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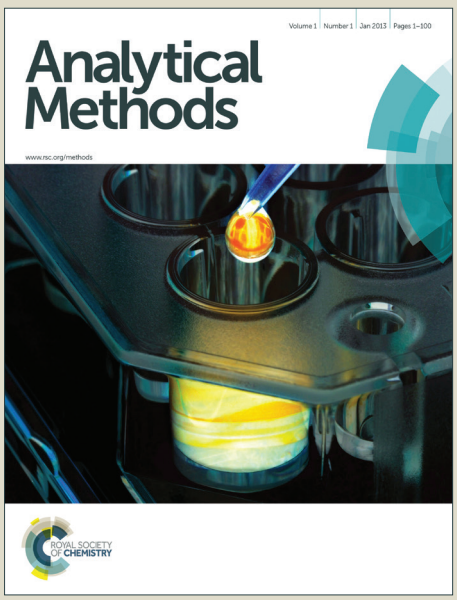

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Human Papillomavirus Genotyping by Surface-Enhanced Raman Scattering

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The first Human Papilloma Virus (HPV) genotyping assay using surface-enhanced Raman scattering (SERS) is reported. Validated PCR primers were used to generate amplification products from plasmids, a control cell line and clinical specimens enabling subsequent identification of specific HPV genotypes using type specific probes across six channels.

Surface enhanced Raman scattering (SERS) is a powerful, information-rich analytical tool capable of sensitive spectroscopic detection. The analyte-specific fingerprint spectra are particularly suited to the simultaneous detection of multiple targets, making the technique amenable to multiplexing beyond the capabilities of fluorescent alternatives[1]. SERS technology has become central to many bio-diagnostic applications and can be divided into two approaches: 1. intrinsic SERS exploiting the highly conserved molecular vibrations of nucleic acids, lipids and other cellular components to allow contrast agent free differentiation[2], and 2. extrinsic SERS making use of Raman tagged nanoparticles as bio-barcodes, whereby conjugation of a protein or nucleic acid allows the direct detection of target analytes in an analogous methodology to fluorescent labelling strategies[3]. Proof of concept studies have suggested both approaches could form a basis for diagnostic assays however neither approach has yet produced an assay capable of impacting patient management pathways. To fulfil the diagnostic potential of SERS, a new SERS spectroscopy platform, RenDx, has been developed for the detection of nucleic acid targets. A complete systems approach was adopted which included semi-automated processing coupled to amplification, and subsequent detection from a 96-well plate using proprietary multivariate component direct classical least squares (DCLS) analysis, allowing multiplexed detection of up to 10 targets per microwell. Through careful modification of existing PCR assays, SERS detection can differentiate between more targets than fluorescent or colourimetric approaches[1]. In order to demonstrate the versatility and power of this systems approach, the first example of HPV genotyping is reported.

HPV is a DNA virus classified in the *Papillomaviridae* family.[4] There are more than two hundred known HPV genotypes and the majority present minimal or no risk to their human host. HPV genotypes can be broadly classified into Low Risk (LR) types and High Risk (HR) types. LR HPV types are responsible for the most common clinical manifestation of an HPV infection, warts.[5] HR HPV types are oncogenic, and persistent HR HPV infections can cause cancers of the anogenital and oropharyngeal regions.[6, 7] Approximately 99% of all invasive cervical carcinomas (ICCs) harbour at least one type of HR HPV DNA[6] and HPV types 16 and 18 account for 70% of ICC cases worldwide.[8] Most developed countries offer a population based cervical screening programme.[9] Cytology based cervical screening identifies dyskaryotic cervical cells and treats cervical intraepithelial neoplasia (CIN) to prevent progression to invasive disease. Prophylactic HPV vaccination targeting HPV16 and 18 was implemented in the UK in 2008.[10] HPV molecular testing has higher sensitivity for high-grade disease compared to cytology but since HPV infection is common, this is at the expense of lowered specificity.[11] A number of commercial DNA based approaches to detect HPV have been developed, including QPCR (*Roche*), Hybrid Capture II (*Qiagen*), Invader Technology (*Cervista*), Fluorescence in-situ hybridisation (FiSH) and post-PCR genotyping.[12] Fluorescence/ QPCR assays have limited multiplexing capabilities in comparison to Raman spectroscopy and this study aimed to exploit the narrow spectra observed through SERS to allow screening in six channels for different HPV genotypes using the validated HPV GP5+/GP6+ PCR primer and probe set[13].

The SERS HPV utilised the RenDx multiplex assay approach (Figure 1). A modified method of the assay described previously was performed[14]. Briefly, biotinylated PCR products were denatured and target specific SERS probes then hybridised to target amplicons. The process is then fully automated and involves the addition of streptavidin magnetic beads (to capture biotinylated strands with hybridised probes), wash cycles (to remove unbiotinylated template and non-captured probes), elution (removing probes from the magnetically captured amplicons) and addition of eluant to a silver nanoparticle suspension for SERS detection. Analysis of a full plate from amplification to results takes under 6 hours.

The assay used 6 dye-labelled probe channels (see ESI Table S1). HPV type specific channels and associated dye spectra were: HPV16 – **B** , HPV18 - **C**, HPV31 - **D** , HPV45 – **E**, other HR HPV (including HPV33, 35, 39, 51, 52, 56, 58, 59, 66, 68) – **F** and LR HPV (including HPV 6, 11, 40, 42, 43, 44) - **G** (See Figure 2).

The HPV SERS assay was validated on DNA obtained from a range of HR HPV plasmids (16; 18; and 45), the HR HPV16 positive human cell line CaSki, and twenty-five residual liquid-based cytology samples. Residual samples had been screened for the presence or absence of HPV using the GP5+/GP6+ PCR-ELISA, a non-commercial research genotyping HPV assay.

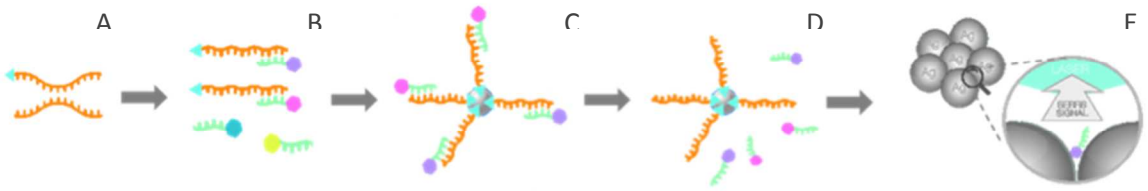

Figure 1. Illustration of the RenDx assay. Denatured biotinylated PCR product (A) is hybridised with SERS probes (B), and captured on streptavidin magnetic beads. After a series of washing steps (C), captured probes are eluted (D) and added to silver nanoparticles, facilitating SERS analysis (E).

The HPV L1 PCR contained 10ng of genomic DNA, 0.5 µM GP5+/GP6+ primers,[13] 200 µM of each dNTP, 1 unit of Hotstar Taq DNA polymerase (Qiagen), Hotstar Taq buffer, total of 3.5 mM $MgCl_2$ and distilled water to a final volume of 25 µl. PCR cycling conditions were 94°C 4 min, 45 cycles of 94°C 30 s, 40°C 90 s, 72°C 60 s followed by 72°C 4 min. To monitor amplification success, products were visualized using 2% agarose gel electrophoresis. For validation of results, routine ELISA (PCR-EIA described in[15]) and SERS tested amplified products were screened in parallel.

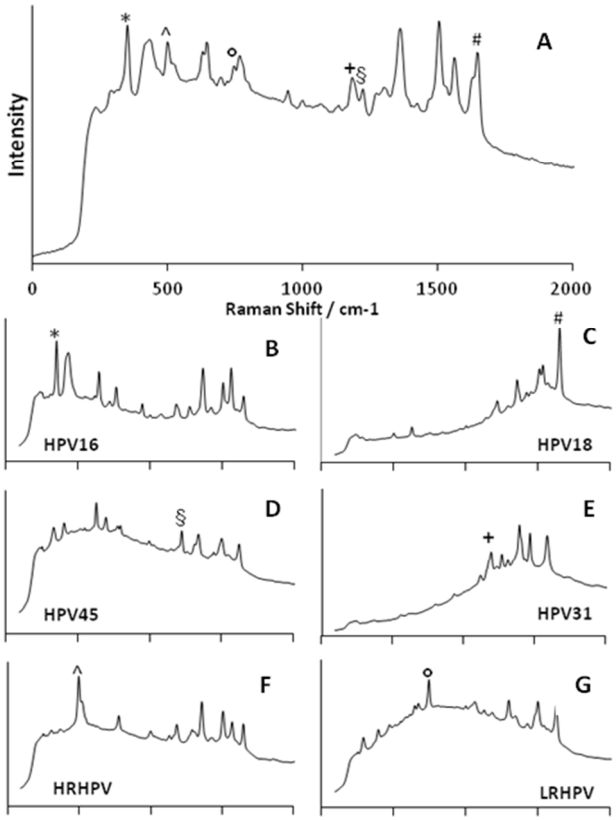

Figure 2. SERS 6-plex. The SERS probes for each of the 6 channels, (HPV 16/18/31/45/HR/LR) are shown multiplexed together (**A**) and individually (**B-G**) using the same X-Axis with Y-normalisation. The RenDx algorithm is designed to deconvolute complex spectra allowing detection of all target probes in a multiplex. *Key*- * -352 cm^{-1} ^ -466 cm^{-1}, ° -745 cm^{-1}, $^{+}$ -1184 cm^{-1}, § -1224 cm^{-1}, $^{\#}$ -1664 cm^{-1}.

Monoplex titrations of plasmids from $1x10^7$ to 10 copies were tested. HPV16 and HPV18 were detected at $1x10^2$ copies' using SERS and PCR-EIA and HPV45 was detected consistently at $1x10^4$ copies. Multiplex titrations of HPV16 + HPV45, HPV18 + HPV45 and HPV16 + HPV18 were tested with equivalent concentrations from $1x10^7$ to 10 copies. In the HPV16 + HPV45 multiplex, HPV16 was detectable at $1x10^2$ copies by both SERS and PCR-EIA but no HPV45 plasmid was identified. In the HPV18 + HPV45 multiplex, HPV18 was detectable at $1x10^2$ copies by both SERS and PCR-EIA but no HPV45 plasmid was identified. The GP5+/GP6+ PCR preferentially amplified HPV16 and HPV18 over HPV45. In the HPV16 + HPV18 multiplex, $1x10^2$ copies of HPV16 and 10 copies of HPV18 were detected by both SERS and PCR-EIA.

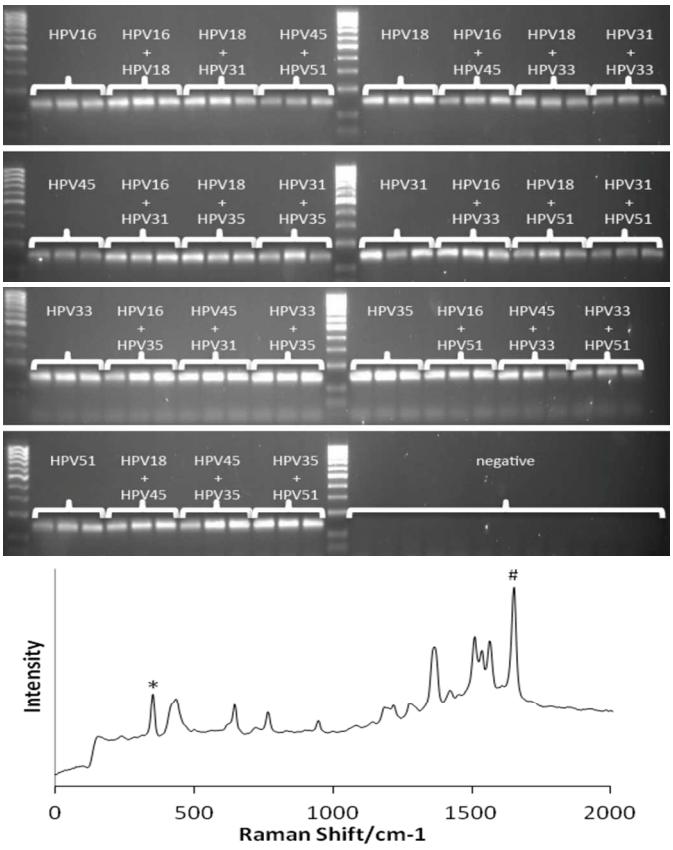

Figure 3. HPV Monoplex and Multiplex Plasmid Amplification. Agarose gels are used to show that PCR amplification was successful. Each agarose band also has respective SERS spectra however for the clarity only a sample HPV16/18 duplex spectra is shown. The duplex uses * to illustrate a unique peak from HPV16 and $^{\#}$ to illustrate a HPV18 peak.

To test the *in vitro* performance of the RenDx HPV assay, a CaSki DNA titration series with estimated HPV copy number ranging from $1x10^7$ to 10 copies was tested to investigate HPV detection in a background of human DNA. Both SERS and PCR- EIA consistently

detected HPV16 in a background of human DNA at $1x10^3$ copies (ESI, Table S2).

To evaluate the performance of the assay in a clinical setting, DNA from twenty-five clinical samples was tested using both SERS and ELISA. Twenty-three of the twenty-five samples gave expected results by SERS and PCR-EIA with samples classified correctly as LR HPV and HR HPV negative or positive. The observed percentage of agreement was 92%, and the Kappa coefficient was 0.83 suggesting substantial agreement with previous tests. The 2/25 discordant results were caused by dual infections and in both cases HPV16 had been previously detected on a single screen using the GP5+/GP6+ PCR-EIA assay. Although the RenDx assay and PCR-EIA correctly identified the second HPV type, both failed to detect the presence of HPV16 in both samples. Sampling differences, low viral load causing a borderline call or an inaccurate initial result could all cause discrepancies between the first and second PCR-EIA analysis.

Conclusions

Here we report a HPV genotyping assay converted from a routine GP5+/GP6+ PCR-EIA method to a SERS based platform. Results indicate comparable detection performance in plasmid, cell line and clinical material. The automated SERS method overcomes many of the time constraints in the manual PCR-EIA assay and facilitates the potential for higher throughput of clinical samples. Further the ability to screen across six channels including; four unique HR HPV types 16/18/31/45, other HR and other LR provides a significant advantage over existing assays. Differentiation between species is clinically advantageous considering future development of an individualised patient management stratified according to the differential type-specific risk associated with HPV genotypes. Without type-specific information, healthcare providers are at risk of either overburdening patients at minimal risk of cancer or under treating high-risk individuals. Providing more comprehensive information should enable the most appropriate patient management. This study also demonstrates ease of transfer of PCR based methods on to a SERS platform. A larger study will be required to assess the full clinical utility of the RenDx HPV assay.

Notes and references

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