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

Lea Hošnjak¹, Kristina Fujs Komloš², Boštjan J. Kocjan¹, Katja Seme¹, Mario Poljak³

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 10⁷ 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 10⁴, 10³, 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

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 vulgaris 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 vulgaris (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 vulgaris 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 vulgaris 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 vulgaris, 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
genome sequences of targeted HPV types retrieved from the GenBank database (accession nos. X55964, EF117890, EF117891, EF362754, EF362755, X74473, AB219933, 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 MFPrimer-2.0 (37). As shown in Table 1, primer combinations 2–27F(59.8)/2R(59.2), 2–27F(59.8)/2R(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-F, HPV27-L2-F, and HPV57-L2-F—were used to amplify targeted regions of HPV2, HPV27, and HPV57 (Fig. 1). 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 MFPrimer-2.0 (37). As shown in Table 1, primer combinations 2–27F(59.8)/2R(59.2), 2–27F(59.8)/2R(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).

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

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Nucleotide position*</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV2-P0(68.25)</td>
<td>ACTGTCACCCCCCAGACATT</td>
<td>HPV2 (4,386–4,404), HPV27 (4,359–4,377)</td>
<td>HPV2 (144-bp)</td>
</tr>
<tr>
<td>HPV2a-X55964</td>
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</tr>
<tr>
<td>HPV2-EF117890</td>
<td>· · · · · · · · · ·</td>
<td>HPV2 (4,529–4,510)</td>
<td>HPV27 (145-bp)</td>
</tr>
<tr>
<td>HPV2-EF117891</td>
<td>· · · · · · · · · ·</td>
<td>HPV2 (4,503–4,483)</td>
<td>HPV27 (157-bp)</td>
</tr>
<tr>
<td>HPV2-EF362754</td>
<td>· · · · · · · · · ·</td>
<td>HPV2 (4,327–4,343)</td>
<td>HPV57 (157-bp)</td>
</tr>
<tr>
<td>HPV2-EF362755</td>
<td>· · · · · · · · · ·</td>
<td>HPV2 (4,407–4,434)</td>
<td>HPV57 (157-bp)</td>
</tr>
<tr>
<td>HPV27-P0(68.55)</td>
<td>TAMCAGCTTTCCTTTCTGGCTTGGCATGACT-3'</td>
<td>HPV2 (4,342–4,456)</td>
<td>HPV57 (4,425–4,448)</td>
</tr>
<tr>
<td>HPV27a-X74473</td>
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</tr>
<tr>
<td>HPV27b-AB211993</td>
<td>· · · · · · · ·</td>
<td>· · · · · · · ·</td>
<td>· · · · · · · ·</td>
</tr>
<tr>
<td>HPV57-P0(65.34)</td>
<td>· · · · · · · · · ·</td>
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</tr>
</tbody>
</table>

Legend/abbreviations: *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. †A single sense primer was used to amplify targeted regions of two HPV types (HPV2 and HPV27).

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.
HPV2/HPV27/HPV57 multiplex real-time PCR

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 × 10^−1 × 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 22 °C ± 0.5 °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 (R²) 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^−1 to 1 × 10 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 10^9 viral genome equivalents per a single reaction. The correlation coefficients (R²) 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. The amplification efficiencies (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.

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 vulgaris. 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 $1 \times 10^8$ to $1 \times 10^9$ 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 $1 \times 10^8$, $1 \times 10^7$, 500, 100, and 10 viral copies of HPV27/HPV57, HPV2/HPV57, and HPV2/HPV27 per reaction, respectively.
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 intraleisional immunotherapy (42). Although they are more prevalent in children, the etiology of common warts does not differ according to the patient’s age group; common warts are most frequently associated with infections with three HPV genotypes: 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 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, direct Sanger sequencing possesses 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 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 cutaneous warts.
Because one of the surrogate markers for determining the etiology of concurrent infections with two or more HPV types can be identifying 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 epidemiological 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**


