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Original article

Enterobacterales gut colonization and late-onset sepsis in neonates: a multicentre prospective study across 18 neonatal intensive care units in six countries

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ABSTRACT

Objectives: Gram-negative bacteria cause a significant proportion of neonatal late-onset sepsis (LOS) and are associated with high mortality. Emerging evidence implicates the gut as a reservoir for invasive pathogens; however, the mechanisms of gut-to-blood translocation and the role of virulence factors remain unclear.

Methods: We conducted a secondary analysis of microbiological samples from the NeoMero-1 trial, a multicentre study of neonatal LOS. Whole-genome sequencing was performed on paired blood and faecal *Enterobacterales* isolates from 22 neonates with gram-negative bacteria bloodstream infection and concurrent gut samples. Genetic relatedness was assessed using multilocus sequence typing and species-specific single-nucleotide polymorphism thresholds. Virulence gene profiles were characterized using the virulence factor database.

Results: In 18 of 22 cases (82%), blood and gut isolates were genetically highly related, supporting gut-to-blood translocation. All invasive *Escherichia coli* (7 over 7) strains consistently harboured haemolysin genes (*hlyA-D*), absent in all the noninvasive strains (2/2 p 0.028). Extremely preterm and low birth weight neonates were overrepresented among those with translocation.

Conclusions: Our findings support the role of gut-derived *Enterobacterales* in the pathogenesis of neonatal LOS. These insights may inform infection control and targeted preventive strategies. Further prospective studies are needed to validate these findings and guide interventions for high-risk neonates.

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Introduction

Neonatal sepsis remains a major global health concern, causing significant mortality and long-term morbidity. Although late-onset sepsis (LOS) is most often caused by coagulase-negative staphylococci, gram-negative bacteria (GNB) account for up to 36% of cases and are associated with higher mortality [1,2].

Increasing evidence suggests the gut as a potential source of neonatal sepsis. Colonization begins shortly after birth, primarily with maternal and environmental flora. Although most *Enterobacterales* are considered commensal organisms, the presence of virulence factors can lead to inflammation and translocation to normally sterile sites [3,4]. Colonization with GNB increases the risk of bloodstream infection (BSI) [5], and outbreak investigations often reveal genetic overlap between colonizing and invasive

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strains [3]. Although mechanisms remain unclear, hypotheses include direct translocation across an immature or compromised intestinal barrier or indirect routes related to immature host defences [6,7].

This study investigates the hypothesis that gut-colonizing *Enterobacteriales* contribute to gram-negative neonatal LOS (GN LOS) through direct bloodstream invasion and explores the virulence factors carried by invasive colonizers. The primary objective was to assess the genetic relatedness between blood and faecal isolates from a cohort of GN LOS. Additionally, the study characterizes the virulome profiles of invasive and colonizing strains.

Methods

Study design and data source

This study is a secondary exploratory analysis based on microbiological samples from the NeoMero-1 trial—a multicentre, randomized, open-label clinical trial comparing meropenem with standard-of-care antibiotic regimens (ampicillin + gentamicin or cefotaxime + gentamicin) in neonates with LOS [8]. LOS was defined by a positive blood culture and clinical/laboratory signs of infection in infants with postmenstrual age ≤ 44 weeks or meeting the Goldstein criteria for sepsis if postmenstrual age > 44 weeks. Between September 2012 and November 2014, the trial enrolled 271 neonates with culture-confirmed LOS across 18 neonatal intensive care units in six countries (Estonia, Greece, Italy, Lithuania, Spain, and Turkey) [8]. Stool or perirectal swabs were collected within 72 hours of baseline, at end-of-treatment (EOT), and at day 28 or neonatal intensive care units discharge to assess gram-negative gut colonization. For this secondary analysis, we selected a convenience sample of all neonates meeting the following criteria:

1. Confirmed GN-BSI (*Enterobacteriales* species) with available blood isolates for sequencing.
2. At least one rectal or faecal sample collected at any time point.
3. Availability of relevant clinical data.

Whole-genome sequencing (WGS) was performed on paired blood and faecal isolates from the same neonates. Sepsis onset was defined by the date of the first positive blood culture. Data analysed included patient demographics, risk factors, antibiotic treatment, clinical outcomes, and resistome/virulome profiles of the isolates.

Microbiological analysis

Bacterial isolates from blood were cultured from frozen stocks (-80°C) on blood agar plates. Stool or perirectal swabs were collected at predefined time points during the NeoMero-1 trial and immediately stored at -80°C . These samples were collected to support future microbiological analyses of GN colonization. For the present study, aimed at assessing gut colonization by *Enterobacteriales*, previously unused swabs were thawed once and directly inoculated onto Brilliance™ UTI chromogenic agar (Oxoid, UK). Colonies were differentiated based on morphology and colour, subcultured on blood agar, and identified by MALDI-TOF mass spectrometry (Bruker, Karlsruhe, Germany) [9]. The isolates included in this study were subjected to WGS using the Illumina MiSeq platform (Illumina, San Diego, CA, USA), with paired-end runs of 2×300 bp, after Nextera XT library preparation. The obtained reads were assembled using SPAdes [10]. For each genome, we determined the ST using an in-house script and the multilocus sequence typing (MLST) schemes and gene allele

sequences available on PubMLST (pubmlst.org). The isolates were further characterized at the genomic level with the identification of resistant and virulence genes using ABRicate (Seemann T, ABRicate, Github <https://github.com/tseemann/abricate>, accessed on: 11 November 2022) and the following databases: The Comprehensive Antibiotic Resistance Database [11] and Resfinder [12] for the resistance genes and VFDB [13] for the virulence genes. The *E. coli* strains were further subjected to in silico O–H–K serotyping determination using the tool ECTyper [14] and fastKaptive. [14]. *Klebsiella pneumoniae* strains were also assessed for virulence using Kleborate [15].

The *E. coli* strains were further subjected to in silico serotyping determination using the tool ECTyper [16], whereas the *K. pneumoniae* strains were assessed for virulence using Kleborate [15].

For each individual, the genome assemblies of the strains isolated from blood were aligned to those obtained from the gut using the tool Nucmer [17] ($-\text{mum} -\text{maxgap} = 500$ setting), and single-nucleotide polymorphisms (SNPs) were determined and counted. Then, for each pair, the likelihood of gut-blood translocation was assessed by combining MLST typing and SNP distance. Bacterial pairs isolated from both the gut were classified as ‘gut only’. Translocation was inferred when strains isolated from blood and gut had the same MLST profile and SNPs distance below the species-specific threshold defined by Hadjirin et al. [18]. More specifically, the authors describe a surveillance study on carbapenemase-producing *Enterobacteriales* in Ireland and provide the following thresholds: 12 SNPs for *K. pneumoniae*, 17 for *Klebsiella oxytoca*, 18 for *E. coli*, and 13 for *Enterobacter aerogenes*, *Enterobacter asburiae*, and *Enterobacter cloacae*. The strains for which the transmission was assessed were classified as ‘invasive’ and the other as ‘not invasive’. For *K. pneumoniae*, *K. oxytoca*, *E. coli*, and *E. cloacae*, the phylogenetic relationships among the strains isolated in this work were assessed by SNP-based phylogenetic analyses as follows. Core SNP calling was performed using the P-DOR tool [19], using the following reference assemblies for their respective species: *K. pneumoniae* strain HS11286 (GCA_000240185), *K. oxytoca* strain FDAARGOS_500 (GCA_003812925), *E. coli* strain K-12 substr. MG1655 (GCA_000005845), and *E. cloacae* strain UCI 24 (GCA_000534275.1). Each core SNP alignment was subjected to maximum likelihood (ML) phylogenetic analysis using IQ-TREE2 software [20]. The phylogenetic trees were annotated using the gplots R library. The genome assemblies generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) under BioProject accession PRJNA1162022.

Ethical consideration

The NeoMero study protocol underwent approval by central ethics review committees for each consortium partner and local ethics approval in all sites [8]. The objectives of the present analysis fell within the scope of the original study.

Results

Description of the sample

Among the patients enrolled during the NeoMero study, 49 infants had a blood culture positive for GNB. Of these, 35 (35/49, 71.4%) infants had blood and rectal swabs collected for analysis and were included in this sub-study. Rectal swabs were obtained from 28 infants on the day of sepsis onset, 5 infants at the EOT, and 2 infants during the follow-up period as the only available sample. Details on the collection time are included in Table S1. Out of the 35 patients, bacteria isolated from the rectal swab (at any time

point) were the same species as those from bloodstream isolates in 22 patients (22/35, 63%). In 10 (10/35, 29%) patients, isolates from faecal flora and bloodstream were of different species. For the remaining three patients (3/35, 9%), isolates from the rectal swab were of poor quality, and identifying the bacteria was impossible (Fig. 1). All information regarding the phenotypic resistance profiles of the isolated species is provided in Table S1.

Genetic relatedness

WGS was performed for the 22 neonates with the same bacteria in their blood and at least one faecal sample. The 50 strains included in the study (Table S1) were grouped into 34 strain pairs based on species and origin, combining strains of the same species isolated from the same patient (Table S2). *Enterobacter spp.* was the most frequently isolated pathogen from the 22 patients involved in the study (13/22 patients, 59%), followed by *Klebsiella spp.* (5/22 patients, 23%) and *E. coli* (4/22 patients, 18%). In silico serotyping of *E. coli* strains showed a total of four different O antigens (O1, O4, O15, and O145), four H antigens (H5, H6, H7, and H18), and four K antigens (KX03, KX54, KX75, and K96). The only patient having strains with different O:H:K typing was patient 14. For more details, see Table S1.

Among the 22 patients, 18 (18/22, 82%) show signals of gut-to-blood translocation, having strains isolated from the gut genetically related to those from the blood. Three patients (3/22, 13%) had faecal and blood isolates with a genetic distance of more than 400 SNPs, suggesting independent gut and blood colonizations. One patient had isolates with the same sequence type and a genetic distance of 42 base pairs. We classified the strains as nonrelated, but

considering the low distance, we cannot exclude a possible gut-to-blood translocation. Of the 22 patients, 18 (18/22, 82%) were classified as associated with gut-to-blood translocation, and 4 patients (4/22, 18%) as nonassociated with gut-to-blood translocation (Table S2). We classified 44 out of 50 strains (44/50, 88%) as 'invasive' (when genetic data supported translocation) and the remaining six strains (6/50, 12%) as 'noninvasive' (Table S3). The SNP-based phylogenetic trees showing the genetic relationships between the strains included in the study are reported in Fig. 2. The trees, annotated with strains' information, are mainly coherent with the SNP distance-based gut-blood transmissions reported above. All the invasive strains isolated from the same patients resulted in being closely related on the tree and harbouring the same panel of resistance genes. Interestingly, a group of invasive strains of *E. cloacae* isolated in Estonia from different patients is grouped in a phylogenetic clade, suggesting a possible inter-patient strain transmission within the hospital. Supporting this hypothesis, the isolates were collected in the same hospital during the period from April 2013 to September 2013.

Virulome profile of invasive and noninvasive isolates

In Fig. 3, the heatmap construction and hierarchical clustering of virulence genes in blood and faecal samples compare the isolates at a single gene level.

Overall, the genome sequencing identified 309 virulence genes among all the pathogens. The analysis of the association between virulence gene presence/absence and the 'invasive' phenotype identified genes potentially involved in the invasive capability of *E. cloacae* and *E. coli*. Specifically, in *E. cloacae*, the *entB* gene, coding

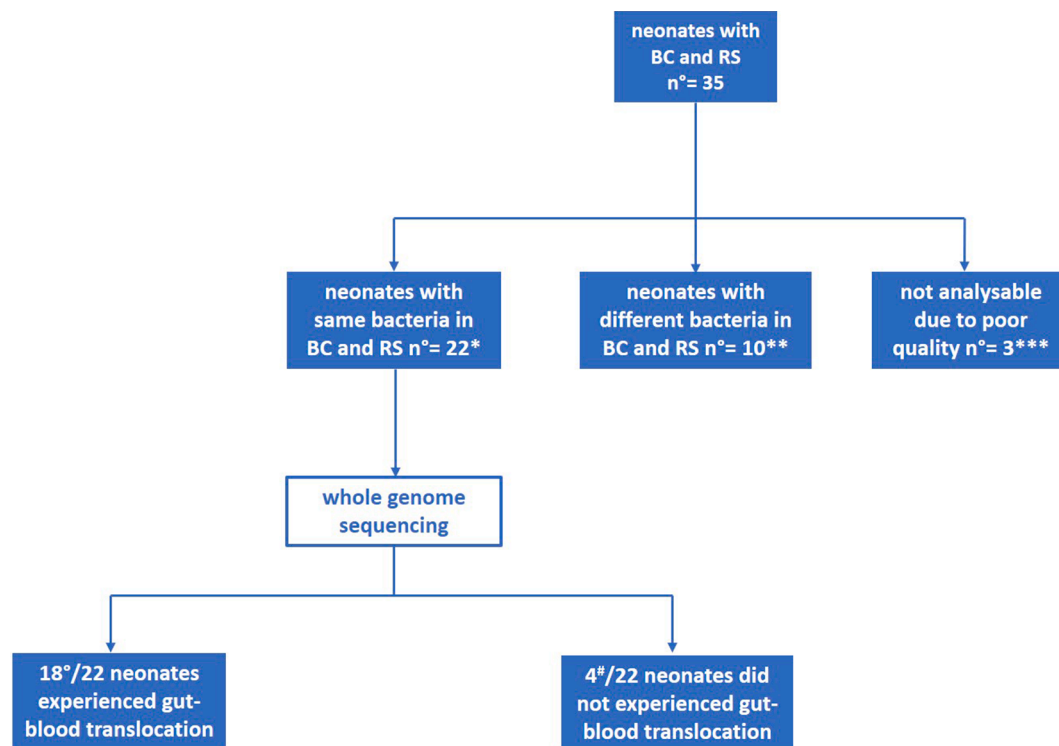


Fig. 1. Study flow chart. BC, blood culture; RS, rectal swab; SNP, single-nucleotide polymorphism.

*Neonates with same bacteria in BC and RS (n = 22): *Enterobacter aerogenes* (1), *Enterobacter asburiae* (1), *Enterobacter cloacae* (11), *Escherichia coli* (4), *Klebsiella oxytoca* (3), and *Klebsiella pneumoniae* (2). **Neonates with different bacteria in BC and RS (n = 10): *Enterobacter asburiae* (1), *Klebsiella oxytoca* (3), *Klebsiella pneumoniae* (4), *Proteus mirabilis* (1), *Serratia marcescens* (1)—isolates from blood culture. ***Not analysable: *Enterobacter asburiae* (1), *Enterobacter cloacae* (2)—isolates from blood culture. Gut-blood translocation (n = 18): *Enterobacter aerogenes* (1), *Enterobacter asburiae* (1), *Enterobacter cloacae* (9), *Escherichia coli* (3), *Klebsiella oxytoca* (2), and *Klebsiella pneumoniae* (2). #No gut-blood translocation (n = 4): *Enterobacter cloacae* (2), *Escherichia coli* (1), and *Klebsiella oxytoca* (1).

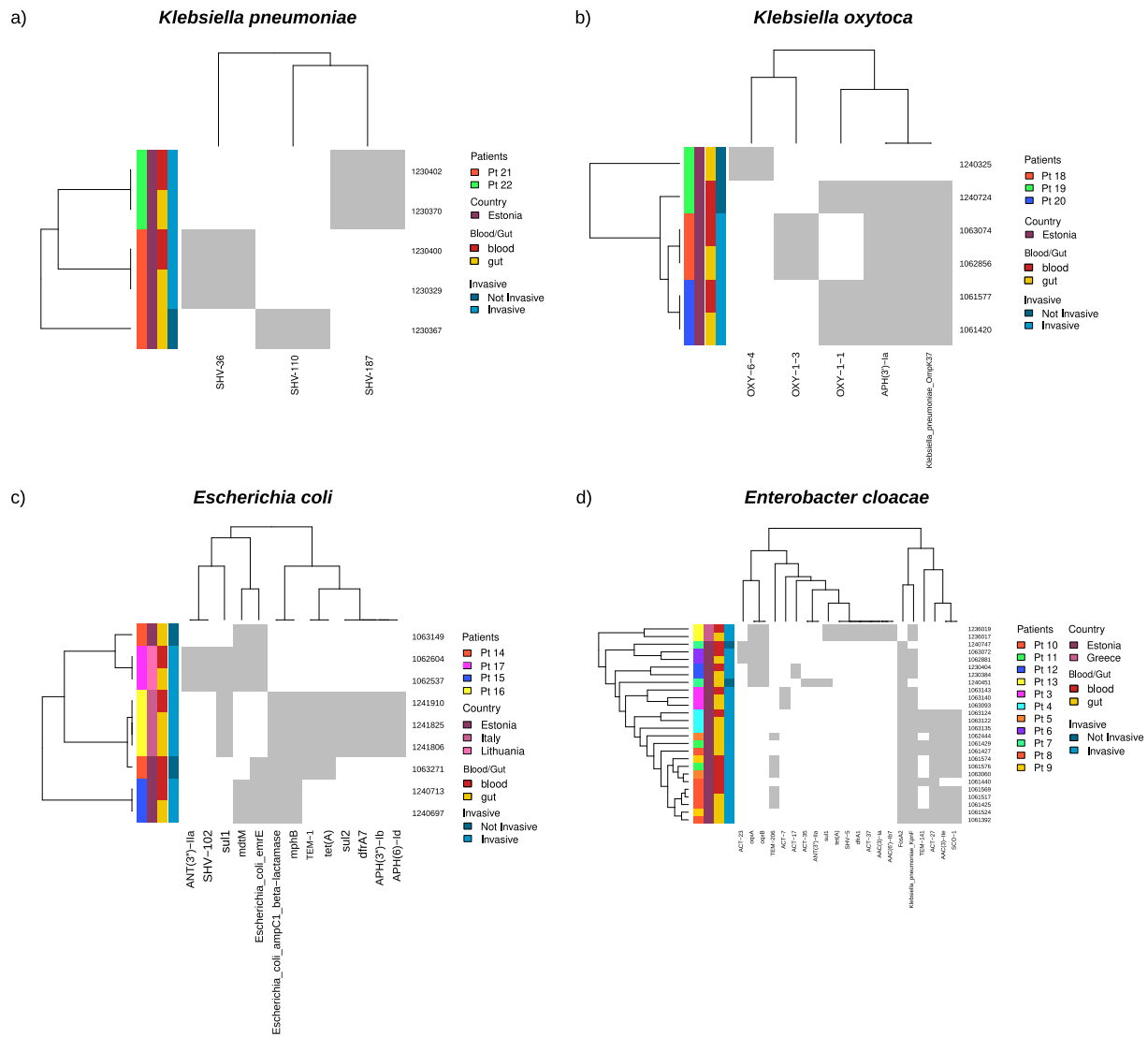


Fig. 2. Heatmap showing the presence/absence of antibiotic resistance genes (ARGs) among the strains included in the study. Each panel refers to a species of *Enterobacteriales* isolated from neonates with Gram-negative LOS: (a) *Klebsiella pneumoniae*; (b) *Klebsiella oxytoca*; (c) *Escherichia coli*; and (d) *Enterobacter cloacae*. On the left side of each heatmap, the core SNP-based phylogenetic tree of the isolates annotated with metadata (species, patient ID, sample type, and collection site). ARGs columns in each heatmap are sorted on the basis of their presence/absence pattern among the strains (represented by the dendrogram on top). AAC(3)-Ia, aminoglycoside 3-N-acetyltransferase type Ia; AAC(3)-IIe, aminoglycoside 3-N-acetyltransferase type IIe; AAC(6')-Ib7, aminoglycoside 6'-N-acetyltransferase type Ib7; ACT, class C beta-lactamase ACT; ampC1, Escherichia coli class C beta-lactamase AmpC1; APH(3'')-Ib, aminoglycoside 3''-phosphotransferase type Ib; APH(6'')-Id, aminoglycoside 6''-phosphotransferase type Id; ANT(3'')-IIa, aminoglycoside 3''-nucleotidyltransferase type IIa; dfrA1, dihydrofolate reductase type A1; dfrA7, dihydrofolate reductase type A7 (trimethoprim resistance); emrE, Escherichia coli small multidrug resistance protein EmrE; FosA2, glutathione S-transferase FosA2; KpnF, small multidrug resistance (SMR) efflux pump KpnF; mdtM, multidrug resistance transporter M; mphB, macrolide 2'-phosphotransferase B; OmpK37, outer membrane porin K37; OXY, Klebsiella oxytoca-derived beta-lactamase; oqxA, resistance-nodulation-cell division (RND) efflux pump subunit OqxA; oqxB, RND efflux pump subunit OqxB; SCO, small multidrug resistance protein SCO; SHV, sulfhydryl variable-type beta-lactamase; sul1, sulfonamide resistance gene 1; sul2, sulfonamide resistance gene 2; TEM, class A beta-lactamase TEM; tet(A), tetracycline resistance gene A (efflux pump).

for Enterobactin synthase component B, was absent in 24 out of 25 (24/25, 96%) invasive strains and present in one of the two non-invasive strains (Fischer's exact test p value 0.08). In *E. coli*, the *hlyA*, *hlyB*, *hlyC*, and *hlyD* genes, part of the operon for the synthesis of the haemolysin, were found in all the invasive phenotype (100%) and not found in all the noninvasive (Fisher's exact test, p value 0.028), whereas the *cvaC* and *kpsD* genes (coding, respectively, for Colicin V and for Polysialic acid transport protein KpsD) were present in noninvasive strains only (Fisher's exact test, P value 0.028). For *K. pneumoniae*, the analysis did not identify virulence genes associated with the gut-to-blood translocation, and the strains were not classified as hypervirulent (i.e. Kleborate virulence score = 0).

Clinical characteristics of patients with potential gut-blood translocation

Among the 35 neonates included in the study, the median age at BSI onset was 15 days (IQR 7.6–27.3). Half of the neonates were extremely preterm (16/35, 46%) and extremely low-birthweight (16/35, 46%). Twelve out of 35 (12/35, 34%) had at least an underlying medical condition, 21 (21/35, 60%) had a central line in situ, and half of them (20/35, 57%) had a previous antibiotic therapy. Table 1 presents a comparison of clinical characteristics and outcomes between neonates colonized by related blood-stream pathogens (derived from gut-blood translocation, $n = 18$) and those with different bacteria in blood and rectal swabs (10

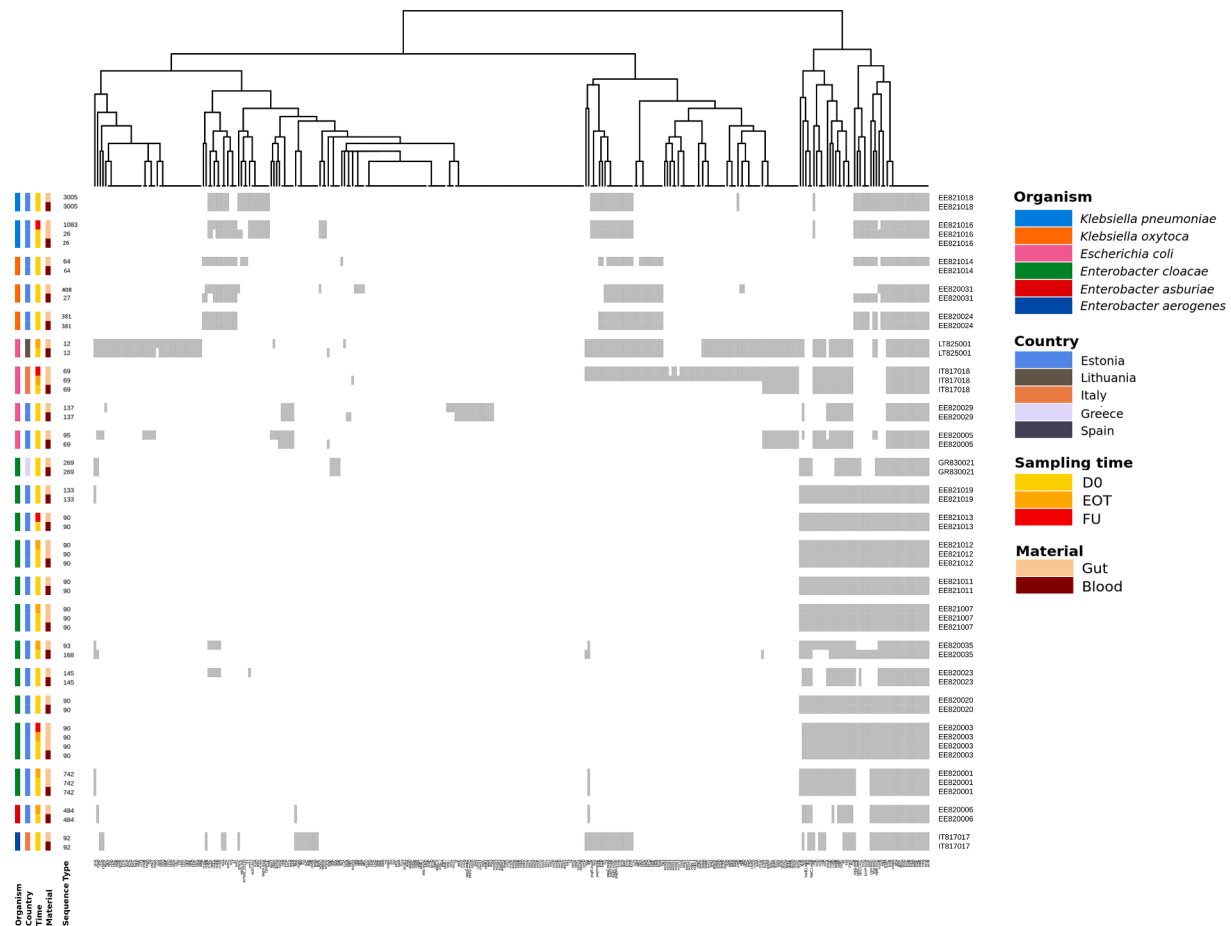


Fig. 3. Heatmap and hierarchical clustering of virulence genes in blood and faecal *Enterobacteriales* isolates. Each row represents a strain (with strains from the same patient grouped together), and each column represents a virulence gene. Grey rectangles indicate gene presence. The top dendrogram clusters genes by similarity in presence/absence patterns, whereas the left-side annotations display strain metadata, including species, country, time of isolation, and sample source, as detailed in the legend.

isolates), or with the same pathogen but with a higher genetic distance, making gut-blood translocation unlikely ($n = 4$). Neonates with probable gut-blood translocation were more frequently extremely preterm (12/18 vs. 3/14) with a median birth gestational age of 25.9 weeks (IQR 25–33.1) versus 31.6 weeks (IQR 28.4–33). Moreover, having extremely low birth weight and having had a relapse of BSI were more frequent among those with probable gut-blood translocation (4/14 vs. 12/18 Fisher's exact test p 0.03, 0/14 vs. 7/18 $p < 0.01$, respectively).

Discussion

This exploratory analysis of microbiological samples from the NeoMero-1 trial examines the relationship between gut colonization, virulence factors, and clinical outcomes in neonates with GN LOS.

Enterobacter spp. was the most isolated pathogen, followed by *Klebsiella* spp. and *E. coli*, consistent with the NeoMero trial's overall GN [8]. This likely reflects the trial's inclusion of LOS cases only, whereas *E. coli* is more common in early onset sepsis due to maternal transmission, and the demanding trial's inclusion criteria resulted in recruitment of very sick patients [8]. The phylogenetic analysis identified a clade of *E. cloacae* isolated from different patients in Estonia, suggesting a possible inter-patient strain transmission.

Among a subset of 35 neonates with paired blood and faecal samples, our findings demonstrate that most sepsis episodes are

caused by gut-colonizing bacteria. These findings align with prior studies showing that gut colonization often precedes sepsis episodes [21,22].

We explored whether virulence genes are associated with the ability of colonizing bacteria to cause invasive disease. In our sample, invasive *E. coli* strains consistently carried the *hlyA*, *hlyB*, *hlyC*, and *hlyD* genes, which encode haemolysin—a toxin known to disrupt host tissues and evade immune responses. These genes were absent in noninvasive strains, suggesting a potential role in gut-to-blood translocation. This finding aligns with previous studies linking haemolysin to invasive neonatal *E. coli* strains when compared with gut flora [23,24]. Studies have reported no such association in sepsis due to urinary tract infections [25] and found a lower prevalence of *hly* genes in isolates from neonates' Cerebrospinal Fluid (CSF) compared with blood isolates [26], indicating that haemolysin may play a more specific role in translocation from the gut rather than in all forms of bacteraemia.

Despite the small number, we observed gut translocation more frequently in extremely preterm and very low birth weight infants. Their physiological immaturity—including reduced gastric acid, mucus, intestinal motility, and secretory IgA—favours bacterial adherence and increases infection risk [27], further emphasizing the critical role of gut barrier immaturity in neonatal sepsis.

By clarifying the relationship between enteric and bloodstream bacteria in neonatal sepsis, the findings of this study may have implications for clinical practice. Demonstrating such a correlation could inform surveillance strategies, such as microclusters to limit

Table 1
Demographic and clinical characteristics of patients, overall and stratified by concordance between faecal and bloodstream isolates

Patients characteristics	All septic newborn N = 35 ^a	GBT unlikely N = 14	Newborns with possible GBT N = 18	p Value
Country				0.45
Estonia	23 (66%)	9 (39%)	14 (61%)	
Greece	4 (11%)	3 (75%)	1 (25%)	
Italy	4 (11%)	2 (50%)	2 (50%)	
Lithuania	1 (3%)	0 (0%)	1 (100%)	
Spain	1 (3%)	0 (0%)	0 (0%)	
Sex				0.20
Female	21 (60%)	7 (35%)	13 (65%)	
Male	12 (34%)	7 (58%)	5 (42%)	
Gestational week, median	28.57 (25–32.71)	31.57 (28.43–33)	25.86 (25–31)	0.12
Gestational age category (weeks of GA)				0.041
<28 0/7	16 (46%)	3 (20%)	12 (80%)	
28 0/7–31 6/7	7 (20%)	5 (71%)	2 (29%)	
32 0/7–33 6/7	5 (14%)	3 (60%)	2 (40%)	
34 0/7–36 6/7	3 (9%)	3 (100%)	0 (0%)	
37 0/7–38 6/7	1 (3%)	0 (0%)	1 (100%)	
39 0/7–40 6/7	1 (3%)	0 (0%)	1 (100%)	
Birth weight grams, median	1077 (730–1879)	1610 (800–1898)	856 (650–1450)	0.12
Birth weight category (grams)				0.034
≥2500	5 (14%)	2 (40%)	3 (60%)	
1500–2500	8 (23%)	7 (88%)	1 (13%)	
1000–1500	4 (11%)	1 (33%)	2 (67%)	
<1000	16 (46%)	4 (25%)	12 (75%)	
Small for gestational age	2 (6%)	1 (50%)	1 (50%)	0.85
Underlying conditions	12 (34%)	4 (33%)	8 (67%)	0.36
Central line	21 (60%)	9 (43%)	12 (57%)	0.89
Surgery	8 (23%)	3 (38%)	5 (63%)	0.68
Previous antibiotic therapy	20 (57%)	10 (50%)	10 (50%)	0.36
Days of life at onset, median	14.78 (7.36–28.34)	11.26 (6.16–57.13)	15.74 (8.52–25.46)	0.79
Relapse	7 (20%)	0 (0%)	7 (100%)	0.008
Dead (at 28 d)	2 (6%)	2 (100%)	0 (0%)	0.098

Categorical variables were analysed using Fisher's exact test and continuous variables with the Wilcoxon test. Analyses were descriptive due to small sample size and performed in Stata BE 17.1. GBT: Gut-blood translocation.

^a Three patients had no analysable gut isolates, see Fig. 1.

the size of the colonization, and support more targeted antibiotic use. Insights into virulence factors like haemolysin may also guide the development of preventive tools, including vaccines or targeted treatments. Since most GNB are commensals, identifying virulence profiles associated with invasiveness could help flag high-risk strains, enabling early interventions or intensified monitoring.

This study has several limitations. It is a secondary data analysis of stored isolates from the largest European cohort of neonates with GN LOS, and it was not designed to establish gut-blood translocation. As an exploratory descriptive analysis, it lacked the statistical power to detect significant differences in the presence of individual virulence genes between paired blood and faecal isolates. Although we could compare virulence profiles between cases with and without evidence of gut translocation, the absence of a control group of colonized but nonseptic neonates limits the generalizability of our findings. The random selection of colonies based on morphology and colour may have led to missed isolates, potentially affecting results in cases without confirmed translocation. The dynamic nature of bacterial genetics and reliance on single time point isolates may have further influenced findings. Additionally, since blood and faecal samples were collected simultaneously at sepsis onset or later, we cannot rule out reverse causality or confirm that colonization preceded infection. Finally, as the study focused on LOS in predominantly premature infants, the findings may not be generalizable to the broader neonatal population.

Conclusion

Rising rates of GN neonatal sepsis and growing antimicrobial resistance call for innovative prevention strategies. Our study highlights the role of gut colonization and bacterial virulence in the pathogenesis of GN LOS and demonstrates the value of WGS in tracing transmission. Larger prospective studies are needed to validate these findings and explore virulence profiles systematically to inform risk stratification and targeted interventions.

CRedit authorship contribution statement

LF, LB, MS, and FC contributed to the conception and design of the study. LF, LB, FC, SP, FFF, LS, EC, AB, ALM, and SP contributed to the acquisition and analysis of the data. FC, LS, and SP performed and interpreted WGS analysis. LF, LB, MS, FC, and GVZ contributed to the interpretation of data. LF, LB, and FC drafted the article. MS, SP, FF, LS, EC, AB, ALM, SP, and GVZ critically revised the paper for important intellectual content. All the authors approved the final version of the paper to be submitted.

Transparency declaration

Potential conflict of interest

The authors declare no conflict of interest related to this work.

Financial report

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cmi.2025.07.025>.

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