Journal of Virological Methods 187 (2013) 345–351

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Simultaneous detection, typing and quantitation of oncogenic human papillomavirus by multiplex consensus real-time PCR

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Article history:
Received 17 January 2011
Received in revised form 11 July 2012
Accepted 30 October 2012
Available online 14 November 2012

Keywords:
HPV
High-risk HPV
Real-time PCR
Cervical cancer
Cervical screening

A B S T R A C T

A consensus multiplex real-time PCR test (PT13-RT) for the oncogenic human papillomavirus (HPV) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 is described. The test targets the L1 gene. Analytical sensitivity is between 4 and 400 GU (genomic units) in the presence of 500 ng of human DNA, corresponding to 75,000 human cells. HPV types are grouped into multiplex groups of 3 or 4 resulting in the use of 4 wells per sample and permitting up to 24 samples per run (including controls) in a standard 96-well real-time PCR instrument. False negative results are avoided by (a) measuring sample DNA concentration to control that sufficient cellular material is present and (b) including HPV type 6 as a homologous internal control in order to detect PCR inhibition or competition from other (non-oncogenic) HPV types. Analysis time from refrigerator to report is 8 h, including 2.5 h hands-on time. Relative to the HC2 test, the sensitivity and specificity were respectively 98% and 83%, the lower specificity being attributable to the higher analytical sensitivity of PT13-RT. To assess type determination comparison was made with a reversed line-blot test. Type concordance was high ($\kappa = 0.79$) with discrepancies occurring mostly in multiple-positive samples.

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1. Introduction

Human papillomavirus (HPV) infection is the primary cause of cervical cancer (Walboomers et al., 1999). Of the 118 types described by de Villiers et al. (2004) 13 have been confirmed as being oncogenic in genital mucosa (IARC, 2007).

HPV testing has been shown to have a higher sensitivity for high-grade cervical dysplasia than the Papanicolaou test, although the specificity is lower due to the high prevalence of benign, transient HPV infections (Cuzick et al., 2006). Many countries have adopted HPV testing as a secondary screening (triage) test for low-grade and equivocal dysplasia (Wright et al., 2002; Waage, 2005) and its use in primary screening has also been suggested (Dillner et al., 2008). The HPV test used most widely is the Qiagen/Digene hybrid capture (HC2) test. HC2 is commercially available worldwide, straightforward to perform and interpret, and supported by a large body of scientific literature making it the baseline against which other tests are compared. The HC2 test classifies the HPV types detected as high-risk or low-risk, without more detailed genotyping. Where genotyping is required more sophisticated tests based on nucleic acid amplification are required.

There are several advantages to genotyping: it distinguishes benign serial transient infections from type-specific persistent infections which have a high probability of neoplastic progression; it distinguishes HPV types of differing oncogenic potential (Schiffman et al., 2005); and it provides epidemiological information which will be of particular value in monitoring the population effects of vaccination against HPV 16 and HPV 18.

Genotyping tests that cover the full range of oncogenic HPV types are available commercially but all the currently available tests involve post-PCR sample handling which necessitates stringent, labour- and resource-intensive molecular hygiene procedures to prevent carry-over contamination. This limits the applicability of such tests in the routine laboratory.

With the aim of providing a more rapid, less labour-intensive HPV typing test that operates in a closed system, a quantitative real-time PCR method using consensus primers and multiplexed type-specific 5′-hydrolysis probes was developed. This test, allows sensitive and specific detection and typing of the 13 oncogenic HPV types HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66. Using standard 96-well instrumentation, clinical samples may be analysed in batches of up to 22 and the entire analysis, including sample processing and reporting can be completed in one working day.

Abbreviations: HPV, human papillomavirus; GU, genomic units; LNA, locked nucleic acid; MGB, minor groove binder; HC2, hybrid capture test.

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http://dx.doi.org/10.1016/j.jviromet.2012.10.021

2. Materials and methods

2.1. Human DNA

All solutions of HPV control DNA were diluted to the appropriate concentrations in a 100 ng/μl solution of human DNA (Sigma D4642, Sigma–Aldrich, St. Louis, MI) in 1× TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) in order to mimic the composition of a cell-rich clinical sample as closely as possible.

2.2. Plasmids

Plasmids containing entire HPV genomes were provided by John Arrand with the permission of Lutz Gissmann (HPV 6, 11, 16 and 18), Gerard Orth (HPV 33 and HPV 39), Attila Lorincz (HPV 31, 35, 45, 52 and 56), Saul Silverstein (HPV 51) and Toshihaki Matsukara (HPV 58 and 59). Plasmids were transformed into *Escherichia coli* DH5-alpha and isolated by caesium chloride density gradient centrifugation (Sambrook et al., 1989) or Qiagen HiSpeed Midi Kit (Qiagen GmbH, Hilden, Germany). DNA concentrations were determined by ultraviolet spectrophotometry (Sambrook et al., 1989). The HPV 66 plasmid was a synthetic sequence corresponding to nucleotides 6320–6600 in the HPV 66 genome (U31794) cloned into pUC57, synthesised by Genscript Corporation, Scotch Plains, NJ. Ten-fold serial dilutions of plasmid DNA in 100 ng/μl human DNA were prepared in order to provide concentrations appropriate for experimental use.

2.3. Clinical samples

Were cervical cytobrush samples sent to Unilabs Telelab from clinical practises in Southeastern Norway for routine HPV analysis subsequent to findings of low-grade dysplasia, equivocal cytology or inadequate sample according to Norwegian guidelines (Waage, 2005). Samples were collected and transported in CYTYC thin prep transport medium (CYTYC, Crawley, UK) according to the manufacturer's recommendations. 10 ml of sample was centrifuged at 3000 rpm for 10 min, the pellet was resuspended in 100 μl phosphate buffered saline and DNA was extracted using the MagNAPure automatic DNA extraction instrument with the MagNAPure LC DNA extraction kit (Roche Diagnostics, Penzberg, Germany). DNA concentration was measured by determining *A*260 and *A*280 using the Eppendorf Biophotometer (Eppendorf AG, Hamburg, Germany) according to the manufacturer's instructions. Samples yielding DNA concentrations < 5 ng/μl were considered to contain too little material and rejected. Samples ($N = 206$) were tested by hybrid capture test, PT13-RT and reverse line-blot analysis.

2.4. Hybrid Capture test

The Digene Hybrid Capture (HC2) test (Digene, Gaithersburg, MD) was performed using 10 ml of sample according to the manufacturers' instructions.

2.5. Reverse line-blot analysis

Biotin-labelled amplicons were generated using primers PTf and 5′ biotin-labelled PTrGr3 (see Table 2), which were designed to have broad specificity for mucosal HPV types and which were found (data not shown) to amplify all the oncogenic HPV types targeted in the real-time PCR reaction. PCR reactions were run in a 50 μl volume containing 5 μl of sample DNA in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using DyNAmo SYBR green qPCR mix with ROX passive control dye (Finnzymes, Espoo, Finland) and the standard PCR program described in Section 2.8. Sample quality was controlled by a parallel amplification of the human globin gene (Saiki et al., 1985) under the same reaction conditions. Denaturation profiles from 60 to 91 °C (temperature increment 1.75 °C/min) were obtained and melting temperatures were determined by the position of peaks in the first derivative plot using the software provided with the instrument. The success of β-globin PCR was assessed according to the presence of a denaturation peak at 81 °C.

Table 1
Probes for reverse line blot analysis.

Target	Sequence
HPV 16	CTGCAAATTTAGCCAGTTCAAA
HPV 18	GCTTCACCTGGCAGCTGTGT
HPV 31	GGCTCCGGTTCAACAGCTAC
HPV 33	TGCCTCTATTCAAAGCAGTGC
HPV 35	GGTACCACTGGCACATTG
HPV 39	GGCACAGATATACGTGCAAA
HPV 45	GGCACTAGCGCTAATATGC
HPV 51	GTAGTGGTAATGGCCGTG
HPV 52	GGGTCTAACTCTGGCAATA
HPV 56	GGTAGCAATGGTAGAGAAC
HPV 58	GGTCCGGTAATACTGCAG
HPV 59	GGTACTGACATACGTGCC
HPV 68	GGCACTGACATACGTGACA

5′ amino-labelled probes (MWG Biotech, Ebersburg, Germany) were bound to carbodiimide-activated nylon membranes (Pall Biodyne C, Pall Europe Ltd., Portsmouth, UK) as described previously (Schouls et al., 1999). Each probe was applied in two 2 mm parallel stripes of 100 and 200 pmol respectively using a lineblot manifold (Miniblotter MN45, Immunetics, Cambridge MA, USA). The probes, which were selected from regions of high intertypic variability without known intratypic polymorphism, are listed in Table 1.

10 μl aliquots of amplified samples supplemented with tracer dye (0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% Orange G, 15% Ficoll-400, 2 mM Tris–HCL pH 7.5, 50 mM EDTA pH 8.0) were diluted in 150 μl 2× SSPE, 0.1% SDS (2× SSPE is 0.3 M NaCl, 0.02 M NaH_2PO_4, 0.2 M EDTA), denatured at 99 °C for 10 min then placed immediately on ice. 140 μl of sample was applied in the Miniblotter MN45 with the sample channels running perpendicular to the probe stripes. The apparatus was sealed and incubated overnight at 45 °C. After aspiration of samples and washing of the sample channels the apparatus was disassembled and the filter was washed twice in 2× SSPE, 0.5% SDS for 10 min at 50 °C, then incubated in 10 ml 2× SSPE, 0.5% SDS containing 2.5 μl (3.75 mU) streptavidin–peroxidase conjugate (Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at 42 °C with rotary mixing. After washing twice in 2× SSPE, 0.5% SDS for 10 min at 42 °C and two brief rinses in 2× SSPE at 42 °C amplicon detection was performed using the Amersham ECL system (Amersham Biosciences, Piscataway, NJ, USA) using a 10 min film exposure.

2.6. Primer design

The PCR primers (see Table 2) were designed to target conserved regions of the L1 gene corresponding to positions 6348–6373 and 6556–6579 in the HPV 16 genome (K02718). A sequence similarity tree of the concatenated primer target sequences provided the initial basis for the grouping of the target types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66, plus the internal control, HPV 6) into four primer target sequence similarity groups. A distance tree of these sequences was constructed using CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The initial groups were: HPV 18, 45, 52 and 59; HPV 39, 51, 56 and 66; HPV 16, 33 and 58; and HPV 31, 35 and HPV 6. A single forward primer, PTf was found to be suitable for all groups. Reverse primers were optimised for each group. Variable positions were accommodated by using inosine as a wildcard base, thymine as a

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