmark) and 3-3′-diaminobenzidine (DAKO, Glostrup, Denmark) as a chromogen substrate to obtain a brown end-product. Subsequently, mouse antihuman monoclonal antibodies for CD3 (DAKO Cytomation, Glostrup, Denmark) were added and visualized using the EnVision Plus-alkaline phosphatase kit (DAKO, Glostrup, Denmark), and New Fuchsin (Merck, Darmstadt, Germany) as a second substrate to yield a red end-product. Finally, sections were mounted by water soluble mounting media.

2.5. Statistical analysis

The statistical analysis was performed using SPSS for windows version 18 (SPSS Inc, Chicago, USA) and analyze it (Analyze-it software Ltd., Leeds, UK). Student's *t*-test was used to compare continuous variables. Quantitative variables were analyzed with the χ^2-test and correlations of ordinal variables using the Spearman rank correlation coefficient. P value <0.05 was considered significant.

2.6. Research ethics

The project has been approved by Al Ain district Human research Ethical committee (Protocol No. 07-145).

3. Results

3.1. Age and gender distribution

In total, 46 cases of tonsillectomy and adenoidectomy, due to tonsillar and adenoid hypertrophy, were selected. All cases were in the 1st and 2nd decade of life with predominant clustering (63%) in the 1st decade. Twenty-seven cases were females and 19 cases were males.

3.1.1. EBV expression correlation between age and gender

Although EBV-positive cases were more ($n = 14$) in the 1st decade than in the 2nd decade ($n = 6$), Cross tab and logistic regression show no significant association between EBV expression and the age ($P = 0.324$) and gender ($P = 0.442$) distributions (Table 1).

Table 1
EBV expression and correlation with age and gender.

Age	EBV[a] positive cases			EBV[a] negative cases			Total cases		
	Female +	Male +	Total +	Female −	Male −	Total −	Female	Male	Total
0–9	6	8	14	6	8	14	12	16	28
10–19	5	1	6	10	2	12	15	3	18
Total	11	9	20	16	10	26	27	19	46

[a] Epstein–Barr virus.

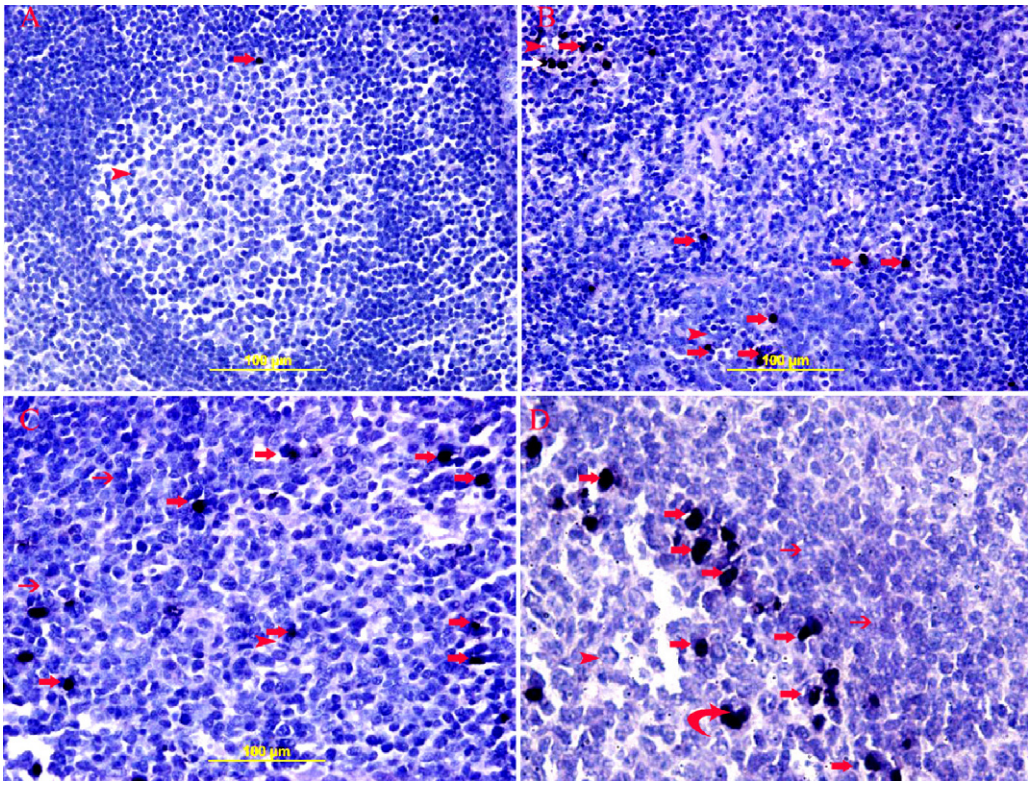

Fig. 1. Expression of EBV in the lymphoid follicles of enlarged tonsils and adenoids. (A) EBER-positive cell (thick arrow) is seen within a lymphoid follicle with a germinal center (arrow head). (B) Many EBER-positive cells (thick arrows) are seen in lymphoid follicles (arrow heads), however, some EBER-positive cells are also seen in the interfollicular area. (C) EBER-positive cells (thick arrows) are seen in the center of lymphoid follicles or having a circumferential distribution in the interphase between germinal center (arrow head) and mantle zone cells (thin arrow). (D) Many EBER-positive cells (thick arrows) are seen in the center of lymphoid follicles or having a circumferential distribution in the interphase between germinal center (arrow head) and mantle zone cells (thin arrow). A large atypical EBER-positive cell (curved arrow) is seen in the germinal center.

3.2. EBV distribution

3.2.1. EBV prevalence in tonsillectomy specimens

In total, 20 (43%) specimens were positive by in situ hybridization method for EBER and show the black nuclear stain. EBER-positive cells are seen within follicular (Fig. 1) and interfollicular (Fig. 2) regions. The frequency of EBV is shown in Table 2. Only 5 (11%) specimens of tonsillectomy show cytoplasmic and membranous positivity for LMP-1 as shown in Fig. 3. LMP-1-positive large cells are seen within the mantle zone of lymphoid follicle and within the interfollicular area, however, LMP-1 expression is seen also in small lymphocytes in the interfollicular area.

3.2.2. EBV prevalence in adenoidectomy specimens

In total, 7 (15%) specimens were positive by in situ hybridization method for EBER and show the black nuclear stain. EBER-positive cells are seen within follicular (Fig. 1) and interfollicular (Fig. 2) regions. The frequency of EBV is shown in Table 2. All those cases were also EBV positive in their tonsillectomy specimens. LMP-1 staining was negative in all 46 case adenoid specimens.

3.3. Distribution of EBV among lymphoid and epithelial cells

Nearly 90% of the EBER-positive cells were found within the T-cell-rich interfollicular regions (Fig. 2) while 10% were present within the B-cell-rich germinal centers of secondary follicles (Fig. 1). All EBER-positive cells were B cells. The EBER-positive cells show immunoreactivity to CD20 only (Fig. 4). There was no immunoreactivity to CD3 (Fig. 4) and cytokeratin (Fig. 2A). Individual EBER positive cells appeared to be randomly distributed within the interfollicular regions (Fig. 3). The distribution of EBER-postive cells within lymphoid follicles is variable. Some follicles have few positive cells within the germinal center (Fig. 1). Some of them were atypical large cells (Fig. 1D). Other follicles show their presence within the mantle zone layer (Fig. 1C and D). There is a third pattern where EBER-positive cells form a circumferential distribution at the interphase between the germinal center cells and the mantle zone cells (Fig. 2C and D).

4. Discussion

Enlargement of tonsils and adenoids is a common finding in children but the etiology remains controversial. In children, there is no correlation between hypertrophic tonsils and the body mass index [13]. The weight of normal tonsils in children is not well documented in anatomy literature; however, the mean weight of the tonsillectomy specimens from pathologic examination was shown to be 7.3 g in children between the ages of 2 and 12 years with 63% of it falls between 5 and 8 g [14]. Tonsillectomy is a common surgical procedure in children, and the most common indication is tonsillar hypertrophy, which leads to obstructive symptoms in the upper airway. There is a poor correlation between enlarged tonsils and the associated symptoms [14].

Recurrent bacterial tonsillitis is considered to be the main reason for the enlargement of the tonsils; however, the bacteriological examinations of the tonsils showed that the relationship is not very clear [15]. Misuse of antibiotic therapy in acute tonsillitis may lead to change in tonsillar microflora and predisposing to recurrent tonsillitis and subsequent colonization by various bacteria and viruses [16]. The role of Actinomycosis is considered in the etiology of recurrent tonsillitis, not as an active infection but

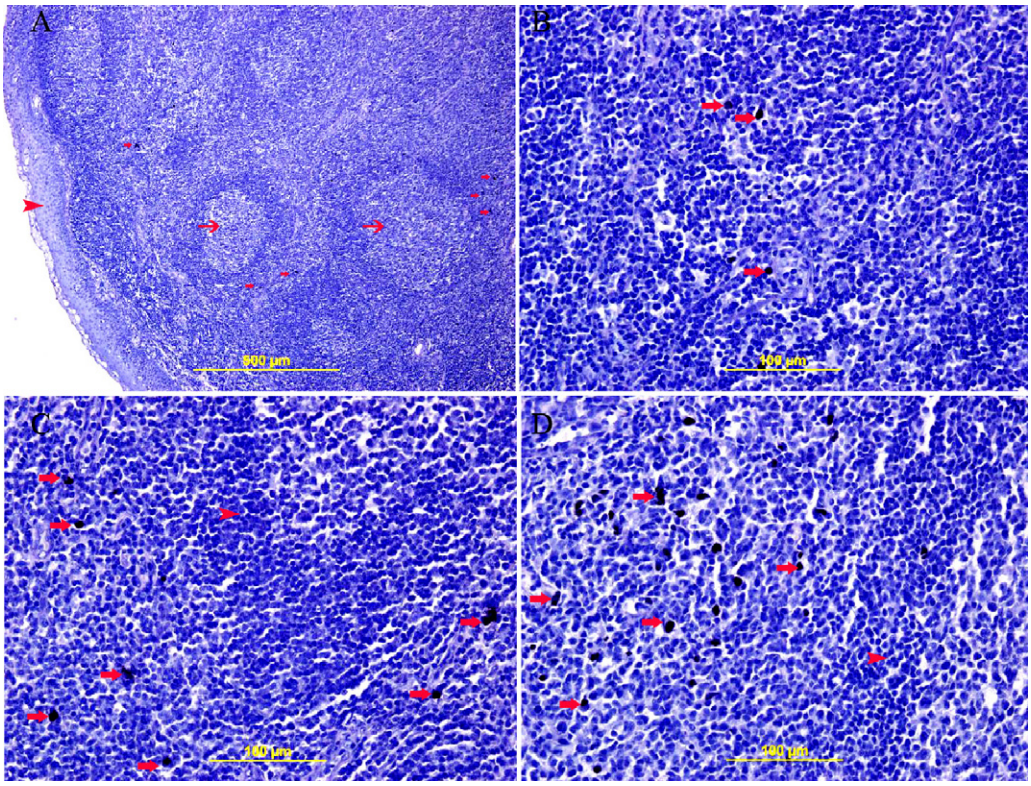

Fig. 2. Expression of EBV in the interfollicular areas of enlarged tonsils and adenoids. (A) There is no expression of EBV in the epithelial cell (arrow head), however, EBER positive cells are seen in the interfollicular areas (thick arrowed) in between lymphoid follicles (thin arrows), EBER in situ hybridization. (B) Many EBER positive cells are seen in the interfollicular areas (thick arrows). (C) A lymphoid follicle (arrow head) is surrounded by many EBER-positive cells in the interfollicular area (thick arrows). (D) Many EBER positive cells are seen in the interfollicular areas (thick arrows), close to a lymphoid follicle with a germinal center (arrow head).

as a factor in the development of lymphoid hyperplasia and hypertrophy [17].

Many viruses are involved in the pathogenesis of pharyngotonsillitis [2,3,5,6]. Colonization of the tonsils by EBV shows no correlation between EBV–DNA quantity and viral core antigen-IgG quantity in the autologous sera [18]. EBV infection is very common, with a seroprevalence rates in excess of 90% worldwide. [19]. Nearly all infections are acquired by oral contact with persons carrying EBV in saliva. Although the exact details of EBV transmission in the oral cavity remain unknown, it is likely that initial infection of either oral epithelial cells or tonsillar B cells is followed by a brief period of replication and lifelong persistence in B lymphocytes [20].

The current study involves tonsils and adenoids of UAE nationals only. Cases were randomly selected from the surgical archive of the pathology department at Tawam hospital for a period June 2004 to May 2005. The study includes patients from different parts of the country. Tawam hospital is the main hospital that provides medical services for UAE nationals. Hence our samples are almost representative of the UAE population.

Tonsils and adenoids were chosen to identify the prevalence of EBV in tissue since they are the initial sites of infection and may reflect the magnitude of EBV infection in the community. In addition, it may help in identifying the correlation between EBV infection and the prevalence of EBV-associated diseases in the community, which might help in the setting of plans to prevent these diseases. In this study we show that EBV is prevalent in 43% of tonsils and 15% of adenoids. It is interesting to see all our cases are children, a finding seen by Endo et al. too [21], indicating that tonsillar and adenoidal hypertrophy occur mainly during this age group. It is well known that EBV has a tropism for the oral and nasopharyngeal tissues, and leads to lymphocytic proliferation [21]. The role of oropharyngeal epithelial cells as a reservoir of EBV was already suggested [21]. We show EBV only inhabits B lymphocytes in tonsils and adenoids, but not T lymphocytes or epithelial cells indicating that B lymphocytes are the main reservoir for EBV. Hudnall et al. [22], showed EBV to be rarely found within epithelial cells and T lymphocytes in EBV-infected tonsils.

Following primary infection in the oropharynx, EBV persists in numerous anatomical sites including pharyngeal tonsils, adenoids, lymph nodes, and peripheral blood. In peripheral blood, the virus is present in small resting memory B cells with latent gene expression limited to EBER, LMP2a, and perhaps EBNA1 [19]. It is well known that the interleukin 10 (IL-10) coding sequence is highly homologous to the EBV open reading frame *BCRF1* [23]. BCRF1 protein, also termed viral IL-10 (vIL-10), inhibits the synthesis of T-helper 1 cytokines [24] and cytotoxic T lymphocytes

Table 2
The prevalence of EBV in tonsillectomy and adenoidectomy specimens.

Specimen	EBV (+) ISH[a] cases	%	EBV (−) ISH[a] cases	%	Total	%
Tonsils	20	43	26	57	46	100
Adenoids	7	15	39	85	46	100

[a] In situ hybridization.

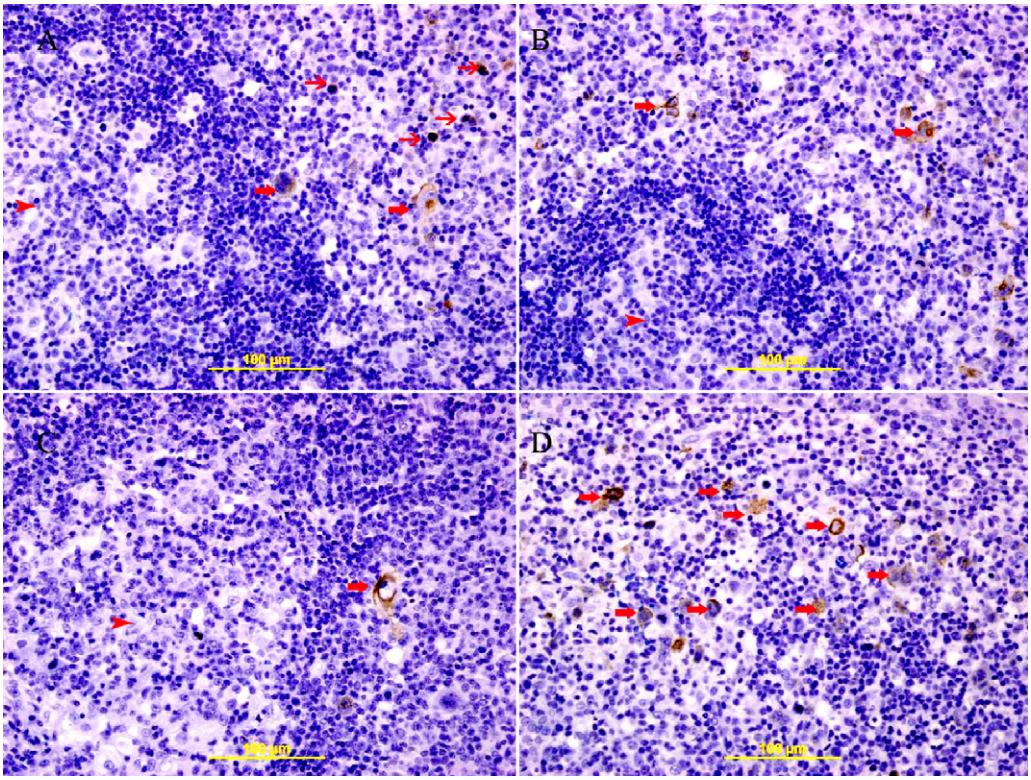

Fig. 3. Expression of EBV-LMP-1 in enlarged tonsils. (A) LMP-1-positive large cells (thick arrows) are seen within the mantle zone of a lymphoid follicle and within the interfollicular area, however, LMP-1 expression is seen also in small lymphocytes in the interfollicular area (thin arrows), streptavidin–biotin immunoperoxidase method. (B) LMP-1-positive cells (thick arrows) are seen within the interfollicular area, close to a lymphoid follicle (arrow head), streptavidin–biotin immunoperoxidase method. (C) LMP-1-positive large cells (thick arrows) are seen within the mantle zone of a lymphoid follicle (arrow head), streptavidin–biotin immunoperoxidase method. (D) Many LMP-1-positive cells (thick arrows) are seen within the interfollicular area, streptavidin–biotin immunoperoxidase method.

(CTL) activity [25]. Therefore, EBV-associated antigen-specific CTL activity might be down-regulated by vIL-10 in EBV-infected areas of the tonsil. Hence, EBV is much more likely to survive in the face of immune surveillance in the tonsils, suggesting that the immune response to EBV in tonsils may be different from that in peripheral blood [25]. The prevalence of EBV infection in tonsils varies according to the detection method. Studies using the ISH for detecting EBER found 26%, [26] 29%, [3] and 65% [27] association of the EBV with tonsillitis. While in our study the prevalence of EBV infection in tonsils is 43%, which is intermediate between these studies and reflects a possible geographical difference in the prevalence of EBV.

It is noteworthy to mention here that the prevalence of EBV (43%) in our specimens is close to its prevalence in Hodgkin lymphoma among UAE national (38%) [28], which may reflect a causal relationship between EBV and Hodgkin lymphoma.

Identification of a high prevalence (43%) of EBV-EBER in tonsillectomy specimens in children suggests that the tonsils are the main reservoir for the EBV, and that this virus may be involved in tonsillar enlargement. On the other hand, the prevalence of EBV in adenoids (15%) is lower than in tonsils (43%), which indicate a lower association between adenoid hypertrophy and EBV infection. This finding is different from that found by Endo et al. [21] which found the prevalence of EBV to be higher in adenoids (57%) than tonsils (29%) by using similar method for detection (ISH), suggesting sampling and geographical differences. In this study, the immunohistochemical staining method was used to detect LMP-1 in tonsils and adenoids with the aim of strengthening our results in combination with EBER-ISH. The results are not encouraging since we detect EBV in only 5 cases (11%) and in few cells within the interfollicular area. This finding is less than that reported by Dias et al. [16], indicating a lesser expression of LMP-1 in EBV infected cells. In addition, it indicates that using EBER-ISH is the gold standard for detecting EBV in tissue. Moreover, the use of EBER-ISH in the detection of EBV is more suitable since we can use it on paraffin-embedded tissue, which allows us to test archive material when required. This makes ISH superior to immunohistochemistry in the detection of EBV in tissue.

All detected EBV-infected cells in this study were CD20-positive B-cells and were located predominantly in the interfollicular regions. Nearly 90% of the EBER-positive cells were located within the T-cell-rich interfollicular regions of the tonsils, while a significant number (10%) were located within the lymphocyte-rich follicular region. These results are consistent with previous studies [22,29].

Almost 10% of EBER-positive cells, all of which stained with CD20 B-cells, were located within the B-cell-rich germinal centers of secondary follicles. Few follicles contained numerous EBER-positive cells. These results are consistent with previous reports of EBER-positive germinal center B-cells in tonsils [30]. It has been suggested that germinal center centroblasts may be a site of EBV persistence [31]. The presence of EBV-positive B-cells in germinal centers is reportedly more common in tonsils from areas endemic for EBV related lymphomas; leading to the suggestion that EBV-infected germinal center B-cells may be more prone to malignant transformation, perhaps due to somatic hypermutation [30]. In this study EBER-positive cells were found within germinal centers of tonsils and adenoids, and some of these cells were atypical and large and might be a precursor for malignant transformation. Although the pattern of distribution of EBER-positive B lymphocytes in the interfollicular regions is random, they have 3 distinct patterns within lymphoid follicles; either within germinal center cells, or mantle zone cells or in the interphase between them. The

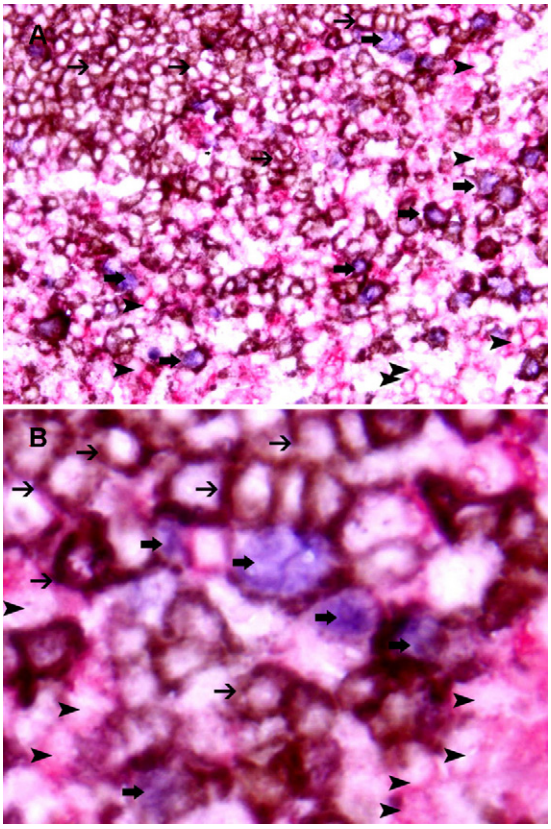

Fig. 4. Triple stain of EBER, CD20 and CD3 in enlarged tonsils and adenoids. B lymphocytes show membranous brown staining for CD20 (thin arrows) while the T lymphocytes show membranous red staining for CD3 (arrow heads). Only some of the B lymphocytes show blue nuclear staining for EBER in situ hybridization (thick arrows): A, 400× and B 1000×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

interphase pattern is very interesting and involves some follicles which have one or more EBER –positive atypical cell within the germinal center. The interphase EBER-positive cells are distributed circumferentially in a round shape at the interphase between germinal center cells and mantle zone cells (Fig. 1C and D) as if they were generated from cell division of EBV-infected germinal center cells and moving outside the lymphoid follicle. Whether this pattern has any role in EBV-persistent infection in tonsils and adenoids or carrying any risk for future malignant transformation needs to be determined in future studies.

On the other hand, EBER was not identified within T-lymphocytes when we use triple staining for EBER, CD20 and CD3 (Fig. 4). Hudnall et al. [22], shows that EBV is rarely found in T lymphocytes in EBV-infected tonsils. There is some controversy regarding the role of oropharyngeal epithelial cells in EBV infection. It has been suggested that primary and persistent EBV infection might be mediated through oropharyngeal epithelial cells [32]. The support for this perception has come mainly from infection detected in desquamated oropharyngeal epithelial cells from patients with infectious mononucleosis by in situ hybridization [33]. However, there is now increasing evidence pointing to B lymphocytes as the likely site of persistent EBV infection, and also as the possible target of primary EBV infection [34]. Several studies have demonstrated EBV replication within the upper epithelial cell layers in oral hairy leukoplakia and are not accompanied by detectable latent infection of basal epithelial cells [35,36]. Thus, these results do not support the idea that EBV persists in epithelial cells. In our study we do not identify EBV in epithelial cells of tonsils and adenoids whereas it is only detected in B lymphocytes. We think that EBV infection might be mediated through oropharyngeal epithelium at early stages of acute infection but later on, the virus might have left epithelial cells or the infected epithelial cells died as a result of host antiviral immunological reaction, hence we do not have EBV persistence and latency within epithelial cells in our samples. On the other hand, we believe that B lymphocytes are sites of primary infection and latency in tonsils and adenoids.

In conclusion, EBV is associated with tonsillar hypertrophy and is prevalent in 43% of our cases. EBV is only detected in B lymphocytes and we believe that B lymphocytes are sites of primary infection and latency. In situ hybridization is the gold standard for the detection of EBV in tissue.

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