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Validation of a standardized extraction method for formalin-fixed paraffin-embedded tissue samples☆

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ABSTRACT

Background: Formalin-fixed paraffin-embedded (FFPE) samples can be DNA-extracted and used for human papillomavirus (HPV) genotyping. The xylene-based gold standard for extracting FFPE samples is laborious, suboptimal and involves health hazards for the personnel involved.
Objectives: To compare extraction with the standard xylene method to a xylene-free method used in an HPV LabNet Global Reference Laboratory at the Centers for Disease Control (CDC); based on a commercial method with an extra heating step.
Study design: Fifty FFPE samples were randomly selected from a national audit of all cervical cancer cases diagnosed in Sweden during 10 years. For each case-block, a blank-block was sectioned, as a control for contamination. For xylene extraction, the standard WHO Laboratory Manual protocol was used. For the CDC method, the manufacturers' protocol was followed except for an extra heating step, 120 °C for 20 min. Samples were extracted and tested in parallel with β-globin real-time PCR, HPV16 real-time PCR and HPV typing using modified general primers (MGP)-PCR and Luminex assays.
Results: For a valid result the blank-block had to be betaglobin-negative in all tests and the case-block positive for beta-globin. Overall, detection was improved with the heating method and the amount of HPV-positive samples increased from 70% to 86% ($p = 0.039$). For all samples where HPV type concordance could be evaluated, there was 100% type concordance.
Conclusions: A xylene-free and robust extraction method for HPV-DNA typing in FFPE material is currently in great demand. Our proposed standardized protocol appears to be generally useful.
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1. Background

Human Papillomavirus (HPV) infections are known to be a major cause of cervical cancer [1]. More than 200 types of HPV have been fully characterized [2]. HPV types are divided in high-risk and low-risk viruses, where the high-risk types, notably HPV16 and HPV18, predominate in HPV-related cancers [1]. In light of current developments in HPV-based screening and HPV vaccination, it is important to develop a robust method for HPV genotyping and detection in cervical tissue, to be able to analyze the HPV-type-specific disease burden and to monitor the effectiveness of HPV prevention programs [3].

Formalin-fixed paraffin-embedding (FFPE) of tissues is suitable for long-term storage of cancer tissue samples and has been globally used in pathology for more than a century. FFPE cancer tissue has often been used for analyzing presence and type of HPV in cancers, for example in order to determine the HPV type-specific burden of disease in various regions of the world and over time [4]. However, the analysis of FFPE specimens includes several potential problems including DNA cross-linking, DNA fragmentation and the presence of PCR inhibitors [4,5]. The goldstandard method for extracting these samples before HPV testing is xylene-based extraction [6]. However, xylene-based extraction is laborious, known to result in suboptimal sensitivity and also involves health hazards for the personnel involved. Recently, a method combining heat

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treatment and a commercially available DNA extraction kit was evaluated compared to xylene-based extraction, showing higher DNA yield and increased sensitivity for HPV testing [5] We wished to further evaluate and investigate the robustness of this method.

2. Objectives

The aim of this study was to evaluate a xylene-free method for the extraction of HPV-DNA in FFPE samples. If found robust, this method could become a new standard method for FFPE extraction before HPV genotyping. We thus compared the xylene-based gold standard method to a commercial column-based extraction with an extra heating step [5].

3. Study design

3.1. FFPE samples

Fifty FFPE-samples from patients with cervical cancer were randomly selected from a national case-control audit encompassing all invasive cervical and unspecified uterine cancers diagnosed from 2002 until 2011 in Sweden, the Advancing Cervical Cancer Eradication Strategies, ACCES, study. The diagnostic slides and the formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from different pathology biobanks throughout Sweden. Here, we selected fifty samples from one biobank, Gävleborg. The diagnostic slides were re-reviewed by an experienced histopathologist and only tissues where presence of cervical cancer could be verified were retained in this study.

3.2. Sectioning

All FFPE blocks were sent to a commercial, accredited laboratory (HistoCenter, Inc., Gothenburg, Sweden) for sectioning. In-between each case-block, a paraffin blank-block was sectioned, as a control for contamination. Every case required new gloves and a new knife. The blank-block was sectioned first. Four 5 μm sections were transferred to a 1.5 mL screw-cap Eppendorf tube using a new, clean, toothpick. The case-block was mounted on the microtome and sectioned with the same knife. Each case was sectioned in six 5 μm sections. The first and last section were set aside for histological review and two times two 5 μm sections were put in two tubes, one tube per extraction method, for HPV testing. The slides for histology were stained with hematoxylin-eosin. The tubes were marked with the blocks specific lab number, B (blank-block) and C (case-block). The slides were marked in the same way, with the specific lab number followed by letters F (first) and L (last). After each case the knife was removed and the microtome was cleaned with DNAZap (Applied Biosystems). Specimen size was determined in millimeters, using a ruler, with size in square millimeters being the product of length and width. Although all cases included in the study had been verified to contain tumor tissue, sections from HPV-negative tumors were re-reviewed a second time and still found to contain tumor tissue.

3.3. DNA extraction

DNA was extracted from the sectioned blocks using two different extraction methods: 1) A method developed at CDC using the Qiagen blood and tissue kit with an extra heating step [5] and 2) the xylene-based gold standard method [6]. The extra heating step has been reported to result in an 8.2 fold increase in PCR-amplifiable cellular DNA and a decrease in inadequate results (5.3%, down from 19.3%) [5].

One tube from each FFPE sample was extracted with xylene. The paraffin was removed by incubation with 1 mL xylene in 50 °C for 30 min followed by vortexing and centrifugation 3000*g*, 10 min, the supernatant was removed with a sterile transfer pipette. This step was repeated once. After removing the xylene the samples was washed twice with pure ethanol and air dried. The air dried pellet was incubated with 100 μL Digestion buffer (50 mM Tris HCl,1 mM EDTA, pH 8,5) with Proteinase K (50 mg/mL) in 37 °C for about 24 h. After incubation the samples were boiled in 100 °C for 10 min to inactivate Proteinase K.

The other tube from each FFPE sample was extracted with the Qiagen/heating method. 180 μL ATL buffer was added to the tube and high-heat treated in 120 °C for 20 min to melt the paraffin. Within the 5 first minutes the tubes were mixed by tapping the tube to make sure that all of the paraffin was under the surface. After 20 min the samples were incubated at room temperature for 3 min, followed by a quick centrifugation. 20 μL proteinase K was added, briefly vortex and incubated in 65 °C for 16 h (the manufacturer's protocol describes 56 °C, but a previous study found 65 °C to be better [5]). The tubes where quickly centrifuged. A solution of 200 μL buffer AL and 200 μL ethanol, per sample, was prepared. After adding 400 μL of the prepared solution immediate vortex was needed. The mixture was added to a DNeasy Mini spin column and centrifuged 1 min at 8000 rpm. The following steps were performed according to the manufacturer's protocol except for the volume in the elution step that was changed to 100 μL AE buffer. In total 200 extractions were performed.

3.4. Quantitative real-time PCR for beta-globin and HPV16

All samples were tested for both beta-globin and HPV16 in real-time PCR, as previously described [7,8]. Samples from the two extraction methods were tested in parallel in both beta-globin and HPV16, undiluted and diluted 1/10 in water.

Briefly, 1 μL extracted sample was used in the PCR of a total volume of 25 μL. A standard program was used, 50 °C, 2.0 min, 95 °C, 10.0 min; 95 °C 15.0 s, 60 °C 1.0 min for 40 cycles. The following primers and probe were used for HPV16, *HPV16 E7 M forward 5′AGCTCAGAGGAGGAGGATGAA 3′, HPV16 E7 M reverse 5′ GGTTACAATATTGTAATGGGCTC 3′ and HPV16 E7 M probe 5′-FAM-CCAGCTGGACAAGCAGAACCGG-TAMRA-3′*.

All samples were analyzed in parallel for quantitative HPV16 real-time PCR, quantitative beta-globin real-time PCR and HPV-genotyping. Samples from each individual case-block, extracted with the two different extraction methods, were analyzed in parallel in the same run in all assays with the same standard curve and positive controls. 1 μL extracted sample was used in all the assays to avoid PCR inhibitors.

For a valid result, the blank-block had to be negative in all three tests and the case-block positive for beta-globin, where a measurement of one copy was considered positive. FFPE samples are known to contain PCR inhibitors and to avoid this, all 50 samples were tested first undiluted and then diluted 1–10.

3.5. HPV genotyping

HPV detection and genotyping of HPV was done using modified general primers, MGP-PCR and Luminex as previously described [9]. Briefly, 1 μL was used as input in the MGP-PCR in a total volume of 25 μL. The probes used in the multiplex Luminex were, HPV6, 11, 16, 18, 18.6650 G variant, 26, 30, 31, 33.2, 35, 35.6624:A variant, 39, 40, 42, 43, 45, 51, 52, 53, 54, 56, 58.1, 59, 61, 66, 67, 68 prototype (discovered by Gerard Orth, also called HPV68a), 68ME.1 (also called HPV68b), 69, 70, 73, 74:911664, 81, 82, 83, 86, 87, 89, 90, 91, universal1 and universal2 (for probes, see Table 1). Samples

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