Tracking over time the developing gut microbiota in newborns admitted to a neonatal intensive care unit during an outbreak caused by ESBL-producing *Klebsiella pneumoniae*

Simona Panelli¹, Marta Corbella², Alessandra Gazzola²,³, Antonio Piralla², Alessia Girello², Simone Rampelli⁴, Marco Candela⁴, Patrizia Cambieri²

¹Centro di Ricerca Pediatrica “Romeo ed Enrica Invernizzi”, Department of Biomedical and Clinical Sciences “L. Sacco”, University of Milano, Milano, Italy; ²U.O.C. Microbiologia e Virologia, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; ³U.O.C. Malattie Infettive, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; ⁴Unit of Holobiont Microbiome and Microbiome Engineering, Department of Pharmacy and Biotechnology, University of Bologna, Italy

**SUMMARY**

The establishment of gut microbiota is reportedly aberrant in newborns admitted to neonatal intensive care units (NICUs), with detrimental long-term health impacts. Here, we vertically tracked the developing gut bacterial communities of newborns hosted in a NICU during an outbreak sustained by ESBL *Klebsiella pneumoniae* and compared colonized and non-colonized patients. Most communities were highly variable from one sampling point to the next, and dominated by few taxa, often *Proteobacteria* and *Enterobacteriaceae*, with marked interindividual variability. This picture was retrieved independently of colonization status or clinical covariates. Our data support the emerging idea of preterm infants as a population in which no defined microbial signatures are clearly associated to clinical status. Instead, the strong pressure of the nosocomial environment, antibiotics and, in this case, the ongoing outbreak, possibly drive the evolution of microbiota patterns according to individual conditions, also in non-colonized patients.

Received April 08, 2020

Accepted October 10, 2020

**Key words:** Infant gut microbiota; Neonatal Intensive Care Unit (NICU); preterm neonates; nosocomial outbreaks; extended spectrum beta-lactamase (ESBL) producing *Klebsiella pneumoniae* (ESBL-Kp).

**Corresponding author:**
Simona Panelli
E-mail: simona.panelli1@unimi.it

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Demographic features, clinical covariates, and days of sampling for the microbiota study are reported in Table 1. Overall, these subjects reflect the heterogeneity of situations normally found within an NICU, here pictured during an outbreak. They thus provide useful insights into the relevant problem of HAIs and their interferences with the proper establishment of the gut microbiota in early life, in a situation in which the evolution of the bacterial colonization is already compromised, as happens for neonates inNICUs. The birth weight of enrolled patients ranged from 921 to 3450 g (median: 1805 g) and the gestational age from 29 to 39 weeks (median: 35). 10/14 infants were born by caesarean section; 2/14 were fed exclusively with breast milk and 4/14 with mixed nutrition. One infant required an endotracheal tube and four required a central vascular catheter, no patient underwent surgery. Seven neonates were positive for ESBL-Kp, as determined by performing a rectal swab at admission and then every seven days. Two of them (patients 9 and 11) were positive from the first rectal swab (at day 6 and 3, respectively). Nine infants had a record of receiving antibiotics during the sampling period, the most common treatments being ampicillin and gentamicin. Infant 11 developed ESBL-Kp conjunctivitis 12 days after colonization. ESBL-Kp colonization did not influence the clinical course of any patient and all the neonates were discharged, on average, after 23 days (range 14-189) of hospitalization.

For the microbiota characterization, at least two faecal samples were obtained from each subject. The first and the second samples were collected within the first week of life and within day 20, respectively. Overall, sampling times varied from 1 to 38 days (Table 1). DNA was extracted from a total of 34 samples using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, DE). V1-V3 regions of the 16S rRNA gene were amplified and sequenced on a 454 GS Junior platform (http://netdocs.roche.com/PPM/GS_FLXplus_Sequencing_XLR70_Kit_Method_Manual_May_2011.pdf). Bioinformatics analyses were based on Mothur (mothur.org) and taxonomy was assigned against the reference database RDP classifier (rdp.cme.msu.edu). High-quality reads were clustered into Operational Taxonomic Units (OTUs) at 97% homology, and resolved into 5 bacterial phyla, 49 families, and 74 genera.

Figure 1 shows the most abundant phyla, families, and genera for each infant along the sampling points, depicting the evolution of the gut microbiota. Most communities are dominated by one or a few taxa, and often undergo dramatic changes over the course of days. Proteobacteria, Enterobacteriaceae and, often, Klebsiella dominate the communities, even in non-colonized newborns. This pattern is consistently reproduced, independent of gestational age, birth weight or other clinical covariates. Many of the most abundant and common taxa are known inhabitants of the hospital environment, such as

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### Table 1 - Clinical characteristics of the enrolled newborn infants.

<table>
<thead>
<tr>
<th>Infant</th>
<th>Gestational age (weeks+ day)</th>
<th>Delivery</th>
<th>Gender</th>
<th>Birth weight (g)</th>
<th>Days of sampling</th>
<th>Feeding</th>
<th>Days (d) of parenteral nutrition</th>
<th>Antibiotics (d=days)</th>
<th>Nasogastric tube</th>
<th>Central vascular catheter</th>
<th>Days of hospitalization</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>CS</td>
<td>M</td>
<td>1760</td>
<td>3, 17</td>
<td>F+BM</td>
<td>8 d</td>
<td>AMP, GEN (2 d)</td>
<td>YES</td>
<td>YES</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>34+5</td>
<td>CS</td>
<td>M</td>
<td>1540</td>
<td>6, 20</td>
<td>F</td>
<td>10 d</td>
<td>YES</td>
<td>YES</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30+6</td>
<td>CS</td>
<td>M</td>
<td>1100</td>
<td>3, 17</td>
<td>BM</td>
<td>11 d</td>
<td>YES</td>
<td>YES</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>36+4</td>
<td>V</td>
<td>M</td>
<td>2289</td>
<td>6, 20, 35</td>
<td>F</td>
<td>4 d</td>
<td>SAM (3 d)</td>
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<td>YES</td>
<td>42</td>
</tr>
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<td>5</td>
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<td>F</td>
<td>1480</td>
<td>1, 16</td>
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<tr>
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<td>V</td>
<td>M</td>
<td>3230</td>
<td>3, 17, 32, 38</td>
<td>F</td>
<td>6 d</td>
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<td></td>
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<td>M</td>
<td>921</td>
<td>1, 9, 16, 37</td>
<td>F+BM</td>
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<td>YES</td>
<td>46</td>
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<tr>
<td>8</td>
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<td>V</td>
<td>F</td>
<td>2240</td>
<td>4, 11</td>
<td>F+BM</td>
<td>9 d</td>
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<td>1850</td>
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<td>F</td>
<td>0 d</td>
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<td>M</td>
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<td>F</td>
<td>16 d</td>
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<td>3, 10</td>
<td>F</td>
<td>5 d</td>
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<td>1695</td>
<td>7, 14</td>
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<td>M</td>
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<td>2595</td>
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<td>0 d</td>
<td>MEM, AK (4 d), AMP (2 d)</td>
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<td>27</td>
<td></td>
</tr>
</tbody>
</table>

aCS=C-section; V=vaginal. Bold characters indicate samples collected when the infant resulted positive for ESBL-Kp following the rectal swab. BM=breast milk; F=formula. AMP=ampicillin, GEN=gentamicin, CEF=cefepime, SAM=ampicillin-sulbactam, AMC=amoxicillin-clavulanic acid, MEM=meropenem, AK=amikacin.
Clostridium XI, which is the phylogenetic cluster containing Clostridoides difficile, as well as other opportunistic pathogens (infants 1, 3, 4, 5, 6, 7, 8), Pseudomonas spp. (infants 2, 8), and Enterococcus spp. (infants 2, 5, 12). Some interesting considerations emerge from the analysis of the colonization routes in Figure 1. Neonates 6 and 7 are both negative for ESBL-Kp at admission and become colonized later. The evolution of their communities is similar; with a decrease in Firmicutes and an increase in Proteobacteria after colonization, even if they present divergent clinical features (see Table 1). On the other hand, patient 4, who was colonized after admission as well, displays a divergent situation, with a microbiota constantly dominated by Enterobacteriaceae and, within it, Klebsiella, along all 3 timepoints (the last one after colonization).

Neonates 9 and 11 are the only patients positive for ESBL-Kp from the first sample. They both present decreasing Proteobacteria and increasing Firmicutes from the first to the second timepoint.

Patients never colonized by ESBL-Kp show different microbiota compositions and evolution trajectories. In newborn 14 (full-term, vaginally delivered, breastfed, not colonized, no invasive procedures), the initially preponderant Proteobacteria (see days 5 and 12) are later substituted by Firmicutes (especially Streptococcus spp.) that dominate the community at day 26. A similar situation characterizes infant 2, even if he differs from patient 14 in many clinical records (e.g., preterm, born by caesarean section, formula fed). On the contrary, other non-colonized neonates present high records of Proteobacteria at all timepoints (patient 13) or a relevant increase of this phylum over time (patients 3 and 5). Finally, in patients 3 and 13, Klebsiella accounts for a relevant proportion of the detected Proteobacteria.

Coherently with the taxonomic picture, community analyses computed using the R libraries Vegan (https://CRAN.R-project.org/package=vegan) and Gplots (https://CRAN.R-project.org/package=gplots) reveal a situation of low within-sample diversity (α-diversity). The main biodiversity indices ranged as follows: Chao1 index: 31.76-92.88; Simpson: 0.72-0.94; Observed Richness: 22.1-62.21, with no increase over days, as normally expected for neonates. The bacterial consortium characterized in infant 7 after colonization (day 16) presents the lowest values for all indexes. Finally, diversity in composition among samples (β-diversity), computed by bootstrap-based clustering and Principal Coordinates Analysis on the unweighted and weighted UNIFRAC dissimilarity matrix, evidenced no clustering of samples based on clinical characteristics (delivery mode, colonization, antibiotics).

Early-life colonization by a correct pioneer gut microbiota is critical for a proper immunological and physiological development, with health repercussions across the lifespan (Arrieta et al., 2014; La Rosa et al., 2014). Even if it is difficult to define a “standard” or “healthy” infant microbiota, general trends are recognizable and linked to the aerobic nature of the newborn gut. The first colonizers are mainly Enterobacteriaceae: in a matter of days, this pioneer flora reduces oxygen levels, allowing colonization by strict anaerobes, dominated by Bifidobacterium, Clostridium, and Bacteroides (Arrieta et al., 2014; Hill et al., 2017). Other abundant taxa at this stage are Enterococcaceae, Streptococcaceae, and Lactobacillaceae (Arrieta et al., 2014).

This route is altered in the premature infant gut, where peculiar factors are at work (Groer et al., 2014; Stewart et al., 2017; Wandro et al., 2018), such as the state of prematurity itself, contact with caregivers, feeding type, antibiotics, invasive medical procedures, and other clinical covariates (Forsgren et al., 2017). The possible onset of outbreaks, facilitated by the peculiar issues of NICUs, contributes as well (Hartz et al., 2015; Escriбанo et al., 2019). Neonates in NICUs are highly vulnerable to colonization and infection by pathogens (bacterial, viral, fungal). This is in turn associated with significant morbidity and mortality, and with lifelong detrimental health outcomes, including through effects on the proper establishment route of the microbiota. The extent of outbreaks in NICUs is remarkable: for example, in a survey conducted in 2017 in various European countries, 10.7% of neonates under care in NICUs were affected by HAIs (Zingg et al., 2017). The burden of antibiotic resistance genes is also implicated: ESBL colonization rates in NICUs is known to reach 39% for the genus Klebsiella and represents a major challenge (Stapleton et al., 2016).

While pathogen gut colonization during nosocomial outbreaks has frequently been reported, the influence of an outbreak on the microbiota establishment in early life is still poorly understood (Escribanio et al., 2019). Our results demonstrated that the neonatal faecal consortia, sequentially analyzed during an outbreak sustained by a multi-drug resistant K. pneumoniae in an NICU, present several peculiarities. They appear to lack beneficial taxa, such as Bifidobacterium spp., whose abundance is expected to increase after the first “aerobic” phase of the neonatal gut (Wandro et al., 2018) and are instead dominated by Proteobacteria (and, within it, by the Enterobacteriacae family), and by genera such as Enterococcus spp. and Staphylococcus spp. Overall, these results reinforce the picture of preterm infants in NICUs carrying gut communities dominated by facultative anaerobes, and thus with a delay in the establishment of the ecosystem, as further witnessed by similar α diversity values over the course of sampling points (La Rosa et al., 2014; Escribano et al., 2019). Regardless of high interindividual variability, Proteobacteria, Enterobacteriaceae, and for many samples Klebsiella often dominate the microbiota in non-colonized neonates as well. Moreover, longitudinal sampling often evidenced dramatic changes in bacterial composition over the course of days. This pattern was observed consistently, independent of clinical variables. Therefore, sharing an environment such as an NICU, even more so during an outbreak sustained by an MDR strain, seemed to drive the evolution of these developing consortia more strongly than clinical covariates and other factors.

The physiological replacement of Proteobacteria by Firmicutes (Arrieta et al., 2014) was infrequent in our cohort, in accord with previous observations on preterm infants (La Rosa et al., 2014; Escribano et al., 2019). Patients 14 and 2 represented two exceptions. They were both non-colonized subjects, but the former’s condition was similar to the physiological status of a neonate “outside the NICU,” whereas the latter was a low-birth-weight preterm infant. In other patients, the ongoing increase of Firmicutes was likely “stopped” by the ESBL-Kp colonization and by the consequent enrichment in Proteobacteria (e.g. patient 7). Finally, an increase of Proteobacteria in successive timepoints was observed as well, even in a non-colonized situation (neonate 5). It is to be noted...
Figure 1 - Top bacterial phyla (A), families (B) and genera (C) making up the gut microbiota of the enrolled neonates during the ESBL-Kp outbreak. Red squares evidence microbial communities analysed following an ESBL-Kp-positive rectal swab. "d"=day of sampling.
that, outside physiological situations as those typical of the first days of life, a Proteobacteria-rich microbiota, or a bloom of this phylum, has been repeatedly associated with intestinal dysbiosis and inflammatory diseases and, in general, is thought to reflect an unstable structure (Arrieta et al., 2014).

In conclusion, our data agree with recent observations according to which preterm neonates admitted to the same NICU are initially colonized by similar microbial communities, whose evolution then appears "personalized," with extreme inter-individual variation mostly attributable to the infant (Wandro et al., 2018, Escribano et al., 2019). Our results also support the emerging picture of preterm infants as a population with no uniform early gut colonization pattern, and no clearly defined microbial signatures associated to clinical status and outcomes and to perinatal exposures. Finally, our data are suggestive of the strong pressure exerted on microbiota patterns by the nosocomial environment, particularly during ongoing outbreaks, even in neonates in which colonization has not been detected. A K. pneumoniae outbreak influence on the establishment of the microbial communities appears to be universal, as previously noted in NICUs for another relevant pathogen such as S. marcescens (Escribano et al., 2019). Adding data on how the conditions of NICUs interfere with the acquisition of gut microbial communities is pivotal to unravelling long-term detrimental health impacts, reportedly associated to the perturbation of “correct” early-life colonization of the gut by a pioneer microbiota (La Rosa et al., 2014). These issues become even more pressing when additional disturbing factors aggravate the picture, as happens when HAI's sustained by MDR bacteria spread in NICUs. All these situations deserve continuous research, as they represent primary causes of infant morbidity and mortality.

Acknowledgments

The authors wish to thank the Fondazione "Romeo ed Enrica Invernizzi". The authors would like to thank Claudio Bandi and Piero Marone for promoting the collaboration between S.P. and M.C., and Edward Christopher Davis for the grammatical review. This work was supported by Ricerca Corrente 2013 from Fondazione IRCCS Policlinico San Matteo to PC (08069213).

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