BRITISH JOURNAL OF BIOMEDICAL SCIENCE
2018, VOL. 75, NO. 3, 110–115
https://doi.org/10.1080/09674845.2017.1411864

Rapid detection and subtyping of human papillomaviruses in condyloma acuminatum using loop-mediated isothermal amplification with hydroxynaphthol blue dye

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ABSTRACT

Objective: Condyloma acuminatum (CA) is a common, viral, sexually transmitted disease worldwide. Human papillomavirus (HPV) genotyping has important clinical implications for the treatment of CA. We developed a loop-mediated isothermal amplification (LAMP) method for the detection of HPV.

Methods: We collected 294 cervical scrape samples, including 30 HPV-6-positive, 30 HPV-11-positive, 22 HPV-16-positive, 20 HPV-42-positve, 30 HPV-43-positive, 20 HPV-44-positive and 142 HPV-negative samples. Tissues from 40 patients with a pathological diagnosis of CA were paraffin-embedded and analyzed by LAMP and Luminex. Hydroxynaphthol blue (HNB) and electrophoresis were used to detect the results of LAMP.

Results: LAMP and Luminex systems were compared in detecting six subtypes of HPV. LAMP reactions were specific for each subtype. The sensitivity of LAMP for HPV-6, as determined by the HNB indicator assay, was 1000 copies/tube. The kappa value between the two methods was 0.98 (HPV-6), 0.94 (HPV-11), 0.89 (HPV-43), 0.87 (HPV-42) 0.79 (HPV-16) and 0.68 (HPV-44). Among the 142 HPV-negative samples determined by the Luminex assay, HPV-6 was detected in eight and HPV-11 in one by LAMP. Among the 40 CA samples, the results of LAMP and Luminex were in agreement in 38 (95%).

Conclusion: The results of this study indicated that the LAMP assay with HNB is superior to the Luminex method in terms of sensitivity and specificity. The specificity of LAMP was 100% and the sensitivity of LAMP was 1000 copies/tube using HNB. LAMP is therefore a useful, quick and accurate method for the clinical diagnosis of HPV subtypes.

ARTICLE HISTORY
Received 9 August 2017
Accepted 15 November 2017

KEYWORDS
Loop-mediated isothermal amplification; LAMP; human papillomavirus; HPV

Introduction

Human papillomavirus (HPV) is a small DNA virus. More than 140 types of HPV have been identified [1] and classified as low-risk or high-risk carcinogenic types, according to their oncogenic potential [2]. The most common high-risk HPVs are types 16 and 18, which are responsible for >70% of cervical cancers and other anogenital carcinomas in women and men, as well as oropharyngeal tumours [3]. Condyloma acuminatum (CA) is a benign tumour caused by infection of the cutaneomucous regions of the genital and anal areas with low-risk HPVs such as HPV-6 or HPV-11 [4]. Histopathological diagnosis and virological testing are required in the clinical diagnosis of CA, and, with identification of the genotype if possible [5]. Various methods are currently used to identify HPV infections. Polymerase chain reaction (PCR) is highly specific and sensitive, but is time-consuming and requires extensive laboratory equipment [6]. Immunohistochemistry is relatively inexpensive, but the sensitivity and specificity are lower than PCR [7, 8]. Luminex suspension array technology, which combines PCR with hybridization to fluorescence-labelled polystyrene bead microarrays, has also been used for the clinical detection of HPV genotypes [9], but is time-consuming, costly and requires specialized equipment. These limitations of existing methods mean that the CA patients need a sensitive, specific, rapid and cost-effective assay for determining HPV genotype [10].

Loop-mediated isothermal amplification (LAMP) is a single-tube gene amplification method that generates large amounts of target DNA [11, 12]. The reaction proceeds at 63–65 °C without thermal cycling, and the procedure can be completed in 1 h. The LAMP assay uses four primers to anneal the target DNA, the positive reaction can be visualized directly by magnesium pyrophosphate precipitation [13]. LAMP is increasingly being

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used together with hydroxynaphthol blue (HNB) dye for the rapid and visual detection and typing of emerging viruses [14, 15]. Although the agarose gel electrophoresis is more sensitive than HNB assay, ease and rapidity of HNB assay make it more appropriate for clinical detection. LAMP can thus be performed without the need for any electrophoresis or PCR equipment, just an ordinary water bath. Additionally, the enzyme used in the LAMP reaction, Bst polymerase, is unaffected by the multiple PCR inhibitors commonly found in blood and saliva, thus avoiding the need for extensive sample purification steps [16]. The results of the LAMP assay could be observed and determined by HNB-mediated visualization with the naked eye, and without opening the tubes after amplification [17]. LAMP could thus be an effective method for the rapid diagnosis of infectious diseases, with sensitivities and specificities equivalent to PCR [18]. Although LAMP has been used to detect high-risk HPV subtypes in cervical carcinomas, genital lesions and anterior tongue carcinomas, it has not been utilized in CA [17].

We developed a LAMP HPV-type-specific DNA amplification method and compared its specificity and sensitivity with the Luminex assay. We aimed to validate LAMP as a rapid, low-cost, accurate test for the detection and identification of the six HPV subtypes (HPV-6, 11, 16, 42, 43, 44).

Materials and methods

A panel of 294 cervical scrape samples, including samples positive for HPV-6 ($n = 30$), HPV-11 ($n = 30$), HPV-16 ($n = 22$), HPV-42 ($n = 20$), HPV-43 ($n = 30$), HPV-44 ($n = 20$) and negative samples ($n = 142$), were collected from Shanghai Tenth People's Hospital and Shanghai Skin Disease Hospital between August 2014 and April 2016. The study was approved by the Ethical Review Board of Shanghai Tenth People's Hospital, Tongji University.

DNA extraction Swabs from genital warts were collected from all participants according to standard sampling procedures at the recruitment sites. Swabs were kept in 3 ml sample transport medium for Tellgenplex HPV DNA Test (Tellgen Life Science, Shanghai, China). According to the kit's guidance, the samples could be stored in the sample transport medium for 1 week at room temperature, 1 month at 2–8 °C and 6 months at −20 °C. To ensure accurate results, all samples were tested for HPV within 24 h. To determine the type-specificity of the LAMP method, DNA from six HPV types (HPV-6, -11, -16, -42, -43 and -44) was extracted from the samples using a QIA prep Spin Miniprep Kit (Qiagen Translational Medicine Co.). After extraction, DNA was eluted in 50 µl distilled water and stored at −20 °C. Extracted DNA was quantified by measurement of the optical density at 260 nm.

Type-specific primers for LAMP amplification of HPV DNA were designed based on the HPV sequences obtained from GenBank (accession numbers: HG793938.1 (HPV-6); KU298879.1 (HPV-11); KP313775.1 (HPV-16); KU298897.1 (HPV-42); HE962401.1 (HPV-43); HE963128.1 (HPV-44)). BLAST software was used to avoid possible cross-reactivity with heterologous HPV viruses. Specifically, LAMP requires four primers (B3, F3, BIP and FIP) to recognize a total of six distinct DNA sequences within the target DNA. LAMP primers for the E6 region of HPV-6, E6 region of HPV-11, E7 region of HPV-16, and L1 regions of HPV-42, HPV-43, and HPV-44 were designed using Primer Explorer V4 Software (Fujitsu, Tokyo, Japan) (Table 1). LAMP reactions were performed using 8 U of Bst polymerase (New England Bio-labs, Beverly, MA, USA), approximately 25 ng of template DNA, 1.4 mM of each dNTP, 6 mM MgSO4, 0.2 µM F3/B3primers, 1.6 µM FIP/BIP, 1× ThermoPol Amplification Buffer (New England Biolabs) in 25 µL total volume. The reactions were carried out at 65 °C for 60 min, followed by 5 min at |90 °C. Each assay was run with negative controls to avoid the contamination. Negative controls consisted of all the above LAMP reagents, but without the template DNA. Each assay was also run with a positive control including all the above LAMP reagents with template corresponding to subtype DNA, to test for successful amplification.

A positive LAMP reaction was identified in two ways: visual detection of HNB (120 µmol/L) relative to negative controls (positive amplification was indicated by a colour change from violet to sky blue), and detection of a ladder-like banding pattern by 2% agarose gel electrophoresis of amplification products at 100 V for 40 min, followed by staining with ethidium bromide. HPV genotyping was carried out using a Luminex-based assay, which combines PCR with hybridization to fluorescence-labelled polystyrene bead microarrays, as the comparison standard (Tellgen Life Science, Shanghai, China). The investigators who performed the LAMP protocols were blinded to the results of the PCR to prevent potential bias.

To determine the specificity of type-specific LAMP, DNA templates of the six single infection subtypes (HPV-6, -11, -16, -42, -43 and -44) were collected, mixed to create a positive template mixture and stored at −20 °C. Every type-specific LAMP used a negative control, and the templates were added to the LAMP system for HPV-6, -11, -16, -42, -43 and -44 subtypes, using the mixture as the DNA template. The total amount of mixture DNA was quantified by measurement of the optical density at 260 nm using a spectrophotometer. The number of templates is obtained according to the molecular weight, and all of the templates were greater than 106. Specificity for the six subtypes was determined by the colour change of HNB and by electrophoresis after LAMP amplification. Agarose gel electrophoresis was performed to verify the specificity of the reaction products. To determine the sensitivity of the assay, the HPV-6 plasmids were serially diluted tenfold from 10^{10}–1 copies with distilled water, as templates for LAMP and Luminex, respectively.

Table 1. Target sequences used as primers for HPV-type-specific LAMP.

Subtype	Primer sequence (5′–3′)	Genome position
HPV-6		
F3	GCATACGTTGCAAATTAATTGTG	72–94
B3	AGTTTCTTCTTCAACAGTTGTT	258–279
FIP	AGGACCTTTAGCTGTTTATATG-CATTGTTTTGCAAGAATG-CACTG	161–137/95–114
BIP	GGCGGCTATCCATATGCAGCG-CATAATCAAAGTGTCTATATTG-GT	168–188/248–224
HPV-11		
F3	AACATCCATAGACCAGTTGT	30–49
B3	CTAAGCAACAGGCACACG	188–205
FIP	CATTCCTGCAAAACACGCACG-CAAGACGTTTAATCTTTCTTTG	109–90/50–72
BIP	ACTGACCACCGCAGAGATAT-GAAAGTTGTCTCGCCACA	111–120/178–161
HPV-16		
F3	TGGAGATACACCCACATTG	6–24
B3	CTTTGTACGCACAACCGA	195–212
FIP	GCTGTCACTTAATTGCTCATAA-CAGGAATATATGTTAGATTTG-CAACCAG	93–69/28–52
BIP	GGACAAGCAGAACCGGACA-GAGCGTAGAGTCACACTTG	127–146/194–177
HPV-42		
F3	AAAATGTCTGCTGAGGCC	697–714
B3	GAACCACTAGGGGTAGGA	876–893
FIP	CCAGCCCTATTAAACAAAT-GACGAAGACAGTATGT-TTTTCTTTTTAAGGC	785–760/720–745
BIP	CGCAATTGGTGAACCTGTAC-CAAATTATGTCTGCCAGATGC	786–806/857–838
HPV-43		
F3	CTGACCCTAATAAATTTGGCTT	227–248
B3	GTGTTTGTTTATAATCCATTGC-TAC	433–457
FIP	ACCAATTTCAACTCCTACGCAT-CACAACACTGGTTACATCA-GAC	318–296/256–276
BIP	TAAGAATGATGACACT-GAAAACCCGTTTCTCTGT-TATCTTGTCCCG	366–390/430–410
HPV-44		
F3	ATTTGTTGGGGAAATCAGTT	955–974
B3	AGCATTCATAGTATGAATATAGGC	1156–1175
FIP	ACTGTGTAGTGGCAGCACAT-AGTTACTGTTGTAGATAC-TACCCG	1039–1019/979–1001
BIP	TAAGCAATACATGCGACATGTT-GAGCATTACCTCCGCCGTTAA	1071–1095/1155–1138

Note: Each set of primers consisted of two outer primers (F3, B3), two inner primers (FIP, BIP).

Paraffin-embedded tissues were collected from 40 patients (13 males, 27 females, mean [SD] age 23.5 [9.5] years) with a diagnosis of CA. DNA was extracted using a paraffin-embedded tissue DNA rapid extraction kit (Tellgen Life Science, Shanghai, China) and determined by LAMP and Luminex, respectively. Agreement between the two methods was quantified by the Kappa test [19].

Results

The specificity of the reaction was determined by checking the cross-reactivity of the assay among HPV types (6, 11, 16, 42, 43 and 44). DNA amplification was only observed when the primer set was reacted with the target HPV DNA. The LAMP products contained inverted repeat structures of various sizes, and positive samples thus produced a ladder pattern in agarose gel electrophoresis. HPV-type-specific LAMP primers only amplified DNA from the respective HPV-type, and no LAMP products were detected in reactions carried out with DNA from other HPV types. The LAMP reactions were thus determined to be specific for each HPV-type (Figure 1).

The sensitivity of HPV-type-specific LAMP was determined using serial dilutions of the plasmids. HPV-6 plasmids at dilutions containing 10^{10}–1 copies/tube were used to determine the detection limit of HPV-type-specific LAMP. The sensitivity of HPV-6 type-specific LAMP was 1000 copies/tube using HNB as an indicator, and 100 copies/tube by agarose gel electrophoresis. The sensitivity of the Luminex assay for HPV-6 was 1000 copies/tube.

Comparison of HPV-type-specific LAMP and Luminex assays was as follows. LAMP was used to detect six subtypes of HPV and compared with the results of Luminex assays. The agreement rates between the two methods were 87% (HPV-11), 72% (HPV-6), 68% (HPV-16), 80% (HPV-42), 83% (HPV-43) and 55% (HPV-44). Among the 142 samples identified as negative by the Luminex assay, HPV-6 was detected in eight and HPV-11 in one by the LAMP assay. The kappa value between the two methods was 0.98 (HPV-6), 0.94 (HPV-11), 0.89 (HPV-43), 0.87 (HPV-42) 0.79 (HPV-16) and 0.68 (HPV-44). Kappa values 0.81–1.00 are regarded as very good, values 0.61–0.80 as good [19].

Evaluation of LAMP for HPV detection in clinical specimens, we examined 40 biopsy specimens from patients with CA and compared the results of HPV-type-specific LAMP and Luminex assays. Among the 40 CA samples, 18 (45%) were positive for HPV-6, seven (14%) for HPV-11, two (5%) for HPV-43 and HPV-44 and one (2.5%) for HPV-16 and HPV-42 according to LAMP. HPV DNA was detected in 30 of the 40 (75%) samples by LAMP, and in 31 of 40 (77.5%) by Luminex. HPV-6 and HPV-44 DNA were detected by LAMP in two samples that were negative by Luminex. The histology and immunohistochemistry showed that the above two cases were characteristic of CA. The agreement between the two detection methods was 95% (38 of 40).

Discussion

LAMP has been reported to detect various types of infectious agents, including varicella-zoster virus, SARS coronavirus, herpes simplex virus, measles virus, mumps virus and influenza virus [20–25]. HPV is detectable in CA and in bowenoid papulosis, and represents a potentially problematic sexually transmitted disease [26]. It is therefore important to identify the causative genital HPV type, from both epidemiological and public health

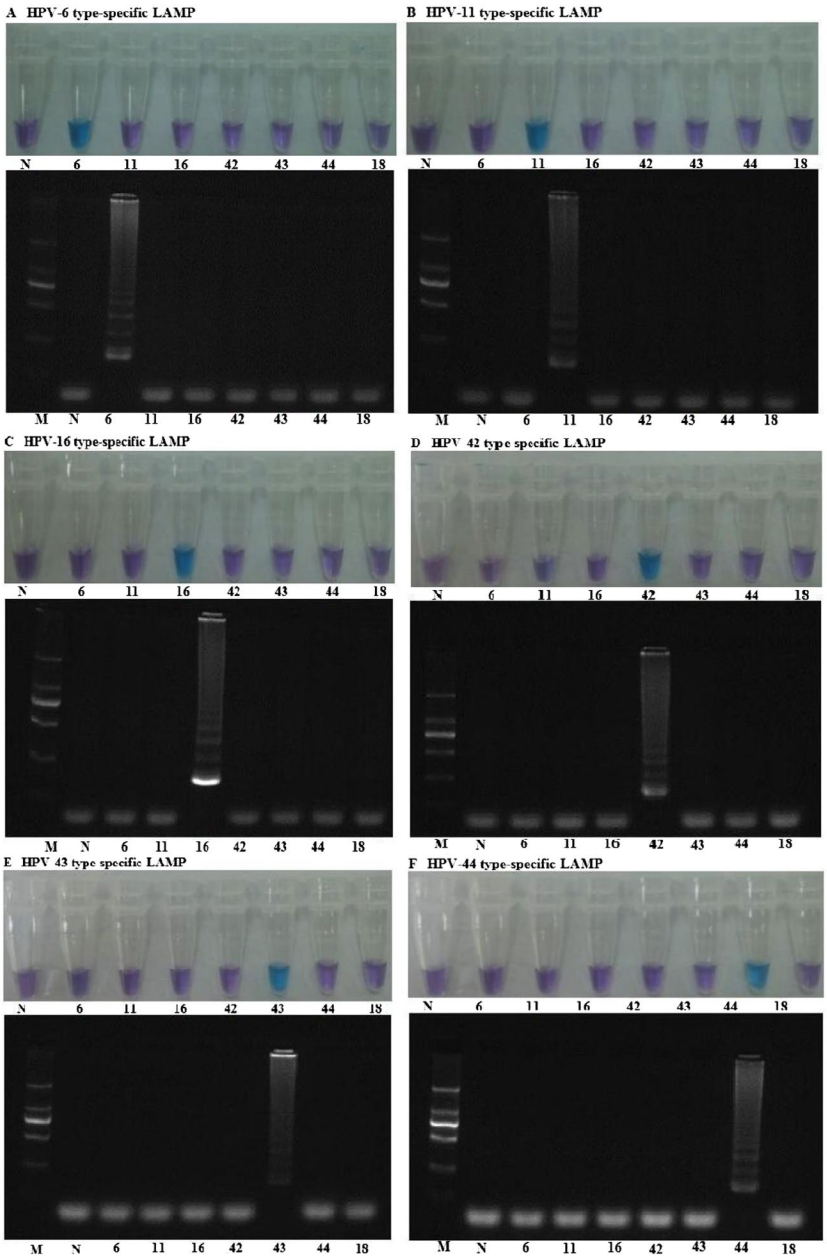

Figure 1. LAMP and molecular genetics**.**

Notes: HPV DNAs were amplified by HPV-6 (A), HPV-11 (B), HPV-16 (C), HPV-42 (D), HPV-43 (E) and HPV-44 (F) type-specific LAMP to determine the specificity of the method. LAMP products were detected by HNB and agarose gel electrophoresis. Positive amplification was indicated by a colour change from violet to sky blue in HNB. M: DL2000, DNA ladder marker; *N*: negative control.

perspectives. The results of the current study suggest that LAMP may be a useful and accurate technique for the detection of HPV subtypes in clinical CA samples.

Although the sensitivities and specificities of LAMP were similar to those of the Luminex method, LAMP has the additional advantages of being rapid and cost-effective. LAMP only amplified the respective type of HPV DNA with no cross-reactivity, and this specificity was confirmed by HNB assays and agarose gel electrophoresis. The LAMP and Luminex assays used different primers, which may have affected the amplification efficiency [27]. The detection limit for HPV-type-specific LAMP using the HNB assay was 1000 copies/tube. These findings demonstrate that the LAMP assay has high specificity and efficiency for the amplification of HPV DNA.

LAMP is an efficient and sensitive assay, and even small amounts of aerosolized amplification product could cause false positive results in subsequent reactions [28]. Fortunately, the presence of HPV DNA can be detected by visual detection alone, and positive LAMP reactions were consistently confirmed by both visual detection and gel electrophoresis in the present study. The ease and rapidity of HNB assay make it more appropriate for clinical monitoring, thus avoiding the need for any specialized electrophoresis or PCR equipment. Luminex assay spent 5 h and cost 25 US dollars per test, in contrast, LAMP with HNB only needed 70 minuses and 4 US dollars each test. Therefore, LAMP with HNB has a better time benefit and more cost-efficiency. Given that LAMP with HNB assessment only requires an ordinary

water bath, the simplicity and cost-effectiveness of this method make it ideal for rapid monitoring in the clinical setting, especially in resource-limited hospitals or rural clinics. The HNB-based assay has an advantage over other colour-based assays in that the HNB is added prior to amplification [29–31], and the assay can also be manipulated to prevent accidental contamination if a reaction tube is broken or accidently opened by clinic or hospital staff.

We evaluated the reliability of HPV LAMP in clinical samples and showed that the results correlated well with those from Luminex analysis (correlation coefficient $r = 0.93$). Most positive samples contained high copy numbers of viral DNA, while no HPV DNA was detected in LAMP-negative samples. The sensitivity of LAMP was greater than that of Luminex, demonstrating the high sensitivity and specificity of LAMP for the analysis of clinical samples. Although there were some discrepancies between the two techniques, it is important to note that the Luminex results themselves may not be perfect. Our results may also have been affected by sample degradation and loss due to inadequate concentrations of source DNA. Unlike PCR, LAMP can reliably amplify DNA directly from tissue without the need for DNA purification. Furthermore, the copy number detection threshold of LAMP allows it to amplify DNA directly from a cytologic brushing of a suspicious lesion [28, 32]. All LAMP-positive samples could be distinguished from negative samples by HNB staining, which represents a potential advantage in terms of large-scale screening.

In summary, LAMP provides a sufficiently robust and accurate method for use as a clinical diagnostic test for subtyping HPV. LAMP demonstrates similar sensitivity to the Luminex assay for detecting viral DNA, with no cross-reactivity and reliable results in clinical specimens. This work represents an advance in biomedical science because the LAMP assay with HNB is thus superior to the Luminex assay in terms of sensitivity, specificity, speed and simplicity, and may be a potentially valuable tool for the detection of HPV DNA in the laboratory.

Summary table

What is known about this subject
- Genital warts and Condyloma acuminatum are attributable to HPV, particularly HPV6 and 11
- Limitations of existing methods call for a sensitive, specific, rapid and cost-effective assay for determining HPV genotype

What this study adds
- The LAMP assay with HNB is superior to the Luminex assay in terms of sensitivity, specificity, speed and simplicity

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Outstanding Young Talent Plan of Shanghai [grant number XYQ2013095]; the National Science Foundation [grant number 81370067], [grant number 81572061]; Shanghai Shenkang Hospital Development Center [grant number SHDC22014006].

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