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THE PATHOLOGY OF NEONATAL NON-ALBICANS CANDIDIASIS: AUTOPSY STUDY AND LITERATURE REVIEW

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Abstract

INTRODUCTION/OBJECTIVES—Non-*albicans Candida* species such as *C. parapsilosis* and *C. glabrata* have emerged as prevalent pathogens in premature infants. The aim of this study was to systematically delineate the histopathologic findings in neonatal non-*albicans* candidiasis.

METHODS—We performed a retrospective clinicopathologic analysis of extremely premature (23-28 weeks gestation) infants diagnosed with invasive candidiasis. Archival autopsy tissues were subjected to periodic acid-Schiff, methenamine-silver and anti-*Candida* (immuno)histochemical stains, as well as dual anti-*Candida* and anti-cytokeratin or anti-CD31 immunofluorescence assays. In addition, we studied the prevalence of intestinal *Candida* colonization in a consecutive autopsy series of extremely premature infants.

RESULTS—Based on positive postmortem blood and/or lung cultures, invasive candidiasis (3 non-*albicans*, 11 *C. albicans*) was diagnosed in 14/187 extremely premature infants examined between 1995 and 2017. In contrast to the well-known inflammatory and tissue-destructive phenotype of congenital *C. albicans* infection, invasive non-*albicans* candidiasis/candidemia caused by *C. parapsilosis* and *C. glabrata* was inconspicuous by routine hematoxylin-eosin-based histopathologic analysis despite a heavy fungal presence detected in intestines, lungs and blood by targeted (immuno)histochemical assays. Intestinal colonization by *Candida* species was identified in 16/26 (61%) extremely premature neonates who had lived for at least one week, as assessed by anti-*Candida* immunostaining.

CONCLUSION—Invasive neonatal non-*albicans* candidiasis/candidemia appears to have no distinct histopathologic signature. Based on the notoriously low sensitivity of fungal blood cultures and the observed high frequency of *Candida* intestinal colonization (>50%), it is likely

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that non-*albicans* candidiasis/candidemia may be underdiagnosed in (deceased) preterm infants. Routine inclusion of targeted (immuno)histochemical fungal detection strategies in the perinatal autopsy may lead to deeper insight into the prevalence and clinical relevance of neonatal non-*albicans* candidiasis.

Keywords

Candida parapsilosis ; *Candida glabrata* ; yeast; prematurity; newborn

INTRODUCTION

Candida species are the leading cause of invasive fungal disease (clinically defined as candidemia (blood stream infection) and/or infection of deep organs (1)) in premature infants (2, 3). The risk for invasive candidiasis is particularly high in extremely-low-birth-weight neonates (birth weight < 1000 g), with mortality rates up to 30% (4). The susceptibility of very preterm infants to *Candida* infections has been attributed to the immaturity of their skin and immune system, as well as multiple risk factors inherent in their intensive care and prolonged hospitalization [summarized in (2)].

Whereas *C. albicans* has historically been the most prevalent cause of fungal infections, infections with non-*albicans Candida* species have increased dramatically over the last two decades (5-7). At present, *C. parapsilosis*, *C. glabrata* (formerly *Torulopsis glabrata*), *C. tropicalis*, and *C. krusei* together represent about half of all *Candida* species isolated from blood cultures. This epidemiological shift has been attributed, in part, to the increased use of azoles and caspofungin (8).

While originally considered to be non-virulent, non-*albicans Candida* species such as *C. parapsilosis* and *C. glabrata* have emerged as significant pathogens, even outranking *C. albicans* as the leading organism in invasive neonatal candidiasis in some centers (9). *Candida albicans* and non-*albicans Candida* species show important differences in morphology, virulence factors, and host interaction. Like most *Candida* species, *C. parapsilosis* and *C. glabrata* exist mainly as spherical or ovoid blastospores or yeast cells and are further capable of producing chains of elongated blastospores termed pseudohyphae (10). *Candida albicans* also exists as elongated filamentous cells known as true hyphae (11). This true hyphal morphogenesis is implicated in the capacity of *C. albicans* to invade and injure epithelial and endothelial cells (12). Interspecies morphological differences also extend to differences in biofilm formation (13, 14) secretory activity (15), and response of the host immune system (16-20).

The aim of this study was to delineate the pathologic findings in premature newborns with invasive non-*albicans Candida* infections. Based on their differences in virulence factors and host response, we speculated that the pathologic findings in invasive neonatal non-*albicans* candidiasis might be distinct from the better-known, tissue-destructive neonatal *C. albicans* infection phenotype.

METHODS

Cases were retrieved from the autopsy files of the Department of Pathology at Women and Infants Hospital (1995–2017) using the search criterion '*Candida*'. Corresponding medical charts were reviewed for relevant perinatal and neonatal information. Archival hematoxylin and eosin (H&E)-stained sections of postmortem tissues were reviewed. Selected sections were further subjected to methenamine-silver nitrate and periodic acid-Schiff staining as well as avidin-biotin-immunoperoxidase staining using a rabbit polyclonal anti-*Candida albicans* antibody (ab53891, Abcam, Cambridge, MA), which cross reacts with other *Candida* yeasts including *C. parapsilosis* and *C. glabrata*. Controls for specificity consisted of omission of the primary antibody, which abolished all immunoreactivity.

The anatomic localization of *Candida* organisms was further assessed by combining anti-*Candida* with anti-cytokeratin (epithelium) and anti-CD31 (endothelium) immunofluorescence staining. Tissue sections were incubated sequentially with polyclonal rabbit anti-*Candida*, AlexaFluor 594-conjugated anti-rabbit IgG (Jackson, ImmunoResearch Laboratories, Inc., West Grove, PA), monoclonal mouse anti-cytokeratin (AE1/3) or anti-CD31 (DakoCytomation, Glostrup, Denmark), and AlexaFluor 488-conjugated anti-mouse IgG (Jackson). Sections were covered with aqueous mounting medium containing 4′,6-diamidine-2'-phenylindole dihydrochloride (DAPI, Vector Laboratories, Inc., Burlingame, CA). Controls consisted of omission of one or both primary antibodies, which abolished the respective immunoreactivities. The sections were viewed by confocal microscopy. Slice or three-dimensional volume reconstruction and projections were generated, as previously described (21)

Colonization of the gastrointestinal tract is one important pathway to subsequent invasive candidiasis (22, 23). To gain insight in the frequency of intestinal colonization by *Candida* species in extremely preterm newborns during their NICU stay, we performed a retrospective autopsy study of a consecutive series of infants born between 23 and 28 weeks' gestation (2006-2017) who had lived for at least one week. Archival sections of lower gastrointestinal tract were subjected to peroxidase-based immunohistochemical analysis using the anti-*Candida* antibody described above and examined for the presence and morphology of fungal organisms.

RESULTS

Invasive non-*albicans* candidiasis.

Of 187 extremely premature infants (23-28 weeks' gestation) autopsied between 1995 and 2017, 14 (7.5%) were diagnosed with invasive candidiasis, as defined by the presence of candidemia (blood stream infection) and/or deep organ involvement. More superficial diseases, such as cutaneous and esophageal candidiasis in the absence of candidemia, were excluded (1). Sixty-six had lived at least one week. There were 3 neonates with non-*albicans Candida* and 11 neonates with *C. albicans* infection. The relevant clinical, placental and postmortem findings of the three infants with invasive non-*albicans* candidiasis are summarized in Table 1. Postmortem examination of the three infants revealed injury patterns associated with complications of extreme prematurity, including early bronchopulmonary

dysplasia, pulmonary hemorrhage, intraventricular hemorrhage and necrotizing enterocolitis. Fungal organisms were inconspicuous by hematoxylin-eosin staining. However, targeted (immuno)histochemical stains used for the current study revealed abundant *Candida* yeast forms in gastrointestinal tract, lungs and most other organs in all three cases. *Candida* organisms were readily detected by traditional histochemical fungal stains such as periodic acid-Schiff and Gomori-methenamine silver in most organs examined (Fig. 1). In liver and intestines, interpretation of these histochemical stains was compromised by the presence of intrinsic pigments and intraluminal debris, respectively. In these organs, detection of *Candida* organisms was greatly enhanced by anti-*Candida* immunohistochemical analysis. A comparative analysis of these various fungal detection methods is illustrated in Figure 2.

Dual anti-*Candida* and anti-cytokeratin immunofluorescence staining confirmed the striking abundance of intestinal yeast forms in all three cases (Fig. 3A-C). Infiltrating yeast cells were often noted in a linear pattern between intestinal epithelial cells and aggregated along the subcellular basement membrane of the glands/crypts (Fig. 3B,C). Similarly, dual immunofluorescence studies of lung sections demonstrated abundant yeast forms in the pulmonary airspaces, displaying variable degrees of degradation and fragmentation (Fig. 3D,E). Whereas the vast majority of *Candida* yeasts were confined to the airspaces, scattered organisms were observed within the pulmonary interstitium, suggestive of invasion (Fig. 3D).

As shown above (Fig. 1), routine histochemical and single anti-*Candida* immunohistochemical analyses were suggestive of widespread visceral involvement, with particularly heavy fungal burden in lungs, liver, kidneys and spleen. Combining anti-*Candida* immunofluorescence with the endothelial marker, anti-CD31, revealed that virtually all of these apparently tissue-invasive visceral *Candida* organisms were confined to the microvasculature, consistent with *Candida* blood stream invasion (candidemia), rather than systemic visceral invasion in the strictest sense (Fig. 3F).

Intestinal colonization by *Candida* species.

We assessed the prevalence of *Candida* organisms in archival sections of intestine obtained from preterm infants born between 23 and 28 weeks' gestation who had lived for at least one week. *Candida*-immunoreactive organisms were identified in intestinal sections of 16/26 (61%) neonates examined. In three cases, the yeast organisms were relatively large-sized and accompanied by true hyphae (Fig. 4A-B), consistent with *C. albicans* commensals. In the remaining 13 cases, variable numbers of smaller yeasts lacking hyphal forms were identified (Fig. 4C-F).

Infants with or without intestinal *Candida* colonization were equivalent with respect to gestational age at birth (median gestational age: 24.5 weeks (range: 23 - 28 weeks) for infants with intestinal *Candida* versus 26 weeks (range: 23 - 28 weeks) for infants without intestinal *Candida*), birth weight (709 g ± 149 g versus 668 g ± 212 g), length of life (39.5 days (range: 14 - 158 days) versus 42.5 days (range: 20 - 289 days)), or presence of histologic acute chorioamnionitis (6/16 versus 5/10). Placental candidiasis (peripheral funisitis associated with fungal elements) was diagnosed in one case from each group.

DISCUSSION

A 22-year-spanning data search of 187 extremely premature infants from our neonatal autopsy archives yielded 11 diagnosed cases of invasive *albicans* and 3 cases of invasive non-*albicans* candidiasis, the latter involving *C. parapsilosis* and *C. glabrata*. In contrast to the distinct inflammatory and tissue-destructive histopathologic changes seen in the majority of neonatal *C. albicans* cases, infection with non-*albicans Candida* organisms was remarkably inconspicuous by routine hematoxylin-based histologic analysis. Using targeted (immuno)histochemical approaches for the purpose of this study, large numbers of *Candida* yeasts were localized in gastrointestinal tract, respiratory tract, and circulation.

Intestinal colonization by *Candida* species is an important risk factor for subsequent disseminated disease, whereby the density of colonization correlates with the risk for candidemia (23-26). Whereas invasion of intestinal epithelial cells by *C. albicans* is mediated, in part, by active, physical penetration by hyphae (12), the mechanisms underlying intestinal penetration by *non-albicans Candida* yeast forms remain incompletely understood. In the present study, confocal microscopy of combined anti-*Candida* and anti-cytokeratin-stained tissue sections revealed a distinct single-file pattern of infiltrating non-*albicans* yeasts (*C. parapsilosis* and *C. glabrata*) in the intestinal epithelium, unaccompanied by histologic evidence of cellular injury or inflammation. This transepithelial migration was associated with linear aggregation of yeast cells along the basement membrane of the intestinal crypts/glands, suggesting this structure, when intact, provides a natural barrier against deeper, systemic invasion by *Candida* yeast forms. These findings are concordant with recent studies in murine models of gut colonization following intragastric inoculation with various *Candida* species. *Candida albicans* readily invaded the epithelium and disseminated from the gut, attributed to its capacity to produce hyphae. Inoculation with yeast forms such as *C. parapsilosis*, in contrast, resulted in persistent colonization without systemic invasion. (27).

Deeper invasion and subsequent hematogenous spread by *Candida* yeast forms are associated with disruption of the intestinal epithelial basement membrane by concomitant traumatic, ischemic or infectious events, such as necrotizing enterocolitis, spontaneous intestinal perforation, and abdominal surgery (24, 28, 29). In the present study, 2/3 neonates with invasive non-*albicans* candidiasis had a history of overt intestinal or abdominal insults, usually in the form of necrotizing enterocolitis.

Fungal-specific stains further revealed the presence of numerous *Candida* yeasts in the pulmonary airways and airspaces in all three neonates with invasive non-*albicans* candidiasis. As in the gastrointestinal tract, the often-massive pulmonary involvement was deceptively insidious by hematoxylin-eosin-based routine histologic examination. This bronchopulmonary (‘air space-invasive’) pattern of pulmonary candidiasis, analogous to that previously described in preterm infants with disseminated *C. albicans* infection (30, 31), may be caused by downward transfer of oropharyngeal commensals by endotracheal intubation or similar oropharyngeal manipulation. None of the cases of non-*albicans* candidiasis displayed a true miliary pattern of pulmonary parenchymal necrosis and inflammation, as may be seen in *C. albicans* pneumonia (30, 31).

The reported frequency of intestinal *Candida* colonization in NICU patients, based on stool cultures, ranges widely from 10% to 60% (32, 33), with *C. albicans* and *C. parapsilosis* as most prevalent commensals (33-36). In the present study, about 60% of deceased extremely preterm neonates displayed evidence of intestinal colonization by *Candida* organisms, as determined by anti-*Candida* immunohistochemical analysis of archival intestinal tissues of a consecutive series of neonates who had lived for at least one week. This result likely underestimates the actual frequency of *Candida* intestinal colonization in extremely premature NICU patients as the available archival material only represented a limited portion of the gastrointestinal tract (estimated less than 5% of the total length), and the sensitivity of the anti-*Candida* immunohistochemical assay may have been affected by autolysis-associated loss of antigenicity.

In addition to allowing culture-independent identification of fungal organisms as *Candida* species, the anti-*Candida* immunohistochemical assays utilized in this study have several advantages over traditional histochemical approaches. Immunostaining, whether peroxidase- or fluorescence-based, facilitated detection of the yeast forms, especially in debris- and pigment-rich backgrounds such as – partially autolyzed – intestines, liver and lungs. Improved visualization by immunostaining further allowed tentative speculation about the type of *Candida* species involved based on their relative size. Among the more common *Candida* species, *C. albicans* yeasts are largest (4-6 x 6-10 μm), followed by *C. parapsilosis* (2.5-4 x 2.5-9 μm) and *C. glabrata* (1-4 μm) (37). In some intestinal samples, the presence of relatively large-sized yeast forms in combination with true hyphae was strongly suggestive of *C. albicans* colonization. In the remaining cases, the exact *Candida* species remained undetermined, although the smaller size in the absence of true hyphae may be suggestive of non-*albicans Candida* species.

In summary, we reported our autopsy experience with neonatal invasive non-*albicans* candidiasis and neonatal intestinal *Candida* colonization. In view of its insidious histopathologic presentation, the postmortem diagnosis of invasive neonatal non-*albicans* candidiasis rests entirely on positivity of blood and/or lung cultures. However, blood cultures are notoriously insensitive in diagnosing disseminated candidiasis, with false-negative rates of up to 50%, especially in small children and following prophylactic or empiric antifungal therapy (38, 39). We speculate that many cases of invasive non-*albicans* candidiasis in (deceased) extremely preterm neonates may go undiagnosed. More generous application of (immuno)histochemical fungal stains in perinatal autopsies may provide important insights into the prevalence, pathophysiology, and clinical relevance of the various forms of candidiasis in the neonatal population, both as commensals and (blood-) invasive organisms.

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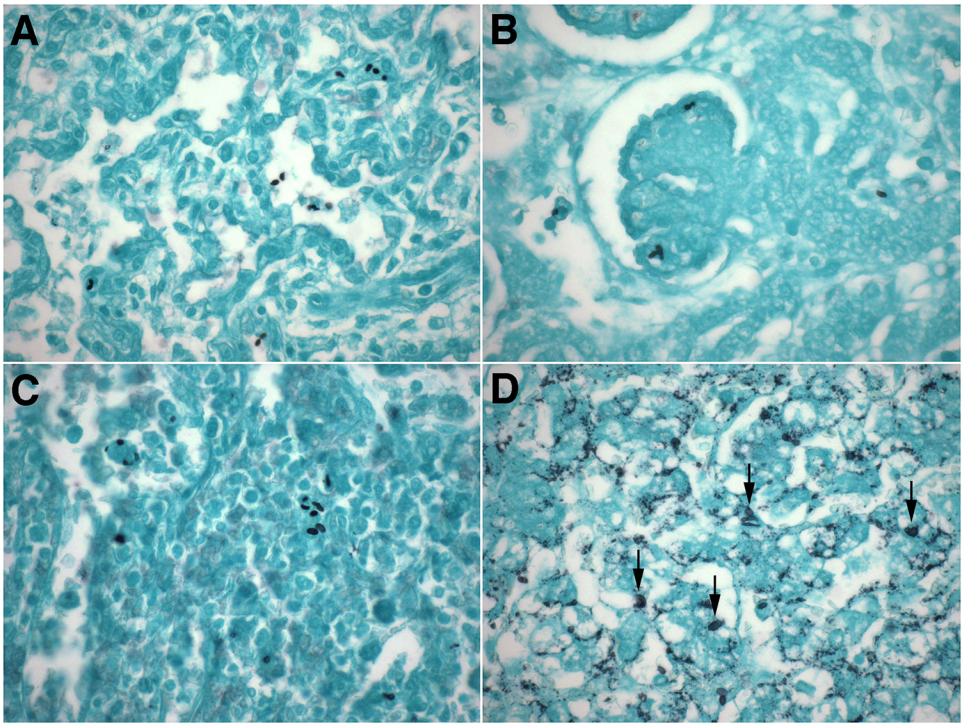

Figure 1. Anatomic distribution of yeasts in invasive neonatal non-*albicans* candidiasis.

A-D. Representative micrographs of lungs (A), kidneys (B), spleen (C) and liver (D) of extremely preterm neonates with invasive non-*albicans* candidiasis showing the ubiquitous presence of *Candida* yeast forms (2-5 μm ovals) in these organs. While readily visible by Gomori-methenamine silver staining in most organs (A-C), the *Candida* yeasts were obscured by intrinsic lipochrome pigment in liver (D, arrows).

(A-D: Gomori-methenamine silver, original magnification: x600).

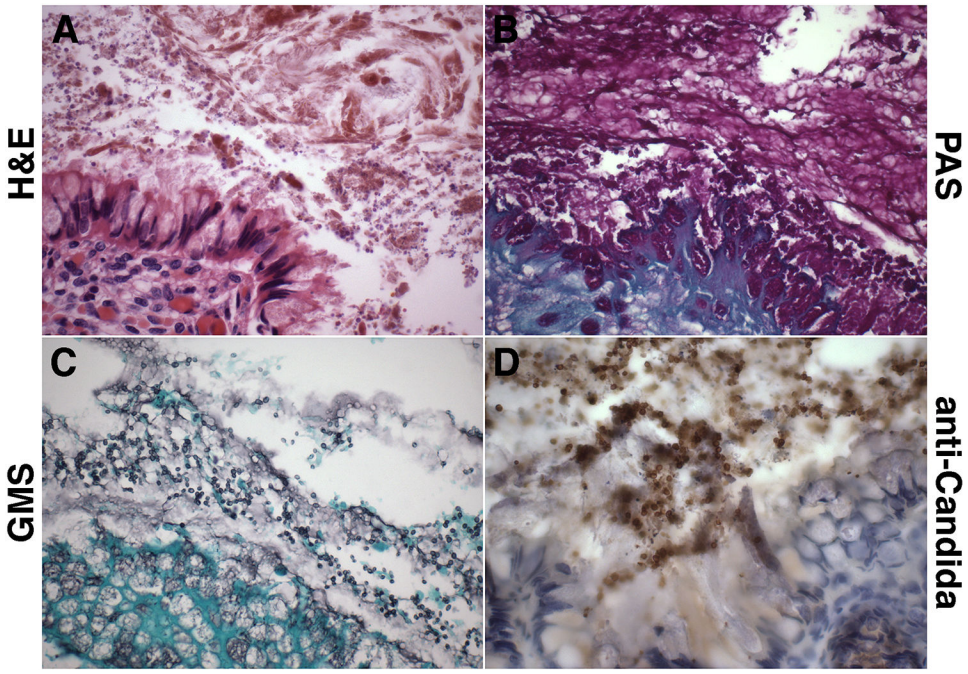

Figure 2. Comparison of (immuno)histochemical fungal detection methods (intestine).

A-D. Representative micrographs of intestine of 23-week gestation neonate (case 1) with invasive *C. parapsilosis* infection showing abundant *Candida* yeasts in the intestinal lumen. In this comparative analysis of (immuno)histochemical fungal detection methods, anti-*Candida* immunohistochemistry (D) was found to be superior for detection of *Candida* yeasts among meconium and intestinal debris, compared with hematoxylin-eosin staining (A), periodic acid-Schiff staining (B), and Gomori-methenamine silver staining (C). (A: hematoxylin-eosin, B: periodic acid-Schiff, C: Gomori-methenamine silver, D: anti-*Candida* DAB immunostaining with hematoxylin counterstain. All: original magnification: x600).

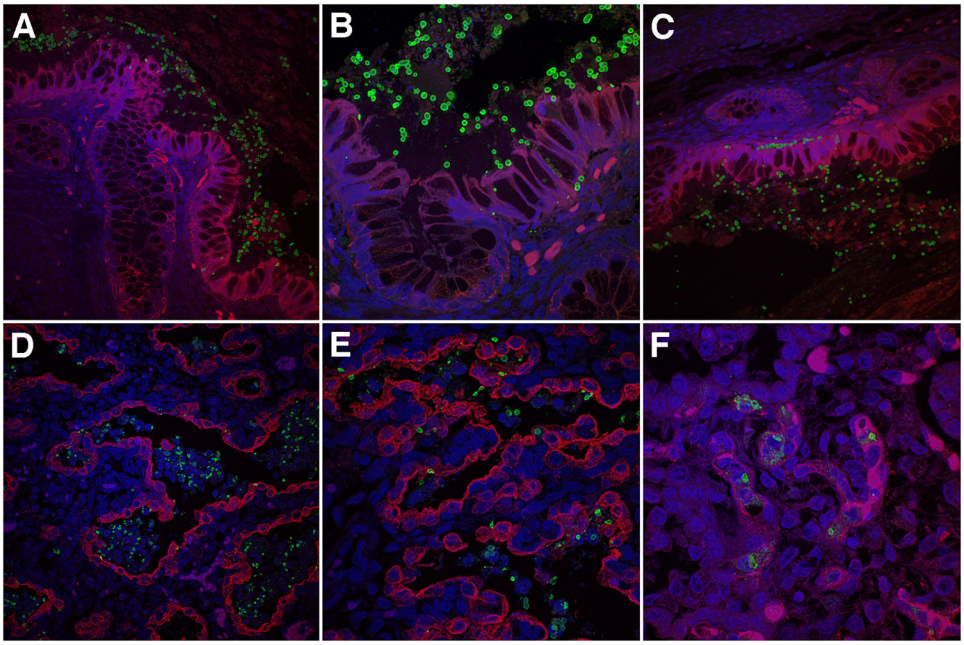

Figure 3. Intestinal and pulmonary features of invasive neonatal non-*albicans* candidiasis.

A-C. Combined anti-*Candida* (green) and anti-cytokeratin (red) immunofluorescence staining of representative lower intestinal tract of extremely preterm neonates with invasive non-*albicans* candidiasis showing abundant *Candida* yeast forms in the intestinal lumen, lining the colonic epithelium (A). Some yeast cells are seen infiltrating the intestinal epithelium in a single-file pattern (B), while others are arranged in a linear pattern along the epithelial basement membrane (C).

D-E. Representative micrographs of lungs of extremely preterm neonates with invasive non-*albicans* candidiasis showing abundant, partially degenerated *Candida* yeast organisms in the distal airspaces and, more sporadically, in the pulmonary interstitium.

F Combined anti-*Candida* and anti-CD31 immunofluorescence staining demonstrating predominant localization of interstitial *Candida* yeasts within the pulmonary microvasculature, rather than interstitial stroma.

(A-F: confocal fluorescence microscopy of intestines and lungs subjected to combined anti-cytokeratin (AE1/3) (A-E) or anti-CD31 (F) (red) and anti-*Candida* (green) immunofluorescence analysis with DAPI counterstain (blue). Original magnification: x400 and x800).

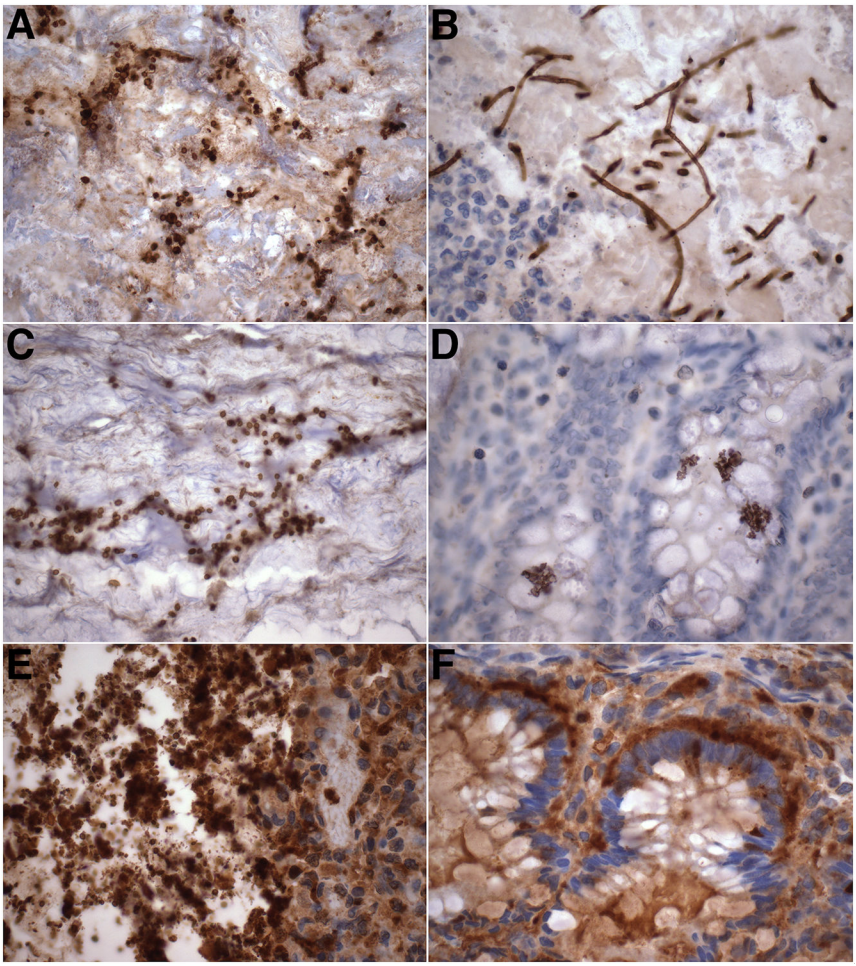

Figure 4. Intestinal colonization by *Candida* species in extremely preterm neonates.

A-B. Micrograph of luminal mucus and debris showing relatively large-sized *Candida*-immunoreactive yeast forms and scattered pseudohyphae (A, arrows) in association with true hyphae (mycelia) (B), consistent with *C. albicans*.

C-D. Representative micrographs showing smaller yeast forms in intestinal lumen (C) and glands (D), not accompanied by hyphal forms, suggestive of non-*albicans Candida* species.

E-F. Intestinal lumen entirely occupied by masses of relatively small *Candida* yeasts (E), linearly arranged around the base of the colonic glands in areas with intact epithelium.

(A-F: anti-*Candida* DAB immunostaining with hematoxylin counterstain. All: original magnification: x600).

Table 1.

Clinical, placental and postmortem findings.

	Case 1	Case 2	Case 3
Age at birth	23 wks	24 wks	25 wks
Birth weight	510 g	640 g	730 g
Delivery mode	C-section (umbilical cord prolapse)	Vaginal (preterm labor)	C-section (placental abruption)
Invasive procedures	Intubation, umbilical A/V catheters, PICC line	Intubation, umbilical A/V catheters, PICC line	Intubation, umbilical A/V and central catheters
Neonatal complications	Necrotizing enterocolitis, multi-system organ failure	Intraventricular hemorrhage, hypertrophic cardiomyopathy, segmental intestinal necrosis	*S. aureus* sepsis, intraventricular hemorrhage, hydrocephaly
Age at death	22 days	13 days	24 days
Cause of death	Sepsis, necrotizing enterocolitis, extreme prematurity	Sepsis, pneumonia, necrotizing enterocolitis, extreme prematurity	Sepsis, extreme prematurity
Antemortem blood cultures	*C. glabrata, C. parapsilosis, S. epidermidis*	Yeast (day 12)	*C. parapsilosis. S. aureus*
Postmortem cultures	*C. glabrata,* coagulase-negative *Staphylococci* (blood); *C. parapsilosis* (lung)	*C. glabrata* (blood, lung, spleen), *S. capitis* (blood)	*C. parapsilosis* (lung, catheter tip), coagulase-negative *Staphylococci* (blood)
Postmortem interval	16 h	12 h	8 h
Placenta	Moderate acute chorioamnionitis, vasculitis and funisitis of cord	Evidence of maternal vascular malperfusion	Evidence of chronic abruption