PCR amplification is more sensitive than tissue culture methods for Epstein–Barr virus detection in clinical material

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In this study we have compared the use of PCR and conventional tissue culture methods to detect Epstein–Barr virus (EBV) in peripheral blood mononuclear cells and throat wash samples. The study population included 29 healthy adult and 20 immunocompromised EBV-seropositive donors. The results show significantly higher EBV detection rates by PCR than the tissue culture methods in throat wash samples from both donor groups ($P < 0{\cdot}01$ in healthy donors and $P < 0{\cdot}009$ in the immunocompromised donors) and in peripheral blood from the immunocompromised but not from the healthy donors ($P < 0{\cdot}008$). Furthermore, when EBV DNA detection rates in throat wash cell pellet and supernatant fluid were compared, a higher positive result was obtained with the cell pellets which reached statistical significance in the immunocompromised group ($P < 0{\cdot}02$). No correlation was found between positivity in throat wash and peripheral blood from the same donors.

Epstein–Barr virus (EBV) is a B lymphocyte-immortalizing virus which has a worldwide distribution with over 90% of the adult population showing evidence of past infection (Henle & Henle, 1966). Primary infection occurs during childhood and is usually asymptomatic. Around 50% of individuals whose primary infection is delayed until adolescence, however, develop acute infectious mononucleosis (Henle *et al.*, 1968).

After primary infection a life-long carrier state is established with low-level virus replication in the oropharynx and shedding of the infectious virus into the oral cavity, and a few circulating B lymphocytes carrying the virus in a latent form (Sixbey *et al.*, 1984; Golden *et al.*, 1973; Nilsson *et al.*, 1971). Conventionally, biological assays have been used to study these two parameters of persistent EBV infection in normal and immunocompromised individuals. When cultured, EBV-carrying B cells are induced into a lytic infection and the virus produced infects other B cells co-resident in the culture, giving rise to 'spontaneous' outgrowth of EBV-immortalized lymphoblastoid cell lines (LCL) (Nilsson *et al.*, 1971; Rickinson *et al.*, 1977). Similarly, EBV can be rescued from throat washings of 10–25% of seropositive individuals by immortalization of lymphocytes from EBV-seronegative donors in culture (Golden *et al.*, 1973; Chang *et al.*, 1973). These biological assays are time-consuming, vary widely in sensitivity and have certain intrinsic problems. Spontaneous outgrowth detects EBV-carrying cells capable of virus production *in vitro* which may represent only a subset of the total, and non-immortalizing or B strain virus, which is a less powerful immortalizing agent than the more common A strain (Rickinson *et al.*, 1987), may not be detected in either assay.

Recently, the highly sensitive PCR method has been used to detect EBV DNA, and in the present study we have compared the sensitivity of conventional tissue culture methods with PCR techniques to detect EBV in paired blood and throat washing samples from healthy adult controls and immunocompromised transplant recipients.

Heparinized peripheral blood and throat washings in 10 ml of saline were collected on a single occasion from 29 EBV-seropositive and 7 seronegative adult healthy controls and on 38 occasions from 20 heart and/or lung transplant recipients from Harefield Hospital, Middlesex, UK. All transplanted patients were receiving cyclosporin A and azathioprine immunosuppressive therapy. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll–Hypaque density gradients (Boyum, 1968). The E negative (B cell-enriched) populations of PBMC were then separated by rosetting with sheep red blood cells using conventional techniques (Kaplan & Clarke, 1974). The E negative (unrosetted) cells were seeded in flat-bottomed microtitre plates at a concentration of 2×10^5 cells per well in a 200 µl volume of tissue culture medium [RPMI 1640 containing glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and foetal calf serum (10%, v/v)], cultured for 6–8 weeks and scored for the appearance of proliferating clumps of immortalized cells.

Following bench centrifugation, the throat wash samples were split into the cell pellet (CP) and the supernatant fluid

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Table 1. Comparison of tissue culture and PCR techniques in detecting EBV in clinical samples

Results are expressed as the number of positive samples/the number of samples tested. The numbers in parentheses denote the percentage of positive samples.

Clinical material	Method	Healthy donors	Transplant patients
PBMC	Spontaneous LCL	12/29 (41)*	18/38 (47)*
	PCR	15/29 (52)	30/38 (79)
Throat washings (TW)	Immortalization assays	4/29 (14)*	9/38 (24)*
	PCR	14/29 (48)	21/38 (55)
Cell pellets from TW	PCR	17/29 (59)	31/38 (81)

* All the samples positive by biological assays were also positive by PCR.

portion (designated TW). CP samples were washed in PBS and the dry pellet used for DNA extraction. TW was filtered through a 0·6 μm sterile syringe filter unit (Millipore) and 10 ml was concentrated by ultracentrifugation at 24000 ***g*** (Ti 50 rotor, Beckman) for 2 h at 4 °C in a sterile tube. The resulting pellet was resuspended in 100 μl of tissue culture medium. To rescue virus, 50 μl of concentrated TW was used to infect 2×10^6 PBMC from a known EBV-seronegative healthy individual for 1 h at 37 °C. The cells were then washed once and seeded in a flat-bottomed microtitre plate at a concentration of 2×10^5 cells per well. After 6–8 weeks, any proliferating foci of immortalized cells were expanded.

DNA was extracted from PBMC and CP by routine methods (Sambrook *et al.*, 1989). Concentrated TW (50 μl) was treated with 100 μg/ml of Proteinase K (Promega) for 1 h at 37 °C and boiled for 10 min. DNA (1 μg) and 20 μl of throat washing were used as PCR templates. Sterile distilled water was always included as DNA template-free control. All samples were amplified on at least two occasions. Human *β*-globin primers were used as internal control to screen all DNA samples (Saiki *et al.*, 1985). Only those DNA samples positive by *β*-globin PCR were used for further analysis. For EBV detection, PCR was carried out using highly sensitive primers which amplify a sequence of the EBV genome within the EBNA 2 gene (Boyle *et al.*, 1991). PCR products (10 μl) were run on 2% agarose gel and Southern hybridized using [α-^{32}P]dCTP-labelled EBNA 2-specific plasmid probes (Adldinger *et al.*, 1985). All samples were found to be positive for type A strain of EBV only. PCR using type-B specific primers did not yield any positive results in any sample (data not shown).

The tissue culture results were compared with those from PCR by a χ^2 test. The EBV negative controls were not included in the calculation. LCL could be grown from peripheral blood B lymphocytes by spontaneous outgrowth from 12 out of 29 (41%) normal seropositive donors and 18 out of 38 (47%) transplant samples (Table 1). Amplification using primers EBNA 2 on PBMC DNA showed positive amplification in 15 of the healthy donors (52%) and 30 transplant samples (79%) (Table 1). All samples from which spontaneous LCLs could be grown also had detectable EBV DNA in their PBMC, whereas in 15 samples, EBV DNA was detected in PBMC though no spontaneous LCL could be grown from B cells. None of the PBMC from the 7 EBV-seronegative donors gave spontaneous outgrowth of LCL or had amplifiable EBV DNA.

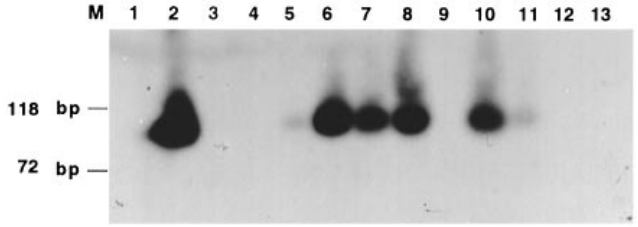

Fig. 1. PCR analysis of DNA from cell pellets of throat washings from healthy adults. DNA was amplified using EBNA 2 primers. Products (10 μl) were separated on 2% agarose gel and Southern hybridized to ^{32}P-labelled EBNA 2-specific probe. Lane M, *Hae*III-digested ϕX174 DNA size markers; lane 1, sterile distilled water; lane 2, B95-8 DNA as EBV-positive control; lanes 3–12, DNA from cell pellets from 10 healthy controls; lane 13, Ramos DNA as EBV-negative control. Throat washing supernatants from controls in lanes 5 and 11 were negative by PCR and by immortalization assay.

Four out of 29 (14%) in the healthy control group and 9 out of 38 (24%) transplant samples had immortalizing EBV in their throat washings which could be rescued by co-culture with B cells from a seronegative donor (Table 1). All samples positive by immortalization assay were also positive by PCR. PCR amplification was successful from TW of 14 out of 29 (48%) of healthy controls and 21 out of 38 (55%) transplant samples (Table 1). The same EBV-specific band was detected in CP from 17 out of 29 (59%) of controls and 31 out of 38 (81%) of transplant samples. Fig. 1 shows the results of PCR on CP DNA from some of the healthy adults. In all samples where TW was positive for EBV DNA, CP also yielded a positive band, whereas 13 additional CP samples gave positive results

although no EBV could be detected in TW. No EBV was found in TW or CP from the 7 EBV-seronegative donors.

The detection of EBV DNA by PCR was significantly higher than the detection of EBV by *in vitro* immortalization for TW and CP samples from in both healthy donors ($P < 0{\cdot}01$ for TW and $P < 0{\cdot}001$ for CP) and transplant recipients ($P < 0{\cdot}009$ for TW and $P < 0{\cdot}000001$ for CP), and in transplant recipients for PBMC ($P < 0{\cdot}008$). In addition, PCR analysis of EBV DNA in CP yielded significantly higher positive detection rate than TW in transplant recipients ($P < 0{\cdot}02$). There was no correlation between positivity in throat wash and PBMC samples from the same individual.

In this study the same throat washing and blood samples were subjected to EBV detection by the *in vitro* immortalization assays and by PCR amplification in order to compare the two methods. This was undertaken because the reported frequency of detection of EBV in PBMC and throat washing by either method differs considerably between different published studies. Thus, for PBMC from normal donors, PCR detection rates range from 0 to 54% (Gopal *et al.*, 1990; Martinez *et al.*, 1995); whereas reports of successful spontaneous outgrowth is variable with up to 96% detection rate where each donor was tested on several occasions over a period of 15 months (Yao *et al.*, 1985). In the case of throat washings from normal donors, published reports using *in vitro* immortalization give between 10 and 25% positive results (Golden *et al.*, 1973; Chang *et al.*, 1973); in contrast, for PCR analysis, the detection rates range from 22 to 70% (Sixbey *et al.*, 1989; Yao *et al.*, 1991).

Our results clearly show that in all cases PCR was more sensitive than the cell culture method and the difference reached statistical significance for PBMC from transplant recipients ($P < 0{\cdot}008$), and throat washings from healthy controls ($P < 0{\cdot}01$ for TW and $P < 0{\cdot}001$ for CP) and from transplant recipients ($P < 0{\cdot}009$ for TW and $P < 0{\cdot}000001$ for CP). Furthermore, PCR detected EBV in all the samples that were positive by biological assays.

The overall detection of EBV DNA by PCR was significantly higher in throat wash cell pellets than filtered concentrated supernatants ($P < 0{\cdot}02$ for transplant samples). The highest published detection rate for EBV in throat wash is 70%, and this is using cell pellets as templates (Yao *et al.*, 1991). This finding probably reflects the fact that, like other herpesviruses, EBV is a cell-associated virus and is therefore more likely to be detected in cells shed from the oropharynx than the cell-free supernatant fluid.

PCR is a rapid, sensitive and specific method which can be used to detect specific DNA or RNA directly on small amounts of non-sterile clinical material. For EBV detection this overcomes the problem of time-consuming and unpredictable tissue culture methods required for the outgrowth of LCLs. However, it must be remembered that the sensitivity of PCR is dependent on the selection of primer pairs and on the optimization of different PCR parameters. We attempted PCR using primers which amplify different regions of the EBV genome (e.g. *Bam*HI W, *Bam*HI K, EBNA 6) and found EBNA 2 primers to be the most sensitive. These EBNA 2 primers were sensitive enough to detect 1–2 copies of EBV genome (from one Namalwa cell) in a background of a million EBV-negative cells (data not shown). Samples with low copy number of EBV genome (< 1 genome per 10^6 PBMC) would therefore not be detected by our PCR. PCR analysis will detect latent cell-associated virus as well as free virus whereas the biological assay detects biologically active virus particles. Therefore, PCR cannot differentiate between immortalizing and non-immortalizing, biologically active and inactive virus, nor can it determine the cell type harbouring the virus.

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