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Hepatitis B viral markers in banked human milk before and after Holder pasteurization

Patricia Ribeiro de Oliveira[a,1], Aparecida Yulie Yamamoto[a,1], Cleonice Barbosa Sandoval de Souza[a,2], Natalia Motta de Araújo[b,3], Selma de Andrade Gomes[b,3], Anália Ribeiro Heck[c,4], José Fernando de Castro Figueiredo[d,5], Marisa Márcia Mussi-Pinhata[a,*]

[a] Department of Pediatrics, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes 3900, 14049-900, Ribeirão Preto, SP, Brazil
[b] Molecular Virology Laboratory, Oswaldo Cruz Institute, Av. Brasil 4365, Manguinhos, Rio de Janeiro, Brazil
[c] Human Milk Bank, University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av. Santa Luzia, 387 Jardim Sumaré, CEP 14025-090, Ribeirão Preto, São Paulo, Brazil
[d] Department of Internal Medicine, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes 3900, 14049-900, Ribeirão Preto, SP, Brazil

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ABSTRACT

Background: Blood screening for hepatitis B virus (HBV) is not universally performed for donor selection in human milk banks.
Objectives: To evaluate the frequency of detection of HBV surface antigen (HBsAg) and HBV-DNA in colostrum of HBV-infected nursing mothers before and after Holder pasteurization.
Study design: Forty-two concentrated breast milk samples were obtained within two postnatal weeks from 24 HBsAg-positive women (4 HBeAg-positive and 20 HBeAg-negative, anti-HBe-positive) were tested for the presence of HBsAg and HBV-DNA before and after Holder pasteurization (30 min at 62.5 °C).
Results: Before pasteurization, HBsAg and HBV-DNA were found in 14/24 (58%), and 20/24 (75%) first milk samples, respectively, obtained by 4 days after delivery. At least one marker was detected in 20/24 (83%) milk samples. Both markers were identified in milk of HBeAg-positive mothers, and most mothers with anti-HBe in blood had at least one HBV marker. Once detected, viral markers were frequently found in milk samples subsequently obtained from the same woman. Holder pasteurization did not affect the probability of detecting HBsAg (8/18, 44%), HBV-DNA (12/18, 67%), or at least one of them (15/18, 83%).
Conclusions: Although the biological implications of these findings remain to be determined, considering that HBV is highly contagious and most recipients of banked human milk are preterm infants, these findings should be taken into account when donors are enlisted for human milk banks without serological screening.

1. Background

After the first demonstration more than 30 years ago of hepatitis B virus (HBV) surface antigen (HBsAg) in the milk of a HBV female carrier,[1] this antigen was detected in none[2] to 71.4%[3,4] of milk samples tested. HBV virions (Dane particles) were identified in 4 of 10 milk samples by electron microscopy.[3] HBe antigen (HBeAg) was also found in human milk.[5] More recently, after the advent of molecular biology techniques, an overall HBV-DNA-positive rate of about 43%[6–9] was detected in milk of HBV-infected women from countries with a high carrier rate.

Donor selection and techniques of milk processing and storage in human milk banks are measures taken in Europe and North America[10,11] to prevent infection in recipient infants in contrast to countries with fewer resources which do not require that milk donors be screened for HBV. Instead, lactating women are usually only verbally screened and milk safety relies on Holder pasteurization[12] (heating to 62.5 °C for 30 min followed by cooling

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B virus "e" antigen; HBc, hepatitis B core; HIV, human immunodeficiency virus; CMV, cytomegalovirus; HTLV-1, human T lymphotropic virus type 1.

* Corresponding author. Tel.: +55 16 36330136/36022479; fax: +55 16 36022700.
E-mail addresses: propat@ig.com.br (P.R. de Oliveira), yulie@fmrp.usp.br (A.Y. Yamamoto), cleonice@fmrp.usp.br (C.B.S. de Souza), nmaraujo@ioc.fiocruz.br (N.M. de Araújo), selma@ioc.fiocruz.br (S. de Andrade Gomes), analia_heck@uol.com.br (A.R. Heck), jfcfigue@fmrp.usp.br (J.F. de Castro Figueiredo), mmmpinha@fmrp.usp.br, mussi.pinhata@pesquisador.cnpq.br, mussi.pinhata@cnpq.pesquisador.br (M.M. Mussi-Pinhata).
[1] Tel.: +55 16 36330136/36022674; fax: +55 16 36022700.
[2] Tel.: +55 16 36330136/36022670; fax: +55 16 36022700.
[3] Tel.: +55 2125621847; fax: +55 2122604866.
[4] Tel.: +55 16 36102649.
[5] Tel.: +55 16 36022468; fax: +55 16 36336695.

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to 7 °C), which inactivates cytomegalovirus (CMV), HIV and type I HTLV.[13] However, there are no data demonstrating the safety of Holder pasteurization for the inactivation of HBV in human milk.

2. Objectives

To evaluate the frequency of HBsAg and HBV-DNA detection in colostrum of HBsAg carriers from a region with a 1% carrier prevalence rate, and to verify the effect of Holder pasteurization on the detection of these viral markers in milk.

3. Study design

3.1. Study population

This study was conducted at the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil. Among 7812 live births from 1999 to 2003, 58 (0.7%) mothers tested positive for HBsAg (Hepanostika HBsAg, Organon Tecnika BV, Boxtel, The Netherlands). HIV-negative mothers who agreed to participate and from whom it was possible to obtain at least one colostrum sample (a minimum volume of 10 ml) within 1 week of delivery were selected for the study. Twenty-four women were enrolled.

3.2. Biological samples and testing for HBV serological markers

Serum samples from all 24 HBsAg-positive mothers, obtained upon admission for delivery, were stored at −20 °C and retested for HBsAg (Hepanostika HBsAg Uni-Forn II, microelisa system, Organon Tecnika BV, Boxtel, Holanda), and tested for anti-HBc and HBe/anti-HBe (Axsym, Microelisa System, Abbott, Wiesbaden, Germany).

Colostrum samples were obtained from these same mothers between 1 and 4 days after delivery. Ten women had more than one collected milk sample (five women gave two samples; two gave three samples; and three gave four samples).

Breast milk samples were collected by manual expression or with a mechanical suction pump (Medela Lactina Select (Lactaset), Medela Inc., McHenry, IL, USA), placed in sterile flasks, and stored frozen (−20 °C) until processing. Bleeding was not apparent during collection. Milk samples were centrifuged at 2000 rpm for 15 min to separate the fatty phase from the intermediate phase (serum) as previously described.[14] This milk serum fraction was concentrated using the Centricon YM-10 kit (Microcon Centrifugal Filter Devices, Bedford, MA, USA) according to the manufacturer's instructions. The mean concentration factor obtained was 3.5 times. For testing after pasteurization, 10 ml of the serum fraction of raw milk was heated to 62.5 °C for 30 min and then cooled to 7 °C (Holder pasteurization process) and 2 ml of pasteurized milk was also concentrated for the laboratory assays. Before and after pasteurization, whole milk aliquots, and concentrated milk serum fractions were stored at −20 °C until testing. Milk samples were tested for HBsAg before and after milk concentration, and before and after milk processing by Holder pasteurization.

3.3. DNA extraction and PCR assays

Concentrated milk samples and serum samples were submitted to DNA extraction and HBV-DNA PCR amplification as previously described.[15,16] Briefly, viral DNA was extracted using phenol/chloroform after treatment of 250 μl of serum or concentrated milk samples with 0.5 mg/ml of proteinase K in the presence of 0.2 M NaCl, 0.25% SDS, for 4 h at 37 °C. After precipitation with ethanol, the pellet was dried and resuspended in 30 μl of distilled water. The pre-S region was amplified by a double step (semi-nested) PCR assay. The first round of amplification was performed using sense primer PS1 (5′-CCATATTCTTGGGAACAAGA-3′, nt position 2826–2845) and antisense primer SR (5′-CGAACCACTGAACAAATGGC-3′, nt position 685–704). The second round of amplification was performed with 1 μl of the first round PCR product, sense primer PS1 and antisense primer PS2 (5′-GGTCCCCAGTCCTCGAGAAG-3′, nt position 124–143). The protocol used for both rounds was: 94 °C, 3 min; 30 cycles at 95 °C (30 s), 52 °C (40 s), 72 °C (2 min), followed by a final elongation step at 72 °C for 7 min. Negative and positive controls were added in both extraction and PCR procedures. The lower limit of DNA detection by this PCR assay was determined using serial dilutions of quantified recombinant plasmids carrying HBV genomes, as previously described.[16] This two-round PCR assay has a sensitivity of about 10–100 DNA molecules per assay. For the 539 bp amplicon detection, an aliquot of the amplified products was submitted to electrophoresis in 2% agarose gel and visualized under UV light after ethidium bromide-staining.

3.4. Data analysis

The proportions of the variables under study were determined by the chi-square test with Yates correction or by the Fisher exact test when indicated. The level of significance was set at $\alpha = 5\%$ for all tests.

4. Results

Mean maternal age was 26.5 years (range: 17–42). Gestational age ranged from 33 to 41 weeks (median = 39 weeks).

Anti-HBc antibodies were detected in the peripheral blood of all 24 HBsAg-positive subjects, and HBe antigen was detected in 4 of them (17%); the remaining 20 participants were anti-HBe positive. HBV-DNA was found in 17 of 20 (85%) blood samples tested (four samples could not be tested due to technical problems). All four HBeAg-positive carriers, and 13/16 (81.2%) HBeAg-negative women were positive for HBV-DNA in blood.

HBsAg was detected in 36% (15 of 42) of non-concentrated milk samples. The frequency of detection increased to 55% (23/42) after concentration ($p < 0.001$). Considering this finding, all the results concerning HBV markers in milk were obtained by testing concentrated samples.

Table 1 shows the frequency of detection of HBV markers in milk samples before and after pasteurization. At least one viral marker (HBsAg and/or HBV-DNA) was detected in most samples, whether considering the first samples obtained from the 24 participants or all samples analyzed. Detection of HBsAg before and after pasteurization was 83% concordant, while the detection of HBV-DNA was 71% concordant. For both markers, most discordance was due to a decrease in positive tests occurring after pasteurization.

Table 1
Detection of HBsAg and HBV-DNA in milk of HBsAg carriers, before and after Holder pasteurization.

HBV markers	Positive/total (%) [95%CI]		*p*
	Before pasteurization	After pasteurization	
Colostrum			
HBsAg	14/24 (58%) [37–78]	8/18[a] (44%) [21–69]	0.37
HBV-DNA	18/24 (75%) [53–90]	12/18 (67%) [41–87]	0.55
At least one marker	20/24 (83%) [63–96]	15/18 (83%) [59–96]	0.67
All milk samples			
HBsAg	23/42 (55%)[£] [39–70]	12/35[b] (34%) [19–52]	0.32
HBV-DNA	29/42 (66%)[£] [53–82]	20/35 (57%) [39–74]	0.39
At least one marker	32/42 (76%) [61–88]	27/35 (77%) [60–90]	0.92

[a] Six samples were not tested.
[b] Seven samples were not tested.
[£] $p = 0.26$, χ^2-test for comparison between proportions of HBsAg and HBV-DNA.