Microevolution of acquired colistin resistance in Enterobacteriaceae from ICU patients receiving selective decontamination of the digestive tract.

Axel B. Janssen^a, Denise van Hout^b, Marc J.M. Bonten^{a,b}, Rob J.L. Willems^a, Willem van Schaik^{a,c}

^a Department of Medical Microbiology, University Medical Center Utrecht, Utrecht University, Heidelberglaan 100, 3584CX, Utrecht, The Netherlands.

^b Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht Universit, Heidelberglaan 100, 3584CX, Utrecht, The Netherlands

^c Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom.

Address correspondence to Willem van Schaik, w.vanschaik@bham.ac.uk, telephone: +44 121 414 6913

Abstract

Colistin is an antibiotic that targets the lipopolysaccharides present in the membranes of Gram-negative bacteria. It is used as last-resort drug to treat infections with multidrug-resistant strains. Colistin is also used in selective decontamination of the digestive tract (SDD), a prophylactic therapy used in patients hospitalised in intensive care units (ICUs) to selectively eradicate opportunistic pathogens in the oropharyngeal and gut microbiota. In this study, we aimed to unravel the mechanisms of acquired colistin resistance in Gram-negative opportunistic pathogens obtained from SDD-treated patients.

Routine surveillance of 428 SDD-treated patients resulted in thirteen strains with acquired colistin resistance (*Escherichia coli* n=9; *Klebsiella aerogenes*, n=3; *Enterobacter asburiae*, n=1) from five patients. Genome sequence analysis showed that these isolates represented multiple distinct colistin-resistant clones, but that within the same patients, colistin-resistant strains were clonally related. We identified previously described mechanisms that lead to colistin resistance, i.e. a G53 substitution in the response regulator PmrA/BasR, and the acquisition of the mobile colistin resistance gene *mcr-1.1*, but we also observed novel variants of *basR* with an 18-bp deletion, and a G19E substitution in the sensor histidine kinase BasS. We experimentally confirmed these variants to contribute to reduced colistin susceptibility. In a single patient, we observed that colistin resistance in a single *E. coli* clone evolved through two unique variants in *basRS*.

We show that prophylactic use of colistin during SDD can select for colistin resistance in species that are not intrinsically colistin-resistant. This highlights the importance of continued surveillance for the emergence of colistin resistance in patients treated with SDD.

Introduction

Selective decontamination of the digestive tract (SDD) is a prophylactic antibiotic regimen used in Dutch intensive care units (ICUs) which lowers the mortality in ICU-admitted patients through the selective eradication of opportunistic pathogens in the oropharyngeal and gut microbiota.¹ One of the targets of SDD are the Enterobacteriaceae, which are collectively responsible for a significant proportion of hospital-acquired infections.²⁻⁶ In SDD, a combination of the antibiotics colistin and tobramycin and the antifungal amphotericin B, is applied to the digestive tract of ICU patients. In addition, during the first four days of ICU stay, patients are also intravenously administered a biliary-excreted third-generation cephalosporin, contributing to the eradication of Gram-negative pathogens from the gut.² The use of SDD is accompanied by surveillance for potential colonisation of the digestive tract by tobramycin- and/or colistin-resistant Enterobacteriaceae.⁷

Colistin is a cationic cyclic polypeptide with a hydrophobic fatty acid acyl chain that specifically acts on Gram-negative bacteria. Colistin electrostatically interacts with the anionic phosphate groups of the lipid A moiety of lipopolysaccharide (LPS) molecules in the outer leaflet of the outer membrane.^{8,9} Through binding to the phosphate groups, and insertion of its hydrophobic domains, colistin destabilizes the outer membrane. After disruption of the outer membrane, colistin targets the LPS that is resident in the cytoplasmic membrane after its synthesis in the cytoplasm. The destabilization of the cytoplasmic membrane ultimately kills the cell.¹⁰⁻¹²

The most frequent mechanisms of colistin resistance involve the reduction of the anionic charges of lipid A, which reduces the electrostatic interactions between colistin and LPS. This is achieved by the covalent linkage of positively charged groups like phosphoethanolamine or 4-amino-4-deoxy-L-arabinose to the phosphate groups of lipid A.¹² Addition of these groups to lipid A is achieved by the products of the EptA-, and Arn-operons, respectively.¹³ The transcriptional activity of these operons is controlled by the two-component regulatory systems PhoPQ and PmrAB (BasRS in *Escherichia coli*). Colistin resistance mutations resulting in the permanent activation of these two-component regulatory systems often occur in

specific hotspots (e.g. Gly-53 and Arg-81 in BasR/PmrA, and Ala-159 in BasS/PmrB).^{14,15} Surveillance for colistin resistance has recently led to the discovery of novel mechanisms of colistin resistance, including Leu-10 substitutions in BasS/PmrB,¹⁶ but most importantly the acquisition of *mcr*-genes.¹⁷ For *E. coli*, the acquisition of *mcr*-carrying mobile genetic elements is a particularly important mechanism through which colistin resistance may occur.¹⁸ Other mechanisms of acquired colistin resistance in Enterobacteriaceae include the production of capsular polysaccharides,¹⁹ and efflux pump activation.²⁰

The use of SDD is not widely accepted,²¹ which is at least partially due to the increasing importance of colistin as a last-resort antibiotic for the treatment of infections caused by multidrug-resistant Gram-negative pathogens.^{22,23} There thus exists an interest in understanding the ability of colistin-resistant strains to emerge and spread during SDD, and to characterise the mechanisms that cause colistin resistance in Enterobacteriaceae from SDD-treated patients. In this work, we analyse thirteen colistin-resistant strains (nine *E. coli* strains, three *Klebsiella aerogenes* strains and one *Enterobacter asburiae* strain) from SDD-treated ICU patients through whole genome sequencing and investigate the mechanisms that have contributed to colistin resistance.

Material and methods

Bacterial strains, growth conditions, chemicals, plasmid isolation, and oligonucleotide primers

Colistin-resistant strains were isolated from SDD-treated patients that were hospitalised in the ICU of the University Medical Centre Utrecht, The Netherlands, between July 2018 to January 2019, as previously described.²⁴ We also included colistin-susceptible strains of the same species that were isolated from the same patient at the same day from which a colistin-resistant strain was isolated. *E. coli* strain BW25113 and the BW25113-derived $\Delta basRS$ strain BW27848 from the Keio collection were obtained from the Coli Genetic Stock Center.^{25,26} All strains were grown in Lysogeny Broth (LB; Oxoid, Landsmeer, The Netherlands) at 37°C with agitation at 300 rpm unless otherwise noted. Strains containing pGRG36 were grown at 30°C.²⁷ When appropriate, kanamycin (50 mg/L; Sigma-Aldrich, Zwijndrecht, The Netherlands), and ampicillin (100 mg/L; Sigma-Aldrich) were used. Colistin sulphate was obtained from Duchefa Biochemie (Haarlem, The Netherlands). L-(+)-arabinose was obtained from Sigma-Aldrich. Plasmids were purified using the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific, Landsmeer, The Netherlands). Oligonucleotide primers (Supplemental Table 1) were obtained from Integrated DNA Technologies (Leuven, Belgium).

Determination of minimal inhibitory concentration

Minimal inhibitory concentrations (MICs) of colistin were determined using a broth microdilution method in line with EUCAST guidelines,²⁸ as previously described.^{15,29} The breakpoint values for colistin resistance ($MIC \leq 2$ mg/L) in Enterobacteriaceae were obtained from the 2019 European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST; http://www.eucast.org/clinical_breakpoints/).

Genomic DNA isolation and whole-genome sequencing

Genomic DNA was isolated, and checked for quality, as described previously.¹⁵ Sequence libraries for Illumina sequencing were prepared using the Nextera XT kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Libraries were sequenced on an Illumina NextSeq 500 system with a 300-cycle (2 × 150 bp) NextSeq 500/550 Mid Output v2.5 kit.

Genome assembly, MLST typing, and identification of antibiotic resistance genes

Illumina sequencing data were assessed (FastQC v0.11.7), and trimmed (nesoni v0.115) for quality, and used for *de novo* genome assembly (SPAdes v3.12.0), as described before.^{15,30} MLST typing was performed using the mlst package v2.10 (<https://github.com/tseemann/mlst>). Assembled contigs were screened for acquired antibiotic resistance genes using ResFinder 3.2 using standard settings.³¹

Construction of core genome phylogenetic trees.

Genome assemblies of the sequenced strains were aligned with publicly available genomes of the same species obtained from NCBI databases (Supplemental Table 2). Conserved regions of genomes were identified and aligned using ParSNP v1.2.³² FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) was used to visualize the phylogenetic tree.

To construct the phylogenetic tree for the clonal strains obtained from patient 31, the genomes of these strains were aligned using snippy v4.4.2 (<http://github.com/tseemann/snippy>). The alignment was filtered for recombination using gubbins v2.4.1.³³ Polymorphic sites were subsequently identified through snp-sites v2.5.1.³⁴ A phylogenetic tree was constructed using FastTree v2.1.10.³⁵ The phylogenetic tree was visualized as described above.

Determination of SNPs and indels through short-read sequences

Read-mapping of Nesoni-filtered reads was performed using Bowtie2.³⁶ SNP and indel-calling was performed using SAMtools 0.1.19³⁷ through the settings described before.³⁸ Identified SNPs and indels were manually linked to features in the Prokka annotation, and inspected for synonymous versus

non-synonymous mutations. Identified mutations SNPs and indels were confirmed by performing PCR and Sanger sequencing (Macrogen, Amsterdam, The Netherlands)

Genome comparisons through Enterobase

To identify mutations that could potentially contribute to colistin resistance in the *E. coli* strains 260 and 263, Raw Illumina sequence reads were uploaded to, and assembled by, Enterobase³⁹ under barcode ESC_OA6301AA and ESC_OA6302AA respectively. The *E. coli* cgMLST scheme was used to define a closest relative in Enterobase and the assemblies of these strains were then used in comparative analyses. Identified sequence variations in the colistin-resistant strains were confirmed by PCR and Sanger sequencing (Macrogen, Amsterdam, The Netherlands)

Construction of chromosomal *basRS* transgene insertions

Single-copy chromosomal transgene insertion mutants of *basRS*, derived from clinical strains, were constructed in BW27848 using the Tn7-transposon system located on the pGRG36 plasmid,^{27,40} as previously described.¹⁵

Determination of maximum specific growth rate

To determine the maximum specific growth rate, a Bioscreen C instrument (Oy Growth Curves AB, Helsinki, Finland) was used. Overnight cultures were used to inoculate 200 µl fresh LB medium 1:1000. Incubation was set at 37°C with continuous shaking set to have maximum amplitude and fastest speed. Growth was observed by measuring the absorbance at 600 nm every 10 minutes.

Data availability

Sequence data has been deposited in the European Nucleotide Archive (accession number PRJEB34028).

Statistical analysis

Where applicable, statistical significance was determined using parametric one-way ANOVA tests. Correction for multiple comparison testing was performed for with a Tukey's (for the all-versus-all comparison of *E. coli* strains of patient 31), or a Dunnett's (for the *K. aerogenes* strains of patient 37) multiple comparison test. Family-wise significance was defined as a p-value < 0.05.

Results

Strains with acquired colistin resistance are rarely isolated during SDD.

As described in our previous work,²⁴ 388 Gram-negative strains were isolated from 1105 rectal swabs, from 428 patients receiving SDD. Of these, 102 strains belonged to species that are intrinsically resistant to colistin. The remaining 286 isolates were tested for colistin susceptibility on Sensititre™ FRCOL plates (Thermo Fisher Scientific, Wesel, Germany). A total of ten *E. coli* strains, one *E. asburiae* strain, and three *K. aerogenes* strains were found to be resistant to colistin through this method. We then tested these strains for colistin susceptibility in a standardized broth microdilution assay and all strains were phenotypically resistant to colistin with the exception of *E. coli* strain 89, which was excluded from further analyses (Table 1). Thus, we found thirteen strains (4.5% of the non-intrinsically-resistant isolates) to be colistin-resistant. The colistin-resistant *K. aerogenes* and *E. coli* strains had MIC values up to 32 mg/L colistin. The MIC of the *E. asburiae* strain was found to reach values up to 8192 mg/L colistin. For patients 27 and 37, colistin-susceptible strains of the same species were also isolated from rectal swabs during surveillance (Figure 1).

In total, five of the 428 patients (1.2%) tested positive for an isolate with acquired colistin resistance. Of the ten *E. coli* strains, seven were isolated from one patient, the remaining three strains originated from three other patients. The three *K. aerogenes* strains were isolated from a single patient. None of the patients carried multiple colistin-resistant species. Of the five patients from whom a strain with acquired colistin resistance was isolated, only patient 307 carried a colistin-resistant strain (*E. asburiae*) at the start of ICU admission, suggesting that this patient had acquired the colistin-resistant strain prior to SDD treatment (Figure 1). This strain was no longer present on any of the following time-points. Patients 311 and 27 were transiently colonised by colistin-resistant strains at 2 and 3 days after ICU admission, respectively. In patient 37 colistin-resistant *K. aerogenes* were detected at day 15, and day 18 of ICU hospitalisation, while strains with acquired colistin resistance were not cultured during subsequent screening from day 22 to discharge from the ICU on day 75. Colistin-resistant *E. coli* were

first detected in patient 31 at day 9 of ICU hospitalisation and remained colonised with colistin-resistant *E. coli*, until the patient was lost to follow up at day 24, with the exception of a negative culture result on day 20.

Colistin-resistant strains from ICU patients have a diverse genetic background and carry a variety of acquired antibiotic-resistance genes

Clonal relatedness of colistin-resistant *E. coli* and *K. aerogenes* strains colonising the ICU patients was assessed by constructing core genome phylogenies, using the genome sequences generated in this study and a collection of publicly available genomes. We observed that three distinct clones of colistin-resistant *E. coli* colonised three individual patients (Figure 2A). The colistin-susceptible *E. coli* strain 137 from patient 27 did not cluster with colistin-resistant *E. coli* strain 138 isolated from the same patient. All *K. aerogenes* strains isolated from patient 37 belonged to a single clone (Figure 2B).

We screened the assembled genomes of the colistin-resistant strains for acquired antibiotic resistance genes (Figure 2C). We identified that strain *E. coli* strain 138 was positive for the *mcr-1.1* gene, which was the sole antibiotic resistance gene on a 59.5 kbp contig that contained an IncI2-type replicon. The sequence of this contig shared 99% identity with plasmid sequences obtained from multiple sources, including from a *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain from a patient from China (accession number MH522416.1),⁴¹ and an *E. coli* strain isolated from chicken faeces in Thailand (accession number MG557851.1),⁴² illustrating the global spread of this plasmid. In addition, we found that the colistin-resistant *E. coli* strains carried between 2 and 12 antibiotic resistance genes, including genes conferring resistance to aminoglycosides and β -lactams. The *K. aerogenes* strains only carried the fosfomycin resistance gene *fosA*, and the single *E. asburiae* strain had *fosA* and the AmpC-type β -lactamase *blaACT-4*.

In-patient microevolution of colistin resistance in *Escherichia coli* during SDD.

To investigate the microevolution towards colistin resistance during gut colonisation, we investigated the genetic diversity between the four clonally related *K. aerogenes* strains from patient 37, and the seven colistin-resistant *E. coli* strains isolated from patient 31. By comparing the genome of the single colistin-susceptible *K. aerogenes* strain with the genomes of the colistin-resistant strains, we determined that all colistin-resistant strains had a G53S substitution in the PmrA transcriptional regulator of the PmrAB two-component regulatory system. This substitution has previously been described to cause colistin resistance in *K. aerogenes*.⁴³ In addition, we observed an insertion of a single guanine nucleotide in the gene encoding the sulphate adenylyltransferase subunit 2 CysD, which is involved in sulphate assimilation. CysD has not been previously described in relation to colistin resistance. No mutations differentiating the three colistin-resistant strains were observed.

The seven clonally related colistin-resistant *E. coli* isolates from patient 31 were obtained on four separate days, over a two-week period (Table 1, Figure 2A). Within these strains, we observed that the strains isolated on 1 and 5 November 2018 (strains 260, 262, and 274) had a G53A substitution in the BasR transcriptional regulator of the BasRS two-component regulatory system. This substitution has previously been experimentally proven to contribute to colistin resistance in *E. coli*.¹⁵ The strains isolated after November 5, 2018 (strains 281, 292, 296, and 297) however, did not encode the G53A BasR substitution. Instead, we observed an 18-bp deletion (nucleotides 10 through 27) in *basS*, leading to the deletion of six amino acids (4 through 9) at the N-terminal end of BasS.

Through phylogenetic analysis of these seven clonally related strains, we observed that the strains carrying the G53A BasR substitution, and those with the 18-bp deletion in *basS*, were intermingled (Figure 3A). Three strains that were all isolated on 14 November 2018 (strains 292, 296, and 297) had a mucoid phenotype (Figure 3B) and carried an I424S substitution in Yrff that was absent in the non-mucoid strains. Yrff is a homolog of IgaA in *Salmonella*, and functions as a negative regulator of the Rcs phosphorelay system, and is thus involved in regulation of capsule production.⁴⁴

To establish the impact of colistin resistance on fitness, we determined the strains' maximum specific growth rate. Among the strains from patient 31, we observed a reduction of about 15% in

maximum specific growth rate in the strains harbouring the 18-bp deletion in *basS*, compared to those harbouring the G53A BasR substitution (Figure 3C). A similar decrease in maximum specific growth rate is observed in the strains that have the I424S substitution in YrfF. For the *K. aerogenes* strains isolated from patient 37 that had the G53S PmrA substitution, we did not observe a change in the maximum specific growth rate (Supplemental Figure 1).

Mutations in *basS* contribute to reduced susceptibility to colistin.

To identify mutations that potentially contributed to colistin resistance in *E. coli* strain 263, for which we lacked an isogenic, colistin-susceptible counterpart, we used the Enterobase database to identify the strain (eo2071, which was not reported to be colistin-resistant; Enterobase barcode ESC_BA7113AA) that is most closely related to strain 263. We then compared the sequence of *basRS* of strain eo2071 to *basRS* of strain 263, leading to the identification of a G19E substitution in BasS in the latter strain.

We next aimed to investigate the relevance of the 18-bp deletion in *basS* and the mutation leading to the G19E substitution for colistin resistance in *E. coli*. Due to the multidrug-resistant nature of the colistin-resistant strains, we constructed chromosomal integration mutants of the genes encoding the mutated BasRS two-component regulatory system in the *attTn7* site in the BW25113 derived Δ *basRS* strain BW27848, as described previously.¹⁵ MIC determinations of the chromosomal integration mutants showed that introduction of the *basRS* alleles of the colistin-resistant strains led to reduced susceptibility to colistin (Table 2). The MIC value of the BW27848 strain with the insertion of *basRS* encoding the 18-bp deletion in *basS* increased 4-fold. The MIC value of the BW27848 strain with the *basRS* encoding the G19E substitution in *basR* increased 2-fold. Restoring the deleted 18 base-pairs to *basS*, or reversing the G19E BasR substitution, returned colistin susceptibility to BW25113 levels.

Discussion

In this study, we genomically characterized strains with acquired colistin resistance from ICU patients treated with SDD. Thirteen strains, from three species with acquired colistin resistance were isolated. The carriage rate of strains with acquired colistin resistance among SDD-treated ICU patients (1.2%) was similar to rates previously reported for Dutch ICUs.^{6,15,45} We found multiple distinct clones of colistin-resistant strains among patients. Colistin resistance in the *E. coli* strains could be explained by the acquisition of *mcr-1.1*, a G53A or G19E substitution in BasR, or an 18-bp deletion in *basS*. The 18-bp deletion in *basS* was associated with a loss of fitness. Colistin resistance in the *K. aerogenes* strains was associated with a G53S substitution in PmrA. The mechanism of colistin resistance in the *E. asburiae* strain was not identified.

Through longitudinal sampling, we are able to observe selection and microevolutionary processes related to prolonged exposure to colistin. We found that colistin resistance in a clonal *E. coli* population may emerge through different mutational trajectories in *basRS*, as observed in the strains obtained from patient 31. The placement of strains with different mutations in *basRS* (either causing a G53A BasR substitution or a 18-bp deletion in *basS*) on distinct branches of the phylogenetic tree, suggests that strains with both mutations have emerged independently. The observation of two distinct variants in *basRS* that contribute to colistin resistance in a clonal *E. coli* population, reflects the ability of *E. coli* to quickly adapt to novel niches and selective pressures through multiple evolutionary pathways.^{46–48} This *E. coli* clone was likely able to persistently colonise the patient because it carried mechanisms that confer resistance to all antibiotics (cephalosporins, tobramycin, and colistin) used in SDD. Through longitudinal sampling of isogenic colistin-susceptible and colistin-resistant *E. coli* strains from patients 27 and 37, we observed that colistin resistance emerged *de novo* in these strains. For the strains isolated from patients 307 and 311, we cannot exclude the possibility that these strains could have already acquired colistin resistance before they colonised the patient.

We observed transient colonisation by an *mcr-1.1* carrying *E. coli* in a single patient receiving SDD. Transient gut colonisation by *mcr*-carrying *E. coli* has previously been observed in the context of international travel, and may reflect the reduced fitness of *E. coli* strains carrying *mcr*-genes.^{49–51} High-level colistin resistance was observed in an *Enterobacter asburiae* strain, a member of the *E. cloacae* complex,⁵² and a clonal population of colistin-resistant *K. aerogenes*. A limited number of high-level resistant *E. asburiae*,^{53–57} and *K. aerogenes*^{43,58–60} strains have been described before. However, the exact mechanisms through which colistin resistance evolves in these species remain poorly studied.^{43,61}

Prolonged exposure to antibiotics will select for resistance, which generally comes at a fitness cost. Fitness of an antibiotic-resistant clone can subsequently increase due to the accumulation of compensatory mutations.⁶² Interestingly, we observed a reduction of the maximum specific growth rate in the *E. coli* strains in which *yrfF* was mutated, which likely contributed to the mucoid phenotype in these strains. The increased biosynthesis of capsular polysaccharides is likely to come at a cost that will negatively impact maximum specific growth rate. While mucoidy has been linked to colistin resistance in *K. pneumoniae*, and *Neisseria meningitidis*,^{19,63,64} we did not observe a difference in the colistin MICs of clonally related non-mucoid and mucoid *E. coli* strains. Mucoidy in *E. coli* is a relatively poorly understood phenotype, but it is likely to contribute to increased survival upon humoral and cellular immune responses.^{65,66}

In this study, we find that Gram-negative opportunistic pathogens carried in the gut of patients can acquire colistin resistance, either through mutation of genes that regulate lipid A modifications or by the acquisition of the *mcr-1.1* gene. However, the low prevalence of colistin-resistant strains in ICU patients suggests that the evolution of colistin resistance is currently of minor concern for the implementation of SDD in Dutch hospitals. As the prevalence of multidrug-resistant Gram-negative bacteria in the Netherlands is low, strains colonising patients will be generally susceptible to one or more of the antibiotics used in SDD. Indeed, in four patients, strains with acquired colistin resistance were rapidly eradicated from the gut. However, the long-term colonisation of patient 31 with an *E. coli* clone

that is colistin resistant and carries genes conferring resistance to the other antibiotics used in SDD, indicates that SDD can select for multidrug-resistant Gram-negative bacteria in the gut of ICU patients. The risk of the emergence of colistin resistance in the patient gut may be more pronounced in countries where higher rates of circulating antibiotic resistant bacteria are observed, or in settings with failing infection control.^{6,22,67,68} Continuous surveillance is thus vital to thwart selection and spread of multidrug-resistant strains upon SDD. Further studies are required to better understand the diversity of mechanisms of acquired colistin resistance in clinical Enterobacteriaceae isolates, particularly in species like *E. asburiae*, and *K. aerogenes* in which colistin resistance mechanisms have so far been poorly studied.

Acknowledgements

We thank Johanna C. Braat, Ellen C. Brouwer, Malbert R.C. Rogers, Moniek Salomons, and the Department of Genetics at the Wilhelmina Children's Hospital, for their expertise on Illumina NextSeq sequencing. We thank Rob Rentenaar, and Judith Vlooswijk for providing the strains that were studied here.

Funding

This work was supported by The Netherlands Organisation for Scientific Research through a Vidi grant (grant number 917.13.357); and a Royal Society Wolfson Research Merit Award (grant number WM160092), both to W.v.S. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Transparency

The authors disclose no conflicts of interest.

Author contributions

A.B.J. conceived and designed experiments, performed experiments, analysed data, and wrote the manuscript. D.v.H. reviewed patients records and microbiological data. M.J.M.B. wrote the manuscript. R.J.L.W. wrote the manuscript. W.v.S. conceived and designed experiments, wrote the manuscript, and supervised the study. All authors reviewed and approved the final version of the manuscript.

References

1. Plantinga NL, de Smet AMGA, Oostdijk EAN, *et al.* Selective digestive and oropharyngeal decontamination in medical and surgical ICU patients: individual patient data meta-analysis. *Clin Microbiol Infect* 2018; 24: 505–13.
2. de Smet AMGA, Kluytmans JAJW, Cooper BS, *et al.* Decontamination of the digestive tract and oropharynx in ICU patients. *N Engl J Med* 2009; 360: 20–31.
3. Sievert DM, Ricks P, Edwards JR, *et al.* Antimicrobial-resistant pathogens associated with healthcare-associated infections summary of data reported to the national healthcare safety network at the Centers for Disease Control and Prevention, 2009–2010. *Infect Control Hosp Epidemiol* 2013; 34: 1–14.
4. Peleg AY, Hooper DC. Hospital-acquired infections due to Gram-negative bacteria. *N Engl J Med* 2011; 362: 1804–13.
5. Wittekamp BH, Plantinga NL, Cooper BS, *et al.* Decontamination strategies and bloodstream infections with antibiotic-resistant microorganisms in ventilated patients: a randomized clinical trial. *J Am Med Assoc* 2018; 320: 2087–98.
6. Oostdijk EAN, Kesecioglu J, Schultz MJ, *et al.* Effects of decontamination of the oropharynx and intestinal tract on antibiotic resistance in ICUs a randomized clinical trial. *J Am Med Assoc* 2014; 312: 1429–37.
7. Wittekamp BHJ, Oostdijk EAN, Cuthbertson BH, Brun-Buisson C, Bonten MJM. Selective decontamination of the digestive tract (SDD) in critically ill patients: a narrative review. *Intensive Care Med* 2019; 46: 343–9.
8. Velkov T, Thompson PE, Nation RL, Li J. Structure-activity relationships of polymyxin antibiotics. *J Med Chem* 2010; 53: 1898–916.
9. Domingues MM, Inácio RG, Raimundo JM, Martins M, Castanho MARB, Santos NC. Biophysical characterization of polymyxin B interaction with LPS aggregates and membrane model systems. *Biopolymers* 2012; 98: 338–44.
10. Putker F, Bos MP, Tommassen J. Transport of lipopolysaccharide to the Gram-negative bacterial cell

370 surface. *FEMS Microbiol Rev* 2015; 39: 985–1002.

371 **11.** Sabnis A, Klöckner A, Becce M, *et al.* Colistin kills bacteria by targeting lipopolysaccharide in the
372 cytoplasmic membrane. *bioRxiv* 2018: 479618.

373 **12.** Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. Molecular mechanisms of antibiotic
374 resistance. *Chem Commun* 2011; 47: 4055–61.

375 **13.** Simpson BW, Trent MS. Pushing the envelope: LPS modifications and their consequences. *Nat Rev*
376 *Microbiol* 2019; 17: 403–16.

377 **14.** Bourrel AS, Poirel L, Royer G, *et al.* Colistin resistance in Parisian inpatient faecal *Escherichia coli*
378 as the result of two distinct evolutionary pathways. *J Antimicrob Chemother* 2019; 74: 1521–30.

379 **15.** Janssen AB, Bartholomew TL, Marciszewska NP, *et al.* Nonclonal emergence of colistin resistance
380 associated with mutations in the BasRS two-component system in *Escherichia coli* bloodstream isolates.
381 *mSphere* 2020; 5: e00143-20.

382 **16.** Cannatelli A, Giani T, Aiezza N, *et al.* An allelic variant of the PmrB sensor kinase responsible for
383 colistin resistance in an *Escherichia coli* strain of clinical origin. *Sci Rep* 2017; 7: 5071.

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

387 **18.** Wang R, van Dorp L, Shaw LP, *et al.* The global distribution and spread of the mobilized colistin
388 resistance gene *mcr-1*. *Nat Commun* 2018; 9: 1179.

389 **19.** Llobet E, Tomás JM, Bengoechea JA. Capsule polysaccharide is a bacterial decoy for antimicrobial
390 peptides. *Microbiology* 2008; 154: 3877–86.

391 **20.** Padilla E, Llobet E, Doménech-Sánchez A, Martínez-Martínez L, Bengoechea JA, Albertí S.
392 *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence.
393 *Antimicrob Agents Chemother* 2010; 54: 177–83.

394 **21.** Timsit JF, Bassetti M. Antipathy against SDD is justified: Yes. *Intensive Care Med* 2018; 44: 1165–8.

395 **22.** Halaby T, Al Naiemi N, Kluytmans J, van der Palen J, Vandenbroucke-Grauls CMJE. Emergence of

colistin resistance in Enterobacteriaceae after the introduction of selective digestive tract decontamination in an intensive care unit. *Antimicrob Agents Chemother* 2013; 57: 3224–3229.

23. Lübbert C, Fauchaux S, Becker-Rux D, *et al.* Rapid emergence of secondary resistance to gentamicin and colistin following selective digestive decontamination in patients with KPC-2-producing *Klebsiella pneumoniae*: a single-centre experience. *Int J Antimicrob Agents* 2013; 42: 565–70. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24100228>. Accessed November 12, 2013.

24. van Hout D, Janssen AB, Rentenaar RJ, Vlooswijk JPM, Boel CHE, Bonten MJM. The added value of the selective SuperPolymyxinTM medium in detecting rectal carriage of Gram-negative bacteria with acquired colistin resistance in intensive care unit patients receiving selective digestive decontamination. *Eur J Clin Microbiol Infect Dis* 2019.

25. Baba T, Ara T, Hasegawa M, *et al.* Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2006; 2: 2006.0008.

26. Zhou L, Lei X, Bochner BR, Wanner BL. Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. *J Bacteriol* 2003; 185: 4956–72.

27. McKenzie GJ, Craig NL. Fast, easy and efficient: site-specific insertion of transgenes into Enterobacterial chromosomes using Tn7 without need for selection of the insertion event. *BMC Microbiol* 2006; 6: 39.

28. European Committee on Antimicrobial Susceptibility Testing (EUCAST). *Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group*. 2016.

29. Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001; 48: 5–16.

30. Bankevich A, Nurk S, Antipov D, *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; 19: 455–77.

31. Zankari E, Hasman H, Cosentino S, *et al.* Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 2012; 67: 2640–4.

32. Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol* 2014; 15: 524.
33. Croucher NJ, Page AJ, Connor TR, *et al.* Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res* 2015; 43: e15.
34. Page AJ, Taylor B, Delaney AJ, *et al.* SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. *Microb genomics* 2016; 2: e000056.
35. Price MN, Dehal PS, Arkin AP. FastTree: Computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 2009; 26: 1641–50.
36. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 2012; 9: 357–9.
37. Li H, Handsaker B, Wysoker A, *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009; 25: 2078–9.
38. van Mansfeld R, de Been M, Paganelli F, Yang L, Bonten M, Willems R. Within-host evolution of the Dutch high-prevalent *Pseudomonas aeruginosa* clone ST406 during chronic colonization of a patient with cystic fibrosis. *PLoS One* 2016; 11: e0158106.
39. Zhou Z, Alikhan NF, Mohamed K, Fan Y, Achtman M. The Enterobase user’s guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. *Genome Res* 2020; 30: 138–52.
40. Choi K-H, Schweizer HP. mini-Tn7 insertion in bacteria with single *attTn7* sites: example *Pseudomonas aeruginosa*. *Nat Protoc* 2006; 1: 153–61.
41. Lu X, Zeng M, Xu J, *et al.* Epidemiologic and genomic insights on *mcr-1*-harbouring *Salmonella* from diarrhoeal outpatients in Shanghai, China, 2006–2016. *EBioMedicine* 2019; 42: 133–44.
42. Yang Q, Li M, Spiller OB, *et al.* Balancing *mcr-1* expression and bacterial survival is a delicate equilibrium between essential cellular defence mechanisms. *Nat Commun* 2017; 8: 2054.
43. Diene SM, Merhej V, Henry M, *et al.* The rhizome of the multidrug-resistant *Enterobacter aerogenes* genome reveals how new ‘killer bugs’ are created because of a sympatric lifestyle. *Mol Biol Evol* 2013; 30: 369–83.

448 **44.** Wall E, Majdalani N, Gottesman S. The complex Rcs regulatory cascade. *Annu Rev Microbiol* 2018;
449 72: 111–39.

450 **45.** de Jonge E, Schultz MJ, Spanjaard L, *et al.* Effects of selective decontamination of digestive tract on
451 mortality and acquisition of resistant bacteria in intensive care: a randomised controlled trial. *Lancet*
452 2003; 362: 1011–6.

453 **46.** Barroso-Batista J, Sousa A, Lourenço M, *et al.* The first steps of adaptation of *Escherichia coli* to the
454 gut are dominated by soft sweeps. *PLoS Genet* 2014; 10: e1004182.

455 **47.** Lescat M, Launay A, Ghalayini M, *et al.* Using long-term experimental evolution to uncover the
456 patterns and determinants of molecular evolution of an *Escherichia coli* natural isolate in the
457 streptomycin-treated mouse gut. *Mol Ecol* 2017; 26: 1802–17.

458 **48.** Giraud A, Arous S, De Paepe M, *et al.* Dissecting the genetic components of adaptation of
459 *Escherichia coli* to the mouse gut. *PLoS Genet* 2008; 4: e2.

460 **49.** Kantele A, Kuenzli E, Dunn S, *et al.* Real-time sampling of travelers shows intestinal colonization by
461 multidrug-resistant bacteria to be a dynamic process with multiple transient acquisitions. *bioRxiv* 2019:
462 827915.

463 **50.** Vasquez AM, Montero N, Laughlin M, *et al.* Investigation of *Escherichia coli* harboring the *mcr-1*
464 resistance gene — Connecticut, 2016. *Morb Mortal Wkly Rep* 2016; 65: 979–80.

465 **51.** Tietgen M, Semmler T, Riedel-Christ S, *et al.* Impact of the colistin resistance gene *mcr-1* on bacterial
466 fitness. *Int J Antimicrob Agents* 2018; 51: 554–61.

467 **52.** Brenner DJ, McWhorter AC, Kai A, Steigerwalt AG, Farmer JJ. *Enterobacter asburiae* sp. nov., a
468 new species found in clinical specimens, and reassignment of *Erwinia dissolvens* and *Erwinia*
469 *nimipressuralis* to the genus *Enterobacter* as *Enterobacter dissolvens* comb. nov., and *Enterobacter*
470 *nimipressuralis* comb. nov.. *J Clin Microbiol* 1986; 23: 1114–20.

471 **53.** Pena I, Picazo JJ, Rodríguez-Avial C, Rodríguez-Avial I. Carbapenemase-producing
472 Enterobacteriaceae in a tertiary hospital in Madrid, Spain: high percentage of colistin resistance among
473 VIM-1-producing *Klebsiella pneumoniae* ST11 isolates. *Int J Antimicrob Agents* 2014; 43: 460–4.

474 **54.** Rossi F, Girardello R, Cury AP, Di Gioia TSR, Almeida JN de, Duarte AJ da S. Emergence of colistin
475 resistance in the largest university hospital complex of São Paulo, Brazil, over five years. *Brazilian J*
476 *Infect Dis* 2017; 21: 98–101.

477 **55.** Bradford PA, Kazmierczak KM, Biedenbach DJ, Wise MG, Hackel M, Sahm DF. Correlation of β -
478 lactamase production and colistin resistance among Enterobacteriaceae isolates from a global surveillance
479 program. *Antimicrob Agents Chemother* 2016; 60: 1385–92.

480 **56.** Boo TW, O’Connell N, Power L, *et al.* First report of IMI-1-producing colistin-resistant *Enterobacter*
481 clinical isolate in Ireland, March 2013. *Eurosurveillance* 2013; 18: pii=20548.

482 **57.** Olaitan AO, Morand S, Rolain JM. Emergence of colistin-resistant bacteria in humans without
483 colistin usage: a new worry and cause for vigilance. *Int J Antimicrob Agents* 2016; 47: 1–3.

484 **58.** Bollet C, Raoult D, Page J. Successive emergence of *Enterobacter aerogenes* strains resistant to
485 imipenem and colistin in a patient. *Antimicrob Agents Chemother* 2005; 49: 1354–8.

486 **59.** Bedenić B, Vranić-Ladavac M, Venditti C, *et al.* Emergence of colistin resistance in *Enterobacter*
487 *aerogenes* from Croatia. *J Chemother* 2018; 30: 120–3.

488 **60.** Landman D, Salamera J, Quale J. Irreproducible and uninterpretable polymyxin B MICs for
489 *Enterobacter cloacae* and *Enterobacter aerogenes*. *J Clin Microbiol* 2013; 51: 4106–11.

490 **61.** Telke AA, Olaitan AO, Morand S, Rolain JM. *soxRS* induces colistin hetero-resistance in
491 *Enterobacter asburiae* and *Enterobacter cloacae* by regulating the *acrAB-tolC* efflux pump. *J Antimicrob*
492 *Chemother* 2017; 72: 2715–21.

493 **62.** MacLean RC, Vogwill T. Limits to compensatory adaptation and the persistence of antibiotic
494 resistance in pathogenic bacteria. *Evol Med Public Heal* 2015; 2015: 4–12.

495 **63.** Campos MA, Vargas MA, Regueiro V, Llompart CM, Albertí S, Bengoechea JA. Capsule
496 polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infect Immun* 2004; 72: 7107–14.

497 **64.** Spinosa MR, Progida C, Talà A, Cogli L, Alifano P, Bucci C. The *Neisseria meningitidis* capsule is
498 important for intracellular survival in human cells. *Infect Immun* 2007; 75: 3594–603.

499 **65.** Cress BF, Englaender JA, He W, Kasper D, Linhardt RJ, Koffas MAG. Masquerading microbial

pathogens: capsular polysaccharides mimic host-tissue molecules. *FEMS Microbiol Rev* 2014; 38: 660–97.

66. Russo TA, Davidson BA, Carlino-MacDonald UB, Helinski JD, Priore RL, Knight PR. The effects of *Escherichia coli* capsule, O-antigen, host neutrophils, and complement in a rat model of Gram-negative pneumonia. *FEMS Microbiol Lett* 2003; 226: 355–61.

67. de Smet AMGA, Kluytmans JAJW, Blok HEM, *et al.* Selective digestive tract decontamination and selective oropharyngeal decontamination and antibiotic resistance in patients in intensive-care units: an open-label, clustered group-randomised, crossover study. *Lancet Infect Dis* 2011; 11: 372–80.

68. Monaco M, Giani T, Raffone M, *et al.* Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. *Eurosurveillance* 2014; 19: 20939.

69. McNally A, Cheng L, Harris SR, Corander J. The evolutionary path to extraintestinal pathogenic, drug-resistant *Escherichia coli* is marked by drastic reduction in detectable recombination within the core genome. *Genome Biol Evol* 2013; 5: 699–710.

70. Malek A, McGlynn K, Taffner S, *et al.* Next-generation-sequencing-based hospital outbreak investigation yields insight into *Klebsiella aerogenes* population structure and determinants of carbapenem resistance and pathogenicity. *Antimicrob Agents Chemother* 2019; 63: e02577-18.

Tables

Strain	Date of isolation	Patient	Species	Colistin MIC (mg/L)
24	6 August 2018	37	<i>Klebsiella aerogenes</i>	0.25
25	6 August 2018	37	<i>Klebsiella aerogenes</i>	32
26	9 August 2018	37	<i>Klebsiella aerogenes</i>	32
27	9 August 2018	37	<i>Klebsiella aerogenes</i>	32
32	7 September 2018	307	<i>Enterobacter asburiae</i>	8192
89	26 July 2018	337	<i>Escherichia coli</i>	0.25
137	30 August 2018	27	<i>Escherichia coli</i>	0.125
138	30 August 2018	27	<i>Escherichia coli</i>	8
260	1 November 2018	31	<i>Escherichia coli</i>	16
262	1 November 2018	31	<i>Escherichia coli</i>	16
263	1 November 2018	311	<i>Escherichia coli</i>	4
274	5 November 2018	31	<i>Escherichia coli</i>	16
281	8 November 2018	31	<i>Escherichia coli</i>	16
292	14 November 2018	31	<i>Escherichia coli</i>	16
296	14 November 2018	31	<i>Escherichia coli</i>	16
297	14 November 2018	31	<i>Escherichia coli</i>	16

Table 1. Strains isolated in this study and relevant metadata. The species of each isolate was determined by MALDI-TOF on a Bruker microflex system (Leiderdorp, The Netherlands). The MIC value of the broth microdilution method represents the median value of three independent replicates of colistin susceptibility testing, performed in triplicate.

Strain	Colistin MIC (mg/L)
BW25113	0.125
BW27848	0.25
BW25113::Tn7 Empty	0.125
BW27848::Tn7 Empty	0.125
BW27848::Tn7 BW25113	0.125
BW27848::Tn7 281	1
BW27848::Tn7 281m	0.125
BW27848::Tn7 263	0.25
BW27848::Tn7 263m	0.125

Table 2. Colistin MICs of strains generated in this study. *E. coli* strain BW27848 is the $\Delta basRS$ mutant of BW25113.²⁶ The *basRS* alleles of colistin-resistant strains from this study were inserted into the *attTn7* site of BW27848. The addition of “m” to a strain name indicates that the construct has been modified through inverse PCR site-directed mutagenesis to reverse the mutation associated with colistin resistance.

Figure legends

Figure 1. Timeline of rectal swabs collected from SDD-treated ICU patients and isolation of colistin-susceptible, or colistin-resistant strains. Filled circle: isolation of colistin-resistant strain from rectal swab. Filled triangle; a colistin-susceptible strain of the same species as the colistin-resistant strain was isolated from the rectal swab. Open diamonds, no naturally colistin-susceptible Gram-negative bacteria were isolated. The symbols are colour coded according to the species of the isolated strain: *Klebsiella aerogenes*, blue; *Enterobacter asburiae*, green; *Escherichia coli*, yellow, orange and red. Multiple symbols on the same day indicate the isolation of multiple strains from the same swab. The length of ICU admission is indicated by a line, and discharge is indicated by a circle at the end of the line.

Figure 2. Phylogeny and acquired resistance genes of colistin-resistant *E. coli* and *K. aerogenes*. A) The phylogenetic tree of *E. coli* represents the core genome alignment (2.1 Mbp) of 198 genomes (178 genomes from public databases, the genomes of the nine colistin-resistant strains, and the two colistin-susceptible strains). One representative reference strain per *E. coli* phylogroup is indicated ⁶⁹. Phylogroup A is coloured dark blue; B1, yellow; B2, light blue; D, green; E, purple; F, pink. Colistin-susceptible and -resistant strains are indicated by filled triangles and circles respectively. The studied strains are highlighted with colours corresponding to Figure 1. B) The phylogenetic tree of *K. aerogenes* represents a core genome alignment (1.6 Mbp) of 56 publicly available genomes, and the three colistin-resistant strains described in this study. Colistin-susceptible and -resistant strains are indicated by filled triangles and circles respectively. The studied strains are highlighted with colours corresponding to Figure 1. The genomes of the dominant *K. aerogenes* ST4 and ST93 lineages associated with infections have a red and green background respectively ⁷⁰. C) Acquired antibiotic resistance genes of colistin-resistant strains. Strains are grouped according to the patient from which they were isolated. Species and MLST type are indicated per strain. Antibiotic resistance genes in the genomes of the colistin-resistant strains were detected by ResFinder 3.2 ³¹. Classes of antibiotic resistance genes are

abbreviated as follow: PMX, polymyxin resistance; BLA, beta-lactam resistance; QLN, quinolone resistance; FOS, fosfomycin resistance; AGC, aminoglycoside resistance; MCL, macrolide, lincosamide, and streptogramin B resistance; PHE, phenicol resistance; SUL, sulfonamide resistance; TET, tetracycline resistance; TMP, trimethoprim resistance. N.D.; not determined.

Figure 3. *In vivo* microevolution of colistin-resistant *E. coli* strains isolated from patient 31. A) Mid-point rooted phylogenetic tree representing a recombination-filtered core genome alignment of the strains isolated from patient 31. Branches with the BasR G53A substitution, the BasS Δ aa4-9 deletion, and the I424S substitution in Yrff are indicated through a green, orange, and purple background respectively. Other mutations are indicated next to the relevant branch. B) Growth phenotypes of the *E. coli* strains isolated from patient 31 on TSA plates with 5% sheep blood after overnight growth at 37°C. C) Maximum specific growth rate of colistin-resistant *E. coli* strains isolated from patient 31. The values presented represent mean with standard deviation, of three independent experiments, performed in duplicate. Statistical significance testing was performed using a parametric one-way ANOVA test with Tukey's multiple comparisons test. Family-wise significance was defined as a p-value < 0.05. The growth rates of strains marked by the same letter differ statistically significantly from those with other letters.

Supplemental materials

Supplemental Figure 1. Maximum specific growth rate of *K. aerogenes* strains. Maximum specific growth rate of the colistin-susceptible, and colistin-resistant *K. aerogenes* strains isolated from patient 37. The values presented represent the means with standard deviations of three independent experiments performed in triplicate. Statistical significance testing was performed by comparing the maximum specific growth rate of the colistin-susceptible strain 24, with those of the colistin-resistant strains through a parametric one-way ANOVA test, with a Dunnett's multiple comparisons test. Family-wise significance was defined as a p-value < 0.05. No statistical significant differences were observed between the maximum specific growth rates of the strains.

Supplemental Table 1. Oligonucleotides used in this study.

Supplemental Table 2. Strains used for phylogenetic analyses. A total of 178 *E. coli* and 56 *K. aerogenes* genome sequences obtained from NCBI databases were used for construction of phylogenetic trees. When multiple assemblies had the same strain name, a numerical indicator was added.

Timeline plot showing days since ICU admission (0 to 75) for five patients (Patient 37, Patient 307, Patient 27, Patient 31, Patient 311). The plot displays various events (circles, triangles) corresponding to the timeline.

Days since ICU admission	Patient 37	Patient 307	Patient 27	Patient 31	Patient 311
0		Green circle			
1	White diamond			White diamond	
2					Orange circle
3		White diamond	Orange triangle		
4	White diamond				
5					
6		White diamond		White diamond	
7			White diamond		
8	White diamond				
9				Red circle	White diamond
10		Black dot		Red circle	
11	White diamond				
12					
13				Red circle	
14					
15	Blue triangle				
16				Red circle	
17					
18	Blue circle				
19	Blue circle				
20					
21					
22	White diamond				
23				Red circle	
24				Red circle	
25				Red circle	
26				Red circle	
27				Red circle	
28				Red circle	
29				Red circle	
30				Red circle	
31				Red circle	
32				Red circle	
33				Red circle	
34				Red circle	
35				Red circle	
36				Red circle	
37				Red circle	
38				Red circle	
39				Red circle	
40				Red circle	
41				Red circle	
42				Red circle	
43				Red circle	
44				Red circle	
45				Red circle	
46				Red circle	
47				Red circle	
48				Red circle	
49				Red circle	
50				Red circle	
51				Red circle	
52				Red circle	
53				Red circle	
54				Red circle	
55				Red circle	
56				Red circle	
57				Red circle	
58				Red circle	
59				Red circle	
60				Red circle	
61				Red circle	
62				Red circle	
63				Red circle	
64				Red circle	
65				Red circle	
66				Red circle	
67				Red circle	
68				Red circle	
69				Red circle	
70				Red circle	
71				Red circle	
72				Red circle	
73				Red circle	
74				Red circle	
75	Black dot