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ORIGINAL ARTICLE

Mammary candidiasis: molecular-based detection of *Candida* species in human milk samples

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Abstract In this prospective and monocentric study, we investigated the performance of a commercialized real-time polymerase chain reaction (RT-PCR) test system for the specific detection of DNA from *Candida albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis* in human milk samples of patients suspicious of mammary candidiasis. For this purpose, 43 breast-feeding women with characteristic symptoms of mammary candidiasis and 40 asymptomatic controls were enrolled. By culture, *Candida* spp. were detected in 8.8 % (4/46) and 9.3 % (4/43) of patient and control samples, respectively. *Candida albicans* (2/46), *C. parapsilosis* (1/46), and *C. guilliermondii* (1/46) were present in patient samples, and *C. lusitaniae* (3/43) and *C. guilliermondii* (1/43) were present in the controls. After RT-PCR was applied, *Candida* spp. were found to be present in 67.4 % (31/46) and 79.1 % (34/43) of patient and control samples investigated, respectively. PCR detection of *C. albicans* and *C. parapsilosis* revealed only a low sensitivity and specificity of 67.4 % and 41.9 %, respectively. Our data do not support the use of *Candida* RT-PCR for sensitive and specific diagnosis of mammary candidiasis.

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Introduction

Candida infection of the lactating mammary may cause substantial symptoms, including shooting breast pain when feeding, with and without involvement of the areola skin [1, 2]. The management is difficult because diagnosis is most often based on subjective signs and symptoms. The correlation between clinical symptoms and proof of *Candida* infections is low.

Ninety-five percent of all *Candida* infections are caused by five species: *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* [3]. Superficial and localized infections with *C. albicans* in lactating women may be a cause of sore nipples and shooting breast pain during lactation [4]. So far, women suffering from these symptoms who are treated without proof of fungal involvement responded to antifungal treatment with fluconazole [5]. The diagnosis of candidiasis based on clinical symptoms is difficult and most infections are associated with *Staphylococcus aureus* [6]. Milk cultivation techniques are of limited value, as lactoferrin may mediate fungistatic effects on *C. albicans* [7]. Culture-independent polymerase chain reaction (PCR) techniques may be promising for the rapid, sensitive, and accurate detection and identification of fungal pathogens [8, 9]. However, clinical data on PCR and mammary *Candida* infections are limited; the study by Panjaitan et al. [10] lacked in showing the usefulness of PCR as a diagnostic tool for *Candida* infections in breast-feeding women. The study population was too small and standard microbial culture was not performed. The CASTLE (*Candida* and *Staphylococcus* Transmission: Longitudinal Evaluation) project investigated the role of *Candida* spp. in nipple pain using real-time PCR (RT-PCR) technology [11, 12]. The study showed *Candida* spp. being associated with burning nipple pain and breast pain. However, breast milk was not investigated.

Here, we evaluated a commercial *Candida*-specific quantitative RT-PCR for the sensitive detection of *Candida* spp. in human milk in patients suspicious of suffering from mammary candidiasis. Various preanalytical sample processing methods were applied to improve culture detection.

Materials and methods

Study design

This monocentric and prospective study was approved by the Institutional Review Board in Innsbruck (UN4801) and informed consent was obtained from breast-feeding mothers (age ≥ 18 years). Breast-feeding women were suspicious of suffering from mammary candidiasis showing one or more of the following symptoms: (1) burning, itching, or shooting pain in the mammilla/areola plus deep breast pain radiating to the shoulder; (2) areola showing a flush or white coating, dry or desquamate skin. Study enrollment was from January to October 2013. Human milk samples from asymptomatic breast-feeding women and without any clinical evidence of mammary candidiasis or mastitis served as controls. Samples were collected from different clinical sites in Austria and Germany, various birth houses in Germany, and by registered doctors and midwives in Austria and Germany. A case report form (CRF) documented the detailed characteristics of symptoms (pain and skin manifestations). Midstream breast milk samples (9 ml) were aseptically collected undergoing standard hygienic procedures and kept on ice during transport to the laboratory.

Microbial cultivation and DNA extraction

Fifty microliters of native human milk samples were routinely cultured on BBL Columbia blood agar (Becton, Dickinson and Company, Heidelberg, Germany) and Sabouraud dextrose agar (SDA) supplemented with gentamicin and chloramphenicol (Becton, Dickinson and Company, Heidelberg, Germany) for 2 and 7 days at 37 °C. Quantification was done by counting the colony-forming units (CFU).

In addition, three preanalytical processing methods (A–C) were applied to improve microbial cultivation and PCR detection. Thus, milk samples were equally divided into three 2-ml portions and underwent a centrifugation for 25 min and 4500 g at room temperature (Fig. 1). Pretreatment A centrifuged the human milk, discarded the supernatant, and resuspended the pellet with 1 ml Tris-EDTA buffer (TE buffer; Biozym, Vienna, Austria); 50 μl was plated on SDA and incubated at 37 °C for 7 days. Pretreatment B resuspended the pellet with 2 ml Sabouraud dextrose broth supplemented with gentamicin and chloramphenicol (Becton, Dickinson and Company, Heidelberg, Germany) and incubated at 37 °C for 24 h. Afterwards, 50 μl was cultivated on SDA at 37 °C for 7 day. Then, the SDA broth was centrifuged and the pellet was resuspended with 1 ml TE buffer. Pretreatment C added ferrous sulfate $Fe(II)SO_4^{2-}$ (Sigma-Aldrich, Vienna, Austria) to 2-ml milk aliquots (200 μg iron/ml milk) and incubated at 37 °C for 24 h. Then, 50 μl of the milk was cultivated on SDA at 37 °C for 7 day. Finally, the milk was centrifuged and the pellet was fully resuspended in 1 ml TE buffer.

The fungal genomic DNA was isolated from TE buffer pellets using the QIAamp UltraSens Virus DNA Kit (QIAGEN, Hilden, Germany), following the manufacturer's recommendations. Purified DNA extracts were stored at −20 °C.

Multiplex PCR

PCR reactions for the detection of *Candida* DNA were performed using the MycoReal *Candida* kit (ingenetix, Vienna, Austria), following the manufacturer's recommendations. This highly sensitive and specific RT-PCR assay amplifies the internal transcribed spacer region 2 (ITS2) and allows the simultaneous detection of seven medically important species of *Candida*, namely *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis* [13]. A sample was considered positive for a *Candida* species upon a positive amplification curve and a biprobe-specific melting peak. To exclude false-negative PCR results, an internal control (IC) was coamplified. In case of PCR inhibition, the assay was repeated with diluted DNA eluates. Each PCR included a negative control consisting of water without template DNA to monitor contamination. DNA extracts from patient samples were analyzed in parallel with an extraction control and a PCR-positive control containing fungal DNA from *C. albicans*. All PCRs were run on a LightCycler 2.0 Instrument (Roche, Indianapolis, IN, USA).

Statistics

Descriptive statistics included measures of central tendency and spread of continuous variables. Samples from breast-feeding mothers with and without clinical symptoms of mammary candidiasis were used to assess PCR assays and cultivation performance. The performance of the PCR test was evaluated by calculating the sensitivity and specificity (95 % confidence interval) using clinical diagnosis of typical symptoms of mammary candidiasis as the gold standard. Unless specified, a p-value less than 0.05 was considered to be statistically significant. For overall statistical analysis, only samples processed with all mentioned cultivation and DNA extraction variants (pretreatments A–C) were included. For frequency calculation, positive bacterial cultivation was only taken into account when a microbial count $>10^4$ CFU ml^{-1} was obtained. Statistical analysis was conducted using GraphPad Prism v5.01.

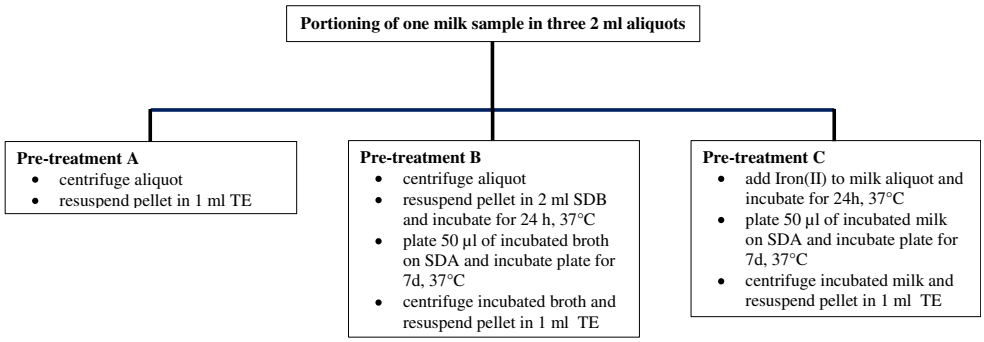

Fig. 1 Workflow for DNA extraction and cultivation of *Candida* in human milk. *TE* Tris-EDTA buffer; *SDA* Sabouraud dextrose agar (supplemented with gentamicin and chloramphenicol); *SDB* Sabouraud dextrose broth (supplemented with gentamicin and chloramphenicol)

Results

Study population

Forty-six milk samples from 43 breast-feeding women with clinical symptoms of mammary candidiasis were enrolled in this study. Forty-three milk samples of 40 asymptomatic breast-feeding women (all collected at the Internal Human Milk Bank from The University Hospital of Innsbruck) served as controls. Clinical signs and symptoms were documented, as shown in Table 1. Seven women received a topical antifungal therapy with nystatin.

Cultivation of *Candida*

Cultivation techniques revealed fungal positivity in 8.8 % (4/46) and 9.3 % (4/43) of samples from symptomatic and asymptomatic breast-feeding women. *Candida albicans* (2/46), *C. parapsilosis* (1/46), and *C. guilliermondii* (1/46) were present in patient samples, and *C. lusitaniae* (3/43) and *C. guilliermondii* (1/43) were present in the controls (Table 2).

PCR detection of *Candida*

PCR was positive in 67.4 % and 79.1 % of patients and controls, respectively (see Table 3). When RT-PCR was applied, *Candida* spp. were observed as being present in 67.4 % (31/46) and 79.1 % (34/43) of patient and control samples investigated, respectively. *Candida albicans* and *C. parapsilosis* were detected in both groups, while *C. lusitaniae* was detected only in the controls. *Candida guilliermondii* failed to be detected in the PCR panel. RT-PCR and culture techniques revealed discordant results; *C. albicans* was detected by culture only in patients and not in controls, while RT-PCR showed *C. albicans* as being present in both patients and controls.

Cultivation of bacterial pathogens

Staphylococcus epidermidis and *S. aureus* were found in 19.6 % (9/46) and 21.7 % (10/46) of patient samples, respectively. Further species included *Acinetobacter johnsonii* 19.6 % (9/46), *Bacillus cereus* 4.3 % (2/46), *Enterococcus faecalis/faecium* 13.0 % (6/46), *Enterobacter* spp. 4.3 % (2/46), *Klebsiella oxytoca* 10.8 % (5/46), and *Streptococcus* spp. 4.3 % (2/46). Control samples displayed *S. aureus* in 7.0 % (3/43), *S. epidermidis* 2.3 % (1/43), *A. johnsonii* 2.3 % (1/43), *K. oxytoca* 2.3 % (1/43), and *Enterobacter* spp. 11.6 % (5/43).

Table 1 Pain characteristics, skin alterations, and localization of symptoms reported from women ($n = 43$) with symptoms of mammary candidiasis (presumptive clinical suspicion). Inclusion criteria for our study were the occurrence of one or a composition of the following clinical symptoms: (1) burning, itching, or shooting pain in the mammilla/areola and in the deep breast, radiating to the shoulder; (2) areola with flush or white coating, dry, or desquamate skin

Documentation of symptoms (no. of patients)		
Pain symptoms	Pain characteristics	Skin alterations
Shooting (19)	During breast-feeding (22)	Reddening (15)
Burning (8)	Between and after breast-feeding (19)	Shiny (9)
Itching (12)	Continuous (1)	Scaling (9)
Different (4)	Unclear (1)	Sore (10)

Table 2 Frequency of *Candida*-positive cultural detection obtained from patient ($n = 46$) and control samples ($n = 43$). Effect of pretreatments A–C on microbial growth: + (<20 CFU), ++ (20–100 CFU), +++ (>100 CFU). *PA* patient samples; *CO* control samples

Sample no.	*Candida* species	Pretreatment		
		A	B	C
CO1	*C. lusitaniae*	+	++	+
CO2	*C. guilliermondii*	+	++	+
CO3	*C. lusitaniae*	+	++	+
CO4	*C. lusitaniae*	+	++	+
PA1	*C. albicans*	+	++	++
PA2	*C. albicans*	+	+++	++
PA3	*C. parapsilosis*	+	+++	++
PA4	*C. guilliermondii*	–	+++	–

Table 3 Frequency of positive *Candida* polymerase chain reaction (PCR) results obtained from patient ($n = 46$) and control samples ($n = 43$); calculation of sensitivity and specificity in % (95 % confidence interval) and Fisher's exact p-value

	Patient samples	Control samples	Sensitivity	Specificity	p-Value
C. albicans	26.1 % (12/46)	44.2 % (19/43)	26.1 (14.8, 41.4)	55.8 (40.0, 70.6)	0.081
C. parapsilosis	56.5 % (26/46)	41.9 % (18/43)	56.5 (41.2, 70.1)	58.1 (42.2, 72.6)	0.205
C. lusitaniae	0.0 % (0/46)	20.9 % (9/43)	0.0 (0, 9.6)	79.1 (63.5, 89.4)	0.001
Candida total[a]	67.4 % (31/46)	79.1 % (34/43)	67.4 (51.9, 80.0)	20.9 (10.6, 36.5)	0.241
Candida total[b]	67.4 % (31/46)	58.1 % (25/43)	67.4 (56.7, 77.6)	41.9 (30.5, 52.8)	0.389

[a] Includes all *Candida*-positive PCR results

[b] Includes only PCR results exclusively positive for *C. albicans* and/or *C. parapsilosis*

Discussion

Early recognition and treatment of *Candida* nipple and/or breast infections is essential to support successful long-term breast-feeding. Fungal diagnosis is difficult and the new RT-PCR technology applied in this study failed to show superiority when compared to the conventional technique.

Of 43 patients suffering from possible candidiasis (presumptive clinical diagnosis), proof of fungal infections was given in only 8.8 % using culture techniques. So far, *S. epidermidis* and *S. aureus* are the dominant pathogens underlying lactating mastitis [14]. Concordant with these data, we observed each species in about 20 % of patient samples but only in a few control samples.

The various pretreatment protocols applied did not improve fungal culture and RT-PCR positivity. The application of Fe^{2+} (pretreatment C) did not increase the yield of *Candida*-positive cultures but improved fungal growth in general (Table 2). In another study, a higher detection rate of 28 % was reached by the addition of Fe^{2+} to milk samples counteracting the lactoferrin-mediated growth inhibition [15]. Precultivation of milk samples in antibiotic-supplemented SDA liquid medium led to nearly the same results as with the addition Fe^{2+}.

Overall, the application of PCR methodology led to high detection rates of *Candida* spp. in human milk samples. DNA from *C. albicans* and *C. parapsilosis* were found in 67 % of patient samples and 58 % of control samples. Hence, the clinical interpretation of *Candida*-positive PCR study results is difficult. When culture remains negative, the clinical role of a PCR-positive result is completely unclear; it is likely that positive PCR data reflect *Candida* skin colonization rather than serving as a major fungal pathogen.

Hale et al. were unable to identify *Candida* in the breast milk of women suspicious of mammary candidiasis using culture and the β(1,3)-D-glucan detection assay [16]. *Candida* was present in the nipple but not in the milk. Sixteen patients claimed to have sore, inflamed or traumatized nipples, burning and radiating pain, as well as painful feeding. One can imagine that patients afflicted with pain suffer from bacterial infections, maternal vasospasm, or infant posterior tongue-tie rather than from fungal mammary.

Our study has some limitations. Firstly, we tested neither samples taken serially nor samples taken before and after antifungal treatment. Secondly, we did not collect nipple swabs, which would give information about the colonization of the mamilla and/or areola.

In conclusion, our study shows that the positive detection rate of *Candida* spp. in human milk considerably increases using specific RT-PCR methodology. However, *Candida* was detected in symptomatic as well as asymptomatic women. Therefore, a positive *Candida* DNA detection is diagnostically less conclusive as an indicator of mammary candidiasis. Any preprocessing of human milk samples did not enhance fungal detection significantly. Also, cultural data were not that significant. Hence, it seems that the state-of-the-art method in diagnosing mammary infections is still under construction.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in this study.

References

1. Heinig MJ, Francis J, Pappagianis D (1999) Mammary candidosis in lactating women. J Hum Lact 15(4):281–288
2. Francis-Morrill J, Heinig MJ, Pappagianis D, Dewey KG (2004) Diagnostic value of signs and symptoms of mammary candidosis among lactating women. J Hum Lact 20(3):288–295
3. Vollmer T, Störmer M, Kleesiek K, Dreier J (2008) Evaluation of novel broad-range real-time PCR assay for rapid detection of human pathogenic fungi in various clinical specimens. J Clin Microbiol 46(6):1919–1926

4. Amir LH, Garland SM, Dennerstein L, Farish SJ (1996) *Candida albicans*: is it associated with nipple pain in lactating women? Gynecol Obstet Invest 41(1):30–34
5. Moorhead AM, Amir LH, O'Brien PW, Wong S (2011) A prospective study of fluconazole treatment for breast and nipple thrush. Breastfeed Rev 19(3):25–29
6. Livingstone VH, Willis CE, Berkowitz J (1996) *Staphylococcus aureus* and sore nipples. Can Fam Physician 42:654–659
7. Andersson Y, Lindquist S, Lagerqvist C, Hernell O (2000) Lactoferrin is responsible for the fungistatic effect of human milk. Early Hum Dev 59(2):95–105
8. Lass-Flörl C, Mutschlechner W, Aigner M, Grif K, Marth C, Girschikofsky M, Grander W, Greil R, Russ G, Cerkl P, Eller M, Kropshofer G, Eschertzhuber S, Kathrein H, Schmid S, Beer R, Lorenz I, Theurl I, Nachbaur D (2013) Utility of PCR in diagnosis of invasive fungal infections: real-life data from a multicenter study. J Clin Microbiol 51(3):863–868
9. Sugawara Y, Nakase K, Nakamura A, Ohishi K, Sugimoto Y, Fujieda A, Monma F, Suzuki K, Masuya M, Matsushima Y, Wada H, Nobori T, Katayama N (2013) Clinical utility of a panfungal polymerase chain reaction assay for invasive fungal diseases in patients with haematologic disorders. Eur J Haematol 90(4):331–339
10. Panjaitan M, Amir LH, Costa AM, Rudland E, Tabrizi S (2008) Polymerase chain reaction in detection of *Candida albicans* for confirmation of clinical diagnosis of nipple thrush. Breastfeed Med 3(3):185–187
11. Amir LH, Cullinane M, Garland SM, Tabrizi SN, Donath SM, Bennett CM, Cooklin AR, Fisher JR, Payne MS (2011) The role of micro-organisms (*Staphylococcus aureus* and *Candida albicans*) in the pathogenesis of breast pain and infection in lactating women: study protocol. BMC Pregnancy Childbirth 11:54
12. Amir LH, Donath SM, Garland SM, Tabrizi SN, Bennett CM, Cullinane M, Payne MS (2013) Does *Candida* and/or *Staphylococcus* play a role in nipple and breast pain in lactation? A cohort study in Melbourne, Australia. BMJ Open 3(3). pii: e002351
13. Schabereiter-Gurtner C, Selitsch B, Rotter ML, Hirschl AM, Willinger B (2007) Development of novel real-time PCR assays for detection and differentiation of eleven medically important *Aspergillus* and *Candida* species in clinical specimens. J Clin Microbiol 45(3):906–914
14. Delgado S, Arroyo R, Martín R, Rodríguez JM (2008) PCR-DGGE assessment of the bacterial diversity of breast milk in women with lactational infectious mastitis. BMC Infect Dis 8:51
15. Morrill JF, Pappagianis D, Heinig MJ, Lönnerdal B, Dewey KG (2003) Detecting *Candida albicans* in human milk. J Clin Microbiol 41(1):475–478
16. Hale TW, Bateman TL, Finkelman MA, Berens PD (2009) The absence of *Candida albicans* in milk samples of women with clinical symptoms of ductal candidiasis. Breastfeed Med 4(2):57–61