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Development of a novel multiplex type-specific quantitative real-time PCR for detection and differentiation of infections with human papillomavirus types HPV2, HPV27, and HPV57

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Abstract

Introduction: The present study describes the development and evaluation of the first multiplex type-specific quantitative real-time PCR (RT-PCR), enabling simple, rapid, sensitive, and specific concurrent detection and differentiation of human papillomavirus (HPV) types HPV2, 27, and 57 in a single PCR reaction.
Results: The HPV2/27/57 multiplex RT-PCR with a dynamic range of seven orders of magnitude (discriminating 10 to 10^8 viral genome equivalents/reaction) has an analytical sensitivity of at least 10 viral copies of each targeted HPV type/reaction, and no cross-reactivities were observed among the included targets. All three primer/probe combinations were efficient in amplifying 500 copies of targeted DNA in a background of 10^8, 10^7, 500, 100, and 10 copies of non-targeted viral DNA/reaction, and the performance of the HPV2/27/57 multiplex RT-PCR was additionally not affected by the presence of background human genomic DNA. When testing DNA isolates obtained from fresh-frozen tissue specimens of various children's warts, the results of the HPV2/27/57 multiplex RT-PCR were completely in line with the results of the conventional Low-risk *Alpha*-PV PCR.
Conclusion: The newly developed HPV2/27/57 multiplex RT-PCR is an appropriate test for use in routine clinical laboratory settings and for studies focusing on the molecular epidemiology, pathogenesis, and natural history of HPV2/27/57-related lesions.

Keywords: human papillomavirus types HPV2, HPV27, and HPV57, detection, differentiation, multiplex type-specific quantitative real-time PCR, development

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Introduction

Human papillomavirus (HPV) types 2, 27, and 57, clustering within the species *Alphapapillomavirus* (*Alpha*-PV) 4, are etiologically associated with more than 65% of verrucae vulgares or common warts, the most frequent HPV-associated benign lesions of the skin, with the highest prevalence in children and immunosuppressed patients (1–9, 11, 12). Two other *Alpha*-PV types, HPV6 and HPV11, are in contrast the main etiological agents of condylomata acuminata or anogenital warts, the most frequent HPV-related benign lesion in the anogenital region of both sexes. However, common warts caused by HPV2, HPV27, and HPV57 can also frequently be found in the anogenital region, especially in children, as a result of autoinoculation from common warts from other parts of the body or infection transmitted from common warts of their parents or household members, and could be clinically misdiagnosed as condylomata acuminata (11–19). Such a misdiagnosis could have potential serious consequences because the appearance of new wart(s) in a child's anal or genital region can be considered an indicator of sexual abuse and can potentially trigger legal action against the parents or household members. Thus, although routine detection of HPV types present in tissue specimens or swabs of both condylomata acuminata (anogenital warts) and verrucae vulgares (common warts) is not generally recommended, it could be very helpful in some clinical circumstances and/or for legal purposes, especially in children. However, to be used for such purposes, diagnostic test(s) for detecting and distinguishing HPV types causing condylomata acuminata versus verrucae vulgares should be highly reliable and accurate.

In addition to *in situ* hybridization methods (20–22), several conventional broad-spectrum polymerase chain reactions (PCR)—which enable detection and differentiation of HPV types that are etiologically associated with condylomata acuminata and verrucae vulgares by subsequent laborious and time-consuming typing of PCR products using agarose gel electrophoresis, hybridization on strips/microtiter wells, and direct Sanger sequencing—have been described previously (23–31). Because *in situ* hybridization and conventional PCRs are suboptimal methods, de Koning et al. (32) and Schmitt et al. (33) developed broad-spectrum HPV typing bead-based xMAP Luminex suspension arrays, which are able to detect and differentiate 23 and 19 HPV types, respectively, that are most frequently found in common warts, including HPV2, HPV27, and HPV57. In addition, Köhler et al. (7) developed a multiplex type-specific quantitative real-time PCR (RT-PCR), which enables detection and differentiation of infections with HPV27 and HPV57. However, to the best of our knowledge, no quantitative real-time PCR allowing simultaneous amplification and differentiation of HPV2, HPV27, and HPV57 has been developed so far.

This study describes the development and analytical and clinical evaluation of a novel multiplex type-specific quantitative RT-PCR, allowing rapid, sensitive, and specific concurrent detection and differentiation of infections with HPV2, HPV27, and HPV57 in a single PCR reaction. The HPV2/27/57 multiplex RT-PCR was evaluated on a collection of fresh-frozen tissue specimens of condylomata acuminata and verrucae vulgares, obtained from children in a routine clinical laboratory setting.

Materials and methods

To determine the most suitable viral genomic region(s) for designing a multiplex RT-PCR, enabling reliable detection and differentiation of infections with HPV2, HPV27, and HPV57, ten complete

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genome sequences of targeted HPV types retrieved from the GenBank database (accession nos. X55964, EF117890, EF117891, EF362754, EF362755, X74473, AB211993, X55965, U37537, and AB361563) were aligned using the MAFFT v6.846 algorithm (34), as described previously (35). After evaluating the multiple alignment of complete HPV2, HPV27, and HPV57 genome sequences, the HPV L2 gene was selected as the most appropriate target region. Type-specific RT-PCR primers and hydrolysis probes (Table 1), allowing amplification of 144-, 145-, and 157-bp fragments of the respective L2 genes, were designed using Vector NTI Advance v11 software (Thermo Fisher Scientific, Carlsbad, CA) and subsequently revised for thermodynamic features of primer/probe and the potential of binding to non-targeted DNA sequences using the web-based applications NetPrimer (PREMIER Biosoft International, Palo Alto, CA), Primer3Plus (36), BLAST (National Center for Biotechnology Information, US National Library of Bethesda, MD), and MFEprimer-2.0 (37). As shown in Table 1, primer combinations 2–27F(59.8)/2R(59.2), 2–27F(59.8)/27R(57.6), and 57F(57.8)/57R(57.9) were used to amplify targeted regions of HPV2, HPV27, and HPV57, respectively. Type-specific hydrolysis probes—HPV2-P0(68.25), HPV27-P0(68.55), and HPV57-P0(65.34) (Table 1)—hybridized completely (100%) only with targeted HPV types and presented several (up to seven) nucleotide mismatches with non-targeted nucleotide sequences, enabling reliable discrimination between infections with HPV2, HPV27, and HPV57 (Fig. 1).

In order to optimize the amplification conditions and to evaluate the sensitivity, specificity, and efficiency of the HPV2/27/57 multiplex RT-PCR, plasmid standards containing viral sequences with binding sites of type-specific primers and probes were generated as follows. Three respective sense primers—HPV2-L2-FW (5'-CCCATTGTGTGATATTTGC-3'), HPV27-L2-FW (5'-CACCCTCATTGGCTTATTA-3'), and HPV57-L2-FW (5'-CGTCTGCTGCAGTAGTGTAC-3')—were used in combination with the consensus antisense primer HPV2,27,57-L2-RW (5'-TGACATAGACATCCGTACTGA-3') to amplify 1,730- , 1,599- , and 1,580-bp fragments of HPV2, 27, and 57, respectively. The obtained PCR amplicons were puri-

Table 1 | Nucleotide sequences of primers and hydrolysis probes designed for amplification of partial L2 genes of HPV2, HPV27, and HPV57.

Primer/probe	Nucleotide sequence (5'–3')	Nucleotide position[a]	Amplicon size
2–27F(59.8)[b]	TACCTGCCCCCCAGACATT	HPV2 (4,386–4,404), HPV27 (4,359–4,377)	HPV2 (144-bp)
2R(59.2)	GGAATGTACCCAGTACGCCC	HPV2 (4,529–4,510)	HPV27 (145-bp)
27R(57.6)	AGGAATATACCCGGTACGTCC	HPV27 (4,503–4,483)	
57F(57.8)	GCAAGCAGGCTGGAACG	HPV57 (4,327–4,343)	HPV57 (157-bp)
57R(57.9)	GGTATGTAGCCTGTGCGTCC	HPV57 (4,483–4,464)	
HPV2-P0(68.25)	TEX-CCCAAGAGTGGAACAGAACACTTTAGCA-BBQ	HPV2 (4,407–4,434)	
HPV27-P0(68.55)	YAK-CTAGGGGTCTTCTTTGGCGGTCTTG-BBQ	HPV27 (4,432–4,456)	
HPV57-P0(65.34)	FAM-TTCGGTGGCCTCGGTATAGGTACT-BBQ	HPV57 (4,425–4,448)	

Legend/abbreviations: [a]Nucleotide positions of primers and probes were compared to HPV2, HPV27, and HPV57 reference sequences (GenBank accession nos. X55964, X74473, and X55965, respectively), which were adjusted to start with a first nucleotide of respective E6 genes. [b]A single sense primer was used to amplify targeted regions of two HPV types (HPV2 and HPV27).

HPV2-P0(68.25)	C	C	C	A	A	G	A	G	T	G	G	A	A	C	A	G	A	A	C	A	C	T	T	T	A	G	C	A
HPV2a-X55964	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
HPV2-EF117890	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
HPV2-EF117891	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
HPV2-EF362754	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
HPV2-EF362755	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
HPV27-X74473	T	•	•	•	•	•	G	C	•	A	•	•	•	•	•	A	•	•	•	•	•	C	•	•	G	•	•	•
HPV27b-AB211993	T	•	•	•	•	•	G	C	•	•	•	•	•	•	•	A	•	•	•	•	•	C	•	•	G	•	•	•
HPV57-X55965	A	•	•	T	•	•	G	•	•	•	•	•	•	•	•	•	G	•	•	•	•	A	•	•	•	•	•	T
HPV57b-U37537	A	•	•	T	•	•	G	•	•	•	•	•	•	•	•	•	G	•	•	•	•	A	•	•	•	•	•	T
HPV57c-AB361563	A	•	•	T	•	•	G	•	•	•	•	•	•	•	•	•	G	•	•	•	•	A	•	•	•	•	•	T
HPV27-P0(68.55)	C	T	A	G	G	G	G	T	C	T	T	C	T	T	T	G	G	C	G	G	T	C	T	T	G			
HPV2a-X55964	T	•	•	•	•	T	•	•	G	•	•	T	•	•	•	•	•	G	•	•	•	•	•	A	•			
HPV2-EF117890	T	•	•	•	•	T	•	•	G	•	•	T	•	•	•	•	•	G	•	•	•	•	•	A	•			
HPV2-EF117891	T	•	•	•	•	T	•	•	G	•	•	T	•	•	•	•	•	G	•	•	•	•	•	A	•			
HPV2-EF362754	T	•	•	•	•	T	•	•	G	•	•	T	•	•	•	•	•	G	•	•	•	•	•	A	•			
HPV2-EF362755	T	•	•	•	•	T	•	•	G	•	•	T	•	•	•	•	•	G	•	•	•	•	•	A	•			
HPV27-X74473	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•			
HPV27b-AB211993	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•			
HPV57-X55965	•	•	G	•	•	•	•	•	•	•	•	T	•	•	C	•	•	T	•	•	C	•	•	C	•			
HPV57b-U37537	•	•	•	•	•	•	•	•	•	•	•	T	•	•	C	•	•	T	•	•	C	•	•	C	•			
HPV57c-AB361563	•	•	•	•	•	•	•	•	•	•	•	T	•	•	C	•	•	T	•	•	C	•	•	C	•			
HPV57-P0(65.34)	T	T	C	G	G	T	G	G	C	C	T	C	G	G	T	A	T	A	G	G	T	A	C	T				
HPV2a-X55964	•	•	T	•	•	G	•	•	T	•	•	A	•	•	•	•	•	•	•	•	C	•	•	C				
HPV2-EF117890	•	•	T	•	•	G	•	•	T	•	•	A	•	•	•	•	•	•	•	•	C	•	•	C				
HPV2-EF117891	•	•	T	•	•	G	•	•	T	•	•	A	•	•	•	•	•	•	•	•	C	•	•	•				
HPV2-EF362754	•	•	T	•	•	G	•	•	T	•	•	A	•	•	•	•	•	•	•	•	C	•	•	•				
HPV2-EF362755	•	•	T	•	•	G	•	•	T	•	•	A	•	•	•	•	•	•	•	•	C	•	•	C				
HPV27-X74473	•	•	T	•	•	C	•	•	T	•	•	T	•	•	•	•	•	•	•	•	C	•	•	•				
HPV27b-AB211993	•	•	T	•	•	C	•	•	T	•	•	T	•	•	•	•	•	•	•	•	C	•	•	•				
HPV57-X55965	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•				
HPV57b-U37537	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•				
HPV57c-AB361563	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•				

Figure 1 | Schematic diagram showing hybridization of HPV2, HPV27, and HPV57 type-specific hydrolysis probes to respective L2 gene sequences. The figure was obtained from a multiple sequence alignment of type-specific hydrolysis probes and complete genome sequences of respective HPV types that were retrieved from the GenBank database (GenBank accession numbers are provided next to all full genome sequences included). Dots show the nucleotide positions of hydrolysis probes identical to the targeted regions of HPV2, HPV27, and HPV57.

fied using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into plasmid vectors with the TOPO XL PCR Cloning Kit (Thermo Fisher Scientific). Plasmid HPV2/27/57 DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen), verified by direct Sanger sequencing and quantified at 260 nm using a NanoDrop ND-2000c spectrophotometer (NanoDrop Technologies, Oxfordshire, UK). The quantified plasmids contained 1.31×10^{10}, 3.30×10^{10}, and 2.59×10^{10} copies of HPV2, 27, and 57 DNA per µl, respectively, and were subsequently serially diluted, as described previously (38). All commercially available reagents were used according to the manufacturers' instructions.

The HPV2/27/57 multiplex RT-PCR was performed in a 96-well plate on a LightCycler 480 Instrument II using a LightCycler 480 Probes Master kit (Roche Diagnostics, Mannheim, Germany). The RT-PCR protocol was designed following the manufacturer's instructions and adjusted to (i) characteristics of targeted nucleotide sequences and synthesized primers/probes and (ii) estimated length of RT-PCR amplicons. The thoroughly optimized reaction mixture consisted of 10 µl of 2 × LightCycler 480 Probes Master (Roche Diagnostics), 0.5 µM of each RT-PCR primer (Table 1), with the exception of the 2–27F(59.8) primer, which was used in a concentration of 1 µM, 0.1 µM of each probe, 5 µl of template DNA (50–100 ng of DNA extracted from clinical samples and $1 \times 10 - 1 \times 10^8$ DNA copies/reaction of plasmid standards), and PCR-grade water up to the final reaction volume of 20 µl. The amplification of targeted nucleotide sequences was performed as follows: (i) initial denaturation of template DNA at 95 °C for 10 min (temperature transition rate of 4.4 °C/s), (ii) followed by 40 amplification cycles consisting of three incubation steps: 95 °C for 10 s (4.4 °C/s), 60 °C for 30 s (2.2 °C/s), and 72 °C for 1 s (4.4 °C/s; fluorescent signal acquisition), and (iii) a final cooling step at 2.2 °C/s to 40 °C with a 30 s hold. Since type-specific hydrolysis probes were labeled with three different 5' fluorophores (TEX, YAK, and FAM; Table 1), real-time monitoring of the fluorescent signal was performed on 610, 560, and 530 nm channels, indicating amplification of HPV2, HPV27, and HPV57, respectively. In addition, due to the slight overlap of the emission spectra of the dyes, the software's color compensation function was applied during the analysis of all RT-PCR experiments. Moreover, the specificity of all HPV2/27/57 RT-PCR amplicons was further confirmed by direct Sanger sequencing with the same primers as used for the RT-PCR, as described previously (39).

The performance of the HPV2/27/57 multiplex RT-PCR in the routine clinical laboratory setting was evaluated on 35 fresh-frozen tissue samples, obtained from the same number of children, 2 to 18 years old, with common warts (10 samples) and anogenital warts (25 samples) that were referred to the Laboratory for Molecular Microbiology and Slovenian HIV/AIDS Reference Centre, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, in the last 10 years.

The tissue samples were first processed for total DNA extraction with a QIAamp DNA Mini Kit (Qiagen) and spectrophotometric analysis of eluted DNA, as described previously (40). Up to 100 ng of extracted DNA was used for downstream PCR analyses. The integrity of the extracted DNA was determined by the quantitative RT-PCR, enabling amplification of the 150-bp fragment of human beta-globin gene. Briefly, the beta-globin RT-PCR was performed on a LightCycler 2.0 Instrument (Roche Diagnostics) using a QuantiTect SYBR Green PCR + UNG Kit (Qiagen). The reaction mixture consisted of 12.5 µl of 2 × QuantiTect SYBR Green PCR Master mix, 0.5 µM of each primer (41), 5 µl of extracted DNA, and PCR-grade water up to the final reaction volume of 25 µl. The amplification of human DNA was performed as follows: (i) initial denaturation of template DNA at 95 °C for 15 min (temperature transition rate of 20 °C/s), (ii) followed by 45 amplification cycles consisting of three incubation steps: 94 °C for 15 s (20 °C/s), 60 °C for 20 s (20 °C/s), and 72 °C for 20 s (2 °C/s; fluorescent signal acquisition at 530 nm), (iii) a melting curve analysis, consisting of three incubation steps: 95 °C for 0 s (20 °C/s), 50 °C for 30 s (20 °C/s), and 95 °C for 0 s (0.1 °C/s), and (iv) a final cooling step at 20 °C/s to 40 °C with a 30 s hold. Testing triplicates of 10-fold serially diluted standards of commercially available human DNA (Human Genomic DNA; Promega, Madison, WI), spanning from 100 ng to 1 pg of DNA per reaction, showed that the beta-globin RT-PCR had a sensitivity of at least 10 pg of human DNA per reaction. The correlation coefficient (R^2) of the standard curve estimated from amplification of human DNA standards over six orders of magnitude and the efficiency of human DNA amplification (E) were estimated at 0.996 and 91.4%, respectively. Only beta-globin-positive DNA isolates (melting peaks between 80.5 and 81.5 °C) were considered adequate for further analyses.

To detect low-risk *Alpha*-PVs associated with various mucosal and cutaneous warts, a PCR protocol targeting an approximately 190-bp fragment of the E1 gene of HPV2, HPV3, HPV6, HPV7, HPV10, HPV11, HPV13, HPV27, HPV28, HPV29, HPV32, HPV40, HPV42, HPV43, HPV44, HPV57, HPV74, HPV77, HPV78, HPV91, HPV94, HPV117, and HPV125 was performed, as described elsewhere (31), and HPV types were subsequently determined by direct Sanger sequencing of all eligible PCR products, as described previously (39). Furthermore, a FRET-based HPV6/11 RT-PCR (40), enabling reliable detection and differentiation of 25.3, 42.9, and 43.4 DNA copies of HPV11 and prototypic and non-prototypic HPV6 genomic variants, respectively, was additionally used to determine the causal agents of condylomata acuminata.

Results

Testing replicates of 10-fold serially diluted plasmids containing targeted fragments of HPV2, HPV27, and HPV57 in concentrations spanning from 1×10^8 to 1×10 DNA copies per reaction, in a background of 100 ng of Human Genomic DNA, showed that the HPV2/27/57 multiplex RT-PCR is able to detect at least 10 viral copies of each targeted HPV type per a single reaction (Fig. 2; A1, B1, C1). The dynamic range of HPV2/27/57 multiplex RT-PCR was seven orders of magnitude for all targeted HPV types, enabling reliable discrimination of 10 to 10^8 viral genome equivalents per a single reaction. The correlation coefficients (R^2) of standard curves estimated from amplification of plasmid standards containing fragments of HPV2, HPV27, and HPV57 were 0.999, 0.999, and 0.998, respectively. The amplification efficiencies (E) were estimated at 95.2, 92.0, and 92.2% for HPV2, HPV27, and HPV57, respectively, and the performance of the HPV2/27/57 multiplex RT-PCR was not affected by the presence of background human genomic DNA. In addition, as shown in Fig. 2 (A2, B2, and C2), no cross-reactivities of HPV27/HPV57, HPV2/HPV57, and HPV2/HPV27 were observed when using primer/probe combinations targeting HPV2, HPV27, and HPV57, respectively. Moreover, all three primer/probe combinations were efficient in amplifying 500 copies of targeted DNA in a background of 1×10^8, 1×10^7, 500, 100, and 10 copies of non-targeted viral DNA per reaction (Fig. 2; A3, B3, C3).

As shown in Table 2, the targeted fragment of human beta-globin gene was successfully amplified from all 35 DNA isolates obtained from fresh-frozen tissue specimens of condylomata acuminata and verrucae vulgares. HPV2, HPV27, and HPV57 were

detected in 7/10 (70.0%) tested verrucae vulgares using both Low-risk *Alpha*-PV PCR and HPV2/27/57 multiplex RT-PCR; and in all seven HPV-positive cases both PCRs identified the same HPV type (Table 2; samples nos. 1–7). The results of both PCRs were additionally completely concordant when testing different warts from the anogenital region, since HPV2, HPV27, and HPV57 were detected in 13/25 (52.0%) tested samples, irrespective of the method used. Furthermore, in seven condylomata acuminata that were previously HPV6-positive using Low-risk *Alpha*-PV PCR, the presence of HPV6 was confirmed with the HPV6/11 RT-PCR and all seven samples tested HPV2/27/57-negative using the HPV2/27/57 multiplex RT-PCR (Table 2, samples nos. 24–30). Using the PCR protocols mentioned above, *Alpha*-PV DNA was absent in three and five samples of tested verrucae vulgares and condylomata acuminata, respectively (Table 2, samples nos. 8–10 and nos. 31–35, respectively).

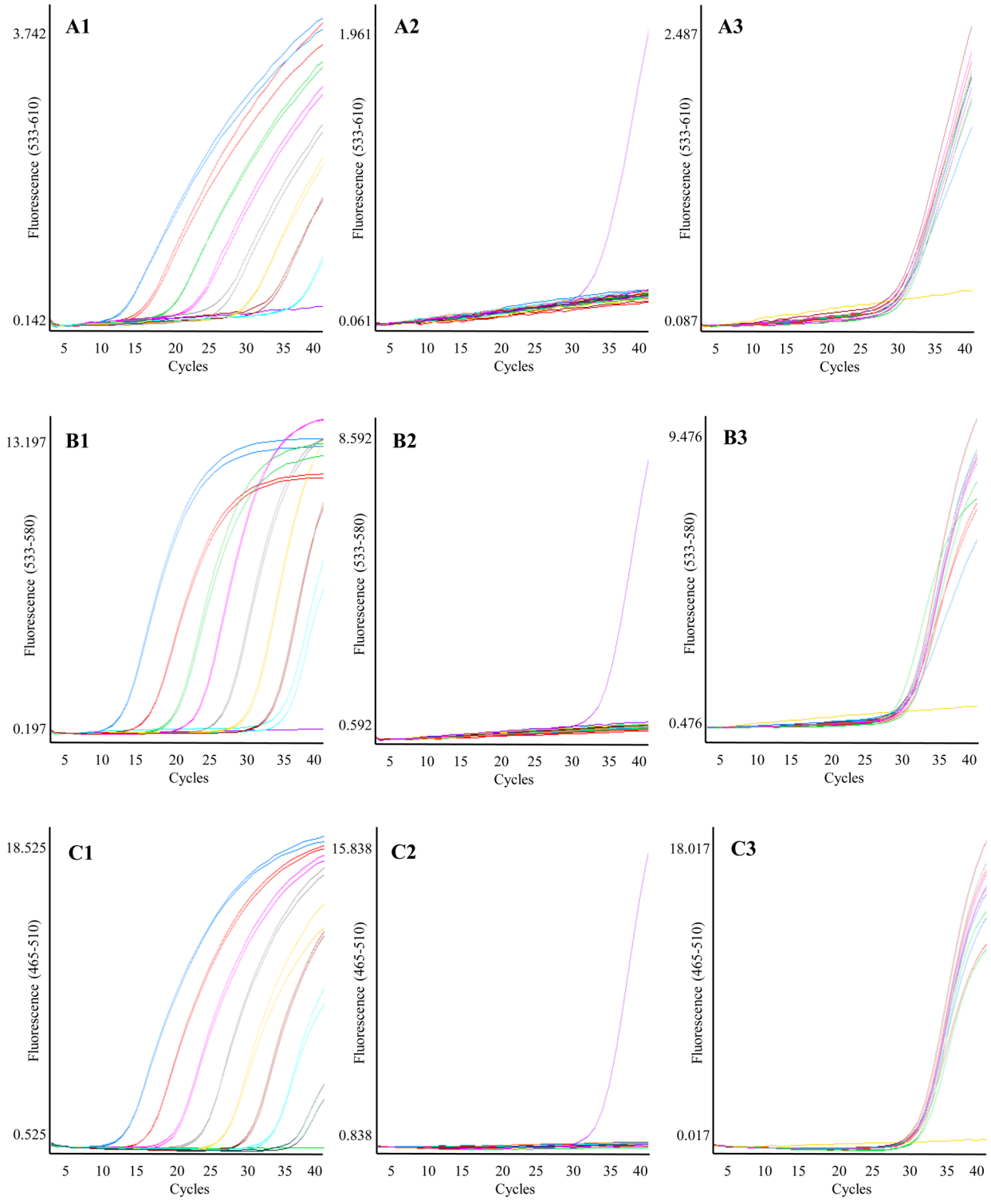

Figure 2 | Evaluation of the performance of HPV2/27/57 multiplex RT-PCR based on the amplification of plasmid standards containing targeted nucleotide sequences of HPV2, HPV27, and HPV57. (A1, B1, and C1) RT-PCR amplification plots of replicates of 10-fold serially diluted plasmids containing targeted fragments of HPV2 (A1), 27 (B1), and 57 (C1) in concentrations spanning from 1×10^8 to 1×10 DNA copies per reaction, in a background of 100 ng of commercially available human DNA (Human Genomic DNA; Promega, Madison, WI), showing that the HPV2/27/57 multiplex RT-PCR is able to detect at least 10 viral copies of each targeted HPV type per a single reaction. (A2, B2, and C2) No amplification of HPV27/HPV57 (A2), HPV2/HPV57 (B2), and HPV2/HPV27 (C2) was observed when using primer/probe combinations targeting HPV2, HPV27, and HPV57, respectively. (A3, B3, and C3) RT-PCR amplification plots showing that all three primer/probe combinations are efficient in amplifying 500 copies of HPV2 (A3), HPV27 (B3), and HPV57 (C3) in a background of 1×10^8, 1×10^7, 500, 100, and 10 viral copies of HPV27/HPV57, HPV2/HPV57, and HPV2/HPV27 per reaction, respectively.

Table 2 | Clinical samples of condylomata acuminata and verrucae vulgares used to evaluate the performance of the HPV2/27/57 multiplex RT-PCR in the routine clinical laboratory setting.

Patient no.	Age (years)	Beta-globin RT-PCR[a]	Low-risk *Alpha*-PV PCR[b]	HPV6/11 RT-PCR[c]	HPV2/27/57 multiplex RT-PCR
1	9	positive	HPV2	negative	HPV2
2	5	positive	HPV2	negative	HPV2
3	10	positive	HPV27	negative	HPV27
4	12	positive	HPV27	negative	HPV27
5	7	positive	HPV57	negative	HPV57
6	13	positive	HPV57	negative	HPV57
7	14	positive	HPV57	negative	HPV57
8	18	positive	negative	negative	negative
9	12	positive	negative	negative	negative
10	13	positive	negative	negative	negative
11	4	positive	HPV2	negative	HPV2
12	8	positive	HPV2	negative	HPV2
13	5	positive	HPV2	negative	HPV2
14	6	positive	HPV2	negative	HPV2
15	9	positive	HPV2	negative	HPV2
16	7	positive	HPV2	negative	HPV2
17	6	positive	HPV57	negative	HPV57
18	11	positive	HPV57	negative	HPV57
19	4	positive	HPV57	negative	HPV57
20	5	positive	HPV57	negative	HPV57
21	8	positive	HPV57	negative	HPV57
22	5	positive	HPV57	negative	HPV57
23	12	positive	HPV57	negative	HPV57
24	5	positive	HPV6	HPV6	negative
25	8	positive	HPV6	HPV6	negative
26	6	positive	HPV6	HPV6	negative
27	7	positive	HPV6	HPV6	negative
28	7	positive	HPV6	HPV6	negative
29	4	positive	HPV6	HPV6	negative
30	3	positive	HPV6	HPV6	negative
31	5	positive	negative	negative	negative
32	7	positive	negative	negative	negative
33	2	positive	negative	negative	negative
34	3	positive	negative	negative	negative
35	4	positive	negative	negative	negative

Legend/abbreviations: [a]The integrity of the extracted DNA was determined by the quantitative RT-PCR, enabling amplification of the 150-bp fragment of human beta-globin gene. [b]A previously published Low-risk *Alpha*-PV PCR (31) was used to detect *Alpha*-PVs that are most frequently associated with various mucosal and cutaneous warts. [c]A FRET-based HPV6/11 RT-PCR (40), enabling reliable detection and differentiation of HPV11 and prototypic and non-prototypic HPV6 genomic variants, respectively, was used to determine the causal agents of condylomata acuminata. Tissue samples obtained from patients nos. 8–10 and 31–35 were additionally tested for the presence of *Gamma/Mu*-PVs and/or MCV (data not shown).

Discussion

Verrucae vulgares or common warts constitute the most frequent benign HPV-associated skin condition, especially in children and immunosuppressed patients (5, 6). Most common warts resolve spontaneously within several months, have a benign nature, and are successfully treated with various regimens or procedures such as cryotherapy, salicylic acid, and topical and intralesional immunotherapy (42). Although they are more prevalent in children, the etiology of common warts does not differ according to patient's age group; common warts are most frequently associated with infections with three HPV genotypes: HPV2, HPV27, and HPV57 (1–12). In contrast, the etiology of warts found in the anogenital region differs between children and adults. Sexually transmitted HPV6 and HPV11 are by far the most common HPV types identified in warts in the anogenital region of adult patients of both genders because the great majority of these warts are indeed condylomata acuminata and only rarely verrucae vulgares (11, 12, 16–18, 43). In contrast, up to two-thirds of warts found in the anogenital region of children are actually verrucae vulgares, which are most frequently etiologically associated with infections with HPV2, HPV27, and HPV57 (13–15, 19). Even though both HPV6 and HPV11 are associated with a small proportion of warts found in the anogenital region of children, the routes of transmission of condylomata acuminata in this population are mostly non-sexual, including vertical transmission and indirect transmission through contaminated objects or surfaces, and are only rarely a result of sexual abuse (13–15, 19).

For years, warts identified in the anogenital region of patients of all ages (including children) referred to our molecular diagnostics laboratory had first been tested for the presence of *Alpha*-PVs using the Low-risk *Alpha*-PV PCR (31), with a turnaround time of at least 370 min, including the analysis of PCR products using direct Sanger sequencing. Although very sensitive and specific, Low-risk *Alpha*-PV PCR is quite laborious, has a long turnaround time, and is therefore inappropriate for use in a routine clinical laboratory setting. The newly developed HPV2/27/57 multiplex RT-PCR is able to specifically detect at least 10 viral copies per a single reaction of each targeted HPV type, irrespective of potentially high concentrations of other HPV types present in a sample (concurrent HPV infection with several HPV types), and its performance is also not affected by the presence of a high background of human genomic DNA. Furthermore, HPV2/27/57 multiplex RT-PCR has a relatively short turnaround time of approximately 70 min, rendering it appropriate for routine diagnostics. Therefore, when testing warts found in the anogenital region of a child, the HPV2/27/57 multiplex RT-PCR recently became the method of choice in our laboratory. HPV2/27/57-negative children's warts are subsequently tested for the presence of HPV6 and HPV11 using the HPV6/11 RT-PCR (40), and when both of these RT-PCRs are negative the conventional Low-risk *Alpha*-PV PCR is used as a supportive method due to its ability to detect several other cu-

taneous and mucosal wart-associated *Alpha*-PV types (31). In contrast, because more than 90% of warts identified in the anogenital region of adult patients are etiologically associated with sexually transmitted HPV6 and HPV11 (11, 12, 16–18, 43), when testing this patient population the method of choice in our laboratory is the HPV6/11 RT-PCR (40), followed in the case of a negative result by HPV2/27/57 multiplex RT-PCR and Low-risk *Alpha*-PV-PCR (31). *Alpha*-PV-negative wart tissue samples are additionally tested in our laboratory for research purposes only for the presence of several *Gamma*- and *Mu*-PVs that cause sporadic cutaneous warts (7, 9, 11, 12, 26, 44, 45). All HPV-negative warts identified in the anogenital region of patients of all ages and both sexes are additionally tested in our laboratory for the presence of molluscum contagiosum virus (MCV) using the MCV FRET RT-PCR (45) because, due to the similar clinical appearance of lesions, up to 10% of molluscum contagiosum lesions can be misdiagnosed as condylomata acuminata or verrucae vulgares and vice versa (45–48).

Because HPV2, HPV27, and HPV57 are associated with a large fraction of verrucae vulgares in immunosuppressed patients, in which they often occur ubiquitously and confluently, Senger et al. provided a basis for the development of virus-like particle-based vaccines against cutaneous *Alpha*-PVs (49, 50). Our HPV2/27/57 multiplex RT-PCR could therefore be additionally applicable for large epidemiological studies of the etiology of common warts in immunosuppressed patients and for potential evaluation of the efficacy of the future vaccine(s) against HPV2, HPV27, and HPV57.

In contrast to previously described conventional PCRs (23–30), which amplify up to 835-bp fragments of HPV DNA, the HPV2/27/57 multiplex RT-PCR targets significantly shorter HPV DNA fragments (144–157-bp), also rendering it very appropriate for HPV typing in archival tissue specimens (51). Furthermore, the majority of conventional broad-spectrum PCRs are not suitable for detecting viral targets present in low concentrations, and Sanger sequencing of PCR products hinders the identification of concurrent HPV infections. Namely, in sporadic cases of common warts concurrent infections with two or more HPV types can be identified, including their well-known etiological agents, such as HPV1, HPV2, HPV4, HPV7, HPV27, HPV57, and HPV65 (3, 7, 10, 26, 32). Because one of the surrogate markers for determining the etiology of common warts is the estimation of the viral load of each HPV type present in the lesion of question (7, 35, 52), HPV2/27/57 multiplex RT-PCR can be used in combination with other quantitative HPV type-specific RT-PCRs to identify the HPV type with the highest HPV viral load and consequently the highest probability of being a "true" etiological agent of the investigated common wart.

In conclusion, the newly developed HPV2/27/57 multiplex RT-PCR, which enables simple, rapid, sensitive, and specific concurrent detection and differentiation of infections with HPV2, HPV27, and HPV57 in a single PCR reaction, is an appropriate test for use in routine clinical laboratory settings and for studies focusing on the molecular epidemiology, pathogenesis, and natural history of HPV2/27/57-related lesions.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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References

1. Rübben A, Krones R, Schwetschenau B, Grussendorf-Conen EI. Common warts from immunocompetent patients show the same distribution of human papillomavirus types as common warts from immunocompromised patients. Br J Dermatol. 1993;128:264-70.
2. Jabłońska S, Majewski S, Obalek S, Orth G. Cutaneous warts. Clin Dermatol. 1997;15:309-19.
3. Rübben A, Kalka K, Spelten B, Grussendorf-Conen EI. Clinical features and age distribution of patients with HPV2/27/57-induced common warts. Arch Dermatol Res. 1997;289:337-40.
4. Majewski S, Jablonska S. The role of HPVs in benign and malignant cutaneous proliferations. Papillomavirus Report. 2003;14:1-10.
5. Bouwes Bavinck JN, Euvrard S, Naldi L, Nindl I, Proby CM, Neale R, et al. Keratotic skin lesions and other risk factors are associated with skin cancer in organ-transplant recipients: a case-control study in The Netherlands, United Kingdom, Germany, France, and Italy. J Invest Dermatol. 2007;127:1647-56.
6. van Haalen FM, Bruggink SC, Gussekloo J, Assendelft WJ, Eekhof JA. Warts in primary schoolchildren: prevalence and relation with environmental factors. Br J Dermatol. 2009;161:148-52.
7. Köhler A, Meyer T, Stockfleth E, Nindl I. High viral load of human wart-associated papillomaviruses (PV) but not beta-PV in cutaneous warts independent of immunosuppression. Br J Dermatol. 2009;161:528-35.
8. Cardoso JC, Calonje E. Cutaneous manifestations of human papillomaviruses: a review. Acta Dermatovenerol Alp Pannonica Adriat. 2011;20:145-54.
9. de Koning MN, Khoe LV, Eekhof JA, Kamp M, Gussekloo J, ter Schegget J, et al. Lesional HPV types of cutaneous warts can be reliably identified by surface swabs. J Clin Virol. 2011;52:84-7.
10. Bruggink SC, de Koning MN, Gussekloo J, Egberts PF, ter Schegget J, Feltkamp MC, et al. Cutaneous wart-associated HPV types: prevalence and relation with patient characteristics. J Clin Virol. 2012;55:250-5.
11. Cubie HA. Diseases associated with human papillomavirus infection. Virology. 2013;445:21-34.
12. Doorbar J, Egawa N, Griffin H, Kranjec C, Murakami I. Human papillomavirus molecular biology and disease association. Rev Med Virol. 2015;25:2-23.
13. Syrjänen S, Puranen M. Human papillomavirus infections in children: the potential role of maternal transmission. Crit Rev Oral Biol Med. 2000;11:259-74.
14. Marcoux D, Nadeau K, McCuaig C, et al. Pediatric anogenital warts: a 7-year review of children referred to a tertiary-care hospital in Montreal, Canada. Pediatr Dermatol. 2006;23:199-207.
15. Aguilera-Barrantes I, Magro C, Nuovo GJ. Verruca vulgaris of the vulva in children and adults: a nonvenereal type of vulvar wart. Am J Surg Pathol. 2007;31:529-35.

16. Aubin F, Prétet JL, Jacquard AC, Saunier M, Carcopino X, Jaroud F, et al. Human papillomavirus genotype distribution in external acuminata condylomata: a large French national study (EDiTH IV). Clin Infect Dis. 2008;47:610-5.
17. Garland SM, Steben M, Sings HL, James M, Lu S, Railkar R, et al. Natural history of genital warts: analysis of the placebo arm of 2 randomized phase III trials of a quadrivalent HPV (types 6, 11, 16, 18) vaccine. J Infect Dis. 2009;119:805-14.
18. Komloš KF, Kocjan BJ, Košorok P, Luzar B, Meglič L, Potočnik M, et al. Tumor-specific and gender-specific pre-vaccination distribution of human papillomavirus types 6 and 11 in anogenital warts and laryngeal papillomas: a study on 574 tissue specimens. J Med Virol. 2012;84:1233-41.
19. Varma S, Lathrop E, Haddad LB. Pediatric condylomata acuminata. J Pediatr Adolesc Gynecol. 2013;26:e121-2.
20. Nuovo GJ, Lastarria DA, Smith S, Lerner J, Comité SL, Eliezri YD. Human papillomavirus segregation patterns in genital and nongenital warts in prepubertal children and adults. Am J Clin Pathol. 1991;95:467-74.
21. Anderson KM, Perez-Montiel D, Miles L, Allen CM, Nuovo GJ. The histologic differentiation of oral condylomata acuminatum from its mimics. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2003;96:420-8.
22. Purdie KJ, Surentheran T, Sterling JC, Bell L, McGregor JM, Proby CM, et al. Human papillomavirus gene expression in cutaneous squamous cell carcinomas from immunosuppressed and immunocompetent individuals. J Invest Dermatol. 2005;125:98-107.
23. Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells. 1989;7:209-14.
24. Shamanin V, Delius H, de Villiers E. Development of a broad spectrum PCR assay for papillomaviruses and its application in screening lung cancer biopsies. J Gen Virol. 1994;75:1149-56.
25. Forslund O, Antonsson A, Nordin P, Stenquist B, Hansson BG. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J Gen Virol. 1999;80:2437-43.
26. Harwood CA, Spink PJ, Surentheran T, Leigh IM, de Villiers EM, McGregor JM, et al. Degenerate and nested PCR: a highly sensitive and specific method for detection of human papillomavirus infection in cutaneous warts. J Clin Microbiol. 1999;37:3545-55.
27. Lai JY, Doyle RJ, Bluhm JM, Johnson JC. Multiplexed PCR genotyping of HPVs from plantaris verrucae. J Clin Virol. 2006;35:435-41.
28. Jeney C, Takács T, Sebe A, Schaff Z. Detection and typing of 46 genital human papillomaviruses by the L1F/L1R primer system based multiplex PCR and hybridization. J Virol Methods. 2007;140:32-42.
29. Lei YJ, Gao C, An R, Shi Q, Chen JM, Yuan YK, et al. Development of a multiplex PCR method for detecting and typing human papillomaviruses in verrucae vulgaris. J Virol Methods. 2008;147:72-7.
30. Sasagawa T, Mitsuishi T. Novel polymerase chain reaction method for detecting cutaneous human papillomavirus DNA. J Med Virol. 2012;84:138-44.
31. Odar K, Kocjan BJ, Hošnjak L, Gale N, Poljak M, Zidar N. Verrucous carcinoma of the head and neck—not a human papillomavirus-related tumour? J Cell Mol Med. 2014;18:635-45.
32. de Koning MN, ter Schegget J, Eekhof JA, Kamp M, Kleter B, Gussekloo J, et al. Evaluation of a novel broad-spectrum PCR-multiplex genotyping assay for identification of cutaneous wart-associated human papillomavirus types. J Clin Microbiol. 2010;48:1706-11.
33. Schmitt M, de Koning MN, Eekhof JA, Quint WG, Pawlita M. Evaluation of a novel multiplex human papillomavirus (HPV) genotyping assay for HPV types in skin warts. J Clin Microbiol. 2011;49:3262-7.
34. Katoh K, Toh H. Parallelization of the MAFFT multiple sequence alignment program. Bioinformatics. 2010;26:1899-900.
35. Hošnjak L, Kocjan BJ, Pirš B, Seme K, Poljak M. Characterization of two novel gammapapillomaviruses, HPV179 and HPV184, isolated from common warts of a renal-transplant recipient. PLoS One. 2015;10:e0119154.
36. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. Nucleic Acids Res. 2012;40:e115.
37. Qu W, Zhou Y, Zhang Y, Lu Y, Wang X, Zhao D, et al. MFEprimer-2.0: a fast thermodynamics-based program for checking PCR primer specificity. Nucleic Acids Res. 2012;40:W205-8.
38. Kocjan BJ, Poljak M, Seme K. Universal ProbeLibrary based real-time PCR assay for detection and confirmation of human papillomavirus genotype 52 infections. J Virol Methods. 2010;163:492-4.
39. Kocjan BJ, Poljak M, Seme K, Potočnik M, Fujs K, Babic DZ. Distribution of human papillomavirus genotypes in plucked eyebrow hairs from Slovenian males with genital warts. Infect Genet Evol. 2005;5:255-9.
40. Kocjan BJ, Seme K, Poljak M. Detection and differentiation of human papillomavirus genotypes HPV-6 and HPV-11 by FRET-based real-time PCR. J Virol Methods. 2008;153:245-9.
41. van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA, et al. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. Int J Cancer. 2002;98:590-5.
42. Sterling J. Treatment of warts and molluscum: what does the evidence show? Curr Opin Pediatr. 2016;28:490-9.
43. Majewski S, Jablonska S. Human papillomavirus-associated tumors of the skin and mucosa. J Am Acad Dermatol. 1997;36:659-85.
44. Ure AE, Forslund O. Characterization of human papillomavirus type 154 and tissue tropism of gammapapillomaviruses. PLoS One. 2014;9:e89342.
45. Hošnjak L, Kocjan BJ, Kušar B, Seme K, Poljak M. Rapid detection and typing of Molluscum contagiosum virus by FRET-based real-time PCR. J Virol Methods. 2013;187:431-4.
46. Hanson D, Diven DG. Molluscum contagiosum. Dermatol Online J. 2003;9:2.
47. Tyring SK. Molluscum contagiosum: the importance of early diagnosis and treatment. Am J Obstet Gynecol. 2003;189:12-6.
48. Trama JP, Adelson ME, Mordechai E. Identification and genotyping of molluscum contagiosum virus from genital swab samples by real-time PCR and pyrosequencing. J Clin Virol. 2007;40:325-9.
49. Senger T, Becker MR, Schädlich L, Waterboer T, Gissmann L. Identification of B-cell epitopes on virus-like particles of cutaneous alpha-human papillomaviruses. J Virol. 2009;83:12692-701.
50. Senger T, Schädlich L, Textor S, Klein C, Michael KM, Buck CB, et al. Virus-like particles and capsomeres are potent vaccines against cutaneous alpha HPVs. Vaccine. 2010;10;28:1583-93.
51. Kocjan BJ, Hošnjak L, Poljak M. Detection of alpha human papillomaviruses in archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens. J Clin Virol. 2016;76 Suppl 1:S88-97.
52. Bzhalava D, Johansson H, Ekström J, Faust H, Möller B, Eklund C, et al. Unbiased approach for virus detection in skin lesions. PLoS One. 2013;8:e65953.