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Characterization of antimicrobial-resistant Gram-negative bacteria that cause neonatal sepsis in seven low- and middle-income countries

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Antimicrobial resistance in neonatal sepsis is rising, yet mechanisms of resistance that often spread between species via mobile genetic elements, ultimately limiting treatments in low- and middle-income countries (LMICs), are poorly characterized. The Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS) network was initiated to characterize the causes and burden of antimicrobial resistance in neonatal sepsis for seven LMICs in Africa and South Asia. A total of 36,285 neonates were enrolled in the BARNARDS study between November 2015 and December 2017, of whom 2,483 were diagnosed with culture-confirmed sepsis. Klebsiella pneumoniae (n = 258) was the main cause of neonatal sepsis, with Serratia marcescens (n = 151), Klebsiella michiganensis (n = 117), Escherichia coli (n = 75) and Enterobacter cloacae complex (n = 57) also detected. We present whole-genome sequencing, antimicrobial susceptibility and clinical data for 916 out of 1,038 neonatal sepsis isolates (97 isolates were not recovered from initial isolation at local sites). Enterobacterales (K. pneumoniae, E. coli and E. cloacae) harboured multiple cephalosporin and carbapenem resistance genes. All isolated pathogens were resistant to multiple antibiotic classes, including those used to treat neonatal sepsis. Intraspecies diversity of K. pneumoniae and E. coli indicated that multiple antibiotic-resistant lineages cause neonatal sepsis. Our results will underpin research towards better treatments for neonatal sepsis in LMICs.

Although there has been a substantial decrease in infant mortality over the past 20 years, the burden remains substantial, with the mortality rate of children under 5 years of age at 38 per 1,000 live births in 2019, and 98% of recorded neonatal deaths now occurring in LMICs. The World Health Organization (WHO) has declared neonatal sepsis a global concern and the burden of neonatal infectious diseases a major challenge. Effective management of sepsis is not always possible when resources are limited, and the steady increase of antimicrobial resistance (AMR) worldwide further compromises sepsis management.

Despite the burden of neonatal sepsis, accurate information on the causes and consequences of neonatal sepsis in LMICs is scarce. Most studies in LMICs are from a single site, are of limited sample size, or lack accurate methods for sepsis diagnosis, pathogen identification and antibiotic susceptibility measurements. In 2015 and 2016, two multicentre neonatal sepsis studies in LMICs were published. However, neither study combined antimicrobial susceptibility testing and whole-genome sequencing (WGS), making it difficult to determine the extent of genomic diversity (which would usually be done by comparing the lineages across geographical
Fig. 1 | Flow chart detailing the enrolment of neonates and incidence of sepsis (both clinically diagnosed and confirmed by culture) per site. The numbers of neonates with missing clinical data for the onset and outcome of sepsis are shown per site. The numbers of isolates collected from neonatal blood cultures are shown per site, with a breakdown by preliminary characterization (as determined by Gram stain) in the flow chart below. The final numbers of isolates included for analysis are highlighted in orange. BB, baby blood; ID, identification (of species); ND, not determined.

Results

Enrolment in BARNARDS and isolation of pathogenic bacteria. The numbers of neonates recruited, clinically diagnosed with sepsis and with a confirmation of sepsis by positive blood culture are outlined in Fig. 1. Of 36,285 infants ≤60 d old (termed herein as neonates) enrolled in the BARNARDS study from November 2015 to December 2017, 2,483 had culture-confirmed sepsis. All 12 clinical sites used the same criteria for clinical diagnosis of sepsis (Supplementary Fig. 1). We found that cases were mainly EOS for both sites in Pakistan, one site in Bangladesh (BC; see Methods for definitions of all two-letter site abbreviations) and the single site in Ethiopia. In Nigeria, India and South Africa, there were mainly LOS cases. In Rwanda, neonatal sepsis cases were equally split between EOS and LOS (Fig. 2).

Automated blood culture systems were used to detect microbial growth, with 2,620 microbial isolates recovered. These 2,620 isolates comprised 1,266 Gram-positive bacteria (GPB) isolates, 1,038 GNB isolates, 22 fungal isolates and 294 unassigned isolates (Fig. 1). The methods for collection and identification were standardized across all sites, with equipment and reagents purchased from uniform suppliers. The primary aim of the BARNARDS study was to characterize the extent of β-lactam resistance in GNB causing clinically diagnosed sepsis in infants <60 d old (see Supplementary Figs. 2 and 3). However, at month 17 (during a BARNARDS network event), we anecdotally noted high rates of isolation of *Staphylococcus* species and therefore collected all of these isolates for analysis (to be reported elsewhere). Of 1,038 isolates, 916 GNB were analysed using WGS. For 122 isolates, identification beyond GNB was not possible because the isolate was lost and/or purification for DNA extraction was unsuccessful (Fig. 1).

In total, 58 different species of GNB were identified across all sites by WGS (Supplementary Table 1), including *K. pneumoniae* (n = 258), *Serratia marcescens* (n = 151), *Klebsiella michiganensis* (n = 117), *Enterobacter* species (n = 80), *E. coli* (n = 75), *Burkholderia* species (n = 61), *Acinetobacter* species (n = 49), *Pseudomonas*...
species \( (n = 36) \) and *Ralstonia mannitolilytica \( (n = 21) \) \( (\text{Fig. 2}) \). Among the GNB characterized herein, 401 were Klebsiella, with six species identified: *K. pneumoniae*, *K. variicola*, *K. quasipneumoniae*, *K. aerogenes*, *K. oxytoca* and *K. michiganensis* \( (\text{Supplementary Fig. 4}) \). *Burkholderia cenocepacia*, *K. michiganensis*, *R. mannitolilytica* and *S. marcescens* were mainly isolated from samples obtained from single sites in Pakistan, Nigeria and Bangladesh \( (\text{Fig. 2}) \).

Overall, *R. mannitolilytica*, *K. michiganensis*, *Burkholderia* species and *Pseudomonas* species caused more cases of EOS than other species \( (\text{Supplementary Table 2 and Supplementary Fig. 5}) \). Of note, similar proportions of fatal sepsis cases were caused by GNB on each continent \( (92/353 \text{ cases in Africa (21%)} \) and 118/475 cases in Asia (20\%))\). We found that *Burkholderia* species or *K. michiganensis* sepsis infections were the most likely to be fatal \( (\text{Supplementary Table 2 and Supplementary Fig. 5}) \). However, there was a large proportion of missing data from certain clinical sites \( (\text{due to data collection and entry error; Fig. 1}) \), which limited the strength of conclusions.

**Interspecies and intraspecies diversity across clinical sites.** To understand the extent of inter- and intraspecies diversity, we aimed to perform WGS on all GNB. Multilocus sequence typing \( (\text{MLST}) \) was used primarily as an unambiguous tool to examine bacterial relatedness. As MLST can be performed easily via PCR of housekeeping genes, in addition to in silico MLST via WGS, assessing relatedness via this tool \( (\text{where applicable}) \) provides a dataset that can be extrapolated to existing data on neonatal sepsis where WGS may not be available.

MLST revealed substantial intraspecies diversity, and 40 previously unknown STs were assigned in 12 species \( (\text{Table 1 and Supplementary Table 2}) \). Fourteen STs were assigned in the *Klebsiella* genera \( (\text{Table 1}) \), including all three *K. aerogenes* STs \( (\text{all from Africa}; n = 2 \text{ from Nigeria and } n = 1 \text{ from South Africa}; ST194–196) \). *K. michiganensis* was mainly ST180 \( (\text{from PP in Pakistan; Table 1}) \). Such low ST diversity and close phylogenetic relatedness, as shown by the core genome phylogeny \( (\text{Supplementary Fig. 6}) \), warrant further investigation. Similarly, we noticed that a single, previously unknown, *B. cenocepacia* ST \( (\text{ST1621}) \), also from PP, was dominant \( (\text{Table 1 and Supplementary Fig. 7}) \). *B. cenocepacia* ST1621 from PP and the *S. marcescens* isolates from BC were indistinguishable during core genome analysis \( (\text{Supplementary Figs. 7 and 8}) \). All local-level clusters will be studied further.

*Enterobacter cloacae* complex isolates were identified belonging to *E. cloacae*, *E. hormaechei*, *E. kobei*, *E. asburiae* and *E. ludwigi*. In total, seven different *Enterobacter* species \( (n = 80) \) with 28 STs were identified. The majority of *Enterobacter* species were found in Pakistan \( (n = 39) \), Nigeria \( (n = 19) \) and Rwanda \( (n = 14) \) \( (\text{Fig. 1 and Supplementary Fig. 9}) \). ST171 was common across sites in Africa and Asia; however, ST346 was only detected in Rwanda, ST523 was only detected in Pakistan and ST850 was only detected in Nigeria. Within *Enterobacter* species, 13 STs were assigned \( (\text{Table 1}) \) to *E. cloacae*, *E. hormaechei* and *E. ludwigi* isolates.

*Acinetobacter* species were recovered from ten out of 12 clinical sites in both Africa and South Asia, and 38 out of 49 \( (78\%) \) were *Acinetobacter baumannii*. Of these, 17 out of 38 \( (45\%) \) belonged to international clones \( (\text{Pasteur MLST}) \) ST1 and ST2 \( (\text{Supplementary Tables 1 and 2}) \).

**AMR of pathogens causing neonatal sepsis.** One aim of the BARNARDS study was to describe the AMR profiles of pathogens causing neonatal sepsis. For this, we performed agar dilution to determine the minimum inhibitory concentrations \( (\text{MICs}) \) of 19 antibiotics, including the current recommended first-line empirical...
treatments for neonatal sepsis, as well as carbapenems (the incidence of carbapenem-resistant GNB is increasing at an alarming rate), on 885 GNB (31 isolates were not recovered following storage at −80 °C after genomic DNA extraction for WGS; Supplementary Table 2 lists the isolates recoverable for WGS and/or MIC testing). Current data in LMICs focus on profiling AMR within certain species only, and are therefore not exhaustive across all pathogens causing sepsis.

GNB (n=885) were resistant to ampicillin (95%), cefotaxime (83%) and ceftiraxone (80%), whereas they were sensitive to meropenem (13%), imipenem (15%) and tigecycline (16%) (Fig. 3a). The MICS required to inhibit the growth of 50% of organisms (that is, MIC\textsubscript{90} values) of piperacillin/tazobactam, carbapenems (imipenem, meropenem and ertapenem), amikacin, fosfomycin, quinolones (ciprofloxacin and levofloxacin) and colistin were lower than their resistance breakpoints. The MIC\textsubscript{90} values of all antibiotics tested were higher than their resistance breakpoints, with the exception of tigecycline, for which the MIC\textsubscript{90} was lower than the epidemiological cut-off value for Providencia and Proteus species\textsuperscript{30,31}.

Overall, GNB isolates resistant to at least one of the cephalosporins tested were less likely to cause LOS than EOS (P=0.017; odds ratio (OR)=0.63; 95% confidence interval (CI)=0.43–0.92; Supplementary Table 3). For the statistical analysis, the outcome measurement was MFBS (deceased as response, alive as reference). Concomitant resistance to the three cephalosporins tested versus isolate susceptibility to all produced an odds ratio of 0.626; 95% CI 0.23–0.98; P=0.017. In this way, concomitant resistance to the three cephalosporins tested among GNB isolates was less likely among infants who stayed alive compared to those who were deceased.

As a marker of extended-spectrum β-lactamase antibiotic resistance gene (ARG), bla\textsubscript{KPC}, was inspected. It was detected in at least nine species (n=523 isolates) and found in isolates from all study sites. We also screened genomes for genes coding for carbapenem resistance (bla\textsubscript{NDM}, bla\textsubscript{OXA-48}-like variants and bla\textsubscript{KPC}) 146 single carbapenemase genes in ten species (n=128 isolates), and two carbapenem resistance gene homologues were present in 24 isolates. bla\textsubscript{NDM-1} (n=90; Bangladesh, n=23; India, n=6;...
Fig. 3 | AMR of neonatal sepsis-causing pathogens. a. Percentages of antimicrobial-resistant aetiologic agents of neonatal sepsis, coloured according to bacterial species/group (n = 885 isolates of GNB). The MICs of the antibiotics were determined by agar dilution and the results were interpreted according to EUCAST guidelines and documents. AMC, amoxicillin/clavulanate; AMK, amikacin; AMP, ampicillin; ATM, aztreonam; CAZ, cefazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CST, colistin; CTX, cefotaxime; ETP, ertapenem; FEP, cefepime; FOF, fosfomycin; GEN, gentamicin; IPM, imipenem; TZP, piperacillin/tazobactam. b, Sunburst diagram detailing the class A (red), B (yellow) and D (green) carbapenemase resistance genes detected. The second ring from the centre shows the carbapenemase genes identified. The distributions across species and clinical sites are shown in the outer rings. ABU, Acinetobacter baumannii; CFI, Citrobacter freundii; ECO, Escherichia coli; ENT, Enterobacter cloacae complex; KMI, Klebsiella michiganensis; KPN, Klebsiella pneumoniae; KQI, Klebsiella quasipneumoniae; PRO, Providencia rettgeri; SER, Serratia marcescens.

Fig. 4 | Sankey diagram showing the distribution of the different plasmid types detected linking to the GNB in which they were found. Plasmid types (left) found to carry carbapenemase AMR genes are colour coded and linked to the GNB species (right) in which the plasmid type was identified. Particular carbapenemase genes are shown on the far right.
Nigeria, n = 16; Pakistan, n = 43; Rwanda, n = 1; South Africa, n = 1) and bla<sub>NDM-5</sub> (n = 3; Bangladesh, n = 1; India, n = 1; Nigeria, n = 1) were mainly detected in clinical sites in South Asia, whereas bla<sub>NDM-7</sub> was predominately recovered from Nigeria and Pakistan (n = 19; Bangladesh, n = 1; Nigeria, n = 11; Pakistan, n = 7) (Fig. 3b). In 79 GNB, bla<sub>CTX-M-15</sub> plus bla<sub>NDM</sub> and/or a bla<sub>OXA-48</sub>-like variant were found. In total, 30 GNB carried a variant of the bla<sub>OXA-48</sub>-like family, with bla<sub>OXA-181</sub> being the most frequent (Pakistan, n = 22; India, n = 1; Fig. 3b). bla<sub>OXA-23</sub> was only found in Bangladesh (n = 5), and the two isolates carrying bla<sub>OXA-48</sub> (one K. michiganensis and one S. marcescens) were from PP. bla<sub>NDM</sub> was found in three Pseudomonas aeruginosa isolates (Bangladesh, n = 2; BC, n = 1; BK, n = 1); India, n = 1). Both bla<sub>NDM</sub> variants from Bangladesh were bla<sub>NDM-1</sub>, whereas the variant detected in India was bla<sub>NDM-6</sub>.

In Enterobacter species, bla<sub>CTX-M-15</sub> was found in isolates recovered from both Africa and South Asia (n = 19; Africa, n = 16; South Asia, n = 3); however, Enterobacter containing carbapenemase genes (n = 18) were largely recovered from South Asia (South Asia, n = 16; Africa, n = 2). Five Acinetobacter (A. baumannii, n = 3; Acinetobacter berezzini, n = 1; Acinetobacter nosocomialis, n = 1) were found to have bla<sub>KPC</sub> (Africa, n = 2; South Asia, n = 3) (Fig. 3b). Additionally, we found bla<sub>OXA-23</sub> within 20 A. baumannii isolates.

The total number of ARGs possessed by each bacterial isolate is shown in Supplementary Table 4. For each ARG increase among E. cloacae, we observed a 13.2% decrease in the likelihood of neonates having LOS (P = 0.016; 95% CI = 0.77–0.97). No other associations between ARG and onset were found (Supplementary Table 3).

Worryingly, we found that 529 (60%) of the GNB isolates tested were resistant to the first-line empirical treatment for neonatal sepsis (both ampicillin and gentamicin).

Plasmids and carbapenemase genes. As many ARGs are carried on mobile genetic elements such as plasmids, we searched for plasmid replicon types in isolates from the different geographical areas and, where possible, analysed linkages between plasmid type and the carriage of specific carbapenemase genes. We detected 1,124 plasmids with 45 incl gene variants, which we categorized into 18 plasmid groups. From these, 1,093 were found within E. coli (n = 169), K. pneumoniae (n = 623), K. michiganensis (n = 142), K. quasipneumoniae (n = 28), Enterobacter species (n = 87) and S. marcescens (n = 44) (Fig. 4). There were 12 plasmid types found within the seven S. enterica isolates, seven among Citrobacter species and five among K. variicola isolates.

Within the six dominant plasmid carriers (Fig. 4), the most frequently detected incl type was IncFIB, with 255 out of 440 hits within K. pneumoniae genomes. bla<sub>NDM-1</sub> was found in IncA/C2 plasmids from K. pneumoniae and Enterobacter species from PP, as well as in IncFIB plasmids from K. pneumoniae from India (Fig. 4 and Supplementary Fig. 10). We found IncX3 plasmids carrying bla<sub>NDM-5</sub> in K. pneumoniae from NK, Nigeria, and multiple bla<sub>NDM-1</sub> in K. pneumoniae and Enterobacter species from Nigeria and Pakistan (Fig. 4 and Supplementary Fig. 10). Col plasmid types were identified within 82 genomes. We found ColKP3 plasmids carrying bla<sub>SK34</sub> in isolates of three different species: E. coli, K. pneumoniae and Enterobacter species (Fig. 4 and Supplementary Fig. 10).

Our bioinformatics analysis relied on the interrogation of short-read sequencing data; therefore, it was not possible to analyse the genetic context to link carbapenemase genes and incl type for all genomes. Instead, a representative genome of each species/ST with the largest contig carrying the carbapenemase gene was chosen to maximize the analysis of other genetic material present, including the incl gene (n = 9; Supplementary Fig. 10). This analysis demonstrated cases where the same carbapenemase gene variant was detected in the same plasmid type across different GNB, suggesting that successful dissemination and acquisition within multiple species may be occurring. We also found cases where the same carbapenemase ARG was detected in multiple different plasmids, further evidencing the spread of AMR.

Characterization of K. pneumoniae. K. pneumoniae is an important cause of neonatal sepsis in LMICs; however, there are few data analysing this species beyond antimicrobial susceptibility testing. Here, we have shown that K. pneumoniae was the most frequently identified GNB; therefore, the genomic diversity of this collection was scrutinized to contextualize these isolates, both within this study collection and within previously known collections of K. pneumoniae. K. pneumoniae (n = 258) was found at all clinical sites (Figs. 1 and 5, Supplementary Table 2 and Supplementary Fig. 4)—predominantly, Ethiopia (n = 95), Nigeria (n = 57) and Pakistan (n = 44).

Genomics analysis within both the global (19,20,23) (Fig. 5a; see Supplementary Table 5 for literature search inclusion criteria) and neonatal sepsis context (19,20) (Fig. 5b) revealed high diversity of K. pneumoniae, with 156 STs from 17 countries spanning five continents. BARNARDS isolates clustered with previously reported neonatal isolates, including ST45, ST48 and ST348 (refs. 19,20,22,23), but we also revealed distinct and new genetic lineages. The major AMR-related K. pneumoniae clades in Asia (ST11) and Europe (ST147 and ST307) were also identified in this study. We only found ST307 in Rwanda (n = 6) and Nigeria (n = 2). However, ST258, a North American clade frequently associated with bla<sub>KPC</sub>, was absent, which accords with the absence of bla<sub>KPC</sub> in this study. We did, however, detect one bla<sub>KPC-2</sub> gene in a K. quasipneumoniae from Bangladesh (Supplementary Fig. 10). While other studies have suggested that bla<sub>KPC</sub> K. pneumoniae causes neonatal sepsis, especially during nosocomial outbreaks, there is currently little evidence from countries in Africa or South Asia.

BARNARDS’ K. pneumoniae were disseminated throughout the global phylogeny, with 57 STs (Table 1 and Fig. 5a). ST35 and ST37 were predominantly found in Ethiopia (n = 38/39 and n = 29/30, respectively). We found four ST35 K. pneumoniae from other neonatal sepsis publications; however, these sit on a distinct branch in the core genome phylogeny (Fig. 5b) and were more closely related to the single ST35 isolated from RU, Rwanda. ST15 isolates were largely isolated from Pakistan and all carried both bla<sub>NDM-1</sub> and bla<sub>OXA-181</sub> (n = 22/27; Fig. 5b). ST15 was almost exclusively found at the South Asian clinical sites, with a single ST15 found at NN, Nigeria. ST442 (n = 6) and ST464 (n = 8) were only found in NN and all isolates contained either bla<sub>NDM-1</sub> (ST442) or bla<sub>OXA-181</sub> (ST464).

Multiple different capsule types (n = 47 KL loci and n = 12 O loci) were identified in silico. ST15 isolates in Pakistan (n = 23) and India (n = 1) were all the O1v1:K112 serotype, whereas single ST15 isolates in Bangladesh and Nigeria had different serotype combinations of O3b:K38 and O1v1:K48, respectively. Similarly, the ST35 isolates from Ethiopia were all O1v2:K108, whereas K. pneumoniae from RU in Rwanda were O2v1:K113. Of the eight ST348 isolates, of which seven were from South Africa and one was from

**Fig. 5 | Core genome characterization of K. pneumoniae isolates.** a. Five-hundred-and-fifty-nine isolates incorporating a global collection. 21 Blue shading indicates K. pneumoniae isolates from the BARNARDS collection. The branch labels are coloured according to country of origin. b. Detailed core genome characterization of 309 K. pneumoniae isolates (n = 258 BARNARDS). Yellow shading indicates isolates from other studies22 causing neonatal sepsis. The outermost rings represent infant outcome (orange) and onset of sepsis (green), followed by the ST, where asterisks represent previously unknown STs. The leaf labels are the code names (coloured according to the study site) of the isolates. The branch symbols in the centre denote the carriage of carbapenemase ARGs (bla<sub>NDM</sub> variants (circles) and bla<sub>OXA-48</sub> group variants (squares)). NA, not applicable.
Onset of sepsis
EOS (≤72 h)
LOS (>72 h)

Infant outcome
Not reported
deceased
Deceased
ND
NA

Carbapenemase ARG
\(\beta\text{la}_{\text{NDM-1}}\)
\(\beta\text{la}_{\text{OXA-181}}\)
\(\beta\text{la}_{\text{OXA-232}}\)
\(\beta\text{la}_{\text{NDM-5}}\)
\(\beta\text{la}_{\text{NDM-7}}\)

Study site
BC
BK
ES
IN
NK
NN
NW
PC
PP
RU
RK
ZAT

Bangladesh (BC), BARNARDS
Bangladesh (BK), BARNARDS
Ethiopia (ES), BARNARDS
India (IN), BARNARDS
Nigeria (NK), BARNARDS
Nigeria (NN), BARNARDS
Nigeria (NW), BARNARDS
Pakistan (PC), BARNARDS
Pakistan (PP), BARNARDS
Rwanda (RK), BARNARDS
South Africa (ZAT), BARNARDS
Australia
Austria
Indonesia
Laos
Singapore
Tanzania
Taiwan
United Kingdom
United States
Vietnam
Unknown

Not reported
deceased
ND
NA
NW (Nigeria), all were O1v1:KL62 and all contained the yersinia-bactin virulence gene.

In total, 115 isolates had a virulence score of 1, 3 or 4, indicating the presence of yersinia-bactin and/or aerobactin/salmochelin virulence genes (see the Kleborate repository17 and Source Data Fig. 5). The odds of LOS were 89% lower for infants with sepsis due to K. pneumoniae who had a virulence score of 3 or 4 compared with those with a score of 0 ($P = 0.04$; 95% CI 0.014–0.90). Additionally, the odds of the outcome deceased were 14 times higher for infants with sepsis due to K. pneumoniae who had a virulence score of 3 or 4 compared with those with a virulence score of 0 ($P = 0.001$; 95% CI 2.76–68.77; Supplementary Table 3). These results suggest that these genes may be involved in quicker onset and MFBS. Alternatively, they may reflect transmission of distinct isolates from the mother’s microbiota and from the clinical environment.

K. pneumoniae harboured multiple β-lactamase genes (Source Data Fig. 5). blaCTX-M-15 was found in 220 out of 258 isolates across diverse STs and at all clinical sites, representing 42% (220/523) of total GNB. Over one-quarter (26%; 69/258) harboured a variant of blaNDM (Figs. 4b and 5b). blaNDM-1 ($n = 15$), blaNDM-5 ($n = 1$) and blaNDM-9 ($n = 9$) were found in Nigeria (mainly NN), whereas blaNDM-1 was the dominant variant in South Asia (Fig. 4b).

All K. pneumoniae were resistant to ampicillin, cefotaxime, ceftriaxone and ceftazidime. K. pneumoniae concomitantly resistant to the three cephalosporins tested ($n = 255$) were significantly more likely to cause EOS than LOS ($P = 0.045$; OR 0.41; 95% CI 0.17–0.98; Supplementary Table 3). Also, 144 isolates had >15 ARGs. For each additional ARG, we observed a 5.7% ($P = 0.028$; 95% CI 0.89–0.99) decrease in the odds of neonates having LOS compared to EOS.

Collectively, this analysis reveals a large degree of intraspecies diversity within K. pneumoniae pathogens causing neonatal sepsis in LMICs. While certain ST groups previously shown to be dominant in particular geographic regions were found during the BARNARDS study, we also detected several different ST groups carrying different carbapenemase ARGs and virulence determinants.

Characterization of E. coli. E. coli has previously been reported as a dominant GNB cause of neonatal sepsis across many different LMICs. In light of this, we aimed to further characterize the BARNARDS E. coli isolates and to compare the results with existing WGS datasets (Supplementary Table 5 displays the literature search inclusion criteria). The 2014 enterotyphogenic E. coli collection displayed large diversity and, when analysed with the BARNARDS dataset, 90 STs across four continents and 21 countries were detected (Fig. 6a). The E. coli analysed herein fell within four main clades of the extended phylogeny. The greatest numbers of E. coli in the BARNARDS study ($n = 75$) were in Nigeria ($n = 33$; 31%) and Rwanda ($n = 17$; 23%), although E. coli neonatal sepsis was identified in 11 of the 12 clinical sites (excluding BK, Bangladesh). A phylogenetic analysis of BARNARDS E. coli revealed four main groups, each containing multiple clades (Fig. 6b), with 37 STs detected. ST10 ($n = 9$), ST131 ($n = 6$), ST410 ($n = 5$) and ST69 ($n = 4$) were the most common. In the Mentzer et al. collection, ST10 was found in five countries across Africa, Asia and South America. Of the nine ST10 E. coli characterized here, seven were from clinical sites within Africa (Ethiopia, $n = 1$; Nigeria, $n = 5$; Rwanda, $n = 1$) and two were from PP, Pakistan. Although all ST10 E. coli belonged to the same phylotype (that is, group A), each isolate had a different O:H serotype profile and the phylogenetic tree (Fig. 6b) shows variability in the branch length, indicating genomic diversity within this ST group. Generally, we found highly variable O:H serotype classification ($n = 57$ O:H combinations), irrespective of the ST or phylgroup (A–F) (Source Data Fig. 6). Of the phylotypes, A ($n = 22$), B1 ($n = 15$) and B2 ($n = 18$) were the most common.

β-lactam and aminoglycoside ARGs were most commonly found in the South Asian isolates, with the exception of blaCTX-M-15, which was also detected in E. coli from Africa. In total, 21 E. coli harboured blaCTX-M-15 and belonged to a variety of STs, including ST131, ST405, ST410, ST10 and ST167. Carbapenemase genes were detected in only three out of 75 E. coli. The three isolates were from South Asia, and all concomitantly carried blaCTX-M-15 and/or blaNDM-5 (ST167; $n = 2$) or blaOXA-1 (ST410; $n = 1$).

No significant associations were found between phenotypic or genotypic AMR-related traits of E. coli and the clinical data assessed herein (Supplementary Table 3).

E. coli isolates were extremely diverse, suggesting that there are probably several important and worrisome lineages.

Discussion

In the BARNARDS study, we established a methodological framework to capture and extensively characterize GNB species causing neonatal sepsis in LMICs. We isolated 916 isolates of GNB, characterized them to species level, used WGS to probe genome composition and MLST to assess intra- and interspecies diversity, and documented extremely high rates of AMR.

Most of the Gram-negative isolates from neonates with sepsis were resistant to at least one β-lactam and one aminoglycoside (597/885; 67%), as has been reported previously for cohorts in India29 and 26 countries in Africa.31 World Health Organization guidelines stipulate ampicillin plus gentamicin as the first line of empirical treatment for neonatal sepsis and third-generation cephalosporins as the second line of treatment. Of note, many of the blood culture isolates from our study were resistant to both lines of treatment, meaning that treatment options are unlikely to be curative.

The identification of 58 different GNB species suggests that the aetiology of neonatal sepsis is complex. We report multiple different lineages causing infection within single species, many of which carry either resistant or putatively virulent mechanisms and several of which have previously been shown to cause neonatal sepsis (for example, ST35 and ST37 K. pneumoniae)23,32. The identification of high-risk clones, such as ST15 in K. pneumoniae and the global clones ST1 and ST2 in A. baumannii, which are notorious for nosocomial infection, indicates the spread and persistence of problematic lineages in LMICs. In addition, through our comprehensive analysis, we identified 40 previously unknown STs, suggesting that well-known and previously unidentified lineages/ST groups are both co-existing and evolving.

A limitation of our study was the inability to follow up all neonates to 60 d (necessitating the exclusion of neonates who were lost to follow-up), which impacted our outcome data (Fig. 1). It is likely that additional local factors (such as the management of sepsis) contributed to mortality; therefore, we cannot attribute MFBS singularly to the presence/absence of genomic traits. Our statistical analyses were exploratory and should be interpreted as hypothesis generating only.

Fig. 6 | Core genome characterization of E. coli isolates. a. Three-hundred-and-sixty isolates incorporating a global collection9. Blue shading indicates E. coli isolates from the BARNARDS collection ($n = 75$). The branch labels are coloured according to country of origin. b. Detailed core genome characterization of 87 E. coli isolates ($n = 75$ BARNARDS). Yellow shading represents isolates from other studies10–12 causing neonatal sepsis. The colours on the right represent infant outcome (orange) and onset of sepsis (green), followed by the ST. The numbers and code names (coloured according to study site) of isolates are also given. The branch symbols denote the carriage of carbapenemase ARGs (blaNDM-5 (circles) and blaOXA-1 (squares)). NF, not found.
In summary, *Klebsiella*, *E. coli* and *Enterobacter* were the main GNB species responsible for sepsis in neonates. We report that 54% of isolated bacteria were resistant to at least one antibiotic within four to six classes of antibiotics, and observed widespread carriage of both resistance genes and virulence factors in GNB causing neonatal sepsis in LMICs. This large, observational study will inform
future research into effective antimicrobial therapies for neonatal sepsis, and may underpin improved infection control practices and could be useful in the development of vaccines for neonatal sepsis in LMICs.

Methods
Study design and processing of blood cultures at clinical sites. A prospective cohort study was conducted through the BARNARDS network consisting of 12 clinical sites in seven countries in Africa and South Asia (Chattogram, Maa-O-Shishu Hospital, Chattogram (BC) and Kumudini Women’s Medical College, Mirzapur (BK) in Bangladesh; St. Paul’s Hospital Millennium Medical College, Addis Ababa (ES) in Ethiopia; the Division of Bacteriology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata (IN) in India; National Hospital Abuja, Abuja (NW), Wuse District Hospital, Abuja (NW) and Murtala Mohammed Specialist Hospital, Kano (NK) in Nigeria; Pakistan Institute of Medical Sciences, Islamabad (PP) and Bhara Kahu Rural Health Centre, Bhara Kahu (PC) in Pakistan; University Central Hospital of Kigali, Kigali (RU) and Kabgayi Hospital, Kabgayi (RK) in Rwanda; and Tygerberg Hospital, Cape Town (ZAT) in South Africa). Ethical approval was obtained from the local ethics committee at each site before the start of the study (Supplementary Table 6). Between November 2015 and December 2017, women in labour or immediately postpartum were recruited prospectively following consent, and their neonates were followed up for the first 60 d of life (at 3, 7, 14, 28 and 60 d of life) or until study withdrawal/death. Neonates admitted to clinical sites showing signs of sepsis were also enrolled. The BARNARDS sample collection workflow is shown in Supplementary Fig. 1. Although it was not strictly a neonatal population, during this study, we employed the term neonate for all enrolments, including those between 30 and 60 d post-birth.

Documentation detailing the parameters for clinically diagnosing sepsis is available in Supplementary Fig. 1. The standard operating procedures for the laboratory processing of blood cultures and subsequent identification of bacteria, which were followed by all clinical sites (following agreement between clinical partners before the start of enrolment), are shown in Supplementary Fig. 2. Laboratory reagents (Liofilchem)—importantly, both agar media and antibiotic discs (used at the clinical sites)—were standardized throughout the network. Bacterial identification performed at each site was confirmed by WGS at Cardiff University. Antimicrobial susceptibility testing was performed twice: initially, at the local sites to guide treatment using antibiotic discs; and then at Cardiff University using the agar dilution method to establish the MICs (details below). The collected clinical data included onset of sepsis (EOS or LOS) and patient outcome following biological sepsis. Neonates that were lost to follow up were categorized along with neonates confirmed alive as ‘not reported deceased’. For the purpose of this study, EOS and LOS were defined as sepsis occurring ≤72 h and >72 h after birth, respectively. If neonates showed clinical signs of sepsis at multiple time points within the first 60 d of life, additional blood cultures were analysed. All viable bacterial species were stored on charcoal swabs (Deltalab) for transport under UN3373 regulations to Cardiff University.

Data availability
Sequence reads have been submitted to the European Nucleotide Archive under project number PRJEB33565. Individual accession numbers and additional genomics data can be accessed in Supplementary Table 4 and the source data. The databases used for this study included VFDiB (http://www.mgc.ac.cn/VFs/download.htm), NCBI (https://github.com/teemann/abricate/tree/master/db/ncbi), ResFinder (https://github.com/teemann/abricate/tree/master/db/resfinder), PlasmidFinder (https://bitbucket.org/genomicepidemiology/plasmidfinder/src/master), MLST (https://github.com/teemann/mlst/tree/master/db/pubmlst), MGE (https://bitbucket.org/mlhki/mge_finder/src/master/mge_finder/), SerotypeFinder (https://bitbucket.org/genomicepidemiology/serotypefinder/src/master), and SeqSero (http://www.denglab.info/SeqSero). Previously published data can be downloaded from the open source Nucleotide Archive repository and used for comparative genomics analysis have the identifiers PRJEB2111, PRJEB28581 and PRJEB20875. The following genomes were downloaded from NCBI: PHGEO10000000–PHGEO100000000, ATNV00000000 and ATNV000000000. Source data are provided with this paper.

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Author contributions
K.S. and M.J.C. designed and guided the study, performed the analysis and wrote the manuscript. K.S., E.P. and J.M. performed the WGS experiments. K.S. and R.A. performed the bioinformatics analysis. M.J.C. contributed to the bioinformatics analysis. E.P. and K. Thomson contributed equally. K. Thomson performed the MIC experiments. K. Thomson and M.J.C. analysed the MIC dataset. K.S. and M.J.C. produced the figures. C.D., R.M., D.G., K. Taiyari and K.H. provided the finalized epidemiological and clinical dataset. M.J.C., D.G. and K. Taiyari performed the statistical analysis. K.S., M.J.C., E.P., K. Thomson, C.D., C.A., A.F., T.H., J.M. and M.N. performed the microbiology culture and sample processing at Cardiff University. G.J.C., D.B., S.S., P.C., S.M., K.I., F.M., S.U., R.Z., H.S., A.M., J.-B.M., A.R., L.G., S.M., A.N.H.B. and A.W. collected and processed the blood cultures and collected clinical data at the clinical sites. T.R.W., M.J.C., R.M., G.J.C., S.B., K.I., R.Z., J.-B.M. and S.M. designed the BARNARDS study.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to K.S. or M.J.C.

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A full list of members and their affiliations appears in Supplementary Table 9.
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- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

No software was used in the data collection. At the low middle income countries, research nurses completed questionnaires with the women approaching labor. These questionnaires were either transcribed onto paper, due to availability of resources/infrastructure, i.e. Internet access, and later uploaded into Bristol Online survey (BOS) or directly entered into BOS using a tablet device provided by the project.

Data analysis

CLIMB (v1.0)
Trimgalore (v0.4.3)
fastqc (v0.11.2)
MultiQC (v1.7)
Flash (v1.2.11)
SPAdes (v3.9.0)
BWA (v0.7.15)
samtools (v1.3.1)
Pilon (v1.22)
quast (v2.3)
Blast nt (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (v2.2.25)
PathogenWatch (v.3.13.10; https://pathogen.watch)
srst2 (v0.2.0)
ABRicate (v0.9.7)
BIGSdb (v2.25.1)
Kaptive (v0.7.0)
and Kleborate (v0.2.0)
SerotypeFinder (v2.0)
SeqSero (v1.0)
ClermonTyping (v.1.3.0)
Prokka (v1.12)
Roary (v3.12.0)
FastTree (v2.1.11)
ITOL (v5.7)
networkD3 package, Rv3.6.2
Geneious prime (2020.1.2)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Sequences reads were submitted to the European Nucleotide Archive (ENA) and given the project number PRJEB33565. A list of individual accession numbers for 916 Gram-negative bacteria can be found in the source data (to accompany figure 4,5 & 6).

Databases used within this study:
VFDB: http://www.mgc.ac.cn/VFs/download.htm
NCBI: https://github.com/tseemann/abricate/tree/master/db/ncbi
Resfinder: https://github.com/tseemann/abricate/tree/master/db/resfinder
Plasmidfinder: https://bitbucket.org/genomicepidemiology/plasmidfinder/src/master
mlst: https://github.com/tseemann/mlst/tree/master/db/mlst
MGE: https://bitbucket.org/mhkj/mge_finder/src/master/me_finder/
Serotype finder: https://bitbucket.org/genomicepidemiology/serotypefinder/src/master
Seqsero: http://www.denglab.info/SeqSero

Previously published datasets downloaded from the ENA repository used for comparative genomics analysis: PRJEB2111, PRJEB2581 and PRJEB20875. Genomes were downloaded from NCBI: PHGE01000000-PHGR01000000, ATNV00000000, ATNV00000000.

Field-specific reporting

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Life sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
The sampling method was purposive and a formal sample size calculation was not conducted.

Based on previous studies led by PI Professor Timothy Walsh (unpublished studies/awaiting publication), BARNARDS anticipated the enrollment level between 500-2000 neonates per clinical site for the duration of the study (depending on geographical location i.e. smaller rural site would have a smaller catchment area). With the anticipated sepsis rate (based on limited public accessible data), this would capture between 1,000-2,500 bacterial (both Gram-negative and Gram-positive) blood culture positive sepsis cases across seasonal variation with an estimated 1,000 Gram-negative sepsis cases.

Data exclusions
The following exclusion criteria was pre-defined: the sepsis case infant/mother sampling pair was excluded in the case of a still born. Following this, data was retrospectively excluded based on the following criteria:
- Incomplete questionnaire; missing multiple data points in the epidemiological dataset
- Mother asked for infant withdrawal
- Error/Substantial inconsistencies in the questionnaire - laboratory sampling match up.

Replication
N/A - Findings were not replicated as this manuscript is a full genomics characterisation of sepsis causing bacteria from low middle income countries (LMICs). All viable isolates eligible for sequencing were included into the analysis.

Randomization
Randomization was not relevant to the study. All women approaching labor were (following consent) enrolled onto the study. If the neonate/infant presented with sepsis, the infant was also enrolled to allow a blood culture to be taken.

Blinding
Blinding was not necessary for this study as we were characterizing all blood culture isolates along with the corresponding infant's clinical data.
Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

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|-----|-----------------------|
| ☒   | Antibodies            |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology and archaeology |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data         |
| ☒   | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq              |
| ☒   | Flow cytometry        |
| ☒   | MRI-based neuroimaging |

Human research participants

Policy information about studies involving human research participants

BARNARDS was a multi-site international prospective observational study incorporating two recruitment pathways:

i.) Birth-Cohort: All mothers in labour admitted to clinical-sites were recruited prospectively and their infant(s) followed up until 60-days old or death.

ii.) Infant Admissions (IA): Infant(s) admitted to clinical-sites showing signs of suspected sepsis in the first 60-days of life until 60-days old or death.

General population characteristics of the mothers’ (outside of the scope of this manuscript): <10% previously had stillbirth, approx. 25% were first time mothers’, 75% were aged between 21-35 years old. Infants’ presenting with sepsis were followed up for 60 days of life. Onset of sepsis was recorded; early onset (EOS) <72h, and late onset (LOS) >72h.

Recruitment

BARNARDS recruited from 12 clinical sites from Rwanda, Bangladesh, Ethiopia, Nigeria, Pakistan, India and South Africa. Where possible, large public hospitals were chosen. Following the presentation of information regarding the study, mothers in labour were enrolled into the study. Consent was collected by trained research staff and using local languages. Corresponding neonates presenting with clinical signs of sepsis were then enrolled into the study. Additionally, neonates not born within the clinical sites that were admitted with clinical signs of sepsis were also enrolled into the study following consent from the mother. The corresponding mothers were also enrolled into the study for the collection of demographic data. Neonatal follow-up was carried out at day 3, 7, 14, 28, and 60 by research nurses either face-to-face or by telephone. Neonates remained in the study until 60 days old, withdrawal, or death. This study incorporated two recruitment pathways to include both neonates born within the clinical sites, and also neonates in the larger catchment areas presenting to the hospital with signs of sepsis.

Ethics oversight

Site committees Named PI Reference(s) Approval date(s)

BC - Ethical Review Committee, Bangladesh Institute of Child Health Samir Kumar Saha BICH-ERC-4/3/2015 15/09/2015

BK - Ethical Review Committee, Bangladesh Institute of Child Health Samir Kumar Saha BICH-ERC-4/3/2015 15/09/2015

ES - Boston Children’s Hospital Grace Chan IRB-P00023058 11/08/2016

IN - Institutional Ethics Committee, National Institute of Cholera and Enteric Diseases and Institute of Post Graduate Medical Education and Research, IPGM&E&R Research Oversight Committee Sulagna Basu A-I/2016-IEC and Inst/IEC/2016/508 17/11/2016 and 04/11/2016

NK - Kano State Hospitals Management Board Kenneth Iregbu 8/10/1437AH 13/07/2016

PW - Health Research Ethics Committee (HREC), National Hospital, Abuja Kenneth Iregbu NHA/EC/017/2015 27/04/2015

PC - Shaheed Zulfiquar Ali Bhutto Medical University, Pakistan Institute of Medical Sciences (PIMS) Islamabad Rabaab Zahra NA, signed letter from Prof. Tabish Hazir 27/05/2015

PP - Shaheed Zulfiquar Ali Bhutto Medical University, Pakistan Institute of Medical Sciences (PIMS) Islamabad Rabaab Zahra NA, signed letter from Prof. Tabish Hazir 27/05/2015

RK - Republic of Rwanda, National Ethics Committee Jean-Baptiste Mazarati No342/RNEC/2015 10/11/2015

RU - Republic of Rwanda, National Ethics Committee Jean-Baptiste Mazarati No342/RNEC/2015 10/11/2015

ZAT - Stellenbosch University and Tygerberg Hospital, Research projects, Western Cape Government Shaheen Mehtar N15/07/063 04/12/2015 and 02/02/2016

Note that full information on the approval of the study protocol must also be provided in the manuscript.