Evidence of HPV subtypes linked with cervical cancer in Nepal

Chop L Bhusal\textsuperscript{a}, Sulochana Manandhar\textsuperscript{b}, Meeta Singh\textsuperscript{c}, Aarati Shah\textsuperscript{d}, Sushharma Neupane\textsuperscript{a}, Dibesh Karmacharya\textsuperscript{b}, Kate Cuschieri\textsuperscript{e}, Heather Cubie\textsuperscript{e}, Duncan C Gilbert\textsuperscript{f}, Sameer M Dixit\textsuperscript{b}

**Objectives:** Cervical cancer is the commonest malignancy among women in Nepal but data are limited on which subtypes of human papillomavirus (HPV) are associated with cancer in this population. Now that vaccines against HPV types 16 and 18 are available, this evidence is of vital importance in obtaining further support for a vaccination programme.

**Methods:** Cervical swabs from 44 histologically confirmed invasive cervical cancer cases were obtained from two tertiary referral hospitals in Nepal. Evidence of HPV subtypes was identified using an HPV multiplex polymerase chain reaction (PCR), and confirmed at the Scottish HPV Virus Reference Laboratory.

**Results:** HPV types 16 and 18 were present in 70% of samples, along with other high-risk subtypes. HPV 6 and 11 were not observed. Epidemiological data assessment appeared to indicate that patient age, age of marriage and age of first pregnancy were associated with increased HPV infection in patients.

**Conclusions:** This study provides further evidence of the importance of HPV types 16 and 18 in cervical cancer in Nepal and adds support to a nationwide vaccination programme and the use of HPV detection in screening programmes.

**Key words:** Cervical cancer, HPV, Nepal, multiplex polymerase chain reaction

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**Introduction**

Cancer of the cervix is responsible for the greatest number of cancer deaths among women in Nepal\textsuperscript{1}, with up to 2000 deaths per annum. Unlike in more developed countries, where comprehensive screening programmes have contributed to reduced incidence and mortality,\textsuperscript{2} cancers in Nepal tend to present at advanced stage with significant associated morbidity and mortality.

It is appreciated that worldwide the majority of cervical cancers are associated with infection with high-risk, oncogenic subtypes.
of human papillomavirus (HPV),\textsuperscript{3,4} acquired through sexual contact. Viral oncogenes E6 and E7 downregulate p53 and pRb, respectively, resulting in cellular proliferation and a malignant phenotype.

The development of vaccines for HPV\textsuperscript{5,6} has brought into focus the precise nature of this association in terms of which subtypes are prevalent in which populations. Preliminary data from Nepal from 54 women with invasive cervical cancer demonstrated HPV-16 in 68.5% and HPV-18 in 22.2%, the next most frequent subtype being HPV-45 (in 5.6% patients).\textsuperscript{7} This is broadly in keeping with similar population studies from India\textsuperscript{8} and Pakistan\textsuperscript{9} and in a wider context the United States of America and the United Kingdom. Provisional experiences of instigating an HPV vaccination programme in Nepal have been encouraging\textsuperscript{10} with 99.3% of 1096 girls completing the course of 3 doses of Gardasil®.

To further support the introduction of HPV vaccination in Nepal, we present here data on the presence of oncogenic high-risk HPV subtypes from 44 invasive squamous cell carcinomas of the uterine cervix from Nepal.

**Methods**

This work has approval from the Ethical Review Board of the Nepal Health Research Council, Ministry of Health and Population, Government of Nepal.

Cervical swab samples were collected from the cervix of women fulfilling inclusion criteria using sterile cytobrushes and immediately inserted into a container containing phosphate buffer saline (PBS) as transport medium and stored at 4–8 °C. Sample vials were brought to room temperature and vortexed vigorously to detach the exfoliated cells. Deoxyribonucleic acid (DNA) was extracted using the GeneALLRibo-Spin VRD Kit (GeneAll, China). Briefly cells were lysed by lysis buffer VL followed by vortexing. DNA was precipitated and captured on spin columns, then washed with buffers RBW and RNW, each followed by centrifugation. Finally the purified DNA was eluted in DNase/RNase free water. The DNA was stored at -20 °C until used for polymerase chain reaction (PCR).

HPV-specific multiplex PCR was carried out using Seeplex HPV4A ACE screening kit. Following the manufacturer’s instructions, 3 μl of undiluted extracted DNA was used as template DNA in sample tubes to make the final volume of 20 μl. Negative and positive controls provided were included in each assay. PCR thermocycling was performed: 5 μl of each PCR product was electrophoresed in 2% agarose gel along with the DNA marker provided with the kit. The result was visualized under ultraviolet illumination and the bands were analysed in the reference of marker DNA (Figure 1). The result was considered valid only in the presence of the distinct band of the internal control indicating PCR success.

Results for HPV types 16 and 18 were validated and samples further genotyped at the Scottish HPV Virus Reference Laboratory, Edinburgh. Samples were assessed using a multimetric HPV genotyping kit.

**Results**

Of the 44 cervical samples analysed, 82% (36) showed HPV infection by PCR, of which 80% were HPV-16 or 18. Eleven HPV types, including nine high-risk groups, were observed in this study. Types 42 and 43 are not considered high-risk but were identified in the samples. HPV 16 and 18 DNA was identified in 68% of the total sample and 83% of HPV positive samples. The prevalence of other types in the population were observed at a much lower percentage (Table 1).
In terms of histological subtypes, 35 of 44 were squamous cell carcinomas. Of these, 78% were positive for HPV DNA while for adenocarcinoma ($n=9$), HPV infection rates were 22%. The majority of cases in this cohort ($n=39$) were stage I or II with only five cases in advanced stage (III/IV) carcinoma.

The majority of patients in this study had married at an early age with 29 of the 41 patients, where data were available, having been married prior to the age of 18 (Nepal’s legal age of marriage until 2002 was 16). Out of these 29 patients, 25 had evidence of HPV infection (86%). Eight of the 12 respondents who reported marrying later than aged 18 showed evidence of HPV (67%), though this does not reach statistical significance (Fisher’s exact test $p=0.2$). More respondents reported having a child under ($n=22$), as opposed to
older ($n=19$) than 18 years of age. Within the two groups, those that became pregnant at an earlier age had a higher HPV infection prevalence (61%) than those in the second group (39%).

**Discussion**

The data presented here confirm earlier findings that the majority of cancers of the cervix in Nepal are associated with HPV infection, and furthermore that HPV-16 and -18 represent the major subtypes responsible. Specifically, 36 of 44 (82%) cases demonstrated the presence of HPV infection. HPV-16 was present in 22 cases (61% of HPV-positive tumours) and HPV-18 in 8 cases (22%); the remaining cases demonstrated the involvement of multiple subtypes.

Combined with the limited previous data, this comprises 98 cases of invasive cervical cancer from Nepal. Of these, 83 (85%) were associated with HPV infection, namely HPV-16 in 59 cases (71% HPV positive, 60% all cervical cancers) and HPV-18 in 20 cases (24% and 20% respectively). Therefore, 80% of cervical cancers in Nepal are theoretically preventable with currently available vaccines.

Marriage and pregnancy at an early age both appear to be linked to a higher likelihood of HPV infection in cervical cancer cases in this study. This concurs with data from a previous publication, where early age at first sexual intercourse (AFSI), early age at first marriage (AFM) and early age at first pregnancy (AFP) were linked to higher incidence of HPV infection and cervical carcinoma in women of developing countries.

While effective screening programmes have reduced morbidity and mortality from cancer of the cervix in the developed world, a multitude of challenges beset the introduction of such a programme across Nepal. Indeed, a previous study reported low rates of compliance with colposcopy following abnormal screening results. As a result, mortality and morbidity from cervical cancer remain unacceptably high. The development and successful clinical introduction of HPV vaccines, however, present a major opportunity to make inroads into cancer prevention in Nepal.

Furthermore, HPV DNA typing can benefit screening programmes. DNA typing in cervical cancer screening is used in many countries; results from large randomized controlled trials testing for HPV DNA suggest that most women with cervical cytologic abnormalities should be assessed using this form of screening. The role of HPV screening in neighbouring India has been widely discussed.

In summary, these results are consistent with those from elsewhere in South Asia and the wider world and should provide further support for the introduction of an HPV vaccination programme across Nepal. Work to assess HPV infection rates in the healthy female population of Nepal should be extended to identify high-risk HPV types in the population and facilitate early screening to achieve comprehensive prevention of cancer of the cervix.

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References


