Journal of Virological Methods 173 (2011) 374–377

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Development of optimal liquid based cytology sample processing methods for HPV testing: Minimising the 'inadequate' test result

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Article history:
Received 24 August 2010
Received in revised form 16 January 2011
Accepted 26 January 2011
Available online 3 February 2011

Keywords:
HPV testing
HPV typing
Cervical screening
Beta-globin (β-globin) negative

A B S T R A C T

Incorporation of HPV testing into cervical screening is anticipated and robust methods for DNA extraction from liquid based cytology (LBC) samples are required. This study compared QIAamp extraction with Proteinase K digestion and developed methods to address DNA extraction failure (β-globin PCR negative) from clinical specimens.

Proteinase K and QIAamp extraction methods in paired LBC samples were comparable with adequate DNA retrieved from 93.3% of clinical specimens. An HPV prevalence cohort (n = 10,000) found 7% (n = 676) LBC samples tested negative for β-globin, and were classified as inadequate. This 'failure' rate is unsuitable for population screening, particularly as the sampling method is intrusive. 379/676 samples were assessed to determine the cause of test failure. Re-testing confirmed adequate DNA in 21.6% of the original extracts; re-extraction from stored material identified 56.2% samples contained adequate material; dilution to overcome sample inhibition (1:10) resolved 51.7% cases in original extracts and 28% in new extracts.

A standardised approach to HPV testing with an optimal DNA concentration input rather than standard volume input is recommended. Samples failing initial DNA extraction should be repeat extracted and assessed for sample inhibition to reduce the 7% of HPV tests being reported as inadequate and reduce the need for retesting of those women to <1%.

Human Papillomavirus (HPV) (Group 1 dsDNA, order unassigned, family Papillomaviridae, genus Papillomavirus) testing is being evaluated to assess its possible role in the UK Cervical Screening Programme. Studies internationally have looked at the use of HPV testing as a primary screening tool (Kitchener et al., 2009a,b) and as part of follow up after treatment of cervical intraepithelial neoplasia (Sharp et al., 2009; Zielinski et al., 2004). Although the optimal DNA extraction method and HPV test is not yet defined the potential problem associated with DNA extraction failure is important for molecular HPV testing. DNA extraction methods and tests to confirm DNA adequacy need to be robust in order to ensure reliable reporting of clinical results and to avoid false negative or false positive HPV results.

A number of commercial DNA extraction methods are available and protocols have been designed for enhanced DNA recovery, dependent upon starting material. QIAamp (Qiagen, Hilden, Germany) is designed for the extraction of DNA from liquid based cytology (LBC) samples, whilst less expensive methods including crude Proteinase K digestion have also proven effective (de Roda Husman et al., 1995). The quality of DNA recoverable from cervical samples has been investigated in archival cervical material (Boulet et al., 2008; de Roda Husman et al., 1995) and in LBC medium (Powell et al., 2006). Routine methods utilise PCR for the confirmation of DNA recovery from cellular material with the amplification of fragments of the human β-globin gene (Canfell et al., 2004) and a β-globin negative sample indicates insufficient DNA recovery. Parallel testing for HPV and β-globin on each specimen aims to eliminate potential false negative results i.e. HPV negative and β-globin negative.

A South Wales cross-sectional study (South Wales HPV study (n = 10,000)) determined the HPV prevalence in women attending routine cervical screening (Hibbitts et al., 2008). PCR for the human house-keeping β-globin gene was carried out to determine DNA extraction efficiency, and HPV prevalence was only reported in β-globin positive samples (Hibbitts et al., 2008). The South Wales HPV study identified 7% (n = 676/10,000) samples as β-globin negative and these specimens were not analysed further.

Specimens used in HPV testing generally require the patient to undergo a vaginal examination similar to cervical cytology sampling. It is known that this is associated with psychological morbidity (Jones et al., 1996). A test that does not yield a valid result in 7% cases, particularly when the test is intrusive, could be deemed unsuitable for use in clinical practice.

The aim of the present study was to compare the efficacy of the commercial QIAamp extraction method with Proteinase K digestion and to develop methods to address DNA extraction failure.

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doi:10.1016/j.jviromet.2011.01.020

Strategies were explored in a clinical setting and an algorithm for sample management was developed for retesting in order to minimise false negative HPV results.

Residual LBC samples (ThinPrep: Cytyc Corp., Marlborough, USA and SurePath: BD, TriPath Imaging, Burlington, USA) were obtained following routine cytological analysis within Cervical Screening Wales (CSW). For each clinical specimen, all residual LBC material received (ca 10 ml) was centrifuged at 3354 × *g* for 10 min; the supernatant was decanted and the cell pellet re-suspended in 2 ml 10 mM Tris (pH 7.4). The cell suspension was divided equally into 2 tubes and then centrifuged at 11,337 × *g* for 5 min. The supernatant was removed leaving the cell pellet. One cell pellet was stored directly at −80 °C. 500 μl 10 mM Tris (pH 7.4) was added to the second cell pellet and the sample resuspended. Two 100 μl aliquots were transferred into single use vials ready for DNA extraction and the remainder of the cell suspension was stored at −80 °C.

For DNA extraction using Proteinase K, 10 μl Proteinase K (14–22 mg/ml, Roche, Lewes, UK) was added to each 100 μl sample, vortexed and incubated at 56 °C for 2 h (vortexing every 15 min). Samples were incubated at 80 °C for 10 min and then at 4 °C for 10 min. The samples were centrifuged at 11,337 × *g*, 4 °C for 10 min and the supernatant transferred into a 96-microwell plate.

The QIAamp MinElute kit (Qiagen, Hilden, Germany) is designed specifically for the purification of nucleic acids from LBC samples. DNA was extracted from 100 μl of the control material or clinical specimen according to the manufacturer's instructions. DNA was eluted into a final volume of 100 μl.

The β-globin PCR amplifies a 200 bp amplimer (de Roda Husman et al., 1995) and the mastermix includes 0.2 mM dNTPs (Invitrogen), PCR buffer (Invitrogen), 1.75 mM $MgCl_2$ (Invitrogen), 0.5 mM PCO3 primer, 0.5 mM PCO5 primer, 0.5 U Taq polymerase (Invitrogen) and water up to 20 μl/sample (Canfell et al., 2004) and 5 μl of DNA (ca 250–500 ng) from each sample. PCR conditions were as follows: preheating for 4 min at 94 °C, 40 cycles of 30 s each at 94 °C, 55 °C and 72 °C, and a final extension for 4 min at 72 °C. The PCR products were analysed by agarose gel electrophoresis (2%) and visualised on a transilluminator. β-Globin results obtained for the specimens were recorded electronically.

High Risk (HR) HPV typing was performed using two molecular methods: HPV PCR ELISA (PCR-EIA) and Greiner Bio-One PapilloCheck® HPV-assay (PapilloCheck®). The PCR-EIA method using the GP5/6+ primers was performed on all specimens with minor modifications (Hibbitts et al., 2008; Jacobs et al., 1997). PapilloCheck® HPV assay was carried out according to the manufacturer's instructions.

Proteinase K and QIAamp DNA extractions were carried out on cell-line derived control material and clinical samples collected from women with low grade cytological abnormalities (low grade cytology study (n = 883)). All DNA extracts were analysed by β-globin PCR. The control DNA samples were prepared from a 10-fold dilution series of the SiHa cell line (ATCC number HTB-35) (Plummer et al., 1998) from 5000 to 5 cells/ml. At each titration point, cells were spiked in triplicate into a background of 10 mM Tris (pH 7.4) and two LBC mediums: SurePath and ThinPrep. ThinPrep LBC clinical samples were collected from women (aged 20–65) in the low grade cytology study in collaboration with Cervical Screening Wales. The low grade cytology study extracted DNA using both QIAamp and Proteinase K. Full HPV typing results obtained in the low grade cytology study on β-globin positive samples have been published (Jones et al., 2009).

The β-globin results comparing the QIAamp and Proteinase K DNA extraction methods of the SiHa titration series are shown in Table 1. QIAamp extracted DNA efficiently with β-globin detection to 500 cells/ml in both LBC mediums and at 50 cells/ml in a background of 10 mM Tris (pH 7.4). The Proteinase K method gave comparable results to the commercial assay and both techniques gave similar DNA extraction efficiencies in the two different LBC media. Table 2 outlines the β-globin results obtained from the low grade cytology study clinical specimens (n = 883) extracted by QIAamp and Proteinase K. 93.3% of samples gave a consistent β-globin positive result after extraction using the two methods and one specimen was β-globin negative by both methods. Inconsistent β-globin results were detected in 6.7% of specimens tested.

Table 1
Comparison of DNA extraction methods in control material. β-Globin results from the SiHa 10-fold dilution titration series cell input from 5000 to 0.5 per reaction in 10 mM Tris (pH 7.4), SurePath and ThinPrep LBC medium. Each titration point was performed in triplicate. Positive results (+) are highlighted in grey and represent a consistent result for each of the triplicate samples investigated.

	Prot K	QIAamp	Prot K	QIAamp	Prot K	QIAamp
5000	+	+	+	+	+	+
500	+	+	+	+	+	+
50	–	+	+	–	–	–
5	–	–	–	–	–	–
0.5	–	–	–	–	–	–

To address DNA extraction failure, samples from the South Wales HPV study were investigated further. The South Wales HPV study comprised 10,000 SurePath LBC samples collected from the routine cervical screening population and DNA was extracted using Proteinase K with analysis by β-globin PCR. 676 of the 10,000 (7%) samples were β-globin negative in the previously published dataset (Hibbitts et al., 2008). A representative subset (n = 379/676) of the β-globin negative samples from the South Wales HPV prevalence study were processed as follows to determine the cause of the test failure: (i) error in original β-globin PCR: the original β-globin negative DNA extract was re-tested by β-globin PCR; (ii) DNA extraction failure: β-globin negative samples were re-extracted from the original cell sample suspension using Proteinase K followed by β-globin PCR; and (iii) possible sample inhibition: DNA extracts from (i) and (ii) were diluted 1:10 and 1:100 in water, followed by repeat β-globin PCR. Samples confirmed as β-globin positive following (i), (ii), or (iii) and had dyskaryotic cervical cytology were tested for HPV (n = 44). The HPV PCR-EIA was performed on the same sample that yielded a positive β-globin result.

10% of samples were re-tested for internal quality control (QC). Fisher's exact test was used to calculate the P values and 95% confidence intervals (CI) are quoted where appropriate.

Table 3 highlights the results following re-analysis of 379/676 β-globin negative samples from the South Wales HPV study. Repeat β-globin PCR, with standard DNA input of the original DNA extract identified 21.6% (n = 82; 95% CI 17.59–26.13) β-globin positive samples. A new Proteinase K extraction was performed on fresh material to determine whether the clinical specimen was a factor in DNA extraction failure. 56.2% (n = 213; 95% CI 51.04–61.26) of the new DNA extracts were β-globin positive. Dilution of the original DNA extracts 1:10 increased β-globin detection significantly with a further 51.7% (n = 196; 95% CI 46.56–56.85) cases β-globin positive (P < 0.0001). In the new DNA extracts, sample inhibition (1:10 and 1:100) was an influencing factor on β-globin positivity, but not as pronounced, with an additional 30.1% (n = 114; 95% CI 25.50–34.97) β-globin positive samples identified.

Table 2
Comparison of DNA extraction methods in clinical material (n = 883) following extraction through Proteinase K and QIAamp are given.

	QIAamp DNA extraction % (n)		Total
	β-Globin positive	β-Globin negative	
Proteinase K extraction % (n)			
β-Globin positive	93.3 (824)	3.6 (31)	96.8 (855)
β-Globin negative	3.1 (27)	0.1 (1)	3.2 (28)
Total	96.4 (851)	3.6 (32)	100 (883)