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Cross-sectional seroprevalence of antibodies against 6, 11, 16 and 18 human papilloma virus (HPV) types among teenagers and young women in Italy

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Abstract

Background. Little is known about human papilloma virus (HPV) seroprevalence in the healthy Italian population. The aim of the study was to assess seroprevalence of antibodies against HPV 6, 11, 16 and 18 among girls and young women in Italy.

Methods. Sera were tested with a commercially available ELISA assay detecting specific IgG.

Results. Seroprevalence was 54.1% in girls between 11 to 18 years old and 8.2% in over 18s. Overall seropositivity in birth cohorts invited for free immunization reached 72.8% and was significantly higher than in other age subgroups. The highest levels of antibodies were detected in girls of 12 years old that should have just completed the vaccination schedule.

Conclusion. A standardized seroepidemiological survey on HPV represents a useful tool for identifying groups at risk in which immunization is recommended, monitoring of vaccinated women, assessing whether booster vaccination is required.

Key words
- human papilloma virus
- seroprevalence
- adolescents
- young adults

INTRODUCTION

Public health is required to notice and adopt innovative tools to ensure the best practices in disease prevention. In the field of cervical cancer prevention, organized and opportunistic screening programmes have been very successful succeeding in reducing incidence of the disease in many developed countries. The behaviour of women, however, besides the limitations of the test itself, did not consent to date to eliminate all cases of disease. Recent release of two vaccines against human papilloma virus (HPV) gave rise to many expectations in primary prevention. At the present over 100 of HPV genotypes have been identified: some strains, defined low-risk types, like HPV 6 or 11, lead to genital warts while others, considered high-risk types, like HPV 16 or 18 or 45, are implicated in lesions that promote the development of cervical cancer [1, 2]. Oncogenic HPV types are also involved in vulva, vagina, anus, penis, oral cavity, pharynx, and larynx cancers [3].

Dealing with one of the most common sexually transmitted infections worldwide, frequently many young women and men become rapidly infected after first intercourse. The consistent use of a condom achieves only partial protection [4]. During their lifetime, a large part of women can become infected several times, but usually with different strains. Age-specific distribution of HPV infection presents a first peak in younger ages (before 25 years old), just after the onset of sexual relations, in all regions of the world, and a second one in perimenopausal ages, more clearly evident in the Americas, Africa and Europe [5]. Most infections are subclinical and transient and when cervical samples are collected, they may show the presence of HPV-DNA and mild cervical intraepithelial lesions. In more than 90% of women the viral DNA and the low-grade colposcopically detectable lesions disappear within one-two years due to cell-mediated immunity, not always associated to seroconversion [6, 7]. Although many infections clear with time, unfortunately a minority of women (10-15%) do not produce a successful immune response and they remain HPV-DNA positive with a stable viral infection [8, 9]. Women persistently infected with high risk HPV strains are at risk of the development of high grade cervical intraepithelial neoplasia (CIN) and invasive cervical cancer (CC). All cases of CC are attributable to persistent HPV infections, leading to the conclusion

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that it is a necessary, but not a sufficient, cause of the disease [10, 11].

Cervical cancer is, worldwide, the third most common malignancy in women and the seventh overall with an estimated 530 000 new cases in 2008. More than 85% of the global burden occurs in developing countries, where it accounts for 13% of all female cancers [12]. In Italy estimates indicate a total of 3418 new cases diagnosed every year with an incidence rate of 9.8 new cases per 100 000 females, remaining a considerable cause of morbidity and mortality causing approximately 1000 deaths yearly [13].

The development of cervical cancer is a long process, leading to pre-cancerous lesions but the progression of disease can be modified through early detection and treatment of cytological abnormalities by established practice of periodical Pap test. Almost all Italian regions have implemented screening programmes based on cervical smear. According to PASSI study 2011, following organized programmes and spontaneous activity, 73% of women between 25-64 years-old have had a Pap smear performed in the previous 3 years [14].

Cervical cytology analysis is a highly effective and established practice in many developed countries but HPV-DNA testing has consistently been shown to be superior in terms of sensitivity and positive predictive value. Screening protocols will therefore necessarily change and, in near future, HPV-DNA detection and Pap test will be closely associated [15].

Recent release and implementation of two prophylactic HPV vaccines (the bivalent against high-risk types 16 and 18 and the quadrivalent directed also against 6 and 11 low-risk types) will similarly lead to revise the attitude of preventing cervical cancer. HPV is particularly effective in eluding detection by the immune system while during virus replication there is no cell damage or necrosis and subsequently absence of inflammation. The lack of viraemic phase contributes to the generation of a weak and inconsistent immune response [16]. Clinical trials evaluating existing vaccines were primarily designed to demonstrate as efficacy endpoint the prevention of incident vaccine-related HPV infection and preneoplastic lesions caused by incident persistent infections related to vaccine HPV types. For immunogenicity assessment, several methods were applied measuring different subsets of antibodies induced by vaccination with specific cut-off definitions, precluding direct comparison of serological data. To date no standardized serological assay method has been acknowledged and no immune responses correlating with protection against infection or disease following subsequent natural exposure to HPV have been defined. It is well known that antibody levels induced by natural infection do not protect against reinfection whereas titres elicited by immunization exceed 100-fold those arising from infection [17]. In Italy vaccination against HPV is offered free of charge to all girls in the twelfth year of life since 2008.

Data on immune status against HPV in a general healthy population are very limited. In this study, we investigated the presence of antibodies against HPV 6, 11, 16, 18 types in a large sample of female population living in Ferrara, a province of the Emilia-Romagna Region (North-East Italy), composed of teenagers and young women.

METHODS

Study population

Serum samples were collected at the laboratory analysis of the S. Anna University Hospital of Ferrara from October to December 2011. At the end of routine analytical sessions, all tubes containing an adequate amount of surplus serum were selected and samples from children of both sexes from 1 to 10 years and female subjects aged 11 to 26 years were systematically harvested. All sera were rendered anonymous and were identified by a code. No additional information about vaccination against HPV, health status, use of drugs, lifestyle and sexual behaviour was gathered. Sera from immunocompromised individuals and repeat sera from the same individuals were excluded. Selected specimens were harvested in two aliquots which were stored at -20 °C until analysis. The study protocol was approved by Local Ethics Committee.

HPV antibody detection

Total anti-HPV immunoglobulin G (IgG) antibodies detection was performed by enzyme linked immunosorbent assay ELISA using a commercial kit supplied by DRG Diagnostic GmbH (Germany). The plate microwells were coated with virus-like particles derived from L1 protein of viral types 6, 11, 16 and 18 expressed in *Saccharomyces cerevisiae*. All the assays were performed solely with one lot of the ELISA kit within its declared shelf life period. After dilution 1:100, samples were analysed according to manufacturer's recommendations using a continuous and automatic in line analysis device (BEP2000 Advance® System, Siemens Healthcare, Germany). According to the manufacturer's instructions, cut-off value was defined as optical density (OD) of negative control plus 0.250. Seropositivity was defined as immune status ratio (ISR) parameter calculated as the ratio between sample OD and cut-off: a serum sample was considered positive if ISR value was ≥1, if ISR value was <1 the sample was considered negative. For quantification of antibodies concentration (expressed as EU/ml) the ratio between sample OD and positive control OD multiplied by 100 was applied. The obtained parameter was suitable for expressing geometrical mean titres (GMT). The concentration of positive control was set to be equal to 100 EU/ml.

Statistical methods

All data were recorded in a database file using Microsoft Excel 2007. Seroprevalence data was summarized as percentages, and positive antibody titres were presented as geometric means. Differences between percentages were assessed by the Chi square test, whereas differences between geometric mean titres were assessed by Student's t-test of logarithmically transformed values. Statistical analysis was performed using StatView® 5.0.1 software (Abacus Concepts, Berkeley, CA, USA). Statistical significance was set at $p < 0.01$ for all tests.

Table 1
Human papilloma virus (HPV) seroprevalence

Age group (mean ± sd)	Presence of HPV antibodies		
	Negative (ISR < 1) N (%)	Positive (ISR ≥ 1) N (%)	Total N (%)
1-10 years (5.4 ± 3.1)	261 (100.0)	0 (0.0)	261 (32.7)
11-18 years (15.2 ± 2.1)	124 (45.9)	146 (54.1)	270 (33.8)
19-26 years (22.1 ± 2.3)	245 (91.8)	22 (8.2)	267 (33.5)
Total	**630 (78.9)**	**168 (21.1)**	**798 (100.0)**

ISR: immune status ratio.

RESULTS

A total of 798 sera were collected from a cross-section of subjects 1-26 years old divided into three groups, as summarized in *Table 1*, according to age: the control group was composed of 261 subjects of which 110 boys and 151 girls, aged between 1 and 10 years; the second group consisted of 270 girls aged between 11 to 18 years (mean age 15.2) and the last group included 267 young women aged 19 to 26 years old (mean age 22.1). All samples belonging to the control group yielded no antibodies titres. On the whole, sera found to have HPV antibodies were 168. More than half (54.1%) of girls between 11 and 18 years were positive against a rate of 8.2% reported in the older age group. Seroprevalence against 6, 11, 16 and 18 HPV types was remarkably higher in teenagers than in young women with a statistically significant difference ($p < 0.0001$).

The levels of total IgG anti-HPV are distributed in a rather wide range that varies from a minimum of about 14.5 EU/ml in both groups to 130 EU/ml in older girls up to over 200 EU/ml in adolescents. Girls from 11 to 18 years old showed a GMT of 68.9 EU/ml (95% CI, 65.7-72.0 EU/ml) reaching values of almost 2.5 times higher than in young women with a GMT of 29 EU/ml (95% CI, 18.9-39.0 EU/ml), showing a statistically significant difference ($p < 0.0001$).

The trend of the antibody titres by single year of birth is shown in *Figure 1*: 11 year old girls have been excluded because, at the time of the serum collection, they represented the cohort being actively called for HPV immunization without having yet received all scheduled doses. The lowest concentration of immunoglobulin was detected in over 18s while the highest level was found in 12 year old girls. From 13 to 16 years, a de-

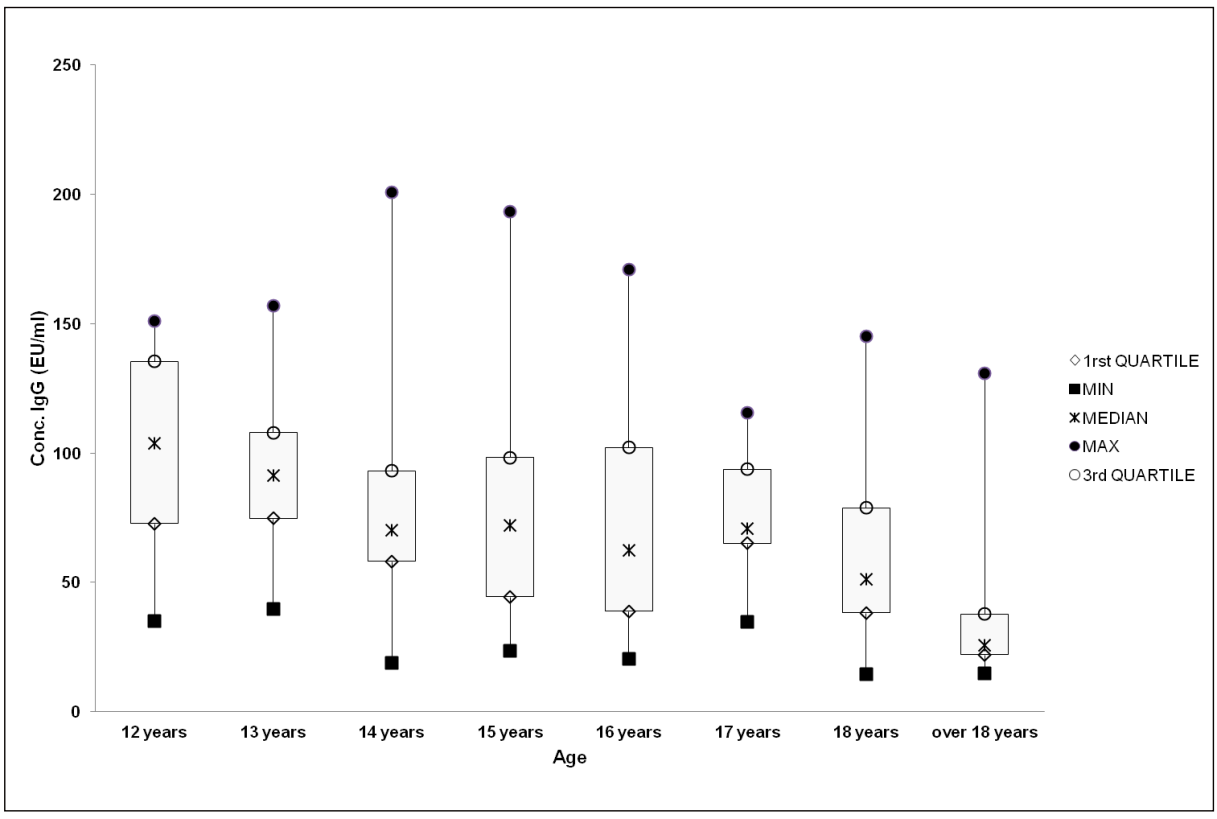

Figure 1
Age-specific trends in anti-HPV IgG concentration.

creasing tendency of anti-HPV IgG median titres has been evidenced, while at the same time, these birth cohorts evidenced that single values are distributed in a very wide range with peaks markedly elevated, whereas a slight increase was recovered in 17 year olds.

When considering seroprevalence and GMT of total IgG anti-HPV, the group of adolescents were further divided into two subgroups based on membership to the birth cohorts covered by the active offer of anti-HPV vaccination *(Table 2)*. Overall seropositivity considering young people invited for immunization (aged 12 to 15 years) reached 72.8% compared to only 37.9% of girls aged 16-18 years ($p < 0.0001$). Teenagers showed an increasing trend in seroprevalence, starting from over half of the 12 year old girls suddenly reaching more than 70% in following birth cohorts with a peak of 80% in 15 year old girls. These values are comparable and consistent with data on vaccination coverage concerning the local health unit of Ferrara released by the Emilia-Romagna Region and updated to 31 December 2011 [18]. In following age groups, seroprevalence considerably decreased to 49% at 16 and gradually reduced to 26.5% in 18 year old girls. In all birth cohorts of teenagers seroprevalence was considerably higher compared to the over 18's showing a statistically significant difference ($p < 0.0001$). The highest levels of total IgG anti-HPV (92.3 EU/ml) were detected in 12 year old girls who should have just completed 3 doses of vaccination. GMT remained very elevated even in the next birth cohort but following values were found to progressively decrease with the exception of 17 year old girls that showed a value of 74.6 EU/ml. Statistical comparison has revealed a significant difference between each of the birth cohorts of adolescents and girls over 18 ($p < 0.0001$ in all comparisons, except 18 year old-over 18 $p = 0.0037$) and also when the two adolescents subgroups were separately compared with over 18 girls ($p > 0.0001$).

Table 2
Serology results in positive samples by age

Age (years)	Positive (ISR ≥ 1) (%)	EU/ml GMT (95% CI)
12-15	**72.8**	**74.7 (66.9-82.5)**
12	55.6	92.3 (67.1-117.6)
13	72.7	85.8 (72.7-98.9)
14	74.3	69.2 (54.7-83.6)
15	80.0	66.6 (52.6-80.6)
16-18	**37.9**	**61.6 (51.3-71.8)**
16	49.0	60.5 (44.3-76.6)
17	37.5	74.6 (61.3-87.9)
18	26.5	53.5 (31.8-75.3)
Over 18	**8.2**	**29.0 (18.9-39.0)**

ISR: immune status ratio.
GMT: geometrical mean titres.

DISCUSSION

Seroepidemiological surveys on HPV have mainly considered illness conditions like several sites cancers and precancerous lesions [19-21] or particular populations considered at risk as HIV positive subjects, men having sex with men, sex workers [22-27]. The studies that took into consideration the general healthy population [28-32] or the effect of immunization [33, 34] used various approaches and diverse methods. This makes it impossible to carry out comparisons of seroprevalence among age groups and countries.

Previous investigations on HPV serology on Italian women have only considered pathological conditions, using a in-house ELISA method based on five recombinant HPV16 proteins. The first one [35] involved women with abnormal PAP smear and demonstrated that the assay was able to detect antibodies in patients infected with HPV16 and it was not genotype-specific as it could discover antibodies also in women infected by other genital HPVs. The same test was successfully applied in evaluating antibodies in HIV-positive women [24], showing that no significant correlation could be found between HPV DNA detection and seropositivity.

The present study represents the first application of a standardized ELISA test able to assess antibody levels against all HPV strains contained in the preparation of the quadrivalent vaccine. As reported in a survey on the Australian population [36], no cases of HPV seropositivity were detected in children above 10 years of age. Teenagers showed seroprevalence rates and antibodies titres considerably higher than young women. Unfortunately in absence of information about sexual behaviour and vaccination, this finding can only be assumed as the result of the extensive immunization, since in birth cohorts affected by the free offer of vaccine higher GMTs have been detected. The subsequent peak in seroprevalence recorded in 17 year-old girls may be put in relation with the beginning of sexual activity. Low seroprevalence rate and antibody titres in young women, on the contrary, may be the result of a drop below detection threshold in the absence of a persistent antigenic exposure in subjects with a previous natural infection. Long-term persistent HPV infection tends to yield polyclonal immunoglobulin G serological reactivity, which can be measured by using the ELISA technique based on type-specific virus-like particles (VLPs) as antigens [37]. Although the specificity of HPV VLP ELISA is reasonably adequate at over 90%, its sensitivity is relatively low [38]. According to a relatively recent study [39], intra- and inter-assay performance variation is difficult to control, especially in cohort studies that require the testing of specimens over extended periods. Being mindful of this, we set out to standardize the VLP ELISA protocol that we used in the present prevalence study.

The use of a commercially available technique, although not able to discriminate antibodies produced by the single viral strain, that employs a well-known technique as the ELISA assay we adopted has several advantages: first, the ability to carry out the determination on a large number of samples; second, it is an easy-to-use technique which is suitable to automation and standardization; third, it does not require over-expensive investments; fourth, it provides the opportunity to reveal the immune status of the population and then

to be able to follow over time, with repeated measurements, the effects of vaccination campaigns, as well as of natural infection.

HPV serology has not been used yet in the clinical setting because of the variety of techniques and biological limitations, moreover sensitivity is relatively low [38]. Most studies relied on the detection of HPV DNA demonstrated that mucosal DNA does not persist in most persons after infection [40] and attempts to detect it are prone to sampling error. In contrast, the results of serological assays appear to better correlate with cumulative post-exposure than DNA-based approaches [41]. The implementation of a standardized and commercially available assay as the ELISA method we adopted would be certainly valuable in broadening knowledge on the natural spread of infection. Therefore a further study taking into account women belonging to older age groups as well as the male population aimed to describe HPV epidemiology and identify groups at greatest risk will be useful.

On the level of public health, a wide seroepidemiological survey represents an important tool in guiding prevention programmes identifying best age groups for immunization because HPV exposure in a population may greatly differ depending on the sexual behaviour of its members and, in the future, will contribute to the monitoring of vaccinated women measuring the duration of vaccine induced-protection and would potentially enable the assessment of whether booster vaccinations are required. Although many prevention strategies are available for cervical cancer, continued public health efforts should be focused on increasing vaccine coverage in the target age groups and cervical cancer screening for women at appropriate intervals with suitable method, considering the need of additional evaluation to ensure appropriate use of health resources, as the vaccinated women will become eligible for screening at a later date.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationship with other people or organizations that could inappropriately bias conduction and findings of this study.

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