

World Journal of *Clinical Pediatrics*

World J Clin Pediatr 2020 November 19; 9(3): 44-62



MINIREVIEWS

- 44 Influenza B infections in children: A review
Bhat YR

ORIGINAL ARTICLE**Basic Study**

- 53 Identification and characterization of resistance and pathogenicity of *Enterococcus spp.* in samples of donor breast milk
Santana LAM, Andrade NNN, da Silva LSC, Oliveira CNT, de Brito BB, de Melo FF, Souza CL, Marques LM, Oliveira MV

ABOUT COVER

Editorial board member of *World Journal of Clinical Pediatrics*, Dr. Rafael Denadai is a board-certified craniofacial and plastic surgeon, who offers both general and specialist cleft-craniofacial and plastic surgical care in Brazil. Dr. Denadai has been recognized among his national and international peers as a highly productive surgeon-scientist, who is involved in a broad range of research within the cleft-craniofacial field, ultimately aiming to improve the delivery of high-level surgical care. Dr. Denadai's extensive research experience includes outcome-based research, patient-reported outcome, patient-centered care, and patient-specific surgical planning and execution. He remains passionate about using patient-focused and data-driven elements to implement changes in private and public surgical practices throughout the care cycle within the fields of cleft-craniofacial, pediatric plastic surgery, and reconstructive plastic surgery. (L-Editor: Filipodia)

AIMS AND SCOPE

The primary aim of the *World Journal of Clinical Pediatrics (WJCP, World J Clin Pediatr)* is to provide scholars and readers from various fields of pediatrics with a platform to publish high-quality clinical research articles and communicate their research findings online.

WJCP mainly publishes articles reporting research results and findings obtained in the field of pediatrics and covering a wide range of topics including anesthesiology, cardiology, endocrinology, gastroenterology, hematology, immunology, infections and infectious diseases, medical imaging, neonatology, nephrology, neurosurgery, nursing medicine, perinatology, pharmacology, respiratory medicine, and urology.

INDEXING/ABSTRACTING

The *WJCP* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: Yun-Xiaoqian Wu, Production Department Director: Xiang Li, Editorial Office Director: Jia-Ping Yan.

NAME OF JOURNAL

World Journal of Clinical Pediatrics

ISSN

ISSN 2219-2808 (online)

LAUNCH DATE

June 8, 2012

FREQUENCY

Irregular

EDITORS-IN-CHIEF

Toru Watanabe, Consolato M Sergi

EDITORIAL BOARD MEMBERS

<https://www.wjgnet.com/2219-2808/editorialboard.htm>

PUBLICATION DATE

November 19, 2020

COPYRIGHT

© 2020 Baishideng Publishing Group Inc

INSTRUCTIONS TO AUTHORS

<https://www.wjgnet.com/bpg/gerinfo/204>

GUIDELINES FOR ETHICS DOCUMENTS

<https://www.wjgnet.com/bpg/GerInfo/287>

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH

<https://www.wjgnet.com/bpg/gerinfo/240>

PUBLICATION ETHICS

<https://www.wjgnet.com/bpg/GerInfo/288>

PUBLICATION MISCONDUCT

<https://www.wjgnet.com/bpg/gerinfo/208>

ARTICLE PROCESSING CHARGE

<https://www.wjgnet.com/bpg/gerinfo/242>

STEPS FOR SUBMITTING MANUSCRIPTS

<https://www.wjgnet.com/bpg/GerInfo/239>

ONLINE SUBMISSION

<https://www.f6publishing.com>

Basic Study

Identification and characterization of resistance and pathogenicity of *Enterococcus spp.* in samples of donor breast milk

Luana Andrade Mendes Santana, Nívea Nara Novais Andrade, Lucas Santana Coelho da Silva, Caline Novais Teixeira Oliveira, Breno Bittencourt de Brito, Fabrício Freire de Melo, Cláudio Lima Souza, Lucas Miranda Marques, Márcio Vasconcelos Oliveira

ORCID number: Luana Andrade Mendes Santana 0000-0001-7527-3140; Nívea Nara Novais Andrade 0000-0001-9372-7548; Lucas Santana Coelho da Silva 0000-0003-0555-0696; Caline Novais Teixeira Oliveira 0000-0003-3094-4363; Breno Bittencourt de Brito 0000-0002-1831-7909; Fabrício Freire de Melo 0000-0002-5680-2753; Cláudio Lima Souza 0000-0002-8094-8357; Lucas Miranda Marques 0000-0002-8276-8149; Márcio Vasconcelos Oliveira 0000-0002-8959-0478.

Author contributions: Santana LAM, Andrade NNN, da Silva LSC, Oliveira CNT, de Brito BB, de Melo FF, Souza CL, Marques LM, and Oliveira MV contributed to the conception and design of the study, literature review and analysis, drafting and critical revision and editing. All authors contributed equally to approval of the final version.

Institutional review board

statement: This study was reviewed and approved by the Ethical Committee of Research in Human Beings of the Multidisciplinary Institute of Health - Campus Anísio Teixeira from the Federal University of Bahia (IMS-CAT/ Universidade Federal da Bahia) under the

Luana Andrade Mendes Santana, Nívea Nara Novais Andrade, Caline Novais Teixeira Oliveira, Breno Bittencourt de Brito, Fabrício Freire de Melo, Cláudio Lima Souza, Lucas Miranda Marques, Márcio Vasconcelos Oliveira, Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Vitória da Conquista 45029-094, Bahia, Brazil

Lucas Santana Coelho da Silva, Campus Soane Nazaré de Andrade, Universidade Estadual de Santa Cruz, Ilhéus 45662-900, Bahia, Brazil

Corresponding author: Fabrício Freire de Melo, MSc, PhD, Postdoc, Professor, Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Rua Hormindo Barros, 58, Quadra 17, Lote 58, Vitória da Conquista 45029-094, Bahia, Brazil. freiremelo@yahoo.com.br

Abstract**BACKGROUND**

Breast milk is the primary source of nutrition for newborns. Hospitalized babies frequently need nutritional support from Human Milk Banks. As bacterial species of the genus *Enterococcus* are part of the microbiota of healthy donors, they may contaminate samples of pumped breast milk.

AIM

To identify and characterize the bacterial virulence and resistance in samples isolated from the nipple-areolar region, hands, and breast milk aliquots from donors at the Human Milk Bank of Municipal Hospital Esaú Matos in the city of Vitória da Conquista, Bahia State, Brazil.

METHODS

The personal hygiene and sanitation of donors were analyzed with the aim of identifying possible reasons for contamination of pumped milk. Cutaneous samples as well as aliquots of unpasteurized and pasteurized milk from 30 participants were obtained. Each *Enterococcus spp.* isolate underwent a disk diffusion susceptibility test and molecular biology techniques to determine resistance and virulence genes.

RESULTS

Enterococcus spp. were identified in 30% of donors ($n = 9$), and 11 specimens were

protocol number
80800717.8.0000.5556 and
committee opinion number
2.475.023.

Conflict-of-interest statement: The authors of this manuscript have no conflicts of interest to disclose.

Data sharing statement: There are no additional data available.

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Specialty type: Infectious diseases

Country/Territory of origin: Brazil

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: August 17, 2020

Peer-review started: August 17, 2020

First decision: September 13, 2020

Revised: September 17, 2020

Accepted: September 25, 2020

Article in press: September 25, 2020

Published online: November 19, 2020

P-Reviewer: Berardi A, Liu H

S-Editor: Zhang L

L-Editor: Webster JR

P-Editor: Wu YXJ



isolated. Resistance to tetracycline was highly prevalent, being detectable in 63% of the isolates ($n = 7$) and followed by intermediate sensitivity to ciprofloxacin, observed in 27% of the specimens ($n = 3$). The *efaA* gene was found in 63% ($n = 7$) of the isolates, while the *ace* gene was detected in 27% ($n = 3$).

CONCLUSION

This study illustrates the importance of microbiological monitoring by Human Milk Banks and the need for alternatives to prevent the presence of *Enterococcus spp.* in hospital settings.

Key Words: *Enterococcus spp.*; Breast milk; Virulence; Human milk; Pasteurization; Antimicrobial resistance

©The Author(s) 2020. Published by Baishideng Publishing Group Inc. All rights reserved.

Core Tip: Human milk is the primary source of nutrition for newborns. Hospitalized newborns frequently require nutritional support from a Human Milk Bank. *Enterococcus spp.* can contaminate human milk, as they are part of the microbiota of healthy donors. This microbial genus is important in human diseases, mainly among newborns; therefore, this study aimed to identify and characterize the virulence and resistance of *Enterococcus spp.* isolated from samples from the nipple-areolar region, hands, and raw and pasteurized milk aliquots from donors at a Brazilian Human Milk Bank.

Citation: Santana LAM, Andrade NNN, da Silva LSC, Oliveira CNT, de Brito BB, de Melo FF, Souza CL, Marques LM, Oliveira MV. Identification and characterization of resistance and pathogenicity of *Enterococcus spp.* in samples of donor breast milk. *World J Clin Pediatr* 2020; 9(3): 53-62

URL: <https://www.wjgnet.com/2219-2808/full/v9/i3/53.htm>

DOI: <https://dx.doi.org/10.5409/wjcp.v9.i3.53>

INTRODUCTION

There is no ideal substitute for human milk (HM) during early life despite current technological advances in baby food production, processing, preservation, and preparation^[1]. Unquestionably, HM supplies newborns (NBs) with all the nutrients that adequately meet the physiological and metabolic needs in this age group^[2].

Premature births and hospitalizations in neonatal intensive care units as well as maternal illness, death, and low milk production can hinder the introduction and maintenance of breastfeeding. In this context, the use of donated HM has become an efficient alternative to breastfeeding^[2] and allows NBs to be fed with milk obtained from Human Milk Banks (HMBs)^[3]. This strategy involves the collection, processing, and quality control of colostrum, transition milk, and mature milk for later distribution to NBs under a doctor or nutritionist prescription^[4].

Enterococcus species are associated with important infections in NBs and are potential contaminants of milked human milk (MHM) samples. *Enterococcus spp.* are part of the gastrointestinal tract microbiota in healthy humans^[5] and may be present in significant numbers on the hands and utensils of milk donors. The presence of these bacteria in human milk indicates that they are due to direct or indirect contact with fecal material, compromising the quality of the MHM^[6]. Some *Enterococcus spp.*, especially *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*), are highly pathogenic in NBs and must be effectively inactivated through pasteurization at HMBs.

To date, there are no published data on the isolation of enteric bacteria with hygienic-sanitary conditions of household collections performed by MHM donors. Considering the propensity of donated milk to be contaminated by these microorganisms, the clinical importance of such pathogens, and their ability to be dispersed through vectors such as health professionals and insects^[7], it is important to investigate their virulence and dispersion capacity. Moreover, it is crucial to evaluate

the quality of the collection, preservation, and processing of the HM offered to NBs. These procedures are essential for a better understanding of the pathogenicity of *Enterococcus spp.* present in raw and pasteurized human milk samples and in samples taken from the hands and nipple-areolar region of donors linked to HMBs.

MATERIALS AND METHODS

Study design, period, region, and population

This is a cross-sectional study performed using samples from the nipple-areolar region, hands, and aliquots of raw and pasteurized MHM from healthy donors registered at the HMB of the Municipal Hospital Esaú Matos in the city of Vitória da Conquista, State of Bahia, Brazil.

The present study recruited donors registered at the above-mentioned HMB who had a donation history with a mean weekly volume of donated milk greater than or equal to 400 mL. Donated samples of raw milk with a volume less than 400 mL were pooled with milk samples from other donors for pasteurization, thus losing the traceability and specificity of the sample from a specific donor. The exclusion criteria were: donors with wounds on the hand or nipple-areolar region and donation of raw milk samples with a milk volume lower than that required for individual pasteurization.

The microbial isolates analyzed in the present study were obtained by the single collection of samples from the nipple-areolar region and hands as well as aliquots of MHM before and after pasteurization from 30 donors between March and August 2018. The collections were carried out at the donors' residences during scheduled visits. In addition to microbiological evaluation of the above-mentioned biological samples, questionnaires on hygienic-sanitary issues regarding the collection, packaging, and preservation of the donated milk were completed by the participants.

Ethical considerations

The study was approved by the Ethics Committee on Research with Human Beings of the Multidisciplinary Institute of Health of the Universidade Federal da Bahia, under the Certificate of Presentation for Ethics Apprehension number 80800717.8.0000.5556 and committee opinion number 2.475.023. The study followed all the conditions established by the Resolution 466/2012 of the Brazilian National Health Council.

Isolation, identification, evaluation of antimicrobial activity, and detection of constitutive, virulence, and resistance genes in *Enterococcus spp.*

After collection, the biological samples were immediately inoculated into enrichment brain heart infusion broth (HIMEDIA®) and incubated at 35-37°C for 24 h. After that, they were placed in Petri dishes with 5% Blood Agar Medium (Base Agar, HIMEDIA®) and Chromagar Orientation (BD™ CHROMagar™ Orientation) in a bacteriological oven at 35-37°C for 24 h. Both methods were important for increasing the isolation and identification of microorganisms of interest in the present study.

The colonies with presumptive characteristics of *Enterococcus spp.* selected in both media underwent Gram staining to evaluate the morphological characteristics as well as to perform the catalase test and subsequent tests to identify the genus *Enterococcus*, namely: hydrolysis of esculin in the presence of bile and growth in sodium chloride (NaCl) 6.5% media.

All *Enterococcus spp.* isolates underwent evaluation of antimicrobial activity using the agar diffusion method^[8-10], according to the recommendations of the Clinical and Laboratory Standards Institute (2017)^[11].

With the aim of detecting the constitutive^[12], virulence^[13-16], and resistance^[17,18] genes in *Enterococcus spp.*, all samples that were positive in the identification step underwent polymerase chain reaction (PCR) after reactivation in brain heart infusion medium.

Statistical analysis

A descriptive analysis of the study variables was carried out from a database constituted in Microsoft Excel through the Statistical Program EPI INFO (version 7.2.2.6). For the frequency comparison, the chi-square test was used, with values of $P < 0.05$ (95% confidence interval) considered statistically significant.

RESULTS

Milk samples were collected from 30 donors registered at the HMB of the Municipal Hospital Esaú Matos, in Vitória da Conquista, Bahia State, Brazil. A total of 81 samples were collected: 30 samples from swabs containing skin samples from the nipple-areolar region and donors' hands, 30 samples of raw MHM, and 21 samples of pasteurized MHM. Following the methodological criteria, it was not possible to obtain an appropriate aliquot of pasteurized milk from 9 (30%) participants as they did not donate enough milk volume to perform pasteurization in a single bottle.

Enterococcus spp. strains were isolated from 9 donors (30%), and a total of 11 strains were identified: 7 strains (63.6%) from skin samples and 4 strains (36.4%) from raw MHM samples. Although *Enterococcus spp.* were not detected in the pasteurized MHM samples, in 38% ($n = 8$) of these samples other microorganisms, mainly *Staphylococcus aureus*, were isolated.

The study participants were questioned about socioeconomic aspects and hygienic-sanitary conditions in order to link selected variables to the route of human milk contamination by *Enterococcus spp.* In this context, most participants were aged 30 years or less, were married or living with a partner, had a family income above two minimum wages, had one child, and were educated to high school level.

With regard to the hygienic-sanitary procedures performed by the donors for milk collection, most participants reported that they always performed the following procedures: hand washing before the collection of milk (96%); held back hair before milking (80%); breast washing before milking (86.7%); used a mask while milking (86.7%); avoided talking during milking (60%); discarded the first jet of milk (53.3%); milk obtained using a manual/electric pump (80%). However, only 12 (80%) participants declared that they checked the size of their nails and trimmed them when necessary before milking (Table 1).

Socioeconomic variables of HM donors such as age (above or below 30 years), marital status (single or living as a couple), family income (higher or lower than 2 minimum wages), number of children (one or more than one), and educational level (high school or graduate) as well as variables related to hygienic-sanitary procedures including hand washing before milking, talking during milking, and discarding the first milk jet at the beginning of collection were not significantly associated with the detection of *Enterococcus spp.* in samples ($P < 0.05$) (Table 1).

Among the 11 isolates of *Enterococcus spp.*, antimicrobial resistance was found in 10 isolates (91%). Resistance to tetracycline was most common (63% of isolates, $n = 7$), and intermediate sensitivity to ciprofloxacin was found in 27% of the isolates ($n = 3$). Resistance to quinolones (ciprofloxacin, norfloxacin, and levofloxacin) was observed in 9% of the isolates ($n = 1$) (Table 2). All isolates were sensitive to vancomycin. All specimens were analyzed by PCR to detect quinolone resistance genes (*gyrA* and *parC* genes), but no positive results were obtained.

The *Enterococcus spp.* isolates were analyzed by PCR for detection of the constitutive *ddl* gene encoding the D-alanine ligases, which are species-specific for *E. faecalis* and *E. faecium*. This analysis showed that among the 11 enterococcal strains from 9 participants, 6 isolates were identified as *Enterococcus spp.* and 5 isolates were identified as *E. faecalis*. However, three *E. faecalis* isolates were found to be simultaneously colonized by *E. faecium* strains. Thus, three donors had *E. faecalis* and *E. faecium* strains. Such detection was possible because, after the initial identification of the *Enterococcus* genus, the stored colonies were collected from the CHROMagar Orientation medium (BD™ CHROMagar™ Orientation), which identified the genus and not the species of *Enterococcus*; thus, colonies of both species were collected.

All isolates of *Enterococcus spp.* underwent PCR for detection of the virulence genes *ace*, *efaA*, *gelE*, *as*, *cylA*, *hyl*, *esp*, *as*, *cylA*, *hyl*, and *esp* (Table 3). Among the 11 isolates, 7 isolates (63%) had the *efaA* gene that encodes the surface antigen A, which is commonly associated with biofilm formation and endocarditis, and 3 isolates (27%) had the *ace* gene, which encodes the protein that adheres to collagen and laminin, favoring bacterial adhesion commonly associated with endocarditis. The following enterocin genes were also genotypically investigated by PCR in *Enterococcus spp.*: Enterocin A, Enterocin B, Enterocin P, and Enterocin 31, also known as Bacteriocin. However, none of the isolates were positive for these genes.

DISCUSSION

Enterococcus spp. are present in the human microbiota and are associated with

Table 1 Descriptive analysis of the socioeconomic aspects and hygienic-sanitary conditions related to the collection of human milk and to human milk donors ($n = 30$) registered in the Human Milk Bank of the Municipal Hospital Esaú Matos in Vitória da Conquista, Bahia State, Brazil

Descriptive analysis of socioeconomic aspects of HM donors		
Variable	<i>n</i>	(%)
Age group		
Age less than 30 yr	19	63.3
Over 30 yr of age	11	36.7
Marital status/Conjugal state		
Married/living with spouse	20	66.7
Single or divorced/without a partner	10	33.3
Family income		
Less than 2 minimum wages ¹	9	30.0
Above 2 minimum wages	21	70.0
Number of children		
One child	16	53.3
Two or more children	14	36.7
Schooling		
High school	21	70.0
Graduate	9	30.0
Total	30	100.0
Descriptive analysis of hygienic-sanitary conditions of HM donors		
Variable	<i>n</i>	(%)
Hand washing prior to collection		
Sometimes/never	1	3.3
Always	29	96.7
Nail size check/trim before collection		
Sometimes/never	18	60.0
Always	12	40.0
Hair holding habit before collection		
Sometimes/never	6	20.0
Always	24	80.0
Breast lavage before collection		
Sometimes/never	4	13.3
Always	26	86.7
Use of mask during collection		
Sometimes/never	4	13.3
Always	26	86.7
Avoid talking during collection		
Sometimes/never	12	40.0
Always	18	60.0
Discard first jet of milk at the beginning of collection		
Sometimes/never	14	46.7

Always	16	53.3
Milk collection		
Manual/ electric pump	24	80.0
Manual milking	6	20.0
Cleaning frequency of the pump ²		
Sometimes/never	0	0
Always before using	24 ¹	100.0 ¹
Total	30	100.0

¹Minimum wage in the period: BRL954,00. ²Question applied only to donors who reported performing milking by hand and using an electric pump.

Table 2 Sensitivity profile of the isolates ($n = 11$) of *Enterococcus spp.* found in relation to the list of antimicrobials evaluated following the recommendations of the Clinical and Laboratory Standards Institute (2017)

Antimicrobials	Sensitive, n (%)	Intermediate, n (%)	Resistant, n (%)
Ampicillin 10 µg	11 (100.0)	0 (0)	0 (0)
Penicillin 10 un	9 (81.8)	0 (0)	2 (18.2)
Tetracycline 30 µg	4 (36.4)	0 (0)	7 (63.6)
Ciprofloxacin 5 µg	7 (63.6)	3 (27.3)	1 (9.1)
Norfloxacin 10 µg	9 (81.8)	0 (0%)	2 (18.2)
Levofloxacin 5 µg	10 (90.9)	0 (0)	1 (9.1)
Nitrofurantoin 300 µg	9 (81.8)	0 (0)	2 (18.2)
Linezolid 30 µg	11 (100.0)	0 (0)	0 (0)
Vancomycin 30 µg	11 (100.0)	0 (0)	0 (0)

un: Units.

Table 3 Distribution of the virulence genes of *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus spp.*

Virulence genes	<i>E. faecalis</i>		<i>E. faecium</i>		<i>Enterococcus spp.</i>		Total	
	N (isolated)	%	N (isolated)	%	N (isolated)	%	N (isolated)	%
<i>Ace</i>	1 ^a	33	1 ^a	33	2	67	3	27
<i>efaA</i>	3 ^b	43	2 ^b	28	3	43	7	63
<i>gelE</i>	0	-	0	-	0	-	0	-
<i>As</i>	0	-	0	-	0	-	0	-
<i>cylA</i>	0	-	0	-	0	-	0	-
<i>Hyl</i>	0	-	0	-	0	-	0	-
<i>Esp</i>	0	-	0	-	0	-	0	-

^a*ace* gene found in 1 isolate (containing strains of *Enterococcus faecalis* and *Enterococcus faecium*). ^b*efaA* gene found in three *Enterococcus faecalis* isolates (being a single isolate of *Enterococcus faecalis* and two isolates of *Enterococcus faecalis* and *Enterococcus faecium*). *E. faecalis*: *Enterococcus faecalis*; *E. faecium*: *Enterococcus faecium*.

important infections in NBs, and are potential contaminants of HM samples^[6]. To date, there are no studies correlating NB infections and infant colonization with *Enterococcus spp.* in the city where this study was carried out. Moreover, there are no data associating socioeconomic aspects and hygienic-sanitary conditions regarding HM donors with the isolation of enteric bacteria. In the present study, *Enterococcus spp.*

were isolated from 30% of the participants. Similarly, *Enterococcus spp.* were isolated from raw MHM samples within four^[19] and seven^[20] days after delivery. In such studies, gram-positive bacteria, including *E. faecalis*^[21], *E. faecium*^[21,22], *Staphylococcus spp.*^[23] and fecal coliforms^[2] were identified in MHM^[19], but they were not detected after milk pasteurization; however, other microorganisms continued to be detected after pasteurization.

Similar findings were observed in other HMBs in which the isolation rates were 2%^[2], 6%^[2], 7.5%^[21], and even 70.4%^[3] in non-pasteurized MHM samples and was 50.7% in pasteurized MHM^[3] samples. These data reinforce the importance of strict hygienic-sanitary procedures in HMBs as well as the microbiological control of HM samples.

Among the 11 *Enterococcus spp.* isolated in this study, different types of resistance to antibiotics were found in 91% of the isolates ($n = 10$). In the phenotypical analysis of antimicrobial resistance, most isolates were susceptible to ampicillin, linezolid, and vancomycin, but resistance to tetracycline (63%), ciprofloxacin (27%), norfloxacin, levofloxacin, nitrofurantoin (9%), and penicillin was detected. *Enterococcus spp.* resistance has been previously reported in samples from miscellaneous clinical materials^[22], food, and clinical samples^[23], with resistance rates of 5% to tetracycline^[22] and of 3%^[22] and 70.5%^[23] to ciprofloxacin.

Despite the identification of quinolone resistance, the resistance genes *gyrA* and *parC* were not detected. This phenomenon may be associated with interferences, including differences in the pH of the Mueller Hinton media, in mean temperature, and in the density of the inoculum applied. Due to *Enterococcus spp.* resistance to antimicrobials such as penicillin, ampicillin, aminoglycosides, tetracycline, and vancomycin and its association with nosocomial infections^[24], it is crucial to identify these bacteria in different types of biological samples, including HM.

In this study, the presence of virulence factors in the isolated strains was also evaluated, and the genes *efaA* and *ace* were found in 63% and 27% of the isolates, respectively. These genes are associated with enterococcal biofilm formation and endocarditis, making the treatment of infections caused by these microorganisms difficult. With regard to species identification, of the three isolates with the *ace* gene, two were *Enterococcus spp.* and one had *E. faecium* and *E. faecalis* strains. Among the seven isolates carrying the *efaA* gene, three were *Enterococcus spp.*, two were *E. faecalis*, and two had *E. faecalis* and *E. faecium* strains.

The genes *ace* and *efaA* have been previously detected in *E. faecalis* and *E. faecium*^[22] hospital strains and the *ace* gene has also been found in *Enterococcus spp.* of clinical origin^[23]. The pathogenicity mechanisms of *Enterococcus spp.* are not yet fully understood. It is known that these microorganisms have virulence factors that are associated with the severity and duration of human infections as well as with the dissemination of these microorganisms in the hospital environment, where they mainly cause urinary infections, endocarditis, and bacteremia. The most frequently reported virulence factors are related to the adhesion to host tissues, invasion, abscess formation, modulation of the host inflammatory responses, and secretion of toxic products^[25].

The high percentage of *Enterococcus spp.* isolates in HM samples associated with the high antimicrobial resistance rate (mainly to tetracycline and ciprofloxacin) and the positivity of virulence factors (detection of the genes *ace* and *efaA*) found in this study confirm the importance of carrying out strict control from the collection to the distribution of MHM. In this context, it is necessary to take utmost care during milk processing in order to avoid the dispersion of microorganisms, as the entry of these microorganisms into the hospital environment can increase the risk of infection in hospitalized patients, mainly in those with a weakened immune system.

CONCLUSION

The present study demonstrates the necessity for microbiological control in HMBs and the requirement to develop alternatives to avoid the entry of *Enterococcus spp.* species into the hospital environment, which can potentially lead to important NB infections that increase the length of hospitalizations and, consequently, health service costs. Considering that most premature NBs are transferred to intensive care units, thereby requiring food supplementation with MHM, and that the consumption of contaminated MHM can lead to mild-to-severe infections, the accurate identification of bacterial pathogens is an essential requirement for the detection of microorganism reservoirs, infection sources, HM distribution monitoring, and decision taking aiming to reduce health risks in NBs. Moreover, strict control from the collection to the

distribution of donated HM may reduce the dispersion rates of virulent and multidrug-resistant microorganisms that can cause several problems in a hospital environment, leading to increases in morbidity, mortality, and costs related to health care.

ARTICLE HIGHLIGHTS

Research background

Human milk is the primary source of nutrition for newborns. Hospitalized babies often require nutritional support from Human Milk Banks. As *Enterococcus spp.* are part of the microbiota of healthy donors, they are potential contaminants of pumped breast milk.

Research motivation

Donor Human Milk is an important alternative that allows the appropriate feeding of hospitalized newborns. A number of steps are needed to ensure the safe collection, processing, and quality control of colostrum, transition milk, and mature milk for subsequent distribution to newborns under a doctor or nutritionist prescription. However, such milk can be contaminated by *Enterococcus spp.* species that are highly pathogenic in newborns, and can increase morbidity, hospitalization time, and mortality among neonates.

Research objectives

The present study intended to identify and describe the bacterial virulence and resistance in samples obtained from the nipple-areolar region, hands, and breast milk aliquots of donors at the HMB of Municipal Hospital Esau Matos in the city of Vitória da Conquista, Bahia State, Brazil.

Research methods

This cross-sectional study used samples from the nipple-areolar region, hands, and aliquots of raw and pasteurized milked human milk from healthy human milk donors. The study recruited donors who had a donation history of a mean weekly volume of donated milk equal to or higher than 400 mL. The microbial isolates from milk samples were analyzed before and after pasteurization. A total of 30 donors were assessed between March and August 2018.

The samples were immediately inoculated into enrichment brain heart infusion broth (HIMEDIA®) and incubated at 35-37°C for 24 h after collection. Thereafter, they were placed in Petri dishes with 5% Blood Agar Medium (Base Agar, HIMEDIA®) and Chromagar Orientation (BD™ CHROMagar™ Orientation) in an incubator at 35-37°C for 24 h.

Colonies in which *Enterococcus spp.* were observed were selected from both media and underwent Gram staining to evaluate the morphological characteristics and to perform the catalase test and subsequent tests to identify the genus *Enterococcus*.

All *Enterococcus spp.* isolates underwent evaluation of antimicrobial activity by the agar diffusion method, according to the recommendations of the Clinical and Laboratory Standards Institute (2017).

To detect the constitutive, virulence, and resistance genes in *Enterococcus spp.*, all samples that were positive in the identification step underwent polymerase chain reaction after reactivation in brain heart infusion media.

A descriptive analysis of the study variables was carried out from a database constituted in Microsoft Excel through the Statistical Program EPI INFO (version 7.2.2.6). For the frequency comparison, the chi-square test was used, with values of $P < 0.05$ (95% confidence interval) considered statistically significant.

Research results

Eighty-one samples were collected, with 30 samples from swabs containing nipple-areolar region skin samples and donors' hands, 30 samples of raw milked human milk, and 21 samples of pasteurized milked human milk. *Enterococcus spp.* strains were isolated from 30% of the donors and 11 strains were identified: 7 strains (63.6%) from skin samples and 4 strains (36.4%) from raw MHM samples.

The majority of participants declared that they washed their hands before the collection of milk (96%), held back hair before milking (80%), washed the breasts before milking (86.7%), used a mask while milking (86.7%), avoided talking during milking

(60%), discarded the first jet of milk (53.3%), and obtained milk using a manual/electric pump (80%).

No statistically significant associations between demographic and hygienic characteristics and the detection of *Enterococcus spp.* were observed. Antimicrobial resistance was identified in 10 isolates (91%), mainly to tetracycline.

Research conclusions

Here we demonstrate the importance of appropriate microbiological control in Human Milk Banks in order to prevent *Enterococcus spp.* in hospitals as these bacteria can lead to important newborn infections that increase morbidity, mortality, and hospitalizations among these patients, and increase health service costs.

Research perspectives

This study adds information to the microbiological control of donated milk, which, if well-performed from human milk collection to its distribution, may reduce the dispersion rates of virulent and multidrug-resistant microorganisms in a hospital environment, avoiding unwanted clinical outcomes among newborns. Putting such measures into practice may improve the health of children around the world.

REFERENCES

- 1 **Silva VAAL**, Caminha MFC, Silva SL, Serva VMSBD, Azevedo PTACC, Filho MB. Maternal breastfeeding: indicators and factors associated with exclusive breastfeeding in a subnormal urban cluster assisted by the Family Health Strategy. *J Pediatr (Rio J)* 2019; **95**: 298-305 [PMID: 29530663 DOI: 10.1016/j.jped.2018.01.004]
- 2 **Borges MS**, Oliveira AMM, Hattori WT, Abdallah VOS. Quality of human milk expressed in a human milk bank and at home. *J Pediatr (Rio J)* 2018; **94**: 399-403 [PMID: 28859913 DOI: 10.1016/j.jped.2017.07.004]
- 3 **Serafini AB**, André MC, Rodrigues MA, Kipnis A, Carvalho CO, Campos MR, Monteiro EC, Martins F, Jubé TF. [Microbiological quality of human milk from a Brazilian milk bank]. *Rev Saude Publica* 2003; **37**: 775-779 [PMID: 14666308 DOI: 10.1590/s0034-89102003000600013]
- 4 **de Almeida JA**, Novak FR. [Breastfeeding: a nature-culture hybrid]. *J Pediatr (Rio J)* 2004; **80**: S119-S125 [PMID: 15583761 DOI: 10.2223/1242]
- 5 **Murray PR**. Medical Microbiology. 4th ed. Guanabara Koogan, 2004: 220-223
- 6 **Novak FR**, Almeida JA, Asensi MD, Moraes BA, dos Prazeres Rodrigues D. [Antimicrobial resistance of coliform isolates from expressed human milk]. *Cad Saude Publica* 2001; **17**: 713-717 [PMID: 11395808 DOI: 10.1590/s0102-311x2001000300026]
- 7 **Oliveira PS**, Souza SG, Campos GB, da Silva DC, Sousa DS, Araújo SP, Ferreira LP, Santos VM, Amorim AT, Santos AM, Timenetsky J, Cruz MP, Yatsuda R, Marques LM. Isolation, pathogenicity and disinfection of *Staphylococcus aureus* carried by insects in two public hospitals of Vitória da Conquista, Bahia, Brazil. *Braz J Infect Dis* 2014; **18**: 129-136 [PMID: 24216155 DOI: 10.1016/j.bjid.2013.06.008]
- 8 **RAZIL**. National Health Surveillance Agency. Brazilian Pharmacopoeia. 5th ed. Anvisa: 2010: 59-73
- 9 **Fracalanza SA**, Scheidegger EM, Santos PF, Leite PC, Teixeira LM. Antimicrobial resistance profiles of enterococci isolated from poultry meat and pasteurized milk in Rio de Janeiro, Brazil. *Mem Inst Oswaldo Cruz* 2007; **102**: 853-859 [PMID: 18060316 DOI: 10.1590/s0074-02762007005000120]
- 10 **Valenzuela AS**, Benomar N, Abriouel H, Cañamero MM, Gálvez A. Isolation and identification of *Enterococcus faecium* from seafoods: antimicrobial resistance and production of bacteriocin-like substances. *Food Microbiol* 2010; **27**: 955-961 [PMID: 20688238 DOI: 10.1016/j.fm.2010.05.033]
- 11 **CLSI**. M100-S26: Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. Clinical and Laboratory Standards Institute, 2017
- 12 **Dutka-Malen S**, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* 1995; **33**: 24-27 [PMID: 7699051 DOI: 10.1128/JCM.33.1.24-27.1995]
- 13 **Mannu L**, Paba A, Daga E, Comunian R, Zanetti S, Duprè I, Sechi LA. Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *Int J Food Microbiol* 2003; **88**: 291-304 [PMID: 14597001 DOI: 10.1016/s0168-1605(03)00191-0]
- 14 **Vankerckhoven V**, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H. Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *J Clin Microbiol* 2004; **42**: 4473-4479 [PMID: 15472296 DOI: 10.1128/JCM.42.10.4473-4479.2004]
- 15 **Shankar V**, Baghdayan AS, Huycke MM, Lindahl G, Gilmore MS. Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. *Infect Immun* 1999; **67**: 193-200 [PMID: 9864215 DOI: 10.1128/IAI.67.1.193-200.1999]
- 16 **Vancanneyt M**, Lombardi A, Andrighetto C, Knijff E, Torriani S, Björkroth KJ, Franz CM, Foulquié Moreno MR, Revets H, De Vuyst L, Swings J, Kersters K, Dellaglio F, Holzapfel WH. Intraspecies genomic groups in *Enterococcus faecium* and their correlation with origin and pathogenicity. *Appl Environ Microbiol* 2002; **68**: 1381-1391 [PMID: 11872491 DOI: 10.1128/aem.68.3.1381-1391.2002]
- 17 **el Amin NA**, Jalal S, Wretling B. Alterations in *GyrA* and *ParC* associated with fluoroquinolone resistance in *Enterococcus faecium*. *Antimicrob Agents Chemother* 1999; **43**: 947-949 [PMID: 10103206 DOI: 10.1128/AAC.43.5.947-949.1999]

- 10.1128/AAC.43.4.947]
- 18 **Brisse S**, Fluit AC, Wagner U, Heisig P, Milatovic D, Verhoef J, Scheuring S, Köhrer K, Schmitz FJ. Association of alterations in ParC and GyrA proteins with resistance of clinical isolates of *Enterococcus faecium* to nine different fluoroquinolones. *Antimicrob Agents Chemother* 1999; **43**: 2513-2516 [PMID: 10508034 DOI: 10.1128/AAC.43.10.2513]
 - 19 **Martín R**, Langa S, Reviriego C, Jiménez E, Marín ML, Xaus J, Fernández L, Rodríguez JM. Human milk is a source of lactic acid bacteria for the infant gut. *J Pediatr* 2003; **143**: 754-758 [PMID: 14657823 DOI: 10.1016/j.jpeds.2003.09.028]
 - 20 **Martín R**, Heilig HG, Zoetendal EG, Jiménez E, Fernández L, Smidt H, Rodríguez JM. Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Res Microbiol* 2007; **158**: 31-37 [PMID: 17224259 DOI: 10.1016/j.resmic.2006.11.004]
 - 21 **Silveira LAM**, D'Amorim MFG, Silva VRD, Terra APS. Controle Microbiológico do Leite Humano de um Hospital Universitário. *Revista Baiana de Saúde Pública* 2012; **36**: 844 [DOI: 10.22278/2318-2660.2012.v36.n3.a560]
 - 22 **Kang M**, Xie Y, He C, Chen ZX, Guo L, Yang Q, Liu JY, Du Y, Ou QS, Wang LL. Molecular characteristics of vancomycin-resistant *Enterococcus faecium* from a tertiary care hospital in Chengdu, China: molecular characteristics of VRE in China. *Eur J Clin Microbiol Infect Dis* 2014; **33**: 933-939 [PMID: 24463723 DOI: 10.1007/s10096-013-2029-z]
 - 23 **Gomes BC**, Esteves CT, Palazzo IC, Darini AL, Felis GE, Sechi LA, Franco BD, De Martinis EC. Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. *Food Microbiol* 2008; **25**: 668-675 [PMID: 18541165 DOI: 10.1016/j.fm.2008.03.008]
 - 24 **Hörner R**, Liscano MGM, Maraschin MM, Salla A, Meneghetti B, Nara L, Frasson Dal Forno NLF, Righi RA. Suscetibilidade antimicrobiana entre amostras de *Enterococcus* isoladas no Hospital Universitário de Santa Maria. *J Bras Patol Med Lab* 2005; **41**: 391-395 [DOI: 10.1590/S1676-24442005000600004]
 - 25 **Eaton TJ**, Gasson MJ. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl Environ Microbiol* 2001; **67**: 1628-1635 [PMID: 11282615 DOI: 10.1128/AEM.67.4.1628-1635.2001]



Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA

Telephone: +1-925-3991568

E-mail: bpgoffice@wjgnet.com

Help Desk: <https://www.f6publishing.com/helpdesk>

<https://www.wjgnet.com>

