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Candida Virulence Properties and Adverse Clinical Outcomes in Neonatal Candidiasis

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Abstract

Objective—To determine if premature infants with invasive *Candida* infection caused by strains with increased virulence properties have worse clinical outcomes than those infected with less virulent strains.

Study design—Clinical isolates were studied from 2 populations; premature infants colonized with *Candida* (commensal, n=27), and those with invasive candidiasis (n=81). Individual isolates of *C. albicans* and *C. parapsilosis* were tested for virulence in each of 3 assays: phenotypic switching, adhesion, and cytotoxicity. Invasive isolates were considered to have enhanced virulence if they measured more than 1 SD above the mean for the commensal isolates in at least 1 assay. Outcomes of patients with invasive isolates with enhanced virulence were compared with those with invasive isolates lacking enhanced virulence characteristics.

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Results—61% of invasive isolates of *C. albicans* and 42% of invasive isolates of *C. parapsilosis* had enhanced virulence. All *C. albicans* cerebrospinal fluid (CSF) isolates (n=6) and 90% of urine isolates (n=10) had enhanced virulence, compared with 48% of blood isolates (n=40). Infants with more virulent isolates were younger at the time of positive culture and had higher serum creatinine.

Conclusions—Individual isolates of *Candida species* vary in their virulence properties. Strains with higher virulence are associated with certain clinical outcomes.

Keywords

C. albicans; *C. parapsilosis*; mortality; neurodevelopmental impairment; phenotypic switch; adhesion; cytotoxicity

Candida species are the third leading cause of late-onset sepsis in premature infants and are associated with an increased risk of mortality and neurodevelopmental impairment among survivors.[1, 2] Historically, *C. albicans* was the most frequent cause of bloodstream infections, accounting for 70–80% of isolates.[3] However, infections caused by *C. parapsilosis* are increasing in frequency worldwide,[4] with a significantly higher prevalence in neonates than in other at-risk populations.[5] In some centers, *C. parapsilosis* has emerged as the leading cause of invasive candidiasis.[6]

Colonization with *Candida* is common in premature infants and is an important risk factor for invasive disease.[7–10] Unlike other fungal pathogens that are primarily acquired from the environment, systemic infection with *C. albicans* and *C. parapsilosis* occurs primarily with commensal organisms colonizing mucosal surfaces or skin.[11] A number of virulence factors have been studied extensively in *C. albicans*, and to a lesser extent in *C. parapsilosis*. [4, 5, 12] These include the capacity to undergo phenotypic switching, expression of surface adhesions that facilitate attachment to host structures, and elaboration of hydrolytic enzymes. Expression of the genes involved in these traits varies between the commensal and disease state and contributes to the adaptability of the organism.[13]

The shift from the commensal state to disseminated disease occurs in part due to alterations in host factors, alterations in competing microflora secondary to antibiotic use, or compromise in the integrity of mucosal surfaces. Unique properties of the commensal strain colonizing an individual patient also may play a role in the likelihood of dissemination and clinical outcomes. The absence of large collections of clinical isolates with accompanying clinical data has made testing this hypothesis difficult. In this study, we took advantage of two collections of clinical isolates from premature infants at risk for disseminated candidiasis. The first collection was obtained from a study designed to determine the route of colonization with *Candida* in very low birth weight (VLBW) infants.[8] These isolates were not associated with invasive disease. The second collection was obtained from a multicenter, prospective study of invasive candidiasis in extremely low birth weight (ELBW) infants.[14] This collection represents a large set of prospectively collected invasive isolates of *Candida* with associated robust clinical data in premature infants. Virulence attributes of the invasive isolates were compared with isolates from colonized uninfected infants to ask two basic questions. First, to what extent do invasive clinical isolates of *Candida* vary expression of virulence attributes? Second, are clinical isolates that demonstrate enhanced virulence associated with worse clinical outcomes among the patients from whom they were isolated?

METHODS

Candida isolates used in this study were obtained from two separate clinical studies (Figure 1), both approved by institutional review boards and performed under informed consent. The first set, termed “commensal”, were obtained from infants with gestational age < 32 weeks and birth weight < 1500 g, from March 1996 through September 1997.[8] Cultures were obtained from oral, rectal, and inguinal samples from infants until death, hospital discharge, or until they were 12 weeks of age. Any *Candida* isolate obtained from a colonized infant was retained, and the stock cultures that were still viable were included in this study (Figure 1). None of the commensal isolates were from normally sterile sites. One infant subsequently developed candidemia and meningitis from the colonizing strain; this isolate was excluded from the commensal set. The second set, termed “invasive”, was obtained from a cohort of infants born at Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Neonatal Research Network (NRN) sites who were 1000 g birth weight, from March 2004 through July 2007.[14] Clinical data were collected on each subject at enrollment and prospectively throughout the hospitalization. The following definitions were used for morbidities: Bronchopulmonary dysplasia (BPD) was defined as a requirement for supplemental oxygen at 36 weeks postmenstrual age. Retinopathy of prematurity (ROP) was defined as the presence of ROP at any stage in either eye on any eye examination. Periventricular leukomalacia (PVL) was defined as the presence of any echolucencies on brain ultrasound examination in the white matter around the lateral ventricles. Proven necrotizing enterocolitis (NEC) was defined as Bell’s stage 2 or greater. Neurodevelopmental follow-up assessments were conducted at 18–22 months corrected gestational age by certified examiners who were unaware of infection status of the subjects. Neurodevelopmental impairment (NDI) was defined, in part, based on the results of developmental testing with the Bayley instrument. During the evaluation of this cohort, the method in use changed from the Bayley II to the Bayley III. Therefore, the definition of NDI differs slightly within the cohort, based on the Bayley instrument used. For those evaluated with the Bayley II, NDI was defined as having one or more of the following: moderate to severe cerebral palsy, mental developmental index (MDI) < 70, psychomotor developmental index (PDI) < 70, blindness (no useful vision) in both eyes, or hearing impairment requiring hearing aids in both years. For those evaluated with the Bayley III, NDI was defined as having one or more of the following: moderate to severe cerebral palsy, gross motor function (GMF) impairment of level 2 or more, cognitive score < 70, blindness with no useful vision or some functional vision only in both eyes, or permanent hearing loss despite cochlear amplification with cochlear implant or hearing aids. An important difference between these assessment tools has been suggested in that cognitive scores on the Bayley III tend to be higher than the MDI score on the Bayley II.[15]

When subjects were evaluated for possible infection throughout their hospital course, cultures positive for *Candida* species were identified, and isolates were retained. Invasive candidiasis was defined as a positive culture from a normally sterile body fluid such as blood, urine (in/out catheterization or suprapubic aspiration), or cerebrospinal fluid (CSF). In both the commensal and invasive collections, all isolates of *C. albicans* and *C. parapsilosis* were identified. The first invasive isolate of either species from blood, urine, and/or CSF in an individual patient was selected and tested in virulence assays. Of the 137 infants with invasive infection, isolates from 81 infants are included in this study (Fig. 1). The other isolates were from sites other than blood, urine or CSF, were species other than *C. albicans* or *C. parapsilosis*, or were not viable.

Virulence Assays

Candida isolates from both collections were tested individually in each of three assays to determine virulence properties: phenotypic switching, adhesion to human epithelial cells,

and damage to human epithelial cells (cytotoxicity). Because the properties of *C. albicans* and *C. parapsilosis* are quite distinct, the method for each virulence assay differed based on the species as described below. Means and standard deviations for each assay were calculated from the data for the commensal isolates. Invasive isolates were then defined as having “enhanced virulence” if an individual isolate measured more than one standard deviation above the mean of the commensal isolates in at least one of the three assays. This definition was established prior to data collection. Investigators conducting the virulence assays were blinded to all patient clinical data.

Phenotypic Switching

C. albicans has the capacity to switch between two cell types, white and opaque.[16] To determine switching frequency, a single colony of white cells from each of the individual strains was suspended in YEPD broth (1% yeast extract, 2% peptone, 2% dextrose) and grown overnight at 30°C with vigorous agitation. Yeast cells were diluted and plated on YEPD agar containing phloxine B (5 µg/ml) at a density of 50–200 yeast per plate. Plates were incubated at 30°C for 48 h and the percentage of colonies that had undergone switching from white to opaque was calculated.

Phenotypic switching in *C. parapsilosis* was determined by colony morphology. Four distinct morphologies have been described: crepe, concentric, smooth and crater.[17] To determine switching frequency, a single smooth colony from each of the individual strains was grown overnight in YEPD broth as above. Yeast cells were plated on YEPD agar containing phloxine B (5 mg/ml) at a density of 50–200 yeast per plate. Plates were incubated at 30°C for 48 h and the percentage of colonies that had undergone switching from smooth to any other morphology was calculated.

Adhesion Assays

Adhesion of individual strains of *C. albicans* or *C. parapsilosis* to human cells was assessed using the FaDu epithelial cell line derived from an adult hypopharyngeal tumor as described previously.[18] Briefly, yeast strains from overnight cultures were washed, counted and suspended at uniform density in RPMI tissue culture media for 60 min at 37°C. Yeast cells were then added to a confluent monolayer of FaDu cells and incubated for 30 min. After a set of standardized washes to remove non-adherent yeast, cells were stained with the yeast-specific fluorescent dye, calcofluor white, and fluorescence, proportional to the number of adherent yeast was measured. Assays for each isolate were conducted in quadruplicate at minimum.

Cytotoxicity Assays

The capacity of individual *Candida* strains to damage human epithelial (FaDu) cells was quantified using the CytoTox-Glo cytotoxicity assay (Promega, Madison, WI). This assay measures protease activity released from mammalian cells following loss of membrane integrity through cleavage of a luminogenic substrate. FaDu cells were maintained in Eagle's minimal essential medium (EMEM), seeded into 96-well plates at 1×10^4 cells per well and incubated for 24 hours at 37°C in 5% CO₂. *Candida* strains were grown overnight at 37°C as described above, washed and resuspended in EMEM. Yeast (1×10^6) were then seeded onto FaDu cells and incubated at 37°C in 5% CO₂. Because cytotoxicity occurred faster with *C. albicans* than with *C. parapsilosis*, *C. albicans* strains were coincubated with FaDu cells for 6 h, and *C. parapsilosis* strains were coincubated for 48 h. After the incubation period, CytoTox-Glo assay reagent was added, the plate was incubated for 15 min at 22°C, and luminescence was measured on a luminometer (“Luminescence before lysis” reading). Protease activity attributable to the presence of yeast was calculated by subtracting protease activity from FaDu cells incubated in the absence of yeast. To determine the total protease

activity per well, lysis reagent was added, and the incubation and luminescence reading was repeated ("Luminescence after lysis" reading). Percent cytotoxicity for each strain was then calculated according to the following formula: $[(\text{Luminescence before lysis}) - (\text{Luminescence from FaDu only})] / [(\text{Luminescence after lysis}) - (\text{Luminescence of media only})]$. Assays for each isolate were conducted in quadruplicate at minimum. Although *Candida* is known to produce proteases as well, yeast cells incubated under identical conditions in the absence of FaDu cells produced negligible luminescence in this assay (data not shown). Virtually all luminescence was therefore attributable to loss of FaDu membrane integrity.

Statistical analyses

Analyses of invasive and commensal isolates were performed at the isolate level. Analyses of clinical characteristics associated with enhanced virulence among subjects with invasive candidiasis were performed at the infant level. Culture specific measures were analyzed with reference to the first positive culture. Demographic characteristics and risk factors were contrasted between infants with and without isolates with enhanced virulence, using Fisher χ^2 exact tests for categorical measures and Kruskal-Wallis tests for continuous measures. The prevalence of comorbidities (BPD, ROP, PVL) and death (by discharge and by 18 months' adjusted age) were also compared between the two infant groups using Fisher χ^2 exact tests. Bayley cohort was defined as born prior to 2006 or evaluated with BSID II, vs. born in 2006 or later or evaluated with BSID III. The prevalence of NDI, and of NDI or death, was compared between virulence groups using logistic regression modeling, adjusting for Bayley cohort. Due to sparse data, modeling of NDI outcomes was not adjusted for clustering of children within research center. Results with a *P*-value less than 0.05 were considered statistically significant. Statistical analyses were performed using *SAS* software, version 9.2.

RESULTS

Virulence Attributes of Invasive Isolates

Values for invasive isolates were compared with the mean value of the commensal isolates ($n = 13$ and 14 commensal isolates for *C. albicans* and *C. parapsilosis*, respectively; Figure 1; available at www.jpeds.com) in the three virulence assays. Phenotypic switching by commensal isolates of *C. parapsilosis* was more frequent than for *C. albicans* (mean 27% vs. 2.6%). In contrast, *C. albicans* commensal isolates had mean adhesion values that were 6-fold higher than *C. parapsilosis* commensals. Commensal isolates of both species had low cytotoxicity (mean 3.5% for *C. albicans*; 12.1% for *C. parapsilosis*).

The source of invasive isolates is summarized in Fig. 1. The majority of invasive isolates for both species were recovered from blood cultures; 40/56 for *C. albicans* and 23/26 for *C. parapsilosis*. Urine cultures provided 10 additional isolates of *C. albicans* and 3 additional isolates of *C. parapsilosis*. Only *C. albicans* was recovered from cultures of CSF (6 additional isolates). All (6/6) of the *C. albicans* strains recovered from CSF demonstrated enhanced virulence as did 90% (9/10) of the urine isolates. 48% (19/40) of *C. albicans* blood isolates had enhanced virulence. Relative to blood isolates, CSF isolates and urine isolates had a significantly higher rate of enhanced virulence ($p=0.02$ and 0.03 , respectively). Of the 3 isolates of *C. parapsilosis* recovered from urine, 2 also showed enhanced virulence, and 39% (9/23) of *C. parapsilosis* blood isolates demonstrated enhanced virulence.

The rate at which isolates of each species from blood, urine, and CSF were in the enhanced virulence range is depicted in Figure 2. Phenotypic switching was a relatively infrequent event for *C. albicans*, similar to the commensal strains, and accounted for a low proportion

(2/34) of the strains with enhanced virulence. 4 of the 6 *C. albicans* CSF isolates had both high adhesion and high cytotoxicity. Phenotypic switching was somewhat more common among isolates of *C. parapsilosis*, but a small proportion (3/11) had enhanced virulence based on this attribute alone. However, of the 3 isolates from urine, 1 had enhanced adhesion and 1 had both enhanced adhesion and cytotoxicity.

Clinical Characteristics Associated with Enhanced Virulence

Demographic data of the 81 subjects with invasive candidiasis and analysis of demographic factors that may be associated with virulence are depicted in Table I. Subjects were extremely premature, with a median gestational age of 24 weeks and a median birth weight of 670 g. Sex was well matched with 52% male. Subjects with invasive candidiasis caused by isolates with enhanced virulence were similar to subjects with isolates lacking enhanced virulence in terms of gestational age, birth weight, sex, and race. There was no difference in the presence of a central catheter or the rate at which all cultures ultimately cleared. As expected, infants who developed invasive candidiasis in the absence of a central catheter represented a minority of the cohort. However, these infants may represent a group colonized by more virulent isolates. Among the 16 infants without a central catheter in place at the time of positive culture, 10 (63%) had isolates in the enhanced virulence group. Infants in the enhanced virulence group developed infection at a younger age (17 vs. 23 days, $p=0.03$). There was no difference in time to clearing infection between the groups. Although the number of subjects being cared for in a humidified environment did not differ between groups, in the subset of those in a humidified environment when the culture was obtained, a higher percent humidity was associated with isolates having enhanced virulence (62% vs. 41%, $p=0.02$). Other potential risk factors for invasive candidiasis—including exposure to antenatal antibiotics or steroids, route of delivery, postnatal corticosteroids, days of parenteral nutrition, early sustained postnatal antibiotic exposure, or gastrointestinal surgery—were not different between groups.

Laboratory test results from around the time of positive cultures were compared between infants with and without isolates with enhanced virulence (Table II). There was no difference in platelet count, serum glucose, or CSF findings. Patients in the enhanced virulence group had a higher serum creatinine (1.1 vs. 0.8 mg/dL, $p=0.01$). Comorbidities and outcomes among patients in each group also were compared (Table III). No differences in rates of BPD, ROP, PVL or proven NEC were detected. However, infants with isolates showing enhanced virulence had a trend toward higher mortality (44% vs. 25%, $p=0.1$) with approximately 80% of deaths being attributed to infection in both groups. Rates of NDI among survivors at 18 months corrected gestational age were not different between groups (43% vs. 27%, $p=0.33$). Because NDI and death are competing variables, a combined outcome of death or NDI at 18 months was evaluated. The enhanced virulence group exhibited a trend toward higher rates of death or NDI at 18 months corrected gestational age (71% vs. 53%, $p=0.1$).

DISCUSSION

Invasive infections with *Candida* occur when the defense mechanisms of the host are compromised, allowing a colonizing strain to access the blood and deeper structures. Virulence attributes of the colonizing organism are also likely contribute to the risk of disseminated infection. A number of studies have evaluated virulence factors associated with infection.[19–24] These studies demonstrate considerable variation among isolates of the same species in terms of adhesion, biofilm formation, hydrolytic enzyme production, and virulence in animal models. In this study we utilized the extensive clinical information available for the patients from whom the invasive isolates were isolated as well as well-established assays to determine virulence properties of organisms. We then separated the

invasive isolates into a group that had similar properties to the commensals and a group that had enhanced virulence properties. We hypothesized that the former group comprises organisms that disseminated primarily due to alterations in host defense, whereas the latter group has virulence characteristics that make them more likely to disseminate and therefore would lead to worse clinical outcomes. This study is unique in that *Candida* isolates frequently are not banked, and where collections exist, comprehensive clinical data generally are lacking.[19]

This study investigated three virulence traits in *C. albicans* and *C. parapsilosis*; phenotypic switching, adhesion, and cytotoxicity. The best studied phenotypic switch in *C. albicans* is the white-opaque transition.[16] A potential role in virulence has been suggested by the observation that the two cell types interact differently with host cells in terms of adhesion, secretion of chemoattractants, and susceptibility to phagocytosis, neutrophils and oxidants. [25–28] In *C. parapsilosis*, four distinct colony morphologies have been described.[17] Cells from these colonies differ in their capacity to invade an agar substrate and in their extent of biofilm formation. Phenotypic switching was observed more commonly among both commensal and invasive *C. parapsilosis* isolates than *C. albicans* isolates, but few *C. parapsilosis* isolates were classified as having enhanced virulence based on this attribute alone. Thus, in this collection phenotypic switching among invasive isolates was seldom more frequent than the commensal isolates and did not contribute substantively to enhanced virulence. However, this study cannot exclude the possibility that differences in rates of phenotypic switching in vivo may occur and offer a pathogenic advantage to the fungus.

The ability of the fungus to adhere to host mucosal surfaces is paramount to its ability to colonize the host, cause mucosal damage and translocate to deeper structures.[29, 30] Relative to invasive isolates recovered from blood, *C. albicans* isolates found in CSF showed a high rate of enhanced adhesion. Enhanced adhesion also was common among urine isolates of both species. This finding is consistent with a study comparing 19 blood isolates of *C. parapsilosis* with 14 isolates from other sources, including 6 from urine.[31] The urine isolates had nearly twice the adhesion rate of the blood isolates. These observations suggest that strains with increased capacity for adhesion may have a higher likelihood to invade the central nervous system (CNS) or establish infection of the urinary tract where sheer stress from movement of fluid is common.

C. albicans is well known to produce extracellular hydrolases that could have a damaging impact on host cells.[32] Extracellular hydrolases have also been described in *C. parapsilosis*. [4] Cytotoxicity generally was low among the commensal isolates. Most notably, CSF isolates of *C. albicans* showed a high rate of enhanced cytotoxicity, similar to the results of adhesion assays. Taken together, these findings suggest that successful infection of the CNS by *C. albicans* may be achieved most reliably by strains that are best adapted to adhesion and elaboration of hydrolytic enzymes that facilitate penetration of the blood-brain barrier.

Our study has several limitations. The colonization study differed from the invasive study in relevant ways. Commensal isolates were collected from VLBW infants in a single center, whereas the invasive isolates were from ELBW infants in multiple centers. The risk of invasive candidiasis between these groups differs based on birth weight and center differences. We were also somewhat limited in number of isolates. In total, 82 invasive *Candida* isolates from 81 patients were available for study. The contribution of fungal virulence factors to any clinical outcome is potentially difficult to detect given the complex and multifactorial nature of the outcomes measured. In this context, our sample size is small. Despite this limitation, statistically significant elevations in serum creatinine and earlier age at positive culture were identified as associated with virulence factors. Additionally, trends

were detected that were in the expected direction for the isolates with enhanced virulence, including a trend toward higher mortality of 44% vs. 25% ($p=0.1$). Isolates that are more virulent may be able to move from colonization to invasive disease more quickly, leading to a younger age at positive culture. Isolates that are inherently more adhesive and cytotoxic were more likely to be found in the CSF and urine. Involvement of the urogenital system may include kidney involvement, leading to higher creatinine values. Any combination of these factors also may have contributed to higher mortality.

The results from this study of a substantive collection of clinical isolates colonizing or infecting premature neonates lead to several conclusions. First, measures of potential virulence factors show substantial variability among clinical isolates of the same species. Second, the finding that isolates from sites other than blood tended to have more virulence than did commensal strains or isolates from the bloodstream implies that virulence as measured in vitro may have some relevance to extent of disease. Finally, expression of virulence traits was associated with relevant clinical outcomes in these patients.

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Abbreviations

VLBW	very low birth weight
ELBW	extremely low birth weight
CSF	cerebrospinal fluid
BPD	bronchopulmonary dysplasia
ROP	retinopathy of prematurity
PVL	periventricular leukomalacia
NEC	necrotizing enterocolitis
NDI	neurodevelopmental impairment
CNS	central nervous system

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Figure 1. Source of commensal and invasive isolates

Clinical isolates of *C. albicans* (*C. alb*) and *C. parapsilosis* (*C. para*) were obtained from two independent studies. In all cases the first isolate was included for any individual patient. Commensal isolates were from infants colonized orally, rectally, or on the skin, but without invasive infection.[8] One infant was colonized with both species. Thirteen *C. albicans* and fourteen *C. parapsilosis* isolates were available at the time of the current study. Invasive isolates came from blood, CSF, or urine.[14] One infant had a positive blood culture for both *C. albicans* and *C. parapsilosis*. Both isolates are reported, for a total of 82 positive cultures.

Figure 2. Rates of enhanced virulence by assay and species

Each isolate of *C. albicans* (*C.a.*) and *C. parapsilosis* (*C.p.*) obtained from cultures of blood, urine, or CSF was evaluated in each of three assays. The rate at which isolates from each source measured in the enhanced virulence range relative to the commensal strains in each assay is depicted.

Table 1

Univariate analysis for demographic factors associated with virulence

Demographic	Total Infants	Enhanced Virulence Attributes		p-value ^a
	N=81	0 (N=36)	1 (N=45)	
Gestational Age (weeks) ^b	24 (24, 25)	24 (24, 25)	24 (24, 25)	0.32
Birth weight (grams) ^b	670 (610, 820)	671 (610, 778)	670 (620, 839)	0.79
Male – n (%)	42 (51.8)	18 (50.0)	24 (53.3)	0.83
Race – n (%)				
Black	38 (46.9)	18 (50.0)	20 (44.4)	0.58
White	41 (50.6)	18 (50.0)	23 (51.1)	
Other	2 (2.5)	0 (0.0)	2 (4.4)	
Age at positive culture (days) ^b	20 (12, 35)	23 (16, 41)	17 (10, 31)	0.03
CVC present – n (%) ^c	63 (79.8)	29 (82.9)	34 (77.3)	0.59
All cultures cleared	53 (65.4)	25 (69.4)	28 (62.2)	0.64
Days to clearance ^d	(N=40) 5.5 (3.0, 10.0)	(N=24) 5.5 (3.0, 9.0)	(N=16) 5.5 (3.0, 7.0)	0.88
Days to clearance after CVC removal ^{d,e}	(N=30) 4.0 (2.0, 7.0)	(N=20) 4.0 (2.0, 7.0)	(N=10) 3.0 (2.0, 7.0)	0.77
Environment humidified on day of culture – n (%) ^f	19 (27.5)	7 (21.9)	12 (32.4)	0.42
Percent humidity ^g	(N=17) 50 (41, 69)	(N=7) 41 (40, 50)	(N=10) 62 (50, 78)	0.02
Antenatal Antibiotics – n (%) ^g	50 (62.5)	20 (57.1)	30 (66.7)	0.49
Antenatal Corticosteroids – n (%) ^h	63 (78.8)	28 (80.0)	35 (77.8)	1.00
Cesarean delivery (%)	43 (53.1)	20 (55.6)	23 (51.1)	0.82
Systemic corticosteroids 24 hours prior to culture – n (%) ⁱ	6 (8.2)	3 (8.8)	3 (7.7)	1.00
Days of parental nutrition ^b	44 (30, 61)	45 (33, 60)	44 (27, 63)	0.93
Sustained early antibiotics ^j	28 (43.1)	15 (46.9)	13 (39.4)	0.62
GI surgery resulting in short gut	3 (3.7)	2 (5.6)	1 (2.2)	0.58

^aP-value for difference between enhanced virulence groups was determined by the Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables

^bMedian (Q1, Q3) displayed for continuous variables

^cCVC (central venous catheter) present at initial culture positive. There are 2 infants missing data for CVC; 1 in the 0 attributes group and 1 in the 1 attributes group.

^dAmong infants with initial positive infection in blood.

^eSame as overall days to clearance for infants who did not have a CVC in place at initial culture positive.

^fHumidified versus not humidified regardless of open/closed environment. There are 12 infants missing data; 4 in the 0 attributes group and 8 in the 1 attributes group.

^gData collected from cultures drawn in a closed, humidified environment; 1 infant in the 1 attributes group was missing data.

^hOne infant in the 0 attributes group was missing data.

ⁱThere are 8 infants missing data for postnatal steroids, 2 in the 0 attributes group and 6 in the 1 attributes group.

^jBroad-spectrum antimicrobial treatment sustained for 5+ days from birth. There are 16 infants missing data, 4 in the 0 attributes group and 12 in the 1 attributes group.

Table 2

Univariate analysis of disease factors associated with virulence

Disease Factor	Number of Enhanced Virulence Attributes				p-value ^a
	N	0 Median (Q1, Q3)	1 Median (Q1, Q3)	N	
Laboratory test results at time of culture					
Lowest platelet count	32	117 (53, 241)	36	140 (59, 235)	0.59
Lowest glucose level	29	95 (88, 123)	38	97 (79,136)	0.68
Highest glucose level	29	129 (89, 162)	38	142 (98, 176)	0.59
Serum creatinine level	32	0.8 (0.5, 1.1)	35	1.1 (0.8, 1.7)	0.01
CSF protein level	8	184 (150, 227)	13	194 (139, 345)	0.97
CSF glucose level	8	54 (36, 89)	1	61 (45, 80)	0.86

CSF: Cerebrospinal fluid

^aP-value for difference between enhanced virulence groups was determined by the Kruskal-Wallis test

Table 3

Analysis of comorbidities and outcomes associated with virulence

Morbidity	Number of Enhanced Virulence Attributes				p-value ^a
	0	1	N	n (%)	
BPD	28	17 (60.7)	31	24 (77.4)	0.26
ROP	30	28 (93.3)	32	26 (81.3)	0.26
PVL	36	3 (8.3)	45	1 (2.2)	0.32
NEC	36	7 (19.4)	45	9 (20.0)	1.00
Death	36	9 (25.0)	45	20 (44.4)	0.10
NDI at 18-mo	22	6 (27.3)	21	9 (42.9)	0.33 ^b
Death by 18-mo	34	12 (35.3)	42	20 (47.6)	0.35
NDI or Death by 18-mo	34	18 (52.9)	41	29 (70.7)	0.10 ^b

BPD: bronchopulmonary dysplasia; ROP: retinopathy of prematurity; PVL: periventricular leukomalacia; NEC: proven necrotizing enterocolitis; NDI: Neurodevelopmental impairment (see text for definition)

^aP-value for difference between enhanced virulence groups was determined by the Fisher's exact test.

^bP-value for difference between enhanced virulence groups was from logistic regression modeling, adjusting for Bayley cohort (see text for definition). Due to sparse data, models were not adjusted for clustering of children within research center.