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Risk factors associated with gut and nasopharyngeal colonization by common Gram-negative species and yeasts in neonatal intensive care units patients

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ABSTRACT

Aim: To characterize dynamics of mucosal colonization of neonates by common aerobic Gram negative species and *Candida* spp. and to identify independent perinatal, neonatal, and environmental factors influencing the colonization process.

Study design: The nasopharyngeal (n = 1145) and rectal (n = 1242) swabs were collected on admission and thereafter twice a week in neonates with risk factors of early onset sepsis (n = 276) admitted within the first 72 h of life. The association between colonization by different microbes and a total of 22 predefined risk factors was assessed using univariate and multiple logistic regression analyses.

Results: Throughout the study about half of the patients had rectal (55.8%) or nasopharyngeal colonization (42.8%) with common Gram-negative microorganisms. Colonization dynamics and risk factors were in general similar for a given bacterial species in both mucosal sites; nonfermentative microbes more often found in nasopharyngeal swabs and *Enterobacteriaceae* in rectal swabs. All organisms except *Escherichia coli* were influenced by the duration of intensive care unit stay but other risk factors were species specific, perhaps reflecting their mode of acquisition. While colonization by *E. coli* and *Candida albicans* was associated with perinatal factors like term birth, vaginal delivery, and breast milk feeding; colonization by *Klebsiella pneumoniae*, *Enteribacter cloacae*, *Acinetobacter spp.* and non-albicans *Candida* spp. were mostly determined by hospital environment (treatment unit and period, artificial interventions and their duration) and gestation age ≤28 weeks.

Conclusions: The knowledge of risk factor profiles may permit the development of strategies to prevent heavy colonization and subsequent invasive disease in high risk infants.

1. Introduction

Colonization of mucosal surfaces with Gram negative microorganisms starts right after birth. We and others have previously shown that by the end of first week of life 52–83% neonates in the neonatal intensive care units (NICU) are colonized with Gram negative microorganisms half of which are ampicillin-resistant (AR) [1,2]. On one hand mucosal colonization by Gram negative microorganisms is a normal process but on the other hand it could also serve as a source for invasive infection [1–7]. Therefore a better understanding of the factors associated with the colonization process should contribute to improved infection control-strategies and eventually improve outcomes for critically ill neonates [7,8].

Numerous studies have looked at factors associated with early mucosal colonization; various factors like maternal microbiota [9,10], intrapartum use of antibiotics [6], premature rupture of membranes (PROM) [6], route of delivery [5,6,9–11], gestational age (GA) [5,9,10,12], surrounding environment [9,10], feeding habits [9–11], and antibiotic use [5,9–12] have been identified. However, most of the studies have either included only a limited number of infants [13,14], have focused on gut colonization alone [6,11,13–15], included healthy infants [5,9,10,16] or looked at only a few microbial species or risk factors at a time [8,15–20] or concentrated on Gram-negative organisms rather than individual species [11], despite that the majority of factors are highly interrelated. The number of studies in critically ill neonates admitted to NICU and looking at multiple factors and species simultaneously is very limited.

We aimed to characterize the dynamics of mucosal colonization by common aerobic Gram negative microorganisms and *Candida* spp. in neonates admitted to NICU and to identify independent perinatal, neonatal, and environmental factors influencing the colonization process.

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2. Methods

2.1. Study design and data collection

This analysis was incorporated into a prospective, cluster-randomized, two-center study comparing the efficacy and safety of penicillin G to ampicillin (both combined with gentamicin) in neonates at risk of early onset sepsis (EOS) admitted into two Estonian third level NICUs from August 2, 2006 until November 30, 2007. Both units admit mostly patients requiring invasive ventilatory support and have facilities for high frequency ventilation and neonatal surgery. Neonates were included in the study if they were (1) younger than 72 h; (2) needed early empiric antibiotic treatment on clinical suspicion and/or due to risk factors of infection (i.e. maternal fever, chorionamnionitis, PROM for more than 18 h, and preterm labor of <35 weeks of gestation) and (3) were expected to stay in unit for >24 h [1,2]. In the first part of the study (period 1; from 2 August 2006 to 20 March 2007) in unit A all patients received ampicillin and in unit B penicillin G. After enrolling half of the patients required to prove clinical equivalence of the two antibiotic regimens the penicillins were switched (period 2; from March 21, 2007 to November 30, 2007) so that in unit A penicillin G was used and in unit B ampicillin was used. If no clinical or laboratory signs of invasive infection appeared and initial blood cultures remained negative, antibiotic therapy was stopped. In case of clinical or culture proven infection initial antibiotic regimen could be continued if susceptible pathogens were involved or changed to a prespecified regimen depending on the antibacterial susceptibility of the isolate. For the empiric therapy of LOS cefuroxime, cefotaxime, ampicillin/sulbactam, or piperacillin/tazobactam alone or in combination with gentamicin was recommended. In neonates with birth weight (BW) below 800 g and vascular catheter(s) in place vancomycin was added until infection caused by coagulase negative staphylococci was excluded by negative cultures. [1,2].

For each patient the following data were recorded: (1) GA categorized to ≤28 weeks — extremely preterm, 29–36 weeks — late preterm, and ≥37 weeks — term infants, BW, mode of delivery, multiple birth, maternal age, presence of chorionamnionitis, PROM for more than 18 h before delivery, administration of antenatal steroids and antibiotics, and intrapartum antibiotic use; (2) type and duration of empiric antibiotic regimen, feeding regimen and character of enteral feeds categorized as total parenteral nutrition (TPN), formula feeding or breastfeeding (breast milk was frozen but neither pasteurized nor fortified except in ELBW neonates after reaching enteral volume of 100 ml/kg), use of broad spectrum antibiotics prior to colonization categorized to carbapenems, third and fourth generation cephalosporins, and beta-lactamase resistant penicillins, duration of NICU stay, artificial lung ventilation (ALV), and central-venous and arterial catheters; and (3) study period and participating unit.

2.2. Mucosal sampling

Rectal and NP or tracheal samples (in ALV patients) were collected with transport swabs (Nuova Aptaca, Canelli, Italy) on admission and then twice a week until discharge from NICU or until Day 60 whichever occurred first. The swabs were immediately transferred to −20 °C; stored there for a maximum of two weeks and processed in batches. In the pilot study conducted to compare the recovery rates of frozen swabs with freshly cultured samples the sensitivity and specificity of frozen samples were 100% (95% CI 79; 100) and 81.2% (95% CI 54; 95), respectively and the recovery rate was 86.4%. Normally sterile body fluids (e.g. blood and cerebrospinal fluid) were cultured on admission and then if clinical condition deteriorated and symptoms suggestive of neonatal sepsis appeared.

2.3. Laboratory methods

After thawing the swabs were directly plated onto blood agar, MacConkey agar and MacConkey agar with 16 μg/mL of ampicillin. Rectal swabs were also plated onto Saboraud agar. The blood and MacConkey agar plates were incubated at 37 °C for 24 to 48 h in ambient air and Saboraud agar plates at 25 °C for at least one week. Each morphologically different colony type was Gram stained and identified on species and genus level according to the Clinical and Laboratory Standards Institute criteria [21]. Commercial kits were employed for the final identification of enterobacteria and yeasts (API 20E and API 20C AUX; Biomérieux, and Marcy l'Etoile, France, respectively) and together with selective media CHROMagar™ Candida (BD BBL, Heidelberg, Germany) also for yeasts. Any Gram-negative microorganism that grew on MacConkey agar with 16 μg/ml of ampicillin was termed ampicillin resistant (AR). The following Gram-negative species were considered common and included into analysis: *Klebsiella pneumoniae*, *Klebsiella oxytoca, Enterobacter cloaceae, Escherichia coli, Serratia* spp., *Acinetobacter* spp., *Stenotrophomonas* spp., and *P. aeruginosa.* Sterile body fluid samples were processed immediately by monitoring with the BACTEC 9240 or VITEK 2 system in the microbiology laboratories of the University Clinics of Tartu or North Estonian Medical Centre.

2.4. Statistical analysis and calculation of risk factors

The software programs Sigma Stat for Windows 2.0 (Jandel Corporation, USA); and R 2.6.2 (A Language and Environment, http://www.r-project.org) were used. In order to identify independent risk factors of colonization separate analysis was performed for each species as well as for the respective AR strains. All variables significant in univariate logistic regression analysis at a p-value of ≤0.1 were entered into multiple logistic regression model with backward stepwise removal in order of insignificance. To avoid confounding by simultaneous inclusion of highly interrelated variables only one was included. There was a high correlation between BW and GA ($r = 0.933$) and between the duration of NICU stay and postnatal age (PNA) ($r = 0.996$). GA was considered a better determinant of immaturity than BW [22,23] and the duration of NICU stay more important than PNA in influencing mucosal colonization. Variables like use of empirical antibacterial therapy, ALV, central venous and arterial catheters were studied by including binomial and duration characteristics. Wherever, feasible continuous variables involving the impact of duration were given a priority. In multivariate analysis the p value of <0.05 was considered statistically significant.

The study was approved by the Ethics Committee of the University of Tartu.

3. Results

3.1. Patients and study samples

A total of 283 neonates were included into the parent study; colonization data were available for 276 (97.5%). The study population with half of the neonates with BW <1500 g, half born via cesarean section, three quarters mechanically ventilated and/or with indwelling catheters, and a quarter with culture proven neonatal sepsis is characteristic for a third level NICU (Table 1).

A total of 1242 rectal and 1145 NP swabs with a per patients median of 3 (rectal and NP interquartile ranges [IQR] 2–6 and 2–5, respectively) for each site were collected. In addition, 60 culture positive tracheal samples were pooled with NP swabs. During the study about half of the patients developed rectal (55.8%) or NP (42.8%) colonization with Gram-negative microorganisms; 38.8% had colonization of both sites. Of all positive rectal and NP samples 73.4% and 60.2%, respectively were termed AR.

Table 1
Demographic and clinical characteristics of the study population.

Neonatal factors	
Duration of NICU stay days; median (IQR)	6.7 (3.8–17)
GA (week); mean/ SD (min/ max)	31.3/5.1 (22/42)
GA <28 weeks; n (%)	92 (33.3)
GA ≥37 weeks; n (%)	55 (19.9)
BW (g) median (IQR)	1495.0 (950.0–2436.5)
≤1000 g; n (%)	75 (27.2)
≤1500 g; n (%)	142 (51.5)
≥2500 g; n (%)	67 (24.3)
Male/female; n	157/ 119
Feeding regimen; n (%)	
Total parenteral nutrition; n (%)	58 (21.0)
Breast milk containing regimen; n (%)	71 (25.7)
Formula; n (%)	146 (52.9)
Additional AB, n (%)	
Beta-lactam + betalactamase inhibitors	48 (17.4)
III and IV generation cephalosporins	19 (6.9)
Carbapenems	31 (11.2)
Sepsis n (%)	67 (24.3)
Early onset sepsis	14 (5.1)
Late onset sepsis	53 (19.2)
Artificial lung ventilation; n (%)	210 (76.1)
Central venous catheters; n (%)	210 (76.1)
Arterial catheters; n (%)	219 (79.4)
Maternal factors	
Multiple birth; n (%)	51 (18.5)
Cesarean section; n (%)	157 (56.9)
Antenatal steroids; n (%)	115 (56.2)
Antenatal antibiotics; n (%)	61 (22.1)
Intrapartum antibiotics; n (%)	95 (34.4)
Maternal chorionaminionitis; n (%)	55 (19.9)
Prolonged rupture of membranes >18 h; n (%)	51 (18.4)
Mother's age; median (min/max)	28 (16/44)

NICU — neonatal intensive care unit, BW — birth weight, GA —gestational age, AB — antibiotic; and IQR —interquartile range.

Altogether the most common mucosal colonizer including AR strains was *E. cloacae* followed by *K. pneumonia*, *K. oxytoca* and *Acinetobacter* spp. All *Enterobacteriaceae* except *Serratia* spp. were more frequently colonizing rectum than NP but *Acinetobacter* spp. were more commonly recovered in the NP (Fig. 1). On admission the vast majority of patients had rectal (94.2%) or NP (98.2%) cultures negative for GN microbes. Thereafter the proportion of patients colonized by *E. cloacae* and *Klebsiella* spp. increased steadily until leveling off on 25% (NP) to 30% (rectal) by weeks 4 and 5. Colonization by *Serratia* spp. showed the same trend, but colonization by *E. coli* was

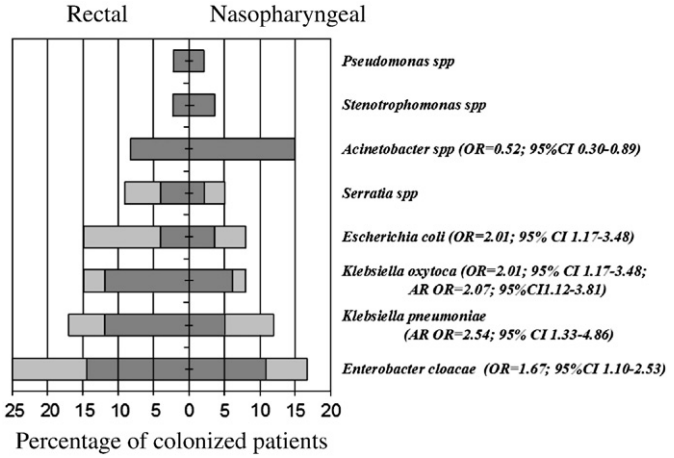

Fig. 1. The percentage of patients having rectal (on the left) or nasopharyngeal (on the right) colonization with Gram-negative microorganisms at least once during the study. Light gray bars indicate ampicillin-susceptible *Entrobacteriaceae* and dark gray bars AR strains. OR together with the 95% CI represent significant differences between rectal and nasopharyngeal colonization rates. AR — ampicillin-resistant strains.

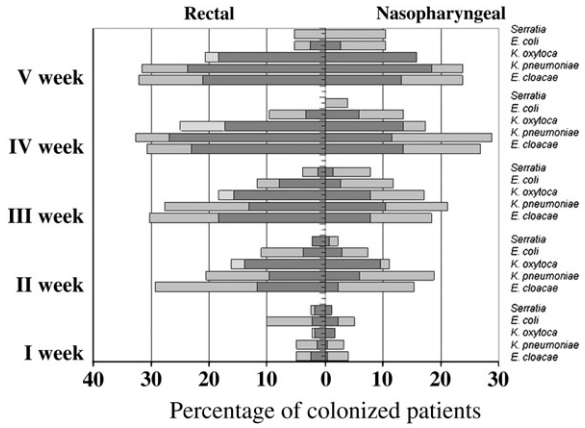

Fig. 2. The percentage of patients colonised by different species of *Enterobacteriaceae* during the first five weeks of NICU stay. The rectal colonization is shown on the left and nasopharyngeal on the right. Colonization by AR strains is presented by darker-gray and ampicillin susceptible strains by light-gray.

not influenced by the duration of NICU stay; at the 1st to 3rd week about 10% of patients became colonized (Fig. 2). The dynamics of AR resistant strains followed similar trends (data not shown).

Throughout the study rectal colonization by *Candida* spp. was observed in 49 (17.8%) patients led by *Candida albicans* (n = 37) and followed by *Candida parapsilosis* (n = 10), *Candida lusitaniae* (n = 5) and *Candida tropicalis* (n = 1). Four patients had *C. albicans* and *C. parapsilosis* concomitantly. Colonization by *C. albicans* started at birth and increased steadily thereafter whereas a sharp rise in non-albicans *Candida* spp. was seen at the fifth week of NICU stay. Extremely preterm babies (22% and 20% for *C. albicans* and non-albicans spp., respectively) were more commonly colonized than late preterm (7% vs 3%) and term babies (15% vs 0) (Fig. 3).

3.2. Risk factors associated with the mucosal colonization

In a univariate regression analysis a total of 22 factors (3 environmental, 5 maternal and 14 neonatal) were associated with colonization by various Gram negative microorganisms at a p value of ≤0.1 (Appendix A). Further only risk factors remaining associated with Gram-negative colonization in multiple regression analysis will be presented.

3.3. Environmental factors — participating unit, treatment period and duration of NICU stay

The duration of NICU stay (median 6.7 days; IQR 3.8–17) was associated with rectal and NP colonization with *Acinetobacter spp.* and all *Enterobacteriaceae* except *E. coli,* and with rectal colonization by

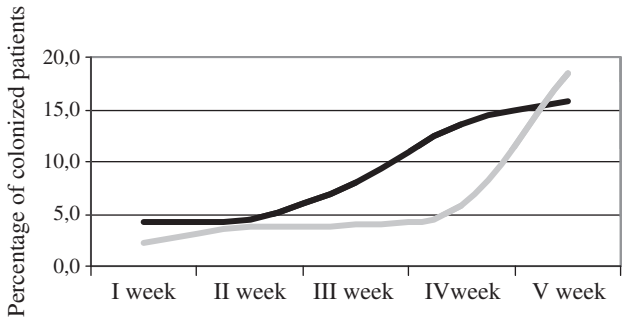

Fig. 3. Percentage of patients with rectal colonization by *Candida* spp. during the first five weeks of NICU stay. Colonization rate by *C. albicans* is presented in black, non-albicans *Candida spp.* in gray lines.

Table 2
Factors influencing rectal and nasopharyngeal colonization by various Gram-negative opportunistic microbes — results of multiple logistic regression.

Influencing factor	*K. pneumoniae*		*K. oxytoca*		*E. cloaceae*		*E. coli*		*Serratia spp*		*Acinetobacter*	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Rectal												
NICU stay days	1.04	1.02–1.07	1.05	1.03–1.07	1.06	1.04–1.08			1.03	1.01–1.05	1.06	1.03–1.08
Unit B vs unit A	0.19	0.09–0.44							6.21	2.16–17.91		
Second period vs first	0.39	0.18–0.83										
Vaginal delivery vs cesarean							4.44	1.97–10.00				
PROM >18 h	2.30	1.01–5.26										
GA ≥37 vs 29–36 weeks							3.13	1.18–8.27				
Breast milk vs parenteral			5.00	1.57–15.92			8.90	2.27–34.70				
Duration of prim AB — Amp days					0.85	0.74–0.98						
Duration of Ci/vC days	1.06	1.01–1.12									1.07	1.00–1.15
Ci/aC	8.06	1.04–62.53										
Duration of i/aC days											0.84	0.73–0.97
Nasopharyngeal												
NICU stay days			1.07	1.04–1.09	1.07	1.04–1.10			1.05	1.02–1.08	1.06	1.04–1.08
Unit B vs unit A	0.20	0.07–0.57			0.42	0.19–0.91			6.12	1.54–24.27	0.38	0.17–0.85
Vaginal delivery vs cesarean							2.69	1.01–7.18			0.40	0.17–0.90
PROM >18 h			4.33	1.54–12.21			4.43	1.66–11.78				
GA ≤28 vs 29–36 weeks	6.64	2.44–18.05										
GA ≤28 vs ≥37 weeks	9.00	1.93–42.08			8.48	1.04–68.89						
Amp vs Pen	6.75	2.51–18.18									0.33	0.15–0.75
Duration of prim AB-Amp days			0.65	0.46–0.91								
Duration of ALV days	0.94	0.90–0.99										

NICU — neonatal intensive care unit; GA — gestation age; PROM — prolonged rupture of membranes; ALV — artificial lung ventilation, Ci/vC — central intravascular catheter; i/aC — intra-arterial catheter; AB — antibiotic; Amp —ampicillin; Pen — penicillin; and prim — primary.

C. albicans and non-albicans *Candida* spp. (OR = 1.05; 95% CI 1.03–1.07 and OR = 1.07; 95% CI 1.04; 1.10, respectively). Depending on the species each day in NICU increased colonization risk by 5% to 8% (Table 2; Fig. 3). Rectal colonization by *K. pneumoniae* and *Serratia* spp. and NP colonization by *K. pneumoniae*, *E. cloacae*, *Serratia* spp., and *Acinetobacter* spp. were also dependent on the participating unit (Table 2) suggesting potential cross-colonization and translocation. In fact there was an outbreak of invasive *K. pneumoniae* infection during the first study period in unit A in which 59 patients were colonized and five developed invasive disease with clonally related strains [24].

3.4. Perinatal factors — gestational age, mode of delivery, and PROM

About 80% of all subjects were preterm and a third were extremely preterm (Table 1). Term neonates were more likely colonized by *E. coli* compared with late preterm but not with the extremely preterm babies. The latter, however, had higher risk of developing NP colonization by *K. pneumoniae* as compared with both other age categories and by *E. cloacae* compared to term newborns (Table 2).

About half of the patients were born by cesarean section and 18% of mothers had PROM (Table 1). Vaginal delivery was associated with fourfold higher risk of rectal and threefold of NP colonization by *E. coli* and twice as high risk of *C. albicans* (OR 2.27, 95% CI 1.06; 4.86; Fig. 3) as compared with cesarean section. At the same time the risk of NP colonization by *Acinetobacter* spp. was reduced to 0.4 (Table 2). PROM was associated with increased risk of rectal colonization by *K. pneumonia* and NP colonization by *K. oxytoca, E. coli* and *Stenotrophomonas* spp (OR = 19.00; 95% CI 1.39; 260.21) (Table 2).

3.5. Neonatal factors — feeding, antibiotics, and indwelling catheters

During the first week of life 53% of patients were formula fed, 26% received breast milk containing regimen and 21% were on TPN (Table 1). Feeding regimen affected rectal but not NP colonization. Neonates on TPN were less likely colonized with *K. oxytoca* and *E. coli,*

Table 3
Factors influencing mucosal colonization by common Amp-resistant *Enterobacteriaceae* and *Acinetobacter* spp — results of multiple logistic regression analyses.

Influencing factor	*K. pneumoniae*		*K. oxytoca*		*E. cloaceae*		*E. coli*		*Serratia spp*		*Acinetobacter*	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Rectal — AR												
NICU stay days	1.03	1.01–1.06	1.06	1.04–1.08	1.05	1.03–1.07					1.08	1.04–1.12
Unit B vs unit A	0.28	0.10–0.74			0.46	0.21–0.98			9.83	1.23–78.65		
GA ≥37 vs 29–36 weeks			3.13	1.02–9.57								
GA ≤28 vs 29–36 weeks	3.34	1.14–9.74										
AB during labor	2.26	1.13–6.07										
Breast milk vs parenteral			5.81	1.65–20.44								
Duration of prim AB days											1.43	1.06–1.93
Nasopharyngeal — AR												
NICU stay days	1.06	1.03–1.10	1.07	1.05–1.10	1.06	1.04–1.08					1.05	1.02–1.08
Unit B vs unit A	0.18	0.03–0.95			0.15	0.05–0.46						
PROM >18 h	5.41	1.44–20.25	4.83	1.44–16.18	1.40	0.56–3.46	5.94	1.58–22.42				
Amp vs Pen					2.50	1.02–6.11						
Duration of prim AB - Pen days											1.32	1.10–1.59
Duration of prim AB - Amp days			0.62	0.42–0.92								

GA — gestational age; PROM — prolonged rupture of membranes; AB — antibiotic; Amp — ampicillin; Pen — penicillin; prim — primary; AR — ampicillin-resistant; and NICU — neonatal intensive care unit.

but more likely with *C. albicans* (OR = 2.91; 95% CI 1.02–8.26) than those receiving breast milk. No difference between breast milk and exclusively formula fed neonates was seen (Table 2).

In addition to empiric ampicillin or penicillin given to all patients with a median duration of 72 h (IQR 53.6–136 h) 96 neonates (35%) received additional broad spectrum antibiotics (Table 1). Compared with penicillin empiric ampicillin therapy was associated with increased risk of NP colonization by *K. pneumoniae* but decreased risk of *Acinetobacter* spp (Table 2). Longer duration of empiric ampicillin therapy decreased risk of rectal colonization by *E. cloaceae*, and NP colonization by *K. oxytoca* both susceptible and AR strains. On the other hand longer duration of penicillin therapy favored NP colonization by AR *Acinetobacter* strains. However, despite additionally administrated broad spectrum antibiotics being associated with higher *Candida* spp. colonization rate in univariate analysis none of them remained independently associated with any organism in multiple regression analysis.

More than 75% of subjects were mechanically ventilated and/or had indwelling catheters (Table 1). The presence of arterial lines was associated with increased risk of rectal and NP colonization by *K. pneumoniae* and each day of a central venous catheter (CVC) increased the risk of rectal colonization with *Acinetobacter* spp. by 7% and non-albicans *Candida* spp. by 6% (OR 1.06, 95% CI 1.00; 1.13). The duration of ALV was associated with increased risk of NP colonization by *K. pneumoniae* (Table 2).

3.6. Factors associated with colonization by AR strains

Risk factors of colonization with AR microorganisms were largely similar to those seen in all strains (Table 3). While rectal colonization by AR *K. oxytoca* more likely occurred in term babies AR *K. pneumoniae* was three times more often isolated in extremely immature as compared with late preterm babies. Compared to TPN breast milk feeding increased the risk of colonization by AR *K. oxytoca.* Empiric ampicillin therapy was associated with increased risk of NP colonization by AR *E. cloacae*. Mode of delivery did not influence colonization by AR strains, but PROM >18 h increased risk of NP colonization by AR *K. pneumonia, K. oxytoca,* and *E. coli.* Intrapartum antibiotic use favored rectal colonization by AR *K. pneumoniae.*

3.7. Invasive infections

A total of 27 cases of bloodstream infections (BSI) caused by GN microorganisms and four by *Candida* spp. occurred (Fig. 4). In 22 cases of the 27 mucosal and invasive strains were phenotypically identical and in 16 of 22 colonization with matching organisms preceded invasive disease.

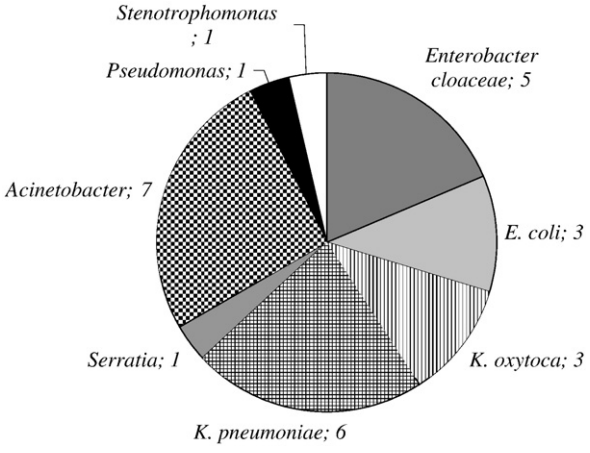

Fig. 4. The distribution of Gram negative microorganisms causing bloodstream infections in neonates.

4. Discussion

In this prospectively conducted study in NICU we show that factors involved in mucosal colonization are species specific likely reflecting the origin of the particular organism. While colonization by *E. coli, C. albicans* and to a lesser extent *K. oxytoca* is mostly of maternal origin being associated with early perinatal factors like PROM, vaginal delivery and/or breast milk feeding, colonization by *K. pneumoniae, E. cloacae* including AR strains and by non-fermentative organisms *Acinetobacter* spp. and non-albicans *Candida* spp. predominantly rises from hospital environment and is influenced not only by the participating unit and study period, duration of NICU stay and invasive interventions but also by immaturity. With some exceptions such as nonfermentative organisms more commonly found in the NP mucosa [25], *Enterobacteriaceae* more commonly found in rectal cultures and feeding regimen more commonly affecting colonization of the gut but not the NP, we found the colonization pattern and interfering risk factors to be similar for a given bacterial species in the two studied mucosal sites. This suggests that when performing colonization studies extrapolations from one site to the other are feasible.

The factors associated with hospitalization such as duration of stay, participating unit and invasive interventions have by far the greatest influence on colonization by Gram-negative organisms and yeasts with the exception of *E. coli, K. oxytoca* and *C. albicans* likely originating from the mother [3,10]. Still, this is not surprising since opportunistic microorganisms *K. pneumoniae, E. cloaceae* and *Acinetobacter* spp. are known to circulate in hospital environment, are transmitted via contaminated equipment or hands of medical personnel and as a result have been associated with invasive disease in NICU [3,19,26–31]. As an example of this we observed cross-colonization of *K. pneumoniae* in one of the participating units involving 59 patients, lasting for more than 6 months and resulting in BSI in five patients. These findings definitely support the previous recommendations that hygiene oriented interventions may enable to avoid or reduce colonization by potentially pathogenic microorganisms and could eventually result in diminished numbers of invasive disease [19,25,28,29,31].

Indeed PNA almost completely overlapping with NICU stay in this study could also have influenced mucosal colonization [31]. By comparing NP carriage rates in healthy and age-matched hospitalized infants over the first 6 months of life Baltimore et al. [32] suggested PNA's primary role in colonization by Gram-negative microorganisms. The results of this study suggest the same although we were unable to confirm whether the PNA or the duration of NICU stay plays the primary role as only the latter variable was entered into multiple regression model.

Not surprisingly the route of feeding as well as the character of enteral feeds is critical for early gut colonization [8,10,29,33]. As in the previous studies enterally fed babies (breast milk or formula) were more likely to colonize with *E. coli, K. oxytoca* and *C. albicans* than those on TPN suggesting the likely maternal origin of these microorganisms. At the same time feeding regimen did not influence colonization by other *Enterobacteriacea*, nonfermentative organisms or non-albicans *Candida* spp. supporting once again the idea that the spread of these organisms occurs via cross-colonization including contamination of nasogastric tubes as demonstrated recently by Hurrell et al. [34]. This further underlines the potential of hospital hygiene oriented interventions in avoiding or reducing colonization by these potentially pathogenic microorganisms.

GA was a host-related determinant of early gut colonization [9,15,28,29]. As shown previously term infants were more likely

colonized with *E. coli* [10], whereas extremely preterm babies had greater risk of colonization by *K. pneumonia* and *E. cloacae* [35]. Previously suggested reasons in preterms include reduced maternal contact, altered intestinal environment rising from different dietary intake, peristalsis and glandular secretions as well as the immune response of intestinal mucosa [12,23,36], host cell gene expression [12] resulting in lower total counts of bacteria [14,37] and limited numbers of species [9,14]. Potential cross-colonization due to longer NICU stay and increased susceptibility or resistance to certain microorganisms due to underdeveloped mucosal barrier cannot be excluded.

Similar to other studies [26,27] we showed in univariate analysis an inverse correlation between GA and number of babies colonized with *Candida* spp. especially by non-albicans *species*. However, this association was not significant in multivariate analysis indicating that low GA only partly explains the greater colonization by *Candida* spp. We suggest that prevention of *Candida* infection should include multiple measures and not be limited to fluconazole prophylaxis in extremely preterm babies.

Use of antibiotics has been associated with the selection of specific organisms like *Candida* spp. [7,26,27,38] or antibiotic resistant strains [5,39,40]. More recently, the role of antibacterial therapy in inducing colonization by resistant *Enterobacteriaceae* has been questioned [28,41]. By conducting multiregression analysis we found only limited associations between early empiric antibiotic therapy and mucosal colonization – NP colonization by *K. pneumoniae* and AR *E. cloacae* were favored by ampicillin treatment – with no effect detected neither for the use of additional broad spectrum antibiotics nor for the duration of antibacterial therapy. These findings confirm the results of two previous studies showing that early empiric exposure to different penicillins and gentamicin does not increase overall colonization by antibiotic-resistant Gram-negative microorganisms [28,29] although ampicillin use may be associated with increase in beta-lactam resistance of *E. cloacae* strains [42].We hypothesize that not antibacterial therapy itself but poor hospital hygiene will enable circulation and transmission of multiresistant strains which then requires use of broad spectrum antibacterial agents and results in longer NICU stay and greater potential for colonization by resistant organisms. Still these findings should be treated with caution as the study lasted for only 18 months which may be too short to change hospital microbial environment.

Some limitations of the study should be noted. First, in this study we focused on the collection of potentially pathogenic opportunistic organisms and did not look at the non-pathogenic bacteria like *Lactobacilli, Bifodobacteria* and *Bacteroides*. However, we appreciate their enormous role in mucosal colonization that may be interfered by all risk factors identified in this study. Second, quantitative cultures which may be even more important when development of invasive disease is concerned were not performed. Still, the stool samples from ELBW babies were collected, analyzed by using qRT-PCR and the results will be published separately [37]. Another disadvantage was not having a control group of antibiotic-naïve subjects. Such a group of neonates would have been extremely difficult to recruit in a third level NICU as almost all critically ill neonates will be exposed to antibiotics and recruiting controls with very different disease severity may have biased the comparisons [12,14,31]. Environmental cultures as well as maternal colonization were also not evaluated. Finally, the associations between colonization and BSI were assessed and are analyzed and described separately [43]. Still we believe that this did not significantly interfere with our conclusions.

5. Conclusions

Risk factors influencing NP and rectal colonization including AR strains are similar and species-specific and are closely inter-related making extrapolations from one site to the other feasible. Colonization by *E. coli, K. oxytoca* and *C. albicans* is mainly influenced by maternal and early perinatal factors, while *K. pneumonia, E. cloaceae* and *non-albicans Candida* spp. are predominantly affected by the hospital environment and prematurity. The impact of antibacterial therapy on colonization with Gram-negative microorganisms including AR strains may have been over-estimated. Better understanding of the risk factor profiles of Gram-negative and fungal colonization in NICU patients may permit the development of strategies to prevent heavy colonization and subsequent invasive disease in high risk infants.

Conflict of interest

Financial disclosure and conflict of interest: all authors have no conflict.

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Appendix A. Table

		Univariate logistic regression		
Influencing factor	Microbe	OR	95% CI	p
Rectal				
Amp vs Pen	*K. pneumoniae*	1.94	1.02–3.72	0.0449
Duration of prim AB — Amp days	*E. cloaceae*	0.87	0.77–0.98	0.0275
Second period vs first	*K. pneumoniae*	0.44	0.23–0.86	0.0156
Unit B vs unit A	*K. pneumoniae*	0.20	0.09–0.43	<0.0001
	E. coli	2.31	1.15–4.62	0.0183
	E. cloacae	0.51	0.29–0.90	0.0191
	Serratia	4.81	1.75–13.21	0.0023
	C. albicans	0.40	0.19–0.84	0.0162
GA ≤28 vs 29–36 weeks	*K. pneumoniae*	4.72	2.26–9.86	<0.0001
	K. oxytoca	4.20	1.90–9.31	0.0004
	E. coli	2.51	1.08–5.81	0.0323
	E. cloacae	2.55	1.39–4.68	0.0024
	C. albicans	3.70	1.60–8.57	0.0022
	Other *Candida*	4.24	1.31–13.78	0.0162
GA ≥37 vs 29–36 weeks	*E. coli*	4.46	1.86–10.72	0.0008
GA ≤28 vs ≥37 weeks	*K. pneumoniae*	4.84	1.75–13.38	0.0024
	E. cloacae	3.14	1.37–7.19	0.0069
Vaginal delivery vs Cesarean	*E. coli*	5.18	2.42–11.07	<0.0001
	C. albicans	2.15	1.06–4.35	0.0337
Breast milk vs parenteral	*K. oxytoca*	2.96	1.31–5.81	0.0076
	E. coli	6.23	1.73–22.37	0.0051
	Serratia	5.69	1.22–26.59	0.0269
Breast milk vs formula	*E. coli*	14.0	1.05–4.37	0.0366
	Serratia	2.50	1.04–5.98	0.0401
Parenteral vs breast milk	*C. albicans*	3.78	1.36–10.50	0.0108
Pareteral vs formula	*C. albicans*	2.83	1.29–6.21	0.0092
Formula vs brest milk	*K. pneumoniae*	2.69	1.06–6.80	0.0373
NICU stay days	*K. pneumoniae*	1.05	1.03–1.07	<0.001
	K. oxytoca	1.05	1.03–1.07	0.000
	E. cloaceae	1.06	1.04–1.08	0.000
	Serratia	1.03	1.01–1.05	0.0114
	Acinetobacter	1.06	1.03–1.08	0.000
	Stenotrophomonas	1.06	1.02–1.11	0.002
	C. albicans	1.05	1.03–1.07	0.000
	Other Candida	1.07	1.04–1.10	0.000
ALV	*K. pneumoniae*	3.15	1.15–8.07	0.0246
	K. oxytoca	3.32	1.14–9.68	0.0284
	E. cloacae	2.97	1.34–6.59	0.0075
	Acinetobacter	7.61	1.01–57.52	0.0493
	C. albicans	6.40	1.50–27.38	0.0123

Appendix A (*continued*)

Influencing factor	Microbe	Univariate logistic regression OR	95% CI	p
Rectal				
Duration of ALV days	*K. pneumoniae*	1.03	1.01–1.06	0.0076
	K. oxytoca	1.03	1.01–1.05	0.0098
	E. cloaceae	1.03	1.01–1.05	0.0162
	Acinetobacter	1.02	1.00–1.04	0.0284
	C. albicans	1.07	1.04–1.11	0.000
	Other Candida	1.03	1.01–1.05	0.0145
Ci/vC	*K. pneumoniae*	3.99	1.38–11.57	0.0109
	K. oxytoca	4.64	1.38–15.56	0.0129
	E. cloacae	3.53	1.53–8.16	0.0032
Duration of Ci/vC days	*K. pneumoniae*	1.11	1.06–1.15	<0.0001
	K. oxytoca	1.07	1.03–1.11	0.000
	E. cloacae	1.08	1.05–1.12	0.000
	Acinetobacter	1.09	1.05–1.14	0.000
	Stenotrophomonas	1.13	1.06–1.21	0.000
	Pseudomonas	1.08	1.02–1.15	0.0125
	C. albicans	1.06	1.03–1.10	0.000
	Other Candida	1.12	1.06–1.18	0.000
i/aC	*K. pneumoniae*	14.89	2.01–110.43	0.0002
	K. oxytoca	5.96	1.39–25–47	0.016
	E. cloacae	4.29	1.64–11.24	0.003
Duration of Ci/aC days	*K. pneumoniae*	1.20	1.11–1.29	<0.0001
	K. oxytoca	1.13	1.05–1.21	0.000
	E. cloacae	1.20	1.12–1.28	0.000
	Acinetobacter	1.08	0.99–1.17	0.0712
	Stenotrophomonas	1.14	1.01–1.27	0.0287
	Pseudomonas	1.18	1.04–1.35	0.0131
	C. albicans	1.17	1.08–1.26	0.000
	Other Candida	1.23	1.11–1.36	0.000
PROM >18 h	*K. pneumoniae*	2.20	1.07–4.51	0.0311
AB during labor	*K. pneumoniae*	3.20	1.68–6.09	<0.0001
Antenatal steroids vs none	*K. pneumoniae*	2.65	1.31–5.36	0.0068
	E. coli	0.44	0.22–0.87	0.0185
	E. cloacae	1.96	1.1–3.48	0.022
	Other *Candida*	11.91	1.54–91.93	0.0175
Additional broad spectrum antibiotics				
Carb vs none	Other *Candida*	5.17	1.63–16.43	0.0053
Ceph vs none	Other *Candida*	4.77	1.20–19.01	0.0268
Bet vs none	*K. pneumoniae*	3.36	1.62–6.98	0.0011
	Other *Candida*	3.79	1.27–11.27	0.0162
Nasopharyngeal				
Amp vs Pen	*K. pneumoniae*	5.26	2.10–13.20	<0.0001
	Acinetobacter	0.46	0.23–0.91	0.0268
Duration of primal AB — Pen days	*K. pneumoniae*	0.76	0.61–0.94	0.0136
	Acinetobacter	1.14	1.02–1.27	0.0212
Duration of primal AB — Amp days	*K. oxytoca*	0.74	0.56–0.98	0.0348
	Acinetobacter	0.77	0.64–0.93	0.0078
Unit B vs unit A	*K. pneumoniae*	0.16	0.06–0.42	0.0002
	E. cloacae	0.35	0.18–0.71	0.0033
	Serratia	4.14	1.13–15.20	0.032
	Acinetobacter	0.38	0.19–0.79	0.009
Period-second per vs first	*K. pneumoniae*	0.23	0.09–0.54	0.0008
	Stenotrophomonas	4.12	0.86–19.78	0.0767
GA ≤28 vs 29–36 weeks	*K. pneumoniae*	7.65	2.99–19.57	<0.0001
	K. oxytoca	5.62	1.99–15.86	0.0011
	E. cloacae	3.25	1.64–6.44	0.0007
	Acinetobacter	2.25	1.09–4.65	0.0287
	Stenotrophomonas	13.88	1.73–111.59	0.0134
GA ≤28 vs ≥37 weeks	*K. pneumoniae*	9.89	2.24–43.64	0.0025
	E. cloacae	24.86	3.28–188.54	0.0019
	Acinetobacter	2.96	1.05–8.37	0.0410
Vaginal delivery vs Cesarean section	*E. coli*	3.09	1.22–7.84	0.0176
	Acinetobacter	0.50	0.24–1.02	0.0556
Breast milk vs formula	*K. oxytoca*	3.83	1.33–10.99	0.0127
NICU stay days	*K. pneumoniae*	1.06	1.04–1.08	0.000
	K. oxytoca	1.06	1.04–1.09	0.000
	E. cloacae	1.06	1.04–1.08	0.000
	Serratia	1.05	1.02–1.08	0.000
	Acinetobacter	1.06	1.04–1.08	0.000
	Stenotrophomonas	1.06	1.02–1.09	0.000
ALV	*K. pneumoniae*	5.54	1.29–23.82	0.0214
	E. cloacae	3.87	1.33–11.25	0.0128
	Acinetobacter	3.32	1.14–9.68	0.0284

Appendix A (*continued*)

Influencing factor	Microbe	Univariate logistic regression OR	95% CI	p
Duration of ALV days	*K. pneumoniae*	1.02	1.00–1.04	0.0845
	K. oxytoca	1.03	1.01–1.05	0.0137
	Acinetobacter	1.04	1.01–1.07	0.0033
	Stenotrophomonas	1.03	1.00–1.05	0.0196
Ci/vC	*K. pneumoniae*	11.69	1.57–87.20	0.0165
	E. cloacae	8.48	2.00–36.02	0.0038
	Acinetobacter	4.64	1.38–15.56	0.0129
Duration of Ci/vC days	*K. pneumoniae*	1.07	1.03–1.11	0.000
	K. oxytoca	1.09	1.05–1.14	0.000
	E. cloacae	1.09	1.05–1.14	0.000
	Acinetobacter	1.10	1.06–1.14	0.000
	Stenotrophomonas	1.12	1.06–1.19	0.000
	Pseudomonas	1.06	1.01–1.13	0.0323
i/aC	*K. pneumoniae*	9.58	1.28–71.69	0.0277
	E. cloacae	14.48	1.59–107.46	0.0089
	Acinetobacter	5.96	1.39–25.47	0.016
Duration of i/aC days	*K. pneumoniae*	1.19	1.10–1.29	0.000
	K. oxytoca	1.15	1.06–1.25	0.001
	E. cloacae	1.20	1.11–1.29	0.000
	Acinetobacter	1.18	1.10–1.27	0.000
	Stenotrophomonas	1.13	1.02–1.25	0.0226
	Pseudomonas	1.17	1.04–1.32	0.0087
AB during labor	*K. pneumoniae*	2.98	1.42–6.26	0.0039
	E. coli	3.03	1.24–7.38	0.0146
	Acinetobacter	2.03	1.04–3.97	0.0384
PROM >18 h	*K. oxytoca*	3.49	1.40–8.70	0.0072
	E. coli	6.62	2.26–16.37	<0.0001
Antenatal steroids vs none	*K. pneumoniae*	5.11	1.91–13.67	0.0012
	E. cloacae	4.59	2.05–10.26	<0.0001
Additional broad spectrum antibiotics				
Carb vs none	*K. oxytoca*	2.95	1.00–8.75	0.0505
	Stenotrophomonas	9.23	2.51.34.00	<0.0001
Bet vs none	*K. oxytoca*	5.86	2.37–14.50	<0.0001
	E. cloacae	3.03	1.45–6.35	0.0033
Maternal age	*K. pneumoniae*	1.11	1.02–1.21	0.0151
Rectal — AR resistant				
Unit B vs unit A	*K. pneumoniae*	0.20	0.08–0.5	0.0006
	E. cloacae	0.40	0.19–0.83	0.0131
	Serratia	11.37	1.44–90.07	0.0213
GA ≤28 vs 29–36 weeks	*K. pneumoniae*	7.65	2.99–19.57	0.0000
	K. oxytoca	6.44	2.49–16.65	0.0001
	E. cloacae	2.97	1.42–6.25	0.0040
GA ≤28 vs ≥37 weeks	*K. pneumoniae*	9.89	2.24–43.64	0.0025
	K. oxytoca	3.14	1.11–8.86	0.0304
	E. cloacae	4.25	1.38–13.05	0.0115
Parenteral vs breast milk	*K. pneumoniae*	3.09	1.01–9.48	0.0487
Breast milk vs formula	*K. oxytoca*	2.99	1.32–6.80	0.0089
Breast milk vs parenteral	*K. oxytoca*	2.84	0.96–8.36	0.0581
NICU stay days	*K. pneumoniae*	1.05	1.03–1.08	0.000
	K. oxytoca	1.06	1.04–1.08	0.000
	E. cloacae	1.05	1.03–1.07	0.000
	Acinetobacter	1.08	1.04–1.12	0.000
ALV	*K. oxytoca*	3.50	1.03–11.87	0.0443
	E. cloacae	3.21	1.10–9.38	0.033
Duration of ALV days	*K. pneumoniae*	1.03	1.01–1.05	0.0145
	K. oxytoca	1.03	1.01–1.06	0.0054
	E. cloacae	1.02	1.00–1.04	0.0278
	Acinetobacter	1.02	1.00–1.05	0.0233
Ci/vC	*K. pneumoniae*	3.50	1.03–11.87	0.0443
	K. oxytoca	5.54	1.29–23.82	0.0214
	E. cloacae	3.21	1.10–9.38	0.033
Duration of Ci/vC days	*K. pneumoniae*	1.08	1.04–1.12	0.000
	K. oxytoca	1.07	1.03–1.11	0.000
	E. cloacae	1.08	1.04–1.13	0.000
	Acinetobacter	1.09	1.03–1.14	0.0015
i/aC	*K. pneumoniae*	9.37	1.27–71.69	0.0277
	K. oxytoca	4.53	1.05–19.55	0.0426
	E. cloacae	5.77	1.35–24.70	0.0181
Duration i/aC days	*K. pneumoniae*	1.18	1.09–1.28	0.000
	K. oxytoca	1.13	1.05–1.21	0.0016
	E. cloacae	1.18	1.09–1.27	0.000
AB during labor	*K. pneumoniae*	4.00	1.87–8.55	0.000
PROM >18 h	*K. pneumoniae*	2.99	1.36–6.57	0.0064

(*continued on next page*)

Appendix A (*continued*)

Influencing factor	Microbe	Univariate logistic regression		
		OR	95% CI	p
Rectal — AR resistant				
Antenatal steroids vs none	*K. pneumoniae*	3.28	1.37–7.85	0.0075
	E. cloacae	2.3	1.10–4.82	0.0272
Nasopharyngeal AR resistant				
Amp vs Pen	*E. cloacae*	2.53	1.11–5.75	0.0264
Duration of primal AB days	*E. cloacae*	0.83	0.69–1.00	0.0474
Duration of primal	*E. cloacae*	0.81	0.66–1.00	0.045
AB-Pen days	*Acinetobacter*	1.27	1.08–1.50	0.0046
Duration of primal	*K. oxytoca*	0.75	0.55–1.02	0.0697
AB-Amp days	*E. coli*	1.23	1.01–1.49	0.0425
Unit B vs unit A	*K. pneumoniae*	0.16	0.04–0.75	0.0197
	E. cloacae	0.14	0.05–0.41	0.0003
GA ≤28 vs 29–36 weeks	*K. pneumoniae*	21.06	2.7–164.15	0.0036
	K. oxytoca	7.54	2.10–27.07	0.0020
	E. cloacae	3.94	1.71–9.08	0.0013
Vaginal delivery vs Cesarean	*E. coli*	5.59	1.16–26.81	0.0316
Breast milk vs parenteral	*K. oxytoca*	2.94	1.08–8.00	0.0343
Breast milk vs formula	*K. oxytoca*	5.15	1.53–17.37	0.0082
NICU stay days	*K. pneumoniae*	1.07	1.04–1.10	0.000
	K. oxytoca	1.07	1.05–1.10	0.000
	E. cloacae	1.06	1.04–1.08	0.000
	Acinetobacter	1.05	1.02–1.08	0.000
ALV	*E. cloacae*	4.92	1.14–21.25	0.0327
Duration of ALV days	*K. oxytoca*	1.03	1.01–1.05	0.0072
	Acinetobacter	1.02	1.00–1.04	0.0487
Ci/vC	*E. cloacae*	10.41	1.39–77.95	0.0225
Duration of Ci/vC days	*K. oxytoca*	1.10	1.05–1.15	0.000
	E. cloacae	1.08	1.04–1.12	0.000
	Acinetobacter	1.07	1.02–1.12	0.0077
i/aC	*E. cloacae*	8.55	1.14–64.14	0.0369
Duration of i/aC days	*K. oxytoca*	1.18	1.08–1.30	0.000
	E. cloaceae	1.14	1.06–1.23	0.000
AB during labor	*K. pneumoniae*	7.77	2.11–28.58	0.002
PROM >18 h	*K. pneumoniae*	4.95	1.65–14.83	0.0042
	K. oxytoca	3.42	1.23–9.47	0.018
	E. coli	7.37	2.00–27.17	0.0027
Antenatal steroids vs none	*K. pneumoniae*	10.99	1.42–85.20	0.0218
	E. cloacae	8.30	2.45–28.07	<0.0000
Additional broad spectrum antibiotics				
Carb vs none	*K. oxytoca*	4.28	1.38–13.20	0.0116
Bet vs none	*K. oxytoca*	8.31	2.98–23.17	<0.0000
	E. cloacae	3.23	1.37–7.50	0.0064

NICU — neonatal intensive care unit; GA — gestation age; PROM — prolonged rupture of membranes; ALV — artificial lung ventilation, Ci/vC — central intravascular catheter; i/aC — intra-arterial catheter; AB — antibiotic; Amp — ampicillin; Pen — penicillin; prim — primal; Ceph — cephalosporins; Carb — carbapenems, Bet — beta-lactam + betalactamase inhibitor combinations.

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