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2 **Characterization of *Klebsiella pneumoniae* isolates from a mother-child**  
3 **cohort in Madagascar.**

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15

16 **Abstract**

17 **Objectives:** To define characteristics of *Klebsiella pneumoniae* (*Kp*) isolated from carriage and  
18 infections in mothers and their neonates belonging to a paediatric cohort in Madagascar.

19 **Methods:** A total of 2000 mothers and their 2001 neonates were included. For each mother, vaginal  
20 and stool samples were collected at birth. Additionally, upon suspicion of infection, samples were  
21 collected from suspected infected body sites in 121 neonates. Genomic sequences of all isolated *Kp*  
22 were used for phylogenetic analyses and to investigate genomic content of antimicrobial resistance  
23 genes, virulence genes and plasmid replicon types.

24 **Results:** Five percent (n=101) of mothers were *Kp*-positive. Of 251 collected *Kp* isolates, 102 (40.6%)  
25 were from mothers and 149 (59.3%) from neonates. A total of 49 (19.5%; all from infants except one)  
26 isolates were from infected body sites. Multilocus sequence typing (MLST) identified 108 different  
27 sequence types (ST) distributed over the six *Kp* phylogroups Kp1 to Kp6. We found 65 (25.8%)  
28 extended-spectrum beta-lactamase (ESBL) producers and a total of 101 (40.2%) multidrug resistant  
29 isolates. The most common ESBL gene was *bla*<sub>CTX-M-15</sub> (in 99.3% of isolates expressing ESBL). One  
30 isolate co-harbored ESBL *bla*<sub>CTX-M-15</sub> and *bla*<sub>NDM-1</sub> genes. Three isolates from infected body sites  
31 belonged to hypervirulent-associated ST23 (n=1) and ST25 (n=2). We observed two cases of mother-  
32 to-child transmission and sustained *Kp* carriage was identified in ten neonates, with identical isolates  
33 observed longitudinally over the course of 18 to 115 days.

34 **Conclusions:** This study revealed substantial genetic diversity and a high rate of antimicrobial  
35 resistance among *Kp* isolated from both carriage and infections in Madagascar.

36

37 **Introduction**

38 *Klebsiella pneumoniae* (*Kp*) is a non-motile and encapsulated member of the Enterobacteriaceae. The  
39 phylogeny of *Kp* (*sensu lato*) is organized into seven phylogroups (Kp1 to Kp7), which were recently  
40 redistributed into distinct taxa. Whereas *K. pneumoniae sensu stricto* now corresponds to Kp1, *K.*  
41 *quasipneumoniae* corresponds to Kp2 (subsp. *quasipneumoniae*) and Kp4 (subsp. *similipneumoniae*),

42 *K. variicola* to Kp3 (subsp. *variicola*) and Kp5 (subsp. *tropica*), “*K. quasivariicola*” to Kp6 and *K.*  
43 *Africana* to Kp7.<sup>1-3</sup> Nevertheless, these taxa are typically all still identified as *Kp* in clinical  
44 microbiology laboratories.

45 *Kp* resides as a normal member of human mucosal flora and is common in the gut. However, *Kp* also  
46 causes severe opportunistic infection in some carriers, and has emerged as an important bacterial  
47 pathogen causing hospital-acquired infections such as septicemia, pneumonia and urinary tract  
48 infections that are resistant to multiple commonly used antibiotics.<sup>4,5</sup> *Kp* can also cause community-  
49 acquired infections, such as pyogenic liver abscesses sometimes complicated by meningitis or  
50 endophthalmitis, and soft tissue abscesses. Community infections are often caused by virulent  
51 clones.<sup>6,7</sup>

52 *Kp* can be transmitted from mother to infant and poses a high risk to colonized neonates.<sup>8-10</sup> An  
53 estimated 20% of neonatal sepsis-related deaths due to treatment failure in the developing world  
54 are attributed to *Kp*.<sup>11</sup> High fatality rates in neonatal *Kp* infections have been reported in India and in  
55 Thailand.<sup>11</sup> Among hospitalized children in Madagascar 21.2% of intestinal carriage isolates were  
56 reported to be ESBL-producers.<sup>9</sup> Chereau *et al.*, studying fecal carriage in pregnant women, reported  
57 a prevalence of 18.5 % of ESBL-producing Enterobacteriaceae, among which *Kp* was one of the most  
58 frequently reported species.<sup>12</sup>

59 The aim of the present work was to determine the microbiological characteristics, particularly  
60 including in particular antimicrobial resistance phenotypes, of *Kp* isolates from pregnant women and  
61 their neonates during a longitudinal study conducted between 2012 and 2016 in urban and rural sites  
62 in Madagascar. We characterized all *Kp* strains isolated from infected body sites and from genital and  
63 fecal samples using phenotypic and genotypic methods. We also investigated the occurrence of  
64 mother-to-child transmission and prolonged carriage of *Kp* in neonates.

65  
66  
67

68 **Materials and Methods**

69

70 ***Patients and bacterial isolates***

71 This study was conducted in the context of an international pediatric cohort, the BIRDY (Bacterial  
72 Infections and antibiotic Resistance Disease among Young children in low-income countries) project,  
73 in Antananarivo (Madagascar's largest city with about 1.4 million of inhabitants) and Moramanga (a  
74 rural area located 116 km east of Antananarivo with about 28,000 inhabitants) between 2012 and  
75 2016.

76 Ethical approvals were obtained by the Ethics Committee of the Madagascar Ministry of Public  
77 Health (reference numbers 68MSANP/CE, N°75MSANP/CE and N°150MSANP/CERBM).

78 Inclusion criteria for mothers and their neonates were previously described.<sup>13</sup> Pregnant women were  
79 recruited during their routine third trimester antenatal visits. A vaginal swab was performed to  
80 detect group B *Streptococcus*, and fecal samples or rectal swabs were taken perinatally to detect  
81 extended-spectrum  $\beta$ -lactamase (ESBL)–producing *Enterobacteriaceae*. Neonates were included at  
82 birth and followed-up during their 18<sup>th</sup> months. At birth, neonates were examined and risk factors for  
83 infection were assessed (protocol online as Technical Appendix,  
84 <https://wwwnc.cdc.gov/EID/article/24/4/16-1977-Techapp1.pdf>). The presence of an infectious risk  
85 factor resulted in the taking of different samples (deep ear canal and anal swabs) to detect any  
86 aetiological agent. In the presence of certain clinical indications, other samples were also collected as  
87 appropriate, such as blood, cerebrospinal fluid, urine, etc<sup>14</sup>. Routine follow-up visits were conducted  
88 twice during the first week of life, weekly during the first month, every two weeks until the third  
89 month, monthly until the 12<sup>th</sup> month, and every two months until the 18<sup>th</sup> month. During follow-up,  
90 additional samples were collected if signs of infection were indicated during the examination by the  
91 physician. All clinical cases with a *Kp*-positive culture were reviewed by a medical doctor and  
92 categorised according to the medical history as a strain isolated from carriage or from an infected  
93 body site.

94 Sample specimens were immediately (within 24h of collection) plated on non-selective culture media  
95 used in clinical laboratory procedures (French reference methods REMIC recommandations).<sup>15</sup> No  
96 enrichment media were used before inoculation. All suspected *Kp* isolates were purified on medium  
97 Simmons Citrate Agar with Inositol (SCAI).<sup>16,17</sup> *Klebsiella pneumoniae sensu lato* was initially identified  
98 using MALDI-TOF mass spectrometry (Biotyper version 3.3, Bruker Daltonics, Champs-sur-Marne,  
99 France) and species identity was refined using whole genome sequences (see below).

100

### 101 ***Antimicrobial susceptibility testing***

102 Antimicrobial susceptibility of isolates was assessed by disk diffusion on Mueller-Hinton agar plates.  
103 Antimicrobials included beta-lactams (cefalotin, ceftazidime, cefepime,  
104 amoxicillin-clavulanate, ticarcillin-clavulanate and piperacillin-tazobactam), a monobactam  
105 (aztreonam), carbapenems (imipenem and ertapenem), aminoglycosides (gentamicin, tobramycin,  
106 amikacin and netilmicin), (fluoro)quinolones (nalidixic acid, levofloxacin and ciprofloxacin),  
107 trimethoprim-sulfamethoxazole and tetracycline. CASFM-EUCAST 2016-defined breakpoints for  
108 *Enterobacteriaceae* were used to interpret susceptibility data for *Kp* ([http://www.sfm-](http://www.sfm-microbiology.org)  
109 [microbiology.org](http://www.sfm-microbiology.org)). Production of ESBL was confirmed by the standard double disk synergy test with  
110 and without cloxacillin (250mg/L) (Sigma-Aldrich, Steinheim, Germany) (CASFM/EUCAST, 2016).

111

### 112 ***WGS***

113 DNA was extracted using the DNeasy blood and tissue kit (Qiagen SAS, Courtaboeuf, France),  
114 according to the manufacturer's instructions. Library preparation was conducted using Nextera XT  
115 technology, and sequencing was performed on a NextSeq-500 instrument (Illumina, San Diego, USA).  
116 STs, virulence genes and K-types (approximated by the *wzi* allele)<sup>18</sup> were assigned using the online  
117 BIGSdb-*Kp* database available on the Institut Pasteur MLST and whole genome MLST website  
118 (<https://bigsdb.pasteur.fr/klebsiella/>).<sup>19</sup> Briefly, housekeeping genes, including *gapA*, *inf*, *mdh*, *pgi*,  
119 *phoE*, *rpoB*, and *tonB*, were scanned and compared to the MLST allele profiles available at the Institut

120 Pasteur MLST website. Furthermore, cgMLST was performed using the scgMLST scheme of 634 core  
121 genes.<sup>20</sup> Identical isolates were defined as isolates with cgMLST profile differing by less than 7 alleles  
122 (excluding missing loci). In addition, we used Kleborate to detect other K-loci (KL) and *Kp* virulence  
123 determinants (<https://github.com/katholt/Kleborate>).<sup>21</sup>  
124 Resistance genes and plasmid replicons were detected using Resfinder and PlasmidFinder,  
125 respectively (<http://www.genomicepidemiology.org>).<sup>22</sup>  
126 A maximum likelihood phylogenetic tree was built from the concatenation of 634 cgMLST gene  
127 alignments with RAxML using a general time reversible (GTR) evolutionary model and a gamma  
128 correction for among-site rate variation.<sup>23</sup> One hundred bootstrap replicates were conducted to  
129 quantify the significance of nodes in the maximum likelihood tree. Gubbins was used to identify  
130 genome regions that had undergone homologous recombination. This tool detects recombination  
131 events based on an elevated SNP density.<sup>24</sup> The resulting trees were visualized using iTol (Version  
132 4.2.3) (<http://itol.embl.de/itol.cgi>).<sup>25</sup>

133

### 134 **Statistical analysis**

135 Analyses were performed with Stata version 12 (StataCorp, LLC, College Station, TX, USA). We  
136 performed univariate analysis to compare differences in proportion by using the  $\chi^2$  or  $\chi^2$  exact tests,  
137 when appropriate. P-values <0.05 were considered to be statistically significant.

138

## 139 **Results**

### 140 **Isolates**

141 Two thousand women and their 2001 neonates were included in the present study (Figure 1). Five  
142 percent (n=101/2000) of women carried *Kp*, with one woman harboring two different *Kp* isolates,  
143 recovered simultaneously from a vaginal swab and a fecal swab (Table 1).

144 In total, 22.7% (n=455/2001) of sampled neonates had clinical signs or risk factors at birth, of whom  
145 1.09% (n=5/455) were *Kp*-carriers at birth (four born by caesarian section and one born vaginally).

146 During follow-up, six percent (n=121/2001) of infants were identified as carrying *Kp*. Twenty-two  
147 infants harbored two different *Kp* isolates, and three harbored three different *Kp* isolates. Overall,  
148 38% (n=46/121) of *Kp*-carrying infants and one *Kp*-carrying mother (0.9%) had a documented  
149 infection with *Kp* (Figure 1).

150 A total of 251 *Kp* isolates were collected during the study, 40.6% (n=102/251) from mothers and  
151 59.3% (n=149/251) from their neonates. A total of 52.1% (n=131/251) originated from the rural site  
152 (Figure 1). Sample origins are shown in Table 1. Among the 251 *Kp* isolates, 19.5% (n=49/251) were  
153 isolated from infected body sites in infants (urine, diverse pus (eyes, umbilical, ear and skin), and  
154 stool) and one from a mother's blood culture (Table 1).

### 155 ***Antimicrobial susceptibility***

156 Antimicrobial susceptibility testing revealed that only 49% (n=123/251) of isolates were susceptible  
157 to all tested antibiotics (Table S1). A total of 25.8% (n=65/251) of isolates were ESBL producers,  
158 among which 86.1% (n=56/65) were carriage isolates. There were no significant differences in  
159 antimicrobial resistance between carriage and infection isolates, with the exception of ciprofloxacin,  
160 for which carriage isolates from mothers were more resistant (Table 2). ESBL-producing isolates were  
161 resistant to first-line antibiotics typically usually used to treat neonatal infections (ampicillin,  
162 cefotaxime and gentamicin), indicating cross-resistance to aminoglycosides. In addition, a particularly  
163 high rate of resistance to trimethoprim/sulfamethoxazole was observed (49.4%; n=124/251). Rates  
164 of resistance to other antibiotics were 40.6% for tetracycline, 30.2% for gentamicin, 32.2% for  
165 tobramycin, 20.7% for ciprofloxacin, 10.7% for ertapenem, and one isolate was imipenem-resistant.  
166 A total of 40.2% (n=101/251) of isolates were categorized as multidrug-resistant (MDR; resistant to =  
167 3 drug classes).

168

### 169 ***WGS and phylogenetic analysis***

170 The genome sequence-derived phylogeny showed that *Kp* isolates were distributed into six  
171 phylogroups (Figure 2), with a large majority (78.4%; n=197) belonging to Kp1. The Kp2, Kp3, Kp4,



172 Kp5 and Kp6 phylogroups corresponded respectively to 1.1% (n=3), 11.9% (n=30), 4.7% (n=12), 2.3%  
173 (n=6) and 1.1% (n=3) of isolates. The genetic diversity of our isolates was illustrated by MLST: 108  
174 different STs were found. Twenty-nine new STs (from ST3301 to ST3329) were discovered and  
175 defined, corresponding to 41 isolates. Due to incomplete profiles, 16 isolates were not assigned to a  
176 defined ST. No single ST dominated; the most frequent STs were ST45 (5.1%), ST37 (4.7%), and ST348  
177 (3.5%) in the Kp1 phylogroup and ST3326 (2.7%) in the Kp3 phylogroup.

178

### 179 **Identification of antimicrobial resistance genes and replicons**

180 Genomic sequences were searched for previously described resistance genes (Table S1; Table 3).  
181 Among ESBL-producing isolates (n=65), 99.3% harboured the *bla*<sub>CTX-M-15</sub> gene. Among these, 75.3%  
182 (n=49/65) carried a single *bla*<sub>CTX-M-15</sub> gene, and 12% (n=8/65) carried it in combination with either  
183 *bla*<sub>CTX-M-14</sub> (6%; n=4/65), *bla*<sub>SHV2-A</sub> (3%; n=2/65) or *bla*<sub>SHV-42</sub> (3%; n=2/65). One ESBL producer isolated  
184 from a mother's rectal swab co-harbored the carbapenem resistance gene *bla*<sub>NDM-1</sub>. Non-ESBL *bla*<sub>SHV</sub>  
185 variants were found in 72.1% (n=181/251) of isolates. *bla*<sub>LEN</sub> variants and *bla*<sub>OKP-D,-B</sub> were found,  
186 respectively, in 11.9% (n=30/251) and 1.9% (n=5/251) of isolates. Aminoglycoside resistance  
187 determinants *aph(6)-Id* and *aac(3)-IIa* were detected in 37% (n=93/251) and 24.3% (n=61/251) of all  
188 isolates, respectively (Table 3). Other infrequent aminoglycoside resistance determinants were  
189 *aph(3')-Ia*, *aph(3'')-Ib*, *aac(6')-Ib-cr* and *ant(3'')-Ia/aadA5*. Full resistance to ciprofloxacin was  
190 observed in 2.3% (n=6/251) of isolates, which exhibited *gyrA* (S83I, S83F and D87A) and *parC* (S80I)  
191 mutations; 17.1% (n=43/251) of isolates harbored *qnrB* variants such as *qnrB1*, *qnrB6* and *qnrB9* or  
192 *qnrS1*. Sulphonamide resistance determinants *sul1* and *sul2* were detected in 43% (n=108/251) of  
193 isolates, respectively; and their combination in 11.5% (n=29/251). Trimethoprim resistance gene *dfrA*  
194 variants were identified in 39.8% (n=100/251) of isolates, with *dfrA14* in 26.6% (n=67/251) and *dfrA5*  
195 in 4.3% (n=11/251). Other infrequently observed variants included *dfrA(1/12/15/17/22/27)*.  
196 Tetracycline resistance genes were efflux genes found in 27.4% (n=69/251) of isolates and were  
197 mainly of classes A and D. Other infrequent determinants for tetracycline resistance were *tet(B)* or

198 *tet(K)* (Table 3). Other identified resistance determinants are detailed in Table S1. There were few  
199 differences between mother and infant isolates in terms of antimicrobial resistance genes (Table 3).  
200 As expected, resistance genes were strongly correlated with phenotypic resistance (Figure 3).  
201 Plasmid analysis revealed a high diversity of incompatibility (Inc) groups. IncFIB(K) was found in  
202 37.8% (n=95/251) of isolates. Other IncFI members (8.7%; n=22/251) included IncFIA(HI1),  
203 IncFIB(AP1918), IncFIB(Mar), IncFIB(pKPHS1) and IncFIB(pQil). IncFII was another frequently detected  
204 replicon, with IncFII(K) detected in 28.2% (n=71/251) of *Kp* isolates. IncR was identified in 8.7%  
205 (n=22/251) of isolates. Plasmid replicons that were infrequently identified included IncA/C2, IncHI1B,  
206 IncN, IncP, IncQ1 and IncY.

207

#### 208 **Identification of virulence determinants**

209 A major virulence determinant of *Kp* is the capsule. Here, capsular types were approximated by their  
210 *wzi* allele or KL locus. A total of 15.9% (n=40/251) of isolates were K undefined. A total of 59 distinct  
211 K types were identified among the remaining (n=211/251) isolates, including K locus KL1 only in 0.3%  
212 (n=1/251) and KL2 in 2.39% (n=6/251). Therefore, these K types typically associated with virulence  
213 were rarely observed. Other virulence determinants were also analyzed (Table 4). The yersiniabactin  
214 siderophore synthesis gene cluster *ybt-irp-fyu*, which is located on the integrative conjugative  
215 element (ICE*Kp*), was detected in 32.2% (n=81/251) of isolates; interestingly these elements were  
216 more frequent in carriage isolates (Table 4). Colibactin (*clb*) was found in 1.5% (n=4/251) of isolates.  
217 The aerobactin siderophore production gene cluster (*iucABCD*) was found in 5.1% (n=13/251) of  
218 isolates, the salmochelin operon (*iroBCDN*) in 2.3% (n=6/251), the ferric uptake operon system  
219 (*kfuABC*) in 26.2% (n=66/251), and the allantoinase cluster (*allABCDRS*) in 1.5% (n=4/251) (Table 4).  
220 Salmochelin (*iro*) and aerobactin (*iuc*) were more frequent among infection isolates. Other virulence  
221 determinants are detailed in Table S1. A total of 5.1% (n=13/251) of isolates harbored several  
222 virulence factors (virulence scores 2 and 3; see Table S1). Among them, eight were carriage isolates  
223 and corresponded to ST2715 (n=4), ST29 (n=1), ST35 (n=1), ST2058 (n=1) and ST3074 (n=1). Among

224 infectious isolates, we identified two isolates: an ST2058 isolate (n=1) and an ST23-KL1 isolate (n=1).  
225 The latter carried virulence genes coding for yersiniabactin (*ybt1*), colibactin (*clb2*), aerobactin  
226 siderophore production (*iucABCD*) and the salmochelin operon *iroBCDN*. In addition, among five  
227 ST25-KL2 serotype isolates, two were considered hypervirulent as they contained virulence genes  
228 encoding aerobactin, salmochelin and the regulator of mucoid phenotype gene *rmpA*; they were all  
229 isolated from urinary tract infections (UTI).

230

### 231 ***Mother-to-child transmission and persistence of Kp in neonates***

232 We identified two distinct episodes of mother-to-child *Kp* transmission (comprising six isolates) and  
233 ten distinct episodes of long-term *Kp* fecal carriage in neonates (comprising 57 isolates) based on  
234 nearly-identical genomic (cgMLST) background (Table 5). Figure 4 summarizes a timeline of these  
235 episodes. Transmission pair #1 was inferred from three *Kp* ST502 isolates: one from a mother's  
236 placental biopsy, one from her neonate's gastric fluid at Day 0, and one from the neonate's urine at  
237 Day nine (Table S2). Transmission pair #2 was inferred from three isolates of a new ST (ST3319), all  
238 recovered at Day 0 from a mother's stool and the gastric fluid and stool of her neonate (Table S2).  
239 The neonates from both transmission pairs were born vaginally.

240 Ten neonates screened at several time points were found to carry or be infected by the same *Kp*  
241 strain longitudinally over a duration of 18 to 115 days. *Kp* isolates originating from two neonates  
242 born by caesarean section and belonging to ST70 and ST711 lost a plasmid harboring antimicrobial  
243 determinants during long-term carriage. Three neonates carried *Kp* isolates that persisted for more  
244 than 60 days and were subsequently re-isolated from an infected body site (Figure 4). Long-term *Kp*  
245 carriage in these neonates was observed for 90, 109 and 115 days, with isolates belonging to ST25,  
246 ST711 and ST3326, respectively. The ST25 isolates were resistant to aminoglycosides,  
247 trimethoprim/sulfamethoxazole and tetracycline; the ST711 isolates were ESBL producers and were  
248 isolated from a baby born by caesarean section; and the ST3326 isolates were susceptible to all

249 tested antimicrobial agents. The ST25 and ST711 isolates harbored IncFIB(K) and IncFII(K) plasmid  
250 replicons.

251

## 252 **Discussion**

253 In this study we investigated a population of *Kp* isolates from a paediatric cohort across two  
254 community settings in Madagascar. We isolated and sequenced 251 *Kp* isolates and analyzed genetic  
255 subtypes, virulence genes, resistance gene and plasmid content. We also investigated episodes of  
256 mother-to-child transmission and long-term carriage in neonates.

257 *Kp* is known to be a highly diverse species.<sup>26</sup> In this first study of the population structure of *Kp*  
258 originating from human communities in Madagascar, six of seven *Kp* phylogroups were represented.

259 Based on MLST, a high degree of genetic diversity was observed. Additionally, sequence types known  
260 to be epidemiologically prevalent in other world regions (ST23, ST45, ST101 and ST25) and/or  
261 associated with multidrug-resistance (ST14, ST15, ST17, ST101 and ST147) were found.

262 Given the high diversity of STs, statistical comparison of their relative prevalence between infectious  
263 and carriage isolates was not possible. However, among other STs, infectious isolates belonged to  
264 ST15, ST17, ST37, ST48 and ST348, which have previously been reported as causes of neonatal sepsis  
265 and outbreaks in neonatal ICUs (NICUs).<sup>27,28</sup>

266 The prevalence of ESBL producers among observed *Kp* isolates (25.8%) was similar to the prevalence  
267 reported in a previous community study conducted in Madagascar during 2015-2016 (24.4%).<sup>29</sup> The  
268 majority of ESBL isolates we found were instances of asymptomatic carriage. This supports the  
269 hypothesis that MDR *Kp* is likely to maintain sustained prevalence in the community and may  
270 become endemic. Among ESBL genes, *bla*<sub>CTX-M-15</sub>, was the most represented (99.3%) and one isolate  
271 co-harbored the carbapenem resistance gene *bla*<sub>NDM-1</sub>. These results are concordant with other  
272 studies in Madagascar, which found that ESBL-producing *Enterobacteriaceae* mostly carry the CTX-M-  
273 15 type.<sup>30,31</sup> Regarding plasmids, IncFI and IncFII were the most frequent replicons detected. This is

274 not surprising because *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-14</sub> are primarily carried on IncF plasmids, as exemplified  
275 by a study in Morocco in which the presence of IncFII was associated with *bla*<sub>CTX-M-15</sub>.<sup>32</sup>  
276 *Kp* has gradually accumulated various antibiotic resistance genes over time, making *Kp* infections  
277 increasingly difficult to treat. The most common associations observed in our study were resistances  
278 to 3<sup>rd</sup> generation cephalosporins, aminoglycosides, and to a lesser extent to  
279 trimethoprim/sulfamethoxazole and tetracycline. The emergence of MDR *Kp*, especially ESBL and/or  
280 carbapenemase producers, has elevated morbidity and mortality rates, as well as health care costs  
281 associated with *Kp* infections to highly burdensome levels.<sup>27,33,34</sup> This emergence is considered to be a  
282 major, global problem for public health.

283 Three hypervirulent isolates were identified during this study: one ST23-K1 isolate from the pus of a  
284 baby's eye and two ST25-K2 isolates from UTIs. The main biomarker genes (aerobactin and *rmpA*)  
285 recognized to differentiate hypervirulent *Kp* from classical *Kp* were identified in these isolates.<sup>35</sup>  
286 Although the presence of genes encoding resistance or virulence factors in an isolate does not  
287 necessarily indicate gene activity in that isolate, previous work has demonstrated the functional  
288 implications of these genes.<sup>36</sup>

289 Among 101 mothers carrying *Kp*, two were observed to transmit to their baby at Day 0. Initial  
290 colonisation of the newborn gut is usually believed to occur when the baby initiates transit through  
291 the labour channel via contamination by maternal vaginal and fecal bacteria.<sup>37</sup> However, some  
292 studies support the presence of bacteria in foetal meconium, amniotic fluid and in blood of the  
293 umbilical cord, suggesting that a baby's first contact with bacteria may occur not at birth, but earlier,  
294 while still in utero.<sup>38</sup> Regardless, the mode of delivery is an important determinant of early intestinal  
295 colonization in neonates. Infants born vaginally are colonized by the maternal vaginal and faecal flora  
296 during delivery, whereas those born by caesarean section are predominantly exposed to bacteria  
297 from the hospital environment.<sup>39</sup> Our data support that *Kp* efficiently colonizes the neonatal gut. We  
298 also observed that *Kp* colonization is significantly more associated with vaginal than stool samples,

309 supporting the hypothesis that mode of delivery has a major influence on mother-to-child  
300 transmission of *Kp*.

301 *Kp* is the causative agent of diverse types of infection.<sup>40</sup> Common *Kp* colonization sites include the  
302 gastrointestinal tract, eyes, respiratory tract, and genito-urinary tract.<sup>41,42</sup> Furthermore, colonization  
303 with ESBL-producing *Kp* can persist and be associated with infection after NICU discharge,<sup>43</sup> and ESBL  
304 *Kp* colonization duration may be considerably longer in infants after colonization during  
305 hospitalization.<sup>44</sup>

306 In this study, we found that persisting *Kp* belonged to ST14, ST15, ST35, ST37, ST45 and ST70, all of  
307 which have previously been reported to cause infections.<sup>27,45,46</sup> Further, three of ten neonate carriage  
308 episodes exceeding 60 days resulted in infections by the same infectious strain (isolated from UTIs  
309 and pus). The duration of *Kp* carriage was long for some neonates, especially for *Kp* ST711, which  
310 was found several times in UTIs for a period up to 115 days, and for *Kp* ST25-K2, a hypervirulent  
311 isolate carried for 109 days in one neonate. Infants born by caesarean section may be at particularly  
312 risk for long-term fecal carriage of ESBL-producing Enterobacteriaceae,<sup>44</sup> as illustrated by the baby  
313 who was infected by ESBL-producing *Kp* ST711 until one year of age. During an outbreak in an NICU  
314 in Norway, gut carriage of a *Kp* ST17 strain with a pKPN3-like CTX-M-15-encoding IncFII persisted for  
315 up to two years.<sup>47</sup> Another study revealed colonization lasting up to 4.5 years, with phenotypic  
316 diversification and parallel selection of pathoadaptive mutations potentially contributing to long-  
317 term carriage and virulence of a KPC-positive ST258 strain.<sup>48</sup> However, although unlikely, and  
318 although *Kp* carriage has been observed to last for up to years at a time, it cannot be excluded that  
319 neonates in this study with persistent carriage could have re-acquired the same clone on multiple  
320 occasions.

321 We acknowledge the presence of limitations to this study. The study design was not appropriate for  
322 studying all episodes of mother-to-child transmission and persistent carriage in neonates. In addition,  
323 plasmids could not be assembled and their transmission could not be studied, as we used short-read  
324 Illumina sequencing technology sequencing.

325 In conclusion, this study has revealed a high diversity of *Kp* isolates circulating in a community cohort  
326 in Madagascar. *Kp* isolates displayed high levels of antimicrobial resistance, including ESBL and  
327 carbapenemase production, and a few hypervirulence gene-carrying isolates were found. We  
328 observed two episodes of mother-to-child transmission and ten episodes of prolonged *Kp* carriage in  
329 neonates. Awareness of *Kp* carriage in both mothers and their neonates is crucial to better  
330 understand risk factors for infection and to improve neonatal care and follow-up.

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342 **Availability of data and materials**

343 The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the BioProject  
344 PRJNA548846.

345 **Transparency declarations**

346 None to declare

347 **Supplementary data**

348 Table S1 and S2 are available as Supplementary data at *JAC* Online.



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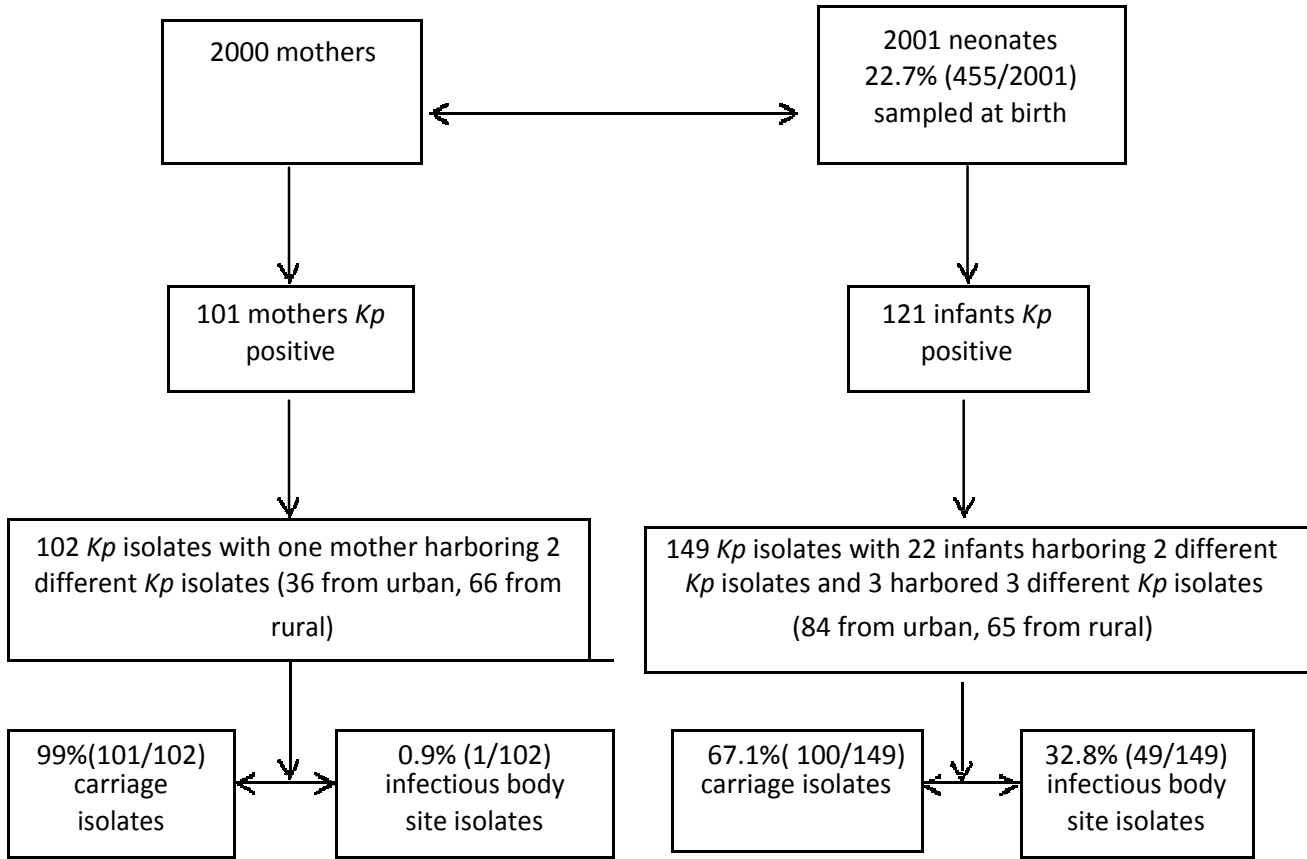
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**Figure 1:** Flow-chart of the study participants with the features of isolates

Tree scale: 0.01

**Sites**

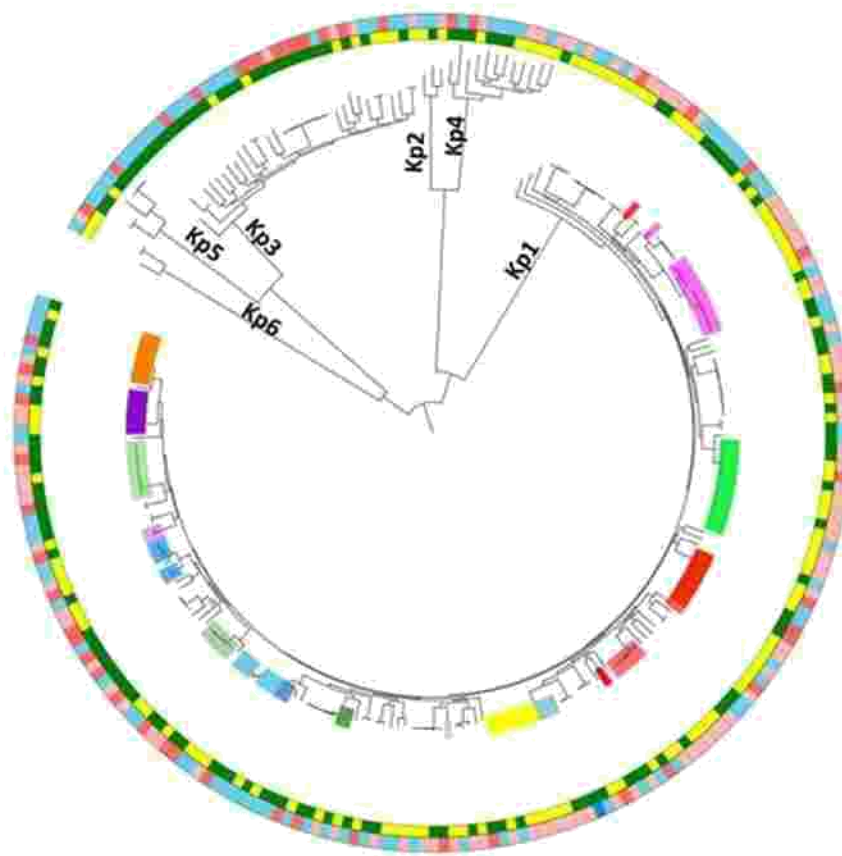
- Urban
- Rural

**Sequence type (ST) (inner ring)**

- ST14
- ST15
- ST17
- ST20
- ST23
- ST25
- ST29
- ST34
- ST35
- ST36
- ST37
- ST39
- ST45
- ST48
- ST54
- ST70
- ST101
- ST138
- ST147

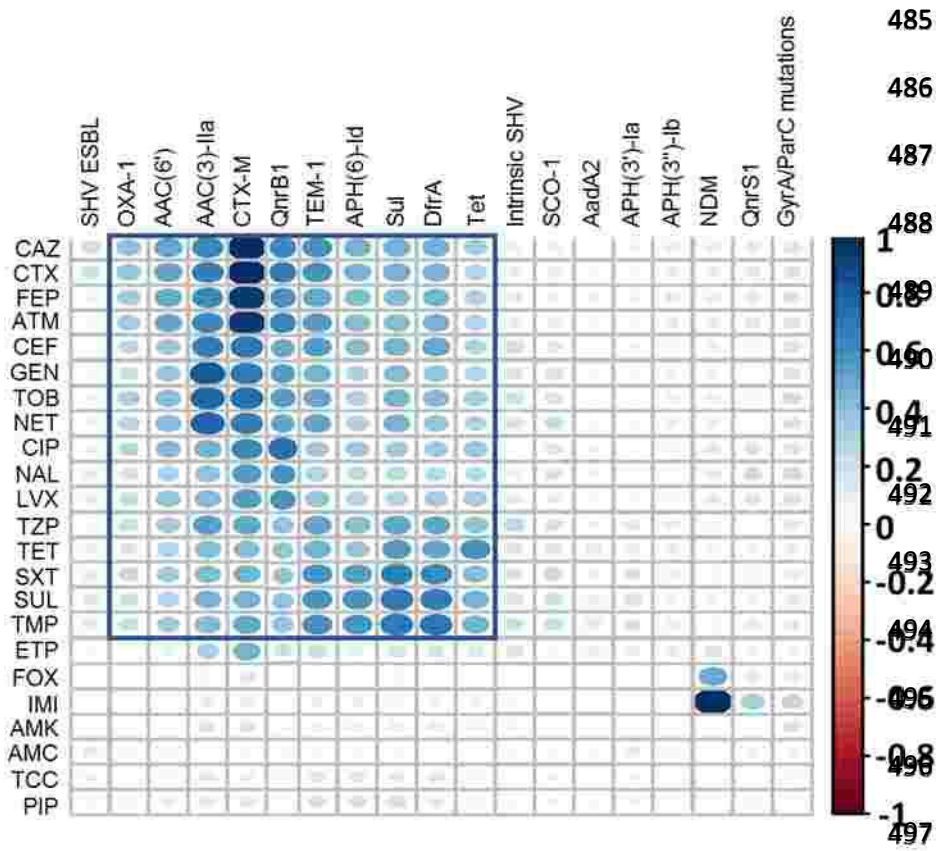
**Isolates**

- Baby infection
- Baby carriage
- Mother infection
- Mother carriage

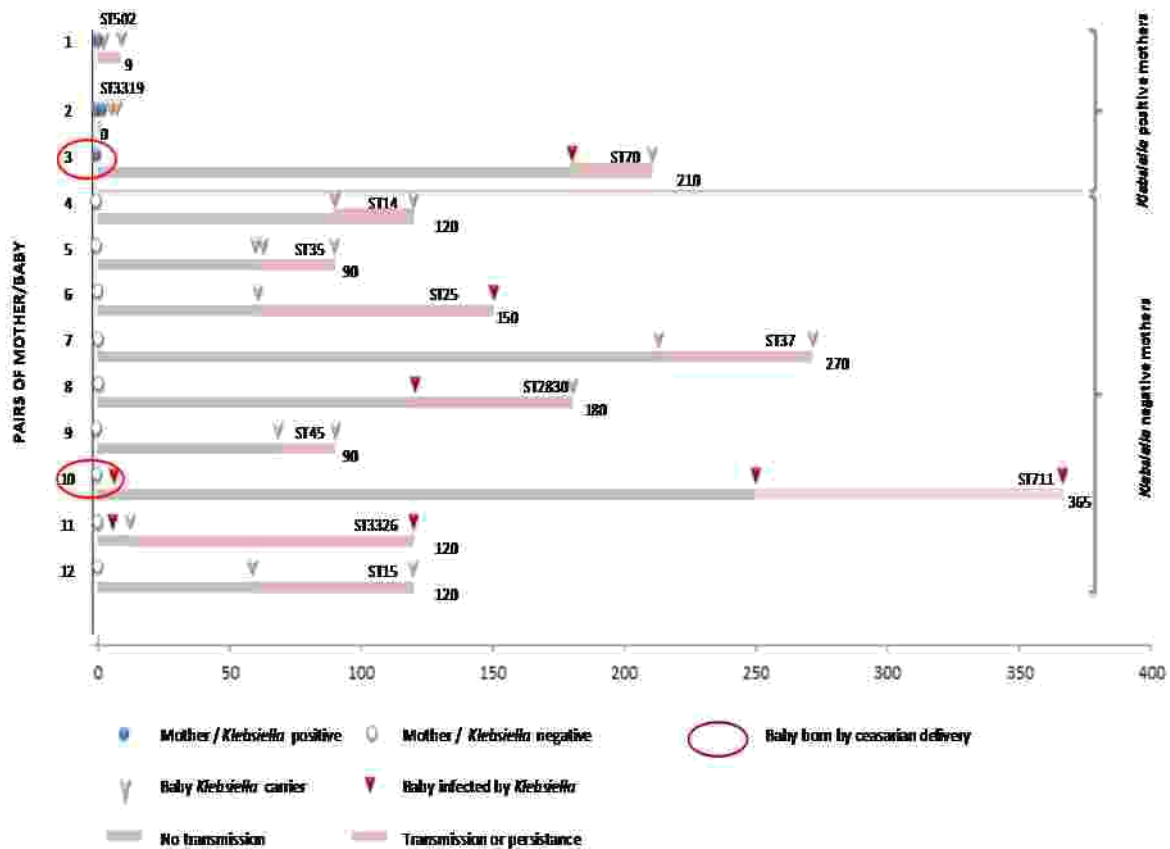


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482 **Figure 2:** Unrooted phylogenetic tree constructed with all 251 isolate sequences. Each phylogroup was clearly separated from others and is indicated along its branch. This  
483 figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.



498 **Figure 3:** Correlation heatmap between antibiotic-resistant phenotype and resistome genotype. X axis:  
499 resistant genes; Y axis: phenotypes. This figure appears in colour in the online version of *JAC* and in black and  
500 white in the print version of *JAC*.



502 **Figure 4:** Timeline representation of 2 pairs of mother-baby transmission and prolonged carriage in 10  
 503 neonates. This figure appears in colour in the online version of JAC and in black and white in the print version of  
 504 JAC. Mothers are represented by circles at day 0, in blue circles when she carried *Kp* isolate and white circles  
 505 when no *Kp* isolate was isolated from mother. Infants infected by *Kp* are represented by red triangles and  
 506 infants *Kp*-carrier are represented by pink triangles. Infants number #6 (ST25), #10 (ST711) and #11 (ST3326)  
 507 were *Kp*-carrier for more than 60 days. Infants number #6 and #11 were carrying a *Kp* isolate at birth; the same  
 508 *Kp* isolate became afterwards infectious; all infectious isolates were from urine samples. The baby number #10  
 509 after a caesarian delivery was positive for *Kp* (signs of infection: *Kp* isolated from pus of his eyes). Another *Kp*  
 510 isolate (ST711) was found at day 250 (isolated from urinary tract infection UTI) and the same *Kp* isolate was  
 511 found again at one year after birth (from a UTI).

512 **Table 1:** Isolates origin among *Kp* positive isolates

Isolates origin	Carriage (n, %)	Infectious body site (n, %)	Total
Mothers	N=101	N=1	N=102
Blood culture	0	1 (100)	1
Placental biopsy	3 (3)	0	3
Stool or anal swab	36 (35.6)	0	36
Vaginal	62 (61.4)	0	62
Infants	N=100	N=49	N=149
Urine	77 (77)	21(42.9)	98
Pus	0	26 (53.1)	26
Stool or anal swab	6 (6)	1 (2)	7
Sputum	11(11)	0	11
Gastric fluid	7(7)	0	7



513 **Table 2:** Antimicrobial resistant isolates

		Infection		Carriage		
		(N=49) (n, %)	(N=101) (n, %)	(n, %)		
AMC	1	43 (87.8)	88 (87.1)	84 (84)		0.5
TIC	1	49 (100)	101 (100)	100 (100)		-
TCC	1	47(95.9)	93 (92.1)	93 (93)		0.8
PIP	1	48 (98)	94 (93.1)	94 (94)		0.8
TZP	1	14 (28.6)	40 (39.6)	50 (50)		0.1
CEF	1	14 (28.6)	42 (41.6)	36 (36)		0.4
FOX	0	1 (2)	2 (2)	1 (1)		1 <sup>§</sup>
CTX	1	8 (16.3)	31 (30.7)	25 (25)		0.4
CAZ	1	8 (16.3)	32 (31.7)	24 (24)		0.2
FEP	1	8 (16.3)	30 (29.7)	21 (21)		0.2
ATM	1	8 (16.3)	29 (28.7)	22 (22)		0.3
IPM	0	0 (0)	1 (1)	0 (0)		-
ETM	0	5 (10.2)	13 (12.9)	9 (9)		0.4
TOB	1	12 (24.5)	34 (33.7)	32 (3)		0.8
NET	0	13 (26.5)	32 (31.7)	29 (29)		0.7
GEN	0	13 (26.5)	34 (33.7)	29 (29)		0.5
AMIK	0	1 (2)	0 (0)	1 (1)		-
NAL	1	6 (12.2)	20 (19.8)	14 (14)		0.3
CIP	1	8 (16.3)	34 (33.7)	16 (16)		0.004**
LVX	1	8 (16.3)	27 (33.3)	16 (16)		0.4
SXT	1	20 (40.8)	47 (53.5)	56 (56)		0.2
TET	1	14 (30)	44 (43.6)	43 (43)		0.9
SUL	1	19 (38.8)	46 (45.5)	56 (56)		0.1
TMP	1	19 (38.8)	49 (48.5)	58 (58)		0.2

514 §  $\chi^2$  exact test

515

516 **Table 3:** Antimicrobial resistance (AMR) genes distribution among *Kp* isolates

Gene	Known AMR phenotype	Mothers isolates N=102 (n, %)	Infants isolates N=149 (n, %)	p-values
<i>bla</i> <sub>CTX-M</sub>	ESBL	32 (31.4)	33 (22.2)	0.1
<i>bla</i> <sub>SHV2-A/SHV42</sub>	ESBL	3 (2.9)	1 (0.7)	0.3 <sup>S</sup>
<i>bla</i> <sub>NDM-1</sub>	ESBL	1 (0.9)	0	-
<i>bla</i> <sub>SHV non-ESBL</sub>	β-lactams	63 (61.8)	118 (79.2)	0.002**
<i>bla</i> <sub>LEN</sub>	β-lactams	19 (18.6)	11 (7.4)	0.007**
<i>bla</i> <sub>OKP-D/-B</sub>	β-lactams	1 (1)	4 (2.7)	0.7 <sup>S</sup>
<i>bla</i> <sub>OXA-1/TEM-1/-40/SCO-1/LAP-2</sub>	β-lactams	33 (32.4)	63 (42.3)	0.1
<i>aph(6)-Ia</i>	aminoglycosides	37 (36.3)	56 (37.6)	0.8
<i>aac(3)-IIa</i>	aminoglycosides	24 (23.5)	37 (24.8)	0.8
<i>aph(3')-Ia/aph(3'')-Ib/ant(3'')-Ia/aadA5</i>	aminoglycoside	16 (15.7)	13 (8.7)	0.09
<i>aac(6')-Ib-cr</i>	aminoglycosides	17 (16.7)	11 (7.4)	0.02**
<i>gyrA</i> (S83I, S83F and D87A) and <i>parC</i> (S80I)	fluoroquinolones	2 (2)	4 (2.7)	1 <sup>S</sup>
<i>qnrB1/B6/B9, qnrS</i>	fluoroquinolones	22 (21.6)	21 (14.1)	0.1
<i>sul1/2</i>	sulphonamides	42 (41.2)	66 (44.3)	0.6
<i>dfrA</i>	trimethoprim	36 (35.3)	64 (42.9)	0.2
<i>Tet(A)/(B)/(D)/(K)</i>	tetracycline	36 (35.3)	33 (22.2)	0.02**

517 \$ ?<sup>2</sup> exact test

519 **Table 4:** Distribution of virulence determinants among *Kp* isolates

<b>Virulence prediction</b>			
	Carriage N=201 (n, %)	Infection N=50 (n, %)	p-value
<i>ICEKp</i>	63 (31.3)	8 (16)	0.03**
<i>clb</i>	1 (0.5)	3 (6)	0.02 <sup>\$</sup>
<i>Ybt-fyu-irp</i>	70 (34.8)	11 (22)	0.08
<i>iro</i>	2 (1)	4 (8)	0.02 <sup>\$</sup>
<i>iuc</i>	7 (3.5)	6 (12)	0.03 <sup>\$</sup>
<i>iut</i>	6 (3.5)	4 (8)	0.2 <sup>\$</sup>
<i>rmpA</i>	12 (6)	1 (2)	0.5 <sup>\$</sup>
<i>kfu</i>	54 (26.9)	12 (24)	0.7
<i>allS</i>	0	4 (8)	-
<i>mrk</i>	198 (98.5)	48 (96)	0.3
<i>kvg</i>	6 (3)	3 (6)	0.4 <sup>\$</sup>
<i>mce</i>	0	1 (2)	-
<i>clp</i>	7 (3.5)	3 (6)	0.4 <sup>\$</sup>

520 \$  $\chi^2$  exact

521 **Table 5:** Characteristics of mother and neonates isolates in cases of transmission and long-term carriage

Mother/ baby pair	Isolate_Id	Individual	isolation_date	Infection/ carriage	ST	Resistance-genes	Replicons
1	01-662MPLA	Mother1	May, 2015	Carriage	ST502	<i>bla<sub>SHV-62</sub>, bla<sub>TEM-1</sub>, ant(3'')-Ia, cml</i>	
	01-662-1LGAS	Baby1	May, 2015	Carriage	ST502	<i>bla<sub>SHV-62</sub>, bla<sub>TEM-1</sub>, strA, strB, aph(6)-Id, ant(3'')-Ia, cml</i>	
	01-662-2ECBU	Baby1	May, 2015	Carriage	ST502	<i>bla<sub>SHV-62</sub></i>	
2	02-719-MPLA	Mother2	November, 2014	Carriage	ST3320	<i>bla<sub>SHV-42</sub>, bla<sub>CTX-M-15</sub>, bla<sub>TEM-1</sub>, strA, strB, aph(6)-Id, aac(3)-IIa, IncFIB(pKPHS1) dfrA14, oqxA</i>	
	02-719MSEL	Mother2	November, 2014	Carriage	ST3319	<i>bla<sub>SHV-1</sub>, aac(6')-Ib-cr, aac(3)-IIa, oqxA, oqxB32, OmpK36</i>	IncFII(K)
	02-719SEL	Baby2	November, 2014	Carriage	ST3319	<i>bla<sub>SHV-42</sub>, bla<sub>CTX-M-15</sub>, bla<sub>TEM-1</sub>, strA, strB, aph(6)-Id, aac(3)-IIa, dfrA14, qnrB1, oqxA</i>	IncFII(K)
	02-719LGAS	Baby2	November, 2014	Carriage	ST3319	<i>bla<sub>CTX-M-15</sub>, strA, strB, dfrA14, qnrB1</i>	IncFII(K)
3	02-746MBV	Mother3	October, 2014	Carriage	ST347	<i>bla<sub>SHV-11</sub>, bla<sub>TEM-1</sub>, bla<sub>OXA-1</sub>, aph(6)-Id, aac(6')-Ib-cr, aac(3)-IIa, tetR, tetA, oqxA, oqxB19</i>	
	02-746-1ECBU	Baby3	May, 2015	Carriage	ST70	<i>bla<sub>CTX-M-15</sub>, bla<sub>SHV-32</sub>, bla<sub>TEM-1</sub>, strA, strB, aph(6)-Id, aac(6')-Ib-cr; aac(3)-IIa, tetR, tetA, dfrA14, qnrB1, oqxB19, oqxA10</i>	IncFIB(K), IncFII(K)
	02-746-2ECBU	Baby3	May, 2015	Infection	ST70	<i>bla<sub>CTX-M-15</sub>, strA, strB, dfrA14, qnrB1</i>	IncFIB(K), IncFII, IncFII(K)
4	01-314-1ECBU	Baby4	April, 2014	Carriage	ST14	<i>bla<sub>CTX-M-15</sub>, bla<sub>SHV-28</sub>, bla<sub>TEM-1</sub>, bla<sub>OXA-1</sub>, strA, strB, aph(6)-Id, aac(6')-Ib-cr, aac(3)-IIa, dfrA14, oqxA</i>	IncFIB(K), IncFII(K)
	01-314-2ECBU	Baby4	May, 2014	Carriage	ST14	<i>bla<sub>CTX-M-15</sub>, bla<sub>SHV-28</sub>, bla<sub>TEM-1</sub>, strA, strB, aph(6)-Id, aac(3)-IIa, dfrA14, oqxA</i>	IncFIB(K), IncFII(K)

5	01-358-1ECBU	Baby5	May, 2014	Carriage	ST35	<i>bla<sub>SHV-33</sub>, oqxB19, oqxA3</i>	IncFIB(K), IncFII(pRSB17)
	01-358-2ECBU	Baby5	May, 2014	Carriage	ST35	<i>bla<sub>SHV-33</sub>, oqxB19, oqxA3</i>	IncFIB(K)
	01-358-3 ECBU	Baby5	June, 2014	Carriage	ST35	<i>bla<sub>SHV-33</sub>, oqxB19, oqxA3</i>	IncFIB(K), IncFII(pRSB17)
6	01-368-1ECBU	Baby6	July, 2014	Carriage	ST25	<i>bla<sub>SHV-11</sub>, bla<sub>TEM-1</sub>, aac(3)-IId, Tet(D), dfrA5, oqxB17, oqxA10</i>	IncFIB(K), IncFII(K)
	01-368-2ECBU	Baby6	October, 2014	Infection	ST25	<i>bla<sub>SHV-11</sub>, bla<sub>TEM-1</sub>, aac(3)-IId, Tet(D), dfrA5, oqxB17, oqxA10</i>	IncFIB(K), IncFII(K)
7	01-443-1ECBU	Baby7	March, 2015	Carriage	ST37	<i>bla<sub>SHV-11</sub>, oqxA, OmpK36</i>	
	01-443-2ECBU	Baby7	April, 2015	Carriage	ST37	<i>bla<sub>SHV-11</sub>, oqxA, OmpK36</i>	
8	01-467-1ECBU	Baby8	December, 2014	Infection	ST2830	<i>bla<sub>OKP_D_1</sub>, bla<sub>TEM-1</sub>, aph(6)-Id, Tet(D), dfrA14, dfrA8</i>	IncFII(29), IncR
	01-467-2ECBU	Baby8	February, 2015	Carriage	ST2830	<i>bla<sub>OKP_D_1</sub>, bla<sub>TEM-1</sub>, strA, strB, aph(6)-Id, Tet(D), dfrA14, dfrA8</i>	IncFII(29), IncR
9	01-595-1ECBU	Baby9	March, 2015	Carriage	ST45	<i>bla<sub>SHV-1</sub>, strA, strB, aph(6)-Id, dfrA14, oqxB19, oqxA11</i>	IncFIB(K), IncFII(K), IncQ1
	01-595-2ECBU	Baby9	March, 2015	Carriage	ST45	<i>bla<sub>SHV-1</sub>, bla<sub>TEM-1</sub>, strA, strB, aph(6)-Id, dfrA14, oqxB19, oqxA11</i>	IncFIB(K), IncFII, IncFII(K), IncQ1IncR
10	02-766-1ECBU	Baby10	October, 2015	Infection	ST711	<i>bla<sub>CTX-M-15</sub>, bla<sub>SHV-83</sub>, bla<sub>TEM-1</sub>, strA, strB, aph(6)-Id, aac(3)-IIa, dfrA14, oqxA, oqxB25</i>	ColpVC, IncFIB(K), IncFII(K)
	02-766-2ECBU	Baby10	October, 2015	Infection	ST711	<i>bla<sub>CTX-M-15</sub>, bla<sub>SHV-83</sub>, bla<sub>TEM-1</sub>, bla<sub>OXA-1</sub>, strA, strB, aph(6)-Id, aac(6')-Ib-cr, aac(3)-IIa, dfrA14, oqxA, oqxB25</i>	ColpVC, IncFIB(K), IncFII(K)
11	02-891-1PUS	Baby11	March, 2015	Infection	ST1584	<i>strA, strB, aph(6)-Id</i>	

	02-891-2ECBU	Baby11	March, 2015	Carriage	ST3326	<i>bla</i> <sub>LEN-17</sub> , <i>oqx</i> <i>B16</i>		522
	02-891-3PUS	Baby11	July, 2015	Infection	ST3326	<i>bla</i> <sub>LEN-17</sub> , <i>oqx</i> <i>B16</i>		523
12	02-1056-1ECBU	Baby12	October, 2015	Carriage	ST15	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>SHV-28</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>strA</i> , <i>strB</i> , <i>aph(6)-Id</i> , <i>aac(6')-Ib-cr</i> , <i>aac(3)-IIa</i> , <i>dfrA14</i> , <i>ParC-80I</i> ; <i>GyrA-83F</i> ; <i>GyrA-87A</i> , <i>oqx</i> <i>A</i> , <i>OmpK36</i>	IncFIA(HI1), IncFIB(K), IncFII, IncR	524
	02-1056-2ECBU	Baby12	December, 2015	Carriage	ST15	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>SHV-28</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>strA</i> , <i>strB</i> , <i>aph(6)-Id</i> , <i>aac(6')-Ib-cr</i> , <i>dfrA14</i> , <i>ParC-80I</i> ; <i>GyrA-83F</i> , <i>GyrA-87A</i> , <i>oqx</i> <i>A</i> , <i>OmpK36</i>	IncFIB(K), IncFII, IncR	

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