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HPV16 detection by qPCR method in relation to quantity and quality of DNA extracted from archival formalin fixed and paraffin embedded head and neck cancer tissues by three commercially available kits

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The aim of the present study was to compare HPV16 detection by quantitative polymerase chain reaction (qPCR) in relation to the quantity and quality of DNA isolated from 21 formalin fixed and paraffin embedded (FFPE) head and neck cancer tissues by three commercially available kits: EX-WAX™ DNA Extraction Kit (M) (Merck Millipore, Darmstadt, Germany), QIAamp® DNA FFPE Tissue (Q) (Qiagen, Hilden, Germany) and ReliaPrep™ FFPE gDNA Miniprep System (P) (Promega, Madison, USA). Quantity of extracted DNA was assessed spectrophometrically and fluorometrically. Its quality was analyzed using A260/280 and A260/230 ratios and the *β-actin* fragment amplifiability in qPCR. HPV16 presence was detected by qPCR, using specific primers and TaqMan probe. HPV infection was found in 8 DNA samples extracted with M kit (38.1%) and in 7 (33.3%) isolated with Q and P kits. Three samples from M and Q kits were characterized by HPV16 positivity and lack of *β-actin* amplifiability. They had significantly lower A260/280 ratio (M: 1.6 ± 0.0, $p = 0.044$ and Q: 1.7 ± 0.0, $p = 0.016$) compared to samples with both fragments amplification (M: 1.7 ± 0.0 and Q: 1.9 ± 0.0). Therefore, for HPV detection by qPCR in FFPE tissues we recommend ReliaPrep™ FFPE gDNA Miniprep System.

1. Introduction

Persistent infection with high-risk human papillomavirus (HPV), predominantly HPV16, is considered as a one of the most important risk factors for development of various type of malignancies (cervical cancers, some head and neck tumours, vaginal, vulvar, penile or anal cancers) (Doorbar et al., 2015). Moreover, there is some evidence demonstrating relation between HPV presence and patients' outcome after radiotherapy or concurrent chemoradiotherapy and suggesting its prognostic or predictive potential (Melkane et al., 2014; Psyrri et al., 2014). Thus, it is extremely important to develop reproducible and sensitive method for HPV detection. Some reports indicate that conventional polymerase chain reaction (PCR), or its quantitative version (qPCR) meets these conditions (Klozar and Tachezy, 2014). Such molecular studies are

Abbreviations: bp, base pair; ds, double stranded; FFPE, formalin-fixed and paraffin-embedded; HPV, Human Papillomavirus; M, EX-WAX™ DNA Extraction Kit; P, ReliaPrep™ FFPE gDNA Miniprep System; PCR, polymerase chain reaction; Q, QIAamp® DNA FFPE Tissue; qPCR, quantitative version of polymerase chain reaction; Qs, Quantus; SCC, squamous cell carcinoma; SE, standard error; S, spectrophotometer.

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Table 1
Characteristics of the 3 commercially available DNA isolation kits for formalin-fixed paraffin embedded tissues used in this study.

DNA isolation kit	Manufacturer (Cat. no.)	Deparaffinization	Digestion			Cross-linking removal	Purification method	Elution volume [μl]
			Method, buffer	Time [min]	Temp [°C]			
EX-WAX™ DNA Extraction Kit	Merck Millipore Darmstadt, Germany (S4530)	none	Protein Digesting Enzyme Solution, Digestion Solution	overnight	50	none	Precipitation Solution, ice cold 100% ethanol	50
QIAamp® DNA FFPE Tissue	Qiagen Hilden, Germany (56404)	xylene, ethanol	proteinase K, Buffer ATL	overnight	56	90 °C, 60 min	QIAamp MinElute column	50
ReliaPrep™ FFPE gDNA Miniprep System	Promega Madison, USA (A2352)	mineral oil, heating	proteinase K, Lysis Buffer	overnight	56	80 °C, 60 min	Binding Column, RNase treatment	50

often based on DNA extracted from formalin-fixed and paraffin-embedded (FFPE) tissues. These samples offer unique opportunity to perform histological and pathological analysis of fixed material even after many years and to obtain long term follow-up data. However, DNA preservation in FFPE tissues over long time period can be limited due to its fragmentation and formation of DNA-protein cross linking (Kokkat et al., 2013). These effects considerably decrease DNA yield as well as its amplifiability and depend mainly on fixation type and storage conditions (time and temperature) (Frankel, 2012). However, the quality and quantity of isolated DNA are also related to extraction method (Kocjan et al., 2015a, 2015b). Currently, DNA is mostly extracted on the basis of ready to use commercially available kits provided by a number of companies. Thus, the aim of the present study was to compare HPV16 detection by qPCR in relation to the quantity and quality of DNA extracted from FFPE samples stored up to 9 years, by three commercially obtainable kits: from Merck Millipore (Darmstadt, Germany), Qiagen (Hilden, Germany) and Promega (Madison, USA). The study was performed in the group of 21 patients with squamous cell carcinoma (SCC) of head and neck.

2. Material and methods

2.1. Patients and material

The study was performed in the group of 21 patients with SCC of the mouth floor (n = 5), tongue (n = 5), tonsil (n = 5) and oropharynx (n = 6). There were 12 patients with tumours in clinical stage T3N2 (57.1%), 4 in T4N1(19.0%), 2 in T4N1 (9.5%) and single cases in stages T2N2, T3N3 and T4N2. Dominant among cancers were these in grade G2 (n = 18, 85.7%) and non-keratinizing (n = 12, 57.1%,). No direct contact with patients and use of their personal (patients') data were necessary, because the study was not intended to modify the diagnostic or treatment procedures. Therefore, no informed consents from patients were required. All samples were anonymized.

All procedures were performed using tumour samples obtained during surgery, fixed in buffered formalin, embedded in paraffin and stored at room temperature for 5–9 years (mean and median storage period 7 years ± 0.2 (standard error (SE)). Before cutting, in order to confirm histology and grading, all samples were reviewed by a pathologist on the basis of sections stained with hematoxylin and eosin.

2.2. DNA isolation

DNA was extracted from FFPE tissues using 3 commercially available kits: EX-WAX™ DNA Extraction Kit (M) (cat. no. S4530, Merck Millipore, Darmstadt, Germany), QIAamp® DNA FFPE Tissue (Q) (cat. no. 56404, Qiagen, Hilden, Germany) and ReliaPrep™ FFPE gDNA Miniprep System (P) (cat. no. A2352, Promega, Madison, USA) (Table 1). All paraffin blocks were cut three times, separately for every kit tested. In case of all three isolation, the same number of 4 μm paraffin sections from single block was used (3–7 sections depending on sample size) was used. In order to prevent cross contamination, the microtom and other accessories were cleaned with DNA Away (cat. no. 7010, Thermo Fisher Scientific, Inc., Waltham, USA) and fresh blade was installed before cutting each block. The first 2–3 sections were discarded. During cutting, excess paraffin was trimmed away and then the sections were placed in 1.5 mL Eppendorf tubes and spinned briefly. All extractions were performed according to manufactures' instructions with our own modifications (M kit: overnight incubation with Digestion Solution and Protein Digesting Enzyme Solution; Q kit: deparffinization step (xylenes for 3 × 10 min), overnight incubation with proteinase K, P kit: overnight incubation with proteinase K).

2.2.1. EX-WAX™ DNA Extraction Kit

For isolation with M kit, first 100% ethanol was added to each tube and gently vortexed. After removing of ethanol, pellets were dried at 50 °C until residual alcohol was no longer present. Then, overnight incubation at 50 °C, with mixture of Digestion Solution and Protein Digesting Enzyme Solution was performed. Next, Extraction Solution was added and the suspension was gently mixed and spinned (12 000 rpm, 10 min). The supernatant was collected in the fresh tube and one hour incubation with the mixture of Precipitation Buffer and ice cold 100% ethanol was performed at −20 °C. After centrifugation (12 000 rpm, 10 min), the supernatant was discarded. The pellets were dried and incubated with Resuspension Solution for 1 h at 50 °C.

2.2.2. QIAamp® DNA FFPE Tissue Kit

DNA extraction with Q kit was started with deparaffinization with xylene (3 × 10 min) and rehydration trough graded ethanol steps. After drying at room temperature, pellets were incubated overnight with solution of Proteinase K in ATL Buffer at 56 °C. Next day, the samples were incubated at 90 °C for 1 hour and afterwards the mixture of AL Buffer and 100% ethanol was added. After short centrifugation, the entire lysate was transferred to the QIAamp MinElute column, washed twice and centrifuged (1400 rpm, 3 min.) DNA was eluted from the column by ATE buffer.

2.2.3. ReliaPrep™ FFPE gDNA Miniprep System

Using P kit, the sections were first incubated in 300–500 mL (depending on the section size) of mineral oil for 1 min, at 80 °C. After adding Solution Buffer and centrifugation (12 000 rpm, 15 s) two phases were observed: aqueous (lower) and oil (upper). The samples were incubated with solution of Proteinase K in Lysate