Development of field-applicable endogenous internally controlled recombinase-aided amplification (EIC-RAA) assays for the detection of human papillomavirus genotypes 6 and 11 using sample releasing agent

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HIGHLIGHTS

- The EIC-RAA assays for HPV6 and 11 possess high sensitivity and specificity.
- The endogenous human β-globin can effectively eliminate false-negatives or invalid amplification results, increasing the reliability of the EIC-RAA assay.
- The EIC-RAA assays for the detection of HPV 6 and 11 using sample releasing agent show the advantages of fast detection speed and simple operation.

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ABSTRACT

Objective: Human papillomavirus (HPV) 6 and 11 are the two most common low-risk HPV subtypes, accounting for more than 90% of condyloma acuminatum. A simple, accurate and rapid screening method to be applied in community-level hospitals is in high demand.

Methods: Endogenous internally controlled recombinase-assisted amplification (EIC-RAA) assays for HPV6 and 11 were performed in a single closed-tube at 39 °C within 30 min. The sensitivity and specificity of EIC-RAA were examined using recombinant plasmids and pre-tested HPV DNA. A total of 233 clinical samples were collected, and the DNA was extracted by traditional multi-step extraction, or sample releasing agent, before analysis by EIC-RAA. For comparison, HPV detection via Quantitative real-time PCR (qPCR) was also performed.

Results: The sensitivity of EIC-RAA analysis was 10 copies/reaction for HPV6, 100 copies/reaction for HPV11, and 100 copies/reaction for the human β-globin gene. No cross-reaction was observed with other HPV subtypes. Clinical performance of the EIC-RAA assay achieved a 100% of concordance rate with the commercial HPV qPCR kit. Further, the EIC-RAA assay achieved a 100% of concordance rate when using multi-step extracted DNA and sample releasing agent-processed DNA.

Summary: The EIC-RAA assay for HPV 6 and 11 detection possesses the advantages of accuracy, simplicity and rapidity, and demonstrates great potential to be used in community-level hospitals for field investigation.

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1. Background

The human papillomavirus (HPV) includes more than 200 different subtypes, divided into the high- and low-risk categories according to their biological characteristics and carcinogenic potential (Zhu et al., 2019). Although the high-risk subtypes are the main pathogenic agents, the low-risk subtypes can cause condyloma acuminatum and low-grade cervical intraepithelial neoplasia, which still pose a considerable health burden to people’s lives. HPV6 and HPV11 are the two most common low-risk subtypes, accounting for more than 90% of condyloma acuminatum (Sturegard et al., 2013). Condyloma acuminatum has become a secondary sexually transmitted disease in sexually active adults, with about 1 million new cases every year in the world (Yu et al., 2018). Moreover, about one-third of condyloma acuminatum cases experience relapse after treatment, resulting in huge medical costs, serious psychological and physical burden to patients. Epidemiological data show that subclinical infection, with no obvious symptoms, is the main form of HPV6 and 11 infections, and is closely related to the high recurrence rate of condyloma acuminatum (Flores-Diaz et al., 2017). Therefore, early, simple and rapid screenings of HPV6 and HPV11 infection and timely treatment of condyloma acuminatum are in high demand, particularly in community-level hospitals.

At present, culturing HPV in vitro remains challenging. Owing to the lack of in vitro culture systems for etiological detection and reliable immunological assays, the detection of HPV is almost entirely dependent on molecular assays (Jamshidi et al., 2012). Currently, the way to test for HPV6 and HPV11, including genotype specific polymerase chain reaction (PCR), Quantitative real-time PCR (qPCR), polymerase chain reaction-restricted fragment length polymorphisms (PCR-RELP), droplet digital PCR (ddPCR), loop-mediated isothermal amplification (LAMP) and hybrid capture 2 test (HC2) (Table 1), these kits possess high specificity and sensitivity, but require high skilled technical personnel, high cost, long detection time, well-equipped laboratories, cumbersome temperature-control devices and sophisticated analysis, which limit the wide adoption of these methods in resource-poor areas (Gree et al., 2000; Hawkins et al., 2013; Lindh et al., 1992; Oliveira et al., 1994; Yu et al., 2015). Therefore, it is essential to develop a more efficient method for rapid screening of HPV6 and HPV11 infections to promote early detection and treatment.

Recombinase-aided amplification (RAA) is an isothermal amplification reaction containing recombinant enzymes extracted from bacteria or fungi, single-stranded DNA binding (SSB) protein, DNA polymerase and specific primers. At an isothermal temperature of 39 °C, the recombinase enzymes can tightly combine with the primers to form the polymer of the enzyme and the primer. With the help of SSB protein the primer searches the template DNA for a complete match of the complementary sequence, the double-stranded structure of the template DNA then is opened. Under the action of DNA polymerase, a new DNA complementary chain is formed. The RAA detection process can usually be completed in less 30 min. In our previous studies, RAA has been successfully applied to the detection of various pathogens and bacteria (Bai et al., 2020; Shen et al., 2019; Wang et al., 2019; Zhang et al., 2019). However, the method required addition of an exogenous reference plasmid as an internal control, which also competed with the target gene.

In this study, we further optimized the RAA method for HPV detection by utilizing the human β-globin gene as an endogenous control, along with specific HPV6 and HPV11 probes. Addition of sample releasing agent further simplifies the sample extraction method and improves the utility of this method in common clinical settings. The endogenous internally-controlled RAA (EIC-RAA) assay was compared with commercial HPV detection kits to evaluate the feasibility of the EIC-RAA assay for field use.

2. Materials and methods

2.1. Clinical sample information

A total of 191 cervical exudative cell samples, and 42 condyloma acuminatum swab samples were collected from Tangshan Gongren Hospital (Hebei, China) and Hebei General Hospital (Hebei, China). The average age of patients was 32.32 ± 8.55 years old. HPV DNA typing kit (qPCR method, Sansure, Hunan, China) was used to screen these samples for 26 HPV subtypes. Among them, 62 cases were positive for HPV6, 12 cases were positive for HPV11, and 8 cases were positive for both HPV6 and HPV11. The remaining 118 cases were positive for other HPV subtypes (HPV16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 67, 68 and 73), and 33 samples were negative for HPV. This study was approved by the Institutional Review Boards of the National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China. The patients gave informed consent to the purpose of the study.

2.2. DNA extraction and sample releasing agent treatment

DNA was extracted from clinical samples using viral RNA/DNA isolation kits (Tianlong, Suzhou, China) in strict accordance with the manufacturer’s instructions. The extracted DNA was stored at −80 °C until further use. In parallel, the samples were treated with sample releasing agent kit (Sansure, Hunan, China) as follows: 200 μL of concentrated solution and 200 μL of the original raw sample were mixed and centrifuged at 12,000 rpm for 10 min. After discarding the supernatant, 50 μL of nucleic acid releasing agent was added and left to stand for 10 min. The prepared nucleic acids were kept at 4 °C, and preferably used on the same day.

2.3. Design of primers and probes

Thirty complete genome sequences of HPV6, HPV11 and human β-globin were downloaded from NCBI GenBank (Table 2) Vector NTI (Thermo Fisher Scientific, USA) was used to align their gene sequences

Table 1. Comparison of HPV DNA detection methods.

| Methodology             | Advantages                                      | Disadvantages                                |
|-------------------------|-------------------------------------------------|----------------------------------------------|
| Nucleic acid amplification technology | PCR, qPCR, PCR-RELP | High sensitivity and specificity | Highly skilled technical personnel, well-equipped laboratories, temperature-control devices and sophisticated analysis |
|                         | LAMP                                            | Short reaction time (30-60 min), High sensitivity and specificity, | Complicated primer design, high cost |
|                         |                                                 | Simple portable equipment                    |                                |
|                         | RAA                                             | Short reaction time (5-30 min),              | Only 2 detection channels, Non-quantitative detection |
|                         |                                                 | High sensitivity and specificity             |                                |
|                         | ddPCR                                           | Very high sensitivity and specificity        | Expensive equipment          |
|                         |                                                 | Simple portable equipment, low cost         | Higher cost |
|                         |                                                 | Accurate quantitation                       |                                |
|                         |                                                 | Longer reaction time                        |                                |
separately, and the Oligo7 (Molecular Biology Insights, USA) software was used to design probes and primers for the highly conserved gene regions of HPV6, HPV11, and human β-globin, according to the design principles of RAA (Bai, 2020). The specificity was further assessed by BLAST on the NCBI website. The finalized primers and probes listed in Table 3 were synthesized by Sangon (Shanghai, China).

### 2.4. Preparation of recombinant plasmids

A 351 bp length (NT6200-6550 bp, GenBank accession No. AF092932.1) of HPV6, a 391 bp length (NT5950-6350 bp, GenBank accession No. M14119.1) of HPV11, and a 300 bp length (NT352-651 bp, GenBank accession No. MK476504.1) of human β-globin were cloned into the pUC57 vector. The plasmid DNA concentration was quantified using a Qubit 2.0 fluorometer and Qubit dsDNA BR Assay Kit (Life Technologies, Warrington, UK). The DNA concentrations were converted to genome copies using the following formula: DNA copy number (copy number/μL) = [6.02 x 10^23 DNA concentration (ng/μL) × 10^-6]/[DNA length in nucleotides × 660] (Ma et al., 2017). The plasmids were diluted 10-fold from 10^6 to 10^2 copies/μL, and stored at -20 °C.

#### 2.5. Sensitivity and specificity of singleplex RAA assay

Singleplex real-time RAA tests for HPV6, HPV11 and human β-globin were performed separately in reaction volumes of 50 μL using the RAA EXO kit (Qitian, Jiangsu, China). The reaction components included 25 μL of buffer, 16.7 μL of DNase free water, 2.1 μL of HPV6, HPV11, or human β-globin gene forward and reverse primers (10 μM) respectively, 0.6 μL of HPV6 or human β-globin gene-specific RAA probe (10 μM), and 1 μL of DNA template or 1 μL of DNase free water. A 47.5 μL of the reaction mixture was added to the lyophilized RAA particle kit containing all necessary enzymes (SSB, 800 ng/μL; UvsX, 120 ng/μL; DNA polymerase, 30 ng/μL), and 2.5 μL of 280 mM magnesium acetate was transferred into the tube. The capped tube was placed in the RAA-B6108 instrument for 4 min of pre-amplification at 39 °C, followed by 30 min in the QT-RAA-F1620 real-time fluorescence detection system (Qitian, Jiangsu, China), at 39 °C for detection. Positive results were determined by the equipment QTRAA-F1620 by setting the slope K value to be greater than or equal to 20 and was simultaneously recorded in the FAM and HEX detection channels.

A serial dilution of the recombinant plasmid to 10^5, 10^4, 10^3, 10^2, 10^1 copies/μL was used to determine the sensitivity of singleplex RAA for HPV6, HPV11 and human β-globin. The negative control reaction was carried out in parallel with each experiment. The specificity of the RAA test was evaluated with cross-reactivity to 118 other HPV positive samples.

#### 2.6. Optimization of EIC-RAA assay protocol

Standard recombinant plasmids of HPV6, HPV11, and human β-globin were prepared in the dilution range of 10^-1 to 10^4 copies/μL. In order to optimize the EIC-RAA analysis so that the amplification of human β-globin in the HEX channel has minimal influence on the sensitivity of the amplification of target genes in the FAM channel, the target plasmids of 10^-1 to 10^4 copies were tested in the presence of 10^-1 to 10^4 copies of human β-globin to examine whether the human β-globin could interfere with detection of HPV6 and HPV11. The optimized EIC-RAA assay was then performed in a 50 μL reaction volume consisting of 25 μL of rehydration buffer, DNase-free water, 2.1 μL of target-specific forward and reverse primers (10 μM), 0.6 μL of HPV6 or HPV11 probe (10 μM), human β-globin-specific forward and reverse primers (10 μM) and 2.5 μL of 280 mM magnesium acetate.

#### 2.7. Optimization of DNA extraction with sample releasing agent

A sample releasing agent kit (Sansure, Hunan, China) consists of concentrated solution and nucleic acid releasing agent. They were used to concentrate the sample, denature proteins, and release nucleic acid. As the use of different concentrated solution and sample input volumes may affect the efficiency and the applicability to the EIC-RAA experimental method, we tested the EIC-RAA assay under different working conditions. A total of 100 μL, 200 μL or 300 μL of concentrated solution was mixed with 100 μL or 200 μL of sample, respectively, into a 1.5 mL EP tube and centrifuged at 12,000 rpm for 10 min. After discarding the supernatant, 50 μL of nucleic acid releasing agent was added and left to stand for 10 min. One to five microliters of the reaction mixture was then tested by EIC-RAA analysis to determine the optimal template input concentration.
2.8. Assessment of the EIC-RAA assay using clinical samples and comparison with a commercial qPCR kit

EIC-RAA and qPCR were used to detect nucleic acids in 233 samples. The HPV nucleic acid detection kit (PCR-fluorescence probe method) was purchased from Sansure (Hunan, China) and was able to detect 26 HPV subtypes and identify specific genotypes (HPV6,11,16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 67, 68 and 73). qPCR reactions were performed according to the manufacturer’s instructions using a CFX96 qPCR System (BIO-RAD, USA).

2.9. Statistical data analysis

IBM SPSS Statistics, version 21 (IBM Corporation, NY, USA) was used to perform all of the statistical analysis. The results were analyzed using kappa tests, and a P-value less than 0.05 was considered statistically significant.

3. Results

3.1. Sensitivity and specificity of singleplex RAA assay

The sensitivity of HPV6, HPV11 and human β-globin was as low as 10 copies/reaction, and no abnormality was found in the negative control. Moreover, the results also showed no cross-amplification with other HPV genotypes (HPV16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 67, 68 and 73; data not shown).

3.2. Sensitivity of EIC-RAA assay

The concentration of internal gene has different effects on the amplification efficiency of target genes and different concentrations of target gene have different effects on the amplification of internal gene because of the co-consumption of RAA reagents in the reactions. The sensitivity of target gene was 10 copies/reaction for HPV6 (Figure 1.1A), 100 copies/reaction for HPV11 (Figure 1.2A) in the presence of $10^5$ copies human β-globin plasmid, indicating the internal gene did not significantly affect the amplification sensitivity of the target gene, while the internal gene was steadily detected in the presence of target gene with a concentration range of $10^1$–$10^5$ copies/reaction in the HEX channel (Figure 1.1B and 1.2B).

3.3. Optimal conditions for EIC-RAA assay using samples treated with sample releasing agent

The amplification curves of the combination of 200 μL of concentrated solution, 200 μL of sample, and 50 μL of nucleic acid releasing agent showed the shortest threshold time (the initial peak time of the fluorescence curve) and the highest fluorescence value to obtain the best amplification efficiency for HPV6 (Figure 2.1A) and HPV11 (Figure 2.1B). Similarly, the best amplification efficiency of EIC-RAA was obtained by loading 2 μL of reaction mixture (Figure 2.2A and B).

3.4. Comparison of EIC-RAA and qPCR detection of HPV in clinical samples

qPCR detection gave Ct values ranging from 17.01 to 37.15 for HPV6, 20.50 to 36.48 for HPV11, and 19.68 to 37.86 for human β-globin (according to the instruction of Sansure kit, qPCR-positive Ct...
The cut off value was set to 39. For 233 previously defined samples, the comparison result between EIC-RAA and qPCR detection of HPV6, HPV11 and human β-globin, were shown in Table 4. The concordance rate between EIC-RAA assay and RT-PCR was 100%, with kappa value of 1 (P < 0.01). Among these condyloma acuminatum swab samples, the internal reference, human β-globin, was not detected in 3 specimens. For the EIC-RAA assay, the coincidence rate between target genes and internal reference was 100%, whether samples were treated with sample releasing agent or multi-step DNA extraction, and the Kappa value was 1 (P < 0.01).

Table 4. Detection of HPV6 and HPV11 in clinical samples.

|                     | qPCR | Sensitivity (%) | Coincidence (%) | Kappa |
|---------------------|------|----------------|-----------------|-------|
|                     | Pos  | Neg            |                 |       |
| EIC-RAA for HPV6    |      |                |                 |       |
| Extracted DNA       | Pos  | 70             | 0               | 100   |
|                     | Neg  | 0              | 163             | 100   |
|                     | Total| 70             | 163             | 1     |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
| Samples treated with sample releasing agent | Pos  | 70             | 0               | 100   |
|                     | Neg  | 0              | 163             | 100   |
|                     | Total| 70             | 163             | 1     |
| EIC-RAA for HPV11   |      |                |                 |       |
| Extracted DNA       | Pos  | 20             | 0               | 100   |
|                     | Neg  | 0              | 213             | 100   |
|                     | Total| 20             | 213             | 1     |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
| Samples treated with sample releasing agent | Pos  | 20             | 0               | 100   |
|                     | Neg  | 0              | 213             | 100   |
|                     | Total| 20             | 213             | 1     |
| EIC-RAA for human β-globin | Extracted DNA | Pos  | 230 | 0 | 100 | 100 | 1 |
|                     | Neg  | 0              | 3               | 100   |
|                     | Total| 230            | 3               | 1     |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
|                     |      |                |                 |       |
| Samples treated with sample releasing agent | Pos  | 230             | 0               | 100   |
|                     | Neg  | 0              | 3               | 100   |
|                     | Total| 230            | 3               | 1     |
4. Discussion

In this study, we established the EIC-RAA assay for the accurate detection of HPV6 and HPV11, using the human β-globin gene as internal control. To our knowledge, this is the first study to detect HPV6 and HPV11 using RAA technology. The EIC-RAA test for HPV6 and 11 was proved to be highly specific, as no cross-reactivity with other HPV subtypes was observed. A total of 233 specimens were assessed by EIC-RAA and qPCR. The results of EIC-RAA and qPCR were consistent. When DNA extractions of the samples were performed with sample releasing agent versus viral RNA/DNA isolation Kit, the results of the two methods were consistent. Sample releasing agent can greatly simplify the steps of nucleic acid extraction, reduce the use of large-scale instruments, shorten the nucleic acids extraction time, and make EIC-RAA detection more suitable for screening in resource-poor areas.

The EIC-RAA assay is a simple and rapid isothermal amplification technique, and the amplification results can be obtained at 39 °C in 30 min. The sensitivity of EIC-RAA was 10 copies/reaction for HPV6 and 100 copies/reaction for HPV11. In comparison, HPV-LAMP, was previously reported to detect HPV6 and HPV11 with a sensitivity of 1 000 copies/reaction for HPV11. In comparison, HPV-LAMP, was previously reported to detect HPV6 and HPV11 with a sensitivity of 1 000 copies/reaction for HPV11. Furthermore, HPV-LAMP requires 4 primers, which is more complicated than the RAA primer design. The EIC-RAA assay is therefore superior to HPV-LAMP in terms of time and sensitivity. As for commercial RT-PCR kits, which are widely used in the clinics, the reported sensitivity is in the range of 44–200 copies/reaction (Kocjan et al., 2008; Micalessi et al., 2011; Wang et al., 2021). Thus, the EIC-RAA assay is more sensitive for the detection of HPV6 and HPV11.

Human β-globin is expressed stably in all types of tissues and cells of the human body. A well-characterized house-keeping gene is often used as an internal control (IC) for molecular diagnosis (Adeyemi et al., 2017; Mori et al., 2008). The use of human β-globin gene as an endogenous internal control for the EIC-RAA assay is similar to the internal control for Cobas (Roche, Switzerland) and BD onclarity (Becton, Dickinson and Company, USA), HPV testing methods approved by the US Food and Drug Administration (FDA) (Salazar et al., 2019). Unlike exogenously added competitive internal controls, which can only monitor the gene amplification reaction, the endogenous human β-globin can also monitor the sample collection and nucleic acid extraction process. The EIC-RAA can therefore effectively eliminate any false-negative or invalid amplification results, increasing the reliability of the assay. In our study, 3 condyloma acuminatum swabs were negative for human β-globin by qPCR and EIC-RAA. We repeatedly tested the extracted nucleic acid, the results did not change, ruling out errors in the course of the experiment. Then, nucleic acid was extracted from the original sample and tested, and the results remained unchanged, ruling out the possibility of nucleic acid extraction failure. We speculate that manual operation error may have occurred during sample collection.

The nucleic acid extraction methods mainly include column extraction, boiling, and magnetic beads, which are a time-consuming and complex multi-step process, often requiring skilled personnel (Hauknes and Kvar, 1993; Hirama et al., 2015; Zhang et al., 2019). In contrast, the sample releasing agent allows DNA extraction with just two steps, agent lysis and high-speed centrifugation, performed at room temperature. This provides significant advantages of simplified operation, reduced time and equipment requirement. However, the optimal working condition of the sample releasing agent needs to be determined prior to sample extraction, as incorrect pH in the concentrated solution or nucleic acid releasing agent may inhibit enzymatic activity in the RAA system. Our results demonstrated a 100% consistency rate between sample releasing agent-treated DNA and multi-step extracted DNA, indicating that the EIC-RAA assay has the potential for field detection of HPV6 and 11 in resource-poor areas.

One shortcoming of this study is that the number of condyloma acuminatum swab samples was small, and more samples of different types, such as surgical condyloma acuminatum sample, should be evaluated by the EIC-RAA assay. In addition, the RAA detection instrument currently has only two fluorescent channels, preventing simultaneous detection of HPV6, HPV11 and human β-globin in the same reaction. Other portable thermostat with four fluorescent channels are worthy of testing to expand this assay.

5. Conclusion

We developed a novel EIC-RAA assay for the detection of HPV6 and HPV11 in this study. With comparable sensitivity, specificity to qPCR, the EIC-RAA method is superior in detection speed and operation simplicity.

Declarations

Author contribution statement

Anna He: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Cheng Fang, Yue Ming, He Tan, Mengyi Zhang and Yaxing Hu: Contributed reagents, materials, analysis tools or data.

Ruiqing Zhang, Jingyi Li, Mingzhu Nie, and Fengyu Li: Analyzed and interpreted the data.

Xuejun Ma, Xinxin Shen and Xiuge Rong: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

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