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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 cause 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). Enterobacteriales (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.

Despite the burden of neonatal sepsis, accurate information on the causes and consequences of neonatal sepsis in LMICs is scarce6-9. 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 measurements9-10. In 2015 and 2016, two multicentre neonatal sepsis studies in LMICs were published11,12. 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

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areas) and resistance. The studies that have taken this approach were of single sites and often used WGS to investigate specific outbreaks. In LMICs, the epidemiology of early-onset sepsis (EOS) and late-onset sepsis (LOS) is not well defined, unlike in high-income countries, where group B Streptococcus is usually considered the main cause of EOS. A systematic review of the causes of blood culture-positive neonatal sepsis in Sub-Saharan Africa by Okomo et al. found that Klebsiella species, Escherichia coli, Enterobacter species and Pseudomonas species accounted for 38% of cases. Other single-site reports showed concordant findings. However, these studies did not specifically determine whether certain species, or sequence type (ST) groups, are more likely to harbour resistance or virulence determinants, how this compares between different geographical areas and whether there is any relation to sepsis onset or outcome.

Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS; www.barnards-group.com) is a network of 12 clinical study sites in four African (Ethiopia, Nigeria, Rwanda and South Africa) and three South Asian countries (Bangladesh, India and Pakistan). The aim of the BARNARDS study is to assess the burden of AMR in neonates in these LMIC. Here, we report on the isolation and characterization of Gram-negative bacteria (GNB) causing neonatal sepsis in seven LMICs, including their AMR profiles. We report associations between phenotypic and genotypic data and sepsis onset and mortality following biological sepsis (MFBS). We also analyse whole-genome sequences from isolates that cause neonatal sepsis.

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 a Gram stain 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

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.
species (n = 36) and Ralstonia mannitolilytica (n = 21) (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 (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 (Fig. 2).

Overall, R. mannitolilytica, K. michiganensis, Burkholderia species and Pseudomonas species caused more cases of EOS than other species (Supplementary Table 2 and Supplementary Fig. 5). Of note, similar proportions of fatal sepsis cases were caused by GNB on each continent (92/353 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 (Supplementary Table 2 and Supplementary Fig. 5). However, there was a large proportion of missing data from certain clinical sites (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 (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 (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 (Table 1 and Supplementary Table 2). Fourteen STs were assigned in the Klebsiella genera (Table 1), including all three K. aerogenes STs (all from Africa; n = 2 from Nigeria and n = 1 from South Africa; ST194–196). K. michiganensis was mainly ST180 (from PP in Pakistan; Table 1). Such low ST diversity and close phylogenetic relatedness, as shown by the core genome phylogeny (Supplementary Fig. 6), warrant further investigation. Similarly, we noticed that a single, previously unknown, B. cenocepacia ST (ST1621), also from PP, was dominant (Table 1 and Supplementary Fig. 7). B. cenocepacia ST1621 from PP and the S. marcescens isolates from BC were indistinguishable during core genome analysis (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. ludwigii. 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) (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 (Table 1) to E. cloacae, E. hormaechei and E. ludwigii 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 (Pasteur MLST) ST1 and ST2 (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 (MICs) of 19 antibiotics, including the current recommended first-line empirical
### Table 1 | STs for the most commonly identified species with a recognized MLST scheme

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolates characterized by WGS*</th>
<th>Number identified per clinical siteb</th>
<th>Number of STs found</th>
<th>Prominent STs</th>
<th>STs predominantly found in specific clinical sites</th>
<th>Previously unknown STs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. baumannii complex</td>
<td>41 (38 ABA and 3 ANO)</td>
<td>BC (n = 8), BK (n = 2), ES (n = 7), IN (n = 3), NK (n = 3), PC (n = 1), PP (n = 8), RK (n = 2), RU (n = 3) and ZAT (n = 3)</td>
<td>15</td>
<td>ST1, ST2, ST57 and STT106</td>
<td>ST2 (BC) and STT1106 (PP)</td>
<td>ST1326 and ST1327</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>56</td>
<td>BC (n = 1), PC (n = 1) and PP (n = 54)</td>
<td>5</td>
<td>ST1621</td>
<td>ST1621 (PP)</td>
<td>ST1621 and ST1623</td>
</tr>
<tr>
<td>E. coli</td>
<td>75</td>
<td>BC (n = 3), ES (n = 11), IN (n = 2), NK (n = 15), NN (n = 7), NW (n = 1), PC (n = 3), PP (n = 10), RK (n = 15), RU (n = 2) and ZAT (n = 6)</td>
<td>37</td>
<td>ST10, ST69, ST131, ST410 and ST517</td>
<td>ST410 (PC) and ST517 (RK)</td>
<td>ND</td>
</tr>
<tr>
<td>E. cloacae complex</td>
<td>78 (1 EAS, 57 ECL, 18 EHO, 1 EKO and 1 ELU)</td>
<td>BC (n = 2), NK (n = 7), NN (n = 9), NW (n = 1), PC (n = 39), RK (n = 14), RU (n = 5) and ZAT (n = 1)</td>
<td>34</td>
<td>ST84, ST93, ST171, ST346, ST523 and ST980</td>
<td>ST84 (PP), ST93 (PP), ST346 (RK), ST523 (PP) and ST980 (RK)</td>
<td>ST1236 and ST1238–ST1248</td>
</tr>
<tr>
<td>K. michiganensis/ K. oxytoca</td>
<td>122 (117 KMI and 5 KOX)</td>
<td>ES (n = 5), PC (n = 4), PP (n = 111) and RK (n = 2)</td>
<td>5</td>
<td>ST180</td>
<td>ST180 (PP)</td>
<td>ST268 (KMI) and ST243–ST244 (KOX)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>258</td>
<td>BC (n = 17), BK (n = 2), ES (n = 95), IN (n = 5), NK (n = 16), NN (n = 37), NW (n = 4), PC (n = 2), PP (n = 42), RK (n = 15), RU (n = 7) and ZAT (n = 16)</td>
<td>57</td>
<td>ST15, ST35, ST37, ST39, ST218, ST307, ST348, ST443, ST464 and ST985</td>
<td>ST15 (PP), ST35 (ES), ST37 (ES), ST218 (ES), ST307 (RK), ST442 (NN), ST464 (NN) and ST985 (ES)</td>
<td>ST4008, ST4410 and ST4411</td>
</tr>
<tr>
<td>K. quasipneumoniae</td>
<td>13</td>
<td>BC (n = 6), BK (n = 1), ES (n = 1), NK (n = 2), NN (n = 2) and PP (n = 1)</td>
<td>10</td>
<td>ST4405</td>
<td>ST4405 (BC)</td>
<td>ST4405–ST4407 and ST4409</td>
</tr>
<tr>
<td>K. variicola</td>
<td>5</td>
<td>BC (n = 1), PP (n = 1), RK (n = 2) and RU (n = 1)</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ST4404 and ST4412–ST4414</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>23</td>
<td>BC (n = 1), BK (n = 4), IN (n = 1), NK (n = 2), NN (n = 3), PC (n = 1), PP (n = 9) and RK (n = 2)</td>
<td>14</td>
<td>ST3235, ST1285 and ST3311</td>
<td>ST1285 (BK) and ST3311 (PP)</td>
<td>ST3311</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>7</td>
<td>NK (n = 6) and NN (n = 1)</td>
<td>4</td>
<td>ST313</td>
<td>ST313 (NK)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Numbers of isolates characterized. For complexes, a breakdown by species is given. **Numbers of isolates from each clinical site. ABA, A. baumannii; ANO, A. nosocomialis; EAS, Enterobacter asburiae; ECL, E. cloacae; EHO, Enterobacter hormaecheii; EKO, Enterobacter kobel; ELU, Enterobacter ludwigii; KMI, K. michiganensis; KOX, K. oxytoca.

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 0.426–0.918; 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<sub>CTX-M-15</sub> 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<sub>NDM</sub>, bla<sub>OKA</sub>, bla<sub>IMP</sub>, bla<sub>GES</sub>, bla<sub>VEBB</sub>, and bla<sub>SK</sub>). There were 146 single carbapenemase genes in ten species (n = 128 isolates), and two carbapenem resistance gene homologues were present in 24 isolates. bla<sub>NDM</sub> (n = 90; Bangladesh, n = 23; India, n = 6;...
**Fig. 3** | AMR of neonatal sepsis-causing pathogens. **a**, Percentages of antimicrobial-resistant aetiological 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. **b**, Sunburst diagram detailing the class A (red), B (yellow) and D (green) carbapenemase resistance genes detected. 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 blaOXA-48 (n = 3; Bangladesh, n = 1; India, n = 1; Nigeria, n = 1) were mainly detected in clinical sites in South Asia, whereas blaNDM-7 was predominantly recovered from Nigeria and Pakistan (n = 19; Bangladesh, n = 1; Nigeria, n = 11; Pakistan, n = 7) (Fig. 3b). In 79 GNB, blaCTX-M-15 plus blaNDM and/or a blaOXA-48-like variant were found. In total, 30 GNB carried a variant of the blaOXA-48-like family, with blaOXA-181 being the most frequent (Pakistan, n = 22; India, n = 1; Fig. 3b). blaOXA-232 was only found in Bangladesh (n = 5), and the two isolates carrying blaOXA-48 (one K. michiganensis and one S. marcescens) were from PP. blaNDM was found in three Pseudomonas aeruginosa isolates (Bangladesh, n = 2 (BC, n = 1; BK, n = 1); India, n = 1). Both blaNDM variants from Bangladesh were blaVIM-2, whereas the variant detected in India was blaVIM-4.

In Enterobacter species, blaCTX-M-15 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 berezini, n = 1; Acinetobacter nosocomialis, n = 1) were found to have blaNDM (Africa, n = 2; South Asia, n = 3) (Fig. 3b). Additionally, we found blaOXA-232 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 Inc 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 carriiers (Fig. 4), the most frequently detected inc type was IncFIB, with 255 out of 440 hits within K. pneumoniae genomes. blaNDM-1 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 blaNDM-3 in K. pneumoniae from NK, Nigeria, and multiple blaNDM-1 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 ColK3P3 plasmids carrying blaOXA-181 or blaOXA-232 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 inc 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 inc 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, furthermore 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 collections11,12,14,27. 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 global14,26 (Fig. 5a; see Supplementary Table 5 for literature search inclusion criteria) and neonatal sepsis context14 (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. 13,15,20), but we also revealed distinct and new genetic lineages. The major AMR-related K. pneumoniae clades in Asia (ST11) and Europe (ST147 and ST307)14 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 blacPC, was absent, which accords with the absence of blacPC in this study. We did, however, detect one blacPC gene in a K. quasipneumoniae from Bangladesh (Supplementary Fig. 10). While other studies have suggested that blacPC K. pneumoniae causes neonatal sepsis, especially during nosocomial outbreaks29, 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 ST73 were predominately 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 blaNDM-1 and blacOXA-232 (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 blacNDM-1 (ST442) or blacNDM-1 (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:KL112 serotype, whereas single ST15 isolates in Bangladesh and Nigeria had different serotype combinations of O3b:KL38 and O1v1:KL48, respectively. Similarly, the ST35 isolates from Ethiopia were all O1v2:KL108, whereas K. pneumoniae from RU in Rwanda were O2v1:KL113. 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 collection21. 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 studies19,22 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 isolates. The branch symbols in the centre denote the carriage of carbapenemase ARGs (blaNDM variants (circles) and blaOXA-48 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
- \( \beta\)-lactamase

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
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 India58 and 26 countries in Africa59. World Health Organization guidelines60 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)61,62. The identification of high-risk clones, such as ST15 in K. pneumoniae63 and the global clones ST1 and ST2 in A. baumannii, which are notorious for nosocomial infection64, 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 days (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 to the presence/absence of genomic traits. Our statistical analyses were exploratory and should be interpreted as hypothesis generating only.
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 further interventions.
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 Bhaba Kuru Rural Health Centre, Bhaba Kuru (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 bacterial identification were followed by all clinical sites (following agreement between clinical partners before the start of enrolment), are shown in Supplementary Fig. 2. Laboratory reagents (BioMérieux) and LPS detection methods 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 laboratory confirmation. 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. Antimicrobial susceptibility testing was performed twice: initially, at the clinical sites—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.

Statistical analyses. Statistical associations between clinical outcomes (onset of sepsis (EOS/LOS) and MFS (alive/deceased)) and phenotypical and genotypic traits were explored using univariable logistic regression models with the Wald test in SPSS version 26. The outcomes for the analyses regarding sepsis onset were EOS and LOS and those for MFS were alive and deceased. Depending on whether predictor variables were continuous (AMR or ARG) or categorical (resistance versus non-resistance to ampicillin and gentamicin, concomitant resistance versus non-concomitant resistance to the three cephalosporins tested, resistance to at least one of the three cephalosporins tested versus to none, resistance versus non-resistance to etrapenem (a marker for carbapenem resistance) or Klebsiella species virulence scores), they were treated as covariates or factors, respectively. For species group analyses, only groups with n ≥ 50 isolates were included, except for B. cenocepacia isolates, which did not carry ARGs. Statistical significance was taken at P ≤ 0.05, and estimated ORs are presented along with 95% CIs.

Reporting Summary. Further information on research design is available in the Nature Research Reporting summary linked to this article.

Data availability

Sequence reads have been submitted to the European Nucleotide Archive 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 VFDI (http://www.mgc.ac.cn/VFS/ download.htm), NCBI (https://www.ncbi.nlm.nih.gov/Blast.cgi; ‘input = contigs’) and PathogenWatch (version 3.13.10; https://pathogen.watch; input = contigs). MLST, virulence and plasmid genomic profiles were characterized using SRST2 (version 0.2.0) and the associated databases PlasmidFinder and VFDB. Genomes were screened for ARGs using ARGfinder (version 0.9.7) (database NCBI) and ResFinder. Novel alleles and novel ST profiles were submitted to BIGSDb (version 1.25.1). The O:K locus profiles for all Klebsiella species were determined using Kaptive (version 0.2.0) and Kleborate (version 0.2.0; https://github.com/katholt/Kleborate). The O:H serotype profiles for all E. coli isolates were determined using SerotypeFinder (version 2.0.2), and SeqSero (version 1.0) was used to determine serotypes for Salmonella. In silico E. coli plyphtotyping was performed using ClermonTyping (version 1.3.0). Genomes were annotated using Prokka (version 1.12). Strain relatedness analysis was performed using Roary (version 3.12.0) to create a core genome alignment and FastTree (version 2.1.11) to generate a maximum likelihood phylogenetic tree. Phylogenetic trees were mid-rooted, visualized and annotated using iTOL (version 5.7). The plasmid Sankey diagram was generated using the networkDS package in R version 3.6.2. The immediate genetic context around carbapenemase genes was performed aligning outputs from ResFinder and PlasmidFinder (in ABRicate) with Mobile Element Finder (version 1.0.1) hosted by the Center for Genomic Epidemiology (https://bitbucket.org/genomicepidemiology/Plasmidfinder/src/ master), and the associated data are provided with this paper.

Received: 21 January 2020; Accepted: 22 January 2021; Published online: 29 March 2021

References


Bioinformatics analyses. Bioinformatics analyses were performed using a high-performance computing cluster at Cardiff University (Advanced Research Computing at Cardiff (ARCCA)) and CLIMB (version 1.0). Paired-end reads (FASTQ) were subjected to quality control checks before downstream analysis. Trim Galore (version 0.4.3) was used to remove the Nextera adapter sequences and low-quality bases. Reports before and after read trimming were generated using FastQC (version 0.11.2) and collated using MultiQC (version 1.17). The mean read length and number of sequences provided on the MultiQC reports were used to determine the sequence coverage. Paired-end reads were overlapped using Flash (version 1.2.11) and assembled into contigs using SPAdes (version 3.9.0). The trimmed FASTQ reads were mapped to the contigs using BWA (version 0.7.15) and SARTools (version 1.3.1). Pilon (version 1.22) was used to assess any misassemblies/errors in base calling in the resulting mapped BAM file. Final genome assembly metrics were generated using QUAST (version 2.1.3). Bacterial species were identified using both BLAST nt (version 2.2.25; https://blast.ncbi.nlm.nih.gov/Blast.cgi; input = contigs) and PathogenWatch (version 3.13.10; https://pathogen.watch; input = contigs). MLST, virulence and plasmid genomic profiles were characterized using SRST2 (version 0.2.0) and the associated databases PlasmidFinder and VFDB. Genomes were screened for ARGs using ARGfinder (version 0.9.7) (database NCBI) and ResFinder. Novel alleles and novel ST profiles were submitted to BIGSDb (version 1.25.1). The O:K locus profiles for all Klebsiella species were determined using Kaptive (version 0.7.0) and Kleborate (version 0.2.0; https://github.com/katholt/Kleborate). The O:H serotype profiles for all E. coli isolates were determined using SerotypeFinder (version 2.0.2), and SeqSero (version 1.0) was used to determine serotypes for Salmonella. In silico E. coli plyphtotyping was performed using ClermonTyping (version 1.3.0). Genomes were annotated using Prokka (version 1.12). Strain relatedness analysis was performed using Roary (version 3.12.0) to create a core genome alignment and FastTree (version 2.1.11) to generate a maximum likelihood phylogenetic tree. Phylogenetic trees were mid-rooted, visualized and annotated using iTOL (version 5.7). The plasmid Sankey diagram was generated using the networkDS package in R version 3.6.2. The immediate genetic context around carbapenemase genes was performed aligning outputs from ResFinder and PlasmidFinder (in ABRicate) with Mobile Element Finder (version 1.0.1) hosted by the Center for Genomic Epidemiology (https://bitbucket.org/genomicepidemiology/Plasmidfinder/src/ master), and the associated data are provided with this paper.

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ARTICLES

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30. Wick, R. R., Heinz, E., Holt, K. E. & Wyres, K. L.Kaptive Web: user-friendly products to enable standard operating procedures across the clinical sites. We acknowledge Liofilchem for continued support in the distribution of microbiology test products to enable standard operating procedures across the clinical sites. We acknowledge Liofilchem for continued support in the distribution of microbiology test products to enable standard operating procedures across the clinical sites.

31. Acknowledgements

We acknowledge the following for continued support in the distribution of microbiology test products to enable standard operating procedures across the clinical sites. We acknowledge Liofilchem for continued support in the distribution of microbiology test products to enable standard operating procedures across the clinical sites. We acknowledge Liofilchem for continued support in the distribution of microbiology test products to enable standard operating procedures across the clinical sites. We acknowledge Liofilchem for continued support in the distribution of microbiology test products to enable standard operating procedures across the clinical sites. We acknowledge Liofilchem for continued support in the distribution of microbiology test products to enable standard operating procedures across the clinical sites. We acknowledge Liofilchem for continued support in the distribution of microbiology test products to enable standard operating procedures across the clinical sites.
We acknowledge Wales Gene Park and ARCCA for continued bioinformatics support and infrastructure availability. Bioinformatics analysis was largely undertaken using the supercomputing facilities at Cardiff University, which were operated by Advanced Research Computing at Cardiff (ARCCA) on behalf of the Cardiff Supercomputing Facility and HPC Wales and Supercomputing Wales projects. We acknowledge support from Supercomputing Wales, which is partly funded by the European Regional Development Fund via the Welsh Government. We thank the team of curators for the databases hosted on PubMLST (https://pubmlst.org/databases/). We also thank the curators of the Institut Pasteur MLST and Whole-Genome MLST databases for curating the Klebsiella data and making them publicly available at http://biggsdb.pasteur.fr. We thank M. Islam for providing access to the clinical sites and epidemiology data in Bangladesh. We acknowledge R. Kamran, the microbiologist from the Padmashree Institute of Management and Sciences, who sadly passed away in 2018. We thank the team at the Bill & Melinda Gates Foundation; namely, P. Srikantiah, R. Izadnegahdar, K. Klugman and S. Vernam. The BARNARDS study was funded by two awards (US$4.28 million (OPP1119772) and US$849,000 (OP1191522)) from the Bill & Melinda Gates Foundation.

Author contributions

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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BARNARDS Group
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A full list of members and their affiliations appears in Supplementary Table 9.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- 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 (v.0.7.15)
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)
BIGSbd (v2.25.1)
Kaptive (v0.7.0)
and Kleborate (v0.2.0)
SerotypeFinder (v2.0)
SeqSero (v1.0)
ClermonTyping (v.1.3.0)
Data

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/pubmlst
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, ATNW0000010000, ATNV0000010000.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

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 consenst) 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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Methods

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Human research participants

Policy information about studies involving human research participants

Population characteristics

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.

For this study, isolates recovered from blood cultures were included into the characterisation of infant sepsis irrespective of cohort pathway.

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


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

NN - 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.