Molecular detection and incidence of human papillomavirus in neonates: Methodology and a pilot study in a medical center

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Neonate;
Polymerase chain reaction

Background/Purpose: Human papillomavirus (HPV) infection can cause laryngeal papillomas in children. Vertical transmission has been confirmed. This study aimed to establish a sensitive molecular diagnostic method and understand the incidence of the HPV-6 and HPV-11 in neonates with intubation.

Methods: We enrolled 108 newborns between October 2007 and January 2010. All neonates were intubated due to underlying disease. The specimens were collected via endotracheal aspiration. DNA of HPV types 6 and 11 was detected by real-time PCR and nested PCR.

Results: HPV-DNA was detected in eight of the 108 newborns studied. Seven respiratory specimens tested positive for HPV-11 and one was positive for HPV-6. The HPV 6/11 detection rate in neonates was 7.4% (8/108).

Conclusion: A rapid, sensitive, specific, and reproducible RT-PCR method and nest PCR were developed for the detection and differentiation of HPV-6 and HPV-11 genomic variants in a single PCR reaction. The assays are of great value for clinical and epidemiologic studies of HPV-6 and HPV-11 infections. Neonatal HPV colonization may be related to juvenile-onset recurrent respiratory papillomatosis. The transmission route may be from mother to child. The clinical significance of neonatal carriage of HPV-6 or HPV-11 warrants further study.

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Introduction

Human papillomavirus (HPV) is a well-known cause of anogenital warts and cervical cancer. In children, HPV is associated with laryngeal and conjunctival papillomas.\(^1,2\) HPV is mainly transmitted through sexual routes, but nonsexual route transmission is also possible.\(^3\) This includes mother–child transmission during the perinatal period and casual physical contact.\(^4\) Vertical transmission may occur when an infant passes through an infected birth canal, through ascending infection, at the time of sperm fertilization, or hematogenously.\(^3,5,6\) However, these observations have yet to be well-established.

HPV-6 and 11 are responsible for recurrent respiratory papillomatosis (RRP), a disease with a peak incidence between 2 and 4 years of age.\(^7\) Several studies showed that most children with RRP were born to mothers who had history of genital condylomata based on clinical observation.\(^8\)\(^9\) This cross-section and prospective study aimed to evaluate the incidence of HPV in newborns with intubation. We used the molecular method to detect the tracheal aspirate of neonates and discussed the risk of RRP in neonates with HPV colonization.

Patients and methods

Population

Between October 2007 and January 2010, 108 neonates were enrolled in National Taiwan University Hospital. The Taiwan Association of institutional review board had approved

![Figure 1. Alignment of HPV6 and HPV11 E6 genes. The figure shows the E6 gene sequence of HPV 6 and 11 (GenBank accession number: FM87037, FM875943, FM875980, PPH11, EU918768, ->PPHL1E671V). The thick black arrows point to the common sequence of HPV 6 and 11. The thin black arrows point to the specific sequence of HPV6 and 11.](image-url)
the research. All parents signed an informed consent form before their children were entered into the study. All neonates were younger than 30 days of age and had been intubated due to underlying illness (such as congenital heart disease, prematurity, respiratory distress, and perinatal asphyxia). Respiratory specimens were collected by tracheal aspiration through an endotracheal tube. Neonatal gestational age, comorbidity, and maternal HPV status were not studied in this study.

Specimens

Before specimens were collected, a chest X-ray was performed to confirm that the endotracheal tube was positioned properly. A 6.5 French sterile suction catheter was then inserted through the endotracheal tube. One-3 mL room-temperature 0.9% saline was installed and aspirated back into a collecting chamber. Warm saline was used instead for neonates with very low birth weight. The above procedure was repeated three consecutive times if tolerated. The specimen was then kept frozen at −80°C until DNA could be extracted.

Laboratory methods

Virus and nucleic acid extraction

Nucleic acid was extracted from tracheal aspirates using a Magna Pure LC 2.0 instrument (Roche Molecular, Mannheim, Germany).

Real-time PCR conditions

DNA of HPV-6 and 11 was detected by real-time polymerase chain reaction (PCR) using HPV type-specific primers based on the HPV E6 genes (quantitative HPV type-specific PCR). The sequences were retrieved from GenBank (Fig. 1). Primers sequences for the HPV-6 were E6-159 5'-CCTgTTTCgAggCgCTATCCAT-3' and E6-377R 5'-ACCgTgCCTTggTTAgTATgTg-3'. The primer of HPV-11 were E6-159F 5'-TgTgTggCgAgACAACTTTCCCT-3' and E6-377R 5'-gCgTgCCTTTCCCAATgTgC-3'. (Table 1). A protocol of 50 cycles of PCR amplification was performed at 95°C for 5 seconds, 58°C for 10 seconds, and 72°C for 10 seconds using

<table>
<thead>
<tr>
<th>Table 1 Primers sequence used for real-time PCR detection of HPV 6 and 11 types</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target type</strong></td>
</tr>
</tbody>
</table>
| HPV 6 | Forward: E6-159 5'-CCTgTTTCgAggCgCTATCCAT-3'  
Reverse: E6-377 5'-ACCgTgCCTTggTTAgTATgTgT-3' |
| HPV 11 | Forward: E6-159 5'-TgTgTggCgAgACAACTTTCCCT-3'  
Reverse: E6-377 5'-gCgTgCCTTTCCCAATgTgC-3' |

Figure 2. The standard curve of HPV6 real-time PCR. Amplification plots of serially diluted HPV-6 corresponding to an input of 4 × 10^0, 4 × 10^1, 4 × 10^2, 4 × 10^3, 4 × 10^4, 4 × 10^5, and 4 × 10^6 viral copies per assay. Values on the y-axis show the fluorescence dependent on the PCR cycle number on the x-axis.
LightCycler RNA Amplification Kit SYBR Green I (Roche) and carried out in LightCycler 1.5 (Roche).

Nest PCR conditions

The assay was performed in two steps, including first round and second round PCR. The first round PCR was designed to amplify the common sequence of HPV-6 and HPV-11 E6 gene. The second round PCR was designed to identify the specific sequence of HPV-6 and HPV-11, respectively, within the first PCR product. The first round primers were HPV6&11-E6-35F (5' CYATAgACCAgTTgTgCAAg 3') and HPV6&11-E6-431R (5' CATgCATgTTgTCCAgCAGTgT 3'). The second round primers were the same primers used in real-time PCR, as described earlier (Table 1). The visualization of the amplified product was carried out using electrophoresis with 2% agarose gel (Onestar, Taiwan).

Results

Detection limits and sensitivities of real-time PCR assays

The fluorescence values versus cycle number are displayed in Figs. 2 and 3. $4 \times 10^1$ copies of the DNA of HPV-6 can be reproducibly detected after 33.47 cycles of real-time PCR. The assay detected the same concentration of HPV-11 at 34.25 cycles.

Melting curve analysis

Melting curve analysis of the PCR products identified a unique melting peak for HPV-11 and HPV-6 genomic variants, allowing for unambiguous differentiation. The prototypic HPV-6 variant showed a specific melting peak at 83°C, and HPV-11 showed a specific melting peak at 81.5°C (Fig. 4).

The electrophoresis of real-time PCR and nest-PCR

All positive specimens were visible with gel electrophoresis and southern blot hybridization. Six positive signals were detected in 108 clinical specimens by real-time PCR; five were HPV-11 and one was HPV-6. The nested PCR revealed two more samples with positive sequences of HPV-11. Fig. 5 shows the typical PCR reaction profiles of HPV-11. The figure also shows the positive nested PCR of HPV-11 and the specific sequence (PPH11).

Clinical manifestation

HPV-DNA was detected from respiratory secretion in eight of the 108 newborns who underwent intubation soon after birth. One carried HPV-6 and 7 carried HPV-11. The median

![Figure 3.](image-url)
gestation age was 36 weeks. Of the 108 newborns, 77 were born via Cesarean section.

All the patients with positive HPV result were intubated due to respiratory distress. Six patients had underlying disease including congenital anomaly, trisomy 13, tetralogy of Fallot, transposition of the great arteries, and biliary atresia. Three patients died of underlying diseases. No stridor was noted in any of the five surviving children during the first 2 years of their life; four patients were born via normal spontaneous delivery, and the other four were born via Caesarean section. No genital condyloma was found in any of their mothers before delivery (Table 2).

Discussion

Recent studies have confirmed that HPV may be passed by vertical transmission from mother to child. Renato and others suggested that perinatal transmission of HPV-DNA occurred in 24.5% of delivery. Gajewska and colleagues detected a high rate (70%) of HPV transmission from mother to neonate. HPV DNA has been detected in amniotic fluid, cord blood, and fetuses. These studies demonstrated that the virus can infect children during the perinatal period. In addition to vertical transmission, horizontal spread of HPV can occur from other family members and those in close contact with the child. Marjut and colleagues presented a prospective study about transmission of high-risk HPV between the parents and infant. The study showed that a persistent presence of HPV in the cervix and oral cavity of a mother was associated with a risk of HPV infection of the infant.

In our study, after analyzing samples of tracheal aspirate within the first 30 days of birth, it was observed that eight neonates (7.4%) tested positive for HPV-DNA. This is the first study to evaluate neonatal HPV carriage in Taiwan. The acquisition of HPV DNA was likely vertical from mothers. This study also showed the efficacy of the multiple methodology in HPV DNA detection with real-time PCR and nest PCR, eliminating false-negative results.

The real-time PCR method has a number of advantages over the assays for HPV-6 and HPV-11. The assay has high sensitivity and specificity. The primers were designed on the basis of HPV-6 and HPV-11 E2 sequences, and the assay is considered to be capable of recognizing the broadest spectrum of genomic variants of HPV-6 and HPV-11.

The HPV transmission is including sexual transmitted route, close personal contact with skin or mucus, and nosocomial transmission. Close personal contact is an important transmission route of cutaneous warts, and nosocomial transmission may result from fumes with the infectious virus released from lesion during treatment with carbon dioxide laser or electrocoagulation. The neonates in our study were all intubated due to underlying disease. The specimens were collected from tracheal aspirate. The transmitted route was not above the three routes. Thereafter, perinatal

Figure 4. The real-time PCR melting curve of HPV6 and 11. Melting curve analysis detected amplicons of real-time PCR by SYBR green. Two separately generated melting peaks were separated from the dissociation curve of amplicons. Values on the y-axis represent the ratio of the first negative derivative of the change in fluorescence to the variation in temperature.
transmission was the main route of our patients. Because all of our study participants were admitted to neonatal intensive care unit soon after birth, the transmission route appears to be passage through birth canal or ascending infection. At this point, the route of HPV vertical transmission remains unclear. The routes may exist during pregnancy (transamniotic ascending infection), during labor (ascending infection after the amniotic membranes are ruptured), or during delivery (by the fetus passing through a contaminated birth canal). In our study, four (50%, n = 4/8) patients were

Table 2  Clinical manifestation of neonates with HPV colonization

<table>
<thead>
<tr>
<th>Case</th>
<th>Gestation age (weeks)</th>
<th>Delivery route</th>
<th>Underlying disease</th>
<th>HPV type</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39</td>
<td>C/S</td>
<td>Biliary atresia</td>
<td>11</td>
<td>Survival</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>C/S</td>
<td>Perinatal asphyxia</td>
<td>11</td>
<td>Survival</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>NSD</td>
<td>Tetralogy of Fallot</td>
<td>11</td>
<td>Survival</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>NSD</td>
<td>Congenital anomaly</td>
<td>11</td>
<td>Death</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
<td>C/S</td>
<td>Transposition of the Great Arteries</td>
<td>11</td>
<td>Death</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>C/S</td>
<td>Transposition of the Great Arteries</td>
<td>11</td>
<td>Survival</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>NSD</td>
<td>Perinatal asphyxia</td>
<td>11</td>
<td>Death</td>
</tr>
<tr>
<td>8</td>
<td>34</td>
<td>NSD</td>
<td>Prematurity</td>
<td>6</td>
<td>Survival</td>
</tr>
</tbody>
</table>

NSD: normal spontaneous delivery; C/S: Cesarean-section delivery.
born via normal spontaneous delivery and four patients (50%, n = 4/8) were born via Caesarean section. The transmission routes of those born via normal spontaneous delivery included transamniotic ascending infection, ascending infection after the amniotic membranes were ruptured, or by the fetus passing through a contaminated birth canal. The transmission routes of those born via Caesarean section included all ascending infection routes and excluded the possibility of a contaminated birth canal. These results emphasize that there may be more transmission routes via ascending infection in comparison with a contaminated birth canal. The patient group and specimens differed from other studies; in other studies, specimens were collected from nasopharyngeal aspirate, the surface of the body, genital organs, or arterial blood taken from the umbilical cord.3,11

The presence of HPV in tracheal aspirate signifies that it can colonize in the trachea via the mother-child vertical transmission. In our study, three patients were premature, and the relationship between maternal HPV infection and neonatal prematurity remains unclear. Mamm and colleagues27 provided maternal HPV infection that may be related to neonatal prematurity.

Colonization of HPV in newborns may result in later respiratory papillomatosis and an increased risk of carcinogenic potential. The virus primarily infects epithelial cells. RRP has a variable incubation period, which may be as short as several weeks or as long as several years. It may develop to the subclinical form or reactivate, leading to chromosomal mutations in host cells. The result after this accumulated latent carcinogenic potential of certain types of HPV during childhood would be the development of neoplasm for life. The natural history of papillomavirus infection is characterized by regression in a period that varies from months to years.5,18 In our study, none of the surviving neonates with HPV colonization progressed to RRP. This may have been due to the relatively small amount of cases and short follow-up period.

Currently, two HPV prophylactic vaccines have been developed successfully: quadrivalent and bivalent HPV vaccines. Both vaccines provide protection against persistent infection with high-risk HPV-16 and 18. The quadrivalent HPV vaccine also provides prevention for HPV-6-related and HPV-11-related genital warts and juvenile-onset recurrent respiratory papillomatosis.19–22 The vaccine has no effect on pre-existing genital infections or lesions, but it prevents new genital infections through one of the four vaccine types as well as the epithelial lesions induced by them. Pawliita and colleagues23 hypothesize that HPV vaccination could have a therapeutic effect in RRP by preventing new papilloma formations at additional sites. Erik and colleagues24 showed that a quadrivalent HPV vaccination program in Hungary could reduce the incidence of cervical cancer, cervical intraepithelial neoplasia, and genital warts. The cost-per-QALY ratio within the range was defined as cost effective. Jane and others25 suggest that the cost-effectiveness of HPV vaccination will depend on the duration of vaccine immunity and will be optimized by achieving high coverage in preadolescent girls, closing the gap among nonvaccinated women younger than 21 years of age, and revising screening policies.

To conclude, eight neonates were detected as having HPV from tracheal aspirate. No clinical symptoms of persistent hoarseness or stridor were noted after extubation, signifying that these patients had HPV colonization in the trachea. Neonates with HPV colonization may be associated with juvenile-onset recurrent respiratory papillomatosis. The route of neonate HPV infection may be through a vertical transmission from mother to child. Our study revealed a 7.4% infection rate by ascending and passage through the birth canal. To prevent vertical transmission of HPV, targeted immunization may be an effective strategy. Real-time PCR assay has proven its value for clinical and epidemiologic studies of HPV-6 and HPV-11 infections.

References