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# Detection of herpes simplex virus-1 by nested PCR. An experimental model[1]

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**Abstract**

Nested polymerase chain reaction (nested PCR) was performed using a reaction mix batch-prepared and kept frozen in single reaction tubes at −20°C until use. Twenty-one New Zealand white rabbits were infected with herpes simplex virus type 1 (HSV-1). Eleven animals were killed on day seven and the other ten were sacrificed on day 21. Viral culture and nested PCR was used to determine the presence of HSV-1 in samples from the tongue, HSV-1 was detected in 90.47% of the animals; in 84.21% by nested PCR and in 52.63% by culture. Nested PCR assay had greater sensitivity than culture in animals sacrificed on day seven with significative difference ($p < 0.05$). Higher sensitivity and faster results were obtained with this method, so we found it reliable and useful in the setting of a clinical laboratory dealing with diagnosis of herpes virus infections. 

*Keywords:* HSV-1; Facial palsy; Nested PCR; Viral culture

## 1. Introduction

A broad spectrum of diseases has been related to HSV-1 infection [1]; some of these are self-limiting like herpes labialis, some are mild compromising normal function of involved organs such as keratoconjunctivitis, but others like herpes encephalitis are life-threatening. In some cases, the relationship between HSV-1 infection and disease has not been well established and is only supported by indirect evidence or by animal models. This is the case of Bell's palsy [2–5].

We have used the animal model previously described [2] in New Zealand white rabbits to investigate some of the presumptive paths of viral

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progression from a peripheral focus of infection and the best method to be used in determining the presence of HSV-1. In the clinical laboratory setting it is of the utmost importance to optimize and make suitable diagnostic procedures to the conditions and limitations in each laboratory. DNA amplification techniques are now broadly used in clinical laboratories as a common diagnostic tool. The powerful sensitivity of PCR makes it suitable in the diagnose of diseases in which the amount of pathogenic agents involved is scanty and traditional procedures lack of sensitivity.

We described the performance characteristics in our laboratory of a nested PCR based method previously described [6] and slightly modified by us in order to simplify the process and to reduce the rate of contamination. Our main innovations was to use a frozen stock of amplification mixture and only one tube to perform the whole process.

## 2. Material and methods

Twenty-one New Zealand White rabbits were injected into the tongue with 300 $\mu$l of HSV-1 FP-16 strain (TCID50 10–3.5, Kärber method [7]). After been experimentally infected, animals were observed each day for the presence of facial palsy.

Eleven rabbits were killed on day seven ('A' group) and the other ten were sacrificed on day 21 ('B' group). One rabbit, injected with 300 $\mu$l of Eagle minimal essential medium without HSV-1, was included in both groups ('A' and 'B') as controls. A tongue scrapping from each animal was collected immediately before inoculation. After the rabbits were killed, samples were collected from the tongue, geniculate ganglia, trigeminal ganglia and medulla on both sides.

All samples were homogenized and decontaminated with 50 IU of penicillin per ml and 50 $\mu$g of streptomycin per ml. A portion of each sample was inoculated onto Vero cells and the bulk was kept frozen at $-40$°C until amplification was performed.

### 2.1. Cell culture

200 $\mu$l of each sample were inoculated onto Vero cells and incubated at 37°C in a 5% $CO_2$ for 15 days. After this time, or when a cytopathic effect (CPE) was observed, the monolayer was scraped and smears were prepared, dried at room temperature, fixed in cold acetone and stained with a FITC-labeled monoclonal antibody (Syva 'Microtrak' HSV-1).

### 2.2. Preparation of DNA for PCR

Samples were homogenized and pelleted by centrifugation at $1800 \times g$ for 5 min and supernatant removed. Then, 100 $\mu$l of lysis solution (10 mM Tris–HCl pH 8.3, 50 mM potassium chloride, 2.5 mM magnesium chloride, 0.5% Nonidet P-40, 0.5% Tween 20 and 10 $\mu$g of proteinase K) was added, mixed and incubated 45 min at 56°C and then 10 min at 96°C to inactivate proteinase K. The amount of DNA in samples was determined using a spectrophotometer (Pharmacia LKB-Ultrospec III) and adjusted to a final concentration of 0.1 $\mu$g/$\mu$l.

### 2.3. Nested PCR method

A 138 bp fragment belonging to the glycoprotein D encoding-gen of HSV-1 was amplified using oligonucleotide primers [6] (Fig. 1). Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer according to the manufacturer's instructions. Reaction mixture containing 10 mM Tris–HCl pH 8.3, 50 mM potassium chloride, 1.5 mM magnesium chloride, 1 U recombinant Taq DNA polymerase (Gibco), 125 $\mu$M each deoxynucleoside triphosphate, and 0.25 $\mu$M each of the oligonucleotides (BJHSV1.1: 5′-ATCACGGTAGCCCGGCCGTGTGACA-3′ (19–43) and BJHSV1.2: 5′-CATACCGGAACG-CACCACACAA-3′ (239–218)) were batch-prepared. 40 $\mu$l of this mixture were distributed into 500 $\mu$l reaction-tubes, overlaid with 30 $\mu$l of mineral oil and stored at $-20$°C for a maximum of 90 days. Aliquot tubes were thawed immediately before use and 1 $\mu$g DNA was added. Duplicate samples of sterile distilled water were

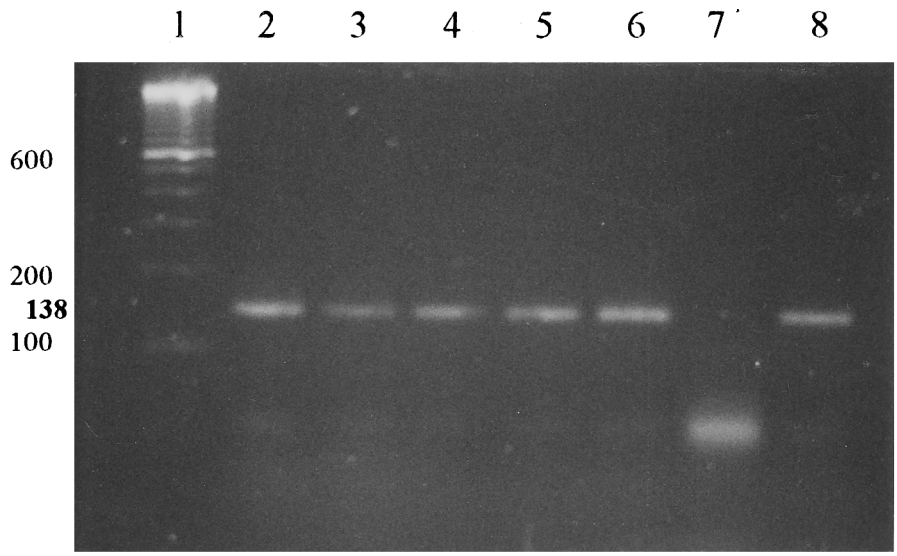


Fig. 1. Nested PCR amplification of a 138 bp fragment of the glycoprotein D encoding-gen of HSV-1 obtained by nested PCR. Lane 1, molecular weight markers (100 bp ladder Gibco); lane 2, sample from the medulla; lanes 3 and 4, samples from geniculate ganglia; lanes 5 and 6, samples from trigeminal ganglia; lane 7 negative control (primers); lane 8, positive control (from HSV-1 cell culture).

prepared at the same time as the animal specimens and served as contamination controls for each PCR experiment and standard precautions [8] were taken to ensure that the PCR assay remained free from DNA contamination. Contamination was not seen in any of the PCR assays described here. The first-round amplification comprised 22 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 1 min followed by an additional final extension step of 10 min at 72°C. After first-round of amplification, 1 U of recombinant Taq DNA polymerase (Gibco) and 25 $\mu$M each of the inner oligonucleotides [BJHSV1.3: 5′-CCAACCGACCACACCGACGA-3′ (51–71) and BJHSV1.4: 5′-GGTAGTTGGTCGTTCGCGCTGAA-3′ (188–166)] were added into the same tube and a second-round amplification was performed. This second-round comprised 35 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, and elongation 72°C for 1 min followed by a final extension step at 72°C for an additional 10 min. 10 $\mu$l of the second-round PCR products were subjected to electrophoretic migration in 3% agarose gel containing ethidium bromide and were examined with UV light. Negative controls and positive controls (performed using DNA extracted from viral cell cultures of HSV-1) were included.

### 2.4. Single PCR method

35 cycles of amplification with 25 $\mu$M of each primer BJHSV1.1 and 1.2 (221 bp product) or BJHSV1.3 and 1.4 (138 bp product) were performed using same conditions described for nested PCR. Contamination controls were included and standard precautions were taken [8] in each PCR experiment as described above. All PCR products were stored at 4°C until hybridization.

### 2.5. Hybridization assay

5 $\mu$l of the reaction products were denatured by treatment with 200 $\mu$l denaturation buffer (NaOH 0.4 N, EDTA 10 mM) at room temperature for 15 min. Each sample was then applied to kyboad-N nylon membrane (Bio-Rad) with a dot blot apparatus. The filter was subsequently washed in 2 × SSC and fixed with UV light. On the day of hybridization assay, the filter was incubated in

Table 1
Detection of HSV-1 in different samples (by nested PCR or cell culture)

| Animals | Tongue | Geniculate ganglia | Trigeminal ganglia | Medulla | Total |
|---|---|---|---|---|---|
| 'A' group | 4/7 (36.36) | 2/9 (18.18) | 2/6 (25) | 3/8 (27.27) | 11/30 (26.82) |
| 'B' group | 5/5 (50) | 6/4 (60) | 6/4 (60) | 2/8 (20) | 19/21 (47.5) |
| Total | 9/12 (42.85) | 8/13 (39.08) | 8/10 (44.44) | 5/16 (23.8) | 30/51 (37.03) |

Positive/negative (%).

prehybridization buffer (2% sodium dodecyl sulfate, 2 × PBS, 5 × Denhardt's solution, 1 mM EDTA, 40 $\mu$g of salmon sperm DNA per ml) at 65°C for 30 min and was hybridized 60 min at 59°C in hybridization buffer (2% sodium dodecyl sulfate, 2 × PBS, 5 × Denhardt's solution, 1 mM EDTA, probe 4 $\mu$M) with probe BJHSV1: 5′-TACGAGGAGGAGGGGTATAA-CAAAGTCTGT-3′ (96–125) (5′-labeled with [$^{32}$P]ATP and T4 polynucleotide kinase). The filter was subsequently washed in 2 × PBS, 1 mM EDTA at room temperature for 10 min, then twice in 2 × PBS, 1 mM EDTA, 1% sodium dodecyl sulfate at hybridization temperature for 10 min and once more in PBS at room temperature for 5 min. Finally the filter was autoradiographed for 24 h at − 70°C with Kodak XRP. A test were considerated positive if the film showed a visible signal. Statistical analysis: Fisher's exact test was used to compare proportions, $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Animals

HSV-1 infection was detected in 19 of 21 (90.47%) animals (at least in one sample) either by nested PCR or cell culture. Out of 19, nested PCR was positive in 16 (84.21%), and cell culture was positive in 10 (52.63%). Infection was detected in nine of 11 animals (81.81%) belonging to 'A' group, seven (63.63%) had positive nested PCR and two (18.18%) had positive cell culture ($P < 0.05$, Fisher's test). This difference was not found into 'B' group: the infection was detected in all animals, 90% were nested PCR positive and 80% had positive cultures. There were no differences of sensitivity of nested PCR between 'A' and 'B' groups (63.63 versus 90%), but in viral cell culture there were significant differences (18.18 versus 80%; $P < 0.05$, Fisher's test). No infection-related symptoms were observed in the animals along follow-up.

### 3.2. Samples

HSV-1 was detected neither in samples obtained before the inoculation nor in controls. HSV-1 was detected in 30 of 81 (37.03%) post-mortem samples. Out of 30, 25 (83.33%) were nested PCR positive and 15 (50%) yielded positive cultures. In animals killed on day seven ('A' group), HSV-1 was detected in 11 of 41 (26.82%). Out of 11, nine (81.81%) by nested PCR and two (18.18%) by culture. The difference in sensitivity between nested PCR and culture was significant ($P < 0.05$, Fisher's test). In animals sacrificed on day 21 ('B' group), HSV-1 was detected in 19 of 40 (47.5%) samples. Out of 19, 16 (84.21%) by nested PCR and 13 (68.42%) by cell culture ($P > 0.05$). The sensitivity of viral culture was 18.18% (two of 11) in 'A' group and 68.42% (13 of 19) in 'B' group ($P < 0.05$, Fisher's test). When the anatomical source of samples is considered, the animals belonging to 'B' group showed a higher rate of positive results in samples from geniculate and trigeminal ganglia than animals belonging to 'A' group (geniculate ganglia 60 versus 18.18% and trigeminal ganglia 60 versus 25%) (Table 1).

### 3.3. Performance of frozen mixture reaction

The results obtained with the frozen-stored mixture reaction were compared with that ob-

tained using freshly prepared one and both performed equally.

### *3.4. Sensitivity comparison between nested PCR and single PCR*

The previous standardization of the nested PCR procedure in our laboratory always gave specific products to HSV-1 when where simultaneously tested with HSV-2, Varicela Zoster, Citomegalovirus, Epstein Barr virus, papovavirus and adenovirus.

Single PCR was able to detect HSV-1 in nine of 25 samples (36%) that were nested PCR positive. All samples found positive by single PCR were also positive by nested PCR. The minimal amount of HSV-1 DNA detected was presumably 3–25 ng in nested procedure and 100 ng in single PCR.

## 4. Discussion

Methods based in cell culture are useful in the diagnosis of HSV-1 infection in some clinical situations, but they are time-consuming and have a low sensitivity when a low amount of viable viruses are expected. In head and neck it could be a powerful diagnostic tool in all process suspected to be caused by HSV-1 such as herpes encephalitis, keratoconjunctivitis, recurrent herpes (labialis or nasal), otitis, gingivostomatitis, pharyngitis and esophagitis. Moreover the HSV-1 has been related with many diseases such as Bell's palsy, Guillain-Barré syndrome, transverse myelitis, Ménière disease, adquire or congenital hearing losses, otosclerosis, etc. In research, molecular characterization of viral infections, oncogenes or other etiologic agents, nested PCR could provide important information regarding the etiopathogenesis of many diseases. Nucleic acids amplification methods may be a useful diagnostic approach in these situations.

The results presented here show that PCR has greater sensitivity than viral culture. Into 'A' group, PCR significatively detected more positive samples than culture, but this difference was not significant in the 'B' group. Viral culture was more often positive in samples from animals killed on day 21 than in those sacrificed on day 7. PCR had equal sensitivity in both groups. These results suggest that in clinical situations where a low viral load are expected, methods based on nucleic acid amplification should be chosen.

In our experience, nested PCR has greater sensitivity than single PCR because all samples positive by single PCR were also positive by nested PCR but the single PCR was only able to detect HSV-1 in nine of 25 samples (36%) that were nested PCR positive. The minimal amount of DNA required to obtain a positive result in nested PCR is four to 33 times smaller than in the single procedure.

Nested PCR procedure always gave specific products and further hybridization did not increased either sensitivity or specificity. Further hybridization of nested PCR products is not essential, so this imply faster, cheaper and easier procedures with reliable results for clinical laboratory.

We want to emphasize the great advantage of using a reaction mixture previously prepared and kept frozen until use with same performances as fresh prepared one. This simplifies to a great extent the procedure and make this technique feasible to clinical laboratories. In order to prove that HSV-1 infection has a aethiological relationship with facial palsy, some animal model of experimental infection has been described [4,5,10–12] but only two succeed [2,13].

The follow-up of our animals showed no facial palsy in any of the cases and no animal had vestibular disturbances or secondary neurological effects.

All kind of samples gave similar results with all methods and we did not find any significant difference in yield between samples from geniculate and trigeminal ganglia [5,9]. We think that the absence of facial palsy in our model could be attributed to a lesser neurovirulent strain of HSV-1 (we have not found any death or limb palsy in our animals) or to the use of a different animal model. However, our findings suggest that HSV-1 infects the majority of geniculate and trigeminal ganglia and medulla of the rabbits that were inoculated in the tongue. In our animal model this infection was asymptomatic, and the nested PCR

was a useful tool for detection of the HSV-1 genome even in latent state.

In a recent report Murakami et al. [14] had found HSV-1 genomes in samples of facial nerve endoneural fluid from the 79% (11/14) of patients with Bell's palsy treated surgically. This findings are a strong evidence of the relationship between HSV-1 and Bell's palsy. As the authors suggest, this rate could be improved with a more sensible tool like the nested PCR.

## 5. Conclusion

We described a simplified procedure for nested PCR in which the reaction mixture is bulk-prepared and stored at $-20$°C during a maximum of 90 days without any reduction in performances. However, despite its advantages traditional nested amplification involving the open transfer of first round products from tube to tube is not recommended for routine clinical laboratory use. The procedure described above avoid this step, with the subsequent reduction in the incidence of contamination along the process. Our procedure allowed us to apply this powerful tool in clinical samples with a low viral load. Nested PCR gave higher sensitivity than single PCR and no further hybridization of the products is needed.

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