Semi-quantitative Digital Analysis for Human Papillomavirus Detection from Environmental Specimens

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Abstract

Background: Recently, human papillomavirus (HPV) deoxyribonucleic acid (DNA) has been detected in urban wastewater, indicating that the virus can reach the sewer and, eventually, other water environments. This study aimed to develop a semi-quantitative assay for HPV DNA detection from environmental specimens using the PCR gel electrophoresis method.

Methods: This was an experimental descriptive qualitative study conducted from July to November 2019 in a standard molecular laboratory and non-laboratory administration room without air conditioner. Three brands of PCR reagents and different annealing temperatures were compared to identify the best condition for conventional PCR of plasmid DNA containing the HPV L1 gene. The semi-quantitative data were obtained from densitometry digital analysis using an imaging software. The optimized protocol was then applied on DNA serial dilutions to seek for the lower limit of detection (LLOD) value and the linear range of the assay. To evaluate the robustness of the assay, the protocol was further applied to spiked specimens of wastewater. Finally, several wastewater samples were tested for the presence of HPV DNA using this protocol.

Results: A broad linear range and HPV L1 gene detection ability were observed with an LLOD of less than 2pg plasmid DNA in field condition. Although the assay successfully detected HPV DNA from several spiked wastewater samples, certain wastewater could interfere with the assay and gave false negative result.

Conclusions: A semi-quantitative conventional PCR method to detect HPVDNA from environmental samples has been established and proven to be robust in field condition with non-optimum cold chain.

Keywords: Environmental specimens, human papillomavirus, PCR, semi-quantitative

Introduction

Human papillomavirus (HPV) is a virus from the papillomaviridae family with the double-stranded deoxyribonucleic acid (dsDNA) genome.[1] The most known high-risk HPV infection can cause cervical cancer, where transmission occurs through sexual contact. A recent study has reported that HPV can remain reactive outside stem cells, and thus there is a possibility of transmission of HPV non-sexually. The ability of HPV can remain reactive outside of host cells because of its characteristics, which are very stable and resistant from environmental conditions.[2] Various types of HPVs have been found from river water samples, especially rivers for waste disposal.[3] Some countries have also recognized the presence of HPVs in water and have been used as an indicator of polluted water.[4]

The HPV detection is most often performed using a PCR detection tool. The latest development of PCR is a portable PCR.[5] The emergence of a portable PCR is one of the solutions to overcome the constraints of PCR, which usually can only be used in laboratories, and it causes the PCR process to be longer. Furthermore, it costs money to transport environmental samples to the laboratory. The amplification process using a portable PCR allows researchers to perform PCR in remote areas, where environmental samples are taken.[6,7]

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The purpose of this study was to develop a semi-quantitative assay for electrophoresis gel of HPV DNA detection from environmental specimens, such as wastewater. Furthermore, this study aimed to validate the protocol for detecting HPV DNA from environmental samples using a portable PCR machine with the less cold-chain condition.

Methods

This research was an experimental study with a descriptive qualitative research design. The research process was carried out from July to November 2019 in the standard molecular laboratory and the administration room which was a non-laboratory room without an air conditioner. All reagents were put in the cooler box with ice-gel, and the pipetting was done on the bench at room temperature, after cleaning the surface using household ethanol. This research activity had a research permit from the Research Ethics Committee Universitas Padjadjaran with the number 1350/UN6.KEP/EC/2019.

The HPV DNA plasmid already containing the HPV 52 L1 gene was obtained from the Laboratory of Genetics and Molecular Biotechnology, Institute of Technology Bandung.[8] The L1 gene in the plasmid was the result of cloning from a biopsy sample of a patient with cervical cancer obtained from Dr. Hasan Sadikin Hospital, Bandung.[9] Upon arrival, the plasmid was sequenced with primer MY09 and MY11 to confirm the presence of the HPV52 L1 gene in the plasmid.

In this study, water samples from the environment were directly used as templates in the PCR or were first spiked with the HPV plasmid followed by the DNA extraction process. Water samples were taken from five points in Bandung city areas; namely, samples A, B, C, D, and E. Sample A was river water from the Taman Hewan area, sample B was the open sewage water from the Sukasari area, sample C was the open sewage water from Sadang Serang area, sample D was river water from Sederhana Market area and sample E was the tap water from Pelesiran area.

Spiking samples were then made by adding a certain amount of HPV DNA to those water samples. The purpose of making spiking samples in this study was as a simulation for the detection of HPV from water samples in the environment. In parallel, other water samples referred as original water samples were prepared by using those A to E samples as they were in the PCR.

This study used a portable PCR machine (MiniPCRTM) in optimizing the protocol for HPV DNA detection in the field and the Eppendorf Gradient Cycler (Eppendorf, Germany) for the standard PCR procedure in the lab. The protocol tested three PCR kits [kitP (Promega), kitB (Biosystem), and kitM (MiniPCR)] and three different annealing temperatures (50°C, 55°C, and 58°C), in the field condition. The P and B reagents were usually used in PCR for HPV DNA detection in our laboratory, while M kit was the original kit for the portable machine and being used at room temperature, without cooling in ice. Three PCR protocols were derived from the original PCR protocol of HPV DNA detection, varied in the annealing temperatures.

The HPV plasmid (514 ng/µl) diluted with nuclease free water at 1000x dilution served as the positive control, and nuclease free water served as the negative control. We used MY09/MY11 primers, one of the primers mostly used for the L1 gene amplification of HPV that would give a 450 bp amplicon.

Table 1 PCR Conditions

Step	Temperature	Duration	Cycle
Early denaturation	95°C	60 seconds	hold
Denaturation	95°C	30 seconds	
	50°C	30 seconds	
Annealing	55°C	30 seconds	35 cycles
	58°C	30 seconds	
Extension	72°C	60 seconds	
Final extension	72°C	5 minutes	1 cycle

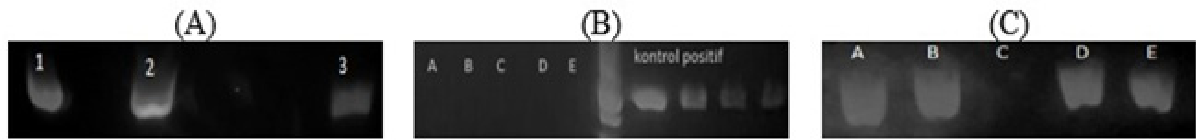

Figure 1 PCR Reaction Results in The Optimization Process. (A) PCR Reaction in The First Laboratory Experiment: 1. Sample with P Reagent at An Annealing Temperature of 50°C; 2. Samples with Reagent B at An Annealing Temperature of 50°C; 3. Samples with Reagent B at Annealing Temperature of 55°C. (B) PCR Reaction Outside The Laboratory on Spiking Samples (A, B, C, D, E) and Positive Control of Dilution Results 1x, 2x, 4x, 8x with M Reagents, and Annealing Temperature of 55°C. (C) PCR Reaction in Spiking Samples with Reagent B and Annealing Temperature of 50°C.

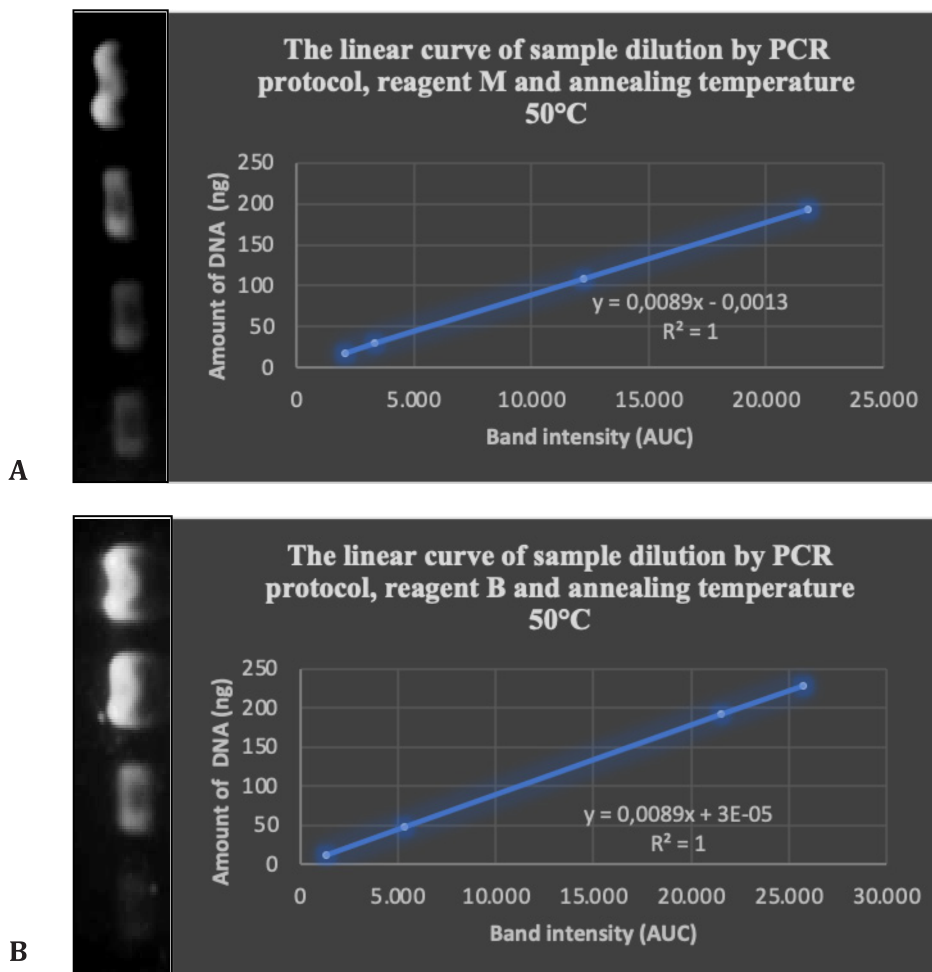

Figure 2 Standard Curves of HPV DNA Plasmid Dilution Samples. (A) PCR Reaction with M Reagents and Annealing Temperature of 55°C Resulted in Amplicons with The Concentration at Dilution 1x = 193.64 ng/µl; 2x = 108.82 ng/µl; 4x = 29.59 ng/µl; 8x= 18,38 ng/µl. (B) PCR Reaction with Reagent B and Annealing Temperature of 50 °C Resulted in The Amplicons with The Concentration of t 8x dilution=228.75 ng/µl; 80x=191.78 ng/µl; 800x=47.38 ng/µl; 8000x=11.48 ng/µl.

These primers could be used at relatively high annealing temperatures.[10] The sequences were 5'-CGTCCMARRGGAWACTGATC-3' for the forward primer and 5'-GCMCAGGGWCATAAYAATGG-3' for the reverse primer.[11] PCR master mixes was made according to the manufacturer's instructions. The PCR profiles were shown in Table 1. The PCR reaction was carried out three times with three different annealing temperatures to see the most stable conditions. PCR amplicons were stored at -20°C. The amplicons from the PCR reaction at this initial stage were sequenced to confirm the correct target by using the Basic Local Alignment Search Tool (BLAST) program, which was accessed through the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The process of developing the assay was done through serial dilution of HPV DNA plasmid samples. Serial dilutions started from 1x, 2x, 4x, 8x, 80x, 800x, 8000x dilutions. The diluted samples were amplified using the optimized PCR protocol. The image of amplicons resulted from this PCR were analyzed with a densitometric procedure using the Image J application software, and the data obtained were further processed in Microsoft Excel software.

Results

The BLAST result on the sequenced amplicon of the first spiked sample showed that the sample was, indeed, containing the L1 gene from HPV type 52 with 100% identity as expected. The standard PCR optimization procedure in the lab was applied initially on the HPV DNA samples using three kinds of reagents and three different annealing temperatures. The most optimal protocol obtained in the PCR process was reagent B and annealing temperature of 50°C (Figure 1).

The results of densitometry on the agarose gel picture showed that there was a decreasing intensity of the band signal (AUC) along with the increasing number of dilutions for each PCR reaction given to the sample. The linear curve shows that curve A and curve B had the same slope value at 0.0089. With the optimal PCR reaction, the amplification process in this study detected samples up to 2.7x10-5ng template DNA, which produced 11.47857037 ng amplicon DNA with 35 PCR reaction cycles (Figure 2).

Discussion

The sequencing result from the plasmid and initial spiked sample with MY09/MY11 primers was confirmed as the HPV52 L1 gene as expected. Genotype 52 HPV is one of the most common types of HPV, causing multiple infections in cervical cancer patients in Bandung, Indonesia.[12,13]

This plasmid sample has been detected for the PCR optimization process and through the spiking experiments using optimal protocols with B reagents and annealing temperature of 50°C. The optimal protocol that has been obtained has been further developed for the field condition. The parameters specified in the development process are LLOD (2.66638x10-5ng), ULOD (2.1331x10-1ng), and reproducibility/robustness of the system. DNA quantification in this system also shows an accurate efficiency seen from the value of R2 = 1 and two standard curves that show the same slope value.

The amplification in a PCR reaction is greatly influenced by many factors, such as the annealing temperature, reagent component such as enzymes, dNTPs, MgCl, and primers as well as the number of PCR cycles, and also the skill of the operator.[14] In this study, we have considered the annealing temperature factor and reagents used. The optimal annealing temperature for a PCR reaction can be determined by knowing the melting temperature (Tm) of the primer used.[14] The formula commonly used to calculate Tm is 4°C (G+C) + 2°C (A+T). From the calculation, the forwarding primary Tm is 50°C and the reverse primary Tm is 52°C. The optimal annealing temperature range based on Tm is Tm-5°C to Tm+2°C which results in annealing temperature 45°C - 54°C. Our study shows that the optimal temperature in this experiment is at 50°C.

A comparison of three different reagents shows that the reagent P is the first reagent to be excluded. In the initial experiments, the P reagent is only able to detect DNA at 50°C, whereas the reaction with reagent B is able todetect up to 55°C annealing temperature. The optimal temperature would show a higher band intensity on the agarose. For B and M reagents at an annealing temperature of 55°C, the PCR worked well for pure plasmid DNA. However, in the spiked samples of wastewater, M reagent did not give amplicon. For reagent B, four out of five spiked samples gave positive results. This result shows that the PCR kit is one of the essential elements that would influence the results, especially in the field condition.[14] Based on the results of the PCR on spiked samples with reagent B, it could be seen

that the PCR reaction was influenced by water quality, even after the DNA extraction process. The possibility of DNA disrupting substances contained in the wastewater sample C which is open sewage water may cause this sample to be the only false negative among the four spiked samples that gave positive signals.

The PCR reaction to the results of dilution of HPV DNA plasmids is an assay development process carried out to determine the LLOD, ULOD, and reproducibility or robustness of the HPV DNA detection system using a portable PCR in the field condition. Reproducibility means that the detection system is precise or has the smallest variation possible when repeated, while robustness means that the testing system is not too affected by changes in sample preparation and handling.[15]

Based on data obtained from the plasmid dilution process, we found that the LLOD value in this study was below 2 pg DNA templates, and the value for ULOD was higher than 0.21 ng DNA templates. LLOD values from this optimized protocol could be further tested to find new lower values. The reproducibility parameters of the optimal protocol in this study were fulfilled by the positive results on repeated PCR experiments. The Robustness parameter met with the discovery of positive results by showing that the protocol could be applied on extracted and non-extracted samples, also when it was performed in the field condition outside the laboratory with almost no cold-chain condition.

On the linear curve, curve A and curve B have the same slope value and the value R2= 1. The value of slope and R2= 1 showed the DNA quantification with densitometry to be quite accurate. Through these standard curves, it could be concluded that if the number of templates of a water sample within the linear range of the assay, then the quantification of the sample with digital densitometry using ImageJ could be also accurate (Figure 2).

The limitation of this study is that the protocol has only been applied to spiking experiments of plasmid DNA. Further testing and validation are needed on raw samples known to be containing DNA of HPV, such as wastewater containing the faecal specimen from the HPV positive-patients.

To conclude, a semi-quantitative conventional PCR method for the detection of HPV DNA in environmental samples such as wastewater has been established and proofed to be robust, showing a stable performance when conducted in the field condition, outside the conventional laboratory.

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References

1. Bernard H, Burk RD, Chen Z, Doorslaer K Van, Villiers E De. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology. 2010;401(1):70–9.
2. Ryndock EJ, Meyers C. A risk for non-sexual transmission of human papillomavirus? Expert Rev Anti Infect Ther. 2014;12(10):1165–70.
3. Iaconelli M, Petricca S, Libera S Della, Di Bonito P, La Rosa G. First detection of human papillomaviruses and human polyomaviruses in River Waters in Italy. Food Environ Virol. 2015;7(4):309–15.
4. La Rosa G. Papillomavirus. In: Rose JB, Jiménez-Cisneros B, (eds) Global Water Pathogen Project Part 3. [cited 2020 January 6] Available from: https://www.waterpathogens.org/book/papillomavirus
5. Abreu ALP, Souza RP, Gimenes F, Consolaro MEL. A review of methods for detect human Papillomavirus infection. Virol J. 2012;9:262.
6. Guevara EE, Frankel DC, Ranaivonasy J, Richard AF, Ratsirarson J, Lawler RR, et al.. A simple , economical protocol for DNA extraction and amplification where there is no lab. Conservation Genet Resour. 2017;10(1):119–25.
7. Boguraev A, Christensen HC, Bonneau AR, Pezza JA, Nichols NM, Giraldez AJ, et al. Successful ampli fi cation of DNA aboard the International Space Station. NPJ Microgravity. 2017;3:26.
8. Hanifa VR. Construction of L1 HPV 52 Gene on pPICZA and pPICZα for expression of virus -like partices (VLP) protein on Pichia pastorisGS115. 2014. [cited 2020 January 6] Available from: http://repositori.sith.itb.ac.id/download.php?iden=NvP76y8Wze.
9. Suhandono S, Kencana Ungu DA, Kristianti T, Sahiratmadja E, Susanto H. Cloning, expression and bioinformatic analysis of human papillomavirus type 52 L1 capsid gene from Indonesian patient. Microbiol Indones. 2014;8(3):94–102.

10. Venceslau EM, Bezerra MM, Lopes ACM, Souza ÉV, Onofre ASC, de Melo CM, et al. HPV detection using primers MY09/MY11 and GP5+/GP6+ in patients with cytologic and/or colposcopic changes. J Bras Patol Med Lab. 2014;50(4):280–5.
11. Manos MM, Waldman J, Zhang TY, Greer CE, Eichinger G, Schiffman MH, et al. Epidemiology and partial nucleotide sequence of four novel genital human papillomaviruses. J Infect Dis. 1994;170(5):1096–9.
12. Sahiratmadja E, Tobing MDL, Dewayani BM, Hernowo BS, Susanto H. Multiple human papilloma virus infections predominant in squamous cell cervical carcinoma in Bandung. Univ Med. 2014;33(1):58–64.
13. Panigoro R, Susanto H, Novel SS, Hartini S, Sahiratmadja E. HPV genotyping linear assay test comparison in cervical cancer patients: Implications for HPV prevalence and molecular epidemiology in a limited-resource area in Bandung, Indonesia. Asian Pac J Cancer Prev. 2013;14(10):5843–7.
14. Lorenz TC. Polymerase chain reaction: basic protocol plus Troubleshooting and optimization strategies. J Vis Exp. 2012;63:e3998.
15. Broadway N. Assay development: 5 considerations and 8 fundamentals. Mater Methods. 2012;2:121.