DOI 10.1111/apm.12720

First environmental sample containing plasmid-mediated colistin-resistant ESBL-producing *Escherichia coli* detected in Norway

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Jørgensen SB, Søraas A, Arnesen LS, Leegaard T, Sundsfjord A, Jenum PA. First environmental sample containing plasmid-mediated colistin-resistant ESBL-producing *Escherichia coli* detected in Norway. APMIS 2017.

We hereby report the detection of the plasmid borne *mcr-1* gene conferring colistin resistance in an extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* ST10 strain retrieved from seawater at a public beach in Norway. The sample was collected in September 2010 and was investigated by whole-genome sequencing in 2016. This report illustrates that *E. coli* strains carrying plasmid-mediated colistin resistance genes have also reached areas where this drug is hardly used at all. Surveillance of colistin resistance in environmental, veterinary, and human strains is warranted also in countries where colistin resistance is rare in clinical settings.

Key words: *mcr-1*; multidrug resistance; one health; antibiotic resistance; ST10

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This finding emanates from the ESCAPE II project which aims to investigate the prevalence of ESBL-A-producing *Escherichia coli* isolates in seawater and freshwater at public beaches close to the Norwegian capital, and compare these with clinical isolates from humans. As the strains have been subject to whole-genome sequencing, more resistance genes and virulence factors have been revealed.

The plasmid borne *mcr-1* gene conferring colistin resistance was first described in China in 2015 (1). Prior to this discovery, resistance to colistin had only been known to be mediated by chromosomal mutations. During 2016, there have been reports from many parts of the world that *mcr-1* frequently occurs in combination with CTX-M type β-lactamases, rendering the isolates resistant to both cephalosporins and colistin. Co-resistance to carbapenems has also been found (2). As resistance toward cephalosporins and carbapenems increases globally, colistin has been regarded as a last resort drug, and it is on the WHO list of critically important antimicrobials for human medicine (3). The occurrence of a resistance mechanism easily transmitted between clones and species by several different plasmids has caused great concern (4).

Received 7 February 2017. Accepted 15 April 2017

MATERIALS AND METHODS

Collection of samples and bacterial culture

Environmental samples were collected on five different dates during the summer season (May–September 2010) at one freshwater and three saltwater beaches located close

to the Norwegian capital Oslo. Samples were collected in sterile containers from a depth of 0.5–1 m, and each container was rinsed three times at the sampling site before the first sample was collected. Portions containing 10–500 mL of water were vacuum-filtered and the filters were grown on Brilliance® agar and ChromeID® ESBL plates for 40–48 h. Blue colonies representing coliform bacteria on Brilliance plates were counted, and all purple colonies representing potential ESBL-producing *E. coli* isolates on ChromeID ESBL plates were individually frozen for later analysis. This method allowed us to estimate the total amount of coliforms per 10 mL, 100 mL, 500 mL, and 1000 mL of water, and to calculate a ratio of ESBL-producing *E. coli* colony forming units/total number of coliform colony forming units.

Bacterial identification and antimicrobial susceptibility testing

Bacterial identification was done by MALDI-TOF (Bruker Daltonics, Bremen, Germany). Antibiotic susceptibility testing for colistin was performed on isolates containing *mcr-1* by the broth microdilution method using in-house designed premade Sensititre microtiter plates (TREK Diagnostics, Cleveland, OH, USA). All isolates were tested on the VITEK-2 system (BioMérieux, Marcy l'Etoile, France) for susceptibility to a selection of other drugs. Interpretation was according to EUCAST breakpoints version 6.0 (http://www.eucast.org).

Molecular analyses

All isolates were examined by a multilocus variable number of tandem repeats assay (MLVA) protocol originally described by Lindstedt et al. and modified by Løbersli et al. (5, 6). From each site and sampling date, we clustered the isolates according to MLVA types and antimicrobial resistance patterns, and selected one isolate from each cluster for whole-genome sequencing. These strains were investigated on the Illumina HiSeq platform, generating 150 base pairs paired end reads. *De novo* assembly, identification of known resistance genes, plasmids, and virulence factors were performed using the database at Center for Genetic Epidemiology (https://cge.cbs.dtu.dk/services/) (7–9).

RESULTS

After 20 rounds of environmental sampling at four different beaches, ESBL-positive samples were detected on eight occasions, and the ratio of ESBL-producing *E. coli* to coliform bacteria varied from 0 to 0.53%.

A total of 82 strains were sequenced. The *mcr-1* gene was found in two isolates sampled on one location (Kalvøya, 59° 53′ 2.39″ N, 10° 32′ 11.99″ E) on September 15th 2010, as a 100% match to the sequence originally described (1). Both were of MLVA type 5, -2, 6, 3, 2, 5. They were phenotypically non-susceptible to ampicillin, cefuroxime, cefotaxime, ceftazidime, aztreonam and trimethoprim–sulfamethoxazole, but sensitive to mecillinam, piperacillin-tazobactam, gentamicin, meropenem and ciprofloxacin. One isolate was interpreted as susceptible to amoxicillin-clavulanate (estimated MIC 8 mg/L), while the other was interpreted as resistant (estimated MIC 16 mg/L). All isolates had a colistin MIC of 4 mg/L (resistant).

The MLVA-results indicate that the two isolates belong to the same strain of ST10 (10, 11). The following acquired resistance genes were present in both: *aadA5*, $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-1B}}$, *dfrA17*, *mcr-1*, *strA*, *strB*, *sul2*, *tet(B)*. Virulence factors detected were *celb*, *cma*, *iro*N, *iss*, and the plasmids were IncFII, IncI1, IncFIB(AP001918), Col156, IncX4, and ColRNAI. Plasmid MLST categorized the strain as IncF F24:A-:B1 and IncI1 ST-3. The same plasmids in different combinations were found in various other strains which did not contain the *mcr-1* gene. ST10-strains were isolated from two different sites and had several different MLVA types.

DISCUSSION

Reports of *mcr-1* in isolates from humans and farm animals are plentiful, as many collections of previously collected samples have been investigated during the last years. There are also reports on findings in wild birds and in the environment (2, 12, 13). The mechanism has been detected in strains isolated in China as early as in the 1980s and yearly in European farm animals since 2004 (14). Until now, only one previous case has been reported from Norway, in a clinical sample from a patient with diarrhea returning from India (15). A small collection of Norwegian veterinary isolates of *E. coli*, *Salmonella* and *Shigella* from 2010 to 2015 have been examined retrospectively, with no detection of *mcr-1* (16). If we look to the other Scandinavian countries, there is only one report on human bloodstream infection with *mcr-1*-contaning *E. coli*; in Denmark in 2015, plasmid mediated colistin resistance combined with $bla_{\text{CTX-M 55}}$ and $bla_{\text{CMY-II}}$ was found in a patient with no known travel history (17). Interestingly, *mcr-1* was also detected in *Salmonella typhimurium* isolates from four Danish patients from 2014 and 2015 (18), and also in five *E. coli* isolates from imported chickenmeat (17). In Sweden, the *mcr-1* has been described in an *E. coli* strain retrieved from a fecal screening sample in a traveler returning from Asia. According to a press release from the Public Health Agency of Sweden in February 2016 (https://www.folkhalsomyndigheten.se/nyheter-och-press/nyhetsarkiv/2016/

februari/bakterie-resistent-mot-sista-behandlingsalternativet-funnen/) several Swedish *Enterobacteriaceae* collections have been examined for *mcr-1* with only one additional finding. This was in an Enterohaemorrhagic *E. coli* (EHEC) isolate of Asian origin.

National and regional surveillance programs for antimicrobial resistance, including colistin, have existed for several years, and an initiative for a global surveillance program, GLASS, has been launched by the World Health Organization (19). However, phenotypic antimicrobial susceptibility testing of polymyxins is complicated, and questions have been raised regarding the reliability of agar dilution, disk diffusion and gradient diffusion. The current recommendation from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) is that only the ISO-standard broth microdilution be used, until further studies have been conducted (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf). Phenotypic testing is necessary to target molecular surveillance, and to detect new resistance genes. Another plasmid-mediated colistin resistance gene, *mcr-2* was detected in Belgium in June 2016 (20). It has 77% nucleotide identity to *mcr-1*, and was found in porcine colistin-resistant *E. coli* isolates collected in 2011-2012. Whether *mcr-2* also has a global dissemination, remains to be revealed.

The vast dissemination of *mcr-1* may be related to frequent use of colistin for treatment of poultry and pigs. However, colistin has not been used in Norwegian veterinary medicine, and to a very limited extent in human medicine (16, 21).

The origin of the current *mcr-1*–positive seawater isolate is unclear. Humans bathing in the fjords, contamination from boat toilets, farm animals, fertilizers used in agriculture, or migrating birds are all possible sources. The MLST type is common among strains isolated from animals, and strains of ST10 have been described to dominate in a collection of *mcr-1*–positive strains from several European countries (14). Recently, *mcr-1* positive ST10 *E. coli* have also been reported from an infected migrating Magellanic penguin, supporting that this lineage can survive and spread in the marine environment (22). There are also reports of *mcr-1* positive ST10 *E. coli* from well water in China (23) and German swine farm areas (24). Our neighboring country Denmark has pig farms with some consumption of colistin (21), and *mcr-1* has been found in various strains there, although not in ST10 (17). However, the occurrence of *mcr-1* on easily transferrable plasmids makes it likely that the dissemination is both clonal and plasmid mediated. Further investigations to establish whether *mcr-1* in our isolates is locatedon the IncX4 or on other plasmids would also be of interest. However, transfer experiments were beyond the scope of this study. The European Medicine Agency updated their advice on colistin use in animals in 2016, and discussed the variation between the EU member states' colistin consumption, encouraging high- and moderate consumers to target the levels of the low consumers (25).

CONCLUSION

This report illustrates that *E. coli* strains carrying plasmid-mediated colistin resistance genes have also reached areas where this drug is hardly used at all. Surveillance of colistin resistance in environmental, veterinary and human strains is warranted also in countries where colistin resistance is rare in clinical settings.

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[Correction added on 06 July 2017, after online publication: Information in Discussion section was not the final version and has been corrected in this version with additional references added.]