Colistin-resistant Enterobacterales among veterinary healthcare workers and in the Dutch population

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Objectives: Plasmid-mediated colistin resistance can be transferred from animals to humans. We investigated the prevalence of carriage of mcr-mediated colistin-resistant Escherichia coli and Klebsiella pneumoniae (ColR-E/K) in veterinary healthcare workers and in the general population in the Netherlands.

Methods: Two cross-sectional population studies were performed: one among veterinary healthcare workers and one in the general population. Participants sent in a faecal sample and filled in a questionnaire. Samples were analysed using selective enrichment and culture. Mobile colistin resistance genes (mcr) were detected by PCR and ColR-E/K were sequenced using Illumina and Nanopore technologies.

Results: The prevalence of mcr-mediated ColR-E/K was 0.2% (1/482, 95% CI 0.04%–1.17%) among veterinary personnel and 0.8% (5/660, 95% CI 0.3%–1.8%) in the population sample. mcr-1 was found in E. coli from four persons, mcr-8 in K. pneumoniae from one person and another person carried both mcr-1 and mcr-8 in a K. pneumoniae isolate. mcr-1 was found on different plasmid types (IncX4, IncI1 and IncI2), while mcr-8 was found on IncN plasmids only.

Conclusions: mcr-mediated ColR-E/K resistance was uncommon in both populations. Professional contact with animals does not increase the chance of carriage of these bacteria in the Netherlands at present. mcr-8 was found for the first time in the Netherlands. Surveillance of colistin resistance and its underlying mechanisms in humans, livestock and food is important in order to identify emerging trends in time.

Introduction

Colistin or polymyxin E is an antibiotic belonging to the class of polymyxins and has been on the market since the 1950s.¹ In human medicine, it is a last-resort drug for treatment of MDR Gram-negative bacterial infections and its use has increased during the last decade.² Polymyxins are categorized as highest priority critically important antimicrobials by WHO.³

Colistin is also used in animals for treatment of gastrointestinal infectious diseases mainly in calves, pigs and poultry.⁴,⁵ For a long time it was believed that the mechanism of resistance to colistin was limited to chromosomal mutations. However, in 2015 a mobile colistin resistance gene (mcr-1) on a plasmid was found in Enterobacterales from pigs, retail meat and an inpatient in China.⁶ The emergence of plasmid-mediated, transmissible colistin resistance is of great concern as it facilitates the spread between different bacterial isolates and species as well as between animals and humans. To date, 10 mcr genes (mcr-1 to mcr-10) and many subvariants have been described.⁷ These genes have been detected in zoonotic pathogens and it is likely that they are transferred between animals and humans.¹,⁸ The more frequent isolation of mcr genes among animal isolates compared with human ones, together with the higher use of colistin in livestock compared with human medicine in China has been suggestive of the direction of transmission from animals to humans.⁶

Although prevalence data and colistin use suggest that plasmid-mediated colistin resistance is transferred from animals to humans, it is unknown whether individuals with professional animal contact carry colistin-resistant Enterobacterales more often. Therefore, the objective of the present study was to investigate the prevalence of mcr-mediated colistin resistant Escherichia coli and Klebsiella pneumoniae (ColR-E/K) carriage among the Dutch population as well as among veterinary healthcare workers.
Material and methods

Ethics

Samples from two population studies were used: one study was part of the Antibiotic Resistant Bacteria in Dutch Veterinarians study (Dutch acronym: AREND) and the other study was part of the national sero-epidemiological study (Dutch acronym: PIENTER3). The University Medical Centre Utrecht designated the AREND study not to be subject to the Medical Research Involving Human Subjects Act or WMO (Decision number 18-389/C), while the medical research ethics committee Noord-Holland approved PIENTER3 (METC number M015-022). All participants signed an informed consent form.

Population studies

The AREND study was conducted between August 2018 and March 2019 and included 482 veterinarians, veterinary technicians and veterinary assistants. Participants were recruited at the annual Dutch veterinary conference in 2018, via articles in newsletters and journals for veterinarians, and by information about the study sent directly to veterinary clinics by postal mail. Criteria for inclusion were age 18 years or older and working in veterinary care. Persons working with companion animals, horses and/or livestock were included. Participants were asked to complete a web-based questionnaire about their contact with animals at work and at home, hygiene, health and medication use and leisure activities such as travel behaviour. Furthermore, they were asked to collect a faecal sample at home and send it to the laboratory by regular mail. On the day of arrival, the faecal samples were kept in the fridge (4°C) until further processing, which took place within 2 days.

The PIENTER3 study was conducted in 2016–17. This was a nationwide cross-sectional population study in the Netherlands, to investigate the protection level against infectious diseases included in the National Immunization Programme. For that purpose, a two-stage cluster sampling technique was used to draw a random sample of persons aged 0–89 years from Dutch municipalities. Subpopulations were oversampled: these included people living in low vaccination coverage areas, people with a non-Western migration background and people with a migration background from Suriname, Aruba and the former Dutch Antilles (SAN). A total of 7600 persons in the Netherlands were enrolled in the study. Participants were invited to give a blood sample and were among others also asked to collect a faecal sample in 15% glycerol-saline solution and send it to the survey location by the participant. The faecal sample was transported under frozen conditions to the laboratory, where the samples were kept at –70°C. For more information about the study design of PIENTER3 see Verberk et al., 2019. In the present study, samples derived from participants aged ≥18 years and stored in the –70°C freezer were used for genome analysis. No further selection criteria were applied. The samples included 660 faecal samples, derived from twenty municipalities, from a total of 2779 persons from 48 municipalities in the Netherlands that had provided a faecal sample.

Laboratory methods

To determine ColR-E/K presence in the faecal samples a sterile cotton swab (AREND) or a 10 μL loop (PIENTER3) of the faecal material was suspended in a sterile glass tube with 5 mL of Buffered Peptone Water (BioTrading) with 2 mg/L colistin (colistin sulphate salt, Sigma). After over-night incubation at 37°C, the enrichment was inoculated on ChromID Colistin R Agar (bioMérieux) with a 10 μL inoculation loop. The plates were incubated overnight at 37°C, and afterwards one colony per colour (pink to burgundy or blue/green) per person was isolated and inoculated on Columbia Agar with sheep blood (Oxoid). Species were confirmed using MALDI-TOF MS (Bruker) according to the manufacturer’s instructions. For ColR-E/K suspected isolates the MICs were analysed by broth microdilution using MIC colistin strips (Merlin) according to the manufacturer’s instruction, following ISO 20776-1:2019. The clinical breakpoint of >2 mg/L recommended by CLSI and EUCAST was used to determine resistance. The ColR-E/K suspected isolates were screened for mcr-1 to -9 genes by two multiplex PCRs. If mcr genes were suspected, the PCR was repeated in singleplex format with the same primers for confirmation. The primers are described previously.

Genome analysis of mcr-mediated ColR-E/K

One colony was inoculated in 1.5 mL BHI broth and incubated overnight at 37°C. The overnight culture was centrifuged at 13,000 g for 3 min. After removal of the supernatant, the cell pellet was washed in 500 μL physiological saline (0.9%) and subsequently centrifuged again for 3 min. The cell pellet was resuspended in 450 μL DNA/RNA-Shield (Zymo Research) and stored at 4°C. Genomic DNA was isolated using a Zymo Research kit (BaseClear, Leiden, the Netherlands). An Illumina genomic nextera XT 2 × 150 bp paired-end DNA library was prepared and sequenced on an Illumina NovaSeq 6000 (BaseClear). Raw reads were trimmed and de novo assembled using SPAdes 3.10.0.

The isolates were analysed by long read Nanopore sequencing as described in Hendriks et al., 2020. Illumina and Nanopore sequences were combined for a hybrid assembly using Unicycler v0.4.8 with modified settings for depth_filter (set to 0.1) and min_fasta_length (set to 1000). Plasmids 260-1 and 132-1 were assembled with the depth_filter set to 0.01. Phylogenetic groups, serotypes and STs were assigned using in-house implemented task templates in Ridom SeqSphere+ (Version 7.1.0 (2020-06)). Plasmids were characterized with Abricate v0.9.3 (https://github.com/tseemann/abricate), using the PlasmidFinder database. Resistance genes were assigned with ResFinder version 4.0 using ≥98% identity and 100% coverage of genes. Plasmids were compared using blastn.

All the genomic sequences are available at the European Nucleotide Archive at the European Molecular Biology Laboratory (accession no. PRJEB45559, see Table S1, available as Supplementary data at JAC-AMR Online).

Results

The 482 veterinary healthcare workers that participated in the AREND study were aged between 20 and 70 years, the average age was 40 years (median 38 years) and 85% were female. The 660 included participants from PIENTER3 study were aged between 18 and 85 years, the average being 50 years (median 52 years). Thirty-three percent were born in a non-Western country, 61% were female and 7.8% reported direct contact with pets and/or farm animals at work.

mcr genes were detected in an E. coli isolate from a veterinary technician participating in the AREND study (0.2% (1/482, 95% CI 0.04%–1.17%) and in three E. coli and two K. pneumoniae isolates from five persons from the PIENTER3 population (0.8% (5/660, 95% CI 0.3%–1.8%), including four with a migration background from Suriname. Three of these six persons reported travel to Asia, two did not report any travel in the past 6 months and for one person the travel history was unknown. All E. coli isolates harboured mcr-1 (three on IncX4 plasmids, and one on IncI1 plasmid). One K. pneumoniae isolate
harboured mcr-8 on an IncF plasmid, while the other K. pneumoniae isolate carried mcr-1 as well as mcr-8 on IncI1 and IncF plasmids, respectively. An overview of the characteristics of the mcr-positive isolates, the participants and the potential risk factors is shown in Table 1.

The three IncX4 plasmids found in E. coli isolates in this study were not identical to each other and were 33, 34, and 41 kb in size. The mcr-1 genes were organized in the same commonly found embedding structure: IS26-hypothetical protein (hp)-hp-par4-hp-hp-mcr-1-par2-hp-pir. This genetic structure was previously found on a 33 kb IncX4 plasmid (pLV23529) in an E. coli isolate originating from swine cecum in Portugal. The genetic region around mcr-1.1 harboured mcr-8 on an IncF plasmid, while the other K. pneumoniae isolate carried mcr-1 as well as mcr-8 on IncI1 and IncF plasmids, respectively. An overview of the characteristics of the mcr-positive isolates, the participants and the potential risk factors is shown in Table 1.

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The two IncF plasmids 91-1 and 137-1_IncF in K. pneumoniae (Table 1) were different in size (103 kb and 161 kb, respectively). Both of them contained an IncFIIK replicon and plasmid 91-1 an IncFIA as well. The genetic region around mcr-8 of plasmid 137-1_IncF was identical to the region in a 300 kb K. pneumoniae plasmid pk9 isolated from a patient in Lebanon (sasA-CopR-mcr-8-hp-hp). This region in plasmid 91-1 was almost identical (sasA-CopR-mcr-8-hp-hp-Kpn43) to that of pk9 and 137_1_incF. Plasmid 137-1_IncF carried, besides the mcr-8 gene, resistance determinants to aminoglycosides (aadA2), β-lactams (blaTEM-18) macrolides [mhp(A)], sulphonamides (sul1), tetracycline (tet(A)) and trimethoprim (dfrA12). Plasmid 91-1 did not carry additional antibiotic resistance genes.

The IncI1 plasmid 137-1_IncI1 of plasmid ST8, carrying the mcr-1 gene, was 51 kb in size. This plasmid differed in size from an mcr-1-carrying IncI1 plasmid (pMCR-E2899) recently isolated from turkey meat (107 kb) in the Netherlands. The genetic region around mcr-1 of plasmid 137-1_IncI1 was identical to the region in pMCR-E2899 (ISAp1-tpap2-mcr1.1-ISApl1). Besides mcr-1, plasmid 137-1_IncI1 carried genes for resistance to aminoglycosides (aadA12), macrolides [erm(B)] and sulphonamides (sul1) in contrast to the published pMCR-E2899, which harboured resistance genes to only β-lactams (blaTEM-18) and colistin (mcr-1).

IncI2 plasmid 88-1 (Table 1) was 60 kb in size and carried the mcr-1.1 gene also surrounded by the genes nik8 and pap2 as described in a 64 kb plasmid PE26 carrying mcr-1.9 found in an enterotoxigenic E. coli isolate from a patient in China. Plasmid 88-1 like PE26 only carried resistance to colistin (i.e. mcr-1.1).

Besides mcr and the other resistance genes carried on the identified plasmids, multiple additional antibiotic resistance determinants were present in several of the isolates investigated (Table 1) either on the chromosome or on other plasmids present.

Discussion

In the present culture-based cross-sectional study, the prevalence of intestinal carriage of mcr-mediated ColR-E/K resistance was low in veterinary healthcare workers as well as in a subset of the general Dutch population. This might be a result of low veterinary colistin usage in the Netherlands. Consequently, mcr-mediated resistance is identified only incidentally in E. coli from different livestock species (<2% in 2019). Colistin is almost never used in companion animals in the Netherlands, therefore carriage of ColR-E/K in pets is also expected to be low. This indicates that at present professional contact with animals is not an important transmission route for humans in the Netherlands. The Dutch population might also be exposed to mcr genes through consumption of (imported) food products. This was illustrated in 2018, when a marked difference was found between the mcr-1 prevalence among caecal samples of broilers fattened in Germany but slaughtered in the Netherlands (24.4%) compared with the mcr prevalence among Dutch broilers (0.3%). To date, the prevalence of mcr-positive E. coli in meat sold in the Netherlands is low, except for turkey meat (13.3% in 2019). Additional studies are needed to evaluate the transfer of mcr genes from animals and food to humans in different countries with different prevalence.

The mcr-prevalence in our study is comparable to that found in patients in other studies from Europe. Among patients attending a tertiary care hospital in the Netherlands prevalence was 0.35% (2/576 persons mcr-1 positive) in 2014–15 and among inpatients in hospitals in Paris it was 0.57% (7/1217 patients mcr-1 positive). In a study among 1091 healthy individuals in Switzerland no mcr-related resistance was found. In China, however, much higher prevalence of carriage of mcr-1-mediated colistin resistance in E. coli was reported in persons visiting hospitals for routine examinations. In 2006, this prevalence was found to be 14.6%. In 2019, after the ban of the use of colistin as growth promotor in animals in China, it declined to 6.3%, which is still much higher than in our study.

Three of the six persons carrying mcr-mediated ColR-E/K in the present study had travelled to Asia during the 6 months prior to sampling. In a study among inpatients in hospitals in Paris three of the seven persons had been hospitalized abroad in Asia in the previous year. Travel to Asia, Africa and Latin America has been identified as a risk factor for carriage of other MDR bacteria, such as ESBL-producing Enterobacteria, and this might play a role for mcr-producing isolates as well.

Four of the six persons carrying mcr genes (including the two mcr-8 carriers) in our study were first generation immigrants from Suriname, but none of them reported travelling to Suriname during the last 6 months. It should be noted that first generation immigrants from Suriname were oversampled in the PIENTER3 population study and this might partly explain our finding. To illustrate: in 2019, 3.0% of the population at large were first or second generation immigrants from Suriname, Aruba and the former Dutch Antilles, against 29.2% of PIENTER3 participants.

In the present study, the mcr-1 genes were found on different plasmid types, namely IncI2 and IncX4 in E. coli and IncI1 in K. pneumoniae. The plasmid families IncHI2, IncI2 and IncX4 account for more than 90% of plasmids carrying the mcr-1 genes from different sources worldwide. IncI1 plasmids carrying mcr-1 are less commonly reported, although they have been isolated recently from wastewater samples of German pig and poultry slaughterhouses. Interestingly, mcr-1 in a similar resistance region on another IncI1 plasmid has been reported in an E. coli isolate originating from a retail turkey meat sample in the Netherlands in 2015. All plasmids described in this study differ from each other, but have mcr-surrounding genes that have been described previously in similar plasmid families all over the world.

To the best of our knowledge this is the first time that mcr-8 genes were found in the Netherlands. We found only two mcr-8 isolates and therefore no conclusions can be drawn about the origin. However, until now mcr-8 has not been described in Europe,
except for one patient in France, who was repatriated from Morocco.\textsuperscript{39} The mcr-8 gene was first described by Wang et al.\textsuperscript{15} in 2018 in K. pneumoniae isolates on IncFII conjugative plasmids derived from chickens and pigs in China and in a patient in a hospital. To date mcr-8 has been described in K. pneumoniae and other bacteria (Stenotrophomonas sp. and Raoultella ornithinolytica) isolated from poultry, sewage water from a poultry farm, pigs and patients in China.\textsuperscript{40–43} In addition, besides the patient in France mentioned earlier, mcr-8 was also found in Algeria (in a patient), Laos (in healthy humans) and Bangladesh (in patients), often located on IncF plasmids.\textsuperscript{44–46}

### Table 1. Characteristics of mcr-positive isolates and participants carrying these colistin-resistant E. coli/K. pneumoniae

| Species | K. pneumoniae | E. coli | K. pneumoniae | E. coli | E. coli | E. coli |
|---------|---------------|---------|---------------|---------|---------|---------|
| ST      | ST4065        | ST106   | ST231         | ST636   | ST93    | ST93    |
| ST      | O2v2: KL-     | O17:H18 | O1v2:KL51     | O21:H7  | O5:H4   | O7:H4   |
| ST      | n.a.          | D1      | n.a.          | B23     | A0      | A0      |
| ID number mcr-carrying plasmid | 91-1 | 132-1 | 137-1 | 88-1 | 260-1 | 308-1 |
| mcr gene type | mcr-8.1 | mcr-1.1 | mcr-1.1 | mcr-8.1 | mcr-1.1 | mcr-1.1 |
| Plasmid type | IncF | IncX4 | IncF | IncF | IncX4 | IncX4 |
| Plasmid size, kb | 103 | 33 | 51 | 161 | 60 | 34 |
| Additional resistance genes on this plasmid | None | None | ermB, sulI | None | bla\textsubscript{TEM-1}\textsubscript{B}, dfrA12, mph(A), sul1 | None |
| Additional resistance genes in the isolate\textsuperscript{a} | bla\textsubscript{SHV-172}\textsubscript{-like}, oqxA, oqxB, tet(A), strA, strB | None | bla\textsubscript{SHV-212}\textsubscript{-like}, oqxA, oqxB | bla\textsubscript{SHV-172}\textsubscript{-like}, oqxA, oqxB, strA, strB, sul2, tet(B) | bla\textsubscript{SHV-172}\textsubscript{-like}, oqxA, oqxB, strA, strB, sul2, tet(B) | bla\textsubscript{SHV-172}\textsubscript{-like}, oqxA, oqxB, strA, strB, sul2, tet(B) |
| Participants | | | | | | |
| Age (category), years | 70–89 | 60–69 | 60–69 | 50–59 | 30–39 | 30–39 |
| Sex | Male | Male | Female | Male | Female | Male |
| Country of birth | Suriname | Suriname | Suriname | Suriname | Netherlands | Netherlands |
| Ethnicity | First gen. Suriname | First gen. Suriname | First gen. Suriname | First gen. Suriname | Other Western | UNK |
| Urbanization level\textsuperscript{b} | Very high | Very high | Very high | Very high | Very high | Very low |
| Has children attending daycare centre | No | No | No | No | No | No |
| Job | Retired | Retired | Healthcare | Maritime industry | Government | Veterinary technician |
| Has weekly or more often contact with animals at work | No | No | No | No | No | No |
| Travel in last 6 months | No travel | Southern Asia | Western Asia | UNK | Eastern Asia | No travel |
| Contact with animals in last 4 weeks | No contact | No contact | Cat | UNK | Dog, guinea pig | Dog, cat, horse |
| Use of stomach protectors | Yes | Yes | No | No | No | No |
| Antibiotic use last 3 months | No | Yes | Yes | Yes | Yes | Yes |
| Which type of antibiotic? | n.a. | Amoxicillin | Co-trimoxazole | UNK | n.a. | n.a. |
| Hospital visit in last 6 months | Yes | Yes | No | UNK | No | UNK |
| Number of hospitalizations in last 6 months | No | No | No | UNK | No | Yes |

n.a., not applicable; gen., generation; UNK, unknown.

\textsuperscript{a}ResFinder results with 100% coverage and >98% identity. The bla\textsubscript{SHV-172}\textsubscript{-like} variant differs by two non-synonymous SNPs from the reference sequence: A256C (Q86K) and T756G (D252E). The bla\textsubscript{SHV-212}\textsubscript{-like} variant differs by one non-synonymous SNP to the reference sequence: A238G (M80V).

\textsuperscript{b}Very high: ≥2500 addresses/km\textsuperscript{2}; high/moderate: 1000–2500 addresses/km\textsuperscript{2}; low/very low: <1000 addresses/km\textsuperscript{2}.
Generally, the mcr-carrying plasmids reported in this study did not harbour as many resistance determinants as some plasmids reported in previous studies that have a similar mcr gene genetic environment. Nevertheless, nearly all isolates carried (multiple) other antibiotic resistance genes. This could complicate treatment in case of an infection.

In conclusion, professional contact with animals does not increase the chance of mcr carriage in humans in the Netherlands at present. mcr-8 was detected for the first time in the Netherlands. Although carriage of mcr-positive ColR-E/K is still very rare in both populations studied, different mcr genes were carried on four different plasmid types. Therefore surveillance of colistin resistance and its underlying mechanisms in food, humans and livestock is important in order to identify emerging trends in time.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC-AMR Online.

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