

Evaluation of non-radioactive temperature gradient SSCP analysis and of temperature gradient gel electrophoresis for the detection of HPV 6-variants in condylomata acuminata and Buschke-Loewenstein tumours

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Abstract. A modified non-radioactive single strand conformation polymorphism analysis incorporating a temperature gradient (TG-SSCP) and temperature gradient gel electrophoresis (TGGE) were evaluated for the detection of human papillomavirus type 6 (HPV 6)-variants in 41 condylomata acuminata and 5 Buschke-Loewenstein tumours. TG-SSCP and TGGE analysed part of the transforming ORF E6 of HPV 6 spanning nucleotides 10 to 495. TG-SSCP distinguished between 8 HPV 6-variants whereas TGGE demonstrated 6 different DNA-species. HPV

6-strains found in Buschke-Loewenstein tumours did not vary in the analysed portion of the E6 ORF as compared to ordinary condylomata acuminata. TG-SSCP and TGGE further showed absence of double infection with different HPV 6-strains in the analysed samples. Our results demonstrated that both methods may be successfully used for the detection of different strains of microbiological agents, although TG-SSCP seemed to provide easier execution and to confer a higher degree of flexibility than TGGE.

Key words: Human papillomavirus (HPV), Sequence variants, Single strand conformation polymorphism (SSCP), Temperature gradient gel electrophoresis (TGGE)

Introduction

Human papillomaviruses (HPV) induce benign and malignant fibroepithelial tumours in man. Among the HPV types predominantly infecting mucosa, HPV 6 and 11 are mainly associated with benign condylomata acuminata, with clinically malignant Buschke-Loewenstein tumours and with laryngeal papillomas [1–4]. HPV 6/11-induced tumours are of significant importance with regards to the public health in the western world. They are considered to be the most common viral sexually-transmitted disease in the USA and prevalence has increased significantly in late years [5, 6].

The understanding of the pathways of HPV infection is a prerequisite of an effective disease prevention. The study of infectious pathways requires the identification of particular strains of HPV. Sequence analysis enables the characterization of viral genomes but the method is time-consuming and costly. Restriction fragment length polymorphism analysis (RFLP) is an alternative to sequencing but it detects only sequence changes affecting restriction enzyme sites. In recent studies single-strand conformation polymorphism (SSCP) analysis [7, 8] and temperature gradient gel electrophoresis (TGGE) [9–11] have been proposed as methods for the identification of sequence variations in alleles of the human genome and in HPV. SSCP and TGGE detect DNA-sequence-

dependent conformational changes of nucleic acid segments by changes in electrophoretic mobility which result in different migration patterns and bandshifts.

In SSCP, single-stranded nucleic acid segments are separated in a non-denaturing polyacrylamide gel. Sequence mutation which alter the conformation of the single stranded DNA are detected by bandshifts.

In TGGE, double-stranded nucleic acid segments migrate through a polyacrylamide gel which displays a temperature gradient perpendicular or parallel to the direction of the electrophoresis. At a sequence-dependent specific temperature, the double-stranded DNA starts to separate. The transition to single-stranded DNA results in migration retardation. Sequence mutations which alter the thermal stability of the nucleic acid segment are detected by bandshifts (parallel TGGE) or by shifted melting profiles (perpendicular TGGE). Both methods are very sensitive as they are able to demonstrate single nucleotide substitutions in homologue DNAs.

The aim of the present study was to establish and evaluate single-strand conformation polymorphism analysis and temperature gradient gel electrophoresis for the detection of HPV 6 sequence variants in condylomata acuminata and Buschke-Loewenstein tumours which had been previously classed into variants by RFLP [4]. The E6 open reading frame (ORF) of HPV 6 was chosen for analysis as the E6

ORF is involved in transformation and immortalization of infected cells and we wished to study whether this gene differed in Buschke-Loewenstein tumours as compared to ordinary condylomata acuminata.

In order to enhance sensitivity, SSCP was modified by adding a highly reproducible temperature gradient to the gel. The rationale of this modification lies in the fact that during SSCP conformational differences between homologue yet base-substituted single-stranded fragments are only expressed within a limited temperature range which is difficult to predict. Electrophoresis applying a temperature gradient should, therefore, enhance the possibility that one DNA-fragment adopts a conformation which differs from the homologue DNA resulting in a different migration pattern.

Materials and methods

Source of HPV 6 DNA. Tissue specimens were obtained from patients attending the Department of Dermatology of the RWTH-Aachen. 41 patients had anogenital condylomata acuminata and 5 patients had Buschke-Loewenstein tumours. Viral DNA was typed as HPV 6 by PCR with primers amplifying part of the ORF L1, the upper regulatory region (URR) and part of the ORF E6 of HPV 6 between nucleotides (nt) 6487-320 of the published HPV 6b sequence [12] as previously described [4].

RFLP-analysis. The previously described variants ACV1-ACV4 of HPV 6 were detected by restriction enzyme cleavage of the PCR-amplified HPV 6 sequence between nt 6487-320 with the endonucleases Rsa I (Pharmacia), Dde I (Pharmacia), Pst I (Boehringer), Hpa II (Pharmacia), Hae II (Pharmacia) and Hae III (Pharmacia) [4].

Temperature gradient-SSCP (TG-SSCP). DNA templates were generated by PCR using primers amplifying between nt 10 and nt 495 of the E6 ORF of HPV 6 according to Saiki et al. [13]. The primer binding sites were chosen after analysis of the E6 gene by Poland's algorithm [14] for optimal melting characteristics during TGGE. The sequence of the primers from 5' to 3' were as follows:

- sense primer, CAATCTTGTTTAAAAAATA;
- antisense primer, TACAATTTAGCTTTATGAACCGC.

Amplification was performed with 35 cycles of 1 min denaturation at 92 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C. Amplification terminated with an extension step of 7 min duration after the last cycle. 10 µl of each PCR-product were then digested with 1 U of the endonuclease Taq I (Pharmacia) at 69 °C for 2 hours. 2 µl of the digested product were mixed with 9 µl of 95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol

and denatured at 100 °C for 1 min and then rapidly chilled on ice for 5 min. 6 µl of the mixture were filled in a single well. Electrophoresis was performed in a Hydrolink gel matrix (AT Biochem, Malvern, PA) according to the manufacturer's instructions which was bound on one side to a GelBond PAG film (FMC, Rockland, ME). A linear temperature gradient from the cathode to the anode was established with a commercially available temperature gradient horizontal gel electrophoresis device (DIAGEN TGGE System, Hilden, Germany). Electrophoresis was carried out in 1X TBE buffer at 400 V for 4 hours. Staining of nucleic acids with silver was done as previously described [10].

TGGE-analysis. TGGE was performed in the same horizontal gel electrophoresis device using a 5% polyacrylamide gel with 2% glycerol and 48% (w/v) urea. For parallel TGGE, 5 µl of non-digested PCR product were mixed with 0.6 µl of 10X MOPS-buffer, 0.05% bromophenol blue and 0.05% xylene cyanol and loaded in a single well. Electrophoresis was carried out with a temperature gradient ranging from 25 °C at the cathode to 70 °C at the anode. For perpendicular TGGE, 30 µl each of non-digested PCR-product of two different samples were mixed with 20 µl 10X MOPS-buffer, 0.05% bromophenol blue and 0.05% xylene cyanol and loaded in a long slot parallel to the cathode. Electrophoresis was first carried out without temperature gradient at 500 V. Up to three different sample preparations were loaded consecutively onto the gel at 15 min intervals. After the last sample had migrated for at least 30 min, a temperature gradient was established perpendicular to the migration direction of the nucleic acids ranging from 25 to 70 °C. Electrophoresis was then continued for 1 hour at 400 V. In both cases, visualization of nucleic acids was done with silver staining.

Sequencing analysis. The most frequent variants of HPV 6 detected by TG-SSCP and TGGE were sequenced by dideoxy sequencing using the T7sequencing Kit™ (Pharmacia) following the manufacturer's instructions. Single-stranded templates were generated by PCR using one biotin-bond primer and by subsequent separation of the single-stranded PCR-products with avidin-coated magnetic beads (Dynabeads, Dynal).

Results

41 HPV 6-samples were obtained from biopsies of condylomata acuminata and 5 samples originated from Buschke-Loewenstein tumours. All samples were grouped by restriction fragment length polymorphism (RFLP) of PCR-amplified DNA (nt 6487-320) into the variants ACV1, ACV2, ACV3 and ACV4 (Table 1) as it was previously described [4].

Table 1. HPV 6 variants detected by RFLP, TGGE and TG-SSCP in condylomata acuminata and Buschke-Loewenstein (B-L) tumours

RFLP	Variant		Diagnosis	
	TGGE-profile	TG-SSCP-pattern	Condyloma acuminatum	B-L tumour
HPV 6b	1	1		
ACV2	1	1	7	
ACV4	2	2	6	
ACV4	3	3	1	
ACV1	3	4	7	1
ACV1	3	5	13	3
ACV1	4	6 ^a	1	
ACV3	5	4	4	1
ACV3	5	7	1	
ACV3	6	8 ^b	1	
Total			41	5

^a Additional Taq I restriction site.

^b Missing Taq I restriction site.

HPV 6b DNA has a different RFLP-pattern due to a deletion within the analysed DNA segment resulting from a cloning artefact [4].

Temperature gradient single strand conformation polymorphism analysis (TG-SSCP) and temperature gradient gel electrophoresis (TGGE) were performed with the PCR-amplified DNA segment covering most of the E6 ORF of HPV 6 (nt 10–495). The DNA fragment were subsequently sequenced in order to validate the TG-SSCP- and TGGE-data.

Before performing TG-SSCP, the PCR-products were digested with the endonuclease Taq I in order to produce two fragments of 259 and 219 bp length. The optimal temperature range for TG-SSCP was determined by comparing SSCP results at different temperatures. This was done using a temperature-gradient gel with different start positions along the temperature gradient. Figure 1 displays the SSCP-patterns at different temperature ranges obtained by 2 hour electrophoresis of the cleaved PCR products. As expected, separation of single-stranded bands was influenced by the selected temperature range. In order to allow maximum separation of variant DNA-segments, we applied a wide temperature gradient ranging from 5 °C at the cathode to 30 °C at the anode and performed electrophoresis at 400 V for 4 hours for comparison of all specimens. Figure 2 shows a typical silver-stained gel displaying 7 different migration patterns. The upper bands are formed by the sense and antisense strand of the 259 bp fragment generated through the Taq I digestion whereas the lower bands originate from the 219 bp

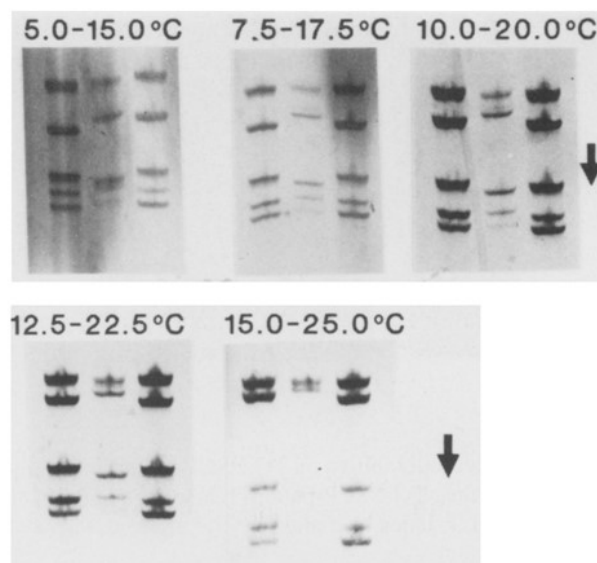


Figure 1. TG-SSCP at different temperature ranges (low value = temperature of the gel at start position; high value = temperature of the gel at stop position of electrophoresis) using Taq I digested PCR-products (nt 10–nt 495) of HPV 6b (left) and two patient samples (TG-SSCP-pattern 2 (middle) and TG-SSCP-pattern 1 (right)). Arrows indicate direction of electrophoresis.

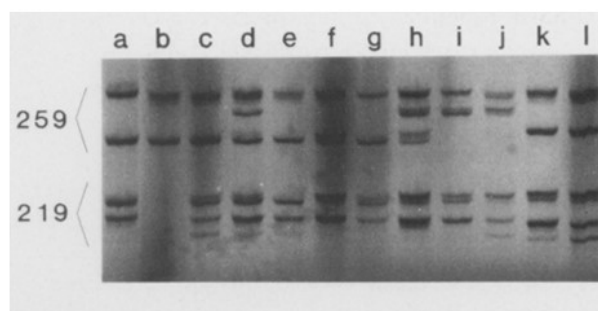


Figure 2. Variants of HPV 6 demonstrated by TG-SSCP with Taq I digested PCR-products (nt 10 – nt 495). TG-SSCP-pattern generated with HPV 6b: lane l; TG-SSCP-pattern 1: lane k; TG-SSCP-pattern 2: lanes i and j; TG-SSCP-pattern 3: lane h; TG-SSCP-pattern 4: lane f; TG-SSCP-pattern 5: lanes a, c, e and g; TG-SSCP-pattern 6: lane b; TG-SSCP-pattern 7: lanes d and h.

fragment. One sample DNA harboured an additional Taq I restriction site on the 219 bp fragment. The resulting TG-SSCP-pattern 6 (Figure 2, lane b), therefore, demonstrates the lack of lower bands. One sample yielded bands of higher molecular weight as it did not contain a Taq I restriction site (TG-SSCP-pattern 8). Patterns generated by TG-SSCP were highly reproducible. Figure 3 shows the resulting patterns of three consecutive runs.

Sequencing of the most frequent variants from nt 1–485 demonstrated that TG-SSCP was able to distinguish variants differing by 1 to 5 base changes (Table 2). Nevertheless, TG-SSCP was not as sensitive in all cases as it did not distinguish between the variants ACV1.TGGE3.TG-SSCP4 and ACV1.-

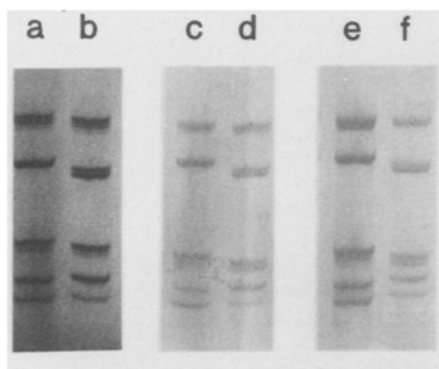


Figure 3. Reproducibility of TG-SSCP-patterns in three consecutive runs. TG-SSCP-pattern 1: lanes a, c and e; TG-SSCP-pattern 4: lanes b, d and f.

Table 2. Sequence data of the major variants with regards to the published sequence of HPV 6b

Variant	Sequence changes with regards to HPV 6b
ACV4	nt 156 A > T
TGGE-profile 2	nt 221 A > T
TG-SSCP pattern 2	nt 251 C > G nt 271 G > C nt 323 A > C nt 365 A > T nt 473 G > A
ACV4	nt 221 A > T
TGGE-profile 3	nt 251 C > G
TG-SSCP-pattern 3	nt 323 A > C nt 365 A > T nt 473 G > A
ACV1	nt 221 A > T
TGGE-profile 3	nt 251 C > G
TG-SSCP-pattern 4	nt 271 G > T nt 323 A > T nt 365 A > T nt 392 C > T nt 473 G > A
ACV1	nt 221 A > T
TGGE-profile 3	nt 251 C > G
TG-SSCP-pattern 5	nt 323 A > C nt 365 A > T nt 392 C > T nt 473 G > A
ACV3	nt 221 A > T
TGGE-profile 5	nt 251 C > G
TG-SSCP-pattern 4	nt 323 A > T nt 365 A > T nt 392 C > T nt 404 A > C nt 473 G > A

TGGE5.TG-SSCP4 which differ by two base changes.

Gels of parallel TGGE showed multiple unspecific bands which greatly hindered interpretation of the resulting patterns (data not shown). Parallel TGGE was, therefore, dispensed with. Although perpendicular TGGE also demonstrated some unspecific bands, they did not influence interpretation as unspecific PCR-products showed completely different melting profiles compared to the specific bands. Perpendicular TGGE only compares two samples at a time. The melting profiles of different DNA-samples superimpose if they have the same thermal stability (Figure 4a) and separate if the thermal stability differs (Figure 4e).

Comparison of all samples demonstrated 6 different melting profiles (Table 1). As with TG-SSCP, the prototype HPV 6b strain could not be distinguished from the clinical specimens demonstrating the ACV2 RFLP-pattern and the TG-SSCP-pattern 1 (Figure 4a lower profile (lp)). TG-SSCP-patterns 1, 2, 3, 6 and 8 were also clearly separated by TGGE (Figures 4f upper profile (up), 4c(lp), 4d(up) and 4i). TGGE failed to distinguish between TG-SSCP-patterns 3–5 (Figures 4b(lp) and 4h middle profile (mp)) which differ in sequence by 1 to 3 base changes (Table 2), and TGGE did not differentiate between the variants ACV3.TGGE5.TG-SSCP4 and ACV3.TGGE5.TG-SSCP7 (Figure 4d(lp)). In contrast to TG-SSCP, TGGE was able to separate the variants ACV1.TGGE3.TG-SSCP4 and ACV3.TGGE5.TG-SSCP4 (Figure 4e(up)).

Results from RFLP, TGGE and TG-SSCP are summarized in Table 1. Analysis of part of the E6 ORF of HPV 6 did not reveal different variants in the Buschke-Loewenstein tumours as compared to ordinary condylomata acuminata. TGGE and TG-SSCP analysis further demonstrated that each sample DNA contained only one HPV 6-variant.

Discussion

With our study we wanted to establish and evaluate TG-SSCP and TGGE for the detection of HPV 6 variants differing in the E6 ORF in condylomata acuminata and Buschke-Loewenstein tumours.

Our results demonstrated that both methods may be used to distinguish between different HPV 6 strains. By either technique we could show that the analysed portion of the E6 ORF of HPV 6 does not vary in Buschke-Loewenstein tumours as compared to ordinary condylomata acuminata. This finding supports the assumption that host factors are probably responsible for the development of Buschke-Loewenstein tumours and that mutations of the viral DNA may only play a minor role in tumour progression [4]. A previous study of Beckmann and colleagues [15] showed the absence of dual infec-

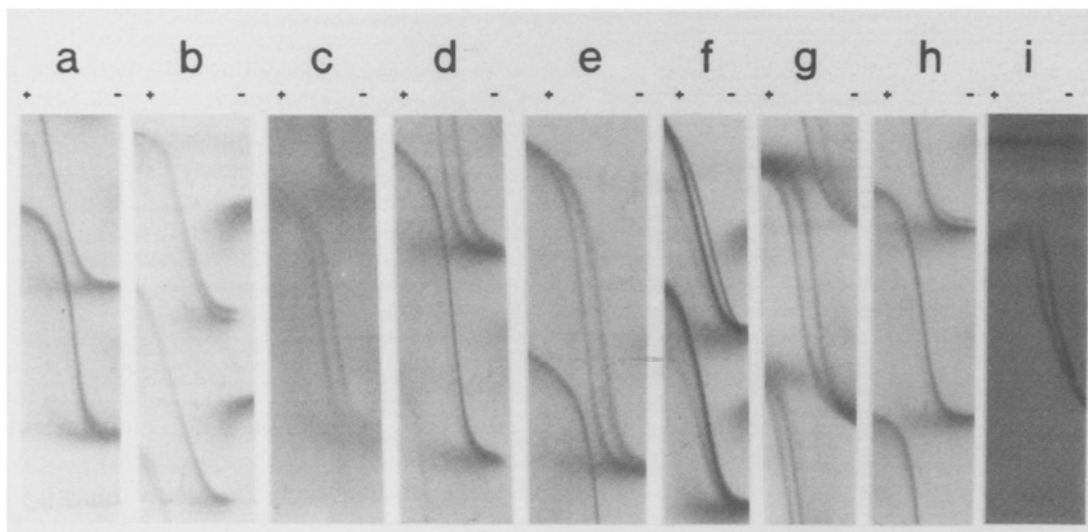


Figure 4. Comparison of PCR-products of HPV 6 (nt 10–nt 495) by TGGE. **a(lp):** HPV 6b / TG-SSCP1; **b(up):** TG-SSCP4 / TG-SSCP1; **b(lp):** TG-SSCP4 / TG-SSCP5; **c(lp):** TG-SSCP4 / TG-SSCP6; **d(up):** TG-SSCP2 / TG-SSCP4; **d(lp):** ACV3.TG-SSCP4 / ACV3.TG-SSCP7; **e(up):** ACV3.TG-SSCP4 / ACV4; **f(up):** TG-SSCP2 / TG-SSCP1; **g(mp):** ACV3 / ACV4; **h(mp):** TG-SSCP4 / TG-SSCP3; **i:** ACV3.TG-SSCP4 / ACV3.TG-SSCP8. *Key:* up = upper profile; mp = middle profile; lp = lower profile; + = high temperature; – = low temperature.

tions with different human papillomaviruses in condylomata acuminata. Our results further suggest that dual infections with different HPV 6-variants are equally absent.

In our study TG-SSCP seemed to have a slight advantage over TGGE as it discriminated two more HPV 6-variants. Still, this apparent difference is not significant as in SSCP and TGGE the ability to discriminate between different DNA samples is highly dependent on the choice of the analysed DNA-sequence and a different primer selection may have resulted in a slight advantage of the other technique.

The major differences between the two methods were found in the setup and the flexibility of the technique and the ease of execution. Set-up and execution of TG-SSCP analysis was straightforward and fast.

TG-SSCP was performed using the same commercially available electrophoresis apparatus as TGGE. The best temperature range for effective separation of variant DNAs in SSCP could be easily determined by applying a temperature gradient to SSCP. The good and reproducible results obtained by adding a wide temperature gradient to SSCP supports our assumption that electrophoresis through a temperature gradient may enhance the possibility that DNA-fragments adopt sequence specific conformations which allow separation of variant DNA-species. Although TG-SSCP should, in principle, be superior to SSCP analysis without a temperature gradient, a temperature gradient may not improve the performance of SSCP in all applications as the sensitivity of SSCP is mainly dependent on the distribution of the base changes within the analysed DNA-sequence

and a temperature gradient may not be necessary to distinguish between all variants.

In contrast to TG-SSCP, TGGE analysis was much more laborious. In our hands, parallel TGGE, which would have allowed the direct comparison of multiple samples, did not confer reliable results and we, therefore, had to perform perpendicular TGGE which can only compare two DNA-species simultaneously. It may be argued that the failure of parallel TGGE was due to an inappropriate choice of the analysed fragment as other studies have shown that careful calculation of DNA melting maps and the use of GC-clamps may significantly enhance the detection of mutations [9]. On the other hand, exact calculation of DNA melting maps is only possible if the location of sequence mutations is known in advance. This was not the case in our study and will probably not be the case in epidemiological studies analysing bacterial or viral DNA. Moreover, the need of carefully selecting sequence and primers for TGGE analysis reduces by itself the ease of execution and the flexibility of the technique.

In conclusion, the evaluation of TG-SSCP and TGGE demonstrated that although both methods may be used successfully for the detection of variant strains of a viral agents, TG-SSCP seems to be easier in execution and to confer a higher degree of flexibility than TGGE. These advantages together with good reproducibility, the availability of a commercial temperature gradient electrophoresis device and the ease of silver-staining, make TG-SSCP a promising technique for epidemiological studies which rely on the genetic heterogeneity of micro-biological agents.

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