bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Multifactorial Chromosomal Variants Regulate Polymyxin Resistance in Extensively Drug-Resistant *Klebsiella pneumoniae*

Miranda E Pitt[1], Alysha G Elliott[1], Minh Duc Cao[1], Devika Ganesamoorthy[1], Ilias Karaiskos[2], Helen Giamarellou[2], Cely S Abboud[3], Mark AT Blaskovich[1], Matthew A Cooper[1]* and Lachlan Coin[1*]

[1]Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia

[2]6th Department of Internal Medicine, Hygeia General Hospital, Athens, Greece

[3]Instituto Dante Pazzanese de Cardiologia, São Paulo, Brazil

Running Title: Polymyxin Resistance in XDR *K. pneumoniae*

*Address correspondence to authors: Matthew A Cooper (m.cooper@uq.edu.au) & Lachlan Coin (l.coin@imb.uq.edu.au)

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

SYNOPSIS

Objectives: Infections facilitated by extensively drug-resistant *Klebsiella pneumoniae* (XDR-KP) cause high mortality and are disseminating globally. Identifying the genetic basis underpinning resistance allows for rapid diagnosis and treatment.

Methods: XDR isolates sourced from Greece and Brazil, including nineteen polymyxin-resistant and five polymyxin-susceptible strains, underwent whole genome sequencing.

Results: Approximately 90% of polymyxin resistance was enabled by alterations upstream or within *mgrB*. The most common mutation identified was an insertion at nucleotide position 75 in *mgrB* via an IS*Kpn26*-like element in the ST258 lineage and IS*Kpn13* in one ST11 isolate. Three strains acquired an IS*1* element upstream of *mgrB* and another strain had an IS*Kpn25* insertion at 133 bp. Other isolates had truncations (C28STOP, Q30STOP) or a missense mutation (D31E) affecting *mgrB*. Complementation assays revealed all *mgrB* perturbations contributed to resistance. Missense mutations in *phoQ* (T281M, G385C) were also found to facilitate resistance. Several variants in *phoPQ* co-segregating with the IS*Kpn26*-like insertion were identified as partial suppressor mutations. Three ST258 samples were found to contain subpopulations with different resistance conferring mutations, including the IS*Kpn26*-like insertion colonising with a novel mutation in *pmrB* (P158R), both confirmed via complementation assays. We also characterized a new multi-drug resistant *Klebsiella quasipneumoniae* strain ST2401 which was susceptible to polymyxins.

Conclusions: These findings highlight the broad spectrum of chromosomal modifications which can facilitate and regulate resistance against polymyxins in KP.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

INTRODUCTION

Klebsiella pneumoniae (KP) strains classified as extensively drug-resistant (XDR) are rapidly emerging due to the dissemination of aminoglycoside, β-lactam, fluoroquinolone and carbapenem plasmid-encoded resistance genes. [1] Notably, carbapenem-resistant KP (KPC) have been linked to high morbidity and an overall mortality of 48% in infected patients. [2] Polymyxin B and colistin (polymyxin E) are now one of the last viable therapeutic options. [3] Unfortunately, resistance to this last line antibiotic class is an increasing global burden, with high prevalence reported in Asia (Korea [4,5], India [6,7]), Europe (Greece [8,10]), Italy [10,11]) and Latin America (Brazil [12,13]). Depending on early detection, mortality associated with polymyxin-resistant KP infections ranges from 20 to 100%. [14]

Polymyxins infiltrate Gram-negative bacteria via initial binding to the basal component of lipopolysaccharide, lipid A. This causes the displacement of Mg^{2+} and Ca^{2+}, disrupting bacterial outer membrane integrity and allowing the polymyxins to enter and act on intracellular targets. An extended exposure in KP triggers the activation of the two-component regulatory systems, PmrAB and PhoPQ. [15-17] This pathway modulates *pmrC* and the *pmrHFIJKLM* operon facilitating the addition of phosphoethanolamine (pEtN) and/ or 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A phosphate groups, impairing polymyxin binding interactions. [18-20] Disruption of *mgrB*, the negative regulator of PhoPQ, has been commonly observed in isolates of clinical origin. [8,21] This constitutive up-regulation incurs no apparent fitness cost and appears to be stable, with minimal reports of reversions. [22,23] Heteroresistant populations, where only a subset of bacteria are resistant, are prevalent in KP which complicates diagnosis. [24] This situation is further exacerbated by the recently reported plasmid gene *mcr-1*, which encodes a pEtN transferase enzyme [25] and identification of pandrug-resistant KP. [26]

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

This study aimed to investigate XDR-KP clinical isolates arising in Greece and Brazil during 2012 to 2014 to identify and validate genetic variants contributing to resistance. These alterations were further compared to prior clinical isolates to decipher if these mutations have been previously detected.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

MATERIALS AND METHODS

Bacterial isolates

KP clinical isolates were acquired from Hygeia General Hospital, Athens, Greece and Instituto Dante Pazzanese de Cardiologia, Brazil from patients in 2012 to 2014. Cultures were supplied as stabs/slants or on agar, and were subsequently cultured in Nutrient Broth. Cultures were made to 20% glycerol and stored at -80 °C. When required for assay or extraction, glycerol stocks were struck out to obtain single colonies on either Nutrient Agar or Tryptic Soy Agar with 5% defibrinated sheeps blood. Reference strains included *Escherichia coli* (ATCC 25922) and *Klebsiella spp*. (ATCC 13883, ATCC 700603, ATCC BAA-2146), which were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Antimicrobial susceptibility assays

Species identification and susceptibility profiles of clinical isolates from Greece and Brazil were evaluated in the clinic using VITEK®2 (bioMérieux) and further validated at the Institute for Molecular Bioscience (IMB) (The University of Queensland, Australia) by determination of MICs using the standard Clinical & Laboratory Standards Institute (CLSI) approved broth microdilution (BMD) methods with Mueller Hinton Broth (MHB). Resistance was determined as per The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Version 5.0, 2015) (see http://www.eucast.org). The break point for tetracycline was in concordance to CLSI guidelines. [27] Categorisation of drug resistance level was determined through guidelines previously outlined. [28]

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

115 **DNA extraction**

116 DNA was extracted from overnight cultures using the DNeasy Blood and Tissue Kit
117 (Qiagen) with the additional enzymatic lysis buffer pre-treatment as per manufacturer's
118 instructions. DNA was quantified with Qubit®3.0 (ThermoFisher Scientific).

119 **DNA library preparation and sequencing**

120 Library preparation was accomplished using the Nextera XT kit (Illumina) with 1 ng input
121 of DNA as per manufacturer's instructions. Quality of libraries were checked using a 2100
122 Bioanalyzer (Agilent Technologies). Libraries were sequenced on an Illumina MiSeq with 300 bp
123 paired end sequencing reads and >100X coverage per sample.

124 **Sequencing analysis**

125 Paired end reads were trimmed with Trimmomatic [29] and assembled using SPAdes. [30] The
126 Rapid Annotation using Subsystem Technology (RAST) was utilised to annotate assembled
127 genomes. [31] Assemblies were also uploaded to the Centre for Genomic Epidemiology (CGE) to
128 identify STs (MultiLocus Sequence Typing Server 1.8 [32]) and acquired resistance which included
129 *mcr-1* detection (ResFinder 2.1 [33]). Phylogroups of STs were established by constructing a
130 phylogenetic tree along with 117 KP STs as reported previously. [34] The 7 MLST defining loci
131 were aligned with MUSCLE [35] and used to build a tree using Neighbour-Joining implemented in
132 MEGA6. [36]

133 **Variant detection**

134 Alterations both in and flanking the genes *pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB* were
135 examined and sequence reads of all strains were aligned to the assembly of 20_GR_12, a
136 polymyxin-susceptible ST258 strain with the least number of contigs, using BWA-MEM. [37] The
137 alignment was analysed through FreeBayes [38] to identify single nucleotide and small indel

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

variation, using a diploid analysis in order to identify potential heterogeneity. Sites with more than 20% of reads mapping to the minor allele were considered potentially heterogeneous. The effects of variations were determined by snpEff. [39] The impact on protein sequence was further confirmed by the Protein Variation Effect Analyzer (PROVEAN). [40] For the analysis of large chromosome changes, the gene sequences including 300 bp flanking were extracted from the assemblies. A multiple alignment of each gene was constructed from the pair-wise alignment to the longest gene sequence.

Insertion sequence element validation

ISFinder [41] was used for the identification of IS elements. To confirm disruptive IS elements, *mgrB* was amplified with primers displayed in Table S1 via 2X Phusion HF master mix (Invitrogen) under the following cycling conditions: 98 °C 10 seconds, 50 °C 30 seconds and 72 °C 60 seconds (35X). Amplicons were examined using Sanger Sequencing, aligned through Clustal Omega [42] and a tree constructed as described above.

Complementation assays

Contribution of variants to resistance were validated through complementation assays as previously described. [43] Briefly, genes (Table S1) were amplified from a polymyxin-susceptible isolate, 20_GR_12, and cloned into the pCR-BluntII-TOPO vector via the Zero Blunt TOPO PCR cloning kit (Invitrogen). Chemically competent *E. coli* TOP10 cells were transformed and selected by the addition of 50 mg/L kanamycin in MHB agar. Isolation of plasmids were via the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) and transformed into KP strains via electroporation (25 µF, 200 Ω, 1.25 kV/cm) with a Gene Pulser (Bio-Rad Laboratories). Selection was accomplished through supplementation of ≥500 mg/L zeocin in MHB agar plates. Transformed colonies (n=≥2) were acquired and placed in MHB containing 1500 mg/L zeocin and 1 mM isopropyl *β-D*-1-

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

thiogalactopyranoside (Sigma Aldrich). If polymyxin susceptibility was not restored upon complementation, genes harbouring mutations were further amplified and introduced into 20_GR_12. To discern the impact of additional mutations in *phoPQ* and *pmrB* segregating with disrupted *mgrB*, mutant genes were introduced into a polymyxin-resistant isolate only harbouring an IS element *mgrB* disruption, 7_GR_13. Controls included transformation of WT genes into 20_GR_12, sequencing of amplicon prior to incision in vector and KP transformed strains undergoing a plasmid extraction and further PCR of the multiple cloning site. Antimicrobial testing against polymyxin B were conducted as above however, using cation-adjusted MHB.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study have been deposited under BioProject PRJNA307517.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

RESULTS

Characterisation of clinical isolates

KP isolates were all characterised in the hospital microbiology facility using VITEK®2 (Table 1). Subtle variations were detected between VITEK and broth microdilution (BMD) results (Table S2), predominantly the level of resistance towards gentamicin, tetracycline and tigecycline. A major dissimilarity was polymyxin susceptibility in 6_GR_12 (sensitive in BMD, resistant in VITEK) and resistant in 23_GR_13 (resistant in BMD, sensitive in VITEK). Polymyxin resistance was identified in 19 of the isolates. An abundance of acquired resistance genes (Table 2) were detected and this presence corresponded to the antimicrobial testing phenotype. This analysis did not identify *mcr-1* in these strains. Only 18_GR_14 and 19_GR_14 were not identified as extended-spectrum beta-lactamase producers amongst the polymyxin-resistant strains. Consequently, all polymyxin-resistant strains harboured non-susceptibility to at least one antibiotic in 14 or more of the 16 antimicrobial categories hence were defined as XDR.

Sequence type determination

Two thirds of the Greece clinical strains were found to belong to ST258 and the remaining were ST11, ST147 or ST383 (Table 1). While 5_GR_13 and 6_GR_12 were both ST383, only 5_GR_13 was resistant to polymyxin. Among the two strains from Brazil, 11_BR_13 was ST437 and 12_BR_13 was ST11. 21_GR_13 had a profile previously undefined and has been newly designated ST2401. Clustering analysis further revealed this multidrug-resistant polymyxin-susceptible strain as *Klebsiella quasipneumoniae* (KQ) (Figure S1).

MgrB disruption

In approximately 90% of strains, *mgrB* was impacted either by missense mutations, nonsense mutations or insertion sequence (IS) elements (Table 3). Both 5_GR_13 and 19_GR_14

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

harboured a truncation while an amino acid change, D31E, was apparent in 3_GR_13. IS element disruption was prevalent in 53% of strains and commonly an IS*5*-like element was integrated at nucleotide position 75 (Figure S2). Sequencing revealed this element was closely related to IS*Kpn26*, herein known as IS*Kpn26*-like, except for 12_BR_13 which matched IS*Kpn13* (Figure 1). IS*1R* was detected upstream of *mgrB* in 11_BR_13 and an IS*1R*-like (A>C, 393 bp; C>T, 396 bp) element in 16_GR_13 and 17_GR_14. Strain 15_GR_13 had a deletion of the *mgrB* locus from nucleotide position 133 onwards. The 127 bp flanking region mapped to IS*Kpn25* with the transposase in the same orientation as *mgrB*. All 3 of IS*1* element insertions, but only one of the 8 IS*Kpn26*-like element insertions had their transposases in the same orientation as *mgrB*.

Single, multiple and heterogeneous mutations

Aberrations in genes commonly identified to confer polymyxin resistance in KP include *mgrB*, *phoPQ* and *pmrAB*. Several non-synonymous mutations were identified across the isolates, however, not all were predicted to be deleterious (Table S3). ST383 contained several mutations in *pmrA* and *pmrB* although only Q30STOP in polymyxin-resistant 5_GR_13 was predicted to have an impact. Similarly, neutral changes in all four of these genes were detected in polymyxin-susceptible KQ strains ATCC 700603 and 21_GR_13. 8_GR_13 and 9_GR_12 harboured a single detrimental missense mutation in *phoQ*. Alterations in *mgrB* were accompanied by one or more missense mutations in *phoP*, *phoQ* and/ or *pmrB*. Predicted deleterious variants segregating with disrupted *mgrB* included *pmrB* (T140P, P158R), *phoP* (P74L, A95S) and *phoQ* (N253T, V446G), which were commonly in the ST258 lineage. V446G (*phoQ*) and P158R (*pmrB*) were heterogeneous in 13_GR_14 (65, 66% variant allele frequency) and 14_GR_14 (52, 57% variant allele frequency). Assembly revealed 23_GR_12 harboured an IS*Kpn26*-like disrupted *mgrB* alongside the intact version with alterations in *phoP* and *phoQ* in 57% of the sample.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Role of *mgrB* disruptions and presence of heteroresistance via complementation assays

Complementation of the WT gene elucidated the role of these mutations in resistance (Table 3). Introduction of pTOPO-*mgrB* restored susceptibility in all resistant isolates with *mgrB* coding mutations or upstream disruptions, with the exception of two strains heterogeneous for the *mgrB* disruption and a *pmrB* coding mutation (13_GR_14 and 14_GR_14). For these two strains, pTOPO-*mgrB* restored susceptibility in zero of three 13_GR_14 colonies and one of three 14_GR_14 colonies. Transformation of 1 out of 3 colonies for both 13_GR_14 and 14_GR_14 strains with pTOPO-*pmrB* restored susceptibility and *mgrB* amplification of these colonies revealed an intact *mgrB* locus (data not shown). This indicates that the mutation in *pmrB* (P158R) is conferring resistance to polymyxin and coexisting with the *mgrB* disrupted population. 23_GR_12 was also observed to have a heterogeneous *mgrB* disruption but did not carry a corresponding *pmrB* mutation however, harboured similar mutations to 2_GR_12 in *phoPQ*. Amplification of *mgrB* identified two of three 23_GR_12 transformed colonies contained the IS element disruption and were reverted to susceptible upon complementation with pTOPO-*mgrB*.

Validation of resistance conferring mutations in *phoQ*

Strains 8_GR_13 and 9_GR_12 harboured a single mutation in *phoQ* potentially conferring resistance (Table 3). When these isolates were transformed with pTOPO-*phoQ*, results remained variable where a lack of growth was present in a susceptible range (MIC: ≤2 mg/L) however, several wells containing high PMB concentrations exhibited growth. To resolve this, the mutated gene was introduced into a polymyxin-susceptible isolate, 20_GR_12, and resistance was apparent.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Identification of suppressor mutations in *phoPQ*

Several mutations co-segregating with disrupted *mgrB* were detected including *phoP* (P74L, A95S), *phoQ* (N253T, V446G) and *pmrB* (T140P). Complementation of WT genes in these isolates commonly facilitated a ≥2-fold increase in MIC with the exception of 10_GR_13, which had an additional predicted neutral mutation in *phoQ* (A225T) (Table S2). To evaluate the potential influence of these mutations on polymyxin resistance, mutated genes were placed into a strain only containing the *mgrB* IS element disruption, 7_GR_13. Complementation of the mutant *phoQ* (N253T) decreased the MIC by 2-fold (Table 3), confirming the role of this mutation as a resistance suppressor. Initially, the *phoQ* (V446G) mutation was anticipated to segregate with the *mgrB* disrupted population in 13_GR_14 and 14_GR_14 as a suppressor mutation however, when *phoQ* was amplified from a colony reverted to susceptible via pTOPO-*mgrB* complementation, the WT *phoQ* was observed (Figure S3). The *phoQ* (V446G) mutation was successfully amplified from a 14_GR_14 colony containing the *pmrB* (T158R) mutation and upon complementation in 7_GR_13, resulted in a 2-fold reduction in MIC. Although this mutation did not segregate with disrupted *mgrB*, it may act as a partial suppressor mutation when a resistance conferring mutation is present in *pmrB*. Mutations in *phoP* (P74L, A95S) reduced the MIC in 7_GR_13 by ≥8-fold which also identifies these as partial suppressor mutations. Complementation of mutant *pmrB* (T140P) into 7_GR_13 did not lead to an observable corresponding reduction in MIC however, once transformed into 20_GR_12, a 2-fold increase in MIC was apparent implying this is not a suppressor mutation.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

DISCUSSION

Polymyxin resistance in XDR-KP is of grave concern, especially the alarming increase reported in Greece and Brazil. [10, 12-14, 44] We evaluated the genetic basis of polymyxin resistance in a series of Greek and Brazilian clinical isolates from patients in 2012 to 2014 and found alterations in genes *mgrB*, *phoPQ* and *pmrAB*.

Inactivation of *mgrB* was highly prevalent in these strains with an IS*Kpn26*-like element being the predominant cause of resistance, as indicated by complementation restoring susceptibility in all isolates. Several other studies have observed an IS*5*-like element integration in the same position, including reports from Greece, Italy, France, Turkey and Colombia. [8,9,45,46] The IS*Kpn26*-like element resembled the same sequence from Greece isolates previously described. [46] We identified that this mutation still persisted in 2014, after being first detected in 2012. [9] The IS*Kpn26*-like forward insertion at nucleotide 75 in ST147, IS*Kpn13* integration at nucleotide 75 in ST11 and the potential *mgrB* disruption with IS*Kpn25* was not previously reported. We identified IS*1R* or IS*1R*-like elements positioned upstream of *mgrB* in 3 isolates (11_BR_13, 16_GR_13, 17_GR_14) which were reverted upon complementation indicating an impact on the promoter region.

Truncations identified at position 28 and 30 of *mgrB* have also been previously detected, although these were identified in differing STs indicating mutations potentially have arisen independently in Greece. [21] Complementation of these changes had not previously been conducted and with our study now providing definitive evidence that these mutations to cause resistance. This study further identified that the amino acid change D31E in 3_GR_13 is a polymyxin resistance conferring change. These findings support the notion that intact MgrB is required to confer negative feedback on PhoPQ. [8]

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

297 Single predicted detrimental mutations were observed in the *phoQ* histidine kinase region,
298 critical for phosphorylation and interaction with *phoP*, in 8_GR_13 (G385C) and 9_GR_12
299 (T281M). The G385C mutation had been detected [21] however, in a differing ST. Complementation
300 revealed an inconsistent MIC for these strains, although when a polymyxin-susceptible isolate was
301 transformed with the mutated gene, full resistance was restored. Dominance of mutated *phoQ* has
302 recently been highlighted and these results may imply the inability of pTOPO-*phoQ* to override
303 the resistance caused by these mutations. [47]

304 Several non-synonymous changes were identified to be not deleterious according to
305 PROVEAN analysis. Notably, these were abundant in KQ strains ATCC 700603 [48] and
306 21_GR_13. This was further identified in KP ST383 lineages and PROVEAN accurately detected
307 these neutral changes. However, this does not negate the possibility of previously resistance
308 conferring alterations being acquired in these loci with subsequent reversion mutations to give rise
309 to a susceptible phenotype.

310 Heterogeneity was apparent in several isolates. In near equal ratios, 13_GR_14 and
311 14_GR_14 possessed the IS*Kpn26*-like *mgrB* disruption and a new alteration conferring resistance
312 in *pmrB*, P158R as determined by complementation. 23_GR_12 consisted of approximately half
313 the reads mapping to the undisrupted genes and the other to the IS*Kpn26*-like strain with several
314 additional predicted deleterious mutations. This heterogeneity may explain the initial clinical
315 detection for this isolate to be polymyxin-susceptible.

316 Several isolates harbouring IS*Kpn26*-like element disrupted *mgrB* were accompanied by
317 mutations in *phoP*, *phoQ* and/ or *pmrB*. These changes were present in ≥98% of reads to render
318 the involvement of heterogeneity unlikely. Once complemented, an increase in resistance was
319 commonly recorded. This potentially reflects partial suppressor mutations as strains which solely

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

possessed this IS element disruption commonly exhibited a heightened MIC of ≥64 mg/L. Once these mutations were introduced into a strain only harbouring the *mgrB* disruption, a reduction in MIC was apparent especially for *phoP* and *phoQ*. The involvement of additional mutations in PhoPQ to influence the level of polymyxin resistance has yet to be reported in KP. Previous research by Miller *et al* [49] determined additional mutations in PhoPQ alters polymyxin resistance in *Pseudomonas aeruginosa*. This prior study describes *phoP* mutations with the capacity to partially or fully suppress resistance causing mutations in *phoQ*. These mutations in *phoP* were near or within the DNA binding site which differs to our results where the alterations are impacting the response regulatory region that interacts with PhoQ. Conversely, all mutations partially suppressing the MIC were identified to be targeting the HAMP and histadine kinase component of PhoQ. These were in regions similar to revertant *P. aeruginosa* strains identified by Lee and Kwan. [50] We postulate these mutations are perturbing the critical transfer of phosphoryl groups from the histadine kinase of PhoQ to PhoP and subsequent *pmrD* expression. Whether these mutations constitute to a fitness advantage due to the reduction of metabolism required for the production of LPS modifications is yet to be discerned.

Interestingly, the *pmrB* T140P had formerly been identified in an ST258 lineage but even when the resistant gene was complemented, the MIC increased by 2-fold but was not defined as clinically resistant. [21,51] When this mutation was introduced into a strain with only the disrupted *mgrB*, an elevation in MIC was witnessed, however, the original MIC for this isolate is 8-fold lower. This may suggest the presence of additional suppressor mutations in other yet to be identified loci.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Rapid and accurate detection of mutations attributed to polymyxin resistance remains a longstanding burden. Our research has contributed to the current understanding of the dissemination and evolution of this resistance in KP. This study highlights several issues of solely interrogating genomes for resistance detection including ST specific non-synonymous changes, heterogeneity and provides the first report of suppressor mutations. Through complementation assays, we have discerned the role of these modifications and have identified resistance-causing alterations for future genome-based diagnostics.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

ACKNOWLEDGEMENTS

We thank Dr Aurélie Jayol and Professor Patrice Nordmann for providing their complementation assay methodology. The authors thank Angela M. Kavanagh for technical support with susceptibility assays. We acknowledge the sequencing services provided by the Australian Genome Research Facility. We thank the team of the curators of the Institut Pasteur MLST system (Paris, France) for importing novel alleles, profiles and/or isolates at http://bigsdb.web.pasteur.fr.

Funding

LC is an ARC Future Fellow (FT110100972). MAC is an NHMRC Principal Research Fellow (APP1059354). MEP is an Australian Postgraduate Award scholar. AGE and MATB are supported in part by a Wellcome Trust Strategic Award 104797/Z/14/Z. Research was supported by NHMRC grants (APP1005350, APP1045326), NIH grant (R21AI098731/R33AI098731) and AID sequencing Grant (2013) as well as funding from the Institute for Molecular Bioscience Centre for Superbug Solutions (610246).

Transparency declarations

None to declare.

Author Contributions

AGE, LC, MAC, MATB conceived the study. MEP, AGE, MDC, DG performed the experiments and analysed the data. MEP and MDC performed the sequencing analysis. IK, HG, CSA provided the bacterial isolates, clinical information on the strains and clinical perspective in writing the paper. MEP wrote the paper with input from all authors.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

388 **REFERENCES**

389 1. Ramirez MS, Traglia GM, Lin DL *et al*. Plasmid-Mediated Antibiotic Resistance and
390 Virulence in Gram-Negatives: the *Klebsiella pneumoniae* Paradigm. *Microbiol Spectr*
391 2014; **2**: 1-15.

392 2. Patel G, Huprikar S, Factor SH *et al*. Outcomes of carbapenem-resistant *Klebsiella*
393 *pneumoniae* infection and the impact of antimicrobial and adjunctive therapies. *Infect*
394 *Control Hosp Epidemiol* 2008; **29**:1099-106.

395 3. Biswas S, Brunel JM, Dubus JC *et al*. Colistin: an update on the antibiotic of the 21st
396 century. *Expert Rev Anti Infect Ther* 2012; **10**: 917-34.

397 4. Suh JY, Son JS, Chung DR *et al*. Nonclonal emergence of colistin-resistant *Klebsiella*
398 *pneumoniae* isolates from blood samples in South Korea. *Antimicrob Agents Chemother*
399 2010; **54**: 560-62.

400 5. Kim SY, Shin J, Shin S *et al*. Characteristics of carbapenem-resistant *Enterobacteriaceae*
401 isolates from Korea. *Diagn Microbiol Infect Dis* 2013; **76**: 486-90.

402 6. Mohamudha Parveen R, Harish BN *et al*. Emerging carbapenem resistance among
403 nosocomial isolates of *Klebsiella pneumoniae* in South India. *International Journal of*
404 *Pharma and Bio Sciences* 2010; **1**: 1-11.

405 7. Goel G, Hmar L, Sarkar De M *et al*. Colistin-resistant *Klebsiella pneumoniae*: report of a
406 cluster of 24 cases from a new oncology center in eastern India. *Infect Control Hosp*
407 *Epidemiol* 2014; **35**: 1076-7.

408 8. Cannatelli A, Giani T, D'Andrea MM *et al*. MgrB inactivation is a common mechanism of
409 colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob*
410 *Agents Chemother* 2014; **58**: 5696-703.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

411 9. Mavroidi A, Katsiari M, Likousi S *et al*. Characterization of ST258 Colistin-Resistant,
412 blaKPC-Producing *Klebsiella pneumoniae* in a Greek Hospital. *Microb Drug Resist* 2016;
413 **22**: 392-8.
414 10. Giamarellou H. Epidemiology of infections caused by polymyxin-resistant pathogens. *Int*
415 *J Antimicrob Agents* 2016; **48**: 614-21.
416 11. Monaco M, Giani T, Raffone M *et al*. Colistin resistance superimposed to endemic
417 carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy,
418 November 2013 to April 2014. *Euro Surveill* 2014; **19**: pii=20939.
419 12. Pereira GH, Garcia DO, Mostardeiro M *et al*. Outbreak of carbapenem-resistant *Klebsiella*
420 *pneumoniae*: two-year epidemiologic follow-up in a tertiary hospital. *Mem Inst Oswaldo*
421 *Cruz* 2013; **108**: 113-5.
422 13. Gaspar GG, Bellissimo-Rodrigues F, Andrade LN *et al*. Induction and nosocomial
423 dissemination of carbapenem and polymyxin-resistant *Klebsiella pneumoniae*. *Rev Soc*
424 *Bras Med Trop* 2015; **48**: 483-7.
425 14. Ah YM, Kim AJ, Lee JY. Colistin resistance in *Klebsiella pneumoniae*. *Int J Antimicrob*
426 *Agents* 2014; **44**: 8-15.
427 15. Groisman EA, Kayser J, Soncini FC. Regulation of polymyxin resistance and adaptation
428 to low-Mg^{2+} environments. *J Bacteriol* 1997; **179**: 7040-5.
429 16. Velkov T, Thompson PE, Nation RL *et al*. Structure-activity relationships of polymyxin
430 antibiotics. *J Med Chem* 2010; **53**:1898-916.
431 17. Cheng HY, Chen YF, Peng HL. Molecular characterization of the PhoPQ-PmrD-PmrAB
432 mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *J*
433 *Biomed Sci* 2010; **17**: 60.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

18. Helander IM, Kato Y, Kilpelainen I *et al*. Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O3. *Eur J Biochem* 1996; **237**:272-8.

19. Velkov T, Deris ZZ, Huang JX *et al*. Surface changes and polymyxin interactions with a resistant strain of *Klebsiella pneumoniae*. *Innate Immun* 2014; **20**: 350-63.

20. Wright MS, Suzuki Y, Jones MB *et al*. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother* 2015; **59**:536-43.

21. Olaitan AO, Diene SM, Kempf M *et al*. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. *Int J Antimicrob Agents* 2014; **44**: 500-7.

22. Arena F, De Angelis LH, Cannatelli A *et al*. Colistin resistance caused by inactivation of the MgrB regulator is not associated with decreased virulence of ST258 KPC carbapenemase-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2016; **60**: 2509-12.

23. Lee JY, Choi MJ, Choi HJ *et al*. Preservation of Acquired Colistin Resistance in Gram-Negative Bacteria. *Antimicrob Agents Chemother* 2015; **60**: 609-12.

24. Meletis G, Tzampaz E, Sianou E *et al*. Colistin heteroresistance in carbapenemase-producing *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2011; **66**: 946-7.

25. Liu YY, Wang Y, Walsh TR *et al*. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016; **16**: 161-8.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

457 26. Zowawi HM, Forde BM, Alfaresi M *et al*. Stepwise evolution of pandrug-resistance in
458 *Klebsiella pneumoniae*. *Sci Rep* 2015; **5**: 15082.
459 27. Clinical and Laboratory Standards Institute. *Methods for dilution antimicrobial*
460 *susceptibility tests for bacteria that grow aerobically – Ninth Edition: Approved standard*
461 *M07-A9*. CLSI, Wayne, PA, 2012
462 28. Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-
463 resistant and pandrug-resistant bacteria: an international expert proposal for interim
464 standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268-81.
465 29. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence
466 data. *Bioinformatics* 2014; **30**: 2114-20.
467 30. Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its
468 applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455-77.
469 31. Overbeek R, Olson R, Pusch GD *et al*. The SEED and the Rapid Annotation of microbial
470 genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 2014; **42**: D206-14.
471 32. Larsen MV, Cosentino S, Rasmussen S *et al*. Multilocus sequence typing of total-genome-
472 sequenced bacteria. *J Clin Microbiol* 2012; **50**: 1355-61.
473 33. Zankari E, Hasman H, Cosentino S *et al*. Identification of acquired antimicrobial resistance
474 genes. *J Antimicrob Chemother* 2012; **67**: 2640-4.
475 34. Maatallah M, Vading M, Kabir MH *et al*. *Klebsiella variicola* is a frequent cause of
476 bloodstream infection in the stockholm area, and associated with higher mortality
477 compared to *K. pneumoniae*. *PLoS One* 2014; **9**: e113539.
478 35. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high
479 throughput. *Nucleic Acids Res* 2004; **32**: 1792-7.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

480 36. Tamura K, Stecher G, Peterson D *et al*. MEGA6: Molecular Evolutionary Genetics
481 Analysis version 6.0. *Mol Biol Evol* 2013; **30**: 2725-9.
482 37. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform.
483 *Bioinformatics* 2009; **25**: 1754-60.
484 38. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. *arXiv*
485 *preprint arXiv* 2012; 12073907.
486 39. Cingolani P, Platts A, Wang le L *et al*. A program for annotating and predicting the effects
487 of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila*
488 *melanogaster* strain w1118; iso-2; iso-3. *Fly (Austin)* 2012; **6**: 80-92.
489 40. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino
490 acid substitutions and indels. *Bioinformatics* 2015; **31**: 2745-7.
491 41. Siguier P, Perochon J, Lestrade L *et al*. ISfinder: the reference centre for bacterial insertion
492 sequences. *Nucleic Acids Res* 2006; **34**: D32-6.
493 42. Sievers F, Wilm A, Dineen D *et al*. Fast, scalable generation of high-quality protein
494 multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 2011; **7**: 539.
495 43. Jayol A, Poirel L, Brink A *et al*. Resistance to colistin associated with a single amino acid
496 change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin.
497 *Antimicrob Agents Chemother* 2014; **58**: 4762-6.
498 44. Neonakis IK, Samonis G, Messaritakis H *et al*. Resistance status and evolution trends of
499 *Klebsiella pneumoniae* isolates in a university hospital in Greece: ineffectiveness of
500 carbapenems and increasing resistance to colistin. *Chemotherapy* 2010; **56**: 448-52.
501 45. Poirel L, Jayol A, Bontron S *et al*. The *mgrB* gene as a key target for acquired resistance
502 to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2015; **70**: 75-80.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

503 46. Cannatelli A, D'Andrea MM, Giani T *et al*. *In vivo* emergence of colistin resistance in
504 *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional
505 inactivation of the PhoQ/PhoP *mgrB* regulator. *Antimicrob Agents Chemother* 2013; **57**:
506 5521-6.
507 47. Halaby T, Kucukkose E, Janssen AB *et al*. Genomic characterization of colistin
508 heteroresistance in *Klebsiella pneumoniae* during a nosocomial outbreak. *Antimicrob*
509 *Agents Chemother* 2016; **60**: 6837-43.
510 48. Elliott AG, Ganesamoorthy D, Coin L *et al*. Complete Genome Sequence of *Klebsiella*
511 *quasipneumoniae subsp. similipneumoniae* Strain ATCC 700603. *Genome Announc* 2016;
512 **4**: e00438-16.
513 49. Miller AK, Brannon MK, Stevens L *et al*. PhoQ mutations promote lipid A modification
514 and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic
515 fibrosis patients. *Antimicrob. Agents Chemother* 2011; **55**: 5761–9.
516 50. Lee JY, Ko KS. Mutations and expression of PmrAB and PhoPQ related with colistin
517 resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn. Microbiol. Infect. Dis*.
518 2014; **78**: 271–6.
519 51. Deleo FR, Chen L, Porcella SF *et al*. Molecular dissection of the evolution of carbapenem-
520 resistant multilocus sequence type 258 *Klebsiella pneumoniae*. *Proc Natl Acad Sci U S A*
521 2014; **111**: 4988-93.
522
523
524
525

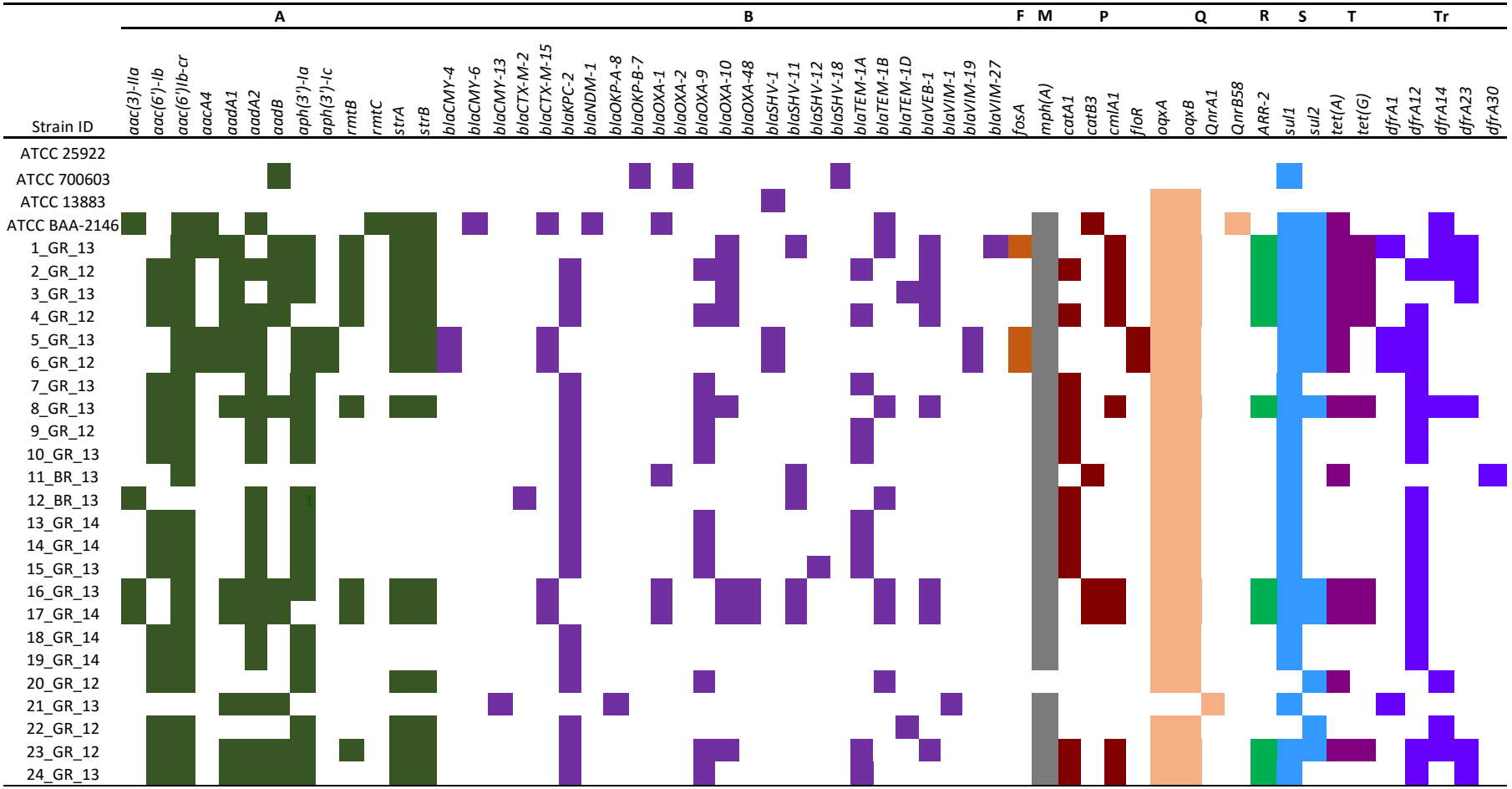
TABLE 1 Characteristics of *Klebsiella* clinical isolates in this study

Strain ID[a]	Source	MLST	Resistance Profile[b]															
			AMG	APβI	CARB	CEPH 1/2	CEPH 3/4	CEPH	QUIN	FOL	GLY	MON	PEN	PENβI	PHEN	PHOS	POL	TET
1_GR_13	Stool	147	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2_GR_12	Urine	258	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R
3_GR_13	Sputum	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4_GR_12	Blood	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
5_GR_13	Stool	383	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
6_GR_12	Stool	383	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
7_GR_13	Stool	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
8_GR_13	Stool	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
9_GR_12	Bronchial secretion	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
10_GR_13	Blood	258	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	R
11_BR_13	Urine	437	I	R	R	R	R	R	R	N	I	R	R	R	N	R	R	N
12_BR_13	Secretion	11	I	R	R	R	R	R	R	N	R	R	R	R	N	R	R	N
13_GR_14	Bronchial secretion	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I
14_GR_14	Urine	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
15_GR_13	Stool	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I
16_GR_13	Stool	11	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	R
17_GR_14	Stool	11	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	R
18_GR_14	Stool	258	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	I
19_GR_14	Stool	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
20_GR_12	Stool	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
21_GR_13	Urine	2401	R	R	R	R	R	R	I	R	S	R	R	R	S	S	S	S
22_GR_12	Sputum	258	R	R	R	R	R	R	R	R	I	R	R	R	R	I	S	I
23_GR_12	Stool	258	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R
24_GR_13	Stool	258	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

543 [a] Strain identification, numerical order catalogued at IMB_Country (GR:Greece, BR:Brazil)_last
544 two digits of isolation year.
545 [b]Multilocus sequence type as identified through MultiLocus Sequence Typing Server 1.8.
546 [c]Antibiotic resistance as determined by VITEK®2 according to EUCAST guidelines (CLSI for
547 tetracycline). Resistance called if identified for one antibiotic in class. AMG, Aminoglycosides;
548 APβI, Antipseudomonal penicillins + β-lactamase inhibitors; CARB, Carbapenems; CEPH1/2,
549 Non-extended spectrum cephalosporins (1st and 2nd generation); CEPH3/4, Extended-spectrum
550 cephalosporins (3rd and 4th generation); CEPH, Cephamycins; QUIN, Fluoroquinolones; FOL,
551 Folate pathway inhibitors; GLY, Glycylcyclines; MON, Monobactams; PEN, Penicillins; PENβI,
552 Penicillins + β-lactamase inhibitors; PHEN, Phenicols; PHOS, Phosphonic acids; POL,
553 Polymyxins; TET, Tetracyclines; R, Resistant; I, Intermediate; S, Susceptible; N, Not tested.
554
555
556
557
558
559
560
561
562
563
564
565

TABLE 2 Acquired resistance genes detected through ResFinder



A, Aminoglycoside; B, Beta-lactam; F; Fosfomycin; M, Macrolide; P, Phenicol; Q, Quinolone; R, Rifampicin; S, Sulphonamide; T, Tetracycline; Tr, Trimethoprim. Shading indicates detection of gene (≥98% homology, ≥60% sequence length).

TABLE 3 Complementation of WT genes from polymyxin-susceptible isolate 20_GR_12 into resistant strains and mutated genes identified in resistant isolates also transformed into *mgrB* disrupted polymyxin-resistant strain 7_GR_13

Strain ID	MLST	Genotype[a]	Polymyxin B MIC (mg/L)[b]											
			Initial	*mgrB*	*phoP*			*phoQ*					*pmrB*	
				WT	WT	P74L	A95S	WT	N253T	T281M	G385C	V446G	WT	T140P
1_GR_13	147	*mgrB* (N25ΔIS*Kpn26*-like[F]), *pmrB* (T140P)	8-16	0.25									32	
2_GR_12	258	*mgrB* (N25ΔIS*Kpn26*-like[R]), *phoP* (A95S), *phoQ* (N253T)	8	0.25	64			64						
3_GR_13	258	D31E	8	0.5-1										
4_GR_12	258	*mgrB* (N25ΔIS*Kpn26*-like[R]), *phoP* (P74L), *phoQ* (N253T)	16-32	0.25	32			>64						
5_GR_13	383	*mgrB* (Q30STOP)	16	0.25										
7_GR_13	258	*mgrB* (N25ΔIS*Kpn26*-like[R])	64->64	0.25-0.5	64	16	16	64	32			32	64	>64
8_GR_13	258	*phoQ* (G385C)	32-64					1-16						
9_GR_12	258	*phoQ* (T281M)	8-16					2-8						
10_GR_13	258	*mgrB* (N25ΔIS*Kpn26*-like[R]), *phoQ* (N253T)	32-64	0.25				32						
11_BR_13	437	*mgrB* (-35ΔIS*1R*[F])	64	0.125										
12_BR_13	11	*mgrB* (N25ΔIS*Kpn13*[R])	>64	0.5-2										
13_GR_14 (Het)	258	*mgrB* (N25ΔIS*Kpn26*-like[R]), *phoQ* (V446G), *pmrB* (P158R)	16	16				>64					≤0.06-32	
14_GR_14 (Het)	258	*mgrB* (N25ΔIS*Kpn26*-like[R]), *phoQ* (V446G), *pmrB* (P158R)	16-32	0.125-32				>64					≤0.06-64	
15_GR_13	258	*mgrB* (I45ΔIS*Kpn25*[F])	32-64	2-4										
16_GR_13	11	*mgrB* (-19ΔIS*1R*-like[F])	64	≤0.06-0.125										
17_GR_14	11	*mgrB* (-19ΔIS*1R*-like[F])	32	0.125										
18_GR_14	258	*mgrB* (N25ΔIS*Kpn26*-like[R])	64	1										
19_GR_14	258	*mgrB* (C28STOP)	64	0.25-0.5										
20_GR_12	258	WT	0.125-0.25	0.125	0.25	0.125	0.125	0.125	0.125	8	32	0.125	0.125	0.5
23_GR_12 (Het)	258	*mgrB* (N25ΔIS*Kpn26*-like[R]), *phoP* (A95S), *phoQ* (N253T)	4-8	0.125	8			16						

[a]Predicted deleterious mutations in genes when compared against polymyxin-susceptible isolate, 20_GR_12; represented as *gene* (change); change defined as initial amino acid, position and new alteration; -, upstream nucleotide position from gene start where first dissimilarity occurs; [F/R], IS element in forward (F) or reverse (R) orientation to *mgrB* defined by transposase direction.

[b]Minimal inhibitory concentration of PMB before and after complementation with specified WT and mutated genes; -, MIC range as per at least 2 transformed colonies and tested in duplicate; Susceptible (≤2 mg/L); 2 to 8-fold above EUCAST clinical breakpoint; ≥16-fold above EUCAST clinical breakpoint

(Het), Heterogeneity detected in isolate.

bioRxiv preprint doi: https://doi.org/10.1101/134684; this version posted May 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

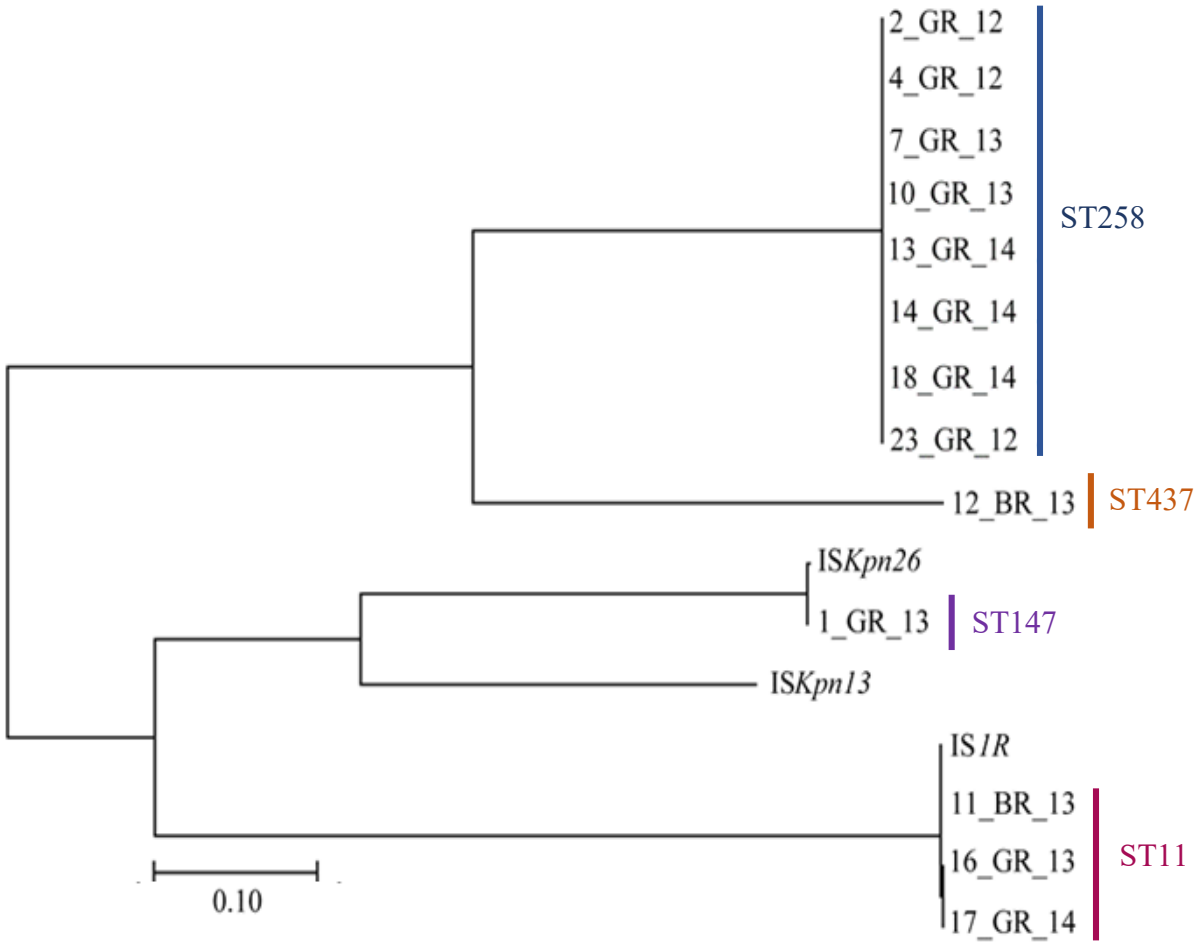


Figure 1. Clustering of IS elements disrupting *mgrB* detected in clinical strains together with reference IS element sequences. Neighbour-joining clustering of the IS element amplicon in relation to reference sequences for IS*Kpn13*, IS*Kpn26* and IS*1R* with transposase in the forward orientation. Scale represents number of base substitutions per site.