A MULTIDISCIPLINARY APPROACH AGAINST ANTIMICROBIAL RESISTANCE: INTEGRATING IMMUNITY, MICROBIOLOGICAL FACTORS AND CLINICAL CARE TO FACE THE THREAT OF KLEBSIELLA PNEUMONIAE-CARBAPENEMASE (KPC)-PRODUCING KLEBSIELLA PNEUMONIAE

Doctoral Thesis in Translational Medicine

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Summary

INTRODUCTION

ANTIMICROBIAL RESISTANCE

Global epidemiology

The global spreading of antimicrobial resistance (AMR) is a complex issue that affects not only the fields of infectious diseases and microbiology, but has potentially dramatic health and economic consequences. In 2019, the *World Health Organization* (WHO) declared that AMR is one of the top 10 global public health threats facing humanity, causing at least 700.000 deaths each year (World Health Organization 2020). In 2015, in the European Union (EU) were estimated 671.689 infections by multidrug-resistant organisms (MDROs), with 33.110 attributable deaths and 874.541 disability-adjusted life-years (DALYs) (Cassini 2019). Of note, in that report the authors concluded that the burden of AMR had steadily increased since 2007, affected particularly infants and people aged 65 years or older, and was the highest in Italy and Greece. It has been estimated that by 2050, 10 million lives a year and a cumulative 100 trillion USD of economic output will be at risk due to infections by MDROs if we do not find now proactive solutions to slow down the rise of AMR (Review on Antimicrobial Resistance 2016).

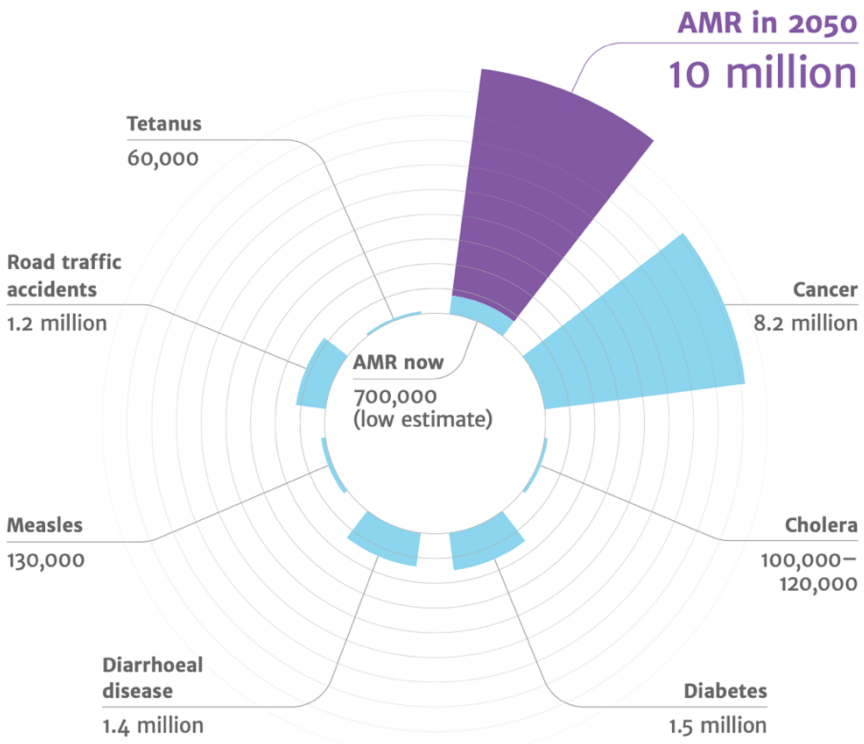


Figure 1. Deaths attributable to AMR every year. Comparison between 2016 and 2050 and between AMR and other causes of deaths (Review on Antimicrobial Resistance 2016)

Carbapenem resistance and KPC-*Kp*

Among all the MDROs, carbapenem-resistant *Enterobacterales* (CRE) are one of the most feared. Carbapenems represent last-line antibiotics against gram negative bacteria, and resistance against this class of drugs confers higher mortality compared to infections caused by susceptible stains (Review on Antimicrobial Resistance 2016). In the 2019, the US *Centers of Disease Control and Prevention* (CDC) defined CRE as "urgent threats" at the highest level of priority (Centers for Diseases Control and Prevention 2019). In the study published by Cassini et al., among the 16 AMR-bacterium combinations analyzed between 2007 and 2015 in the EU, the burden of carbapenem-resistant *Klebsiella pneumoniae* (*Kp*) increased the most (by 6,16 times) in terms of number of infections and number of deaths, with the median number of attributable deaths rising from 341 to 2094 (Cassini 2019). According to the most recent annual epidemiological report on AMR in the EU by the *European Centers of Disease Control and Prevention* (ECDC), for most gram-negative bacteria – antimicrobial group combinations resistances remained stable at high levels over the last 5 years (European Centers for Diseases Control and Prevention 2020). Several European countries reported carbapenem resistance percentages above 10% in *K. pneumoniae*, with an EU mean of 7,9% but wide variation depending on geographical region (from 0% in Estonia and Latvia to 58% in Greece). In Italy, almost a third (28,5%) of invasive isolates of *K. pneumoniae* were reported resistant to carbapenems (European Centers for Diseases Control and Prevention 2020).

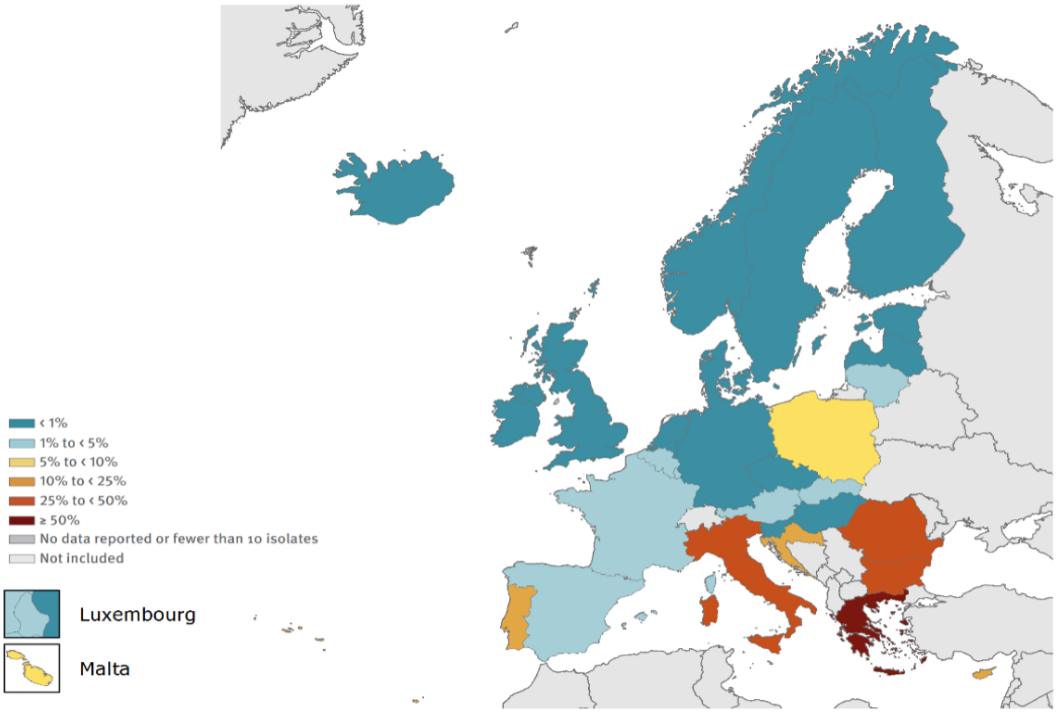


Figure 2. Percentage of invasive isolates of Klebsiella pneumoniae resistant to carbapenems in EU, by country, 2019 (European Centers for Diseases Control and Prevention 2020)

The widespread diffusion of carbapenem resistance in *Enterobacterales* is caused by plasmid-mediated transmission of resistance genes that encode enzymes able to hydrolyze carbapenems (*i.e.,* carbapenemases) (Cui 2019). Based on amino acid homology in the Ambler classification, carbapenemases can be divided into classes A, B, and D. Class A (*i.e., Klebsiella pneumoniae* carbapenemase – KPC) and class D (OXA-type carbapenemases) enzymes share a serine residue in the active site and therefore are called serine β-lactamases. Class B carbapenemases (*i.e.,* New Delhi metallo-β-lactamase - NDM or Verona integron-encoded metallo-β-lactamase - VIM) require the presence of zinc for activity and hence are referred to as metallo-β-lactamases (Bush 2010) (Ruppé 2015)

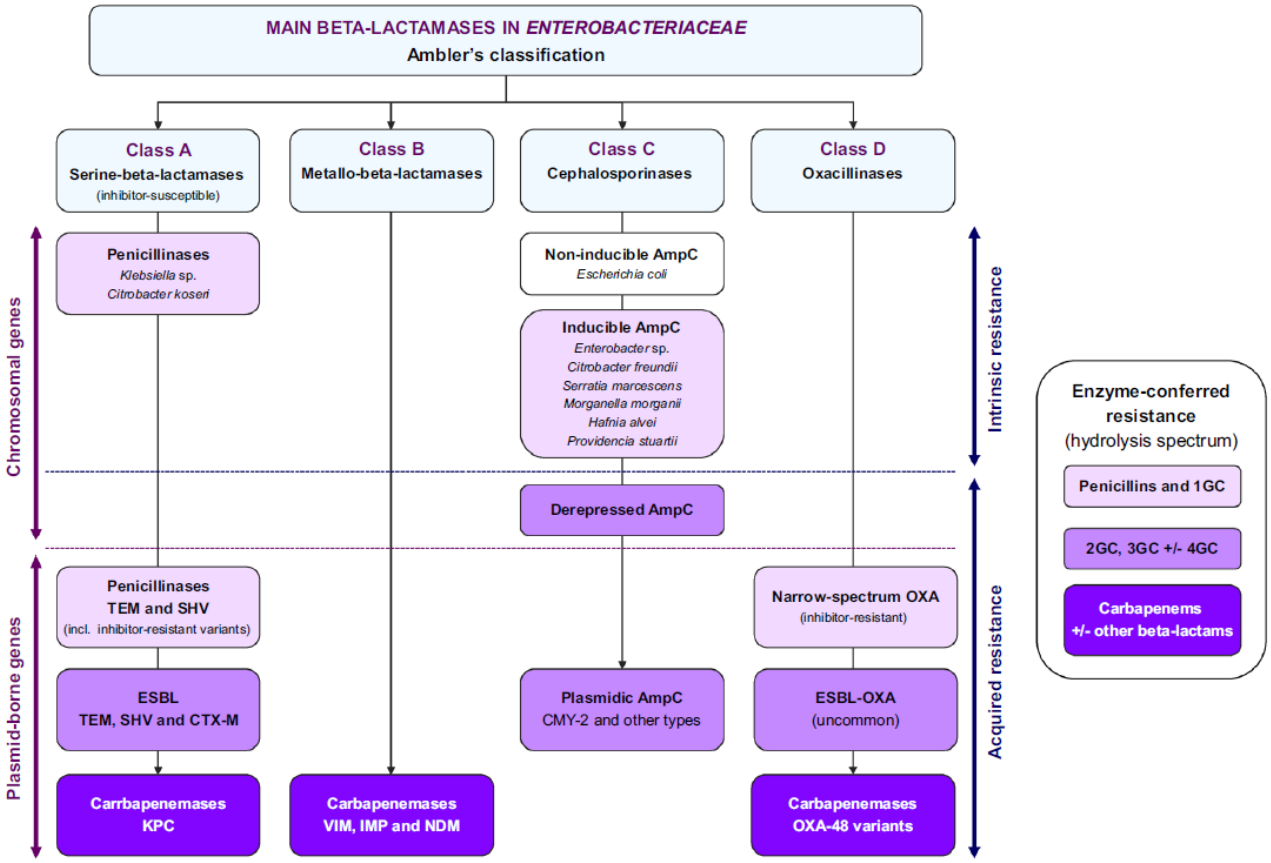


Figure 3. Chromosomal and plasmidic ß-lactamases of major clinical interest in Enterobacterales (formerly Enterobacteriaceae) (Ruppé 2015)

According to the 2017 European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE), in Italy 95,9% of carbapenemase-producing *K. pneumoniae* harbored KPC enzyme, while OXA-48-like, NDM, VIM and other mechanisms of carbapenem resistance accounted for less than 5% altogether (Grundmann 2017).

KPC-*Kp* COLONIZATION & INFECTION: MICROBIOLOGY

Taxonomy and phylogenetic analyses

Klebsiella pneumoniae is a rod-shape, non-motile, lactose-fermenting, encapsulated gram-negative bacterium that belongs to the family *Enterobacterales* (formerly *Enterobacteriaceae*) (American Society for Microbiology 2015). It is an enteric bacterium that in the community setting colonizes 5% to 38% of the intestinal tract and 1% to 6% of the nasopharynx of healthy individuals (Podschun 1998). Colonization is much higher in hospitalized patients, among whom reported rates of *K. pneumoniae* carriers are as high as 77% in the stool, 19% in the pharynx, and 42% on the hands (Podschun 1998). This increase in prevalence is clinically important, since nosocomial infections by

K. pneumoniae are found more frequently in carriers compared with noncarriers (see below “KPC-*Kp* colonization & infection: beyond microbiology – clinical aspects). *K. pneumoniae* can be spread through person-to-person contact (for example, via the contaminated hands of healthcare workers) or, less commonly, by contamination of the environment, and can lead to healthcare associated infections (HAIs) and outbreaks (UpToDate 2021). As stated above, *K. pneumoniae* has emerged as a major clinical and public health threat because of the increasing prevalence in the healthcare setting of multidrug-resistant strains. At the same time, severe community-acquired infections caused by "hypervirulent" *K. pneumoniae* (hv*Kp*) have been reported especially in the Asia-Pacific area, associated with strains expressing multiple acquired virulence factors (K. L. Wyres 2020).

K. pneumoniae genomes are 5–6 Mbp in size, encoding approximately 5.000 – 6.000 genes: 1.000 – 2.000 genes are conserved in all members of the species (core genes), whereas the remaining are variably present (accessory genes). Phylogenetic analyses conducted in the pre-genomic era identified 77 distinct capsular (K) serotypes, 9 lipopolysaccharide (O) types and variable AMR profiles within the *K. pneumoniae* population (Lam 2021). Genomic studies based on allelic variation in core genes have characterized hundreds of subpopulations of *K. pneumoniae* strains. These can be defined as sequence types (STs) by multi-locus sequence typing (MLST) of 7 chromosomally encoded housekeeping genes or, more recently, as clonal groups (CGs) by core-genome MLST (cgMLST), which is based on nucleotide sequence variation in hundreds of genes (Diancourt 2005) (K. L. Wyres 2020).

K. pneumoniae clones responsible for HAIs are widely geographically distributed, and localized infections or outbreaks are often caused by distinct lineages. However, a few clones are more frequently involved in MDR HAIs, hence are referred to as “MDR clones”: CG258, CG15, CG20, CG29, CG37, CG147, CG101 and CG307 (K. L. Wyres 2020). In contrast to HAIs, hv*Kp* infections are dominated globally by the same subset of lineages: the most common is CG23, followed by CG65 and CG86 (K. L. Wyres 2020). In a recent Italian study conducted from 2016 to 2018 across 15 hospitals in Lombardy, of the 989 KPC-*Kp* strains analyzed, 32 different STs were identified (Rossi 2021). The most frequent clones were ST512 and ST307 in 45% and in 33% of cases, respectively. While the former was evenly distributed across all centers, ST307 was found in smaller and more localized clusters.

Pathogenicity and virulence factors

A number of pathogenicity and virulence factors of *K. pneumoniae* are known to contribute to the pathogenesis of infections (K. L. Wyres 2020) (UpToDate 2021).

Pathogenicity factors are core chromosomally encoded in all bacterial strains and are required to progress from colonization to infection. These include the loci for the biosynthesis of the core siderophore enterobactin (encoded by the *Ent* locus), type 1 and type 3 fimbriae (encoded by *firm* and *mrk, respectively*), as well as variable capsule (K antigen) and lipopolysaccharide (O antigen) biosynthesis loci (K. L. Wyres 2020).

Capsule production occurs via the Wzx/Wzy-dependent pathway (biosynthesis loci *wzi*, *wza*, *wzb*, *wzc*, *wzx* and *wzy*) (Whitfield 2020). More than 130 capsular loci (*cps*) encoding distinct capsule types have been identified. Of them, only 77 have been distinguished by traditional serological typing (K types). Capsular polysaccharide may contribute to *K. pneumoniae* pathogenicity by protecting against phagocytosis by host immune cells (Cortés 2002). Worldwide, K2 is the most common capsular type of clinical isolates, despite prevalence varies widely in different regions (UpToDate 2021). Capsule types K1 and K2 are associated with invasive diseases in animal models and are more often found in bacteria with hypermucoviscous phenotype (see below) (UpToDate 2021).

Twelve distinct O-loci, along with other genes localized in other regions of the genome, concur to define the 9 lipopolysaccharide (LPS) O serotypes (K. L. Wyres 2020). LPS confers resistance against complement-mediating killing and also increases bacterial virulence by acting as an endotoxin and triggering cytokine response that can eventually lead to multiorgan failure in septic shock (UpToDate 2021). Serotypes O1 and O2 are the most frequent among clinical *K. pneumoniae* isolates (K. L. Wyres 2020).

Virulence factors are encoded by accessory genes and their presence increases the propensity to cause infection and/or the disease severity. These include the acquired sideropohores yersiniabactin, aerobactin and salmochelin (encoded by *ybt*, *iuc* and *iro*, respectively) which force intracellular storage of important co-factor such as iron, the genotoxin colibactin that induces DNA damage (encoded by the *clb* locus) and the hypermucoviscous phenotype (K. L. Wyres 2020).

Hypermucoidy is one of the most well-known virulence phenotype and is characterized by “sticky” growth of *K. pneumoniae* colonies on agar plates. It is commonly associated with capsule overproduction due to accessory regulator genes *rmpA* or *magA* and correlates clinically with invasive infections (K. L. Wyres 2020) (UpToDate 2021).

Resistance factors

K. pneumoniae genome harbors several AMR genes that define the bacterial resistome (Wright 2007). Acquired AMR mechanisms are in part due to mutations in core chromosomal gens, but the majority are associated with horizontally acquired (mostly plasmid-encoded) genes (K. L. Wyres 2020) (Navon-Venezia 2017) (K. L. Wyres 2016).

The major core gene mutations involved in AMR are the following: *ompK35* and *ompK36*, which can confer resistance to carbapenems through reduced permeability via membrane porin modification; *phoPQ*, *pmrAB*, *mgrB* and *ccrAB*, which can confer resistance to colistin through modification of lipid A (component of LPS); *gyrA*, *gyrB*, *parC* and *parE*, which can confer resistance to fluoroquinolones through topoisomerase modification; *rpsJ*, which can confer resistance to tigecycline through ribosomal modification; *oqxAB*, *acrAB* (*ramAR*, *rarA*, *soxS* and *marA*), which can confer resistance to carbapenems, fluoroquinolones and tigecycline through induced expression of intrinsic efflux pumps (K. L. Wyres 2020) (Navon-Venezia 2017).

Horizontally acquired genes are non-randomly distributed in *K. pneumoniae* population. Their number follows a bimodal distribution, with most bacterial strains carrying either zero acquired AMR genes (*i.e.*, wild-type strains) or more then 6 acquired AMR genes encoding resistance to multiple drug classes (*i.e.*, MDROs) (K. L. Wyres 2020). Whole genome sequencing studies performed in the healthcare setting have detected direct transfer of AMR plasmids between distinct *K. pneumoniae* strains and between *K. pneumoniae* and other *Enterobacterales*, presumably driven by selection secondary to exposure to a range of antimicrobials (Conlan 2014) (A. J. Mathers 2015) (Bosch 2017). Of particular clinical concern are the dissemination of extended spectrum beta-lactamases (ESBLs) and carbapenemases.

ESBLs genes encode for enzymes able to hydrolyze third-generation cephalosporins and monobactams, and to various extent four-generation cephalosporins (cefepime) and piperacillin/tazobactam (Bush 2010). Based on Ambler classification, ESBLs belongs to class A (CTX-M, SHV, TEM) and class D (OXA-types ESBLs) serine β-lactamases (Bush 2010) (Ruppé 2015). Within ESBLs, the dissemination of the $bla_{CTX\text{-}M}$ gene is that of the most clinical concern (K. L. Wyres 2016). Carbapenemases classification has been described above (see above: “ANTIMICROBIAL RESISTANCE”). Within carbapenemases, the dissemination of the bla_{KPC} gene in *K. pneumoniae* (*i.e.,* KPC-*Kp*) is that of the most clinical concern, particularly in Europe and Italy. Worldwide, the rapid dissemination of KPC-*Kp* has mostly been caused by the clonal expansion of strains of CG258, including ST258 and related variants such as ST512 (Munoz-Price 2013) (Kwong 2018). In 2016, in

the most recent Italian report on carbapenem-resistant *K. pneumoniae* with 17 laboratories collecting all the consecutive non-replicate samples from invasive infections, the vast majority (97%) of samples were positive for the bla_{KPC} gene (Conte 2016). In that study, KPC-*Kp* population was largely oligoclonal with the wide diffusion of ST512 lineage carrying *cps-2* capsular type and producing the KPC-3 enzyme. In the aforementioned study by Rossi et al. on KPC-*Kp* outbreak in Lombardy in 2016-2018, 2 KPC variants (bla_{KPC-2} and bla_{KPC-3}) were identified in 68% of isolates. Of note, bla_{KPC-2} gene was absent in ST512 but predominant in ST307 and ST258 (Rossi 2021).

Plasmid-borne colistin-resistance was reported for the first time in China in 2015 through the gene *mcr -1* in *Escherichia coli* (Liu 2016). The gene *mcr* encodes for an enzyme that changes lipid A in LPS, suppressing its binding to colistin. In later years, plasmid-mediated *mcr* has been found worldwide and 9 different variants have been described. According to the last ECDC report, in EU plasmid-related colistin-resistance is still uncommon in *Enterobacterales,* despite its prevalence is predicted to increase (European Centers for Diseases Control and Prevention 2016).

KPC-*Kp* COLONIZATION & INFECTION: BEYOND MICROBIOLOGY

Clinical aspects: clinical presentation and risk factors

K. pneumoniae is a frequent cause of HAIs, which occur primarily in patients with impaired host defenses. The most common clinical syndromes are urinary tract infections, pulmonary infections (both in patients with mechanical ventilation and in non-ventilated patients), intra-abdominal infections and primary bloodstream infections (BSI) (UpToDate 2021). The presence of KPC enzyme increases morbidity and mortality of *Klebsiella pneumoniae* infections. Indeed, mortality rates above 50% have been reported in patients with carbapenem-resistant *Kp* bloodstream infections (Grundmann 2017) (Logan 2017). Impaired host defenses likely play an important role in the pathogenesis of infections, with some risk factors being associated to an increased rate of *K. pneumoniae* infections, namely: diabetes mellitus, hepatobiliary disease, chronic obstructive pulmonary disease, renal failure, parenteral nutrition, malignancy and immunosuppressive therapy (Lee 1994) (Kang 2006) (Opilla 2008) (Huang 2015).

As the other CRE, KPC-*Kp* colonization is a major risk factor for a subsequent infection. In a prospective cohort of 1,765 patients screened for rectal colonization with *K. pneumoniae*, Martin and colleagues reported an adjust odds ratio (OR) of 4.0 (95% confidence interval [CI] 2.1-7.7) for an extra-intestinal infection in colonized patients over a 3-month period (Martin 2016). A 2017 study

by Gorrie and colleagues among 498 ICU patients found that *K. pneumoniae* intestinal carriage was significantly associated with a subsequent infection (risk 16% in colonized vs 3% in non-colonized patients, OR 6.9, CI 2.3-19.7), and that almost half (49%) of *K. pneumoniae* infections were caused by the patients' colonizing strain characterized by whole genome sequencing (Gorrie 2017). In a systematic review of 10 studies and over 1.800 CRE-colonized patients, Tischendorf and colleagues found a cumulative rate of infection of 16.5%, in one study up to 89% (Tischendorf 2016). Progression from CRE colonization to infection are indeed higher in specific at-risk categories: 27% in ICU patients, 25.8% in patients undergoing autologous HSCT and 40% in those undergoing allogeneic HSTC (Girmenia 2015). Of note, in patients with onco-hematologic diseases up to 70% of bacteremia are gut-borne due to microbial translocation of pathogens (Samet 2013).

Over the last 10 years, several studies have analyzed the risk factors for KPC-*Kp* colonization and/or infection and for disease mortality. All these studies have contributed to addressed important clinical issues, such as the validation of scores to guide infection control measures or adequate empiric antimicrobial therapies.

In 2014, by a retrospective multicenter analysis of 5 Italian University Hospitals, Tumbarello and colleagues derived 2 clinical scores: one for KPC-*Kp* colonization and one for KPC-*Kp* infection (Tumbarello M 2014) (Table 1).

Variable	OR (95% CI)	P-value	Variable	OR (95% CI)	P-value
KPC Kp Isolation [‖]			**KPC Kp Infection** [‖]		
≥ 2 previous acute-care hospitalizations [‡]	5.92 (4.40-7.98)	<0.001	≥ 2 previous acute-care hospitalizations ‡	4.26 (3.02-6.01)	<0.001
Indwelling central venous catheter *	1.66 (1.29-2.12)	<0.001	Indwelling central venous catheter *	2.59 (1.91-3.50)	<0.001
Recent carbapenem therapy [†]	2.98 (2.19-4.05)	<0.001	Recent carbapenem therapy [†]	3.59 (2.46-5.23)	<0.001
Recent fluoroquinolone therapy [†]	1.69 (1.29-2.21)	<0.001	Recent fluoroquinolone therapy [†]	2.22 (1.59-3.10)	<0.001
Previous Intensive Care Unit admission [‡]	5.13 (3.49-7.53)	<0.001	Charlson score ≥ 3 [§]	7.49 (5.46-10.27)	<0.001
Indwelling urinary catheter *	3.89 (3.03-4.99)	<0.001	Recent surgical procedures †	2.03 (1.48-2.76)	<0.001
Hematological cancer	1.90 (1.27-2.83)	0.002	Neutropenia *	3.19 (1.50-6.78)	0.003
Surgical drain *	1.62 (1.16-2.45)	0.004			

[*] At the time of the index culture (cases) or at any time during hospitalization (controls); [†] 0-30 days before index culture (cases) or at any time during hospitalization (controls); [‡] within the 12 months preceding index culture (cases) or at any time during hospitalization (controls); [§] within the 3 months preceding index culture (cases) or at any time during hospitalization (controls). [‖] Risk factors identified as significant in this model remained significant (i.e., no significant change in ORs or P values) if the specific antimicrobials were replaced with a variable *recent exposure to ≥ 3 different antimicrobial*

Table 1. Tumbarello KPC score, with risk factors for KPC-Kp colonization (left) and infection (right). For each item, the respective Odds Ratio values are reported (Tumbarello M 2014)

The identified models showed good predictive value, with an area under the receiver operating characteristic (AUROC) curve for KPC-*Kp* colonization and infection of 0.82 (95% CI 0.80-0.84) and 0.82 (95% CI 0.80-0.85), respectively.

In the same year, Giannella et al. published the results of a multicenter prospective study (5 Italian University Hospitals for a period of 2 years) with the analysis of risk factors for development of BSIs by carbapenemase-resistant *K. pneumoniae* in patients with known rectal colonization (Giannella M 2014). Based on the independent variables that most correlated with the risk of BSI, the Giannella risk score with sensitivity, specificity, positive predictive value, and negative predictive value of 93%, 42%, 29%, and 93%, respectively, was proposed at the optimal cut-off ≥2 (Table 2).

	OR (95% CI)	P-value	Risk score point
Admission to ICU	1.65 (1.05–2.59)	0.03	2
Invasive abdominal procedures	1.87 (1.16–3.04)	0.01	3
Chemotherapy/radiation therapy	3.07 (1.78–5.29)	<0.0001	4
Colonization at site besides stool (risk per each additional site)	3.37 (2.56–4.43)	<0.0001	5 per site

ICU, intensive care unit; OR, odds ratio.

Table 2. Giannella risk score, with risk factors for BSI from carbapenem-resistant K. pneumoniae in patients with known rectal colonization. For each item, risk points and Odds Ratio values are reported (Giannella M 2014)

In 2016, Gutiérrez-Gutiérrez and colleagues of the ESCMID - ESGBIES group (ESCMID Study Group for Bloodstream Infections, Endocarditis and Sepsis), from the results of a large multinational derivation/validation study (INCREMENT project) developed a predictive score for mortality at 14 and 30 days in patients with BSI due to carbapenem-producing *Enterobacterales* (CPE) (Gutiérrez-Gutiérrez 2016). The score, called INCREMENT-CPE score, identifies 3 different risk bands (low 0-8, medium 9-13, high 14-17) and shows an AUROC at 14 days of 0.80 (95% CI 0.74-0.85 in the derivation cohort, 0.73-0.88 in the validation cohort) with overlapping results at 30 days (Table 3).

Variable	Regression coefficient (95% CI)	Score
Severe sepsis or septic shock	1.76 (1.01-2.50)	5
Pitt score ≥6	1.39 (0.54-2.25)	4
Charlson comorbidity index ≥2	0.93 (0.09-1.78)	3
Source of BSI other than urinary or biliary tract	0.92 (0-1.85)	3
Inappropriate early targeted therapy	0.69 (0.07-1.31)	2
Total points		*17*

BSI = bloodstream infection.

Table 3. INCREMENT-CPE score, with risk factors for 14- and 30-day mortality in patients with BSI caused by CPE. For each item, specific risk points assigned based on regression coefficients are reported (Gutiérrez-Gutiérrez 2016)

A subsequent study combined the Giannella risk-score (GRS) and the INCREMENT-CPE score (ICS) to externally validate the two scores in order to recommend appropriate empiric therapy in KPC-*Kp*–

colonized patients (Cano 2018). With the optimal cutoff point fixed at <7 and ≥7 (92.9% sensitivity, 84.8% specificity), the GRS showed an AUROC curve for KPC-*Kp* infection of 0.92 (95% CI 0.87-0.98). Among patients with infections, the ICS showed an AUROC of 0.78 (95% CI, .65–.91) for 30-day all-cause mortality.

Recently, an Italian multicenter retrospective study analyzed the 30-day mortality and associated risk factors of KPC-*Kp* infections treated with the new β-lactam/β-lactamase inhibitor combination ceftazidime/avibactam (CAZ-AVI) (Tumbarello 2021). Within a cohort of 577 patients (391 BSI and 165 nonbacteremic infections), all-cause mortality rate at 30 days after infection onset was 25%. In the multivariate analysis, mortality was associated with septic shock at presentation, neutropenia, INCREMENT-CPE score ≥8, a lower respiratory tract infection, and with CAZ-AVI dose adjustment for renal function (Table 4).

	Adjusted for the Propensity Score Matching for Combination Therapy?			
	No		Yes	
Variables	*P*	OR (95% CI)	*P*	OR (95% CI)
INCREMENT score ≥8	.01	2.06 (1.18–3.59)	.005	2.23 (1.27–3.91)
Septic shock at infection onset	.002	2.72 (1.45–5.09)	.003	2.59 (1.37–4.89)
Neutropenia	<.001	6.37 (2.42–16.74)	<.001	6.86 (2.55–18.42)
Lower respiratory tract infection	.04	1.90 (1.03–3.53)	.008	2.48 (1.26–4.86)
CAZ-AVI by prolonged infusion	.003	.52 (.34–.79)	.006	.54 (.34–.83)
CAZ-AVI dose adjustment for renal function	.001	2.39 (1.42–4.03)	.01	2.01 (1.15–3.48)

Abbreviations: CAZ-AVI, ceftazidime-avibactam; CI, confidence interval; OR, odds ratio.

Table 4. Multivariate analysis of factors associated with 30-Day mortality in KPC-Kp infection (M. R. Tumbarello 2021)

Host factors: immune markers associated with KPC-*Kp* and other bacterial colonization/infections

Several host factors of both cellular and humoral immunity have been studied in bacterial colonization/infections and in sepsis. Yet, no immune biomarker has been specifically associated to KPC-*Kp* colonization or infection status.

As for cellular immunity, T helper (Th) CD4+ subsets play a critical role in host defense against pathogens, as cytokine production modulates immune response both at systemic and mucosal level. In addition to the classical Th1 and Th2 subsets, depending on cytokine stimulation naive CD4 T cells may polarized into other three lineages: Th17, regulatory T cells (Treg) and follicular helper T cells (Tfh) (Zhu 2010). During infections, Tregs control inflammation and maintain mucosal homeostasis, while Th17 serve as proinflammatory cells and enhance effector responses (Sehrawat 2017). Predominant products of Th17 are IL-17 (in six isoforms), IL-21, IL-22, which have a key role in the induction of innate immune defenses. Several recent studies have indicated that IL-17A is a

biomarker as well as a therapeutic target in sepsis (Ge 2020) (Chamoun 2020). Tfh have a central role in helping B cells to produce antibody against pathogens. They are mainly located in secondary lymphoid organs but can also be identified in the circulation (Olatunde 2021).

Innate lymphoid cells (ILCs) and human mucosal-associated invariant T (MAIT) cells are populations of lymphocytes that reside in mucosal sites such as skin, oral, intestinal, respiratory, and urogenital tracts. These cells are exposed to a variety of both commensal and pathogenic bacteria and under non-pathological conditions they are involved in protection and maintenance of tissue homeostasis by interactions with the microbiome (Beck 2020) (Meermeier 2018). ILCs are divided into three subgroups: ILC1, ILC2, and ILC3, which are considered as innate analogues of the three major CD4+ T effector cells Th1, Th2, and Th17 because of their similar functions. By analogy to Th1 cells, ILC1 mediate type 1 immunity against intracellular pathogens such as viruses and certain bacteria through production of interferon gamma (IFN-γ) and tumor necrosis factor (TNF)-α. ILC2 mediate a type 2 immune response, characterized by the production of IL-4, IL-5, IL-9, and IL-13 cytokines. ILC3 are the equivalent of Th17 cells due to the production of IL-22 and IL-17. ILC3 are highly abundant in the intestine where they play a crucial role in intestinal immunity including the maintenance of mucosal barrier integrity and microbiota-host homeostasis. During infectious events, ILCs contribute to host defense by responding rapidly to the pathogens as well as orchestrating other immune cells (Beck 2020). MAIT cells are the most abundant T-cell subset in humans and have a pivotal role in the early phase of mucosal protection. MAIT cells combine critical features of innate and adaptive immunity such as a rapid expansion in response to bacterial infection, a selective recognition of antigens broadly expressed by many bacterial and fungi and an expression of pro-inflammatory cytokines IFN-γ, TNF-α and IL-17 as well as cytolytic products (Meermeier 2018). Despite being mainly located in mucosal tissues and secondary lymphoid organs, both ILCs and MAIT cells can be also detected in peripheral blood.

As for circulating soluble inflammatory markers, besides classical biomarkers of systemic inflammation (*i.e.*, C-reactive protein (CRP), procalcitonin) that are routinely used in the management of bacterial infections and sepsis, novel inflammatory markers are being studied for the diagnosis of infectious conditions and the assessment of disease severity. Long pentraxin-3 (PTX3) belongs to the superfamily of pentraxins, highly-conserved mediators of innate immunity which also includes CRP and serum amyloid P. PTX3 is produced by several cell types in response to pro-inflammatory signals and microbial recognition, and acts as a rapid and important mediator of innate immunity against pathogens and as a regulator of inflammation, by modulating complement

activation and cell extravasation, and facilitating pathogen recognition by immune cells (Porte 2019). PTX-3 has been extensively studied in sepsis and other infectious conditions. In sepsis, PTX3 plasma levels are associated with disease severity, patient survival, and response to therapy. In a recent single-center prospective observational study of 75 patients with septic shock at ICU admission, the AUROC for in-hospital mortality of PTX3 was 0.70 and resulted higher than PCT (0.43) and CRP (0.48). Adding PTX3 to the routinely used severity scores SAPS II, SOFA and APACHE II increased their prognostic capacity (AUC 0.814, 0.795, and 0.741, respectively) (Perez-San Martin 2020). In 958 patients with sepsis/septic shock of the Albumin Italian Outcome Sepsis (ALBIOS) trial, circulating PTX3 levels of resulted abnormally high (72 [33-186] ng/mL), and were associated with disease severity, occurrence of new organ failures and death (Caironi 2017). Compared to patients with pulmonary sepsis of the ALBIOS trial, COVID-19 patients in the ICU tend to have a lower plasma concentration of PTX3. Similarly to patients with bacterial sepsis, 28-day non-survivors COVID-19 patients presented higher circulating PTX3 than survivors (30 [19-64] ng/mL vs 18 [11-36] ng/mL, p 0.030) (Protti 2021). Finally, in animal models of severe *K. pneumoniae* infections, PTX3 deficiency was associated with higher bacterial burden, higher release of pro-inflammatory cytokines and mortality (Asgari 2021). These findings indicate that PTX3 may contribute to the control of severe infections (and specifically of *K. pneumoniae* infection) by modulating inflammatory responses and tissue damage.

Another promising biomarker in critically ill patients with severe infection is the soluble interleukin (IL)-1 decoy receptor type 2 (sIL-1R2). IL-1 is a proinflammatory cytokine with a primary role in the innate response and in the activation and orientation of adaptive immunity, being critical to the pathogenesis of a variety of inflammatory and infectious conditions. The IL-1 system comprises several negative regulators including the decoy receptor IL-1R2, which is able to efficiently recognize and bind IL-1 but not structurally able to signal or activate the IL-1 pathway (Molgora 2018). IL-1R2 exists in both membrane bound and soluble forms (sIL-1R2). IL-1R2 shedding can occur slowly (hours) in response to anti-inflammatory mediators such as Th2 cytokines (IL-4, IL-13), IL-10 and glucocorticoids, and rapidly (minutes) in response to LPS, TNF, reactive oxygen species (Peters 2013). Plasma levels of sIL-1R2 are 5-10 ng/ml in healthy donors and increase in critically ill patients with infectious conditions such as sepsis (Müller 2002) or acute respiratory distress syndrome (Kovach 2015), often correlating with the severity of the disease.

Gut microbiome composition and function have been extensively examined in healthy individuals and during bacterial colonization/infections and sepsis. However, to date very few studies have specifically assessed microbiome profile in KPC-*Kp* colonization or infection.

At gut level, the total number of prokaryotes located as ecological niches includes $14x10^{13}$-10^{14} bacteria, defined as gut microbiota. This is composed by more than 1000 bacterial species with an amount of genetic material of $3x10^{6}$ (100 times higher the value of the human genome) defined as gut microbiome. Although the two terms are different, they are often used interchangeably by the scientific community (Ancona 2021).

Besides maintaining intestinal health and helping the digestion process, gut microbiome has several important functional properties. Indeed, it plays a crucial role in the prevention of opportunistic pathogenic infections (*i.e.*, *Clostridioides difficile* infection), production of metabolites and signaling molecules, immune cell differentiation and immune homeostasis, glucose and lipid metabolism (Ghosh 2021). Gut microbiome of healthy adults typically harbors more than 1000 species of bacteria belonging to a relatively few known bacterial phyla with *Bacteroidetes* and *Firmicutes* being the dominant phyla (70% of the total microbiota), and has different distribution according to the gastrointestinal tract (Figure 4) (Arumugam 2011) (Shreiner 2015) (Ghosh 2021).

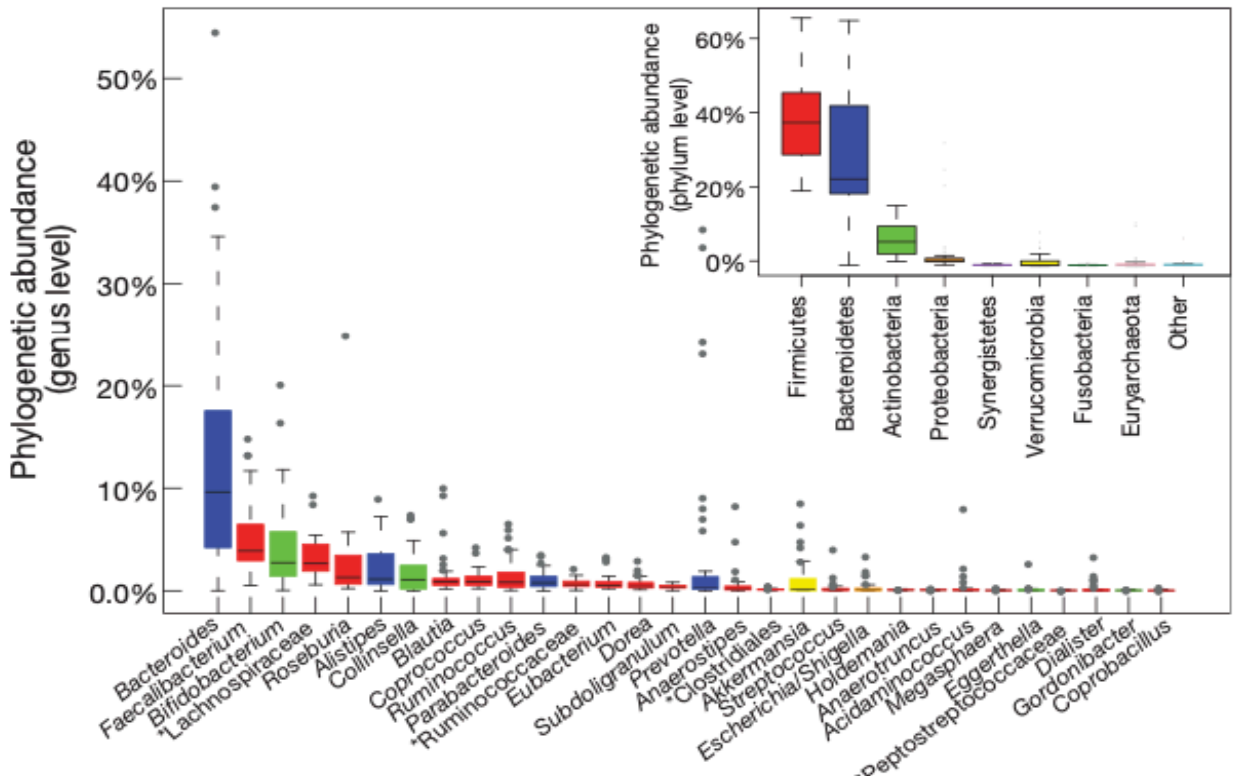


Figure 4. Genus abundance variation box plot for the 30 most abundant genera of the human gut microbiota, as determined by metagenomic sequencing of human fecal samples. Inset shows phylum abundance box plot. Genera are colored by their respective phylum. Genus- and phylum-level abundances are measured using reference-genome-based mapping. Unclassified genera under a higher rank are marked by asterisks (Arumugam 2011).

Several factors influence bacterial composition. Physiologically, gender, age, body mass index (BMI), diet and the use of antibacterial agents have a strong impact on gut microbiome. In addition, several medical conditions such as gastrointestinal disorders, metabolic or autoimmune diseases, and other systemic inflammatory or infectious conditions are characterized by different microbiome compared to health individuals (Ancona 2021) (Shreiner 2015).

The concepts of microbial abundance, richness, evenness and diversity are crucial to describe microbial composition and to examine differences and similarities of bacterial communities between samples (or conditions). Briefly, richness refers to the number of species in a given community (or sample), and is unaffected by species abundances. Relative species abundance measures how common or rare a species is relative to other species in a given community (or sample). It is calculated in operational taxonomic units (OTUs, defined by a 97% identity threshold of the 16S gene sequences to distinguish bacteria at the genus level) or grouped into Sequence Variants (SVs) (Lin 2020). Evenness focuses on species abundances and indicates how evenly distributed the numbers of each species are. Species evenness is highest when all species in a given community (or sample) have the same abundance (Cox 2013) (Figure 5).

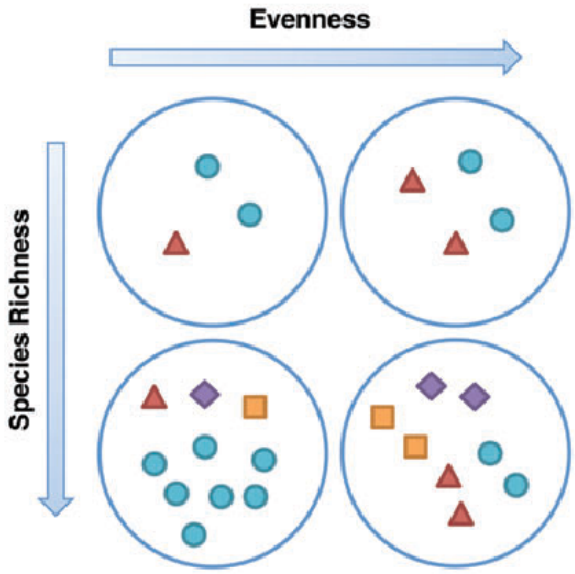


Figure 5. A diagram demonstrating species richness and evenness and how they describe the composition of a community. Each shape represents an individual and the colour and nature of the shape represents a different type of organism. Increased numbers of different types of organism is described as increased species richness. When no one organism is dominant, the community is described as even (Cox 2013).

Species diversity is a measurement of species richness combined with evenness, and generally increases together with increasing richness and evenness. Diversity indices measure the overall community heterogeneity. Alpha diversity ("within sample diversity") is a measure of microbiome diversity within a given community (or sample). Some of the most widely used alpha diversity

measures in microbiome research are the Shannon index, the Simpson's index and the Pielou's evenness index (that measures diversity along with species richness). Beta diversity ("between sample diversity") quantifies similarity or dissimilarity between communities (or samples). Some of the most popular beta diversity measures include Bray-Curtis index and the Unifrac distances (that considers the phylogenetic tree information) (Cox 2013) (Wagner 2018).

In recent years, with the emergence of AMR as a great concern to global health and of infections by MDROs as a major clinical challenge (see above: "ANTIMICROBIAL RESISTANCE"), growing attention has been put on microbiome-mediated mechanisms of AMR and possible strategies for MDROs colonization control. In fact, the gut microbiome hosts a large reservoir of AMR genes called "resistome" (Casals-Pascual 2018), which consists of 2 components: a resident resistome carried by commensal bacteria, and a transitory resistome carried by bacteria that only periodically are present in the gut. These latter bacteria can transfer their resistance to the commensal microbiota or become permanent microbiota residents themselves, thus increasing the gut resistome (Baron 2018). Studies in humans and animal models have found that particular commensal species seem to inhibit the growth of specific MDROs, suggesting innovative solutions with gut microbiome-modifying approaches for decolonization strategies (Gargiullo 2019) (Feehan 2020). Apart from the risk of AMR, studies on critically-ill patients have shown that microbiome dysbiosis (*i.e.*, a dysregulation between the different bacterial species that compose the gut microbiota) can lead to worse clinical outcomes (Bassetti 2020). Commonly, gut microbiome of ICU patients with sepsis demonstrates loss of microbial richness and diversity, dominance of few taxa (often potential pathogens), and loss of site-specificity with isolation of the same organism at multiple sites (Yeh 2016). The transition of a healthy microbiome into a "pathobiome" has been hypothesized to be a driver of severe outcome in critically patients, both for increasing the risk of infections and for negatively impact on host immunity (Bassetti 2020). Gut microbiome-modifying therapies such as the fecal microbiota transplantation (FMT) and the use of probiotics or bacteriophages have been therefore proposed for individuals with MDROs colonization, immunocompromised host and for critically-ill and septic patients (Alagna 2020) (Wuethrich 2021) (Korach-Rechtman 2020) (Ancona 2021) (Bassetti 2020).

To date, very few studies have specifically assessed microbiome profile in KPC-*Kp* colonization or infection. In 2018, Seekatz and colleagues conducted a case-control study of long-term acute care hospital patients to identify gut microbiome profiles and clinical features associated with colonization by KPC-*Kp* (Seekatz 2018). Case (n = 32) and control (n = 99) patients had distinct

fecal microbiota communities, but neither alpha nor beta diversity clustering distinguished case and control specimens. Two OTUs (belonging to genus *Desulfovibrio* and family *Ruminococcaceae*) and the presence of a decubitus ulcer were independently associated with case status. Combining the presence of the 2 OTUs and clinical features increased the likelihood of KPC-*Kp* colonization to >38%. In the same year, Shimasaki and colleagues demonstrated the association between increased relative abundance of KPC-*Kp* in the intestinal tract and KPC-*Kp* bacteremia in long-term acute care hospital patients (Shimasaki 2018). KPC-*Kp* colonization was detected in 255/562 (45.4%) admissions and KPC-*Kp* bacteremia in 11/562 (4.3%). A relative abundance cutoff of 22% predicted KPC-*Kp* bacteremia with sensitivity 73%, specificity 72%, and relative risk 4.2. In a multivariable Cox regression model adjusted for age, Charlson comorbidity index, and medical devices, carbapenem receipt was associated with achieving the 22% relative abundance threshold. Finally, in 2021 Sun and colleagues showed an association between *Klebsiella* spp. colonization density (assessed by quantitative PCR (qPCR) assay in rectal swab) and subsequent infections in a case-control study (Sun 2021). Cases were patients with a clinically- and microbiologically- confirmed infection and a preceding or coincident colonization isolate with the same *wzi* capsular sequence type. Controls were colonized patients without subsequent infection. *Klebsiella* relative abundance by qPCR highly correlated with 16S sequencing. The median *Klebsiella* relative abundance was higher in cases (15.7%) than in controls (1.01%). Adjusting for multiple clinical covariates, a *Klebsiella* relative abundance of >22% was associated with infection overall (OR 2.87) and with bacteremia (OR 4.137).

STUDY HYPOTESIS AND STUDY QUESTIONS

So far, very few studies on KPC-*Kp* colonization/infection have integrated clinical data with pathogens factors (*i.e.*, KPC-*Kp* clone, virulence and resistance factors), host immunity (*i.e.*, cellular and humoral immune response) or gut microbiome profile, revealing important aspects of this complex condition.

In 2019, Kim and colleagues assessed the impact of resistance and virulence factors of *Klebsiella pneumoniae* on the 30-day mortality of 579 patients with *K. pneumoniae* BSI (Kim 2019). Mortality rate was 16.9%. Among the clinical factors, increased SOFA score and leucopenia exhibited strong associations with increased mortality (adjusted Hazard Ratio HR of 1.28 and 2.26, respectively). Among the pathogenic factors, carriage of the *pks* gene (responsible for the synthesis of colibactin) was a risk factor with adjusted HR 1.80, especially when accompanied by MDR status. *K.*

pneumoniae isolates of the *wzi50* capsular type frequently harboured *pks* and *ybtA* (responsible for the synthesis of yersiniabactin) and mostly exhibited MDR, resulting in increased 30-day mortality (adjusted HR 2.66). In contrast, hypermucoviscous *K. pneumoniae* isolates showed an inverse association with 30-day mortality (adjusted HR 0.55). In 2020, Iwanaga and colleagues studied the role of host factors and possible therapeutic strategies in animal models of ST258 KPC-*Kp* pulmonary infection (Iwanaga 2020). Using immunocompromised $Rag2^{-/-}$ $Il2rg^{-/-}$ mice, the authors identified that lymphoid cell populations expressing type 1 and type 17 cytokines (*i.e.*, NK cells and ILCs) were critical for host resistance and that immunotherapy with recombinant IL-22 (physiologically produced by NK cells and ILC3) can improve pulmonary immunity to ST258 KPC-*Kp* infection. Finally, in 2021 Kiner and colleagues published important findings on the impact of gut microbes on mucosal CD4+ T cells differentiation (Kiner 2021). By analyzing mice under germ-free conditions, with normal commensal microbiota or infected with pathogens that elicit biased T effector (T_{eff}) responses, the authors proved that mucosal T_{eff} cells clustered according to the type of infection, rather than by the cytokine pattern that might have been expected from the TH paradigm.

Focusing on host immunity, pathogen genetic characteristics and gut microbiome, the present study aimed to develop an integrated approach to better understand KPC-*Kp* colonization/infection. In particular, main aims of the study were to: i) evaluate clinical parameters and immunological and microbiological biomarkers that can distinguish colonization from infection; ii) evaluate clinical parameters and immunological and microbiological biomarkers associated with progression from colonization to invasive infection, and iii) investigate clinical risk factors and immunological or microbiological biomarkers associated with mortality.

In view of the spread of epidemic strains of KPC-*Kp* to a large number of hospitals in Italy, a better understanding of clinical, microbiological and immunological factors associated to the development of severe infections could prompt to early detection of high-risk patients and to implement strategies to better prevent or manage infections. Also, the integrated approach to KPC-*Kp* colonization/infection could serve as translational model to study other infectious diseases or complex medical conditions.

METHODS

STUDY DESIGN, SETTING AND PATIENTS

Study design

We conducted an observational, prospective, multicenter study which included 3 University Hospitals in Lombardy (San Gerardo Hospital in Monza, Policlinico Hospital in Milan and Humanitas Research Hospital in Milan). From March 2019 to August 2021, patients with ≥1 KPC-*Kp* isolate during their hospital stay who met the following eligibility criteria were enrolled. Inclusion criteria were: age 18 and over, ability to sign informed consent; exclusion criteria were: colonization or infection by *Enterobacterales* other than *K. pneumoniae* harboring bla_{KPC} or by *K. pneumoniae* with mechanisms of carbapenem resistance other than KPC (*i.e.* MBL, OXA producing, ESBL/AmpC + porin loss). All patients were identified through the microbiology laboratory surveillance systems and enrolled by trained personnel. After enrollment, blood and fecal samples were obtained together with the routine samples needed for the clinical management. In a subgroup of patients, KPC-*Kp* strains were collected for genomic analyses. Enrolled patients were followed from enrollment to the time of hospital discharge or death. Furthermore, in order to assess the outcome after discharge, a subgroup of patients (Policlinico Hospital) was contacted by phone 1 and 3 months after discharge. For patients hospitalized multiple times during the study period, if they do not develop KPC-*Kp* infection we only considered the first detection of KPC-*Kp* colonization. For patients that progressed from colonization to infection (either within the same hospitalization period or during different hospitalizations), a second blood and fecal sample was taken (Figure 6).

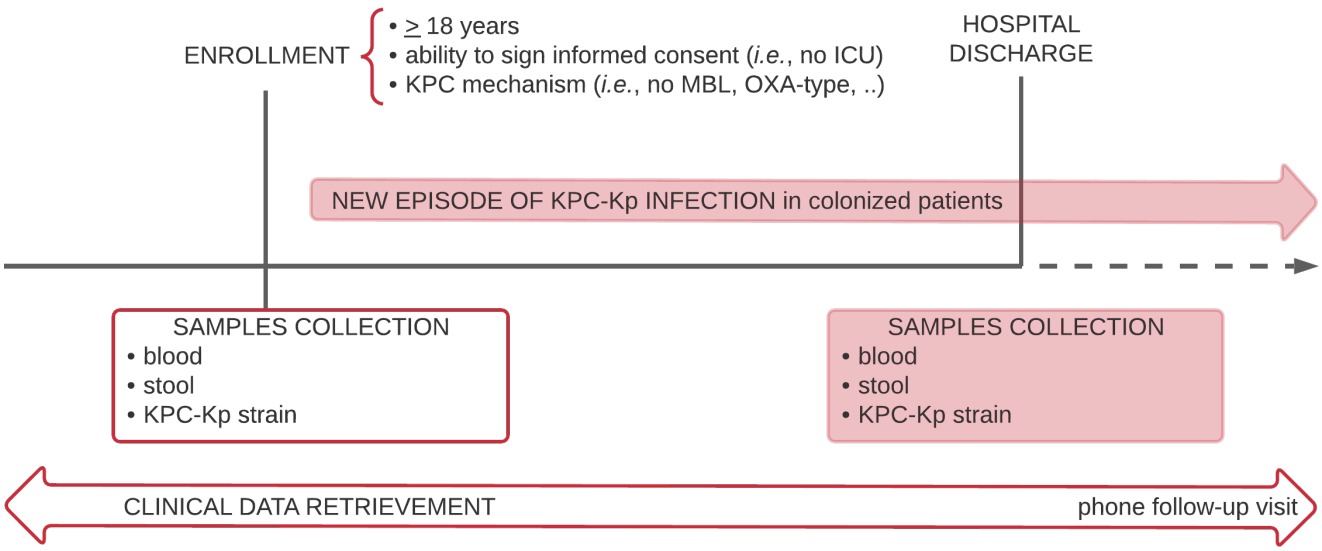
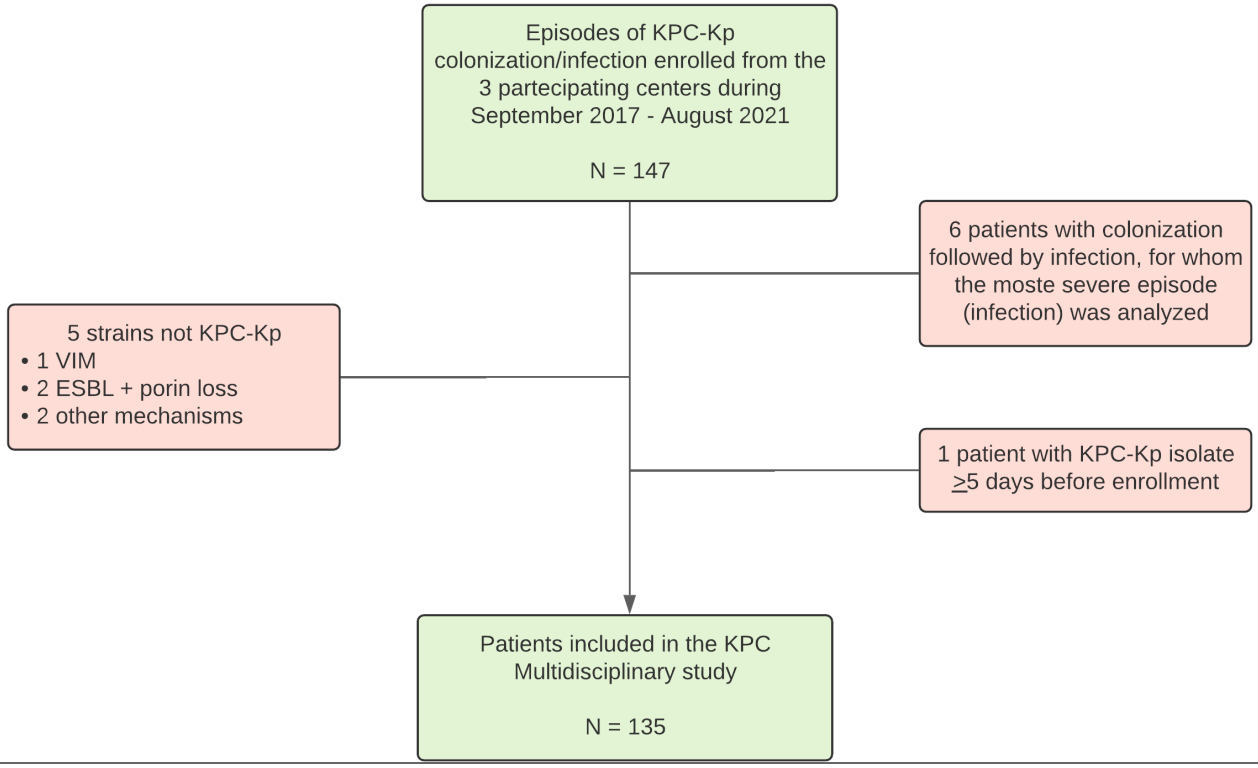


Figure 6. KPC Multidisciplinary study design

Because this was an observational study, treatment of KPC-*Kp* infections was at the discretion of the attending physicians and no change to the center-specific surveillance protocol was required.

The study protocol was first approved by the Research Ethics Committee of the coordinating center (San Gerardo Hospital) and was subsequently approved by the ethics committees of the other participating centers. This study is part of the project *Ricerca Finalizzata 2016* (GR-2016-02362572), which derived from a monocentric study already ongoing at San Gerardo Hospital (approved on 09/08/2017 and deliberated on 12/09/2017). Patients enrolled from September 2017 to May 2018 in the monocentric study (n=27) were therefore included in the analysis. During the study period, only 6 patients present KPC-*Kp* colonization followed by a documented infection. Due to the limited number of this group, only the most severe episode (KPC-*Kp* infection) was included in the analysis. Figure 7 summarizes patient enrollment and laboratory analyses across the participating centers.

Due to COVID-19 emergency, between March – June 2020 and November 2020 – February 2021 (first and second pandemics waves), patient enrollment was temporary interrupted in all the participating centers.



	San Gerardo Hospital	Policlinico Hospital	Humanitas Research Hospital	Overall
enrollment period	09/2017 – 05/2018 10/2019 – 08/2021	03/2019 – 08/2021	10/2019 – 08/2021	09/0217 – 05/2018 03/2019 – 08/2021
patients enrolled	41 24/41 (58.5%) KPC_{COL} 17/41 (41.5%) KPC_{INF}	68 52/68 (76.5%) KPC_{COL} 16/68 (23.5%) KPC_{INF}	26 13/26 (50%) KPC_{COL} 13/26 (50%) KPC_{INF}	135 89/135 (65.9%) KPC_{COL} 46/135 (34.1%) KPC_{INF}
KPC-*Kp* genome analysis	7/41 (17.1%)	30/68 (44.1%)	0/26	37/135 (27.4%)
host immunity analysis	39/41 (95.1%) soluble markers 7/41 (17.1%) cellular markers	54/68 (79.4%) soluble markers 32/68 (47%) cellular markers	25/26 (96.1%) soluble markers 18/26 (69.2%) cellular markers	118/135 (87.4%) soluble markers 57/135 (42.2%) cellular markers
gut microbiome analysis	26/41 (63.4%)	26/68 (38.2%)	17/26 (65.4%)	69/135 (51.1%)

Figure 7. Flow chart of the study population and laboratory analyses

Patient classification and clinical data collection

KPC-*Kp* infections were diagnosed *as per* clinical practice by infectious disease specialists involved in the study protocol. For doubtful cases, we referred to the US CDC criteria (Centers for Diseases Control and Prevention 2014). Infections were classified as KPC-*Kp* bacteremia when: i) a blood

culture was positive for a KPC-*Kp* strain with or without KPC-*Kp* positive cultures from other body sites and ii) the patient had clinical signs of systemic inflammatory response syndrome requiring antimicrobial drug treatment. We defined nonbacteremic KPC-*Kp* infections by: i) documented KPC-*Kp* isolate from nonblood cultures, such as intra-abdominal, urinary, or respiratory samples, ii) absence of KPC-*Kp* positive blood culture during the index hospitalization, and ii) clinical signs of infection.

We classified cases as colonization when: i) $\geq$1 biological sample resulted positive for KPC-*Kp* (surveillance swabs or cultures other than blood) and ii) the patient had no clinical signs of infections caused by KPC-*Kp* requiring antimicrobial treatment.

At enrollment and during hospitalization, data relevant to the definition of clinical status (infection/colonization) and to the outcome (development of invasive infection in colonized patients and mortality/survival) were obtained and recorded in a centralized database. In particular, the following variables were collected: i) center from which the patient was enrolled; ii) patient characteristics: diagnosis at admission, date of admission, age, gender, BMI, ward of admission/KPC-*Kp* isolation, comorbidities (age-adjusted Charlson Comodbidity Index, CCI), immunosuppressive therapy, previous antibiotic therapy, previous hospitalization (or long term facilities stay), previous colonization by MDROs, previous invasive procedures (including surgery or mechanical ventilation), devices in place at enrollment (*i.e.*, naso-gastric tube, central venous catheter, urinary catheter), type of nutrition at enrollment (oral diet/enteral feeding or total parenteral nutrition), duration of hospitalization, time elapsed since hospitalization to KPC-*Kp* isolation, acute medical conditions concomitant to KPC-*Kp* isolation, severity parameters (SOFA score, white blood cells (WBC), C reactive protein (CRP) and procalcitonin (PCT)) at the time of KPC-*Kp* isolation, date of blood and stool sample, severity parameters (SOFA score, WBC, CRP and PCT) at the time of blood sample collection, outcome of the infection, diagnosis at discharge, cause and date of death, date of discharge or transfer to long term facility; iii) infection characteristics: colonization/infection status, date of KPC-*Kp* isolation, in case of colonization reason of screening (*i.e.,* active surveillance, risk factors, contact with infected patients, ongoing outbreak), source of infection.

During the follow-up phone calls at month 1 and 3 after discharge (Policlinico Hospital), patients were asked if any further hospitalization or infection had occurred and the cause of the hospitalization/infection. In this way any episode of KPC-*Kp* infection (including those managed in non-participating centers) in patients previously known to be colonized was reported.

LABORATORY ANALYSES

KPC-*Kp* strains whole genome sequencing

Genetic characterization of KPC-*Kp* strains was conducted at the Microbiology and Virology Unit of ASST Grande Ospedale Metropolitano Niguarda and the University of Milan.

Total DNA extraction was performed for the selected isolates using the QIAamp DNA mini kit (Qiagen, Italy) according to manufacturer's instructions. DNA was sequenced using the Illumina Nextseq 550 platform (Illumina Inc., San Diego, CA, United States), with paired-end runs of 2 × 250 bp, after Nextera XT library preparation, obtaining a 99.98% of mapped reads with a deep of 250x for genome reconstruction and characterization. Paired end files have been preprocessed with FASTP software (Chen 2018) in order to remove low quality reads or artificial sequences such as primer and adapters, which could negatively affect genome reconstruction and characterization. To achieve this, a minimum quality Phred score threshold of 28 has been imposed such that reads ends with lower quality or reads with average lower quality are discharged. Since *K. pneumoniae* clinical isolates present a greatly varying genomic content, we opted for a *de-novo* genome assembly thanks to which additional genome content (putatively involved in clinical manifestations and not yet deposed in public database) can be assembled and characterized. Since one of the most important factors involved in a *de-novo* genome reconstruction consist of read's length, pre-processed reads have been merged in longer strings thanks to Flash2 software (Magoč 2011) which exploits read quality information in order to produce the longest merge among all the paired reads . Reads merging lead to a smaller subset of longer sequences which are more suitable for a *de-novo* reconstruction since merged reads length is maximized and low complex and thus confounding sequences are removed. Longer merged reads have been then used to reconstruct original genome structure with SPADEs software (Bankevich 2012) which provide a *de-novo* genome assembly based on reads terminal overlapping information which are treated as node of a De-Brujin Graph and the most probable path into this graph return the most probable genome assembly. Since the most important parameter in a *de-novo* genome assembly consist of k-mer length, we launched SPADEs with a k-mer grid search of 33,47,59,71,79 in order to allow the automatic detection of the most suitable value. Each assembled genome has been passed to PILON software (Walker 2014) which improves genome assemblies by using intrinsic reads quality information in combination with reconstructed genome in order to resolve genome ambiguous locations which are the main bottleneck for long scaffold reconstruction.

Clinically relevant features of KPC-*Kp* strains, such as virulence and resistance genes have been characterized thanks to Kleborate software (Lam 2021). Clonal typing of retrieved isolates is also provided by this software which rely on well-known typing schema such as MLST and antigenic KO locus typing to determine phylogenetic relationship between isolates and support, in such a way, downstream route of transmission analysis. Antimicrobial resistance mechanisms for each isolated have also been checked with ABRicate software (https://github.com/tseemann/abricate), which provides assembly mass screening for antimicrobial resistance or virulence genes deposed in public databases such as CARD (Jia 2017), ARG-ANNOT (Gupta 2014), Resfinder (Bortolaia 2020), MEGARES (Doster 2020), PlasmidFinder (Carattoli 2020) and VFDB (Chen L 2005).

Using Kleborate software, virulence and resistance scores were calculated as follow. Virulence score ranges from 0 to 5 (0 = negative for all of yersiniabactin (ybt), colibactin (clb), aerobactin (iuc), 1 = yersiniabactin only, 2 = yersiniabactin and colibactin (or colibactin only), 3 = aerobactin (without yersiniabactin or colibactin), 4 = aerobactin with yersiniabactin (without colibactin), 5 = yersiniabactin, colibactin and aerobactin). In the present analysis, KPC-*Kp* strains were divided in two groups (virulence score 0 vs $\geq$1). Resistance score ranges from 0 to 3 (0 = no ESBL, no carbapenemase (regardless of colistin resistance), 1 = ESBL, no carbapenemase (regardless of colistin resistance), 2 = Carbapenemase without colistin resistance (regardless of ESBL genes or OmpK mutations), 3 = Carbapenemase with colistin resistance (regardless of ESBL genes or OmpK mutations)). In the present analysis, KPC-*Kp* strains were divided in two groups (resistance score 2 vs 3).

Phylogenetic relationships among isolates has been investigating through a core single nucleotide polymorphism (SNP) genome alignment used to infer the most probable phylogenetic tree. Core SNP alignment has been produced with kSNP3 software (Gardner 2015), which detects a subset of k-mers identical in all strains except for a single nucleotide (SNPs) in their middle which vary among them and that can be used as phylogenetic signal. SNPs are concatenated into a FASTA sequence and the sequences are aligned each other with MAFFT software (Katoh 2002) and resulting multiple sequence alignment is used as input for IQtree2 (Chernomor 2016) to produce a phylogenetic tree with branch topology robustness described by support values.

Cellular and humoral immune markers analysis

Analyses of innate and adaptive immune responses were conducted at the Department of Inflammation and Immunology of Humanitas Research Hospital.

For the *ex vivo* immunophenotyping experiments, frozen samples were used and were thawed in R10 supplemented with 20 µg/ml DNase I from bovine pancreas (Sigma-Aldrich). After extensive washing with PBS (Sigma-Aldrich), the cells were stained immediately with the Zombie Aqua Fixable Viability kit (BioLegend) for 15 min at room temperature. Then, the cells were washed and stained with the combination of mAbs purchased from either BD Biosciences, BioLegend, or eBioscience. mAbs were previously titrated to define the optimal concentration. Chemokine receptors were stained for 20 min at 37°C, while all other surface markers were stained for 20 min at room temperature. All data were acquired on a FACS Symphony A5 flow cytometer (BD Biosciences) equipped with five lasers (UV, 350 nm; violet, 405 nm; blue, 488; yellow/green, 561 nm; red, 640 nm; all tuned at 100 mW, except UV tuned at 60 mW) and capable to detect 30 parameters. Flow cytometry data were compensated in FlowJo by using single stained controls (BD Compbeads incubated with fluorescently conjugated antibodies). Flow Cytometry Standard (FCS) 3.0 files were imported into FlowJo software version 9 and analyzed by standard gating to remove aggregates and dead cells, and identify different cell populations.

PTX3 plasma levels were measured by means of a home-made Sandwich ELISAs assay based on original reagents developed in Humanitas Research Hospital (detection limit 0.1 ng/mL, inter-assay variability from 8% to 10%), as previously described (Brunetta 2021). No cross-reaction with other members of the pentraxin superfamily (*i.e.*, CRP) is reported. Soluble IL-1R2 (sIL-1R2) was measured with a commercial ELISA assay (Human IL-1R2 Quantikine ELISA kit, code DR1B00, R&D Systems) according to instructions provided by supplier. Each plasma sample was measured in duplicate by staff blinded to patient's characteristics.

Gut microbiome sequencing

Gut microbiota profiling was characterized at the Department of Inflammation and Immunology of Humanitas Research Hospital.

Total microbial DNA from all the fecal samples collected has been extracted as previously described (Consolandi 2015), since the protocol herein described was specifically modified to allow an efficient and unbiased bacterial DNA extraction from human fecal samples. Genomic DNA quality was assessed by using the TapeStation 2200 system (Agilent, Santa Clara, CA, USA). Only samples having a DNA Integrity Number (DIN) > 4 were used for successive analyses.

For each sample, the V3–V4 region of the 16S rRNA gene was PCR-amplified by using primers carrying overhanging adapter sequences (primer selection originally described in (Klindworth

2013)), following the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina MiSeq System 2013) (Illumina, San Diego, CA, USA), and libraries were barcoded using dual Nextera® XT indexes (Illumina). Indexed libraries were pooled at equimolar concentrations and sequenced on a HiSeq 2500 Illumina sequencing platform generating 2 × 250 bp paired-end reads, according to manufacturer's instructions (Illumina).

The obtained 16S data from fecal samples were filtered of contaminant reads from the host with mapping on GRCh38 human genome with Bowtie2 software and were trimmed and quality checked with Cutadapt and fastqc tools. The microbiota analysis was performed with QIIME2 software. Alpha diversity values were expressed using Pielou's eveness index. Comparisons were assessed through Wilcoxon Rank-sum test or Kruskal-Wallis test, depending on the number of groups. Beta diversity was assessed as principal coordinates analysis (PCoA) of Unweighted Unifrac distances, with pairwise permanova test for comparison between groups. The figures were generated with R software.

STATISTICAL ANALYSIS

Categorical variables are presented as frequency and proportion (%), and continuous variables as mean, median and interquartile range (IQR: Q1-Q3). Normality of continuous variables was assessed graphically and tested through Kolmogorov-Smirnov test and Shapiro-Wilk test, depending on sample size. We used $\chi 2$ or Fisher's exact tests to compare categorical variables and the T-test or Wilcoxon Rank-sum test to compare continuous variables, depending on variables distribution.

For immunological and microbiome data, the comparisons between the two groups of infected and colonized patients was also performed taking into account the time elapsed between KPC-*Kp* isolation and blood or stool sample as potential confounder. The time-adjusted analysis of covariance was used to evaluate differences between the two groups, with rank-transformed variables when the distributions were not normal (Rank ANCOVA).

Values of $p \leq 0.05$ were considered statistically significant; SAS 9.4 software (Inc., Cary, NC, USA) was used for all analysis.

RESULTS

PATIENT CHARACTERISTICS

Overall, 135 patients were enrolled from the three participating centers. The median age among patients was 71 (Q1-Q3: 60–81) years, 66.7% were males and 33.3% females, median Charlson comorbidity index (CCI, indicating patient frailty for underlying medical conditions) was 6 (Q1-Q3: 4-7). Fifty-eight patients (43.9%) were immunocompromised, mostly from onco-hematological diseases (34.5%), solid organ transplant (31%) or high-dose (prednisone >20mg for ≥4 weeks) steroid treatment (32.8%). Most patients were hospitalized in the year before KPC-*Kp* isolation (104/132, 78.8%) and received antibiotic therapy in the previous 30 days (114/131, 87%). Slightly less than half of the study population (58/124, 46.8%) was known for previous colonization or infection by MDROs. A considerable proportion of patients had undergone major surgery and mechanical ventilation in the 30 days prior to KPC-*Kp* isolation (33/135, 24.4% and 34/132, 25.7%, respectively).

At KPC-*Kp* detection, median time of hospitalization was 15 (Q1-Q3: 6-32) days. The most frequent inpatient setting were medical wards (69/135, 51.1%), followed by surgical and sub-intensive care units (33/135, 24.45 and 27/135, 20%, respectively). Onco-haematological units accounted for only 6/135 (4.5%) cases. Site of KPC-*Kp* isolation varied depending on clinical severity (see below), with surveillance swabs (65/135, 48.2%), urine (36/135, 26.7%) and blood (18/135, 13.3%) being the most frequent. At the time of KPC-*Kp* detection, 102/119 (85.7%) patients suffered from acute medical conditions, either non-infectious (*i.e.,* early postoperative period, acute kidney failure, decompensated diabetes, cardiogenic pulmonary edema) or infections due to pathogens other than KPC-*Kp*. Broad spectrum antibiotic therapy was ongoing in 93/128 (72.7%) patients, and 48/123 (39%) of them had a concomitant infection or colonization by MDROs other than KPC-*Kp*. Median time length from KPC-*Kp* detection to blood samples and fecal samples collections for immunological and gut microbiome analysis was 5 (Q1-Q3: 4-7) days and 6 (Q1-Q3: 4-9) days, respectively. During hospital stay, 6/130 (4.6%) patients died. For survived patients, median length of hospitalization was 15 (Q1-Q3: 8-25) days. Among the subgroup of patients that undergone follow up phone calls, death or new hospital admittance occurred in 8/59 (13.5%) within month 1

and in 13/45 (28.9%) within month 3. Supplementary Table 1 summarizes demographic and clinical characteristics of the study population.

Among the 135 patients enrolled, 89/135 (65.9%) were colonized by KPC-*Kp*, 46/135 (34.1%) were infected. Table 5 describes demographic and clinical characteristics of patients with KPC-*Kp* colonization and infection. Time between hospital admittance and enrollment resulted longer in colonized compared to infected patients, with median length of 18.5 (Q1-Q3: 8-37) days vs 10 (Q1-Q3: 5-24) days, respectively (p 0.004). This result partly reflects the fact that we have included no patients from the ICU, where surveillance is intended to be weekly on all admitted patients, and only 4.5% patients from onco-haematological units, where active screening is often adopted. In the hospital settings in analysis, MDROs surveillance was carried out for various reasons depending on hospital protocols (*i.e.*, active screening, risk factors, possible contact with colonized patients), which may therefore lead to later identification. As expected, biological samples where KPC-*Kp* was isolated differed significantly between the two groups (p <0.001). Colonized patients were identified mostly through surveillance swabs (64/89, 71.9%) or urinary samples (20.2%). About 78% of KPC-*Kp* infections were BSI or urinary tract infections (18/46, 39.1% per site). In one case KPC-*Kp* infection was diagnosed through surveillance swab (*i.e.*, neutropenic fever in hematological patient with no other isolates and clinical response to anti-KPC-*Kp* therapy).

Invasive procedures in the 72 hours prior to KPC-*Kp* isolation (*i.e.*, surgery, endoscopic procedures, gastrostomy or chest tube insertion) were more frequent in infected than in colonized patients (16/44, 36.4% compared to 16/89, 18%, p 0.03). Conversely, concomitant infection or colonization by MDROs other than KPC-*Kp* resulted higher in colonized than in infected patients (38/84, 45.2% compared to 10/39, 25.6%, p 0.038). This is likely related to the different biological samples used to detect KPC-*Kp* between the two groups. KPC-*Kp* colonization was largely diagnosed during surveillance screening, which can detect not only KPC-*Kp* but also other concomitant MDROs. On the contrary, in infected patients KPC-*Kp* was diagnosed in invasive biological samples, which only reported the pathogen(s) responsible for the acute infection. Notably, the number of patients with acute medical conditions at KPC-*Kp* isolation resulted similar in both groups at high percentages (67/78 (85.9%) of colonized patients and 35/41 (85.5%) of infected patients), as was the number of patients with ongoing antibiotic therapy (60/85 (70.6%) of colonized patients and 33/43 (76.7%) of infected patients). Among infected patients, sepsis occurred in 18/44 (40.9%) of cases at presentation. Compared to those with colonization, patients with KPC-*Kp* infection were characterized by significantly higher levels of inflammatory markers (white blood cells count, C-

reactive protein (CRP) and procalcitonin) and worse values of renal function both at time of KPC-*Kp* isolation and at collection of samples for immunological and microbiome analyses (Table 5). In-hospital mortality resulted low in both groups with no significant differences (3/89 (3.4%) in colonized patients and 3/46 (6.5%) in infected patients). Among survived patients, length of hospital stay was longer for those with KPC-*Kp* infection than for colonized ones, with 22 (Q1-Q3: 15-28) days compared to 14 (Q1-Q3: 7-21) days from KPC-*Kp* isolation (p<0.001).

	N	KPC_{COL}	N	KPC_{INF}	*p value°*
Epidemological characteristics					
Age	89	69 (58-79)	46	76.5 (64-84)	ns
Gender female	89	31 (34.8)	46	14 (30.4)	ns
BMI	35	24.5 (21.6-27)	19	24 (22-28)	ns
Charlson comorbidity index (age-adjusted)	89	5 (4-7)	46	6 (4-7)	ns
Anamnestic factors					
Hospitalization for more than 48 hours in the 12 months prior to KPC-*Kp* isolation	87	67 (77)	45	37 (82.2)	ns
Major surgery in the 30 days prior to KPC-*Kp* isolation	89	23 (25.8)	46	10 (21.7)	ns
Mechanical ventilation in the 30 days prior to KPC-*Kp* isolation	88	24 (27.3)	44	10 (22.7)	ns
Invasive procedures in the 72 hours prior to KPC-*Kp* isolation	89	16 (18)	44	16 (36.4)	0.030
Treatment with broad-spectrum antibiotics in the 30 days prior to KPC-*Kp* isolation	88	79 (89.8)	43	35 (81.4)	ns
Previous colonization/infection with MDROs	84	42 (50)	40	16 (40)	ns
Immunocompromised in the 30 days before KPC-*Kp* isolation • AIDS • steroids • oncohaematologic diseases & chemotherapy • SOT & immunosuppressive therapy	88	41 (46.6) 1 (2.5) 12 (29.3) 14 (34.1) 14 (34.1)	44	17 (38.6) 0 7 (41.2) 6 (35.3) 4 (23.5)	ns
Diagnosis at hospital admittance • infection • acute noninfectious condition • chronic noninfectious condition	89	50 (56.2) 22 (24.7) 17 (19.1)	46	25 (54.3) 13 (28.3) 8 (17.4)	ns
Clinical characteristics at KPC-*Kp* isolation					
Days between hospital admittance and enrollment	88	18.5 (8-37)	41	10 (5-24)	0.004
Ward of KPC-*Kp* isolation • Medical Units • Surgical Units • Sub-intensive care Units • Onco-haematological Units	89	46 (51.7) 18 (20.2) 21 (23.6) 4 (4.5)	46	23 (50) 15 (32.6) 6 (13) 2 (4.4)	ns
Site of KPC-*Kp* isolation • blood • urine	89	0 18 (20.2)	46	18 (39.1) 18 (39.1)	<0.001

• respiratory samples • abdominal samples • surveillance swab		3 (3.4) 4 (4.5) 64 (71.9)		3 (6.6) 6 (13) 1 (2.2)	
Nutrition support • Oral diet/enteral feeding • Parenteral nutrition	76	 71 (93.4) 5 (6.6)	36	 33 (91.7) 3 (8.3)	ns
Presence of invasive devices	86	66 (76.7)	43	38 (88.4)	ns
Acute medical condition	78	67 (85.9)	41	35 (85.4)	ns
Ongoing antibiotic therapy	85	60 (70.6)	43	33 (76.7)	ns
Colonization/infection with other MDROs	84	38 (45.2)	39	10 (25.6)	0.038
Severity scores and parameters at KPC-*Kp* isolation					
SOFA score • 0-1 • 2-8	44	 21 (47.7) 23 (52.3)	17	 7 (41.2) 10 (58.8)	ns
If KPC-*Kp* infection, sepsis at presentation		-	44	18 (40.9)	-
Leucocytes, n/mmc	88	7655 (5030-10185)	43	10530 (7170-14940)	0.002
C-reactive protein, mg/dl	84	5.2 (1.7-10.3)	42	9.9 (5.4-17)	<0.001
Procalcitonin, ng/ml	18	0.21 (0.07-1.49)	15	1.96 (0.35-11.2)	0.004
Creatinine clearance (CKD EPI), ml/min	87	80 (62-97)	42	52.3 (34-81)	0.001
Severity scores and parameters at sample collection					
SOFA score • 0-1 • 2-8	39	 20 (51.3) 19 (48.7)	15	 8 (53.3) 7 (46.7)	ns
Leucocytes, n/mmc	88	6520 (4605-9285)	44	7940 (5280-11660)	ns
Lymphocytes, n/mm	83	1230 (760-1820)	40	1215 (970-1855)	ns
C-reactive protein, mg/dl	83	2 (1-4.7)	43	7.7 (3.8-13.5)	<0.001
Procalcitonin, ng/ml	20	0.15 (0.1-‘0.68)	18	2.06 (0.37-3.02)	0.002
Creatinine clearance (CKD EPI), ml/min	84	81.3 (55.7-99)	44	64 (49.2-87.6)	0.050
Days between KPC-*Kp* isolation and blood sample collection	89	5 (4-7)	46	5.5 (4-7)	ns
Days between KPC-*Kp* isolation and fecal sample collection	75	6 (4-9)	34	7 (4-12)	ns
Outcome					
In-hospital mortality*	86	3 (3.5)	44	3 (6.8)	ns
Length of hospital stay in survived patients, days from KPC-*Kp* isolation*	83	14 (7-21)	41	22 (15-28)	<0.001
Death or new hospital admittance at month 1^	45	4 (8.9)	14	4 (28.6)	ns
Death or new hospital admittance at month 3^	35	9 (25.7)	10	4 (40)	ns

Legend: KPC_{COL} patients with KPC-*Kp* colonization, KPC_{INF} patients with KPC-*Kp* infection, BMI body mass index, MDROs multidrug resistant organisms, AIDS acquired immune deficiency syndrome, SOT solid organ transplant, SOFA score sequential (sepsis-related) organ failure assessment score, CKD EPI chronic kidney disease epidemiology collaboration (equation to estimate glomerular filtrate rate). Categorical variables are expressed as counts and percentages and continuous variables as medians and interquartile ranges. °Chi-square or Fisher exact test p-value for categorical variables and Wilcoxon Rank-sum test p-value for continuous variables, *Five patients (3 colonized, 2 infected) were still hospitalized at the time of analysis and were removed from the analysis of outome, ^subgroup analysis of patients who underwent follow up phone calls

Table 5. Demographic and clinical characteristics of colonized and infected patients

DISTRIBUTION, PHYLOGENY, AND VIRULENCE AND RESISTANCE GENES OF KPC-*Kp* CLONES

Thirty-seven KPC-*Kp* strains were analyzed. Of them, 30/37 (81%) were provided by Policlinico Hospital while 7/37 (18.9%) by San Gerardo Hospital (Figure 7).

Eight different STs were identified. The most numerous clones were ST307 in 12/37 (32.4%), ST512 in 9/37 (24.3%) and ST20 in 8/37 (21.7%) isolates. Two KPC variants were found, KPC-2 in 13/37 (35.1%) and KPC-3 in 24/37 (64.9%) isolates. KPC-2 was absent in ST512 and ST20 but predominant in ST307 and ST258. Table 6 reports STs distribution stratified according to *wzi* locus for capsular biosynthesis and bla_{KPC} enzyme variants among the 37 KPC-*Kp* strains analyzed.

Sequence Type	*wzi* variant	bla_{KPC} variant	number of isolates (%)
ST20	*wzi*24	KPC-3	8 (21.7)
ST45	*wzi*101 *wzi*149	KPC-3 KPC-3	1 (2.7) 1 (2.7)
ST101	*wzi*137	KPC-2	1 (2.7)
ST258	*wzi*29	KPC-2	3 (8.1)
ST307	*wzi*173	KPC-2 KPC-3	9 (24.3) 3 (8.1)
ST405	*wzi*143	KPC-3	1 (2.7)
ST512	*wzi*154	KPC-3	9 (24.3)
ST2648	*wzi*490	KPC-3	1 (2.7)

Legend: ST sequence type, *wzi* locus for capsular biosynthesis, bla_{KPC} enzyme variants of KPC carbapenemases

Table 6. Correspondence among ST, wzi and blaKPC enzyme variants produced by the 37 KPC-Kp isolates analyzed

Phylogenetic tree of the KPC-*Kp* genomes using core SNP genome alignment is represented in Figure 8 and supplementary Figure 1. In order to contextualize phylogenetic relationships between our strains and publicly deposed ones, additional KPC-Kp isolates have been retrieved from NCBI by considering European strains found to be of clinical interest and collected in the years 2019-2020 (Figure 9). These strains IDs are reported in supplementary Table 2).

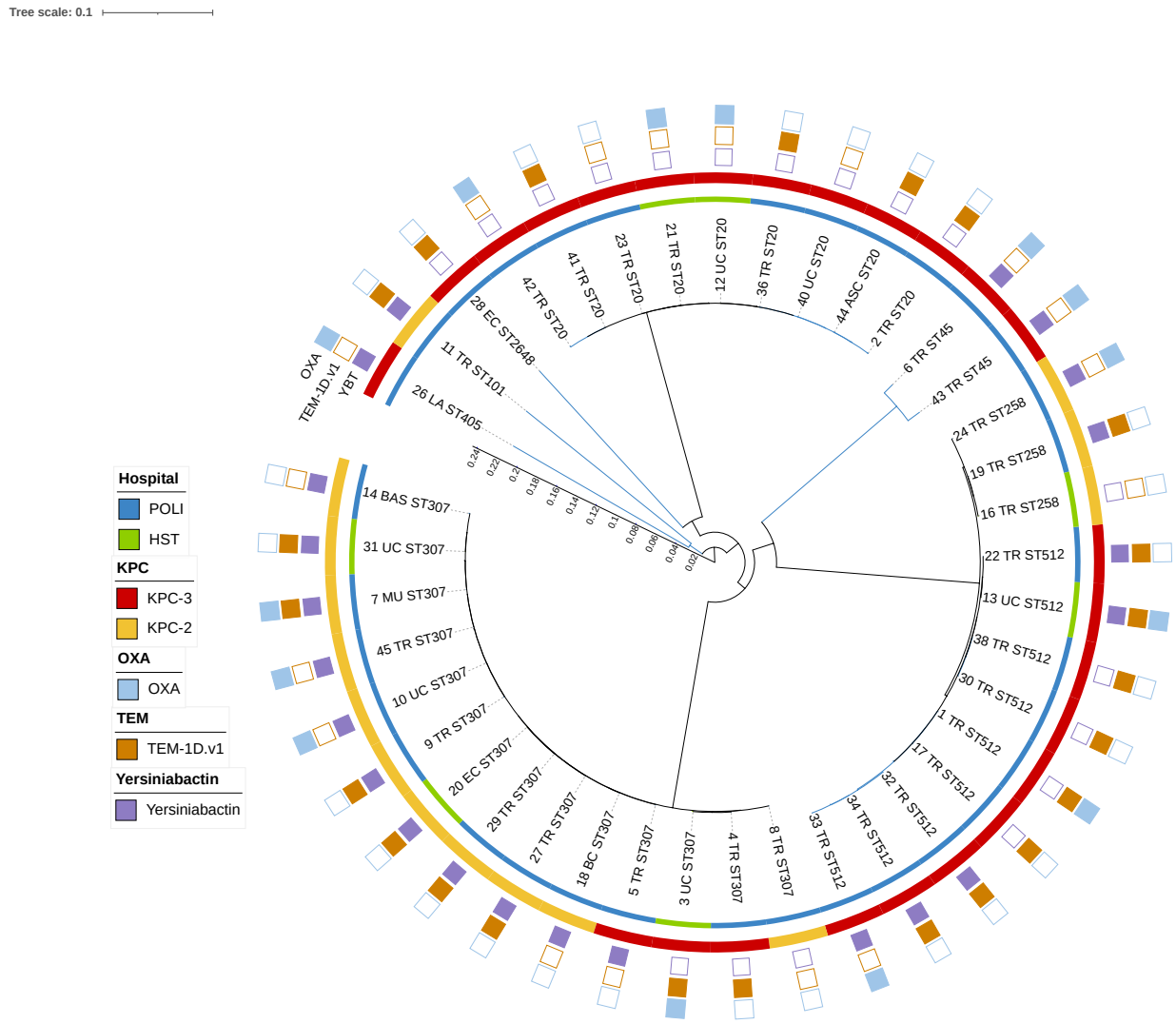


Figure 8. Phylogenetic tree base on core SNPs alignment of the 37 KPC-Kp isolates analyzed. Inner circle shows hospitals from which strains were isolated; middle circle shows the KPC-Kp mechanism identified; and outer circle shows the presence of selected resistance factors (betalactamases OXA and TEM) and the virulence factor Yersiniabactin (YBT) (see: supplementary Tables 2 and 3)

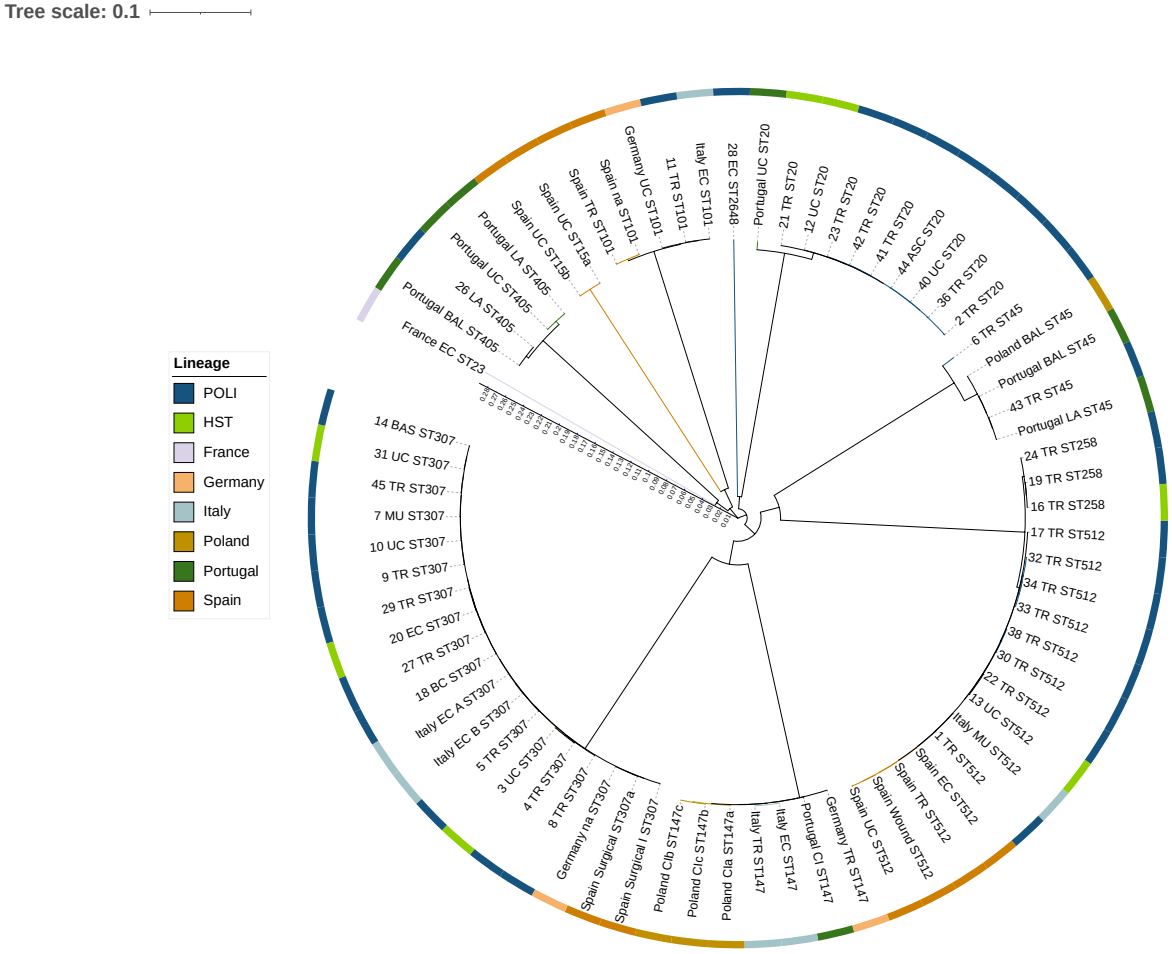


Figure 9. Phylogenetic tree base on core SNPs alignment of the 37 KPC-Kp isolates analyzed, together with clinically relevant KPC-Kp isolates retrieved from literature (see: supplementary Table 1).

For each KPC-*Kp* strain, major virulence and resistance genes were characterized (supplementary Table 3 and supplementary Table 4) and virulence and resistance scores were calculated as explained above (see: METHODS – LABORATORY ANALYSES: "KPC-*Kp* strains whole genome sequencing"). Seventeen out of 37 (45.9%) KPC-*Kp* strains did not present any of the virulence factors in analysis (virulence score 0), while for 20/37 (54.1%) isolates virulence score was $\geq$1. Table 7 describes clinical and bacterial genome characteristics in the two groups. Interestingly, carbapenemase *bla*$_{KPC}$ enzyme distribution differed between groups, with KPC-2 in 2/17 (11.8%) isolates with virulence score 0 and 11/20 (55%) isolates with virulence score $\geq$1, KPC-3 in 15/17 (88.2%) isolates with virulence score 0 and 9/20 (45%) isolates with virulence score $\geq$1.

	virulence score 0 N=17	viruence score ≥1 N=20
Demographic and clinical characteristics		
Age	64 (56-80)	68 (59-79)
Charlson comorbidity index (age-adjusted)	5 (4-6)	6 (4-7.5)
Hospitalization for more than 48 hours in the 12 months prior to enrollment	14 (87.5)	20 (100)
Treatment with broad-spectrum antibiotics in the 30 days prior to enrollment	15 (88.2)	17 (85)
Previous colonization/infection with MDROs	9 (52.9)	8 (40)
Site of KPC-*Kp* isolation		
• blood	1 (5.9)	2 (10)
• urine	2 (11.7)	3 (15)
• respiratory samples	0	2 (10)
• abdominal samples	1 (5.9)	1 (5)
• surveillance swab	13 (76.5)	12 (60)
Ongoing antimicrobial therapy	13 (81.2)	12 (63.2)
Colonization/infection with other MDROs	7 (53.8)	8 (47.1)
Leucocytes at KPC-*Kp* isolation, n/mmc	7800 (5170-10530)	5820 (3445-8870)
C-reactive protein at KPC-*Kp* isolation, mg/dl	4.3 (2.2-10.6)	9.1 (1.9-11.6)
Leucocytes at sample collection, n/mmc	7390 (4540-7610)	6210 (3660-8110)
C-reactive protein at sample collection, mg/dl	2.6 (1.7-4)	5.8 (2.4-10.1)
Death	0	1 (5)
KPC-*Kp* genome characteristics		
Sequence Type		
• ST20	8 (47)	0
• ST45	0	2 (10)
• ST101	0	1 (5)
• ST258	1 (5.9)	2 (10)
• ST307	3 (17.6)	9 (45)
• ST405	0	1 (5)
• ST512	4 (23.6)	5 (25)
• ST2648	1 (5.9)	0
K locus		
• KL8	1 (5.9)	0
• KL17	0	1 (5)
• KL24	8 (47.1)	1 (5)
• KL62	0	1 (5)
• KL102	3 (17.6)	9 (45)
• KL106	1 (5.9)	2 (10)
• KL107	4 (23.5)	5 (25)
• KL151	0	1 (5)
O type		
• O1	9 (52.9)	1 (5)
• O2	8 (47.1)	16 (80)
• O4	0	1 (5)
• other	0	2 810)
wzi locus		
• *wzi*24	8 (47.1)	0

• *wzi*29	1 (5.9)	2 (10)
• *wzi*101	0	1 (5)
• *wzi*137	0	1 (5)
• *wzi*143	0	1 (5)
• *wzi*149	0	1 (5)
• *wzi*154	4 (23.5)	5 (25)
• *wzi*173	3 (17.6)	9 (45)
• *wzi*490	1 (5.9)	0
bla_{KPC}		
• KPC-2	2 (11.8)	11 (55)
• KPC-3	15 (88.2)	9 (45)

Legend: MDROs multidrug resistant organisms, SOFA score sequential (sepsis-related) organ failure assessment score. Categorical variables are expressed as counts and percentages and continuous variables as medians and interquartile ranges (IQRs)

*Table 7. Demographic and clinical parameters and KPC-*Kp *genome characteristics according to virulence score*

As for resistance factors, 12/37 (32.4%) isolates presented mutations associated with colistin resistance in addition to carbapenemases (resistance score 3), while the remaining 25/37 (67.6%) did not (resistance score 2). Table 8 describes clinical and bacterial genome characteristics in the two groups. Interestingly, carbapenemase bla_{KPC} enzyme distribution differed between groups, with KPC-2 in 13/25 (52%) isolates with resistance score 2 and 0 isolates with resistance score 3, KPC-3 in 12/25 (24%) isolates with resistance score 2 and 12/12 (100%) isolates with resistance score 3 (p 0.002).

	resistance score 2 N=25	**resistance score 3 N=12**
Demographic and clinical characteristics		
Age	69 (60-80)	60.5 (52.5-81.5)
Charlson comorbidity index (age-adjusted)	6 (4-7)	5 (4-7.5)
Hospitalization for more than 48 hours in the 12 months prior to enrollment	23 (92)	11 (100)
Treatment with broad-spectrum antibiotics in the 30 days prior to enrollment	20 (80)	12 (100)
Previous colonization/infection with MDROs	11 (44)	6 (50)
Site of KPC-*Kp* isolation		
• blood	3 (12)	0
• urine	4 (16)	1 (8.3)
• respiratory samples	2 (8)	0
• abdominal samples	1 (4)	1 (8.3)
• surveillance swab	15 (60)	10 (83.4)
Ongoing antimicrobial therapy	17 (70.8)	8 (72.7)
Colonization/infection with other MDROs	12 (60)	3 (30)
Leucocytes at KPC-*Kp* isolation, n/mmc	6970 (3650-10050)	5890 (3800-9450)
C-reactive protein at KPC-*Kp* isolation, mg/dl	8.4 (2.2-11.4)	3.4 (1.9-17.5)
Leucocytes at sample collection, n/mmc	7110 (4880-8060)	7230 (3660-8290)

C-reactive protein at sample collection, mg/dl	4.87 (1.8-9.8)	2.9 (1.7-4)
Death	1 (4)	0
KPC-*Kp* genome characteristics		
Sequence Type		
• ST20	0	8 (66.7)
• ST45	2 (8)	0
• ST101	1 (4)	0
• ST258	3 (12)	0
• ST307	12 (48)	0
• ST405	1 (4)	0
• ST512	5 (20)	4 (33.3)
• ST2648	1 (4)	0
K locus		
• KL8	1 (4)	0
• KL17	1 (4)	0
• KL24	1 (4)	8 (66.7)
• KL62	1 (4)	0
• KL102	12 (48)	0
• KL106	3 (12)	0
• KL107	5 (12)	4 (33.3)
• KL151	1 (4)	0
O type		
• O1	2 (8)	8 (66.7)
• O2	20 (80)	4 (33.3)
• O4	1 (4)	0
• other	2 (8)	0
wzi locus		
• *wzi*24	0	8 (66.7)
• *wzi*29	3 (12)	0
• *wzi*101	1 (4)	0
• *wzi*137	1 (4)	0
• *wzi*143	1 (4)	0
• *wzi*149	1 (4)	0
• *wzi*154	5 (20)	4 (33.3)
• *wzi*173	12 (48)	0
• *wzi*490	1 (4)	0
bla_{KPC}		
• KPC-2	13 (52)	0
• KPC-3	12 (48)	12 (100)

Legend: MDROs multidrug resistant organisms, SOFA score sequential (sepsis-related) organ failure assessment score. Categorical variables are expressed as counts and percentages and continuous variables as medians and interquartile ranges (IQRs)

Table 8. Demographic and clinical parameters and KPC-Kp genome characteristics according to resistance score

KPC-*Kp* genome characteristics of the 30 isolates from patients enrolled at Policlinico Hospital, overall and divided for colonized (26/30) and infected (4/30) patients, are reported in supplementary Table 5.

HOST IMMUNITY

Circulating soluble inflammatory markers PTX3 and sIL-1R2 were measured in 118 patients, 79 with KPC-*Kp* colonization and 39 with KPC-*Kp* infection. For 17 samples, results were not available (analysis not yet concluded or not performed due to low quality of samples). Immunophenotype of circulating cells was assessed in 58 patients, 37 with KPC-*Kp* colonization and 21 with KPC-*Kp* infection. For 77 samples, results were not available (analysis not yet concluded or not performed due to low quality of samples). Values of immunological parameters of the entire study population are reported in supplementary Table 6.

Among KPC-*Kp* patients, median values of PTX3 and sIL-1R2 were 16.13 (Q1-Q3: 10.04-25.57) ng/ml and 17.02 (Q1-Q3: 11.98-24.95) ng/ml, respectively. No significant differences were found between colonized and infected patients (PTX3: 14.63 (Q1-Q3: 9.78-23.36) vs 18.87 (Q1-Q3: 10.58-39.51) ng/ml; sIL-1R2: 16.01 (Q1-Q3: 11.6-24.2) vs 20.64 (Q1-Q3: 13.63-25.04) ng/ml).

Immunophenotypic analysis revealed differences in subpopulations of circulating T helper cells in the peripheral blood of colonized and infected patients, with KPC-*Kp* infection associated with lower percentages of circulating Th1, Th2 and Th17 compared to KPC-*Kp* colonization (Th1: 4.43 (Q1-Q3: 1.16-9.01) vs 8.48 (Q1-Q3: 4.29-17.1) % CD4+ T cells, p 0.017; Th2 4.26 (Q1-Q3: 2.40-9.73) vs 9.38 (Q1-Q3: 4.29-17.1) % CD4+ T cells, p 0.037; Th17 7.89 (Q1-Q3: 3.55-15.42) vs 16.6 (Q1-Q3: 7.09-38.8) % CD4+ T cells, p 0.035). No significant differences were found in the fraction of T regulatory cells and follicular helper T cells, nor in MAIT cells and in the subpopulations of innate lymphoid cells ILC1, ILC2, ILC3 (Table 9).

	KPC_{COL}	**KPC_{INF}**	***p value°***
Circulating soluble inflammatory markers*			
PTX3, ng/ml	14.63 (9.78-23.36)	18.87 (10.58-39.51)	ns
sIL-1R2, ng/ml	16.01 (11.6-24.2)	20.64 (13.63-25.04)	ns
Circulating cell immunophenotype^			
Th1, % CD4+ T cells	8.49 (4.29-17.1)	4.04 (1.04-8.12)	0.009
Th2, % CD4+ T cells	9.38 (4.14-21.8)	4.08 (2.21-9.47)	0.023
Th17, % CD4+ T cells	16.6 (7.09-38.8)	6.99 (2.8-15.1)	0.020
Treg, % CD4+ T cells	5.08 (3.03-6.68)	5.95 (5.23-7.94)	0.052
Tfh, % CD4+ T cells	4.78 (2.51-7.39)	4.66 (2.11-6.32)	ns
MAIT cells, % TCRab	0.68 (0.25-1.38)	0.47 (0.24-1.75)	ns

ILC1, %LIN neg CD127+CD161+	11.7 (8.61-24.5)	16.7 (11.12-26.47)	ns
ILC2, %LIN neg CD127+CD161+	10 (6.35-13.5)	5.34 (1.46-11.67)	ns
ILC3, %LIN neg CD127+CD161+	1.78 (0.8-4.07)	1.46 (1.11-3.93)	ns

Legend: * analysis of soluble inflammatory markers is available in 79 colonized patients and 39 infected patients, ^analysis of circulating cell immunophenotype is available in 37 colonized patients and 21 infected patients, PTX3 long pentraxin 3, sIL-1R2 soluble IL-1 receptor type 2, Th T helper cells, Treg T regulatory cells, Tfh follicular helper T cells, MAIT mucosal-associated invariant T cells, ILC innate lymphoid cells. Variables are expressed as medians and interquartile ranges. ° Wilcoxon Rank-sum test p-value

Table 9. Immunological parameters of colonized and infected patients

Similar results were obtained when the time elapsed from KPC-*Kp* isolation to blood samples collection was considered as covariate (Rank ANCOVA) (p values: 0.007 for Th1, 0.01 for Th2, 0.01 for Th17, 0.054 for Treg).

GUT MICROBIOME ANALYSIS

Gut microbiome analysis was performed in 69 patients distributed across the 3 participating centers, 46/69 (66.7%) with KPC-*Kp* colonization and 23/69 (33.3%) with KPC-*Kp* infection (Figure 7).

No differences in alpha diversity were observed between hospitals and wards of KPC-*Kp* isolation (supplementary Figure 2). Patients with KPC-*Kp* infection were characterized by alpha diversity values significantly lower than those with KPC-*Kp* colonization (median Pielou's eveness index of 0.56 (Q1-Q3: 0.51-0.69) vs 0.68 (Q1-Q3: 0.6-0.74), p 0.019). Alpha diversity values differed significantly also comparing patients with and without broad-spectrum antibiotic therapy at KPC-*Kp* isolation (0.64 (Q1-Q3: 0.51-0.70) vs 0.68 (Q1-Q3: 0.60-0.75), p 0.021) and, within patients with KPC-*Kp* infection, comparing those presenting with and without sepsis at presentation (0.50 (Q1-Q3: 0.48-0.54) vs 0.65 (Q1-Q3: 0.51-0.7), p 0.033). We then repeated the three comparisons considering only the samples collected either <7 days or $\geq$7 days from KPC-*Kp* isolation. Interestingly, statistical significance remained only for samples collected within 7 days from KPC-*Kp* isolation (Figures 10-12).

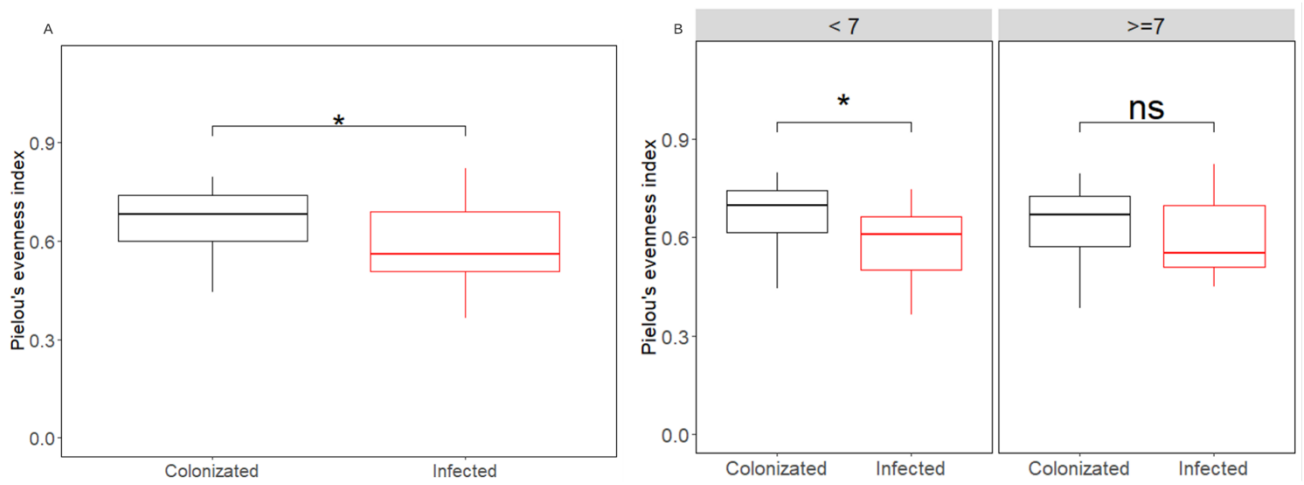


*Figure 10. Alpha diversity values (Pielou's eveness index) of gut microbiome of patients with KPC-Kp colonization (black) or infection (red). Comparison between groups irrespective of time elapsed from KPC-Kp isolation to fecal sample collection (A) and splitting between samples collected before or after 7 days from KPC-Kp isolation (B). *Wilcoxon Rank-sum test p-value <0.05, ns not significant*

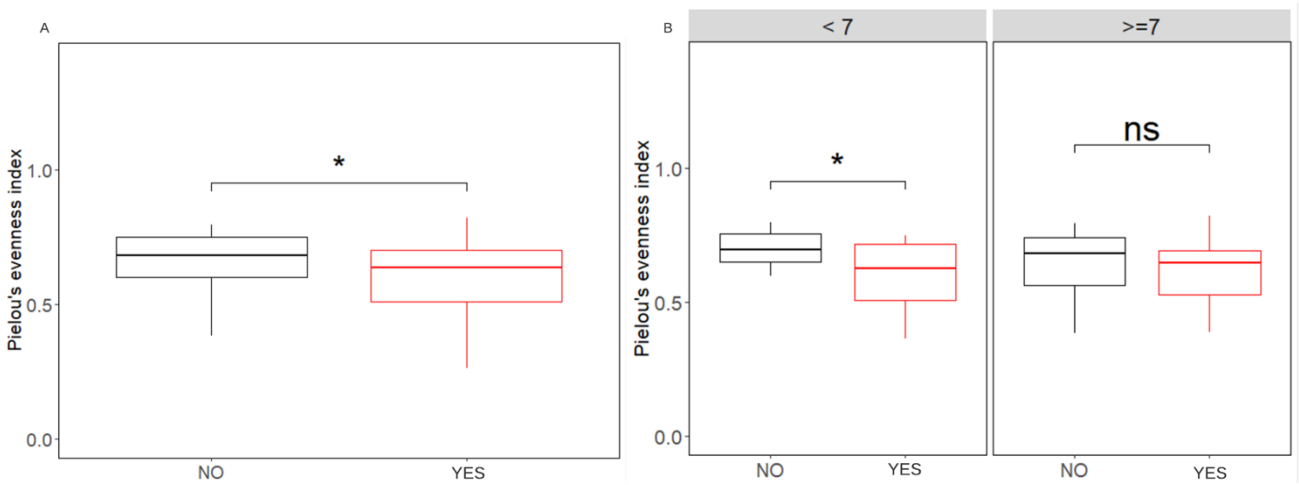


*Figure 11. Alpha diversity values (Pielou's eveness index) of gut microbiome of patients with (red) or without (black) broad-spectrum antibiotic therapy at KPC-Kp isolation. Comparison between groups irrespective of time elapsed from KPC-Kp isolation to fecal sample collection (A) and splitting between samples collected before or after 7 days from KPC-Kp isolation (B). *Wilcoxon Rank-sum test p-value <0.05, ns not significant*

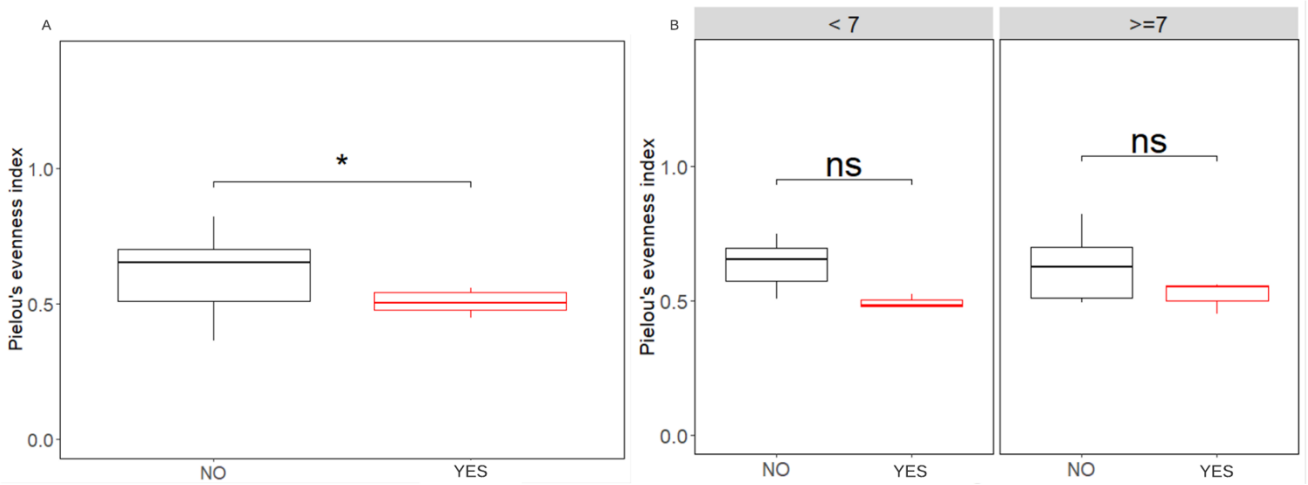


*Figure 12. Alpha diversity values (Pielou's eveness index) of gut microbiome of KPC-Kp infected patients with (red) or without (black) sepsis at infection onset. Comparison between groups irrespective of time elapsed from KPC-Kp isolation to fecal sample collection (A) and splitting between samples collected before or after 7 days from KPC-Kp isolation (B). *Wilcoxon Rank-sum test p-value <0.05, ns not significant*

No significant differences in alpha diversity values were found considering the following variables: time from KPC-*Kp* isolation to sample collection (<7 vs ≥7 days), age classes (21-65 vs 66-79 vs 80-93 years), CCI classes (<5 vs ≥5), treatment with broad-spectrum antibiotics in the 30 days prior to KPC-*Kp* isolation, previous colonization/infection with MDROs, immunosuppression in the 30 days before KPC-*Kp* isolation, acute medical condition at KPC-*Kp* isolation, bla_{KPC} enzyme variant, virulence score (o vs ≥1), resistance score (2 vs 3) (data not shown).

Beta diversity differed significantly between age classes (21-65 years vs 80-93 years, p value 0.024) (Figure 13), but not comparing hospitals and wards of KPC-*Kp* isolation nor KPC-*Kp* infection or colonization status. Of note, PCoA did not differ considering the time elapsed from KPC-*Kp* isolation to fecal sample collection (<7 vs ≥7 days), suggesting that this has limited impact on beta diversity (Figure 14 and supplementary Figures 3 and 4).

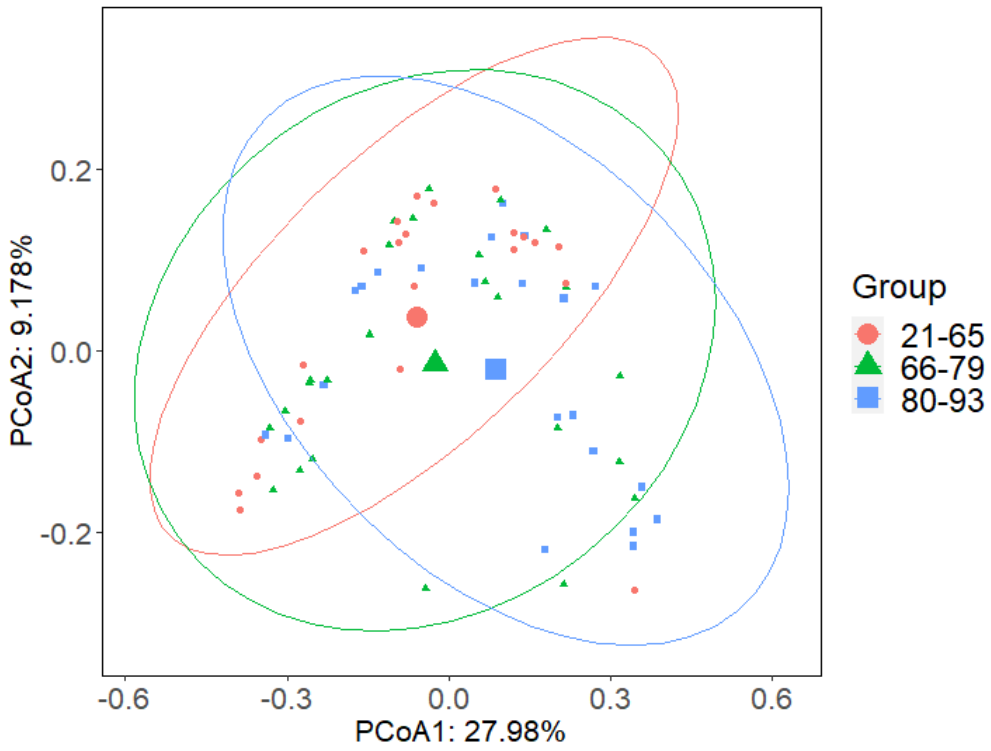


Figure 13. Beta diversity values (Unweighted Unifrac) of gut microbiome of KPC-Kp patients depending on age class. PCoA differs significantly between 21.65 years and 80-93 years (p value 0.024)

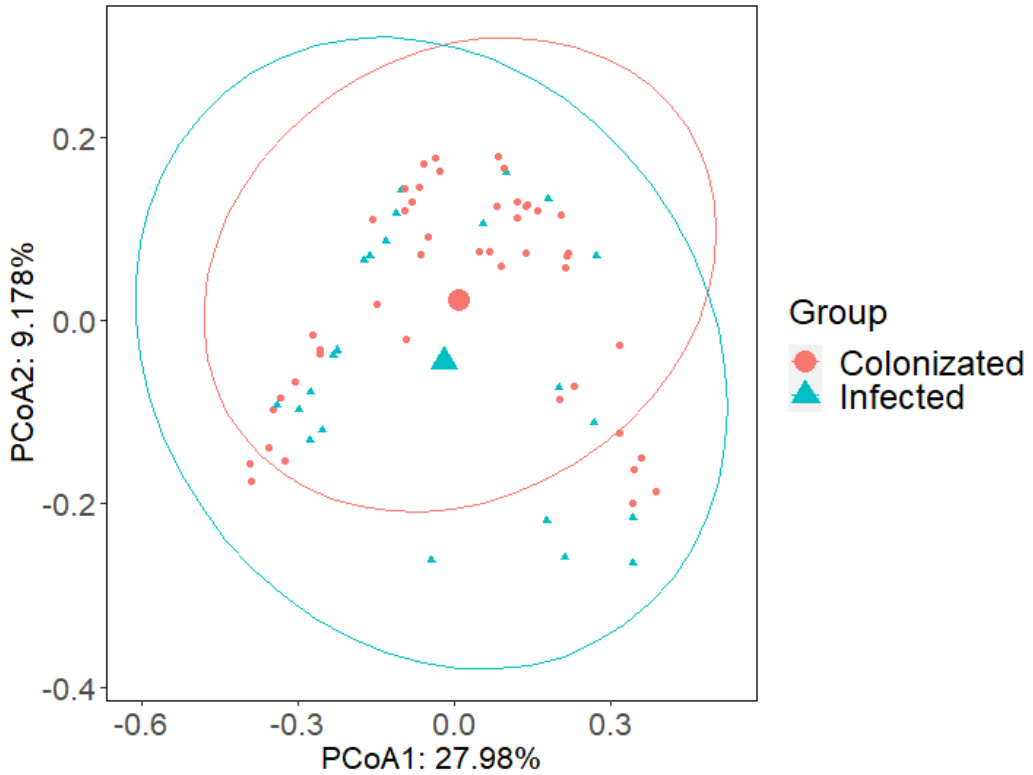


Figure 14. Beta diversity values (Unweighted Unifrac) of gut microbiome of KPC-Kp patients depending on infection/colonization status. No significant differences were found between the two groups

DISCUSSION & CONCLUSIONS

This study provides a detailed picture of clinical, immunological and microbiological characteristics of patients with KPC-*Kp* colonization and infection, and highlights differences in circulating immune markers and gut microbiome between groups. Also, it analyses KPC-*Kp* isolates through WGS, finding correlations between carbapenemase variants and virulence and resistance factors.

K. pneumoniae is a frequent cause of HAIs, affecting primarily patients with impaired host defenses for immunosuppression or recent surgery (Kang 2006) (Opilla 2008) (Huang 2015). The presence of KPC enzyme increases morbidity and mortality of Kp infections because of higher failure of empiric antibiotic therapy and limited therapeutic options (Grundmann 2017) (Logan 2017). The diagnosis of KPC-*Kp* colonization is also clinically important, since it represents a major risk factor for subsequent infection (Martin 2016) (Gorrie 2017) (Tischendorf 2016) and can easily spread across patients therefore requiring strict infection control measures (Review on Antimicrobial Resistance 2016) (Centers for Diseases Control and Prevention 2019).

In agreement with literature results, our study population consisted in elderly (median age 71 years) and frail patients (median age-adjusted CCI 6), with a relevant percentage of immunocompromised (43.9%). In our cohort, KPC-*Kp* represented a typical healthcare-associated pathogen, with the majority of patients with previous hospitalization in the year before enrollment (78.8%) and broad-spectrum antibiotic therapy ongoing at KPC-*Kp* isolation (72.7%) or in the 30 days before (87%). Notably, in a significant percentage of cases KPC-*Kp* detection followed (46.8%) or was concomitant (39%) to the diagnosis of other MDROs colonization/infection.

We found a higher percentage of recent invasive procedures in infected (36.4%) than colonized patients (18%), which is consistent with published clinical scores that assess the risk of KPC-*Kp* infection in colonized patients (Tumbarello M 2014) (Giannella M 2014), where the presence of central venous catheter, recent surgical or intra-abdominal procedures are taken into account. Despite the diagnosis of sepsis in 40% of infected patients at presentation, we did not find significant differences in hospital mortality between infected and colonized patients (6.5% vs 3.4%). This differs from the multicenter study conducted in Lombardy from 2016 to 2018 by Rossi and colleagues, which found in-hospital mortality of 14% in KPC-*Kp* colonized patients and 11.9% and 28% in mild and severe infections (Rossi 2021). Of note, while median age, CCI and underlying conditions of

enrolled patients did not differ between the two studies, in the study by Rossi at al. 25.7% of the population was composed by ICU patients (excluded in our study).

We identified 8 different STs of KPC-*Kp*, with ST307 (32.4%), ST512 (24.3%) and ST20 (21.7%) being the most frequent as confirmed by the Maximum Likelihood phylogenetic tree. This is in line with recent epidemiological studies in Italy and Lombardy region (Conte 2016) (Rossi 2021). Those epidemic clones have previously been associated with outbreaks and are reported to have an increased capacity to acquire drug resistance (Navon-Venezia 2017) (Snitkin 2012) (A. J. Mathers 2015). We identified 2 bla_{KPC} enzyme variants, KPC-2 (35.1%) and KPC-3 (64.9%). Consistently with the findings by Rossi and colleagues, KPC-2 was absent in ST512 but predominant in ST307 and ST258 (Rossi 2021).

Core SNP phylogenetic tree analysis including KPC-*Kp* strains from European hospitals (Figure 9 and supplementary Table 2) describes an epidemiological picture worth of further investigations. Indeed, although we exploited more than 33.000 core SNPs shared among bacterial strains of different countries and years, we observed surprisingly closed phylogenetic relationships among them even if uncorrelated under a geographic and temporal point of view. Is the case of MDP-P018 and MDP-P032 (sample IDs 26 and 43 in Figure 9), which present a surprisingly tight relationship with Portuguese samples SAMN13915695 and SAMN13915730. Also, the Italian sample SAMN18011696 collected in Foggia in 2019 share high identity percentages with MDP-P007 (sample ID 11 in Figure 9), while KPC-Kp strains SAMN09813401 and SAMN09813398 collected in Palermo in 2017 well cluster with our isolates in ST307 clade.

Thanks to Kleborate software (Lam 2021), we calculated virulence and resistance scores of each KPC-*Kp* isolate, and we assessed their correlation to clinical and microbiological characteristics (Tables 7 and 8). Interestingly, KPC-*Kp* strains expressing KPC-2 were more frequently characterized by virulence score $\geq$1 (11/13, 84.6%) and by resistance score 2 (13/13, 100%). On the contrary, KPC-*Kp* strains expressing KPC-3 were more frequently characterized by virulence score 0 (15/24, 62.5%) and by resistance score 3 (12/24, 50%). This could be due to common plasmid-mediated transmission of carbapanemases and virulence or resistance factors. Further studies are warranted to explore this interesting correlation. Only 7 patients with KPC-*Kp* infection underwent bacterial genome analysis, with 3 presenting sepsis at infection onset. Of note, all of them were characterized by a virulence score $\geq$1 and a resistance score of 2. These findings open interesting perspectives on the use of WGS of KPC-*Kp* in real-life situations to stratify patients and guide antimicrobial and

supportive therapies, as already happens in other fields of clinical microbiology and infectious diseases such as sepsis (Mangioni 2019) (Mangioni D 2020). Further studies are warranted to clarify the extent of such relationship.

We did not find differences in circulating soluble inflammatory markers PTX3 and sIL-1R2 between KPC-*Kp* colonization and infection groups (Table 9). This is probably due to the high percentage of patients with acute medical conditions at KPC-*Kp* isolation in both groups (85.9% of colonized and 85.4% of infected patients) and to the exclusion of critically ill ICU patients from the present study. Accordingly, median levels of PTX3 of the study population (16.13 ng/ml) are similar to those of hospitalized febrile patients (14.2 ng/ml) but significantly lower than those of ICU patients with sepsis (44.4-72 ng/dl) (de Kruif 2010) (Caironi 2017), median levels of sIL-1R2 of the study population (17.02 ng/ml) are significantly lower than those of ICU patients with sepsis (78.7 ng/dl) (Müller 2002). Both PTX3 and sIL-1R2 median levels of the study population result higher than those of external cohorts of healthy controls (PTX3 2.30 ng/ml, sIL-1R2 <10 ng/ml) (de Kruif 2010) (Müller 2002).

KPC-*Kp* infected and colonized patients differed significantly in the proportion of circulating T cell immunophenotype. Percentages of pro-inflammatory Th1, Th2 and Th17 cells resulted higher in patients with KPC-*Kp* colonization, while percentages of anti-inflammatory Tregs resulted higher in infected patients. These findings are consistent with data reported in the literature. Both experimental studies from animal infection models and human studies demonstrated the protective effects of Th1 and Th17 cells in immunity to bacterial pathogens, and particularly to *Klebsiella* spp (Curtis 2009) (Bengoechea 2019). By contrast, septic patients show suppression of Th1, Th2 and Th17 cell function and increased proportion of regulatory T cells, which all can concur to the decreased T cell effector function that characterize sepsis and eventually to immune paralysis in septic shock (van der Poll 2017). Also, induction of the anti-inflammatory cytokine IL-10 (produced by T reg cells) has been demonstrated in *K. pneumoniae* infections (Yoshida 2001), and its over-expression correlates with decreased survival in animal models of gram-negative pneumonia (Dolgachev 2014).

Interestingly, dysregulation of host immune system and aberrant mucosal immunity have been associated to microbiome alterations during infections, systemic autoimmune diseases and cancer, such as reduced bacterial diversity, marked shifts in abundance of certain bacterial taxa and altered microbiome-associated metabolite composition, (Zheng 2020).

We did find significant differences in alpha ("within sample") diversity between colonized and infected patients, with the latter group characterized by a lower measure. By contrast, beta ("between sample") diversity did not differ between groups (Figures 10 and 14).

The central role of gut microbiome in MDROs colonization and infection is acquiring increasing interest. Commensal bacteria can act both directly and indirectly through immune system regulation to the maintenance of a healthy microbiota and the immune homeostasis or, on the contrary, as a reservoir for potentially pathogens (dysbiosis) which concurs to the dysregulation of the immune system (Wuethrich 2021). Several findings have proved that a high alpha diversity is associated with a healthy microbiota (Pickard 2017) (Kowalska-Duplaga 2019) and therefore microbiota-based treatments such as fecal microbiota transplant, administration of probiotics/prebiotics, and phage therapy have been proposed (Wuethrich 2021). So far, very few recent studies have investigated microbiome composition in infected and colonized subjects, none of them evaluating KPC-*Kp*. Sánchez-Pellicer and colleagues compared patients with colonization or infection by *Clostridioides difficile* and healthy donors. Infected and colonized subjects presented a gut microbiome different from that of healthy controls (loss of alpha diversity and richness), although similar to each other (Sánchez-Pellicer 2021). Zhao and colleagues investigated nasal microbiome in *Staphylococcus aureus* colonized and non-colonized neonates, finding that neonates with bacteremia had an increased relative abundance of S. aureus sequences and lower alpha diversity measures compared to colonized neonates and controls (Zhao 2021).

We did not find differences in alpha and beta diversity among samples from different hospitals and wards of KPC-*Kp* isolation (supplementary Figures 2 and 3). Likewise, no differences emerged considering the time elapsed from KPC-*Kp* isolation to sample collection (supplementary Figure 4). We reasonably conclude that these factors had limited impact in the differences between KPC-*Kp* colonized and infected patients.

In accordance with several studies on antibiotic-induced dysbiosis (Wuethrich 2021) (Ran 2020), we found differences in alpha diversity comparing samples of patients with and without broad-spectrum antibiotic therapy at KPC-*Kp* isolation, with the former group characterized by lower values. Alpha diversity differed also comparing, within KPC-*Kp* infected patients, those with and without sepsis at presentation, with the former group characterized by a lower measure. This is consistent with studies conducted in ICU patients that revealed a remarkable loss of microbial diversity during sepsis with increased abundance of microbes tightly associated with inflammatory markers (Bassetti 2020) (Agudelo-Ochoa 2020) (Yang 2021).

The key strength of the present study is its integrated approach to patients with KPC-*Kp* colonization/infection. Focusing on both clinical, immunological and microbiological (pathogen isolates and gut microbiota) factors, we identified host- and microbiome-related features that could differentiate KPC-*Kp* colonization from infection. Also, this approach allowed us to find a possible association between specific KPC-*Kp* genomic characteristics (carbapenemase variants, virulence and resistance score) and the severity of KPC-*Kp* infection at presentation.

Yet, the findings in this study are subject to a number of limitations. First, delays in patient enrollment and laboratory analyses during COVID-19 pandemic periods have limited the expected sample size, and therefore results of KPC-*Kp* genome sequencing and circulating immunophenotype as well as subgroup comparisons in gut microbiome analysis should be confirmed in larger cohorts. Second, enrollment interruption during COVID-19 outbreaks and the absence of KPC-*Kp* active surveillance protocols in all the hospital wards examined challenge the possibility to obtain an accurate phylogenetic analysis of KPC-*Kp*. At this regard, enrollment is still ongoing, thus permitting in the next months to consolidate our findings and to proceed traking KPC-*Kp* outbreaks and their origin by a Bayesian approach. Third, one of the purposes of the study was to evaluate the development of KPC-*Kp* infection in colonized patients, but this occurred in only a minority of case (6, for which only the infectious episode was analyzed). This probably depends on the limited number of hematological patients enrolled and in the exclusion of ICU patients as *per protocol*, which are two of the categories at most risk to develop infections. Also, we cannot exclude that during COVID-19 emergency periods, enrolled patients with a new infection episode did not seek care in one of the participating centers but rather accessed to hospitals outside the Milan area for safety reasons. An amendment to the study has recently been proposed to include patients with temporary inability to give consent (i.e., ICU patients or septic patients), pending their study acceptance once the condition is resolved. Finally, due to the study design the collection of blood and fecal samples was not concomitant to KPC-*Kp* isolation, rather it followed by a few days (median of 5 and 6 days, respectively). Immunological and gut microbiome results were corrected through time-adjusted analysis, yet some possible confounders remain.

Overall, the results of this study indicate that the integration of clinical variables with microbiological and immunological data allow a more comprehensive picture of KPC-*Kp* colonization/infection, with relevant implications for the prevention and management of these conditions. We ultimately obtained a better understanding of the relationship between host-related

and pathogen-related variables and their interplay in the pathogenesis of KPC-*Kp* infections. These data could allow decision-makers and clinicians to plan preventive and therapeutic strategies, and ultimately to make a real contribution to the control of outbreak and its impact on the health of our patients.

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SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. Demographic and clinical characteristics of the KPC Multidisciplinary study population

		KPC_{Pts}
Epidemological characteristics		
Age	N Mean, Median (Q1-Q3) [min-max]	135 69.8, 71 (60-81) [21-93]
Gender female	n/N (%)	45/135 (33.3)
BMI	N Mean, Median (Q1-Q3) [min-max]	54 25.1, 24.2 (21.6-27.4) [17-38.9]
Charlson comorbidity index (age-adjusted)	N Mean, Median (Q1-Q3) [min-max]	135 5.6, 6 (4-7) [0-12]
Anamnestic factors		
Hospitalization for more than 48 hours in the 12 months prior to KPC-*Kp* isolation	n/N (%)	104/132 (78.8)
Major surgery in the 30 days prior to KPC-*Kp* isolation	n/N (%)	33/135 (24.4)
Mechanical ventilation in the 30 days prior to KPC-*Kp* isolation	n/N (%)	34/132 (25.8)
Invasive procedures in the 72 hours prior to KPC-*Kp* isolation	n/N (%)	32/133 (24.1)
Treatment with broad-spectrum antibiotics in the 30 days prior to KPC-*Kp* isolation	n/N (%)	114/131 (87)
Previous colonization/infection with MDROs	n/N (%)	58/124 (46.8)
Immunocompromised in the 30 days before KPC-*Kp* isolation • AIDS • steroids • oncohaematologic diseases & chemotherapy • SOT & immunosuppressive therapy	n/N (%)	58/132 (43.9) 1/58 (1.7) 19/58 (32.8) 20/58 (34.5) 18/58 (31)
Diagnosis at hospital admittance • infection • acute noninfectious condition • chronic noninfectious condition	n/N (%)	 75/135 (55.6) 35/135 (25.9) 25/135 (18.5)
Clinical characteristics at KPC-*Kp* isolation		
Days between hospital admittance and enrollment	N Mean, Median (Q1-Q3) [min-max]	129 24.4, 15 (6-32) [0-150]
Ward of KPC-*Kp* isolation • Medical Units • Surgical Units • Sub-intensive care Units • Onco-haematological Units	n/N (%)	 69/135 (51.1) 33/135 (24.4) 27/135 (20) 6/135 (4.5)

Site of KPC-*Kp* isolation	n/N (%)	
• blood		18/135 (13.3)
• urine		36/135 (26.7)
• respiratory samples		6/135 (4.4)
• abdominal samples		10/135 (7.4)
• surveillance swab		65/135 (48.2)
Nutrition support	n/N (%)	
• Oral diet/enteral feeding		104/112 (92.9)
• Parenteral nutrition		8/112 (7.1)
Presence of invasive devices	n/N (%)	104/129 (80.6)
Acute medical conditions	n/N (%)	102/119 (85.7)
Ongoing antibiotic therapy	n/N (%)	93/128 (72.7)
Colonization/infection with other MDROs	n/N (%)	48/123 (39)
Severity scores and parameters at KPC-*Kp* isolation		
SOFA score	n/N (%)	
• 0-1		28/61 (45.9)
• 2-8		33/61 (54.1)
Leucocytes, n/mmc	N Mean, Median (Q1-Q3) [min-max]	131 9404, 8580 (5730-116009 [110-38340]
C-reactive protein, mg/dl	N Mean, Median (Q1-Q3) [min-max]	126 8.8, 6.5 (2.9-11.3) [0.01-59.1]
Procalcitonin, ng/ml	N Mean, Median (Q1-Q3) [min-max]	33 3.72, 0.35 (0.16-3.95) [0.03-26.1]
Creatinine clearance (CKD EPI), ml/min	N Mean, Median (Q1-Q3) [min-max]	129 72.2, 76.3 (49.4-95) [8-190]
Severity scores and parameters at sample collection		
SOFA score	n/N (%)	
• 0-1		28/54 (51.8)
• 2-8		26/54 (48.2)
Leucocytes, n/mmc	N Mean, Median (Q1-Q3) [min-max]	132 8156, 7260 (4755-10110 [0-32080]
Lymphocytes, n/mm	N Mean, Median (Q1-Q3) [min-max]	123 1391, 1230 (840-1820) [143-4330]
C-reactive protein, mg/dl	N Mean, Median (Q1-Q3) [min-max]	126 5.2, 3 (1.3-7.2) [0.06-31.2]
Procalcitonin, ng/ml	N Mean, Median (Q1-Q3) [min-max]	38 3.44, 0.43 (0.13-2.22) [0.04-87.9]
Creatinine clearance (CKD EPI), ml/min	N Mean, Median (Q1-Q3) [min-max]	128 75, 78.5 (52.9-98.9) [10.9-192]
Days between KPC-*Kp* isolation and blood sample collection	N Mean, Median (Q1-Q3) [min-max]	135 6.6, 5 (4-7) [1-30]
Days between KPC-*Kp* isolation and fecal sample collection	N Mean, Median (Q1-Q3)	109 7.5, 6 (4-9)

	[min-max]	[1-31]
Outcome		
In-hospital mortality	n/N (%)	6/130 (4.6)
Lengths of hospital stay in survived patients, days from KPC-*Kp* isolation	N Mean, Median (Q1-Q3) [min-max]	126 19, 15 (8-25) [2-73]
Death or new hospital admittance at month 1	n/N (%)	8/59 (13.5)
Death or new hospital admittance at month 3	n/N (%)	13/45 (28.9)

Legend: KPC_{Pts} patients with KPC-*Kp* isolation, BMI body mass index, MDROs multidrug resistant organisms, AIDS acquired immune deficiency syndrome, SOT solid organ transplant, SOFA score sequential (sepsis-related) organ failure assessment score, , CKD EPI chronic kidney disease epidemiology collaboration (equation to estimate glomerular filtrate rate)

Supplementary Table 2. Publicly deposed KPC-Kp isolates used in the phylogenetic tree (Figure 9). These isolates were retrieved from NCBI by considering European strains found to be of clinical interest and collected in the years 2019-2020

SampleName	Assembly	Biosample	ST_assignment	Location	IsolationSource
Italy_EC_ST101	ASM1858896v1	SAMN18011696	ST101	Italy	blood
Germany_UC_ST101	ASM190251v1	SAMN05412456	ST101	Germany	urine
Spain_na_ST101	ASM886773v1	SAMN12572044	ST101	Spain	-
Spain_TR_ST101	ASM974632v1	SAMN13337936	ST101	Spain	rectal swab
Poland_CIa_ST147	ASM1362524v1	SAMN15015463	ST147	Portugal	clinical isolate
Poland_CIa_ST147	ASM1487713v1	SAMN16327541	ST147	Poland	clinical isolate
Poland_Cib_ST147	ASM1487769v1	SAMN16327562	ST147	Poland	clinical isolate
Poland_Cic_ST147	ASM1487775v1	SAMN16327560	ST147	Poland	clinical isolate
Italy_EC_ST147	ASM1610738v1	SAMN16386434	ST147	Italy	blood
Italy_TR_ST147	ASM1610740v1	SAMN16386433	ST147	Italy	rectal swab
Germany_TR_ST147	ASM1978108v1	SAMN14996483	ST147	Germany	tracheal secretion
Spain_UC_ST15a	ASM1921856v1	SAMN19795652	ST15	Spain	urine
Spain_UC_ST15b	ASM886762v1	SAMN12572048	ST15	Spain	urine
Portugal_UC_ST20	ASM1664340v1	SAMN13915673	ST20	Portugal	urine
France_EC_ST23	ASM1570001v1	SAMN15415360	ST23	France	blood
Italy_EC_A_ST307	ASM1532148v1	SAMN09813401	ST307	Italy	blood
Italy_EC_B_ST307	ASM1532154v1	SAMN09813398	ST307	Italy	blood
Germany_na_ST307	ASM1758433v1	SAMN18311233	ST307	Germany	-
Spain_Surgical_ST307a	ASM1924945v1	SAMN19795650	ST307	Spain	wound

Spain_Surgical_ST307b	ASM1983628v1	SAMN15091882	ST307	Spain	wound
Portugal_LA_ST405	ASM1652882v1	SAMN13915689	ST405	Portugal	intra-abdominal sample
Portugal_UC_ST405	ASM1664315v1	SAMN13915717	ST405	Portugal	urine
Portugal_BAL_ST405	ASM1664372v1	SAMN13915695	ST405	Portugal	low respiratory tract
Poland_BAL_ST45	ASM1178490v1	SAMN12715042	ST45	Poland	low respiratory tract
Portugal_BAL_ST45	ASM1652707v1	SAMN13915732	ST45	Portugal	low respiratory tract
Portugal_LA_ST45	ASM1652715v1	SAMN13915730	ST45	Portugal	intra-abdominal sample
Italy_MU_ST512	ASM1532155v1	SAMN09813396	ST512	Italy	sputum
Spain_TR_ST512	ASM1924943v1	SAMN19795649	ST512	Spain	rectal swab
Spain_UC_ST512	ASM1924956v1	SAMN19795646	ST512	Spain	urine
Spain_Wound_ST512	ASM886754v1	SAMN12586631	ST512	Spain	wound
Spain_EC_ST512	ASM886782v1	SAMN12572037	ST512	Spain	blood

Supplementary Table 3. Virulence factors of the 37 KPC-*Kp* isolates analyzed

ID	ST	K locus	O type	wzi	virulence score	Yersiniabactin	Colibactin	Aerobactin	Salmochelin	RmpADC	rmpA2
MDP-P005/2	ST307	KL102	O2	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-P006	ST20	KL24	O1	wzi24	0	-	-	-	-	-	-
MDP-P007	ST512	KL107	O2	wzi154	0	-	-	-	-	-	-
MDP-P008	ST307	KL102	O2	wzi173	0	-	-	-	-	-	-
MDP-P009	ST307	KL102	O2	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-P010	ST512	KL107	O2	wzi154	0	-	-	-	-	-	-
MDP-P011	ST45	KL62	O2	wzi149	1	ybt 10; ICEKp4	-	-	-	-	-
MDP-P012	ST307	KL102	O2	wzi173	0	-	-	-	-	-	-
MDP-P013	ST101	KL17	O1	wzi137	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-P014	ST307	KL102	O2	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-P015	ST307	KL102	O2	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-P016	ST512	KL107	O2	wzi154	0	-	-	-	-	-	-
MDP-P017	ST258	KL106	O2	wzi29	1	ybt 13; ICEKp2	-	-	-	-	-
MDP-P018	ST307	KL102	unknown	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-P020	ST512	KL107	O2	wzi154	1	ybt 13; ICEKp2	-	-	-	-	-
MDP-P021	ST20	KL24	O1	wzi24	0	-	-	-	-	-	-
MDP-P022	ST258	KL106	unknown	wzi29	1	ybt 13; ICEKp2	-	-	-	-	-
MDP-P023/2	ST2648	KL8	O1	wzi490	0	-	-	-	-	-	-
MDP-P024	ST512	KL107	O2	wzi154	0	-	-	-	-	-	-
MDP-P025	ST405	KL151	O4	wzi143	1	ybt 0; ICE?	-	-	-	-	-
MDP-P026	ST307	KL102	O2	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-P027	ST512	KL107	O2	wzi154	4	ybt 16; ICEKp12	-	iuc 1	-	-	rmpA2_6*-49%
MDP-P028	ST512	KL107	O2	wzi154	4	ybt 16; ICEKp12	-	iuc 1	-	-	rmpA2_6*-49%
MDP-P030	ST20	KL24	O1	wzi24	0	-	-	-	-	-	-
MDP-P031	ST512	KL107	O2	wzi154	4	ybt 16; ICEKp12	-	iuc 1	-	-	rmpA2_6*-61%
MDP-P032	ST20	KL24	O1	wzi24	0	-	-	-	-	-	-

MDP-P033	ST20	KL24	O1	wzi24	0	-	-	-	-	-	-
MDP-P034	ST307	KL102	O2	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-P035	ST20	KL24	O1	wzi24	0	-	-	-	-	-	-
MDP-P036	ST45	KL24	O2	wzi101	1	ybt 10; ICEKp4	-	-	-	-	-
MDP-S001	ST20	KL24	O1	wzi24	0	-	-	-	-	-	-
MDP-S003	ST512	KL107	O2	wzi154	1	ybt 13; ICEKp2	-	-	-	-	-
MDP-S005	ST258	KL106	O2	wzi29	0	-	-	-	-	-	-
MDP-S006	ST307	KL102	O2	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-
MDP-S007	ST307	KL102	O2	wzi173	0	-	-	-	-	-	-
MDP-S008	ST20	KL24	O1	wzi24	0	-	-	-	-	-	-
MDP-S009	ST307	KL102	O2	wzi173	1	ybt 9; ICEKp3	-	-	-	-	-

Legend: ST sequence type, K locus capsular polysaccharide (K-antigen) biosynthesis locus, O type Lipopolysaccharide (LPS, O-antigen) serotype, *wzi* locus for capsular biosynthesis, RmpADC/rmpA2 genes associated with the hypermucoidy phenotype

Supplementary Table 4. Resistance factors of the 37 KPC-*Kp* isolates analyzed

ID	ST	resistance score	acquired AMR genes*								chromosomal AMR gene mutations*		
			AGly	Flq	Tet	Tmt	Sul	*bla*	bla_{ESBL}	bla_{CARBA}	Omp	Col R	Flq R
MDP-P005/2	ST307	2	aac(6')-Ib-cr.v2	qnrB1.v2^	-	dfrA14.v2*	-	OXA-1;TEM-1D.v1^	CTX-M-15	KPC-2	-	-	GyrA-83I;ParC-80I
MDP-P006	ST20	3	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	PmrB-9%	-
MDP-P007	ST512	2	aac(6')-Ib'.v1;aadA2^;aph3-Ia.v1^	-	-	dfrA12	sul1	-	-	KPC-3	OmpK35-25%;OmpK36GD	-	GyrA-83I;ParC-80I
MDP-P008	ST307	2	aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1	qnrB1.v2^	tet(A).v1	dfrA14.v2*	sul2	OXA-1	CTX-M-15	KPC-2	-	-	GyrA-83I;ParC-80I
MDP-P009	ST307	2	aac(6')-Ib-cr.v2	qnrB1.v2^	-	dfrA14.v2*	-	OXA-1;TEM-1D.v1^	CTX-M-15	KPC-2	-	-	GyrA-83I;ParC-80I
MDP-P010	ST512	2	aac(6')-Ib'.v1*;aadA2;aph3-Ia.v1^	-	-	-	sul1	-	-	KPC-3	OmpK35-25%;OmpK36GD	-	GyrA-83I;ParC-80I
MDP-P011	ST45	2	-	qnrB1.v2^	-	dfrA14.v2*	-	TEM-1D.v1^	-	KPC-3	-	-	-
MDP-P012	ST307	2	aac(6')-Ib-cr.v2;strA.v1^;strB.v1	qnrB1.v2^	tet(A).v1	dfrA14.v2*	sul2	OXA-1	CTX-M-15	KPC-3	-	-	GyrA-83I;ParC-80I
MDP-P013	ST101	2	armA	-	-	-	-	TEM-1D.v1^	-	KPC-2	OmpK35-17%;OmpK36TD	-	GyrA-83Y;GyrA-87N;ParC-80I
MDP-P014	ST307	2	aac(6')-Ib-cr.v2	qnrB1.v2^	-	dfrA14.v2*	-	OXA-1	CTX-M-15	KPC-2	-	-	GyrA-83I;ParC-80I
MDP-P015	ST307	2	aac(6')-Ib-cr.v2	qnrB1.v2^	-	dfrA14.v2*	-	OXA-1;TEM-1D.v1^	CTX-M-15	KPC-2	-	-	GyrA-83I;ParC-80I
MDP-P016	ST512	3	aac(3)-IIa.v1^;aac(6')-Ib'.v1*;aac(6')-Im;aph(2'')-IIa*;strA.v1^;strB.v1	qnrB1.v2^	-	dfrA14.v2*	sul2	-	CTX-M-15	KPC-3	OmpK35-25%;OmpK36GD	MgrB-6%	GyrA-83I;ParC-80I
MDP-P017	ST258	2	aac(6')-Ib'.v1	-	-	-	-	-	-	KPC-2	OmpK35-25%	-	GyrA-83I;ParC-80I
MDP-P018	ST307	2	aadA2^;aph3-Ia.v1^;strA.v1*;strB.v1*	qnrS1	-	dfrA12	sul1;sul2	TEM-1D.v1^	CTX-M-15	KPC-2	-	-	GyrA-83I;ParC-80I
MDP-P020	ST512	2	aac(6')-Ib'.v1;aadA2	-	-	-	sul1	-	-	KPC-3	OmpK35-25%;OmpK36GD	-	GyrA-83I;ParC-80I
MDP-P021	ST20	3	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	PmrB-9%	-
MDP-P022	ST258	2	aac(6')-Ib'.v1;aadA2^;aph3-Ia.v1^	-	-	dfrA12	sul1	TEM-1D.v1^	-	KPC-2	OmpK35-25%	-	GyrA-83I;ParC-80I
MDP-P023/2	ST2648	2	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	-	-
MDP-P024	ST512	2	aac(6')-Ib'.v1;aadA2^;aph3-Ia.v1^	-	-	dfrA12	sul1	-	-	KPC-3	OmpK35-25%;OmpK36GD	-	GyrA-83I;ParC-80I
MDP-P025	ST405	2	aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1	-	-	dfrA14.v2*	sul2	OXA-1	CTX-M-15	KPC-3	-	-	-

MDP-P026	ST307	2	aadA2^	qnrS1	-	dfrA12	sul1	OXA-9.v1	-	KPC-2	-	-	GyrA-83I;ParC-80I
MDP-P027	ST512	3	aac(6')-Ib'.v1;aadA*?;aadA2;ant(2'')-Ia	-	-	-	sul1	TEM-1D.v1^	-	KPC-3	OmpK35-25%	MgrB-62%	GyrA-83I;ParC-80I
MDP-P028	ST512	3	aac(6')-Ib'.v1;aadA*?;aadA2;ant(2'')-Ia	-	-	-	sul1	TEM-1D.v1^	-	KPC-3	OmpK35-25%	MgrB-62%	GyrA-83I;ParC-80I
MDP-P030	ST20	3	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	PmrB-9%	-
MDP-P031	ST512	3	aac(6')-Ib'.v1;aadA*?;aadA2;ant(2'')-Ia	-	-	-	sul1	TEM-1D.v1^	-	KPC-3	OmpK35-25%	MgrB-62%	GyrA-83I;ParC-80I
MDP-P032	ST20	3	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	PmrB-9%	-
MDP-P033	ST20	3	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	PmrB-9%	-
MDP-P034	ST307	2	aac(6')-Ib-cr.v2	qnrB1.v2^	-	dfrA14.v2*	-	OXA-1	-	KPC-3	OmpK35-70%	-	GyrA-83I;ParC-80I
MDP-P035	ST20	3	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	PmrB-9%	-
MDP-P036	ST45	2	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	-	-
MDP-S001	ST20	3	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	PmrB-9%	-
MDP-S003	ST512	2	aac(6')-Ib'.v1;aadA2	-	-	-	sul1	-	-	KPC-3	OmpK35-25%;OmpK36GD	-	GyrA-83I;ParC-80I
MDP-S005	ST258	2	aac(3)-IId^;aac(6')-Ib'.v1	-	tet(D)	-	-	TEM-1D.v1^	SHV-12	KPC-2	OmpK35-25%	-	GyrA-83I;ParC-80I
MDP-S006	ST307	2	aadA2^	qnrS1	-	dfrA12	sul1	OXA-9.v1	SHV-12	KPC-2	-	-	GyrA-83I;ParC-80I
MDP-S007	ST307	2	aac(6')-Ib-cr.v2;strA.v1^;strB.v1	-	tet(A).v1	dfrA14.v2*	sul2	OXA-1	CTX-M-15	KPC-3	-	-	GyrA-83I;ParC-80I
MDP-S008	ST20	3	-	-	-	-	-	TEM-1D.v1^	-	KPC-3	-	PmrB-9%	-
MDP-S009	ST307	2	aac(6')-Ib-cr.v2	qnrB1.v2^	-	dfrA14.v2*	-	OXA-1	CTX-M-15	KPC-2	-	-	GyrA-83I;ParC-80I

Legend: *resistance results are grouped by drug class according to the ARG-Annot database, with beta-lactamases divided into Lahey classes according to the betalactamase database BLDB. No acquired resistance genes were found for colistin (Col), fosfomycin (Fcyn) and tigecycline (Tgc) in the strains examined. Resistance genes for the following drug classes are not reported due to limited clinical interest: glycopeptide (Gly), macrolide, lincosamide and streptogramin B (MLS), phenicol (Phe), rifampin (Rif). AGly aminoglycoside, Flq fluoroquinolone, Tet tetracycline, Tmt trimethoprim, Sul sulphonamide, bla acquired narrow-spectrum beta-lactamases, bla_{ESBL} extended-spectrum beta-lactamases, bla_{CARBA} carbapenemases, Omp osmoporins mutation resulting in reduced susceptibility to beta-lactamases, Col R mutations of core genes MgrB or PmrB resulting in reduced susceptibility to colistin, Flq R mutations of core genes GyrA-83/-87 and ParC-80/-84 resulting in reduced susceptibility to fluoroquinolone

Supplementary Table 5. Distribution of KPC-*Kp* genome characteristics in patients enrolled at Policlinico Hospital, overall and divided for colonized and infected patient

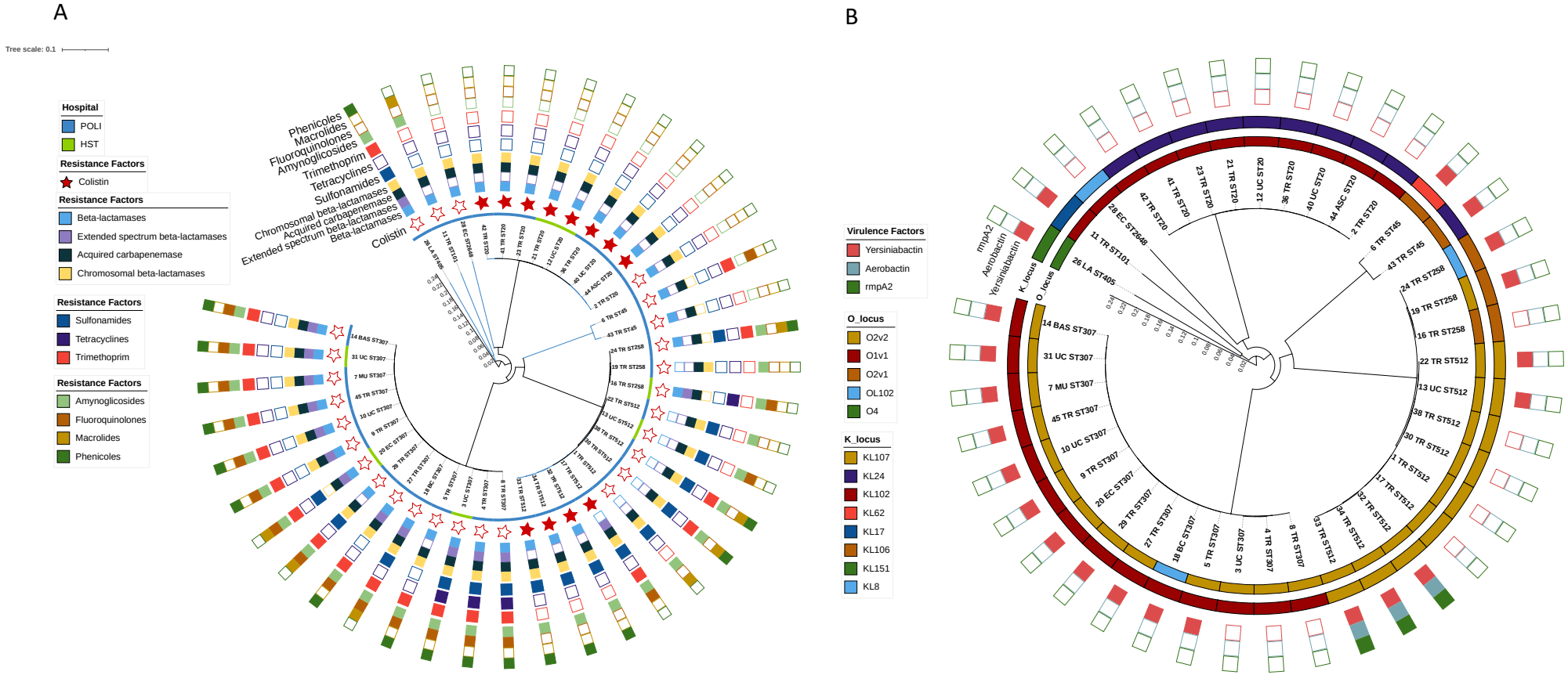
	KPC_{Pts} N=30	**KPC_{COL} N=26**	**KPC_{INF} N=4**
Sequence Type			
• ST20	6 (20)	5 (19.2)	1 (25)
• ST45	2 (6.7)	2 (7.7)	0
• ST101	1 (3.3)	1 (3.8)	0
• ST258	2 (6.7)	2 (7.7)	0
• ST307	9 (30)	7 (26.9)	2 (50)
• ST405	1 (3.3)	1 (3.9)	0
• ST512	8 (26.7)	8 (30.8)	0
• ST2648	1 (3.3)	0	1 (25)
K locus			
• KL8	1 (3.3)	0	1 (25)
• KL17	1 (3.3)	1 (3.8)	0
• KL24	7 (23.3)	6 (23.2)	1 (25)
• KL62	1 (3.3)	1 (3.8)	0
• KL102	9 (30)	7 (26.9)	2 (50)
• KL106	2 (6.7)	2 (7.7)	0
• KL107	8 (26.8)	8 (30.8)	0
• KL151	1 (3.3)	1 (3.8)	0
O type			
• O1	8 (26.7)	6 (23.2)	2 (50)
• O2	19 (63.3)	18 (69.2)	1 (25)
• O4	1 (3.3)	1 (3.8)	0
• other	2 (6.7)	1 (3.8)	1 (25)
wzi locus			
• *wzi*24	6 (20)	5 (19.3)	1 (25)
• *wzi*29	2 (6.7)	2 (7.7)	0
• *wzi*101	1 (3.3)	1 (3.8)	0
• *wzi*137	1 (3.3)	1 (3.8)	0
• *wzi*143	1 (3.3)	1 (3.8)	0
• *wzi*149	1 (3.3)	1 (3.8)	0
• *wzi*154	8 (26.8)	8 (30.8)	0
• *wzi*173	9 (30)	7 (26.9)	2 (50)
• *wzi*490	1 (3.3)	0	1 (25)
bla_{KPC}			
• KPC-2	10 (33.3)	8 (30.8)	2 (50)
• KPC-3	20 (66.7)	18 (69.2)	2 (50)
Resistance score			
• 2	20 (66.7)	17 (65.4)	3 (75)
• 3	10 (33.3)	9 (34.6)	1 (25)
Virulence score			
• 0	13 (43.3)	11 (42.3)	2 (50)
• $\geq$1	17 (56.7)	15 (57.7)	2 (50)

Supplementary Table 6. Immunological parameters of the study population

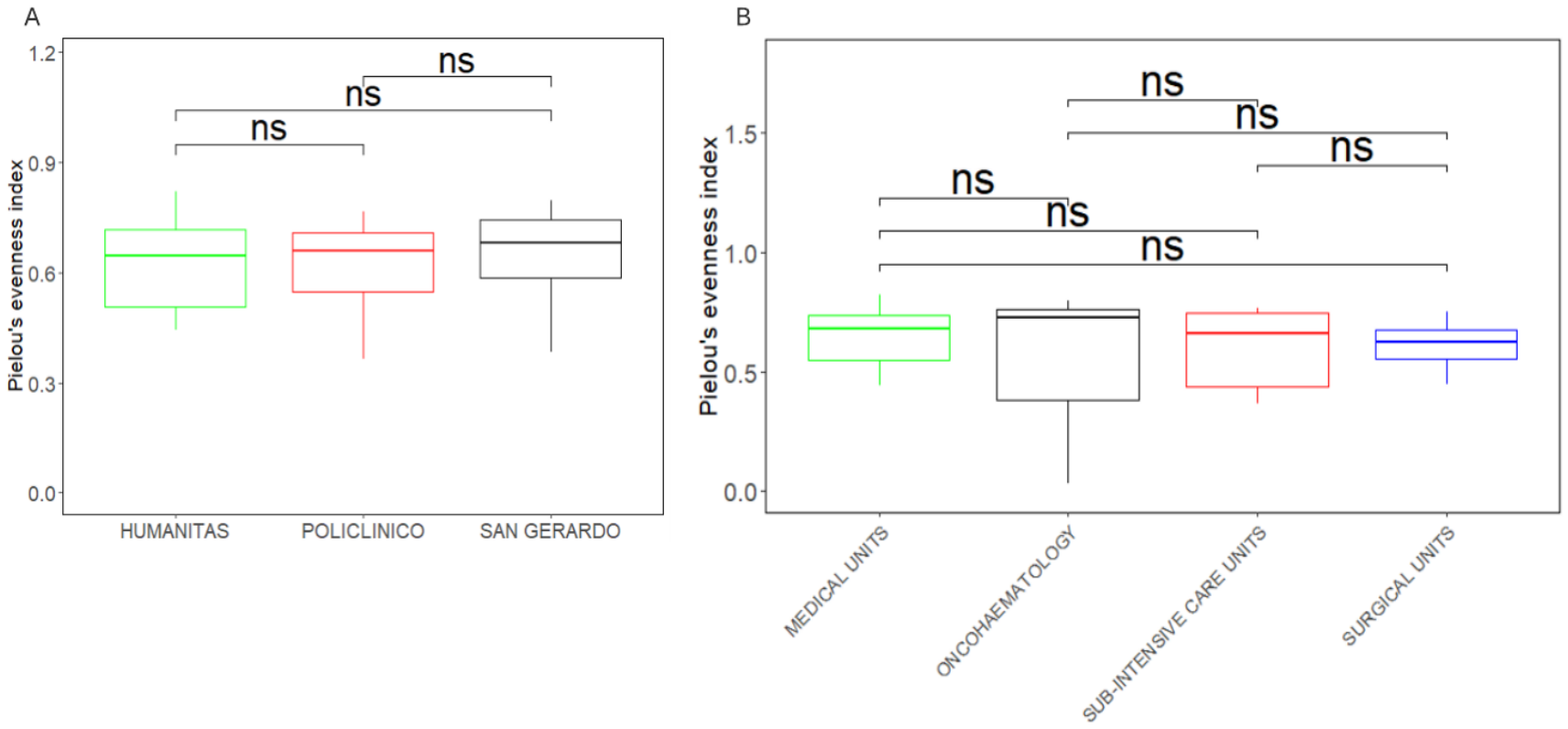
	KPC_{Pts}						
	N	Mean	Median	Lower Quartile	Upper Quartile	Minimum	Maximum
Circulating soluble inflammatory markers*							
PTX3, ng/ml	118	25.98	16.13	10.04	25.57	4.38	233.93
sIL-1R2, ng/ml	104	20.1	17.02	11.98	24.95	0	82.59
Circulating cell immunophenotype^							
Th1, % CD4+ T cells	58	9.96	7.27	3.64	15.3	0.12	33.9
Th2, % CD4+ T cells	58	11.75	5.46	2.96	19	0.12	38.1
Th17, % CD4+ T cells	58	18.35	11.8	4.8	34	0.31	50.5
Treg, % CD4+ T cells	58	5.65	5.6	3.49	7.58	0.02	14.1
Tfh, % CD4+ T cells	58	5.24	4.67	2.16	7.11	0	18.3
MAIT cells, % TCRab	58	1.13	0.62	0.25	1.66	0.03	8.74
ILC1, %LIN neg CD127+CD161+	58	19.56	14.8	9.09	27.1	1.44	100
ILC2, %LIN neg CD127+CD161+	58	8.59	9.15	2.34	13.5	0	20.7
ILC3, %LIN neg CD127+CD161+	58	3.31	1.71	1.01	4.23	0	24.1

Legend: *for 17 samples, analysis of soluble inflammatory markers is not available (not yet concluded or not performed due to low quality of samples), ^for 77 samples, analysis of circulating cell immunophenotype is not available (not yet concluded or not performed due to low quality of samples), PTX3 long pentraxin 3, sIL-1R2 soluble IL-1 receptor type 2, Th T helper cells, Treg T regulatory cells, Tfh follicular helper T cells, MAIT mucosal-associated invariant T cells, ILC innate lymphoid cells

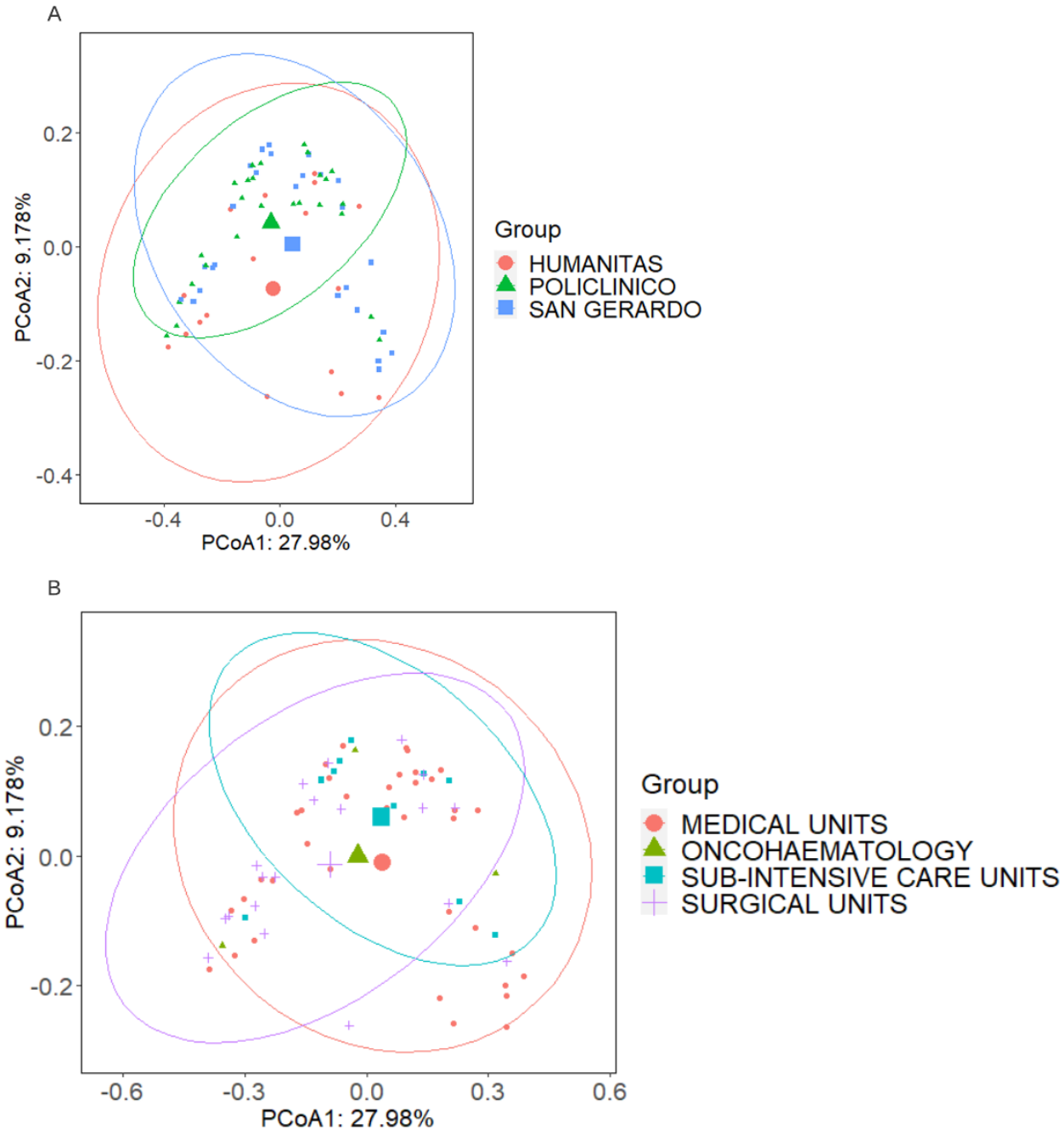
Supplementary Figure 1. Phylogenetic tree based on core SNPs alignment of the 37 KPC-Kp isolates analyzed. Panel A: inner circle shows hospitals from which strains were isolated; stars show the presence of mutations associated to colistin resistance; outer circles show betalactamases and other genes of antibiotic resistance. Panel B: inner circle shows O (lipopolysaccharide) locus; middle circle shows K (capsular) locus; outer circles show genes associated to virulence factors



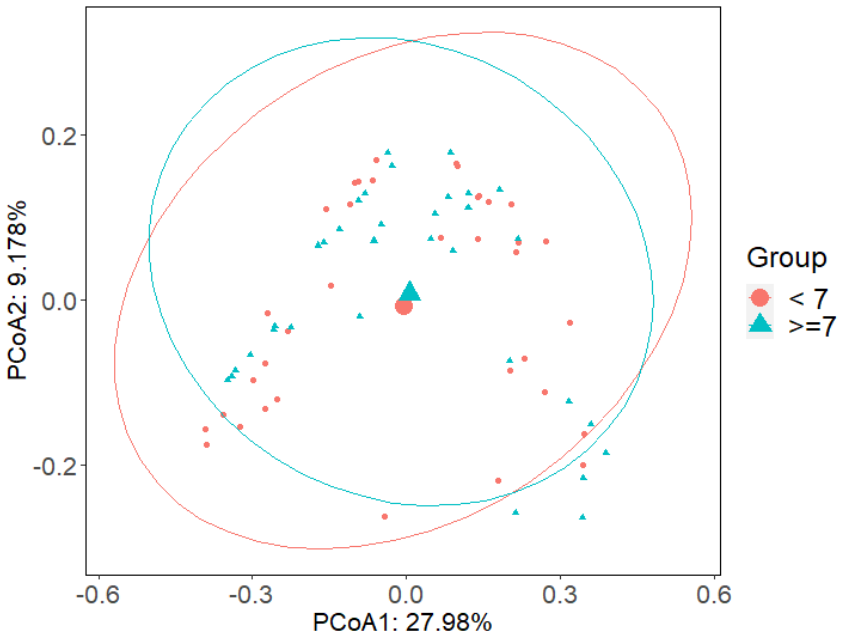
Supplementary Figure 2. Alpha diversity values (Pielou's eveness index) of gut microbiome of patients enrolled in the KPC MDP study. No differences were found comparing samples from the three participating centers (A) nor comparing different wards of KPC-*Kp* isolation (B)



Supplementary Figure 3. Beta diversity values (Unweighted Unifrac) of gut microbiome of patients enrolled in the KPC MDP study. No differences were found comparing samples from the three participating centers (A) nor comparing different wards of KPC-*Kp* isolation (B)



Supplementary Figure 4. Beta diversity values (Unweighted Unifrac) of gut microbiome of KPC-*Kp* patients depending on time elapsed from KPC-*Kp* isolation to fecal sample collection (<7 vs ≥7 days). No significant differences were found between the two groups.



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