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Influência da colonização por Enterobacterales produtoras de carbapenemases no desfecho clínico de pacientes transplantados

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"Hay, en verdad, dos cosas diferentes: saber y creer que se sabe. La ciencia consiste en saber; en creer que se sabe está la ignorancia"

Hipócrates

Resumo

Os transplantes têm o propósito de prolongar e melhorar a qualidade de vida de pacientes com doenças terminais. De acordo com o *Global observatory on donation and transplantation* (GODT), em 2018 foram realizados 146.840 transplantes de órgãos, e, segundo a *World Health Organization* (WHO), mais de 50.000 transplantes de células tronco hematopoiéticas são realizados no mundo anualmente. Os pacientes transplantados são um grupo de risco para contrair infecções por agentes bacterianos, principalmente associadas às Enterobacterales. As Enterobacterales fazem parte da microbiota intestinal humana, e aquelas produtoras de carbapenemases (EPC) estão frequentemente associados com infecções adquiridas no ambiente hospitalar, sendo considerados um dos principais patógenos associados a infecções em pacientes transplantados. Representados principalmente por *Klebsiella pneumoniae*, as EPCs são uma preocupação mundial de acordo com autoridades como o *Centers for Disease Control and Prevention* (CDC) e a *World Health Organization* (WHO) no tratar-se de resistência antimicrobiana. Tal preocupação se deve ao fato de estes microrganismos serem uma causa emergente de infecções associadas à saúde pública. Quando presentes, as infecções por EPCs são difíceis de serem tratadas devido à resistência a diversos antimicrobianos (MDR), estando associadas a um aumento na mortalidade. Neste estudo, primeiramente detectamos um caso clínico de transmissão plasmidial de resistência aos carbapenêmicos em uma infecção abdominal. Relatamos pela primeira vez a transferência do gene $bla_{\mathrm{KPC\text{-}2}}$ de um isolado de *K. pneumoniae* para um de *Kluyvera ascorbata* através da confirmação por testes de conjugação. Também detectamos que o plasmídeo responsável por carrear o gene $bla_{\mathrm{KPC\text{-}2}}$ era da família de incompatibilidade IncN através de sequenciamento de genomas completos (WGS). Em um segundo trabalho, selecionamos 80 isolados de CPE para realizar a técnica de WGS. Detectamos a presença de 4 clones epidêmicos de *K. pneumoniae* produtores de carbapenemases (Kp-PC): ST11/KPC-2, ST16/KPC-2, e ST15/NDM-1, todos responsáveis por surtos inter e intra-hospitalares, e ST437/KPC-2 o qual esteve presente em apenas em um hospital. Identificamos o resistoma e o viruloma destes clones, que possuem em média 9 genes de resistência adquirida e 27 genes de virulência. Ainda, identificamos plasmídeos responsáveis por carrear as carbapenemases, sendo IncN o principal associado a $bla_{\mathrm{KPC\text{-}2}}$, e possivelmente o maior responsável pela disseminação deste gene no Brasil. Por outro lado, a presença do gene $bla_{\mathrm{NDM\text{-}1}}$ esteve associada de maneira restrita ao plasmídeo IncFIB. Em um terceiro trabalho, buscamos metodologias alternativas e promissoras para a detecção de resistência a polimixina B em isolados de EPC. Este trabalho foi dividido em três etapas: a) avaliação da praticidade e acurácia do método de eluição de disco de polimixina B em caldo (PBDE), que se mostrou um método acurado e tão confiável quando o padrão ouro (BMD), com excelente concordância categórica (CA, 99,5%), aceitáveis *very major error* (VME, 1,11%) e nenhum *major error* (ME); b) Avaliação das metodologias de *screening test* e diluição em agar, que apesar da baixa sensibilidade, as metodologias baseadas em agar utilizando a polimixina B tiveram uma boa performance considerando todos os outros parâmetros de qualidade (CA, 93,4%, sensibilidade – Sen, 86,2%, especificidade - Spe, 98,7%, valor preditivo positivo - PPV, 98%, valor preditivo negativo - NPV, 90,7% , e índice *kappa* – κ, 0,86 para o *screening test*; e CA, 92,7%, Sen, 86,2%, Spe, 97,5%, PPV, 96,1%, NPV, 90,6%, e κ, 0,85 para o agar dilution); c) Avaliação da acurácia do teste polimixina NP utilizando colônias semeadas a partir de hemoculturas, e também, amostras colhidas diretamente de frascos de hemoculturas. . Em ambos os testes, a partir da colônia (NP-*colony*) e dos frascos (NP-*bottle*) obtivemos excelentes performances (CA, 96,2%, Sen, 98,9%, Spe 94,4%, PPV, 92,2%, NPV, 99,3% para NP-*colony*; e CA, 93,4%, Sen, 94,1%, Spe, 93,7%, PPV, 94,1% e NPV, 93,7%, para NP-*bottle*). Estes resultados sugerem que ambos os testes podem ser fortes candidatos para implementação em laboratórios clínicos de microbiologia. Por fim, avaliamos as características da coorte, em um modelo prospectivo dos pacientes transplantados. Também avaliamos as características dos isolados de EPC oriundos destes pacientes e concluímos que a transferência sucessiva de pacientes de uma unidade para a outra dentro do Complexo Hospitalar favorece a disseminação e promoção de surtos por EPC e outros microrganismos muitirresistentes (MDR). Além disto, variáveis como hospitalização por mais de 30 dias, uso de ventilação mecânica ($\geq$ 24h), transferências sucessivas entre unidades, e colonização por *K. pneumoniae* são fatores de risco independentes para os pacientes desenvolverem uma infecção por estes patógenos. E, como esperado, estar infectado por EPC é um fator de risco para óbito. Essa tese gerou resultados relevantes, apresentando problemas de saúde pública que necessitam ser discutidos de maneira extensiva e pesquisados através de estudos adicionais para melhor entendermos a estrutura organizacional, os fatores de resistência e virulência de EPC, especialmente *K. pneumoniae* isolados de pacientes transplantados, com o objetivo de prevenir e controlar novos surtos, uma vez que sabemos o grande impacto econômico e sanitário relacionado a estes temas. E por fim, também produzimos importantes resultados para o avanço nas metodologias diagnósticas relacionadas a detecção de susceptibilidade a polimixina B em ERCs.

Palavras-chave: Transplantes, Enterobacterales, carbapenemases, polimixina B, sequenciamento de genomas completos

Abstract

Transplants are intended to prolong and improve the quality of life of patients with terminal illnesses. According to the Global observatory on donation and transplantation (GODT), 146,840 organ transplants were performed in 2018, and the World Health Organization (WHO) reported that more than 50,000 hematopoietic stem cell transplants are annually performed worldwide. Transplanted patients are a high-risk group to contracting infections by bacterial agents, mainly associated with Enterobacterales. The Enterobacterales are common in human intestinal microbiota, and those producing carbapenemases (CPE) are often associated with hospital acquired infections and are considered one of the most important pathogens responsible for infections in transplanted patients. According to the Centers for Disease Control and Prevention (CDC) and the WHO, CPEs, mainly *Klebsiella pneumoniae*, are a worldwide public health concern due its emergence on causing Hospital infections. CPE infections are difficult to treat due their antimicrobial multidrug resistance (MDR) characteristic, and to be associated with an increase in mortality. In this study, firstly we detected a clinical case of a plasmid mediated transfer of carbapenem resistance during abdominal infection. We reported for the first time the transfer of the $bla_{\text{KPC-2}}$ gene from a *K. pneumoniae* to a *Kluyvera ascorbata* mediated by a IncN plasmid. Secondly, we selected 80 CPE isolates to perform the WGS technique. We detected 4 carbapenemase-producing *K. pneumoniae* (CP-Kp) epidemic clones: ST11/KPC-2, ST16/KPC-2, and ST15/NDM-1, responsible for intrahospital and interhospital outbreaks; And ST437/KPC-2 which was detected in only one hospital. We identified resistome and virulome of these clones, that are composed by a mean of 9 acquired resistance genes and 27 virulence genes. We identified plasmids carrying carbapenemase genes. IncN was the most frequently associated to $bla_{\text{KPC-2}}$ and possibly the main responsible for its dissemination in Brazil. On the other hand, $bla_{\text{NDM-1}}$ gene was restrict associated to the IncFIB plasmid. Thirdly, we searched for alternative methods to detect polymyxin B resistance in CPE isolates. We divided this moment in 3 phases: a) Evaluation of accuracy and feasibility of the polimixin B disk elution (PBDE) method, which was accurate and as reliable as the gold standard (BMD), with excellent categorical agreement (CA, 99.5%), acceptable *very major error* (VME, 1.11%) and no *major error* (ME); b) Evaluation of screening test and agar dilution, which even both presenting low sensitivity, agar-based methodologies using polymyxin B had great performance when considering all the other quality parameters (CA, 93.4%, sensitivity – Sen, 86.2%, specificity - Spe, 98.7%, positive predictive value - PPV, 98%, negative predictive value - NPV, 90.7% , and *kappa* index – κ, 0.86 to *screening test*; and CA, 92.7%, Sen, 86.2%, Spe, 97.5%, PPV, 96.1%, NPV, 90.6%, and κ, 0.85 to agar dilution); c) Evaluation of the polymyxin NP test accuracy using bacterial colonies and directly from blood culture bottles, both tests from colony (NP-colony) and bottles (NP-bottle) showed excellent performances (CA, 96.2%, Sen, 98.9%, Spe 94.4%, PPV, 92.2%, NPV, 99.3% to NP-*colony*; and CA, 93.4%, Sen, 94.1%, Spe, 93.7%, PPV, 94.1% and NPV, 93.7%, to NP-*bottle*), suggesting that they can be strong candidates to be used in microbiology clinical laboratories after more studies. Finally, we evaluated the cohort characteristics, in a prospective model, of the transplanted patients. Also, we evaluated the characteristics of the CPE isolated from these patients. We concluded that patient successive transfer to different Hospital Units favors dissemination and promotion of CPE outbreaks and other MDR microorganisms in this Hospital Complex. Variables as hospitalization (> 30 days), mechanical ventilation (≥ 24h), successively transfer to different units, and colonization by *K. pneumoniae* are independent risk factors to develop a CPE infection. And, as expected, to be infected by a CPE is a risk factor for death. This thesis generated relevant results, presenting public health problems that need to be extensively discussed and researched. Additional studies may help to better understand the organizational structure, resistance, and virulence features in CPE, especially *K. pneumoniae* originated from transplanted patients. Thus, preventing and controlling new outbreaks that are known for their great sanitary and economic impact worldwide. And lastly, we also produced important results to the advance of diagnostic methodologies in relation to the CRE polymyxin B susceptibility detection.

Palavras-chave: Transplants, Enterobacterales, carbapenemases, polymyxin B, whole genome sequencing

Lista de Abreviações

ABTO - Associação Brasileira de Transplante de Órgãos

BKC - *Brazilian Klebsiella pneumoniae*

bla - Gene beta-lactamase

BMD - *Broth microdilution* (Microdiluição em caldo)

BrCAST - *Brazilian Committee on Antimicrobial Susceptibility Testing*

CAT - colistina em agar

CBDE – *Colistin broth disk elution* (Eluição de disco de colistina em caldo)

CC - Complexo clonal

CDC - *Centers for Disease Control and Prevention*

CLSI - *Clinical and Laboratory Standards Institute*

EPC - Enterobacterales produtores de carbapenemases

ERC - Enterobacterales resistentes aos carbapenêmicos

ERC-não-PC - Enterobacterales resistentes aos carbapenêmicos não-produtores de carbapenemases

ERC-PC - Enterobacterales resistentes aos carbapenêmicos produtores de carbapenemase

ESBL - Beta-lactamases de amplo espectro

EUA – Estados Unidos da América

EUCAST - *European Committee on Antimicrobial Susceptibility Testing*

FAO - *Food and Agriculture Organization of the United Nations*

GODT - *Global observatory on donation and transplantation*

IMP - Imipenemase

Inc - I*ncompatibility family* (Família de incompatibilidade)

IS – *Insertion sequences* ou Sequências de Inserção

KPC - *Klebsiella pneumoniae* carbapenemase

Kp-KPC - *Klebsiella pneumoniae* produtoras de KPC

Kp-NDM - *Klebsiella pneumoniae* produtoras de NDM

L-Ara4N - 4-amino-4-deoxy-L-arabinose

LPS - Lipopolissacarídeo

MBL - Metallo-beta-lactamase

mcr-1 - *Mobile colistin resistance*

MDR - Multirresistentes

ME - *Major error*

MIC - Concentração inibitória mínima

MRSA - *Staphyloccocus aureus* resistentes à meticilina

NDM - *New Delhi* metallo-beta-lactamase

OMS ou WHO - Organização mundial da Saúde ou *World Health Organization*

OTR - Pacientes receptores de órgãos

OXA – Oxacillinase

OIE - *World Organisation for Animal Health*

PCR - *Polymerase Chain Reaction*

PEtN - fosfoetalonamina

PK/PD - Farmacocinética e farmacodinâmica

RBT - Registro Brasileiro de Transplantes

ST - *Sequence Type*

TCTH - Tecidos e células tronco hematopoiéticas

Tn - Transposon

tnp - Transposase

TOS - Transplantes de órgãos sólidos

UTI - Unidade de terapia intensiva

VIM - Verona imipenemase

VME - *Very major error*

VRE - *Enterococcus* resistentes à vancomicina

WGS - *Whole genome sequencing* (Sequenciamento de genoma completo)

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Sumário

1. Revisão da literatura

1.1. Transplantes

No início do século XX foi realizada uma série de estudos experimentais que representaram o ponto de partida para os transplantes de órgãos em humanos. Em 1912 Alexis Carrel foi contemplado com o prêmio Nobel em Fisiologia ou Medicina, reconhecimento baseado no pioneirismo em pesquisas com cirurgias experimentais e transplantes de órgãos e tecidos (4).

O primeiro procedimento concluído com sucesso foi um transplante renal realizado em 1954 envolvendo dois irmãos em Boston, Estados Unidos da América (EUA). E no Brasil os transplantes tiveram início em 1964, também com um transplante renal como pioneiro, e logo, transplantes cardíacos, hepáticos, intestinais e pancreáticos (5).

Nas últimas décadas os transplantes têm se tornado uma prática disseminada mundialmente. Estes procedimentos têm melhorado significativamente a qualidade de vida de milhares de pessoas. Os contínuos avanços na tecnologia médica levaram a uma diminuição de complicações pós transplante e um aumento na confiança das pessoas em relação ao procedimento, refletindo em um aumento de doadores vivos nos últimos anos (6).

Atualmente são realizados transplantes de órgãos sólidos (TOS), tecidos e células tronco hematopoiéticas (TCTH). Estes procedimentos são feitos a partir de um doador, pessoa viva ou falecida, para um receptor (7). De acordo com o *Global observatory on donation and transplantation* (GODT), foram realizados 17 transplantes por hora no ano de 2018, traduzidos em 146,840 órgãos transplantados (Figura 1), 5.6% a mais do que em 2017, no entanto, representam menos de 10% da necessidade da população mundial (1). No Brasil a tendência desse aumento anual se repete, o número total de transplantes em 2019 (9,232) foi 4.36% maior se comparado com o ano de 2018 (8).

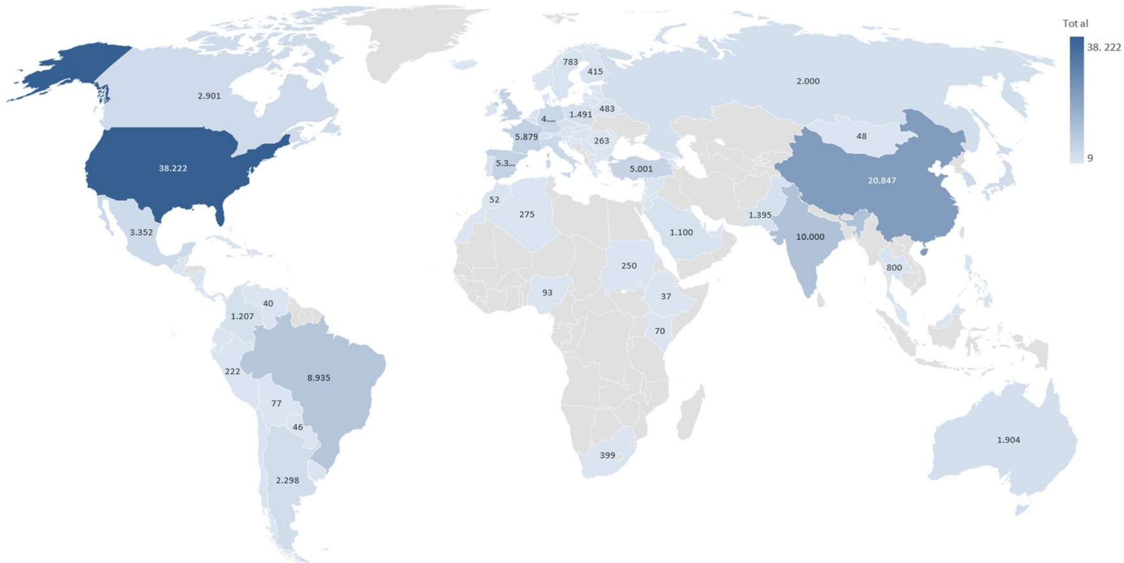

Figura 1. Mapa com o número total de transplantes de órgãos sólidos em cada país em 2018. Adaptado de GODT (2018) (1).

O manejo dos pacientes após o transplante é crucial, uma vez que estes, possuem alterações fisiológicas e anatômicas, permanecem internados por tempo prolongado em Unidades de Terapia Intensiva (UTI), e são frequentemente expostos a antimicrobianos de amplo espectro (9). Desta forma, estão sujeitos a infecções por agentes bacterianos que podem ser transmitidos pelo doador ou pelo ambiente hospitalar, ocasionando complicações agravadas pelo fato de os pacientes estarem imunodeprimidos (10). Portanto, estes pacientes constituem um grupo de alto risco para desenvolver complicações como rejeição do enxerto, retorno de doença de base, e principalmente, infecções bacterianas por importantes patógenos como as Enterobacterales (11–15) principal causa de mortalidade e morbidade neste grupo (10).

Como comentado anteriormente, os transplantes são procedimentos cirúrgicos e podem ser identificados de acordo com o sítio ou material biológico a ser transferido do doador para o receptor. O item a seguir, 1.1.1, irá abranger os TOS, os quais são os procedimentos mais frequentes no Brasil e no Mundo.

1.1.1. Transplantes de órgãos sólidos (TOS)

Após o transplante, há uma expectativa de melhora no desfecho clínico dos pacientes. Esta melhora está diretamente relacionada com os constantes avanços tecnológicos na área médica e científica, tais como: melhor compreensão da resposta imune, conhecimento na área de preservação de órgãos, uso de imunossupressores mais potentes e seguros, prevenção e tratamento de rejeição do enxerto e protocolos específicos de prevenção e tratamento de infecções (16).

Segundo o GODT, no ano de 2018 foram realizados 95.479 transplantes renais, 34.074 hepáticos, 8.311 cardíacos, 6.475 pulmonares, 2.338 pancreáticos e 163 intestinais (17). Já no Brasil, de acordo com o Registro Brasileiro de Transplantes (RBT) da Associação Brasileira de Transplante de Órgãos (ABTO), entre janeiro e setembro de 2020 foram realizados 5.357 transplantes de órgãos sólidos. Sendo que destes, 3.486 transplantes renais, 1.506 hepáticos, 218 cardíacos, 108 pancreáticos e 39 pulmonares (18).

Os procedimentos cirúrgicos se tornaram mais seguros, e há uma taxa mundial crescente de transplante de órgãos, porém, complicações pós-cirúrgicas são recorrentes em receptores de órgãos. O principal risco de complicações está relacionado a infecções nosocomiais que ocorrem na fase inicial pós-transplante (19). Podem ocorrer infecções por patógenos oportunistas nas fases intermediária e final pós-transplante e assim como as da fase inicial, devem ser detectadas precocemente. Desta maneira, tratamento precoce é a chave para ter melhor prognóstico em receptores de órgãos sólidos com infecções severas (19).

No entanto, embora os transplantados sejam suscetíveis a infecções por uma quantidade significativa de patógenos, manifestam sinais e sintomas diminuídos de infecção invasiva. Portanto, o diagnóstico de infecção é dificultado nestes indivíduos se comparados aos indivíduos imunocompetentes (20). As infecções representam a maior causa de morte dentro de 1 ano após transplantes de coração ou pulmão, respectivamente 32% e 35% receptores vão a óbito (19).

Os receptores de órgãos sólidos são suscetíveis à infecções devido a uma série de fatores: 1) fatores pré-transplante (condições imunológicas e doenças críticas); 2) tipo de órgão transplantado; 3) características intraoperatórias (duração prolongada do procedimento cirúrgico, necessidade de transfusão de sangue); 4) e fatores pós-transplante (grau de imunossupressão, profilaxia ajustada e possível infecção por citomegalovírus) (19,21).

Durante o primeiro mês após o transplante, infecções provenientes de complicações na cirurgia, do doador, pré-existentes no receptor, e nosocomiais são mais comuns. E aqueles receptores de coração, pulmão e fígado possuem maior risco de desenvolver infecções quando comparados com pacientes receptores renais (19). Entretanto, pacientes receptores renais que apresentam vazamentos anastomóticos ureterais, perfusato contaminado, fazem uso de cateteres ureterais e cateteres venosos centrais, possuem um alto risco de contrair infecções no período inicial do pós-operatório (19).

Estas infecções ocorridas no período pós-transplante são agravadas na presença de microrganismos multirresistentes (MDR), especialmente bacilos gram-negativos (22). Por definição, organismos MDR são aqueles resistentes a, pelo menos 1 agente, de 3 ou mais diferentes classes de antimicrobianos (23). Incluem-se possivelmente neste grupo, bactérias não-fermentadoras como *Pseudomonas aeruginosa*, *Burkholderia* spp., ou *Acinetobacter baumannii* resistente aos carbapenêmicos (CRAB), bem como Enterobacterales produtores de β-lactamases de espectro estendido (ESBL), e Enterobacterales resistentes aos carbapenêmicos (ERC), especialmente *Klebsiella pneumoniae* produtora de carbapenemases (24,25).

A incidência de infecções por Enterobacterales ESBL MDR em receptores de transplantes de fígado varia de 5,5% a 7%, sendo as espécies mais comuns *K. pneumoniae* e *E. coli.* Por outro lado, as infecções por ERC MDR neste mesmo grupo variam de 6 a 12,9% dos casos (26). Já em transplantados renais, grupo que apresenta uma maior taxa de infecções pós-transplante, estima-se que a prevalência de infecções do trato urinário é de aproximadamente 38% (27,28). *E. coli* ESBL é responsável por 12% das infecções (29), e no caso das *K. pneumoniae* resistentes aos carbapenêmicos a incidência de infecção varia de 3 a 10% (15,30–33), sendo que 50% das infecções urinárias estão associadas com recorrência nestes pacientes (34). CRABs podem causar pneumonia, particularmente associada a ventilação mecânica (P-VM), e são responsáveis por pelo menos 3% dos episódios de infecções sanguíneas após transplante renal (35,36).

Infecções pós transplante de coração são principalmente associadas a pneumonia, infecções no trato urinário e bacteremia, sendo os agentes mais comuns ERCs, *P. aeruginosa* MDR e CRAB (37). Um estudo publicado por Zhou *et al.*(38) reportou a presença de infecções bacterianas causadas por organismos MDR, como *K. pneumoniae* ESBL (26,1% dos casos), CRAB (0,4%) *E. coli* ESBL (3,1%), e *P. aeruginosa* (0,8%), em pacientes receptores de transplante cardíaco. Em relação aos pacientes receptores de transplante de pulmão, pneumonia associada a *P. aeruginosa* é frequente, sendo este não-fermentador responsável por aproximadamente 25% dos casos (39). Um resumo dos patógenos mais frequentes responsáveis por infecções em pacientes transplantados é apresentado na tabela 1.

Tabela 1. Patógenos mais frequentes de acordo com o tipo de transplante.

Patógeno		Tipo de Transplante					
		Renal	Pancreático	Intestinal	Hepático	Pulmonar	Cardíaco
Bactéria	Enterobacterales	x	x	x	x	x	x
	Enterococcus spp.		x	x	x		
	Staphylococcus spp.		x	x	x	x	x
	Pseudomonas spp.					x	
	Burkholderia spp.					x	
	Anaeróbios		x	x			
Fungo	*Pneumocystis jirovecii*	x	x	x	x	x	x
	Candida spp.	x	x	x	x		
	Aspergillus spp.	x	x	x	x	x	x
Vírus	CMV	x	x	x	x	x	x
	HSV/VZV	x	x	x	x	x	x
Protozoário	*Toxoplasma*						x

CMV, Citomegalovírus; HSV, Herpes simplex vírus; VZV, Varicella zoster vírus

Adaptado de Timsit *et al.* 2019 (19).

1.1.2. Transplantes células tronco hematopoiéticas (TCTH)

Assim como mencionado anteriormente para os TOS, houve um grande progresso no que diz respeito aos TCTH nas últimas décadas. Estes avanços resultaram em alterações nas políticas de fontes de doadores de células tronco, nas condições de regime para os transplantados, e nos tratamentos de suporte (40,41). De acordo com a Organização Mundial da Saúde (OMS), o transplante é eficaz no tratamento de 90% dos pacientes com doenças hematológicas malignas e não-malignas em estágios precoces, caso haja um doador compatível (42).

Mais de 50.000 transplantes são realizados anualmente no mundo inteiro e há uma tendência de aumento ano após ano (42). No Brasil, de acordo com o RBT da ABTO, foram realizados 2.146 transplantes de janeiro a setembro de 2020. Sendo que 1.270 foram autólogos (procedimento utiliza as células tronco do próprio paciente) e 876 alogênicos (procedimento utiliza células tronco de outro indivíduo) (18).

No entanto, uma das principais complicações que estes pacientes enfrentam, é a infecção por bactérias multirresistentes (MDR), as quais contribuem significativamente para o aumento de mortalidade, que podem chegar a 64,4% quando pacientes de TCTH apresentam infecções por ERCs, especialmente as produtoras de carbapenemases (40,41,43,44). Infecções da corrente sanguínea, pneumonia e infecções gastrointestinais são mais frequentes nestes pacientes. Infecções do trato urinário são menos frequentes e normalmente associadas ao uso de cateteres (14).

Estas infecções podem ser ocasionadas por uma série de patógenos, dentre eles, *Staphyloccocus aureus* resistentes à meticilina (MRSA), *Enterococcus* resistentes à vancomicina (VRE), *Clostridium difficille, Mycobacterium tuberculosis*, e vírus respiratórios adquiridos na comunidade (45). Entretanto, de acordo com Michael Satlin e Thomas Walsh (46), as três maiores ameaças para os receptores de TCTH são, Enterobacterales MDR, incluindo aqueles produtores de beta-lactamases de amplo espectro (ESBL) e os resistentes aos carbapenêmicos (ERC), *Pseudomonas aeruginosa*, e também os VREs. Estas bactérias são consideradas de tal maneira, porque são frequentes causadoras de infecções nos TCTH, e bacteremias causadas por estes microrganismos estão associadas a altas taxas de mortalidade (46).

1.2. Enterobacterales

As bactérias da ordem Enterobacterales são bacilos Gram-negativos, anaeróbios facultativos, não formadores de esporos. Membros desta ordem, principalmente da família *Enterobacteriaceae*, são amplamente reconhecidos como patógenos para humanos e animais. Exemplos que podem ser citados são as espécies *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Salmonella entérica*, e *Yersinis pestis* (47).

Estas bactérias fazem parte da microbiota intestinal humana e estão frequentemente associados com infecções adquiridas no ambiente hospitalar, que têm origem nos tratos respiratório, gastrointestinal e urinário, assim como na corrente sanguínea (48). Também podem ser associadas a infecções comunitárias, nas quais *E. coli* é uma das maiores causadoras de infecções no trato urinário e bacteremias (49).

As Enterobacterales, como mencionado no item 1.1, estão entre os principais patógenos causadores de infecções em pacientes transplantados. Um tema preocupante é a resistência aos antibióticos apresentada por este grupo de microrganismos. Estas bactérias podem disseminar seus mecanismos de resistência rapidamente através de elementos genéticos móveis como plasmídeos e transposons (50), limitando as opções de tratamento e aumentando as probabilidades de falha terapêutica.

Os antibióticos da classe dos β-lactâmicos, tais como cefalosporinas de 3° e 4° geração e penicilinas são frequentemente utilizadas como primeira escolha no tratamento de infecções causadas por Enterobacterales. Após o uso substancial destes agentes surgiram as primeiras evidências de cepas resistentes a estes antibióticos. Esta resistência estava diretamente relacionada com a produção de enzimas β-lactamases de espectro extendido (ESBL), as quais são responsáveis por conferir resistência à grande parte dos β-lactâmicos, porém ainda se mantém sensíveis aos carbapenêmicos, cefamicinas, e inibidores de β-lactamases, como ácido clavulânico e tazobactam (51).

Devido ao aumento da quantidade de cepas produtoras de ESBL, e também ao seu espectro ampliado e sua estabilidade frente à β-lactamases (52), os carbapenêmicos tornaram-se os antibióticos de escolha no tratamento de infecções graves por Enterobacterales. Esta classe de antibióticos, tendo como primeiro representante o imipenem, foi introduzida e aprovada na década de 1980, para uso clínico (53). Subsequentemente foram introduzidos meropenem, ertapenem e doripenem, e até o final da década de 1990 havia uma baixa frequência de resistência aos carbapenêmicos (54). No entanto, sua utilização indiscriminada nas últimas décadas, somada com a habilidade de alguns microrganismos em transferir elementos genéticos móveis que conferem resistência, impulsionaram a emergência de bactérias resistentes a esta classe (55). Isto gera uma grande preocupação para os profissionais da saúde, uma vez que a realização de transplantes é considerada um fator de risco para contrair infecções causadas por Enterobacterales Resistentes aos Carbapenêmicos (ERC), principalmente devido ao período prolongado de internação hospitalar e a frequente exposição a terapias antimicrobianas de amplo espectro (56–59). Patel *et al.*(60) descreveram após uma análise estatística multivariável, que em pacientes transplantados foi encontrado um risco aumentado em 3,71x (IC 95%; 1,41 – 9,73; p = 0,008) de desenvolver uma infecção por ERC, especialmente *K. pneumoniae*, quando comparado com os controles (infecção por Enterobacterales sensíveis aos carbapenêmicos - ESC).

1.2.1. Enterobacterales *resistentes aos carbapenêmicos (ERC)*

A definição de ERC recomendada pelo *Centers for Disease Control and Prevention* (CDC) refere-se a isolados de Enterobacterales que apresentam resistência ao ertapenem, imipenem, meropenem ou doripenem, de acordo com as *guidelines* do *Clinical and Laboratory Standards Institute* (CLSI) (61). Há uma ressalva relacionada aos membros dos gêneros *Providencia* spp.*,* *Proteus* spp.*,* e *Morganella* spp. , em que a resistência unicamente ao imipenem não confere o critério de ERC, uma vez que estes organismos tem concentrações inibitórias mínimas (MICs) intrinsecamente elevadas ao referido antimicrobiano (62). Assim como o CLSI, o *European Committee on Antimicrobial Susceptibility Testing* (EUCAST) é referência mundial para métodos de detecção e pontos de corte ideais em determinado grupo bacteriano (61,63). Recentemente, o *Brazilian Committee on Antimicrobial Susceptibility Testing* (BrCAST)(64) também passou a ser utilizado no Brasil tendo como referência o EUCAST.

Tabela 2. Pontos de corte clínicos de concentração inibitória mínima para carbapenêmicos em Enterobacterales de acordo com CLSI, EUCAST e BrCAST.

Carbapenêmicos	**CLSI 2020 (S; I; R)**	**EUCAST 2021 (S; R)**	**BrCAST 2021 (S; I; R)**
Doripenem	≤1; 2; ≥4	≤1; >2	≤1; 2; >2
Ertapenem	≤0,5; 1; ≥2	≤0,5; >0,5	≤0,5; -; >0,5
Imipenem	≤1; 2; ≥4	≤2; >4[1] ≤0,001; >4[2]	≤2; 4; >4[1] ≤0,001; 0,002-4; >4[2]
Meropenem	≤1; 2; ≥4	≤ 2; >8	≤2; 4-8; >8

CLSI, *Clinical and Laboratory Standards Institute*; EUCAST, *European Committee on Antimicrobial Susceptibility Testing*; BrCAST, *Brazilian Committee on Antimicrobial Susceptibility Testing*; S, sensível; I, intermediário; R, resistente.
* Todos os MICs estão listados em µg/mL
[1]Enterobacterales exceto *Morganellaceae*
[2]*Morganellaceae*. A atividade intrinsecamente baixa do imipenem contra *Morganella morganii*, *Proteus* spp. e *Providencia* spp. requer alta exposição ao imipenem.

Os ERC, principalmente *K. pneumoniae*, são considerados uma preocupação mundial de acordo com diversas organizações consideradas autoridades no combate a resistência antimicrobiana. Alcançaram o nível máximo de importância segundo o documento *Urgent Resistance Threats* do *CDC* (65); são considerados prioridade no programa *5-Year antimicrobial resistance national action plan* do *UK's Department of Health and Social Care* (66); e são considerados também prioridade na *priority list of resistant pathogens* da *WHO (World Health Organization)* (67).

Tal preocupação se deve ao fato de estes microrganismos serem uma causa emergente de infecções associadas à saúde pública (68). E, quando presentes, as infecções por ERCs são difíceis de serem tratadas devido à sua característica de apresentar resistência à diversos antimicrobianos, e serem associadas a um aumento na mortalidade. Nos Estados Unidos estima-se que as infecções por ERC resultam em 26% de mortalidade e um custo financeiro de aproximadamente 275 milhões de dólares anualmente (69). Em uma análise realizada em países de baixa e média renda (PANORAMA) foi concluído que ERCs causando infecções de corrente sanguínea foram associados com o aumento de 75% de probabilidade de mortalidade hospitalar, uma diminuição próxima a 40% na probabilidade de alta hospitalar com vida e um aumento no tempo de hospitalização de 3,7 dias (70). Além disto, como mencionado na sessão 1.2., estes microrganismos têm a capacidade de disseminar resistência através de elementos genéticos (71).

Infecções ocasionadas por ERCs estão frequentemente relatadas em pacientes receptores de órgãos (OTR). As taxas de mortalidade neste grupo de pacientes variam de 30-50% quando desenvolvem uma infecção causada por uma ERC (15,72). E quando observados dados apenas relacionados a *K. pneumoniae*, o risco de morte pode aumentar em até 10 vezes (73,74), refletindo em um problema de saúde pública mundial (11–15). Além disto, as infecções por ERCs levam o paciente a permanecer por tempo prolongado internado, aumentam custos financeiros e são associadas com maior mortalidade se comparadas com Enterobacterales sensíveis aos carbapenêmicos (Ben-David *et al.* - 48% vs 17%; Patel *et al* – 38% vs 12%) (60,75,76).

As ERCs são divididas de acordo com seu mecanismo de resistência em dois grupos: 1) ERCs produtoras de carbapenemase (ERC-PC); 2) e ERCs não-produtoras de carbapenemases (ERC-não-PC), e os mecanismos de resistência mais frequentes serão abordados no item 1.2.2.

1.2.2. Mecanismos de resistência aos carbapenêmicos em Enterobacterales

Enquanto algumas cepas possuem resistência intrínseca aos carbapenêmicos, outras contêm elementos genéticos móveis, como plasmídeos e transposons, que carreiam genes responsáveis pela resistência a esta classe de antimicrobianos. Dentre estes diferentes mecanismos, três destacam-se por sua ampla disseminação em Enterobacterales: a produção de enzimas, a hiperexpressão de bombas de efluxo e a mutação de porinas (77).

1.2.2.1. ERCs-PC

A produção de carbapenemases é reconhecida como o principal mecanismo de resistência aos carbapenêmicos em Enterobacterales. Estas enzimas pertencem a família das β-lactamases que têm a capacidade de hidrolisar essencialmente todos os β-lactâmicos, inclusive os carbapenêmicos (15).

Frequentemente, estes genes produtores de carbapenemases estão localizados em elementos genéticos móveis junto de outros genes de resistência, resultando em co-resistência ou até multirresistência (MDR) a diversas classes de antibióticos (68,78). Ainda, estas enzimas podem ser transmitidas entre diferentes espécies bacterianas (79).

Sabe-se que as ERC-PC podem produzir uma vasta variedade de carbapenemases que auxiliam na classificação destes microrganismos de acordo com Ambler (80). β-lactamases de classe A, B e D são reconhecidas pela sua relevância clínica, por outro lado as de classe C não foram associadas à casos clínicos até o momento (50). As enzimas KPC (*Klebsiella pneumoniae* carbapenemase), NDM (New Delhi metallo-β-lactamase), VIM (Verona imipenemase), OXA (Oxacillinase) e IMP (Imipenemase) destacam-se pela dispersão mundial e forte associação com surtos hospitalares (12,50,81–83).

As enzimas KPC são pertencentes a classe A e frequentemente carreadas por plasmídeos, os quais serão abordados no capítulo 1.2.3.1. Têm a capacidade de hidrolisar carbapenêmicos e são parcialmente inibidas pelo ácido clavulânico (84). São enzimas disseminadas mundialmente e frequentemente associadas com MDR (85). O primeiro estudo que reportou uma cepa produtora de KPC no Brasil foi publicado em 2009 (86). Monteiro *et al* descreveram a presença da variante KPC-2 em isolados de *K. pneumoniae* oriundos de 4 pacientes situados na cidade de Recife - PE, fato ocorrido 10 anos após a primeira detecção de KPC-2 no mundo, em 1996, na Carolina do Norte nos Estados Unidos da América (EUA) (50,87). Subsequentemente, inúmeras espécies de Enterobacterales produtoras de KPC-2 têm sido descritas distribuídas geograficamente por todo o país, no entanto, *K. pneumoniae* é a espécie mais frequente. Existem até o momento, 79 variantes de KPC descritas mundialmente (88), porém no Brasil a variante KPC-2 é predominante.

A enzima BKC, chamada *Brazilian Klebsiella carbapenemase*, teve sua primeira descrição em 2015, portanto se comparada ao ano de publicação de suas antecessoras, pode ser considerada uma das mais novas enzimas de classe A das carbapenemases. Esta enzima foi detectada primeiramente no Brasil, e até o momento apresenta uma baixa frequência possivelmente pelo fato de que o gene $bla_{\text{BKC-1}}$ localiza-se em um plasmídeo não-conjugativo (89,90). Estas enzimas já foram detectadas em isolados de *K. pneumoniae, Enterobacter hormaechei*, *C. freundii* e *E. coli* (90–92). Apenas 2 variantes destas enzimas foram reportadas até a presente data (88).

A segunda principal família de enzimas são as metallo-β-lactamases (MBL), da classe B. São enzimas que possuem íons de zinco no seu sítio ativo (82). As NDM estão disseminadas no mundo e estão são geralmente presentes em microrganismos MDR. Estudos demonstram que plasmídeos que carreiam o gene bla_{NDM} também levam consigo outros determinantes de resistência a quinolonas, aminoglicosídeos e β-lactamases de amplo espectro (ESBL) (93) . Atualmente, foram descritas 31 variantes destas enzimas que também se disseminam através de elementos genéticos móveis e possuem propriedades hidrolíticas eficazes contra a maioria dos β-lactâmicos, exceto monobactams (88,94).

Em contraste com a KPC, a enzima NDM foi primeiramente detectada em 2008, em um isolado de *K. pneumoniae* oriundo de um de uma infecção urinária de um paciente da Suécia que havia viajado para Nova Delhi, Índia (95). Cinco anos passados, foi descrita a primeira detecção de um isolado produtor de NDM no Brasil. Tratava-se de um isolado de *Providencia rettgerii* oriundo de um paciente situado na cidade de Porto Alegre – RS (96). Após este episódio, NDM tem sido descrita em outras Enterobacterales distribuídos no país inteiro (96–98). Isolados de Enterobacterales chamados de co-produtores, produzindo ambas enzimas KPC e NDM também foram detectados no país (97–100).

Ainda na família das MBL, também merecem destaque VIM e IMP. Ambas são originárias de *P. aeruginosa* e foram transferidas para membros da ordem Enterobacterales. Além disto, partilham de similaridades relacionadas aos plasmídeos as quais são carreadas e no seu mecanismo de ação, ambas hidrolisam β-lactâmicos exceto monobactam e são suscetíveis aos inibidores de β-lactâmicos (101). As IMP foram as primeiras carbapenemases descritas, no princípio dos anos 1990, e até o momento foram reportadas 89 variantes deste grupo, enquanto que as VIM foram descritas em 1997 e possuem 73 variantes (88).

As enzimas de classe D clinicamente relevantes são representadas principalmente pelo grupo OXA-48-*like*. Estas enzimas são ativas contra penicilinas, possuem atividade intermediária contra cefalosporinas e baixa eficácia contra carbapenêmicos (50). São enzimas também carreadas por plasmídeos e tem a habilidade de se mutar e expandir seu espectro de atividade. Atualmente foram reportadas mais de 1000 variantes do grupo das oxacilinases(88). Particularmente, na cidade de Porto Alegre em 2013, foi detectada pela primeira vez uma nova variante do grupo das OXA-48-*like, a* OXA-370. Esta enzima foi detectada em um isolado de *E. hormaechei* resistente aos carbapenêmicos oriundo de um swab retal de um paciente (102). Esta enzima já foi detectada identificada também em *K. pneumoniae* isolados no estado do Rio de Janeiro e em outras cidades no sul do Brasil (103,104). Na figura 2 é apresentada uma linha do tempo referenciando o surgimento de carbapenemases no mundo.

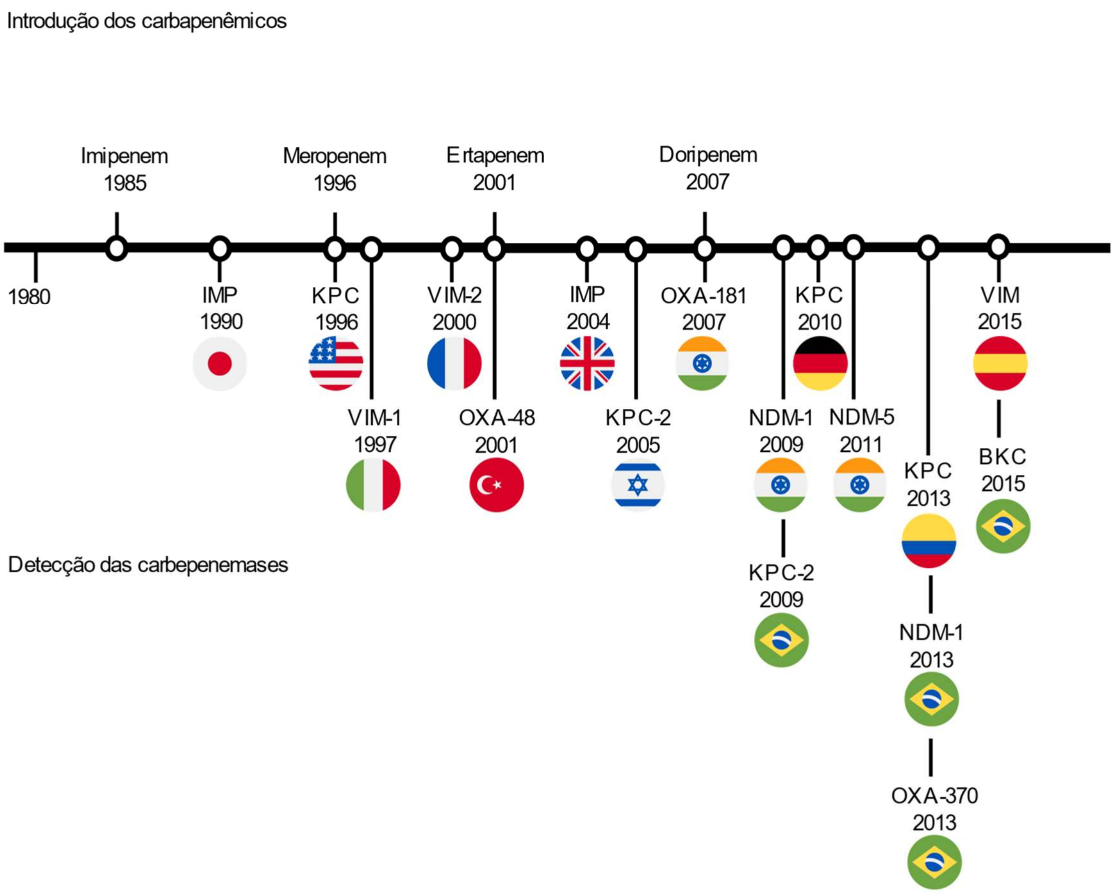

Figura 2. Linha do tempo da introdução dos carbapenêmicos na prática clínica e o surgimento de carbapenemases no mundo. Adaptado de Suay-Garcia *et al.* (2).

1.2.2.2. ERCs-não-PC

Mecanismos de resistência aos carbapenêmicos não relacionados com a produção de carbapenemases também podem ser observados entre as Enterobacterales. A produção de outras β-lactamases como AmpC-*type*, a perda de porinas e a hiperexpressão de bombas de efluxo são mecanismos alternativos que quando presentes frequentemente desencadeiam fenótipos bacterianos MDR (105). Estes mecanismos podem apresentar-se em um conjunto no qual a produção de carbapenemases pode ou não estar presente (106).

As β-lactamases AmpC-*type* são pertencentes a classe C de Ambler. São maioritariamente enzimas codificadas por genes cromossomais (cAmpCs) mas que em alguns casos também podem ser mediadas por aquisição plasmidial (pAmpCs). Não degradam os carbapenêmicos, porém são capazes de impedir fisicamente a ligação entre antibiótico e seu alvo nestes microrganismos (107,108). As Enterobacterales produtores de pAmpC podem exibir fenótipos MDR devido a co-expressão com múltiplos determinantes de resistência a não β-lactâmicos, incluindo quinolonas e aminoglicosídeos, mediados por plasmídeos, limitando o número de opções eficazes para o seu tratamento (109). Por outro lado, aqueles produtores de cAmpCs frequentemente possuem altos níveis de susceptibilidade às fluoroquinolonas e aminoglicosídeos (110,111).

A hiperexpressão de bombas de efluxo esta geralmente associada com MDR em Enterobacterales. Estes sistemas são codificados por genes que podem ser facilmente transferidos para outros microrganismos através de elementos genéticos móveis como plasmídeos (112). As bombas de efluxo que conferem MDR encontradas nos genomas bacterianos pertencem as famílias ABC, MFS, SMR, MATE, PACE e RND (113). A família RND contribui com um dos maiores mecanismos de efluxo associado a membranas em Enterobacterales, e o complexo mais relevante descrito em isolados clínicos pertence ao sistema AcrAB-TolC (114,115).

O terceiro mecanismo de resistência não relacionado com a produção de carbapenemases é a alteração da síntese porinas. Esta alteração atua inibindo os carbapenêmicos de invadir a parede celular bacteriana (116). Estas alterações na expressão das porinas auxiliam na resistência aos β-lactâmicos em bactérias MDR, uma vez que já foram descritas em *K. pneumoniae* junto de outros mecanismos de resistência como produção de AmpC e carbapenemases (117). As porinas OmpK35 e OmpK36 degradam diferentes classes de antibióticos e estão extremamente difundidas em *K. pneumoniae*. Estas porinas apresentam alta permeabilidade para compostos lipofílicos e estruturalmente grandes como, respectivamente, Benzilpenicilina e Cefepime (118). Isto sugere que OmpK35 e OmpK36 formam canais mais largos e permeáveis quando comparados com seus homólogos OmpF e OmpC em *E. coli*, possivelmente explicando o porquê de serem reportados frequentemente casos de resistência por perda de porinas em isolados clínicos de *K. pneumoniae* (118).

1.2.3. Klebsiella pneumoniae produtora de carbapenemases (Kp-PC)

As Kp-PC são Enterobacterales resistentes aos carbapenêmicos considerados um problema de saúde pública internacional por pelo menos quatro razões de acordo com a OMS (119): 1) são um dos principais causadores de infecções adquiridas nos hospitais, tais como pneumonia, infecções da corrente sanguínea, e em pacientes internados em UTIs; 2) são responsáveis pela maior parte das infecções em pacientes imunossuprimidos, principalmente em OTR; 3) são altamente resistentes aos antibióticos; 4) e tem um potencial elevado de disseminação mundial através de elementos genéticos móveis (68,71).

1.2.3.1. Filogenia e plasmideos em Kp-PC

Atualmente existem mais de 100 *Sequence Types* (ST) de *K. pneumoniae* produtoras de KPC (Kp-KPC), e o seu perfil de disseminação pandêmica está diretamente relacionado com membros do complexo clonal 11/258 (CC11/258) (83). Os clones internacionais de alto risco ST11 e ST258 compõe o CC11/258, assim como ST340, ST437 e ST512, sendo estes todos estas variantes *single-locus* da ST258 que não possuem um poder de disseminação tão significativo em relação as dois STs mais predominantes e que dão nome ao CC (83,120,121).

Existe uma vasta variedade de STs de *K. pneumoniae* produtoras de NDM (Kp-NDM, porém, não existe uma linhagem amplamente predominante neste grupo. Este fato pode sugerir que não há, até o momento, um único clone de alto risco nestes isolados (122). Portanto, percebe-se uma discrepância quando se trata de distribuição de clones ao comparar Kp-KPC com Kp-NDM. As ST11, ST14, ST15 e ST147 são as mais frequentes em Kp-NDM e têm sido reportadas em diferentes continentes (122).

O clone internacional de alto risco *K. pneumoniae* ST11 tem sido comumente reportado como um patógeno eficaz, associado a importantes fatores de virulência (123,124) e resistência, incluindo a produção de KPC e NDM (125–130). Este mesmo padrão também é observado no Brasil, onde *K. pneumoniae* ST11, junto da ST258, permanecem sendo as cepas detectadas com maior frequência, seguida por ST437, que também pertence ao CC11/258 (131). A dispersão generalizada destes clones tem sido diretamente associada com a transmissão de fatores de resistência, principalmente carbapenemases, as quais são carreadas por elementos genéticos móveis como plasmídeos e transposons, facilitando a ocorrência de surtos em diversas regiões do mundo (50). Outras STs como ST 101, ST340 e ST442 tem sido reportadas esporadicamente no país (121,132,133).

O plasmídeo predominante que carreia o gene *bla*$_{KPC}$ em *K. pneumoniae* CC11/258 é pertencente à família de incompatibilidade (Inc) IncF com replicons FIIk (120,134). Este plasmídeo frequentemente contém genes adicionais de resistência aos antibióticos das classes dos aminoglicosídeos, quinolonas, sulfonamidas, tetraciclinas e trimetoprim (134). Apesar disso, o gene *bla*$_{KPC}$ também já foi reportado em muitos outros plasmídeos, como IncI2, IncX, IncA/C, IncR, IncN, IncM e ColE1 (120,133–136). Até o presente momento, o IncN é o principal plasmídeo responsável por carrear o gene *bla*$_{KPC-2}$ em cepas de *K. pneumoniae* ST11 oriundas do Brasil. (133). O gene *bla*$_{KPC}$ tem sido reportado com frequência flanqueado pelas sequências de inserção ISKpn6 e ISKpn7, localizado em um transposon Tn*4401* que adicionalmente contém genes transposase (*tnpA*) e uma resolvase (*tnpR*) (137).

Os genes *bla*$_{NDM}$ também tem sido reportados em diversos tipos de plasmídeos em *K. pneumoniae*, tais como, IncA/C, IncF, IncR, IncH, IncN, IncL, IncM, e IncX (138–143). Entretanto, o tipo predominante reconhecido como fator imprescindível para a disseminação de *bla*$_{NDM}$ são os plasmídeos da família IncA/C (134,144) os quais possuem a capacidade de carrear vários genes de resistência aos aminoglicosídeos, cefalosporinas e quinolonas (134). De acordo com Wu et al. (122), os principais plasmídeos que carreiam os genes *bla*$_{NDM}$ em Enterobacterales encontradas no Brasil são IncFII e IncX3. Porém em 2020 Raro et al. (93), detectaram a presença destes genes carreados por IncFIB em *K. pneumoniae* isolados de pacientes transplantados no Brasil.

1.2.3.2. Mecanismos de virulência em Kp-PC

Assim como os fatores de resistência, os fatores de virulência também estão implicados diretamente no sucesso da patogenicidade das Enterobacterales, principalmente em *K. pneumoniae,* permitindo que este microorganismo possa superar o sistema imune inato e manter-se infectando um hospedeiro humano (145). Dentre os importantes fatores de virulência que já foram descritos nestes isolados bacterianos incluem-se a cápsula, o LPS (lipopolissácarídeo), o cluster *genotoxin colibactin* (*clbABCDEFGHIJLMNOPQR)*, genes *siderophores* (*irp1* e *irp2*), o cluster *yersiniabactin siderophore* (*ybtAEPQSTUX*), o cluster *fimbriae mannose-resistant Klebsiella-like* type III (*mrkABCDFHIJ*), cluster *fimbriae* type I (*fimACDHK)* o sistema *ferric uptake* (*kfuABC*), o gene *yersiniabactin receptor* (*fyuA*), os quais podem ser peças fundamentais na emergência de cepas de Kp-KPC com potencial de virulência que induz o desenvolvimento de doenças infecciosas mais invasivas (3,146).

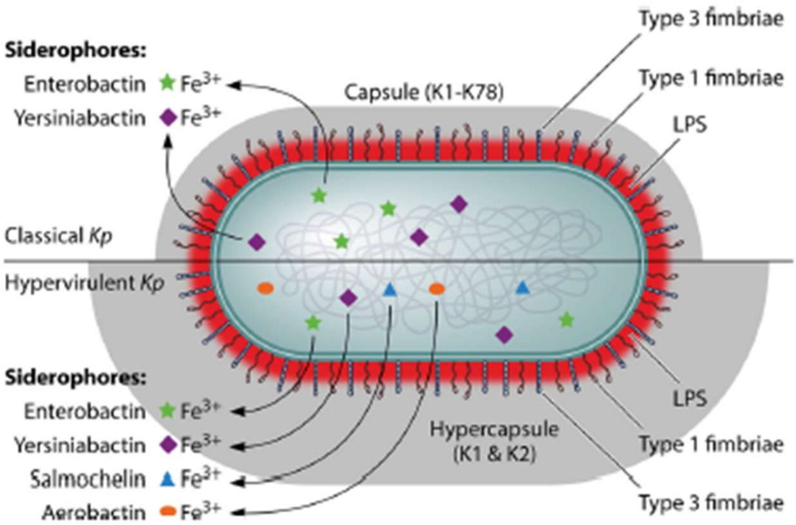

Figura 3 – Quatro fatores de virulência bem caracterizados em isolados de *K. pneumoniae* clássica e hipervirulenta. Referência: Paczosa *et al.* 2016 (3).

O fator de virulencia mais estudado em *K. pneumoniae* é a cápsula, a qual é uma matrix polissacarídica que reveste a célula bacteriana e é extremamente necessária para a virulência deste microrganismo (147,148). A cápsula tem como funções: 1) auxiliar nos processos de prevenção de fagocitose por células imunes do hospedeiro; 2) dificultar a ação bactericida de peptídeos antimicrobianos como beta defensinas humanas 1, 2 e 3 e lactoferrina; 3) bloquear componentes complemento, como C3, de interagir com a membrana resultando na prevenção de opsonização e lise mediada pelo complemento; 4) e evitar a resposta inflamatória diminuindo a produção de espécies reativas de oxigênio (ERO), interleucinas IL-8, IL-6 e TNF-α (*tumor necrosis fator*) (3). Aqueles que não a possuem (acapsulados) tem sua virulência dramaticamente reduzida como já demonstrado em estudos comparativos com cepas encapsuladas infectando modelos animais, principalmente ratos. Neste caso, aqueles acapsulados apresentam-se em menor carga bacteriana nos pulmões, ocasionam uma menor taxa de mortalidade e são incapazes de se disseminar sistematicamente nos ratos (148–151). As cepas de *K. pneumoniae* também podem apresentar uma hipercápsula, se tornando microrganismos conhecidos como hipervirulentos, como acontece em cepas do CC23 (3). Ambos os tipos de cápsula, clássico ou hipercápsula, são compostos por polissacarídeos capsulares cepa-específicos chamados de *K antigens, K types* ou *K locus*, e são conhecidos 78 tipos até o momento (152).

O LPS é o principal componente da membrana celular externa de todas as bactérias Gram-negativas. O LPS é composto por lipídeo A, antígeno O e um *core* oligossacarídeo. Este componente pode trazer benefícios e ao mesmo tempo alguns obstáculos para *K. pneumoniae* durante uma infecção, uma vez que é um importante fator de virulência que protege contra defesas humorais, mas também pode ser um forte ativador do sistema imunológico do hospedeiro (3). O lipídeo A é responsável por se inserir na membrana bacteriana e ter a capacidade de ativar inflamação. A *K. pneumoniae* pode modificar seu lipídeo A para torná-lo menos inflamatório durante uma infecção, além disto, este componente pode proteger contra a ação bactericida de antimicrobianos peptídicos catiônicos. O antígeno O é a subunidade mais externa do LPS e tem papel importante na proteção contra o complemento. Pode se ligar e sequestrar C3b para prevenir a formação de poros na membrana bacteriana (3).

As fimbrias também representam uma classe de fatores de virulência importantes em *K. pneumoniae*, atuando na adesão e formação de biofilme. *Fimbriae* I e III são as maiores estruturas adesivas caracterizadas como fatores de patogenicidade. A *Fimbriae* tipo I são finos filamentos que estão ligados a membrana, tem estruturas adesivas compostas primariamente por subunidades de *FimA* e nas pontas por subunidades de *FimH*. Tem a função de invasão celular na bexiga e formação de biofilme nesse órgão e em superfícies abióticas. É expressa em aproximadamente 90% das cepas de *K. pneumoniae* e em outros membros Enterobacterales clínicos e ambientais (153,154). Já as *fimbriae* tipo III são tipo hélice, ligadas a membrana, possuem estruturas adesivas compostas primariamente por subunidades *MrkA*, com subunidades *MrkD* nas pontas. São importantes no processo de formação de biofilme e ligação em equipamentos médicos, o que facilita sua invasão em pacientes (3). O cluster *fimbriae mannose-resistant Klebsiella-like* type III (*mrkABCDFHIJ*) foi recentemente detectado em isolados de Kp-PC oriundos de pacientes transplantados no Brasil (93).

K. pneumoniae necessita de ferro para sobreviver durante uma infecção. E para conseguir captar este material, este microrganismo utiliza a tática de adquirir ferro através da secreção de sideróforos (*siderophores*), moléculas que possuem uma maior afinidade pelo ferro quando relacionado com proteínas de transporte no hospedeiro. Os sideróforos podem literalmente roubar ferro das proteínas do hospedeiro ou "limpar" o material do ambiente (155). Quando produzem mais de um sideróforo, *K. pneumoniae* possuem um maior poder de colonizar tecidos e/ou evitar neutralização de um sideróforo pelo hospedeiro (155,156). Até o momento já foram descritos diversos sideróforos em *K. pneumoniae*, incluindo *enterobactin*, *yersiniabactin*, *salmochelin* e *aerobactin* (3).

1.3. Resistência a polimixina B

Como mencionado anteriormente, na sua grande maioria, os Kp-PC são MDR, portanto existem poucas opções de antibióticos disponíveis e a falha terapêutica é frequente (157). Em situações onde novas combinações de beta-lactâmicos e inibidores de beta-lactamase não estão amplamente disponíveis, as polimixinas têm sido usadas como último recurso em infecções por Kp-PC, apesar da sua toxicidade (158).

Todavia, a resistência a estes antimicrobianos tem aumentado. Desde a primeira publicação no ano de 2006, a resistência as polimixinas em *Enterobaterales* tem se tornado um grande desafio para os trabalhadores da saúde, e corroborando com esta preocupação, atualmente já existem achados de disseminação clonal intra- e inter-hospitalar de Kp-KPC resistentes as polimixinas no Brasil (131) e em países como Itália (159,160), Estados Unidos (161), Grécia (162), Hungria (163) e Turquia (164). No Brasil a prevalência de resistência as polimixinas varia de acordo com a região geográfica. Enquanto na região sul do país, na cidade de Porto Alegre, estas taxas podem chegar a 35.3% (165), na cidade de São Paulo na região sudeste, a resistência a polimixinas é de aproximadamente 27,1% (166) . E em países Europeus, as taxas podem variar entre 20.8%, 31.7% e 43% na Grécia, Espanha e Itália, respectivamente (167–169).

Os mecanismos mais frequentes de resistência a polimixina em *K. pneumoniae* são devidos a mutações cromossomais. Em *K. pneumoniae*, estas mutações geram modificação no lipopolissacarídeo (LPS), seguida da adição de 4-amino-4-deoxy-L-arabinose (L-Ara4N) no lipídeo A. Este processo desencadeia a diminuição da afinidade das moléculas de polimixina com o LPS (170). As mutações em genes e operons relacionadas com a modificação do LPS descritas até o momento são: 1) mutações em genes responsáveis pela síntese de grupos catiônicos e sua adição no LPS (*pmrC, pmrE* e *pmrHFIJKLM*) (171–173); 2) mutações em genes regulatórios que codificam proteínas envolvidas nos sistemas dois componentes PmrAB e PhoPQ em *K. pneumoniae* e outras Enterobacterales (171,174); 3) e mutações em reguladores destes sistemas, como no gene *mgrB e* operon crrAB , que regulam respectivamente, o sistema PhoPQ e o PmrAB, em *K. pneumoniae* (175–177). O mais frequente mecanismo de resistência encontrado no Brasil é a interrupção do gene *mgrB* pela inserção de sequencias de inserção (SI) ou mutações missense (178). No estudo publicado por Aires *et al*. 100% das cepas de *K. pneumoniae* resistentes às polimixinas apresentaram alterações no gene *mgrB*. Estas cepas foram isoladas em diferentes estados do país (Distrito Federal, Espírito Santo, Pernambuco, Rio de Janeiro, Rio Grande so Sul). E, dentre as alterações identificadas truncando o *mgrB* estão as seguintes SIs: IS*903B*, IS*10L*, IS*5*, IS*Kpn26* e IS*102* (178). Outro estudo que também utilizou cepas de *K. pneumoniae* resistentes às polimixinas oriundas das cidades de Dourados e Campo Grande, na região centro-oeste do Brasil, detectou a presença de interrupções por SI no *mgrB* em 22 de 30 isolados (73%), sendo elas IS*903*, IS*5*, IS*Kpn13*, IS*Ecp1* e IS*Kpn18* (179).

Como mencionado anteriormente, as mutações cromossomais são maioritariamente responsáveis pela resistência das Enterobacterales às polimixinas (B e E). Porém em 2015 Liu *et al*. detectaram a presença do gene *mcr-1* (*mobile colistin resistance*), responsável por codificar um mecanismo de resistência as polimixinas mediado por plasmídeo, designado MCR-1 (128). MCR-1 é uma enzima da família das fosfoetalonaminas (PEtN) transferases que atua codificando uma PEtN que é adicionada ao lipídio A presente nos LPS, aumentando as cargas catiônicas no LPS, consequentemente, reduzindo a afinidade de ligação das polimixinas ao LPS (128).

O gene *mcr-1* foi detectado em *E. coli* isolada de animais de uma fazenda na China. Desde então o gene *mcr-1* se disseminou em diversas espécies da ordem Enterobacterales (*E. coli, K. pneumoniae*, *Enterobacter cloacae, Enterobacter aerogenes*, *Citrobacter freundii*, *Kluyvera ascorbata*, entre outras espécies), e tem sido reportada nos cinco continentes em fazendas, alimentos, esgotos e nos humanos (colonizando e infectando) (180). De acordo com o conceito "One Health", o ambiente desenvolve um papel importante na proteção da saúde pública. E os genes de resistência adquirida (ARG), assim como o *mcr-1,* têm sido amplamente observados em uma variedade de isolados ambientais oriundos de fazendas, cidades, comunidades e ambientes naturais, como mencionado anteriormente. Desta forma, o ambiente torna-se um reservatório importante de ARGs e chama atenção de importantes organizações mundiais como a WHO, o *Food and Agriculture Organization of the United Nations* (FAO), e a *World Organisation for Animal Health* (OIE), os quais promovem práticas para reduzir os níveis de AMR no ambiente (181).

Além disto, o fato do *mcr-1* ser mediado por plasmídeo mobilizou estes órgãos de saúde pública mundial, pois além de ter um significativo potencial de disseminação, o *mcr-1* poderia ser adquirido por EPC gerando microrganismos verdadeiramente Pan-resistentes (PDR) e resultando em infecções impossíveis de serem tratadas (128). Cepas de Enterobacterales produtoras de *mcr-1* já foram descritas no Brasil (182,183), incluindo a co-ocorrência dos genes *mcr-1* e $bla_{\mathrm{KPC\text{-}2}}$ em um isolado clínico de *E. coli* (183).

1.3.1. Detecção de perfil de susceptibilidade a polimixina B em CRE

Atualmente há um grande desafio na detecção de susceptibilidade às polimixinas. Métodos amplamente utilizados como ensaio de difusão de discos e gradiente antimicrobiano não são suficientemente acurados. Sistemas automatizados não são confiáveis para estes antibióticos. Além disto, a Microdiluição em caldo (BMD), padrão ouro recomendado por ambas organizações, CLSI e EUCAST (61,184), é uma técnica trabalhosa e que demanda tempo de análise, representando um problema para os laboratórios de microbiologia clínica.

Existem também outras metodologias como meio de cultura universal para *screening* de gram-negativos resistentes às polimixinas o qual utiliza sulfato de colistina e foi descrito por Nordmann *et al*. em 2016 (185)., bem como o teste colorimétrico *PolymyxinNP*, que também utiliza a colistina, descrito por Nordmann e Poirel (186). Recentemente o CLSI endossou os testes de colistina em agar (CAT) e eluição de disco de colistina em caldo (CBDE) para serem utilizados em laboratórios clínicos (61). Porém, embora similares, a polimixina B e a colistina (polimixina E) são drogas antimicrobianas distintas, que apresentam diferentes perfis farmacocinéticos e farmacodinâmicos (PK/PD) (187). Por esta razão CLSI desaconselha o uso dos pontos de corte de colistina para interpretação de susceptibilidade à polimixina B, e pontos de corte específicos para cada droga foram publicados (61,187).

Diferentes testes baseados em na eluição de discos em caldo, na utilização de agar e inclusive no teste colorimétrico *PolymyxinNP* (186) passaram a ser considerados para a detecção de susceptibilidade à polimixina B em EPC. Estas metodologias, embora já estudadas tendo como foco o uso de colistina, não levam em consideração o uso da polimixina B. Portanto, testes com polimixina B são necessários, como comentado anteriormente e de acordo com Humprhies *et al*.(187), sabemos que a polimixina B possui características diferentes e, portanto, deve ser tratada como tal. Além disto, existem países que não tem acesso a colistina, enquanto outros optam pelo uso de polimixina B ao invés de colistina. Desta forma, faz se necessária a busca por metodologias mais práticas, rápidas, e tão fidedignas quanto a BMD, para que se possa seguir avançando na detecção de susceptibilidade à polimixina B em microrganismos MDR, especialmente os CREs.

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3. Objetivos

3.1. Objetivo geral

Analisar a influência da colonização por Enterobacterales produtoras de carbapenemases (EPC) no desfecho clínico de pacientes transplantados.

3.2. Objetivos específicos

- Determinar a incidência da colonização de EPC isoladas de pacientes transplantados;
- Avaliar as variáveis que conferem risco de colonização por EPC em pacientes transplantados;
- Avaliar o perfil de resistência a polimixina B de isolados produtores de carbapenemases utilizando a técnica de concentração inibitória mínima;
- Propor metodologias alternativas para a detecção do perfil de susceptibilidade a polimixina B em isolados de EPC;
- Confirmar e identificar a presença dos genes bla_{KPC} e bla_{NDM} em isolados de EPC;
- Investigar a presença do gene *mcr-1* através de PCR;
- Realizar o sequenciamento de genoma completo (WGS) de isolados de EPC;
- Verificar a presença de surtos e tipagem de isolados de EPC através do estudo de filogenia por core genome "*Multilocus sequence typing*" (cgMLST) e através de "*Single Nucleotide Polymorphisms*" (SNPs);
- Investigar o conjunto de genes de resistência adquirida presentes nas EPCs (resistoma);
- Investigar o conjunto de genes de virulência presentes nas EPCs (viruloma);
- Detectar os plasmídeos responsáveis por carrear carbapenemases e descrever seus entornos genéticos;
- Comparar a relação clonal de EPCs do sul do Brasil com EPCs de outros continentes.

4. Esquema gráfico dos artigos científicos

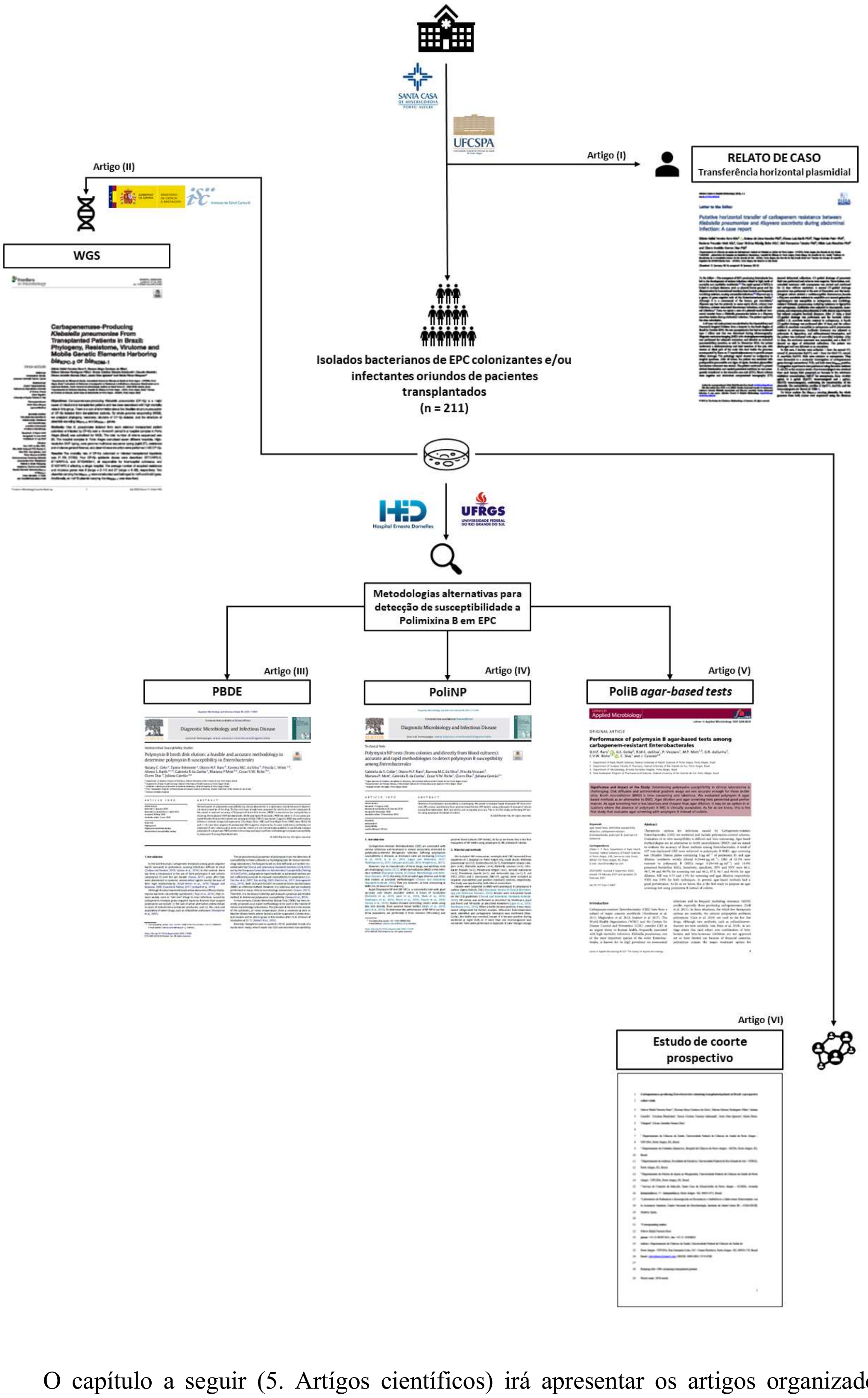
Neste capítulo encontra-se um esquema gráfico temporal dos artigos científicos produzidos ao longo do Doutorado. Ao todo foram incluídos neste estudo 211 pacientes transplantados colonizados que podem ou não ter desenvolvido uma infecção por CPE. De todos estes pacientes foi isolada uma amostra de CPE colonizante, e em caso de infecção, uma ou mais amostras infectantes, totalizando 300 amostras coletadas.

Ao presenciar um caso clínico relevante de possível transmissão plasmidial de resistência aos carbapenêmicos de um isolado de *K. pneumoniae* para um isolado de *Kluyvera ascorbata* presentes em uma infecção abdominal, decidiu-se avaliar os isolados através das técnicas de conjugação e *WGS*, resultando no primeiro artigo científico publicado (**artigo I**).

Em 2018 fui contemplado com uma bolsa de Doutorado Sanduíche da CAPES (PDSE – CAPES), e desenvolvi parte do meu projeto em colaboração com a Dra. María Dolores Pérez Vázquez e com o Dr. Jesús Oteo Iglesias no *Laboratorio de Referencia e Investigación em Resistencia a Antibióticos del Centro Nacional de Microbiología* do *Instituto de Salud Carlos III (ISCIII)*, em Madrid, Espanha. Selecionamos 80 isolados de CPE para enviar ao ISCIII e realizar WGS com o objetivo de analisar a filogenía, resistoma, viruloma e plasmídeos carreadores de resistência às carbapenemases nestes CPEs. Para selecionar estes isolados seguimos os seguintes critérios: a) isolados infectantes obtidos até aquele período, sendo apenas uma cepa por paciente e sempre o isolado mais invasivo (causador de bacteremia) – total de 48 cepas; b) isolados colonizantes: b.1) oriundos de pacientes não incluídos no grupo “a”; b.2) que tivessem variabilidade de carbapenemases (KPC, NDM); b.3) oriundos de pacientes de diferentes tipos de transplante; e b.4) oriundos de pacientes que abrangessem uma proporcionalidade em todo o período da sua coleta – total de 32 cepas. Como resultado inicial desta colaboração obtivemos a publicação do segundo artigo científico (**artigo II**).

A partir do meu retorno do Doutorado Sanduíche, em 2019, iniciamos uma colaboração com a prof. Dra. Juliana Caierão junto a Universidade Federal do Rio Grande do Sul (UFRGS), bem como com os Departamentos de Controle de Infecção e do laboratório de Microbiologia do Hospital Ernesto Dornelles de Porto Alegre. O objetivo geral desta colaboração sempre foi a busca por metodologias alternativas promissoras para a detecção de resistência a polimixina B em isolados de EPC, um grande desafio para laboratórios de microbiologia clínica. Como frutos desta parceria publicamos os artigos científicos **III**, **IV** e **V**.

E, por fim, como um fechamento da tese, o artigo **VI** reporta a estrutura populacional dos pacientes transplantados colonizados e possivelmente infectados por EPC na região Sul do Brasil, além de apresentar variáveis independentes consideradas fatores de risco para o desenvolvimento de infecção por uma EPC. Este artigo será submetido ao *Journal of Global Antimicrobial Resistance*.



O capítulo a seguir (5. Artígos científicos) irá apresentar os artigos organizados por autoria e publicação.

5. Artigos científicos

5.1. Artigos originais publicados como primeiro autor

5.1.1. Artigo original I

Título:

Putative horizontal transfer of carbapenem resistance between *Klebsiella pneumoniae* and *Kluyvera ascorbata* during abdominal infection: A case report

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Letter to the Editor

Putative horizontal transfer of carbapenem resistance between *Klebsiella pneumoniae* and *Kluyvera ascorbata* during abdominal infection: A case report

Otávio Hallal Ferreira Raro MSc[1], Daiana de Lima-Morales PhD[2], Afonso Luis Barth PhD[2], Tiago Galvão Paim PhD[3], Mariana Preussler Mott MSc[1], Cezar Vinícius Würdig Riche MSc[1], Uirá Fernandes Teixeira PhD[4], Fábio Luiz Waechter PhD[4] and Cícero Armídio Gomes Dias PhD[1]

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To the Editor—The emergence of KPC-producing *Enterobacter* has led to the development of serious infections related to high levels of mortality and morbidity worldwide.[1,2] The rapid spread of KPCs is linked to multiple elements, such as plasmid-borne genes and the dissemination by international travelers; these bacteria are frequently multidrug resistant, causing untreatable infections.[3,4] *Kluyvera* spp is a genus of gram-negative rods of the Enterobacteriaceae family.[5] Although it is a commensal of the human gut microbiota,[6] *Kluyvera* spp has the potential to cause septic shock, urinary tract infections, catheter-associated bloodstream infections, and abdominal infections.[7] Here, we report a case of a plasmid-mediated horizontal transfer from a *Klebsiella pneumoniae* isolate to a *Kluyvera ascorbata* isolate during abdominal infection. The patient approved the data submission.

A 43-year-old male patient was admitted to the Hepatobiliary and Pancreatic Surgical Division from a hospital in the South Region of Brazil in October 2016. He was asymptomatic but had an incidental type 1 biliary cyst that was discovered during ultrasonography. Magnetic resonance imaging (MRI) with cholangiopancreatography was performed for adequate evaluation and showed an abnormal pancreatobiliary junction, as well. In November 2016, the patient underwent a cholecystectomy and total resection of the cyst, with closure of distal part of the main bile duct inside the pancreas, accompanied by Roux-en-Y hepaticojejunostomy to provide proper biliary drainage. The pathology report showed no malignancy in surgical specimen. After 48 hours, the patient was evaluated with postoperative pancreatitis and signs of sepsis, therefore piperacillin/tazobactam treatment was started. The patient continued to present clinical deterioration and needed parenteral nutrition; he was consequently transferred to the intensive care unit (ICU). Blood cultures were negative and abdominal computerized tomography (CT) showed abdominal collections. CT-guided drainage of pancreatic fluid was performed and cultures were negative. Nevertheless, antimicrobial treatment with meropenem was started and continued for 14 days without resolution. A second CT-guided drainage procedure was performed at the end of December, and the bacteriological culture yielded a multisusceptible *Enterococcus faecalis*; a *Kluyvera ascorbata* resistant to ampicillin and second-generation cephalosporin but susceptible to carbapenem, and multidrug-resistant *Klebsiella pneumoniae*, including resistance to tigecycline and carbapenem. Antibiotics were adjusted to vancomycin, meropenem, and ertapenem plus polymyxin B with clinical improvement but without complete bacterial clearance. After 17 days, a third CT-guided drainage was performed, and the bacterial culture yielded 1 *K. ascorbata* isolate resistant to carbapenem. A fourth CT-guided drainage was performed after 15 days, and the culture yielded *K. ascorbata* susceptible to carbapenem and *K. pneumoniae* resistant to carbapenem. Antibiotic treatment was adjusted to polymyxin B, tigecycline, and sulfametoxazol-trimetoprim, and the patient was evaluated with signs of controlled infection. After 14 days, the antibiotic treatment was suspended, and a final CT showed no signs of abdominal collections. The patient was discharged and was followed as an outpatient.

In this case, 2 isolates were collected: 1 from the second CT, named *K. pneumoniae* KpOT1, and 1 from the third CT, named *K. ascorbata* KaOT2. Both were resistant to meropenem. They were forwarded to a molecular investigation of carbapenemase genes through conventional PCR, and both were $bla_{\text{KPC-2}}$ positive. Conjugation experiments were performed using the azide-resistant *E. coli* J53 as the receptor strain. One transconjugant was obtained from each isolate; both presented an increase in the minimum inhibitory concentration (MIC)[8] for meropenem, from <0.0625 μg/mL to 2 μg/mL (KpOT1 transconjugant) and 1 μg/mL, (KaOT2 transconjugant), confirming the transferability of the plasmids. The susceptibility profiles of KpOT1, KaOT2, and the transconjugants are shown in Table 1.

To better analyze the $bla_{\text{KPC-2}}$ carrying plasmids, the whole genomes from both strains were sequenced using the Illumina

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Table 1. Antimicrobial Resistance Profile of the *Klebsiella pneumoniae* and *Kluyvera ascorbata* Pathogenic Isolates and the Transconjugants TKp and TKa

Antimicrobial Resistance	Microorganism				
	E. coli J53	*K. pneumoniae* KpOT1	*K. ascorbata* KaOT2	TKp	TKa
AMP	S	R	R	R	R
ASB	S	R	R	R	R
CFZ	S	R	R	R	R
CAZ	S	R	R	S	S
FEP	S	R	R	I	I
SXT	S	R	S	S	S
AK	S	I	S	S	S
GEN	S	I	S	S	S
CIP	S	R	S	S	S
MER	S	R	R	I	I
IMP	S	R	R	R	R
ERT	S	R	R	I	I
PTZ	S	R	R	I	I
MER MIC, g/mL	<0.0625	>32	>32	2	1
Resistance genes					
	...	*aph(3')-Ia* *aac(6')Ib-cr* bla_{KPC-2} bla_{SHV-11} $bla_{CTX-M-15}$ bla_{OXA-1} *oqxA* *oqxB* *fosA* *mph(A)* *catB4* *sul1* *tet(A)* *dfrA30*	bla_{KPC-2} $bla_{CTX-M-56}$	bla_{KPC-2}	bla_{KPC-2}

Note. AMP, ampicillin; ASB, ampicillin-sulbactam; CFZ, cefazolin; CAZ, ceftazidime; FEP, cefepime; SXT, trimethoprim/sulfamethoxazole; AK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; MER, meropenem; IMP, imipenem; ERT, ertapenem; PTZ, piperacillin/tazobactam; MIC, minimum inhibitory concentration; *aph(3')-Ia*, aminoglycoside resistance; *aac(6')Ib-cr*, fluoroquinolone and aminoglycoside resistance; bla_{KPC-2}, β-lactam resistance; bla_{SHV-11}, β-lactam resistance; $bla_{CTX-M-15}$, β-lactam resistance; bla_{OXA-1}, β-lactam resistance; *oqxA*, quinolone resistance; *oqxB*, quinolone resistance; *fosA*, fosfomycin resistance; *mph(A)*, macrolide resistance; *catB4*, phenicol resistance; *sul1*, sulphonamide resistance; *tet(A)*, tetracycline resistance; *dfrA30*, trimethoprim resistance; $bla_{CTX-M-56}$, β-lactam resistance.

MiSeq platform (San Diego, CA). Detailed analyses indicated that the bla_{KPC-2} was located on an IncN plasmid. The carbapenemase resistance gene was flanked by the insertion sequences IS*Kpn7* and IS*Kpn6*, located on a Tn4401 transposon, isoform *b*.[9] The scaffold bearing the bla_{KPC-2} was 50,417 bp long, and no other resistance gene was found in this scaffold. The IncN plasmid was also found in the *K. ascorbata* genome, containing the same bla_{KPC-2} resistance gene and the same genome environment, indicating that a plasmid transference occurred between KpOT1 and KaOT2 when the patient was in the hospital unit. Further in silico analyses indicated that *K. pneumoniae* KpOT1 belonged to the sequence type ST437, one of the most prevalent sequence types among the KPC-producing *K. pneumoniae* and related to the clonal complex 258, which is distributed worldwide.[10,11] Other resistance genes were identified in both isolates (Table 1). This whole-genome shotgun sequencing project has been deposited in the DDBJ/ENA/GenBank (accession no. RHFM00000000 for *Klebsiella pneumoniae* OT1 and accession no. RHFN00000000 for *Kluyvera ascorbata* OT2). The versions described in this article are versions RHFM01000000 and RHFN01000000.

This clinical case highlights the possibility of plasmid-mediated horizontal transfer between species during infections. Furthermore, KPC-2–producing *K. ascorbata* has only been isolated once, from a rectal swab in a surveillance study in Israel[12] and once in China from a patient's biliary drainage.[13] We suggest that the carbapenem-susceptible *K. ascorbata* recovered in the fourth CT-guided drainage procedure could be related to a different clone or a heteroresistance event, but we cannot confirm this hypothesis. As far as we know, this is the first report of a KPC-2–carrying plasmid transference from a multidrug-resistant *Klebsiella pneumoniae* ST 437 to a *Kluyvera ascorbata* during abdominal infection.

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Conflicts of interest. All authors report no conflicts of interest relevant to this article.

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5.1.2. Artigo original II

Título:

Carbapenemase-Producing *Klebsiella pneumoniae* From Transplanted Patients in Brazil: Phylogeny, Resistome, Virulome and Mobile Genetic Elements Harboring *bla*$_{KPC-2}$ or *bla*$_{NDM-1}$

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Carbapenemase-Producing *Klebsiella pneumoniae* From Transplanted Patients in Brazil: Phylogeny, Resistome, Virulome and Mobile Genetic Elements Harboring bla_{KPC-2} or bla_{NDM-1}

Otávio Hallal Ferreira Raro[1,2], Ravena Maya Cardoso da Silva[1], Edison Moraes Rodrigues Filho[3], Teresa Cristina Teixeira Sukiennik[4], Claudio Stadnik[4], Cícero Armídio Gomes Dias[1], Jesús Oteo Iglesias[2] and María Pérez-Vázquez[2]*

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Objectives: Carbapenemase-producing *Klebsiella pneumoniae* (CP-Kp) is a major cause of infections in transplanted patients and has been associated with high mortality rates in this group. There is a lack of information about the Brazilian structure population of CP-Kp isolated from transplanted patients. By whole-genome sequencing (WGS), we analyzed phylogeny, resistome, virulome of CP-Kp isolates, and the structure of plasmids encoding bla_{KPC-2} and bla_{NDM-1} genes.

Methods: One *K. pneumoniae* isolated from each selected transplanted patient colonized or infected by CP-Kp over a 16-month period in a hospital complex in Porto Alegre (Brazil) was submitted for WGS. The total number of strains sequenced was 80. The hospital complex in Porto Alegre comprised seven different hospitals. High-resolution SNP typing, core genome multilocus sequence typing (cgMLST), resistance and virulence genes inference, and plasmid reconstruction were performed in 80 CP-Kp.

Results: The mortality rate of CP-Kp colonized or infected transplanted inpatients was 21.3% (17/80). Four CP-Kp epidemic clones were described: ST11/KPC-2, ST16/KPC-2, and ST15/NDM-1, all responsible for interhospital outbreaks; and ST437/KPC-2 affecting a single hospital. The average number of acquired resistance and virulence genes was 9 (range = 2–14) and 27 (range = 6–36), respectively. Two plasmids carrying the bla_{KPC-2} were constructed and belonged to IncN and IncM types. Additionally, an IncFIB plasmid carrying the bla_{NDM-1} was described.

Conclusion: We detected intrahospital and interhospital spread of mobile structures and international *K. pneumoniae* clones as ST11, ST16, and ST15 among transplanted patients, which carry a significant range of acquired resistance and virulence genes and keep spreading across the world.

Keywords: transplanted patients, bla_{KPC-2}, bla_{NDM-1}, whole-genome sequencing, cgMLST, epidemic clones

INTRODUCTION AND OBJECTIVE

Carbapenemase-resistant Enterobacterales (CRE) infection or colonization is a threat to organ transplant recipients (OTRs). The mortality rates in OTR range from 30% to 50% in infections caused by CRE (Satlin et al., 2014; Xu et al., 2017), and when it is focused only in *Klebsiella pneumoniae*, this risk of death increases 10-fold (Brizendine et al., 2015; Lanini et al., 2015), resulting in a worldwide public health emergency, because these microorganisms have been reported in all continents (Nordmann et al., 2011; Satlin et al., 2014). In 2017, the World Health Organization (WHO) released a report (World Health Organization [WHO], 2017b) that marked carbapenem-resistant *K. pneumoniae* (CR-Kp) as a matter of international concern as one of the major causes of hospital-acquired infections. In addition, CR-Kp was also included in the global priority list of antibiotic-resistant bacteria as a critical pathogen by the WHO (World Health Organization [WHO], 2017a).

The most important mechanisms of carbapenem resistance in Enterobacteriaceae are the plasmid-borne carbapenemases. The most common carbapenemases in Enterobacteriaceae are *K. pneumoniae* carbapenemase (KPC; class A); Verona integron-encoded metallo-β-lactamase, imipenemase, and New Delhi metallo-β-lactamase (NDM; class B); and the OXA-48 types (class D). Many carbapenemase genes are carried in different plasmid types (Poirel et al., 2011; Pitout et al., 2015; Raro et al., 2019).

The first study to report the detection of carbapenemases in Brazil was published in 2009 (Monteiro et al., 2009) and described the presence of KPC-2 in *K. pneumoniae* from four patients in the city of Recife, Pernambuco, Brazil, 10 years after the first detection of KPC-2 in the world, in North Carolina, United States (Yigit et al., 2001; Queenan and Bush, 2007). Subsequently, KPC-2 has been described in other species of Enterobacterales distributed throughout the country, but *K. pneumoniae* is the most frequent species carrying carbapenemases. New Delhi metallo-β-lactamase was first detected in 2008 in a Swedish patient who traveled to New Delhi, India (Yong et al., 2009); 5 years later came the first report of an NDM producer strain in Brazil, which was *Providencia rettgeri* isolated from a patient in the city of Porto Alegre, Rio Grande do Sul, Brazil (Carvalho-Assef et al., 2013). After 2009, sporadic cases of carbapenemase-producing Enterobacteriaceae (CPE) were described in Brazil, including the coproduction of NDM-1 and KPC-2 (Pereira et al., 2015; Quiles et al., 2015).

Dissemination and outbreaks caused by KPC- and NDM-producing *K. pneumoniae* isolates have been reported, but we call attention to the scarcity of reports of CPE isolates colonizing or infecting transplanted patients reported so far (Taglietti et al., 2013; Lee et al., 2018).

In the present study, we aimed to describe the phylogeny, resistome, virulome, and the plasmids encoding bla_{KPC} and bla_{NDM} of CP-Kp strains isolated from transplanted inpatients in a tertiary hospital complex from the city of Porto Alegre, Brazil.

MATERIALS AND METHODS

Clinical Data and Strain Collection

In this study, a total of 80 transplanted inpatients admitted to a 1,000-bed tertiary hospital complex in Porto Alegre, from August 2017 to November 2018 were screened in an active surveillance program to detect carbapenemase-producing *K. pneumoniae*. During this surveillance program, rectal swabs of all patients were collected and screened in admission and once a week for detection of CPE. At the laboratory, a screening procedure was performed using disk diffusion to detect carbapenem resistance. If reduced susceptibility was observed, the isolates were subjected to CarbaNP phenotypic test (Nordmann et al., 2012) following complex hospital's infection control service and microbiology laboratory recommendations. The hospital complex made up of seven different specialist hospital buildings, including a national referral transplant hospital, and is responsible for providing services to the metropolitan region of Porto Alegre, which comprises more than 4 million people in the south of Brazil (Instituto Brasileiro de Geografia e Estatística [IBGE], 2010). The criteria used to include the patients were as follows: (1) to be a solid organ or marrow bone transplanted patient; (2) to be admitted to the hospital complex during August 2017 and November 2018, and (3) to be colonized by CP-Kp after the transplant surgery (post-operative period). Patient movement networks among the hospitals were characterized through Inkscape v0.92.4 vector graphics editor[1].

One CP-Kp isolate per patient was collected always in the post-operative period. Infection isolates were prioritized over colonization isolates when both were present. When multiple isolates were detected from different infection sites from the same patient, isolates collected from invasive infections (blood) were always prioritized over isolates from other non-invasive sites. And, when multiple isolates from the same clinical site were present, the first isolate collected was prioritized. The identification of the strains and carbapenemase production was confirmed using MALDI-TOF MS (matrix-assisted laser desorption ionization time of flight; Bruker Daltonics, BD, Bremen, Germany) and CarbaNP phenotypic test (Nordmann et al., 2012) after detection of reduced susceptibility to at least one carbapenem antibiotic by disk

[1] http://www.inkscape.org/

diffusion. Disk diffusion tests were then performed for other antimicrobial agents (ampicillin, piperacillin-tazobactam, ampicillin-sulbactam, cefazolin, cefuroxime, cefepime, amikacin, gentamicin, ciprofloxacin, norfloxacin, nitrofurantoin, and sulfamethoxazole-trimethoprim) in all clinical isolates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2018). The reference strains used in the disk diffusion technique were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603. For CarbaNP assay, *K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA-1706 were used as positive and negative controls, respectively, according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2018). This strategy was performed according to the complex hospital's infection control service and microbiology laboratory recommendations. Whole-genome sequencing (WGS) was performed in all 80 CP-Kp isolates.

DNA Extraction, Genomic Library Preparation, and Sequence Analysis

DNA from the isolates was extracted using a QIAamp® DNA mini kit (Qiagen®, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA paired-end libraries were generated using the Nextera XT DNA sample preparation kit (Illumina Inc., San Diego, CA, United States). These libraries were sequenced using the Illumina HiSeq 500 next-generation sequencer with 2 × 150-bp paired-end reads (Illumina Inc). Raw sequence data were submitted to the European Nucleotide Archive (PRJEB34380). The quality of the high-throughput sequence data was assessed by FastQC (Andrews, 2010), and these short reads were subsequently assembled *de novo* into contigs using SPAdes 3.9.0 (Bankevich et al., 2012) testing five different kmers under parameters optimized to give the best assembly, which quality was evaluated by QUAST[2] (Gurevich et al., 2013). Scaffolding was performed with SSPACE (Boetzer et al., 2011), and GapFiller was used to close sequence gaps (Nadalin et al., 2012). Automatic *de novo* annotation of draft genomes was done using Prokka v1.12-beta (Seemann, 2014).

Illumina sequence reads of *K. pneumoniae* isolates were mapped to the chromosome of *K. pneumoniae* NTUH-K2044 (accession no. NC_012731.1) using Snippy to detect SNPs among all samples[3]. Additionally, Gubbins software was used to eliminate recombinant regions (Croucher et al., 2015). Sequence reads were mapped to an average of 91.97% of the reference genome, with a mean depth of 153x in mapped regions across the isolates. Finally, we generated a concatenated alignment with 52,139 SNP sites. Whole-genome sequencing quality data are detailed in **Supplementary Table S1**.

Phylogenetic Analysis

A core genome multilocus sequence typing (cgMLST) that relies on species-specific schemes with a fixed number of chromosomal target genes was applied. For *K. pneumoniae*, we applied a published and public scheme of 2,567 genes (Pérez-Vázquez et al., 2019). This scheme was used to compare *K. pneumoniae* Brazilian isolates with all publicly available complete genomes of *K. pneumoniae* of the same STs. A minimum spanning tree was reconstructed through Ridom SeqSphere + software (Ridom GmbH, Münster, Germany) to analyze the results.

A maximum likelihood phylogenetic tree was reconstructed using SNPs within the core genome using RAxML v7.0.4 (Stamatakis, 2006) with a general time-reversible model and gamma correction for among site rate variation. The SNP alignment of each sequence type was used to recalculate individual maximum likelihood phylogenetic trees. The support for the nodes on the trees was assessed using 100 bootstrap replicates. MEGA X software (Kumar et al., 2018) was used to detect SNPs among all samples.

Analysis of Antimicrobial Resistance, Virulence Genes, and Plasmid Reconstruction

Antimicrobial resistance genes were analyzed using ResFindertool (CGE server)[3] with an ID threshold of 98% except for β-lactamase variants, which were determined with a 100% identity. Additionally, SRST2 (Inouye et al., 2014) was used to detect resistance genes and alleles with the ARGannot database (Gupta et al., 2014). Virulence genes were identified using the BIGSdb-Kp database (Institut Pasteur, last accessed March 2019)[4] (Bialek-Davenet et al., 2014). Capsule K-locus and LPS O-antigen typing were characterized using WGS data through Kaptive tool (Wyres et al., 2016; Wick et al., 2018)[5]. To reconstruct the plasmids of each genome, an in-house script was used[6] as described (Pérez-Vázquez et al., 2019).

RESULTS AND DISCUSSION

Clinical and Demographic Data

The 80 selected inpatient gender and mean age (standard deviation) were 33.8 and 66.3%, female and male, respectively, and 54 (±13.8) years old. The patients were submitted to different transplants, with kidney being the most prevalent type of surgery, representing 61.3%, followed by lung and liver in second and third place with 17.5 and 16.3% of frequency, respectively. All transplant types are listed in the **Supplementary Table S1**.

The patients included in the study were admitted to four of the seven hospitals from the complex. The number of patients admitted in each hospital was as follows: hospital A (HA) (61/80, 76.3%), hospital B (HB) (14/80, 17.5%), hospital C (HC) (1/80, 1.3%), and hospital D (HD) (4/80, 5.0%). Complete details of the hospital and units are in the **Supplementary Table S1**. The mortality rate of transplanted patients colonized or infected by CP-Kp was 21.3% (**Supplementary Table S1**).

[2]http://quast.sourceforge.net/quast
[3]https://github.com/tseemann/snippy
[4]http://bigsdb.pasteur.fr
[5]https://github.com/katholt/Kaptive
[6]https://github.com/BU-ISCIII/plasmidID

Bacterial Isolates

The 80 selected CP-Kp samples were collected from different sources, being 40% from surveillance rectal swabs and named as colonizing CP-Kp. Isolates obtained from clinical specimens were 21.2% from urine, 20.0% from the bloodstream, 16.2% from respiratory samples, 1.3% from abdominal liquid, and 1.3% from catheter tip (**Supplementary Table S1**). The carbapenemase genes detected were bla_{KPC-2} (71 isolates, 88.8%), and bla_{NDM-1} (nine isolates, 11.2%). The isolates were assigned to eight different STs: (1) ST11 with 62.5% of the isolates, (2) ST258 with 3.75%, (3) ST437 with 10%, these three STs were part of the clonal complex 11/258 (CC11/258); (4) ST15 with 8.75%, (5) ST4019 with 2.5%, both genetically related and the only carrying bla_{NDM-1}; (6) ST16 with 10%, (7) ST17 with 1.25%, both STs are part of the clonal complex 17 (CC17); and (8) ST39 with 1.25%. Isolates belonging to CC 11/258 are predominant in Brazil, as in the present study. On the other hand, other Brazilian studies described the presence of ST15 CP-Kp isolates, but to the best of our knowledge, isolates of these studies were not associated to bla_{NDM-1}, as described here (Gonçalves et al., 2017; Andrade et al., 2018). ST39 was not reported in previous studies with isolates obtained from patients in Brazil, and only individual cases of ST39 producing bla_{KPC-2} or bla_{NDM-16} were reported in two different studies from China (Liu et al., 2017; Xie et al., 2020).

Antibiotic resistance was observed against ampicillin, piperacillin-tazobactam, ampicillin-sulbactam, cefazolin, cefuroxime, cefepime, imipenem, meropenem, ertapenem, ciprofloxacin, norfloxacin, and nitrofurantoin to all CP-Kp isolates. On the other hand, 93.7% of the isolates were susceptible to amikacin, 18.7% to gentamicin, and 2.1% to sulfamethoxazole-trimethoprim. Susceptibility profiles of all clinical CP-Kp isolates are detailed in **Supplementary Table S1**. We recognize that the use of diffusion tests, instead of microdilution tests, can limit the definition of the resistance profile. However, agar diffusion tests are reliable and endorsed by both European Committee on Antimicrobial Susceptibility Testing and CLSI (Clinical and Laboratory Standards Institute, 2018; The European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2018).

Phylogenetic Analysis of KPC-Kp and NDM-Kp

Genome assemblies of the sequenced *K. pneumoniae* were analyzed by a gene-by-gene approach (Bialek-Davenet et al., 2014), together with all publicly available complete genomes of *K. pneumoniae* of the same STs; the allelic distance from cgMLST was visualized in a minimum spanning tree (**Figure 1A**). The results of allelic distances shown in Brazilian isolates had a population average difference of 836 (0–2,005) alleles and clustering into five groups (clusters 1–5). Four outbreaks (A–D) were detected when the following threshold was applied; less than 15 alleles distance in a pairwise comparison among all isolates (Lepuschitz et al., 2019; Miro et al., 2020). The isolates included in each outbreak presented an average difference of 12 (range = 0–25), 5 (range = 0–11), 4 (range = 0–11), and 2 (range = 0–5) alleles, in outbreak A (ST11/KPC-2), B (ST437/KPC-2), C (ST16/KPC-2), and D (ST15/NDM-1), respectively. Outbreak A was due to 47 isolates (colonizing 32%; clinical specimens 68%). They differed from outbreaks B, C, and D by an average of 355 (range = 346–363), 1,942 (range = 1,915–1,947), and 2,000 (range = 1,968–2,005) alleles. Outbreak B included six isolates (colonizing 66.7%; clinical specimens 33.3%); they differed from C and D by an average of 1,951 (range = 1,951–1,952) and 1.993 (range = 1,990–1,994). Outbreak C was due to seven isolates (colonizing 14.3%; clinical specimens 85.7%) that differed from outbreak D by an average of 1,991 (1,990–1,993) alleles. Outbreak D was due to five isolates (colonizing 80.0%, clinical specimens 20.0%). Both outbreaks A and B could maintain themselves during all the four collection quarters of sample collection. Outbreak A was present in three hospitals of the hospital complex (HA, HB, and HD) while outbreak B was present only in HA. Outbreak C was present in three hospitals (HA, HB, and HC) and started in the second period of sample collection and persisted until the end. Outbreak D was present in two hospitals (HA and HB) and started in the second half of the sample collection period (**Figure 2B**). Three outbreaks (A, C, and D) were present in at least two hospitals, suggesting that these *K. pneumoniae* high-risk clones (ST11/KPC-2, ST16/KPC-2, and ST15/NDM-1) were capable of interhospital spread and could be responsible for interregional and international spread, as reported elsewhere (Munoz-Price et al., 2013; Wu et al., 2019). We also compared CP-Kp isolated in this study with *K. pneumonia*e isolates of the same STs from other geographic regions; no isolate from another country was detected as a related clone with the Brazilian isolates; thus, we can affirm that we have the dissemination of geographically well-settled clones in our country (**Figure 1A**).

A maximum likelihood phylogenetic tree was reconstructed using 52,139 high-quality SNPs that were identified with reference to the sequence of *K. pneumoniae* strain NTUH-K2044 (**Figure 1B**). The general population presented SNP approximation average of 4,609 (0–11,982) SNPs and a clustering in five groups (clusters 1–5), similar to the clusters described previously with cgMLST analysis. The isolates were also grouped into four outbreaks (A–D). The isolates included in each outbreak presented an average difference of 16 (range = 0–31), 5 (range = 0–8), 7 (range = 1–17), and 1 (range = 0–3) SNPs, in outbreaks A (ST11/KPC-2), B (ST437/KPC-2), C (ST16/KPC-2), and D (ST15/NDM-1), respectively. Both techniques used to access the phylogeny of the CP-Kp isolates resulted in the same clusters and outbreaks. When a high-quality SNP approach was used, high diversity was found in isolates that belonged to ST11; this fact could be supported by the idea that some of these SNPs arose because of recombination events [30]. Patient movement networks between hospitals revealed that 45 of 47 patients involved in outbreak A were hospitalized at least once in the HA. Moreover, patient movement networks also revealed that after hospitalization patients could be moved once, twice, or even three times among the hospitals. Hospital A was a source hospital in

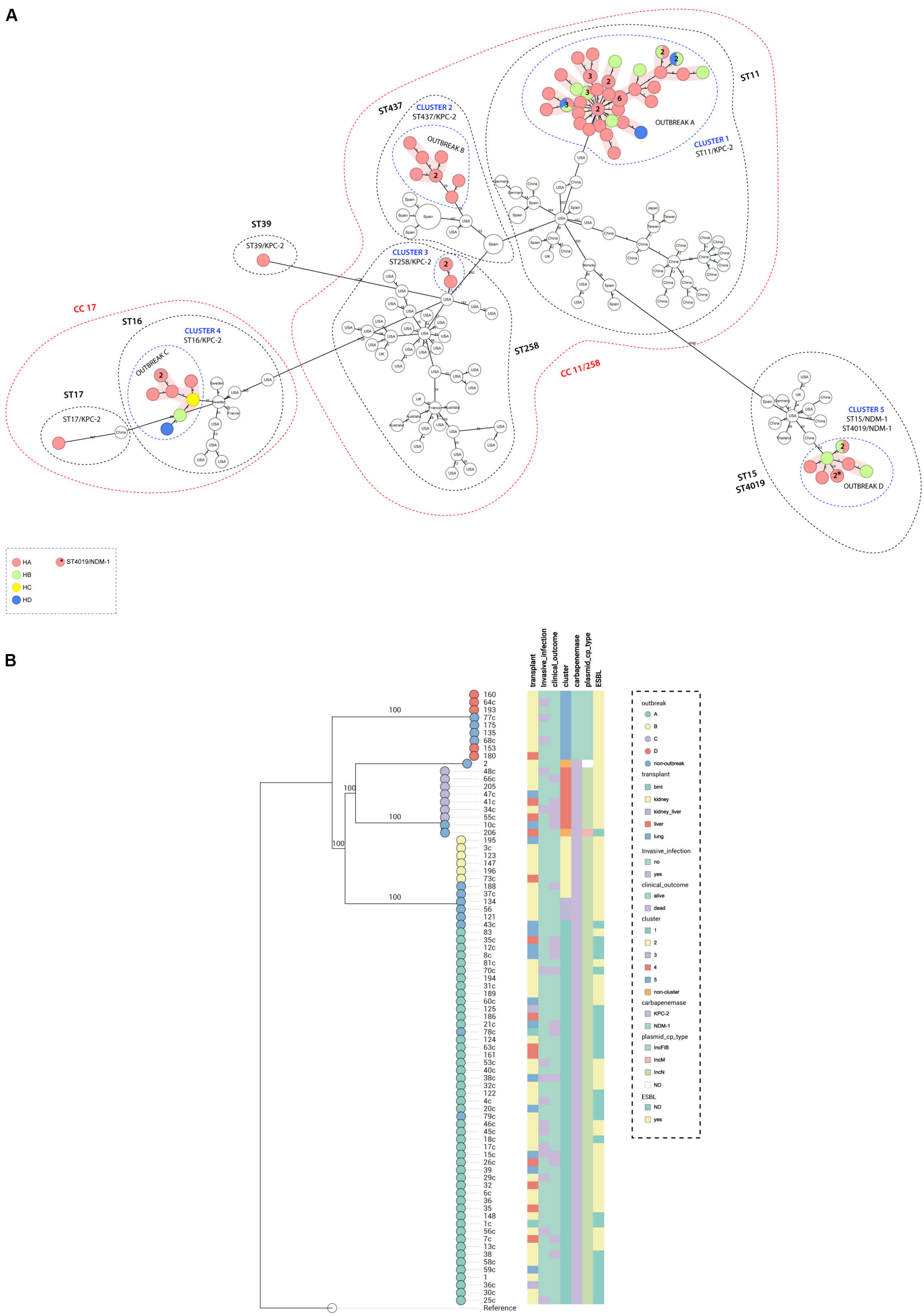

FIGURE 1 | Continued

FIGURE 1 | (A) Minimum spanning tree. Distance based on an *ad hoc cgMLST* of 2567 genes. Each colored circle indicates the hospital origin of the isolates; more than one isolate is indicated with its respective quantities in numbers, and non-colored circles are isolates from other countries. Red shadow indicates outbreak, blue dashed circles indicate cluster, black dashed circles represent sequence types, and red dashed circles represent clonal complex. Distances are not in scale.
(B) Maximum likelihood tree showing the relationship between isolates, branch lengths are indicative of the number of SNPs. Colored strips in (from left to right) transplant type, invasive infection, clinical outcome, cluster, carbapenemase type, plasmid type carrying the bla_{KPC-2} or bla_{NDM-1} genes, and ESBL type. Nodes are colored according to the outbreak caused by the isolates. Reference: *K. pneumoniae* NTUH-K2044 (accession no. NC_012731.1).

64.5% of the patient movements, thus being a significant reservoir to disseminate and acquire resistance features in CP-Kp (**Figure 2A**).

Resistance and Virulence Genes in CP-Kp

In the 80 CP-Kp isolates studied, the average number of acquired resistance genes (ARGs) was 9 (range = 2–14, **Supplementary Table S1** and **Figure 3**). Seventy-one (88.8%) carried bla_{KPC-2}, and nine (11.2%) carried bla_{NDM-1}. Extended spectrum β-lactamases genes (ESBL) identified in these isolates were as follows: 23 (28.7%) isolates carried $bla_{CTX-M-2}$, 20 (25%) $bla_{CTX-M-15}$, 5 (6.2%) $bla_{CTX-M-8}$, 4 (5%) $bla_{CTX-M-14}$, 1 (1.3%) $bla_{CTX-M-35}$, and 1 (1.3%) isolate carried bla_{SHV-40}. Coproduction with ESBL was detected in 62% of bla_{KPC-2} *K. pneumoniae* and in 100% of bla_{NDM-1} *K. pneumoniae* isolates. No plasmid-encoded AmpC genes were identified in our isolates. The predominant genes encoding aminoglycoside-modifying enzymes were N-acetyltransferases, being the most frequent *aac(3′)-IIa* (65%), *aac(6′)-Ib3* (55%), and *aac(6′)-Ib-cr* (17.5%). Three isolates (3.7%) carried acquired 16S rRNA methyltransferase *rmtB*; all these isolates were bla_{KPC-2} producers and belonged to ST258. Plasmid-mediated quinolone resistance qnr-like determinants were detected in 18.8% of the isolates, being *qnrS1* the most frequent (11.3%). One study that performed WGS in 10 KPC-2-producing *K. pneumoniae* selected from different cities and states of Brazil found a high frequency of qnr-like resistant determinants (100%) and bla_{CTX-M} genes (80%) (Fuga et al., 2019). In contrast, we found only 8.4% and 62% of qnr-like determinants and bla_{CTX-M} genes, respectively. Chloramphenicol acetyltransferase *catA1* and *catB4* were the most frequent phenicol resistance mediators, being detected in 57.5 and 22.5% of the isolates, respectively. The dihydropteroate synthases associated with sulfonamide resistance also were detected; 67.5% presented *sul1* and 15.0% *sul2*; moreover, 2.5% of the isolates presented both *sul1* and *sul2* genes. The dihydrofolate reductases associated with trimethoprim resistance were detected in 98.8% of the isolates, being the most frequent *dfrA30* (77.5%). The isolates of outbreaks B and D presented a higher average number of ARGs when comparing with the isolates from the other outbreaks (outbreak B: average = 12, range = 12–12; outbreak D: average = 12, range = 10–13, outbreak C: average = 9, range = 8–10; outbreak A: average = 8, range = 2–9). To summarize, the most frequent ARG profile was as follows: *aac(3′)-IIa*, *aac(6′)-Ib3*, $bla_{CTX-M-2}$, bla_{KPC-2}, $bla\text{-}_{OXA-2}$, bla_{TEM-1B}, *catA1*, *sul1*, and *dfrA30,* detected in isolates responsible by outbreak A (ST11/KPC-2). The most stable ARG profile was found in isolates from outbreak B (ST437/KPC-2), where all isolates carried the same profile: *aac(3′)-IId*, *aph(3′)-Ia*, *aac(6′)-Ib-cr*, $bla_{CTX-M-15}$, bla_{KPC-2}, $bla\text{-}_{OXA-1}$, bla_{TEM-1B}, *mph(A)*, *catB4*, *sul1*, *dfrA30*, *tetA*, and *tetD* (**Supplementary Table S1**). The acquisition and loss of antibiotic resistance genes have been associated to *K. pneumoniae* strains undergoing selective antibiotic pressure (Simner et al., 2018). We noted that isolates within each outbreak did not have many changes in their ARG profile. The most relevant change was in isolates from outbreak A, which we observed an acquisition of bla_{CTX-M} genes in 51.1% of the isolates.

The analysis of the capsular polysaccharide locus showed that all isolates included in each individual outbreak had the same *wzi* allele, not shared within outbreaks. The following K-loci were identified in outbreaks: KL64 (outbreak A), KL36 (B), KL51 (C), and KL24 (D), as shown in **Supplementary Table S1** and **Figure 4**. Notably, KL1 to KL77 are associated with the classical 77 serologically defined K types. The most frequent K-locus detected in this study was the KL64, which was detected in the ST11 isolates. This K-locus is the most prevalent in China in ST11 isolates and has been reported in Brazilian ST11 *K. pneumoniae* isolates since 2018, which in one case was related to a fatal bacteremia, as we detected here in three patients (3.8%) which progressed to death (Andrade et al., 2018; de Campos et al., 2018). The intercontinental ST15 KL24 clone was found in seven isolates that coproduce bla_{NDM-1} and $bla_{CTX-M-15}$; this capsular type is commonly linked to $bla_{CTXM-15}$ but not to bla_{NDM-1} (Holt et al., 2015; Andrade et al., 2018).

The LPS O-antigen typing showed that, as in K-locus analyses, all isolates included in each individual outbreak had the same O-antigen allele, not shared within outbreaks. The O-antigens identified in the outbreaks were as follows: O2v1 (outbreak A), O4 (B), O3b (C), and O1v1 (D), as shown in **Supplementary Table S1** and **Figure 4**. Its estimate that exists nine main O-antigens clusters described, and serotypes, O1, O2, and O3, are associated with almost 80% of the *K. pneumoniae* infections (Follador et al., 2016; Martin and Bachman, 2018). In contrast with the study reported by Wick et al. (2018), we observed a highly conserved O-antigen locus in the CC 11/258 isolates, being O2v1 the most frequent one, involved in the ST11 isolates.

The virulome was performed by WGS (**Supplementary Table S1** and **Figure 4**), and in accordance to the BIGSdb-Kp database (Bialek-Davenet et al., 2014), a total of six virulence features were detected in this study, being the genotoxin colibactin cluster (*clbABCDEFGHIJLMNOPQR*), siderophore genes (*irp1* and *irp2*), the yersiniabactin siderophore cluster (*ybtAEPQSTUX*), the mannose-resistant *Klebsiella*-like (type III)

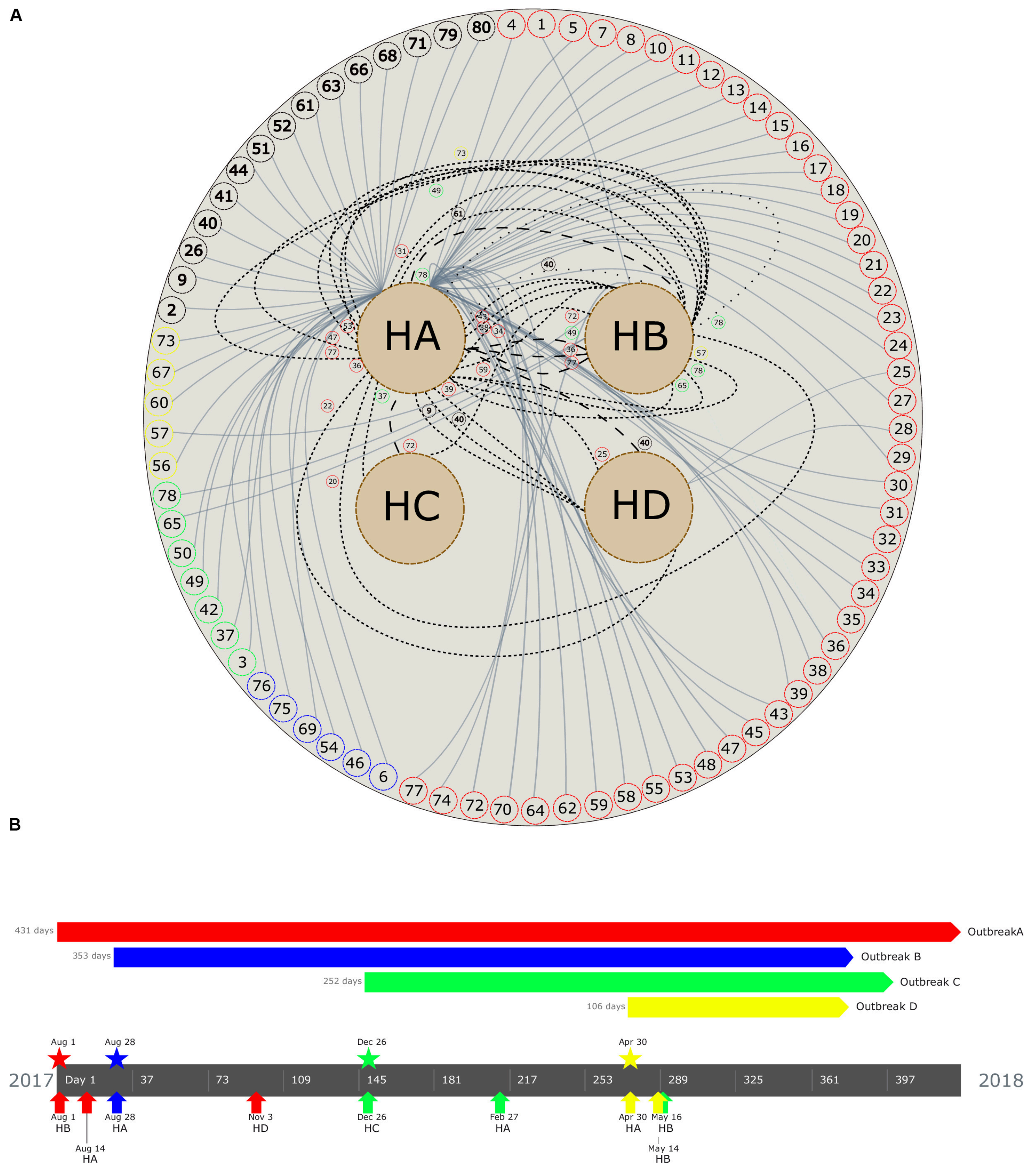

FIGURE 2 | (A) Patient movement networks between hospitals. Outer dashed circles indicate patient's identification being red (patients with isolates grouped in outbreak A), blue (outbreak B), green (outbreak C), yellow (outbreak D), and black (non–outbreak associated). Inner dashed circles indicate hospitals (HA, hospital A; HB, hospital B; HC, hospital C; and HD, hospital D). Continuous lines emerging in each patient represent hospitalization, short dashed lines represent first movement among hospital, long dashed lines represent second movement, and spaced dotted line represents third movement. All patient movements are identified with patient identification. **(B)** Timeline of the four carbapenemase-producing *K. pneumoniae* outbreaks. Outbreaks are represented by colors (outbreak A, red; B, blue; C, green; D, yellow). Pentagons indicates the period of the outbreak, stars represent the beginning of the outbreak, and arrows up indicate the first outbreak's isolate detected in each hospital (see geolocation on https://microreact.org/project/RZLItIMKm).

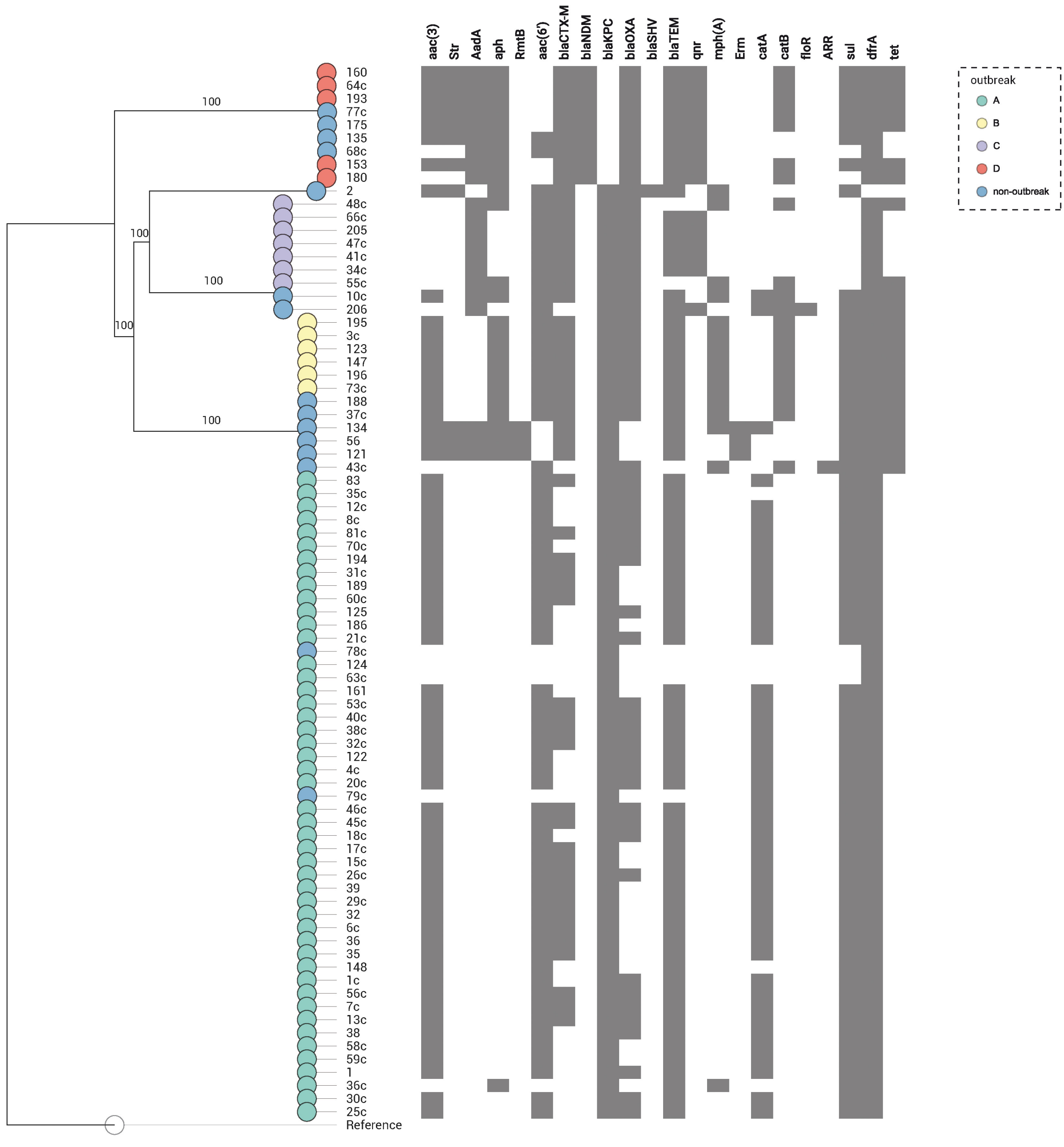

FIGURE 3 | Distribution of the resistance genes in 80 *K. pneumoniae* expressing bla_{KPC} or bla_{NDM} genes. Genes conferring resistance (resistome) are shown at top. A map according to the presence or absence of each gene is shown underneath, where presence is shown in gray and absence in white. Nodes are colored according to the outbreak caused by the isolates. Reference: *K. pneumoniae* NTUH-K2044 (accession no. NC_012731.1).

fimbriae cluster (*mrkABCDFHIJ*), the ferric uptake system (*kfuABC*), and the yersiniabactin receptor gene *fyuA*. In general, the CP-Kp presented an average number of 27 (range = 6–36) virulence genes. A study recently conducted in São Paulo, Brazil, published that ST16 KPC-2 *K. pneumoniae* presented higher virulence than CC11/258 isolates in the *Galleria mellonella* pathogenicity model (Andrey et al., 2019). Although we had the limitation of not performing any pathogenicity animal model, in our study, the isolates of outbreak A (ST11/KPC-2) presented a higher average number of virulence genes when compared with the isolates from the other outbreaks (outbreak A: average = 35, range = 27–36; outbreak D: average = 20, range = 20–20; outbreak C: average = 19, range = 19–19; outbreak B: average = 6,

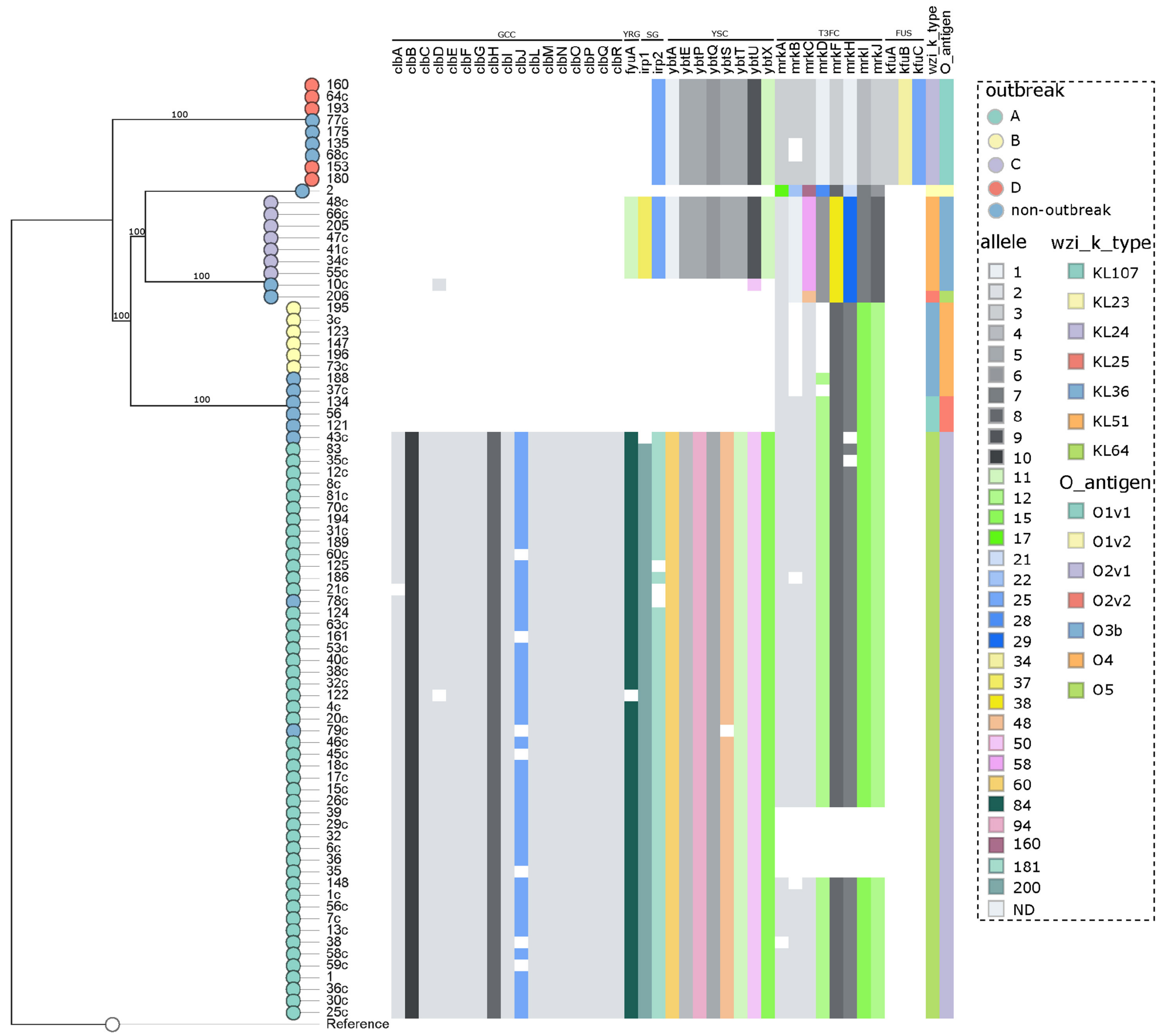

FIGURE 4 | Distribution of the virulence genes in 80 *K. pneumoniae* expressing bla_{KPC} or bla_{NDM} genes. Genes conferring virulence (virulome) are shown at top. A colored heat map according to the allele number of each gene is shown underneath. Nodes are colored according to the outbreak caused by the isolates. Virulence genes groups are shown on top indicating (from left to right) genotoxin colibactin cluster (GCC), yersiniabactin receptor gene (YRG), siderophore genes (SG), yersiniabactin siderophore cluster (YSC), type III fimbriae cluster (T3FC), and ferric uptake system (FUS). Wzi alleles are shown in the top right. Reference: *K. pneumoniae* NTUH-K2044 (accession no. NC_012731.1).

range = 6–6), a fact that may enable ST11/KPC-2 isolates to be responsible for a high rate of invasive infections, as observed here and worldwide (Lee et al., 2016; de Campos et al., 2018). The colibactin cluster genes were only observed in outbreak A, being present in all the isolates of this group, but were disrupted in 17.0%. The colibactin-producing *K. pneumoniae* induce DNA damage and chromosomal instability in eukaryotic cells, which leads to senescence of epithelial cells and apoptosis of immune cells (Faïs et al., 2018), this virulence mechanism is strongly associated to CC23 *K. pneumoniae* (Lam et al., 2018).

The three virulence features included in yersiniabactin loci, *ybtAEPQSTUX*, siderophore genes, and yersiniabactin receptor, were detected in isolates of outbreaks A and C, and in all isolates of outbreak D, yersiniabactin loci lack yersiniabactin receptor and *irp1* gene, which could reduce the biosynthesis and expression of yersiniabactin, the adherence capability, and the pathogenicity of these isolates (Pelludat et al., 1998; Tu et al., 2016). The yersiniabactin cluster enables the scavenging of iron from host transport proteins, thus enhancing the ability of bacteria to replicate within the host and survive, and is mostly related to invasive infections (Holden and Bachman, 2015).

FIGURE 5 | Overview of the IncN plasmid harboring bla_{KPC-2} detected in *K. pneumoniae* ST11, ST16, ST258, and ST437 involved in outbreaks A, B, and C, in this study. The figure represents the bla_{KPC} plasmid according to the homology with highly similar one from database (blue outer ring). Graph represents the Illumina reads mapped against this plasmid with depth of coverage ranging from 0 (red) to 500, colored orange when values are 1 to 20, and green if more than 200 reads. Gray boxes represent cds from automatic annotation, with dark and light color when they were found in forward or reverse strand, respectively. Colored stripes represent a more detailed annotation that includes antibiotic resistance genes in red, IS in blue, and Rep genes in yellow. Homology between constructed plasmid and Illumina assembled contigs is represented in the inner ring, with each contig colored according to its number.

The *ybtAEPQSTUX* cluster was detected in all outbreaks described in the study except only one that was not associated with an invasive infection (outbreak B). The type III fimbriae cluster (T3Fc) is responsible for mediating adherence to the renal tubular, respiratory tract, and lung tissue cells, which is crucial for biofilm formation (Schroll et al., 2010; Ares et al., 2017). Isolates from all outbreaks presented the T3Fc cluster, although in some isolates of outbreaks A and B, this cluster was not complete, and in a few isolates of outbreak A (12,8%, 6/47), T3Fc was not detected. The ferric uptake system (*kfuABC*) was detected only in outbreak D and non-outbreak related ST15 and ST4019 isolates. Interestingly, bla_{NDM-1} carriers seem

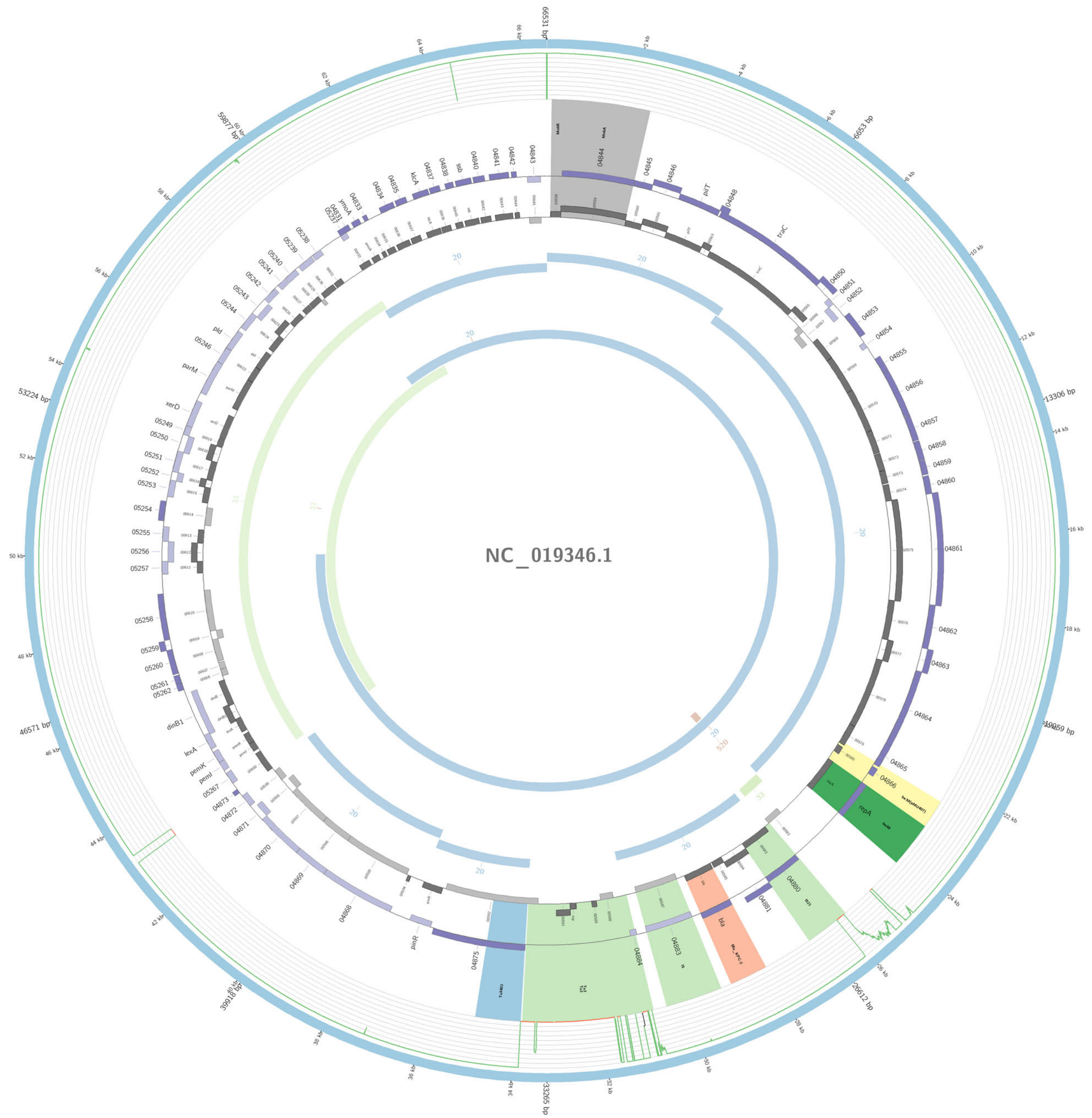

FIGURE 6 | Overview of the IncM plasmid harboring bla_{KPC-2} detected in *K. pneumoniae* ST17 non-outbreak involved in this study. The figure represents the bla_{KPC} plasmid according to the homology with highly similar one from database (blue outer ring). Graph represents the Illumina reads mapped against this plasmid with depth of coverage ranging from 0 (red) to 500, colored orange when values are 1 to 20, and green if more than 200 reads. Gray boxes represent cds from automatic annotation, with dark and light color when they were found in forward or reverse strand, respectively. Colored stripes represent a more detailed annotation that includes antibiotic resistance genes in red, IS in blue, and Rep genes in yellow. Homology between constructed plasmid and Illumina assembled contigs is represented in the inner ring, with each contig colored according to its number.

to be strongly associated with *kfuABC* system in our study. *KfuABC* has been associated with increased virulence by enabling lineages to use iron from diverse human and environmental sources, and it was recently associated to *K. pneumoniae* ST 101 (Roe et al., 2019).

To summarize, the most frequent virulence factor (VF) profile was as follows: *clb*ABCDEFGHIJLMNOPQR, *irp*1, *irp*2, *ybt*AEPQSTUX, *mrk*ABCDFHIJ, and *fyu*A, detected in isolates responsible by outbreak A (ST11/KPC-2). And the most stable VF profile was found in isolates from

FIGURE 7 | Overview of the IncFIB plasmid harboring bla_{NDM-1} detected in *K. pneumoniae* ST15 and ST4019, involved in outbreak D in this study. The figure represents the bla_{NDM} plasmid according to the homology with highly similar one from database (blue outer ring). Graph represents the Illumina reads mapped against this plasmid with depth of coverage ranging from 0 (red) to 500, colored orange when values are 1 to 20, and green if more than 200 reads. Gray boxes represent cds from automatic annotation, with dark and light color when they were found in forward or reverse strand, respectively. Colored stripes represent a more detailed annotation that includes antibiotic resistance genes in red, IS in blue, and Rep genes in yellow. Homology between constructed plasmid and Illumina assembled contigs is represented in the inner ring, with each contig colored according to its number.

outbreak D (ST15/NDM-1), where all isolates carried the same profile: *irp*2, *ybt*AEPQSTUX, *mrk*ABCDFHIJ, *kfu*ABC (**Supplementary Table S1**).

We did not observe great differences when comparing isolates from rectal swabs or clinical specimens in terms of ST or outbreak. However, some differences could be observed as follows: (1) apparently, clinical specimens possessed less acquired resistance genes (ARGs) and more virulence factors (VFs) than surveillance isolates (Clinical specimens, ARGs: mean 8.5, range = 2–14, VFs: 29.9, range = 6–36; Rectal swabs. ARGs: 9.8, range = 2–14, VFs: 22.1, range = 6–36); (2) as expected, 88.2% (15/17) of the patients that died were infected by a CP-Kp, the remaining 2/17 (11.8%) were colonized but did not developed an infection; and (3) clinical specimens were collected from patients hospitalized in all four Hospitals, while the colonizing isolates were collected from patients hospitalized only in two Hospitals from the complex (Hospital A and B).

Characterization of Plasmid Sequences Carrying bla_{KPC} and bla_{NDM} Genes

The plasmidID mapping tool was used for the identification and reconstruction of three plasmids harboring carbapenemase genes (two carrying bla_{KPC-2} and one carrying bla_{NDM-1}). An IncN plasmid (∼53,081 bp), that was almost identical to the NC_021664.2 accession number (average identity = 95.68%; average coverage percentage = 94.57%), which carried a bla_{KPC-2} gene (**Figure 5**), an IncM plasmid (∼66,531 bp) highly similar to the plasmid NC019346.1 accession number (average identity = 98.04%; average coverage percentage = 95.92%), carrying a bla_{KPC-2} (**Figure 6**), and an IncFIB plasmid (∼54,064 bp) almost identical to the plasmid NZ_CP014757.1 accession number (average identity = 98.9%; average coverage percentage = 85.52%) that carried a bla_{NDM-1} gene (**Figure 7**). In addition to the bla_{NDM-1} gene, the plasmid IncFIB carried five ARGs (bla-$_{OXA-1}$, *catB4*, $bla_{CTX-M-15}$, *qnrS1*, and *aph(3′)-VI*), but no additional ARGs were identified in the IncN and IncM KPC-2 plasmids. Thus, the only plasmid that was associated with multidrug resistance (MDR) was the IncFIB, an IncF plasmid's family known by their MDR characteristic (Rozwandowicz et al., 2018). The IncFIB plasmid was the only presenting the bla_{NDM-1} in our study. IncFIB was also reported carrying the bla_{NDM-1} in studies conducted in Myanmar and India, but differently from this study, and as had been described elsewhere, they found a greater number of plasmids harboring NDM-1 diversity (Hudson et al., 2014; Peirano et al., 2014; Conlan et al., 2016; Sugawara et al., 2018).

The IncN plasmid was detected in all isolates of outbreaks A, B, and C and in other non-outbreak related isolates belonging to ST11, ST16, ST258, and ST437; this fact highlighted the wide-spreading capacity of this plasmid among *K. pneumoniae* isolates. The genetic environment of this plasmid had a variant of Tn*4401b*, which carried only the bla_{KPC-2} as ARG. The Tn*4401b* isoform also had the elements *tnpR*, *tnpA*, *istA*, and *istB* upstream of the bla_{KPC-2} and downstream a truncated *tnpA* gene, highly similar to the already described FCF1305 plasmid (GenBank CP004366.2) (Perez-Chaparro et al., 2014). This finding confirms the persistence and movement of the IncN carrying bla_{KPC-2} around the country.

In one ST17 non-outbreak causative isolate, bla_{KPC-2} was harbored by an IncM plasmid. The IncM plasmid was often misidentified and reported as IncL/M group; this may explain why there are very few reports of the IncM harboring KPC-2 in Enterobacterales. The IncL/M group has been more associated with carrying the bla_{OXA-48} gene (Kopotsa et al., 2019), but here in South America, it seems to play a role in the dissemination of the bla_{KPC-2} gene; nevertheless, it was also reported in one study from China to be carrying the gene (Andrade et al., 2011; Liu et al., 2015; Jure et al., 2019). To our knowledge, ST17 *K. pneumoniae* with an IncM plasmid carrying the bla_{KPC-2} has only been reported in this study. The isoform Tn*4401f* was detected in this plasmid carrying the bla_{KPC-2} gene flanked upstream by the elements *istA* and *istB*, and downstream by *tnpA* and *tnpR,* a bla_{KPC} environment similar to the pNE1280 (GenBank JQ837276.1) (Bryant et al., 2013).

The IncFIB(pQil) plasmid was identified among outbreak D and in two additional non-outbreak related isolates belonging to ST15 and two to ST4019; all samples carried the bla_{NDM-1} gene. Similar to pNDM-1fa (accession number NZ-CP014757.1) (Conlan et al., 2016), the IncFIB detected in the study collection has a region that contained a bleomycin resistance gene (ble_{MBL}) upstream of the bla_{NDM-1} gene, and downstream it had two transposases, IS*630* and IS*30*, and an aminoglycoside resistance gene (*aph(3′)-VI*). Furthermore, this plasmid also had another two resistance mediator genes, a fluoroquinolone (*qnrS1*) and an ESBL ($bla_{CTX-M-15}$).

CONCLUSION

Our data reveal that high-risk clones in *K. pneumoniae* (ST11/KPC-2, ST16/KPC-2, and ST15/NDM-1) were responsible for the intrahospital and interhospital spread of bla_{KPC-2} and bla_{NDM-1} genes in transplanted patients from a hospital complex located in the south of Brazil. Patient movement networks among different hospitals may be associated with the dissemination of these clones.

We provided information about the phylogenetics of the study collection, showing a geographically conserved population in Brazil not clonally related to strains of the same MLST types isolated all over the world.

The IncN plasmid was the most frequent in our isolates and is probably a major cause of the widespread presence of bla_{KPC-2} in this country. The dissemination of the bla_{NDM-1} gene in the hospital complex studied was due to an IncFIB plasmid that harbored this carbapenemase gene together with the $bla_{CTX-M-15}$ gene.

Here we present a public health problem that needs to be more discussed and researched through further studies to better understand the spread, structural organization, and resistance/virulence features of the CP-Kp isolated in transplanted patients, in order to control and prevent outbreaks, given the great health and economic impact involved in these themes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the ENA project under accession number PRJEB34380.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee from Santa Casa de Misericórdia de Porto Alegre and by the Plataforma Brazil of the Ministry of Health from Brazil. Number of registration and approval: 2.974.453; CAAE (Ethical appreciation presentation certificate): 67009717.7.0000.5335.

AUTHOR CONTRIBUTIONS

OR contributed to the conception, design, and implementation of the study, acquisition of laboratory and clinical data, analysis of the results, drafting the article, and approval of the final version of the manuscript. RS contributed to the acquisition of laboratory data, analysis of the results, and review and approval of the final version of the manuscript. EF contributed to the design of the study, acquisition of clinical data, analysis of the results, and review and approval of the final version of the manuscript. TS and CS contributed to the design of the study, analysis of the results, and review and approval of the final version of the manuscript. CD, JO, and MP-V contributed to the conception, design, and implementation of the study, analysis of the results, drafting the article, and approval of the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01563/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.1.3. Artigo original V

Título:

Performance of Polymyxin B agar-based tests among carbapenem resistant Enterobacterales

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ORIGINAL ARTICLE

Performance of polymyxin B agar-based tests among carbapenem-resistant Enterobacterales

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Significance and Impact of the Study: Determining polymyxins susceptibility in clinical laboratories is challenging. Disk diffusion and antimicrobial gradient assays are not accurate enough for these antibiotics. Broth microdilution (BMD) is time consuming and laborious. We evaluated polymyxin B agar-based methods as an alternative to BMD. Agar dilution and agar screening tests presented good performances. As agar screening test is less laborious and cheaper than agar dilution, it may be an option in situations where the absence of polymyxin B MIC is clinically acceptable. As far as we know, this is the first study that evaluates agar screening with polymyxin B instead of colistin.

Keywords
agar-based tests, alternative susceptibility detection, carbapenem-resistant Enterobacterales, polymyxin B, polymyxin B resistance.

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Abstract

Therapeutic options for infections caused by Carbapenem-resistant Enterobacterales (CRE) are restricted and include polymyxins-centred schemes. Evaluation of *in vitro* susceptibility is difficult and time consuming. Agar-based methodologies are an alternative to broth microdilution (BMD) and we aimed to evaluate the accuracy of those methods among Enterobacterales. A total of 137 non-duplicated CRE were subjected to polymyxin B BMD, agar screening test (Mueller Hinton plates containing 3 $\mu g\ ml^{-1}$ of polymyxin B) and agar dilution (antibiotic serially diluted 0·25–64 $\mu g\ ml^{-1}$). CRE of 42·3% were resistant to polymyxin B (MICs range: 0·25–>64 $\mu g\ ml^{-1}$) and 16·8% presented borderline MICs. Sensitivity, specificity, PPV and NPV were 86·2, 98·7, 98 and 90·7% for screening test and 86·2, 97·5, 96·1 and 90·6% for agar dilution. ME was 0·73 and 1·5% for screening and agar dilution respectively; VME was 5·8% for both techniques. In general, agar-based methods had a good performance. As far as we know, this is the first study to propose an agar screening test using polymyxin B instead of colistin.

Introduction

Carbapenem-resistant Enterobacterales (CRE) have been a subject of major concern worldwide (Nordmann *et al.* 2011; Magiorakos *et al.* 2012; Jeannot *et al.* 2017). The World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) consider CRE as an urgent threat to human health, frequently associated with high mortality infections. *Klebsiella pneumoniae*, one of the most important species of the order Enterobacterales, is known for its high prevalence on nosocomial infections and its frequent multidrug resistance (MDR) profile, especially those producing carbapenemases (Holt *et al.* 2015). In these situations, for which few therapeutic options are available, the cationic polypeptide antibiotic polymyxins (Cain *et al.* 2018) are used as the last line drugs. Although new antibiotic such as ceftazidime–avibactam are now available (van Duin *et al.* 2018), in settings where this (and other) new combination of beta-lactams and beta-lactamase inhibitors are not approved yet or have limited use because of financial concerns, polymyxins remain the major treatment option for infection caused by CRE (Perez *et al.* 2019). In cases of carbapenem-susceptible isolates, there is no doubt treatment with carbapenems is a much better option than polymyxins. That is the reason why our bacterial sample was restricted to CRE.

Extensive usage of polymyxins preventing infection or promoting growth in animal farms, accelerated the selection of resistant isolates due to chromosomal mutations or plasmid-mediated resistance (Olaitan *et al.* 2014; Liu *et al.* 2016). Thus, a reliable and agile test to evaluate polymyxin resistance in clinical laboratories is a subject of major concern. Disk diffusion and antimicrobial gradient assay are not accurate enough for these antibiotics, and automated systems are currently not reliable. Indeed, broth microdilution (BMD) is the reference method according to the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Clinical and Laboratory Standards Institute 2020; European Committee on Antimicrobial Susceptibility Testing 2020). However, BMD is time consuming and laborious, being a challenge to many clinical microbiology laboratories (Simar *et al.* 2017; Vasoo 2017).

Besides BMD, alternative methodologies are available. An universal commercial culture medium for screening polymyxin-resistant gram-negative isolates using colistin sulphate (3·5 μg ml^{-1}) was described by Nordmann *et al.* (2016). Most recently, CLSI endorsed the colistin agar test (CAT) to be used by clinical laboratories (Clinical and Laboratory Standards Institute 2020).

As highlighted by Humphries *et al.* (2019), although remarkably similar, polymyxin B and colistin (polymyxin E) are distinct drugs with different pharmacokinetic/pharmacodynamic profiles and should be treated as such. Moreover, several countries do not have access to colistin, while others prefer to use polymyxin B over colistin. That is the reason why we aimed to compare the reference BMD with polymyxin B-based alternative methods: (i) 3 μg ml^{-1} agar screening test and (ii) agar dilution.

Results and Discussion

According to BMD, among 137 CRE evaluated, 58 (42·3%) were resistant to polymyxin B and 79 (57·7%) susceptible to this antibiotic. Polymyxin B BMD MICs ranged from ≤0·25 to >64 μg ml^{-1}, with 23 isolates (16·8%) presenting borderline MICs (2 or 4 μg ml^{-1}). Five of those were counted as susceptible (MIC = 2 μg ml^{-1}), and the remaining 18 isolates were included in the resistant group (MIC = 4 μg ml^{-1}).

Carbapenem-resistant Enterobacterales, mainly *K. pneumoniae,* are an urgent threat to human health. They are commonly MDR organisms and a major cause of hospital-acquired infections such as bloodstream infections, pneumonia and infections in newborns and patients in intensive-care units (World Health Organization (WHO) 2018). For these MDR bacteria, there are significantly reduced antibiotic options available, leading to therapeutic failures (Tzouvelekis *et al.* 2012).

Polymyxins have been used as a last resort in infections due to carbapenem-resistant *K. pneumoniae* (Nabarro and Veeraraghavan 2015). However, in the last decades, polymyxins resistance has risen in countries of Europe, with rates near 21, 32 and 43%, respectively, in Greece, Spain and Italy (Zagorianou *et al.* 2012; Monaco *et al.* 2014; Pena *et al.* 2014). In Brazil, polymyxins resistance rates are near 27% in CRE isolates (Sampaio and Gales 2016). Another study in the same country found an increase in polymyxin B resistance among KPC-producing *K. pneumoniae*, rising from 0% in 2011 to 27·1% in 2015 (Bartolleti *et al.* 2016).

Since its first report in Brazil in 2006 (Gales *et al.* 2006), polymyxin resistance among Enterobacterales has become a great challenge to Brazilian healthcare workers, with evidences of interhospital and intrahospital clonal spread (Sampaio and Gales 2016). Of note, ceftazidime–avibactam are available in Brazil since the middle of 2018 and are still expensive to be widely used among healthcare institutions around the country. Imipenem–relebactam and meropenem–vaborbactam are not available in Brazil as in many regions of the world.

We evaluated polymyxin B agar-based methodologies (agar screening test and agar dilution) as alternatives to BMD. The agar screening test presented an "almost perfect" *kappa* index correlation according to Landis and Koch (Landis and Koch 1977), when compared to BMD (κ = 0·86). The test presented good categorical agreement (CA) when compared to BMD (93·4%). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 86·2, 98·7, 98 and 90·7%, respectively, for the screening test. Figure 1 shows the performance of the tests, considering different parameters.

Screening test presented 0·73% (1 *K. pneumoniae*, MIC 0·5 μg ml^{-1}) of major error (ME) and 5·8% (n = 8) of very major error (VME). Of note, 7 of 8 (87·5%) isolates with results classified as VME had borderline MIC (4 μg ml^{-1}) by BMD. If the acceptable ± 1-fold dilution rule is taken into consideration, these isolates could, eventually, be classified as susceptible (MIC = 2 μg ml^{-1}). Indeed, if these challenge isolates are tacked aside, VME would be 0·73% (1 isolate with MIC = 32 μg ml^{-1} defined as susceptible by screening test) (Table 1), highlighting the central role of these isolates in the performance of the tests.

Agar dilution also presented an 'almost perfect' correlation according to Landis and Koch (Landis and Koch

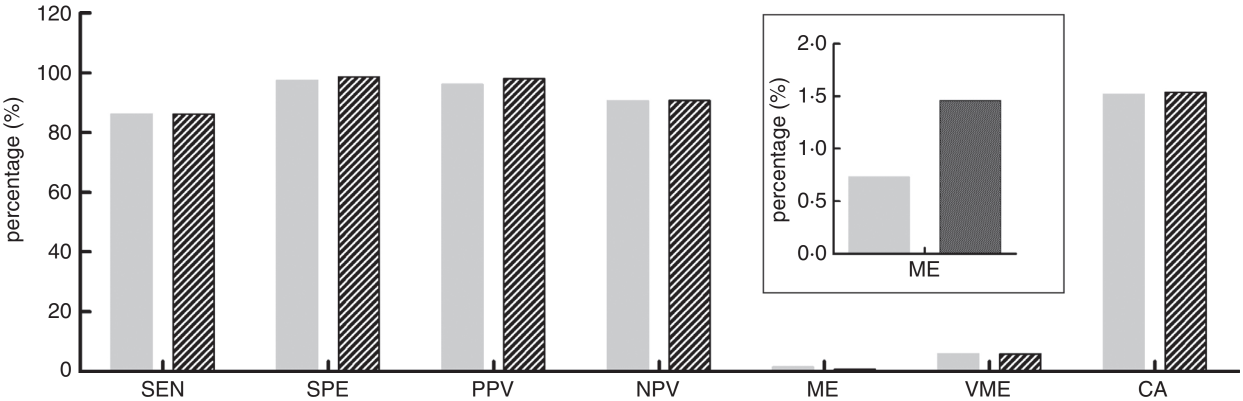

Figure 1 Specific quality values to the agar-based techniques performed when compared to the gold standard BMD. Grey bar indicates polymyxin B screening test and hatched bar indicates agar dilution. An ampliation of the VME value is given in the highlighted box. SEN, sensitivity, SPE, specificity, PPV, positive predictive value; NPV, negative predictive value; ME, major error; VME, very major error; CA, categorical agreement.

Table 1 Discriminatory major error and very major error discrepancies presented by the techniques when compared to the gold standard BMD

Method	Result	BMD (μg ml^{-1})	*N*	Error	Result	BMD (μg ml^{-1})	*N*	Error
PST	NEG	4	7	VME	POS	0·5	1	ME
		32	1	VME				
AD	<0·25	4	1	VME	4	2	1	ME
	0·5	4	3	VME				
	1	4	3	VME	32	0·5	1	ME
	1	32	1	VME				

BMD, broth microdilution; *N*, number of isolates; PST, polymyxin B screening test; AD, agar dilution; ME, major error; VME, very major error.

1977) when compared to BMD (κ = 0·85). CA was 92·7% and the essential agreement (EA) was 54% (Fig. 2). Among disagreements, 27% (17 of 63) occurred with isolates presenting borderline MICs (2 and 4 μg ml^{-1}). In another perspective, among isolates with borderline MICs (n = 23), 73·9% (n = 17) had discordant results, mainly considering those bacteria with MIC of 4 μg ml^{-1} (Fig. 2). Sensitivity, specificity, PPV and NPV were 86·2, 97·5, 96·1 and 90·6% respectively.

Agar dilution had 5·8% of VME. Interestingly, isolates misclassified as susceptible in screening test were also classified as such (false susceptible) in agar dilution. MICs of theses isolates are presented in Table 1, as well as the errors observed. Compared to the screening test, a higher proportion of ME was observed: 1·5%. It corresponded to two isolates with MICs of 2 μg ml^{-1} (borderline) and 0·5 μg ml^{-1}, which, according to agar dilution, had MICs of 4 and 32 μg ml^{-1} respectively. MIC median (interquartile interval) to BMD was of 1 (0·25–8) μg ml^{-1} and to agar dilution was of 1 (0·5–24) μg ml^{-1}. Detailed data about MICs defined by agar dilution and BMD are given in Fig. 2.

CLSI and EUCAST recommend BMD to determine polymyxins susceptibility against Enterobacterales, but this methodology is laborious, time consuming and requires a certain level of expertise in execution and interpretation (Simar *et al.* 2017; Vasoo 2017). Alternative techniques have been suggested and used by researchers, but except for the CAT and colistin broth disk elution (CBDE), which were included in the 2020 M-100 CLSI document (Clinical and Laboratory Standards Institute 2020), none was endorsed by the CLSI or EUCAST. Furthermore, among the methodologies currently recognized by CLSI, BMD is the only recommended specifically to polymyxin B, once CAT and CBDE use colistin.

Interestingly, Humphries *et al.* (2019) reinforces that both, colistin and polymyxin B, are distinct drugs and specific tests must be performed to them. That is the reason why CLSI no longer recommends colistin breakpoints as a surrogate to interpret polymyxin B susceptibility; instead, specific breakpoints were published for both drugs (Humphries *et al.* 2019; Clinical and Laboratory Standards Institute 2020).

Among alternative methodologies, the universal Super-Polymyxin® medium (SPM) developed by Nordmann *et al.* (2016) has shown sensitivity and specificity of 100% when detecting colistin-resistant Enterobacterales. Further studies have also evaluated SPM accuracy and results were

		AD										
		<0,25	0,25	0,5	1	2	4	8	16	32	64	>64
BMD	<0,25	4	0	10	3	0	0	0	0	0	0	0
	0,25	5	0	16	5	1	0	0	0	0	0	0
	0,5	7	0	10	4	2	0	0	0	1	0	0
	1	1	0	3	2	0	0	0	0	0	0	0
	2	2	0	0	1	1	1	0	0	0	0	0
	4	1	0	3	3	0	1	2	2	3	2	1
	8	0	0	0	0	0	1	3	0	2	3	1
	16	0	0	0	0	0	0	3	2	2	3	1
	32	0	0	0	1	0	0	2	1	1	2	3
	64	0	0	0	0	0	0	0	0	0	0	0
	>64	0	0	0	0	0	0	0	0	0	0	9

Figure 2 Scatterplot for agar dilution minimum inhibitory concentrations compared with BMD. In a grey colour scale, EA, DA, ME and VME are presented, respectively, from weakest to strongest grey. BMD, broth microdilution; AD, agar dilution; EA, essential agreement; DA, dilution agreement; ME, major error; VME, very major error; Categorical agreement (CA) are presented in top left and bottom right quadrants.

quite distinct: one group found sensitivity and specificity of 86·8 and 97·5% respectively; while other researchers found 100 and 90·3% of sensitivity and specificity respectively (Girlich *et al.* 2018; Jayol *et al.* 2018). Indeed, SPM seems to be a useful tool to screen colistin-resistant Enterobacterales. However, cost may be an issue for some microbiology laboratories in low-income countries. Also, it does not specifically evaluate susceptibility to polymyxin B, which goes against CLSI current recommendations (Humphries *et al.* 2019; Clinical and Laboratory Standards Institute 2020).

As far as we know, there is no publication evaluating screening test with polymyxin B instead of colistin among Enterobacterales. The screening test had better values of specificity, PPV and NPV than agar dilution.

Even with a great *kappa* and categorical agreement, sensitivity was low for both agar-based methodologies and could be, at least partially, caused by the inoculum (1 µl), which will be discussed below. Noteworthy we must emphasize that both techniques presented the same VME value, which is high considering the recommendations of the Food and Drug Administration. However, almost all VME observed for both agar-based methodologies were among isolates presenting borderline MICs, which would be expected, as they are a nightmare for tests accuracy. As our study had a considerable percentage of isolates with borderline MICs, their influence in the performance became highlighted.

We inoculated 1 µl using a Steers Replicator which is the easiest way to perform screening and agar dilution, especially when considering the routines of large laboratories. However, Humphries *et al.* (2019) recently evaluated colistin agar dilution inoculating 1 and 10 µl. Indeed, the authors observed that VME reduced and CA was improved when a higher volume of inoculum was applied. Further studies must be performed with polymyxin B agar-based methodologies to understand if higher inoculum would, as described for colistin, improve their performance.

Because of their cationic charges, polymyxins are attracted by the negative charges of plastic compounds, such as polystyrene and polypropylene, used in BMD microplates (Bakthavatchalam *et al.* 2018). Assuming that this adsorption would not be enough to influence MIC results, CLSI and EUCAST do recommend BMD in polystyrene plates without polysorbate-80 or other surfactants (The European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2016). However, it would be reasonable to suppose that this adsorption could affect, at least in some level, concentration of antibiotic in some wells. Thus, Enterobacterales could have underestimated MIC values by BMD in our study, which may explain the percentage of ME detected in agar dilution and agar screening test, once agar-based methodologies are not influenced at all by this physicochemical polymyxins issue.

As screening test and agar dilution presented quite similar performance, there are some other peculiarities to be assessed. Screening test is less laborious and cheaper, once only one dilution is evaluated. On the other hand, agar dilution allows to define MIC value. As therapeutic window of polymyxins is narrow, adjusting treatment based on MIC results is not as important as for other antimicrobials, such as beta-lactams (Zavascki and Nation 2017). Thus, we propose that it could be used in microbiology laboratories, instead of agar dilution.

The present study has some strengths that need to be emphasized: (i) it is original since agar screening was never evaluated using polymyxin B instead of colistin before; and (ii) a considerable proportion of isolates presenting borderline MICs was evaluated by agar-based methods.

Despite the low sensitivity, polymyxin B agar-based methods performed well considering all other parameters. Increasing inoculum volume from 1 to 10 μl may be a key point to reduce the occurrence of false-negative results and should be further evaluated. VME were almost exclusively linked to isolates with borderline MICs (4 μg ml^{-1}), which are widely recognized to impair the accuracy of methodologies.

Materials and methods

Bacterial population

We randomly included 137 non-duplicated CRE recovered from inpatients hospitalized from August/2017 to January/2019 in two hospitals from Southern Brazil, where the study was approved by both ethical committees.

Species identification was performed through MALDI-TOF MS (matrix-assisted laser desorption ionization time of flight; Bruker Daltonics, BD, Bremen, Germany) and BD Phoenix™ M50 instrument (Becton, Dickinson and Company, NJ). Isolates were considered CRE when reduced susceptibility in the disk diffusion test was detected to at least one carbapenem antibiotic (ertapenem, imipenem and meropenem) according to CLSI breakpoints (Clinical and Laboratory Standards Institute 2018). All isolates were screened and were positive to colorimetric carbapenemase phenotypic testing Carba NP (Nordmann *et al.* 2012).

Isolates from the following species were included: *K. pneumoniae* (n = 116), *Escherichia coli* (n = 7), *Enterobacter cloacae* complex (n = 3), *Klebsiella oxytoca* (n = 3), *Klebsiella ozaenae* (n = 1), *Citrobacter freundii* (n = 1), *Providencia rettgeri* (n = 2), *Serratia marcescens* (n = 2), *Klebsiella* sp. (n = 1) and *Citrobacter* sp. (n = 1).

All isolates were subjected to the reference BMD using cation-adjusted Mueller Hinton broth (Sigma-Aldrich, St. Louis, MO) with polymyxin B (polymyxin B sulphate; Sigma-Aldrich) in polystyrene 96 wells microplates (#K30-5096 U; Kasvi laboratory, Paraná, Brazil). The antibiotic was serially diluted (0·25–64 μg ml^{-1}) into microplates. Results were interpreted based on the CLSI guidelines: isolates with MIC ≥4 μg ml^{-1} were considered resistant (Humphries *et al.* 2019). MICs ranging from 2 to 4 μg ml^{-1} were considered 'borderline' according to the CLSI breakpoints (Clinical and Laboratory Standards Institute 2020).

Escherichia coli ATCC 25922 was used as negative (susceptible) control. Isolates of *Morganella morganii* and *S. marcescens* from our collection, intrinsically resistant to polymyxins, were used as positive (resistant) controls. *Escherichia coli* ATCC 25922 was tested in all microplates and MIC results ranged from 0·5 to 1 μg ml^{-1}.

Polymyxin B agar dilution

Polymyxin B agar dilution was performed using 90 mm Mueller-Hinton agar (Sigma-Aldrich) plates containing the antibiotic serially diluted (0·25–64 μg ml^{-1}). One Mueller-Hinton plate without antibiotic was used as growth control. Polymyxin B stock solution (2000 μg ml^{-1}) was prepared and maintained in glass tubes at −20°C, while work solution (200 μg ml^{-1}) was freshly prepared each testing day. McFarland bacterial suspensions of 0·5 were prepared and diluted (1 : 10) with 0·9% NaCl and 100 μl of the suspensions were deposited in each well of a Steers Replicator. It was inoculated 1 μl of the suspension at the surface of the plate to obtain an inoculum of approximately 10^4 CFU per ml. Plates were incubated in ambient air for 16–20 h at 37 ± 2°C. The MIC was defined as the lowest concentration of the antibiotic in the plate where there was no bacterial growth. Isolates were considered resistant to polymyxin B when MIC was 4 μg ml^{-1} or higher (Humphries *et al.* 2019; Clinical and Laboratory Standards Institute 2020).

Polymyxin B screening test

Polymyxin B screening test was performed using two 90-mm Mueller-Hinton agar (Sigma-Aldrich) plates per isolate, one without antibiotic (growth control) and the other with a final concentration of 3 μg ml^{-1} of polymyxin B. Polymyxin B solutions and inoculum were prepared as described for the agar dilution test. Plates were incubated in ambient air for 16–20 h at 37 ± 2°C. Isolates were considered susceptible to polymyxin B when no growth was observed in the plate with the antibiotic (MIC ≤3 μg ml^{-1}), while the growth of two or more colonies indicated that the isolate was resistant to polymyxin B (MIC >3 μg ml^{-1}). No growth at the plate without antibiotic made the read of the test unfeasible.

Statistical analyses

The quantitative results were presented through median and interquartile interval, while the qualitative ones through frequency and percentage. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), categorical agreement (CA), essential agreement (EA) and *kappa* index (κ) were performed considering 'resistant' as a 'positive' result and 'susceptible' as 'negative'. Statistical significance of 0·05 was adopted, and analyses were performed using SPSS ver. 20 (IBM, Armonk, NY).

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Conflict of interest

None to declare.

Authors' contribution

Otávio H. F. Raro, Juliana Caierão: conception and design, acquisition and analysis of data, drafting and revising the manuscript and approval of the final submitted version. Gabriela da S. COLLAR, Ravena M. C. da Silva, Priscila Vezzaro, Gabriela R. da Cunha: acquisition and analysis of data, revising the manuscript and approval of the final submitted version. Mariana P. Mott, Cezar V. W. Riche: acquisition and analysis of data, critically revising the manuscript and approval of the final submitted version. Cícero Dias: conception and design, drafting and revising the manuscript and approval of the final submitted version.

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Carbapenemase-producing Enterobacterales colonizing transplanted patients in Brazil: a prospective cohort study

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Carbapenemase-producing *Enterobacterales* colonizing transplanted patients in Brazil: a prospective cohort study

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ABSTRACT

Purpose: Carbapenemase-producing *Enterobacterales* (CPE) are prone to colonize intestinal tract, which might lead to infection, especially in organ (OTR) and hematopoietic (HSCT) stem cell transplant recipients. Our aim was to analyze the influence of CPE colonization or infection in the clinical outcome of transplanted patients (TP) and to gain insight into the microbiological features of CPE detected in these patients attended in hospitals from Brazil's South.

Methods: OTR or HSCT patients hospitalized between August/2017 and November/2018, colonized by CPE, were included in this prospective cohort study. Clinical and demographic data were collected. Polymyxin B susceptibility was accessed. Multiplex PCR for carbatpenemase genes as well as mcr was performed with further sequencing.

Results and Discussion: 211 TP were included, 74.9% of them colonized by a CPE (C-CPE) and 25.1% patients colonized that developed an infection by a CPE (CI-CPE). The all-cause mortality of C-CPE and CI-CPE patients was of 15.8% and 32.1%, respectively (p = 0,018). *Klebsiella pneumoniae* and *bla*$_{KPC-2}$ gene were the most prevalent CPE and carbapenemase gene detected in both groups, respectively. No CPE carried the mcr-1 gene. Hospitalization stay (>30 days), mechanical ventilation (≥24h), hospital patient transfers and colonization with Klebsiella pneumoniae were considered independent risk factors to develop a CPE infection. Moreover, to be infected by a CPE (CI-CPE) was considered a risk factor to death (HR 2,08; 95% CI; 1,13 – 3,86; p = 0,019).

Conclusions: We reported the population structure of OTR and HSCT patients colonized/infected by a CPE from the south of Brazil. We highlight the importance of a CPE surveillance active control to implement measures and protocols to OTR and HSCT patients, especially those with the above-mentioned risk factors, aiming to decrease the mortality caused by CPE acquisition.

Keywords: Transplanted patients; carbapenemase-producing *Enterobacterales*; colonization; infection

1. INTRODUCTION

According to the Global observatory on donation and transplantation, in 2018 140,964 organ transplants were performed, 9.6% more than in 2015; however, these values represent less than 10% of the need of the world's population [1]. In Brazil, the number of transplants increased 10.8% comparing 2015 with 2018, with a total of 8,829 transplants performed in 2018 (http://www.transplant-observatory.org/summary/ last access in 18/03/2020). The organ transplant recipients (OTR) and Hematopoietic stem cell transplanted (HSCT), both a large immunocompromised group of global population, are extremely susceptible to colonization or infection by different microorganisms, including carbapenemase-producing *Enterobacterales* (CPE) [2].

CPE are prone to colonize the intestinal tract of individuals, which leads to infection in a proportion of patients, especially OTR [3]. On the other hand, because of the lack of phagocytes to combat bacterial infections, and also, their damaged gastrointestinal mucosal, the HSCT patients are also a high risk group to develop bacteremia [4,5]. The mortality risk in these patients after CPE infection is up to ten times higher when comparing with carbapenem susceptible *Enterobacterales* [4,6]. Moreover, mortality rate can be as high as 78% in the OTR and 64.4% in HSCT population [7,8]. Thus, it represents a public health worldwide emergency, since these microorganisms have been reported in all continents and are typically resistant to carbapenems and other antimicrobial drugs (multidrug resistant; MDR) available in hospitals and health care services [9].

As CPE are generally MDR organisms, they are a threat since reduces treatment options are available, leading to therapeutic failure most often [3]. In sets where the new combinations of beta-lactams with beta-lactamase inhibitors are not widely available, polymyxins has been used as a last resort in infections due to CPE, despite their recognized toxicity [10]. Consequently, polymyxin resistance has risen in European countries, reaching rates near 20.8%, 31.7% and 43% in Greece, Spain and Italy, respectively, in the last decade [11–13]. In Brazil, polymyxin resistance rates are near 27% in CPE isolates [14]. In some regions of Brazil, prevalence of polymyxin B resistance among CPE are even higher, reaching 35.3% in South Brazil [15].

In this scenario, there is a lack of information about OTR and HSCT patients colonized and/or infected by CPE worldwide, and particularly in Brazil. Hence, the aim of this study was to analyze the influence of CPE colonization/infection in clinical outcome of transplanted patients; and to gain insight into microbiological features of CPE detected in OTR and HSCT population.

2. METHODS

2.1 Study design and patient population

Solid organ or hematopoietic stem cell transplanted inpatients hospitalized between August/2017 and November/2018, which were screened positive in an active surveillance program to detect colonization for carbapenemase-producing *Enterobacterales* (CPE), were included in this prospective cohort study. During this surveillance program, rectal swabs of all patients were collected and screened in admission and once a week for detection of CPE. At the laboratory, screening was performed using disk diffusion to detect carbapenem resistance. If reduced susceptibility was observed, the isolates were subjected to Carba NP test [16] following hospital's infection control service and microbiology laboratory recommendations. The cohort was constituted by patients admitted to a 1,000-bed tertiary hospital complex which is composed by seven different specialized hospital buildings, including a national referral transplant service. This hospital complex, located in Porto Alegre, provides services to more than 11 million people in South of Brazil [17]. Individuals were enrolled in two groups: 1) colonized patients (C-CPE), represented by those from whom a CPE was recovered exclusively in surveillance cultures, from rectal swab; and 2) colonized and infected (CI-CPE), encompassing patients who had the same CPE recovered firstly from the rectal swab, and then from clinical specimens. The study was approved by the research ethics committee from the Complex Hospital and by the Plataforma Brasil of the Ministry of Health from Brazil.

2.2 Clinical and demographic data

Clinical and demographic data were accessed in agreement with the ethical committee, the department of hospital infection control service and the medical head of the transplant service of this Complex Hospital. Thus, a list of all transplanted patients was daily generated, and as soon as a CPE was detected colonizing an OTR or HSCT, clinical and demographic data was collected. Information obtained were: age, gender, transplant type, transplant indication, hospital location (six out of seven hospitals from the complex were involved: Hospital A, HA; Hospital B, HB; Hospital C, HC; Hospital D, HD; Hospital E, HE; Hospital F, HF; Hospital units are numbered, e.g. HA, unit 1 is named HA-1), cause of hospitalization, total hospital stay, overall outcome (all-cause death), mechanical ventilation (MV), and data concerning the use of immunosuppressor . Survival curve and incidence of death curve were also evaluated. Patient movement networks among hospitals were characterized through Inkscape v0.92.4 vector graphics editor (http://www.inkscape.org/). Clinical and demographic data of transplanted patients were followed up for 12 months after the inclusion in the study.

2.3 Bacterial isolates

Only one isolate per patient was included. In the CI-CPE group, clinical specimens were used over rectal swabs specimens. Rectal swabs (surveillance cultures) were cultured using the commercial medium CHROMAGAR KPC (PlastLabor®). Among isolates recovered from clinical specimens, those presenting decreased susceptibility to any carbapenem (ertapenem, imipenem or meropenem) by disk diffusion, according to the Clinical and Laboratory Standard Institute (CLSI) guidelines were included in the study [18]. All isolates were identified by

MALDI-TOF MS (Matrix-assisted laser desorption ionization time-of-flight; Bruker Daltonics BD, Bremen, Germany) and Carba NP test [19]. The collection of isolates occurred in four periods of four months each: first: 01/08/2017-30/11/2017; second: 01/12/2017-31/03/2018; third: 01/04/2018-31/07/2018; and fourth: 01/08/2018-30/11/2018.

2.4 Antimicrobial susceptibility test to polymyxin B

As polymyxin B is one of the last choice antibiotic in cases of CPE infection, and is part of the therapeutic scheme of the hospital complex to treat these infections, susceptibility to this drug was determined by defining minimum inhibitory concentration (MIC) using broth microdilution , according to CLSI guidelines [18].

2.5 DNA extraction, PCR and Sequencing

To further evaluate CPE isolates, chromosomal DNA was extracted by thermic lysis method, and the search for genes encoding carbapenemases ($bla_{\text{OXA-48-like}}$, bla_{KPC}, bla_{VIM}, bla_{IMP} and bla_{NDM}) [20] and the plasmid-mediated polymyxin resistance gene *mcr-1* [21] were determined by multiplex PCR. The sequences of detected genes were confirmed by DNA sequencing.

2.6 Statistical analyses

Quantitative results were presented through mean and standard deviation or median and interquartile interval, while the qualitative ones through frequency and percentage. Outcome associated factors were verified by Student's t-test, Mann-Whitney test, Chi-square test, or Fisher's Exact test when appropriated, and were showed the Prevalence ratio (PR) estimation with a confidence interval of 95% (95% CI), which were obtained through Poisson's regression analyses, including robust variance estimation (RVE). Multivariate analyzes were performed for statistically significant variables observed in univariate analyses. Survival time was calculated as the period from colonization date to death date or the 12 months of follow up time. The time of observation consider to all patients started ("the time zero") in the isolation of a colonizing CPE (patient colonization), until patient die or in the following 12 months limit ("the end of the follow up"). The survival curves were generated by Kaplan-Meier curves, and Hazard ratio measures were obtained with 95% CI, through Cox's regression analyzes. Statistical significance of 0.05 was adopted. Statistical analyses were performed using the SPSS version 20 (IBM,Armonk,NY).

3. RESULTS AND DISCUSSION

3.1 Patients

A total of 211 transplanted inpatients were included in the study, 158 (74.9%) of which were colonized by a CPE (C-CPE). On the other hand, 53 (25.1%) CPE-colonized patients evolved to an infection by the CPE (CI-CPE). There was no significant difference between mean age (standard deviation) ($p = 0.196$) and gender ($p = 0.470$) between the two groups: C-CPE presented mean of 52.9 (± 14.5) years old and 67.1% of males while CI-CPE had 55,8 (± 13) years old as the mean age and 60.4% were males. As expected, kidney transplantation was the most frequent among inpatients, followed by liver and lung transplants. Table 1 presents the clinical and demographic characteristics of transplanted patients from both groups (C-CPE X CI-CPE).

Table 1 – Clinical and demographic characteristics of C-CPE and CI-CPE patients after 1 year-follow up of organ or hematopoietic stem cell transplantation

Variable	C-CPE 158 (74,9%)	CI-CPE 53 (25,1%)	p-value
Age*	52,9 ± 14,5	55,8 ± 13,0	0,196
Male gender	106 (67,1%)	32 (60,4%)	0,470
Transplant (Tx)			0,207
Kidney	84 (53,2%)	30 (56,6%)	
Kidney/Liver	8 (5,1%)	1 (1,9%)	
Liver	38 (24,1%)	8 (15,1%)	
Lung	27 (17,1%)	12 (22,6%)	
Hsct	1 (0,6%)	2 (3,8%)	
Reason[1]			0,487
CKD	79 (50,0%)	26 (49,1%)	
Cirrosis/HCC	17 (10,8%)	3 (5,7%)	
Cirrosis/nHCC	11 (7%)	4 (7,5%)	
Pulm. fibrosis	5 (3,2%)	5 (9,4%)	
COPD	7 (4,4%)	2 (3,8%)	
Other	39 (24,7%)	13 (24,5%)	
Hospitalization			0,666
Hospital A	128 (81%)	45 (84,9%)	
Other	30 (19%)	8 (15,1%)	
Reason[2]			0,059
PO-kidney-Tp	34 (21,5%)	11 (20,8%)	
PO-liver-Tp	15 (9,5%)	5 (9,4%)	
PO-lung-Tp	10 (6,3%)	8 (15,1%)	
Sepsis	9 (5,7%)	4 (7,5%)	
AKF	1 (0,6%)	2 (3,8%)	
Resp. insuf.	2 (1,3%)	3 (5,7%)	
Other	87 (55,1%)	20 (37,7%)	
Total Stay[3#]	22 (16-34)	41 (24,5-63,5)	< 0,001
≤ 30 days	115 (72,8%)	20 (37,7%)	< 0,001
> 30 days	43 (27,2%)	33 (62,3%)	
Empirical[4]	50 (31,6%)	16 (30,2%)	0,979
Adjusted[5]	14 (8,9%)	3 (5,7%)	0,571
MV[#]	5 (2 – 17,25)	36 (6,5 – 53,5)	0,004
MV >24h	16 (10,1%)	13 (24,5%)	0,016
Imunossupressor ≥ 3 drugs	105 (66,5%)	41 (77,4%)	0,188
Movement[#]	1 (1 – 0)	1 (0 – 2)	< 0,001
Movement			< 0,001
0	52 (32,9%)	13 (24,5%)	
1	87 (55,1%)	17 (32,1%)	
2	15 (9,5%)	15 (28,3%)	
≥3	4 (2,5%)	8 (15,1%)	
Global outcome	25 (15,8%)	17 (32,1%)	0,018

[1] Transplant reason; [2] Hospitalization reason; [3] Total hospitalization stay; [4] Empirical therapy; [5]Adjusted therapy. C-CPE, transplanted patients colonized by a CPE; CI-CPE, transplanted patients infected or that had a clinical specimen collected after colonized by a CPE; HSCT, hematopoietic stem cell transplant; Cirrosis/HCC, cirrosis with hepatocellular carcinoma; Cirrosis/nHCC, cirrosis without hepatocellular carcinoma; Pulm. fibrosis, pulmonary fibrosis; COPD, chronic obstructive pulmonary disease; PO-kidney-Tx, post-operative after kidney transplant; PO-liver-Tx, post-operative after liver transplant; PO-lung-Tx; post-operative after lung transplant; AKF, acute kidney disease; Resp. insuf., respiratory insufficiency; MV, mechanical ventilation; * Data given in mean ± standard deviation; [#] Data given in median (interquartile interval, Q1-Q3)

According to Aguado *et al.* [22] and Gutiérrez-Gutiérrez *et al.* [23], the incidence of CPE infection ranged from 3 to 23% in organ transplant recipients (OTR). We observed a quite higher rate: 25.1%. Lung, liver, and hematopoietic stem cell (HSCT) transplanted patients were those who presented higher rates of CPE infection after colonization (CI-CPE group), being 30.8%, 26.3% and 66.7%, respectively. However, we highlighted that these results should be interpreted carefully, as there were few cases of HSCT patients in this cohort (3, 2 of which from the CI-CPE group). Total hospital stays (median, interquartile interval) were significantly longer in CI-CPE (41, 24.5 – 63.5 days) than in C-CPE patients (22, 16 - 34 days) (p < 0.001). Also, the risk of became infected by CPE was increased in 2.69x (95% CI; 1.70 - 4.27; p < 0.001) if patient stayed hospitalized for more than 30 days, which is in accordance with the longer total hospitalization stay observed among those from the CI-CPE group, as mentioned above. Results are detailed in table 2.

Table 2 – Univariate and multivariate statistical analyses of independent risk factors to develop a CPE infection.

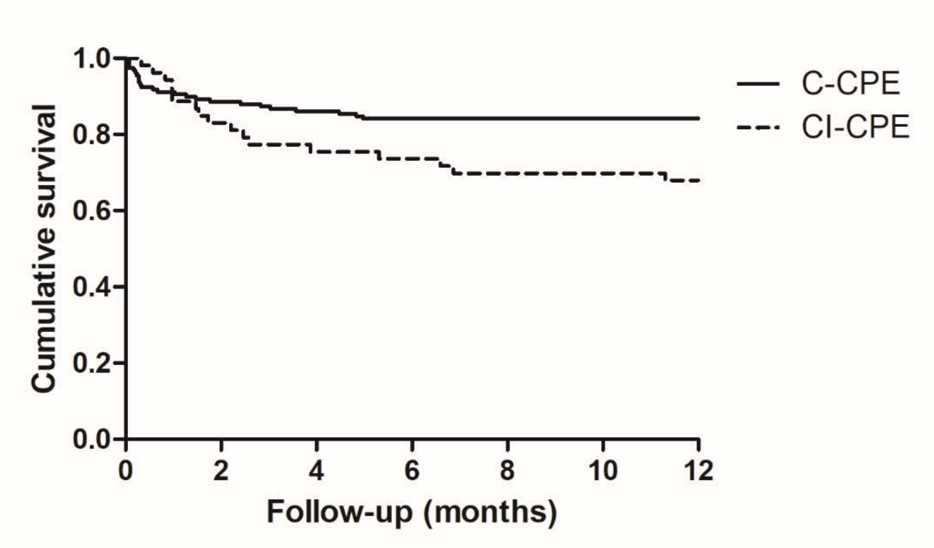
Factor	Univariate		Multivariate	
	PR (95% CI)	p-value	PR (95% CI)	p-value
Total Stay[1]				
≤ 30 days	1		1	
> 30 days	2,93 (1,81 – 4,73)	< 0,001	2,69 (1,70 - 4,27)	< 0,001
MV				
None	1		1	
Yes (> 24h)	2,04 (1,25 - 3,32)	0,004	2,68 (1,65 - 4,37)	< 0,001
Movements/transfers				
0	1		1	
1	0,82 (0,43 - 1,57)	0,544	1,09 (0,61 – 1,93)	0,769
2	2,50 (1,37 – 4,57)	0,003	3,00 (1,65 – 5,46)	< 0,001
≥ 3	3,33 (1,77 – 6,26)	< 0,001	2,95 (1,48 – 5,85)	0,002
Microorganism				
Non-Kp-CPE	1		1	
K. pneumoniae	3,09 (1,02 – 9,32)	0,045	3,09 (1,04 – 9,22)	0,043

[1] Total hospitalization stay; MV, mechanical ventilation; Non-Kp-CPE, Non-*K. pneumoniae* carbapenemase-producing *Enterobacterales*; PR (95% CI), prevalence ratio (confidence interval 95%);

Although few patients were submitted to mechanical ventilation (MV, ≥ 24h), those infected with CPE needed ventilation support more frequently than colonized patients (C-CPE 16, 10.1%; CI-CPE 13, 24.5%; p = 0.016). The risk to be infected by a CPE increased 2.68x when patient was subjected to MV (95% CI; 1.65 - 4.37; p < 0.001). The median days of MV in C-CPE was 5 days (range 2 – 17.25) and in CI-CPE was 7x higher, (36 days, range 6.5 – 53.5) (p = 0.004). These data reinforce the findings of Giannella *et al.* [24] demonstrating that prolonged MV was an independent risk factor for infection with CPE. Of note, 66.7% of the CI-CPE which needed MV are lung recipients. Among C-CPE and CI-CPE patients, 66.5 and 77.4% were treated with ≥ 3 immunosuppressive drugs, respectively, without significant difference between the groups (p = 0.188). The most frequently prescribed immunotherapy was the combination of calcineurin inhibitors, antimetabolites, and corticosteroids.

The overall mortality of the C-CPE and CI-CPE groups was 15.8% (n=25) and 32.1% (n=17), respectively (p = 0.018). Among all patients, the mean estimation time of life in the first 12 months after colonization was of 10.02 months (95% CI; 9.46 - 10.52), being 10.32 months (95% CI; 9.71 – 10.93) in C-CPE and 9.10 months (95% CI; 7.89 – 10.32) in CI-CPE patients. CI-CPE patients presented 2x higher risk of death during the 12 months of follow up when compared to C-CPE patients (HR 2.08; 95% CI; 1.13 – 3.86; p = 0.019) (Figure 1).

Figure 1 – Survival curve between both groups of transplanted inpatients.

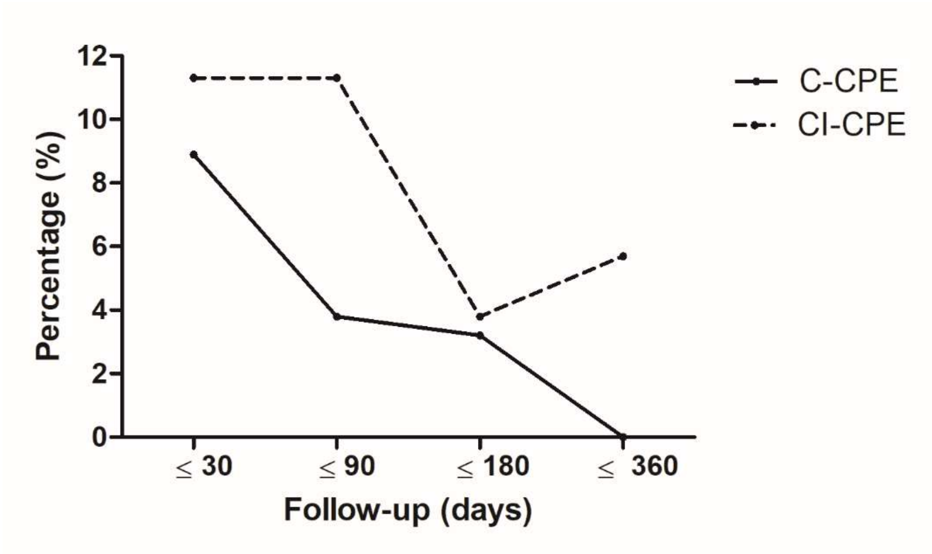


C-CPE, only colonized by carbapenemase-producing *Enterobacterales*; CI-CPE, infected by carbapenemase-producing *Enterobacterales* after colonization; follow up time = 12 months.

In the CI-CPE group, we observed high rates of all-cause mortality among liver, lung and HSCT transplant recipients. Among the liver transplanted CI-CPE patients, the mortality rate was 75%, six out of eight patients who were infected by a KPC-2-producing *K. pneumoniae* had died. Lubbert *et al*. reported similar data: 78% of mortality in liver transplant recipients that were infected by KPC-2-producing *K. pneumoniae* in Germany [7]. Giannella *et al*. [24] and Freire *et al*. [25] reported a little lower, but still worrisome, rates of death in liver transplanted patients infected by a CPE, 42.1% and 59.1%, respectively. Six out of 12 (50%) of lung transplanted CI-CPE patients died. An Italian study evaluated a higher number (n=113) of patients submitted to lung transplantation and found a lower all-cause death rate of 10.6% (12/113) [26]. As reported elsewhere [27], we also observed a mortality rate of 50% (1 out of 2) in the hematopoietic stem cell transplanted CI-CPE. Kidney transplanted patients could present as high as 42% of death rate according to the literature [28–30], but here we detected an overall mortality of 13.3% among the CI-CPE patients. Only one CI-CPE patient had undergone both kidney and liver transplantation and was included among the survivors after one year of follow-up.

The incidence of death was higher in the first 30 days after colonization in the C-CPE group (≤ 30, 8.9%; ≤ 90, 3.8%; ≤ 180 3.1%; and ≤ 360 days, 0%) and in the first 30 and 90 days after the infection in the CI-CPE group (≤ 30, 11.3%; ≤ 90, 11.3%; ≤ 180 3.8%; and ≤ 360 days, 5.7%) (Figure 2), demonstrating the importance of an effective surveillance program to detect CPE colonization and early diagnosis of these organisms in transplanted patients in order to prevent future deaths. We must consider that the high rate of deaths in the first 30 days might be explained not only by the presence of a CPE, but also by many complications that a recently transplanted patient could normally present (graft rejection, infection by other organisms, recurrence of underlying disease, and others).

Figure 2 – Incidence of death among transplanted inpatients included in the study.

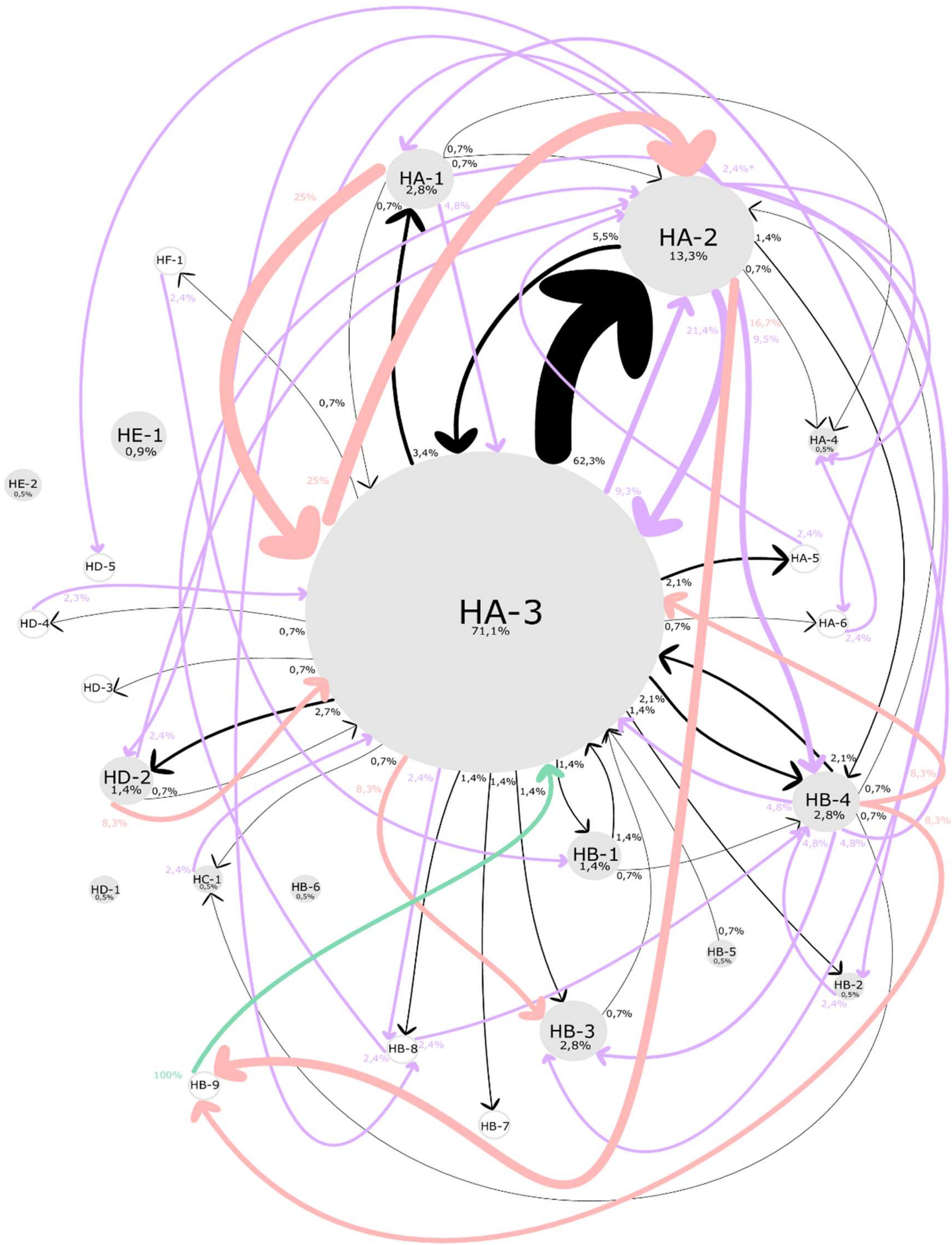


C-CPE, only colonized by carbapenemase-producing *Enterobacterales*; CI-CPE, infected by carbapenemase-producing *Enterobacterales* after colonization; follow up time = 12 months.

3.2 Patient network transfers between hospital units

Movements or transfers of patients among hospitals proved to be important to track dissemination and development of infection by CPE in transplanted inpatients admitted to this hospital complex. This observation was also made in a larger nationwide study performed in England [31]. The median number of patient transfers in our study were 1 (1 – 0) and 1 (0 – 2), respectively, in C-CPE and CI-CPE ($p < 0,001$). Our data showed that the risk to develop a CPE infection is increased in 3x (95% CI; 1.65 – 5.46; $p < 0.001$) and 2.95x (95% CI; 1.48 - 5.85; $p = 0.002$), when patient is transferred 2 or ≥ 3 times, respectively, compared to those that were not transferred at all (Table 2). Overall, transplanted patients were hospitalized mainly in the HA-3 (71.1%), and first transference occurred most frequently from HA-3 to HA-2 (62.3%). Other transfers were the return from HA-2 to HA-3 (21.4%), followed by a second return movement from HA-3 to HA-2 (25%), the movement from HA-1 to HA-3 (25%), and the transfer from HB-9 to HA-3 (only one patient). All transfers can be visualized on Figure 3. We highlight here that there are few studies that address the issue of patient movement during hospitalization and transmission of CPE, especially among transplant recipients.

Figure 3 – Patient movement networks among hospital units.



Gray circles represent hospital units and its sizes indicate patient's hospitalization percentage. Black arrows indicate 1° patient's transfer; violet arrows indicate 2° patient's transfer; red arrows indicate 3° patient's transfer; and green arrows indicates 4° patient's transfer. Arrow's sizes represent patient's movement percentages. Hospital A, HA; Hospital B, HB; Hospital C, HC; Hospital D, HD; Hospital E, HE; and Hospital F, HF; Hospital units are numbered, e.g. HA, unit 1 is named HA-1; *(purple) indicates group of lines that comes from the same point and represents 2.4% of patients each.

3.3 Bacterial Isolates

The most frequent CPE species isolated from both groups was *Klebsiella pneumoniae*, representing 81% (n=128) and 94.3% (n=50) in C-CPE and CI-CPE groups, respectively. The risk to become infected was 3x higher when a *K. pneumoniae* was colonizing when compared with patients colonized with a non-*Klebsiella pneumoniae* carbapenemase-producing *Enterobacterales* (non-Kp-CPE) (95% CI; 1.04 - 9.22; p = 0.043) (Table 2). Non-Kp-CPE, such as *Serratia marcescens*, *Enterobacter cloacae* complex, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella oxytoca*, and *Providencia rettgeri*, were detected less frequently, as showed in the table 3. The high rate of *K. pneumoniae* detected in this study might be explained by its expressive prevalence, being the main CPE organism found not only in transplanted, but also in other patients with opportunistic nosocomial infections, and in hospital outbreaks [32]. In the CI-CPE group, bacteria were recovered from urine (20, 37.7%), blood (17, 32.1%), respiratory tract (12, 22.6%), abdominal liquid (2, 3.8%), pleural liquid (1, 1.9%) and catheter tip (1, 1.9%).

Table 3 – Microbial characteristics of CPE detected in C-CPE and CI-CPE patients

Variable	C-CPE	CI-CPE	p-value
Microrganism[1]			0,321
K. pneumoniae	128 (81%)	50 (94,3%)	
*K. oxytoca**	3 (1,9%)	0 (0%)	
*E. coli**	3 (1,9%)	0 (0%)	
*E. cloacae cpx**	6 (3,8%)	0 (0%)	
*S. marcescens**	11 (7%)	1 (1,9%)	
*P. rettgeri**	2 (1,3%)	1 (1,9%)	
*C. freundii**	5 (3,2%)	1 (1,9%)	
Microrganism[2]			0,036
Non-Kp-CPE	30 (19%)	3 (5,7%)	
K. pneumoniae	128 (81%)	50 (94,3%)	
Origin/source			NA
Rectal swab	158 (100%)	NA	
Urine	NA	20 (37,7%)	
Blood	NA	17 (32,1%)	
Respiratory tract	NA	12 (22,6%)	
Pleural liquid	NA	1 (1,9%)	
Abdominal liquid	NA	2 (3,8%)	
Catheter tip	NA	1 (1,9%)	
Collection period			0,059
First	51 (32,3%)	26 (49,1%)	
Second	40 (25,3%)	13 (24,5%)	
Third	33 (20,9%)	10 (18,9%)	
Fourth	34 (21,5%)	4 (7,5%)	
Polymyxin B			0,403
≥4µg/mL	65 (44,8%)[RS]	27 (52,9%)[RS]	
Multiplex PCR			1,000
KPC-2	145 (91,8%)	49 (92,5%)	
NDM-1	13 (8,2%)	4 (7,5%)	
Others	0 (0%)	0 (0%)	

[1] All CPE detected in this study; [2] *K. pneumoniae* versus non-Kp-CPE detected in this study; * non-Kp-CPE detected in this study; [RS], reduced susceptibility; Periods: first: 01/08/2017-30/11/2017; second: 01/12/2017-31/03/2018; third: 01/04/2018-31/07/2018; and fourth: 01/08/2018-30/11/2018.

Overall, an important percentage of CPE were resistant to polymyxin B in both groups. Indeed, 44.8% and 52.9% of the isolates from C-CPE and CI-CPE had a MIC of ≥ 4µg/mL. *S. marcescens* and *P. rettgeri* species were excluded from the tests because of intrinsic resistance to this antimicrobial [33]. No statistical difference was observed between groups (p = 0.403) (Table 3). Comparing our data with other Brazilian studies, we observed a rise on polymyxin B resistance rates among CPE from 27% in 2016 [14], to 43.6% in 2019 (our overall rate of polymyxin B resistance), which is quite similar to the 43% of polymyxin resistance rate in CPE reported in Italy [11].

All isolates from our study did not carried the *mcr-1* gene (Table 3). Therefore, chromosomal alterations leading to Lipid A changes may be the mechanisms causing resistance to polymyxin B among CPE evaluated, which, indeed, are the most common mechanisms among polymyxins resistant bacteria [34].

As expected, bla_{KPC-2} was the most prevalent gene among CPE (Table 3). It was detected in 91.8% of the C-CPE and 92.5% of the CI-CPE isolates. The KPC-2 is the most prevalent carbapenemase variant detected in the country [14]. The remaining 8.2% and 7.5% isolates in C-CPE and CI-CPE groups, respectively, presented the bla_{NDM-1} gene. We did not perform a phylogeny molecular test on our CPE isolates, but even so, we can suggest the presence of an intra- and inter-hospital spread of *Klebsiella pneumoniae* clones in the hospital complex studied, once we detected 31.2% and 47.2% of the C-CPE and CI-CPE isolates respectively, presenting the same crucial characteristics: 1) identified as *K. pneumoniae* species; 2) polymyxin B non-susceptible (MIC $\geq$ 4µg/mL); 3) KPC-2 producers; 4) collected from patients hospitalized in Hospital A (HA); 5) 78.7% were isolated from patients subjected to at least one unit or hospital transfer. The combination of these characteristics reinforces the hypothesis suggested above.

We recognize that our study has limitations: 1) it does not include in the cohort OTR or HSCT who were previously colonized by a CPE before the transplant surgery; and 2) it was impossible to perform a phylogeny molecular test to all the CPE isolates.

4. CONCLUSIONS

Here we presented the population structure of organ transplant (OTR) and hematopoietic stem cell transplanted (HSCT) recipients colonized and infected by CPE in a complex hospital from the South of Brazil. We also demonstrated the polymyxin B susceptibility profile and genetic features on carbapenem resistance of CPE colonizing or infecting isolates from OTR and HSCT. We suggest intra and inter-hospital dissemination of KPC-2-producing *Klebsiella pneumoniae* clones.

We reinforce that successive transfers of transplanted patients to several hospital units is an important health concern on disseminating and promoting hospital outbreaks by MDR organisms, as CPE. Moreover, we support national and continental studies to screen “high risk” regions or hospitals, helping to prevent and control the spread of these microorganisms.

Univariate and multivariate statistical analyses showed that factors such as > 30 days hospitalization stay, use of mechanical ventilation (≥ 24h), to be continuously transferred to different hospital units, and to be colonized by *Klebsiella pneumoniae* are risk factors for CPE colonized transplanted patients to develop a CPE infection. Also, we demonstrated that, as expected, to be infected by a CPE is a risk factor to death.

We highlight the importance of CPE surveillance active control to implement measures and protocols to OTR and HSCT patients, especially those with the above-mentioned risk factors, aiming to decrease the mortality caused by CPE acquisition.

5. DECLARATIONS

5.1 Funding

No funding was received.

5.2 Conflicts of interest

None to declare.

5.3. Ethics approval

This study has the approval of the research ethics committee from the complex Hospital and by the Plataforma Brasil of the Ministry of Health from Brazil. Number of registration and approval: 2.974.453; CAAE (Ethical appreciation presentation certificate): 67009717.7.0000.5335.

5.4 Consent to participate

Not applicable.

5.5 Consent for publication

Not applicable.

5.6 Availability of data and material

None to declare.

5.7 Code availability

Not applicable.

5.8 Authors' contributions

OR contributed to the conception, design, and implementation of the study, to the acquisition of laboratory and clinical data, to the analysis of the results, to the drafting the article and to the approval of the final version of the manuscript.

RM contributed to the acquisition of laboratory data, to the analysis of the results, and to the review and approval of the final version of the manuscript.

EF contributed to the design of the study, to the acquisition of clinical data, to the analysis of the results, and to the review and approval of the final version of the manuscript.

JC contributed to the analysis of the results, and to review and approval of the final version of the manuscript.

CB contributed to the analysis of the statistical results, and to review and approval of the final version of the manuscript.

TS contributed to the design of the study, to the analysis of the results and to the review and approval of the final version of the manuscript.

JO, MPV and CD contributed to the conception, design, and implementation of the study, to the analysis of the results, to drafting the article and to the approval of the final version of the manuscript.

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5.3. Artigos originais publicados como co-autor

5.3.1. Artigo original III

Título:

Polymyxin B broth disk elution: a feasible and accurate methodology to determine polymyxin B susceptibility in Enterobacterales

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Antimicrobial Susceptibility Studies

Polymyxin B broth disk elution: a feasible and accurate methodology to determine polymyxin B susceptibility in *Enterobacterales*

Naiany C. Cielo [a], Tanise Belmonte [a], Otávio H.F. Raro [b], Ravena M.C. da Silva [b], Priscila L. Wink [c,d], Afonso L. Barth [a,c,d], Gabriela R da Cunha [e], Mariana P Mott [b,e], Cezar V.W. Riche [b,e], Cícero Dias [b], Juliana Caierão [a,*]

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ABSTRACT

Determination of polymyxins susceptibility by clinical laboratories is a nightmare, mainly because of physicochemical properties of the drug. Elution tests have already been proposed for colistin, but not for polymyxin B. We aimed to evaluate accuracy of Polymyxin B broth disk elution (PBDE) to determine the susceptibility to this drug. We evaluated 196 *Enterobacterales* (45.9% polymyxin B-resistant). PBDE was done in 15-mL cation-adjusted Mueller-Hinton broth where one polymyxin B disk (300 U) was eluted (2 μg/mL). BMD was performed as reference method. Categorical Agreement (CA), Major Error (ME) and Very Major Error (VME) were 99.5%, 0% and 1.11% (one false-negative *K. pneumoniae* MIC 4 μg/mL), respectively. As some institutions preferably use polymyxin B over colistin and in some countries colistin are not commercially available, to specifically evaluate polymyxin B is important. PBDE proved to be a cheap and easy to perform methodology to evaluate susceptibility to polymyxin B among *Enterobacterales*.

1. Introduction

In the last few years, carbapenem resistance among gram-negative bacilli increased in prevalence, causing infections difficult to treat (Codjoe and Donkor, 2018; Sabino et al., 2019). In this context, there has been a renaissance in the use of both polymyxin B and colistin (polymyxin E) over the last decade (Vasoo, 2017), years after they were abandoned as systemic antimicrobial agents mainly because of their high nephrotoxicity (Koch-Weser et al., 1970; Falagas and Kasiakou, 2005; Zavascki & Natlon, 2017; Lenhard et al., 2019).

Although the pharmacokinetics/pharmacodynamics efficacy of polymyxins has been consistently questioned (Tsuji et al., 2019), they remain widely used as "last-line" drugs to treat infections caused by carbapenem-resistant gram-negative bacteria. Reasons that support polymyxins use include (i) the lack of active alternative antimicrobials in cases of metallo-beta-lactamase producers, and (ii) the costs and availability of newer drugs, such as ceftazidime-avibactam (Humphries et al., 2019).

The physicochemical properties of polymyxins turn the detection of susceptibility to these antibiotics a challenging task for clinical microbiology laboratories. Techniques based on disk-diffusion are neither recommended by Clinical and Laboratory Standards Institute (CLSI,2019) nor by the European Committee on Antimicrobial Susceptibility Testing (EUCAST,2016), and gradient-based methods or automated systems are not sufficiently accurate to evaluate susceptibility to polymyxins (Lo-Ten-Foe et al., 2007; Tan and Ng, 2007; Poirel et al., 2017; Kulengowski et al., 2019). Both CLSI and EUCAST recommend broth microdilution (BMD) as reference method. However, it is laborious and not routinely performed in many clinical microbiology laboratories (Vasoo, 2017). Therefore, it is necessary to develop and evaluate a practical and reliable method to determine polymyxins susceptibility (Simner et al., 2019).

In this scenario, Colistin Broth Disk Elution Test (CBDE) has been recently proposed as an easier methodology to be used in the routine of clinical microbiology laboratories. The principle of the test is the elution of the antibiotic, at room temperature, from a commercial disk in Mueller-Hinton broth, where bacteria will be suspended. Colistin resistant isolates will be able to grow in this solution after 16 to 20 hours of incubation at 35 °C (Simner et al., 2019).

Recently, Humphries and co-workers (2019) published results of a multicenter study, which made the CLSI antimicrobial susceptibility

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Table 1
Minimal inhibitory concentration distribution among *Enterobacterales* evaluated in this study.

Isolates	Total	MIC (μg/mL)										
		≤ 0,125	0.25	0.5	1	2	4	8	16	32	64	>64
K. pneumoniae	173	10	23	23	16	15	18*	10	18	27	9	4
K. oxytoca	6	3	1		1	1						
E. coli	5	1	2	2								
E. cloacae complex	5	1	2	1	1							
S. marcescens	2											2
P. rettgeri	2											2
C. freundii	2		1		1							
K. ozanea	1	1										

Columns highlighted in blue represent borderline MICs, according to CLSI.
* Includes one *K. pneumoniae* presenting false negative result (ME).

testing (AST) subcommittee to endorse the CBDE for colistin testing of *Enterobacterales* and *Pseudomonas aeruginosa*.

To the best of our knowledge, there are no published studies evaluating polymyxin B, instead of colistin, in elution tests. Some institutions preferentially use polymyxin B; and several countries only have access to polymyxin B. That is the reason why we aimed to propose the Polymyxin B broth disk elution (PBDE) in a single antibiotic concentration, as a screening test to identify polymyxin B resistant *Enterobacterales*, searching for a more practical method with results as reliable as BMD.

2. Material and methods

2.1. Bacterial isolates and susceptibility profile

We evaluated 196 non-duplicated *Enterobacterales* resistant to carbapenems recovered from inpatients of three hospitals in Porto Alegre city, South Brazil. Isolates included mainly *Klebsiella pneumoniae* (n = 173), but also *Klebsiella oxytoca* (n = 6), *Escherichia coli* (n = 5), *Enterobacter cloacae* complex (n = 5), *Serratia marcescens* (n = 2), *Providencia rettgeri* (n = 2), *Citrobacter freundii* (n = 2) and *Klebsiella ozanea* (n = 1). *E. coli* ATCC 25922 and a clinical isolate of *Morganella morganii* from our personal collection, intrinsically resistant to polymyxins (polymyxin MIC >64 μg/mL), were used as susceptible and resistant controls, respectively.

Polymyxin B BMD was performed for all isolates as reference method in 96-well polystyrene plates, following ISO 20776-1:2006 procedures, using cation-adjusted Mueller-Hinton broth (Sigma-Aldrich) and polymyxin B sulfate salt (Sigma-Aldrich). No lyophilized powder was used and plates were prepared right before experiments, with no frozen storage.

Results obtained were interpreted based on CLSI guidelines: bacteria presenting MIC ≥4 μg/mL were considered resistant (Humphries et al., 2019). The 29th Edition of M100 CLSI document does not present polymyxin B breakpoints for *Enterobacterales*. Indeed, it defines colistin breakpoints should be used as a surrogate to interpret polymyxin B MICs. However, the CLSI Subcommittee for Antimicrobial Susceptibility Testing approved specific polymyxin B breakpoints, which is part of the 30th edition of M100 document, in 2020 (Humphries et al., 2019).

2.2. Polymyxin B broth disk elution (PBDE)

PBDE was performed according to Simner et al. (2019), with modifications. Briefly, for each isolate, two glass tubes containing 15 mL of cation-adjusted Mueller-Hinton broth (Sigma-Aldrich) were used. One tube was used as growth control and the other was used to add one 300 U-polymyxin B disk (Oxoid), in order to obtain a concentration of 2 μg/mL of polymyxin B in the 15 mL-solution. This tube was maintained at room temperature for 30 minutes to allow polymyxin B to elute from the disk to the broth, before inoculation. Bacterial suspensions were prepared by adding colonies from an overnight growth on Tryptone Soy Agar (Oxoid) plate in sterile 0.85% saline and adjusting the turbidity to match the 0.5 McFarland standard. A 75 μL aliquot of the bacterial suspension was added to each tube, reaching a final bacterial concentration of approximately 7.5×10^5 UFC/mL.

Results were read visually after 16 to 20 hours of incubations at 35 °C. Isolates were considered resistant when presented visual turbidity (growth) in the tube with 2 μg/mL of polymyxin B. Tests were performed in duplicate and readers were blinded for BMD MIC results.

3. Results

Among the 196 *Enterobacterales* evaluated, 90 (45.9%) were resistant (including 4 intrinsically resistant isolates – 2 *S. marcescens* and 2 *P. rettgeri*), while 106 (54.1%) were susceptible to polymyxin B by BMD. Polymyxin B MICs varied from ≤0.125 to >64 μg/mL, with 34 isolates (17.3%) presenting borderline MICs (2 or 4 μg/mL), according to CLSI breakpoints (Table 1).

PBDE demonstrated an excellent performance, as Categorical Agreement (CA) with BMD was 99.5%. Indeed, 89 out of 90 isolates resistant to polymyxin B (MIC >2 μg/mL) grew in tubes containing the eluted antibiotic, resulting in 1.1% of Very Major Errors (VME). Only one *K. pneumoniae* with polymyxin B MIC of 4 μg/mL was susceptible by PBDE.

Among the 106 susceptible *Enterobacterales* evaluated, no Major Errors (ME) were observed, as all of them presented susceptible results by PBDE, including those with MIC of 2 μg/mL.

4. Discussion

Infections caused by carbapenem-resistant gram-negative bacteria are difficult to treat, raising mortality rates, hospital stay and costs (Federico and Furtado, 2018; Sabino et al., 2019). In such situation, the use of polymyxins as “last-line” therapeutic option has spread dramatically over the last years (Monaco et al., 2014; Giamarellou, 2016; ECDC, 2017; ESPAUR, 2017; Otter et al., 2017; Lenhard et al., 2019).

The method recommended by CLSI and EUCAST to evaluate susceptibility to polymyxins, the BMD, is laborious and may be difficult to be performed in routine of microbiology laboratories (Dafopoulou et al., 2015; Hindler and Humphries, 2013). Broth disk elution methodologies demand ordinary materials (antimicrobial disks and broth) and have been described for antibiotics such as carbenicillin, chloramphenicol, clindamycin, penicillin, tetracycline (Jorgensen et al., 1980) and beta-lactams (Jorgensen et al., 1986; Shungu et al., 1985) since the 1980s. Besides, it has now emerged as a practical and cheap methodology for polymyxins, costing around US$ 0.42 per test (this study).

Elution tests using colistin have been evaluated by few authors. Simner et al. (2019) firstly proposed Colistin Broth Disk Elution Test (CBDE) to evaluate 172 isolates (77.9% susceptible and 22.1% resistant), including *Enterobacterales*, *P. aeruginosa* and *Acinetobacter baumannii* in a multicenter study (three sites). We performed a similar study (n = 196), but evaluated exclusively *Enterobacterales* and included twice as many resistant isolates (54.1% susceptible and 45.9% resistant), in order to better evaluate possible VME. Number of isolates presenting borderline MICs is not clear in the study of Simner and co-workers. Those authors performed elution in concentrations of 1, 2 and 4 μg/mL of colistin and found a CA between the elution method and BMD of 98% that very similar to the results of our study (99.5%). According to their results, three *mcr-1*-producing *E. coli* presented MICs of 4 μg/mL by BMD and 2 μg/mL by CBDE, indicating 7.9% of VME overall.

Dalmolin and co-workers (2019b) also performed elution tests to evaluate colistin susceptibility for 85 isolates of *Enterobacterales* and non-fermentative gram-negative bacilli (40% susceptible and 60% resistant). Compared to Simner et al. (2019), the methodology was performed with some modifications: (i) a unique concentration of colistin (2 μg/mL) was tested; (ii) the colistin elution time at room temperature was increased from 30 to 60 minutes; and (iii) a miniaturized variation of the original tube test was proposed, being performed in polystyrene microtiter plates. For the elution in tubes they observed, overall, three false-susceptible results for isolates with MIC of 4 μg/mL (VME = 5.6%). On the other hand, seven false-resistant results (20.6% of ME) were detected: two isolates with MIC of 4 μg/mL and five isolates with MIC >4 μg/mL. However, when exclusively *Enterobacterales* (n = 68) are taken into consideration,Dalmolin et al. (2019b) found 91.2%, 4.65% and 16% of CA, ME and VME, respectively. The high rates of ME and VME observed may be partially explained by the fact that the authors evaluated a high proportion of isolates with borderline MICs (n = 15; 42.8%).

Recently, Humphries et al. (2019) described results of a multicenter evaluation of CBDE among *Enterobacterales*, *P. aeruginosa* and *A. baumannii*. The method was performed as described by Simner et al. (2019), with minor modifications, evaluating eluted-colistin at the following concentrations: 0 μg/mL, 0.4 μg/mL, 1 μg/mL, 2 μg/mL and 4 μg/mL. The authors performed 627 tests, including 348 among *Enterobacterales* and found 97.9% of CA. Besides, they observed 3.2% and 0.9% of VME and ME, respectively, mainly among *A. baumannii*. Indeed, for *Enterobacterales* CA increased (98.6%), and VME and ME reduced (2.5% and 0%, respectively), with results very similar to ours, except for VME that, in our study, was lower (1.11%). However, the study of Humphries and co-workers tested a very low number of gram-negative bacilli presenting borderline MICs (30 out of 627 tests performed; 4.8%).

Based on the results of Humphries and co-workers (2019), the CLSI antimicrobial susceptibility testing (AST) subcommittee endorsed the CBDE for colistin testing of *Enterobacterales* and *Pseudomonas aeruginosa*. Interestingly, the authors described that one of the limitations of their work is that they did not test polymyxin B. Indeed, as far as we know, our study is the first to evaluate polymyxin B in elution tests.

Although polymyxin B and colistin differ only by one amino acid in the heptapeptide ring, they are distinct drugs considering pharmacokinetics/pharmacodynamics (PK/PD). There are increasing data supporting the fact that the administration of colistin as an inactive prodrug colistimethate may lead to a higher risk of acute kidney injury if compared to polymyxin B (; Ngamprasertchai et al., 2018). This justify, at least partially, why some institutions preferentially use polymyxin B over colistin. Moreover, several countries only have commercial access to polymyxin B (Humphries et al., 2019).

MICs of polymyxin B and colistin are very similar, although they may not be identical (Landersdorfer et al., 2018). Some authors demonstrate that polymyxin B MICs usually trend within ±1 dilution with colistin (Chew et al., 2017; Sader et al., 2015). If it is true to all bacterial population (including ours) and if results of colistin susceptibility tests are extrapolated to polymyxin B in cases of borderline MICs, it may configure ME or VME and, eventually, therapeutic failure may occur. CLSI, reinforcing the need of individual MICs for each drug, no longer recommend colistin breakpoints as surrogate to interpret polymyxin B MICs. Instead, they defined specific values for polymyxin B among *Enterobacterales* (Humphries et al., 2019).

Those above-mentioned arguments reinforce the need for specific polymyxin B tests. We evaluated this antibiotic eluted in the single concentration of 2 μg/mL, which coincides with the CLSI breakpoint. As polymyxin B disks are commercially available containing 300 U of antimicrobial (30 μg), lower concentrations would demand higher volumes of cation-adjusted Mueller-Hinton broth, which we considered not consistent with routine of clinical laboratories.

Since PBDE, as proposed in this study, is a qualitative screening test, it provides “susceptible” or “resistant” results, without MIC values. Due to polymyxin B-induced nephrotoxicity, its therapeutic window is very narrow (Nation et al., 2019), making it difficult to perform significant adjusts in antibiotic dosage, according to MIC values. In this scenario, a quantitative format may not be helpful.

Moreover, this methodology may be adapted, as performed elsewhere (Dalmolin et al., 2019b; Humphries et al., 2019; Simner et al., 2019). If necessary, more than one concentration, especially higher concentrations such as 4 μg/mL (one disk eluted in 7.5 mL of broth) and 8 μg/mL (one disk eluted in 3.75 mL of broth), may be used in order to determine, at least partially, MIC values. The use of higher concentrations, however, may need to be validated in specific studies.

The PBDE demonstrated excellent accuracy, with only one *K. pneumoniae* with MIC of 4 μg/mL presenting false-negative result. Overall, isolates presenting borderline MICs are the ones that compromises sensitivity and specificity of laboratorial techniques increasing VME and ME, once ±1 dilution are universally accepted when MIC values are considered, and this variations for borderline isolates implies in categorical changes (from susceptible to resistant or vice-versa). Other authors evaluating elution techniques also observed VME and ME for those isolates (Dalmolin et al., 2019b; Humphries et al., 2019).

We mainly analyzed *K. pneumoniae* (88.3%), decreasing representativeness of *Enterobacterales* in this study. However, among this species we have a varied population considering MIC values, including 17.3% (n = 34) of isolates presenting borderline MICs.

Testing higher numbers of isolates belonging to other species would be interesting to understand if specific bacterial characteristic would compromise PBDE accuracy. For example, *Enterobacter* spp. is well recognized as a genus where skipped wells in BMD are common, possibly because of polymyxin B heteroresistance (Simar et al., 2017). This is relevant because if the skipped well coincides with the 2 μg/mL well in BMD, it may compromise accuracy of PBDE. Our study only evaluated 5 bacteria belonging to *E. cloacae* complex, with MICs of ≤0.125 (n = 1) 0.25 (n = 2), 0.5 (n = 1) and 1 (n = 1) μg/mL. No skipped wells were observed among them, probably because of population size. It would be interesting further studies with increased number of this and other species (*P. aeruginosa* and *A. baumannii*, which are also related with skipped wells) to understand its influence on PBDE accuracy.

Our study population did not encompass *mcr*-positive bacteria. Indeed, we consider it as a limitation once it is not rare isolates harboring *mcr* genes present borderline MICs (Coppi et al., 2017; Bell et al., 2019). On the other hand, as we already included a significant proportion of *Enterobacterales* with MICs of 2 and 4 μg/mL, this limitation is lessened. Although we have no reasons to believe the mechanism of resistance per se would affect the accuracy of the test, to evaluate *mcr*-positive bacteria is mandatory to confirm or refute this hypothesis.

Because BMD is time-consuming, other simplest methodologies such as Rapid Polymyxin NP test have been tested in multiple studies for determining susceptibility to polymyxins. It usually presents very good sensitivity (98 to 100%) and a little lower specificity (82–100%), which may be worrisome for some bacterial population (Dalmolin et al., 2019a; Jayol et al., 2018; Malli et al., 2018; Poirel et al., 2018; Yainoy et al., 2018).

The major advantage of Rapid Polymyxin NP test over PBDE is the reduced incubation time (up to 4 h). However, this method can only be performed for *Enterobacterales* because it detects glucose metabolism related to bacterial growth. Although our population included exclusively *Enterobacterales*, Simner et al. (2019), Humphries et al. (2019) and Dalmolin et al. (2019b) performed broth disk elution for *Acinetobacter* spp. and *Pseudomonas aeruginosa*. There are no reasons to doubt that PBDE will perform well for glucose non-fermentative bacilli, although further studies must be performed to confirm this statement.

The present study has some strengths that need to be emphasized: (i) it is original, since an elution test was never evaluated for polymyxin B before; and (ii) a high proportion of isolates presenting borderline MICs was employed.

5. Conclusion

Polymyxin B broth disk elution is a cheap and an easy-to-perform methodology to detect polymyxin B susceptibility among *Enterobacterales*. It proved to be an accurate method as reliable as BMD, with excellent CA, acceptable VME and none ME.

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Declarations of interest

None.

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5.3.2. Artigo original IV

Título:

Polymyxin NP tests (from colonies and directly from blood cultures): accurate and rapid methodologies to detect polymyxin B susceptibility among Enterobacterales

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Technical Note

Polymyxin NP tests (from colonies and directly from blood cultures): accurate and rapid methodologies to detect polymyxin B susceptibility among *Enterobacterales*

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A R T I C L E I N F O

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A B S T R A C T

Detection of polymyxins susceptibility is challenging. We aimed to evaluate Rapid Polymyxin NP from colonies (NP-colony) and directly from positive blood bottles (NP-bottle), using polymyxin B instead of colistin among *Enterobacterales*. Both had similar and acceptable accuracy. This is the first study performing NP-bottle using polymyxin B instead of colistin.

1. Introduction

Carbapenem-resistant *Enterobacterales* (CRE) are associated with serious infections and treatment is almost exclusively restricted to polymyxins-centered therapeutic schemes. Defining polymyxins susceptibility is relevant, as resistance rates are increasing (Lenhard et al., 2019; Li et al., 2019; Logan and Weinstein, 2017; Nordmann et al., 2011; Sampaio and Gales, 2016; Wright et al., 2017).

However, due to characteristic of these drugs, susceptibility tests are challenging (Vasoo, 2017). Broth microdilution (BMD) is the reference method (European Society of Clinical Microbiology and Infectious Diseases, 2016). Recently, CLSI included agar dilution and broth disk elution as accepted methodologies (Clinical and Laboratory Standards Institute, 2020). They are, however, as time-consuming as BMD (16-20 hours of incubation).

Rapid Polymyxin NP test (RP-NP) is a colorimetric test with good accuracy and results available within 4 hours of incubation (Dalmolin et al., 2019; Jayol et al., 2018; Malli et al., 2018; Nordmann et al., 2016; Poirel et al., 2018; Shoaib et al., 2020; Yainoy et al., 2018). Studies showed interesting results when using this test directly from positive blood bottles (Malli et al., 2019; Jayol et al., 2016). To determine the performance of RP-NP in our bacterial population, we performed it from colonies (NP-colony) and positive blood cultures (NP-bottle). As far as we know, this is the first evaluation of NP-bottle using polymyxin B (PB) instead of colistin.

2. Material and methods

We included 240 consecutive, nonduplicated CRE recovered from inpatients of 2 hospitals in Porto Alegre city, South Brazil: *Klebsiella pneumoniae* (n=213), *Escherichia coli* (n=7), *Enterobacter cloacae* complex (n=6), *Klebsiella oxytoca* (n=4), *Klebsiella ozaenae* (n=2), *Citrobacter freundii* (n=2), *Providencia rettgeri* (n=2), *Serratia marcescens* (n=2), *Providencia stuartii* (n=1), and *Salmonella* spp. (n=1). *E. coli* ATCC 25922 and *S. marcescens* (MIC=16 μg/mL) were included as negative (susceptible) and positive (resistant) controls, respectively. The study was approved by both ethical committees.

Isolates were subjected to BMD with polymyxin B (polymyxin B sulfate, Sigma-Aldrich, USA) (European Society of Clinical Microbiology and Infectious Diseases, 2016). Results were interpreted based on the CLSI guidelines (Clinical and Laboratory Standards Institute, 2020). NP-colony was performed as described by Nordmann, Jayol and Poirel and NP-bottle as described elsewhere (Jayol et al., 2018; Nordmann et al., 2016). When a bottle became positive, it was maintained refrigerated for further analysis. Whenever Enterobacterales were identified and carbapenem resistance was confirmed (Blue-Carba), the bottle was enrolled, except if it became positive during weekend or night shift, or if more than one microorganism was recovered. Tests were performed in duplicate. If color change (orange

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Table 1
Performance of NP-colony and NP-bottle among *Enterobacterales* when compared to broth microdilution

Parameter	NP-colony (n=240)	NP-bottle (n=33)
kappa	0.92	0.88
CA	96.2%	93.4%
Sensitivity	98.9%	94.1%
Specificity	94.4%	93.7%
PPV	92.2%	94.1%
NPV	99.3%	93.7%
ME	3.3%	3%
VME	0.42%	3%

CA, categorical agreement; PPV, positive predictive value; NPV, negative predictive value; ME, major error; VME, very major error

to yellow) was not perfectly clear, result was considered undetermined.

According to BMD, 39.6% (95/240) were resistant to PB. MICs varied from ≤0.25 to >64 μg/mL, with 13.8% (33/240) of isolates presenting borderline MICs (2 or 4 μg/mL). Fifty CRE were recovered from blood cultures. Thirty-three of these bottles (66%) were submitted to NP-bottle: 53.1% (17/33) with CRE resistant to PB. MICs for cultured bacteria from these bottles varied from 0.25 to > 64 μg/mL, with 2 (6.1%) isolates presenting borderline MICs.

NP-colony and NP-bottle performed well and similarly (Table 1). The only VME in NP-colony and NP-bottle occurred with a *K. pneumoniae*, MIC >64 μg/mL. Among false-resistant isolates in NP-colony (8/240; 3.3%), 37.5% (3.8%) had borderline MICs (2 μg/mL). The only false-resistant isolate in NP-bottle (*K. pneumoniae*, MIC=0.5 μg/mL) was correctly identified as susceptible by NP-colony (Table 2).

Two NP-colony results were undetermined because color change was unclear: *K. pneumoniae*, recovered from urine, MICs of 0.5 and 1 μg/mL. Repeating tests allowed clear read.

Median time for positive results in NP-colony was 2 hours and in NP-bottle, 3 hours. Indeed, 92.6% (87/94) and 47.1% (8/17) of true-positive results demanded 2 hours of incubation in NP-colony and NP-bottle, respectively. Four-hour incubation was exclusively needed for two isolates (MIC of 4 μg/mL). One of those was recovered from blood culture and its NP-bottle also required 4 hours of incubation, which was the unique 4-hour incubation needed in NP-bottle.

Since its first publication (Nordmann et al., 2016), RP-NP was largely evaluated among Enterobacterales with sensitivity ≥90% (Dalmolin et al., 2019; Jayol et al., 2018; Malli et al., 2018; Poirel et al., 2018; Shoaib et al., 2020; Yainoy et al., 2018), and specificity varying from 82 to 100% (Dalmolin et al., 2019; Jayol et al., 2018; Malli et al., 2018; Poirel et al., 2018; Shoaib et al., 2020; Simar et al., 2017; Yainoy et al., 2018), similar to our results.

Some bacteria seem to significantly influence the performance of RP-NP. *Enterobacter* spp. are recognized by its heteroresistance to polymyxins. Some authors demonstrated a sensitivity of RP-NP as low as 25% to this species (Simar et al., 2017). As our population included few *Enterobacter* spp., its influence in test performance was not evidenced.

(Jayol et al. 2018) found 1.9% and 5% of VME and ME, respectively, when evaluating RP-NP among 223 *Enterobacteriaceae*. Although we observed higher percentage of ME (3.3%), our VME was considerably lower (0.42%), with similar population size. (Conceição-Neto et al. 2020) recently analyzed RP-NP using 170 *K. pneumoniae* and found CA of 91.2%, 6.4% of ME and 9.7% of VME. NP-colony in our study performed better.

Isolates with borderline MICs may compromise more significantly the performance of diagnostic tests. When (Conceição-Neto et al. 2020) stratified their results by MIC values, it became evident: CA decreased significantly for isolates presenting MIC of 4 μg/mL (from 91.2% to 38.5%). We also observed the influence of these isolates: 37.5% of ME in NP-colony were among isolates with MICs of 2 μg/mL.

As far as we know, only two studies evaluated RP-NP directly from positive blood cultures, both using colistin. (Jayol et al. 2016) firstly performed this test among Enterobacterales (n=73) and found good results. The other study (Malli et al., 2019) found sensitivity (100%), specificity (100%) and NPV (96.7%) slightly higher than ours, but a PPV much lower (76.5% X 94.1%).

NP-bottle performed similar to NP-colony, with quite lower sensitivity and NPV. It had acceptable ME (3%), but high VME (3%) considering FDA values for acceptable diagnostic tests. It may be explained, at least partially, by the few bottles we tested as VME were represented by only 1 isolate. This is probably the major limitation of our study.

The major strength is its originality, since NP-bottle was never evaluated using PB instead of colistin before. Some countries do not have access to colistin and some institutions prefer PB over colistin. Although similar, they are different drugs and must be treat as such (Humphries et al., 2019). Indeed, CLSI reinforced the need of individual MICs for each drug, no longer recommending colistin breakpoints as surrogate to interpret PB MICs, increasing the importance of testing this antibiotic (Clinical and Laboratory Standards Institute, 2020).

Two NP-colony tests generated uncertain read and were considered inconclusive. It is interesting once one could expect color change issues with NP-bottle, because of natural color of the blood. No inconclusive results were observed for NP-bottle.

In conclusion, both NP-colony and NP-bottle had excellent performances and may be easily implemented in clinical microbiology laboratories. Caution may be necessary with NP-bottle, requiring studies with larger number of specimens.

Table 2
Discriminatory errors presented by NP-colony and NP-bottle when compared to the reference method.

Method	Result (n)	MIC* (n)	Error
NP-colony	NEG (1)	>64 (1)**	VME
	POS (8)	0.25 (1)	ME
		0.5 (2)	ME
		1 (2)	ME
		2 (3)	ME
NP-bottle	NEG (1)	>64 (1)**	VME
	POS (1)	0.5 (1)	ME

ME, major error; VME, very major error.
* MIC, minimal inhibitory concentration in µg/mL defined by broth microdilution.
** Same isolate with results categorized as VME by both NP-colony and NP-bottle.

Declaration of interest

All the authors declare no conflict of interest.

Acknowledgments

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6. Nota do autor – Avanços em metodologias alternativas para detecção de susceptibilidade a polimixina B em CRE

Após trabalhar extensivamente na busca e avaliação de metodologias alternativas para detecção de susceptibilidade a polimixina B em CRE, contando com a colaboração de excelência da professora Dra. Juliana Caierão tivemos a oportunidade de fornecer a comunidade médica e científica informações originais e valiosas a respeito do tema, por intermédio da publicação de três artigos científicos. Portanto, resolvemos adicionar a tese importantes conclusões obtidas em forma de "nota do autor", e que estão mencionadas nos parágrafos seguintes.

O estudo publicado em 2020 por Cielo *et al*. (1) avaliou a praticidade e acurácia do método de eluição de disco de polimixina B em caldo (PBDE) para determinar a susceptibilidade de Enterobacterales estritamente à polimixina B. Esta metodologia já foi descrita anteriormente para outros antibióticos, como carbenicilina, cloranfenicol, clindamicina, penicilina, tetraciclina (2) e beta-lactâmicos (3,4) desde os anos 1980s. A conclusão dos autores é de que PBDE é uma metodologia barata e fácil de ser realizada para a detecção da susceptibilidade a polimixina B entre isolados de Enterobacterales. Segundo os resultados encontrados, é um método acurado e tão confiável quanto o padrão ouro (BMD), com excelente concordância categórica, aceitáveis *very major error* (VME) e nenhum *major error* (ME).

O estudo pioneiro publicado por Raro *et al.*(5), avaliou as metodologias de *screening test* e diluição em agar para isolados de Enterobacterales e descreveu que apesar da baixa sensibilidade, as metodologias baseadas em agar utilizando a polimixina B tiveram uma boa performance considerando todos os outros parâmetros de qualidade (especificidade, valor preditivo positivo, valor preditivo negativo, concordância categórica, índice *kappa*). E que um aumento no volume do inóculo (1 para 10µL) pode ser a chave para reduzir a ocorrência de falsos-negativos. Por fim, concluíram que os VME foram praticamente exclusivos aos isolados com MICs (*minimum inhibitory concentration*) *borderline* (4µg/mL), estes que são reconhecidos por dificultar a acurácia deste tipo de metodologia (5).

Outro teste desenvolvido para a detecção rápida de susceptibilidade a colistina foi desenvolvido por Nordmann, Jayol e Poirel (6). Trata-se de um teste qualitativo bioquímico e colorimétrico que tem demonstrado acurácia para bactérias cultivadas, apresentando resultados em até 4 horas de incubação (7–9). Ainda nesta linha, avaliamos a acurácia do teste polimixina NP em Enterobacterales utilizando colônias e também realizado diretamente de frascos de hemoculturas (10). Este estudo utilizou polimixina B ao invés de colistina e concluiu que ambos testes a partir da colônia (NP-*colony*) e dos frascos (NP-*bottle*) tiveram performances excelentes, sugerindo que podem ser fortes candidatos para implementação em laboratórios clínicos de microbiologia, após a realização de testes e estudos adicionais (10).

De fato, ao iniciarmos essa tese identificamos a necessidade de testes alternativos tão fidedignos quanto a BMD, porém mais rápidos e práticos para a definição da resistência à polimixina B especialmente em EPC. Após investigarmos alguns destes testes alternativos, podemos concluir, devido ao seu bom desempenho observado, que eles demonstraram potencial para serem adotados na rotina de laboratórios clínicos. Trata-se de um tema amplo, e os resultados das publicações contidas nessa tese contribuem para um avanço do conhecimento na área

6.1 Referências

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7. Conclusões

Ao final desta tese de Doutorado foi possível caracterizar a estrutura populacional de pacientes transplantados colonizados e/ou infectados por Enterobacterales produtoras de carbapenemases (EPC), internados em um Complexo Hospitalar de referência no Sul do Brasil, no período de agosto de 2017 até novembro de 2018. Assim como a estrutura populacional dos isolados de EPC oriundos destes pacientes.

Nesta tese podemos detectar a presença de clones de alto risco de *Klebsiella pneumoniae* ST11/KPC-2, ST16/KPC-2 e ST15/NDM-1 que foram responsáveis pela disseminação de $bla_{\text{KPC-2}}$ e $bla_{\text{NDM-1}}$ em pacientes transplantados neste complexo hospitalar, e que possivelmente também estão abrangendo toda ou grande parte da população deste ambiente. Adicionalmente, é possível concluir que essa disseminação através de transmissão horizontal gênica plasmidial está diretamente relacionada com a massiva presença de plasmídeos da família de incompatibilidade IncN os quais foram encontrados sempre carreando o gene $bla_{\text{KPC-2}}$, e IncFIB, que por sua vez estiveram sempre carreando o gene $bla_{\text{NDM-1}}$.

Geramos importantes informações filogenéticas sobre 80 isolados de *K. pneumoniae* que se mostraram geograficamente conservados no Brasil e não relacionados de maneira clonal com outras cepas das mesmas ST *types* recuperadas em diversas regiões do mundo.

Percebemos que sucessivas transferências dos pacientes para diferentes unidades hospitalares formando uma "rede de movimentação dos pacientes", favoreceu de maneira significativa a disseminação intra e inter-hospitalar de clones epidemiológicos de alto risco de *K. pneumoniae* ST11/KPC-2, ST16/KPC-2 e ST15/NDM-1, representando uma preocupação de saúde pública. Portanto sugerimos repensar este modelo visando evitar ou diminuir a disseminação destes clones, bem como futuros surtos deste e de outros organismos MDR.

Tivemos a oportunidade de avaliar um caso clínico específico de transferência horizontal de resistência aos carbapenêmicos mediada pelo gene $bla_{\text{KPC-2}}$ o qual estava sendo carreado por um plasmídio da família IncN. Fato este que reforça o grande potencial de disseminação destes fatores de resistência aos carbapenêmicos entre indivíduos de Enterobacterales de diferentes espécies. Este plasmídeo foi transferido de uma *K. pneumoniae* para uma *Kluyvera ascorbata*, ocasionando uma co-infecção abdominal em um paciente de 43 anos de idade.

Tendo em vista as dificuldades na detecção de susceptibilidade as polimixinas em ERC, avaliamos metodologias para fins diagnósticos na tentativa de propor novas e fidedignas rotinas laboratoriais neste tema. Alavancados pela falta de estudos com testes utilizando a polimixina B ao invés de colistina, avaliamos metodologias alternativas utilizando especificamente este antimicrobiano. Avaliamos testes baseados em eluição de discos em caldo, testes baseados em agar e inclusive o teste colorimétrico polyNP. E obtivemos resultados promissores que sugerem que estes testes têm o potencial para serem futuramente utilizados em laboratórios de microbiologia clínica.

Geramos resultados relevantes, apresentando problemas de saúde pública que necessitam ser discutidos de maneira extensiva e pesquisados através de estudos adicionais para melhor entendermos a estrutura organizacional, os fatores de resistência e virulência de EPC, especialmente *K. pneumoniae*, isolados de pacientes transplantados, com o objetivo de prevenir e controlar novos surtos, uma vez que sabemos o grande impacto econômico e sanitário relacionado à estes temas. E, por fim, produzimos importantes resultados para o avanço das metodologias diagnósticas relacionadas a detecção de resistência a polimixina B em ERCs.

Como perspectivas futuras gostaríamos de desenvolver uma base de dados fidedigna para detecção de mecanismos de resistência, baseada em mutações cromossomais, à polimixina B utilizando a ferramenta *Srst2* (*Short Read Sequence Typing for Bacterial Pathogens* - https://github.com/katholt/srst2). Seguindo nesta linha, pretendemos utilizar esta e outras ferramentas como *Artemis* (https://github.com/sanger-pathogens/Artemis) para identificar os mecanismos de resistência à polimixina B para os isolados de *K. pneumoniae* que foram submetidos ao WGS. Por fim gostaríamos de estudar outros fatores envolvidos na manutenção do maquinário celular bacteriano destas cepas sequenciadas.

7. Anexos

7.1 Parecer consubstanciado da última versão da apresentação do projeto

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Influência da colonização por Enterobactérias produtoras de carbapenemases no desfecho de pacientes transplantados

Pesquisador: Cicero Armidio Gomes Dias

Área Temática:

Versão: 3

CAAE: 67009717.7.0000.5335

Instituição Proponente: ISCMPA

Patrocinador Principal: CNPQ

DADOS DO PARECER

Número do Parecer: 2.974.453

Apresentação do Projeto:

A avaliação anterior não se altera em razão da emenda.

Objetivo da Pesquisa:

Objetivo da emenda:

Solicitar a extensão do cronograma.

Avaliação dos Riscos e Benefícios:

A avaliação anterior não se altera em razão da emenda.

Comentários e Considerações sobre a Pesquisa:

A avaliação anterior não se altera em razão da emenda.

Considerações sobre os Termos de apresentação obrigatória:

Conforme justificativa apresentada a emenda trata-se de uma solicitação de extensão de cronograma.

Conclusões ou Pendências e Lista de Inadequações:

A pesquisa encontra-se de acordo com a Norma vigente Resolução 466/12 para pesquisa em seres humanos.

Considerações Finais a critério do CEP:

Após avaliação das alterações efetuadas no estudo acima descrito, o presente Comitê não encontrou óbices quanto à implementação das mesmas.

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Telefone: (51)3214-8571 **Fax:** (51)3214-8571 **E-mail:** cep@santacasa.tche.br

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1196736_E2.pdf	19/10/2018 10:42:25		Aceito
Outros	solicitacao_extensao_coleta.pdf	19/10/2018 10:39:04	Cicero Armidio Gomes Dias	Aceito
Cronograma	cronograma_adendo.pdf	19/10/2018 10:37:24	Cicero Armidio Gomes Dias	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_adendo.pdf	19/10/2018 10:37:08	Cicero Armidio Gomes Dias	Aceito
Outros	termo_compromisso_UFCSPApdf.pdf	17/05/2017 19:15:38	Otávio Hallal Ferreira Raro	Aceito
Outros	utilizacao_de_dados_de_prontuarios_e_uso_de_publicacao.pdf	06/04/2017 10:24:29	Otávio Hallal Ferreira Raro	Aceito
Outros	utilizacao_de_dados_de_material_biologico.pdf	06/04/2017 10:23:21	Otávio Hallal Ferreira Raro	Aceito
Outros	isencao_de_onus.pdf	06/04/2017 10:20:03	Otávio Hallal Ferreira Raro	Aceito
Outros	inscricao_de_projeto_de_pesquisa.pdf	06/04/2017 10:18:52	Otávio Hallal Ferreira Raro	Aceito
Outros	confidencialidade_do_sujeito_no_estudo.pdf	06/04/2017 10:16:07	Otávio Hallal Ferreira Raro	Aceito
Outros	Autorizacao_da_chefia.pdf	06/04/2017 10:09:36	Otávio Hallal Ferreira Raro	Aceito
Orçamento	orcamento.pdf	23/03/2017 11:54:23	Otávio Hallal Ferreira Raro	Aceito
Folha de Rosto	folha_de_rosto.pdf	23/03/2017 11:53:15	Otávio Hallal Ferreira Raro	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 22 de Outubro de 2018

Assinado por:
ELIZETE KEITEL
(Coordenador(a))

Endereço: R. Profª Annes Dias,295 Hosp.Dom Vicente Scherer
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7.2 Parecer consubstanciado do envio de relatório final

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Influência da colonização por Enterobactérias produtoras de carbapenemases no desfecho de pacientes transplantados

Pesquisador: Cicero Armidio Gomes Dias

Área Temática:

Versão: 3

CAAE: 67009717.7.0000.5335

Instituição Proponente: ISCMPA

Patrocinador Principal: CNPQ

DADOS DA NOTIFICAÇÃO

Tipo de Notificação: Envio de Relatório Final

Detalhe:

Justificativa: Relatório enviado conforme solicitado pelo CEP/UFCSPA.

Data do Envio: 04/12/2020

Situação da Notificação: Parecer Consubstanciado Emitido

DADOS DO PARECER

Número do Parecer: 4.480.751

Apresentação da Notificação:

Relatório final do projeto emitido em 04/12/2020.

Objetivo da Notificação:

Notificar este CEP sobre o encerramento do presente projeto na instituição.

Avaliação dos Riscos e Benefícios:

De acordo.

Comentários e Considerações sobre a Notificação:

Relatório emitido no modelo CEP, preenchido e assinado pelo pesquisador responsável.

Considerações sobre os Termos de apresentação obrigatória:

De Acordo.

Endereço: R. Profª Annes Dias,295 Hosp.Dom Vicente Scherer
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IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Continuação do Parecer: 4.480.751

Conclusões ou Pendências e Lista de Inadequações:

A pesquisa encontra-se de acordo com a Norma vigente Resolução 466/12 para pesquisa em seres humanos.

Considerações Finais a critério do CEP:

O presente Comitê não encontrou óbices no relatório final, considerando o posicionamento do Investigador Responsável, este Comitê não se opõe ao encerramento do Protocolo de Estudo.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Envio de Relatório Final	FORMULARIO_RELATORIO_FINAL.pdf	04/12/2020 11:06:57	Cicero Armidio Gomes Dias	Postado

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 22 de Dezembro de 2020

Assinado por:
Carlos Eugênio Santiago Escovar
(Coordenador(a))

Endereço: R. Profª Annes Dias,295 Hosp.Dom Vicente Scherer
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7.3. Regras para os autores das revistas (Instruction for Author)

7.3.1. Regras artigo original I

https://www.cambridge.org/core/journals/infection-control-and-hospital-epidemiology/information/instructions-contributors?

7.3.2. Regras artigo original II

https://www.frontiersin.org/about/author-guidelines

7.3.3. Regras artigo original III

https://www.elsevier.com/journals/diagnostic-microbiology-and-infectious-disease/0732-8893/guide-for-authors

7.3.4. Regras artigo original IV

https://www.elsevier.com/journals/diagnostic-microbiology-and-infectious-disease/0732-8893/guide-for-authors

7.3.5. Regras artigo original V

https://sfamjournals.onlinelibrary.wiley.com/hub/journal/1472765x/homepage/forauthors.html

7.3.6. Regras artigo original VI

https://www.elsevier.com/journals/journal-of-global-antimicrobial-resistance/2213-7165/guide-for-authors