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The effect of maternal flora on *Candida* colonisation in the neonate

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Summary

Colonisation may be the first step for the development of *Candida* infection. The source of neonatal colonisation is thought to be the hospital environment or the maternal vaginal tract. This study investigated to what extend *Candida* isolates in neonates are similar to isolates from their mother's vaginal tract. Vaginal samples were collected from 347 pregnant women within 48 h before delivery. Samples from oral and rectal mucosa of their neonates were collected within 24–72 h after delivery, were cultured and yeast species were identified. Antifungal susceptibility tests against six antifungal agents were performed. All paired isolates from mother and infant were genotyped by pulse field gel electrophoresis. A total of 82 mothers and of 16 infants were found colonised by *Candida* spp. *C. albicans* was the most common species in pregnant women (n = 68) followed by *C. glabrata* (n = 11). Only *C. albicans* was isolated from infants, mainly (14/16) from rectal site. All colonised neonates were born to mothers colonised by *C. albicans*. *Candida* genotyping revealed identical strains in all investigated neonate–mother pairs. All isolates were susceptible to amphotericin B. Our findings strongly suggest that vertical transmission has the principal role in the neonatal colonisation by *C. albicans* in the very first days of life.

Key words: *Candida*, molecular typing, infant colonisation, antifungal susceptibility.

Introduction

Candida constitutes a large family of about 200 species, of whom only a few are of clinical significance, including *C. albicans, C. parapsilosis, C. krusei, C. tropicalis, C. glabrata, C. guilliermondii, C. lusitaniae, C. kefyr, C. stellatoidea, C. intermedia* and others.[1] The most common and more virulent is *C. albicans*, responsible for 40–80% of neonatal candidiasis cases.[1,2] The organism colonises the gastrointestinal tract, the vagina, the skin and the upper respiratory tract. Vulvovaginal candidiasis can be present in 75% of all women during their reproductive years. During pregnancy, asymptomatic candidal colonisation of the vagina is common, affecting 30–40% of women. The phenomenon is possibly attributed to increased levels of estrogens that promote yeast adhesion and penetration into the vaginal mucosa.[3]

Neonates may acquire *Candida* species vertically through the vagina during labour, or horizontally from the hospital environment, especially from hands of health care workers.[4,5] Colonised neonates are asymptomatic. However, colonisation could be the first step for the development of mucocutaneous candidiasis or systemic disease.[1,6] Systemic *Candida* infections are common in neonatal intensive care units, especially among preterm and very low birth neonates. It is estimated that 15% of these neonates are colonised from

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their mother, whereas the rest 85% are colonised horizontally inside the units.[7] However, not much is known about the timing and extends of neonatal vertical and horizontal colonisation.

The objective of this study was to investigate the association between maternal and neonatal *Candida* colonisation.

Materials and methods

Study design

This study included 347 pregnant women admitted to the Obstetrics Departments of the University General Hospital and Venizeleion General Hospital, both in Heraklion, Crete, Greece, from February 2005 to April 2009. Eligible for enrolment were pregnant women who at the time of sampling, i.e. within 48 h before delivery, expected to give birth by vaginal route. Pregnant women who finally gave birth by caesarean section were still included in the study. The selection of pregnant women was at random order. These 347 pregnant women represented 2% of the total births in the prefecture of Heraklion during the 4-year study period. *Candida* colonisation was investigated both in mothers and in their neonates. Demographic and clinical data were collected by the same investigator from hospital registries and mother-retrieved questionnaires. Mothers were informed about the aims of the study and about the sample collection from both themselves and their offspring. Ethical approval for the study was obtained from the relevant Institutional Committee.

Sample collection

Maternal samples were obtained from vaginal mucosa within 48 h before delivery. Neonatal samples were obtained from oral (cheek, lip, ventral and dorsal surface of tongue) and rectal mucosa within 24–72 h after delivery. In cases of symptomatic neonates colonised by *Candida*, repeated samples were collected from the same sites on days 14 and 28 after birth.

Culture and mycological studies

A sterile fibre-tipped swab was used to collect the samples. The specimens were inoculated onto Sabouraud dextrose agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated for 72 h at 36 °C under aerobic conditions. Results were categorised semiquantitatively as 1+, 2+, 3+ and 4+ (yeast colonies limited to quadrant 1, 2 and 3 or extended to all quadrants of Petri plate respectively). Yeast isolates were identified to species level using the API 20 CAUX system (BioMérieux, Marcy L' Etoile, France). Antifungal susceptibility testing against amphotericin B, 5-fluorocytosine, fluconazole, ketoconazole, itraconazole, voriconazole, caspofungin, anidulafungin and micafungin was performed by the *E*-test method as recommended by the manufacturer (BioMérieux). The plates were incubated at 35 °C and read at 24 and 48 h. The minimal inhibitory concentration (MIC) was read as the lowest concentration at which the border of the elliptical zone of growth inhibition intersected the scale on the test strip. For the azoles an 80% inhibition in growth was used as the MIC cut-off (microcolonies were ignored), and for 5-fluorocytosine and amphotericin B the MIC endpoint was defined as the lowest concentration with nearly complete (90%) and complete (100%) inhibition respectively. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 served as quality control strains. For all antifungal agents tested, interpretative breakpoints followed those published as part of the M27-A3 document.[8]

Molecular typing (PFGE karyotyping and *Bss*HII restriction fragment analysis)

The isolates from colonised mother–infant pairs were further analysed for their genetic relatedness. The pulsed-field gel electrophoresis (PFGE) method was conducted as previously described by Chen *et al.* [9] with slight modifications. Briefly, yeast cells were grown on Sabouraud dextrose agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) for 48 h at 37 °C. Colonies were then suspended in cell suspension buffer (100 mmol l^{-1} Tris/HCl, 100 mmol l^{-1} EDTA, pH 8.0) to a final concentration of 10^9 CFU ml^{-1}, treated with 100 µl of lyticase (1250 unit ml^{-1} in 50% glycerol; Sigma-Aldrich Co., St. Louis, MO) at 37 °C for 30 min and embedded in plugs of 1% InCert agarose (Lonza Rockland Inc., Rockland, ME). The plugs were then treated overnight at 50 °C with 5 ml of cell lysis buffer (100 mmol l^{-1} Tris/HCl, pH 8.0, 0.45 mol l^{-1} EDTA, pH 8.0, 1% *N*-lauroylsarcosine, 1 mg ml^{-1} proteinase K). Plugs were washed twice with double-distilled H_2O at 50 °C for 15 min and six times with TE buffer at 50 °C for 10 min. For karyotyping, electrophoresis was performed with a Gene Navigator system (GE Healthcare Bio-Sciences, Uppsala, Sweden) at pulse time 60–700 s, 90 V in 0.8% agarose gel with 0.5X TBE for 66 h. For *Bss*HII digestion, plugs were incubated into 200 µl of appropriate buffer solution for 1 h at 50 °C.

The plugs were then transferred to 200 μl of buffer solution containing 4 units of *Bss*HII (New England Biolabs, Inc. Ipswich, MA) and incubated at 50 °C overnight. Electrophoresis was performed at pulse time 6–50 s, 180 V in 0.8% agarose gel for 36 h. BssHI has been reported by Chen *et al.* [9] to exhibit the highest discriminatory power.

Statistical analysis

Analyses were performed by two-tailed unpaired *t*-test, and Fisher's exact test, except if stated otherwise. Risk ratios (RR) and 95% confidence intervals were calculated. The values of $P < 0.05$ were defined as significant.

Results

Maternal colonisation

Among the 347 mothers, 82 (23.6%) were colonised by *Candida* species and one (0.29%) by *Saccharomyces cerevisiae* (Table 1). The predominant species was *C. albicans* followed by *C. glabrata.* No significant differences were observed regarding colonisation rates or *C. albicans* predominance among mothers in the caesarean section or vaginal delivery groups. Risk factors for maternal *Candida* colonisation are shown in Table 2. Colonised mothers tended to be younger (mean ± SEM, 25.2 ± 0.52 vs. 26.9 ± 0.32 years, $P = 0.011$), smokers (25.6% vs. 15.5%; RR 1.65, 95% CI 1.05–2.39; $P = 0.05$) and with a history of sexual intercourse during pregnancy (72.0% vs. 15.5%; RR 2.73, 95% CI 1.77–4.22; $P < 0.0001$). No significant differences were observed regarding the remaining analysed variables.

Table 1 Yeast species in 83 colonised mothers and 16 colonised infants.

	Total	Vaginal birth	Caesarean birth
Colonised mothers	*n* = 83	*n* = 69	*n* = 14
Candida albicans	68 (81.9)	58 (84)	10 (71.4)
Candida glabrata	11 (13.2)	8 (11.6)	3 (21.4)
Candida tropicalis	1 (1.2)	1 (1.4)	0 (0)
Candida famata	1 (1.2)	1 (1.4)	0 (0)
Candida parapsilosis	1 (1.2)	1 (1.4)	0 (0)
Saccharomyces cerevisiae	1 (1.2)	0 (0)	1 (7.1)
Colonised neonates	*n* = 16	*n* = 15	*n* = 1
Candida albicans	16 (100)	15 (100)	1 (100)

Values in parenthesis are expressed in percentage.

Infant colonisation

Among all infants, 16 (4.61%) were found colonised; in 14, *Candida* was isolated from rectal and in two from oral swabs (Table 1). All colonised neonates were born to colonised mothers and in all 16 mother–infant pairs *C. albicans* was the isolated species. A single neonate with rectal colonisation developed oral thrush 10 days after birth. Oral and rectal samples were again obtained in the 14th day of life, while still on

Table 2 Risk factors for colonisation of mother and infant by *Candida* species.

Factors	Colonised	Non-colonised	*P*-value; RR (95% CI)
Mothers	*n* = 82	*n* = 265	
Ethnicity (Greek; immigrant)	46; 36	180; 85	0.06; 0.68 (0.47–1.0)
Age in years (mean ± SEM)	25.2 ± 0.52	26.9 ± 0.32	0.011
Previous pregnancies	44	141	1.0; 1.0 (0.69–1.48)
History of pregnancy			
Use of antibiotics	12	58	0.16; 0.68 (0.39–1.18)
Use of antifungals	14	34	0.36; 1.28 (0.78–2.09)
Use of steroids	1	3	1; 1.06 (0.19–5.84)
Diabetes	8	16	0.31; 1.45 (0.80–2.65)
Yogurt consumption	42	134	1; 1.02 (0.70–1.49)
Sexual intercourse	59	109	<0.0001; 2.73 (1.77–4.22)
Tobacco use	21	41	0.047; 1.58 (1.05–2.39)
Premature membranes rupture	19	74	0.48; 0.82 (0.532–1.30)
Infants	*n* = 16	*n* = 331	
Maternal colonisation	16	66	<0.0001; na
Vaginal delivery	15	266	0.22; 3.52 (0.47–26.2)
Gender (male)	9	168	0.80; 1.23 (0.47–3.24)
Gestational weeks (mean ± SEM)	39.2 ± 0.23	38.7 ± 0.09	0.25
Birthweight in kg (mean ± SEM)	3.33 ± 89	3.27 ± 26	0.73
Breastfeeding	9	181	1.0; 1.06 (0.40–2.79)
Use of antibiotics	0	12	1.0; na
Medical condition	1	28	1.0; 0.73 (0.10–5.33)

RR, risk ratios; CI, confidence intervals.

Table 3 Antifungal susceptibility of the yeast isolated from 83 colonised mothers and 16 colonised neonates.

	Susceptible isolates, *n* (%)					
	Ampho B	5-fluorocytosine	Ketoconazole	Fluconazole	Itraconazole	Voriconazole
Isolates from mothers						
C. albicans	68 (100)	66 (97)	65 (96)	65 (95.6)	64 (94)	67 (98.5)
C. glabrata	11 (100)	11 (100)	1 (9.1)	2 (18.2)	1 (9.1)	5 (45.4)
C. tropicalis	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
C. parapsilosis	1 (100)	1(100)	1 (100)	1 (100)	1 (100)	1 (100)
C. famata	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
C. cerevisiae	1 (100)	1 (100)	0	0	0	1 (100)
Total	83 (100)	81 (98)	69 (83)	70 (84)	68 (82)	76 (92)
Isolates from infants						
C. albicans	16 (100)	15 (93.8)	16 (100)	16 (100)	15 (93.8)	16 (100)
MIC range (μg ml^{-1})						
C. albicans	0.016–0.25	0.023 ≥ 32	0.004 ≥ 32	0.025 ≥ 256	0.004 ≥ 32	0.004 ≥ 32
C. glabrata	0.094–0.5	0.012–0.125	0.25–12	1.5–48	16 ≥ 32	0.25–3

oral nystatin. *C. albicans* was found in both samples. On 28th day of life oral thrush had disappeared. Among the 16 colonised infants, 15 belonged to the vaginal delivery group and one to the caesarean section group; the latter was colonised in the oral mucosa. Risk factors for infant *Candida* colonisation are shown in Table 2. The single factor that contributed to infant colonisation was the colonisation of the mother (100% vs. 19.9; $P < 0.0001$).

Concomitant maternal and infant colonisation

From the 16 colonised neonates, 14 (87.5%) were born to mothers colonised with significant amount of *C. albicans* (3+ or 4+). Among 25 mothers with colonisation grade 4+, nine colonised infants were born, in contrast to 19 mothers with colonisation grades 1+ and 2+, two colonised infants were born (36% vs. 10.6%, RR 1.40, 95% CI 1.00–1.95, one-tailed $P = 0.05$).

Genetic relatedness of *C. albicans* isolates from mother–infant pairs was investigated by PFGE of *Bss*HII-digested genomic DNA (Fig. 1). In all 16 colonised neonates, the pulsotypes of *C. albicans* were identical to their mothers'. Electrophoretic karyotyping of maternal *C. albicans* isolates displayed seven isolates with identical bands suggesting clonal relatedness (data not shown).

Antifungal susceptibility

The antifungal susceptibility of yeast species against amphotericin B, 5-fluorocytosine, fluconazole, ketoconazole, itraconazole and voriconazole in strains isolated from mothers and neonates is shown in Table 3. Caspofungin, anidulafungin and micafungin were only

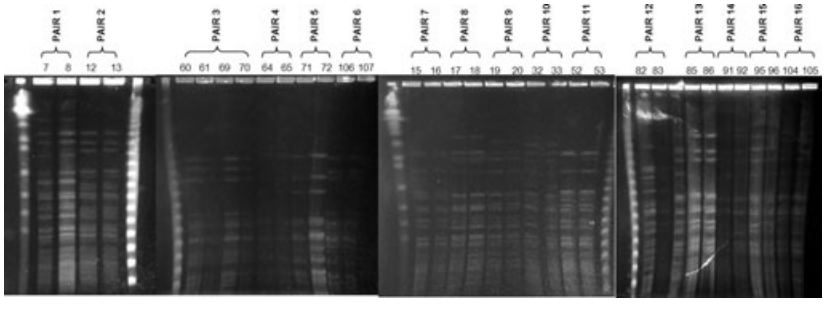

Figure 1 Genetic relatedness of *Candida albicans* isolates from 16 mother–infant pairs as shown by pulsed-field gel electrophoresis of *Bss*HII restriction endonuclease analysis of genomic DNA. By pair 1, 2, etc. are depicted the paired *Candida* isolates from mother and infant. The four isolates in pair 3 depict the paired *Candida* isolates 60 and 61 from mother and infant and furthermore the isolate 69 and 70 from neonatal rectal and oral swab 14 days after birth.

Table 4 Distribution of minimal inhibitory concentration (MIC) values of antifungal agents for the 84 *Candida albicans* isolates.

	No. of isolates with MIC (μg ml^{-1})													
Antifungal agent	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	≥32	64	≥256
Amphotericin B	74	5	5											
5-fluorocytosine	63	7	2	1	6	1		1				3		
Ketoconazole	81								1			2		
Fluconazole	15	9	20	17	12	3		2		2	1		2	1
Itraconazole	74	4					3					2		
Voriconazole	74		4	2	1	2						1		

tested against the *Candida* isolated from the mother–infant pairs and all 32 isolates were found to be susceptible to these echinocandin compounds. MIC values of antifungal agents against *C. albicans* and *C. glabrata* strains isolated from mothers and infants and distribution of MIC values of the antifungal agents tested for *C. albicans* isolates are similarly shown in Table 4. All isolates were susceptible to amphotericin B, whereas the least susceptibility was observed for itraconazole. *C. glabrata* isolates were confirmed to be naturally resistant to the azoles, as previously documented,[10] but were all sensitive to amphotericin B and 5-fluorocytosine.

Discussion

In our study, vaginal *Candida* colonisation of pregnant women was 23.6%, in accordance with reported rates which widely range from 5.6% to 69.2%.[11,12] The most common species was *C. albicans* followed by *C. glabrata,* which is again in agreement with the reported frequencies of *C. albicans, C. glabrata* and *C. tropicalis* in the vaginal flora.[3,11,13] Furthermore, our study showed that tobacco use and sex intercourse during pregnancy are risk factors for maternal vaginal *Candida* colonisation. Smoking has been already related to oral candidosis and bacterial vaginosis, but not to vaginal candidosis.[14,15] Other risk factors that have been suggested including pregnancy, oral contraceptives, systemic or vaginal antibiotics and diabetes mellitus.[3] Despite the fact that mothers did not present with symptoms of *Candida* infection, antifungal susceptibility testing was performed for the better understanding of the *Candida* ecology in our area, and as in the context of the study the antifungal susceptibility phenotype would constitute a rough estimation of the similarity of maternal-infant strains.

Candida colonisation was found in 4.6% of neonates and the only *Candida* species isolated was *C. albicans*. The rectal mucosa was significantly more colonised than oral mucosa. It is known that *Candida* colonises the gastrointestinal tract of 4.8–10% neonates and that *C. albicans* is the predominant species,[13] but not much is known about the process of the oral and rectal colonisation.[11,16–18] Oral colonisation seems to increased from birth up to the 18th month of age and then decreased.[11] Rectal colonisation seems to be more frequent.[16,17] Our findings, derived from swabbing very early in life, do not confirm the hypothesis that the earliest site colonised is the oral cavity.[18] These differences may be attributed to different study design and setting as well as to the age of sampling.

In this study, neonates were only colonised by *C. albicans*, which is observed mainly in vertical transmission, whereas *C. parapsilosis* has been observed in horizontal transmission in the neonatal intensive care unit setting.[19] It is of great interest that all non-colonised mothers gave birth to non-colonised neonates, that all colonised neonates were born from colonised mothers and furthermore that *C. albicans* was the only species isolated from 16 mother–infant pairs. The molecular typing study showed that in all colonised neonates the pulsotype of *C. albicans* was identical to the pulsotype of their mothers. According to PFGE-*Bss*HII typing method, the 16 maternal *C. albicans* isolates were different. Electrophoretic karyotyping of the maternal *C. albicans* isolates displayed seven isolates with identical bands suggesting clonal relatedness. However, this method has a less discriminatory power than PFGE-*Bss*HII.[9]

These findings suggest that colonised neonates may acquire *C. albicans* via vertical transmission. These *C. albicans* colonised neonates met criteria for vertical transmission according to the research of Bliss *et al.* [4] had been born by *C. albicans* colonised mother, developed *C. albicans* colonisation by 1 week of age and had *C. albicans* isolate identical to the maternal isolate. All colonised neonates were full term and healthy, except for one of vaginal delivery with oral colonisation, who was admitted to Neonatal Intensive Care Unit because of respiratory distress. It is

interesting that neonatal *Candida* colonisation is mostly investigated among preterm neonates in Neonatal Intensive Care Units, where horizontal transmission may be more possible; Bliss *et al.* [4] demonstrated that 41% of *C. albicans* colonising very low-birthweight infants was due to vertical transmission; Waggoner-Fountain *et al.* [5] demonstrated that 14% of mother–preterm infant pairs were colonised with the identical strain of *C. albicans*. According to Caramalac *et al.* [11] vaginal mucosa was not the main route of *Candida* transmission to full-term neonates.

Our observation that the vast majority of colonised neonates were born to mothers vaginally colonised with a large quantity of *C. albicans* colonies may suggest correlation between candidal colony counts in the vagina of mother and *Candida* colonisation in the neonate. Perinatal risk factors for neonatal colonisation were maternal colonisation and vaginal delivery. It has been reported that low gestational age (<32 week) and very low birthweight (<1500 g) are risk factors for neonatal *Candida* colonisation.[5,18,20] We did not confirm these findings, but in our cohort there was only one neonate with very low birthweight (1420 g) and two neonates with low gestational age (lower gestational age 32 weeks).

Our study demonstrated that early *Candida* colonisation of the neonate seems to occur through vertical transmission in the first 72 h of life. However, we did not investigate horizontal transmission from other sources. Furthermore, we did not swab all infants later on (especially on 7th day) to explore the full process of colonisation. Nevertheless, our findings strongly suggest that early neonatal colonisation by *C. albicans* occurs through vertical transmission, during or immediately after birth, and that horizontal transmission is not the principal mode of colonisation in the very first days of life.

Conflict of interest

None for Anthoula Filippidi, Emmanouil Galanakis, Sofia Maraki, Irene Galani, Maria Drogari-Apiranthitou, Maria Kalmanti, Elpis Mantadakis. Dr G. Samonis has received fees for speaking, for organising education, reimbursement for attending symposiums, funds for research, fees for serving on an advisory board from companies Pfizer, Gilead, Astellas and MSD.

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