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Detection of Human Papillomavirus (HPV) and other Sexually Transmitted Pathogens in cervical and self-collected specimens of women referred to colposcopy

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Abstract: High-risk Human Papillomavirus (hrHPV) persistent infection is acknowledged as the primary cause of cervical cancer. Coinfection with other sexually transmitted infections (STIs) may be associated with a higher risk of cervical lesion progression. This study investigated the detection of hrHPV and 7 other STIs in matched clinician-collected cervical samples and self-taken vaginal and urine specimens of 345 asymptomatic women referred to colposcopy for abnormal cervical cytology. The association of sexually transmitted coinfections with cervical dysplasia was evaluated. Detection of hrHPV and 7 other sexually transmitted pathogens was carried out using Anyplex™II HR and Anyplex™II STI-7e, respectively. The positivity of hrHPV infections in cervical, vaginal and urine samples were respectively 67.0%, 71.3% and 68.1%, while STIs were detected in 47.9%, 57.9% and 56.4%. A good analytical agreement was observed between cervical and self-taken samples for hrHPV and STIs detection. STIs positivity rate was found to be higher in hrHPV-positive compared to hrHPV-negative women, but no association was found between STIs coinfections and the grade of cervical lesions. In conclusion, self-collected specimens proved to be a valid non-invasive alternative to cervical samples to detect hrHPV and STIs. Longitudinal studies are required to evaluate the role of STIs coinfections in lesions progression.

Keywords: High-risk Human Papillomavirus (hrHPV); Sexually transmitted infections (STIs); Self-sampling

1. Introduction

Cervical cancer represents a serious threat to women's health globally, with an age-standardized incidence rate of 14.1 per 100,000 women. It is the fourth most common cancer among women, with 661,000 new cases and 348,000 deaths reported in 2022, according to GLOBOCAN data [1]. Persistent infection with high-risk Human Papillomavirus (hrHPV) is widely acknowledged as the primary cause of cervical cancer.

To date, nearly 200 different HPV types have been identified, including twelve classified by the International Agency for Research on Cancer (IARC) as oncogenic or "high-risk" HPV types belonging to the Group 1 (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), with HPV 16 and HPV 18 being the two most common genotypes associated with cervical cancer development [2]. Although HPV infections are widespread among the female population, most of them are transient and spontaneously cleared by the host. Persistent infection with one of the carcinogenic hrHPV types is responsible for the onset of cervical precancer and cancer [3]. Several factors, such as sexual habits, smoking, high parity, long-term use of oral contraceptives and the presence of other sexually

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transmitted infections (STIs), may increase the risk of cervical dysplasia progression in HPV-positive women.

Coinfections of hrHPV with other sexually transmitted pathogens has been proposed as a risk factor for the development of cervical cancer in HPV-positive women but thier role is controversial and requires to be further investigated [4,5]. Interactions between HPV and other sexually transmitted pathogens that share similar anatomical sites could accelerate cervical lesions progression, enhancing HPV replication and persistence of infection [6].

Although STIs affect both sexes, women are at greater risk due to the anatomy of their reproductive system [7]. While the treatment of symptomatic STIs is effective, many asymptomatic infections are overlooked [8]. The failure to diagnose and treat asymptomatic STIs is responsible for persistence and/or dissemination of infections to sexual contacts. The diagnosis of STIs usually requires screening and/or diagnostic procedures, which may be difficult due to the social stigma associated with this kind of infections, to the limited access to healthcare, particularly in low-middle income countries, and to the invasive nature of the diagnostic procedures [9,10]. The delayed diagnosis and treatment of STIs often increases the risk of long-term health complications, including pelvic inflammatory disease (PID) and infertility in women [11].

Self-samples are currently being used in cervical cancer screening programs worldwide [12]. The accuracy of PCR-based HPV assays on self-collected samples has been demonstrated to be similar to that of clinician-collected cervical specimens [13,14]. Recently, the VALHUDES (**Val**idation of **Hu**man Papillomavirus Assays and Collection **De**vices for **S**elf-samples and Urine Samples) protocol has been developed to evaluate the clinical accuracy of HPV tests in combination with collection devices for HPV testing on self-collected vaginal and urine samples in women referred to colposcopy following cervical cancer screening, in whom higher HPV positivity rates are expected [15].

Self-sampling, as non-invasive and easy-to-perform procedure, could also be applied to the diagnosis and management of other STIs, allowing women who feel uncomfortable with conventional clinical practices to access treatment [9]. Moreover, the use of self-collected samples may represent a more convenient way to screen women for both hrHPV and other STIs from a single clinical sample. A recent review found that self-sampling increased the uptake of STIs testing services compared to samples collected by healthcare professionals [11].

The aim of the present study is to investigate the prevalence and the rates of coinfections of hrHPV genotypes and 7 other sexually transmitted pathogens (*Ureaplasma parvum (UP), Ureaplasma urealyticum (UU), Mycoplasma genitalium (MG), Mycoplasma hominis (MH), Chlamydia trachomatis (CT), Neisseria gonorrhoeae (NG), Trichomonas vaginalis (TV)*) in clinician-collected cervical samples, self-collected vaginal and urine specimens in women referred to colposcopy. In addition, we evaluated the distribution of STIs among hrHPV-positive and hrHPV-negative women and investigated the association of coinfections between hrHPV and other sexually transmitted pathogens with the severity of cervical dysplasia.

2. Results

2.1. Study Population

Three hundred and forty-five women with a prior abnormal Pap smear were recruited at the first colposcopy referral visit. Their median (interquartile range, IQR) age was 37 (29–46) years. Supplementary Table 1 shows the age distribution of the study population, most of the women (31.3%) were aged 30-40.

Cytology results show that 47.5% of women had low-grade squamous intraepithelial lesions (LSIL), followed by those with atypical squamous cells of undetermined significance (ASCUS) (24.9%) and high-grade squamous intraepithelial lesion (HSIL) (13.6%). 127 women were positive on colposcopic examination, while 218 patients tested negative. Patients underwent cervical biopsy and/or treatment with conization according to clinical judgement and to local clinical protocols. Histological results showed 4 cases of cervical cancer: 2 cases of squamous cell carcinomas, one of adenocarcinoma “in situ” and one cervical carcinoma with dual histological components (Table 1).

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Table 1. Clinical data of the study group.

Cytology (n = 345)	**N**	**%**
HSIL	47	13.6%
ASCH	26	7.5%
LSIL	164	47.5%
ASCUS	86	24.9%
AGC	14	4.1%
NILM	8	2.3%
Colposcopy (n = 345)		
POS	127	36.8%
NEG	218	63.2%
Histological outcome (n = 84)		
Negative	11	13.1%
CIN 1	13	15.5%
CIN 2	12	14.3%
CIN 3	44	52.4%
Cervical cancer	4	4.8%

HSIL (High-grade squamous intraepithelial lesion); ASCH (Atypical squamous cells—cannot exclude HSIL); LSIL (Low-grade squamous intraepithelial lesion); ASCUS (Atypical squamous cells of undetermined significance); AGC (Atypical glandular cells); NILM (Negative for intraepithelial lesion or malignancy); POS (Positive); NEG (Negative); CIN 1 (Cervical intraepithelial neoplasia grade 1); CIN 2 (Cervical intraepithelial neoplasia grade 2); CIN 3 (Cervical intraepithelial neoplasia grade 3).

2.2. HPV Detection and Genotyping

Among the study population (n = 345), three women were excluded from the analysis because one of their matched samples tested invalid twice. As a result, 342 women were included in the following analysis.

The prevalence of hrHPV, in this cohort of women referred to colposcopy for abnormal cervical cytology, was 67.0% (229/342), 71.3% (244/342), and 68.1% (233/342) in cervical, vaginal, and urine samples, respectively, with HPV16 and HPV31 being the most common types, followed by HPV58 and HPV66 (Figure 1).

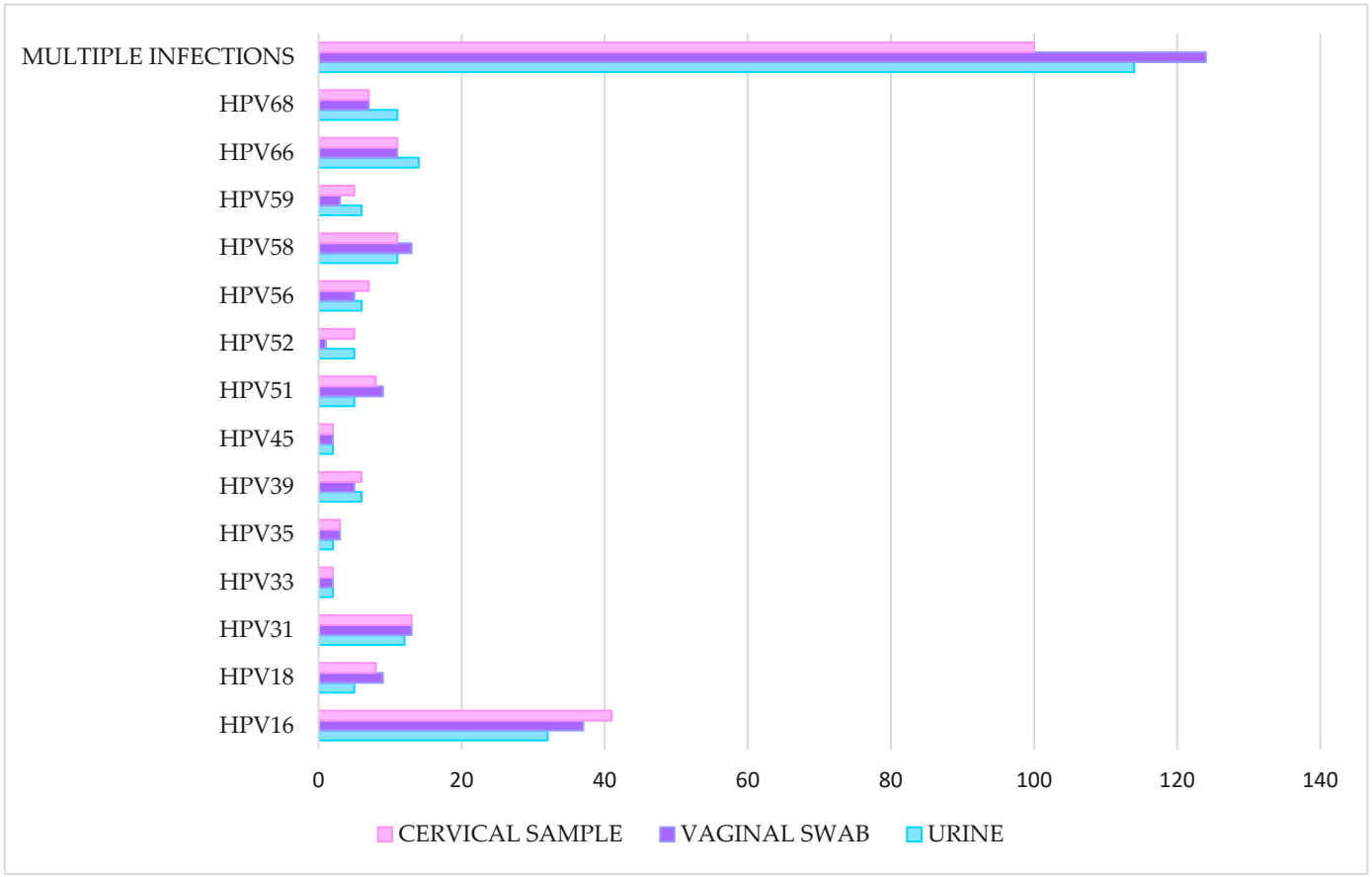

Figure 1. Prevalence of hrHPV genotypes in different samples.

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In total, 56.3% (129/229), 49.2% (120/244) and 51.1% (119/233) of the women showed single infection with one hrHPV type in cervical swab, vaginal self-sample and urine, respectively, whereas multiple hrHPV infections were detected in 43.7% (100/229), 50.8% (124/244) and 48.9% (114/233) of patients in cervical sample, vaginal swab and urine, respectively (Table 2).

Table 2. Single and multiple infections caused by hrHPV.

	Cervical sample n. (%) n = 229	**Vaginal swab n. (%) n = 244**	**Urine n. (%) n = 233**
Single hrHPV	129 (56.3%)	120 (49.2%)	119 (51.1%)
2 hrHPV	58 (25.3%)	77 (31.6%)	65 (27.9%)
3 hrHPV	30 (13.1%)	24 (9.8%)	27 (11.6%)
More than 3 hrHPV	12 (5.2%)	23 (9.4%)	22 (9.4%)

A good/substantial agreement was observed between cervical and self-taken samples in detecting hrHPV (κ = 0.870 and κ = 0.773 for vaginal and urine specimens, respectively) (Table 3 and Table 4).

Table 3. Type-specific agreement and test concordance between cervical and vaginal self-samples.

	HPV type	+/+[1]	+/-	-/+	-/-	**Agreement [%]**	**Kappa[2] [95% CI]**
Total population (n = 342)	hrHPV	227	2	17	96	94.4	0.870 (0.814 - 0.927)
	HPV16	79	1	7	255	97.7	0.936 (0.893 - 0.980)
	HPV18	18	0	4	320	98.8	0.894 (0.791 - 0.997)
	HPV31	44	2	9	287	96.8	0.870 (0.795 - 0.945)
	HPV33	10	5	3	324	97.7	0.702 (0.506 - 0.898)
	HPV35	7	0	4	331	98.8	0.772 (0.556 - 0.988)
	HPV39	19	1	2	320	99.1	0.922 (0.835 - 1.000)
	HPV45	10	2	5	325	98.0	0.730 (0.539 - 0.922)
	HPV51	26	0	10	306	97.1	0.823 (0.717 - 0.929)
	HPV52	27	3	3	309	98.2	0.890 (0.804 - 0.977)
	HPV56	25	0	4	313	98.8	0.920 (0.842 - 0.998)
	HPV58	24	2	8	308	97.1	0.812 (0.699 - 0.925)
	HPV59	19	0	4	319	98.8	0.899 (0.800 - 0.997)
	HPV66	33	1	8	300	97.4	0.865 (0.779 - 0.951)
	HPV68	27	2	8	305	97.1	0.828 (0.724 - 0.932)

Table 4. Type-specific agreement and test concordance between cervical and urine self-samples. .

	HPV type	+/+[1]	+/-	-/+	-/-	**Agreement [%]**	**Kappa[2] [95% CI]**
Total population (n = 342)	hrHPV	214	15	19	94	90.06	0.773 (0.701 - 0.845)
	HPV16	68	12	11	251	93.3	0.812 (0.738 - 0.886)
	HPV18	14	4	3	321	98.0	0.789 (0.638 - 0.941)
	HPV31	43	2	6	291	97.7	0.901 (0.834 - 0.969)
	HPV33	10	5	3	324	97.7	0.702 (0.506 - 0.898)
	HPV35	7	0	6	329	98.2	0.692 (0.459 - 0.924)
	HPV39	16	4	4	318	97.7	0.788 (0.645 - 0.930)
	HPV45	9	3	2	328	98.5	0.775 (0.584 - 0.966)
	HPV51	23	3	11	305	95.9	0.745 (0.617 - 0.872)
	HPV52	24	6	2	310	97.7	0.775 (0.584 - 0.966)
	HPV56	25	0	5	312	98.5	0.901 (0.816 - 0.987)
	HPV58	21	5	8	308	96.2	0.743 (0.609 - 0.877)
	HPV59	18	1	4	319	98.5	0.870 (0.758 - 0.982)
	HPV66	31	3	11	297	95.9	0.793 (0.689 - 0.897)
	HPV68	23	6	17	296	93.3	0.630 (0.492 - 0.769)

hr (high-risk); HPV (Human Papillomavirus); CI (confidence interval); n (number). [1] +/+ positive on self- and cervical samples, +/- positive only on cervical samples, -/+ positive only on self-samples, -/- negative on both sample types. [2] Kappa concordance between the self- and clinician-collected cervical samples is presented as

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follows: slight ($0.00 < \kappa < 0.20$), fair ($0.21 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$) and almost perfect ($0.81 < \kappa < 1.00$).

2.3. STIs Prevalence

47.9% (164/342), 57.9% (198/342), and 56.4% (193/342) of investigated women resulted positive for at least one of the seven STIs under investigation in their cervical, vaginal and urine samples, respectively.

UP was the most prevalent pathogen in all samples, followed by UU and MH. No women showed infection with NG. In general, for most of the sexually transmitted pathogens, the detection rate was lower in cervical swabs (Table 5 and Supplementary Figure 1), but statistically significant differences were found only for UP positivity rates in vaginal ($p< 0.01$) and urine ($p< 0.05$) specimens compared to cervical samples (Supplementary Tables S2 and S3).

The majority of women with positivity for the STIs panel were infected by a single pathogen in all sample types (Table 6).

Table 5. Distribution of STI pathogens in the three sample types.

	UP n (%)	***UU*** n (%)	***MH*** n (%)	***MG*** n (%)	***CT*** n (%)	***NG*** n (%)	***TV*** n (%)
Cervical sample (n = 342)	129 (37.7%)	34 (9.9%)	31 (9.1%)	8 (2.3%)	11 (3.2%)	0 (0%)	4 (1.2%)
Vaginal swab (n = 342)	165 (48.2%)	40 (11.7%)	40 (11.7%)	11 (3.2%)	13 (3.8%)	0 (0%)	3 (0.9%)
Urine (n = 342)	158 (46.2%)	38 (11.1%)	39 (11.4%)	10 (2.9%)	11 (3.2%)	0 (0%)	3 (0.9%)

Table 6. Single and multiple STI infections.

	Cervical sample n. (%) n = 164	**Vaginal swab n. (%) n = 198**	**Urine n. (%) n = 193**
Single STI	122 (74.4%)	143 (72.2%)	141 (73.1%)
2 STIs	32 (19.5%)	38 (19.2%)	39 (20.2%)
More than 2 STIs	10 (6.1%)	17 (8.6%)	13 (6.7%)

The agreement between cervical and self-taken samples for detecting STIs was found to be significant ($\kappa = 0.779$ and $\kappa = 0.738$ for vaginal and urine specimens, respectively), with almost perfect agreement between urine and vaginal swab specimens (95%, $k = 0.899$) (Table 7 and Table 8).

Table 7. Type-specific agreement and test concordance between cervical and vaginal self-samples.

	STI	+/+[1]	+/-	-/+	-/-	**Agreement [%]**	**Kappa[2] [95% CI]**
Total population (n = 342)	STI	162	2	36	142	88.9	0.779 (0.714 - 0.844)
	UP	127	2	38	175	88.3	0.764 (0.697 - 0.831)
	UU	30	4	10	298	95.9	0.788 (0.681 - 0.895)
	MH	31	0	9	302	97.4	0.859 (0.769 - 0.949)
	MG	8	0	3	331	99.1	0.838 (0.657 - 1.000)
	CT	11	0	2	329	99.4	0.914 (0.795 - 1.000)
	NG	0	0	0	342	100	Not Applicable
	TV	3	1	0	338	99.7	0.856 (0.576 - 1.000)

Table 8. Type-specific agreement and test concordance between cervical and urine self-samples.

	STI	+/+[1]	+/-	-/+	-/-	**Agreement [%]**	**Kappa[2] [95% CI]**
Total population	STI	156	8	37	141	86.8	0.738 (0.668 - 0.808)
	UP	121	8	37	176	86.8	0.732 (0.660 - 0.804)
	UU	26	8	12	296	94.1	0.690 (0.562 - 0.817)
	MH	27	4	12	299	95.3	0.746 (0.627 - 0.864)
	MG	8	0	2	332	99.4	0.886 (0.729 - 1.000)

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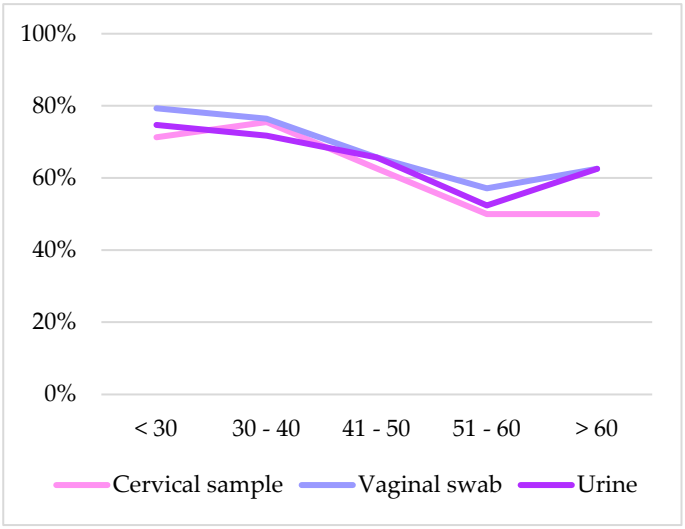
(n = 342)	*CT*	9	2	2	329	98.8	0.812 (0.632 - 0.992)
	NG	0	0	0	342	100	Not Applicable
	TV	3	1	0	338	99.7	0.856 (0.576 - 1.000)

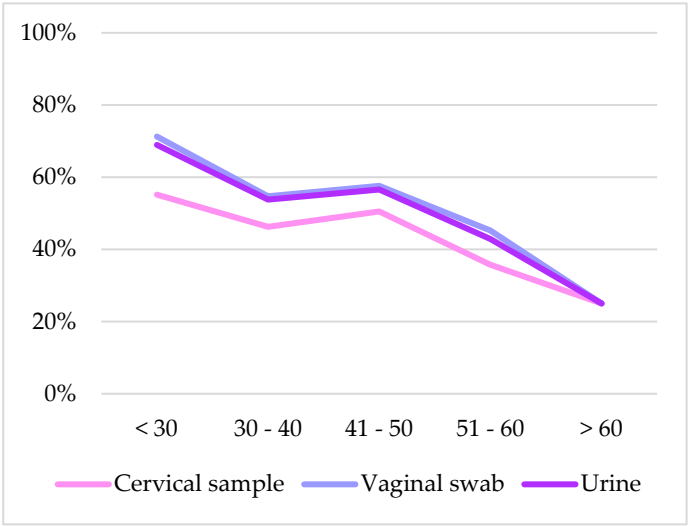
STI (sexually transmitted infection); CT (Chlamydia trachomatis), NG (Neisseria gonorrhoeae), TV (Trichomonas vaginalis), MH (Mycoplasma hominis), MG (Mycoplasma genitalium), UU (Ureaplasma urealyticum) and UP (Ureaplasma parvum); CI (confidence interval); n (number). [1] +/+ positive on self- and cervical samples, +/- positive only on cervical samples, -/+ positive only on self-samples, -/- negative on both sample types. [2] Kappa concordance between the self- and clinician-collected cervical samples is presented as follows: slight ($0.00 < \kappa < 0.20$), fair ($0.21 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$) and almost perfect ($0.81 < \kappa < 1.00$).

2.4. hrHPV and STIs Prevalence in the Different Age Groups

Figure 2 shows the comparison between hrHPV and STIs prevalence in cervical, vaginal and urine samples across the different age groups of women referred to colposcopy for abnormal cervical cytology. hrHPV prevalence was higher in the younger age groups in all sample types. In cervical samples, the highest hrHPV prevalence (75.5%) was observed in the 30-40 age group of women.

Younger women had also higher prevalence of STIs. In the <30 years of age group 55.2% of cervical samples, 71.3% of vaginal self-samples and 69.0% of urine were STIs-positive. The STI rates decreased with age, with the lowest prevalence in the >60 years of group, where all sample types showed a prevalence of 25%.





(a) **(b)**

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Figure 2. Comparison of hrHPV (a) and STI (b) prevalence by age group.

2.5. hrHPV and STI Co-Infections

Table 9 shows the comparison between STIs prevalence in hrHPV-positive and hrHPV-negative women. For almost all the STIs investigated, the positivity rate among hrHPV-positive women was higher as compared to the hrHPV-negative group in all sample types. The difference was statistically significant for UP, MH in all sample types and for MG in vaginal swabs (Supplementary Table S4).

Table 9. Distribution of STI pathogens in hrHPV-positive and negative women in the three sample types. *p < 0.05; **p < 0.01; ***p < 0.001.

		UP		***UU***		***MH***		***MG***		***CT***		***NG***		***TV***	
		n (%)	*p*	n (%)	*p*	n (%)	*p*	n (%)	*p*	n (%)	*p*	n (%)	*p*	n (%)	*p*
Cervical samples	hrHPV-positive (n = 229)	102 (44.5%)	***	27 (11.8%)	0.15	29 (12.7%)	***	8 (3.5%)	0.06	10 (4.4%)	0.11	0 (0%)	1	3 (1.3%)	1
	hrHPV-negative (n = 113)	27 (23.9%)		7 (6.2%)		2 (1.8%)		0 (0%)		1 (0.9%)		0 (0%)		1 (0.9%)	
Vaginal swabs	hrHPV-positive (n = 244)	137 (56.1%)	***	32 (13.1%)	0.27	37 (15.2%)	**	11 (4.5%)	0.04*	12 (4.9%)	0.12	0 (0%)	1	2 (0.8%)	1
	hrHPV-negative (n = 98)	28 (28.6%)		8 (8.2%)		3 (3.1%)		0 (0%)		1 (1.0%)		0 (0%)		1 (1.0%)	
Urine	hrHPV-positive (n = 233)	125 (53.6%)	***	30 (12.9%)	0.18	34 (14.6%)	**	9 (3.9%)	0.18	10 (4.3%)	0.18	0 (0%)	1	2 (0.9%)	1
	hrHPV-negative (n = 109)	33 (30.3%)		8 (7.3%)		5 (4.6%)		1 (0.9%)		1 (0.9%)		0 (0%)		1 (0.9%)	

Table 10 shows the distribution of hrHPV infections in cervical samples according to the clinical data, while Table 11 reports the distribution of hrHPV and STIs infections in the same groups of women.

hrHPV infections showed a high prevalence among young women (<30 years: 71.3%; 30-40 years: 75.5%), women with positive colposcopy outcome (82.4%), and high-grade cytologycal lesions (HSIL: 80.9%; ASCH: 80.8%). In women with histologically confirmed high-grade cervical lesions (≥CIN 2), 96.7% of them showed hrHPV infection (Table 10).

The highest prevalence of co-infections with hrHPV and other sexually transmitted pathogens was found in women under 30 years (46.0%), the lowest in women over 60 years (25.0%). Among women with positive and negative colposcopy outcomes, 45.6% and 33.6% of women showed co-infections with hrHPV and STIs pathogens, respectively. Rates of co-infections with hrHPV and sexually transmitted pathogens among women with low-grade cervical lesions ranged from 21.4% to 43.8%, while among those with high-grade cervical lesions from 34.0% to 50.0%. In the group of women with histologically confirmed ≥CIN 2 lesions, 96.6% of women were hrHPV-positive however no difference was observed between those with or without concomitant coinfections with other sexually transmitted pathogens (48.3% vs 48.3%), as illustrated in Table 11.

Table 10. Distribution of hrHPV-positive cervical samples according to clinical data.

	hrHPV positivity on cervical sample n (%)
Age in years (n = 342)	

 doi:10.20944/preprints202412.1919.v1

<30	62/87 (71.3%)
30-40	80/106 (75.5%)
41-50	61/99 (61.6%)
51-60	21/42 (50.0%)
>60	4/8 (50.0%)
Cytology (n = 342)	
NILM	1/8 (12.5%)
ASCUS	52/85 (61.2%)
AGC	5/14 (35.7%)
LSIL	112/162 (69.1%)
ASCH	21/26 (80.8%)
HSIL	38/47 (80.9%)
Colposcopy (n = 342)	
Negative	126/217 (58.0%)
Positive	103/125 (82.4%)
Histology (n = 84)	
< CIN 2	16/24 (66.7%)
≥ CIN 2	58/60 (96.7%)

Table 11. Distribution of hrHPV and STI co-infection in cervical samples.

	hrHPV+ / STI+	hrHPV+ / STI-	hrHPV- / STI+	hrHPV- / STI-	Total
Total population	130 (38.0%)	99 (28.9%)	34 (9.9%)	79 (23.1%)	342
Age in years (n = 342)					
<30	40 (46.0%)	22 (25.3%)	8 (9.2%)	17 (19.5%)	87
30-40	40 (37.7%)	40 (37.7%)	9 (8.5%)	17 (16.1%)	106
41-50	34 (34.3%)	27 (27.3%)	16 (16.2%)	22 (22.2%)	99
51-60	13 (31.0%)	8 (19.0%)	2 (4.8%)	19 (45.2%)	42
>60	2 (25.0%)	2 (25.0%)	0 (0%)	4 (50.0%)	8
Cytology (n = 342)					
NILM	0 (0%)	1 (12.5%)	1 (12.5%)	6 (75.0%)	8
ASCUS	26 (30.6%)	26 (30.6%)	10 (11.8%)	23 (27.0%)	85
AGC	3 (21.4%)	2 (14.3%)	1 (7.1%)	8 (57.1%)	14
LSIL	71 (43.8%)	41 (25.3%)	15 (9.3%)	35 (21.6%)	162
ASCH	13 (50.0%)	8 (30.8%)	0 (0%)	5 (19.2%)	26
HSIL	16 (34.0%)	22 (46.8%)	7 (14.9%)	2 (4.3%)	47
Colposcopy (n = 342)					
Negative	73 (33.6%)	53 (24.4%)	28 (12.9%)	63 (29.1%)	217
Positive	57 (45.6%)	46 (36.8%)	6 (4.8%)	16 (12.8%)	125
Histology (n = 84)					

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< CIN 2	10 (41.7%)	6 (25.0%)	2 (8.3%)	6 (25.0%)	24
≥ CIN 2	29 (48.3%)	29 (48.3%)	2 (3.4%)	0 (0%)	60

3. Discussion

In this cross-sectional study, we evaluated the detection of hrHPV and 7 other sexually transmitted pathogens in self-collected vaginal and urine samples compared to cervical swabs of women referred to colposcopy. A high prevalence of STIs was detected in hrHPV-positive women, but no association was found between women with the high-grade cervical dysplasia and concomitant coinfections with hrHPV and one or more of the other investigated sexually transmitted pathogens.

The VALHUDES study protocol is currently being used for the evaluation of the clinical accuracy of HPV tests in self-collected vaginal and first-void urine samples [16]. However, there are no guidelines for the correct assessment of other STIs in self-collected samples. In the present study, we observed a good analytical agreement between cervical samples and self-collected vaginal and urine samples for the detection of hrHPV. The concordance rate of cervical and vaginal specimens was higher than that of cervical and urine samples, consistent with what was reported in previous studies [17, 18]. HPV16 and HPV31 were the most frequently detected genotypes in all sample types. Cervical samples showed a higher prevalence of single hrHPV infections as compared to self-collected samples, as previously reported by our group [18]. The results of this study suggest that self-collected specimens can be considered as a valid non-invasive alternative to cervical samples for the detection of hrHPV, contributing to improving women's participation to HPV screening. There has recently been a strong emphasis focusing on self-sampling as a key strategy to accelerate the global fight against cervical cancer [19]. Furthermore, self-collection has shown good acceptability in low resource settings, improving success in cervical cancer elimination [20, 21] by reducing public health costs and alleviating feelings of shame and discomfort offering women privacy [20]. Acceptability, however, depends on socio-cultural aspects and women's perceptions. Currently, countries such as New Zealand, Australia, the Netherlands, France and Sweden have included self-sampling in their national cervical cancer screening programs [12, 14].

Furthermore the present work has demonstrated a substantial agreement between self-collected samples and cervical specimens for the detection of other STIs in asymptomatic women referred to colposcopy. Vaginal swabs showed the highest STI detection rate, followed by urine specimens. The difference in positivity rates between cervical and self-collected samples was statistically significant for *UP.* These results are in alignment with other studies reporting that vaginal swabs are more sensitive than urine in detecting these pathogens [22, 23]. For the screening of STIs in women, the CDC's recommendations, published in 2014, have indicated vaginal swabs as the optimal sample type for the laboratory-based detection of both *CT* and *NG* [24]. The result of the present study further confirm the accuracy in the use of vaginal swabs for the detection of STIs.

The present study has also reported higher rates of multiple STI infections among self-collected specimens as compared to cervical samples, suggesting that some of the investigated pathogens may be responsible for infections of the lower genital tract. Bacteria belonging to *Mycoplasmataceae* family, including the two genera *Mycoplasma* spp. and *Ureaplasma* spp., are the most common STI pathogens. They are important opportunistic pathogens of the female lower genital tract [25] and they can be isolated in the urogenital system of many healthy individuals as commensals [26]. This may explain the lower detection rates in cervical swabs for most of the investigated sexually transmitted pathogens. Analyzing the distribution of each potential STIs pathogen across the different samples, *UP* was the most prevalent *Mycoplasmataceae* detected, followed by *UU* and *MH*. This is in line with findings from previous studies conducted in Greece [27], in Australia [28], in China [29] and by our group [6] where *UP* was found to be the most prevalent microorganism among healthy women. In our study *MG*, *CT*, *NG* and *TV* were detected at lower rates, probably due to the fact that our participants were asymptomatic and recruited when referred to colposcopy for an abnormal Pap smear.

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In the present study, STIs infections were more frequently detected among hrHPV-positive than hrHPV-negative women, but no association was found between the grade of the cervical lesion and the frequency of STIs coinfections detected. While persistent hrHPV infections have been demonstrated to be the necessary cause of the onset of cervical precancer and cancer, the potential role of other STIs as cofactors in the development of cervical lesions is still controversial. While some studies have suggested an association between the presence of STIs and a higher risk of occurrence of high-grade cervical lesions [4 , 5, 30, 31, 32], others have reported no association [33, 34] as also indicated by the results of the present cross-sectional study. Even if the role of STIs other than hrHPV and cervical lesions development remains to be clarified, changes in local microbiota and infections with other sexually transmitted pathogens have been proposed to act as cofactors with HPV infection, potentially facilitating its entry and persistence through chronic cervical inflammation and ulceration of the cervical epithelium as well as through a reduction in host cell–mediated immunity [25]. Persistent cervicitis might also enhance the progress of undetected precancerous cervical lesions [35, 36]. In fact, previous published reports indicate that cervical carcinogenesis is associated with inflammation [35, 36], driven by the hormonal milieu, regulatory cytokines and chemokines, as well as multiple cervicovaginal microorganisms [37]. The chronic inflammation caused by *Ureaplasma* spp. infections might favor the entry of other microorganisms or induce chromosomal alterations that might lead to carcinogenesis of epithelial cells [38]. In an early study, Lukic et al. postulated that *UU* is related to the persistence of HPV infection and early cervical cytological changes [39] through several inflammatory responses, involving the production of reactive oxidative metabolites, increased expression of cytokines, chemokines and angiogenic factors, decreased cell-mediated immunity and the generation of free radicals [32, 40]. Moreover, it is well known that the immunological status of a patient may influence HPV infection persistence. Surprisingly however the present study showed no association between hrHPV and STIs coinfections and the underlying severity of cervical lesions.

In this study, statistically significant differences were found in the distribution of *UP* between hrHPV-positive and hrHPV-negative women in the cervical, vaginal swab and urine samples. Similar results have been previously reported in cervical samples with a significantly higher *UP6* infection rate in the HPV-positive group than in the HPV-negative group [41]. In a previous study, Parthenis et al. recruited 345 asymptomatic patients attending a gynecology clinic for routine cervical screening, and *Ureaplasma* spp., detected in 30.2% of hrHPV-positive women, was the most frequently isolated pathogen [42]. Verteramo et al. showed an increased infection rate of *UU* in HPV-positive women, with a significant association found in the presence of high colonization rate of *UU* at cervical level [25]. Surprisingly, the present study showed statistically significant differences in the *MH* positivity rate for hrHPV-positive women compared to hrHPV-negative women in all sample types, as well as *MG* positivity in vaginal swabs. *Mycoplasma* infections have been linked to “in vitro” chromosomal changes and cell transformation [43, 44]. No significant difference was found for the other investigated STI pathogens which were rarely detected in our population.

The association between *CT* and cervical cancer has been widely investigated [25, 31, 40, 45, 46]. *CT* might increase susceptibility to HPV causing micro-abrasions or cervical epithelial cells and molecular alterations, facilitating the entry of virions [25, 31]. However, in this study, there wasn’t a significant difference between *CT* distribution in hrHPV-positive women and hrHPV-negative women, probably because of the low frequency of *CT* infections in our population.

The results of the present study further confirm the high prevalence of STIs in young women, as previously described in the literature [47, 48]. The risk of acquiring sexually transmitted infections decreases with age, as well as the probability of clearing the infection. Since this study is conducted in a colposcopy setting, as expected the prevalence of hrHPV infections was found to be very high. For this reason, the high hrHPV positivity rate found in older women may not reflect the trend of hrHPV infections distribution in the different age groups in the general population.

In our cross-sectional study we found a higher STI rate infection in hrHPV-positive women compared to hrHPV-negative women, but this result was not correlated with the clinical outcome underlying severity of cervical lesions, underlining the controversial role of HPV and STIs

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coinfections in the development of cervical dysplasia. Further longitudinal studies including a larger number of women will allow in the future to better investigate the possible role of hrHPV-STIs coinfections in cervical lesion development.

In conclusion, the present study demonstrated that self-taken specimens may be a good alternative for screening of both hrHPV and other STIs. Due to the increasing prevalence according to ECDC data [49], it may be important in the future to implement laboratory-based detection of STIs on self-collected samples among women who participate to cervical cancer screening programs or during subsequent visits for the treatment of cervical lesions.

4. Materials and Methods

4.1. Study Design and Sample Collection

From May 2017 to September 2024, 345 women have been enrolled at the Colposcopy Clinics of Fondazione IRCCS San Gerardo dei Tintori (Monza, Italy) following the signing of an informed consent. Women were referred to colposcopy because of a recent abnormal cervical cytology result.

Prior to gynaecological examination, all women were asked to autonomously collect a first-void urine sample using a Colli-Pee® device (Novosanis, Belgium) and a vaginal swab using FLOQSwab® 552.80 (Copan Italia Spa, Brescia, Italy).

Before performing the colposcopy, the physician collected a cervical specimen from each woman using an L-shaped Endo/Esocervical FLOQSwab® (Copan Italia Spa, Brescia, Italy) that was immediately resuspended into 20 ml of ThinPrep® PreservCyt® Solution (HOLOGIC™, Marlborough, MA, USA).

Based on the colposcopy findings and clinical judgment, women underwent biopsy and/or treatment with conisation.

The classification of cytological lesions was conducted according to the Bethesda system [50], whilst the histological outcomes were classified according to World Health Organization (WHO) histological classification of tumors [51]. Histological lesions worse than cervical intraepithelial neoplasia grade 2 (≥CIN2) were considered as high-grade lesions.

The study was conducted following the approval of the Ethics Committee of the University of Milano-Bicocca (Protocol n. 0037320/2017 and update n. 0086409/2018).

4.2. Pre-Analytical Samples Processing and Nucleic Acids Extraction

All samples were processed at the Laboratory of Clinical Microbiology of the University of Milano-Bicocca, Monza, Italy. On their arrival at the laboratory, cervical samples were vortexed for 30 seconds, aliquots of 1.5 mL were dispensed into sterile cryotubes and stored at -20°C until testing.

First-void urine collected using Colli-Pee® was also shaken on the vortex for 30 seconds and aliquots of 1.5 mL were stored at -20°C in sterile cryotubes until testing.

Vaginal self-samples were transported dry at the laboratory where they were suspended in 5.5 mL of ThinPrep® PreservCyt® Solution. 1 ml was then dispensed into sterile cryotubes and stored at -20°C until testing.

200 µl of all sample types were used to perform nucleic acid extraction using STARMag 96x4 Universal Cartridge Kit (Seegene, Seoul, Republic of Korea) on the MicroLab Nimbus workstation (Hamilton) with a final elution volume of 100 µl.

4.3. HPV and STIs Detection

Nimbus platform allows real-time PCR plate preparation of Anyplex™ II HR HPV (Seegene, Seoul, Republic of Korea) and Anyplex™ II STI-7e (Seegene, Seoul, Republic of Korea) assays.

Anyplex™ II HR HPV is a full genotyping HR-HPV assay that individually detects 14 different genotypes of hrHPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and a cellular gene target by melting curves analysis. The analysis is performed on the CFX96 (Bio-Rad, Hercules, USA) with 5 µl of template DNA in a total volume of 20 µl.

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Anyplex™ II STI-7e allows the detection of 7 sexually transmitted pathogens, Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, Mycoplasma hominis, Mycoplasma genitalium, Ureaplasma urealyticum and Ureaplasma parvum. According to the manufacturer's instructions, the real-time PCR analysis is performed on the CFX96 using 5 µl of template DNA in a total volume of 20 µl.

Data interpretation of the results obtained with both assays was done using the Seegene Viewer software according to the manufacturer's instructions.

Samples that were invalid according to the software interpretation were retested. After two invalid results, samples were excluded from the analysis.

4.4. Statistical Analysis

Qualitative and quantitative variables were summarized using absolute and relative (percentage) frequencies and medians (interquartile ranges, IQR), respectively. Concordance between the results of cervical and self-collected specimens with the two assays was evaluated using Cohen's kappa (κ) statistics and defined as follows: slight ($0.00 < \kappa < 0.20$), fair ($0.21 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$) and almost perfect ($0.81 < \kappa < 1.00$). Statistical analyses were performed with R software (R Core Team 2021). Statistical significance between positivity rates was calculated using Pearson's Chi-squared (X^2) test with Yates correction or Fisher's exact test, as appropriate (Tables S2, S3 and S4).

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Prevalence of STI pathogens in different samples; Table S1: Age distribution of the study population; Table S2: Tests used to calculate statistical significance differences between positivity rates of UP, UU, MH and MG in the three sample types; Table S3: Tests used to calculate statistical significance differences between positivity rates of CT, NG and TV in the three sample types; Table S4: Tests used to calculate statistical significance differences between positivity rates of STI pathogens in hrHPV-positive and negative women in the three sample types.

Author Contributions: Conceptualization, C.E.C. and F.L.; methodology, C.G., M.M., M.R., R.C.N., F.P. and R.M.; formal analysis, C.G., M.M. M.R. and G.M.; investigation, C.E.C.; data curation, C.G, M.M, M.R. and M.L.D.M.; writing—original draft preparation, C.G., M.M. and M.R.; writing—review and editing, C.G., M.M., M.R., M.L.D.M., R.C.N., F.P., G.M., R.F., F.L. and C.E.C.; supervision, C.E.C.; project administration, C.E.C. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Final study datasets generated by the study are stored locally and securely at the University of Milano-Bicocca. Anonymized data will be available by request to the corresponding author on a case-by-case basis pending approval by the University of Milano-Bicocca.

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Conflicts of Interest: C.E.C. is a minority share-holder of Hiantis. C. G., M. M., M. R., M. L. D. M., R. C. N., F. P., G. M., R. M., R. F., F. L. declare no conflicts of interest.

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