The relationships between candidemia and candidal colonization and virulence factors of the colonizing strains in preterm infants

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Premature infants are at high risk of developing candidal infections originating from their own normal flora or from the hospital environment. This study involves the surveillance cultures and blood cultures of candidemic preterm infants with low birth weights who have been analyzed for colonization period and status, and for virulence factors such as acid proteinase and phospholipase.

Arbitrarily primed-polymerase chain reaction (AP-PCR) was applied to the blood culture isolates of the babies with candidemia and their last colonizing strains in order to determine whether the source of fungemia was the rectum.

Of 65 colonized infants, 6.2% developed candidemia with identical strains originating from their rectum according to their PCR patterns.

Our findings indicate that the properties of the colonizing yeasts such as increase in number-although not statistically significant because of the small sample size-and/or exhibition of strong hydrolytic enzyme activities through a long duration of colonization might contribute to the development of candidemia in preterms.

Key words: low birth weight, Candida species, colonization, candidemia, virulence factors.

Systemic candidiasis may develop in patients with risk factors such as hematological malignancies, gastrointestinal system operations, and preterm births.

According to the report of the National Nosocomial Infections Surveillance System (NNISS), isolation of Candida spp. from nosocomial systemic infections has risen from 8th to 4th in rank between 1984 and 1988[1]. Among these cases endogenous infections are higher in frequency than exogenous ones[2].

Sixty percent of newborns colonize Candida spp. on their skin and mucous membranes and this may lead to invasive infections, especially in preterm infants, and may result in death despite aggressive antifungal therapy[3].

In addition to the high number of colonizing yeasts, some hydrolytic enzymes of the strains were suggested to have an important role in the pathogenesis in cases who develop severe candida infections[4]. However, in low birth weight (LBW) infants, the importance of two virulence factors for both colonization and candidemia have not been discussed sufficiently in the literature.

In order to define which property (or properties) had of role in colonization and the following systemic infection, we analyzed the arbitrarily primed-polymerase chain reaction (AP-PCR) patterns of the Candida strains isolated from colonized body sites and blood stream infections, which give information about the source of the strains, their numbers in colonizing status and their acid proteinase and phospholipase activities.

Material and Methods

A total of 134 preterm infants with LBW who were admitted to Marmara University Children's Hospital, Newborn Intensive Care Unit, and Social Security Organization –Göztepe Children's Hospital, Premature Service between 1999 and 2002 were included in this study.

Swabs were obtained from their oropharynx, axilla, umbilicus and rectum in the first 24 hours of life, twice a week during the first two weeks, and once in each following week during their stay in the hospital. Colony count estimations were made for each positive culture by performing serial dilutions followed by inoculations on Sabouraud dextrose agar (SDA) and cycloheximide-chloramphenicol containing SDA, incubated for a week at 30°C. The results were quantified as cfu/ml.

Blood cultures were obtained from the infants suspected to develop systemic infection and the cultures were followed up using BACTEC-9240 (Becton-Dickonson, U.S.) system. From the positive cultures, Gram-stained preparations were performed and SDA inoculations were made when yeasts were seen under microscope.

The detection of species of the strains was made by performing germ tube and chlamydospore formation tests, and using ID 32 C (bioMerieux, France) assimilation[5].

Acid proteinase activities of the strains were detected on bovine serum albumin (BSA) containing agar (pH: 5.0) plates, and the results were read according to millimetric width of the transparent zones around the colonies occurring on previously opaque plates and were reported as strong (++), mild (+) or negative (–). CBS 2730 C. albicans standard strain kindly provided by Dr. R. Rüchell was used as positive control[6].

Phospholipase activities of the strains were assayed on egg yolk containing agar (pH: 4.3). The results were reported as negative (–), mild (+), strong (++) or very strong (+++), as described previously. During the test procedure SC 5314 C. albicans strain kindly provided by Dr. M. Ghannoum was used as positive control[7,8]. Chi Square test was used for the statistical evaluation of the association between hydrolytic enzyme production and development of candidemia.

DNAs of the strains were extracted using NucleoSpin (Macherey-Nagel) kit. AP-PCR reactions were performed using T3B primer (5'-AGG TCG CGG GTT CGA ATC C-3'…….) as cited in previous studies[9].

The criteria described by Hedderwick et al.[2] to define the colonization status of the strains. Colonization times reported as early or late were used as described in previous studies[10].

Results

The gestational ages of the preterm infants in this study were 24-36 weeks and the birth weights were 640-2500 g; 49.3% had birth weights below 1500 g (very low birth weight=VLBW), and 50.8% had birth weights between 1500 and 2500 g (low birth weight=LBW).

In 69 (51.5%) babies no significantly positive surveillance culture was detected despite their long stay in the hospital. In the remaining 65 (48.5%), colonizations were detected on different body sites on 125 culture episodes. Rectum was the commenest colonized site as reflected in 57 (45.6%) infants, followed by oropharynx (32.8%), axilla (16.0%) and umbilicus (5.6%).

Colonization status of the infants is given in Table I.

Table I. Clonization Status of 65 Infants According to Their Involved Body Sites

Body Site	Culture Episodes	%
Rectum	57	45.6
Oropharynx	41	32.8
Skin	20	16.0
Umbilicus	7	5.6
	125	100

Candida albicans was the most frequently isolated species (80% of the total), mostly from rectum (44 isolates), followed by oropharynx (33 isolates), axilla (17 isolates), and umbilicus (6 isolates). Candida prapsilosis was isolated from rectum[7], oropharynx[3], and axilla[2], comprising 9.6% of the total. Candida tropicalis was third, isolated from rectum[5], oropharynx[4], and from axilla[1], accounting for 8% of the total. Candida guilliermondii was isolated from rectum[1], oropharynx[1] and from umbilicus[1], for 2.4% of the total.

"Early" colonization was seen in 76 (60.8%) infants, in rectum (31.2%), oropharynx (20.8%), axilla (7.2%), and umbilicus (1.6%), while "late" colonization was detected in 49 (39.2%), in rectum (14.4%), oropharynx (12%), axilla (8.8%), and umbilicus (4%), respectively.

Candidemia developed in only four of 65 (6.2%) colonized infants during their stay in hospital. The blood culture and the last colonizing strains at that time were same with respect to species in all patients. In the first fungemic patient, although the number of the colonizing yeasts

was not high ($1x10^3$ cfu/ml), C. albicans was isolated from blood culture due to "persistent" rectal colonization. In the second candidemic patient, C. albicans was isolated from the blood culture due to the "persistent" and "probably persistent" colonizations in relatively high numbers ($2x10^6$ cfu/ml) in rectum and oropharynx, respectively. In the third patient, fungemia was detected due to C. albicans, associated with the high number of colonizing yeasts ($5x10^6$ and $7x10^5$ cfu/ml) in rectum and oropharynx, respectively, and in the presence of "intermittent" colonizations at both sites. In the fourth patient, fungemia was caused by C. parapsilosis, consistent with the "persistent" colonization of rectum with higher number ($7x10^6$ cfu/ml) of yeasts.

In the infants other than candidemics, the numbers of the rectal colonizing strains were between $3x10^2$-$6x10^6$.

The AP-PCR results of the strains isolated from the last surveillance cultures at the time of candidemia of each patient were found to be identical. In the first and second patients both rectal and blood strains yielded quite similar patterns. The C. albicans strains isolated from the third patient's rectum and blood cultures were identical, but exhibited a small difference on one band from the first two patients (Fig. 1).

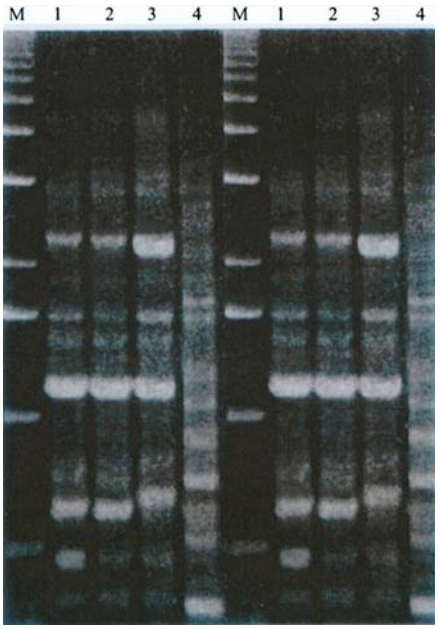

Fig. 1. Arbitrarily primed-polymerase chain reaction (AP-PCR) results of the four fungemic patients. Left: rectal colonization isolates; Right: blood culture isolates.

The last colonzinig isolates at the time of candidemia and blood culture isolates of the four candidemic patients had strong acid proteinase (++) and strong phospholipase (+++) activities.

The analysis of the hydrolytic enzyme activities of the remaining 121 strains isolated from the nonfungemic but colonized infants yielded the following results:

Out of the 50 C. albicans strains isolated from "transient" colonizations, 18 and (++), 27 had (+) and 5 had (–) acid proteinase activity while 9 had (++), 31 had (+) and 10 had (–) phospholipase activity. Out of 28 strains of "intermittent" colonizations, 21 had (++), 6 had (+), 1 had (–) acid proteinase activity; 14 had (++) 7 had (+) and 7 had (–) phospholipase activity. Out of a total of 11 C.albicans strains isolated from "probably persistent" colonizations, 8 had (++) and 3 had (+) acid proteinase, and 1 had (+++) 7 had (++), and 3 had (+) phospholipase activities. Of the 8 "persistent" C. albicans strains, all had (++) acid proteinase, and 1 had (+++) and 7 had (++) phospholipase activity. Out of a total of 11 Candida parapsilosis strains isolated from "transient", "intermittent" and "persistent" colonizations, 4 had (++), 2 had (+), and 5 had (–) acid proteinase and phospholipase activity. Of a total of 10 C. tropicalis strains 4 had (++), 5 had (+) and 1 had (–) acid proteinase and 3 had (++), 5 had (+), and 2 had (–) phospholipase activity. Out of 3 C. guilliermondii strains isolated only from "transient" colonizations, all were negative for either enzyme activity (Fig. 2a, 2b, 3).

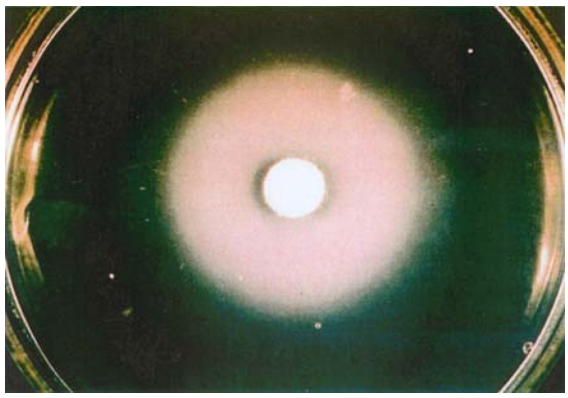

Fig. 2a. Negative acid proteinase activity on albumin containing medium.

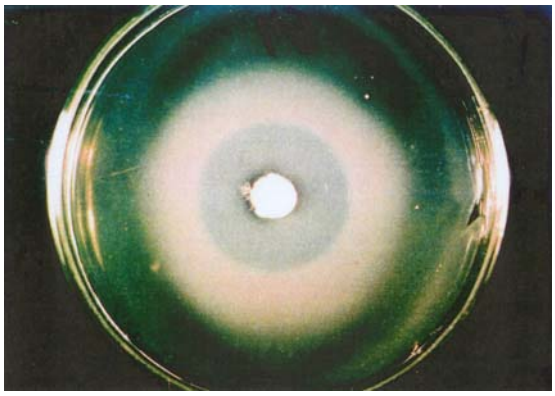

Fig. 2b. Strongly positive (++) acid proteinase activity on albumin containing medium.

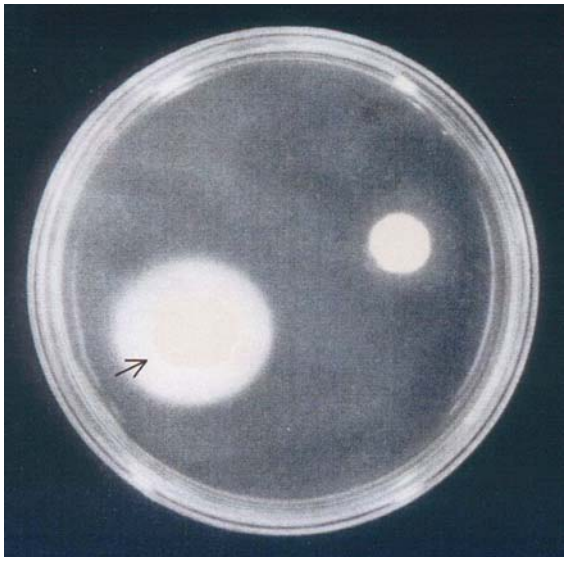

Fig. 3. Negative phospholipase activity on right side. Strongly positive (+++) phospholipase activity on left side (arrow).

Discussion

According to the findings in the literature, 60% of the infants with risk factors such as LBW, presence of central venous catheter (CVC) and long-term antibacterial treatment may become colonized with yeasts on their skin and mucous membranes. This may lead to invasive fungal infections despite antifungal therapy. Especially in infants with VLBW (<1500 g), the mortality rate has been reported to be 28% for those who develop sepsis, while it is 7% in those without sepsis[3,11].

In our study the preterm infants were found to be colonized at a rate of 48.5%, which is higher than the findings of Baley et al.[10] and Saiman et al.[12], who reported 26.7% and 23%, respectively. Our findings indicate that the most frequently colonized body site was the rectum, followed by oropharynx, axilla and umbilicus. This finding complies with some other studies[13-15]. This distribution may be due to the use of antibacterial agents, which may lead to overgrowth of the yeasts in the intestines. We also detected a high rate (60.8%) of early colonization in the first two body sites, as stated in the literature[10,14]. The body site with the weakest colonization was the umbilicus, as explained in other studies by the time of detachment of the umbilicus in the very early period of life[3].

In the literature, definition of the colonization status has been offered as an important parameter in the epidemiology of invasive fungal infections[2,16]. Although the rates differed due to the body sites involved, in the present study transient colonization rate (49.6%) was found to be the highest, followed by intermittent, persistent and probable persistent colonizations when evaluated with respect to the total colonization number (Table I). This finding did not agree with the findings in a large extensive prior study in which transient colonization was found in only 4.3% of 92 patients[2].

In our study Candida albicans was found to be the commonest isolated species (80%) from the infants followed by C. parapsilosis in 125 culture episodes. Candida albicans was detected in rectal cultures mostly, followed by oropharynx, axilla and umbilicus cultures. The findings for the two species are in agreement with a prior study[12]. We isolated C. guilliermondii spp. at a rate of 2.4% a finding that we have not observed in other studies.

In the present study 6.2% of the infants developed candidemia during the colonization period. In the first patient, in spite of the presence of persistent and intermittent colonizations with C. albicans in rectum and oropharynx, the number of colonizing yeasts were $1x10^3$ and $3x10^4$, respectively, and did not increase at the hime of candidemia. In this case intestinal persistent colonization may be the reason for development of the fungemia. In the second patient the number of yeasts, which led to intermittent colonization in the rectum, increased to $5x10^7$ at the time of candidemia due to C. albicans. In the third patient as with the second, at the time of candidemia due to C. albicans, the number of the yeasts increased

to $5x10^6$ in the intermittently colonized rectum, and was associated with resultant fungemia. In the fourth infant who developed candidemia, there was a persistent colonization in the rectum by C. parapsilosis which rose to $7x10^6$ and was associated with fungemia. In preterm infants, intestinal colonization with Candida spp. has been suggested to be a source for sepsis. In a prior study including 40 VLBW infants, 28% of 21 babies with intestinal C. albicans colonization developed sepsis. The commonly shared characteristic was the presence of $\geq 8x10^6$ cfu/g threshold number of yeasts in the stool of these infants[17]. Our patients, with the exception of the first infant, had gradually increasing numbers of yeasts in the rectum at the time of candidemia, consistent with the cases above.

Despite these findings, it is not easy to define a threshold value for colonizing yeasts in various body sites, although it is accepted that a high number of yeasts may be associated with invasive infections. This led the authors to suggest suppressing the number of yeasts in the oral cavity and intestines[18,19].

In the present study the rate of candidemia was relatively low when compared to other reports in which the rates were given as 10% and 20%, respectively[3,13]. This low percentage in our study was associated mainly with the decrease in use of antibacterial agents in Marmara University Children's Hospital.

We also examined the AP-PCR results of rectal and blood isolates of the candidemic patients in order to detect the source of blood stream infection. The C. albicans strains of the first and second infants from both sites exhibited identical patterns between the strains and the patients. This result showed that the source of candidemia in the two infants was the rectum; this similarity between the two babies may be related to their stay in the same ward at the same time (Fig. 1).

Additionally, we investigated the hydrolytic enzyme activities of the strains in terms of association with virulence factors and colonization and/or candidemia. We detected strong acid proteinase and phospholipase activities in both colonizing and blood stream isolates of the four candidemic patients (Figs. 2b, 3), However, the number of fungemic patients was too low to make a suggestion regarding the strains leading to fungemia because of their high virulence. Although we have not come across a study which reports any association between the virulence factors and colonization or candidemia in preterm babies, there are several animal experiments which suggest that besides abundance, the passage of yeasts to the blood stream from the intestines requires strong hydrolytic activities in order to cleave intestinal villi and microvilli[20]. When we examined the virulence factors of the isolates from the non-candidemic but colonized babies only, the strains obtained from persistent and probable persistent colonizations exhibited stronger enzymatic activities compared to the strains recovered from intermittent and transient colonizations. However, these results were not of statistical significance in terms of association between the virulence factors and colonization, due to the small sample sizes in the comparison groups.

In addition to the numbers and the virulence factors of the colonizing strains, the immune system of the infected organism is considered to be very important for the colonization of the yeasts[21]. Especially in the infants with weakened phagocytic activities, the risk for sepsis is expected to be increased[22].

In conclusion, especially for the three patients with VLBW, we suggest that the candidemia may have been due to their insufficient immune systems, accompanied by a long duration of colonization with the Candida spp. in an increased amount and with strong hydrolytic activities.

Further studies must be carried out in order to define the properties of the yeasts which cause colonization in pre-term babies, in terms of threshold numbers and virulence factors, and to determine the correct approach in the prevention of candidemia in such patients.

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