Genomic Epidemiology of Colistin-resistant Enterobacterales from Dutch Patients: A Prospective Matched Case-control Study

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Genomic epidemiology of colistin-resistant Enterobacterales from Dutch patients: a prospective matched case-control study

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Abstract

Colistin is a last-resort treatment option for infections with multidrug-resistant Gram-negative bacteria. However, colistin resistance is increasing. A six-month prospective matched case-control study was performed in which 22 Dutch laboratories with 32 associated hospitals participated. Laboratories were invited to send a maximum of five colistin-resistant *Escherichia coli* or *Klebsiella pneumoniae* (COLR-EK) isolates and five colistin-susceptible isolates (COLS-EK), matched on patient location, material of origin and bacterial species. After confirmatory tests, 72 COLR-EK/COLS-EK pairs (75% *E. coli* and 25% *K. pneumoniae*) were included. Twenty-one percent of COLR-EK patients had received colistin, in contrast to 3% of COLS-EK patients (OR>2.9). Of COLR-EK isolates, five contained *mcr-1* and two *mcr-9*. One isolate lost *mcr-9* after repeated sub-culturing, but retained colistin resistance. Among 46 sequenced COLR-EK isolates, genetic diversity was large and 19 (41.3%) isolates had chromosomal mutations potentially associated with colistin resistance. In conclusion, colistin resistance is not rare in the Netherlands and caused by the *mcr* gene in a minority of COLR-EK isolates.

Introduction

Multidrug-resistant Gram-negative bacteria are rapidly emerging worldwide.[1-3] Several countries in Europe report carbapenem resistance percentages above 10% in *Klebsiella pneumoniae*. It is observed less frequently in *Escherichia coli*.[3] The polymyxin colistin is a last resort treatment option against severe infections by multi-drug resistant Gram-negative organisms (MDRO) and is increasingly used. However, colistin is potentially neuro- and nephrotoxic when administered parenterally.

Colistin has been used for decades for the prevention and treatment of infections caused by Enterobacterales in livestock.[4,5] In humans in the Netherlands, colistin is mainly used as part of the treatment of infections with *Pseudomonas aeruginosa* in nebulised form in patients with pulmonary diseases such as cystic fibrosis, as well as for topical treatment of otitis externa and ophthalmic infections.[4,6] In addition, colistin is used in prophylactic antibiotic regimens as a component of selective decontamination of the digestive tract (SDD) or selective oropharyngeal decontamination (SOD) with the aim to reduce infections and mortality in ICU-admitted patients and in neutropenic patients with a haematological disease.[7] Colistin is also used as last-resort treatment for MDRO.

Colistin resistance is increasing worldwide [8-10] and this poses problems in treatment of infections with MDRO. *K. pneumoniae* is the species most commonly involved in the development of colistin

resistance.[11] Among 646 carbapenem-resistant *K. pneumoniae* found in Europe in 2013-2014, 28% was tested colistin-resistant.[12] Outbreaks of carbapenemase (*bla*$_{NDM-1}$ and *bla*$_{OXA-48}$)-producing and colistin-resistant *K. pneumoniae* have been reported in Europe and highlight the emerging threat that humans are currently facing.[13] The prevalence and incidence of colistin resistance is difficult to assess, as colistin susceptibility testing is usually not part of the initial routine testing panel for Enterobacterales and is methodologically challenging with several methods producing unreliable results.

Several chromosomal mutations in bacteria can lead to colistin resistance. For *K. pneumoniae*, chromosomal mutations leading to colistin resistance have been intensively studied. Mutations in the *pmrAB, phoPQ, mgrB* and *crrB* genes are important mechanisms leading to resistance. In *E. coli*, evidence on the role of chromosomal mutations in colistin resistance is scarce.[14] Colistin resistance in *E. coli* strains has been linked to *phoPQ* and *pmrAB* genes, but experimental validation is mostly lacking.[14] The risk for spread of colistin resistance is further increased by transferable plasmid-mediated colistin resistance (*mcr*) genes that can transmit colistin resistance more easily between bacteria, including bacteria from different species.[15] Until now, *mcr* genes 1 to 10 have been discovered. Notably, *E. coli* is the most abundant *mcr*-containing species.[16,17] Colistin resistance by chromosomal mutations and *mcr* genes is mostly caused by adding cationic groups to the lipid A moiety of lipopolysaccharide (LPS) which establishes resistance to the cationic colistin.[18] Colistin resistance may be triggered directly by selection during treatment with colistin[11] or indirectly during treatment with other antibiotics by co-transfer of the *mcr* gene with other resistance genes on the same plasmid or different plasmids.[19,20]

Little is known about the genomic epidemiology of colistin resistance in the Netherlands. One outbreak with colistin-resistant carbapenemase-producing *K. pneumoniae* has been described.[21]

The objectives of this study were to determine the incidence, molecular characteristics and risk factors of colistin-resistant *E. coli* or *K. pneumoniae* (COLR-EK) isolates and/or transmissible resistance elements in the Netherlands.

Results

Twenty-two Dutch laboratories, providing services for 32 hospitals and/or primary care, participated in this project, covering all but two NUTS-2 (nomenclature of territorial units for statistics) regions in the Netherlands (NL23 and NL21). Colistin resistance was found in nine of ten participating NUTS-2 regions.

Characteristics of patients

Of the 22 participating laboratories, 17 have found COLR-EK isolates that met the inclusion criteria for this study. Of these 17 laboratories, COLR-EK isolates obtained from 72 patients with matching colistin-susceptible *E. coli* or *K. pneumoniae* (COLS-EK) isolates from 72 other patients were confirmed to be colistin-resistant at the RIVM and were included in this study, implying a median of 3 (IQR 2-6) patients per laboratory. Some laboratories sent more than the maximum of five COLR-EK isolates and these were also included when inclusion criteria were met. Characteristics of the patients are shown in Table 1. Most isolates were derived from urine samples (n=55 per group, 76.4%). Furthermore, 42 (58.3%) isolates were collected in hospitals, 24 (33.3%) in general practices and 6 (8.3%) in other healthcare facilities. Of 43 COLR-EK patients with available information on colistin use, 9 (20.9%) had received colistin in the previous six months, significantly more than 40 COLS-EK patients in which only a single patient (2.5%) had received colistin, resulting in a univariate odds ratio (OR) of 58.3 (95% confidence interval (CI) 2.9–1158.7; p=0.008). Of those ten patients who had used colistin recently, two developed an infection caused by COLR-EK, located in the urinary tract. Colistin was used for SDD/SOD in all ten cases. Among 26 hospitals that provided information on colistin use in SDD/SOD, 20 (76.9%) hospitals provided SDD/SOD medication with colistin. Use of other antibiotics in the previous six months was observed in 73.2% of COLR-EK and 55.9% of COLS-EK patients (OR 2.8; 95% CI 0.9–8.8). In the COLR-EK group, 22.2% had a malignancy, which was significantly higher than in the COLS-EK group with 10.3% (OR 4.7; 95% CI 1.1 – 19.6; p=0.033). However, the effect disappeared after correction for colistin use (OR 2.6; 95% CI 0.4-15.8). Four of 14 (28.6%) patients with known malignancies and data on colistin use had used colistin compared to none among 38 patients without malignancies.

Testing for colistin resistance

Among the 22 participating laboratories, colistin susceptibility testing policies differed substantially. Nine laboratories tested all Enterobacterales for colistin resistance. Seven laboratories tested for colistin resistance only for special reasons, i.e. when colistin was considered as treatment option (n=2) or in case of a combination of factors, such as specific bacterial species and certain patient groups (n=5). One laboratory never tested for colistin resistance except for this study. No information on the testing

policies was available for the five remaining laboratories. Table 2 depicts the tests in use and the indications.

Among 18 participating laboratories that reported numbers of detected COLR-EK, 8,243 *K. pneumoniae* and 45,508 *E. coli* isolates were identified during the study period of which 81.3% and 82.2% were tested for colistin susceptibility, respectively. Among 6,705 tested *K. pneumoniae* isolates, 42 (0.6%) were found colistin-resistant by participating laboratories. Among 37,392 tested *E. coli* isolates, 130 (0.3%) were tested colistin-resistant. When combined, 0.4% were tested colistin-resistant from both species.

For selection of COLR-EK and COLS-EK isolates for the current study, some laboratories used other colistin susceptibility testing policies than those used for regular practice. Table 3 shows the used colistin susceptibility testing methods for the submitted isolates and the number of submitted isolates with the percentage of isolates that were confirmed at the Dutch National Institute for Public Health and the Environment (RIVM). Fifteen of 22 laboratories based isolate submission only on screening test results (mostly automated tests). As expected, these laboratories had a lower percentage of confirmed isolates compared to laboratories that based submission of isolates on subsequent confirmation tests. In total, 119 presumed COLR-EK and 92 presumed COLS-EK isolates of 17 laboratories were received by the RIVM. Of these, 72 COLR-EK (60.5%) were confirmed to be colistin-resistant and 72 COLS-EK (78.3%) to be susceptible by broth microdilution (BMD) test and were included in the study. This included 18 *K. pneumoniae* and 54 *E. coli* per group. In addition, six COLR-EK isolates, including two *K. pneumoniae* and four *E. coli* isolates, were only excluded because there was no matching COLS-EK isolate. A flow chart is provided in Figure 1.

The minimal rate of carbapenem-susceptible colistin-resistant *K. pneumoniae* and *E. coli* was 0.01 and 0.03 per 10,000 person years in 21 hospitals with available data, whereas the rate in inpatients in 30 hospitals with available data was 0.20 and 0.41 per 10,000 patient days, respectively.

Characteristics of isolates

Thirty-three *E. coli* and 13 *K. pneumoniae* COLR-EK/COLS-EK pairs were analysed by next-generation sequencing (NGS). Whole genome multilocus sequence typing (wgMLST) indicated a diverse population for both *E. coli* and *K. pneumoniae* (Figure 2 and 3). The median allelic differences in the minimum spanning trees were large, namely 825 (IQR 294-2,921) for *E. coli* and 3,548 (IQR 3,360-

3,583) for *K. pneumoniae*. Only a single genetic cluster of two colistin-resistant *K. pneumoniae* isolates of patients from different regions submitted by two different laboratories was found.

Microbiological characteristics of all sequenced isolates are shown in Supplementary Table 1 and 2. Among the total of 26 sequenced *K. pneumoniae* strains, the capsular serotypes (K-antigen) and MLST types were highly diverse. LPS (O-antigen) serotypes were less diverse. The only detected gene encoding a virulence factor for *K. pneumoniae* in this study was yersiniabactin, a siderophore, detected in five COLR-EK isolates (38.5%) and two COLS-EK isolates (15.4%; OR 3.6 (95% CI 0.5-24.2)). The 66 sequenced *E. coli* strains were also highly diverse in serotypes and MLST types. The well-known multi-drug resistant ST131 was observed in eight isolates (n=5 COLR-EK of which one with *mcr-1*, n=3 COLS-EK), of which seven were derived from urine samples. Numerous different genes encoding virulence factors were observed in *E. coli* isolates, but there were no significant differences between the colistin-resistant and -susceptible group. When only urine samples were included (n=46; 23 per group), there were also no significant differences.

Colistin resistance

The median colistin minimum inhibitory concentration (MIC) by BMD was 8.0 mg/L (IQR 4.0-12.0) in the COLR-EK and 0.25 mg/L (IQR 0.25-0.5) in the COLS-EK group.

Colistin resistance caused by mcr *genes*

Of 72 COLR-EK isolates, *mcr* genes were found in seven (9.7%) isolates. Of 18 colistin-resistant *K. pneumonia* isolates, two contained *mcr-9* and one *mcr-1*, whereas, of 54 colistin-resistant *E. coli* isolates, four contained *mcr-1*. The isolates containing *mcr-1* had a colistin MIC of 2, 4 or 16 mg/L with BMD, whilst both *mcr-9* isolates had a colistin MIC of 32 mg/L. We attempted to cure the latter two isolates from their *mcr-9* genes by repeated subculture and were successful for one. Despite the loss of the *mcr-9* gene, the isolate retained its high MIC for colistin. No new chromosomal mutations in the *pmrAB*, *phoPQ* and *mgrB* genes were found after curing. NGS and third-generation sequencing (TGS) of the isolate revealed that 39,237 base pairs including the *mcr-9* gene were deleted from the plasmid. Molecular characterisation of the five *mcr-1* positive isolates, revealed that *mcr-1* of one isolate (MIC=4 mg/L) was two base pairs shorter than the reference. Whether the gene was still functional is unknown.

Table 4 shows the results of NGS and hybrid assembly of the isolates with *mcr* genes. The isolates with *mcr* genes all had different MLST types, capsular serotypes and LPS serotypes, which indicates different genetic backgrounds. Figure 4 shows characteristics of the five *mcr*-containing plasmids with available hybrid assembly data (IDs starting with RIVM) and previously found plasmids from the NCBI database that highly or completely resembled them. The plasmids with *mcr* genes from this study contained varying %G+C content and varying replicon families, more specifically *mcr-1* plasmids included replicons IncHI2 or no replicon, and the *mcr-9* plasmids replicons IncHI2, IncHI2A and IncR. The *mcr* plasmids were unrelated (<64% identity). The *mcr-9* plasmids from two *K. pneumoniae* isolates appeared different, since the antimicrobial resistance (AMR) genes and the plasmid size differed substantially, though they both contained ESBL gene $bla_{\text{CTX-M-9}}$. The *mcr-1* plasmids of *E. coli* did not contain other AMR genes and differed in size, whereas the *mcr-1* plasmid of *K. pneumoniae* contained several other AMR genes and was considerably larger than the *mcr-1* plasmids from *E. coli*. The previously identified plasmids from the NCBI database were found in Spain, USA, Qatar and Brazil. Two patients with isolates harbouring both *mcr-1* visited a foreign country in the previous six months (Egypt and an unknown country) and they had both been hospitalised in that country.

Chromosomal mutations potentially associated with colistin resistance

Table 5 shows the isolates with *mcr* genes or chromosomal mutations in the *pmrAB, phoPQ, mgrB* and *crrB* genes, that are potentially associated with colistin resistance. Chromosomal mutations that were present in COLS-EK isolates only or in both COLS-EK and COLR-EK isolates were removed and the entire list of chromosomal mutations can be found in Supplementary Table 3. Of 46 sequenced COLR-EK isolates, 19 (41.3%) had a chromosomal mutation potentially associated with colistin resistance. Of these, two also had an *mcr* gene, i.e. one with *mcr-1* and one with *mcr-9*. Among the 13 sequenced colistin-resistant *K. pneumoniae* isolates, eight isolates with chromosomal mutations were found. Mutations were mainly found in the *mgrB* gene, with the majority being insertions. Among the 33 sequenced colistin-resistant *E. coli* isolates, 11 chromosomal mutations were found, mainly in the *pmrB* gene.

Antimicrobial resistance for other antibiotics

There were significantly more MDRO in the COLR-EK group (17/72 (23.6%)) compared to the COLS-EK group (6/68 (8.8%)), with an OR of 3.9 (95% CI 1.3-12.2; *p*=0.019). Of COLR-EK isolates, 19.7% (14/71) was ESBL-producing, which was significantly more than the 7.4% (5/68) of the COLS-EK group (OR 3.5; 95% CI 1.1-11.6; *p*=0.037). For *E. coli*, the 33 sequenced COLR-EK isolates contained a median of 3 (IQR 1:7) AMR genes per isolate and 1 (IQR 1:4) per isolate in the COLS-EK group. Similarly, the 13 *K. pneumoniae* isolates had a median of 5 (IQR 4:11) AMR genes per isolate for the COLR-EK isolates and a median of 4 (IQR 4:7) for the COLS-EK isolates.

Discussion

This study showed that colistin resistance among Enterobacterales cultured from patients is not uncommon in the Netherlands. Patients with COLR-EK isolates had more frequently used colistin in SDD/SOD, compared to COLS-EK patients. WgMLST revealed there was no comprehensive dissemination of highly similar colistin-resistant strains. Only seven (9.7%) of the COLR-EK isolates carried *mcr* genes, including five with *mcr-1* and two with *mcr-9*, whereas 19 (41.3%) sequenced COLR-EK isolates had a chromosomal mutation potentially associated with colistin resistance. Interestingly, the *mcr-9* gene did not elicit phenotypical colistin resistance in our cohort. Furthermore, the COLR-EK group contained more MDRO compared to the COLS-EK group. This could potentially be explained by more previous antibiotic use (at any moment in time) in the COLR-EK group.

In this study, 0.4% of tested isolates were found colistin-resistant by participating laboratories. This was in complete accordance with data from the Dutch Infectious Disease Surveillance Information System-Antibiotic Resistance (ISIS-AR)[22]. In the same study period, 40 laboratories that provided results from screening tests and/or confirmation tests for ISIS-AR identified 0.4% (290/71,839) of tested *K. pneumoniae* and *E. coli* isolates as colistin-resistant. This indicates that the laboratories that participated in this study were a good representation of all laboratories in the Netherlands.

This study provides important insights into the epidemiology of colistin resistance in the Netherlands. Most previous studies examining colistin resistance in humans in the Netherlands focused on the *mcr-1* gene,[23-25] specific patient populations[26-31] or travelers.[32-34] Similar to the current study, a higher percentage of colistin resistance caused by chromosomal mutations compared to *mcr* genes was also found by Bourrel *et al.,*[35] who screened patients in six Parisian hospitals for rectal carriage of colistin-resistant *E. coli*.

Interestingly, we found the presence of *mcr-9* not to be associated with phenotypical resistance. Some other studies also reported a minor impact of *mcr-9* on the colistin MIC,[36-38] although the MIC may be increased in the presence of colistin by the higher expression of *qseC* and *qseB* genes.[39] The isolate in our study, that was cured from the *mcr-9* gene also had a mutation in the *mgrB* gene, which led to an amino acid difference (C39Y). Possibly, this mutation caused colistin resistance, but information on this mutation is scarce.[14,40]

We found several chromosomal mutations presumed to be associated with colistin resistance. For *K. pneumoniae*, most chromosomal mutations that were only found in colistin-resistant isolates were verified in experimental settings in previous studies. The most frequent observed chromosomal mutation was an insertion in the chromosomal *mgrB* gene, which is often described as cause of colistin resistance in *K. pneumoniae*.[40-45] For *E. coli*, the involvement of chromosomal mutations in colistin resistance is mostly not confirmed by laboratory experiments.[14]

We found that previous colistin use was associated with colistin resistance, which is in accordance with several previous studies.[11] In our study, colistin was used only as component of SDD/SOD to prevent development of infections in specific patient groups, such as patients with a haematological disease or patients staying at the intensive care. Though most studies showed SDD/SOD is effective in reducing the number of infections in specific patients groups,[46] it is only used in a small number of countries.[47,48] The use of SDD/SOD remains a controversial topic mainly because of the potential selection and spread of multi-drug resistant bacteria. Numerous studies found no difference in presence of colistin resistance during SDD/SOD compared to controls without SDD/SOD,[28,49-53] but there are also studies that suggest an increase in colistin resistance during SDD/SOD.[30,54] Most studies examined colistin resistance during or shortly after the use of SDD/SOD, but our data suggest that a longer period is needed to detect colistin resistant bacteria, which may be relevant for future clinical trials.

Another factor found to be associated with colistin resistance in the univariate analysis in this study was malignancy, but this effect disappeared after correction for colistin use. Colistin is also used as SDD in patients with haematological malignancies, which may explain the increased colistin resistance in these patients. A previous study suggested that the use of colistin may be involved in the pathogenesis of colorectal cancer by stimulating the production of colibactin in procarcinogenic bacteria,[55] but the design of our study does not provide sufficient data to evaluate this. We examined the presence of colibactin-

producing genes in our 66 sequenced *E. coli* isolates and found a *pks*-island in 21 isolates, of which 12 were colistin-resistant. However, all *pks*-islands were not completely present.

The detection of 72 confirmed colistin-resistant isolates from 17 of 22 participating laboratories in this study, with an underestimation of the occurrence due to the exclusion of CRE and restricted testing in participating laboratories, shows that colistin-resistant isolates are not rare in the Netherlands and therefore there is a need for surveillance of routine diagnostic AST. This is further emphasised by the increasing colistin resistance worldwide and has been recognised by ECDC.[8-10] As a result, standardisation of colistin susceptibility testing will become more important. Unfortunately, colistin-susceptibility testing with EUCAST and CLSI-recommended BMD[56] is frequently not part of the routine antimicrobial susceptibility testing (AST) panel for Enterobacterales,[3] as also observed in this study. Furthermore, only 52.9% of the participating laboratories screened all Enterobacterales for colistin resistance. These laboratories used automated systems as screening test, which are inferior compared to BMD.[57] Unfortunately, many laboratories are reluctant to use BMD for all *E. coli* and *K. pneumoniae* isolates because the application is very labour intensive. However, high very major error rates (producing false susceptible results) are reported for automated systems and they are therefore not suitable as screening test. Major error rates (producing false-resistant results) are low.[57,58] In contrast, 24.4% (29/119) of by participating laboratories presumed COLR-EK isolates were rejected in this study due to colistin MIC ≤2 mg/L and only 2.2% (2/92) COLS-EK isolates were rejected due to MIC>2 mg/L. The majority of the laboratories sent in isolates for this study based on automated testing methods. This suggests a high major error rate of automated systems in Dutch medical microbiology laboratories (MMLs). In addition to surveillance of routine diagnostic AST, testing of all *E. coli* and *K. pneumoniae* isolates for colistin resistance with methods that are more reliable dan automated testing methods, is needed. Possibly, the Rapid Polymyxin NP test may be a good alternative for automated testing, since it is less labour intensive.[40,59]

This study provided important insights into the presence of COLR-EK in humans in the Netherlands, the current susceptibility testing policies, distribution of strains and genomic characteristics of colistin-resistant Enterobacterales. However, there were also a few limitations. This study may have underestimated the true incidence of colistin resistance, since carbapenem-resistant Enterobacterales (CRE) isolates were not included. However, data from ISIS-AR showed that only 1.4% of colistin-resistant isolates were carbapenem-resistant with an MIC >8 mg/L. Second, several laboratories only

tested a selection of isolates for colistin resistance and provided a maximum of five COLR-EK isolates per laboratory. Finally, persons that filled in the questionnaires were not blinded for colistin susceptibility testing results and therefore data on colistin use that were missing not at random cannot be ruled out. In conclusion, COLR-EK isolates are present in the Netherlands and colistin resistance is caused by *mcr* genes in a minority of isolates. This study suggests that diverse COLR-EK populations for both *E. coli* and *K. pneumoniae* are present. In the future, surveillance of routine diagnostic AST with detection of *mcr* genes and molecular typing should be implemented to monitor and control the occurrence and spread of colistin resistance and *mcr* genes. For this, testing of all *E. coli* and *K. pneumoniae* isolates with more reliable testing methods by local laboratories is needed.

Methods

A prospective matched case-control study with density-based sampling was performed. This project took place between May 2019 and February 2020 and was part of a pan-European multicentre study on colistin- and carbapenem-resistant Enterobacterales of the European Centre for Disease Prevention and Control (CCRE survey).[60]

Participating laboratories

Twenty-two Dutch MMLs providing services for 32 hospitals, participated in this project using the infrastructure of a web-based laboratory network, called Type-Ned, which is used for the national carbapenemase-producing Enterobacterales surveillance in the Netherlands.[61] Laboratories were selected based on NUTS-2 regions of the associated hospitals. In the Netherlands, the provinces represent the NUTS-2 regions. At least one hospital site per NUTS-2 region had to be included.[62] Participating hospitals had to offer acute care services.

Study population and isolates

MMLs were requested to send a maximum of five COLR-EK isolates with a MIC >2 mg/L for colistin and/or a *mcr* gene that were collected in a six-month period. In line with the European CCRE survey guidelines, only isolates not producing carbapenemases and with a meropenem MIC ≤0.25 mg/L were included. Controls were selected with density-based sampling: for each COLR-EK, the first following COLS-EK with a colistin MIC ≤ 2mg/L and a meropenem MIC ≤ 0.25 mg/L matched with the COLR-

EK on patient location (sender of the isolate: from community or hospital), patient material and bacterial species, was requested. Only a single isolate per patient was included in the study. MMLs were asked to send isolates which they classified as COLR-EK and COLS-EK, based on their routine susceptibility testing.

Confirmatory antimicrobial susceptibility testing

Microbiological confirmation of all submitted isolates was performed at the RIVM. Species assignments were confirmed by MALDI-TOF (Microflex LT System; Bruker, Leiderdorp, Netherlands). Colistin resistance was confirmed using a standardised BMD (Micronaut MIC strip colistin, Merlin) using an ECDC-recommended protocol[63] including one positive control (NCTC 13846) and three negative controls (ATCC 25922, ATCC 27853 and ATCC 700603). The presence of *mcr* genes was assessed by using two specific in-house multiplex PCRs for *mcr-1* to *mcr-5* genes and for *mcr-6* to *mcr-8* genes. The absence of carbapenem resistance and carbapenemase production/genes was assessed as described before.[61] AST of colistin and meropenem was performed according to EUCAST detection guidance and breakpoints.[56,64]

COLR-EK and COLS-EK isolates were classified as MDRO or non-MDRO, based on antibiograms received by participating laboratories (mostly results of VITEK automated testing). MDRO was defined according to definitions of the Dutch Working Group on Infection Prevention [65]: *E. coli* or *K. pneumoniae* that are ESBL-producing, that are resistant to both a fluoroquinolone and an aminoglycoside or that produce carbapenemases. ESBL-production was defined as resistance to ceftazidime and/or either cefotaxime or ceftriaxone with additional resistance to cefepime or susceptibility to cefoxitin. When results of Etest ESBL strips or combination disc methods[66] were available, these were used to define ESBL-producers.

Genomic analysis

Forty-six COLR-EK/COLS-EK pairs were subjected to NGS to assess genetic relatedness of strains, serotypes and presence of chromosomal mutations leading to colistin resistance, AMR genes, plasmid replicons and virulence factors. The selection of isolates for NGS was based on a minimal time between sample dates of a colistin-resistant and the matched colistin-susceptible isolate, good representativeness of both *E. coli* and *K. pneumoniae* isolates and a diverse selection of geographic

locations. NGS, TGS and wgMLST were performed as described before.[67] In short, genetic relatedness was assessed by wgMLST and genetic clusters were defined as collections of isolates with a maximum of 25 alleles differences for *E coli a*nd 20 for *K. pneumoniae*.

AMR genes were identified via ResFinder software and only AMR genes with ≥97% sequence identity with the reference sequences were included. The presence of *mcr* genes 1 to 10 was also analysed using BLAST (CLC Genomics Workbench version 20.0.3; Qiagen Bioinformatics, Aarhus, Denmark). The presence of plasmid replicons was assessed using PlasmidFinder software, including only replicons with 100% sequence identity and that were completely present. For the identification of serotypes and virulence factors, VirulenceFinder, SerotypeFinder and Kleborate (https://github.com/katholt/Kleborate) were used. Raw NGS sequence data of all sequenced isolates were deposited in the European Nucleotide Archive at the European Molecular Biology Laboratory-European Bioinformatics Institute under accession number PRJEB46966 and plasmids with *mcr* genes in GenBank of the National Centre for Biotechnology Information (NCBI) under BioProject ID PRJNA754858.

Isolates carrying *mcr* genes were subjected to TGS (Oxford Nanopore, Oxford, United Kingdom). The NGS and TGS data were used in Unicycler hybrid assemblies[68] to reconstruct chromosomes and plasmids, which were annotated by Prokka[69]. Only contigs of >2.5 kb were analysed in this study. Plasmids containing *mcr* genes were compared with each other using chromosome comparison in BioNumerics and with other previously found *mcr*-containing plasmids in NCBI BLAST.

Isolates with the *mcr-9* gene were repeatedly subcultured, with the aim to cure the isolates from the *mcr-9* gene. The absence of the *mcr-9* gene was examined using PCR. In case absent, BMD was performed simultaneously on the cured and non-cured isolate and both isolates were subjected to NGS and TGS.

To identify mutations that may potentially lead to colistin resistance, NGS reads were mapped in CLC Genomics Workbench version 20.0.3 against the gene sequences of *pmrAB, phoPQ, mgrB* and *crrB*, present in the NCBI sequence database (references for *K. pneumoniae*: *crrB* - KY587106, *mgrB* - MN187248, *phoQ* - KY587110, *pmrA* - MG243721, *pmrB* - KJ626267, *phoP* - KY587067; *E. coli*: PhoP - NZ_CP038353-Eco, PhoQ - NZ_CP038353-Eco, PmrA - NZ_CP038353-Eco, PmrB - NZ_CP038353 Eco).

Metadata collection

Clinical and epidemiological data were extracted from the electronic patient medical records by the participating laboratories and entered into Type-Ned. Microbiological data (including no of detected, tested and positive *K. pneumoniae* and *E. coli* isolates and the test on which submission of isolates was based), extracted from the local laboratory information system, and general hospital data (denominator data and use of colistin in SDD/SOD) were entered into web-based questionnaires. In addition, a questionnaire on local laboratory testing policies was composed in collaboration with ISIS-AR prior to start of this study and these data were extracted from ISIS-AR for participating laboratories after this study.[22]

The minimal rate of carbapenem-susceptible COLR-EK isolates was calculated as the number of colistin-resistant *E. coli* and *K. pneumoniae* isolates, confirmed for this study, divided by the number of patient days or person years, provided by the participating laboratories (cases of hospitals without provided denominator data were subtracted).

Ethical permissions and privacy. The medical ethical committee of the University Medical Centre Utrecht has defined this study (19/262) as not falling under the scope of the Dutch law 'Wet medische-wetenschappelijk onderzoek' ("Niet WMO-plichtig"). The collection and storage of data complied to the General Data Protection Regulation (EU 2016/679).

Statistics and data presentation

Data are presented as n (%) for categorical variables and mean (standard deviation) or, for variables that have a skewed distribution, median and interquartile range (IQR) [first quartile-third quartile] for numerical variables. Categorical variables with characteristics of matched COLR-EK and COLS-EK isolates were compared using univariate logistic regression with correction for variables used for matching (patient location, material of origin and bacterial species) and the OR with 95% CI were calculated. When necessary, a multivariate logistic regression was performed with additional independent variables. For numerical variables with non-normal distribution, the Wilcoxon signed rank test was used. For the comparison of virulence factors between colistin-resistant and -susceptible *E. coli*, data was corrected with a Bonferroni multiple testing correction. A two-sided p-value of <0.05 was considered statistically significant. In all analyses, a complete case analysis was performed. The

number of patients with available data per variable are mentioned. Our hypothesis is that missing data were mostly missing at random, due to the substantial workload or difficulties to find certain information in the electronic patient files (most missing data were observed in variables, such as antibiotic use or colistin use in the previous six months and a profession with direct patientcare). However, persons that filled in the questionnaire were not blinded for colistin susceptibility testing results and therefore missing not at random cannot be ruled out. STATA SE version 15.1 (StataCorp, College Station, TX, USA) was used for data-analysis.

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Author contributions

The study was conceived and supervised by LMS and EJK. KEWV coordinated the study, performed part of the analysis and drafted the study protocol and the manuscript. ADH performed and coordinated the laboratory experiments and performed TGS and hybrid assembly. SW analysed the NGS-data. APAH analysed *mcr* plasmid data. APAH, DWN, PB, AFS, SCDG and CCHW aided in composing the study protocol. JJG provided advise on the statistics. CCHW, AFS and SCDG provided advise on epidemiological analysis. All authors critically reviewed the manuscript. The ColRE survey study group collected and sent the isolates and provided isolate, patient, hospital and laboratory data.

Competing interests

The authors declare no competing interests.

Data availability

Raw NGS sequence data of all sequenced isolates were deposited in the European Nucleotide Archive at the European Molecular Biology Laboratory-European Bioinformatics Institute under accession number PRJEB46966 and plasmids with *mcr* genes in GenBank of the National Centre for Biotechnology Information (NCBI) under BioProject ID PRJNA754858. Microbiological characteristics of all sequenced isolates are shown in Supplementary Table 1 and 2. The epidemiological datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request. Laboratory and hospital data from questionnaires included in this study and from ISIS-AR are available from the corresponding author upon reasonable request and with permission of the participating laboratories.

Code availability

The STATA SE syntax to reproduce the epidemiological analyses reported in this paper are available from the corresponding authors upon request.

Figures

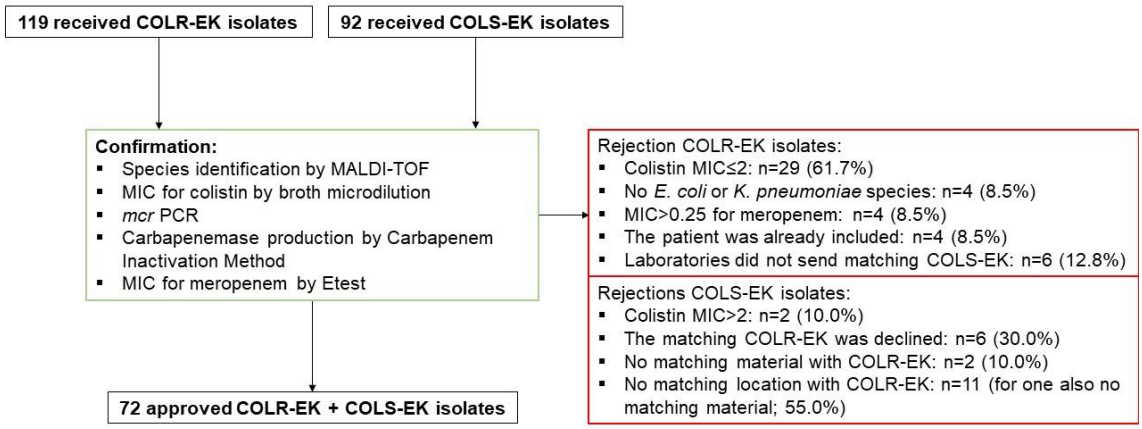


Figure 1. Flow chart. Abbreviations: COLR-EK: colistin-resistant *E. coli* or *K. pneumoniae*, COLS-EK: colistin-susceptible *E. coli* or *K. pneumoniae*, MALDI-TOF: matrix assisted laser desorption/ionisation time-of-flight analyser, MIC: minimum inhibitory concentration, PCR: polymerase chain reaction.

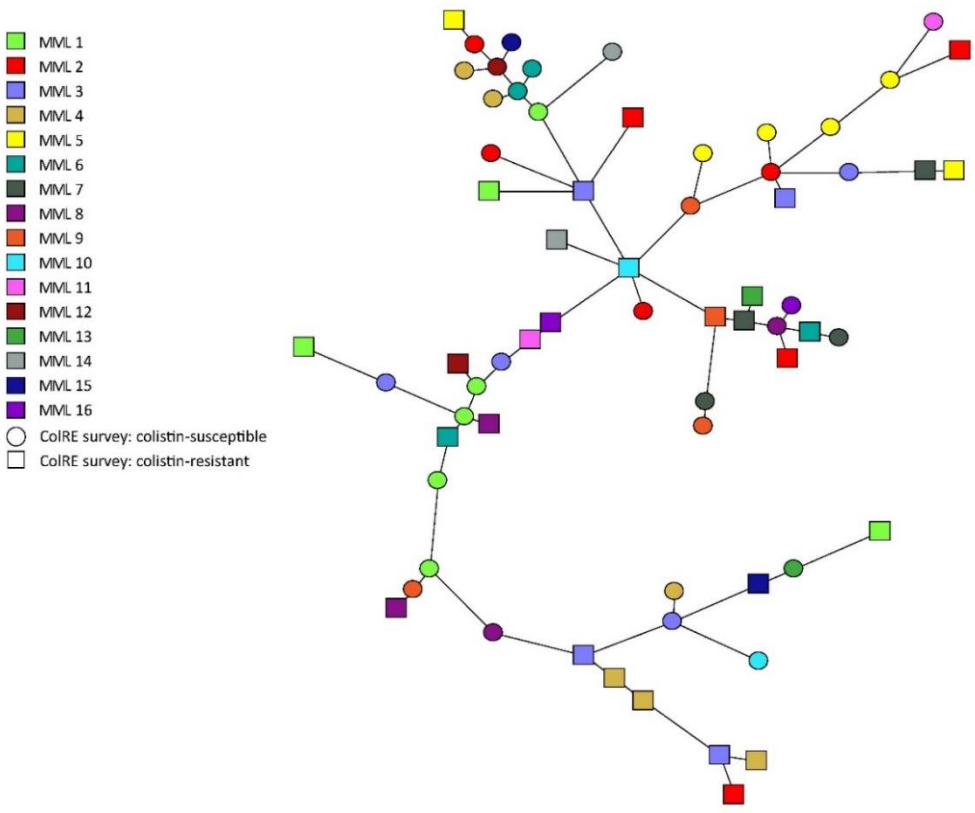


Figure 2. Minimum spanning tree of wgMLST results of 66 *E. coli* isolates. Circles and squares represent one isolate, which is connected to the closest relative. The length of the lines in between the isolates is proportional to the allelic distance. The colours represent the different participating medical microbiology laboratories. A cluster was defined as ≥2 isolates with an allelic difference of ≤25. There were no clusters observed for *E. coli*. Abbreviations: COLRE: colistin-resistant Enterobacterales, MML: medical microbiology laboratory

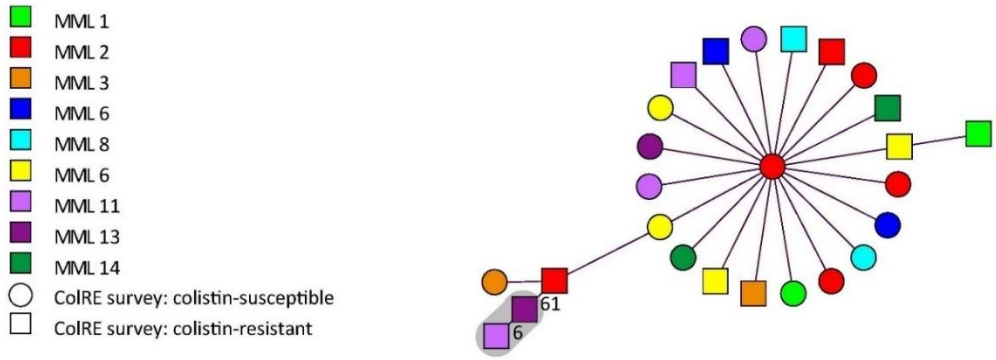


Figure 3. Minimum spanning tree of wgMLST results of 26 *K. pneumoniae* isolates. Circles and squares represent one isolate, which is connected to the closest relative. The length of the lines in between the isolates is proportional to the allelic distance. The colours represent the different participating medical microbiology laboratories. A cluster is indicated by a grey halo and is defined as ≥2 isolates with an allelic difference of ≤20. Abbreviations: COLRE: colistin-resistant Enterobacterales, MML: medical microbiology laboratory.

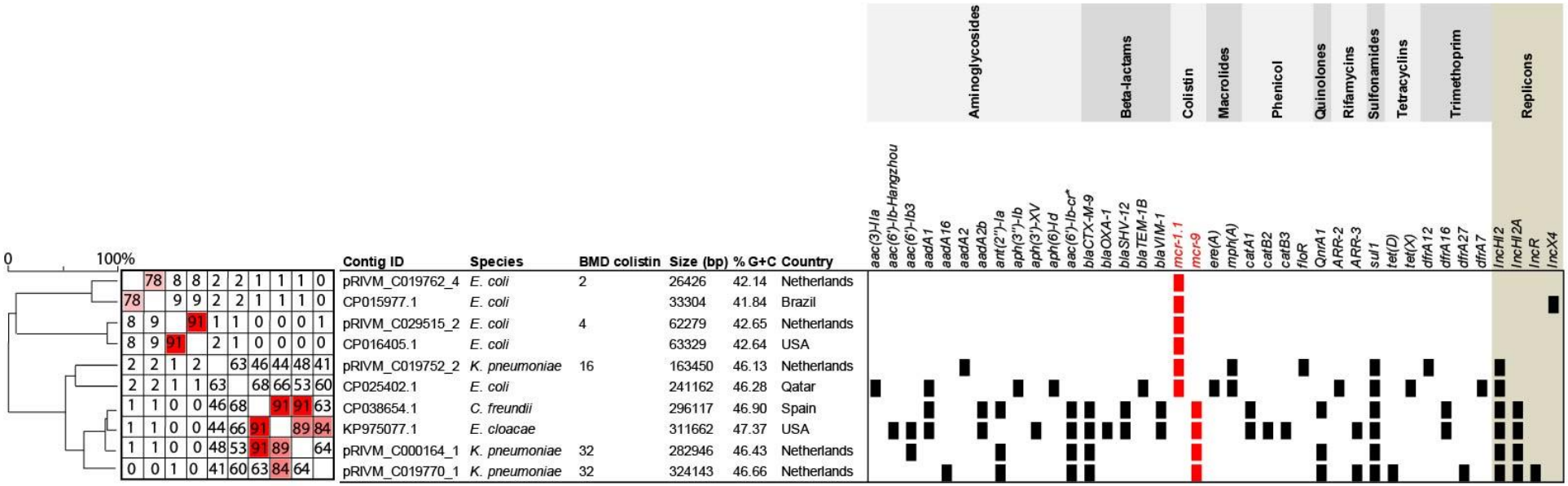


Figure 4. Comparison of plasmids containing *mcr* genes. Isolates of which the Contig ID starts with 'RIVM' are isolates from the current study. The other isolates are the closest resembling plasmids from the NCBI database. Abbreviations: BMD: broth microdilution, bp: base pairs, USA: United States of America, % G+C: guanine-cytosine content.

744 # Tables

745 **Table 1. Characteristics of patients carrying COLR-EK or COLS-EK isolates**

	COLR-EK (N=72)			COLS-EK (N=72)			Univariate logistic regression with correction for matched variables	
	N	N total	%	N	N total	%	Odds ratio*	95% CI
Median age (interquartile range)~		72	73.5 (IQR 56.0-83.0)		72	72.0 (IQR 51.5-78.0)		
Female	53	72	73.6%	53	72	73.6%	1.0	0.4 - 2.2
Species								
E. coli	54	72	75.0%	54	72	75.0%		
K. pneumoniae	18	72	25.0%	18	72	25.0%		
Material								
Urine	51	72	70.8%	52	72	72.2%		
Urine in case of bladder catheter	4	72	5.6%	3	72	4.2%		
Rectum/perineum swab	10	72	13.9%	10	72	13.9%		
Faeces	3	72	4.2%	3	72	4.2%		
Wound secretion	1	72	1.4%	1	72	1.4%		
Blood	2	72	2.8%	2	72	2.8%		
Throat swab	1	72	1.4%	1	72	1.4%		
Sender of isolate								
Hospital	42	72	58.3%	42	72	58.3%		
- inpatient[#]	27	42	64.3%	36	42	85.7%	0.2	0.1 - 0.7
- outpatient	15	42	35.7%	6	42	14.3%		
General practitioner	24	72	33.3%	24	72	33.3%		
Nursing home/Elderly home/Care centre	6	72	8.3%	6	72	8.3%		
Specialty								
Intensive care	5	72	6.9%	6	72	8.3%	0.7	0.1 – 3.5
Surgery	3	72	4.2%	1	72	1.4%	4.3	0.3 – 58.4
Other	33	72	45.8%	35	72	48.6%	0.7	0.2 - 2.4
Not admitted to the hospital	31	72	43.1%	30	72	41.7%		
Infection	54	69	78.3%	51	69	73.9%	2.7	0.5 – 15.0
Comorbidity								
Renal insufficiency	2	45	4.4%	3	58	5.2%	0.9	0.1 – 6.0
Immunosuppression	3	45	6.7%	5	58	8.6%	0.3	0.0 - 7.2
Type 2 diabetes mellitus	3	45	6.7%	5	58	8.6%	0.7	0.1 – 3.3
Chronic obstructive pulmonary disease	3	45	6.7%	1	58	1.7%	5.0	0.5 – 55.8
Malignancy[#]	10	45	22.2%	6	58	10.3%	4.7	1.1 – 19.6
Foreign visits								
<2 months ago >24 hours treated in foreign hospital	2^	72[§]	2.8%	0	71[§]	0.0%		
<6 months ago foreign visit without hospital visit or admission	0	72[§]	0.0%	2^^	71[§]	2.8%		
<1 year ago contact with foreign hospital, but not <2 months ago >24 hours treated in foreign hospital	0	72[§]	0.0%	1[$]	71[§]	1.4%		
Previous antibiotic use								
Use of colistin in past 6 months[$$ #]	9	43	20.9%	1	40	2.5%	58.3	2.9 – 1158.7
Other antibiotic use in past 6 months	30	41	73.2%	19	34	55.9%	2.8	0.9 – 8.8

Resident of nursing home/elderly home/rehabilitation centre	16	71	22.5%	9	69	13.0%	4.2	1.0 - 17.8
Contact with professionally held animals	0	72[§]	0.0%	0	71[§]	0.0%		
Profession with direct patient care	0	42	0.0%	0	41	0.0%		

* Resembling the incidence rate ratio. ~ Wilcoxon signed rank test: no significant difference. # $p<0.05$ in univariate logistic regression with correction for matched variables. ^ Egypt and unknown country. ^^ Sweden and Austria. $ Ghana. $$ all selective intestinal/oropharyngeal decontamination with colistin. § the total number of patients with known data is not certain, because there was no 'unknown' option in the questionnaire for this variable. Abbreviations: COLR-EK: colistin-resistant *E. coli* or *K. pneumoniae*, COLS-EK: colistin-susceptible *E. coli* or *K. pneumoniae*, IQR: interquartile range.

Table 2. Colistin susceptibility testing policies and methods for Enterobacterales

	Colistin susceptibility testing method		
Policy for colistin susceptibility testing	Initial test	Confirmation test	Num. of labs
Always	VITEK	BMD[1-3]	7
		Etest[4]	1
		Unknown[3]	1
Only when considering colistin as treatment	BMD	None	1
	Unknown	Unknown[3]	1
In case of a combination of factors	BMD	None	2
	Etest or BMD	None	1
	VITEK or BMD	BMD if VITEK was used[1]	1
		BMD and when ColR NGS[1]	1
Only for this study			1
No data available			5
Total			22

Confirmation test is performed when: [1] colistin is considered as treatment, [2] in case of a combination of criteria, [3] the isolate is colistin-resistant in the initial test and has certain characteristics or [4] the isolate is colistin-resistant in the initial test.

Abbreviations: BMD: broth microdilution, ColR: colistin-resistant, NGS: next-generation sequencing.

Table 3. Colistin susceptibility testing policies and confirmation of submitted isolates

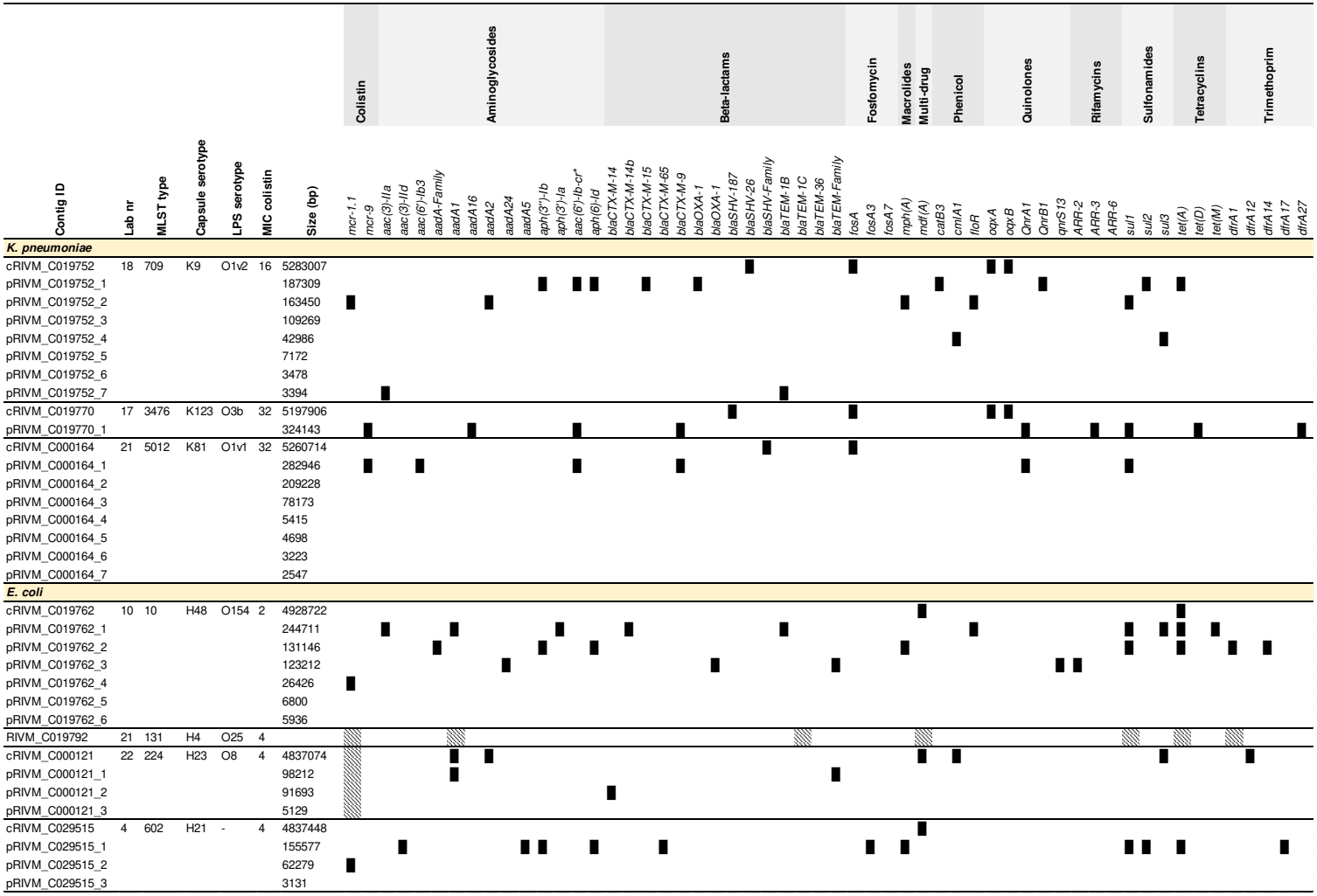
Used test result for isolate selection	Num. of labs	COLR-EK				COLS-EK			
		Colistin susceptibility testing method[1]	Submitted	Confirmed	%	Colistin susceptibility testing method[1]	Submitted	Confirmed	%
Screening test	15	Automated[2]	106	61	57.5%	Automated[3]	80	61	76.3%
Confirmation test	7	BMD/Etest	13	11	84.6%	Automated	12	11	91.7%
	22		119	72	60.5%		92	72	78.3%

Of note: table 3 also includes isolates that are rejected based on other reasons than an incorrect colistin MIC.

[1] Unknown method for the five laboratories with no submitted isolates. [2] Also two submitted isolates with disk diffusion and two isolates with an Etest. [3] Also one submitted isolate with disk diffusion.

Abbreviations: BMD: broth microdilution, COLR-EK: colistin-resistant *E. coli* or *K. pneumoniae*, COLS-EK: colistin-susceptible *E. coli* or *K. pneumoniae*.

764 **Table 4. Molecular characterisation of seven isolates with *mcr* genes.**



765

766 Contig IDs, including chromosome IDs (cxxxx) and plasmid IDs (pxxxx) are depicted on the Y-axis, with numbers after the underscore indicating the plasmid numbers (_1, _2, _3, etc), and antimicrobial

767 resistance genes with antibiotic classes are depicted on the X-axis. The presence of antimicrobial resistance genes is indicated with black boxes. Striped boxes indicate that only next-generation

768 sequencing (NGS) data is available or that a gene was only found with NGS. Abbreviations: bp: base pairs, LPS: lipopolysaccharide, mdf(A): multidrug resistance, MLST: multilocus sequence typing.

Table 5. Overview of isolates with *mcr* genes or non-silent chromosomal mutations, potentially involved in colistin resistance

Isolate ID	MIC colistin	*mcr*	*pmrA*	*pmrB*	*mgrB*	*crrB*	*phoP*	*phoQ*
K. pneumoniae								
RIVM_C019776	16	no			first 39 bp are absent			
RIVM_C019778	8	no			insertion of ISEc68 on p74			
RIVM_C019785	8	no			G37S(G109A)			
RIVM_C019837	16	no		T157P(A469C)				
RIVM_C000156	64	no			absent	deletion of 955 bp		
RIVM_C000119	64	no			insertion of IS-like element on p46			
RIVM_C019878	16	no			insertion of IS-like element on p70			
RIVM_C019770	32	*mcr-9*			C39Y(G116A)			
RIVM_C019752	16	*mcr-1.1*						
RIVM_C000164	32	*mcr-9*						
E. coli								
RIVM_C019737	8	no		V128E(T382A)				
RIVM_C019749	4	no		T159M(C475T)				
RIVM_C019767	8	no		V91E(T272A)				
RIVM_C019769	8	no						E464D(G1392T)
RIVM_C019789	8	no		extra triplet (GCG) with amino acid A (p266)				
RIVM_C019808	4	no		L105Q(T314A)				
RIVM_C019825	4	no	N67K(C200A+C201A); D68E(C204A)	M4I(G12C)				
RIVM_C019864	8	no		V91E(T272A+A273G)				
RIVM_C019866	16	no		first 48 bp are absent			V88A(T263C)	
RIVM_C028932	4	*no*		M4I(G12C)				
RIVM_C029515	4	*mcr-1.1*						I175F(A523T)
RIVM_C000121	4	*mcr-1.1*						
RIVM_C019762	2	*mcr-1.1*						
RIVM_C019792	4	*mcr-1.1*						

Point mutations are indicated by the amino acid substitution (nucleotide substitution) with the number representing the location. Silent mutations and mutations that were present in (both colistin-resistant and) colistin-susceptible isolates were not included in this table. Abbreviations: bp: base pairs, MIC: minimum inhibitory concentration, p: position.

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