

Contribution of maternal gut carriage to neonatal acquisition of extended-spectrum beta-lactamase-producing Enterobacterales in Madagascar and Cambodia.

Corresponding Author: Dr Anne-Lise Beaumont

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study analyzed 499 mother-neonate pairs from community cohorts in Madagascar and Cambodia to investigate early neonatal colonization with ESBL-producing Enterobacterales. Using short- and long-read sequencing, the authors found that only 16.5% of neonatal colonizations could be attributed to maternal gut carriage. Most cases originated from other sources, with primiparity, cesarean section, neonatal resuscitation, and wet season identified as key risk factors. These findings challenge the traditional view that maternal transmission is the main source of early neonatal ESBL-PE acquisition.

This is a well-conducted and valuable study that addresses an important public health issue in low- and middle-income countries. The inclusion of cohorts from Madagascar and Cambodia is a major strength, providing critical data from settings that are often underrepresented in the literature. Although the study has many strong points, I have several comments that I believe could help further improve the clarity, methodological transparency, and overall impact of the work.

I have few main comments

Although the initial cohort size is relatively large, with 499 mother-neonate pairs included, the number of informative pairs for transmission analysis is much smaller. 96 pairs were both colonized which substantially reduce the statistical power for evaluating maternal transmission pathways. This should be discussed.

The variability and early timing of neonatal sampling, with some neonates sampled as early as day 1 and others at day 3 of life is presented as a strength by authors but could also be a major limitation. Early gut colonization in newborns is a progressive process, with rectal or stool cultures often becoming positive only after 48 to 72 hours of life. Neonates sampled earlier (24-48h) may have been falsely classified as non-colonized. This introduces a bias, potentially underestimating the true colonization rate, especially from maternal sources. A uniform and later sampling point, such as at one week of life, would have provided a more accurate assessment of acquisition dynamics and allowed better differentiation between maternal and environmental contributions.

The findings are derived from low-income countries where hygiene standards, infection control measures, and environmental contamination with ESBL-producing Enterobacterales are markedly different from those in high-income countries. In such environments, it is expected that environmental and healthcare-associated sources play a predominant role. In contrast, in high-income settings, maternal transmission may remain a much larger contributor to neonatal colonization. Therefore, while the study provides critical insights into neonatal acquisition in resource-limited contexts, caution is needed when extrapolating these results to settings with different hygiene practices and healthcare infrastructure.

--> Although the authors compare their results to studies from various countries, I believe it is important to fully discuss how differences in income level and healthcare infrastructure might influence transmission patterns, which is crucial for interpreting the generalizability of their findings.

More specific comments :

Line 58: this is an old reference. nothing more recent ?

Line 62 : you can find more recent studies in Madagascar with similar population and higher colonization rates (Milenkov et al 2024)

Line 116: Authors give very few informations regarding how genetic comparisons between isolates were performed. The authors do not specify whether SNP distances were calculated across a core-genome defined at the species level or restricted to isolates belonging to the same sequence type (ST). This makes a huge difference on how many SNPs are compared. These methodological choices critically influence the interpretation of relatedness between isolates. Additionally, the method used to construct the phylogenetic trees is only briefly mentioned (NJ ? Maximum likelihood ?), with no indication of the number of bootstrap replicates or the statistical support for the inferred trees.

Lines 125-127 + 220: are these plasmids frequent in Madagascar or Cambodia ? Can we imagine that they are successful plasmids that can be shared by many different ESBL-producing Enterobacterales ?

Lines 329-344 :

Authors used two different selective culture media for isolating ESBL-producing Enterobacterales: CHROMagar ESBL in Madagascar and Drigalski plates supplemented with cefotaxime in Cambodia. These media have different sensitivities and specificities for detecting ESBL-producing colonies, and CHROMagar facilitates identification of diverse ESBL-E colonies compared to Drigalski-based methods. This discrepancy could have influenced species diversity, and potentially the number of isolates selected for further analysis. This methodological heterogeneity should be discussed.

Table : A table comparing carriers and non-ESBL carriers should be added

Reviewer #2

(Remarks to the Author)

The authors aim to quantify the role of maternal transmission of ESBL-PE in settings in Cambodia and Madagascar. There are not many studies which aim to estimate the importance of different transmission routes, let alone in LMICs.

I feel this is a rather straightforward, but well-described and executed study which clearly shows the limited impact of maternal transmission in ESBL-PE acquisition by neonates.

Minor details: line 392: Was there a pair of identical isolates with identical plasmids. If so, the definition of p and c should be changed to avoid pairs are counted twice.

Reviewer #3

(Remarks to the Author)

The manuscript reports on two mother-newborn cohorts (Cambodia, Madagascar) regarding commonalities/differences between ESBL-PE in mothers and neonates, with the objective to clarify/challenge the common believe that neonatal sepsis in newborns is principally caused by ESBL-PE carried out and transmitted by the mother. Investigations included high-resolution approaches such as short- and long-read sequencing, which allowed to compare clones and plasmids. This is the first study using this type of methods to assess the role of mother microbiota in neonatal sepsis. In the end, the authors provide evidence that less than 20% of neonatal colonization refers to maternal transfer, highlighting the importance to consider other (environmental) factors, especially in LMICs.

The paper is well written and the study well-conducted. The outcome is highly relevant

I only have minor comments:

- Increased prevalence of Enterobacter in neonates compared to mothers: I wonder why. The point has not been discussed. Something to do with environmental sources ?

- The Cambodia/Madagascar binome has not been really exploited. I understand that it reflects more previous projects. But I would suggest to take the opportunity of having these two situations to have a specific paragraph in the discussion section to elaborate on the data, whatever similar (which may indicate general features) or different (which may indicate specific local situations). The authors have only discussed ceasarian sections but I guess that many other similar/different characteristics of the countries may provide valuable info (including weather, habitats, practices, ...).

- Yes working in the community is a strength avoiding bias due to the diversity of settings. It is also a limitation since the environment of each mother-child pair is different from the other so that identifying which are the other responsible sources is highly challenging. Considering that the main message that mother gut is not the main factor is now known, I wonder whether getting back in a further study to a less diverse environment would not be valuable. Maybe the authors could propose this somewhere in the perspectives ?

- I would not emphasize that much the possible role of breast milk. First of all, breastfeeding is a valuable practice in my view for children health. Second, the ref 36 study used metagenomics and I am not so convinced that the 70% pool of ARG genes really correlates with ESBL-PE colonization by clones and plasmids ... Even though I fully agree that curbing AB use during pregnancy is a good idea. To be appreciated by the authors

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed most of the previous comments and have made clear efforts to explain and moderate their findings in a pedagogical and constructive manner.

However, I still have some concerns:

- Given the kinetics of intestinal microbiota establishment, one may question the sensitivity of ESBL-E detection from a rectal swab taken on day 1 of life. So, I remain skeptical about the impact of sampling at different time points and continue to believe this could introduce a bias that may affect the interpretation of the study. To further address this concern, the authors could consider adding a table or a paragraph comparing maternal or other sources of acquisition according to the day of sampling (day 1, 2, or 3). This would provide valuable data to support the interpretation of their findings.

- Since the study involved a limited number of centers (two sites per country), it is plausible that some ESBL-E transmissions resulted from ongoing outbreaks within certain maternity wards or neonatal units. To rule out the possibility that the observed transmissions are driven by local epidemics—particularly in Cambodia—it would be helpful to include the SNP distance matrix from the core genome comparison of all the isolates by species. Supplemental figures 3, 6 and 7 suggest that some strains are closely related. Do these represent an outbreak cluster? This could be clarified by adding the SNP matrix and could be discussed.

- Lines 180 and 184 : wrong figure and supp table numbering

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Authors have addressed main comments

made.

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Responses to reviewers comments:

Reviewer #1 (Remarks to the Author):

This study analyzed 499 mother-neonate pairs from community cohorts in Madagascar and Cambodia to investigate early neonatal colonization with ESBL-producing Enterobacterales. Using short- and long-read sequencing, the authors found that only 16.5% of neonatal colonizations could be attributed to maternal gut carriage. Most cases originated from other sources, with primiparity, cesarean section, neonatal resuscitation, and wet season identified as key risk factors. These findings challenge the traditional view that maternal transmission is the main source of early neonatal ESBL-PE acquisition.

This is a well-conducted and valuable study that addresses an important public health issue in low- and middle-income countries. The inclusion of cohorts from Madagascar and Cambodia is a major strength, providing critical data from settings that are often underrepresented in the literature. Although the study has many strong points, I have several comments that I believe could help further improve the clarity, methodological transparency, and overall impact of the work.

We would like to thank Reviewer #1 for the time allocated to the reading and revision of our work.

I have few main comments

Although the initial cohort size is relatively large, with 499 mother-neonate pairs included, the number of informative pairs for transmission analysis is much smaller. 96 pairs were both colonized which substantially reduce the statistical power for evaluating maternal transmission pathways. This should be discussed.

Thank you for this relevant comment. However, while 98 out of 499 mother-neonate pairs were co-colonized, our analysis does not rely solely on co-colonized pairs. Rather, it is based on all 170 colonized neonates, including both concordant and discordant colonized pairs. Notably, the 72 discordant cases (where the neonate was colonized but the mother was not) provide important information on potential non-maternal acquisition routes. The limitation is therefore the size of the colonized subgroup itself (n=170), rather than the proportion of co-colonized pairs. We have added the following sentence to the manuscript (lines 341-342) as a limitation: *“Additionally, the limited number of colonized neonates, reduced the statistical power of our analysis and underscores the need for further studies in larger cohorts.”*

The variability and early timing of neonatal sampling, with some neonates sampled as early as day 1 and others at day 3 of life is presented as a strength by authors but could also be a major limitation. Early gut colonization in newborns is a progressive process, with rectal or stool cultures often becoming positive only after 48 to 72 hours of life. Neonates sampled earlier (24-48h) may have been falsely classified as non-

colonized. This introduces a bias, potentially underestimating the true colonization rate, especially from maternal sources. A uniform and later sampling point, such as at one week of life, would have provided a more accurate assessment of acquisition dynamics and allowed better differentiation between maternal and environmental contributions.

We agree with your remark, this could indeed represent a limitation of our study. However, we would like to emphasize that our methodological choice was guided by two arguments:

- First, we selected this early sampling window because most cases of neonatal sepsis occur within the first three days of life. Since infections are typically preceded by a phase of colonization, this suggests that colonization can happen rapidly. Our findings may therefore help guide timely interventions to prevent early-onset infections.
- Second, we chose an early sampling point because using a later one would have made it more difficult to determine whether acquisition was directly originated from the mother. By one week of life, neonates are typically exposed to a broader range of potential sources. For example, they are also in contact with other household members who could have acquired the bacteria from the mother (or from a common source, such as food) and then transmitted it to the neonate. This could have introduced greater ambiguity in attributing the source of colonization.

To clarify our rationale, we have added this explanation to the Discussion section (lines 320-325) : *“The early timeframe (first three days of life) was selected as it aligns with the period when most cases of neonatal sepsis occur and facilitates clearer attribution of colonization to maternal transmission. Delayed sampling would increase the likelihood of alternative transmission pathways, making it more challenging to distinguish direct maternal acquisition from indirect acquisition through other household members colonized with the same strain or shared environmental sources, such as water, for example. »*

In addition, experimental data support this approach. The BARNARDS study (Carvalho et al., Nat Microbiol 2022, doi: 10.1038/s41564-022-01184-y) did not observe a significant difference in the prevalence of antibiotic resistance genes between neonates sampled within 0–72 hours versus those sampled later (72 hours–60 days ; Supplementary Table 2b). While these findings support our overall hypothesis, it is important to note that the BARNARDS study used resistome-based methods, which may not be directly comparable to our culture-based approach. In our study, among neonates in whom acquisition was attributed to the mother, 81.5% were sampled before 48 hours of life. This proportion is not significantly different from that of the other neonates, of whom 66.4% were also sampled before 48 hours ($p = 0.16$). This suggests that the timing of sampling did not introduce a major bias in detecting maternal transmission within our study population.

That said, we acknowledge that colonization is a progressive process, and that an ideal design would have included multiple standardized sampling timepoints for all

participants, to better capture the dynamics of colonization, as well as sampling of others potential sources. Therefore, we have added this sentence to the discussion (lines 342-353) : *“Because neonatal colonization is a dynamic process, early sampling may have underestimated the colonization rate and, consequently, maternal acquisition. However, later sampling might also reflect transmission from other sources. Future studies should include multiple sampling points during the first week of life and consider additional potential sources.”*

The findings are derived from low-income countries where hygiene standards, infection control measures, and environmental contamination with ESBL-producing Enterobacterales are markedly different from those in high-income countries. In such environments, it is expected that environmental and healthcare-associated sources play a predominant role. In contrast, in high-income settings, maternal transmission may remain a much larger contributor to neonatal colonization. Therefore, while the study provides critical insights into neonatal acquisition in resource-limited contexts, caution is needed when extrapolating these results to settings with different hygiene practices and healthcare infrastructure.

--> Although the authors compare their results to studies from various countries, I believe it is important to fully discuss how differences in income level and healthcare infrastructure might influence transmission patterns, which is crucial for interpreting the generalizability of their findings.

Thank you for raising this important point, which may indeed not be immediately apparent to all readers and deserves to be emphasized. In response to your comment and the one of another reviewer, we expanded the Discussion section to provide a more detailed comparison between Madagascar and Cambodia, and also to discuss the generalizability of our findings to other settings, by including the following text (lines 277-284) : *“Differences in income level and geographical context are often associated with substantial variation in infection prevention and control practices, as well as in levels of environmental contamination, notably within healthcare settings. In Madagascar, approximately one-third of deliveries took place at home, leading to markedly different environmental exposures. As a result, transmission dynamics in high-income countries are likely to differ from those observed in our study, and our findings may not be generalizable to such settings. In contexts where hospital-based ESBL-PE contamination is low and hygiene protocols are strictly enforced, as is often the case in high-income countries, the mother may remain a key source of early neonatal acquisition.”*

More specific comments :

Line 58: this is an old reference. nothing more recent ?

We have replaced the reference with a more recent source: the study *“Global burden of bacterial antimicrobial resistance 1990-2021: a systematic analysis with forecasts*

to 2050,” published in *The Lancet* in 2024 (lines 57-58): “*This contributed to an estimated ~430,000 neonatal deaths associated to antimicrobial resistance in 2021* »

Line 62 : you can find more recent studies in Madagascar with similar population and higher colonization rates (Milenkov et al 2024)

Thank you for this correction, we corrected the introduction accordingly (lines 61-62: “*reaching up to 80% in Cambodia and 34% in Madagascar (Milenkov 2024).*”

Line 116: Authors give very few informations regarding how genetic comparisons between isolates were performed. The authors do not specify whether SNP distances were calculated across a core-genome defined at the species level or restricted to isolates belonging to the same sequence type (ST). This makes a huge difference on how many SNPs are compared. These methodological choices critically influence the interpretation of relatedness between isolates. Additionally, the method used to construct the phylogenetic trees is only briefly mentioned (NJ ? Maximum likelihood ?), with no indication of the number of bootstrap replicates or the statistical support for the inferred trees.

We agree that our Methods section may not have provided sufficient detail on these points.

First, regarding the method used to calculate SNP distances, we applied an approach in which the core genome was defined specifically for each mother–neonate pair, thereby maximizing the size of the shared core genome and increasing the sensitivity of SNP detection.

The following sentence was added in the Material and Methods section (lines 460-464): “*For each mother-neonate pair involving ESBL-PE isolates from the same species, the reads sequenced from the neonatal isolate were aligned against the assembled maternal isolate genome using bwa-mem2 v2.2.1 (github.com/bwa-mem2/bwa-mem2) in order to estimate the number of Single Nucleotide Polymorphisms (SNPs) between the two isolate genomes.*”

Second, the phylogenetic trees were constructed using JolyTree at the genus level, a distance-based method (related to the minimum evolution criterion), and then using IQ-TREE at the species level (which optimizes maximum-likelihood criteria). These tools were mentioned in the associated figure captions, but we have now expanded the Material and Method accordingly (lines 469-470) : “*At the genus level, genome-based phylogenetic trees were inferred using the distance-based method JolyTree v2.1 (Supplementary Figures 3, 6 and 7). At the species level, maximum likelihood phylogenetic trees were inferred using IQ-TREE*”

Then, branch supports of the genus trees were estimated using the rate of elementary quartets as estimated by JolyTree, and this information has been added to the relevant Supplementary Figure captions. Branch supports of the species trees were estimated using the UFboot approach (1,000 replicates) as implemented in IQ-TREE, but not displayed in the Figure 2 for better reading, as almost all branch supports were 100%; however, this last information was added in the Figure 2

caption : *“Almost all internal branches are 100% supported as assessed by the UFboot approach (1,000 replicates) implemented in IQ-TREE”*

Lines 125-127 + 220: are these plasmids frequent in Madagascar or Cambodia ?
Can we imagine that they are successful plasmids that can be shared by many different ESBL-producing Enterobacterales ?

We understand your concern that the presence of similar plasmids in both maternal and neonatal isolates does not definitively establish direct maternal transmission, as certain plasmids may be widely circulating within the local bacterial population. We had already acknowledged this limitation (lines 337-338): *“Furthermore, it is still unknown how often identical plasmids could occur without a transmission, as “core plasmids” of some lineages”*. To investigate on the frequency of the five identical plasmids, we compared our data to published databases. According to the PLSDB plasmid database (<https://ccb-microbe.cs.uni-saarland.de/plsdb2025>), using the mash dist option with default settings, the three plasmids identified in Cambodia displayed at least 80% similarity to plasmids commonly found in that country. In contrast, the two plasmids identified in Madagascar had matches in the database, but none originating from Madagascar, although some metadata associated with the matches were unavailable. To further investigate, plasmid sequences from Madagascar were compared to assemblies from project PRJEB56633 in the Enterobase Escherichia database

(<https://enterobase.warwick.ac.uk/species/index/ecoli>), which includes 114 ESBL-carrying plasmids recently characterized in Madagascar (Milenkov et al., The Lancet Microbe, 2025, DOI: 10.1016/S2666-5247(24)00065-X). No matches were found, even at a lower threshold (60% coverage). Taken together, these findings suggest that the coincidental co-occurrence hypothesis is unlikely in the Madagascar setting but cannot be excluded in Cambodia. However, we believe that the short time interval between sampling and the close mother–neonate relationship support maternal transmission as the most plausible explanation. We have now made this limitation more explicit (line 337-340) : *“Furthermore, it is still unknown how often identical plasmids could occur without a transmission, as “core plasmids” of some lineages, potentially leading to a mistaken conclusion of transmission when it may merely reflect a coincidental co-occurrence. However, the narrow spatiotemporal window between the two samples makes this alternative explanation less likely.”*)

Lines 329-344 :

Authors used two different selective culture media for isolating ESBL-producing Enterobacterales: CHROMagar ESBL in Madagascar and Drigalski plates supplemented with cefotaxime in Cambodia. These media have different sensitivities and specificities for detecting ESBL-producing colonies, and CHROMagar facilitates identification of diverse ESBL-E colonies compared to Drigalski-based methods. This discrepancy could have influenced species diversity, and potentially the number of

isolates selected for further analysis. This methodological heterogeneity should be discussed.

This is a very good point. The heterogeneity in the selective media used was due to technical constraints but we agree that using CHROMagar ESBL in both countries would have been better. We added this sentence to the discussion (lines 331-334) : *“Heterogeneity in the selective media used in Madagascar and Cambodia may have introduced differential bias beyond detection performance, as chromogenic media facilitate the identification of morphologically distinct ESBL-producing strains.”*

Table : A table comparing carriers and non-ESBL carriers should be added

This table has been added to the Supplementary Material (Supplementary Table 1) and is now referenced in the main text at lines 113–114.

Reviewer #2 (Remarks to the Author):

The authors aim to quantify the role of maternal transmission of ESBL-PE in settings in Cambodia and Madagascar. There are not many studies which aim to estimate the importance of different transmission routes, let alone in LMICs.

I feel this is a rather straightforward, but well-described and executed study which clearly shows the limited impact of maternal transmission in ESBL-PE acquisition by neonates.

Minor details: line 392: Was there a pair of identical isolates with identical plasmids. If so, the definition of p and c should be changed to avoid pairs are counted twice.

Thank you for reviewing our work and for this comment. In fact, c refers to the number of pairs with identical isolates, while p corresponds to those with different isolates but identical plasmids. Therefore, no double counting occurred. To clarify this point, we have added this information to the Methods section (lines 493-496): *“The contribution of maternal versus other sources to ESBL-PE acquisition in newborns was estimated as $(c+p)/n$ and $1-(c+p)/n$, respectively, where c is the number of pairs with identical isolates, p the number of pairs with different isolates but identical plasmids, and n the total number of newborns colonized with ESBL-PE.”*

Reviewer #3 (Remarks to the Author):

The manuscript reports on two mother-newborn cohorts (Cambodia, Madagascar) regarding commonalities/differences between ESBL-PE in mothers and neonates, with the objective to clarify/challenge the common believe that neonatal sepsis in newborns is principally caused by ESBL-PE carried out and transmitted by the mother. Investigations included high-resolution approaches such as short- and long-read sequencing, which allowed to compare clones and plasmids. This is the first study using this type of methods to assess the role of mother microbiota in neonatal

sepsis. In the end, the authors provide evidence that less than 20% of neonatal colonization refers to maternal transfer, highlighting the importance to consider other (environmental) factors, especially in LMICs.

The paper is well written and the study well-conducted. The outcome is highly relevant

Many thanks for the time you allocated to revising our work.

I only have minor comments:

- Increased prevalence of *Enterobacter* in neonates compared to mothers: I wonder why. The point has not been discussed. Something to do with environmental sources ?

You raise a very important point. We fully agree that the difference in species distribution between mothers and neonates is striking and deserves further discussion. The higher prevalence of *Enterobacter* in neonates likely results from a combination of factors. Healthcare settings are well-recognized reservoirs for *Enterobacter*, which is a common cause of NICU outbreaks. While mothers are also exposed to the hospital environment, neonates encounter specific exposures within the maternity ward, such as surfaces or medical equipment, that can serve as concentrated reservoirs for *Enterobacter* species. Neonatal susceptibility might also play an important role in colonization. The reduced microbial diversity and immaturity of the neonatal microbiota may result in lower colonization resistance, thereby facilitating *Enterobacter* establishment. We added the following text within the discussion (lines 263-267): *“Furthermore, the distinct species distribution between mothers and neonates, with a substantially higher proportion of Klebsiella spp. and Enterobacter spp. in neonates, also suggests the involvement of sources other than the mother. This finding points towards the role of environmental reservoirs as likely contributors to early colonization, notably hospital reservoirs as these two bacterial genera are frequently associated with nosocomial transmission.”*

- The Cambodia/Madagascar binome has not been really exploited. I understand that it reflects more previous projects. But I would suggest to take the opportunity of having these two situations to have a specific paragraph in the discussion section to elaborate on the data, whatever similar (which may indicate general features) or different (which may indicate specific local situations). The authors have only discussed cesarean sections but I guess that many other similar/different characteristics of the countries may provide valuable info (including weather, habitats, practices, ...).

The multicentric design of our study indeed warrants a more thorough comparison of the cohorts. To address this, we added a dedicated paragraph in the Results section highlighting the main similarities and differences between settings (lines 102-108):

“The proportion of primiparous women, as well as the number of low-birth-weight neonates or those requiring neonatal resuscitation, was similar across the two studies. However, the level of infrastructure differed between countries: in Cambodia, a significantly higher proportion of households had access to electricity ($p < 0.001$) and indoor toilet facilities ($p < 0.001$), and mothers were more often engaged in paid employment ($p < 0.001$). Another major difference concerned the place of delivery: 82% of Cambodian mothers gave birth in a hospital or clinic, compared to less than 15% in Madagascar, where a large proportion of deliveries occurred at home.”

In addition, we further discussed these findings in the Discussion section as follows (line 277-295):

“Differences in income level and geographical context are often associated with substantial variation in infection prevention and control practices, as well as in levels of environmental contamination, notably within healthcare settings. As a result, transmission dynamics in high-income countries are likely to differ markedly from those observed in our study, and our findings may not be generalizable to such settings. In contexts where hospital-based ESBL contamination is low and hygiene protocols are strictly enforced, as is often the case in high-income countries, the mother may remain a key source of early neonatal acquisition. However, this hypothesis has yet to be formally confirmed.

Marked socio-demographic differences were observed between the two countries, likely reflecting their respective World Bank income classifications: Cambodia has been designated a lower-middle-income country since 2015, whereas Madagascar remains a low-income country. In addition to socio-demographic differences, the prevalence of ESBL colonization was significantly higher in Cambodia, consistent with the literature, both among mothers and neonates and in the general population. Part of the explanation may lie in Cambodia’s geographic and economic context: Cambodia shares land borders and trade routes with several countries in Southeast Asia, a region known for intense antibiotic use and circulation of substandard antimicrobial drugs, factors that may contribute to higher antimicrobial resistance pressure. In contrast, Madagascar’s insular geography may act as a partial barrier to such cross-border circulation. Yet despite these contextual differences, the proportion of acquisitions attributable to maternal sources did not differ significantly between countries.”

- Yes working in the community is a strength avoiding bias due to the diversity of settings. It is also a limitation since the environment of each mother-child pair is different from the other so that identifying which are the other responsible sources is highly challenging. Considering that the main message that mother gut is not the main factor is now known, I wonder whether getting back in a further study to a less diverse environment would not be valuable. Maybe the authors could propose this somewhere in the perspectives ?

Yes, we fully agree. Our group has in fact conducted such a study in Madagascar, and the data are currently under analysis. We have added the following sentence to

the Perspectives section (lines 361-364): *“Notably, potential sources of transmission may differ substantially across contexts, between countries, delivery settings, or rural and urban environments. Transmission pathways should therefore be assessed within each specific epidemiological setting.”*

- I would not emphasize that much the possible role of breast milk. First of all, breastfeeding is a valuable practice in my view for children health. Second, the ref 36 study used metagenomics and I am not so convinced that the 70% pool of ARG genes really correlates with ESBL-PE colonization by clones and plasmids ... Even though I fully agree that curbing AB use during pregnancy is a good idea. To be appreciated by the authors

We understand your concern: given the well-established benefits of breastfeeding for child health, it would indeed be inappropriate to raise concerns without supporting evidence, especially considering the limitations of resistome-based studies in this context. However, our team is currently investigating this issue, and preliminary (unpublished) data suggest that a significant proportion of breast milk samples and nipple swabs are culture-positive for ESBL-producing Enterobacterales. While this does not necessarily imply that breast milk is the source of neonatal colonization, we believe this potential route of transmission warrants further investigation.

In response to your comment, we have revised the sentence to better reflect this uncertainty, to introduce other plausible sources of transmission, and to re-emphasize the well-documented health benefits of breastfeeding (lines 364-374): *“Alternative maternal sources like breastmilk, hands, and nipples, should also be investigated. While breastfeeding is widely recognized to confer ⁵⁸ important health benefits and may protect against antimicrobial resistance⁵⁹, it has also been shown to carry antimicrobial resistance genes (ARGs), with one metagenomic study reporting that 70% of ARGs detected in breastmilk are also found in infant feces³⁵. Because resistome-based approaches cannot formally establish transmission pathways, culture-based methods are needed to determine whether transmission through breast milk occurs at the clonal level.*

In addition, a study from Tanzania found that 18.5% of mothers’ hands were contaminated with Gram-negative bacteria resistant to third-generation cephalosporins⁶⁰. This suggests that transmission could also occur via direct contact with the mother’s hands or nipple, which is frequently handled during breastfeeding. »

Responses to reviewer comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed most of the previous comments and have made clear efforts to explain and moderate their findings in a pedagogical and constructive manner.

Thank you for your comment and for revising our work a second time.

However, I still have some concerns:

- Given the kinetics of intestinal microbiota establishment, one may question the sensitivity of ESBL-E detection from a rectal swab taken on day 1 of life. So, I remain skeptical about the impact of sampling at different time points and continue to believe this could introduce a bias that may affect the interpretation of the study. To further address this concern, the authors could consider adding a table or a paragraph comparing maternal or other sources of acquisition according to the day of sampling (day 1, 2, or 3). This would provide valuable data to support the interpretation of their findings.

We understand your concern and agree that our manuscript should include these additional data. Accordingly, we have added Supplementary Table 1, which presents a cross-tabulation of sampling day (day 1, 2, or 3) with ESBL-E colonization prevalence, maternal acquisition, and acquisition from other sources.

	Day of neonatal sampling			p-value*
	Day 1	Day 2	Day 3	
N	397	28	74	
Neonatal ESBL-PE colonization (n, %)	143 (36%)	6 (21.4%)	21 (28.4%)	0.15
ESBL-PE acquisition from the mother	24 (6.0%)	1 (3.6%)	3 (4.1%)	0.92
ESBL-PE acquisition from another source	119 (30.0%)	5 (17.9%)	18 (24.3%)	0.27

**Pearson's Chi-squared test; Fisher test*

No significant differences in these three parameters were observed across sampling days, based on Chi-squared or Fisher's tests. To refer to this table, we have introduced the following additions in the main text:

- *"The majority of neonates (397) were sampled on day 1, 28 were sampled on day 2 and 74 on day 3 (Supplementary Table 1). Neonatal colonization prevalence was not significantly different if sampling was performed on day 1, 2 or 3 of life ($p=0.15$)."* (lines **107-109**)

- “Neonatal acquisition from maternal source ($p=0.92$) or from another source ($p=0.27$) were not significantly associated with the day of sampling (Supplementary Table 1)”. (line **154-156**)

However, although this is not evident from our dataset, we agree that early sampling may represent a limitation that is difficult to assess in the absence of multiple sampling points. Therefore, we retained the following sentence in the discussion, which we had introduced in response to your initial review: “*Because neonatal colonization is a dynamic process, early sampling may have underestimated the colonization rate and, consequently, maternal acquisition. However, later sampling might also reflect transmission from other sources. Future studies should include multiple sampling points during the first week of life and consider additional potential sources.*” (line **351-356**)

- Since the study involved a limited number of centers (two sites per country), it is plausible that some ESBL-E transmissions resulted from ongoing outbreaks within certain maternity wards or neonatal units. To rule out the possibility that the observed transmissions are driven by local epidemics—particularly in Cambodia—it would be helpful to include the SNP distance matrix from the core genome comparison of all the isolates by species. Supplemental figures 3, 6 and 7 suggest that some strains are closely related. Do these represent an outbreak cluster? This could be clarified by adding the SNP matrix and could be discussed.

This is a highly relevant comment. However, although there were only two sites per country, deliveries took place in diverse settings (including hospitals, primary care centers, or at home) which inherently reduces the likelihood that the observed transmissions were driven by outbreaks confined to one single healthcare facility in each site.

To further investigate the possibility of local outbreaks, we computed SNP distance matrices from core genome alignments of all isolates within each species (provided as Supplementary Table 3).

For interpretation, we defined a possible outbreak as a cluster of isolates that: (i) originated from the same site (e.g., Kampong Speu in Cambodia), (ii) were sampled from the same type of delivery setting (e.g., hospital), and (iii) were collected within a relatively short timeframe (< 1 year). Accordingly, we treated Moramanga (semi-rural site in Madagascar) during the BIRDY study as distinct from Moramanga during the NeoLIC study, since more than a year elapsed between the end of the former and the start of the latter. We defined isolates as closely related if they differed by fewer than 10 SNPs. For clarity, SNP distances were color-coded for species with at least one identical mother–neonate pair, namely *Escherichia coli*, *Klebsiella quasipneumoniae*, *Enterobacter kobei*, and *Enterobacter intestinhominis* (see legend of Supplementary Table 3).

As a result, the number of clusters meeting our possible outbreak criteria was very low (n=3), with consistently very few isolates per clusters, indicating no evidence of a major outbreak. All clusters potentially reflecting an outbreak linked to a case of mother-to-neonate transmission are listed in the 'Cluster_description' sheet of the Supplementary Table 3. The largest cluster comprised only five *Enterobacter kobei* isolates - two from the mother–neonate pair and three additional related isolates. The two remaining clusters, one of *Klebsiella quasipneumoniae* and one of *Escherichia coli*, each included only one additional isolate in addition to the mother–neonate pair.

We therefore interpret this additional analysis as evidence that the likelihood of observed transmissions being driven by local epidemics is very low in our dataset. Nevertheless, for these three small clusters in particular, we cannot formally exclude the possibility that mother–neonate transmission arose from a local outbreak, with both individuals infected by a shared third source. This limitation had already been acknowledged in our discussion: “*While we aimed to trace transmission, it remains impossible to definitively determine whether the observed events were due to direct maternal transmission or exposure to a third common source*” (line **341-343**).

However, it is also important to note that these clusters may indicate the co-occurrence of circulating isolates within a defined geographical population, rather than a true outbreak. Moreover, even in the context of a local outbreak, maternal-to-neonate transmission remains plausible. Finally, if a few rare cases were indeed outbreak-related, this would only reinforce our conclusion regarding the low proportion of neonatal acquisitions from maternal sources.

To include these results in the manuscript, the following modifications have been made:

- In the methods section :
 - “*This design allowed women to be enrolled regardless of their antenatal care status. Therefore, in each site, deliveries could occur in diverse settings, including hospitals, primary healthcare centers, or at home.*” (line **407-409**)
 - “*Using Parsnp⁷⁰ v1.6.2, we also computed pairwise Single Nucleotide Polymorphisms (SNP) matrices from the core genome alignment, constructed from all isolates of each bacterial species. This analysis aimed to estimate the number of SNPs between mother–neonate pairs involving ESBL-PE isolates of the same species and to identify potential local outbreaks linked to identical pairs. We defined a possible outbreak as a cluster of isolates differing by fewer than 10 SNPs, associated with a mother–neonate pair, originating from the same site (e.g., Kampong Speu), sampled from the same type of delivery setting (e.g., hospital), and collected within a relatively short timeframe (< 1 year).*” (line **467-473**)
- In the results section : “*Interestingly, potential outbreaks involving mother–neonate pairs were rare (Supplementary Table 3), with only three clusters identified. Each cluster involved a small number of isolates, with a maximum of*

three additional isolates beyond those from the mother–neonate pair in the largest cluster. The other two clusters included only one additional isolate each.” (line **135-139**)

- In the discussion section : *“However, although such a scenario could be more plausible in the context of a local outbreak, this remains marginal in our dataset and is unlikely to have had a significant influence on our results.”* (line **344-346**)

Besides, despite differences in SNP calculation methods, this additional analysis corroborated our initial findings, showing a minimal number of 0–2 SNPs between genomes of mother–neonate pairs forming a cherry in the phylogenetic trees. To avoid confusion arising from the use of two different SNP calculation methods, we removed the first analysis (i.e., deleted *“For each mother-neonate pair involving ESBL-PE isolates from the same species, the reads sequenced from the neonatal isolate were aligned against the assembled maternal isolate genome using bwa-mem2 v2.2.1 (github.com/bwa-mem2/bwa-mem2) in order to estimate the number of Single Nucleotide Polymorphisms (SNPs) between the two isolate genomes.”*, line **448**) and updated Supplementary Figure 1 using the results from the SNP distance matrices.

- Lines 180 and 184 : wrong figure and supp table numbering

We have corrected the numbering of the figure and supplementary tables, and updated all references in the text to reflect the addition of a new supplementary table.