Optimization of a manual and rapid Silica-based DNA Extraction Method: Applied to Human papillomavirus detection using fresh Cervical Biopsies Samples

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Abstract: Since the advent of the polymerase chain reaction in 1985, the extraction of DNA has become an important step in the diagnosis of certain diseases. To do this, manual extraction methods remain indispensable alternative for limited resources laboratories in developing countries because of the cost of commercially available kits. The main objective in this study was to test and optimize a fast, non-toxical, high reliable and low cost silica-based method of viral HPV-DNA extraction protocol from tissue specimens.
A total of 50 cervical biopsies have been extracted for HPV-DNA simultaneously by the silica-based method and the phenol/chloroform reference method, followed by PCR amplification with the consensus primers MY09/11 and GP5+/6+.
We obtained the DNA of sufficient quality and quantity to perform all PCR amplification with a mean concentration of 210.4 ng/µL and a mean yield of 52 µg/mL. The purity ($DO_{260nm/280nm}$=1.76±0.18) was considered as satisfactory compared to the reference method ($DO_{260nm/280nm}$=1.77±0.13). Both methods used to amplify the viral DNA in the same samples showed perfect concordance results. The silica-based method has proved to be the fastest, easy to implement and does not use toxic chemicals and does not degrade DNA. In contrast, the traditional method with phenol/chloroform remains heavy, slow (2-3 day) and use toxic and irritating products for the manipulator and can degrade DNA.
This method provides a good DNA and could be easily performed in limited-resource laboratories, not requiring sophistical equipment and less expensive.

Keywords: silica-based method, HPV-DNA extraction, cervical biopsies, Congo.

I. INTRODUCTION

The Human Papillomavirus is a DNA virus, which remains at present non-cultivable in routine and only molecular biology methods allow the HPV infection diagnosis. Indeed, the development of molecular biology techniques such as polymerase chain

reaction (PCR) for over 30 years, have greatly facilitated the detection of these viruses in different biological samples [1]. However, most of these techniques require a DNA extraction which is a fundamental first step because it determines the success and quality of the final result compared with the molecular detection techniques to use. Several suitable DNA extraction methods for PCR amplification have been developed, most of which are commercially available in kit form [2]. Other manual, such as the reference phenol/chloroform method is laborious, expensive, and uses toxic chemicals involving several steps and time consuming [3]. Some extraction methods are adapted to the biological sample types with an impact on the molecular biology method that will be used to amplify DNA. It is clear that the extraction method plays an important role in performance in downstream molecular applications [4].

Moreover, in most African countries, laboratories are poorly equipped. It is therefore difficult to use advances molecular biology techniques such as commercial extraction kits that sometimes requires adequate and sophisticated equipment besides their high costs. Apart from these problems related to cost and equipment, in the majority of extraction kits, the number of exploitable samples is limited. Hence, the need for some laboratories to develop their own manual technique called "home technical" appropriate to the nature of the treated samples in their research topics.

In this study, we optimized the manual DNA extraction silica-based method previously described by Boom et al. (1990) [5] from tissue specimens among women population at risk of developing a CC.

II. METHODS

A. Sample collection

The study was conducted on the remaining biopsy samples of women attending the General Hospital of Loandjili, Pointe-noire, southwest of the Republic of Congo after a biopsy for histological examination.

A total of 50 fresh biopsies specimens were collected. All experiments were carried out to Morocco at the Virology, Microbiology and Quality/ETB laboratory of the Faculty of Science and Technology, University Hassan II of Casablanca. Informed consent was obtained from all women whose biopsies were subject to this study. The local ethics committee of Research in Health Sciences of Congo also approved the study.

B. Conduct of the experimental study

The basic protocol used is a modification of that described by Boom et al. (1990) from the silica-based method. The parameters tested in this study and optimized to be suitable for this method and the type of the treated samples were: i) pretreatment samples, ii) lysis cells, iii) volume of silica fraction, iv) washing time of DNA and v) elution buffer concentration [6].

The assessment was conducted compared to the reference phenol/chloroform method. For both methods, the samples pretreatment was done by washing in phosphate buffered saline (PBS).

After subdivided each sample into two parts, three major phases have been framework of this study:(1) the first one was to extract samples by the silica-based method, adapting and modifying certain parameters respecting the technique previously described by Boom on samples of liquid nature. (2) The second phase was to extract the duplicate of each sample by the phenol/chloroform/isoamyl alcohol method used as the reference method in our laboratory. (3) Finally, the third phase was to assess the DNA extract obtained from both methods by testing by PCR the detection of HPV.

C. Evaluation criteria of the DNA extract

Compared to the reference phenol/chloroform method, the following parameters were evaluated to validate our method: (1) Extraction yield. The extracted DNA concentration was determined by measuring the absorbance at 260nm and 280nm. The yield was calculated by performing the ratio between the quantity of DNA obtained and the initial volume of sample used after cell lysis [7]. (2) Purity. Contamination of the DNA extracted proteins was assessed by measuring the ratio $OD_{260\ nm}/OD_{280nm}$. Extracted DNA was considered pure when the ratio was between 1.7 and 1.9 [8]. The quality and the absence of PCR inhibitors were also assessed by amplifying a DNA fragment of 268bp β-globin gene. The PCR-amplified fragments were analyzed by electrophoresis on a 2% agarose gel.

D. Silica-based DNA extraction protocol

- ***Preparation of buffers and silica fraction***

The preparation of buffers and fraction of silica was performed as described by Boom et al. (1990) by adjusting some parameters to the equipment available in our laboratory

- ***Procedure***

Cell Lysis: We added in the lysis buffer 100µL of proteinase K (PK, 20mg/mL) at a PK final concentration of 200µg/mL. 400µL of digestion solution were placed in Eppendorf tubes containing fresh biopsy and then incubated at 56°C for 30 min.

DNA purification and DNA Elution were performed as described by Boom et al. (1990) with some modifications

E. Phenol/chloroform DNA extraction protocol

In this method called "reference", the principle of treating the lysate after enzymatic digestion with phenol/chloroform/isoamyl alcohol (25:24:1), which allows the separation of the phases, the aqueous phase is supposed to contain the DNA. Briefly, after washing with PBS, each sample was digested by mixing 500µL of lysis buffer (1M Tris-HCl pH 8, 0.2 M EDTA, 5 M NaCl, 10% SDS) with 20mg/ml PK. the rest followed the standard protocol.

F. Quantitative and qualitative evaluation of the DNA extract

The DNA concentration was measured by a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, USA). The DNA quality and the absence of PCR inhibitors were assessed by amplifying a 268 bp fragment of the β-globin gene using the PC04 and GH20 primers [9]. All amplification was carried out with 50ng/µL of DNA concentration in a final volume of 25µL in a Perkin Elmer 2400 GeneAmp PCR thermal Cycler (Scientific Support, Inc, Hayward, CA). The GoTaq DNA Polymerase Master Mix (Promega®) was used for all PCR tests.

G. HPV DNA detection by nested-PCR

In this study, Nested-PCR was carried out to detect HPV-DNA using the universal primers MY09/MY11 in the first round and GP5+/GP6+ in the second round. Degenerated primers MY09/MY11 amplify 450bp long fragment in highly conserved region in L1 gene and Consensus primers GP5+/GP6+ generate 150bp into the same region [10]. DNA from the SiHa cell line was used as positive PCR control and Ultra-pure PCR water (Bioline) as negative control to assess the success of the amplification.

III. RESULTS

Several studies have shown that the silica particles might be used in the purification of DNA in a highly concentrated salt environment and Guanidium isothiocyanate (GuSCN) is a powerful chaotropic agent which also plays the deproteinizing role. After several experiments we present the results obtained after the development of the method with samples from fresh biopsies.

A. Evaluation of the extraction Method

- ***Optimization***

In comparison with the Boom et al. (1990) method, Table I shows the different parameters that have been modified and optimized for the treatment of fresh biopsy specimens.

TABLE I. MODIFYING PARAMETERS COMPARED TO BOOM TECHNIQUE

Parameters	This study	Boom et al.
Proteinase K (PK)	200µL/mL vol. final	Absent
Incubation times	30min at 56°C	10min (room T°)
Volume silica particles	30µL	40µL
Centrifugation time	2 /3 min	15s /2min
B3 concentration	10mMTris-HCl - 0,1mMEDTA	10mMTris-HCl - 1mMEDTA

Trials conducted in the cells lysis without PK were inadequate to complete lysis in the samples mainly biopsies. The use of Proteinase K guarantees perfect hydrolysis of histones, thereby releasing the DNA.

- ***Results validation***

The main quantitative results are summarized in Table II. The average yield of DNA extraction was calculated by making the ratio of the quantity of DNA obtained (concentration in ng/µL) of the initial volume of the sample (400µL method for silica and 500µL method for phenol/chloroform). The sample initial volume is considered to be obtained after cell lysis and before the addition of the silica particles.

TABLE II. COMPARISON OF TWO EXTRACTION METHODS OF ANALYZED SAMPLES

Methods	Concentration (ng/µL)	Ratio OD_{260nm}/OD_{280nm}	Yield (µg/mL)
Silica-based method	210.4±101	1.76±0.18	52±2
Phenol/chloroform	373.1±156	1.77±0.13	74±9

The degree of purity and the average concentrations achieved with the two methods are presented in the table 2 and illustrated in figure 1.

The successful amplification of a 268pb of the β-globin gene fragment was performed to assess the quality of the DNA extracted and the absence of PCR inhibitors in the two methods. Figure 2 shows an example of the electrophoretic profile on 2% agarose gel extracted by the silica-based method (1S-3S) than phenol/chloroform (1P-3P).

B. Ability to amplifying HPV DNA

The viral HPV-DNA was detected by nested-PCR using MY09/11 as outer primers and GP5+/6+ as inner primers to amplifying 450pb and 150pb of HPV-L1 gene. Compared to both techniques, figure 3 shows the electrophoretic profiles on 2% agarose gel

IV. DISCUSSION

Given that, HPV is the main cause of cervical cancer, this virus is not cultivable routinely and molecular biology techniques are the only reliable tool for the viral-DNA detection. Thus the first step in this process involves the viral DNA extraction, which must be good quality and suitable for the technique to be used for the virus detection. In this study, to analyze a high number of samples at the same time and getting a clean and good quality DNA, we tested and optimized the silica-based method of DNA-extraction described by Boom et al. (1990). The optimization process was assessed by comparison to the results obtained by the reference phenol/chloroform method [11]. Adjustments to the original method (Boom) allowed obtaining a clean and good DNA quality easily amplifiable by PCR. In this study the amplification of β-globin gene was performed under the right conditions, reflecting an absence of PCR inhibitors with the extraction method used [12-14]. Biological type of our sampling (fresh biopsies specimens) required the addition of PK in the lysis buffer composition. Indeed, although the GuSCN is a powerful chaotropic agent and also deproteinizing, the addition of PK was necessary to complete cell lysis. This addition is a first difference with Boom technique used on liquid samples.

Boom et al. in their method, used 40µL of SiO_2 as the volume of silica fraction to be added which would add more particles to adsorb DNA. However, during our trials we have found that the greater the volume of the fraction added was significant (40µL and 50µL), less we get a reasonable volume during elution when we add 50µL of B3 with the risk of diluting the DNA in samples of low concentration. In this dual concern, we opted for a volume of 30µL of SiO_2 in place of 20µL considered too low or 40µL considered too high. To allow a spin time that corresponds to the maximum speed of 10,000 rpm, we increased the time to 2 min instead of 15s in the Boom method, adapting to our type of centrifuge (SIGMA 2K15C). This modification was also performed and adjusted with centrifugation time for the elution step which was 3 min instead of 2min as determined by Boom. In the Boom method, the elution buffer was 1mM EDTA. Although this concentration is within the normal margin used in the composition of TE buffer (Tris-HCl-EDTA), we have to facilitate the optimization during the PCR reaction (because we used a PCR buffer $MgCl_2$ free, Promega 5X) reduced the concentration of 0.1mM EDTA. Indeed, although the function of the chelating agent EDTA is an inhibitor of DNAse Mg^{2+} dependent, a high concentration in the elution solution may also inhibit the PCR reaction especially the action of Taq polymerase that using Mg^{2+} as a cofactor, which would result in tedious optimizations steps [15].

The beta globin is an ubiquitous gene commonly used in molecular biology to appreciate and evaluate the quality of the DNA extraction and the absence of PCR inhibitors [9]. The results showed that the DNA obtained was pure and good quality regarding the PCR amplification.

Whatever the method of extraction and purification used, we obtained the DNA of sufficient quality and quantity to perform all acts of genic amplification. The extracting means yield was 52ng/µL for silica-based method and 74ng/µL for phenol/chloroform method [16]. The purity was considered as satisfactory compared to the reference method. The OD ratio 260/280 nm was especially excellent with both methods (1.76 and 1.77 respectively). In contrast, DNA concentrations were lower with the silica-based method compared to the reference method. DNA concentration obtained with the silica method averaged 210.4ng/µL relative to the phenol/chloroform method which was 373.1ng /µL. This concentration, although low, is widely adapted to the PCR method. In terms of working time, the test method is faster (about 1h35min of effective work) compared to the phenol/chloroform method (6hours of effective work).

For their ability to detect viral HPV DNA, we performed nested PCR which is a highly sensitive and specific than conventional single PCR [17-19]. The both extracts showed a perfect analogy to the results obtained. This analogy shows that the silica-based method is also entitled to amplify viral DNA that the reference method.

The DNA extraction silica-based method is as efficient as the phenol/chloroform method [20]. However, in its implementation, this technique has some additional advantages such as: speed, simplicity of implementation, the use of non-toxic reagent. According to Boom, the silica-based method is the cheapest per sample, compared with the phenol/chloroform method, although some reagents are less stable than 3 weeks after preparation.

V. CONCLUSION

The present method is an easy and rapid DNA extraction procedure. The procedure is suitable for the extraction of Pap smears or biopsies samples and the detection of HPV-DNA in any research laboratory and in public health programs to screen this virus. This method could be easily performed in limited-resource laboratories, not requiring sophistical equipment, less expensive and provides a DNA as good as the phenol/chloroform method.

COMPETING INTERESTS

The authors declare that they have no competing interest.

AUTHORS' CONTRIBUTIONS

ALMB conducted all handling and the overall design of the experiment. SZA contributed in handling and conducted a review of the manuscript. DM participated in the critical reading of the manuscript and sampling. NT and OM contributed to the development of technical. LH and MME were responsible for the implementation of the project. All authors read and approved the final manuscript.

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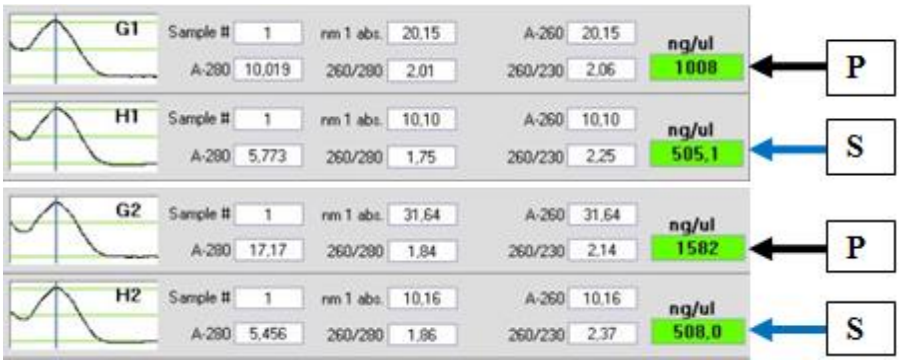

Figure 1: NanoDrop results showing trend curves related to the degree of purity of the extracted DNA, the $DO_{260nm}/_{280nm}$ ratio and the concentration (µg/µL)

P= Phenol/chloroform samples,
S= Silica samples

Figure 2: Electrophoretic profile after amplifying of β-globin gene on 2% agarose gel

M: ladder 100bp,
C-: negative control,
P: Phenol/chloroform samples,
S: Silica samples

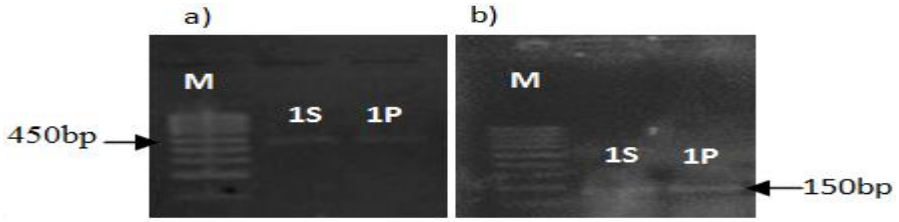

Figure 3: Electrophoretic profile of a 2% agarose gel after amplification of the L1 gene

a) MY09/11 PCR;
b) GP5+/6+ PCR
M: ladder 100pb;
P: Phenol/chloroform samples;
S: Silica samples