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Pervasive transmission of a carbapenem resistance plasmid in the gut microbiota of hospitalised patients

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Introductory paragraph

Infections caused by carbapenemase-producing enterobacteria (CPE) are a major concern in clinical settings worldwide. Two fundamentally different processes shape the epidemiology of CPE in hospitals: the dissemination of CPE clones from patient to patient (between-patient transfer), and the transfer of carbapenemase-encoding plasmids between enterobacteria in the gut microbiota of individual patients (within-patient transfer). The relative contribution of each

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Author contributions
ASM, RLS and BC conceived the study. RC designed and supervised sampling and collection of bacterial isolates. MHG, PRG collected the bacterial isolates and performed bacterial characterization. CDA and NLF collected the epidemiological data and performed preliminary analyses. R-GNOSIS WP5 Study Group designed sampling protocols and facilitated the training and capacity building for the collection of bacterial isolates and preliminary analyses. JdLF, JRB and CdlV performed the experimental work and analysed the results. RLS, BC, PM and TC performed the data analysis. ASM coordinated the study. ASM and RLS wrote the initial draft of the manuscript. ASM, RLS, JdlF, JRB, BC, PM, and TC contributed to the final version of the manuscript. All authors read and approved the manuscript.

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process to the overall dissemination of carbapenem resistance in hospitals remains poorly understood. Here, we used mechanistic models combining epidemiological data from more than 9,000 patients with whole genome sequence information from 250 enterobacteria clones to characterise the dissemination routes of a pOXA-48-like carbapenemase-encoding plasmid in a hospital setting over a two-year period. Our results revealed frequent between-patient transmission of high-risk pOXA-48-carrying clones, mostly of *Klebsiella pneumoniae* and sporadically *Escherichia coli*. The results also identified pOXA-48 dissemination hotspots within the hospital, such as specific wards and individual rooms within wards. Using high-resolution plasmid sequence analysis, we uncovered the pervasive within-patient transfer of pOXA-48, suggesting that horizontal plasmid transfer occurs in the gut of virtually every colonised patient. The complex and multifaceted epidemiological scenario exposed by this study provides insights for the development of intervention strategies to control the in-hospital spread of CPE.

Introduction

Antibiotic resistance is one of the most concerning health challenges facing modern societies[1]. Antibiotic resistance is of particular concern in clinical settings, where resistant pathogens significantly increase the mortality rates of critically ill patients and the costs associated with infection management and control[1,2]. The spread of antibiotic resistance genes between bacteria commonly associated with nosocomial infections is mainly driven by the horizontal transfer of conjugative plasmids[3,4]. However, the frequency with which this occurs in the clinical settings and its importance for the dissemination of resistance at a local level remain poorly defined.

One of the most clinically relevant groups of nosocomial pathogens are enterobacteria that produce carbapenemases (ß-lactamase enzymes able to degrade carbapenem antibiotics). Among carbapenemase-producing enterobacteria (CPE), clones of *Klebsiella pneumoniae* and *Escherichia coli* carrying plasmid-encoded carbapenemases pose the highest clinical threat[5]. Despite their clinical relevance, major gaps remain in our understanding of the epidemiology of CPE and of carbapenemase-encoding plasmids. Previous work has highlighted the importance of in-hospital CPE transmission from patient to patient[6,7] (between-patient transfer). However, the dissemination and evolution of CPE in hospitals present an additional layer of complexity: the transfer of carbapenemase-encoding plasmids between enterobacteria clones in the gut microbiota of individual patients (within-patient transfer)[8–12]. Understanding the relative importance of between-patient and within-patient transfer is of central importance for understanding the epidemiology of CPE and informing intervention strategies to control the spread of carbapenem resistance in clinical settings.

One of the most frequent carbapenemases in enterobacteria is OXA-48[13]. *bla* $_{\text{OXA-48}}$ was first described in a *K. pneumoniae* strain isolated in Turkey in 2001[14] and is now distributed worldwide, with particularly high prevalence in North Africa, Middle Eastern countries, and Europe[13]. The *bla* $_{\text{OXA-48}}$ gene is usually carried by IncL broad-host range plasmids closely related to pOXA-48[15] (Extended Data 1). These plasmids share a highly conserved backbone and differ mainly by the presence/absence of various insertions; for simplicity, they are all referred to as pOXA-48 throughout the text. This plasmid type is frequently

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associated with *K. pneumoniae* high-risk clones[16], such as the sequence types 11 (ST11), ST15, ST101, and ST405[6,15,17,18], which are able to readily spread between hospitalized patients producing outbreaks of infections[19,20]. Previous epidemiological studies strongly suggested the possibility of within-patient pOXA-48 transfer[20–25], indicating that pOXA-48 would be an ideal study system to investigate the nosocomial dissemination of carbapenem resistance.

In the present study, we examined the between-patient and within-patient transfer dynamics of pOXA-48 in a tertiary hospital over a two-year period. For our analysis, we used a large and well-characterized collection of pOXA-48-carrying enterobacteria generated at the *Hospital Universitario Ramon y Cajal* in Madrid as part of the European project R-GNOSIS (Resistance of Gram-Negative Organisms: Studying Intervention Strategies)[26,27]. Using statistical models and combining epidemiological data from more than 9,000 patients with whole-genome sequence information from 250 enterobacteria clones, we aimed to define pOXA-48 transfer dynamics at an unprecedented resolution. Specifically, we aimed to determine the relative contribution of between-patient and within-patient plasmid transfer in the epidemiology of pOXA-48, and to use these data to inform improved intervention strategies to control the spread of carbapenem resistance in hospitals.

Results

Patients colonised by pOXA-48-carrying enterobacteria in the hospital

During the R-GNOSIS project, hospitalised patients were periodically sampled to detect the presence of enterobacteria producing extended spectrum ß-lactamases (ESBL) and carbapenemases in their gut microbiota (see methods). The study enrolled all patients admitted to two medical wards (gastroenterology and pneumology) and two surgical wards (neurosurgery and urology). The full details of the R-GNOSIS study in our hospital, including the study population and CPE characterization, have been previously reported by Hernandez-Garcia *et al* [21,27]. Briefly, from March 2014 to March 2016, 28,089 rectal swabs were collected from 9,275 patients, and 171 enterobacteria strains carrying pOXA-48-like plasmids were isolated and characterised from 105 patients (Figure 1, Extended Data 2–3, and Supplementary Table 1, see methods for details). The proportion of patients who were found to be colonized with pOXA-48-carrying enterobacteria on at least one occasion during their hospital admission was 0.5% in urology (18/3,483), 1.3% in gastroenterology (33/2,591), and 1.5% both in neurosurgery (16/1,068) and pneumology (38/2,559) (Figure 1).

In line with previous reports[13], *K. pneumoniae* was the most frequent pOXA-48-carrying species (n= 108). However, pOXA-48 was detected in an additional 7 enterobacterial species, with *E. coli* being the second most frequent carrier (n= 45, Figure 1C, Supplementary Table 1). In several pOXA-48-carrying patients (18/105), there was co-colonisation of the gut microbiota with more than one species carrying the plasmid, suggestive of within-patient plasmid transfer events (Figure 1B).

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Using epidemiological data to analyse pOXA-48 transfer dynamics

To investigate how pOXA-48 spread in the hospital, we analysed the epidemiological data using a previously described model which enabled us to estimate the daily probability of a patient acquiring pOXA-48-carrying enterobacteria and quantify the effect of covariates on this probability (see methods, Extended Data 4 and Supplementary Table 2)[28]. We performed this analysis independently for the two species with a large number of isolates, *K. pneumoniae* and *E. coli,* and we included two covariates in the model. The first covariate was the number of other patients colonized by pOXA-48-carrying enterobacteria in the ward on the same day, which we expect to be positively associated with the daily risk of acquisition if between-patient bacterial transfer is important. The second covariate was known pre-existing intestinal colonisation of the patient by a pOXA-48-carrying enterobacteria of a different species (*K. pneumoniae* or *E. coli*). If within-patient plasmid transfer is important (from *K. pneumoniae* to *E. coli* and *vice versa*), then we would expect this to also be positively associated with the daily risk of a patient acquiring pOXA-48-carrying enterobacteria. We considered different transmission models including and excluding these covariates and performed model comparisons using the widely applicable information criterion (WAIC, Supplementary Table 2). The model that best fitted our data was the one including both covariates and permitting the risk of between-patient transfer to vary by ward (see Supplementary Table 3 for daily probability values and methods for details).

The baseline daily probabilities for becoming colonised with pOXA-48-carrying *K. pneumoniae* or *E. coli* were 0.1% (95% credible interval [CrI] 0.08%, 0.12%) and 0.04% (95% CrI, 0.02%, 0.05%), respectively (Supplementary Table 3). Results showed that the probability of acquisition of a pOXA-48-carrying *K. pneumoniae* was higher if other patients were already colonised with a pOXA-48-carrying clone in the neurosurgery (Odds ratio [OR] 6.7, 95% CrI 2.5, 11.7) and pneumology wards (OR 2.7, 95% CrI 1.2, 4.6). In the gastroenterology (OR 1.7, 95% CrI 0.4, 4.1) and urology wards (OR 0.6, 95% CrI 0.01, 4.4) there were no clear effects. In contrast, the presence of other patients colonised by pOXA-48-carrying clones was not associated with the probability of acquiring a pOXA-48-carrying *E. coli* in the neurosurgery (OR 0.23, 95% CrI 0.001, 2.0) or urology wards (OR 0.4, 95% CrI 0.002, 2.7), and there was only weak evidence for a positive association in the gastroenterology (OR 1.9, 95% CrI 0.4, 4.7) and pneumology wards (OR 2.6, 95% CrI 0.9, 4.5) (Extended Data 4A). This result suggested that *K. pneumoniae* is more important for between-patient transfer than *E. coli.*

The model also showed that prior colonisation with a pOXA-48-carrying clone of a different species was associated with a dramatic increase in the probability of isolation of a second pOXA48-carrying species (Extended Data 4). This risk was high both when a patient was first colonised by *K. pneumoniae* (OR 23.3, 95% CrI 8.3, 53.4) and when initially colonized with *E. coli* (OR 15.8, 95% CrI 3.8, 42.7). This result underlines the potential importance of within-patient plasmid transfer in the disemination of pOXA-48, a role supported by the high frequency of co-colonised patients (Extended Data 4). However, other explanations may be responsible for this observation, such as independent colonisation events of patients by different pOXA-48-carrying clones.

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Genomic analysis of pOXA-48-carrying enterobacteria

A key limitation of our epidemiological model is that it is based solely on species identification, which restricts the possibility of reconstructing the spread of specific clones and plasmids. To track within-patient and between-patient plasmid transfer at a higher level of resolution, we integrated genomic information by sequencing the genomes of the 171 pOXA-48-carrying isolates represented in Figure 1C. In line with previous studies[26,29], the sequencing results revealed that a small subset of isolates initially identified as *K. pneumoniae* are actually *Klebsiella quasipneumoniae* (n= 2) or *Klebsiella variicola* (n= 3) (Extended Data 5).

We analysed the genetic relatedness of *K. pneumoniae* isolates and of *E. coli* isolates by reconstructing the core genome phylogeny for each species (Figure 2). For *K. pneumoniae* (n= 103), most of the isolates belonged to a few high-risk sequence types: ST11 (n= 64), ST307 (n= 17), or ST15 (n= 9). In contrast, *E. coli* (n= 45) showed a more diverse population structure, with more than three isolates only identified for one sequence type, ST10 (n= 11).

We next considered the distribution of the different clonal groups (defined by the different STs) across colonised patients (Figure 2). Most *K. pneumoniae* isolates belonged to STs present in more than one patient, whereas approximately half of *E. coli* isolates belonged to STs present in only one patient. This result, together with the results of statistical analysis and the genomic data, suggested that a limited number of *K. pneumoniae* high-risk clones are responsible for most of the between-patient transfer events. However, we observed several cases of pOXA-48-carrying *E. coli* STs colonising different patients, suggesting that *E. coli* is also responsible for sporadic between-patient transmission events.

Reconstruction of between-patient transfer dynamics of pOXA-48-carrying clones

To investigate the specific dissemination routes of pOXA-48-carrying clones, we integrated epidemiological and genomic data using SCOTTI[30] (see methods). SCOTTI is a structured coalescent-based tool for reconstructing bacterial transmission, which accounts for bacterial diversity and evolution within hosts, non-sampled hosts, multiple infections of the same host, and direct and indirect transmission events. We analysed the spread of the dominant *K. pneumoniae* and *E. coli* STs within and among the four wards under study (Figure 3, Supplementary Figures 1–4). As expected from the genomic data (Figure 2A), clones belonging to *K. pneumoniae* ST11 were responsible for most of the putative between-patient transmission events. The analysis attributed transmission events of ST11 carrying pOXA-48 on every single ward and even between wards, with neurosurgery being the ward with the highest frequency and probability of transmission of ST11 (Figure 3), as suggested by the epidemiological model. In light of these results, we investigated *K. pneumoniae* ST11/pOXA-48 epidemiology in the neurosurgery ward in more detail by looking at the spatiotemporal distribution of colonised patients (Extended Data 6). The neurosurgery ward includes 11 rooms with 20 beds (9 double rooms and 2 single rooms). Of the 16 colonized patients, 6 had overlapping stays in the same room, suggesting that this room acted as a hotspot for *K. pneumoniae* ST11/pOXA-48 colonisation and transmission.

SCOTTI also predicted transmission events mediated by three further pOXA-48-carrying clones. Two transmission events were attributed to *K. pneumoniae* ST307 in the pneumology ward and three more to *K. pneumoniae* ST15: two in gastroenterology and another one between the gastroenterology and urology wards. In line with the genomic results (Figure 2B), SCOTTI also attributed two between-patient transfer events to *E. coli* ST10, one on the gastroenterology ward and another one between the gastroenterology and urology wards (Figure 3).

Genetic analysis of pOXA-48 confirms pervasive within-patient plasmid transfer

Our results suggest that the high frequency of patient colonisation by two plasmid-carrying species could be due to within-patient pOXA-48 transfer (Figure 1 and Extended Data 4). However, although unlikely, another possibility is independent colonisation events involving different plasmid-carrying clones. To distinguish between these possibilities, we analysed the genetic sequence of pOXA-48 across all isolates with the aim of using specific genetic signatures in the plasmid to provide evidence for or against within-patient plasmid transfer. To increase the resolution of this analysis, we enriched the R-GNOSIS collection by recovering and sequencing the complete genomes of all the pOXA-48-carrying enterobacteria isolated from patients in our hospital since the plasmid was first reported in 2012 (Supplementary Table 1). In total, we determined the sequences of 250 isolates by short read methods and two of these genomes and six pOXA-48 plasmids were also sequenced by long-read methods to allow hybrid assembly (see Extended Data 7 and methods).

The results showed that pOXA-48 is highly conserved (Figure 4A). The core plasmid sequence spanned more than 60 kb (>90% of plasmid sequence) in 219 of the 250 strains (Supplementary Table 1 and methods). Analysis of the core genome among these 219 pOXA-48-like plasmids revealed an identical sequence in 80% of them. In the remaining 20%, we detected a total of 21 SNPs, with each plasmid presenting 1 or 2 SNPs compared with the most common variant in the collection (named pOXA-48_K8, see Figure 4A and Extended Data 1).

Given the low plasmid-sequence variability, we could not track plasmid transmission using the same tools used for bacterial transmission. Instead, we monitored plasmid transfer by using the rare plasmid variants carrying specific core-region SNPs as genetic fingerprints (Figure 4, Extended Data 1). We focused on instances where the same rare, traceable plasmid variant was present in isolates belonging to different species. We considered that isolation of different bacterial species carrying the same rare plasmid variant from the same patient would be a very strong indicator of within-patient plasmid transfer. We found four examples in which the same rare plasmid variant was present in different bacterial species (Figure 4A). The SNPs in these four plasmid variants were located in *xerD*, *traC* and in two predicted genes encoding hypothetical proteins (see Extended Data 1). In all four examples, different species carrying the same plasmid variant were isolated from the same patient (Figure 4B). For example, plasmid variant 3 was detected in 6 bacterial isolates belonging to four clones (one *K. quasipneumoniae*, one *E. coli* and two *C. freundii*), and all of them were recovered from a single patient in the hospital (patient code YUE). Crucially, the chances of

independent patient colonisation with the different bacterial clones carrying these rare plasmid variants are extremely low (variant 1, 6.4×10^{-4}; variant 2, 8.9×10^{-4}; variant 3, 1.1×10^{-8}; variant 4, 2.1×10^{-5}), confirming that these were within-patient plasmid transfer events.

High in vitro *pOXA-48 conjugation rate*

Despite the limitations imposed by the sensitivity and frequency of the sampling method, the four selected pOXA-48 variants with core-region SNPs demonstrated pervasive within-patient plasmid transfer. However, the specific SNPs used as genetic fingerprints might affect the conjugation ability of the plasmid, which would make it impossible to generalize the results with these variants to the most common pOXA-48 variant. In fact, the SNP present in plasmid variant 4 produces a non-synonymous mutation in *traC*, which is a gene involved in conjugative transfer[31] (Extended Data 1). To test this possibility, we experimentally measured the conjugation rates of the most common pOXA-48 variant (pOXA-48_K8), and the four traceable variants, by introducing the plasmids independently into the *E. coli* strain J53 and determining the conjugation rate of each variant in this isogenic background (Figure 5, see methods). We performed mating experiments on three different agar media: (i) the common laboratory medium LB, (ii) MacConkey, which contains bile salts, one of the main selective pressures in the gut environment[32], and (iii) M9 minimal medium supplemented with gluconate (MMG), which is an abundant carbon source for *E. coli* in the gut[33]. Moreover, to better resemble the gut environment we performed the experiments not only in aerobic but also in anaerobic conditions (Figure 5).

Previous reports have shown that pOXA-48 plasmid conjugates at high rates[15,25], due to the insertion of Tn*1999* into the transfer inhibition gene *tir*[34]. In line with these results, the *in vitro* pOXA-48_K8 conjugation ability in our experiments was high (see Figure 5 for conjugation rates and Extended Data 8 for conjugation frequencies). Interestingly, although conjugation rates differed between culture media (ANOVA, effect of medium; F= 43.6, df= 2, $P= 3.9\times10^{-16}$) and oxygen availability (ANOVA, effect of oxygen; F= 356.1, df= 1, $P< 2\times10^{-16}$), they did not differ significantly between the most frequent variant and the plasmid variants carrying traceable SNPs in the conditions tested (ANOVA, effect of plasmid variant; F= 1.2, df= 4, $P= 0.306$). These results confirmed that the common pOXA-48 variant has similar conjugation ability as the traceable variants, suggesting that the horizontal spread of pOXA-48 in the gut microbiota is probably the norm in colonized patients.

Discussion

CPE are emerging as one of the most concerning threats to public health worldwide[5]. Recent studies have highlighted the central relevance of hospitals as hotspots for the dissemination of CPE among patients and for the dissemination of the carbapenemase-encoding conjugative plasmids between enterobacteria clones[6–8]. In this study, we performed a high-resolution epidemiological analysis to uncover the dissemination routes of pOXA-48 (both at the bacterial and plasmid levels). By integrating epidemiological and genomic data, we uncovered frequent between-patient bacterial transfer and pervasive within-patient plasmid transfer.

In light of our results, we propose that in-hospital pOXA-48 dissemination generally adheres to the following dynamics: high-risk pOXA-48-carrying enterobacteria clones, mainly *K. pneumoniae* ST11, spread among hospitalised patients, colonising their gut microbiota (Figures 1, 2 and 3). Once patients are colonised, the plasmid readily spreads through conjugation to other resident members of the gut microbiota (enterobacteria such as *E. coli*, *C. freundii*, and *E. cloacae*, Figures 1, 4 and 5). The plasmid's high conjugation rate increases its chances of becoming established in the gut microbiota because, even if the invading nosocomial clone is eliminated, pOXA-48 can survive in a different bacterial host. Moreover, pOXA-48 produces variable fitness effects in different bacterial hosts[35]. Therefore, the frequent plasmid transfer provides a test bench for new bacterium-plasmid combinations, some of which may present particularly high fitness, being able to persist and even disseminate to new human hosts[4]. An illustrative example of these general dynamics is the case of the patient carrying plasmid variant 4 (Figure 4B; patient code TGY). This patient was first colonised by *K. pneumoniae* ST11/pOXA-48 in October 2014, and 11 days later a pOXA-48-carrying *E. coli* strain was isolated from the same patient (ST457). During a new admission 17 months later, a different pOXA-48-carrying *E. coli* (ST1722) was recovered from the patient's gut microbiota. The pOXA-48 variant in all the clones carries a traceable SNP, confirming that the patient was colonized throughout the period by pOXA-48-carrying enterobacteria, even though the plasmid had moved from its original *K. pneumoniae* ST11 host to *E. coli* clones in the gut microbiota.

Another interesting observation emerging from this study is that most of the events attributed to between-patient transmission originated from a small subset of patients (Figure 3). This result highlights the potential role of super-spreader patients in the nosocomial dissemination of CPE[36]. Unfortunately, given the small number of super-spreader patients, we were not able to associate them with any particular epidemiological aspect, such as age or length of stay.

There are certain limitations associated with our study. First, due to the design of the R-GNOSIS study, only one isolate per bacterial species and time point was recovered from the patients (Supplementary Table 1). Therefore, the degree of intra-patient variation could not be assessed in detail and, although SCOTTI is robust to this limitation[30], the predicted between-patient transfer events results should be interpreted with caution. Second, we could not access metadata on antibiotic usage in the patients under study. Therefore, we could not investigate how antibiotic treatments affected the risks of colonization by pOXA-48-carrying enterobacteria and of intra-patient plasmid transfer. Third, two key aspects for the success of this study were the high degree of sequence conservation of the pOXA-48-like plasmid and the strong link between this plasmid and the *bla* $_{\text{OXA-48}}$ gene. These are particular characteristics of pOXA-48 and *bla* $_{\text{OXA-48}}$, and may prevent the application of our approach to the study of the epidemiology of other carbapenemases. For example, plasmid variation and the frequent between-plasmid *bla* $_{\text{KPC}}$ transposition makes the epidemiological analysis of KPC-coding plasmids difficult[8,9,37]. Finally, we only used long-read sequence data from a small subset of isolates/plasmids (2 complete genomes plus 6 complete pOXA-48-like plasmids). Due to plasmid conservation mentioned above, this limited number of samples was sufficient to reconstruct plasmid epidemiology. However, a larger number of samples analysed by long-read sequencing technology could have improved our analysis.

An important goal of this study is to inform improved intervention strategies aimed at controlling the spread of carbapenem resistance in hospitals. Our results can help in the design of interventions to control OXA-48 dissemination at two levels:

(i) *Between-patient*. We have shown that the spread of pOXA-48-carrying enterobacteria between patients in the hospital is mainly mediated by high-risk clones commonly associated with nosocomial infections. These clones reside in hospital settings and are able to survive in the environment, creating stable reservoirs (often involving room surfaces and sinks[38–41]). Moreover, our results also showed that there are specific colonisation and transmission hotspots, such as individual rooms within wards (Extended Data 6). We therefore propose that measures to detect and control environmental reservoirs and transfer hotspots could prevent between-patient pOXA-48 dissemination. Such measures could represent a useful addition to the strategies based on patient surveillance and standard precautions already applied in hospitals, and could complement and improve the outcome of contact isolation measures[26].

(ii) *Within-patient.* A key finding of our study is the high prevalence of within-patient pOXA-48 transfer, which in turn helps to establish long-term pOXA-48 gut carriers. Preventing within-patient plasmid transfer and gut carriage is thus a particularly promising strategy for containing carbapenem resistance. This goal could be achieved either by blocking plasmid conjugation[42] or, ideally, by specifically clearing pOXA-48 from the gut microbiota of patients by targeted decontamination. Decontamination strategies would aim to remove pOXA-48 plasmid or pOXA-48-carrying enterobacteria from carriers while preserving the integrity of the gut microbiota. New biotechnological advances are being made on this front. For example, CRISPR (clustered regularly interspaced short palindromic repeats) based technology can be used for targeted plasmid elimination[43], and toxin–intein antimicrobials could be engineered to selectively remove pOXA-48-carrying clones from the microbiota[44]. Further work is urgently needed to tailor these emerging technologies into effective intervention strategies against the threat of plasmid-mediated carbapenemases.

Methods

Study design and data collection

We studied samples collected from patients admitted in a Spanish university hospital from March 4th, 2014, to March 31st, 2016, as part of an active surveillance-screening program for detecting ESBL/carbapenemase-carriers (R-GNOSIS-FP7-HEALTH-F3-2011-282512, www.r-gnosis.eu/)[21,26,27,45]. This study was approved by the Ramón y Cajal University Hospital Ethics Committee (Reference 251/13), which waived the need for informed consent from patients on the basis that the study was assessing ward-level effects and it was of minimal risk. This screening included a total of 28,089 samples from 9,275 patients admitted to 4 different wards (gastroenterology, neurosurgery, pneumology and urology) in the Ramon y Cajal University Hospital (Madrid, Spain). We used a randomly generated three letter code for patient anonymization. Rectal samples were obtained from patients within 72 h of ward admission; weekly additional samples were recovered in patients hospitalised ≥7 days, and a final sample at discharge was obtained in those patients with a hospital stay ≥3 days (swabbing interval: gastroenterology, median 2 days, IQR 1, 6 days;

neurosurgery, median 3 days, IQR 1, 7 days; pneumology, median 2 days, IQR 1, 6 days; urology, median 1 day, IQR 1, 3 days. Extended Data 3). This protocol allowed us to obtain a time sequence for each patient in the hospital.

In this paper we have focused on the subset of patients colonised by pOXA-48-carrying enterobacteria within the R-GNOSIS project. More information on patients colonised by pOXA-48-carrying enterobacteria in our hospital, as well as information on other CPE isolated as part of the R-GNOSIS study can be found in previous papers[21,27,45]. Prevalence of colonisation by OXA-48-carrying enterobacteria among patients from 2014 to 2016 was 1.13% (105/9,275 patients). pOXA-48-carrying enterobacteria were the most frequent CPE in the hospital in this period, with 171 positive isolates (Supplementary Table 1). To better characterise pOXA-48 diversity and dissemination, we included in the within-patient pOXA-48 transfer analysis all the pOXA-48-carrying enterobacteria isolated from patients in our hospital since it was first reported in 2012. Specifically, we included 79 additional pOXA-48-carrying enterobacteria not included in the R-GNOSIS project (Supplementary Table 1).

Bacterial characterisation

Samples were initially characterised as previously described, following the RGNOSIS protocol[27]. Briefly, swabs were plated on Chromo ID-ESBL and Chrom-CARB/OXA-48 selective agar media (BioMérieux, France) and bacterial colonies able to grow on these media were identified by MALDI-TOF MS (Bruker Daltonics, Germany). One isolate per species identified on the chromogenic agar was recovered from each sample. OXA-48 production was confirmed with KPC/MBL/OXA-48 Confirm Kit test (Rosco Diagnostica, Denmark). The MicroScan automated system (Beckman Coulter, CA, USA) was used for the antimicrobial susceptibility testing and the results were interpreted according to EUCAST guidelines (EUCAST breakpoint v7.1, www.eucast.org). Furthermore, *bla* $_{\text{OXA-48}}$-like resistance genes were initially identified by multiplex PCR[46]. pOXA-48-like plasmids were detected by PCR assays targeting the IncL replication initiation gene *repC* and *bla* $_{\text{OXA-48}}$-like genes using primers described before[14,47]. Both sets of primers would amplify variants of *bla* $_{\text{OXA-48}}$, but genome sequencing confirmed that *bla* $_{\text{OXA-48}}$ itself was present in all cases. All the isolates with positive results for those PCRs were classified as pOXA-48-carrying enterobacteria and their genomes were subsequently sequenced.

Bacterial culture, DNA extraction, Illumina sequencing and PacBio sequencing

All pOXA-48-carrying enterobacteria isolates (n=250) were grown in Lysogeny broth (LB) medium at 37°C. Genomic DNA of all the strains was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), following manufacturer's instructions. Whole genome sequencing was conducted at the Wellcome Trust Centre for Human Genetics (Oxford, UK), using the Illumina HiSeq4000 platform with 125 base pair (bp) paired-end reads for all isolates. Illumina HiSeq4000 technology, provided a high coverage (>100x) facilitating subsequent genomic and plasmid analyses (Supplementary Table 4). Furthermore, 2 *K. pneumoniae* isolates (K8, carrying the most abundant plasmid variant in the hospital during this period [pOXA48_K8], and K165) and 6 specific pOXA-48-like plasmids (from *K. pneumoniae* K2, K187, K236-1 and K273 and *C. freundii* CF12 and

CF13) were sequenced using the Pacific Biosciences (PacBio) platform (The Norwegian Sequencing Centre; PacBio RSII platform using P5-C3 chemistry). PacBio technology was used in this subset of samples to provide closed chromosomes and plasmids to use as references (GenBank accession numbers for pOXA-48 plasmids: MT441554, MT989343-MT989349).

Assembling and quality control (QC) analysis of sequence data

Trimmomatic v0.33[48] was used to trim the Illumina sequence reads. SPAdes v3.9.0[49] was used to generate *de novo* assemblies from the trimmed Illumina sequence reads with the –cov-cutoff flag set to 'auto', and additional rounds of Pilon were performed following assembly, until no changes were found[50]. We used Unicycler v0.4.8-beta[51] to generate genome assemblies combining Illumina and PacBio sequences. QUAST v4.6.0[52] was used to generate assembly statistics (Supplementary Table 4). All the *de novo* assemblies reached enough quality including total size of 4.5-6.3 Mb (depending on the species). The total number of contigs over 1 kb was lower than 200 and more than 90% of the assembly comprised contigs greater than 1 kb (Supplementary Table 4). Qualimap v2.2.1[53] was used to control the quality of the sequencing data (Supplementary table 4). Prokka v1.5[54] was used to annotate the *de novo* assemblies with predicted genes. 219 of the 250 isolates shared more than 90% of plasmid sequence compared to pOXA-48_K8; 19 isolates presented plasmid sequences covering 30-90% sequence of pOXA-48_K8; and no pOXA-48 sequences (<3%) were recovered from 12 of the 250 isolates characterised as pOXA-48-carrying (Supplementary Table 1). We assume that these isolates lost part or all of the pOXA-48-like plasmid during the culture cycles previous to DNA extraction, because they were positive to the specific pOXA-48 PCRs performed after isolation (see *Bacterial characterisation* section above).

Plasmid annotation was complemented with the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline[55]. In Extended Data 1 we present the sequence analysis of pOXA-48_K8 (from *K. pneumoniae* K8), which represents the most common variant in the collection studied.

Phylogenetic analysis and identification of STs and clustering

First, we used Mash v2.0[56] to determine distances between *K. pneumoniae* and *E. coli* genomes using the raw sequence Illumina reads, to confirm that all genomes belonged to the same species (mash distance <= 0.05). These genomic distances were clustered into trees with mashtree v0.33[57] (Extended Data 5). Second, we analysed the core genome sequences from pOXA-48-carrying *K. pneumoniae* and *E. coli* isolates to study their diversity and to understand the distribution of the different clones across patients. The Snippy tool v2.5 (https://github.com/tseemann/snippy) was used for variant calling and the snippy-core function was used to determine the core genome (the set of common genetic regions present in all the isolates when mapped against the same reference) for each species. The whole genome sequences used as references were *K. pneumoniae* K8 and *E. coli* C728 (Supplementary Table 1). Gubbins[58] was used to detect recombinant regions and to remove them from the Snippy whole genome sequence alignments. We used IQ-TREE to construct a maximum-likelihood tree from the alignments, with the feature of automated detection of

the best evolutionary model[59]. All trees were visualised using the iTOL tool[60]. The outgroups used for tree construction were: for *K. pneumoniae*, *K. quasipneumoniae* K301 (from our collection, Supplementary Table 1) and for *E. coli*: *E. coli* TW10509 (GCF_000190995, selected as outgroup for falling outside the diversity represented by *E. coli* genomes in this work). The outgroups are indicated as a horizontal black line at the root of the tress.

Snippy v2.5 (https://github.com/tseemann/snippy) was also used to establish the core genome sequence of all the pOXA-48 plasmid variants found in the collection. The seven-gene ST of all the isolates was determined using the multilocus sequence typing (MLST) tool (https://github.com/tseemann/mlst).

Transmission mathematical modelling

Our statistical model was designed based on the premises established by Crellen T, *et al*[28]. The objective of our model is to estimate the daily probability of acquisition of a new pOXA-48-carrying enterobacteria by a patient in the hospital. Acquisition can occur through pOXA-48-carrying bacteria acquisition (between-patient transfer), or through pOXA-48 conjugation in the gut microbiota of the patient to a new enterobacteria host (within-patient transfer).

We tracked all the pOXA-48-carrying enterobacteria identified in the hospital during the R-GNOSIS study period (Figure 1). This allows us to estimate and compare the acquisition of the most prevalent pOXA48-carrying species, *K. pneumoniae* and *E. coli*, independently.

When pOXA48-carrying isolates were identified on admission (from the first sample of the patient), these were not considered as nosocomial acquisitions, but the patients were still considered as potential origin of a new transmission (Extended Data 3). Also, re-admitted patients who were previously colonised by pOXA48-carrying enterobacteria were treated as new admissions in terms of colonisation status, since they could have become decolonised between hospital stays.

Each day in the ward, a patient can become colonised by a new pOXA-48-carrying *K. pneumoniae* or *E. coli*. However, as we lacked swabbing results from each day, the timing of new colonisation events with a pOXA-48-carrying clone are interval censored, and the analysis needs to account for this interval censoring[28]. If the probability of becoming colonised on day i for patient j is p_{ij}, the probability of remaining uncolonized is $(1\text{-}p_{ij})$. Therefore, in interval k for patient j consisting of N_{kj} days, the probability of remaining uncolonized is:

$$\prod_{i=1}^{N_{kj}} (1 - pij)$$

And the probability of becoming colonised (v_{kj}) is the complement:

$$v_{kj} = 1 - \prod_{i=1}^{N_{kj}} (1 - p_{ij})$$

The outcome for patient j in interval k (X_{kj}), is either that the patient acquired a new pOXA-48-carrying enterobacteria (X_{kj} =1) or did not (X_{kj} = 0). The likelihood is given by:

$$X_{kj} \sim \text{Bernoulli}\ (v_{kj})$$

The daily probability of becoming colonised (p_{ij}) is related by the logit link function to a linear function of covariates (π_{ij}):

$$\pi_{ij} = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \ldots$$
$$p_{ij} = \exp(\pi_{ij})/(\exp(\pi_{ij}) + 1)$$

Where x represents a vector of predictors (data) and β is a vector of slopes (parameters) that are to be estimated. The β coefficient can be a single parameter, or permitted to vary by ward. The range of values and the prior distributions of the different parameters are described in Supplementary Table 2.

We developed and fitted models to study the probability of acquisition of pOXA-48-carrying *K. pneumoniae* and, separately, *E. coli*. We included the probability of *K. pneumoniae* and *E. coli* transferring the plasmid towards each other in the gut microbiota of colonised patients. To that end, we introduced as covariates: first, the number of other patients already colonised by a pOXA-48-carrying enterobacteria each day, to study the risk of between-patient transfer (β coefficient), and second, if a patient was previously colonised with pOXA-48-carrying *E. coli* or *K. pneumoniae*, to study within patient pOXA-48-transmission (γ coefficient). To study within-patient transmission we took advantage of the weekly swabs recovered from each patient.

We considered five different transmission models to assess transmission of pOXA-48-carrying *K. pneumoniae* and *E. coli*:

1) Where the daily risk of acquiring pOXA-48-carrying *K. pneumoniae* and *E. coli* is constant (intercept only).

2) A constant term plus a between-patient transmission parameter β, where the explanatory variable (n_i) is the number of patients colonised by pOXA-48 enterobacteria in the four wards.

3) As (2) but permitting the transmission parameter β to vary by ward (β_w) and considering the number of patients colonised by a pOXA-48 enterobacteria in each ward (n_{wi}).

4) As (2) but including a γ parameter for the within-patient transmission, and an explanatory variable (x_i), which indicates if a patient had been previously

colonised by a pOXA-48-carrying enterobacteria from a different species (yes, $x_i = 1$; no, $x_i = 0$).

5) As model (4) but permitting the transmission parameter β to vary by ward (β_w) and considering the number of patients colonised by a pOXA-48 enterobacteria in each ward (n_{wi}).

The probability of colonisation for individual j on day i for the respective models is calculated from:

$$\text{Transmission Model 1: } \text{logit}(p_{ij}) = a$$
$$\text{Transmission Model 2: } \text{logit}(p_{ii}) = \alpha + \beta n_i$$
$$\text{Transmission Model 2: } \text{logit}(p_{ij}) = \alpha + \beta_w n_{wi}$$
$$\text{Transmission Model 2: } \text{logit}(p_{ij}) = \alpha + \beta n_i + \gamma x_i$$
$$\text{Transmission Model 2: } \text{logit}(p_{ij}) = \alpha + \beta_w n_{wi} + \gamma x_i$$

We fitted the statistical models using Hamiltonian Markov chain Monte Carlo in Stan (version 2.17.3) within the R environment (v. 3.4.3). Prior distributions were normal distributions using weakly informative priors[28]. Model comparison was performed with widely applicable information criterion (WAIC, Supplementary Table 2). The model that best fits our data is model number 5. We use 95% credible intervals (CrIs) to summarise uncertainty in posterior distributions. Daily probabilities calculated with model 5 are presented in Supplementary Table 3.

Identification of transmission routes among patients

We applied SCOTTI[30], a structured coalescent-based tool for reconstructing transmission, to the dominant *K. pneumoniae* and *E. coli* STs (with more than four isolates: *K. pneumoniae* ST11, ST15, ST307 and *E. coli* ST10), combining epidemiological and genomic data. As input data for SCOTTI, we used the genome alignments (eliminating recombination regions identified using Gubbins[58]), the admission and discharge dates of patients (including information about re-admissions), and the date of isolation of each pOXA-48-carrying bacterium (Supplementary Table 1). During the R-GNOSIS study, patients were sampled periodically so, for many patients, more than one isolate was recovered in a short period of time, which increases the level of resolution of SCOTTI[30]. In fact, for 39 out of the 105 patients colonised with pOXA-48-carrying enterobacteria in the study, more than one pOXA-48-carrying strain was isolated and their genomes sequenced (2 isolates from 21 patients, 3 isolates from 7 patients, 4 isolates from 7 patients, 5 isolates from 2 patients, and 6 isolates from 2 patients, Supplementary Table 1). Crucially, transmission events predicted by SCOTTI for the construction of the transmission network are not necessarily direct transmission events between patients, they can also be indirect transmission events. Indirect transmission may include unobserved and non-sampled intermediate colonised patients or environmental reservoirs in the hospital[30].

Due to the possibility of transmission events between wards, we established a hierarchical analysis. First, we applied SCOTTI to the patients/genomes included in each ward to identify transmission routes within each ward, and second, we analysed the data of the 4

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wards combined to identify additional transmission events between wards (Supplementary Figures 1–4).

To determine the number of SNPs of difference between the clone pairs involved in the putative transmission events predicted by SCOTTI, we used the Snippy tool v2.5 (https://github.com/tseemann/snippy). For this analysis we compared the isolate in the recipient patient with the isolate from the patient at the putative origin of the transmission, which was used as reference.

Identification of within-patient transmission routes of specific plasmid variants

In order to confirm within-patient plasmid transfer we studied specific pOXA-48 variants across the different isolates. The sequences belonging to pOXA-48 plasmid were mapped using the complete sequence of one of the plasmids sequenced by PacBio as reference (from *K. pneumoniae* K8, pOXA-48_K8, Extended Data 1), and the different variants and SNPs were identified using Snippy v2.5 (https://github.com/tseemann/snippy). We first analysed the degree of genetic variation in the plasmid among all the 250 bacterial clones. We compared the pOXA-48 variants sharing a core region of at least 60 kb (>90 % of the whole sequence, n= 219, Supplementary Table 1 and described in Extended Data 1). We investigated cases where a variant of the plasmid carrying a “rare” traceable SNP is present in different clones (from different species). We define “rare” traceable plasmid variants as small subgroups of pOXA-48 plasmids from the collection, with highly similar structure and size compared to the reference pOXA-48_K8 (core plasmid sequence of more than >90% of plasmid sequence), but present one or more specific and unique SNPs (carrying “rare” SNPs, Figure 4). Given the extremely high level of sequence conservation of our collection of pOXA-48 (80% of the 219 plasmids included in this analysis present an exactly identical >60 kb core region), these SNPs can be reliably used to track plasmid transfer between bacterial isolates. The high quality and coverage of our genome sequences allowed us to carry out this analysis and to identify high-confidence SNPs in plasmids (Supplementary Table 1 and 4). Moreover, we confirmed the mutations of the 4 “rare” traceable SNPs used for plasmid tracking using Sanger sequencing.

We found four traceable plasmid variants present in different bacterial species and, in all cases, we found instances of different species carrying the same plasmid variant and isolated from the same patient (Figure 4B). The closed pOXA-48 plasmid from *C. freundii* CF13 was used as a reference to show that there were no detectable differences in the complete sequences of pOXA-48-like plasmids transferred among the isolates found in patient YUE. We screened pOXA-48-like plasmids in the NCBI database (217 plasmids with ≥ 50% identity and query coverage compared to pOXA-48_K8, November 10th, 2020), looking for the specific mutations in these 4 plasmid variants. Mutations in plasmid variants 2, 3 and 4 were unique to our collection. The specific sequence of plasmid variant 1 was observed in other pOXA-48-like plasmids in this database (18 plasmids). However, only 8 out of 250 pOXA-48-like plasmids in our collection presented this specific profile (Figure 4A and Supplementary Table 1), so we consider that it is a reliable genetic signature to track within-patient plasmid transfer within the hospital. For the putative cases of within-patient plasmid transmission, we estimated the probability of these strains being acquired by independent

subsequent transmissions events, assuming a random distribution of plasmid-carrying strains across patients. Analyses were performed using R (Version 3.4.2) (www.R-project.org). Finally, we did not detect conserved pOXA-48 structural variations (insertions/deletions) in our collection that could be used as genetic signatures to identify cases of within-patient plasmid transmission under the requirements explained in the Results section.

Conjugation assays

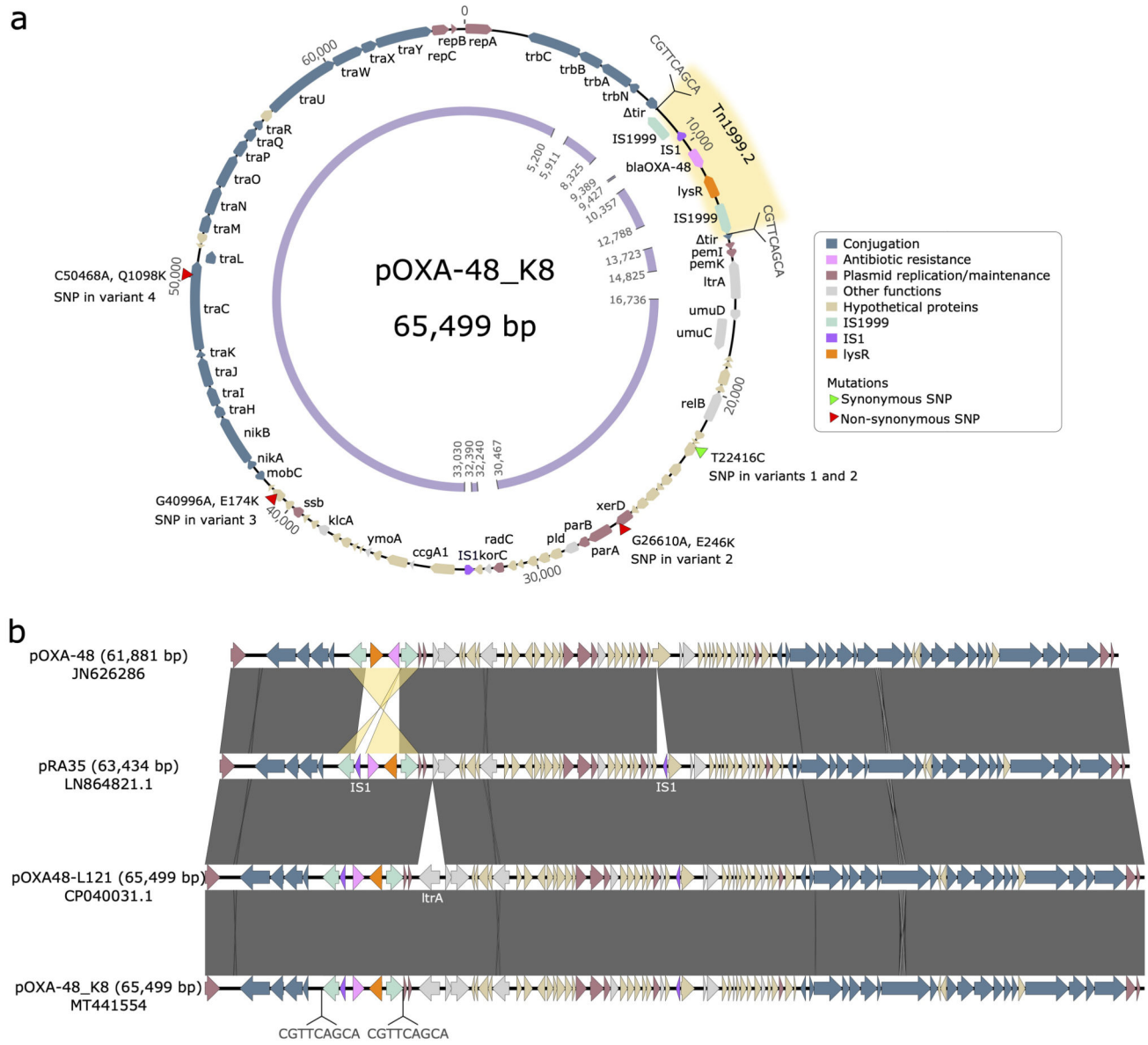
Three different media were used for this experiment: Lysogeny broth (LB) and LB agar (Conda, Spain), MacConkey Broth and agar (Oxoid, England and Difco, Spain, respectively), and M9 minimal salts (Difco, Spain) supplemented with 20 mM Sodium Gluconate (Sigma-Aldrich, MO, USA) and with 0.1% Casein Hydrolysate (Difco, Spain). For simplicity we refer to these medium as Minimal Medium Gluconate (MMG). Additionally, MMG was supplemented with 1.5% European Bacteriological Agar (Conda, Spain) when solid MMG was required. For anaerobic conditions commercial anaerobic atmosphere generation bags were used (GasPack™ EZ, BD, USA).

An initial conjugation round was performed to introduce pOXA-48 plasmids variants into *E. coli* J53[61] (a sodium azide resistant laboratory mutant of *E. coli* K-12). pOXA-48-carrying wild type strains (donors) and *E. coli* J53 (recipient) were streaked from freezer stocks onto solid LB agar medium with antibiotic selection (ertapenem 0.5 μg/ml and sodium azide 100 μg/ml, respectively) and incubated overnight at 37°C. Three donor colonies and one recipient colony were independently inoculated in 2 ml of LB in 15-ml culture tubes and incubated for 1.5 h at 37°C and 225 rpm. After growth, donor and recipient cultures were collected by centrifugation (15 min, 1,500 g) and cells were re-suspended in each tube with 300 μl of sterile NaCl 0.9%. Then, the suspensions were mixed in a 1:1 proportion, spotted onto solid LB medium and incubated at 37°C for 1.5 hours. Transconjugants were selected by streaking the conjugation mix on LB with ertapenem (0.5 μg/ml) and sodium azide (100 μg/ml). The transconjugants were verified by $bla_{\text{OXA-48}}$ gene amplification by PCR as previously described[14] and plasmid variants were verified by Sanger sequencing.

For the isogenic conjugation experiments, the five different *E. coli* J53 carrying pOXA-48 plasmid variants acted as independent donors, and a chloramphenicol resistant version of J53 developed in our lab was used as the recipient strain (J53/pBGC)[35]. 6 colonies of each donor and recipient strains were independently inoculated in 2 ml of LB in 15-ml culture tubes and incubated overnight at 37 °C and 225 rpm. Next day, 50 μl of each culture were used to inoculate 5 ml of LB/MacConkey/MMG in 50-ml culture tubes. After 3.5 hours of incubation at 37°C and 225 r.p.m (no shaking in anaerobic conditions), the pellets were collected by centrifugation (15 min, 1,500 g) and cells were re-suspended in each tube with NaCl 0.9%. 50 μl of donor and recipient suspensions were mixed in a 1:1 proportion and plated on a sterile 0.45 μm nitrocellulose filter (Dorsan, Spain) on LB/MacConkey/MMG agar mediums and incubated at 37°C for 1-2 hours. Simultaneously, serial dilutions of each conjugation mix were plated on agar selecting for donors, recipient and transconjugants as controls (carbenicillin 100 μg/ml, chloramphenicol 50 μg/ml and a combination of both, respectively). After 1 hour of incubation at 37°C, the filter contents were re-suspended in 2 ml of sterile NaCl 0.9%, serially diluted and plated on selective agar for donors, recipient

and transconjugants. Conjugation rates were determined using the end-point method[62,63] (Figure 5), and the frequencies of transconjugants per donor were calculated from the same data (Extended Data 8).

Extended Data



Extended Data Fig. 1. Plasmid pOXA-48

(a) Schematic representation of plasmid pOXA-48_K8 (GenBank Accession Number MT441554). The reading frames for genes are shown as arrows, with the direction of transcription indicated by the arrowhead. Arrow colours indicate the functional classification of the gene (see legend). The *bla* OXA-48 gene is indicated in pink. The IS*1999.2* is highlighted in yellow. The direct repeats flanking the Tn*1999.2* are indicated

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(CGTTCAGCA). pOXA-48_K8 carries a group II intron (indicated by *ltrA*) downstream *pemK*. This intron has also been detected in other pOXA-48-like plasmids, like pOXA48-L121[1]. SNPs positions detected in the pOXA-48 variants that were used to track within-patient transfer are indicated with triangles (green for synonymous SNPs and red for non-synonym SNPs, using pOXA48_K8 as reference). The position of the mutated nucleotides in the plasmid is indicated, as well as the aminoacidic changes for non-synonymous mutations in the proteins. The inner purple circle shows the core region of pOXA-48 variants that shared at least 60 kb (>90 % of the whole sequence, 219 out of 250, Supplementary Table 1), which were used to investigate within-patient plasmid transfer (Figure 4). The numbers in the inner circle indicate the last/first nucleotide position of those regions included in the core sequence, using pOXA48_K8 as the reference. Most of the plasmids were sequenced with short-read technology, therefore the ISs are not included in the core-genome because they are repeated in the plasmid and do not map properly. The group II intron (indicated by *ltrA*) is not part of the core either because it is a highly promiscuous genetic element (see Supplementary Table 1 for details about presence/absence of this intron in the pOXA_48-like plasmids in our collection). Other genes excluded from the core regions are *radC*, *korC* and *trbB*. (b) Comparison of pOXA-48-like plasmid sequences. Compared to the original pOXA-48[2], pOXA-48_K8 carries the central part of Tn*1999* inverted, with a copy of IS*1* inserted in IS*1999*, upstream of $bla_{\text{OXA-48}}$ (named Tn*1999.2*[3]). Moreover, pOXA-48_K8 carries an additional copy of IS*1* close to the *korC* gene, also found in plasmid pRA35[4].

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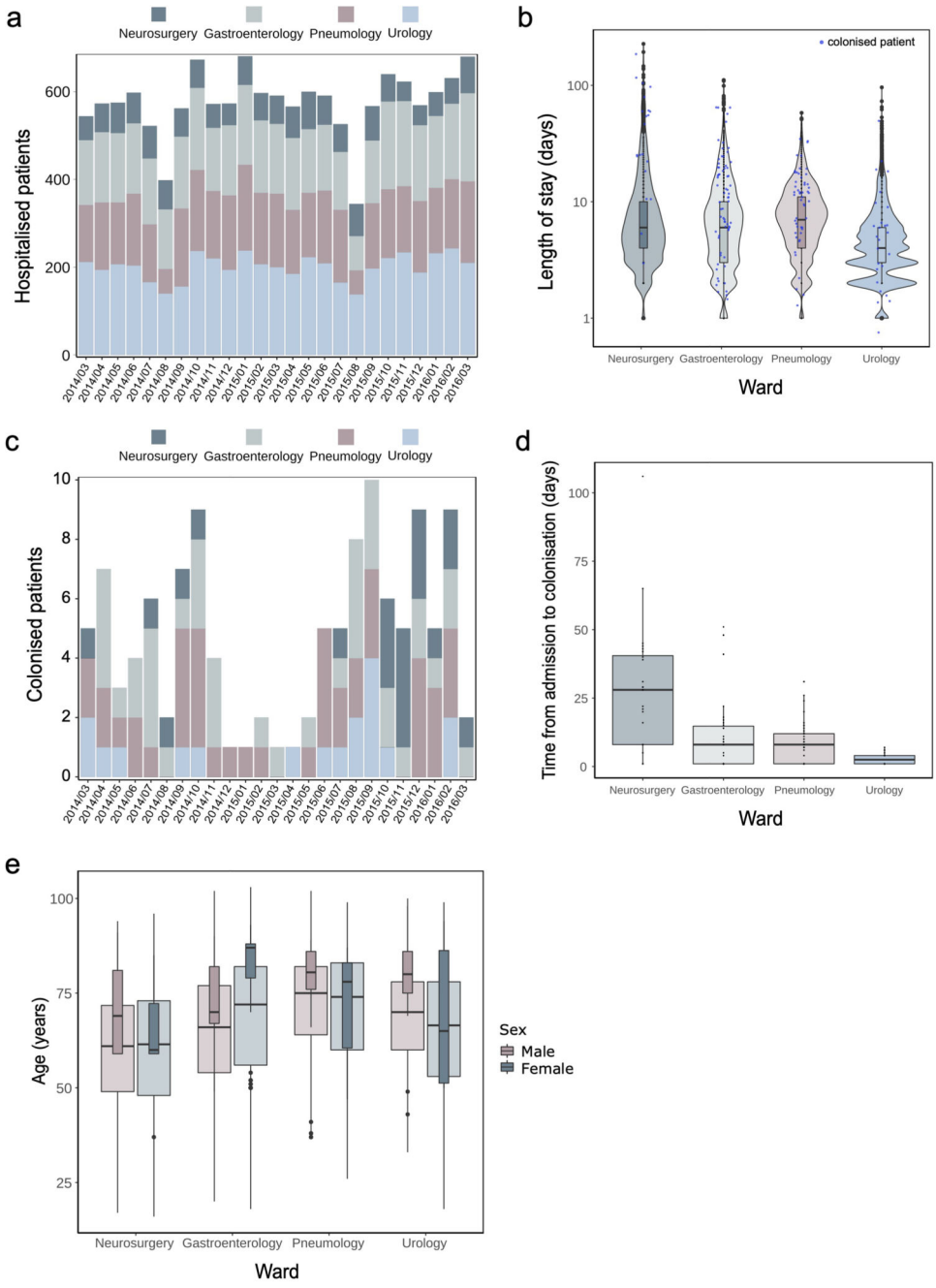

Extended Data Fig. 2. Patients sampled and patients colonised by pOXA-48-carrying enterobacteria over time

(a) Patients sampled during the R-GNOSIS study. Number of hospitalised patients in each ward over the 25-month study period, divided per month. (b) Length of stay of patients per ward. Violin plots represent the distribution of length of stay per ward of all the patients admitted during the study period (neurosurgery, n= 1,068; gastroenterology, n= 2,591; pneumology, n= 2,559; urology, n= 3,483). Blue dots represent the length of stay of those patients colonised by pOXA-48-carrying enterobacteria (neurosurgery, n= 16; gastroenterology, n= 33; pneumology, n= 38; urology, n= 18). Horizontal lines inside boxes

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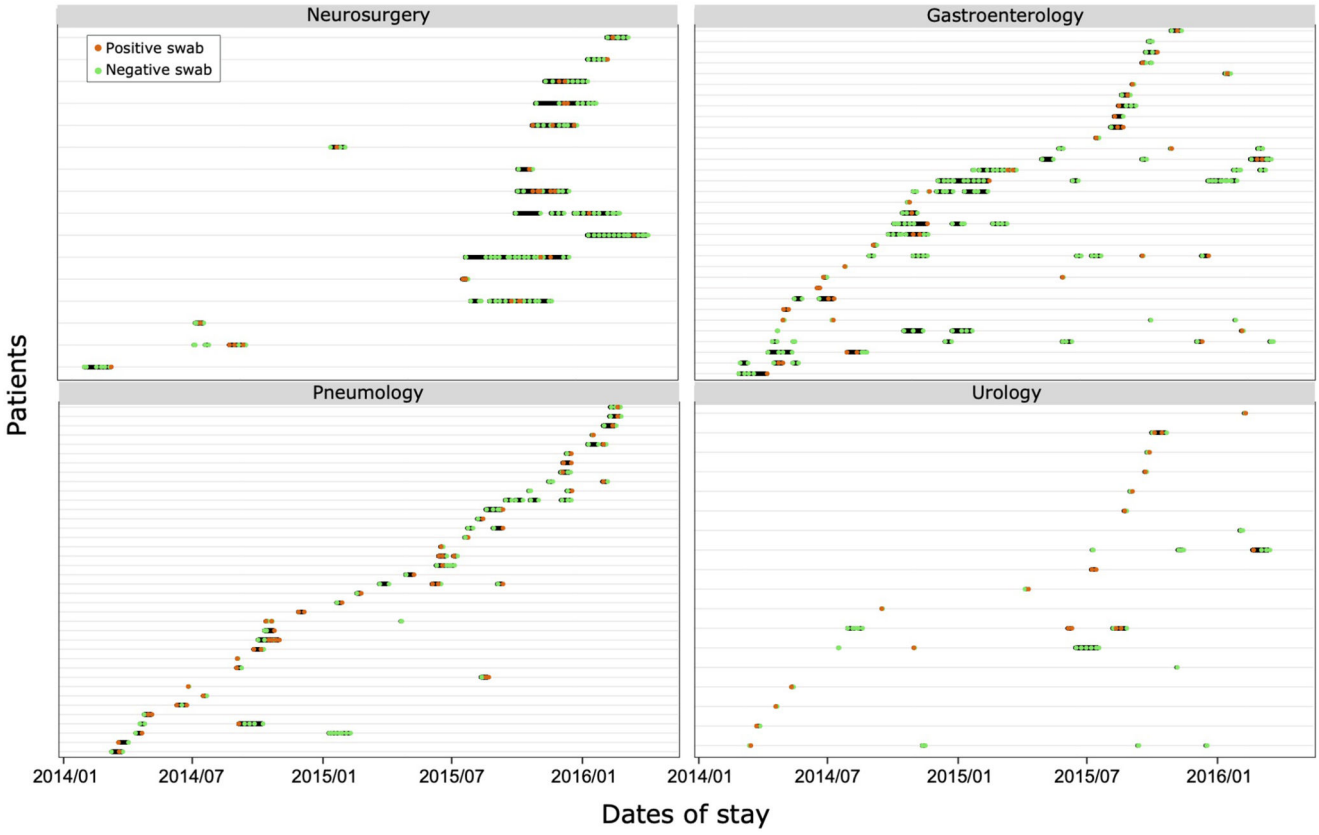
indicate median values, the upper and lower hinges correspond to the 25th and 75th percentiles, and whiskers extend to observations within 1.5 times the interquartile range. (c) Patients colonised by a pOXA-48-carrying enterobacteria during the study. Number of colonised patients in each ward, over the 25-month study period, divided per month (including every colonised patient, not only newly colonised patients). (d) Distribution of days from admission to colonisation in each colonised patient divided per ward, represented as a boxplot (neurosurgery, n= 16; gastroenterology, n= 33; pneumology, n= 38; urology, n= 18). Horizontal lines inside boxes indicate median values, the upper and lower hinges correspond to the 25th and 75th percentiles, and whiskers extend to observations within 1.5 times the interquartile range. (e) Age of patients admitted in the different wards under study represented as boxplots and divided by sex. Lighter boxes represent the ages of all the patients admitted to the wards (neurosurgery: female= 498, male= 570; gastroenterology: female= 1,090, male= 1,501; pneumology: female= 1,131, male= 1,428; urology: female= 912, male= 2,571). Narrow darker boxes represent those patients colonised by a pOXA-48-carrying enterobacteria (neurosurgery: female= 7, male= 9; gastroenterology: female= 13, male= 20; pneumology: female= 18, male= 20; urology: female= 4, male= 14). Horizontal lines inside boxes indicate median values, the upper and lower hinges correspond to the 25th and 75th percentiles, and whiskers extend to observations within 1.5 times the interquartile range.



Extended Data Fig. 3. Swab outcomes from patients colonised by pOXA-48-carrying enterobacteria

Outcome of the samples recovered from the 105 patients colonised by a pOXA-48-carrying enterobacteria in the R-GNOSIS study. The figure shows the distribution of patients colonised by pOXA-48-carrying enterobacteria in the four wards under study over the 25-month study period. Each row represents a patient, and the black segments represent the length of hospital stay (from admission to discharge). Coloured points within the segments indicate the sampling dates. Red points indicate those samples from which a pOXA-48-carrying isolate was recovered (positive swabs), and green points indicate those samples from which no pOXA-48-carrying isolate was recovered (negative swabs). 29 of the 105 colonised patients were admitted in multiple occasions during the study period. The percentage of positive result for a pOXA-48-carrying enterobacteria in the first sample taken after a new admission of these 105 patients was 24.85% (41/165).

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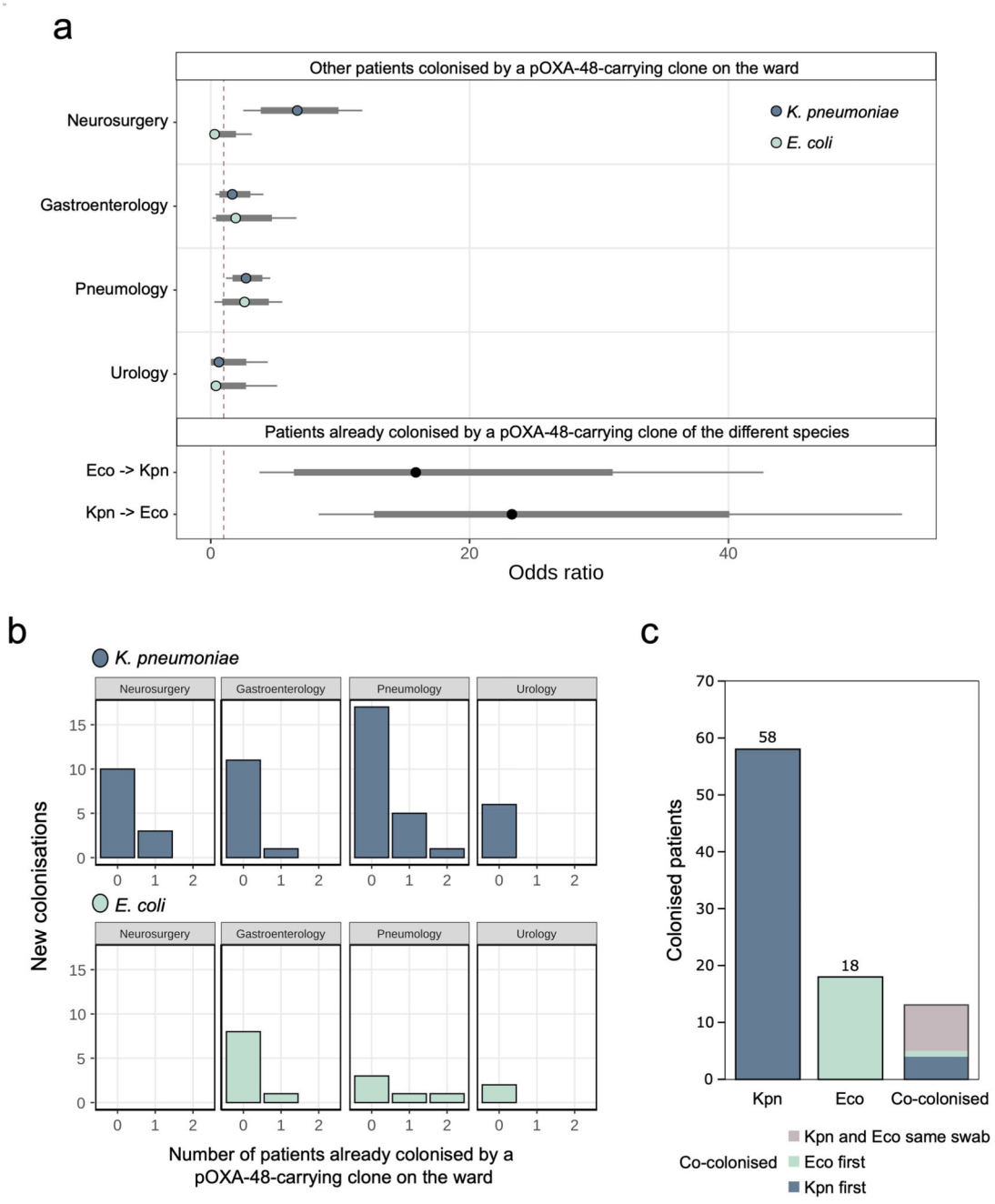
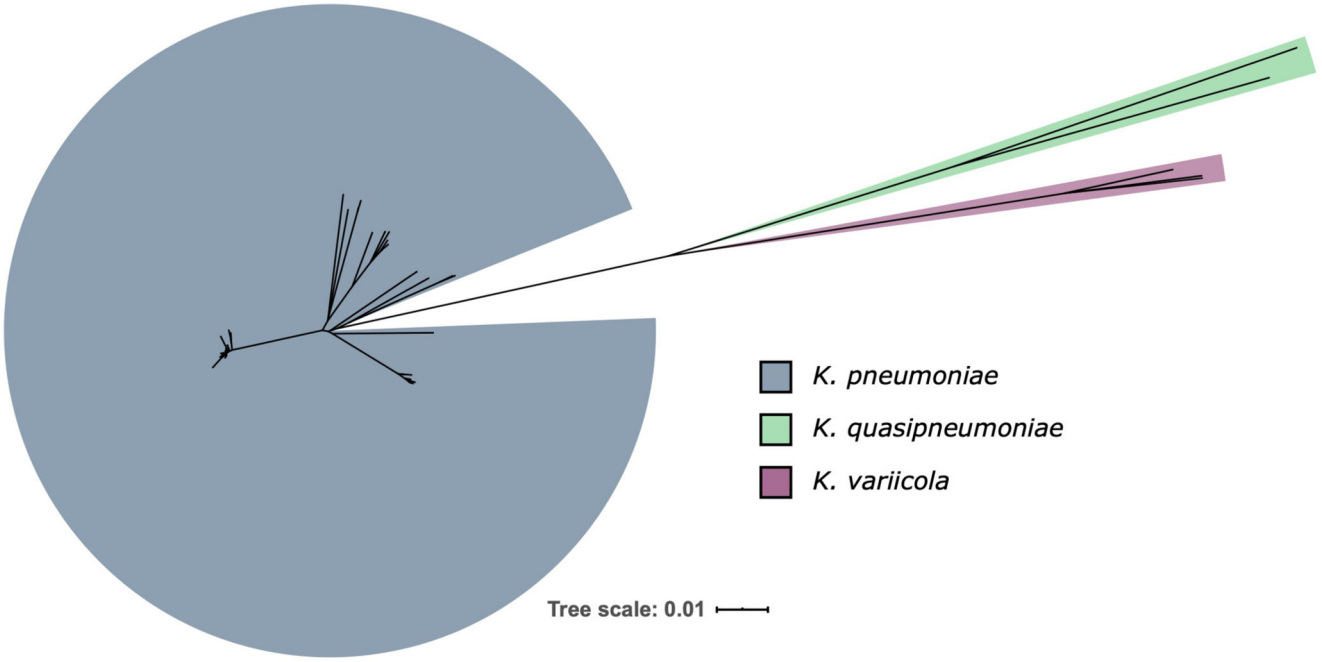


Extended Data Fig. 4. Acquisition of pOXA-48-carrying enterobacteria by hospitalised patients

(a) Posterior distribution of odds ratio for the daily risk of colonisation with a pOXA-48-carrying *K. pneumoniae* or *E. coli* (see methods for details). Two covariates were included. The first is the presence of other patients colonised by a pOXA-48-carrying clone on the ward, (upper part, stratified by ward). If between-patient transfer of the plasmid is important, we expect to see a positive association (odds ratio >1) with the daily probability of acquiring a pOXA-48 clone. Second, pre-existing colonisation with a pOXA-48 clone of a different species (lower part). This covariate measures how being previously colonised by a

pOXA-48-carrying *E. coli* is associated with the daily probability of becoming colonized with a pOXA-48-carrying *K. pneumoniae* clone (Eco -> Kpn) and *vice versa* (Kpn -> Eco). We expect to see a positive association if within-patient transfer of pOXA-48 between different bacterial clones is important. Points represent posterior medians; thick grey lines represent the 80% credible interval (CrI) and thinner black lines represent the 95% CrI. (b) Number of previously uncolonised patients becoming colonised by a pOXA-48-carrying *K. pneumoniae* (top row) or *E. coli* (bottom row) as a function of the number of patients on the ward already colonised by a pOXA-48-carrying clone. (c) Number of R-GNOSIS study patients colonised by pOXA-48-carrying *K. pneumoniae* (Kpn) or *E. coli* (Eco) clones or both (co-colonised). For co-colonised patients, the colour code indicates whether *K. pneumoniae* or *E. coli* were isolated first or whether both species were simultaneously isolated from the same swab.



Extended Data Fig. 5. Phylogenetic analysis of isolates preliminary identified as *K. pneumoniae*

Unrooted phylogeny of 108 whole genome assemblies from the clones phenotypically identified as *K. pneumoniae*. Branch length gives the mash distance (a measure of k-mer similarity) between assemblies. Note the three distinct clusters, which are considered to be separate species (distance > 0.05): *K. pneumoniae* (n= 103), *K. quasipneumoniae* (n= 2) and *K. variicola* (n= 3).

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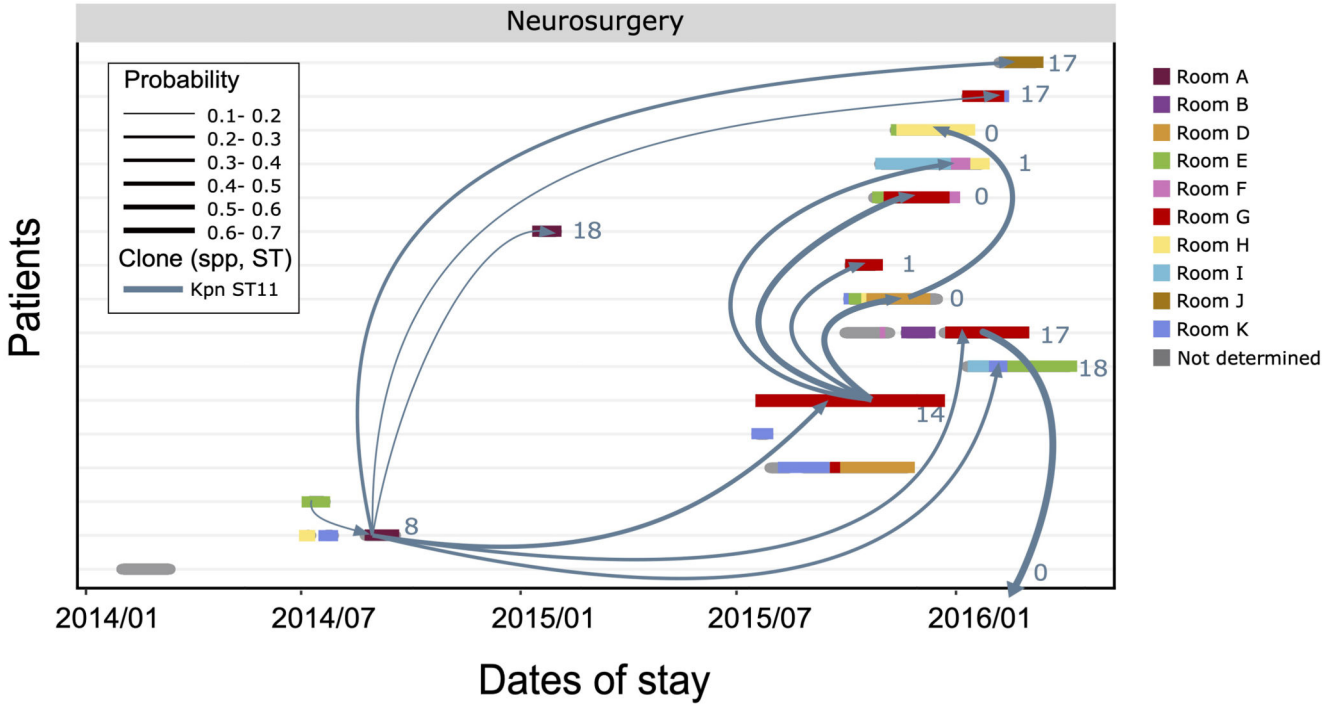

Extended Data Fig. 6. Spatiotemporal distribution of patients colonised by *K. pneumoniae* ST11 in the neurosurgery ward

Distribution of patients colonised by pOXA-48-carrying *K. pneumoniae* ST11 in the neurosurgery ward. Each row represents a patient and the colour segments represent the length of stay in the hospital (from admission to discharge). The colours of the segments represent the different rooms within the ward (see legend). Arrows represent transmission events predicted by SCOTTI. Line thickness represents the probability of the transmission predicted by SCOTTI. The number to the right of the arrowhead indicates the number of SNPs between the complete core genomes of the pair of clones involved in the putative transmission event. Note that 6 out of 16 patients shared room G in overlapping stays.

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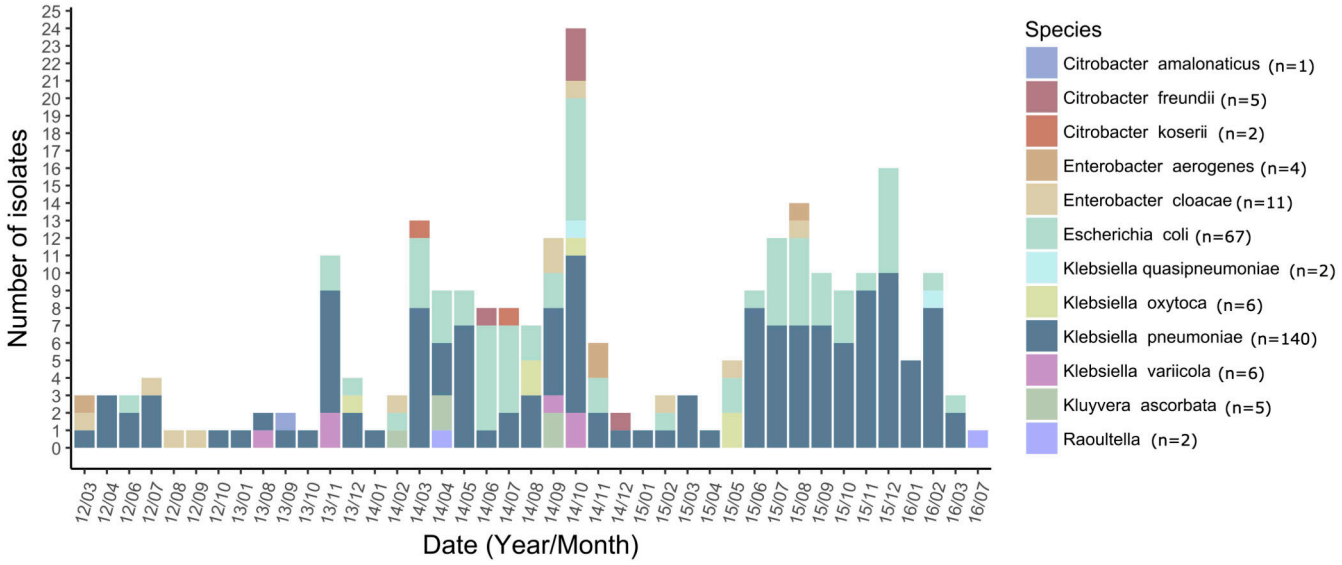
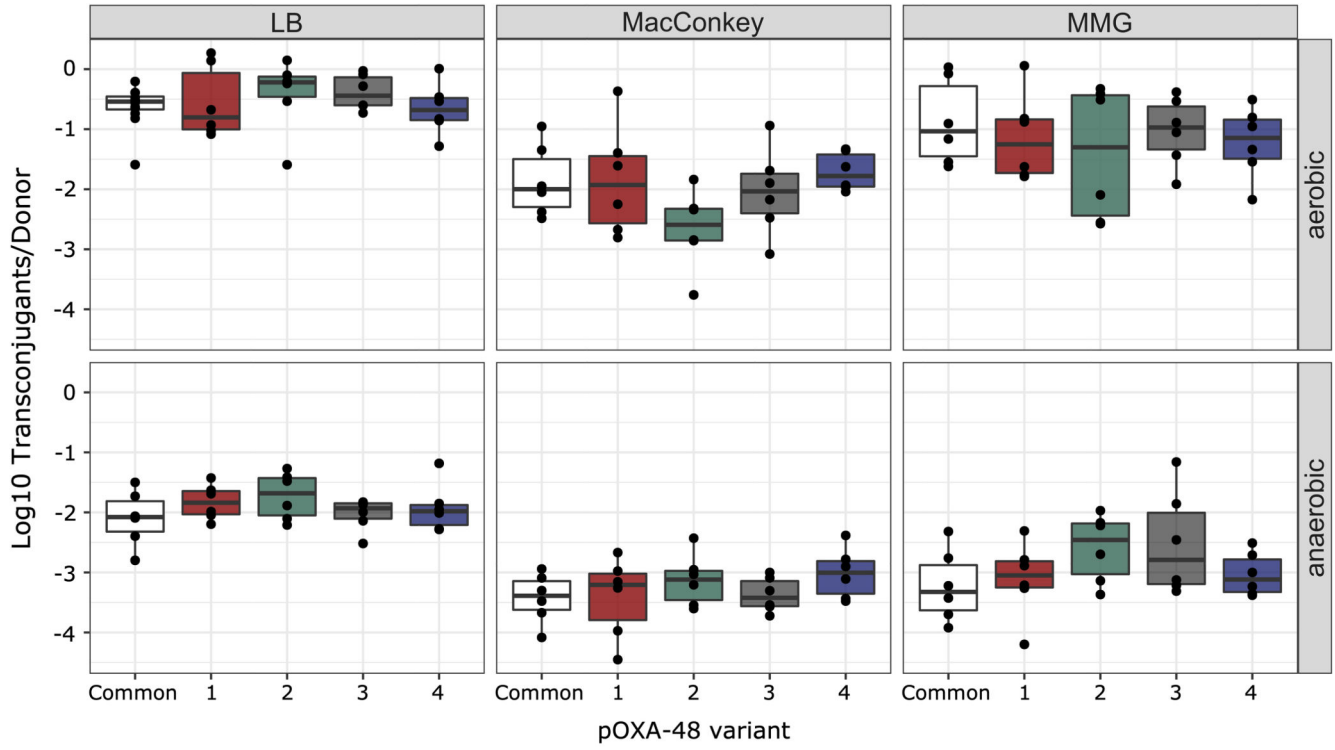


Extended Data Fig. 7. pOXA-48-carrying enterobacteria analysed in this study

Representation of the 250 pOXA-48-carrying clones isolated in the hospital from the first description till the end of the study period. The colour code indicates the species of the pOXA-48-carrying enterobacteria as indicated in the legend.



Extended Data Fig. 8. Conjugation frequency of plasmid pOXA-48

Conjugation frequencies (transconjugants per donor) of the most common pOXA-48 variant in the hospital and the four variants with SNPs in the core region used to track within-patient

plasmid transfer (n= 6 biological replicates). Conjugation experiments were performed on three different agar media: LB, MacConkey and M9 minimal medium supplemented with gluconate (MMG), and both in aerobic and anaerobic conditions. Plasmid variant numbers correspond to those indicated in Figure 4. Horizontal lines inside boxes indicate median values, the upper and lower hinges correspond to the 25th and 75th percentiles, and whiskers extend to observations within 1.5 times the interquartile range. The data presented here is the same as in Figure 5, but represented as conjugation frequency instead of rate. Source data for this figure is available as supplementary information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Data availability

The sequences generated and analysed during the current study are available in the Sequence Read Archive (SRA) repository, BioProject ID: PRJNA626430, http://www.ncbi.nlm.nih.gov/bioproject/626430. The closed, annotated pOXA-48 plasmids generated in this study are available under GenBank accession numbers: MT441554, MT989343-MT989349.

Source data for Figure 5 and Extended Data 8 are available as supplementary information.

Code availability

The code generated during the current study is available in GitHub, http://www.github.com/leonsampedro/transmission_stan_code.

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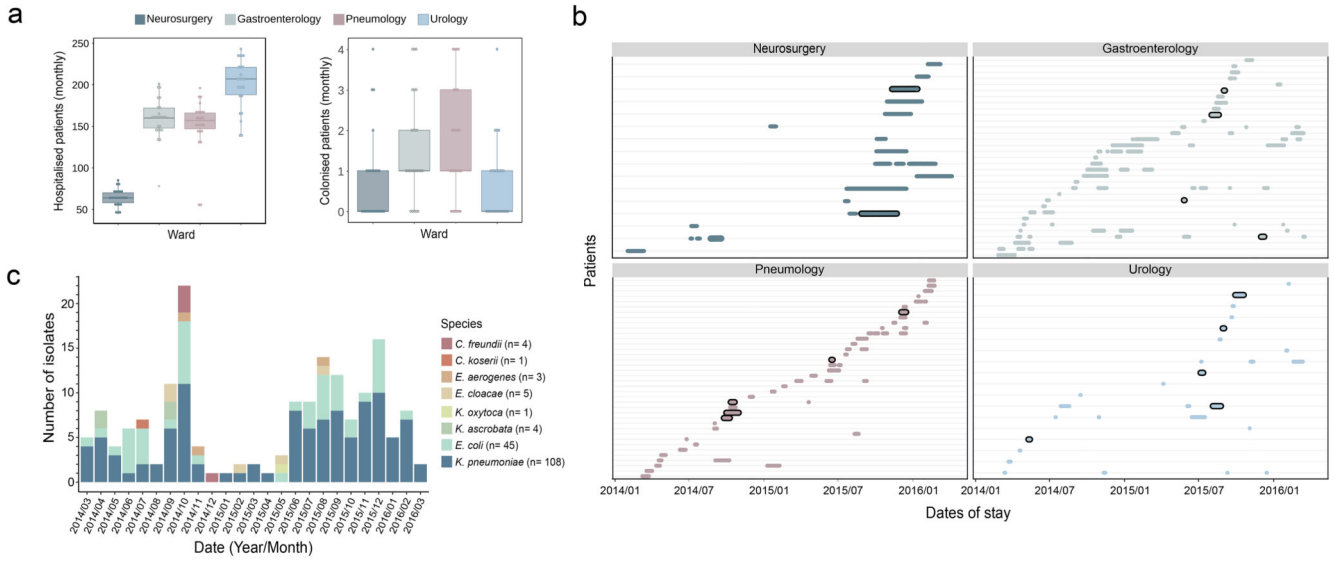

Figure 1. Study population, colonised patients, and pOXA-48-carrying enterobacteria.

(a) Patients hospitalised and patients colonised by pOXA-48-carrying enterobacteria per month during the R-GNOSIS study (n= 25 months). The left panel shows the distribution of hospitalised patients per ward by month as a boxplot. Horizontal lines inside boxes indicate median values, the upper and lower hinges correspond to the 25th and 75th percentiles, and whiskers extend to observations within 1.5 times the interquartile range. The right panel shows the distribution of all colonised patients per ward by month as a boxplot. (b) Distribution of patients colonised by pOXA-48-carrying enterobacteria in the four wards under study over the 25-month study period. Each row represents a patient, and the coloured segments represent the length of hospital stay (from admission to discharge). Black outlining of colour segments indicates patient co-colonisation with more than one pOXA-48-carrying species. (c) Enterobacteria isolates carrying pOXA-48 recovered from the patients during the 25 months of the study. The species of the pOXA-48-carrying isolates are colour-coded as indicated in the legend.

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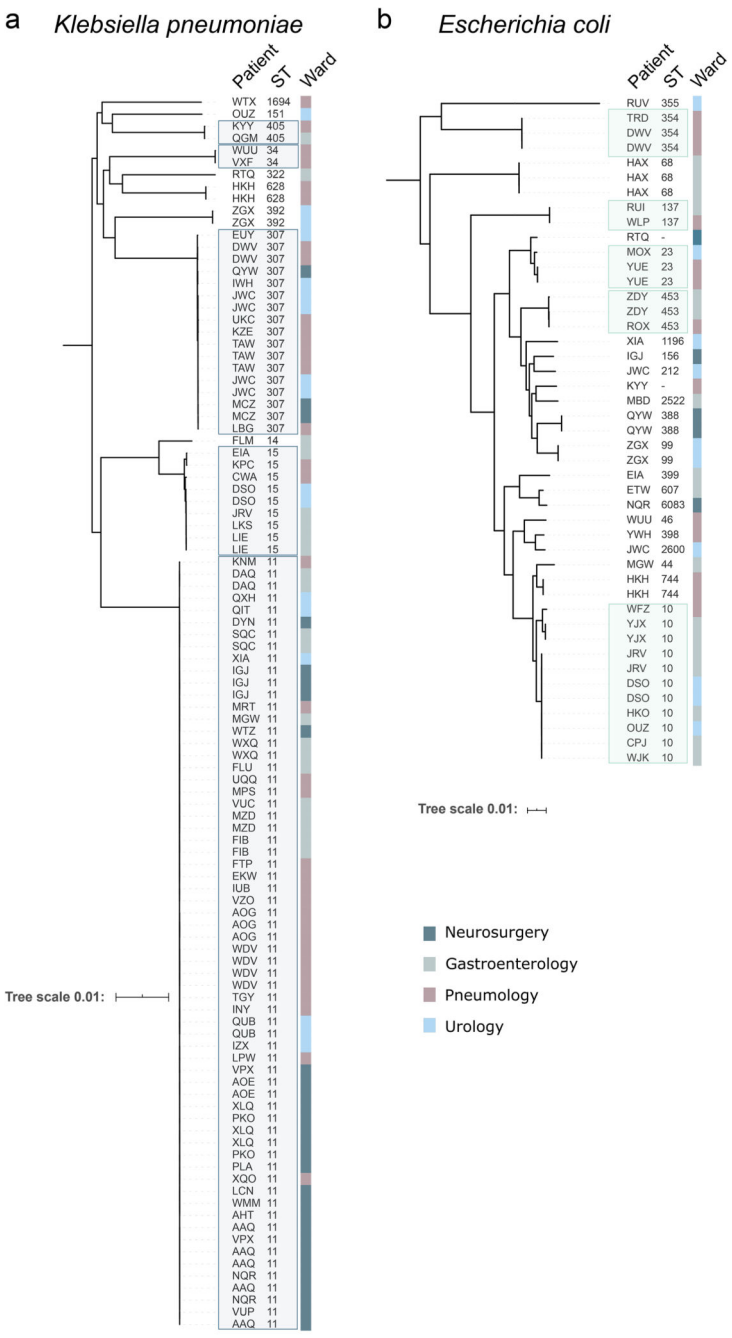

Figure 2. Phylogenetic analysis of pOXA-48-carrying *K. pneumoniae* and *E. coli*.

Genetic relationships among (a) *K. pneumoniae* (n= 103) and (b) *E. coli* (n= 45) isolates carrying pOXA-48 and recovered during the R-GNOSIS study. Tree construction is based on polymorphisms in the core genome (scale: single nucleotide polymorphism [SNPs]/site). The columns to the right of the tree indicate patient code, isolate sequence type (ST), and the ward where the isolate was recovered (colour code in legend). Boxes with colour shading indicate recovery of isolates of the same sequence type (ST) from multiple patients in the hospital.

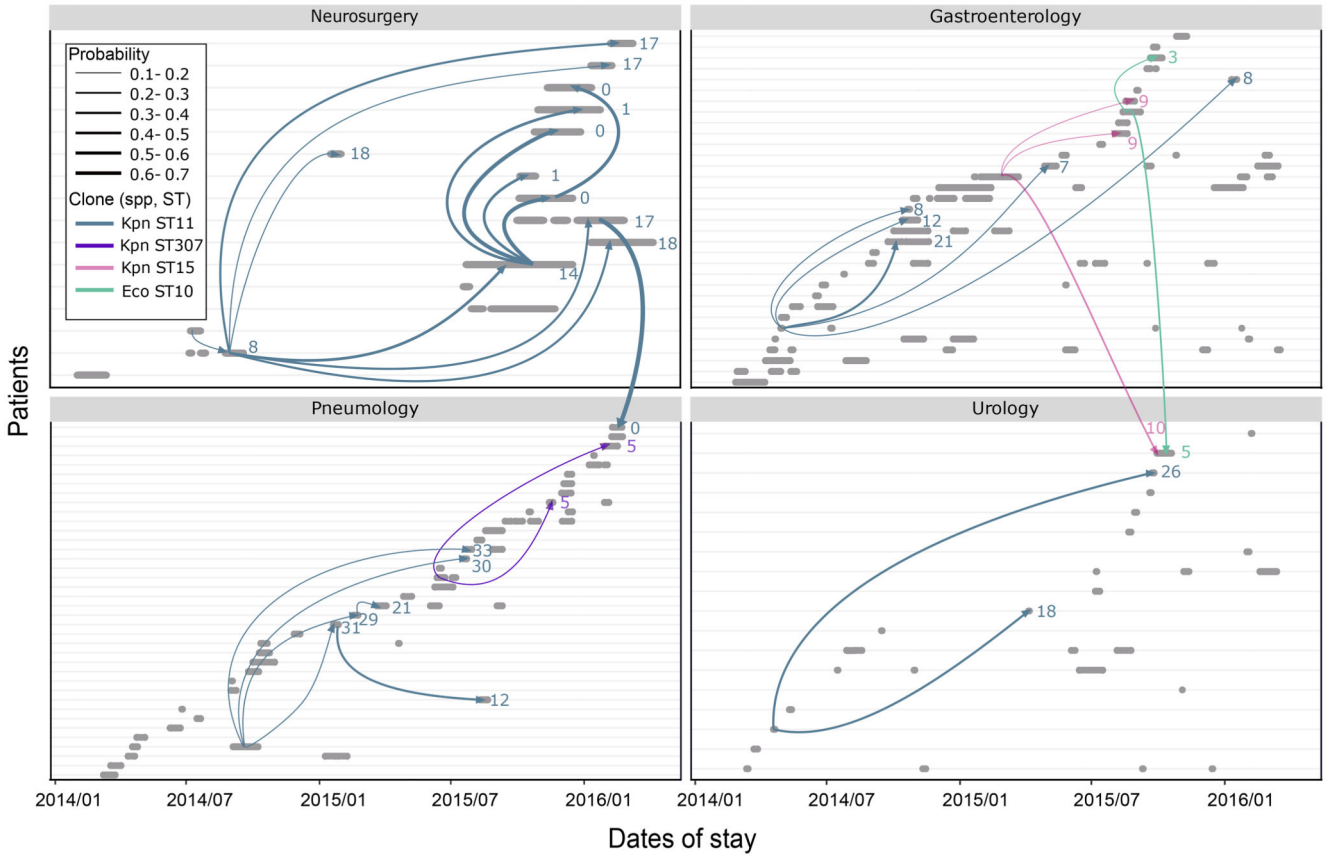

Figure 3. SCOTTI reconstruction of between-patient transfer of pOXA-48-carrying enterobacteria.

The charts represent SCOTTI-attributed between-patient transfer events involving pOXA-48-carrying enterobacteria clones in the hospital, with individual panels representing the distribution of patients colonized by pOXA-48-carrying enterobacteria on the different wards. Each row represents an individual patient, and the grey segments represent the length of stay (from admission to discharge). Coloured arrows represent transmission events predicted by SCOTTI with the highest posterior probability (see Supplementary Figures 1–4). Line colour indicates the clone responsible for the transmission event, and line thickness represents the probability of the SCOTTI-attributed transmission, as indicated in the legend: Kpn, *K. pneumonia*; Eco, *E. coli*; ST, sequence type. Numbers to the right of arrowheads indicate the number of SNPs differentiating the complete genomes of the clone pair involved in the putative transmission event. Note that the transmission events predicted by SCOTTI are not necessarily direct transmission events between patients, they can also be indirect transmission events including unobserved and non-sampled intermediate colonised patients or environmental reservoirs in the hospital.

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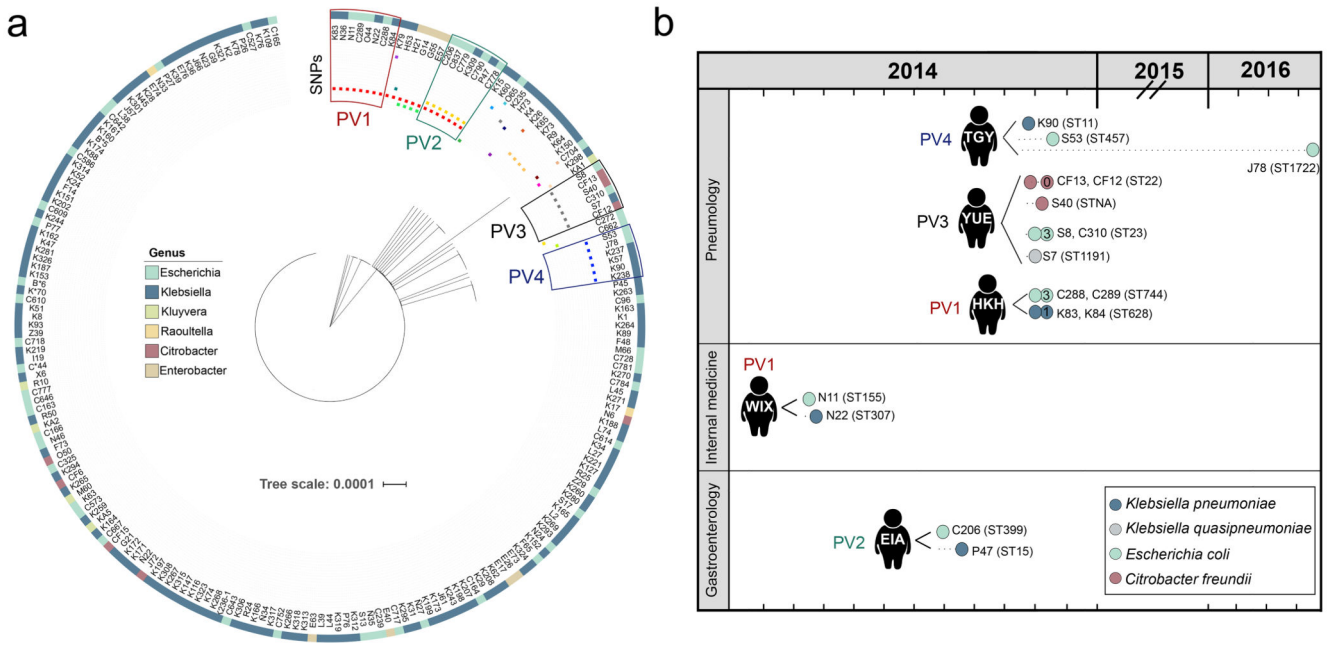

Figure 4. Within-patient pOXA-48 transfer.

(a) Dendrogram constructed from the 21 polymorphisms present in the core region of pOXA-48. The outermost circle indicates the genus of plasmid-carrying isolates according to the colour code in the legend, the second circle indicates the isolate names, and the remaining circles indicate the presence of each plasmid SNP. Coloured boxes indicate the four pOXA-48 plasmid variants (PV1-4) carrying 'rare' SNPs present in clones of different species and used as genetic fingerprints. (b) Representation of patients colonized by clones carrying rare (traceable) plasmid variants. Patients are labelled with their corresponding three letter patient code. Circles represent clones isolated from the patient, with the fill colour indicating the bacterial species (see legend), and the position of the circle indicating the date of isolation. The name and sequence type (ST) of each isolate is indicated. Circles in the same row indicate different isolates of the same clone; the number inside the second circle indicates the number of SNPs accumulated in the complete bacterial genome relative to the first isolation. All isolates within each patient carried the same traceable plasmid mutation, which is indicated in the figure. Note that in patient WIX, the pOXA-48 plasmid carried the group II intron *ltrA* in isolate N22 but not in N11. Interestingly, in N11 *ltrA* is located on a different plasmid, suggesting that *ltrA* can easily excise/insert between genomic locations, precluding its use as a stable plasmid genetic signature (Supplementary Table 1).

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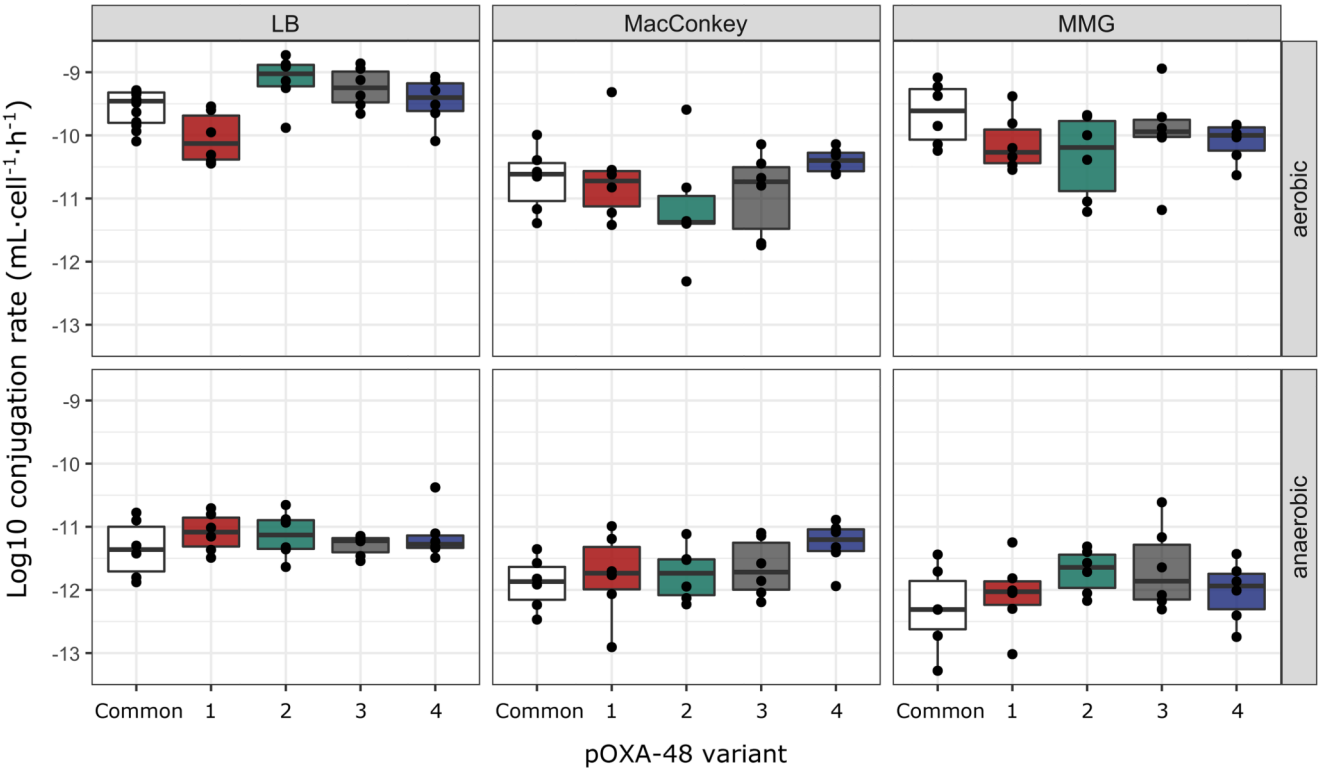

Figure 5. pOXA-48 conjugation rate.

Conjugation rates of the most common pOXA-48 variant in the hospital (pOXA-48_K8) and the four core-region SNP variants used to track within-patient plasmid transfer (6 biological replicates). Conjugation experiments were performed on three different agar media: LB, MacConkey and M9 minimal medium supplemented with gluconate (MMG), and both in aerobic and anaerobic conditions. Plasmid variant numbers correspond to those indicated in Figure 4. Horizontal lines inside boxes indicate median values, the upper and lower hinges correspond to the 25th and 75th percentiles, and whiskers extend to observations within 1.5 times the interquartile range. Source data for this figure is available as supplementary information.

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