One-step typing of Epstein–Barr virus by polymerase chain reaction: predominance of type 1 virus in Japan

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The prevalence of two types of Epstein–Barr virus (EBV) in Japan was studied by using the polymerase chain reaction (PCR). The U2 region encoding EBV nuclear antigen 2 (EBNA-2) was chosen as the target of amplification. Consensus primers were synthesized from sequences common to the two types but encompassing a large stretch of deletion in the sequence of type 1 EBV. The primers were capable of amplifying both types at the same time but allowed differentiation of each type by the size of the amplification products. Thus we could carry out detection and typing of EBV in a one-step PCR. EBV was detected in mouth washings of 21 (23%) of 91 seropositive healthy adults. Twenty samples (22%) contained type 1 and only one (1%) type 2. Seventy-nine patients suffering from various types of tonsillitis were also studied. EBV was detected in mouth washings of 37 patients (47%). Thirty-four (43%) contained type 1 and three (4%) type 2. Double infection was not seen in either healthy donors or patients. These results indicate that type 1 EBV is highly dominant and the type 2 variant is quite rare in Japan.

Epstein–Barr virus (EBV) is a ubiquitous B lymphotropic human herpesvirus. Most people apparently have primary infection during early childhood without noticeable symptoms and the virus persists in the haematopoietic system for the rest of life with intermittent excretion in saliva. Primary infection in young adults, however, often causes a self-limiting lymphoproliferative disease, infectious mononucleosis. EBV is also aetio logically associated with human cancers such as endemic Burkitt's lymphoma, nasopharyngeal carcinoma and EBV-associated lymphoma in immunocompromised patients (Henle & Henle, 1985). Furthermore, the list of EBV-associated diseases has been growing (Leyvraz et al., 1985; Greenspan et al., 1985; Fox et al., 1986; Weiss et al., 1987; Harabuchi et al., 1990).

It is now known that there are two types of EBV. Type 1 (or type A), the prototype of EBV, has been studied for the last 27 years (Epstein et al., 1964). Type 2 (or type B) was recently discovered in some Burkitt's lymphomas of patients from central Africa and New Guinea. This differs from type 1 in the BamHI YH region encoding EBV nuclear antigen 2 (EBNA-2) (Adldinger et al., 1985; Zimber et al., 1986), and speculations such as a restricted geographical distribution and a close association with Burkitt's lymphoma were raised. Recent investigations, however, revealed that the type 2 virus was almost as prevalent as the type 1 virus in normal populations not only in the endemic area of Burkitt's lymphoma but also in the U.S.A. (Young et al., 1987; Sixbey et al., 1989). EBV is known to immortalize human B cells easily in vitro and EBNA-2 functions as the principal element for this activity (Skare et al., 1985; Wang et al., 1987). It has been shown that the type 2 virus is much less efficient in immortalization of B cells than the type 1 virus (Rickinson et al., 1987). This may explain at least partly why the type 2 virus had long remained undiscovered. It is yet to be fully investigated as to whether type 2 EBV differs in any way from type 1 virus in terms of geographical distribution or disease association.

The polymerase chain reaction (PCR) is a powerful technique for detection of a specific DNA sequence in a small amount of sample (Saiki et al., 1988). In the present study, we have established sensitive methods for detection and typing of EBV by PCR. Consensus primers were selected from conserved sequences in the U2 region encoding EBNA-2 (Dambaugh et al., 1984). The two types could be differentiated easily by the size of the PCR products due to a long stretch of deletion in the type 1 sequence. Type-specific primers were also developed from divergent sequences in the EBNA-2 region. The PCRs were employed for detection and typing of EBV excreted in saliva from healthy adults and also from patients with various types of tonsillitis. We have found that the type 1 virus is highly dominant and that the incidence of type 2 virus is quite low in the Kansai area of Japan.

The cell-lines B95-8 (type 1-positive), Raji (type 1-
positive), Jijoye (type 2-positive), AG876 (type 2-positive), Akata (determined as type 1-positive in the present study) (Takada et al., 1991), BJAB and K562 (EBV-negative) were maintained in RPMI-1640 supplemented with 10% foetal bovine serum (FBS) (for information on the EBV types in the cell lines, see Zimber et al., 1986). Akata and AG876 were kindly provided by Dr K. Takada (Nihon University, Tokyo, Japan) and Professor A. B. Rickinson (University of Birmingham, Birmingham, U.K.), respectively. Cellular DNA samples used as standards were prepared by digestion with proteinase K and RNase, extraction with phenol and chloroform–isoamyl alcohol, and precipitation with ethanol as described previously (Sambrook et al., 1989). For estimation of the detection sensitivity of PCR, EBV-positive cells serially diluted 10-fold, were mixed with a constant number of EBV-negative cells, and each cell mixture was pelleted, suspended with 30 µl of buffer (10 mM-Tris–HCl pH 7.4, 1 mM-EDTA) containing 0.05% Triton X-100 and lysed by boiling for 10 min. After centrifugation at 7000 g for 10 min, supernatants were used for PCR. Saliva samples were collected from healthy adults (personnel of the Shionogi Institute for Medical Science, and medical personnel and students of Wakayama Medical College), and patients with various types of tonsillitis (attending Wakayama Medical College Hospital) by mouth washing with 9 ml of PBS. One ml of FBS was added to each sample and low-speed centrifugation was done to remove cell debris. Each supernatant fluid was then passed through a 0.8 µm filter and stored at −80 °C until DNA preparation as follows. The samples were centrifuged at 45 000 g for 1 h at 20 °C and pelleted materials were resuspended in 500 µl of buffer (10 mM-Tris–HCl pH 7.5, 10 mM-EDTA) containing 0.5% SDS and 200 µg/ml of proteinase K. After incubation at 37 °C overnight, DNA was extracted with phenol and chloroform, precipitated with ethanol, and resuspended in 200 µl of distilled water. Twenty µl of each sample was used for each PCR amplification.

Oligonucleotides used for primers and probes were synthesized on a DNA synthesizer (Cyclone, MilliGene/Biosearch) and purified by Nensorb Prep cartridges (E. I. du Pont de Nemours & Co.) and HPLC. PCR was carried out using a Gene Amp kit (Perkin-Elmer Cetus). DNA samples were denatured at 94 °C for 10 min, and placed in 100 µl of reaction mixture containing 50 mM-KCl, 10 mM-Tris–HCl pH 8.3, 1.5 mM-MgCl2, 0.01% gelatin, 0.5 µg of each primer, 0.3 mM (for general PCR) or 0.4 mM (for type-specific PCRs) of each dNTP and 2.5 units of Taq polymerase. Amplification was carried out by 40 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 2 min and extension at 72 °C for 3 min on a DNA Thermal Cycler (Perkin-Elmer Cetus). After amplification, each reaction mixture was extracted once with phenol and once with chloroform–isoamyl alcohol. One-tenth of each reaction mixture was then analysed by electrophoresis on a 6% polyacrylamide gel and staining with ethidium bromide (Sambrook et al., 1989). For hybridization with probes, DNA fragments separated by gel electrophoresis were denatured with a solution of 1·5 M-NaCl and 0·5 M-NaOH and transferred onto a Hybond N nylon membrane (Amersham) with a solution of 1·5 M-NaCl and 0·25 M-NaOH. Prehybridization was carried out at Tm−15 °C (Sambrook et al., 1989) with 5 × saline–sodium phosphate–EDTA (SSPE) buffer, 0·01% SDS, 1 × Denhardt’s solution and 200 µg/ml of

Table 1. Sequences of oligonucleotide primers and probes synthesized from the U2 region of EBV and used for general and type-specific PCRs

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Base positions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>For general PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (sense)</td>
<td>+5’CTGTGGTTGCGACGCTGCA</td>
<td>1425–1447</td>
</tr>
<tr>
<td>B (sense)</td>
<td>+5’TGCCAACACCTTAAGCA</td>
<td>1444–1463</td>
</tr>
<tr>
<td>C (sense)</td>
<td>+5’TTGCTTGTGCTTATGCT</td>
<td>1471–1490</td>
</tr>
<tr>
<td>D (sense)</td>
<td>+5’TGAAACCGTACCTTTC</td>
<td>1566–1583</td>
</tr>
<tr>
<td>E (antisense)</td>
<td>−5’GGCTCTGTTCGGCATCCCT</td>
<td>1835–1813</td>
</tr>
<tr>
<td>F (sense)</td>
<td>+5’TCTCAGATACATGAAC</td>
<td>2771–2789</td>
</tr>
<tr>
<td>G (sense)</td>
<td>+5’CAATGTATCCTTAAATG</td>
<td>2994–3013</td>
</tr>
<tr>
<td>H (antisense)</td>
<td>−5’GTGTTGGAGAAGTAGCTAAC</td>
<td>3095–3077</td>
</tr>
<tr>
<td>I (antisense)</td>
<td>−5’GGCAAGGTGTGAGAACA</td>
<td>3149–3130</td>
</tr>
<tr>
<td>For type 1-specific PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J (sense)</td>
<td>+5’ACAACCACCTATGC</td>
<td>2079–2098</td>
</tr>
<tr>
<td>K (antisense)</td>
<td>−5’ACCGTGTTCTGGAATCT</td>
<td>2338–2319</td>
</tr>
<tr>
<td>For type 2-specific PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L (sense)</td>
<td>+5’GGATTACCTAGCAGCATC</td>
<td>2088–2107</td>
</tr>
<tr>
<td>M (antisense)</td>
<td>−5’GGAGGAGGTGCTGTAAT</td>
<td>2340–2324</td>
</tr>
</tbody>
</table>

*The base positions are those from Dambaugh et al. (1984).
salmon sperm DNA. Hybridization was carried out at
\( T_m-15 ^\circ C \) in the same solution containing probes
labelled by using \([\text{^{32}P}]d\text{dATP} \) and a 3' end-labelling kit
(Amersham). Membranes were washed at \( T_m-15 ^\circ C \) for
1 h with three changes of 5 \( \times \) SSPE and 0.01% SDS.
Autoradiography was carried out overnight at room
temperature using Fuji X-ray films.

A substantial sequence divergence exists in the U2
region of EBV encoding EBNA-2 between strains B95-8
(type 1) and AG876 (type 2) (Dambaugh et al., 1984).
The 5' one-third and the 3' one-third are relatively
conserved, whereas the middle one-third is highly
divergent. Furthermore, there are several long stretches
of deletions in the nucleotide sequences of both types.
We synthesized various oligonucleotides based on
sequences chosen from the U2 region and tested their
suitability as PCR primers for detection and typing of
EBV (Table 1). Different combinations of primers were
tried for specific amplification using DNA samples
prepared from standard human cell lines. General PCR
capable of detecting and typing with a single set of
primers was tried by using primer pairs common to both
types and encompassing a deletion in either type. As
shown in Fig. 1(a), a primer set of F and I produced
amplification products with a size of about 350 bp from
type 1 and about 400 bp from type 2. Faint bands
Corresponding in size to dimers and trimers were also
seen. The specificity of the amplified products was
confirmed by Southern blot hybridization using the
oligonucleotides G and H as probes (Fig. 1b). A primer
set of F and H also produced good specific amplifications
but other combinations of primers were dropped because
of high non-specific amplifications (data not shown). For
a type-specific PCR, primer pairs J-K and L-M
produced good specific amplifications for types 1 and 2,
respectively (data not shown).

DNA samples prepared from cell mixtures which were
composed of 10-fold serially diluted Raji cells (type 1-
positive) or AG876 cells (type 2-positive) and a constant
number of BJAB cells (EBV-negative) were subjected to
amplification using the general and type-specific PCRs.
As shown in Fig. 2, the general PCR was capable of
producing a band clearly detectable by ethidium bro-
mide staining or Southern blot hybridization when the
reaction mixture contained DNA from at least 10 Raji or
AG876 cells. Similarly, the type 1-specific PCR pro-
duced a band clearly detectable by ethidium bromide
staining from a reaction mixture containing DNA from
at least 10 Raji cells, whereas the type 2-specific PCR
detected EBV from a reaction mixture containing DNA
from a single AG876 cell (data not shown).

To determine the prevalence of EBV types 1 and 2 in
the Kansai area of Japan, the general PCR was used to
detect and type EBV excreted in saliva samples obtained
from 100 healthy adults. Fig. 3 illustrates typical
PCR reactions and the results are summarized in Table
2. From standard serological studies for anti-EBNA,
anti-VCA and anti-EA carried out as described previ-
Fig. 2. Detection sensitivity of general PCR. A cell suspension containing type 1-positive Raji cells (a) or type 2-positive AG876 cells (b) was serially diluted 10-fold and mixed with a cell suspension containing a constant number of EBV-negative BJAB cells. Mixed cells were pelleted, lysed and subjected to PCR using primers F and I. After separation by gel electrophoresis, PCR products were analysed by ethidium bromide staining (left) and Southern blot hybridization using 3' end-labelled oligonucleotides G and H as probes (right). Lanes are as follows. M, φX174 DNA digested with HaeIII; 1, 1 × 10^6 Raji or AG876 cells + 2.5 × 10^4 BJAB cells; 2, 1 × 10^4 Raji or AG876 cells + 2.5 × 10^5 BJAB cells; 3, 1 × 10^3 Raji or AG876 cells + 2.5 × 10^5 BJAB cells; 4, 1 × 10^2 Raji or AG876 cells + 2.5 × 10^5 BJAB cells; 5, 10 Raji or AG876 cells + 2.5 × 10^5 BJAB cells; 6, 1 Raji or AG876 cells + 2.5 × 10^5 BJAB cells; 7, 2.5 × 10^5 BJAB cells.

Table 2. Detection and typing of EBV in mouth washings from healthy donors and patients with various types of tonsillitis

<table>
<thead>
<tr>
<th>Donor</th>
<th>Number of donors</th>
<th>Mean age (range)</th>
<th>PCR-positive for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy seronegative</td>
<td>9</td>
<td>25 (21–32)</td>
<td>0 0 0 9</td>
</tr>
<tr>
<td>Healthy seropositive</td>
<td>91</td>
<td>28 (19–51)</td>
<td>20 (22%) 1 (1%) 0 70</td>
</tr>
<tr>
<td>Patients with acute tonsillitis</td>
<td>17</td>
<td>31 (12–48)</td>
<td>8 (47%) 1 (6%) 0 8</td>
</tr>
<tr>
<td>Patients with chronic tonsillitis</td>
<td>19</td>
<td>20 (11–59)</td>
<td>6 (31%) 0 0 13</td>
</tr>
<tr>
<td>Patients with focal tonsillitis with palmoplantar pustulosis</td>
<td>34</td>
<td>44 (17–63)</td>
<td>16 (47%) 2 (6%) 0 16</td>
</tr>
<tr>
<td>Patients with focal tonsillitis with IgA nephropathy</td>
<td>9</td>
<td>28 (13–48)</td>
<td>4 (44%) 0 0 5</td>
</tr>
</tbody>
</table>
with consensus primers F and I. Products were analyzed by gel electrophoresis and ethidium bromide staining. Lanes are as follows. 1, B95-8; 2, BJAB; 3, AG876; Lanes 4 to 11, individual samples from healthy donors; M, φX174 DNA digested with HaeIII.

Fig. 3. PCR detection and typing of EBV in mouth washings. Samples were subjected to PCR amplifications using consensus primers F and I. Products were analyzed by gel electrophoresis and ethidium bromide staining. Lanes are as follows. 1, B95-8; 2, BJAB; 3, AG876; Lanes 4 to 11, individual samples from healthy donors; M, φX174 DNA digested with HaeIII.

ously (Henle & Henle, 1982), nine were found seronegative for EBV. No EBV was detected in mouth washings from these seronegative donors. On the other hand, among the 91 seropositive donors, 21 (23%) were found to excrete EBV. Twenty donors were found to excrete type 1 virus, whereas only one was found to excrete type 2 virus. The typing results obtained by the general PCR were confirmed by type-specific PCRs.

Latent EBV is considered to be reactivated by various immunological or inflammatory stimulations. Therefore, we also tested EBV excretion in saliva samples collected from patients suffering from various types of tonsillitis. Our rationale was that these patients would give a higher rate of EBV excretion than healthy donors and, therefore, assist our study by increasing the total number of EBV-positive samples. Furthermore, a linkage might be demonstrated between a particular disease and EBV type or rate of reactivation. All the tested patients were confirmed to be EBV seropositive. As shown in Table 2, among 17 patients suffering from acute tonsillitis, nine (53%) were found to excrete EBV. Eight patients were excreting type 1, and one type 2. Similarly, among 19 patients with chronic tonsillitis, six (32%) were found positive for type 1. In cases of focal infection of the tonsils with palmoplantar pustulosis, 18 of 34 patients (53%) were excreting EBV. Sixteen were found to excrete type 1, whereas two were excreting type 2. In the case of focal infection of tonsils with IgA nephropathy, four out of nine patients (44%) were found to excrete type 1 EBV. All the typing results obtained by the general PCR were confirmed by the type-specific PCRs.

The serological tests measuring antibody titres against EBV-encoded antigens such as EBNA, EA and VCA (Henle & Henle, 1982; Kure et al., 1986) provide information useful for assessment of the state of EBV infection in the host such as primary infection, latent infection and reactivation. On some occasions, however, a direct demonstration of EBV may be required. Immunological detection of EBV-encoded antigens in infected cells and tissues has been widely used, but possible concerns about specificity and/or sensitivity may sometimes undermine the results obtained. In recent years highly sensitive and specific detection methods employing hybridization techniques on a membrane filter or in situ have become available. Furthermore PCR, which is often capable of detecting even a single copy of a target sequence, has come to be applied more and more for the detection of EBV sequences (Sixbey et al., 1989; Saito et al., 1989; Telenti et al., 1990; Gopal et al., 1990; Chang et al., 1990; Crouse et al., 1990). In the present study, we have developed general and type-specific PCRs useful for detection and typing of EBV. The primers are all chosen from the U2 region of EBV which encodes EBNA-2 and is known to be quite divergent between the two types (Dambaugh et al., 1984). The primer sequences for the general PCR are a consensus for both types but encompass a large stretch of deletion in the type 1 sequence. Therefore, it can amplify both types with a single pair of primers and yet differentiate each type based on the size of amplification products. The primers for type-specific PCRs are selected from a region of EBNA-2 highly divergent between the two types. In the present study, we used the general PCR for primary examinations, and the type-specific PCRs for confirmatory assays. Sixbey et al. (1989) also developed a general PCR for detection and typing of EBV using primers synthesized from the EBNA-2 region. Their primers were chosen from sequences common to both types but encompassing sequences highly divergent between the two types. After PCR amplification, the determination of EBV types had to be done by hybridization analysis using type-specific probes. Virus typing by our general PCR is based on the size of the PCR products and, therefore, can be done at the level of ethidium bromide staining without any need for such hybridization analysis.

Employing PCR for detection and typing of EBV in throat washings, Sixbey et al. (1989) found that the type 2 virus was almost as prevalent as the type 1 virus in healthy adults and immunocompromised patients in Memphis, Tennessee, U.S.A. Also there were people infected with both types. In the present study, we also determined the detection frequency and type distribution of EBV in saliva samples from healthy adults and patients with various types of tonsillitis by using PCR. In
contrast to the results of Sixbey et al. (1989), we found that EBV detected in our samples was predominantly the type 1 virus and the incidence of the type 2 virus was low (Table 2). The detection sensitivities of our general PCR for type 1 and for type 2 are apparently not grossly different (Fig. 2). Our overall positive rate of EBV in saliva samples from seropositive healthy adults is 23%, a value which is quite similar to those obtained by other researchers also using PCR techniques (Sixbey et al., 1989; Gopal et al., 1990). Therefore, it is not likely that the observed low frequency of type 2 virus in our samples is due to low detection sensitivity of our general PCR. It appears, therefore, that, at least in the Kansai area of Japan, more than 90% of seropositive people are infected with the type 1 virus. It is also noteworthy that those rare people harbouring type 2 virus were not doubly infected with type 1 virus. Since most children in Japan are seroconverted within 1 year of age (Kure et al., 1986), transmission between family members seems to be the main route of EBV infection in Japan. Afterwards, reinfection might be mostly prevented by the immune system. Gratama et al. (1990) also indicated the importance of familial transmission through analysis of EBV strains isolated from individual members of seven families in The Netherlands and Sweden. Our results thus put forward a question concerning the geographical and/or ethnic distribution of the type 2 virus.

We also studied the excretion of EBV in patients with various types of tonsillitis. These patients were selected in the present study because infection and/or inflammation of the mesopharynx would reactivate and, therefore, increase the rate of excretion of EBV. Patients with acute tonsillitis indeed showed a rate of EBV excretion about twice that of healthy donors. Therefore, inflammatory processes may enhance reactivation and excretion of EBV in saliva. Patients with chronic tonsillitis, however, showed a less increased rate of excretion than those with acute tonsillitis. Interestingly, patients with focal tonsillar infection accompanied by palmoplantar pustulosis (Thomsen & Osterbye, 1973) or IgA nephropathy (Berger, 1969) showed a much higher rate of EBV excretion than those with conventional chronic tonsillitis. Such differences may suggest unique inflammatory processes in the former two diseases. Indeed, these are considered to be a group of tonsillar diseases with a probable involvement of autoimmune pathological processes (Kuki & Tabata, 1988; Hayashi & Tabata, 1988; Masuda et al., 1988).

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References


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