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Ü. Parm, T. Metsvaht, E. Sepp, M.-L. Ilmoja, H. Pisarev, M. Pauskar, I. Lutsar

Eur J Clin Microbiol Infect Dis (2010) 29:807–816
DOI 10.1007/s10096-010-0931-1

ARTICLE

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Received: 9 November 2009 / Accepted: 3 April 2010 / Published online: 6 May 2010

Abstract The purpose of this study was to compare the impact of ampicillin and penicillin used for empiric treatment of early onset sepsis (EOS) on initial gut colonization by aerobic and facultative anaerobic microorganisms. A cluster-randomized, two-center, switch-over study was conducted in two paediatric intensive care units in Estonia and included 276 neonates. Rectal swabs were collected twice a week until discharge or day 60. Colonizing microbes were identified on species level and tested for ampicillin resistance (AR). The number of patients colonized with Gram negative microorganisms and *Candida* spp was similar in both treatment arms but ampicillin resulted in longer colonization duration (CD) of *K. pneumonia* (p=0.012), AR *Serratia* spp (p=0.012) and *Candida* spp (p=0.02) and penicillin in that of AR *Acinetobacter* spp (p=0.001). As for Gram positive microorganisms penicillin treatment was associated with a greater number of colonized patients and higher CD of *Enterococcus* spp and *S. aureus* but lower ones of *S. haemolyticus* and *S. hominis*. Influence of ampicillin and penicillin on initial gut colonization is somewhat different but these differences are of low clinical relevance and should not be a limiting step when choosing between these two antibiotics for the empiric treatment of EOS.

Ü. Parm (✉) · E. Sepp · M. Pauskar · I. Lutsar (✉)
Institute of Microbiology, Tartu University,
Ravila 19, 50411 Tartu, Estonia
e-mail: ylleparm@nooruse.ee
e-mail: Irja.Lutsar@ut.ee

T. Metsvaht
Clinics of Anaesthesiology and Intensive Care,
University Clinics of Tartu,
Lunini 6, 50411 Tartu, Estonia

M.-L. Ilmoja
Paediatric Intensive Care,
Tallinn Children's Hospital,
Tervise 28, 13419 Tallinn, Estonia

H. Pisarev
Department of Public Health,
Tartu University,
Ravila 19, 50411 Tartu, Estonia

Introduction

Empiric use of antibiotics in neonatal intensive care units is common but their widespread use is not problem-free [1]. The issue that administration of antimicrobial agents causes disturbances in the ecological balance between the host and the microorganisms and thus has a potential to interfere with initial gut colonization in neonates cannot be ignored [2].

A combination of gentamicin with a beta-lactam antibiotic such as penicillin G or ampicillin is the most recommended treatment for early onset sepsis (EOS) [3, 4]. Studies have shown that broad spectrum antibiotics including ampicillin have led to increased rates of colonization by potentially pathogenic members of *Enterobacteriaceae* and *Candida* spp. [5–7]. On the other hand narrow spectrum penicillins like penicillin G have the least potential of interfering with normal gut colonization [8]. Still the number of comparative studies on this issue is limited. De Man et al. [9] compared the effect of amoxicillin plus cefotaxime with penicillin plus tobramycin on gut colonization with special attention to the emergence of resistant *Enterobacteriaceae* in two NICUs over a period of six months and showed that with the first regimen 18.8% of neonates became colonized with bacteria resistant to initial antibiotics compared to only 1.3% with the second regimen. Still, the study did not clarify whether the

differences between the two antibiotic regimens were triggered by amoxicillin or cefotaxime or both. The use of ampicillin in intrapartum prophylaxis of group B streptococcal infection has been associated with the emergence of *Escherichia coli* as a major causative pathogen of EOS and probably an increase in ampicillin-resistance [6, 10], but the data are not conclusive [11].

Based on the above, we assumed that ampicillin as an antibiotic with wider Gram-negative coverage interferes more with initial gut colonization than penicillin and has the potential of inducing emergence of ampicillin-resistant Gram-negative bacteria.

We chose gut colonization studies to compare the two antibiotic regimens because in neonates opportunistic bacteria of the gastrointestinal tract serve as the predominant source of subsequent bloodstream infections [1, 12–14]. Systematic investigations in the field may have a role in understanding the changing spectrum of bacteria involved in late onset sepsis (LOS) and possibly other long-term outcomes [12].

In a cluster-randomized switch-over study in neonates at risk of EOS and requiring third level intensive care, the influence of ampicillin or penicillin, both combined with gentamicin, on gut colonization by aerobic, facultative anaerobic and ampicillin resistant bacteria was compared in an analysis taking into account other factors interfering with gut colonization.

Methods

Bowel colonization assessments were seeded in a study comparing the clinical efficacy of ampicillin and gentamicin to that of penicillin and gentamicin in risk factor based empiric treatment of EOS [15].

Study setting

The study was conducted in two Estonian third level pediatric intensive care units (PICU) from August 2, 2006 until November 30, 2007. Both units admit patients up to 16 years; about 60–65% of them are neonates cared for in a separate area. The units are divided into four and five rooms, respectively; the number of neonates in a room varies from three to six. The nursing staff/infant ratio in the units is 1:2, but can be 1:3 occasionally. Gloves, gowns, caps and masks are used routinely in all aseptic procedures. Both units follow similar hospital infection prevention guidelines and strict antibiotic policy, in which narrow spectrum antibiotics and short courses are preferred. Patients colonised with alert microorganisms are isolated in separate rooms and cared for by separate nurses. Both units practice early introduction of enteral feeding with preference given to breast milk. Formula if needed is prepared centrally; donor milk is not used.

Study population

The study enrolled neonates aged less than 72 h, needing empiric therapy for proven or suspected EOS with penicillin G (25,000 IU/kg 8–12 hourly) or ampicillin (25 mg/kg 8–12 hourly) plus gentamicin (4–5 mg/kg 24–48 hourly, according to gestational age) according to the criteria described by Schrag et al. [16]. Patients who had received a different antibiotic regimen for more than 24 h, had suspicion of meningitis, necrotizing enterocolitis, peritonitis or severe sepsis with a history of isolation of microorganisms resistant to the study regimen from maternal urinary tract or birth canal or had other situations where the treating physician considered a different antibiotic regimen necessary were excluded.

Study design

During the first study period (from August 03, 2006 to March, 20 2007) in unit A ampicillin and in unit B penicillin G was used. After enrolling half of the patients required for proving clinical equivalence of the two antibiotic regimens the penicillins were switched so that during the second period (from March 21, 2007 to November 30, 2007) in unit A penicillin G and in unit B ampicillin was used (Fig. 1). If no clinical or laboratory signs of invasive infection developed and initial blood cultures remained negative, antibiotics were stopped on day 3. 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 were recommended. In case of severe sepsis or septic shock cefotaxime with gentamicin or meropenem with or without vancomycin was to be used. In ELBW neonates with birth weight below 800 g and vascular catheter(s) in place, addition of vancomycin was recommended until culture results became available.

Data collection

Basic demographic and clinical data are shown in Table 1. Feeding regimen was documented on days 1, 3 and 7 with patients categorized into the following groups based on the route of nutrition and the character of enteral feeds: (1) total parenteral nutrition (TPN) when enteral calories constituted

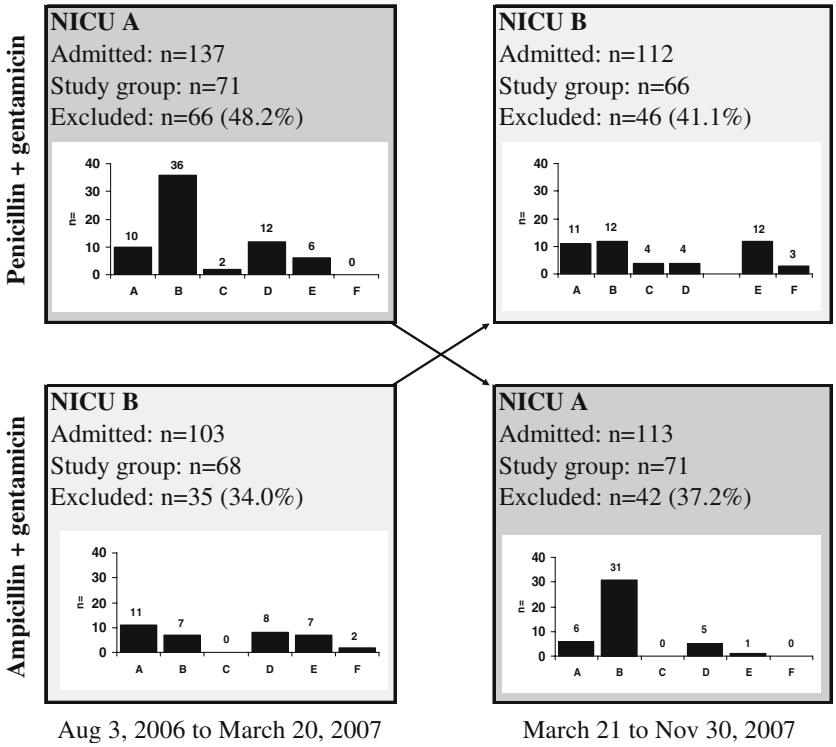

Fig. 1 Study outline and reasons for exclusion. Reasons for exclusion are shown on diagram as follows: *A* age on admission more than 72 h, *B* no need for early empirical antibiotic treatment, *C* different antibiotic regimen for more than 24 h, *D* need for different antibiotic regimen on admission, *E* transfer from neonatal intensive care unit within 24 h, *F* no samples. Number of cases shown on the *y* axis

less than 10% of total daily calories, (2) breastfeeding when breast milk constituted more than 10% of enteral feeds, and (3) formula feeding when formula constituted more than 89% of enteral feeds. Additional parenteral nutrition supplying up to 89% of daily caloric intake was accepted in the two latter groups.

Sampling

Monitoring of gut colonization had been implemented by the infection control services prior to the study with rectal samples collected with transport swabs (Nuova Aptaca, Canelli, Italy) on admission and twice a week thereafter until discharge from PICU or until day 60 whichever occurred first.

Laboratory methods

Transport swabs were stored at −20°C for a maximum of a week and processed in batches. After thawing the swabs were directly plated onto blood agar, MacConkey agar, MacConkey agar with 16 μg/ml of ampicillin and Saboraud agar. The blood and MacConkey agar plates were incubated at 37°C for 24–48 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 [17]. For final identification of enterobacteria and staphylococci API commercial kits (API 20E and API STAF; Biomérieux, and Marcy l'Etoile, France, respectively) and for identification of yeasts CHROMagar™Candida (BD BBL, Heidelberg, Germany) were employed. The *Staphylococcus aureus* strains were further tested for methicillin-resistance (MRSA) by determining *nuc* and *mecA* genes as described elsewhere [18, 19].

In this study any Gram-negative microorganism that grew on MacConkey agar with 16 μg/ml of ampicillin was termed ampicillin-resistant (AR).

Statistical analysis

The two treatment regimens were compared to each other with regards to number of patients colonized and colonization duration (CD); the latter describing the ratio of colonizing days per 100 PICU days. The number of colonizing days was counted from the first until the last positive culture and an extra two days were added to compensate for the sampling interval, which was three to four days.

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 for statistical analysis. Hierarchical mixed effect models corrected for the study centre and treatment period were used in all comparisons. Differences in proportions were compared using Chi-square test. To compensate for the influence of other

Table 1 Demographic data and clinical characteristics of the study population

Description	Amp + gentamicin (n=139)	Pen + gentamicin (n=137)	p
Neonatal factors			
Duration of PICU stay days, median (IQR)	6 (3–14.8)	7.5 (4–17.2)	0.263
GA (week) mean (±SD)	31.2 (±5.1)	31.4 (±5.1)	0.554
GA<28 weeks, *n* (%)	47 (33.8)	45 (33.1)	0.996
GA≥37 weeks, *n* (%)	27 (19.4)	28 (20.1)	0.952
BW (g) mean (±SD)	1952 (±1123)	1824 (±1038)	0.946
≤1000 g, *n* (%)	36 (25.9)	39 (28.4)	0.731
≤1500 g, *n* (%)	72 (51.8)	70 (51.1)	0.997
≥2500 g, *n* (%)	36 (25.9)	31 (22.3)	0.622
Male/female, *n*	76/63	81/56	0.532
Nutritional habits, *n* (%)			
Total parenteral nutrition	28 (20.1)	30 (21.9)	0.834
Breast milk containing regimen	32 (23)	39 (28.5)	0.37
Formula	79 (56.8)	67 (48.2)	0.231
[a]Additional AB, *n* (%)	41 (30.9)	55 (40.1)	0.083
Sepsis, *n* (%)			
Early onset sepsis	6 (4.3)	8 (6.5)	0.763
Late onset sepsis	25 (17.9)	29 (21.2)	0.607
Maternal factors			
Multiple births, *n* (%)	34 (24.5)	23 (16.5)	0.154
Caesarean section, *n* (%)	79 (56.8)	78 (56.8)	0.917
Antenatal AB, *n* (%)	36 (25.9)	25 (18.2)	0.166
Antenatal steroids, *n* (%)	85 (61.2)	70 (50.4)	0.118
Maternal chorionamnionitis, *n* (%)	21 (15.1)	29 (20.9)	0.250
Prolonged rupture of membranes >18 h, *n* (%)	24 (17.3)	27 (19.4)	0.713

PICU paediatric intensive care unit, *BW* birth weight, *GA* gestational age, *AB* antibiotic, *Amp* ampicillin, *Pen* penicillin, *IQR* interquartile range

[a] Only patients with AB treatment for more than 12 h

factors on gut colonization a multivariate mixed effect model adjusted for gestational age, mode of delivery, maternal chorionamnionitis, rupture of membranes for more than 18 h before delivery, use of antenatal steroids and antibiotics, duration of ICU stay, type of feeding, presence of mechanical ventilation and culture proven EOS and use of carbapenems, third and fourth generation cephalosporins and beta-lactamase resistant penicillins [6, 7, 9, 11, 12, 20–30] was performed.

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

Results

Study population and setting

A total of 465 neonates (age 0–28 days) were admitted to both units with 43% and 34% excluded in units A and B, respectively (Fig. 1). Exclusion for no need of early empiric antibiotic treatment was more common in unit A than in unit B (odds ratio [OR] 3.78; 95% CI 2.18–6.53), likely reflecting a difference in admitted population. A total of 283 patients were included, colonisation data were available in 276 (97.5%) of them, thus 139 neonates in the ampicillin and 137 in the penicillin group constituted the study population (Fig. 1).

As shown in Table 1 the population characteristics in both treatment groups were well balanced. The median duration of primary antibiotic regimen was 64 h (interquartile range [IQR] 57–138) in the ampicillin and 72 h (IQR 55–134) in the penicillin arm. The use of additional broad spectrum antibiotic classes in both regimens was similar (Table 2) with the median duration of 220 h (IQR 95–347) in the penicillin and 268 h (IQR 108–446) in the ampicillin group. The median number of rectal samples collected per patient was three; with IQR 2–5 in the ampicillin and 2–6 in the penicillin group. The aetiology of EOS and LOS is presented in Table 3. There was a greater number of LOS cases caused by *S. epidermidis* in the

Table 2 Use of additional broad-spectrum antibiotics and antifungals

Additional treatment	Amp + gentamicin*n*=139 (%)	Pen + gentamicin*n*=137 (%)	*p*
Beta-lactam + betalactamase inhibitor combinations	23 (16.5)	25 (18.2)	0.831
III and IV generation cephalosporins	7 (5.0)	12 (8.8)	0.325
Carbapenems	13 (9.4)	18 (13.1)	0.421
Fluconazole	15 (10.8)	10 (7.3)	0.423

Amp ampicillin, *Pen* penicillin G

penicillin compared with the ampicillin group (2.7 vs 7.6 per 1000 patients days, RR 0.32; 95% CI 0.19–0.55). However, in a multivariate model adjusted for (other) risk factors of LOS the difference between the two treatments became non-significant (p=0.08).

The study population recruited in both units was similar except for the following: in unit A there were more patients with birth weight (BW) <1000 g (53 vs 22; OR=3.22; 95% CI 1.82–5.70) and those receiving TPN during the first week of life (51 vs 7; OR=10.76; 95% CI 4.67–24.81) than in unit B. On the other hand in unit B more patients received breast milk containing regimen within the first week of life (57 vs 14; OR=6.36; 95% CI 3.33–12.17) than in unit A.

During the study two outbreaks of bloodstream infections (BSI) confirmed by pulse field gel electrophoresis (PFGE) were observed. In unit B an outbreak of MRSA infection involving three patients occurred during the penicillin treatment period and in unit A five patients had a BSI caused by *K. pneumoniae* (all strains intermediately resistant to ampicillin) during the ampicillin treatment period. The latter outbreak was likely responsible for the differences in *K. pneumoniae* colonization between units and treatment periods.

Influence of participating units on gut colonization

In unit B more patients were colonized with *E. coli* (OR=2.31; 95% CI 1.15–4.62) and *Serratia* spp (OR=4.81; 95% CI 1.75–13.21), but significantly less patients harbored *E. cloacae* (OR=0.51; 95% CI 0.29–0.90), *K. pneumoniae* (OR=0.20; 95% CI 0.09–0.43), *S. haemolyticus* (OR=0.13; 95% CI 0.06–0.26), and *S. hominis* (OR=0.28; 95% CI 0.11–0.71), compared to unit A. Colonization with the

Table 3 Bacterial aetiology of early and late onset sepsis by treatment group

Microorganisms	EOS episodes, *n*		LOS episodes, *n*	
	Amp	Pen	Amp	Pen
Gram-positive	4	4	17	28
Staphylococcus epidermidis	–	3	5	14
Staphylococcus haemolyticus	–	–	7	4
Staphylococcus hominis	–	1	–	1
Other CoNS	–	–	–	1
Staphylococcus aureus: MSSA	–	–	1	2
MRSA	–	–	1	3
Enterococcus spp.	–	–	3	1
Streptococcus agalactiae	4	–	–	–
Streptococcus salivarius	–	–	–	1
Gram-negative	2	3	13	13
Acinetobacter baumannii	–	–	4	3
Escherichia coli	1	1	2	1
Enterobacter cloacae	1	1	2	2
Klebsiella oxytoca	–	–	–	3
Klebsiella pneumoniae	–	–	5	1
Pseudomonas spp.	–	–	–	1
Stenotrophomonas spp.	–	–	–	1
Serratia spp.	–	–	–	1
Haemophilus influenzae	–	1	–	–
Candida spp	–	1	3	1
TOTAL episodes	6	8	33	42

EOS early onset sepsis, *LOS* late onset sepsis, *Amp* ampicillin plus gentamicin regime, *Pen* penicillin plus gentamicin regime, *CoNS* coagulase negative staphylococci, *MSSA* methicillin susceptible *Staphylococcus aureus*, *MRSA* methicillin resistant *Staphylococcus aureus*

respective AR Gram negative organisms followed a similar pattern, except for *E. coli*, in which AR strains occurred with similar frequency in both units.

Influence of treatment period on gut colonization

The gut colonization pattern in both treatment periods was similar except for *K. pneumoniae* (OR=2.25; 95% CI 1.17–4.34), *S. haemolyticus* (OR=1.92; 95% CI 1.10–3.36), and *S. hominis* (OR=6.46; 95% CI 2.16–19.28), all being more frequent colonizers during the first compared to the second period.

Influence of empiric antibiotic regimen on bowel colonization over time

As presented in Fig. 2a and b Gram-positive microorganisms were the first and most common colonizers of the rectal mucosa, followed by Gram-negative and Gram-negative AR bacteria and yeasts. Both antibiotic regimens had similar effects on early gut colonization with Gram negative microorganisms and *Candida* spp except for greater number of patients colonized by *Acinetobacter* spp on days 13–16, including AR *Acinetobacter* spp between day 10 and 16 in the penicillin compared with the ampicillin arm (Fig. 2b). As for the colonization by Gram positive organisms the following differences between the two antibiotics were observed: penicillin treatment resulted in significantly greater number of patients colonized with *Enterococcus* spp at all time-points from day 6 to 16 and by *S. aureus* between days 3 and 5 than ampicillin (Fig. 2a).

Influence of empiric treatment regimen on gut colonization—model corrected for participating unit and treatment period

As both the treatment period and the participating unit affected bowel colonization, all further analyses were corrected for these two co-variates. First, the penicillin and ampicillin treatments were compared based on the number of colonized patients. With the exception of AR *Acinetobacter* spp found only in the penicillin arm (0 vs 8; p=0.008), the number of patients colonized with all other Gram negative organisms was similar in both treatment arms (Table 4). Again differences were observed among Gram-positive microorganisms. Compared with penicillin, ampicillin treatment was associated with twofold greater odds of colonization by *S. haemolyticus* (OR 2.22; 95% CI 1.2–4.12) and sixfold that of *S. hominis* (OR 6.46; 95% CI 2.12–19.67), whereas the odds of colonisation by *Enterococcus* spp and *S. aureus* were about one half (OR 0.5; 95% CI 0.3–0.83) and one third (OR 0.34; 95% CI 0.13–0.91), respectively (Table 4).

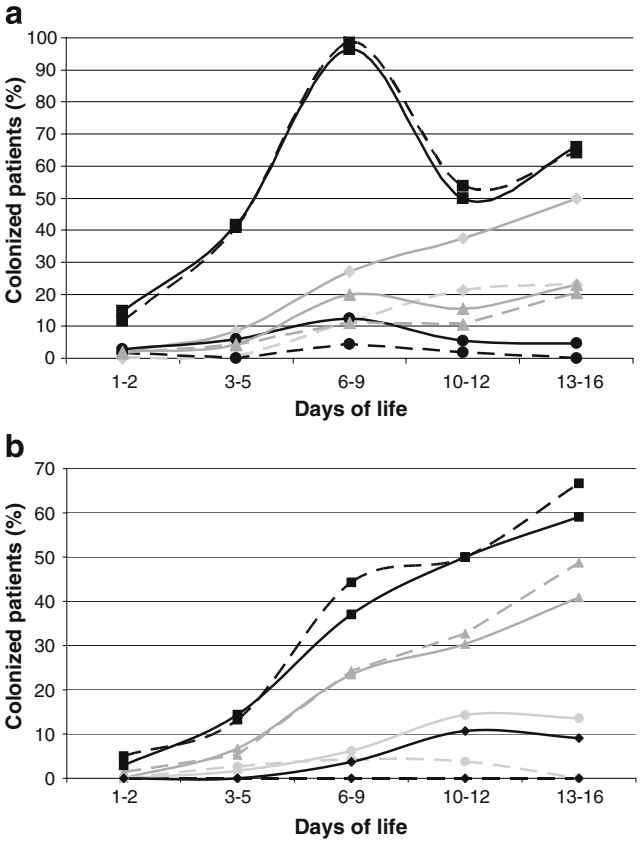

Fig. 2 **a**. Colonization with Gram-positive microorganisms over time. *Continuous lines* present data of the penicillin and *dotted lines* of the ampicillin group. CoNS are represented by *squares*, yeasts by *triangles*, *S. aureus* by *circles*, and *Enterococcus* spp by *diamonds*. The following significant differences were observed: the rate of patients colonised with *Enterococcus* spp was greater in the penicillin as compared with ampicillin arm on days 6–9 (OR=2.99; CI 95% 1.23–7.25) and days 13–16 (OR=3.33; CI 95% 1.29–8.63). The rate of patients colonised with *S. aureus* on days 3 to 5 was greater in the penicillin than in the ampicillin arm (p=0.012). **b**. Colonization with Gram-negative and Gram-negative AR microorganisms. *Continuous lines* present data of the penicillin and *dotted lines* of the ampicillin group. *Enterobacteriaceae* are represented by *squares*, AR *Enterobacteriaceae* by *triangles*, *Acinetobacter* spp by *circles*, and AR *Acinetobacter* spp by *diamonds*. The following significant differences were observed: the rate of patients colonized by *Acinetobacter* spp in the penicillin as compared with the ampicillin arm on days 13–16 (6 vs 0; p=0.009) and AR *Acinetobacter spp* on days 10–12 (6 vs 0; p=0.045) and days 13–16 (4 vs 0; p=0.046)

Second, we compared the CDs (days colonised per 100 ICU days) in the two treatment arms. In this respect penicillin and ampicillin treatments were more distinct than in the per subject analysis. Higher mean CD of *K. pneumoniae* (difference +7.8; p=0.004) was seen in the ampicillin arm and AR *Serratia* spp (−3.4; p=0.011) and AR *Acinetobacter* spp (−2.3; p=0.004) in the penicillin arm (Table 4). Among Gram positive bacteria the CDs followed similar trends as in the per subject analysis; ampicillin was associated with higher mean

Table 4 Number of patients colonized and colonization duration (CD) of different microorganisms—an univariate hierarchical model corrected for participating unit and treatment period

Microorganism	Patients		OR	95% CI	Mean colonization duration (CD) ($n \pm$ SD)		p
	Amp (n)	Pen (n)			Amp ($n \pm$ SD)	Pen ($n \pm$ SD)	
Gram-negatives	78	76	1.03	0.64–1.65	45.8 (40.7)	41.7 (38.9)	0.222
Enterobacter cloacae	31	38	0.58	0.31–1.08	13.5 (20.9)	14.6 (21.2)	0.394
Klebsiella pneumoniae	30	17	1.9	0.85–4.25	14.3 (22.7)	6.5 (11.4)	**0.004**
Klebsiella oxytoca	19	22	1	0.45–2.24	6.1 (10.5)	9.8 (16.5)	0.088
Escherichia coli	17	24	0.78	0.39–1.56	10.3 (18.2)	14.7 (24.3)	0.138
Serratia spp	11	14	0.54	0.19–1.58	5.8 (10.6)	4.5 (8.1)	0.268
Acinetobacter spp	9	14	0.51	0.19–1.32	2.7 (5.0)	3.8 (2.3)	0.239
All nonfermentative	24	28	0.62	0.26–1.48	7.3 (12.1)	8.4 (13.3)	0.332
Gram-positives	108	114	0.7	0.39–1.28	67.8 (35.6)	72.0 (29.5)	0.178
All CoNS	97	107	0.67	0.39–1.14	56.3 (38.4)	63.9 (35.0)	0.053
Staphylococcus epidermidis	57	69	0.69	0.43–1.1	26.3 (38.0)	34.8 (41.7)	**0.039**
Staphylococcus haemolyticus	43	25	2.22	1.2–4.12	21.5 (40.0)	9.3 (23.2)	**0.001**
Staphylococcus hominis	22	4	6.46	2.12–19.67	7.0 (21.2)	1.2 (8.1)	**0.001**
Enterococcus spp	36	55	0.5	0.3–0.83	14.0 (20.8)	36.9 (32.6)	**0.001**
Staphylococcus aureus	6	16	0.34	0.13–0.91	1.9 (3.7)	3.5 (6.4)	0.054
Streptococcus spp	12	11	1.11	0.45–2.7	5.7 (10.4)	3.3 (6.0)	0.118
Candida spp	26	20	1.29	0.64–2.61	12.3 (20.0)	7.7 (13.2)	0.077
Ampicillin resistant strains							
Gram-negatives	58	55	1.06	0.66–1.7	30.2 (36.1)	27.5 (33.4)	0.285
Enterobacter cloacae	21	19	1.09	0.49–2.42	7.3 (12.4)	5.3 (9.2)	0.169
Klebsiella pneumoniae	21	12	1.46	0.62–3.44	6.9 (11.8)	5.2 (9.5)	0.211
Klebsiella oxytoca	17	16	1.24	0.5–3.1	4.6 (8.0)	6.8 (12.0)	0.158
Escherichia coli	4	7	0.66	0.18–2.38	2.5 (4.8)	3.9 (7.4)	0.249
Serratia spp	7	4	2.43	0.41–14.18	3.9 (7.5)	0.5 (1.0)	**0.011**
Acinetobacter spp	0	8	0	**0.008**[a]	0	2.3 (4.3)	**0.004**
All nonfermentative	6	13	0.39	0.07–2.05	0.8 (1.5)	3.5 (6.4)	0.354

CoNS coagulase negative staphylococci, *CD* colonization duration (colonizing days per 100 PICU days)

Significant differences are presented in bold

[a] Presents p value as odds ratio cannot be calculated

CDs of *S. haemolyticus* (difference +12.2; p=0.001) and *S. hominis* (+5.8; p=0.001) but lower CDs of *S. epidermidis* (−8.5; p=0.039) and *Enterococcus* spp. (−22.9; p=0.001). Although colonization by *S. aureus* in per patients analysis was different, in CD analysis this did not reach significance at the p value of <0.05 (p=0.054) (Table 4).

Influence of antibiotic regimen on bowel colonization in multivariate, risk factor adjusted mixed effect model analysis

Next we conducted a multivariate analysis including microorganisms that in the model corrected for the study centre and treatment period which reached significance at a p value of ≤0.1 (Table 4). In per subject analysis the differences that remained significant were the greater number of patients colonized with *S. aureus* (p=0.006) and *Enterococcus* spp (p=0.0009) in the penicillin arm and those with *S. haemolyticus* (p=0.039) and *S. hominis* (p=0.009) in the ampicillin arm (Table 5).

>Similarly to univariate analysis described above, early empiric AB regimen remained an independent risk factor for the CD of all major Gram negative microorganisms (*K. pneumonia*, AR *Serratia* spp, and AR *Acinetobacter* spp) also in multivariate models (Table 5). Among Gram positive organisms ampicillin was still independently associated with higher CD of *S. haemolyticus* (p=0.001) and *S. hominis* (p=0.001) and penicillin with *Enterococcus* spp (p=0.001) and *S. aureus* (p=0.052), but the association between penicillin treatment and higher CD of *S. epidermidis* was of borderline significance (p=0.0725). In addition,

Table 5 Impact of ampicillin and penicillin regimen on gut colonization—results of multifactorial mixed effect model analysis

Regimen	Per patient analysis			Colonization duration (CD) analysis		
	p	Estimator	SE	*p*	Estimator	SE
Ampicillin regimen favours						
Staphylococcus haemolyticus	0.039	0.747	0.361	0.001	11.449	3.810
Klebsiella pneumoniae	0.107	0.670	0.416	0.012	6.646	2.944
Staphylococcus hominis	0.003	1.858	0.642	0.001	6.378	1.998
Candida spp	NA	NA	NA	0.020	6.147	3.048
AR *Serratia* spp	NA	NA	NA	0.012	3.657	1.619
Penicillin regimen favours						
Enterococcus spp	<0.001	−1.218	0.369	0.001	−12.971	3.531
Staphylococcus epidermidis	NA	NA	NA	0.073	−7.182	4.915
Klebsiella oxytoca	NA	NA	NA	0.060	−3.962	2.536
Staphylococcus aureus	0.006	−1.940	0.712	0.052	−2.794	1.719
AR *Acinetobacter* spp	0.996	−49.059	15930.952	0.001	−2.482	0.812
Entrobacter cloacae	1.142	−0.479	0.327	NA	NA	NA
Acinetobacter spp	0.224	−0.637	0.524	NA	NA	NA

SE standard error

NA not applicable since the model was not conducted as the *p* value in the univariate hierarchical model was >0.1

ampicillin treatment, not reaching significance for the CD of *Candida* spp in univariate analysis ($p=0.077$) (Table 4), now became associated with higher CD of *Candida* spp ($p=0.02$) (Table 5).

Discussion

To our best knowledge this is the first prospective study comparing the effect of two widely used antibiotic regimens—penicillin and ampicillin, both combined with gentamicin—on initial mucosal colonization in neonates with suspected EOS, looking specifically at colonization with Gram-negative AR bacteria. We found that the main differences between these two regimens did not involve *Enterobacteraceae* but appeared in colonization with Gram positive microorganisms. In patients receiving penicillin containing regimen, colonization with *S. aureus* and *Enterococcus* spp occurred with greater frequency and that of *S. haemolyticus* and *S. hominis* with lower frequency than in those receiving ampicillin. As for Gram negative microorganisms and *Candida* spp, the number of colonized patients in both treatment arms was similar but greater CDs of *K. pneumonia,* AR *Serratia* spp and *Candida* spp were seen in the ampicillin arm and that of AR *Acinetobacter* spp in the penicillin arm.

Studies have shown that gut colonization of a neonate is a complex process influenced by several intrinsic and extrinsic factors [6, 7, 11, 12, 27–30]. Thus, when evaluating the interference of early antibacterial therapy with this process the methodological considerations are of utmost importance. A major advantage of this study was the switch-over design that enabled accounting for the differences between the participating units and treatment periods. As shown here and by others [25], such differences leading to potential cross-colonization are unavoidable despite both units having fairly similar staffing, following similar treatment guidelines and strict infection control measures. Another strength was the statistical analysis conducted by us. First, to minimize the role of the participating unit and treatment period in the assessment of the antibiotic regimen, all comparisons were corrected for these two variables. Second, not only the number of colonized patients but also the time of colonization and its duration was considered, the latter potentially affecting cross-colonization more than the former [25]. Finally, also maternal, neonatal and environmental factors known to interfere with gut colonization like GA, use of broad spectrum antibiotics, severity of disease, etc. were taken into account [6, 7, 11, 12, 27–30]. We believe that such an approach enabled us to characterize adequately the true differences of these two antibiotic regimens on bowel colonization.

In contrast to the previous EOS study comparing amoxicillin plus cefotaxime regimen to penicillin plus tobramycin, as well as some studies on intrapartum antibiotic prophylaxis, we failed to prove our main hypothesis that ampicillin as an antibiotic with broader Gram-negative coverage than penicillin will select for AR *Enterobacteraceae* except for the greater CD of AR *Serratia* spp [9, 31]. With this our findings are in accordance with Jaureguy et al. who, in an intrapartum prophylaxis study, failed to demonstrate that amoxicillin selects for beta-lactam resistant enterobacteria [11]. At present in most countries all *Enterobacteraceae* but *E. coli* have become uniformly resistant to ampicillin [32, 33], and thus it is not surprising that ampicillin and penicillin have similar influence on gut colonization with Gram-negative bacteria. Still,

one should bear in mind that our study was conducted in a country with low antibiotic resistance [34] including low prevalence of extended spectrum beta-lactamase (ESBL) producing microorganisms in the PICU setting (approximately 2%) [32]. Future studies should identify whether these data also apply to countries with high resistance rates. A recent study from Brazil found prior penicillin use in 87% of patients colonized with ESBL producing *K. pneumoniae* whereas the rate among non-colonized patients was 29%; the respective data for ampicillin were 13% and 9% but the study was too small to be conclusive [35].

We demonstrated that differences between penicillin and ampicillin are mainly seen in colonization by Gram positive microorganisms. The association between antibiotic use other than amoxicillin and increased enterococcal colonization is well documented in literature [33, 36, 37]. The results of our study with increased colonization by enterococci in the penicillin compared with the ampicillin arm affirm this. However, which antibiotic should be preferred in this respect is more controversial. Similarly to Hufnagel et al. we demonstrate that early mucosal colonization is associated neither with increased risk of enterococcal bacteraemia nor with increase in overall mortality [38]. More interestingly, recent in vitro data indicate that some strains of *E. faecalis* could suppress the proliferation of intestinal pathogens and thus have a potential to prevent infection and induction of inflammation suggesting that enterococcal colonization may even be beneficial.

The shift in colonization by coagulase negative staphylococci (CoNS) so that penicillin treatment was associated with greater colonization of *S. epidermidis* (in multivariate analysis the $p=0.07$) and lower colonization by *S. haemolyticus* and *S. hominis* than ampicillin is more difficult to explain and to our knowledge has not been described before. It is likely that these different species of staphylococci compete for the colonization niche with each other but we are not aware of any studies in the subject. The different colonization pattern may still have clinical relevance as mucosal colonisation is a potential source of CoNS in invasive infections [14]. Differences in the pathogenicity of CoNS species have been demonstrated with 71% of *S. epidermidis* but only 35% of *S. haemolyticus* and 26% of *S. hominis* strains being capable of slime production, the latter being associated with invasive disease [39–41]. The relevance of differences in gut colonization observed in this study is further supported by the clinical findings showing a trend towards greater prevalence of LOS caused by *S. epidermidis* in the penicillin compared with the ampicillin arm (7.6 vs 2.7 per 100 patient days, respectively).

Several limitations of the study have to be noted. First, the open label design and involvement of two units only did not allow accounting for the variability potentially occurring in multicenter settings. Second, we did not include all admitted patients but only those who required treatment with ampicillin or penicillin. Third, the duration of each study regimen over eight months is likely too short to identify changes in the circulating microflora within a unit. Fourth, in both arms neonates received additional broad spectrum, which potentially could interfere with gut colonization; but on the other hand, this closely mirrored the clinical scenario in a PICU where the majority of infected neonates receive more than one antibiotic regimen during their stay [6, 12, 24, 25, 28]. Exclusion of these patients from the analysis would most likely introduce a major population bias with the sickest babies at highest risk of adverse colonization being left out. We believe that the use of other antibiotics did not impact the overall conclusions as in both treatment arms their frequency as well as type was similar. In addition, risk factor adjusted multivariate analysis eliminated the potential influence of confounders including broad spectrum antibiotics as much as possible. Finally, we did not evaluate quantities of colonizing microorganisms. However, the stool samples from extremely premature babies for RT-PCR analysis were collected and will be reported separately.

In conclusion, the interference with the initial gut colonization should not be a limiting factor in choosing between ampicillin and penicillin for the empiric treatment of EOS, although one should bear in mind that some clinically less important differences between these two agents occur. The selection should be based mainly on the local distribution of EOS causing microorganisms and their antibiotic susceptibility. Further studies should clarify our understanding and clinical relevance of the differences in early gut colonization by various CoNS species and enterococci.

Acknowledgements The study was supported by Estonian Science Foundation Grant number 6984; Estonian Target Financing number 2726 and ESPID Small Grant Award. The authors are grateful to colleagues from both participating NICUs for their support and help in documenting all clinical decisions, as well as to head nurse Tiina Kilg and all nurses for their help in collecting samples. We would like to thank associated Professor Reet Mändar for her valuable remarks and suggestions while preparing the manuscript.

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