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FUNGAL GENOMICS AND PATHOGENESIS (S SHOHAM, SECTION EDITOR)

Neonatal Candidiasis: New Insights into an Old Problem at a Unique Host-Pathogen Interface

Amanda B. Arsenault[1] · **Joseph M. Bliss**[1,2]

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Abstract *Candida* species are the leading cause of invasive fungal infections in premature infants. Associated with substantial morbidity and mortality, these infections represent serious and sometimes catastrophic complications in the course of hospitalization of a preterm infant in the neonatal intensive care unit. Although virulence factors of *Candida* and the host defense mechanisms that are important in protection from candidiasis have been the subject of intensive study, considerably less is known about the features of this disease that are specific to premature neonates. As animal models for neonatal candidiasis have been developed, efforts to understand the similarities and differences of candidiasis in the neonatal host relative to other immunocompromised patients have begun to provide insights to these questions.

Keywords *Candida* · Neonate · Candidiasis · Animal model · Virulence · Host defense

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✉ Joseph M. Bliss
jbliss@wihri.org

Amanda B. Arsenault
aarsenault@wihri.org

[1] Department of Pediatrics, Women & Infants Hospital of Rhode Island, Warren Alpert Medical School of Brown University, Providence, RI, USA

[2] Department of Pediatrics, Women & Infants Hospital, 101 Dudley St., Providence, RI 02905, USA

Introduction

Commonly found as commensal organisms colonizing skin and mucosal surfaces, *Candida* species have also long been recognized as an important cause of human disease, particularly, but not exclusively, among those with compromised immunity. The infections caused by these organisms can be broadly classified into mucocutaneous and invasive candidiasis. Mucocutaneous disease includes oropharyngeal candidiasis (oral thrush), esophageal candidiasis, and vaginal candidiasis, with the latter a common infection among otherwise healthy women [1]. Invasive candidiasis includes candidemia as well as infection of deeper organs, and these infections are associated with high mortality and morbidity [2]. Patients at risk for candidiasis include those who are immunocompromised on the basis of HIV infection, chemotherapy, and bone marrow or solid organ transplantation, as well as trauma or burn patients and those in intensive care units [2, 3].

Newborn infants are a unique subset of patients who are also susceptible to these infections and their complications. Otherwise healthy, term infants commonly develop mucocutaneous disease in the form of oral thrush and diaper dermatitis [4], whereas premature infants are at risk for disseminated disease [5, 6]. The occurrence of candidiasis in this population is likely due in part to the immaturity and modulation of the immune system in the developing fetus that matures after delivery. Neonatal candidiasis therefore represents a unique host-pathogen interface in which both the virulence mechanisms of the fungus and the response of the infant to infection may be quite distinct from other clinical settings of *Candida* infection. This review will focus on the features of candidiasis in the neonate. The epidemiology and risk factors that are specific to this patient population will be discussed. In addition, recently developed animal models will be reviewed as

well as contemporary studies that provide insights related to the pathogenesis of these infections.

Epidemiology

Fungal organisms have been commonly associated with late-onset neonatal sepsis over the past several decades. In a review published by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Neonatal Research Network (NRN) registry, *Candida albicans* was the third most commonly isolated organism in first episodes of late-onset neonatal sepsis and accounted for 12 % of cases overall [7]. Despite detection and treatment, the mortality rate of invasive candidiasis was 32 %, and among those affected by *C. albicans*, mortality rates reach as high as 44 %. Additionally, rates of severe neurodevelopmental impairment are high among survivors [8].

Invasive neonatal candidiasis began to increase in incidence concurrent with the increase in survival of very low birth weight infants. In a study of 19 NRN centers, the overall rate of invasive candidiasis in extremely low birth weight infants (birth weight <1000 g) was 9 %, with a variation in incidence ranging from 2 to 28 % per center [8]. However, in a recent study from 2014, the incidence of invasive candidiasis showed a decrease in the US from 3.6 per 1000 infants in 1997 to 1.4 per 1000 infants in 2010 [9••]. The greatest decrease in incidence was seen among neonates with a birth weight of less than 750 g. This decrease was likely mediated by a combination of practices, including more judicious use of broad-spectrum antibiotics, improved maintenance of central venous catheters and an increase in prophylactic and empiric use of anti-fungal drugs.

C. albicans and *C. parapsilosis* are the most common species isolated in episodes of invasive disease in neonates [10]. While not traditionally thought to be as virulent, *C. parapsilosis* has emerged as a significant pathogen in neonatal candidiasis and in some centers has surpassed *C. albicans* as the leading organism in invasive disease [11]. Other non-*albicans Candida* species that are seen less commonly include: *C. glabrata*, *C. lusitaniae*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii*. Collectively for *C. albicans* and *C. parapsilosis*, the most common sites of infection are blood, urine, and CSF with a higher proportion of meningitis caused by *C. albicans* [12, 13].

Risk Factors

Risk factors that are well established for development of invasive candidiasis are listed in Table 1. Earlier gestational age and lower birth weight are clearly associated with increased risk for candidiasis [6, 14]. In addition to immaturity of various components of the immune system, immature skin portends increased risk for skin colonization and subsequent invasive disease. Studies assessing rates of colonization of infants with *Candida* have also implicated route of delivery as a risk factor. Colonization with *C. albicans* occurs earlier and is more common after vaginal delivery, suggesting a vertical mode of transmission. However, colonization with *C. parapsilosis* generally occurs later in the course of ICU admission and is the organism more commonly cultured from the hands of health care workers, suggesting horizontal transmission of this species [13]. The more recent finding that receiving breast milk colonized with yeast is associated with yeast colonization in preterm infants, albeit not always by the same species, highlights an additional potential exposure unique to these patients relative to other groups at risk [15].

Table 1 Risk factors for neonatal invasive candidiasis

Nonmodifiable postnatally	Potentially modifiable
Prematurity	Central venous catheters
Low birth weight	Broad-spectrum antibiotics (esp. 3rd generation cephalosporins)
Skin or GI colonization	H2 blockers
Compromise of GI mucosal integrity (NEC, anatomic anomalies)	Parenteral nutrition (IV lipid infusions)

GI gastrointestinal, *NEC* necrotizing enterocolitis, *IV* intravenous

Colonization of the gastrointestinal (GI) tract has also been established as an important risk for subsequent development of disseminated disease. Saiman et al. assessed infants from six NICUs and found rates of colonization ranging from 18 to 26 %. The most prevalent species was *C. albicans* followed by *C. parapsilosis*, which mirrors the most common pathogens present in invasive disease [13]. In another study from the same group, 40 % of cases of invasive candidiasis for whom GI cultures were also available were found to have the same clone of *Candida* at both sites [14]. *Candida* likely gains access to the intravascular space by translocation through the intestinal epithelium. Therefore, GI pathology such as necrotizing enterocolitis or other congenital intestinal anomalies confer additional risk for invasive disease in colonized neonates secondary to compromise of the GI mucosal integrity [16, 17].

Multiple factors inherent in the intensive care and prolonged hospital admissions required by premature infants increase the risk of developing candidiasis [6, 14, 16, 18, 19]. One such factor is the use of central venous catheters. Each additional day of catheter use is associated with further increased risk above that of the presence of the catheter alone [16]. The use of broad spectrum antibiotics, in particular third-generation cephalosporins, is thought to alter the intestinal microbiome in such a way that *Candida* colonization occurs

unopposed by commensal bacteria normally present. Thus, antimicrobial stewardship has likely contributed to the decreased incidence of invasive neonatal candidiasis seen in recent years [9••]. Additional risk factors identified across several studies include the use of H2-receptor blockers, which decrease the pH of the gastric contents, altering the microbial milieu and allowing for the increased survival and reproduction of *Candida* species. The use of parenteral nutrition, particularly IV lipid infusions, provides a lipophilic environment in which *Candida* can thrive. Unlike those factors inherent to prematurity itself, the above factors are potentially modifiable and limiting their use is prudent to decreasing the incidence of neonatal candidiasis.

Animal Models

While the pathogenesis of candidiasis has been studied in depth, there is a relative paucity of literature that addresses the pathogenesis of these infections in a neonatal host. The lack of suitable animal models in which to study this disease process has made this endeavor challenging. Early attempts to establish such a model involving gastric inoculation of newborn mice demonstrated that gastrointestinal colonization and systemic disease could be achieved, but the model was limited by technical challenges as well as variability among strains utilized [20, 21]. However, in 2011, two new models were described: a neonatal rat model [22] and a neonatal mouse model [23]. Importantly, in both models, methods used in adult animals such as pretreatment with broad-spectrum antibiotics or agents to suppress immunity were not necessary to achieve invasive disease. This finding supports the notion that the immaturity of the animals recapitulates the susceptibility of newborn infants and demonstrates their utility to study this disease and its pathogenesis.

In the rat model, 2–3-day-old newborn Sprague-Dawley rats were utilized [22]. Injections of *C. albicans* were administered via intravenous (temporal vein), intragastric, and intraperitoneal routes. Fungal burden was determined from homogenized organs (lung, liver, kidney), and the histopathology of these organs was also assessed. Overall, survival of pups receiving IV infection was significantly shorter than adults (2 vs 6 days). Fungal burden was also higher in pups than adults. No adult rats had liver involvement, and only 2/7 adults were found to have lung involvement 30 days after IV injection. In contrast, all pups had liver involvement and 6/7 had lung involvement at the same time point. Interestingly, a similar number of adult rats and pups had renal involvement. After intragastric inoculation, pups were found to have overall higher fungal burdens, higher dissemination of *Candida* outside of the GI tract and continued to shed yeast in stool longer than adult rats.

In the mouse model, 2-day-old BALB/c mouse pups were infected with *C. albicans* by the intraperitoneal route [23]. Similar to the rat model, mice were monitored for survival, and fungal burden (kidney and lung) and histopathology (kidney, liver, and spleen) were assessed. Fungal burden was higher in the kidney as compared to lungs and was proportional to the dose of yeast injected. Fungal elements were found to be scattered throughout the liver and kidney. Fungi seen in the spleen were in the capsular region with some penetration by hyphae into the parenchyma. Thus, the use of IP injection in this model reliably led to disseminated disease. In contrast, adult mice infected by the intraperitoneal route develop disseminated disease only if immunosuppression is induced prior to infection [24].

One advantage of these models of neonatal candidiasis is that they do not require immunosuppression to induce disseminated infection. The rat model offers an additional benefit of the pups being of larger size which allows for easier manipulation and specimen procurement [22]. However, in order to produce reliable systemic disease, the rat model relies on intravenous injection of the temporal vein which poses technical challenges. Although intragastric infection in rat pups also resulted in disseminated disease, organ fungal burden decreased over the course of the infection, and mortality rates were low for this route. In mouse pups, intraperitoneal injection resulted in widespread disease and mortality, obviating the need for IV injection or establishment of GI colonization. The mouse model also provides the advantage of the ability to draw from the widely available genetic tools characteristic of murine systems. Overall, with slight differences, both animals offer reproducible models in which to study the pathogenesis of neonatal candidiasis. Noted differences from adult models support the notion that studies in neonatal animals are useful to discern mechanisms of disease that may be unique to an immature host.

Pathogenesis of Neonatal Candidiasis

Candida Virulence Factors

Although invasive candidiasis has been an active area of investigation, considerably less attention has been given to the unique host and pathogen factors that make neonates particularly susceptible to disseminated candidiasis. As reviewed by Bendel, colonization of the skin or mucosal epithelium by *Candida* is an important precursor to infection and places infants at risk. The subsequent penetration of these epithelial surfaces by yeast leads to disseminated disease [25]. Virulence factors such as production of proteinases and phospholipases, morphologic switching, and biofilm formation have been extensively studied and contribute to the efficient colonization of epithelial surfaces by *Candida*. Sugar residues on the buccal

mucosa or extracellular matrix proteins of epithelial cells are known ligands to which *Candida* species adhere. Moreover, when the epithelium is damaged and the integrity of the mucosal barriers is compromised, more extracellular matrix proteins are exposed allowing for increased adhesion and dissemination of the organisms.

C. albicans is unique relative to many non-*albicans* species in its capacity to grow as both yeast and true hyphae. Hyphal forms of *C. albicans* invade epithelium and produce invasive disease in neonates more so than other species of *Candida*. This suggests that the ability to produce hyphae is an important virulence factor for dissemination. Multiple studies have shown that mutant forms of *C. albicans* which are unable to produce hyphae are less able to adhere to human epithelial cells and lead to less disseminated disease when intravenously injected. However, mutant strains of *C. albicans* that exist only in the hyphal form are less able to colonize and are not capable of producing disseminated disease in mice [26]. Additionally, when mice were injected with a strain that was modified such that the ability to undergo hyphal growth could be regulated in vivo, mortality was only seen under conditions in which hyphal growth was allowed. However, fungal burdens were similar, suggesting that yeast cells are important for dissemination [27]. Therefore, the ability to produce hyphae alone is not sufficient to establish invasive disease; rather the ability to exist in both yeast and hyphal forms is important for virulence.

In order to further delineate which factors may lead *C. albicans* to be more virulent than other *Candida* species, Gale and colleagues examined interactions of various species with premature GI epithelium, using a cell line derived from small intestine enterocytes of 20–22-week fetuses [28]. *C. albicans* was more frequently located intracellularly as compared to *C. parapsilosis*, *C. glabrata*, and *C. dubliniensis* in these assays. The ability of *C. albicans* to produce hyphae appeared to play a role in this process. Additionally, *C. glabrata* and *C. parapsilosis* were only noted to exist in budding yeast form and were never noted to invade enterocytes. *C. albicans* was also noted to produce more cell injury compared to other species of *Candida*. This difference was also attributed to the ability of *C. albicans* to produce hyphae, as mutant strains unable to produce hyphae caused less injury. Interestingly, *C. albicans* strains differed in their capacity to cause cell injury, suggesting that factors other than hyphal growth are responsible. IL-8 secretion was also higher from cells exposed to *C. albicans* as compared to other species of *Candida* and to uninfected controls. However, despite differences in induction of injury, all strains of *C. albicans* tested resulted in similar levels of IL-8 secretion, leading the authors to conclude that *C. albicans* may induce inflammation in cells through injury-independent mechanisms.

As potential virulence factors for candidiasis are identified, a common approach to evaluate their role in vivo is to generate mutant strains in which the gene of interest is deleted and test the mutant in an animal model of disease. Only a handful of such mutants have been tested in neonatal models. One such protein is Als3p, a member of the agglutin-like sequence family of proteins [29, 30]. Als3p mediates adhesion to a variety of host substrates including endothelial cells, oral epithelium, type IV collagen, fibrinogen, and fibronectin. Heterologous expression of Als3p induces adherence in the nonadhesive yeast, *Saccharomyces cerevisiae* [31]. Moreover, antibodies against Als3p reduce adherence of *C. albicans* to both epithelial and endothelial cells, much like mutant strains of *C. albicans* lacking expression of Als3p [32–34]. In addition to its role as an adhesin, Als3p also plays an important role in mediating endocytosis by host cells. Endocytosis of *C. albicans* by oral epithelial cells and vascular endothelial cells was reduced in *ALS3* null mutants, while endocytosis was induced when latex beads were coated with the N-terminus of Als3p [34]. Aside from its role in adhesion and invasion, Als3p has been noted to have additional roles that may contribute to virulence. It allows hyphal forms of *C. albicans* to bind ferritin and utilize it as a sole source of iron and has a role in biofilm formation [35, 36]. Surprisingly, studies of Als3p in vivo have suggested that it is not critical for disseminated disease following tail-vein injection in adult mice [37]. Mutant strains lacking Als3p were able to bind as effectively as wild-type *C. albicans* to host endothelial cells and produce disseminated infection as effectively as wild-type. Fungal burdens in the brain, kidney, and spleen were similar in mice infected with Als3p mutant strains as in mice infected with wild-type strains and survival curves were indistinguishable. However, additional insight into the role of Als3p in vivo was gained from a study in which *C. albicans ALS3* was heterologously expressed in *C. glabrata*. Following injection intravenously in adult mice, expression of *ALS3* led to increased fungal burden in the brain as well as increased trafficking to and persistence in the kidneys [38•].

In contrast to the results seen in the adult tail-vein model, when neonatal mice were infected with an Als3p-deficient strain, median survival was prolonged relative to pups infected with wild-type *C. albicans* [23]. Additionally, monoclonal antibody directed against Als3p affords protection in infected pups (unpublished data). The different outcomes in the adult and neonatal models may be related to the different routes of infection employed. Infection of pups by the intraperitoneal route requires additional steps for the organism to gain access to the blood stream and disseminate, whereas these are bypassed by tail-vein injection in adults. Als3p may have a role in dissemination from the peritoneal site, where adhesion and invasion of host cells is likely an important step. The neonatal model may therefore have particular relevance to disseminated candidiasis that occurs following gastrointestinal colonization and disruption of the mucosal barrier, whereas the adult model is reminiscent of direct access to the

vascular space as may occur with intravenous catheters. These findings highlight the importance of considering both the host and the features unique to different models when considering the pathogenesis of infection.

Another virulence determinant that has been evaluated in a neonatal model of candidiasis is extracellular secreted lipase. These enzymes, secreted by both *C. albicans* and *C. parapsilosis*, are relevant to pathogenesis through nutrient acquisition, adhesion to host tissues, hydrolysis, and lysing of competing microflora. Previous studies have established the role of secreted lipase in virulence in adult mice [39, 40]. Neonatal rats were infected via the intravenous, intragastric, or intraperitoneal routes with either wild-type or lipase-deficient *C. albicans* or *C. parapsilosis*. Diminished virulence with mutants of both species was demonstrated, although more consistently across all routes of infection for *C. parapsilosis* than for *C. albicans* [22]. The authors speculate that the increased prevalence of *C. parapsilosis* in neonates and this organism's affinity for growth in high-lipid parenteral nutrition solutions commonly used in premature infants may underscore the importance of secreted lipase in virulence of this species. Differences in the manifestation of these infections between adult mice and rat pups were also noted, again suggesting that study of candidiasis in neonatal hosts can identify important differences in pathogenesis.

To determine whether virulence characteristics among different strains of *Candida* have implications in clinical outcomes, a large collection of clinical isolates was used to identify virulence traits of *Candida* that may be associated with disease manifestations in human neonatal candidiasis. Clinical isolates collected from infants with disseminated candidiasis as part of a large trial conducted through the NICHD NRN [6] were compared to a separate collection from infants who were colonized with *Candida* but without invasive disease [41]. Isolates were tested in assays of adhesion, toxicity to cultured cells, and their capacity to undergo phenotypic switching in vitro. Infants infected with invasive isolates that had enhanced activity in any of these assays relative to the isolates from colonized infants ("enhanced virulence") were compared to infants infected with strains that lacked these properties. Infants in the enhanced virulence group were infected earlier in their hospitalization, had a higher serum creatinine, and a trend toward higher mortality (44 vs 25 %; $p=0.1$) [42••]. Additionally, all of the isolates recovered from cerebrospinal fluid had enhanced virulence. These findings suggest that virulence characteristics of the individual infecting strain measured in vitro can have a measurable effect on the manifestations and outcomes of human disease.

Neonatal Host Factors

As discussed above, virulence traits of the organism that are relevant to disease in neonatal hosts are beginning to be identified and may impact disease outcome. Identification of host factors in the neonate that lead to susceptibility to invasive candidiasis is another important approach to understand pathogenesis. Although developmental alterations in immunity that are characteristic of newborns have been studied in some detail [43], very few studies have focused on the neonatal response to *Candida* specifically. Neutrophils are critically important in antifungal host defense, and absolute or functional neutropenia are well-recognized risk factors for invasive infection. Premature infants, however, are generally not neutropenic when invasive candidiasis is diagnosed. This observation led our group to determine whether neonatal neutrophils differed in their ability to undergo phagocytosis or generate an oxidative burst when exposed to *C. albicans* or *C. parapsilosis* as compared to adult neutrophils [44]. Neutrophils were isolated from cord blood of term and preterm infants immediately after delivery and compared to adult neutrophils from peripheral blood of healthy volunteers. No differences were detected in oxidative burst produced by neonatal (term and preterm) as compared to adult neutrophils in response to *C. albicans* or *C. parapsilosis*. Likewise, there was no difference in phagocytosis efficiency of either species of *Candida* among preterm, term, or adult neutrophils. Interestingly, all groups of neutrophils were significantly more efficient in their ability to phagocytose *C. parapsilosis* yeast as compared to *C. albicans* yeast forms. These findings suggest that other factors that modulate neutrophil function against *Candida* in vivo may differ in neonates that would be undetected in the in vitro setting.

The surprising finding that neutrophils undergo phagocytosis of *C. parapsilosis* yeast with much greater efficiency than *C. albicans* yeast led to additional experiments that identified a role for the S-type lectin, galectin-3 (gal3) in this process. Gal3 is one of a family of β-galactoside-binding lectins, which have key regulatory roles in the immune system and in inflammation [45]. Treatment of neutrophils with antibody directed against gal3 decreased phagocytosis, and treatment with recombinant gal3 augmented phagocytosis efficiency of *Candida* [46]. Additional experiments suggested that gal3 is secreted from neutrophils and acts as a proinflammatory signal to augment neutrophil phagocytosis. Gal3-deficient mice were also shown to manifest more severe disease than wild-type mice when infected with either *C. albicans* or *C. parapsilosis*, adding support to the importance of this lectin in disseminated disease [47•]. These findings may be particularly relevant to neonatal candidiasis in that gal3 levels in serum collected from cord blood of human neonates is approximately half the concentration found in adult serum [47•]. Additionally, cultured cord blood cells were found to express lower gal3 relative to adult serum levels, its expression was induced by an invasive strain of group B *Streptococcus*, but not by a colonizing strain, and gal3 expression increased with increasing gestational age [48•].

Identification of host factors such as these that are unique to neonates and affect their susceptibility to invasive infection is an area that is ripe for future investigation.

Conclusions

Although decreasing in incidence in recent years, neonatal candidiasis remains an important cause of morbidity and mortality among premature infants. Improved understanding of its epidemiology and risk factors has led to practice modifications that have mitigated but not eliminated the risk for these infections. Recent development of animal models that are tailored to the neonatal host are likely to provide insights into the unique virulence attributes of the pathogen and unique susceptibilities of the host that contribute to disease in this setting. Current efforts are beginning to shed light on these complex relationships, which may ultimately enable interventions tailored to this host-pathogen interaction and allow improved, targeted preventive and therapeutic strategies.

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Compliance with Ethics Guidelines

Conflict of Interest Amanda B. Arsenault and Joseph M. Bliss declare no conflicts of interest.

Human and Animal Rights and Informed Consent All studies by the authors involving animal and/or human subjects were performed after approval by the appropriate institutional review boards. When required, written informed consent was obtained from all participants.

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