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

Andriniaina RAKOTONDRASOA[1]*, Virginie PASSET[2], Perlinot HERINDRAINY[3], Benoit GARIN[4], Elsa KERMORVANT-DUCHEMIN[5], Elisabeth DELAROCQUE-ASTAGNEAU[6], Didier GUILLEMOT[6], Bich-Tram HUYNH[6#], Sylvain BRISSE[2#], Jean-Marc COLLARD[1#]

[1]Experimental bacteriology unit, Institut Pasteur Madagascar, Antananarivo, Madagascar; [2]Institut Pasteur, Biodiversity & Epidemiology of Bacterial Pathogens, Paris, France; [3]Epidemiology & Public Health Unit, Institut Pasteur Madagascar; [4]Laboratoire Immuno-Hématologie CHU Pointe-à-Pitre, 97159, Abymes, Guadeloupe, France ; [5] Université Paris Descartes et AP-HP, Hôpital Universitaire Necker-Enfants malades, Paris, France; [6]UMR1181 Biostatistique, Biomathématique, Pharmaco-épidémiologie et Maladies Infectieuses (B2PHI), Institut Pasteur, Paris, France.

***Corresponding author. Tel: + 261 34 98 185 32; E-mail: aina@pasteur.mg**

[#]These authors contributed equally to this work

Abstract

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

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

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

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

Introduction

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

K. variicola to Kp3 (subsp. *variicola*) and Kp5 (subsp. *tropica*), "*K. quasivariicola"* to Kp6 and *K. Africana* to Kp7. [1-3] Nevertheless, these taxa are typically all still identified as *Kp* in clinical microbiology laboratories.

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

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

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

Materials and Methods

Patients and bacterial isolates

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

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

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

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

Antimicrobial susceptibility testing

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

WGS

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

Pasteur MLST website. Furthermore, cgMLST was performed using the scgMLST scheme of 634 core genes.[20] Identical isolates were defined as isolates with cgMLST profile differing by less than 7 alleles (excluding missing loci). In addition, we used Kleborate to detect other K-loci (KL) and *Kp* virulence determinants (https://github.com/katholt/Kleborate).[21]

Resistance genes and plasmid replicons were detected using Resfinder and PlasmidFinder, respectively (http://www.genomicepidemiology.org).[22]

A maximum likelihood phylogenetic tree was built from the concatenation of 634 cgMLST gene alignments with RAxML using a general time reversible (GTR) evolutionary model and a gamma correction for among-site rate variation.[23] One hundred bootstrap replicates were conducted to quantify the significance of nodes in the maximum likelihood tree. Gubbins was used to identify genome regions that had undergone homologous recombination. This tool detects recombination events based on an elevated SNP density.[24] The resulting trees were visualized using iTol (Version 4.2.3) (http://itol.embl.de/itol.cgi).[25]

Statistical analysis

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

Results

Isolates

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

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

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

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

Antimicrobial susceptibility

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

WGS and phylogenetic analysis

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

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

Identification of antimicrobial resistance genes and replicons

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

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

Mother-to-child transmission and persistence of Kp in neonates

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

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

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

Discussion

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

Kp is known to be a highly diverse species.[26] In this first study of the population structure of *Kp* originating from human communities in Madagascar, six of seven *Kp* phylogroups were represented. Based on MLST, a high degree of genetic diversity was observed. Additionally, sequence types known to be epidemiologically prevalent in other world regions (ST23, ST45, ST101 and ST25) and/or associated with multidrug-resistance (ST14, ST15, ST17, ST101 and ST147) were found.

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

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

not surprising because *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{CTX\text{-}M\text{-}14}$ are primarily carried on IncF plasmids, as exemplified by a study in Morocco in which the presence of IncFII was associated with *bla*$_{CTX\text{-}M\text{-}15}$.[32]

Kp has gradually accumulated various antibiotic resistance genes over time, making *Kp* infections increasingly difficult to treat. The most common associations observed in our study were resistances to 3rd generation cephalosporins, aminoglycosides, and to a lesser extent to trimethoprim/sulfamethoxazole and tetracycline. The emergence of MDR *Kp*, especially ESBL and/or carbapenemase producers, has elevated morbidity and mortality rates, as well as health care costs associated with *Kp* infections to highly burdensome levels.[27,33,34] This emergence is considered to be a major, global problem for public health.

Three hypervirulent isolates were identified during this study: one ST23-K1 isolate from the pus of a baby's eye and two ST25-K2 isolates from UTIs. The main biomarker genes (aerobactin and *rmpA*) recognized to differentiate hypervirulent *Kp* from classical *Kp* were identified in these isolates.[35]

Although the presence of genes encoding resistance or virulence factors in an isolate does not necessarily indicate gene activity in that isolate, previous work has demonstrated the functional implications of these genes.[36]

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

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

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

In this study, we found that persisting *Kp* belonged to ST14, ST15, ST35, ST37, ST45 and ST70, all of which have previously been reported to cause infections.[27,45,46] Further, three of ten neonate carriage episodes exceeding 60 days resulted in infections by the same infectious strain (isolated from UTIs and pus). The duration of *Kp* carriage was long for some neonates, especially for *Kp3* ST711, which was found several times in UTIs for a period up to 115 days, and for *Kp* ST25-K2, a hypervirulent isolate carried for 109 days in one neonate. Infants born by caesarean section may be at particularly risk for long-term fecal carriage of ESBL- producing Enterobacteriaceae,[44] as illustrated by the baby who was infected by ESBL-producing *Kp* ST711 until one year of age. During an outbreak in an NICU in Norway, gut carriage of a *Kp* ST17 strain with a pKPN3-like CTX-M-15-encoding IncFII persisted for up to two years.[47] Another study revealed colonization lasting up to 4.5 years, with phenotypic diversification and parallel selection of pathoadaptive mutations potentially contributing to long-term carriage and virulence of a KPC-positive ST258 strain.[48] However, although unlikely, and although *Kp* carriage has been observed to last for up to years at a time, it cannot be excluded that neonates in this study with persistent carriage could have re-acquired the same clone on multiple occasions.

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

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

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Availability of data and materials

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

Transparency declarations

None to declare

Supplementary data

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

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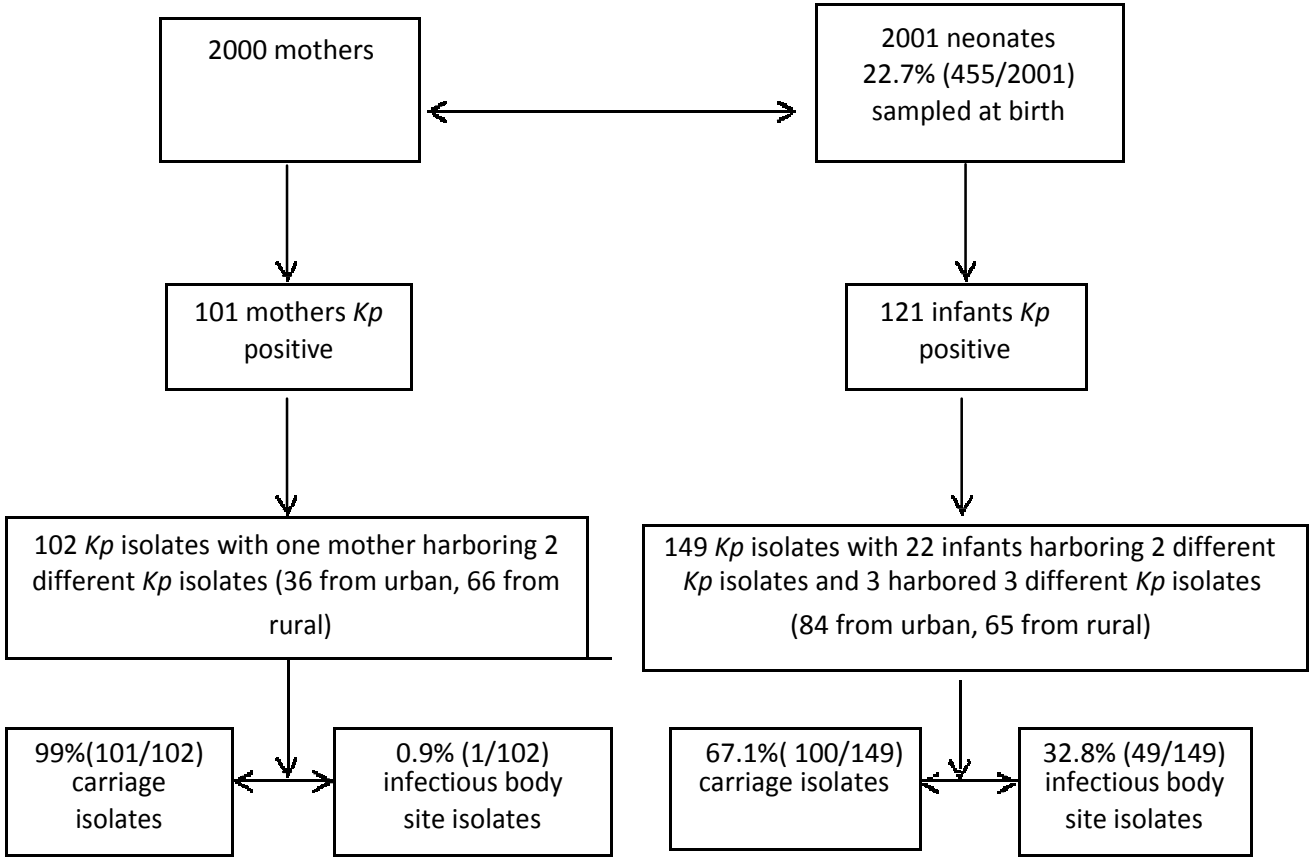

Figure 1: Flow-chart of the study participants with the features of isolates

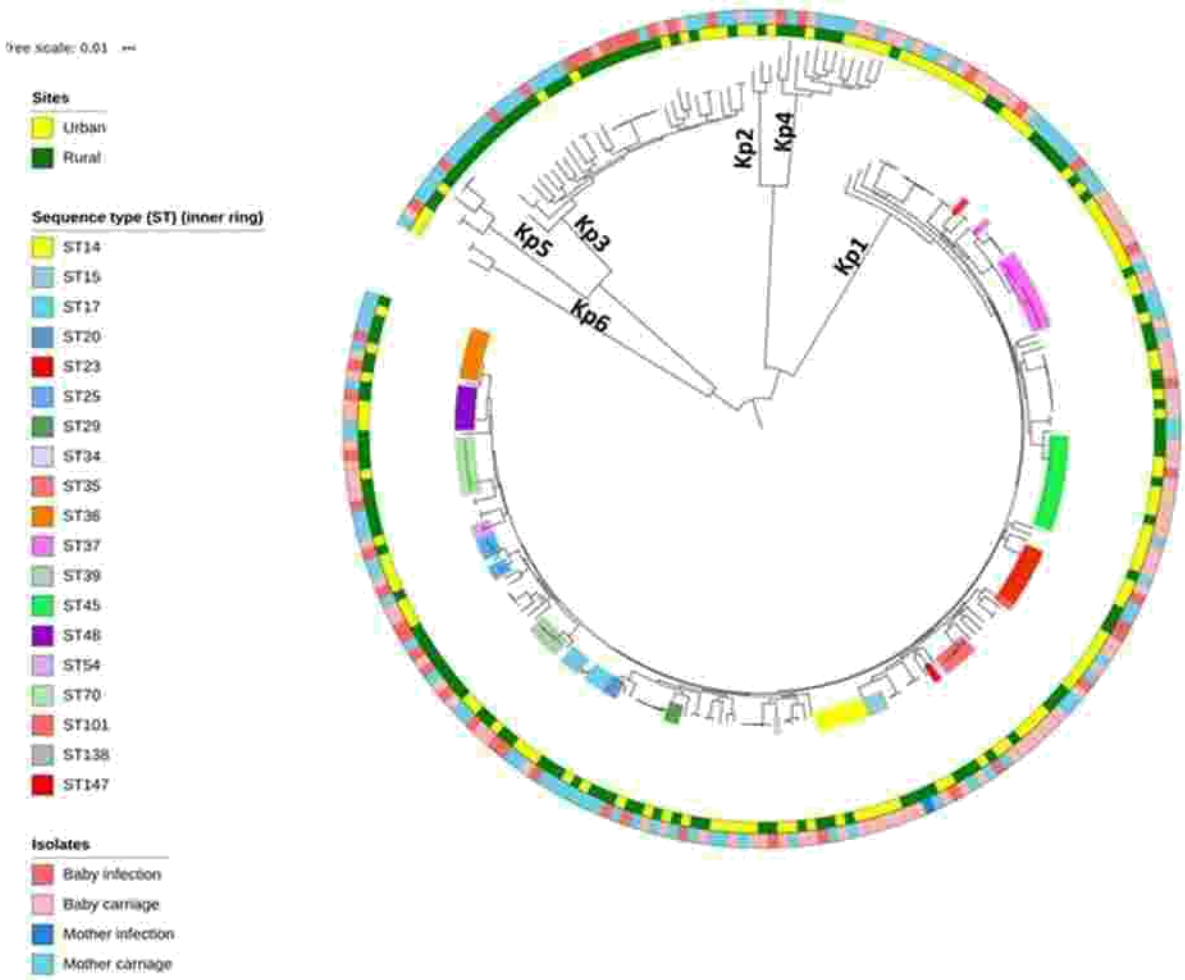

Figure 2: Unrooted phylogenetic tree constructed with all 251 isolate sequences. Each phylogroup was clearly separated from others and is indicated along its branch. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

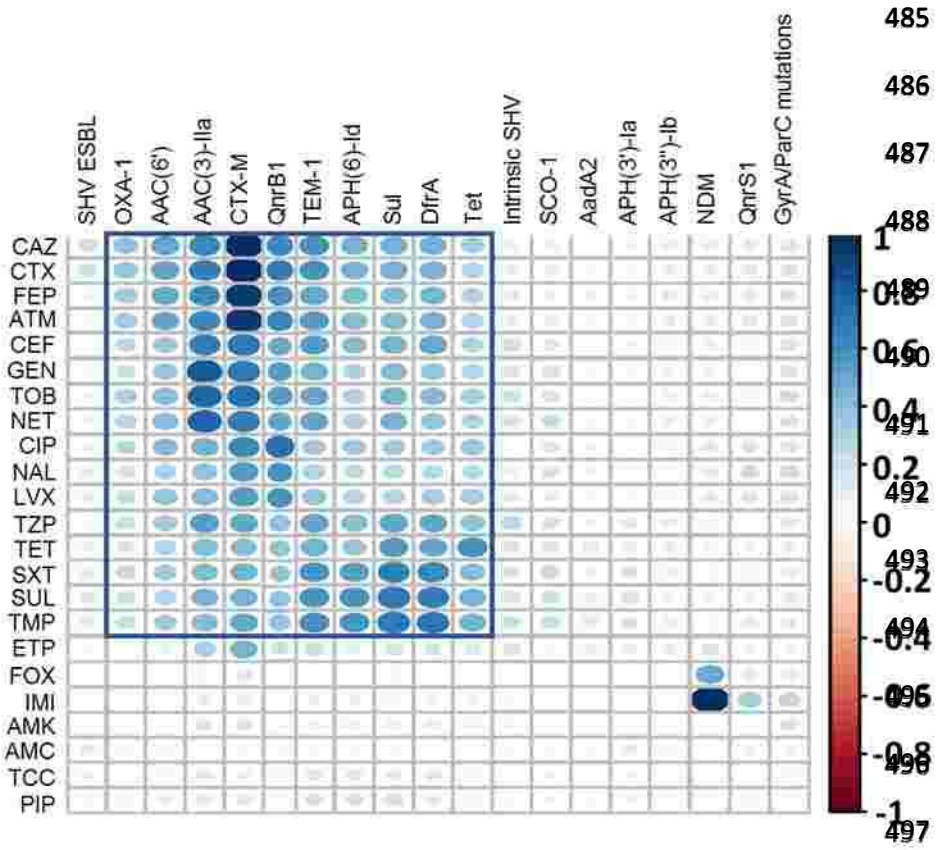

Figure 3: Correlation heatmap between antibiotic-resistant phenotype and resistome genotype. X axis: resistant genes; Y axis: phenotypes. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

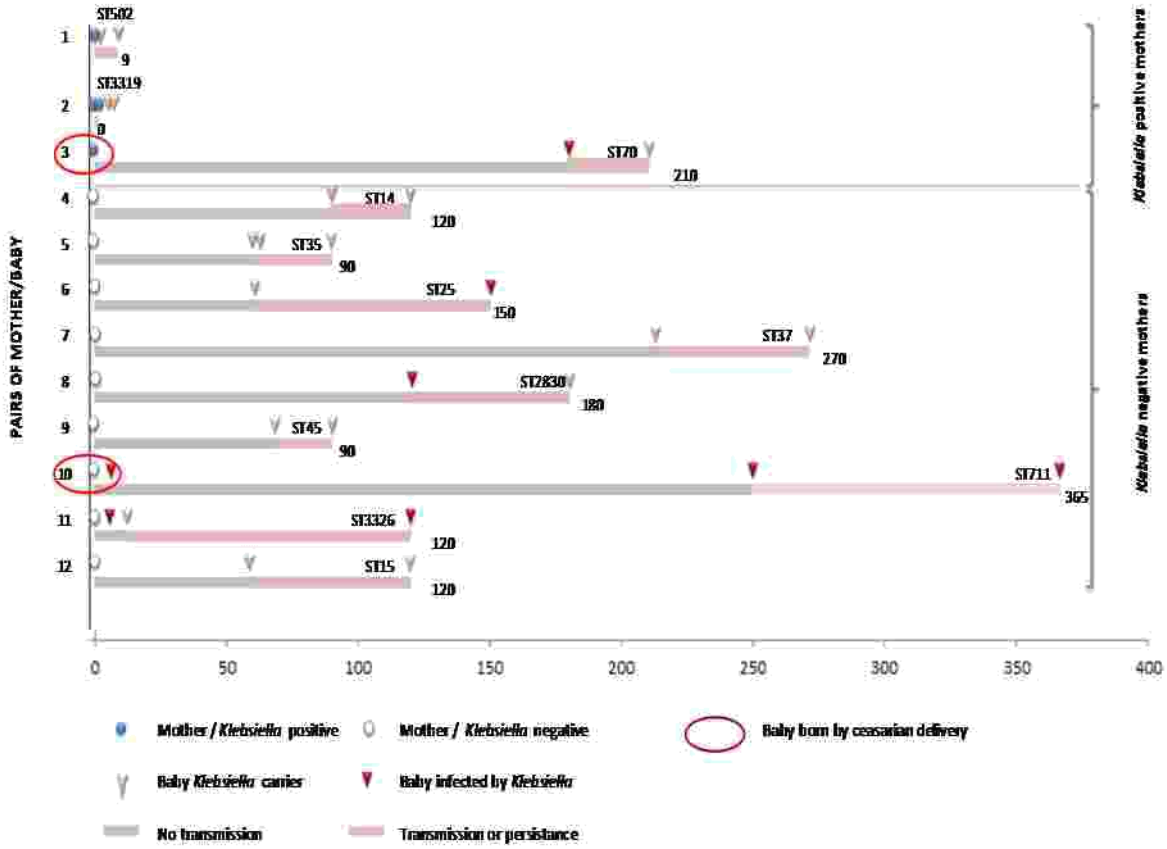

Figure 4: Timeline representation of 2 pairs of mother-baby transmission and prolonged carriage in 10 neonates. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*. Mothers are represented by circles at day 0, in blue circles when she carried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

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$
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 $ $?^2$ 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
bla$_{\text{CTX-M}}$	ESBL	32 (31.4)	33 (22.2)	0.1
bla$_{\text{SHV2-A/SHV42}}$	ESBL	3 (2.9)	1 (0.7)	0.3$^{\$}$
bla$_{\text{NDM-1}}$	ESBL	1 (0.9)	0	-
bla$_{\text{SHV}}$ non-ESBL	ß-lactams	63 (61.8)	118 (79.2)	0.002**
bla$_{\text{LEN}}$	ß-lactams	19 (18.6)	11 (7.4)	0.007**
bla$_{\text{OKP-D/-B}}$	ß-lactams	1 (1)	4 (2.7)	0.7$^{\$}$
bla$_{\text{OXA-1/TEM-1/-40/SCO-1/LAP-2}}$	ß-lactams	33 (32.4)	63 (42.3)	0.1
aph(6)-Id	aminoglycosides	37 (36.3)	56 (37.6)	0.8
aac(3)-IIa	aminoglycosides	24 (23.5)	37 (24.8)	0.8
aph(3')-Ia/aph(3'')-Ib/ant(3'')-Ia/aadA5	aminoglycoside	16 (15.7)	13 (8.7)	0.09
aac(6')-Ib-cr	aminoglycosides	17 (16.7)	11 (7.4)	0.02**
gyrA (S83I, S83F and D87A) and *parC* (S80I)	fluoroquinolones	2 (2)	4 (2.7)	1$^{\$}$
qnrB1/B6/B9, qnrS	fluoroquinolones	22 (21.6)	21 (14.1)	0.1
sul1/2	sulphonamides	42 (41.2)	66 (44.3)	0.6
dfrA	trimethoprim	36 (35.3)	64 (42.9)	0.2
Tet(A)/(B)/(D)/(K)	tetracycline	36 (35.3)	33 (22.2)	0.02**

517 $ $?^2$ exact test

518

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

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

520 $ $?^2$ exact

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	*bla*$_{SHV-62}$, *bla*$_{TEM-1}$, *ant(3'')-Ia*, *cml*	
	01-662-1LGAS	Baby1	May, 2015	Carriage	ST502	*bla*$_{SHV-62}$, *bla*$_{TEM-1}$, *strA*, *strB*, *aph(6)-Id*, *ant(3'')-Ia*, *cml*	
	01-662-2ECBU	Baby1	May, 2015	Carriage	ST502	*bla*$_{SHV-62}$	
2	02-719-MPLA	Mother2	November, 2014	Carriage	ST3320	*bla*$_{SHV-42}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *strA*, *strB*, *aph(6)-Id*, *aac(3)-IIa*, IncFIB(pKPHS1) *dfrA14*, *oqxA*	
	02-719MSEL	Mother2	November, 2014	Carriage	ST3319	*bla*$_{SHV-1}$, *aac(6')-Ib-cr*, *aac(3)-IIa*, *oqxA*, *oqxB32*, *OmpK36*	IncFII(K)
	02-719SEL	Baby2	November, 2014	Carriage	ST3319	*bla*$_{SHV-42}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *strA*, *strB*, *aph(6)-Id*, *aac(3)-IIa*, *dfrA14*, *qnrB1*, *oqxA*	IncFII(K)
	02-719LGAS	Baby2	November, 2014	Carriage	ST3319	*bla*$_{CTX-M-15}$, *strA*, *strB*, *dfrA14*, *qnrB1*	IncFII(K)
3	02-746MBV	Mother3	October, 2014	Carriage	ST347	*bla*$_{SHV-11}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *aph(6)-Id*, *aac(6')-Ib-cr*, *aac(3)-IIa*, *tetR*, *tetA*, *oqxA*, *oqxB19*	
	02-746-1ECBU	Baby3	May, 2015	Carriage	ST70	*bla*$_{CTX-M-15}$, *bla*$_{SHV-32}$, *bla*$_{TEM-1}$, *strA*, *strB*, *aph(6)-Id*, *aac(6')-Ib-cr*; *aac(3)-IIa*, *tetR*, *tetA*, *dfrA14*, *qnrB1*, *oqxB19*, *oqxA10*	IncFIB(K), IncFII, IncFII(K)
	02-746-2ECBU	Baby3	May, 2015	Infection	ST70	*bla*$_{CTX-M-15}$, *strA*, *strB*, *dfrA14*, *qnrB1*	IncFIB(K), IncFII, IncFII(K)
4	01-314-1ECBU	Baby4	April, 2014	Carriage	ST14	*bla*$_{CTX-M-15}$, *bla*$_{SHV-28}$, bla$_{TEM-1}$, *bla*$_{OXA-1}$, *strA*, *strB*, *aph(6)-Id*, *aac(6')-Ib-cr*, *aac(3)-IIa*, *dfrA14*, *oqxA*	IncFIB(K), IncFII(K)
	01-314-2ECBU	Baby4	May, 2014	Carriage	ST14	*bla*$_{CTX-M-15}$, *bla*$_{SHV-28}$, *bla*$_{TEM-1}$, *strA*, *strB*, *aph(6)-Id*, *aac(3)-IIa*, *dfrA14*, *oqxA*	IncFIB(K), IncFII(K)

5	01-358-1ECBU	Baby5	May, 2014	Carriage	ST35	*bla*$_{SHV-33}$, *oqxB19*, *oqxA3*	IncFIB(K), IncFII(pRSB17)
	01-358-2ECBU	Baby5	May, 2014	Carriage	ST35	*bla*$_{SHV-33}$, *oqxB19*, *oqxA3*	IncFIB(K)
	01-358-3 ECBU	Baby5	June, 2014	Carriage	ST35	*bla*$_{SHV-33}$, *oqxB19*, *oqxA3*	IncFIB(K), IncFII(pRSB17)
6	01-368-1ECBU	Baby6	July, 2014	Carriage	ST25	*bla*$_{SHV-11}$, *bla*$_{TEM-1}$, *aac(3)-IId*, *Tet(D)*, *dfrA5*, *oqxB17*, *oqxA10*	IncFIB(K), IncFII(K)
	01-368-2ECBU	Baby6	October, 2014	Infection	ST25	*bla*$_{SHV-11}$, *bla*$_{TEM-1}$, *aac(3)-IId*, *Tet(D)*, *dfrA5*, *oqxB17*, *oqxA10*	IncFIB(K), IncFII(K)
7	01-443-1ECBU	Baby7	March, 2015	Carriage	ST37	*bla*$_{SHV-11}$, *oqxA*, *OmpK36*	
	01-443-2ECBU	Baby7	April, 2015	Carriage	ST37	*bla*$_{SHV-11}$, *oqxA*, *OmpK36*	
8	01-467-1ECBU	Baby8	December, 2014	Infection	ST2830	*bla*$_{OKP\_D\_1}$, *bla*$_{TEM-1}$, *aph(6)-Id*, *Tet(D)*, *dfrA14*,*dfrA8*	IncFII(29), IncR
	01-467-2ECBU	Baby8	February, 2015	Carriage	ST2830	*bla*$_{OKP\_D\_1}$, *bla*$_{TEM-1}$, *strA*, *strB*, *aph(6)-Id*, *Tet(D)*, *dfrA14*, *dfrA8*	IncFII(29), IncR
9	01-595-1ECBU	Baby9	March, 2015	Carriage	ST45	*bla*$_{SHV-1}$, *strA*, *strB*, *aph(6)-Id*, *dfrA14*, *oqxB19*, *oqxA11*	IncFIB(K), IncFII(K), IncQ1
	01-595-2ECBU	Baby9	March, 2015	Carriage	ST45	*bla*$_{SHV-1}$, *bla*$_{TEM-1}$, *strA*, *strB*, *aph(6)-Id*, *dfrA14*, *oqxB19*, *oqxA11*	IncFIB(K), IncFII, IncFII(K), IncQ1IncR
10	02-766-1ECBU	Baby10	October, 2015	Infection	ST711	*bla*$_{CTX-M-15}$, *bla*$_{SHV-83}$, *bla*$_{TEM-1}$, *strA*, *strB*, *aph(6)-Id*, *aac(3)-IIa*, *dfrA14*, *oqxA*, *oqxB25*	ColpVC, IncFIB(K), IncFII(K)
	02-766-2ECBU	Baby10	October, 2015	Infection	ST711	*bla*$_{CTX-M-15}$, *bla*$_{SHV-83}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *strA*, *strB*, *aph(6)-Id*, *aac(6')-Ib-cr*, *aac(3)-IIa*, *dfrA14*, *oqxA*, *oqxB25*	ColpVC, IncFIB(K), IncFII(K)
11	02-891-1PUS	Baby11	March, 2015	Infection	ST1584	*strA*, *strB*, *aph(6)-Id*	

	02-891-2ECBU	Baby11	March, 2015	Carriage	ST3326	*bla*$_{\text{LEN-17}}$, *oqxB16*		522
	02-891-3PUS	Baby11	July, 2015	Infection	ST3326	*bla*$_{\text{LEN-17}}$, *oqxB16*		523
12	02-1056-1ECBU	Baby12	October, 2015	Carriage	ST15	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA-1}}$, *strA*, *strB*, *aph(6)-Id*, *aac(6')-Ib-cr, aac(3)-IIa*, *dfrA14*, *ParC-80I*;*GyrA-83F*;*GyrA-87A*, *oqxA*, *OmpK36*	IncFIA(HI1), IncFIB(K), IncFII, IncR	524
	02-1056-2ECBU	Baby12	December, 2015	Carriage	ST15	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA-1}}$, *strA*, *strB*, *aph(6)-Id*, *aac(6')-Ib-cr, dfrA14*, *ParC-80I*;*GyrA-83F*, *GyrA-87A*, *oqxA*, *OmpK36*	IncFIB(K), IncFII, IncR	