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HPV Prevalence in Anal Warts Tested with the MY09/MY11 *SHARP Signal* System

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Anal warts are, from an aetiological point of view, a diverse category of lesions including condylomata acuminata, fibroepithelial polyps and seborrhoeic keratosis. Human papillomavirus induced anal warts, in contrast to other types of warts, are contagious and not infrequently sexually transmitted, they therefore need to be accurately identified. A total of 24 anal warts were randomly collected and the histopatholgical diagnoses based on microscopy, alone or in combination with a sensitive PCR-based human papillomavirus test, were compared using the *SHARP Signal* system for detection. Three lesions were identified as condyloma acuminatum by morphology alone due to the obvious presence of koiloytotic atypia; 11 warts without koilocytes were identified only after a positive test for anogenital human papillomavirus. One additional lesion contained human papillomavirus DNA of cutaneous type and 9 papillomas were human papillomavirus-negative and tentatively diagnosed as fibroepithelial polyps or seborrhoeic keratosis. All 14 condylomas contained human papillomavirus of low-risk type. Of these, 12 warts showed a positive human papillomavirus reaction with *in situ* hybridization. Morphology alone cannot reveal the true nature of most anal papillomas, even when koilocytotic atypia is considered as a diagnostic hallmark. An optimal diagnosis of anal warts requires a sensitive PCR-based human papillomavirus DNA test. A test for identification of cutaneous human papillomavirus DNA is also worthwhile. *Key words: anogenital; warts; human papillomavirus; condylomas; in situ hybridization; polymerase chain reaction.*

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Anal warts are usually diagnosed using light microscopy. However, the diagnosis is not conclusive with respect to the pathogenic mechanism behind the lesion. For instance, seborrhoeic keratosis and condylomas may show similar morphology, yet display completely different aetiologies (1–2).

The differential diagnosis between condyloma acuminatum and warts of non-viral origin is of clinical importance, since the former is an infectious disease transmitted by contagious events sometimes related to sexual behaviour.

One characteristic feature of some condylomata acuminata is the presence of koilocytotic cells in the upper layers of the squamous epithelium. The koilocytes denote the presence of human papillomavirus (HPV) virons in the cytoplasm of the epithelial cells, but this picture is seen only when the epithelium contains relatively large quantities of virus particles. Koilocytosis is frequently absent even when the cause of the wart is viral.

An evaluation of HPV in anal warts is of diagnostic value, but one of the most commonly employed HPV methods, namely *in situ* hybridization (ISH) has a relatively low sensitivity. About 10–50 viral DNA copies in each cell are necessary for a positive HPV reaction. Consequently, a lesion may be virus-related, yet give a negative HPV ISH test (3–5).

A relationship appears to exist between ISH and koilocytosis, since koilocytotic cells often prove positive for HPV with ISH. On the other hand, non-koilocytotic cells can be both HPV-positive and negative. More sensitive HPV tests are required to distinguish reliably between HPV-related and unrelated anal warts, especially those lacking koilocytotic atypia in the squamous cells (6).

In the present study anal warts with and without koilocytosis were investigated for the presence of HPV of anogenital and cutaneous types. To identify HPV, both the ISH and a sensitive PCR-based HPV identification technique were employed. The aim was to classify the anal warts in more distinct pathogenic terms.

MATERIALS AND METHODS

Data on all histopathological material at the Department of Pathology in Uppsala are collected in a central database, where each specimen is given an individual topographical and morphological code (Systematized NOmenclature of MEDicine, SNOMED). A total of 24 biopsies from the anus recorded as warts (papillomas) at our Department during 1995–1997 were used for the study. They were obtained from 14 males and 10 females, mean age 48 years (males 49 years, females 47 years) and age range 24–87 years. The biopsies were fixed in 4% neutral buffered formaldehyde solution (4.5 g $NaH_2PO_4.H_2O$, 8.3 g $Na_2HPO_4.H_2O$, 110 ml concentrated formaldehyde solution and distilled H_2O to a final volume of 1 litre, pH 7.2) and embedded in paraffin. Sections, about 4 μm thick, from each case were stained with H & E and evaluated histopathologically.

DNA preparation and controls

DNA extraction was performed as described by Lungo et al. (7), except for the incubation time, which was prolonged to 4 h. Empty paraffin blocks and distilled water were used as negative controls. Positive controls were obtained from previously HPV-positive gynaecological biopsies. To exclude false-negative results, all specimens were tested with the β-globin primers PC04 and GH20 resulting in a 268-base pair amplimer (8). HPV DNA amplification was successfully performed on all 24 β-globin-positive specimens (Table I).

Hot start PCR

To ascertain the presence of HPV DNA in the wart specimens, MY09/MY11 (MY11 being biotinylated) were used for the PCR reaction essentially as optimized and described previously (9–10) (Table I). The PCR profile described in the *SHARP Signal* System was slightly modified. The main difference from the earlier application was that 2.5 mM instead of 4 mM magnesium chloride was used and the

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Table I. *Primer pair used for PCR in the present study*

Primer pair	Sequence 5′–3′	Gene position	Length (bp)	Product size (bp)	Ref.
β-globin/PCO4	CAACTTCATCCACGTTCACC	54–73	20		
β-globin/GH20	GAAGAGCCAAGGACAGGTAC	195–176	20	268	8
HPV/MYO9	CGTCCMARRGGAWACTGATC[a]	L1 (HPV6)	20		
HPV/MY11[b]	GCMCAGGGWCATAAYAATGG[a]		20	398	9
HPV/CP-1	ATGGTACARTGGGCATWTGATAA[a]	E1	23		
HPV/CP-IIG	ATATTGTCTGAGCCTCCWAARTT[a]	E1	23		
HPV/CP-IIS	ATGTTAATWXAGCCWCCAAAATT[a]	E1	23	188	12

HPV, human papillomavirus.
[a] Degenerate code M = A + C; R = A + G; W = A + T; X = G + C.
[b] Biotinylated at its 5′ end.

annealing and extension steps were prolonged from 1 to 2 min and 2 to 3 min, respectively. To monitor reproducibility, 10 negative and 10 positive cases were run twice. Hot start PCR was performed with AmpliWax (Perkin Elmer, Norwalk, CN, USA) according to the manufacturer's instructions. Aliquots (10 μl) of each PCR product were resolved by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. All of the cases, even those without a visible band on the agarose gel, were tested with the detection method.

HPV detection of low- and high-risk types with SHARP Signal System

The ELISA-based *SHARP Signal* System (Digene Diagnostics, Beltsville, MD, USA) is a sandwich capture hybridization assay that uses colorimetric detection (11). An aliquot (5 μl) of a PCR reaction containing 5′-biotinylated products is hybridized to a specific single-stranded RNA probe. The resultant RNA-DNA hybrids are captured by means of biotin onto the surface of streptavidin-coated microwells. Immobilized hybrids react with an antihybrid antibody conjugated to alkaline phosphatase and are detected with the colorimetric substrate para-nitrophenylphenol. The intensity of the colour generated is proportional to the amount of biotinylated PCR products, caused by at least one biotinylated primer. Absorbency is read on a plate reader set at 405 or 410 nm. After a hot-start PCR with MY09/MY11-biotin, hybrid capture detection was performed exactly according to the manufacturer's recommendations. Low-risk (types 6, 11, 42–44) and high-risk HPVs (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58) were screened with one appropriate RNA probe for each group.

Detection of HPV of cutaneous types

Two sets of consensus PCR primers consisting of a common 3′ primer and two 5′ primers were used. These two primer pairs enabled the detection of a 188 base pair fragment in the E1 open reading frame of the HPV genome in HPV types 1, 2, 3, 4, 5, 6b, 7, 8, 9, 10a, 11, 12, 14a, 16, 17, 18, 19, 20, 21, 22, 24, 25, 31, 33, 36, 37, 38, 39 and 46 (12) (Table I).

The PCR product was initially identified on a 1.5% agarose gel after staining with ethidium bromide. Thereafter HPV was typed by single-strand conformation polymorphism (SSCP) using semi-automated electrophoresis on polyacrylamide gels (PAGE) combined with sensitive silver staining. To establish a standard for the band patterns of the various cutaneous HPV types, we used HPV plasmid DNA, which allowed us to distinguish the most common cutaneous HPV types (1, 2, 3, 4, 5/48, 10a and 10b). All the types tested are separated from each other, demonstrating diverse band patterns.

Table II. *Determinants of HPV infection in anal warts*

	Koilocytosis	HPV-positive (ISH)[a]	HPV-positive (PCR)[b]	Total
Squamous papillomas	3	12	15[c]	24

[a] ISH, *in situ* hybridization.
[b] PCR, polymerase chain reaction.
[c] 14 papillomas proved positive after application of the primer pair (MY09/MY11) for identification of anogenital HPV type and 1 additional wart with the primer pairs (CP-1, CP-IIG and CP-IIS), which also identify cutaneous HPV type.

In situ hybridization

In order to verify HPV DNA in the squamous cell nucleus of each papilloma, especially in non-koilocytotic cells, all of the HPV PCR positive biopsies were examined with *in situ* hybridization.

The *in situ* hybridization technique employed in our laboratory is described in detail elsewhere (3–4). Briefly, serial 4–6 μm sections on organosilane-coated slides were incubated overnight at 60°C and treated with proteinase K (P-0360, Sigma Biochemicals, St. Louis, MA, USA) at a concentration of 0.1 mg/ml 1 × SSPE (150 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA) for 30 min at 37°C and 3% H_2O_2 for 10 min at room temperature. Denaturation was done for 5 min on a 94–96°C hot-plate. Hybridization was carried out overnight with the *pan*probe (Omniprobe, Digene, Silver Springs, Md.) which detects HPV types 6, 11, 16, 18, 31, 33, 35, 42–45, 51, 52 and 56 and also for 2 h with probe sets 6/11, 16/18 and 31/33/35 (Digene) at 37°C in a moist chamber. Post-hybridization was performed in 30% (Omniprobe) or 50% (probe set) formamide/2 × SSPE (final concentration) for 10–20 min at 37°C, followed by enzymatic detection with biotinylated horseradish peroxidase bound to streptavidin (K 377 A-B, Dakopatts, Copenhagen, Denmark) 1:100 for 30 min at 37°C and visualization with 3,3-diaminobenzidine tetrahydrochloride-H_2O_2. Finally, the slides were counterstained in Mayer's haematoxylin for 15–30 s, rinsed in graded ethanol and xylene and cover slipped. A positive (labelled total DNA to ensure all components were working) and a negative DNA (labelled vector only) hybridization check, provided by the manufacturer, were included to ensure that each ISH step was carried out correctly and that the assay was specific. Further checks were made with known HPV-positive biopsies.

RESULTS

The 24 biopsies of anal warts had a papillary growth pattern with squamous hyperplasia, hyperkeratosis and varying degrees of parakeratosis. One wart partly displayed transitional cell differentiation. Three specimens showed an obvious koilocytotic atypia accentuated towards the superficial area and surface of the lesion. The other cases lacked koilocytotic alterations.

The stroma tissue mostly lacked inflammatory cells or contained few mixed inflammatory cells. Neither spongiosis,

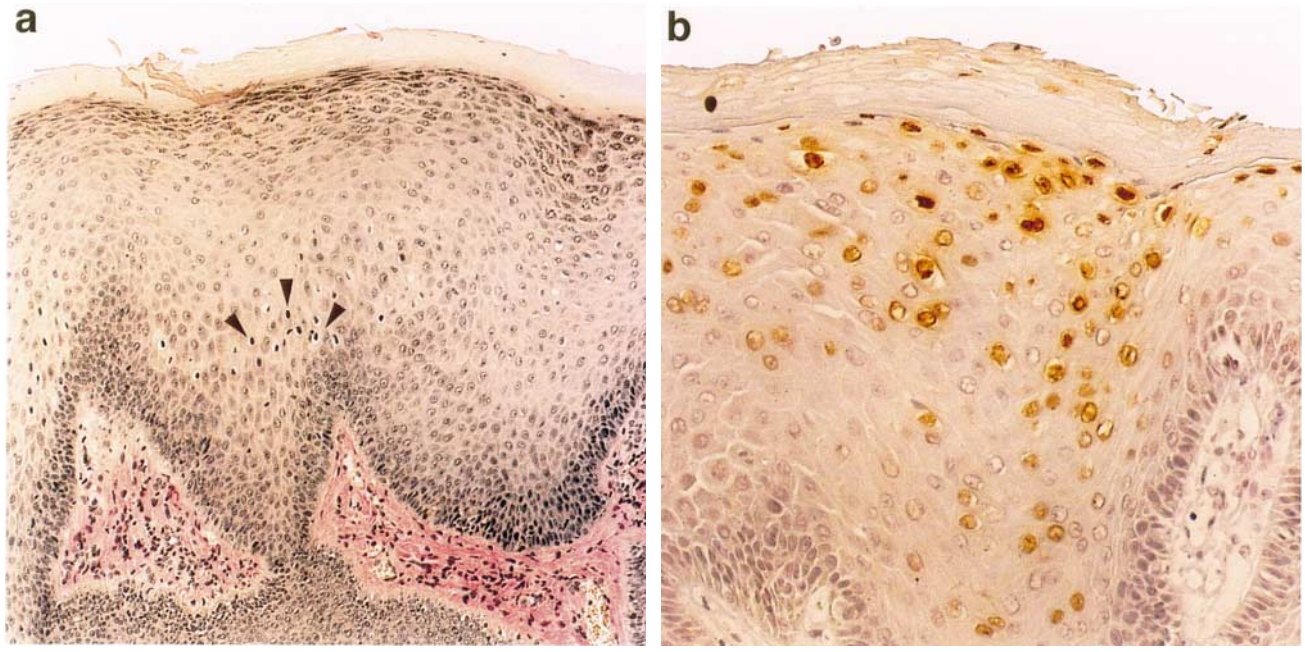

Fig. 1. (*a*) Anal wart excised from a 64-year-old woman, showing squamous hyperplasia, papillomatosis, hyperkeratosis and a slight accumulation of cells with koilocytic atypia (arrow); van Gieson's stain. (*b*) The lesion proved HPV-positive after application of the MY09/MY11 *SHARP Signal* system and *in situ* hybridization disclosed a positive HPV DNA signal in both koilocytic and non-koilocytic squamous cells; PAP stain.

epithelial invasion of granulocytes nor pronounced invasion of plasma cells indicative of condyloma latum was seen.

Analysis of HPV DNA in the tissues with the *SHARP Signal* system revealed a positive HPV reaction in 14 cases, all of which were of low-risk type (6. 11 and 42–44). Eight cases emanated from males (57%) and 6 cases from females (60%). The mean age of the HPV-positive cases was 43 years and of the HPV-negative cases 49 years. Thus, a slight difference was observed between the HPV-positive and HPV-negative individuals with respect to age distribution (Table II).

Twelve of the 14 HPV-positive warts were HPV-positive and 2 were HPV-negative after applying the *in situ* hybridization test with a nuclear staining which was especially distinct in the 3 cases displaying koilocytotic atypia (Fig. 1a and b).

One wart revealed a HPV DNA amplicon after application of the primer pairs identifying cutaneous HPV types. However, the specific type could not be established in that particular case by SSCP analysis.

DISCUSSION

The PCR-based HPV detection systems used in the present study are particularly sensitive for identifying occasional or a few HPV DNA copies per tissue section. Previous studies have indicated that the MY09/MY11 *SHARP Signal* system has a sensitivity at least equal to other PCR-based HPV detection systems (10, 13–15). Thus, a negative result presents strong evidence that the specimen is free from HPV DNA. However, the possibility of false-negative results due to the existence of hitherto unidentified HPV types with which the primer pairs used do not hybridize, must be borne in mind. Furthermore, the HPV DNA may not always be well preserved and may be inconvenient for analysis because of delayed or a poor tissue fixation procedure. Consequently, a positive HPV reaction is of greater diagnostic value than is a negative one.

The natural history of HPV infection in young women has been investigated and has in most cases been found to be transient (16, 17). Most young women with a positive HPV test had after a 24 month period become negative. Women with low-risk HPV types were more likely to show regression than were women with high-risk HPV types. Thus, our finding of HPV-positive anal warts in a group of significantly older patients is interesting, especially since all were of the low-risk type.

The question whether to screen for carriage of high-risk HPV in the anal canal has been raised, especially concerning homosexual men but also in women the incidence of anal cancer has increased in recent decades (18).

It should be pointed out that the detection of HPV DNA in the anal canal is a common phenomenon in both men and women. One study detected anal HPV DNA in 61% of immunocompetent homosexual men (19) and in another study 67% of heterosexual women with normal anal cytology carried HPV DNA (20). Those studies both used PCR technique applied to cell samples but in this study PCR technique was applied to biopsies from what was clinically suspected to be anal warts.

The diagnostic difficulty of distinguishing between anal warts of different origins is especially obvious when distinct diagnostic features are lacking, such as koiloytotic atypia in HPV-related condylomas and pigmentation and occurrence of well-developed horn pseudocysts in seborrhoeic keratosis (senile keratosis, basalcell papilloma). In this study, koilocytotic atypia was a diagnostic adjunct in only 3 of the 15 HPV-positive anal warts.

It is emphasized that in anal warts without distinguishing light microscopy features, a PCR-based HPV test is a valuable diagnostic tool. It is of particular importance when the

question of sexually transmitted disease is raised. A sensitive HPV test can corroborate the existence of sexually transmission but not exclude such a disease. However, in the absence of a HPV test, no conclusion can be drawn about the pathogenesis of most anal warts, since a common morphology does not allow them to be distinguished.

The mean age of those individuals with HPV-related disease was slightly lower than in those without infection. This is consistent with the observation that the relative frequency of condylomas is greater in younger persons. It is also consistent with the fact that seborrhoeic keratosis and fibroepithelial polyps are more frequently seen in older persons (21 – 23).

For some unknown reason, koilocytotic atypia is more often seen in cervical condylomas than in condylomas arising in external genitalia such as the vulva and penis and in the anus. Koilocytosis is mostly an uncertain diagnostic adjunct at these latter sites. ISH gives more relevant information and a PCR-based HPV test is the most accurate procedure when the pathologist wishes to give optimal information with respect to the aetiology of an anal wart (6).

The present findings raise the question as to whether the diagnosis should be based on morphology alone or combined with corroborating molecular biological methods. Obviously, a preliminary light microscopy diagnosis of say, a seborrhoeic keratosis could be altered to a condyloma acuminatum after an additional HPV test. Awareness of this diagnostic enigma gives support to a more liberal application of HPV tests in routine diagnostic pathology at sites where HPV-related squamous alterations occur.

However, diagnosing and treating asymptomatic HPV infection cannot be recommended until we have more knowledge about the infectious potential of the infection in that phase. Interesting results have recently been presented that the median duration of HPV infection was only 8 months, and at 24 months only 9% continued to be infected (16). With this knowledge it seems of doubtful benefit to diagnose an infection for which we have no cure to offer in an asymptomatic patient.

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