Short Communication

A link between the newly described colistin resistance gene mcr-9 and clinical Enterobacteriaceae isolates carrying blaSHV-12 from horses in Sweden

Stefan Börjesson a,b,*, Christina Greko a, Mattias Myrenäs a, Annika Landén a, Oskar Nilsson a, Karl Pedersen a

a Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute (SVA), Uppsala, Sweden
b Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden

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A B S T R A C T

Objectives: The aim of this study was to investigate the occurrence of the newly described transferable colistin resistance gene mcr-9 in extended-spectrum β-lactamase (ESBL)-producing clinical Enterobacteriaceae isolates from horses in Sweden.

Methods: A total of 56 whole-genome sequenced ESBL-producing Enterobacteriaceae isolates from horses were subjected to in silico detection of antimicrobial resistance genes and identification of plasmid replicon types. The colistin minimum inhibitory concentration (MIC) for mcr-9-positive isolates was determined by broth microdilution. Relatedness between Enterobacteriaceae carrying mcr genes was determined by multilocus sequence typing (MLST) and core genome MLST.

Results: Thirty ESBL-producing Enterobacteriaceae isolates from horses were positive for the colistin resistance gene mcr-9. These isolates included Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca and Citrobacter freundii and belonged to diverse MLST sequence types within each species. Two of the mcr-9-containing isolates originated from the same horse. All mcr-9-positive isolates had colistin MICs below or equal to the EUCAST epidemiological cut-off value of 2 mg/L and were negative for the two potential regulatory genes qseB-like and qseC-like for mcr-9. Except for one isolate carrying only blaTEM-1B, all of the isolates carried blaSHV-12 and blaTEM-1B, and were all considered multidrug-resistant as they harboured genes encoding resistance to aminoglycosides, chloramphenicol, fosfomycin, macrolides, quinolones, sulfonamides, trimethoprim and tetracyclines. Plasmid replicon types IncHI2 and IncHI2A were detected in all mcr-9-positive isolates.

Conclusion: The occurrence of mcr-9 was common among clinical ESBL-producing Enterobacteriaceae isolates from horses in Sweden and was linked to the ESBL-encoding gene blaSHV-12 and plasmid replicon types IncHI2 and IncHI2A.

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1. Introduction

Colistin was previously used mainly in food-producing animals for the prevention and treatment of infections caused by Enterobacteriaceae, and globally usage is still common [1]. Recently, colistin has received renewed attention as an important antimicrobial agent for the treatment of infections caused by carbapenem-resistant and multidrug-resistant Gram-negative bacteria in human medicine [2]. Therefore, the recent description of the plasmid-mediated colistin resistance gene mcr-1 in Enterobacteriaceae from China is of concern [3]. Since the description of mcr-1, additional mcr homologues have been described and several of the genes occur worldwide [1,4]. However, the occurrence of mcr genes is rare in Sweden, with only a handful of cases reported from humans [5].

In May 2019, a novel mcr homologue, mcr-9, was described in a Salmonella enterica serotype Typhimurium carrying blaSHV-12 isolated from a human patient [6]. The authors also showed that mcr-9 could be detected in 335 genomes of various species in the National Center for Biotechnology Information (NCBI) database and that the occurrence of mcr-9 could be linked to plasmids

* Corresponding author at: Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute (SVA), Uppsala, Sweden.
E-mail address: stefan.borjesson@liu.se (S. Börjesson).

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belonging to replicon types IncH1 and IncH1A. The same gene and plasmid combination was shortly thereafter also described in an isolate from a human patient in France [7].

As of 2010, the National Veterinary Institute (SVA) in Sweden has encouraged Swedish veterinary laboratories to submit presumptive extended-spectrum β-lactamase (ESBL)- and plasmid-encoded AmpC β-lactamase (pAmpC)-producing Enterobacteriaceae for verification and genotypic characterisation to SVA, and from 2017 all identified ESBL- and pAmpC-producing Enterobacteriaceae are subjected to whole-genome sequencing (WGS). In a retrospective investigation, a bla<sub>SHV-12</sub>-positive <i>Escherichia coli</i> isolated from a horse in 2018 was found to be positive for the mcr-9 gene when subjected to in silico detection of antimicrobial resistance genes (ARGs) using the ResFinder database on 6th of May 2019. Detection of mcr-9 marks the first detection of mcr genes from animals in Sweden and, as a result of this random finding, we decided to investigate whether mcr-9, or other mcr genes, could be detected in additional ESBL/pAmpC-producing Enterobacteriaceae clinical isolates from horses for which WGS data were available.

2. Materials and methods

2.1. Isolates

Between 2017–2018, 55 Enterobacteriaceae isolates from horses were verified phenotypically as ESBL-producers from submission to the SVA of presumptive ESBL- and pAmpC-producing Enterobacteriaceae by Swedish veterinary laboratories [5], and all isolates were subjected to WGS. In addition, one ESBL-isolate from a clinical submission from a horse in 2016 had been subjected to WGS and was also included in the study. Of the total 56 isolates, 32 harboured <i>bla</i>_<sub>SHV-12</sub>, 22 harboured <i>bla</i>_<sub>CTX-M-5</sub>, 1 harboured <i>bla</i>_<sub>CTX-M-15</sub> and 1 harboured only <i>bla</i>_<sub>TEM-1</sub>.

2.2. Genome sequencing, bioinformatics and colistin susceptibility testing

DNA was extracted from bacterial isolates using an EZ1® DNA Tissue Kit (QiAGEN, Hilden, Germany) according to the manufacturer’s protocol, and DNA concentrations were determined using a Qubit<sup>®</sup> HS DNA Kit (Life Technologies, Carlsbad, CA, USA). Sequencing was performed using Illumina-based technology either in-house using an Illumina Nextera XT Kit (Illumina Inc., San Diego, CA, USA) and 250-bp paired-end sequencing on an Illumina MiSeq sequencer (Illumina Inc.) or the DNA was submitted to GATC Biotech GmbH (Konstanz, Germany) or SciLifeLab Clinical Genomics (Göteborg, Sweden). To identify potential contamination and bacterial species, reads were checked using Kraken against the MiniKraken 8GB database (19 October 2017). Reads were trimmed with Trimomatic 0.36 and genome assembly was performed using SPAdes v.3.11.1 with ‘--careful’ parameter followed by Pilon v.1.22. The presence of ARGs as well as plasmid replicon types were determined using Antimicrobial Resistance Identification By Assembly (ARIBA) v.2.13.3 with downloaded databases of ResFinder and PlasmidFinder (8 May 2019). Seven-loci multilocus sequence typing (MLST) and core genome MLST (cgMLST) were performed and visualised in SeqSphere + software (Ridom GmbH, Münster, Germany), whilst plasmid MLST (pMLST) was performed by uploading assemblies to pubmlst.org. The occurrence of the two potential regulatory genes qseB-like and qseC-like for mcr-9 was checked online using BLASTn (https://blast.ncbi.nlm.nih.gov/) by aligning qseB and qseC sequences from Kieffer et al. [7] to assemblies with default settings.

Sequence reads for the included isolates have been deposited in the European Nucleotide Archive (ENA) with accession no. PRJEB32700.

For isolates verified to carry mcr-genes, the colistin minimum inhibitory concentration (MIC) was determined using microdilution with MICRONAUT MIC-Strip Colistin (Merlin Diagnostika, Bornheim-Hersel, Germany) according to the recommendations of the manufacturer. The results were interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values (ECOFFs) [http://www.mic.eucast.org; accessed 17 May 2019].

3. Results and discussion

Of the 56 ESBL-producing Enterobacteriaceae isolates from horses in Sweden, 30 were shown to carry the mcr-9 gene (Table 1). Except for one isolate that carried only the <i>bla</i>_<sub>TEM-1</sub> gene, all mcr-9-positive isolates were also positive for <i>bla</i>_<sub>SHV-12</sub> and <i>bla</i>_<sub>TEM-1</sub>. Only two <i>bla</i>_<sub>SHV-12</sub>-positive clinical isolates from horses lacked the mcr-9 gene. The mcr-9-positive isolates originated from a diverse set of samples, including uterus (n = 10), wound/surgical wound (n = 7), urine (n = 5), abscess (n = 2) and other origins (n = 6) (Table 1). Since mcr-9 was only identified in isolates carrying <i>bla</i>_<sub>SHV-12</sub>, additional isolates from other sources positive for <i>bla</i>_<sub>SHV-12</sub> that had been subjected to WGS at SVA were also checked for the presence of mcr-9. A total of 8 additional isolates [broiler production (n = 6), dog (n = 1) and cat (n = 1)] were available for in silico testing but none of these were positive for mcr-9. The high occurrence of mcr-9 in this set of isolates from horses was unexpected as mcr genes have not previously been described in animals in Sweden. Furthermore, colistin is only used for pigs in Sweden, but polymyxin B may be used topically for companion animals [5]. The colistin MICs for all mcr-9-positive isolates were also below or equal to the EUCAST ECOFFs of 2 mg/L (Table 1). The isolates also lacked the two potential regulatory genes qseB-like and qseC-like, which Kieffer et al. [7] suggested could play a role in the inducibility of mcr-9. Thus, both the selective effect of colistin and the clinical impact of the gene is therefore uncertain. All isolates also carried qac6(6)-Iic and aph(6)-ld conferring resistance to aminoglycosides, ere(A) conferring resistance macrolides, and sul1 and sul2 conferring resistance to sulfonamides. In addition, genes encoding other types of aminoglycoside resistance were identified in all isolates (Table 1). Other ARGs were also identified among the isolates; trimethoprim (87%), chloramphenicol (57%), tetracycline (50%) fosfomycin (43%) and quinolones (17%) (Table 1). In equine medicine in Sweden, benzylpenicillin, gentamicin, streptomycin and trimethoprim/ sulfonamides are the only classes authorised for systemic treatment, leaving fluoroquinolones as the only off-label option [8]. It is therefore possible that spread of the mcr-9-carrying isolates among horses in Sweden has been facilitated by use of the few drug classes authorised for horses or off-label use of cephalosporins. It is important to point out that for infections in horses such as those caused by isolates in this investigation, there would be no alternatives authorised for treatment in Sweden.

The spread of Enterobacteriaceae carrying mcr-9, <i>bla</i>_<sub>SHV-12</sub> and <i>bla</i>_<sub>TEM-1</sub> among horses in Sweden appears likely to be by plasmid dissemination as the genes were identified in isolates belonging to the species <i>Enterobacter cloacae</i> complex (n = 15), <i>E. coli</i> (n = 10), <i>Klebsiella oxytoca</i> (n = 4) and <i>Citrobacter freundii</i> (n = 1), with identified isolates belonging to a diverse set of MLST sequence types (STs) (Table 1). Furthermore, occurrence is not connected to one specific geographic location, animal hospital or stud farm. Although the main spread appears non-clonal, some clonal dissemination was observed. Six <i>E. cloacae</i> isolates from three sites belonged to ST116 and based on cgMLST these isolates could...
some alleles (Table 1). Two Citrobacter, Klebsiella and Escherichia coli isolates were shown to be identical (IncHI2). The isolates was identified from the same hospital (data not shown). Furthermore, at one study farm, five E. coli isolates were shown to belong to two MLST ST1423 (n = 2) and ST1861 (n = 3) (Table 1), with the respective STs being identical using cgMLST (Fig. 1). Interestingly, the isolate that lacked bI_1861 was an E. coli ST9239 that was shown to be essentially identical to another isolate that carried bI_1861 (Fig. 1). Two isolates originated from the same clinic and from the same horse (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Year</th>
<th>Strain</th>
<th>Colistin MIC</th>
<th>MLST</th>
<th>Additional plasmid replicon types (Inc)</th>
<th>ARGs*</th>
<th>Origin/sample site</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>2018</td>
<td>3553</td>
<td>2</td>
<td>ST233</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aph(3')-Ib, bla_CMY-2, catA2, aarA, qnrB10, tetD</td>
<td></td>
<td>Animal hospital A/surgical wound</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>2017</td>
<td>3243</td>
<td>0.25</td>
<td>ST1861</td>
<td>aadA1, aadA2, aph(3')-la, aarA1, aarA9</td>
<td></td>
<td>Stud farm A/uterus</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3283</td>
<td>0.25</td>
<td>ST1861</td>
<td>aadA1, aadA2, aph(3')-la, aarA1, aarA9</td>
<td></td>
<td>Stud farm A/uterus</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3294</td>
<td>0.25</td>
<td>ST1861</td>
<td>aadA1, aadA2, aph(3')-la, aarA1, aarA9, qnrA9</td>
<td></td>
<td>Animal hospital B/surgical wound</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3371</td>
<td>0.25</td>
<td>ST2557</td>
<td>aac(6')-Ib, ant(3')-la, aap(3')-Ib, mfdA, tetD</td>
<td></td>
<td>Horse clinic A/urine</td>
</tr>
<tr>
<td></td>
<td>2018</td>
<td>3468</td>
<td>0.25</td>
<td>ST3293</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital A/urine</td>
</tr>
<tr>
<td></td>
<td>2018</td>
<td>3652</td>
<td>0.25</td>
<td>ST1252 (MGR28)</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital A/blood</td>
</tr>
<tr>
<td></td>
<td>2018</td>
<td>3642</td>
<td>0.25</td>
<td>ST1423</td>
<td>aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Horse clinic A/urine</td>
</tr>
<tr>
<td></td>
<td>2018</td>
<td>3666</td>
<td>0.25</td>
<td>ST4398 (MGR28), F1</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital A/urine</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>2017</td>
<td>3100</td>
<td>0.25</td>
<td>ST113</td>
<td>aac(6')-Ib, aadA1, ant(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital B/post-operative folliculitis</td>
</tr>
<tr>
<td>complex</td>
<td>2017</td>
<td>3114</td>
<td>0.25</td>
<td>ST116</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetB</td>
<td></td>
<td>Lab A/furunculosis</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3229</td>
<td>0.25</td>
<td>ST116</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital A/surgical wound</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3290</td>
<td>0.25</td>
<td>ST116 (MGR28)</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital A/surgical wound</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3401</td>
<td>0.25</td>
<td>ST116</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital A/urine</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3458</td>
<td>0.25</td>
<td>ST102 (MGR28)</td>
<td>aac(6')-Ib, ant(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital B/surgical wound</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3546</td>
<td>0.25</td>
<td>ST113</td>
<td>aac(6')-Ib, aadA1, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital B/surgical wound</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>2016</td>
<td>2798</td>
<td>0.25</td>
<td>ST238</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital B/surgical wound</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3163</td>
<td>0.25</td>
<td>ST37</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital B/thrombophlebitis</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3280</td>
<td>0.25</td>
<td>ST37</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital B/thrombophlebitis</td>
</tr>
<tr>
<td></td>
<td>2017</td>
<td>3320</td>
<td>0.25</td>
<td>ST2</td>
<td>aac(6')-Ib, aadA2, aph(3')-la, aap(3')-Ib, tetD</td>
<td></td>
<td>Animal hospital B/urine</td>
</tr>
</tbody>
</table>

MLST, multilocus sequence typing; ARG, antimicrobial resistance gene.

* All isolates carried aac(6')-Ib, aph(3')-la, aadA1, sul1 and sul2.

b Two isolates collected from the same horse from samples collected 10 days apart, with the later isolate 3846 being negative for bI_{SHV-12}.

c Private laboratories not connected to a specific animal hospital or stud farm.
original study and the current one also carried aac(6’)-Ile, aph(6’)-Id, ere(A), sul1 and sul2. This raises the question of whether it is global spread of specific IncHI2 and IncHI2A plasmids, or a plasmid having both replications, which may also carry multiple genes encoding antimicrobial resistance. The next step would be to establish the full sequence of the detected IncHI2 and IncHI2A plasmids to confirm that they in fact carry mcr-9, blaSHV-12 and blaTEM-1B, and if so how mcr-9 is located compared with the other ARGs.

It appears that the epidemiology of bla\textsubscript{SHV-12}-carrying isolates among horses in Sweden might differ from the rest of Europe. A previous French study showed that bla\textsubscript{SHV-12} isolates from horses was mainly connected to IncX plasmids, and previously IncX3 plasmids have been shown to predominantly carry bla\textsubscript{SHV-12} [9,10]. In the current study, the IncX replicon was not detected in isolates carrying mcr-9 and bla\textsubscript{SHV-12}, although the two Swedish bla\textsubscript{SHV-12} isolates from horses that lacked mcr-9 were positive for the IncX3 replicon.

In conclusion, we were able to show that mcr-9 is common (30/56: 54%) among clinical ESBL-producing Enterobacteriaceae isolates from Swedish horses, with the occurrence linked to multidrug-resistant isolates carrying bla\textsubscript{SHV-12} and bla\textsubscript{TEM-1B} as well as plasmids belonging to replicon types IncHI2 and IncHI2A.

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Competing interests
None declared.

Ethical approval
Not required.

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Fig. 1. Minimum spanning tree (MST) of the core genome multilocus sequence typing (cgMLST) relationship of (A) Escherichia coli and (B) Enterobacter cloacae complex isolates carrying the blar\textsubscript{SHV-12} and mcr-9 genes from horses in Sweden, based on genes present in all compared isolates. Numbers on the lines between isolates indicate differences in alleles between isolates; the lengths of lines are based on the number of gene differences on a log scale; and the diameter of circles is based on the number of isolates on a log scale. (A) MST based on 2483 alleles present in seven E. coli clinical isolates carrying blar\textsubscript{SHV-12} and mcr-9 from horses in Sweden. cgMLST is based on the Enterobase cgMLST database available through SeqSphere + software; the ST denotes the MLST number as defined by the scheme (https://enterobase.warwick.ac.uk/). * Indicates the isolate lacking the blar\textsubscript{SHV-12} gene. (B) MST based on 3873 alleles present in ten E. cloacae complex clinical isolates carrying blar\textsubscript{SHV-12} and mcr-9 from horses in Sweden. cgMLST is based on target genes extracted from the Enterobacter hormaechei 34998 genome (GenBank accession no. CP012167) using SeqSphere + software, with the resulting cgMLST scheme consisting of 4273 target genes for phylogenetic analyses; the ST denotes the MLST number as defined by the scheme at https://pubmlst.org.
to Prof. Laurent Poirel for providing the sequences of the qseB-like and qseC-like genes identified in the study by Kieffer et al. [7].

References


