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Plasmid with Colistin Resistance Gene *mcr-1* in Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* Strains Isolated from Pig Slurry in Estonia

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A plasmid carrying the colistin resistance gene *mcr-1* was isolated from a pig slurry sample in Estonia. The gene was present on a 33,311-bp plasmid of the IncX4 group. *mcr-1* is the only antibiotic resistance gene on the plasmid, with the other genes mainly coding for proteins involved in conjugative DNA transfer (*taxA*, *taxB*, *taxC*, *trbM*, and the *pilX* operon). The plasmid pESTMCR was present in three phylogenetically very different *Escherichia coli* strains, suggesting that it has high potential for horizontal transfer.

A plasmid containing the *mcr-1* gene causing colistin resistance was originally described in *Escherichia coli* strains from animals, animal products, and human samples in South China (1). This plasmid could cause serious problems when transferred into strains for which colistin is the last treatment option. Since the first report, sequence information from several antibiotic resistance programs has been screened for the presence of the *mcr-1* gene (2–25). The gene was found in several samples from around the world. The *mcr-1* genes described are present in several plasmid backbones. In addition to *mcr-1*, two new *mcr* gene variants were detected (14, 20). Although by now we are aware that *mcr-1* is not restricted to China, we need more information for a better

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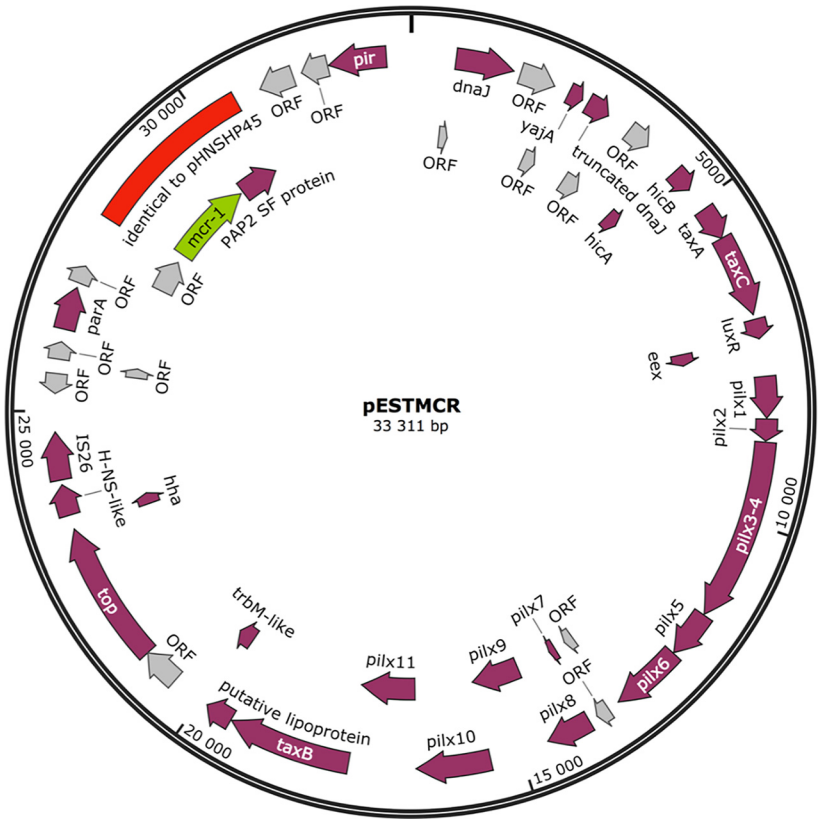

FIG 1 Map of the *mcr-1*-containing plasmid isolated. The region identical to pHNSHP45 where *mcr-1* was originally identified (1) is marked in red. The *mcr-1* gene is marked in green. The figure was created with the SnapGene software. ORF, open reading frame.

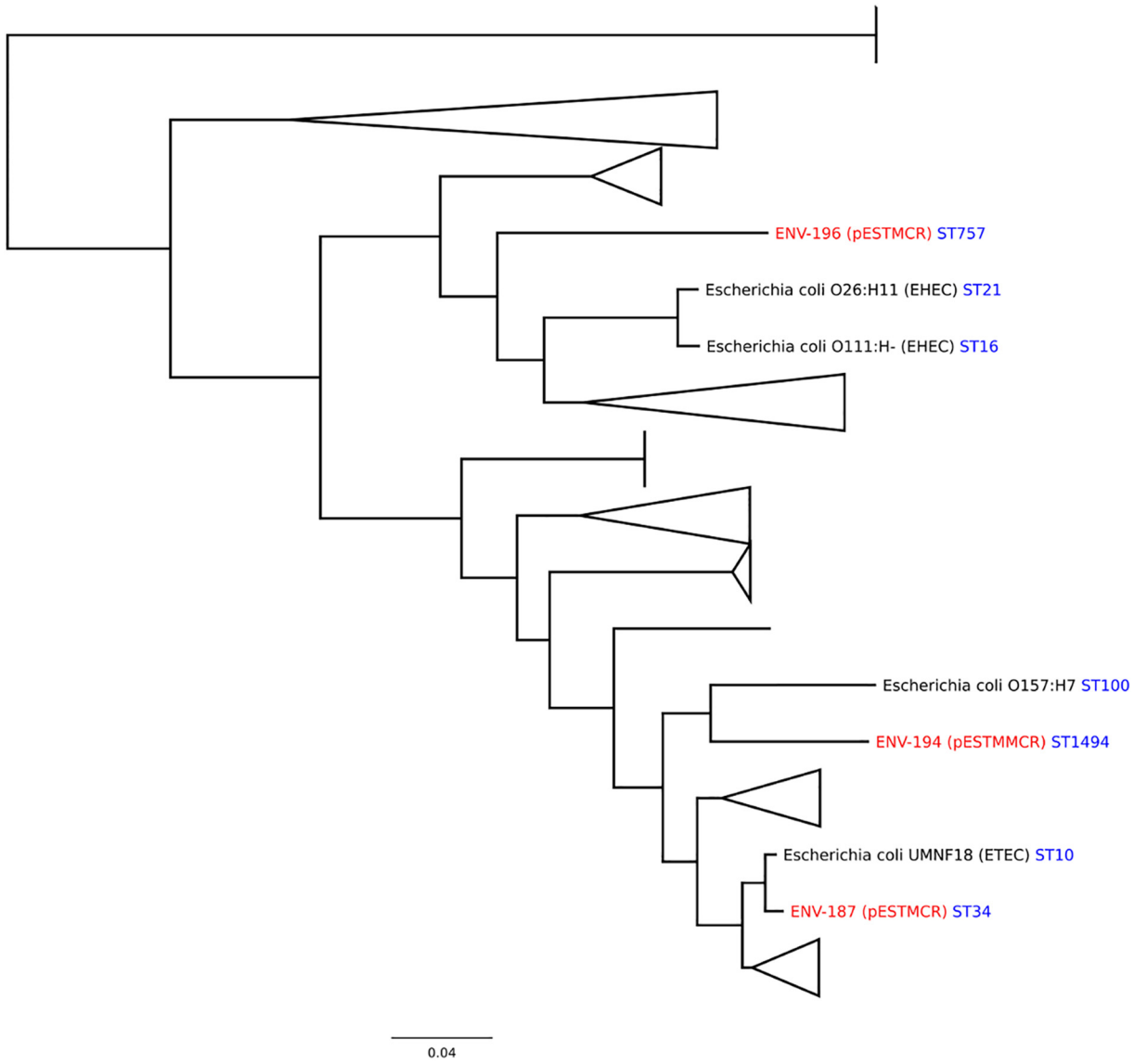

FIG 2 All available *E. coli* complete genomes were downloaded from the NCBI database in September 2015. These genome sequences were compared with the genomes of strains containing *mcr-1* (marked in red). The names of the closest relatives of the *mcr-1*-containing strains are indicated in black. Core genomes were constructed using rapid core genome multialignment (parsnp) (31). The tree was constructed from core alignment using RAxML under the general time-reversible (GTR) Gamma model. The scale bar represents the mean number of nucleotide substitutions per site. Sequence types (ST) for the sequences present in the database (32) are indicated. ST757 is closely related to clonal complex 10.

understanding of how the gene has spread and how big a concern it might be in different countries. The existing collections of strains and sequences are a good source of information that can be retrieved rapidly.

A survey of the spread of antibiotic resistance in Estonia gathered samples during the years 2011 to 2014. It included 347 *E. coli* strains: 144 strains from humans, 88 strains from animals, and 115 strains from the environment. Of the 237 *Pseudomonas aeruginosa* strains, 147 strains were collected from humans, 64 strains were from animals, and 26 strains were from the environment. The collection of *E. coli* strains was specifically targeted at extended-spectrum β-lactamase (ESBL) producers. All strains were characterized by Illumina HiSeq 2500 sequencing of the DNA (Nextera XT libraries, paired-end 150-bp reads), followed by *de novo* assembly of the reads into contigs (26). We searched our data set with BLAST against all three *mcr* sequence variants. The *mcr-1* gene was found in three *E. coli* strains isolated from a single pig slurry sample originating from a farm in November 2013. The farm has a breeding herd of 150 sows and is located in North Estonia. Animals from this farm were not sampled. The strains had been isolated as follows. About 5 g of slurry sample diluted with 0.9% NaCl solution containing ampicillin (10 μg/liter) was incubated overnight at 37°C. *E. coli* selection was made according to ISO 9308-3 standard. As a second selection step, the samples from positive wells were plated as a dilution series onto ESBL agar (Oxoid Brilliance ESBL agar with cefpodoxime). From the plates, isolates having the *E. coli* phenotypes (blue or pink) were purified.

The contigs containing *mcr-1* were around 33 kb long. PCR primers were designed to the ends of the contig, and the missing

part of the plasmid was amplified and Sanger sequenced. All three plasmids were identical (Fig. 1). The plasmid replication origin groups to IncX4 (27). The plasmid contains 47 open reading frames. These include *taxA*, *taxB taxC*, and *trbM* genes and the *pilX* operon, which are probably responsible for movement of the plasmid between different bacterial hosts (28–30). *mcr-1* is the only antibiotic resistance gene in the plasmid.

Interestingly, the plasmid is identical to the contigs described in *Salmonella* isolated from meat samples in France (7) and highly similar to plasmids pMCR1-IncX4 (*Klebsiella pneumoniae*, China) (17), pAf48 (*E. coli*, South Africa) (19), and a new *mcr* variant carried by pMCR1.2-IT (*K. pneumoniae*, Italy) (20). The *mcr-1*-containing *Salmonella* strains were all from one serogroup, O:4. This suggested that the colistin resistance provided by the gene might be limited to certain groups of host strains. To investigate the host spectrum, we compared the genomic sequences of our strains with the completed genome sequences available in the NCBI Genomes database (Fig. 2). All three strains cluster into separate branches of the tree, indicating considerable variation.

All three strains were resistant to colistin, with Etest-based MICs of 2 μg/ml (ENV-196) and 4 μg/ml (ENV-187 and ENV-194). These resistance levels are consistent with the original report (1), where it was found that the colistin MICs for the *mcr-1* gene carrying *E. coli* strains are between 2 and 8 μg/ml. The plasmid was isolated and transformed into laboratory model *E. coli* strain DH5α, where it increased the colistin MIC from 0.25 μg/ml to 4 μg/ml. It has been reported previously that the *mcr-1*-carrying plasmid could be transformed into *Pseudomonas* (1). In our case, transfection into the environmental *Pseudomonas putida* strain PAW85, which could be a recipient of the plasmid after the sludge reaches the receiving field, was also attempted but failed. No increase in the number of colistin-resistant colonies over the background level of spontaneous resistance mutations was observed.

The strains were ESBL producers, and the corresponding β-lactamase genes could be identified in the genomic sequences: CTX-M-1 in ENV-187, CTX-M-1 and TEM-1A in ENV-194, and CTX-M-1 and AmpC in ENV-196. The strains were sensitive to most of the antibiotics tested: meropenem (MIC, <0.016 μg/ml), ciprofloxacin (MIC, 0.008 μg/ml to 0.012 μg/ml), amikacin (MIC, 2 μg/ml to 4 μg/ml), gentamicin (MIC, 1 μg/ml to 1.5 μg/ml), fosfomycin (MIC, 1 to 4 μg/ml), tigecycline (MIC, 0.125 μg/ml to 0.19 μg/ml), and piperacillin-tazobactam (MIC, 1 μg/ml to 2 μg/ml). Resistance to trimethoprim-sulfamethoxazole was observed in one strain (ENV-196; MIC, >32 μg/ml), while two strains were sensitive (MIC, 0.064 μg/ml to 0.094 μg/ml). This general pattern indicates that these particular strains are not likely to be problematic from an antibiotic treatment viewpoint. Still, considering that the three strains hosting the plasmid, isolated from one sample, were genetically very different, it is probable that the plasmid is highly mobile. It could be transferred into strains resistant to a wide range of antibiotics, thereby creating strains that are very hard to treat. Therefore, the epidemiological situation should be closely followed.

Accession number(s). The accession number for the pESTMCR sequence in GenBank is KU743383.

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