IJC
International Journal of Cancer

Urine testing to monitor the impact of HPV vaccination in Bhutan and Rwanda

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Cancer Epidemiology

Bhutan (2010) and Rwanda (2011) were the first countries in Asia and Africa to introduce national, primarily school-based, human papillomavirus (HPV) vaccination programmes. These target 12 year-old girls and initially included catch-up campaigns (13–18 year-olds in Bhutan and ninth school grade in Rwanda). In 2013, to obtain the earliest indicators of vaccine effectiveness, we performed two school-based HPV urine surveys; 973 female students (median age: 19 years, 5th-95th percentile: 18–22) were recruited in Bhutan and 912 (19 years, 17–20) in Rwanda. Participants self-collected a first-void urine sample using a validated protocol. HPV prevalence was obtained using two PCR assays that differ in sensitivity and type spectrum, namely GP5+/GP6+ and E7-MPG. 92% students in Bhutan and 43% in Rwanda reported to have been vaccinated (median vaccination age = 16, 5th–95th: 14–18). HPV positivity in urine was significantly associated with sexual activity measures. In Rwanda, HPV6/11/16/18 prevalence was lower in vaccinated than in unvaccinated students (prevalence ratio, PR = 0.12, 95% confidence interval, CI: 0.03–0.51 by GP5+/GP6+, and 0.45, CI: 0.23–0.90 by E7-MPG). For E7-MPG, cross-protection against 10 high-risk types phylogenetically related to HPV16 or 18 was of borderline significance (PR = 0.68; 95% CI: 0.45–1.01). In Bhutan, HPV6/11/16/18 prevalence by GP5+/GP6+ was lower in vaccinated than in unvaccinated students but CIs were broad. In conclusion, our study supports the feasibility of urine surveys to monitor HPV vaccination and quantifies the effectiveness of the quadrivalent vaccine in women vaccinated after pre-adolescence. Future similar surveys should detect increases in vaccine effectiveness if vaccination of 12 year-olds continues.

National, primarily school-based, HPV vaccination programmes were started in Bhutan in 2010[1] and in Rwanda in 2011.[2] HPV6/11/16/18 vaccine coverage was reported as >90% in the target groups of both countries. In Bhutan, this target was 12 year-old girls, with a one-round catch-up campaign in 2010 for 13–18 year-old girls. In Rwanda, the target was girls attending primary school grade 6 (aged ≥12 years) in 2011, with three-rounds of catch-up vaccination in 2011, 2012 and 2013 for secondary school grade 3 (aged ≥15 years). Efforts were also made to reach out-of-school girls in health centres in both countries.[1,2]

These two early-introducing countries provide the first opportunity to evaluate the impact of HPV vaccination in low/medium income countries (LMIC), of which the most feasible and informative measure in the medium-term (5–15 years) is type-specific HPV infection in sentinel populations of adolescent girls and young women. To this end, the International Agency for Research on Cancer (IARC) has developed long-term monitoring studies with the ministries of health of the two countries, beginning with two cervical cell surveys in the country capitals (Thimphu and Kigali) to establish robust baseline estimates of HPV prevalence among unvaccinated sexually active women aged 18-69. These studies have revealed a relatively high burden of HPV infection both in Bhutan[3] and Rwanda.[4]

However, to obtain earliest indicators of vaccine effectiveness, that is, how well it works in routine circumstances rather than in a randomized clinical trial, HPV prevalence

Key words: HPV vaccination, urine, HPV, effectiveness, Bhutan, Rwanda
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: International Agency for Research on Cancer; the Ministry of Health of Rwanda; and Bill & Melinda Gates Foundation (USA); **Grant number:** 35537 and OPP1053353
DOI: 10.1002/ijc.30092
History: Received 12 Oct 2015; Accepted 17 Feb 2016; Online 17 Mar 2016
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What's new?

The self-collection of urine for human papillomavirus (HPV) testing is a potential alternative to cervical sampling for the monitoring of HPV prevalence and HPV vaccination effectiveness in women. The present study indicates that urine surveys could be especially useful in Bhutan and Rwanda, which were among the first countries in Asia and Africa to implement school-based HPV vaccination programmes. First-void urine samples yielded evidence for lower prevalence of HPV types 6, 11, 16, and 18 in vaccinated versus unvaccinated female students ages 18 to 20. Women in this age group often are reluctant to undergo examination for cervical cytology.

should be measured in age groups of young women who may have initiated sexual activity but are often still reluctant to accept a gynaecological examination for the collection of cervical cells.[5] HPV testing from urine is therefore a possible solution to obtaining representative information in these women. A meta-analysis[6] showed that the sensitivity and specificity of HPV detection in urine compared to the cervix were, respectively, 87 and 94% for any HPV type and 73 and 98% for HPV16 and 18. Sensitivity is substantially improved by using first-void urine rather than random or mid-stream samples[6] and by taking some precautions in sample collection and DNA retrieval.[7]

On account of the relatively high rates of secondary school attendance in Bhutan and Rwanda,[8] we initiated a series of repeated HPV urine surveys among female high-school students to monitor changes in the prevalence of HPV types. The present report describes HPV prevalence in the first of these urine surveys in each country, and most notably, according to history of HPV vaccination. These surveys targeted mainly 18 to 20 year-old female students, a large fraction of whom had already been reached by the catch-up HPV vaccination programme, especially in Bhutan.

Methods

Study population

We aimed to recruit approximately 1,000 18–20 year-old female students in each country. In Bhutan, all secondary schools in Thimphu (five), and one in the nearby town of Paro, were included, of which two were public and four private. In Rwanda (where average school size was much smaller) 22 secondary schools in Nyarugenge district, Kigali, had to be included (8 public and 14 private). Most private schools in Rwanda, but none in Bhutan, had a religious affiliation. Participation was never refused by school authorities or teachers and both gave full support to study logistics.

The survey was undertaken over one month in Bhutan (September 2013) and 14 months (April 2013 to May 2014) in Rwanda. Students in the targeted age groups were invited by school staff to attend study recruitment meetings held during or after school hours. Potential participants were informed about and could raise questions on the study. All students who signed an informed consent form received a device for self-collection of urine (see below). The importance of collecting first-void urine from the first urination of the day, as well as returning the urine sample on same day as collection, was stressed. The large majority of students present at recruitment meetings signed the informed consent form, but exact denominators of students by year of age in each school were not available. A few students younger than 18 or older than 20 years also attended recruitment meetings and were allowed to join the study.

On the day following the recruitment meeting, urine samples were recovered at school entry (see below), and a short questionnaire was filled in directly by the student (Bhutan) or *via* a study interviewer (Rwanda) using an *ad hoc* created portable electronic device. The questionnaire included information on places of birth and living, history of sexual intercourse and HPV vaccination. Urine samples and questionnaires could only be matched through an anonymised identification number.

Urine collection and DNA extraction

Participants self-collected a urine sample using a device (Colli Pee^{TM}, Novosanis) designed to collect the first 14 ml of first-void urine immediately into 7 ml of a urine-conservation medium to avoid DNA degradation,[7] and to allow subsequent urine volume to exit the device into the toilet. Upon arrival at school on the morning of sample taking, urine samples were promptly cooled, transported to the central laboratory and stored at -20° C until shipment to IARC in cold boxes with ice packs. Subsequently, samples were shipped on dry ice to the Centre for the Evaluation of Vaccination, University of Antwerp, Belgium, where DNA extraction was performed as described elsewhere.[7] Briefly, in order to concentrate all DNA, including cell free DNA fragments, 4 ml of urine sample was centrifuged at 4,000*g* for 20 mins in an Amicon Ultra-4 50 K filter device (Merck Millipore, Belgium). Centrifugation was repeated for 10 min if remaining volume on the filter was >1 ml. After filtration, 2 ml of NucliSENS Lysis Buffer (BioMérieux, Benelux) was added to the concentrate retained on the filter and incubated for 10 min at room temperature. All material was subsequently transferred to the NucliSENS Lysis Buffer vial and DNA extraction was performed using the generic easyMAG off-board lysis protocol. DNA was eluted in 55 µl of elution buffer and DNA extracts were shipped back to IARC on dry ice.

HPV testing and genotyping

Two different testing methods of different analytical sensitivity were used for HPV testing to try to allow us to compare

possibly different estimates of HPV vaccine effectiveness by assay and overcome the possible problem of the relative lack of sensitivity of HPV detection in urine.

In the Department of Pathology at the VU University Medical Centre, Amsterdam, β-globin polymerase chain reaction (PCR) analysis was conducted to confirm the presence of human DNA in all specimens[9] and a general primer GP5+/6+ -mediated PCR was used to amplify HPV DNA.[10] HPV positivity was assessed by hybridization of PCR products in an enzyme immunoassay with two oligoprobe cocktails that, together, detect the following 44 mucosal HPV types: HPV6, 11, 16, 18, 26, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 85, 86, 89 and 90. Subsequent HPV genotyping was conducted by reverse-line blot hybridization of GP5+/6+ PCR products as described before.[11] Nine HPV types detectable by GP5+/6+ were never found and a few infections were classified as low or high risk of uncharacterized type (Appendix Tables A1 and A2).

A second type specific E7 PCR bead-based multiplex genotyping assay (henceforth referred to as E7-MPG) was performed in the Infections Section laboratory at IARC, Lyon using a Luminex bead-based platform.[12,13] The assay detects DNA from 21 HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82) (Appendix Tables A1 and A2) and from *Chlamydia trachomatis*.[14] Two β-globin primers are included to control DNA quality. The assay has been validated,[15] and is known to be more sensitive than GP5+/6+ in detecting low viral copy numbers and, in particular, in detecting individual HPV types in multiple-type infections.[12]

Statistical analyses

Ninety-five percent confidence intervals (CI) for HPV prevalence were computed assuming a binomial distribution. All prevalence ratios (PR) for HPV detection and corresponding 95% CIs were computed using binomial regression models with a log link and adjusted for age group ($\leq$18, 19 and $\geq$20 years) and sexual intercourse (never or ever). In particular, we assessed PRs for any HPV type; the combinations of vaccine types (HPV6/11/16/18); the combination of 10 HPV types that were detectable by both assays and belong, like HPV16 and 18, to α9- or α7-species[16] (HPV31/33/35/39/45/52/58/59/68/70); and all other types detectable by either test. The heterogeneity of PRs for HPV positivity among vaccinated and unvaccinated women was evaluated using the Wald χ^2_1 test, but results are not reported on account of the lack of significant or meaningful differences. HPV vaccine effectiveness was computed as 1-PR and the corresponding 95% CIs.

Ethical approval

The present studies had the approval of both the Research Ethical Board of the Ministries of Health of Bhutan and Rwanda and the IARC Ethics Committee.

Results

A total of 1,148 students in Bhutan and 1,130 in Rwanda signed a consent form, but for 84 and 132 students, respectively, a urine sample was not returned, and in 24 and 73 students, β-globin was undetectable by either one or both HPV assays (Fig. 1). A few additional exclusions were due to insufficient urine volume for the second test (E7-MPG), lack of questionnaire or students' uncertainty about HPV vaccination history. Hence a total of 973 and 912 students were included from Bhutan (median age: 19 years, 5–95 percentile: 18–22) and Rwanda (median age 19 years, 5–95 percentile: 17–20), respectively.

Ninety-two percent of students in Bhutan and 43.1% in Rwanda reported to have been vaccinated against HPV and median age at vaccination was 16 (5–95 percentile= 14–18) in both countries. The distribution of students by age group, and selected characteristics, is shown in Table 1, separately by vaccination history. In both countries, vaccinated students were significantly younger than unvaccinated students ($p < 0.001$). Being born in the capital was positively associated with HPV vaccination in Bhutan ($p = 0.031$) but negatively associated in Rwanda ($p = 0.030$). Vaccination status was unrelated to place of living, to history of sexual intercourse (10.3% in Bhutan and 19.3% in Rwanda), and to detection of *Chlamydia trachomatis*. *Chlamydia trachomatis* detection differed substantially by sexual history: prevalence was 8.8% in Bhutan and 6.3% in Rwanda among women who reported sexual intercourse and 2.8 and 1.1%, respectively, among those reported no intercourse (data not shown).

Table 2 shows the prevalence of HPV (any type) according to selected characteristics in Bhutan, separately by HPV assay. Overall HPV prevalence was 9.0% (95% CI: 7.3–11.0) by GP5+/GP6+ and 11.1% (95% CI: 9.2–13.2) by E7-MPG, that included however much fewer types than GP5+/GP6+. Associations with HPV positivity were similar, irrespective of HPV test, namely strong associations with history of sexual intercourse (PR= 4.19; 95% CI: 2.81–6.25 by GP5+/GP6+ and 3.51; 95% CI: 2.43–5.07, by E7-MPG), and *Chlamydia trachomatis* detection (PR> 6 according to both HPV assays). No significant differences were found by vaccination history (PR = 0.88; 95% CI: 0.47–1.65 by GP5+/GP6+ and 0.78; 95% CI: 0.44–1.37 by E7-MPG), age, nor place of birth or living (Table 2).

Table 3 shows corresponding findings for Rwanda. Overall HPV prevalence was 12.2% (95% CI: 10.1–14.5) by GP5+/GP6+ and 17.1% (95% CI: 14.7–19.7) by E7-MPG. Associations with HPV positivity were found for history of sexual intercourse (PR = 4.40; 95% CI: 3.11–6.23, by GP5+/GP6+ and 2.46; 95% CI: 1.85–3.27 by E7-MPG) and Chlamydia trachomatis (PR = 3.02; 95% CI: 2.12–4.30 by GP5+/GP6+ and 2.23; 95% CI: 1.49–3.36 by E7-MPG), whilst age and place of birth or living were unrelated. Overall, HPV prevalence in Rwanda was significantly lower in vaccinated than in

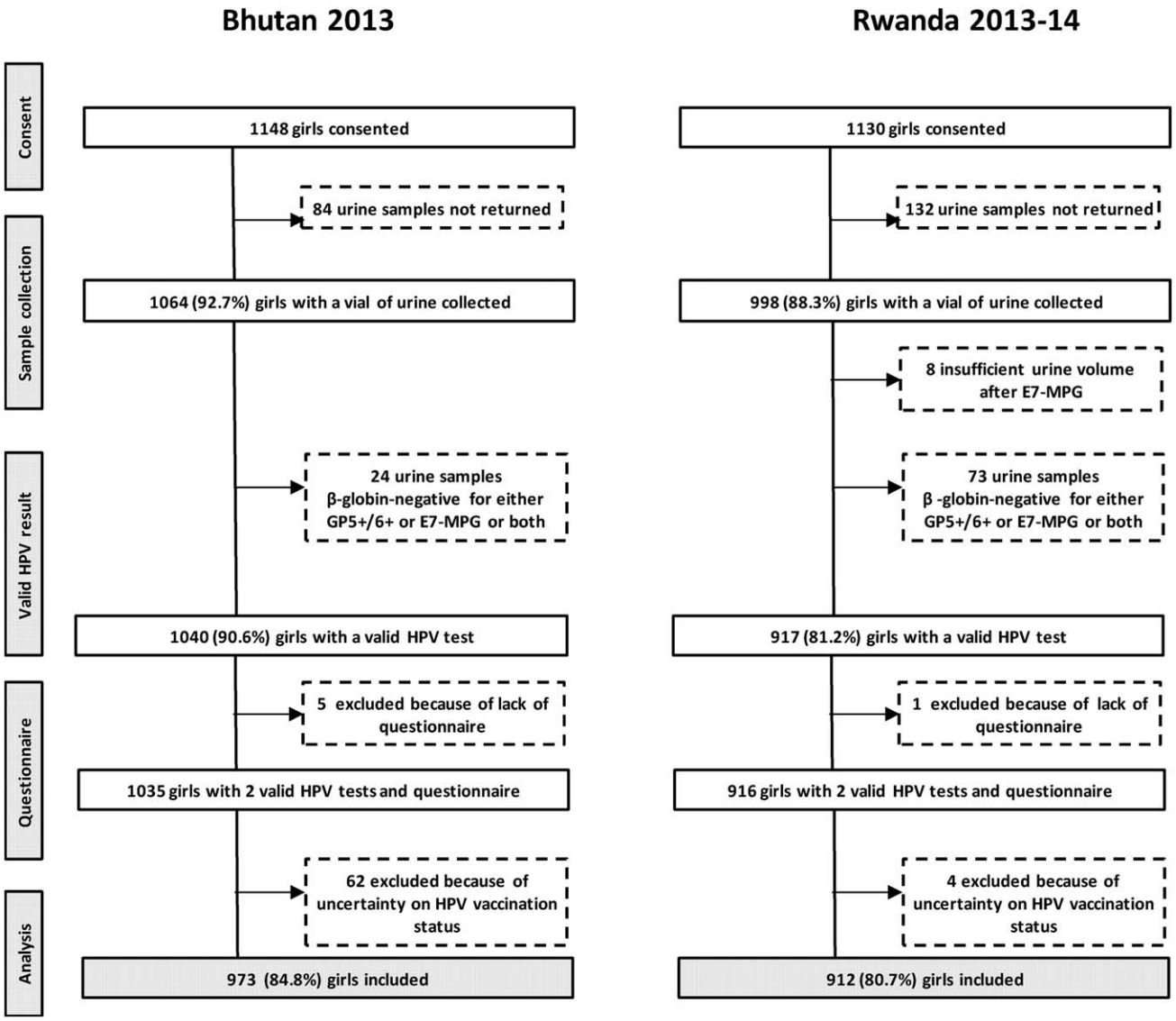

Figure 1. Study flow-chart, Bhutan and Rwanda, 2013–14.

unvaccinated students by E7-MPG (PR = 0.68; 95% CI: 0.50–0.92), but was not significantly different by GP5+/GP6+ (Table 3). In both countries, vaccination preceded sexual intercourse in the majority of students for whom the relevant information was self-reported (data not shown).

Table 4 shows prevalence and PRs for the combination of HPV6/11/16/18, HPV31/33/35/39/45/52/58/59/67/68/70, and other types by vaccination history. In Bhutan, vaccine type prevalence was lower in vaccinated (0.7%) than unvaccinated women (2.6%) by GP5+/GP6+, but the difference did not reach statistical significance (PR = 0.32; 95% CI: 0.06–1.64). This difference was even less clear for E7-MPG (PR = 0.86; 0.11–6.77). PRs for the other two combinations of HPV types were all close to unity and 95% CIs were broad, irrespective of the test.

In Rwanda, HPV6/11/16/18 prevalence was significantly lower in vaccinated than in unvaccinated women (PR = 0.12, 95%CI: 0.03–0.51 by GP5+/6+ and 0.45; 95% CI: 0.23–0.90 by E7-MPG). For HPV31/33/35/39/45/52/58/59/67/68/70 and other types, no statistically significant differences were observed for either GP5+/6+ or E7-MPG. However, for E7-MPG, we observed a decrease of borderline statistical significance in the prevalence of types related to HPV16 or 18 among vaccinated students (PR = 0.68; 95% CI: 0.45–1.01) (Table 4). Analyses in Table 4 were repeated for HPV16/18 reaching findings similar to those for the four vaccine types. PRs for HPV16/18 were 0.32 (0.06–1.64) for GP5+/6+ and 0.64 (0.08–5.20) for E7-MPG in Bhutan, and 0.17 (0.04–0.74) for GP5+/6+ and 0.54 (0.25–1.18) for E7-MPG in Rwanda (data not shown).

Prevalence of individual HPV types in Bhutan and Rwanda according to GP5+/GP6+ and E7-MPG are shown in detail in Appendix Tables A1 and A2, respectively. Most HPV types were more frequently detected in Rwanda than in Bhutan and by E7-MPG than GP5+/GP6+.

Table 1. Comparison of human papillomavirus (HPV) vaccinated and unvaccinated female students by selected characteristics in Bhutan and Rwanda

	Bhutan		Rwanda	
	Vaccinated *N* (%)	Unvaccinated *N* (%)	Vaccinated *N* (%)	Unvaccinated *N* (%)
All	896	77	393	519
Age-group (years)				
≤17	14 (1.6)	1 (1.3)	47 (12.0)	16 (3.1)
18	261 (29.1)	9 (11.7)	164 (41.7)	147 (28.3)
19	319 (35.6)	18 (23.4)	82 (20.9)	192 (37.0)
20	180 (20.1)	14 (18.2)	99 (25.2)	147 (28.3)
≥21	122 (13.6)	35 (45.5)	1 (0.3)	17 (3.3)
χ^2_4	$p<0.001$		$p<0.001$	
Place of birth				
Thimphu/Kigali	293 (32.7)	16 (20.8)	198 (50.4)	299 (57.6)
Outside Thimphu/Kigali	602 (67.3)	61 (79.2)	195 (49.6)	220 (42.4)
χ^2_1	$p=0.031$		$p=0.030$	
Place of living				
With family/relative[1]	733 (81.8)	65 (84.4)	336 (85.5)	427 (82.3)
Boarding school	163 (18.2)	12 (15.6)	57 (14.5)	92 (17.7)
χ^2_1	$p=0.568$		$p=0.192$	
History of sexual intercourse				
Never	804 (89.7)	67 (87.0)	317 (80.7)	403 (77.7)
Ever/Prefer not to answer[2]	92 (10.3)	10 (13.0)	76 (19.3)	116 (22.4)
χ^2_1	$p=0.455$		$p=0.269$	
Chlamydia trachomatis[3]				
Negative	866 (96.7)	74 (96.1)	387 (98.5)	505 (97.3)
Positive	30 (3.4)	3 (3.9)	6 (1.5)	14 (2.7)
χ^2_1	$p=0.799$		$p=0.232$	

[1]Includes 20 girls in Bhutan and 11 in Rwanda who reported living "Alone/with friends."
[2]Includes 43 (Bhutan) and 4 (Rwanda) students who preferred not to answer this question.
[3]Detected by E7-MPG.

Discussion

The present school-based study is the first evaluation of the feasibility and outcome of urine surveys to monitor HPV prevalence in LMICs successfully initiating national HPV vaccination programmes. It clearly demonstrates the effectiveness of the quadrivalent vaccine against HPV6/11/16/18 in Rwanda, and possibly some weak cross-protection against HPV types that are phylogenetically close to HPV16 or 18. Findings from Bhutan are compatible with those from Rwanda but not statistically significant due to the small number of unvaccinated students.

The effectiveness of catch-up HPV vaccination against HPV6/11/16/18 infection in Rwanda is estimated as 88% (95% CI: 49–97) according to a clinically validated HPV assay (GP5+/6+), and 55% (95% CI: 10–77) according to an assay with a higher analytical sensitivity (E7-MPG). Our findings are similar to those that have been reported in by-intention-to treat analyses from the community-based Costa Rican trial of the bivalent vaccine, that is, 69% (95% CI: 53–80%) efficacy against HPV16/18 among 18–19 year-old women.[17] The favourable effect of catch-up HPV vaccination against vaccine types in our study is also consistent with the findings of a recent meta-analysis on nine high-resource countries in which national HPV vaccination programmes have included catch-up campaigns.[18] Two studies in the meta-analysis were based in total[19] or in part[20] on urine samples. The pooled population-based PR for HPV16/18 infection in post- versus pre-vaccination period was 0.36 (95% CI: 0.25–0.53) in girls aged 13–19 and 0.69 (95% CI: 0.47–1.01) in women aged 20–25.[18] In countries with high-coverage, there was also evidence for herd immunity in the post-vaccination period.[18] Indeed, herd immunity may have already reduced HPV6/11/16/18 among unvaccinated women in the current surveys, especially in Bhutan, and would have the effect of under-estimating vaccine effectiveness.

In Rwanda, we found some clues that the quadrivalent vaccine may also provide some cross-protection against a

Table 2. Prevalence ratios (PR) for human papillomavirus (HPV) positivity and corresponding 95% confidence intervals (CI) according to selected characteristics among 973 female students from Bhutan

		GP5+/6+[1]		E7-MPG[2]	
Characteristic	***N***	**HPV-positive N (%)**	**Adjusted PR (95% CI)[3]**	**HPV-positive *N* (%)**	**Adjusted PR (95% CI)[3]**
All	973	88 (9.0)		108 (11.1)	
Age-group (years)					
≤18	285	19 (6.7)	1	27 (9.5)	1
19	337	32 (9.5)	1.50 (0.88–2.55)	40 (11.9)	1.34 (0.85–2.10)
≥20	351	37 (10.5)	1.37 (0.82–2.31)	41 (11.7)	1.14 (0.73–1.78)
χ^2_2 for trend			*p = 0.284*		*p = 0.654*
Place of birth					
Thimphu	309	28 (9.1)	1	36 (11.7)	1
Outside Thimphu	663	60 (9.1)	1.04 (0.68–1.59)	72 (10.9)	1.01 (0.70–1.48)
Place of living					
With family/relative	798	72 (9.0)	1	84 (10.5)	1
Boarding school	175	16 (9.1)	0.96 (0.58–1.59)	24 (13.7)	1.21 (0.80–1.82)
History of sexual intercourse					
Never	871	59 (6.8)	1	77 (8.8)	1
Ever/Prefer not to answer	102	29 (28.4)	4.19 (2.81–6.25)	31 (30.4)	3.51 (2.43–5.07)
***Chlamydia trachomatis*[4,5]**					
Negative	940	69 (7.3)	1	89 (9.5)	1
Positive	33	19 (57.6)	7.65 (5.21–11.2)	19 (57.6)	6.05 (4.22–8.67)
HPV vaccination					
No	77	9 (11.7)	1	11 (14.3)	1
Yes	896	79 (8.8)	0.88 (0.47–1.65)	97 (10.8)	0.78 (0.44–1.37)

[1]44 HPV types are detectable by GP5+/GP6+ (see Methods).
[2]21 HPV types are detectable by E7-MPG (see Methods).
[3]Adjusted for age and history of sexual intercourse as appropriate.
[4]Detected by E7-MPG.
[5]Adjusted for age only due to lack of convergence when history of sexual intercourse was included.

combination of types (HPV31/33/35/39/45/52/58/59/67/68/70) that were chosen *a priori* because they belong to the same species as HPV16 and 18. Our estimates of cross-protection (approximately 30% by both HPV assay) are consistent with the findings in FUTURE I and FUTURE II trials among 16–26 year-old women, in whom the efficacy of the quadrivalent vaccine against 6-month persistent infection from HPV31/33/45/52/58 was 25% (95% CI: 5–41) in initially HPV-negative women[21] and 18% (95% CI: 5–29) in an intention-to-treat analysis.[22] For overall HPV prevalence and prevalence of types other than HPV16, 18, and related types, GP5+/6+ showed PRs closer to unity than E7-MPG probably because the assay included more types that are not expected to be affected by the quadrivalent vaccine.

Ideally, urine HPV surveys should have been put in place before the implementation of HPV vaccination but the very rapid initiation of catch-up campaigns in both countries prohibited such an approach. Our surveys allow, however, an estimation of the effectiveness of HPV vaccination after pre-adolescence by self-reported vaccination history. Surveys were performed three years (Bhutan) and one year (Rwanda) after the end of high-coverage catch-up campaigns. This means that women up to age 21 in Bhutan[1] had a high probability of having been vaccinated, and the percentage of vaccinated students in our urine survey is consistent with the reported 90% coverage.[1] In Rwanda, where a school grade-based approach was used,[2] substantial age variation within school grades, as a result of the reconstruction of the education system during the last decades,[23] is the likeliest explanation for the relatively high level of vaccination coverage among women aged 18 years or older, notably those who were not born in the capital. Unfortunately, students could not accurately report the number of vaccine doses they had received but national data suggest excellent adherence to the 3-dose regimen once vaccination was initiated.[1,2]

Confidence in using urine samples to detect HPV is relatively recent. Systematic reviews have shown a fairly good concordance with cervical cells for HPV positivity in

Table 3. Prevalence ratios (PR) for human papillomavirus (HPV) positivity and corresponding 95% confidence intervals (CI) according to selected characteristics among 912 female students from Rwanda

		GP5+/6+[1]		E7-MPG[2]	
Characteristic	N	HPV-positive N (%)	Adjusted PR (95% CI)[3]	HPV-positive N (%)	Adjusted PR (95% CI)[3]
All	912	111 (12.2)		156 (17.1)	
Age-group (years)					
≤18	374	37 (9.9)	1	53 (14.2)	1
19	274	37 (13.5)	1.07 (0.71–1.61)	49 (17.9)	1.12 (0.79–1.59)
≥20	264	37 (14.0)	0.99 (0.65–1.50)	54 (20.5)	1.19 (0.84–1.68)
χ^2_2 for trend			*p=0.942*		*p=0.324*
Place of birth					
Kigali	497	63 (12.7)	1	91 (18.3)	1
Outside Kigali	415	48 (11.6)	1.01 (0.72–1.41)	65 (15.7)	0.88 (0.66–1.18)
Place of living					
With family/relative	763	97 (12.7)	1	135 (17.7)	1
Boarding school	149	14 (9.4)	0.94 (0.56–1.58)	21 (14.1)	0.89 (0.58–1.36)
History of sexual intercourse					
Never	720	51 (7.1)	1	93 (12.9)	1
Ever/Prefer not to answer	192	60 (31.3)	4.40 (3.11–6.23)	63 (32.8)	2.46 (1.85–3.27)
***Chlamydia trachomatis*[4]**					
Negative	892	98 (11.0)	1	145 (16.3)	1
Positive	20	13 (65.0)	3.02 (2.12–4.30)	11 (55.0)	2.23 (1.49–3.36)
HPV vaccination					
No	519	69 (13.3)	1	105 (20.2)	1
Yes	393	42 (10.7)	0.87 (0.61–1.24)	51 (13.0)	0.68 (0.50–0.92)

[1]44 HPV types are detectable by GP5+/GP6+ (see Methods).
[2]21 HPV types are detectable by E7-MPG (see Methods).
[3]Adjusted for age and history of sexual intercourse as appropriate.
[4]Detected by E7-MPG.

women[6,24–26] and concluded that urine may be a useful alternative to cervical samples to monitor HPV changes in the post-HPV vaccination era.[6,24–26] Theories of the existence of PCR inhibitors in urine have been dispelled, with the need to avoid rapid DNA degradation and improve HPV DNA yield proven to be more important.[7] Essential steps were therefore made in our surveys to standardize and improve the sensitivity of HPV detection in urine.[7,27] These include: (*i*) collection of first-void urine; (*ii*) avoidance of DNA degradation through the use of urine-conservation medium and buffer in both urine collection and processing; (*iii*) sufficient volume of urine to allow subsequent sample concentration; and (*iv*) recovery of cell-free HPV DNA in addition to cell-associated DNA.[7] Few samples had to be excluded because of β-globin-negativity and the meaningfulness of urine samples for HPV is supported by consistent associations with history of sexual intercourse and *Chlamydia trachomatis* detection in our study, irrespective of HPV assay or country.

Comparisons with previous HPV urine surveys in asymptomatic adolescents and young women are hampered by variations in inclusion criteria and HPV testing protocols, notably use of mid-stream instead of first-void urine and lack of used preservatives.[28] An overall HPV prevalence in urine of 9–17% (depending upon the country and HPV test) in our study can be compared with 15% in 15–18 year-old female students in Scotland,[29] 4% in 15–18 year-old girls recruited in schools and sports centres in Italy,[30] or 11 and 19% among young women aged 13–17 and 18–25 years, respectively, in tribal populations in India.[28]

The mean age at first intercourse for young women in national surveys was reportedly 18 years in Bhutan[31] and 19 in Rwanda,[32] but a substantial fraction of female students in our urine surveys (median age = 19 years) declared not to have had sexual intercourse. Among them HPV prevalence in urine was 7–13%, depending upon country and HPV test. Under-reporting of sexual intercourse in this group cannot be excluded but the strong correlation with *Chlamydia trachomatis* is reassuring. In addition, only HPV infections on the cervix are probably transmitted exclusively sexually. As urine samples will detect HPV infections of the vulva and

Table 4. Prevalence ratios (PR) for positivity for human papillomavirus (HPV) 6/11/16/18 or 31/33/35/39/45/52/58/59/68/70 or other types (see Methods) and corresponding 95% confidence intervals (CI) according to HPV vaccination history in Bhutan and Rwanda

HPV Vaccination History	*N*	HPV6/11/16/18-pos	Adjusted PR (95% CI)[1]	HPV-31/33/35/39/45/52/58/59/68/70-pos[2]	Adjusted PR (95% CI)[1]	HPV-other pos	Adjusted PR (95% CI)[1]
Bhutan GP5+/6+[3]	973						
No	77	2 (2.6)	1	4 (5.2)	1	5 (6.5)	1
Yes	896	6 (0.7)	0.32 (0.06–1.64)	45 (5.0)	1.09 (0.41–2.91)	53 (5.9)	0.98 (0.40–2.37)
Bhutan E7-MPG[4]	973						
No	77	1 (1.3)	1	5 (6.5)	1	6 (7.8)	1
Yes	896	11 (1.2)	0.86 (0.11–6.77)	60 (6.7)	1.13 (0.47–2.69)	49 (5.5)	0.73 (0.32–1.66)
Rwanda GP5+/6+[3]	912						
No	519	21 (4.1)	1	28 (5.4)	1	43 (8.3)	1
Yes	393	2 (0.5)	0.12 (0.03–0.51)	15 (3.8)	0.70 (0.38–1.30)	30 (7.6)	1.07 (0.69–1.67)
Rwanda E7-MPG[4]	912						
No	519	33 (6.4)	1	64 (12.3)	1	48 (9.3)	1
Yes	393	11 (2.8)	0.45 (0.23–0.90)	32 (8.1)	0.68 (0.45–1.01)	26 (6.6)	0.72 (0.45–1.14)

[1]Adjusted for age and ever had sexual intercourse.
[2]HPV types belonging to the same species as HPV16 or 18.
[3]44 HPV types are detectable by GP5+/GP6+ (see Methods).
[4]21 HPV types are detectable by E7-MPG (see Methods).

vagina it is plausible that these infections may derive from intimate contacts other than penetrative intercourse or from non-sexual transmission through fingertips or fomites.

An important strength of our present study is represented by the excellent compliance, in both countries, of health and education authorities, female students and their families. This strong societal commitment to HPV vaccination allowed us to have almost comprehensive participation of female students in the targeted study areas. Selection bias should have been attenuated by the good access to secondary education in Bhutan (79% of females in 2011)[8] and steady improvements in Rwanda,[33] and also by the concentration of secondary education establishments in the two capitals. The presence of a well-developed primary care network in both countries is known to overcome to a large extent differences in the access to vaccination by social class.[1,2]

In addition, we have been able to put into place recent improvements in the management of urine samples[7] and to compare findings from two high-quality HPV assays that differ in respect to sensitivity and number of detectable HPV types. Whether a high analytical sensitivity of HPV testing in urine surveys is an advantage or a danger for monitoring the impact of HPV vaccine remains unclear. The use of a highly sensitive assay such as E7-MPG may attenuate the problem of "unmasking," that is, the possibility that the elimination by vaccination of targeted HPV types may increase the apparent prevalence of other types that would have otherwise been undetectable by less sensitive assays such as GP5+/GP6+.[34] Better sensitivity for multiple infections may also improve the statistical power to assess cross-protection against non-vaccine types. On the other hand, estimates of vaccine effectiveness against HPV6/11/16/18 were lower using the more sensitive assay, possibly due to increased detection of low-level HPV DNA that may have no clinical significance. In the long-term, highly sensitive testing of urine may show HPV vaccination programmes with high coverage to eliminate even these low-level infections by interrupting transmission all over the anogenital tract.

In conclusion, our present findings, in combination with abundant data on cervical infection in sexually active women,[3,4] mark the beginning of the monitoring of HPV infection after the introduction of vaccination in Bhutan and Rwanda and support the feasibility of urine HPV surveys for this purpose. In the future, as vaccinated cohorts of women will predominantly represent those vaccinated in preadolescence, similar repeat urine assays can be expected to detect greater increases in HPV vaccine effectiveness than in our present study.

Acknowledgements

We are very grateful to the Ministries of Health and Education, school staff, and study participants from Bhutan and Rwanda. We also thank Mrs M. Mahirwe and E. Mudaheranwa, for field work in Rwanda; S. McKay Chopin and S. Biesmans, for skilful laboratory work; and V. Chabanis and S. Gamon for manuscript preparation. F. Lazzarato provided precious assistance with the programming and management of portable devices. The funding sources did not have any role in the writing process of this manuscript. PVD and AV are co-founders and board members of Novosanis, a spin-off company of the University of Antwerp. PS received speaker fees from Roche, Gen-Probe, Abbott, Qiagen, and Seegene, and has a minority stake in Self-screen B.V., a spin-off of VU University Medical Center. Other authors have no conflicts of interest to declare.

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