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Inactivation of high-risk human papillomaviruses by Holder pasteurization: implications for donor human milk banking

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ABSTRACT

AIMS: Several studies have recently reported the detection of oncogenic human papillomaviruses (HPV) in human milk of a minority of lactating mothers. These findings raised safety concerns in the context of human donor milk banking given the potential risk of HPV transmission to recipient infants. The aim of this study was to investigate whether the Holder pasteurization, a procedure currently in use in human donor milk banks for milk pasteurization, completely inactivate high-risk and low risk HPV. METHODS: HPV pseudoviruses (PsV) were generated, spiked into cell culture medium or donor human milk and subjected to thermal inactivation. HPV PsV infectivity and morphological integrity was analyzed by cell-based assay and by electron microscopy respectively. RESULTS: The Holder pasteurization completely inactivated the infectivity of high-risk (types 16 and 18) and low-risk (type 6) HPV both in cell culture medium and in human milk causing PsV particles disassembly. CONCLUSIONS: The results presented here indicate that the Holder pasteurization is an efficient procedure to inactivate high-risk and low-risk HPV thus preventing the potential risk of their transmission through human donor milk.

INTRODUCTION

Human papillomaviruses (HPV) are non-enveloped, double-stranded DNA viruses belonging to the *Papillomaviridae* family (15). HPV are classified into low-risk and high-risk groups according to their oncogenic potential. Low-risk HPV types, such as HPV-6 and HPV-11, can cause benign lesions of the anogenital areas, oral papillomas, conjunctival papillomas, as well as low-grade squamous intra-epithelial lesions of the cervix. Perinatally acquired low-risk HPV can also cause recurrent respiratory papillomatosis in infants and young children (19). Infection with high-risk HPV types (primarily types 16, 18, 31, and 45) can cause cervical cell abnormalities that are precursors to cancer (21). HPV types 16 and 18 cause about 70% of all cases of invasive cervical cancer worldwide, with type 16 having the greatest oncogenic potential (5). Moreover, they are also implicated in some head and neck cancers. The sexual activity is considered the primary route of transmission of high-risk HPV among adult individuals (18), however non-sexual routes are currently under investigation such as the vertical transmission through maternal milk. Indeed, in 2008 a paper by Sarkola et al. (28) reported that 10 out of 223 breast-milk samples (4.5%) collected from Finnish women tested positive for HPV-16 raising the concern that high-risk HPV infections might be transmitted to infants via breast feeding. Recent studies by Cazzaniga et al., in Italy (9), Yoshida et al., in Japan (33) and Glenn et al., in Australia (13) confirmed the presence of high-risk HPV sequences in the milk of a minority of lactating women. By contrast, Mammas et al., could not detect any HPV sequences in milk samples from Greek women (22). To date, the possibility that HPV may be transmitted to infants through breast feeding causing clinically relevant infections remains to be investigated.

Beside breast feeding, another context in which the potential risk of HPV transmission to recipient infants should be taken in to account is that of human donor milk banks. Donor breastmilk from milk banks is known to contribute to the survival and well being of premature and sick babies, particularly when born extremely premature and with very low birth weight. Moreover, it has been shown that where new milk banks are established, the provision of donor milk can increase the rates

of breastfeeding on discharge from hospital of these babies (2). In this context the microbiological safety of donor milk relies mainly on donor selection and on milk pasteurization with the Holder method (62.5°C for 30 min) (17, 3, 1). Indeed, the Holder procedure has been shown to completely inactivate some viral pathogens that can cause clinically relevant infections to the infants when transmitted by maternal milk. These include the human immunodeficiency virus (HIV), the human T-lymphotrophic virus type I and II (HTLV-I and HTLV-II) and the human cytomegalovirus (HCMV) (12, 25, 31, 11, 14). By contrast, despite the concerns raised by the detection of oncogenic HPV in maternal milk, there are no data demonstrating the safety of Holder pasteurization for the inactivation of HPV. The difficulties to study HPV infectivity are due to the fact that these viruses cannot be cultured using conventional monolayer cell lines as they replicate exclusively in stratified squamous epithelial tissues (15). To overcome this obstacle, John Schiller and coworkers developed systems to efficiently produce high-titer HPV-based gene delivery vectors, known as HPV pseudoviruses (PsV) in cultured cell lines (6, 7). HPV PsV, are made of the two recombinant capsid protein L1 and L2 which assemble into particles that are indistinguishable from authentic HPV capsids and are capable of efficiently delivering reporter plasmids to a wide variety of cell lines. For this reason HPV PsV have rapidly become a useful tool for studying HPV infectivity (8, 10, 20). The aim of this study was to exploit the HPV PsV-based assays and electron microscopy to investigate the effects of Holder pasteurization on high-risk and low risk HPV. The data obtained demonstrate that Holder pasteurization completely inactivates HPV infectivity in cell culture medium and in human milk.

MATERIALS AND METHODS

Milk samples. Breast milk samples were collected from healthy volunteer mothers with normal Papanicolau smear tests. Informed consent for breast milk donation was obtained from lactating mothers of newborns admitted in the Neonatal Care Unit of University of Torino. This study was approved by the Ethical Committee of the Italian Association of Human Milk Banks.

Cells. The 293TT cell line, derived from human embryonic kidney cells transformed with the simian virus 40 (SV40) large T antigen, was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10 % fetal calf serum (FCS; Gibco- BRL), Glutamax-I 1 % (Invitrogen, Carlsbad, CA) and nonessential amino acids 1 % (Sigma Aldrich, Steinheim, Germany). 293TT cells allow high levels of protein to be expressed from vectors containing the SV40 origin due to overreplication of the expression plasmid (Buck et al. 2004). Low-passage-number human embryonic lung fibroblasts (HELFs) were grown as monolayers in minimum essential medium (MEM) (SIGMA) supplemented with 10% fetal calf serum (FCS) (SIGMA), 1 mM sodium pyruvate and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany).

Virus. A (BAC)-derived HCMV strain Towne incorporating the GFP sequence (23) was propagated on HELFs. The GFP marker facilitates identification of infected eukaryotic cells. Viral stocks were prepared by infecting HELFs at a virus-to-cell ratio of 0.01. The cells were incubated in MEM supplemented with 2% heat-inactivated FCS and cultured until a marked cytopathic effect was observed. Stocks were centrifugally clarified and frozen at −80°C. Virus titers were determined by plaque assay on HELFs.

HPV PsV production. Plasmids and 293TT cells used for pseudovirus (PsV) production were kindly provided by John Schiller (National Cancer Institute, Bethesda, MD). HPV-16, HPV-18 and HPV-6 PsVs were produced according to previously described methods (6). Briefly, 293TT cells were transfected with plasmids expressing the papillomavirus major and minor capsid proteins (L1 and L2, respectively), together with a reporter plasmid expressing the secreted alkaline phosphatase

(SEAP), or green fluorescent protein (GFP). Capsids were allowed to mature overnight in cell lysate; the clarified supernatant was then loaded on top of a density gradient of 27 to 33 to 39% Optiprep (Sigma-Aldrich, St. Louis, MO) at room temperature for 4 h. The material was centrifuged at 234,000 X g for 3.30 h at 16 °C in an SW50.1 rotor (Beckman Coulter, Inc., Fullerton, CA) and then collected by bottom puncture of the tubes. Fractions were inspected for purity in 10% sodium dodecyl sulfate (SDS)–Tris–glycine gels, titrated on 293TT cells to test for infectivity by SEAP or GFP detection, and then pooled and frozen at -80 °C until needed. The L1 protein content of PsV stocks was determined by comparison with bovine serum albumin standards in Coomassie-stained SDS-polyacrylamide gels.

HPV inactivation assays. For the SEAP-based assays, 293TT cells were preplated 24 h in advance in 96-well tissue culture-treated flat bottom plates at a density of 20000 cells/well in 100 µL of neutralization buffer (DMEM without phenol red, 10 % heat-inactivated fetal bovine serum, 1 % glutamate, 1 % nonessential aminoacids, 1 % antibiotic-antimycotic solution, and 10 mM Hepes). Diluted PsV stocks equivalent to a final concentration of approximately 1 ng/ml L1 or increased volumes of PsVs (equivalent to approximately 2 and 4 ng/ml L1) were incubated for 30 minutes in ice (4°C), at room temperature (25°C) or at increasing temperatures in a PCR thermocycler (37°C, 45°C, 50°C, 55°C or 62.5°C) and then cooled at 4°C. The PsV solution (100 µL/well) was transferred onto the preplated cells and incubated for 72 h. After incubation, 25 µL of supernatant was harvested. The SEAP content in the supernatant was determined using a Great Escape SEAP Chemiluminescence Kit (BD Clon- tech, Mountain View, CA) as directed by the manufacturer. Thirty minutes after the addition of the substrate, samples were read using a Wallac 1420 Victor luminometer (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA). The SEAP activity is expressed as counts per second (CPS).

For the GFP-based assays diluited PsV stocks were incubated for 30 minutes at 4°C or 62.5°C and then cooled at 4°C. The final concentration of PsV was approximately 1 ng/ml L1. The PsV

141 solution was transferred onto the preplated cells and incubated at 37°C. After 72 h the GFP-
142 expressing infected cells were observed on an inverted Zeiss LSM510 fluorescence microscope.
143 **HCMV inhibition assays.** HELF cells were seeded into 96-well plates at a density of 10^4
144 cells/well, and incubated at 37°C in a 5% CO2 atmosphere for 24 h. Cells were infected with GFP-
145 expressing HCMV, preincubated for 30 minutes at 4°C in ice or at 62.5°C in a PCR thermocycler,
146 at a multiplicity of infection (MOI) of 1 for 2 hours at 37°C in MEM supplemented with 2% of
147 heat-inactivated FCS. Then monolayers were washed three times and medium supplemented with
148 10% FCS was replaced. Three days postinfection, cells infected were examined by an inverted
149 Zeiss LSM510 fluorescence microscopy.
150 **Virus spiking experiments.** Raw milk samples from mothers were spiked with an inoculum of
151 HPV-16 Psv (GFP) preparation (40 ng/ml L1) or cell-free culture supernatant of HCMV (GFP) (10^4
152 pfu per milliliter raw milk) for Holder pasteurization experiments.
153 **Holder pasteurization.** Each 100 μL of spiked milk specimens were incubated for 30 minutes at
154 62.5°C in a water bath, a procedure currently in use in human donor milk banks for milk
155 pasteurization, and then cooled at 4°C. An additional sample of spiked milk was incubated for 30
156 min at 4°C as a control. The virus-milk mixtures were then diluted in cell culture medium (1:2) and
157 added to preplated cells to test virus infectivity, as previously described.
158 **Electron microscopy.** An aliquot of diluted HPV-PsV preparation was allowed to adsorb for about
159 3 min on carbon and formvar-coated grids and then rinsed several times with water. Grids were
160 negatively stained with 0.5% uranyl acetate and excess fluid was removed with filter paper.
161 Observations and photographs were made using a CM 10 electron microscope (Philips, Eindhoven,
162 The Netherlands).
163 **Data analysis.** Each data is the mean ± standard deviation of the mean for three determinations
164 performed in duplicate. For the SEAP-based PsV neutralization assays the mean value obtained at
165 different temperatures was expressed as a percentage of the mean value obtained at 4°C. Statistical
166 analysis was performed by analysis of variance (ANOVA) followed by a Bonferroni test if *P* values

showed significantly differences (P value < 0.05), using GraphPad Prism 5.00 (GraphPad Software).

RESULTS

Characterization of purified HPV-16 PsV. For this study HPV-16 was chosen as a pivotal model because it is the genotype identified most often in human milk samples (28, 9, 33, 13). In preliminary experiments, the quality of the HPV-16 PsV preparations was assessed by SDS-PAGE and electron microscopy analysis. As shown in Fig. 1A, a major band migrating at 55 kDa was detected by SDS-PAGE followed by Coomassie brilliant blue staining (lane 1). The major band was confirmed to be the L1 major capsid protein by Western blot analysis with anti-L1 antibody (lane 2). No L1-reactive proteolytic degradation products were observed at molecular masses below 55 kDa demonstrating the quality of the preparation. Figure 1B shows an electron micrograph of the same PsV stock. The PsV particles exhibited an average diameter of 50 to 60 nm, similar to that of an authentic HPV capsid, and appeared as individual, well-assembled, icosahedral capsids. Similar results were obtained with the other HPV PsV types used in this study (data not shown).

Effect of heat treatment on HPV-16 PsV infectivity in cell culture medium. To assess the effect of heat treatment on HPV infectivity, HPV-16 PsV diluted in cell culture medium, were exposed to increasing temperatures ranging from 4°C to 62.5°C for 30 minutes and then used to infect 293TT cell cultures. Viral infectivity was quantitatively assessed by measuring the SEAP levels in the cell culture supernatants. As shown in Fig. 2, PsV were stable at 4°C and 25°C, whereas exposure to 45°C, 50°C and 55°C resulted in a reduction of infectivity, although to different extents. By contrast, Holder pasteurization (62.5°C for 30 min.) completely abolished PsV infectivity. Of note, similar results were obtained when the Holder pasteurization was applied to PsV of an additional high-risk type such as HPV-18 and to a low-risk type such as HPV-6 (Fig. 3). Moreover, the effect of Holder pasteurization was not dependent on the PsV titer used as it was effective at HPV-16 PsV concentrations up to 4 ng/ml of L1 capsid protein (Fig. 4). To rule out residual infectivity that could not be revealed by the SEAP assay, GFP-expressing PsV were used as they allow the detection of single infected cells by microscopy. As a control of the effectiveness of the Holder procedure, the assay was run in parallel with a GFP-expressing HCMV, a virus which is known to

be completely inactivated by Holder pasteurization (11, 14). The results shown in Fig. 5 demonstrate that no GFP-expressing cells could be detected in cell monolayers infected with HPV-16 PsV (panel A) or HCMV (panel B) that underwent Holder pasteurization. By contrast, a high number of GFP-expressing cell are visible in monolayers infected with viruses maintained at 4°C for 30 min as positive controls.

To further investigate the effect of the Holder pasteurization on HPV we analyzed the heat-treated HPV-16 PsV by electron microscopy. Fig. 6 compares a sample subjected to Holder pasteurization (panel B) to one maintained at 4°C for 30 min (panel A) as a control. As expected, in the control panel several PsV particles are clearly visible. At a higher magnification (panel A, inset) these particles appear as well assembled icosahedral capsids. By contrast, the analysis of the pasteurized PsV revealed that the heat treatment completely destroyed the structural integrity of the viral particles as no individual PsV can be observed (Fig. 6 panel B). Rarely, structures suggestive of denatured, disassembled capsids can be detected at a higher magnification (panel B, inset).

Taken together these results demonstrates that HPV PsV are completely inactivated by Holder pasteurization in a acqueous matrix such as a cell culture medium.

Effect of Holder pasteurization on HPV PsV in human milk. To determine the effectiveness of Holder pasteurization on HPV in human milk, raw milk samples from donor mothers were spiked with an inoculum of GFP expressing HPV-16 PsV or HCMV used a control. The inoculated milk was then pasteurized at 62.5°C for 30 minutes in a water bath, a procedure currently in use in human donor milk bank for milk pasteurization, or kept at 4°C. Diluted samples were then inoculated into cell monolayers and examined for GFP expression. As shown in Fig. 7, no GFP-expressing cells could be detected in cell monolayers infected with pasteurized HPV-16 PsV (panel A) or HCMV (panel B). By contrast, a high number of GFP-expressing cells are visible in monolayers infected with viruses maintained at 4°C for 30 min as positive controls. Similar results were obtained when HPV-18 and HPV-6 PsV were used (data not shown).

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DISCUSSION

The potential risk of infection in recipient infants raised by the recent detection of high-risk HPV in human milk should be taken into account in the context of donor human milk banking. Currently, donor selection and pasteurization of milk are the most important procedures that guarantee the microbiological safety of human donor milk. Donor selection by serology or molecular diagnostic techniques does not seems suitable to prevent a potential risk of HPV transmission through milk as no concordance between cervical DNA detection and co-existent seropositivity was documented (26). Moreover, no correlation was found between mothers' cervical or oral HPV results and HPV detection in their breast milk (28, 22, 33). Besides, serology for HPV is not currently recommended in donor mothers screening (1, 16, 24).

The Holder pasteurization is the most used and recommended method for the heat treatment of milk in donor human milk banks as it allows a good compromise between the nutritional quality of milk and its microbiological safety. Although its effectiveness to inactivate relevant viral pathogens transmitted through milk, like HIV, HTLV-I, HTLV-II and HCMV, is well established, no data are available for HPV. Therefore, the aim of this study was to determine whether the Holder method completely inactivates HPV infectivity. We conducted our experiments both in cell culture medium and human milk since other researchers have demonstrated the protective effects of milk on non enveloped viruses such as hepatitis A virus (27, 4), poliovirus (30) and foot-and-mouth disease virus (32). We did demonstrate that Holder pasteurization abolishes completely the infectivity of high-risk HPV (i.e. HPV-16 and HPV-18) and low-risk HPV (i.e HPV-6) PsV in both matrices indicating that milk fat and the casein proteins do not exert any protective effect on HPV PsV particles.

It has been shown that virus inactivation by pasteurization depends on denaturation of viral proteins, as well as disassembly of virus particles (29). To investigate the mechanism of HPV PsV inactivation by Holder pasteurization we examined heat-treated PsV by electron microscopy. According to what has been reported for other viruses (29) we observed that Holder pasteurization

induced HPV PsV particle disassembly which result in the generation of non infectious subunits. Taken together, the results presented here indicate that the Holder pasteurization is an efficient procedure to inactivate high-risk and low-risk HPV thus preventing the potential risk of their transmission through human donor milk.

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FIGURE LEGENDS

Figure 1. Characterization of purified HPV-16 PsV. (A) An aliquot of purified PsV preparation was analyzed by SDS-PAGE with Coomassie brilliant blue staining (lane 1) or immunoblotting with an anti-L1 antibody (lane 2). (B) Electron micrograph of a purified PV preparation. Bar 100 nm.

Figure 2. Effect of different temperatures on HPV-16 PsV infectivity. PsV were incubated in cell culture medium at 4°C, 25°C, 37°C, 45°C, 50°C, 55°C or 62.5°C for 30 minutes and then used to infect 293TT cells for the measurement of SEAP expression. Results are given as the mean ± standard deviation of triplicates. The mean value was expressed as a percentage of the mean value obtained at 4°C. * *P* value < 0.05.

Figure 3. Effect of Holder pasteurization on HPV-18 and HPV-6 PsV infectivity. PsV were incubated in cell culture medium at 4°C, or 62.5°C for 30 minutes and then used to infect 293TT cells for the measurement of SEAP expression. Results are given as the mean ± standard deviation of triplicates. The mean value was expressed as a percentage of the mean value obtained at 4°C. *P* value < 0.05.

Figure 4. Effect of Holder pasteurization on increasing concentrations of HPV-16 PsV ranging from 1 to 4 ng/ml of L1 capsid protein. PsV were incubated in cell culture medium at 4°C, or 62.5°C for 30 minutes and then used to infect 293TT cells for the measurement of SEAP expression (CPS: counts per second). Results are given as the mean ± standard deviation of triplicates. The mean value was expressed as a percentage of the mean value obtained at 4°C. *P* value < 0.05.

Figure 5. Effect of Holder pasteurization on GFP expressing HPV-16 PsV (panel A) or HCMV (panel B) in cell culture medium. PsV and HCMV were exposed to 4°C or to 62.5°C for 30 min and then used to infect 293TT or HELF cells respectively. Cells were analyzed by confocal laser scanning microscopy. The upper panels show the fluorescence images while the lower panels show phase-contrast images.

Figure 6. Elecron microscopy of purified HPV-16 PsV. (A) An aliquot of purified PsV preparation was analyzed after incubation at 4°C for 30 min. Four distinct PsV particles are visible (indicated by arrows). The inset shows a well assembled icosahedral capsid at a higher magnification. (B) An aliquot of purified PsV preparation was analyzed after Holder pasteurization. No intact PsV are visible. The inset shows a structure suggestive of a dysassembled capsid at a higher magnification. Both images are at 120,000X magnification. This picture is representative of ten microscopic fields examined for each experimental condition.

Figure 7. Effect of Holder pasteurization on GFP expressing HPV-16 PsV (panel A) or HCMV (panel B) in human milk. PsV and HCMV were exposed to 4°C or to 62.5°C for 30 min and then used to infect 293TT or HELF cells respectively. Cells were analyzed by confocal laser scanning microscopy. The upper panels show the fluorescence images while the lower panels show phase-contrast images.