Title:

A rapid DNA extraction method suitable for Human Papillomavirus (HPV) detection

Brian Brestovac[1], Michelle E Wong[1], Paul S Costantino[1], David Groth[1]

[1]School of Biomedical Sciences, CHIRI Biosciences Research Precinct, Faculty of Health Sciences, Curtin University, GPO Box U1987, Perth, Western Australia

Corresponding author:
Brian Brestovac
School of Biomedical Sciences
Curtin University
GPO Box U1987
Perth, Western Australia, 6845
Ph: 61-8-9266 7474
Fax: 61-8-9266 2342
B.Brestovac@curtin.edu.au

Keywords:
Human Papillomavirus (HPV), Cervical Cancer, Liquid Based Cytology, XytXtract

Running title: DNA extraction for HPV.

Abstract:

Infection with oncogenic human papillomavirus (HPV) genotypes is necessary for the development of cervical cancer. Testing for HPV DNA from liquid based cervical samples can be used as an adjunct to traditional cytological screening. In addition there are ongoing viral load, genotyping and prevalence studies. Therefore, a sensitive DNA extraction method is needed to maximise the efficiency of HPV DNA detection. The XytXtract Tissue kit is a DNA extraction kit that is rapid and so could be useful for HPV testing, particularly in screening protocols. This study was undertaken to determine the suitability of this method for HPV detection. DNA extraction from HeLa and Caski cell lines containing HPV 18 and 16 respectively together with DNA from five liquid based cervical samples were used in a HPV PCR assay. DNA was also extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) as a comparison. DNA extracts were serially diluted and assayed. HPV DNA was successfully detected in cell lines and cervical samples using the XytXtract Tissue kit. In addition, the XytXtract method was found to be more sensitive than the QIAmp method as determined by a dilution series of the extracted DNA. While the XytXtract method is a closed, the QIAamp method uses a spin column with possible loss of DNA through DNA binding competition of the matrix, which could impact on the final extraction efficiency. The XytXtract is a cheap, rapid and efficient method for extracting HPV DNA from both cell lines and liquid based cervical samples.

Introduction

Human papillomavirus (HPV) infection is necessary for the development of cervical cancer, which is the most prevalent cancer in women worldwide [zur Hausen et al., 1981; Walboomers et al., 1999; Munoz et al., 2003]. It is recognised that the Papanicolaou screening programmes are expensive with significant limitations [Cuzick, 2002; Goldie et al., 2006]. Therefore, it has been proposed that direct testing for HPV be used in screening for cervical cancer particularly in developing countries [Cuzick, 2002; Ronco and Segnan, 2007; Cuzick et al., 2008; Cox, 2009; Naucler et al., 2009; Darlin et al., 2013]. In addition, urine, self sampling and biopsy samples have also been investigated for their utility in HPV detection [Carcopino et al., 2012; Darlin et al., 2013; Tanzi et al., 2013].

Since HPV is quite difficult to culture [Taichman et al., 1984], DNA technologies have been almost used exclusively for the detection of HPV in cervical samples and a

variety of different methods have been used, however most are based upon the Polymerase Chain Reaction (PCR) [Brestovac et al., 2005a; Klug et al., 2008; Didelot et al., 2011]. Applications are not restricted to detection of HPV, as tests for genotype determination, viral load and prevalence studies all can assist in the understanding of the relationship between HPV and cervical cancer [Brestovac et al., 2005b; Wahlström et al., 2007; Stevens et al., 2009; Broccolo et al., 2013]. Since the study of HPV has been almost performed exclusively by DNA technologies, the extraction of DNA from samples is a critical step. The availability of low cost, efficient and rapid methods being particularly useful for HPV testing in screening protocols [Dunn et al., 2007].

The object of this present study was to determine the suitability of an alkaline-heat based protein degradation method [Rudbeck and Dissing, 1998; Shi et al., 2004; Chomczynski and Rymaszewski, 2006], the direct XytXtract tissue kit (Xytogen, Perth, Australia, http://xytogen.com) for extracting HPV DNA from cytology samples and cervical cancer cell lines. This method was compared to the widely used column based QIAamp DNA mini kit. To test the efficiency of the DNA extraction method, extracted DNA was diluted serially and tested by PCR. The PGMY09/11 primer set developed for a PCR test of mucosal HPV consists of a pool of primers which amplify a 450 bp region of the L1 gene within the HPV genome [Gravitt et al., 2000] and this method was selected since it is used widely. Dilution end points using this PCR were used to compare DNA extraction methods.

Materials and Methods

Cell lines and Samples

Two cervical cancer cell lines were used: HeLa cells containing approximately 50 copies of HPV 18 per cell and CaSki cells containing approximately 500 copies of HPV type 16 per cell [Yee et al., 1985; Guerin-Reverchon et al., 1989]. HeLa cells were suspended in phosphate buffered saline (PBS) at a cell concentration of 2.0×10^6 per mL while Caski cells were similarly suspended at 3.2×10^6 per mL. This represents 1.0×10^6 cells with 5.0×10^7 copies of HPV 18 in a 500 µl aliquot of HeLa cells, and 1.6×10^6 cells with 8.0×10^8 copies of HPV 16 in a 500 µl aliquot of Caski cells. These 500 µl aliquots were then used for DNA extraction.

Five high-grade cervical intraepithelial lesion (HSIL) samples in ThinPrep (Cytyc, Boxborough, MA, USA) medium were also tested. The cells suspended in the ThinPrep medium were mixed by inversion and a 500 µl aliquot was taken and used for DNA extraction.

DNA Extraction

DNA extraction was performed with minor modifications of the manufacturer's protocols.

For XytXtract, the 500 µl sample aliquots were centrifuged at 16,000 x g for 5 minutes and supernatant discarded. The pellets was resuspended in 500 µl of PBS and centrifuged at 16,000 x g for 5 minutes and supernatant again removed. The pellets were resuspended in 1 mL of PCR grade water, centrifuged at 16,000 x g for 5 minutes and supernatant removed. Solution 1A (32 µl) and solution 1B (8 µl) were added to the remaining cell pellets, mixed by vortexing and incubated at 95^{o}C for 15 minutes. Following incubation, 10 µl of solution 2 was added, mixed by vortexing and centrifuged at 16,000 x g for 5 minutes. The supernatant containing the DNA was removed, transferred to a sterile microfuge tube and stored at -20^{o}C until required.

For the QIAamp DNA mini kit, 500 µl aliquots were centrifuged at 16,000 x g for 5 minutes, the supernatant discarded and cells resuspended in 200 µl of PBS. Proteinase K (20 µl) and Buffer AL (200 µl) were added and incubated at 56^{o}C for 10 minutes. Absolute ethanol (200 µl) was added and the mixture applied to the QIAamp spin columns. These were centrifuged at 6,000 x g for 1 minute with waste tube discarded. The column was washed with 500 µl of buffer AW1 and again with 500 µl of AW2. The purified DNA was eluted from the spin column with 200 µl of buffer AE into a clean sterile microfuge tube and stored at -20^{o}C until required.

Polymerase Chain Reaction

A tenfold dilution series of each of the DNA extracts from the cell lines and ThinPrep samples were performed, and the presence of HPV was detected by PCR using the PGMY09/11 primer set which amplifies a 450 bp region of the HPV L1 gene. The amplification mix was composed of 0.2 µM of each primer, 10x PCR buffer (Invitrogen, Carlsbad, CA), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 8 µl of DNA template in a total final volume of 12 µl. Thermocycling conditions consisted of an initial denaturation

step of 94^{o}C for 10 minutes, followed by 45 cycles of denaturation at 94^{o}C for 1 minute, annealing at 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute, with a final extension step of 72^{o}C for 7 minutes. Products were electrophoresed in a 2% agarose gel containing 1x TAE buffer precast with SYBR safe (Invitrogen, Carlsbad, CA) and photographed using a UV transilluminator.

Results

The cell lines and all ThinPrep samples extracted using the XytXtract method amplified a correct sized HPV L1 product from the PCR. However, one ThinPrep sample (sample 3) extracted using the QIAamp method failed to show an amplification product. However, a correct sized PCR fragment was present from the XytXtract extracted DNA for this same sample.

An end point dilution of Hela cells, showed that in the XytXtract extracted DNA, a product could be detected at a 10^{-4} dilution, representing approximately 5 x 10^{3} copies of the HPV 18 genome. The corresponding end point dilution using QIAamp extracted DNA was 10^{-2} representing approximately 5 x 10^{5} copies of the HPV 18 genome. The end point dilution for the XytXtract from Caski cells was 10^{-5} representing approximately 8 x 10^{3} copies of the HPV 16 genome. The corresponding end point for the QIAamp extracted DNA was 10^{-2} representing approximately 8 x 10^{6} copies of the HPV 16 genome. The XytXtract extraction method consistently resulted in a 2 to 3 $\log_{10}$ greater analytical sensitivity for HPV 18 and 16 genomes respectively (Table 1).

The PCR results from the ThinPrep samples were expressed in dilutions since HPV copy number per cell in these samples is not known. XytXtract extraction of DNA resulted in greater analytical sensitivity for the detection of HPV than the corresponding QIAamp method for all but one sample (Table 2). Figure 1 shows the gel comparison of the end points for sample 5, which clearly shows that the sample extracted by the XytXtract method resulted in a 4 $\log_{10}$ greater analytical sensitivity than the QIAamp method. Further to this, the XytXtract extracted DNA from sample 3 amplified a PCR product in the neat (undiluted) extract but no product was observed for the corresponding QIAamp extracted DNA (Table 2).

Discussion

It has been proposed that HPV testing could be used in cervical cancer screening programmes [Cuzick, 2002; Goldie et al., 2006; Ronco and Segnan, 2007; Cuzick et al., 2008; Cox, 2009; Naucler et al., 2009; Darlin et al., 2013]. Furthermore, almost all reports in relation to HPV and cervical cancer have been DNA based, including detection, viral load, genotype determination and prevalence studies [Walboomers et al., 1999; Brestovac et al., 2005a; Brestovac et al., 2005b; Klug et al., 2008; Stevens et al., 2009; Carcopino et al., 2012; Broccolo et al., 2013]. HPV testing has a very strong negative predictive value for cervical cancer and confidence in the negative result is reliant upon the analytical sensitivity. Thus a rapid and efficient DNA extraction method would be important in HPV testing in screening protocols. The XytXtract DNA extraction method is a very rapid technique with results that are at least comparable if not greater in analytical sensitivity for testing HPV by PCR, than extraction using the QIAamp method. The analysis of DNA extracted from the cells lines showed that XytXtract method was 2 to 3 $\log_{10}$ more sensitive in detecting HPV 18 and 16 respectively using the PGMY09/11 PCR system. All XytXtract extracted ThinPrep cytology samples showed greater or equal analytical sensitivity for the detection of HPV DNA. This greater analytical sensitivity would not only be important in screening protocols but also in studies on viral load, genotype determinations and population based distributions. In particular, ThinPrep sample number 3 did not amplify a PCR product for DNA extracted using the QIAamp method and so would be reported as negative for the presence of HPV. In contrast, the DNA from the XytXtract method amplified a product in the undiluted fraction of sample 3 and so the presence of HPV was determined.

Although final volumes of extracted DNA differ between methods (50µl for XytXtract and 200µl for QIAamp), and some concentration effect could be inferred with the XytXtract method, this does not sufficiently explain the large $\log_{10}$ differences in analytical sensitivity observed. The QIAamp system relies upon a spin column for which DNA is captured onto a binding matrix and it is conceivable that competitive binding of HPV and human DNA could result in loss of HPV DNA. In contrast, the XytXtract method is a closed system and therefore has no competitive binding to a matrix, resulting in no loss of DNA in the final extracted fluid. In addition, the XytXtract method uses an alkaline-heat protein degradation system to remove PCR inhibitors [Rudbeck and Dissing, 1998; Shi et al., 2004; Chomczynski

and Rymaszewski, 2006]. It is these factors that probably resulted in the difference in analytical sensitivity of the two DNA extraction methods.

Conclusion

HPV detection, viral load and genotyping are performed primarily by DNA techniques and so the DNA extraction method is critical. The XytXtract DNA extraction method is a closed alkaline-heat protein degradation system that is both rapid and efficient and so very suitable for extracting DNA from cell lines and liquid based cervical samples for the detection of HPV by PCR.

Competing interests

Dr David Groth has financial interest through an indirect royalty payment from XytXtract kits.

Ethical approval

This study was approved by the Human Research Ethics Committee at Curtin University of Technology in relation to the use of human ThinPrep samples (approval number SoBS08/07).

Acknowledgments

The authors acknowledge the provision of research facilities and the scientific and technical assistance of the staff of CHIRI Biosciences Research Precinct core facility, Curtin University. Also to thank Western Diagnostic Pathology for providing samples.

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