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Heliyon

Clinical implementation of a multiplex PCR detection method for HPV causing plantar warts and genotype distribution in different geographical areas of Spain

A Aldana-Caballero [a], E Mingorance-Alvarez [b], R Mayordomo [b,*], F Marcos-Tejedor [c]

[a] Department of Nursing, Physiotherapy and Occupational Therapy, Faculty of Health Sciences, Universidad de Castilla-La Mancha, Talavera de la Reina, Spain
[b] Department of Anatomy, Cellular Biology and Zoology, Universidad de Extremadura, DEDAP Research Group, Plasencia, Spain
[c] Department of Medical Sciences, Universidad de Castilla La Mancha, DEDAP Research Group Collaborator, Talavera de La Reina, Spain

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ABSTRACT

Human papillomavirus is the ethological agent of various tumors, including plantar warts as one of the most frequent clinical presentations. Diagnosis of these warts continues to be mainly clinical, and a significant incidence of misdiagnosis leads to inadequate treatment. The aim of this study is to implement and validate a multiplex PCR detection method in the clinical setting to detect HPV in samples and to study genotype distribution in Spain to improve future molecular diagnostics. Viral DNA was extracted from 128 samples of clinically suspected plantar warts from various locations in Spain. A multiplex PCR was run alongside internal controls, and amplicons were processed for sequencing and HPV genotyping. The method was validated by assessing both inter- and intra-run repeatability. The PCR detection method returned 81.2 % (n = 104) positive results in the samples tested. Inter- and intra-run repeatability tests showed excellent intra-run agreement (κ = 1.00, p < 0.001) and good inter-run agreement (κ = 0.737, p < 0.001). The most frequent HPV type was HPV1, followed by HPV27, showing a statistical difference between the distribution of HPV genotypes in different areas of Spain. Clinical implementation of a DNA PCR detection method for plantar warts can avoid 18.8 % of unnecessary treatments in doubtful cases, and the method is reliable and validated for the purpose. HPV types show an asymmetric geographical distribution that should be considered for diagnosis and treatment.

1. Introduction

Human papillomavirus (HPV) is responsible for cutaneous and mucosal tumors. Its clinical implications range from benign tumors to cancer, depending mainly on the HPV type causing the lesion [1,2], and it is present in many other conditions [3,4]. It can also affect the plantar aspect of the feet, causing lesions that grows into the skin, creates reactive hyperkeratosis, and appears in high pressure-bearing areas, resulting in intense pain and inability to walk [5]. Plantar lesions have an annual incidence of 14 % and mostly affect the young population, but also adults and immunocompromised patients [5]. Patients seek treatment for the symptoms and to avoid infecting other people. However, treatments are painful, as they mostly consist of chemical burning over several visits [5,6].

Correct diagnosis of lesions is therefore of paramount importance to spare patients from unnecessary, painful treatment and avoid the costs involved. However, diagnosis of plantar warts is mainly based on clinical inspection, which can lead to misdiagnosis. Conditions such as fibrokeratoma, heloma, molluscum contagiosum, foreign bodies and other dermatological conditions can have similar macroscopic appearances to HPV lesions. Studies have shown rates of up to 76.6 % of misdiagnosis in some cases related to plantar warts and other conditions alike [7].

One explanation for the failure to sufficiently rely on complementary tests could be scarce research about developing, validating, and implementing diagnostic tools as complementary methods to verify infection. Although some studies have addressed various methods for plantar warts, including DNA detection [8–10], further studies with clinical implementation as a routine procedure to verify infection are needed. This study assesses and validates a multiplex DNA PCR detection method as a complementary test to verify HPV plantar wart diagnoses in the clinical setting, and studies HPV genotype distribution in different areas of Spain to obtain information for the development of future diagnostic tools.

2. Methods

The study was approved by the Bioethics and Biosafety Committee of the University of Extremadura, under the number 123/2018, following the ethical principles for medical research involving human subjects as per the Declaration of Helsinki and current legislation.

2.1. Sample collection

Samples were collected from patients seeking clinical foot care for lesions showing the previously described signs and symptoms, who met the following inclusion criteria:

- First consultation
- No prior treatment or at least one month without treatment
- Consent to participate in the study by allowing sample collection

The sampling method comprised taking scrapings of the skin covering the lesion. This step is routinely performed by the professional and is painless and simple. Before scraping with a scalpel blade, the skin was disinfected with 70 % ethanol. Information was collected about the patient and the sample (sex, age, time since onset, location on the foot, prior treatment, and possible associated risk factors), and samples were kept at −20 °C until processing.

A total of 128 samples from clinically suspected plantar warts were collected from 120 patients attending clinical foot care in different areas of Spain. Table 1 shows the location of the lesions, prior treatments, and risk factors.

2.2. DNA extraction

Using a Genomic DNA extraction kit (Invitrogen Corp., Thermo Fisher Scientific Inc., MA, USA), DNA extraction was performed on 0.025 g of skin scrapings from the samples following the manufacturer's instructions for mammalian tissue, with a 3-h incubation time and a two-step elution with 50 µl and 100 µl elution volumes for a total of 150 µl to improve DNA yield, storing samples at 4 °C until DNA processing.

2.3. Internal controls and plasmid construction for positive control

Plasmids containing HPV1 L1 target sequence from a reference genome (Accession number: V01116) were synthetized (Integrated DNA Technologies Inc., Coralville, IA, USA), transformed into *E. coli* DH5α competent cells, and used as a process positive control.

Table 1
Location, risk factors, and prior treatment of lesions.

RISK FACTOR	PRIOR TREATMENT	LOCATION
None 73.8 % (n = 90)	None 89.5 % (n = 77)	Internal plantar arch 5.6 % (n = 5)
Indoor swimming pool 9 % (n = 11)	Nitric acid 5.8 % (n = 5)	Heel 22.5 % (n = 20)
Outdoor swimming 7.4 % (n = 9)	Cryotherapy 1.2 % (n = 1)	Plantar first toe 15.7 % (n = 14)
Gym 4.1 % (n = 5)	Surgery 1.2 % (n = 1)	Plantar second toe 1.1 % (n = 1)
Locker room 2.5 % (n = 3)	Keratolytic 2.3 % (n = 2)	Plantar fourth toe 3.4 % (n = 3)
Family history 3.3 % (n = 4)		Plantar fifth toe 4.5 % (n = 4)
		Plantar 1MH[a] 16.9 % (n = 15)
		Plantar 2 MH 11.2 % (n = 10)
		Plantar 3 MH 6.7 % (n = 6)
		Plantar 4 MH 3.4 % (n = 3)
		Plantar 5 MH 5.6 % (n = 5)

a. (1–5) MH: (First-fifth) metatarsal head.

Negative control contained ddH$_2$O as a template for the multiplex PCR.

Internal quality control for the PCR consisted in the detection of the human β-Globin gene to assess DNA integrity. Primers used for the detection of the β-Globin gene were PC04 and GH20 [11], resulting in a positive 268 bp band in agarose gels.

2.4. Multiplex HPV DNA PCR detection method

PCRBIO HS Taq DNA Polymerase kit (PCR Biosystems Ltd., London, UK) was used for PCR detection of HPV DNA following the manufacturer's instructions, using 75 ng DNA per sample and 10 μM of previously described primers [10] with the following protocol: Initial start 95 °C for 1 min, then 95 °C for 15 s, 45 °C for 30 s and 72 °C for 30 s, for 35 cycles, followed by final annealing at 72 °C for 3 min.

Amplified products were run by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide or RedSafe™ nucleic acid staining solution (iNtRON Biotechnology, Burlington, MA, USA) at 100 V for 20 min. Gels were visualized under UV light, showing a positive band at 210-238bp. A positive result was considered when showing that positive band alongside a correct positive and negative control.

2.5. Validation assays

Analytical sensitivity was calculated with 10-fold serial dilutions of constructed plasmids from alpha-papillomavirus and mu-papillomavirus types (HPV27 and HPV1 respectively) targeted by the two different set of primers of the multiplex, to check for potential differences due to primer binding or efficiency. Limit of detection (LOD) was calculated following guidelines for laboratory-developed molecular assay validation [12], by running 5–6 different concentrations in 8 replicates over 5 days. Then probit regression analysis was performed to calculate C95 (concentration at which 95 % of the samples test positive).

Precision of the method was assessed by running intra and inter-run repeatability tests. To this end, 10 positive and 10 negative samples, comprising clinical samples and constructed plasmids at different dilutions (strong and weak positive samples), were tested, and repeated on the same day to assess intra-run repeatability, and on another day in another laboratory with different equipment using the same protocol to test inter-run repeatability. The Kappa (κ) statistic was used to determine the level of agreement, with 95 % confidence intervals (CI).

2.6. Genotyping by sanger sequencing

PCR amplification products were processed for purification and DNA sequencing at the Techniques Applied to Biosciences Service (STAB), University of Extremadura. The DNA sequences obtained were visualized using SnapGene Viewer® version 6.0.2 (GSL Biotech LLC, San Diego, CA, USA) and analyzed using L1 Taxonomy Tool from the PaVE (Papillomavirus Episteme) database to search for matches to existing HPV reference genomes (PaVE, http://pave.niaid.nih.gov) [13].

2.7. Statistical analysis

Descriptive and inferential analyses were performed with SPSS Statistic Software version 22.0 (Armonk, NY, IBM Corp.). Statistical figures were prepared using RStudio Version April 1, 1717 (RStudio Team (2021). RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA, USA; URL http://www.rstudio.com/).

Probit regression analysis was used to calculate LOD for analytical sensitivity. Pearson's Chi-square (χ2) was used to assess statistical differences between qualitative variables (genotype distribution, and genotype with sex, risk factors, location of lesions, simple or multiple lesions and prior treatment), and the *t*-test was used to calculate mean differences (age and time since onset) with other factor variables. The Kappa (κ) statistic was applied to calculate the level of agreement for the precision (inter and intra-run) assays. All CI were 95 %.

3. Results

3.1. Study participants

A total of 120 participants were included in the study, 51.9 % male and 48.1 % female, with a mean age of 22.7 ± 15.4 and a mean time since onset (in months) of 7.5 ± 16. By left or right foot, 46.6 % (n = 41) of lesions were found on the right foot and 52.3 % (n = 46) on the left foot, with one participant presenting lesions on both feet. Simple lesions were found in 70.6 % (n = 60) of cases, and 29.4 % (n = 25) presented mosaic warts.

Table 1 shows information about contributing risk factors, prior treatments, and location on the foot.

3.2. DNA PCR detection method

The samples studied were positive in 81.2 % (n = 104) of cases and negative in 18.8 % (n = 24).

Analytical sensitivity with 10-fold serial dilutions of the constructed HPV27 plasmid resulted in an (C95) LOD of 8.69×10^4 copies of viral DNA, and 1.51×10^4 copies in the case of HPV1. The lowest copy number detected was between 5-9.6 $\times 10^3$ of viral DNA.

Precision of the method by assessing intra- and inter-run repeatability showed excellent consistency for intra-run repeatability ($\kappa = 1.00$, $p < 0.001$), and good agreement for inter-run repeatability ($\kappa = 0.737$, $p < 0.001$). Table 2 shows the intra- and inter-run repeatability assays and their level of agreement.

Sample sequences and genotype analysis revealed seven HPV types: HPV1, HPV2, HPV27, HPV57, HPV4, HPV63, and HPV65. The most frequent type was HPV 1 (39.8 %), followed by HPV27 (12.5 %). Table 3 shows the frequency of HPV types in positive samples.

Distribution of HPV genotypes was asymmetric: HPV27, HPV57 and HPV2 were the most common in northern Spain, while in other areas HPV1 and HPV4 were more frequent. Pearson's Chi-square ($\chi2$) revealed a statistical difference between the areas of distribution of HPV genotypes ($p < 0.001$). Fig. 1 shows the genotype geographical distribution in a plotted map.

4. Discussion

A previous study showed that misdiagnosis of HPV plantar warts when relying on visual diagnosis can have a significant influence on unnecessary treatment of lesions that are not caused by this virus infection [7]. In our study, 24 (18.8 %) of the 128 samples analyzed were negative to detectable HPV types by the multiplex PCR detection method used. Clinical implementation of the DNA PCR detection method as a complementary test after initial diagnosis to verify infection will help health professionals to decide, in negative cases, whether to take an observation-based approach or conduct further analyses to make a differential diagnosis.

The multiplex DNA PCR detection method elucidated analytical sensitivity of 1.51–8.69×10^4 copies of the target constructed plasmid in 95 % of the replicates according to the probit regression analysis. This result is slightly different to those of Sasagawa et al. [10], who observed an analytical sensitivity equivalent to detect up to 7.00×10^3 copies of HPV DNA. This discrepancy could be due to differences in the protocol used in our study, which includes reagents and thermocycling differences, or on the approach on calculating the LOD, since in our study LOD is calculated through C95, however, lower detections between 5000 and 9000 copies were possible in some cases (data not shown). It is also worth noting that in our study we used an initial quantity of DNA in reaction tubes of 75 ng or above, which is lower to what [10] used in their study, between 100 and 300 ng of DNA for unknown samples. This is based on the decision after optimizing the protocol to reduce PCR inhibition, however further studies on viral load on these types of samples will provide insightful information to improve these types of DNA detection methods. Moreover, our study validated the precision of the multiplex DNA PCR for plantar warts by assessing inter- and intra-run repeatability, with excellent intra-run agreement ($\kappa = 1.00$), indicating that the results are consistent, and good inter-run agreement ($\kappa = 0.737$), indicating that the assay is valid as a diagnostic tool and its results are highly consistent between laboratories and repeated sets of samples. Missed positives in precision assays could make up for the samples below the 20 % of the LOD used on the precision assays, as suggested in literature [12] and/or the differences of instruments (Thermocycler, pipettes, handling) in a different laboratory.

The sampling method was similarly optimal. Other studies applied a PCR detection method for cutaneous warts, including plantar warts, using biopsies or swabs as the sampling method [10,14–16] with good results. However, biopsy can be detrimental in managing plantar warts because the plantar aspect of the foot is prone to significant scarring, and cost-benefit can be difficult to assess. Skin scrapings, in contrast, provide a considerable concentration of viral DNA as the target for the PCR detection method, as demonstrated in a study by de Planell-Mas et al. [17], who concluded that scraping has the same sensitivity as biopsies or resected warts, and is a less invasive and simpler sampling method. Swabs are a recently studied option, e.g., García-Oreja et al. [9], who compared the skin scraping method and swabs and obtained good results to support the use of swabs, which could be easier and quicker for health professionals.

Genotyping HPV is very important in cervical screening, because different HPV types have implications in the prognosis of lesions [1,18]. In the case of cutaneous warts, HPV genotype may not have such a significant impact, although some authors reported differences in the clinical outcome of lesions depending on the HPV type present [19,20], and therefore it could be worthwhile studying the genotypes affecting plantar warts, especially in recurrent cases or when treatment is unsatisfactory. Our results revealed seven HPV types, of which HPV1 was the most frequent (39.8 %), followed by HPV27 (12.5 %) (Table 3). De Planell-Mas et al. [17] also described HPV1a as the most frequent type (20.9 %) in their sample, followed by HPV2 (15.2 %) and HPV27. Different results were obtained by García-Oreja et al. [9], who found that HPV2 was the most prevalent type, followed by HPV19. These studies addressed other areas of Spain from those in our study, suggesting that certain HPV types are more frequent in some areas. This result is supported by the evidence obtained in our study, as shown in Fig. 1, where, for instance, HPV27 and HPV57 are more frequent than HPV1 in northern Spain, while in central-western areas HPV1 is the most frequent type, with significant statistical differences ($p < 0.001$). Geographical

Table 2
Intra- and inter-run repeatability and level of agreement.

| Repeatability tests					
		Positive	Negative	Kappa[b] (95 % CI)	p
Intra-run	Assay 1	6	14	1.00	<0.001
	Assay 2	6	14		
Inter-run	Assay 1	6	14	0.737	<0.001
	Assay 3	4	16		
TOTAL		N = 20			

b. Value of κ and strength of agreement: <0.20 Poor, 0.21–0.40, 0.41–0.60 Moderate, 0.61–0.80 Good, 0.81–1.00 Very good.
33. Genotype distribution.

Table 3
HPV type frequency.

	HPV TYPE	%
	HPV1	39.8 % (n = 51)
	HPV27	12.5 % (n = 16)
	HPV2	10.2 % (n = 13)
	HPV57	7.8 % (n = 10)
	HPV65	5.5 % (n = 7)
	HPV4	4.7 % (n = 6)
	HPV63	0.8 % (n = 1)
	Negative	18.8 % (n = 24)
TOTAL		100 % (n = 128)

Statistical analysis found no other relation or dependency of the other variables (sex, age, risk factors, location of the lesion, simple or multiple lesions, time since onset and prior treatment) with HPV genotype.
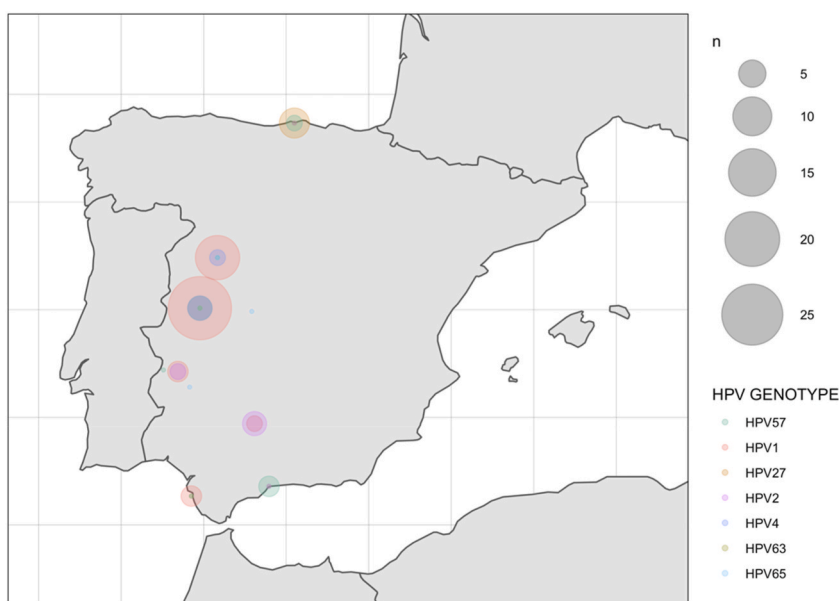
Fig. 1. Geographical genotype distribution.

distribution of HPV types has been identified in cervical screening, especially between western and eastern countries, and provides grounds for adapting diagnostic tools to location-specific types or using new tools that can detect a broader spectrum of HPV types [21]. It is worth mentioning, that one case that was excluded from the sample, showed an inconclusive result that could potentially be a multiple infection with at least two different types (data not shown). In this regard, further methods should be applied in the future to investigate these cases. Sample size is, however, a limitation inherent to this study, due to the logistics difficulty that it has, and for that reason, further studies with larger sample sizes could probably throw more evidence that could support these preliminary results more firmly. Future studies will also benefit from considering the cost-effectiveness and the logistic implications that implementing these types of diagnostic assays for plantar lesions in a larger scale could have, especially considering that samples should be taken by a professional and could not depend on the patients themselves.

Describing the HPV types present in certain areas will provide a solid basis for future treatment and diagnostic developments that better correspond to the context of HPV infection in specific locations, and this, will allow further development of detection techniques for the types commonly found in a particular area. This study shows that genotype distribution of HPV plantar warts is asymmetric in different areas of Spain, and HPV1 and HPV27 tend to be the most prevalent types. The multiplex DNA PCR detection method is a good and reliable tool to verify infection and can avoid unnecessary treatment in as many as 18.8 % of doubtful cases, with excellent implementation in the clinical setting, as demonstrated by the intra- and inter-run repeatability. Further research is needed to make these diagnostic tools readily available to health professionals at an affordable price or potential commercially available assays development.

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CRediT authorship contribution statement

A. Aldana-Caballero: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Mingorance Álvarez E:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Mayordomo R:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **F. Marcos-Tejedor:** Conceptualization, Formal analysis, Investigation, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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