

High abundance of high-risk Human Papillomavirus genotypes in wastewater in Uruguay

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ABSTRACT

The aim of this study was to determine the frequency of Human Papillomavirus (HPV) genotypes in wastewater of Salto city, Uruguay, in order to obtain a general overview of the circulating genotypes in their population. HPV was detected in 34% (32/93) of the wastewater samples collected and analyzed during 2020/21 in Salto city, Uruguay. Thirty-three genotypes were observed, of which 16 presented read abundance higher than 1%, including both high-risk (HR) and low-risk (LR) genotypes. HR genotypes 31, 16, 58, 52, 33 and 59 were detected representing 40% (163,220 reads) of the total read abundance, with genotypes 31 (64,365), 16 (39,337) and 58 (36,332) being the most abundant. LR genotypes 72, 6, 11 and 40 were also detected in a high frequency, accounting for 37% (148,359) of the HPV reads. This study highlights the high frequency of HR genotypes of HPV, circulating in the population of Salto city which is a burden in public health mainly due to the devastating impact of cervical cancer in women.

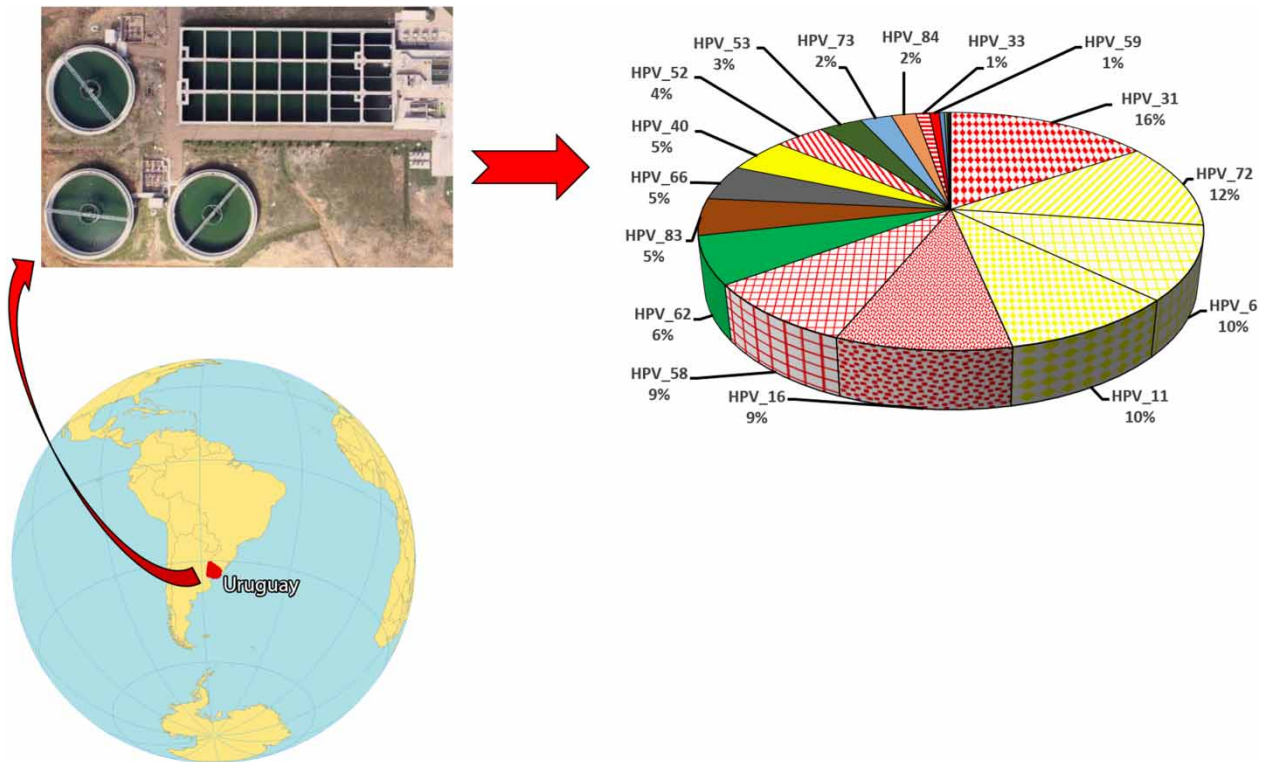
Key words: genotypes, high-risk, Human Papillomavirus, next-generation sequencing, wastewater

HIGHLIGHTS

- This was the first study analyzing the HPV genotype circulation by next-generation sequencing in wastewater in developing countries.
- Thirty-three genotypes of HPV were detected in wastewater in Uruguay.
- High-risk (HR) genotypes represented 40% of the HPV reads.
- Low-risk (LR) genotypes were also detected in a high frequency (37%).
- This study reveals the impact of HR HPV genotypes in public health mainly due to cervical cancer.

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GRAPHICAL ABSTRACT



INTRODUCTION

Human Papillomavirus (HPV) infections are responsible for almost all (more than 95%) cases of cervical cancer worldwide, although the majority of the HPV-infected population eventually clears the infection (WHO 2022). Moreover, this virus is related to nearly 90% of anal cancer, 50% of penile cancer, 70% of vagina cancer and 20–60% of oropharyngeal cancer (Mo *et al.* 2022). HPVs are non-enveloped viruses that contain a circular double-stranded DNA genome between 7.7 and 7.9 bp in length and approximately 60 nm in diameter. These viruses are diverse and classified within the *Papillomaviridae* family divided into genera *alpha-papillomavirus*, *beta-papillomavirus*, *gamma-papillomavirus*, *mu-papillomavirus* and *nu-papillomavirus* (<http://pave.niaid.nih.gov>, Howley *et al.* 2013). Mucosal/genital HPVs belong to *alpha-papillomaviruses* which are classified, based on their association with cancer lesions, into high-risk (HR) genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) and low-risk (LR) genotypes (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP 6108) (Seyoum *et al.* 2022). HPVs have been detected in urban wastewater, demonstrating that these viruses find their way into sewage when washing the skin and mucous membranes and also as a result of shedding into the urine (La Rosa *et al.* 2013).

The aim of this study was to determine the frequency of HPV genotypes in raw wastewater of Salto city, Uruguay, in order to obtain a general overview of the circulating genotypes in their population.

METHODS

Untreated wastewater samples (500 mL) were collected from June 2020 to January 2021 at four sites in Salto (104,028 inhabitants) with a total of 93 grab samples. Two collection sites are located in pumping stations, collecting wastewater from the northern (Site A) and southern (Site B) regions of the city. The other two sites are located in a suburb located 6 km from the city which is not connected to the sewage network (Sites C and D). Based on these wastewater collection sites, nearly 90% of the neighborhood of the city is represented. As a control of the viral concentration process, 50 μL of PP7 bacteriophage (1.0×10^6 genomic copies/ μL) was added to each wastewater sample which was concentrated by the PEG precipitation method, according to the protocol described by Lewis & Metcalf (1988) with modifications (Masachessi *et al.* 2022). Nucleic acid extraction was performed with a QIAmp Viral RNA mini kit according to the manufacturer's

instructions (QIAGEN, Germany). For the detection of PP7, a first reverse transcription reaction was performed with random primers pd(N)6 (Macrogen, South Korea) and Revert Aid reverse transcriptase (Thermo Scientific™, Carlsbad, CA, USA) according to the manufacturer's instructions, then, a qPCR was performed with primers 247F and 320 R, 1X Sensifast Probe No-Rox Kit (Bioline, UK) and 274 probe (Rajal *et al.* 2007).

HPV detection was performed by nested PCR with primers MY09/MY11 and GP5 + /GP6+ toward the L1 region of the genome (Strauss *et al.* 1999). Negative and positive controls (kindly provided by Dr Andrade) were used ranging from nucleic acid extraction to nested PCR reactions and all the extracted nucleic acid were analyzed pure and 1/10 diluted to avoid the inhibition of the enzymatic reactions. Amplicons with the correct size (140 bp) for the nested PCR were purified using the Pure Link Quick Gel Extraction and PCR Purification Combo kit (Invitrogen), following the manufacturer's instructions. Purified amplicons were quantified with the Qubit Fluorometer (ThermoFisher Scientific) and were equimolarly pooled before indexing. Deep amplicon sequencing was performed by Macrogen NGS sequencing service (South Korea); first, the library was generated by using the TruSeq Nano DNA Kit followed by sequencing with the HiSeq2500 platform with 100 bp PE. Geneious Prime® 2021 software was used for the bioinformatic analysis, reads were trimmed, deduplicated and then paired reads were merged and chimeras were removed. A dataset, consisting of 442 reference HPV genome sequences, was retrieved from PapillomaVirus Episteme (PaVE) database (pave.niaid.nih.gov). Finally, the curated reads were mapped to this custom reference dataset.

RESULTS AND DISCUSSION

In this study, the presence of HPV genotypes was assessed in wastewater samples in order to obtain a snapshot of the HR and LR circulating genotypes in the population of Salto, Uruguay. Grab sampling was performed instead of composite sampling which is frequently used for wastewater collection since it is an operationally straightforward option and cheaper than composite collection. The composite collection is neither feasible nor affordable mainly for developing countries due to economic limitations although a higher sensitivity for the detection and quantification of viruses in this matrix is possible. It is likely that the usage of grab sampling in our study introduced a bias in the detection frequency of HPV (Rafiee *et al.* 2021; Acer *et al.* 2022).

All wastewater samples spiked with PP7 presented a positive result by qPCR (100%) and an average viral recovery of 20% with minimum and maximum of 1 and 73%, respectively. HPV was detected in 34% (32/93) of the wastewater samples collected and analyzed during 2020/21 in Salto city, Uruguay (Table 1). In several studies, the frequency of HPV in sewage varied from 17 to 81% (Symonds 2008; La Rosa *et al.* 2013). The HPV frequency detected in this study is similar to that observed in the analysis of wastewater from Egypt where 30% of positivity was detected by using the same PCR assay based on L1 gene fragment amplification (Hamza & Hamza 2018). This virus has also been detected in other environmental waters such as rivers and swimming pools. Moreover, based on this wide HPV dissemination, a waterborne transmission cannot be ruled out; however, these studies were performed detecting only the genomic DNA and unfortunately, there is no information on its infectivity since no standardized cell culture assay is available.

Amplicons of HPV-positive wastewater samples were combined and deep amplicon sequencing was performed in a HiSeq2500 platform observing a high presence of different genotypes. Overall, 33 genotypes were observed after this analysis, of which 16 presented read abundance higher than 1%, including both HR and LR genotypes (Figure 1). Previous studies

Table 1 | Distribution of HPV according to the site and month of collection of wastewater samples from Salto, Uruguay from June 2020 to January 2021

Collection Site	2020							2021
	June	July	August	September	October	November	December	January
Site A	–(0/1)	+(2/5)	+(1/5)	+(1/4)	+(2/4)	+(1/4)	+(3/4)	+(3/4)
Site B	–(0/1)	+(2/4)	+(4/5)	+(2/4)	+(2/4)	+(1/2)	NP	NP
Site C	+(1/1)	+(1/1)	+(1/5)	+(1/4)	–(0/3)	–(0/3)	–(0/2)	–(0/2)
Site D	–(0/1)	–(0/1)	–(0/5)	+(1/4)	+(2/3)	–(0/3)	+(1/2)	–(0/2)
Frequency (%)	25	45	30	31	43	17	50	38

NP, not performed.

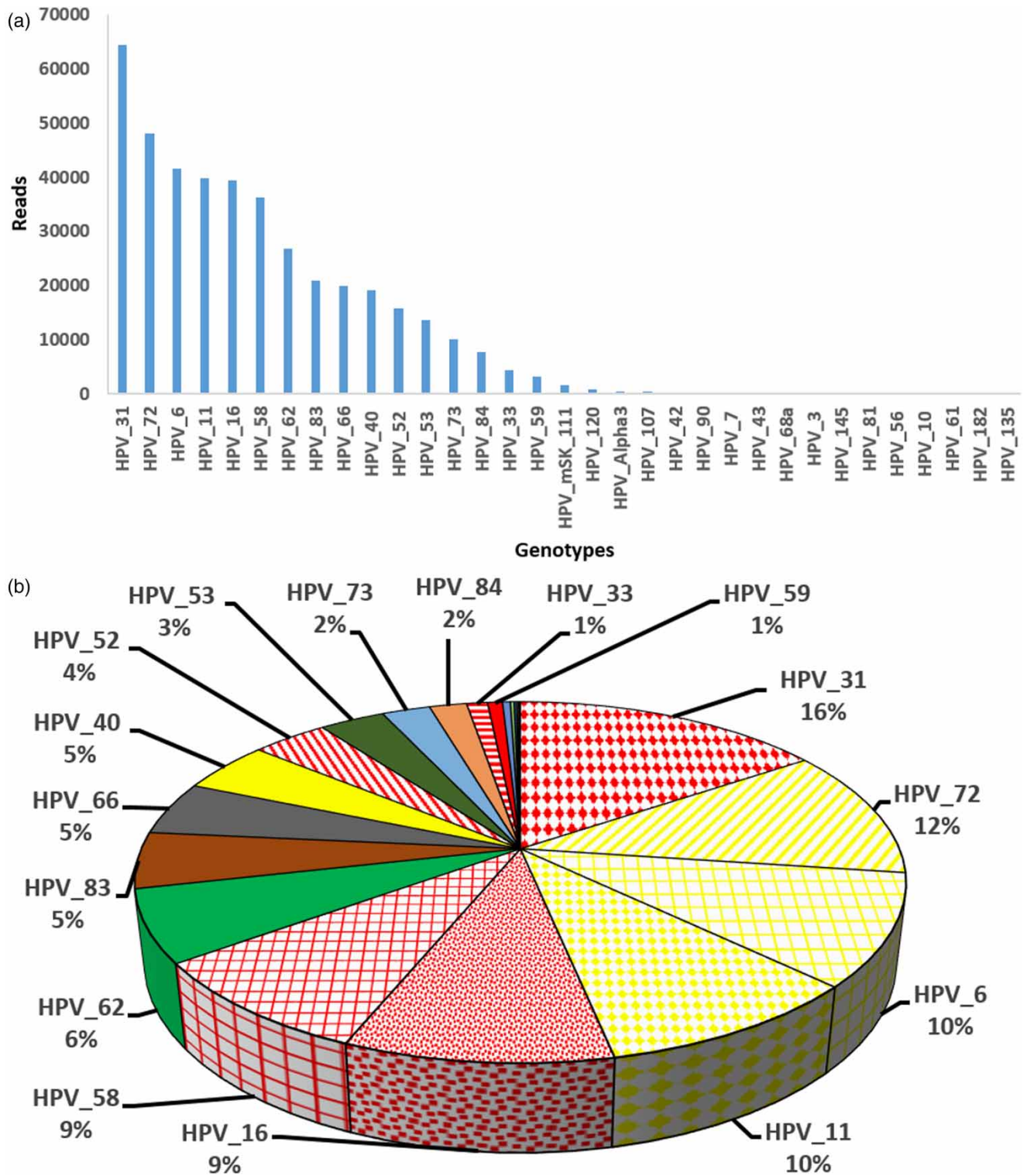


Figure 1 | (a) Absolute read abundance and (b) frequency of the read abundance according to the HPV genotypes. (b) HR and LR genotypes are presented in red and yellow textures, respectively; genotypes with frequencies <1% are not identified. Please refer to the online version of this paper to see this figure in color: <http://dx.doi.org/10.2166/wh.2022.330>.

performed in developed countries also found high co-circulation of different genotypes applying the metagenomic approach (Martínez-Puchol *et al.* 2020; Itarte *et al.* 2021). A comparison of untargeted viral metagenomic with target enrichment sequencing and deep amplicon sequencing revealed that the latter strategy allows the identification of a higher diversity of HPV genotypes similar to the diversity found in our study (Martínez-Puchol *et al.* 2020).

HR genotypes 31, 16, 58, 52, 33 and 59 were detected representing 40% (163,220 reads) of the total read abundance, with genotypes 31 (64,365), 16 (39,337) and 58 (36,332) being the most abundant within this group of genotypes. LR genotypes 72, 6, 11 and 40 were also detected in a high frequency accounting for 37% (148,359) of the HPV reads.

To the best of our knowledge, this is the first study analyzing the HPV diversity in wastewater in developing countries in order to have a picture of the HPV genotype circulation in the population served by the wastewater network. In a recent study performed in a sub-cohort of women testing HPV-positive in seven Latin American countries, HPV 16 was the most frequent genotype detected in all histological lesions grades which increased from $\leq 14.5\%$ in cervical intraepithelial neoplasia (CIN) 1 lesions to 65% in cervical cancer lesions. Moreover, genotypes 31, 52, 58 and 33 were also frequently detected mainly in CIN 3 lesions (Correa *et al.* 2022). These results are similar to those obtained in our work where genotypes 31, 16 and 58 were the most frequent HR genotypes detected with frequencies of 16, 9 and 9%, respectively. Unfortunately, in this study, we could not assess the presence of HPV in clinical samples in order to compare it with the environmental results. In Uruguay, previous studies assessed the burden of HPV infections in women mainly in Montevideo city, the capital of the country, which observed a frequency of HPV ranging from 21 to 42% with genotype 16 as the most frequently detected, evidencing the burden of this virus in this population (Ramas *et al.* 2013; Berois *et al.* 2014). LR genotypes like HPV 6 and 11, as detected in this study, have been previously observed in wastewater from Italy and environmental waters from Russia (La Rosa *et al.* 2013; Stolbikov *et al.* 2022). The study of these genotypes is important since they can produce genital warts and also have been rarely detected in invasive cancer and anal cancer (Egawa & Doorbar 2017). On the other hand, genotypes 40 and 72, which are also associated with anogenital warts, were detected for the first time in wastewater samples in this study (La Rosa 2016).

In Uruguay, the tetravalent HPV vaccine has been included in the immunization schedule since 2019 for both girls and boys older than 13 years; however, there is a low adherence and the coverage of this vaccine was 61% in 2018 (González *et al.* 2021). The fraction of vaccinated individuals is small, which can be reflected in the high frequency of HR genotypes in wastewater in Salto since this vaccine prevents not only epithelial lesions but also persistent infections with HPV genotypes 6/11/16/18 (Garland *et al.* 2016).

CONCLUSION

This study highlights the high frequency of HR genotypes of HPV, circulating in the population of Salto city which is a burden to public health mainly due to the devastating impact of cervical cancer in women. Further studies of the HPV circulation in wastewater along with cutaneous and skin samples will provide important epidemiologic data on the diseases caused by oncogenic viruses.

ACKNOWLEDGEMENTS

We thank the financial support for this project by Fondo Clemente Estable (FCE_1_2017_1_136092) of the National Agency of Research and Innovation (ANII), Uruguay and the OSE staff for technical assistance with the collection of wastewater samples.

DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 9 September 2022; accepted in revised form 30 November 2022. Available online 14 December 2022