Detection of 14 human papillomavirus genotypes in cervical samples in women from a central-southern area of Italy showing different Pap test results

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INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide. Since human papillomavirus (HPV) was first associated with genital lesions (Zur Hausen, 1976), viral DNA has been found in most cervical cancers and today is recognised as a necessary cause for its development (Parkin et al., 1999; Walboomers et al., 1999; Zur Hausen 2008). Although HPV infection is not characterised by overt clinical appearance in most cases, it has been frequently associated with precancerous or cancerous lesions of several body districts such as head and neck, skin, colon and, mainly, cervix (Walboomers et al., 1999; Gillison et al., 2004).

A major role in infectious agent transmission, not limited to the genital lesions, is characteristically played by the patient’s sexual activity, the most important risk factors for HPV infection being an increased number of sex partners (Tarkowski et al., 2004), age at first intercourse and sexual habits.

More than 100 HPV genotypes have been identified and classified on the basis of oncogenicity as low (e.g. HPV6 and 11), intermediate (e.g. HPV31, 33, 35, 51, 52 and 58) and high risk (e.g. 16 and 18), even though in routine clinical practice in-
Intermediate and high risk genotypes are commonly grouped together (Munoz et al., 2003; Castellsagué, 2008).

Until recently, exfoliative cytology (i.e. Pap test), successfully employed in disclosing premalignant as well as malignant epithelial cervical lesions with very low cost-benefit ratio, has been considered the golden standard procedure for cervical cancer screening.

Its regular application, moreover, has led to a significant reduction in the incidence and mortality from cervical cancer in developed countries. With a regular Pap test screening program, the risk of cervical cancer can be reduced up to 90% in a few years (Berg et al., 2004). In addition to this unique diagnostic tool, several molecular tests are now available for the identification of HPV DNA, showing higher sensitivity and higher reproducibility than traditional cytology.

Since some viral genotypes show heterogeneous association and/or cluster distribution even in the same geographical area (Munoz et al., 2003; Vaccarella et al., 2006), knowing HPV genotypes distribution could be very important for the development of new specific vaccines; unfortunately, these data are currently not available.

The aim of this observational study was to evaluate how cytological characteristics shown in Pap test correlate to prevalence of HPV infection in a group of women from a well defined area of central-southern Italy (Molise region). HPV genotype frequencies and possibility of obtaining additional information by combining molecular and morphological examinations were also studied. Results are compared with literature data obtained in other Italian geographical areas.

MATERIALS AND METHODS

Patients

In the period May 2006-December 2007, 364 women (mean age 37.4±8.9; range 21-64 years) referring for routine control gynecology examination (including Pap test and HPV molecular test) to the Outpatient Clinics of the Catholic University of Sacred Heart Medical Centre in Campobasso (Italy, Molise region) entered the present study.

Both analyses were performed at the Department of Laboratory Medicine and Pathology (UNI EN ISO 9001:2000 certification number: 9122.UCSC; expiration: 2010) of the medical centre.

Molecular analysis

Cervical specimens for HPV DNA were collected by endocervical brushing and stored in phosphate buffered saline (PBS) at -20° C until testing. Total DNA extraction was carried out with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

A validated in-vitro diagnostic (IVD) test was used to amplify the HPV DNA in the samples (HPV Low and High Risk Typing amplification kit, Nuclear Laser Medicine, Milan, Italy) according to the manufacturer's instructions. The kit is designed to selectively amplify the DNA of 14 HPV genotypes (2 low risk: 6 and 11; 12 high risk: 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66) in multiplex PCR reactions, using four primers mixtures. Three of the primer mixtures simultaneously amplify a 723-bp fragment of the human beta-globin gene, which serves as an internal control (amplification efficiency).

Amplification was performed on a Biometra T1 thermocycler (Biometra, Goettingen, Germany). Amplicons were run on a 3% agarose gel together with a molecular weight ladder and, after ethidium bromide staining, visualized under UV light. By using a scheme included in the kit, detection of each amplicon specific length (ranging from 227 to 520 bp) allows a correct identification of each genotype.

Morphological assessment of cervical lesions

Cervical smears were automatically stained according to Papanicolau method using a Leica ST5020 multistainer workstation, mounted by means of a Leica CV5030 robotic glass coverslipper (Leica Microsystems, Wetzlar, Germany) and examined under a microscope.

Diagnostics were done following the original Richart criteria (Richart, 1967), integrated by Bethesda modifications (Natl. Canc. Inst. Workshop, 1989). Briefly, cases were classified as normal when cellular abnormalities and/or koilocytosis were not observed.

When mild nuclear alterations and/or koilocytes were observed, the case was classified as low grade squamous intraepithelial lesion (LSIL). When moderate to severe nuclear abnormalities
(dyscaryosis), with or without koilocytosis, were present, cases were classified as high grade squamous intraepithelial lesion (HSIL). When cases showed atypical squamous or glandular cells of undetermined significance, they were classified ASCUS or AGUS, respectively (Bethesda Workshop, 1993). Cervical smears were all considered adequate on the basis of amount and distribution of material and presence of endocervical cells.

Data retrieval and statistical analysis
Data concerning pathology as well as other laboratory investigations, i.e. cases identification codes, date of examination, test result codes, were retrieved and stored in an Excel spreadsheet file (Microsoft Corporation, Redmond, WA, USA). In pooling data, subjects' identities were omitted and results were referred only to accession numbers given in the laboratory. Thus, the data resulted completely anonymous.

Analysis of variance and Student t-test were adopted in comparing means among or between different patient groups, respectively. Frequencies in different groups were compared by Pearson chi-square analysis. Probabilities equal or greater than 5% (p ≥ 0.050) were considered not significant.

RESULTS
Table 1 shows the Pap test results of the 364 women, in three different age groups. Two hundred and twenty patients (60.4%) showed normal results, the vast majority being comprised in age group of 30 years or over (180 out of 220; 81.8%). Conversely, about one third of women under 30 years of age (24 out of 76, 31.6%) presented a HSIL Pap test result. Distribution of cytological features frequencies among age groups, however, showed a significant difference (p=0.011). Interestingly, patients with HSIL Pap test result were significantly younger than those with normal cytology (35.1±7.7 and 38.1±9.3, respectively; p=0.009).

Table 2 shows the prevalence of HPV DNA test results in the same three age groups as in Table 1. We observed 128 positive tests (35.2%), among which 91 (71.1%) showed single and 37 (28.9%) multiple genotype infections, with an overall infections number of 176 (see Table 3). It is noteworthy that HPV positive women were significantly younger than HPV negative ones (35.9±8.4 and 38.2±9.1, respectively; p=0.018). Among HPV positive women, those with multiple genotypes infections were significantly younger than those with single genotype infections (31.7±6.9 and 37.6±8.3, respectively; p=0.0002).

In order to compare the sample with the Molise region population, the resident population on 1st January 2006 was considered (ISTAT, 2006). At that date, 320907 subjects resulted resident in the Molise region, of which 156387 were males (49.0%) and 164520 were females (51.0%). Thus, we studied 0.22% of the overall female population in Molise.

The most common HPV genotype, as shown in Table 3, was HPV16 with an overall frequency of 40.6%, followed by 20.3% HPV6 and 13.3%

<table>
<thead>
<tr>
<th>Pap test</th>
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<tbody>
<tr>
<td>Age groups (years)</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>&lt;30</td>
</tr>
<tr>
<td>30-55</td>
</tr>
<tr>
<td>&gt;55</td>
</tr>
<tr>
<td>All cases</td>
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<tr>
<td>Mean age±SD*</td>
</tr>
</tbody>
</table>

No case of AGUS was present in this group of women. Pearson chi-square in comparing results among all frequencies: p=0.011. *Analysis of variance among all mean ages: p=0.033. **Student-t test between mean ages of normal and HSIL patient groups (a total of 304 observations): p=0.009.
HPV56. HPV18 was present only in 5.5% of infections, never associated with HPV16. The same table also reports the distribution of specific genotypes in single or multiple infections. In order to evaluate the trend of different genotypes to be present alone or in combination, the ratio between single and multiple infections was calculated. The highest ratios were found for HPV39, HPV6 and HPV16 (2.50, 2.25 and 1.89, respectively). HPV11 was found only in two women, but never in combination with other genotypes. On the contrary, the lowest ratios were observed for

### TABLE 2 - Prevalence of HPV negative and HPV positive women in different age groups.

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>DNA test</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>HPV – N (%)</td>
</tr>
<tr>
<td>&lt;30</td>
<td>76 (20.9)</td>
<td>43 (56.6)</td>
</tr>
<tr>
<td>30-55</td>
<td>278 (76.4)</td>
<td>185 (66.6)</td>
</tr>
<tr>
<td>&gt;55</td>
<td>10 (2.7)</td>
<td>8 (80.0)</td>
</tr>
<tr>
<td>All cases</td>
<td>364 (100)</td>
<td>236 (64.8)</td>
</tr>
</tbody>
</table>

Mean age±SD

<table>
<thead>
<tr>
<th>Overall</th>
<th>HPV –</th>
<th>HPV +</th>
<th>Single</th>
<th>Multiple</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.4±8.9</td>
<td>38.2±9.1</td>
<td>35.9±8.4</td>
<td>37.6±8.3</td>
<td>31.7±6.9</td>
</tr>
</tbody>
</table>

Percentage values of HPV negative or positive cases are calculated as the proportion of total patients in each age group whereas percentage of single or multiple infections are calculated as proportion of HPV + patients only, in each age group. °p=0.018; *p=0.0002.

### TABLE 3 - Prevalence of the 14 different HPV genotypes detected by the kit used in single and multiple infections in 128 women positive for at least one HPV specific genotype.

<table>
<thead>
<tr>
<th>HPV genotype</th>
<th>Specific genotype infections (N)</th>
<th>Overall prevalence (%)</th>
<th>Single genotype infections (N)</th>
<th>Multiple genotypes infections (N)</th>
<th>Single/multiple infections ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>52</td>
<td>40.6</td>
<td>34</td>
<td>18</td>
<td>1.89</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>20.3</td>
<td>18</td>
<td>8</td>
<td>2.25</td>
</tr>
<tr>
<td>56</td>
<td>17</td>
<td>13.2</td>
<td>8</td>
<td>9</td>
<td>0.89</td>
</tr>
<tr>
<td>31</td>
<td>15</td>
<td>11.7</td>
<td>5</td>
<td>10</td>
<td>0.50</td>
</tr>
<tr>
<td>58</td>
<td>10</td>
<td>7.8</td>
<td>3</td>
<td>7</td>
<td>0.43</td>
</tr>
<tr>
<td>45</td>
<td>9</td>
<td>7.0</td>
<td>1</td>
<td>8</td>
<td>0.13</td>
</tr>
<tr>
<td>59</td>
<td>9</td>
<td>7.0</td>
<td>4</td>
<td>5</td>
<td>0.80</td>
</tr>
<tr>
<td>52</td>
<td>8</td>
<td>6.2</td>
<td>4</td>
<td>4</td>
<td>1.00</td>
</tr>
<tr>
<td>39</td>
<td>7</td>
<td>5.4</td>
<td>5</td>
<td>2</td>
<td>2.50</td>
</tr>
<tr>
<td>18</td>
<td>7</td>
<td>5.4</td>
<td>3</td>
<td>4</td>
<td>0.75</td>
</tr>
<tr>
<td>66</td>
<td>6</td>
<td>4.6</td>
<td>2</td>
<td>4</td>
<td>0.30</td>
</tr>
<tr>
<td>33</td>
<td>4</td>
<td>3.1</td>
<td>2</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>35</td>
<td>4</td>
<td>3.1</td>
<td>0</td>
<td>4</td>
<td>0.00</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>1.5</td>
<td>2</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4 shows a correlation between HPV DNA test results and cytological classification. It is evident that HPV infection distribution correlates with cytological findings (p=0.00001: 93.6% women with normal Pap test showed negative DNA test, whereas 91.7% of HSIL cases showed positive DNA test. The vast majority of HPV positive cases were represented by high risk genotypes (108 out of 128 cases, 84.4%). By grouping women on the basis of the diagnostic kit design, high risk genotypes were more frequently represented in HSIL group (62.0%). Women with high risk HPV genotype infection (n=108) were significantly younger than those with low risk infection (n=20) (mean ages 35.1±7.7 and 40.5±10.5, respectively; p=0.008). Detailed analysis of HPV positive women, however, did not show significant differences when considering all four cytological classes or limiting the analysis to LSIL and HSIL cases (p=0.23 and 0.76, respectively). On the contrary, interesting results were found when grouping HPV positive women according to HPV 16 and HPV 18 presence or absence. More than 70% women positive for HPV 16 or 18 showed HSIL (p=0.003 among all groups; p=0.0002 in comparing HSIL to LSIL). Interestingly, 14 out of 220 women with normal Pap test resulted positive at DNA tests (6.4%), 11 of which, moreover, showed one or more high risk HPV genotype(s), including HPV16 or 18 in 7 cases.

**DISCUSSION**

HPV DNA is present in the vast majority of cervical cancers (Zur Hausen, 2008) and is strictly related to the onset and progression of the tumor. Moreover, the recent introduction of vaccination against HPV as a preventive measure for cervical cancer (Zur Hausen, 2008) has increased the studies both on HPV infection worldwide diffusion and on HPV genotypes prevalence in different geographic areas, with the aim of better designing the vaccine composition. As a consequence, in the past decade the approach both to diagnosis and to screening programs has been revisited, progressively adding HPV DNA identification to traditional cytology. Data concerning the relationship between HPV infection and Pap test results are, in general, quite discordant in the literature, probably because of different criteria in study population enrolment (age, disease absence or presence, etc.), different molecular methods (including number of screened genotypes) and inter-observer variability in Pap test microscopic examination (Llewellyn, 2000). Even in the same country, such as in Italy, heterogeneous results are frequent (Centurioni et al., 2005; De Francesco et al., 2005; Ronco et al., 2005; Crapolicchio et al., 2006; Gargiulo et al., 2007; Ammatuna et al., 2008; Capra et al., 2008; Del Prete et al., 2008; Menzo et al., 2008). Our data from Molise region show a 35.2% prevalence of HPV infection, close to that...
obtained in a similar study from Puglia, a neighboring area of Southern Italy (30.4%) (Crapolicchio et al., 2006). Interestingly, data from several studies carried out in Northern areas of Italy show a much lower HPV infection prevalence ranging from 6.6% in Brescia (De Francesco et al., 2005) to 15.9% in Genoa (Centurioni et al., 2005). In addition to the above reasons concerning this heterogeneity, these findings could be also explained by different individual behaviour (Menzo et al., 2007).

Despite this variability, HPV16 is widely confirmed as the most frequent genotype and HPV16/18 as strongly associated with HSIL (Table 4) (Spano et al., 2005; Del Mistro et al., 2006; Menzo et al., 2007).

Although in our study cohort the youngest patient was 21 years old, our data are in agreement with literature in that HPV positive women are significantly younger than HPV negative ones. The incidence of HPV infection declines from 43.4% to 20.0% while age increases (Table 2); interestingly, a parallel decline of HSIL cases is also observed (Table 1).

Furthermore, women with multiple infections are even younger than those presenting a single genotype in cervical samples. It is noteworthy that analysis of ratio between single and combined infections for each HPV type (Table 3) seems to show higher propensity for some genotypes to infect as single agent: the higher the ratio, the higher the genotype propensity to infect as an individual agent, such as for HPV39, 6, 16 and 11. On the contrary, lower ratios seem to indicate a tendency to infect together with one or more different genotypes.

However, other conditions, such as host immunological status, number and frequency of partners, could facilitate co-infections. This point is not worthless and might play an important role in vaccine design strategy. In our opinion, specific genotypes overall prevalence among infections in a defined geographical area is more relevant than genotype combinations.

In a significant number of HPV DNA negative cases (30 out of 236 patients; 12.7%), we found morphological alterations (Table 4). This apparently conflicting result might be explained by the absence, in the diagnostic kit, of the HPV genotype(s) present in that case (false negative DNA test result). However, it is possible that at least in some cases the lesion is not HPV-related (true negative DNA test result).

Cases showing HPV positive test and normal Pap test deserve some consideration, too. In the normal Pap test group (n=220) we found 14 HPV positive tests (6.4%), frequently with high risk genotypes (Table 4). The 14 cases were cytologically re-evaluated and confirmed to be normal, because neither significant nuclear alteration nor convincing koilocytosis were found. Apparently the virus was present in normal looking squamous cells.

This finding might be explained by suggesting that, in the early stage of HPV infection, no defined koilocytosis and/or nuclear changes have yet occurred. Furthermore, it is well also known that HPV infections, especially in young women, are often self-limited and without cytologic sequelae. Host genetic control and/or immunological resistance to viral oncogenicity should be taken into account when considering all these observations.

Lack of cytological lesions in HPV cervical infections, moreover, is not surprising and is widely reported in the literature, although with some differences. Oh et al., for example, reported a rate of HPV positive test in normal looking cervical smears as low as 0.7% (8 out of 1144) (Oh et al., 2006), although in that case only four genotypes were investigated (HPV16, 18, 31 and 33). Gupta et al. found 86 HPV16/18 infections among 769 cytologically negative women (11.2%) (Gupta et al., 2001).

A recent meta-analysis on 157879 women showed that, although in variable proportions across world regions, different HPV genotypes can be identified in cervical samples with normal cytology (de Sanjosé et al., 2007). It is noteworthy that PCR detection of HPV16/18 in cutaneous warts of immunocompetent subjects is not associated with dysplastic changes at histological level (Payal et al., 2006), likely due to the different epithelial structure and immunologic status of skin and cervical mucosa. In situ hybridization studies and sequential cytological examinations might help to address this interesting point in women with positive molecular results and negative Pap tests. In conclusion, we confirmed that HPV infection does not always parallel morphology in cervical cytology, even in the presence of high-risk HPV. Consequently, there is a percentage of cases...
where PAP test is not able to ascertain risk of disease progression. For this reason, HPV molecular testing should always be proposed to improve the accuracy of cytological diagnosis and to select those cases that deserve further investigation and/or a more frequent follow-up (Stoler, 2003; Mayrand et al., 2007; Nauleer et al., 2009). Furthermore, a molecular fingerprint such as that obtained by means of HPV DNA typing is required for identification of HPV type eventually present, for the information about number and combination of HPVs and, especially in young women, for the assessment of persistence and/or change in HPVs grouping during the follow-up with a reasonable cost benefit ratio.

Lastly, on the basis of the results presented in this paper, a public health policy focused on young people and aimed at awareness and a local prevention campaign should be strongly encouraged.

REFERENCES


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