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Host defense proteins in breast milk and neonatal yeast colonization

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Abstract

Background—Colonization increases risk for invasive candidiasis in neonates. Breast milk host defense proteins may affect yeast colonization of infants.

Objective—To evaluate breast milk host-defense proteins relative to yeast colonization in infants.

Methods—Infants admitted for longer than 72h to the NICU at Women and Infants Hospital in Providence, RI, were eligible. After consent, expressed breast milk and swabs from oral, rectal, and inguinal sites from infants were cultured weekly for 12 weeks, or until discharge, transfer, or death. Breast milk was tested for levels of human lactoferrin, lysozyme, apolipoprotein J, mucin-1, dermcidin, and soluble CD14 using commercial ELISA. Concentrations of these components were compared in breast milk received by infants who were colonized or not colonized with yeast.

Results—From an original cohort of 130, 61 infants had samples available for this subanalysis. A convenience sample of stored breast milk was analyzed. Median lactoferrin, apolipoprotein J, and mucin-1, did not differ between colonized and uncolonized groups. Soluble CD14 was higher in the surface-colonized group (1.8 µg/ml, n=12) compared with the surface-uncolonized group (1.6 µg/ml, n=12, p=0.02). Median lysozyme levels were higher in the surface-uncolonized group (483.0 ng/ml, n=12) vs. the surface-colonized group (298.3 ng/ml, n=12, p=0.04). Median dermcidin levels were higher in the surface-uncolonized group (19.4 ng/ml, n= 12) vs. the surface-colonized group (8.7 ng/ml, n=12, p=0.04).

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Disclosures:

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Conflicts of Interest:

There are no other financial conflicts of interest to disclose.

Conclusions—This study shows an association between colonization with *Candida* in neonates and lower levels lysozyme and dermcidin in received breast milk. Further study is needed to confirm these findings.

Keywords

breastfeeding; neonatal; *Candida*; colonization

Introduction

Despite nationwide decreases in the incidence of invasive fungal infections in recent years, systemic candidiasis is still an important cause of morbidity and mortality in premature neonates.¹ Heavy colonization with yeast is a risk factor for invasive fungal infection in infants.² Studies on colonization have confirmed that both vertical and horizontal routes of transmission are relevant.³ An association of fungal colonization with receipt of expressed breast milk colonized with yeast has also recently been reported.⁴

Human milk components, including certain oligosaccharides, can influence the human neonatal gut microbiome⁵ and diet variation is known to alter the gut microbiome in healthy adult volunteers.⁶ Regarding *Candida* species, diets higher in amino acids, protein, and fatty acids were negatively correlated with *Candida* identified in the microbiome.⁶ In addition, human breast milk is known to have many host-defense components, including immunoglobulins, lactoferrin, mucins, dermcidin, and clusterin,⁷ several of which are fungicidal⁸ or inhibit fungal colonization of human mucosae.⁹ Studies have also demonstrated that administration of bovine lactoferrin to premature infants is associated with reductions in late-onset sepsis and invasive fungal infection without altering *Candida* colonization,¹⁰ but in general data regarding the role of specific human milk components in the colonization of the neonatal gut and development of the neonatal microbiome are emerging.

In this study, we investigate human host defense protein content in expressed human milk and the relationship with infant fungal colonization. We hypothesized that the concentration of host defense proteins in human milk fed to infants would vary between yeast-colonized and yeast-uncolonized infants.

Methods

The study design has been reported previously.⁴ Briefly, infants were enrolled from the neonatal intensive care unit (NICU) at Women & Infants Hospital of Rhode Island. The sole inclusion criterion was admission to the NICU with a minimum stay of 72 hours. Exclusion criteria included death, transfer, or discharge from the NICU at less than 72 hours of age and lack of parental informed consent. Cultures of expressed breast milk and swabs from oropharynx, inguinal, and rectal sites of infants were obtained at enrollment and then weekly for twelve weeks, or until the patient exited the study due to discharge from the hospital, transfer, or death. Breast milk and swabs were cultured for yeast. An infant was defined to be colonized if yeast was recovered from any site at any point during their participation in the study. Yeast isolates were primarily *Candida* species, but *Trichosporon* species were also

isolated. Expressed breast milk (**0.5 ml**) from each infant's mother was obtained from the daily supply, and may have been freshly expressed and refrigerated, or may have been frozen and thawed prior to being refrigerated. No donor breast milk was used in this cohort. Samples were transported in sterile polystyrene tubes. Following culture, residual breast milk samples with volumes of approximately 250–300 μ l were subsequently cryopreserved at -80°C in Nunc cryotubes (Thermo Scientific, Waltham, MA) until analysis. The study was approved by the Institutional Review Board of Women & Infants Hospital, and data extraction and storage complied with the Health Information Portability and Accountability Act of 1996.

Cultures and Identification

Milk and swabbed samples were cultured on YPD agar media (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 100 $\mu\text{g/ml}$ of streptomycin and 50 $\mu\text{g/ml}$ of ampicillin to inhibit bacterial growth, and yeast isolates were identified using the Vitek-2 system (bioMérieux, Marcy L'Étoile, France).

Measurement of Host Defense Proteins

Breast milk samples were originally collected for the purpose of culturing for yeast. Remaining samples from both the colonized and uncolonized cohorts were available for testing in the present study. Midway through the study, the informed consent document was changed to allow for testing of host defense proteins. Samples collected prior to this change could not be used for measurement of host defense proteins. This change excluded 69 infants, leaving 61 infants for eligible for this analysis, which was fewer than anticipated. For some subjects, multiple milk samples from the same infant-mother pair from different time points were available to be tested. A convenience sample of available stored specimens from colonized and uncolonized groups was selected for testing. All available colonized subjects were included when there adequate residual specimen for an assay. Samples from the uncolonized cohort were selected based on having adequate volume for the assay. Commercial ELISA kits were used to test for human lactoferrin, lysozyme, apolipoprotein J (Abcam, Cambridge, Massachusetts), soluble CD14 (Sigma Aldrich, St. Louis, Missouri), human mucin-1, and dermcidin (USCN Life Science, Hubei, China). Samples were tested in duplicate on one assay, with sets of standards run in duplicate or triplicate depending on manufacturers' instructions.

Statistical Analysis

Statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, N.C.). Samples from the colonized cohort and uncolonized cohort were analyzed as individual specimens using negative binomial models with generalized estimating equations adjustment to account for lack of independence between samples attributed to the same patient. For these analyses Proc Genmod was used, specifying an exchangeable correlation structure. A two sided p-value of < 0.05 was considered statistically significant. Results were tabulated both on a per sample basis as well as a per patient basis.

Results

A total of 130 patients from 2276 total admissions during this time period were recruited from February 2011 to November 2012. Follow-up extended through December 2012. Cultures were obtained on 129 subjects; cultures were unable to be obtained from one patient due to medical instability. The yeast colonization rate was 22%. Results from the entire cohort have been reported in detail elsewhere.⁴ Of the original 129 subjects with culture data available, 61 infants were eligible for inclusion in the present study. In this subgroup, 16% were colonized, all with *Candida* species. A total of 5 colonized infants were identified in the sub-cohort: 3 of the 5 infants contributed multiple breast milk samples. Notably, only one infant received breast milk that was colonized with yeast; the organism recovered from breast milk was *C. lusitaniae*, while the organism recovered from the infant was *C. parapsilosis*.

Infants in this NICU during the time of the study were preferentially fed mother's breast milk. Parenteral nutrition was also used until infants achieved full enteral feeds. Days receiving parenteral nutrition are presented as an estimate of when infants achieved full feeds are shown in Table 1. Median duration of parenteral nutrition was slightly longer in the uncolonized group. Weekly breast milk samples were obtained; estimates of transition to exclusive formula feeding were obtained with the total number of breast milk samples, which was longer in the uncolonized group. None of the differences in Table 1 on exclusive breast milk feeding, length of stay, or days on parenteral nutrition were found to be statistically significant.

The number of samples and patients tested for each assay are presented in Table 2. In an attempt to increase power, multiple milk samples attributed to a single subject, but obtained at different time points, were analyzed. Lack of independence among samples attributed to a single subject were taken into account in the statistical modeling. Concentrations of apolipoprotein J, lactoferrin, and mucin-1 did not differ between the colonized and uncolonized groups (Table 2). However, lysozyme, and dermcidin concentrations were significantly higher in the uncolonized group than the colonized group. Conversely, soluble CD14 was higher in the colonized group compared with the uncolonized group. Individual measurements of each component for all samples are depicted graphically in Figure 1.

Discussion

Systemic candidiasis represents an important cause of morbidity and mortality in premature neonates,¹ and recent evidence suggests that breast milk can alter infant fungal colonization.⁴ Data presented here suggest that there may be an association between lysozyme and dermcidin concentration in expressed breast milk and colonization with yeast in a neonatal intensive care setting when individual milk samples were analyzed, taking into account that subjects may have contributed more than one milk specimen. Concentrations of the host defense proteins, lysozyme and dermcidin, were lower in breast milk given to infants colonized with yeast than in the breast milk given to uncolonized infants. This raises the question of whether bioactive content, in addition to timing, caloric content, and nutritional content, should be a consideration in the decisions regarding feeding neonates in

the intensive care setting. More rigorously designed studies are needed to better characterize potential relationships between composition of breast milk and colonization with yeast.

The association of lysozyme levels with yeast colonization is consistent with animal models of the gut microbiome.¹¹ Studies in which goat milk was genetically modified to contain human lysozyme showed alterations in the microbiome of the recipient goat compared to a conventionally fed control animal. At 14 days, the human lysozyme fed goats had higher levels of Bacteroidetes and lower levels of Firmicutes compared to the conventionally fed goats. It is speculated that lysozyme helped select for beneficial bacteria in the goat microbiome.¹¹

Dermcidin is a cationic human host defense peptide secreted by the eccrine glands and was first described by Schitteck and colleagues in 2001.¹² Dermcidin shows no homology to known defensins or cathelicidins. This peptide has *in vitro* antimicrobial activity against *Candida albicans*, as well as *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*.¹² To our knowledge, dermcidin levels in breast milk have not been previously associated with fungal colonization status. Mechanisms of dermcidin's effects on colonization status were not examined in this study.

CD14 was observed to be somewhat higher in the colonized group when analyzed by sample. It is unclear from this study by what mechanism, if any, this association translates to fungal colonization. Soluble CD14 may play a role in establishment of gut bacteria, and has been shown to play a role in activating the innate immune response to lipopolysaccharide (LPS) carried by *Escherichia coli* and other bacteria.¹³ Additionally, LPS has been shown to enhance phagocytosis of *Candida* through induction of expression of dectin-1 in human peripheral blood monocytes.¹⁴ One mechanism may be that higher levels of CD14 reduce *E. coli* and other Gram-negative colonization and thus LPS exposure, making the environment more favorable for *Candida*. There are likely other complex interactions between host bacteria, fungi, and the immune system which are impacted by sCD14. This study was not designed to examine such complex interactions in the human gut.

This study has several important limitations. As an observational study, causal relationships cannot be determined. While the original study sample was sufficiently powered to investigate relationships, specifically the relationship between breast milk colonization and infant colonization, the current study was designed as a *post-hoc* analysis. The investigation was thus limited to an analysis of a much smaller subgroup of patients with cryopreserved samples of infants' mother's milk. The volume of breast milk obtained was not sufficient to allow for separate aliquots for testing of multiple substances, and the samples may have been degraded through repeated freeze-thaw cycles. Internal studies show a degradation of lactoferrin with multiple freeze-thaw cycles. Additional studies on freeze-thaw cycle were not able to be performed for the other substances due to the volume of milk. Nonetheless, degradation would most likely result in a type II error, lending more support that the difference found is a true finding. It is possible that negative findings from this study are false negatives, and that study on fresh specimens would have different, significant findings.

The results presented here provide evidence for a relationship between the host defense protein composition of the breast milk and infant yeast colonization, particularly with respect to lysozyme and dermcidin. The source of the host-defense proteins may not be the breast milk itself, but rather contact with maternal or infant skin secretions, which could not be assessed in this study. Breast milk is also known to change in composition over time. Examination of this change in composition with relation to colonization status would elucidate temporal relations, and help determine causal relationships. Future studies to address these issues would include rigorously designed investigations to examine the relationship between the composition of breast milk and fungal colonization status. Molecular techniques could be used to determine the burden of organisms, along with their relationship with other commensal organisms on the skin and in the gastrointestinal tract.

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Well Established

Breast milk is known to have effects on establishing the bacterial microbiome on infants. Less is known about the fungal microbiome, however fungal colonization is known to occur via both vertical and horizontal routes.

Newly Expressed

This study examines host defense proteins in breast milk in association with infant yeast colonization. This study reports a possible negative association with yeast colonization and levels of lysozyme and dermcidin in breast milk.

Figure 1. Distribution of breast milk measurements

Colonized subjects are unshaded; uncolonized subjects are shaded. Individual measurements are in open circles; bars represent median and interquartile ranges when multiple measurements are available for a subject.

Table 1

Feeding characteristics of tested cohort

	Uncolonized	Colonized
N	9	5
Exclusively fed breast milk	2	1
Median number of weekly breast milk samples	3 (IQR 2–3)	2 (IQR 2–3)
Median days on parenteral nutrition	6 (IQR 4–7)	4 (IQR 1–8)
Median length of stay in days	20 (IQR 13–34)	23 (IQR 18–29)

Table 2

Breast milk host defense protein concentration by yeast colonization status

	Colonized		Uncolonized		P-value ^a
	N	Concentration	N	Concentration	
Apolipoprotein J	10	203.5 µg/ml	10	322.3 µg/ml	0.33
Dermcidin	12	8.7 ng/ml	12	19.4 ng/ml	0.04
Lactoferrin	12	7.9 mg/ml	12	8.2 mg/ml	0.92
Lysozyme	12	298.3 ng/ml	12	483.0 ng/ml	0.04
Mucin-1	12	28.2 ng/ml	12	32.8 ng/ml	0.82
Soluble CD14	11	1.8 µg/ml	9	1.6 µg/ml	0.02

^a Analysis investigated each milk sample taking into account interdependence between samples attributed to the same patient. P-value represents value for the calculated rate ratio.