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WarmStart colorimetric LAMP for the specific and rapid detection of HPV16 and HPV18 DNA

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ABSTRACT

Background and objectives: Persistent infection with High-Risk HPV genotypes is the principal cause for the development of cervical cancer with HPV16 and HPV18 to be the most frequently identified HPV genotypes observed in approximately 70% of cervical cancer cases worldwide. The present study focused on the development of a simple molecular methodology based on WarmStart colorimetric LAMP for the specific identification of HPV16 and HPV18.

Methods: The method was developed by designing LAMP type-specific primer sets that target the E6 gene. The assay was applied using HPV-positive clinical samples along with control cases in order to evaluate the specificity of the newly designed isothermal protocol. In addition, an experimental cutoff value was estimated through reconstitution experiments with HPV-DNA plasmids. LAMP amplicons were visualized by color changes, thus eliminating the requirement for post-amplification processing steps.

Results: The WarmStart colorimetric LAMP facilitates the isothermal amplification of 10 copies per reaction of both HPV16 and HPV18 DNA, while it exhibits 100% specificity for the detection of the corresponding genotypes in LSIL and HSIL cases. Moreover, the assay demonstrates 100% PPV and 100% NPV. Finally, the sensitivity of conventional PCR with the type-specific LAMP primer sets (B3/F3) for the HPV16, HPV18 DNA detection was 100 copies/reaction and 10 copies/reaction, respectively.

Conclusions: The newly established WarmStart colorimetric LAMP can be considered as a powerful molecular tool that it can be easily implemented in small clinical and research laboratories for a rapid and efficient identification of the most tumorigenic HPV genotypes.

1. Introduction

Cervical cancer is the second leading cause of cancer death among women worldwide (Berman and Schiller, 2017). Long-term infection with high-risk Human Papillomavirus genotypes (HPV) is the key event for the subsequent development of cervical disease. HPVs comprise a heterogeneous group of small, non-enveloped, capsid-enclosed dsDNA viruses that infect the mucosal and cutaneous epithelia (zur Hausen, 1996; Doorbar et al., 2012). In particular, the high-risk HPV genotypes HPV16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68 are considered as the key factors for the growth of severe cervical dysplasia and cervical cancer, while HPV16 and HPV18 are the most oncogenic genotypes accounting for approximately 50% and 20% of cervical cancer cases around the world, respectively (Li and Xu, 2017).

Screening for cervical cancer is traditionally based on cytological testing for routine diagnosis (the Papanicolaou, Pap test). The cytological screening test shows variable levels of sensitivity (30–87%) and is considered as a highly subjective, because it is relying on morphological criteria (Sinha et al., 2018; Goodman et al., 2018). Nowadays, molecular detection of HPV DNA is regarded as the gold standard for

Abbreviations: LAMP, loop-mediated isothermal amplification; FIP, forward inner primer; BIP, backward inner primer; F3, forward outer primer; B3, backward outer primer; LF, loop forward primer; LB, loop backward primer; GuSCN, guanidine thiocyanate; HPV, Human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion

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identification of HPV viruses, since the viral genome is detected in 99.7% of studied cervical cancer cases and exhibits higher sensitivity (84–100%) than the conventional cytological screening tests (Clavel et al., 2001; Iacobellis et al., 2018; Del Pino et al., 2017). Technologies for HPV genotyping such as Hybrid Capture 2 (HC2), Cervista, Amplicor and polymerase chain reaction (PCR) though they vary in their methods, are time-consuming and require expensive equipment (Gradíssimo and Burk, 2017; Tsakogiannis et al., 2017). Therefore, it is important to develop a simple, rapid, and cost-effective method for early detection of HPV DNA.

Loop-mediated isothermal amplification (LAMP) is an alternative method for isothermal amplification of small nucleotide targets that gain increasing popularity (Notomi et al., 2000, 2015). LAMP reaction is based on strand displacement by a Bst DNA polymerase under isothermal conditions with a temperature range between 60 °C–70 °C where a set of four (or six) different primers binds to six (or eight) different regions on the target gene making the assay highly specific. This primer set consists of two outer primers (forward outer primer - F3 and backward outer primer - B3), two inner primers (forward inner primer - FIP and backward inner primer – BIP) and eventually two loop primers (loop forward primer - LF and loop backward primer - LB) that may further reduce the amplification time (Nagamine et al., 2002). As a consequence, a minimum set of four primers that strictly recognize six distinct regions on the target sequences can be used spanning between 180bp to 200bp ensuring high specificity. The method generates a large amount of amplification products in positive samples and LAMP products can be detected by agarose gel electrophoresis or by visual inspection of color changes (Fischbach et al., 2015). The reaction can be completed within 60–80 min using simple and inexpensive instrumentation that is available in any laboratory such as a water bath or a heating block. The main advantages of LAMP are: a minimal equipment, high sensitivity of less than 50 copies of viral genomes and a rapid detection time (less than 80 min) (Blomström et al., 2008).

Towards this end, the present analysis focuses on the establishment of a reliable, non-labor and cost effective laboratory protocol based on WarmStart colorimetric LAMP methodology for HPV genotyping for the rapid and specific detection of the most prevalent high-risk HPV genotypes (HPV16, HPV18) in cervical samples through amplification of the E6 gene. The pH based colorimetric LAMP is based on the same principle of LAMP amplification but designed to allow visual detection of positive reactions. The master mix contains the pH sensitive dye phenol red in a low buffer solution that changes color from bright pink to yellow upon DNA target amplification. This system is designed to provide a fast, visual detection of amplification based on the production of protons and subsequent drop in pH that occurs in solution color from pink to yellow. This detection makes possible to use simple equipment such as a water bath or a heating block instead of sophisticated and expensive instrumentation as Real Time LAMP. Our goal was to develop a molecular tool that can be easily implemented in small and of limited resources laboratories. Finally, clinical samples as well as reconstitution experiments with HPV DNA plasmids were used in order to evaluate the sensitivity and specificity of the assay.

2. Materials and methods

2.1. Cervical samples

In the present analysis a total of 173 cervical clinical samples were examined. The women attended the colposcopy clinic of the 3rd Department of Gynecology and Obstetrics in the tertiary care “ATTIKON” University General Hospital between June 2013 and June 2017. The study population did not represent a normally screened population, since most patients attended the outpatient clinics after a referral abnormal cytology and or colposcopy. In particular, seventy-seven samples were diagnosed as low-grade squamous intraepithelial lesions (LSIL), seventy-four samples were diagnosed as high-grade squamous intraepithelial lesions (HSIL), while the control group comprised twenty-two cervical samples with normal cytology and without HPV infection (Table 1). All patients signed an informed consent form, while the study was approved by the Bioethics committee of the hospital (Approval number 5/14 – 06-2013).

Table 1
Distribution of HPV genotypes among LSIL and HSIL clinical cases.

HPV genotype	LSIL (n = 77)	HSIL (n = 74)	Total Number (n = 151)
HPV16	20	21	41
HPV18	14	10	24
HPV31	9	9	18
HPV33	1	1	2
HPV35	2	5	7
HPV45	3	4	7
HPV51	14	8	22
HPV58	4	4	8
HPV66	10	12	22

2.2. DNA isolation and HPV16 and HPV18 DNA detection

Genomic DNA was extracted from ThinPrep samples from patients and controls, respectively, using the chaotropic agent guanidine thiocyanate (GuSCN) (Casas et al., 1995). The HPV genotyping was performed by a previously described methodology of Multiplex PCR assay using L1 type-specific primer sets (Tsakogiannis et al., 2015). The methodology identified the HPV genotypes 16, 18, 45, 35, 66, 33, 51, 58, and 31 (Table 1).

2.3. Design of primers for LAMP

The WarmStart colorimetric LAMP was applied in order to detect the HPV genotypes 16 and 18 through isothermal amplification of the E6 gene. The complete E6 ORF of the reference sequence of HPV16 DNA was obtained from the sequence database PaVE (http://pave.niaid.nih.gov) and from NCBI (GI no. 333,031), while the complete E6 ORF of the reference sequence of HPV18 DNA was obtained from Genbank database (accession number NC_001357), in order to design the E6 type-specific primers for LAMP amplification. The E6 type-specific LAMP primer sets were designed in the present analysis by using the free software PrimerExplorer V5 (http://primerexplorer.jp/lampv5e/index.html). In order to test the specificity of the E6 type-specific LAMP primer sets as well as to find possible cross-reactivity with heterologous HPV sequences, LAMP primers were subjected to NCBI website with the MEGA BLAST algorithm. Each set of E6 LAMP type-specific primers consists of two outer (F3, B3) and two inner (FIP, BIP) primers (Table 2).

2.4. HPV16 - HPV18 DNA detection using F3/B3 primer sets

Cervical samples that were found to be positive for HPV16 and HPV18 DNA through Multiplex PCR assay (Tsakogiannis et al., 2015) were further subjected to conventional PCR using the newly designed E6 type-specific LAMP outer primer sets (F3/B3) for HPV16 and HPV18 DNA detection, respectively. This procedure was followed in order to test the efficacy of the newly designed type specific primer sets in PCR. The HPV16 F3/B3 primer set generated a fragment of 206bp in size, while the HPV18 F3/B3 produced a fragment of 201bp in size (Table 2). Both PCR assays were performed in a final volume of 50 µl. Briefly, each PCR mixture consisted of 25 pmol of each primer set, 10 X PCR buffer (DreamTaq DNA Polymerase, Thermo Fischer Scientific Inc., Massachusetts, USA) containing 2 mM $MgCl_2$, 0.25 mM from each dNTP (Invitrogen, Life Technologies, Paisley, UK) and 1.5U of DreamTaq DNA Polymerase. The cycling conditions were as follows: 40 cycles of 30 s at 95 °C, 30 s at 50 °C, and 1 min at 72 °C. The first cycle was proceeded by

Table 2
Sequences of type-specific primers and the positions they target for the isothermal amplification of the E6 gene of HPV16 and HPV18 through the designed WarmStart colorimetric LAMP assay. The assay incorporates two outer primers (forward outer primer - F3 and backward outer primer - B3) and two inner primers (forward inner primer - FIP and backward inner primer – BIP) for each individual HPV genotype.

LAMP primers	Sequence (5'-3')	Position
HPV16		
HPV16 E6 F3	ATGCACCAAAAGAGAACTGC	83
HPV16 E6 B3	ACAGCATATGGATTCCCATCTC	288
HPV16 E6 FIP	TGTTTGCAGCTCTGTGCATAA-GTTTCAGGACCCACAGGA	106
HPV16 E6 BIP	AGAATGTGTGTACTGCAAGCAA-ATCCCGAAAAGCAAAGTCAT	249
HPV18		
HPV18 E6 F3	AAAAACTAACTAACACTGGGTTA	376
HPV18 E6 B3	ACTTGTGTTTCTCTGCGT	577
HPV18 E6 FIP	GGTGTCTAAGTTTTTCTGCTGGAT-AATTTATTAATAAGGTGCTGCG	402
HPV18 E6 BIP	CGACGATTTCACAACATAGCTGG-GTTGGAGTCGTTCCTGTC	558

a 3 min denaturation step at 95 °C and the final cycle was followed by a 5 min elongation step at 72 °C.

The results of PCR were monitored in a 2% agarose gel stained with 1 μg/ml of ethidium bromide in Tris-borate-EDTA buffer using a 100-bp DNA ladder as a molecular weight marker (Invitrogen, Life Technologies, Paisley, UK).

2.5. Plasmid construction

Two different plasmids were constructed, each containing a partial fragment of HPV16 and HPV18 E6 genes in order to test for the sensitivity and specificity of PCR and LAMP protocols. In particular, the HPV16 and HPV18 E6 plasmids were assembled through PCR amplification of the partial fragment of the E6 gene that was generated through the outer LAMP E6 type-specific primer sets F3/B3, as it was mentioned above (Table 2). Subsequently, amplicons were subjected to cloning using the TOPO TA Cloning KIT (Life Technologies, Carlsbad, CA, USA). The recombinant plasmid DNA was extracted using the Nucleospin plasmid kit (Macherey Nagel, Duren, Germany) following the manufacturer's protocol and the plasmids were sequenced at Macrogen Europe, Amsterdam, the Netherlands. The cloned sequences were characterized by database search at the NCBI website as it was previously described.

2.6. Optimization of WarmStart colorimetric LAMP conditions

Serial ten-fold dilutions of the two type-specific HPV DNA plasmids ranging from 1 to 1×10^5 copies were used in order to normalize WarmStart colorimetric LAMP for HPV16 and HPV18 identification. Briefly, each isothermal reaction, was carried out in 25 μl volume, including 1.6 μM of each inner primer (FIP/BIP), 0.2 μM of each outer primer (F3, B3), the Warm Colorimetric 1X Master Mix which contains a low-Tris reaction buffer with all necessary cofactors at optimized concentrations for LAMP assay, Bst 2.0 WarmStart DNA Polymerase that contains 8 mM MgSO4 and Phenol Red for pH detection of LAMP products (New England Biolabs, Ipswich, MA, USA). This system is designed to provide a fast, clear visual detection of amplification based on the production of protons and subsequent drop in pH that occurs from the extensive DNA polymerase activity in a LAMP reaction, producing a change in solution color from pink to yellow, with readout of positive amplification reactions judged by eye. The reaction temperature was assessed at 65∘C and reaction time was for 60–80 min. Color was visible directly on removal from incubation temperature. The color was intensified by allowing the reaction to cool at room temperature and the results were photographed for recording. Finally, the products were also analyzed by 2% agarose gel electrophoresis and visualized under an UV transilluminator.

2.7. Sensitivity of the WarmStart colorimetric LAMP assay by gel electrophoresis and colorimetric detection

Serial ten-fold dilutions of the two type-specific DNA plasmids ranging from 1 to 1×10^5 copies were used in triplicates in order to determine the minimum HPV DNA copy number that the WarmStart colorimetric LAMP is able to identify. The WarmStart colorimetric LAMP conditions are mentioned above. Moreover, the corresponding serial ten-fold dilutions of the two type-specific DNA plasmids were used in order to asses the sensitivity of PCR with the primer sets F3/B3 for HPV16 and HPV18 DNA identification, respectively. The PCR conditions are mentioned above. Finally, the products of both methodologies were also analyzed by 2% agarose gel electrophoresis and visualized under an UV transilluminator. Finally, the detection limits were then determined for both assays (WarmStart colorimetric LAMP and PCR).

2.8. Specificity of the WarmStart colorimetric LAMP by gel electrophoresis and colorimetric detection

The specificity of the WarmStart colorimetric LAMP assay as well as the specificity of conventional PCR with the newly designed HPV16 and HPV18 type-specific primers was examined by using DNA extracts collected from clinical samples that were negative for HPV16 or HPV18 DNA, respectively, but were positive for HPV genotypes 31, 33, 35, 45, 51, 58 and 66 (Table 1). In order to further evaluate, the specificity of the newly designed LAMP primers the E6 HPV16 and HPV18 recombinant plasmids were also used as templates in order to perform the newly established protocols of WarmStart colorimetric LAMP and conventional PCR, respectively. In particular, the HPV18 E6 plasmids were used as templates in WarmStart colorimetric LAMP and PCR using the HPV16 E6 type-specific primers sets, while the HPV16 E6 plasmids were used now as templates with the HPV18 E6 type-specific primers sets. Finally, the products were also analyzed by 2% agarose gel electrophoresis.

2.9. Evaluation of the WarmStart colorimetric LAMP assay with clinical specimens

To evaluate the WarmStart colorimetric LAMP assay in clinical specimens, a total of forty-one HPV16 and twenty-four HPV18 positive samples that were detected as such by Multiplex PCR (Tsakogiannis et al., 2015) and E6 type-specific primer sets F3/B3 were further subjected to WarmStart colorimetric LAMP. Moreover, the newly established WarmStart colorimetric LAMP protocol was applied in clinical samples that were negative for the HPV genotype of our interest as well as in control group (Table 1). As it was previously described, the reaction temperature was normalized at 65∘C for 60 min. However, clinical samples that did not give a clear yellow color were incubated

for additional 20 min. The amplicons were also analyzed by 2% agarose gel electrophoresis.

Finally, the positive predictive value and negative predictive value (% PPV, % NPV) of the newly established WarmStart colorimetric LAMP and conventional PCR were calculated considering the outcomes derived from the initial HPV genotyping through Multiplex PCR using L1 type specific primer sets (Tsakogiannis et al., 2015).

2.10. Quantitative real-time PCR for HPV16 and HPV18 genotyping

The outer E6 type-specific F3/B3 primer sets were also used to establish a quantitative Real-Time PCR protocol to calculate the copy numbers of viral DNA in clinical samples with poor isothermal amplification signal. Cervical samples were tested in triplicates. Real-Time PCR assay was conducted on the Mx3005P$^{@}$ instrument and were carried out in a final volume of 20 µl. Briefly, each Real-Time PCR mixture contained 5 pmol of outer primer set, 2X Master Mix (SYBR Select qPCR Master Mix Kit, Thermo Fischer Scientific Inc., Massachusetts, USA) and ROX Reference Dye. The cycling conditions were as follows: An initial denaturation step at 95℃ for 2 min followed by 40 cycles in two steps: 95℃ for 15 s, and 60℃ for 1 min. Data acquisition at 510 nm was performed at anneal/extension step (60℃). The melting curve was generated by heating from 55 ˚C to 95 ˚C in increments of 2 ˚C. The total run time was 110 min including the time needed for melting temperature analysis.

In order to confirm the outcomes of Real-Time PCR, the amplicons were monitored in a 2% agarose gel electrophoresis stained with 1 µg/ml of ethidium bromide in Tris-borate-EDTA buffer using a 100-bp DNA ladder as a molecular weight marker (Invitrogen, Life Technologies, Paisley, UK). The HPV16 F3/B3 primer set generated a fragment of 206bp in size, while the HPV18 F3/B3 yielded a fragment of 201bp in size (Table 2).

3. Results

In the present study, a WarmStart colorimetric LAMP assay was developed in order to identify the high-risk HPV genotypes 16 and 18. E6 LAMP type-specific primers sets were designed to target highly conserved regions of the E6 gene of the corresponding HPV genotypes. The methodology was applied in a total of 173 cervical samples, forty-one of which were diagnosed as HPV16 positive and twenty-four were diagnosed as HPV18 positive using the previously described protocol of Multiplex PCR (Tsakogiannis et al., 2015) (Table 1). Moreover, the newly designed E6 type-specific LAMP outer primer sets (F3/B3) were also incorporated in HPV16 and HPV18 identification in order to evaluate the specificity of the corresponding primers as well as to compare the sensitivity of PCR assay with the newly established protocol of WarmStart colorimetric LAMP. The conventional PCR with E6 type-specific primer sets B3/F3 confirmed the initial outcomes of Multiplex PCR with L1 type- specific primer sets (Tsakogiannis et al., 2015) (Table 1). As a result, the outer primer sets for the amplification of HPV16 and HPV18 E6 gene present the same specificity with the previously described L1 type-specific primer sets (Table 3).

To determine the optimal condition for HPV detection, LAMP was conducted with different reaction temperatures while reaction time was constant at 60 min. The condition of WarmStart colorimetric LAMP assay was determined in plasmids harboring the partial fragment of E6 gene of HPV16 and HPV18 DNA. The absence or presence of the bands after gel electrophoresis was used to assess the optimum condition of each reaction as well as the clear visual detection of amplification based on the production of protons and subsequent drop in pH, thus producing a change in solution from pink to yellow, with readout of positive amplification reactions judged by eye. On the basis of the above results, LAMP conditions were optimized in a 25 µl reaction volume, containing 1.6 µM of each inner primer (FIP/BIP), 0.2 µM of each outer primer (F3, B3), the WarmStart Colorimetric 1X Master Mix, Bst 2.0 WarmStart DNA Polymerase that contains 8 mM MgSO4 and Phenol Red for pH detection of LAMP products (New England Biolabs, Ipswich, MA, USA). The reaction temperature was normalized at 65∘C and reaction time was 60 min (Fig. 1). Notably, the clinical samples that did not give a clear yellow color were incubated for additional 20 min. If desired the time can be extended for additional 20 min in samples with very low copies number as was the case of the two clinical samples that after the additional time of 20 min gave questionable results for HPV18.The copy number of these samples was determined previously only by Real Time PCR and found to be 5 copies/reaction, which is under the detection limit of colorimetric LAMP, Multiplex PCR and conventional PCR for HPV18 (10 copies/reaction), (Table 3). However conventional PCR with the newly designed E6 type-specific LAMP outer primer sets (F3/B3) HPV18 DNA provided no amplification signal.

The sensitivity of WarmStart colorimetric LAMP was considered through the previously described plasmid constructs. In particular, serial ten-fold dilutions of the HPV16 and HPV18 plasmids ranging from 1 to 1×10^5 copies were used in triplicates and the sensitivity of LAMP assay was found to be 10 copies/reaction for HPV16 as well as for HPV18 DNA detection (Fig. 2). Moreover, the corresponding serial ten-fold dilutions of plasmids were used in triplicates and the sensitivity of conventional PCR with B3/F3 for the HPV16 DNA detection was 100 copies/reaction (Fig. 2), while the sensitivity of conventional PCR for the detection of HPV18 DNA was 10 copies/reaction (Table 3, Fig. 2).

The specificity of WarmStart colorimetric LAMP and conventional PCR with B3/F3 was determined by evaluating the cross-reactivity of the assay between different HPV genotypes by using clinical samples that have been previously diagnosed as negative for HPV16 or HPV18, respectively and positive for 31, 33, 35, 45, 51, 58 and 66 (Fig. 3). Moreover, HPV16 plasmids were amplified with the newly designed HPV18 type-specific primers sets, while HPV18 plasmids were amplified using the HPV16 type-specific primers sets (Fig. 4). These results revealed that LAMP and PCR products were only produced when the primer sets reacted with their respective HPV genome, indicating that the primer sets used in the present LAMP were specific only for HPV16 and 18 sequences (Figs. 3 and 4).

Optimal WarmStart colorimetric LAMP assay was evaluated for detection of viral DNA in all clinical specimens that were used in the present study (Fig. 5). The diagnostic performance of the WarmStart colorimetric LAMP was examined using the clinical samples that were previously found to be positive for HPV16 and HPV18 infection (Table 1). Moreover, a total of eighty-six HPV positive samples (45, 35, 66, 33, 51, 58, and 31) and twenty-two HPV negative samples were also incorporated in the present study. Using WarmStart colorimetric LAMP

Table 3
Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) of WarmStart Colorimetric LAMP and Conventional PCR with the newly designed E6 type specific primer sets.

Assay	Sensitivity	Specificity	PPV	NPV
HPV16 WarmStart Colorimetric LAMP	10 copies/reaction	100%	100%	100%
HPV18 WarmStart Colorimetric LAMP	10 copies/reaction	100%	100%	100%
HPV16 Conventional PCR	100 copies/reaction	100%	100%	100%
HPV18 Conventional PCR	10 copies/reaction	100%	100%	98.84%

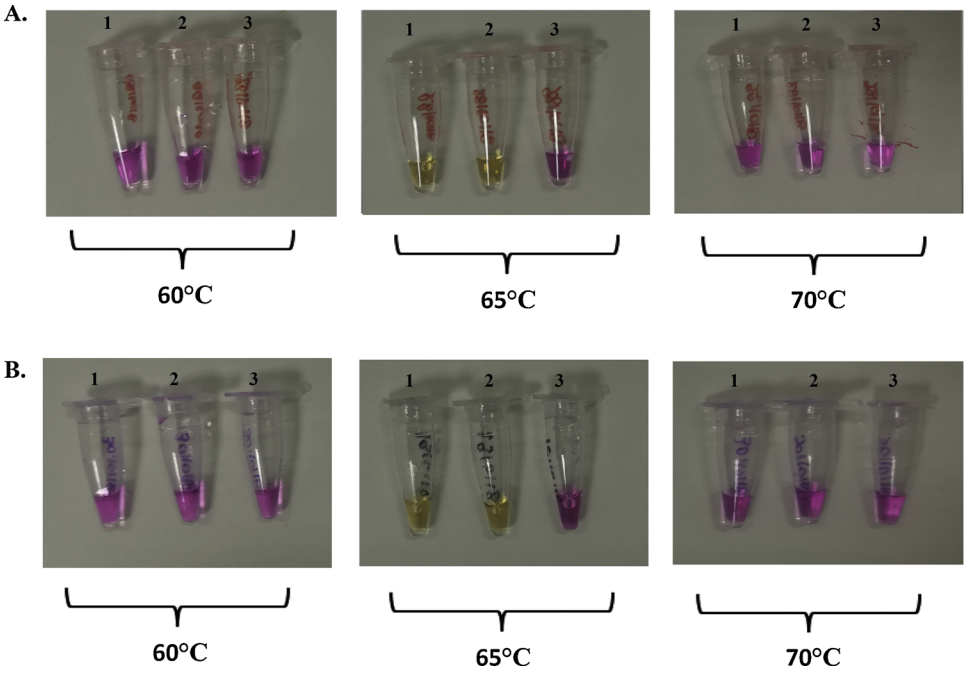

Fig. 1. Different temperature conditions of WarmStart colorimetric LAMP assay using. 10 and 100 copies of HPV16 DNA plasmids. (A) WarmStart colorimetric LAMP for the detection of HPV16 DNA at 60 °C, 65 °C and 70 °C. Tubes 1, 2 contain 100 and 10 copies of HPV16 DNA, respectively, tube 3 contains negative control-ddH_2O. (B) WarmStart colorimetric LAMP for the detection of HPV18 DNA at 60 °C, 65 °C and 70 °C. Tubes 1, 2 contain 100 and 10 copies of HPV18 DNA, respectively, tube 3 contains negative control-ddH_2O.

assay, HPV genotypes were similarly detected as with PCR and Multiplex PCR, while the assay provided negative signal in clinical samples that harbored either another HPV genotype than 16 or 18 or, were negative for HPV infection (Fig. 5). The sensitivity, specificity, positive predictive value and negative predictive value of the LAMP assay were 100%. Thus, with high specificity and sensitivity, the WarmStart colorimetric LAMP assay was effective in detecting viral DNA in clinical samples diagnosed as LSIL and HSIL.

4. Discussion

Molecular detection of HPV16 and HPV18 DNA is a significant tool

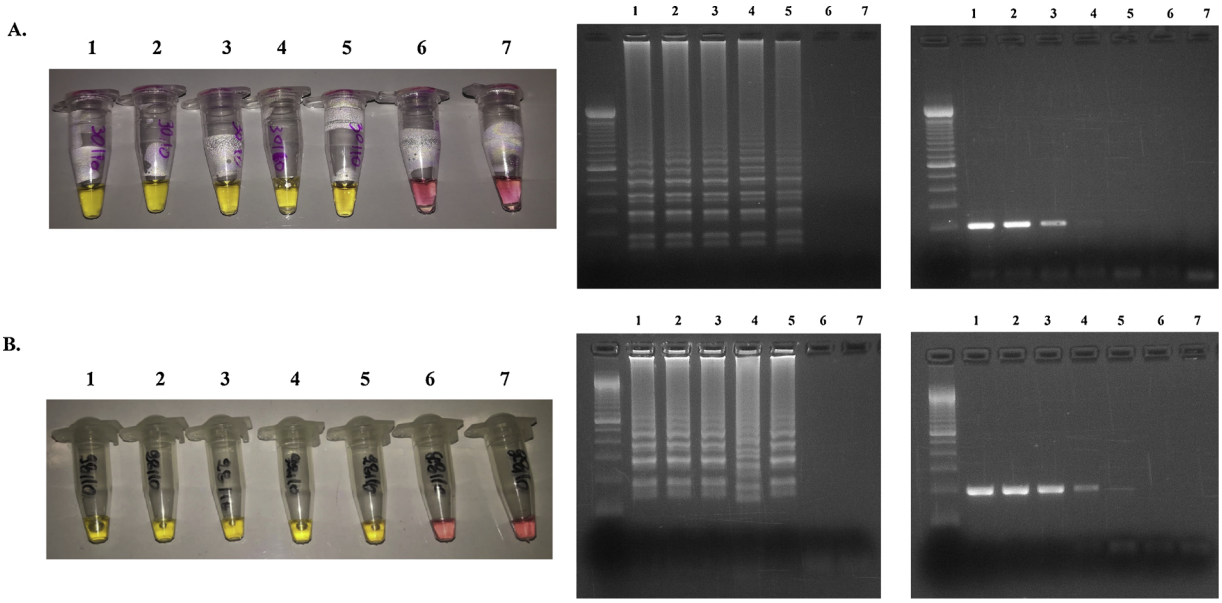

Fig. 2. Sensitivity control of WarmStart colorimetric LAMP and PCR assay using serial ten-fold dilutions of the HPV16 and HPV18 DNA plasmids ranging from 1 to 1×10^5.
(A) Warm WarmStart colorimetric LAMP sensitivity control for the detection of HPV16. Tubes from 1 to 6: 100.000, 10.000, 1000, 100, 10, 1 copy numbers of HPV16 DNA plasmids; tube 7 the negative control-ddH_2O. The outcome of sensitivity control (lanes 1-6: 100.000, 10.000, 1000, 100, 10, 1 copy numbers; lane 7: negative control-ddH_2O) was confirmed by gel electrophoresis. PCR assay using the HPV16 B3/F3 primer sets. Lanes from 1 to 6: 100.000, 10.000, 1000, 100, 10, 1 copy numbers of HPV16 DNA plasmids; lane 7 the negative control-ddH_2O.
B) Warm WarmStart colorimetric LAMP sensitivity control for the detection of HPV18. Tubes from 1 to 6: 100.000, 10.000, 1000, 100, 10, 1 copy numbers of HPV18 DNA plasmids; tube 7 the negative control-ddH_2O. The results of sensitivity control (lanes 1-6: 100.000, 10.000, 1000, 100, 10, 1 copy numbers; lane 7: negative control-ddH_2O) was confirmed by gel electrophoresis. PCR assay using the HPV16 B3/F3 primer sets. Lanes from 1 to 6: 100.000, 10.000, 1000, 100, 10, 1 copy numbers of HPV16 DNA plasmids; lane 7 the negative control-ddH_2O.
In all agarose gels, a molecular weight marker, 100bp–1.500bp with 100bp increments and an additional fragment at 2.017bp was used (Invitrogen, Life Technologies, Paisley, UK).

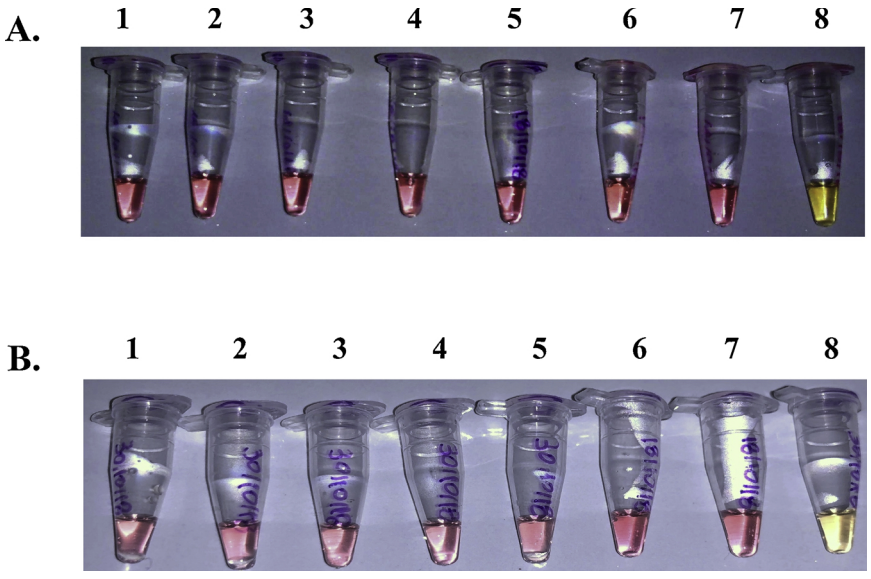

Fig. 3. (A) WarmStart colorimetric LAMP with HPV16 E6 type-specific primer sets in clinical samples negative for HPV16 infection (tube 1; HPV35, tube 2; HPV51, tube 3; HPV51, HPV33, tube 4; HPV31, HPV58 tube 5; HPV18, tube 6; HPV66, HPV45 tube 7; negative control-ddH_2O, tube 8; HPV16 positive clinical sample – positive control). (B) WarmStart colorimetric LAMP with HPV18 E6 type-specific primer sets in clinical samples negative for HPV18 infection (tube 1; HPV35, tube 2; HPV51, tube 3; HPV51, HPV33, tube 4; HPV31, HPV58 tube 5; HPV16, tube 6; HPV66, HPV45 tube 7; negative control-ddH_2O, tube 8; HPV18 positive clinical specimen- positive control).

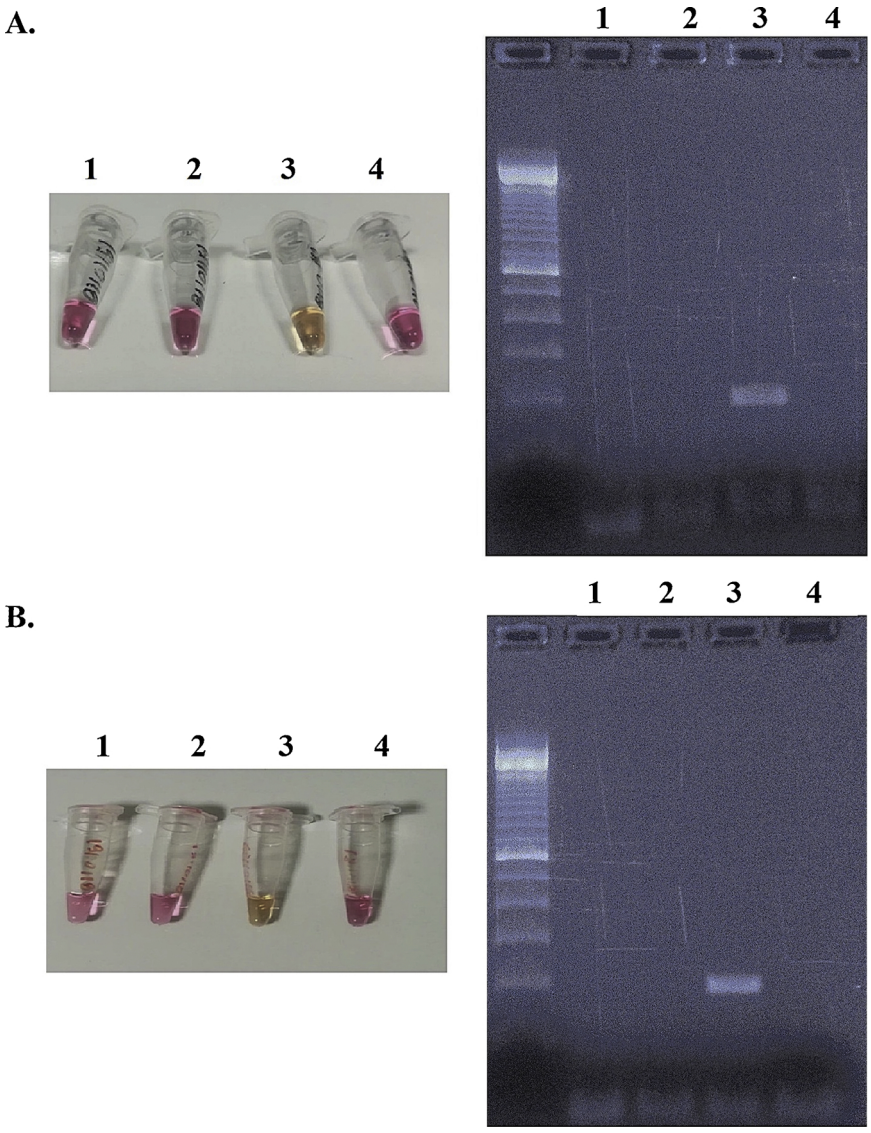

Fig. 4. Specificity control using HPV16 and HPV18 plasmids. (A) On the left, application of WarmStart colorimetric LAMP with HPV16 E6 type-specific primers. The tubes 1 and 2 correspond to HPV18 plasmids (1000 and 100 copies, respectively)-pink color, tube 3 corresponds to HPV16 plasmid (1000 copies)-yellow color and tube 4 corresponds to negative control (ddH_2O)-pink color. On the right, the outcomes of conventional PCR with B3/F3 primer sets in the respective plasmids (lanes 1, 2 correspond to HPV18 plasmids;1000 and 100 copies- negative signal, lane 3 corresponds to HPV16 plasmid;1000 copies-positive signal and lane 4 corresponds to negative control-ddH_2O). (B) On the left, application of WarmStart colorimetric LAMP with HPV18 E6 type-specific primers. The tubes 1 and 2 contain HPV16 plasmids (1000 and 100 copies, respectively)-pink color, tube 3 contain HPV18 plasmid (1000 copies)-yellow color and tube 4 corresponds to negative control-(ddH_2O)-pink color. On the right, the outcomes of conventional PCR with B3/F3 primer sets in the respective plasmids (lanes 1, 2 correspond to HPV16 plasmids;1000 and 100 copies- negative signal, lane 3 corresponds to HPV18 plasmid;1000 copies- positive signal and lane 4 corresponds to negative control-ddH_2O) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

In agarose gels a molecular weight marker, 100bp–1.500bp with 100bp increments and an additional fragment at 2.017bp was used (Invitrogen, Life Technologies, Paisley, UK).

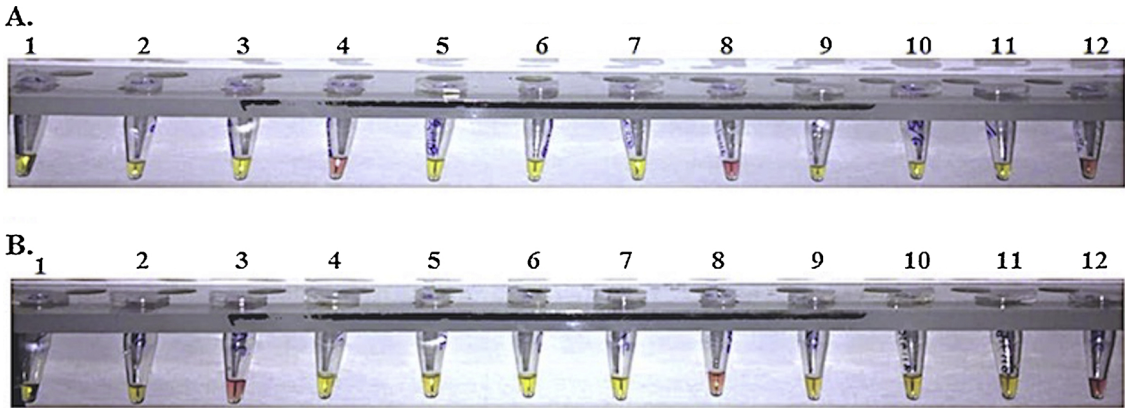

Fig. 5. Application of WarmStart colorimetric LAMP assay in clinical samples. (A) WarmStart colorimetric LAMP using HPV16 LAMP E6 type-specific primers. Tubes 1, 2, 3, 5, 6, 7, 9, 10 (yellow color-positive) include HPV16 positive clinical samples and tube 11 (yellow color-positive) contains positive control (HPV16 E6 plasmid; 100 copies). Tubes 4 and 8 (pink color-negative) contain clinical samples positive for HPV18 and HPV33 respectively, while tube 12 (pink color-negative) contains negative control-ddH_2O. (B) WarmStart colorimetric LAMP using HPV18 LAMP E6 type-specific primers. Tubes 1, 2, 4, 5, 6, 7, 9, 10 (yellow color-positive) harbor HPV18 positive clinical specimens and tube 11 (yellow color-positive) contains positive control (HPV18 E6 plasmid; 100 copies). Tubes 3 and 8 (pink color-negative) contain clinical samples positive for HPV16 and HPV66 respectively, while tube 12 (pink color-negative) contains negative control-ddH_2O. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

for predicting cervical dysplasia especially in patients without abnormal cytological findings. It has been advocated that the identification of HPV16 and HPV18 DNA increases the stratification of risk by selecting individuals who present higher risk for developing HSIL+ (Tsakogiannis et al., 2017; Castle et al., 2011; Cox et al., 2013; Schiffman and Wentzensen, 2016; Halfon et al., 2010). Although molecular technologies for HPV genotyping such as Hybrid Capture 2 (HC2), Cervista, Amplicor and polymerase chain reaction (PCR) are used in routine cervical cancer screening, they are regarded as time consuming and expensive (Gradíssimo and Burk, 2017; Tsakogiannis et al., 2017). The Loop-mediated isothermal amplification (LAMP) that was first established by (Notomi et al. (2000); Notomi et al., 2015), amplifies small, nucleotide-targets under isothermal conditions. The method is regarded as specific, simple, rapid and inexpensive. The capacity of LAMP to amplify nucleotide targets in a short time has introduced LAMP to the field of HPV-DNA identification (Livingstone et al., 2016; Kumvongpin et al., 2016; Luo et al., 2011; Saetiew et al., 2011). In the present study a rapid, sensitive and specific LAMP colorimetric assay was developed for the detection of HR HPV16 and HPV18 genotypes. The assay was established to specifically amplify a conserved region of the E6 gene of the corresponding HPV genotypes, while the monitoring of results was conducted by visual inspection of color change, reducing the time and cost of HPV identification.

The specificity of the WarmStart colorimetric LAMP showed no cross-reaction among the examined HPV genotypes 31, 33, 35, 45, 51, 58 and 66 (Table 3, Figs. 3 and 4). These findings demonstrate that the present WarmStart colorimetric LAMP assay has high specificity. It is noteworthy, that the assay could identify with high efficiency the HPV16 and HPV18 DNA among the different grades of cervical dysplasia (LSIL, HSIL). The WarmStart colorimetric LAMP assay exhibited a considerable amplification sensitivity, as well. An experimental cutoff value was determined for the WarmStart colorimetric LAMP by testing plasmid solutions containing different copy numbers of E6 fragments generated by the outer B3/F3 type-specific primer sets for HPV16 and HPV18, respectively. Moreover, the same plasmid solutions were subjected to conventional PCR using the outer B3/F3 type-specific primer sets, in order to compare the sensitivity of WarmStart colorimetric LAMP assay and PCR (Fig. 2). WarmStart colorimetric LAMP reaction detected 10 copies/test of HPV16 and 18 DNA while by PCR 100 copies/test for HPV16 DNA and 10 copies/test for HPV18 DNA were detected, proving that the sensitivity of LAMP was superior (Table 3, Fig. 2). The value of the WarmStart colorimetric LAMP lies on the monitoring of results (Fischbach et al., 2015). LAMP amplicons were visualized by color changes, thus eliminating the requirement for post-amplification processing steps, including gel electrophoresis and ethidium bromide staining. As a consequence, a non-labor, rapid, specific and sensitive molecular protocol was established that requires non-sophisticated instumentation for HPV16 and HPV18 detection.

It is considerable to highlight that the conventional PCR is not our gold standard for evaluating the performance of the LAMP assay. The initial detection and identification of HPV16 and HPV18 DNA was performed with Multiplex PCR using L1 type-specific primer sets, the sensitivity of which has been previously estimated at 10 copies/reaction (Tsakogiannis et al., 2015). As a result, we observed the same sensitivity between LAMP and Multiplex PCR and consequently no issue for calculation results of 100% PPV and 100% NPV considering LAMP assay was observed. Moreover, it is significant to underline that the conventional PCR was developed in order to examine the efficiency of the newly designed type specific primer sets in both PCR and LAMP methodology and it was not regarded as the gold standard neither for HPV genotyping nor for the evaluating the performance of the LAMP assay.

The colorimetric LAMP assay in HPV genotyping has been previously described in order to identify the HR HPV genotypes 16, 18, 45, 52, and 58, while the visual monitoring of results was achieved with the hydroxynaphthol blue dye (Saetiew et al., 2011). In this study the assay reached a sensitivity of 100 copies/test for HPV16 DNA detection and 10 copies/test for HPV18 detection, while the specificity for HPV16 and HPV18 DNA identification was 100% and 98.5%, respectively (Saetiew et al., 2011). Interestingly, the assay was designed to amplify the E7 gene of HPV16 DNA and the L1 gene of HPV18 DNA at 63 °C for 65 min (Saetiew et al., 2011). In contrast, the newly established protocol of WarmStart colorimetric LAMP targets the E6 gene of the corresponding HPV genotypes. It exhibits a higher sensitivity and specificity than the previously described protocol of colorimetric LAMP assay, since it enables the amplification of 10 copies/test of both HPV16 and HPV18 DNA, while it presents 100% specificity for the identification of HPV16 and HPV18 (Table 3, Figs. 2–4).

In conclusion, a WarmStart colorimetric LAMP assay for identification of the most tumorigenic HR-HPV genotypes HPV16 and HPV18 was developed. The sensitivity, specificity, positive predictive and negative predictive value of this assay in LSIL and HSIL clinical specimens was 100% (Table 3). As a consequence, the newly established molecular protocol of LAMP can be considered as a powerful molecular tool, particularly useful and extremely convenient in a large scale screening projects.

Conflict of interest

All authors declare that they have no conflicting or dual interests.

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