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Moraxella Species as Potential Sources of MCR-Like Polymyxin Resistance Determinants

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ABSTRACT Plasmid-mediated resistance to polymyxins mediated by the MCR-1/2 determinants has been reported in *Enterobacteriaceae* worldwide. Using PCR-based and cloning strategies, a series of *Moraxella* spp. were screened for *mcr*-like genes. *Moraxella* spp. that are mainly animal pathogens but may also be human pathogens were identified as potential reservoirs of *mcr*-like genes.

KEYWORDS polymyxin, plasmid, resistance, reservoir, animal, MCR

Resistance to polymyxins in *Enterobacteriaceae* results mostly from chromosomal mutations in genes involved in modification of lipopolysaccharide (LPS) (1, 2). In particular, it has been shown that mutations, truncations, or insertions into genes encoding LPS-modifying enzymes may be responsible for acquired resistance to polymyxins in *Enterobacteriaceae* (1, 3, 4), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (1, 5). However, the plasmid-mediated polymyxin resistance determinant MCR-1 was recently found in *Escherichia coli* and *Klebsiella pneumoniae* isolates recovered from humans and animals in China (6). The *mcr-1* gene has been reported worldwide in other enterobacterial genera, including *Enterobacter*, *Salmonella*, and *Shigella* (7–11). This resistance was identified in *Enterobacteriaceae* isolated from various animal species, including cattle, chicken, and pigs (12–14), and from river samples and vegetables (15).

MCR-1 is a 541-amino-acid phosphoethanolamine transferase that adds phosphoethanolamine to the lipid A moiety of LPS, leading to a more cationic LPS structure and consequently to resistance to polymyxins (6). Recently, the MCR-2 protein (538 amino acids long, 81% amino acid identity with MCR-1) was identified in Belgium from colistin-resistant *E. coli* (16). Additionally, the MCR-1.2 and MCR-1.3 variants (both exhibiting single amino acid substitutions versus MCR-1) were identified in Italy and China, respectively (17, 18). Several plasmid types carrying the *mcr-1* gene, belonging to the IncI2, IncHI2, or IncX4 incompatibility groups, have been identified (8, 10, 12). In addition, the *mcr-1.2* and *mcr-2* genes were found on IncX4 plasmid scaffolds (16, 17).

It is often suggested that animals treated with polymyxins represent a significant reservoir of polymyxin-resistant Gram-negative isolates, particularly MCR-producing isolates (19). In fact, polymyxins are heavily used in veterinary medicine, and Europeans, particularly in Spain and Italy, have a high consumption of those antibiotics for food-producing animals (20). The frequent identification of MCR-1-producing isolates from animals further supports the hypothesis that the animal world might be the primary source of this resistance determinant.

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TABLE 1 Identity at amino acid (and nucleotide) levels between plasmid-mediated MCR-1/MCR-2 polymyxin resistance determinants and those identified in *M. porci*, *M. osloensis*, *M. lincolnii*, and *M. catarrhalis*

Polymyxin resistance determinant	Identity at amino acid (nucleotide) level (%) with:				
	MCR-1	MCR-2	MCR-POR	MCR-OSL	MCR-LIN
MCR-2	81 (79)				
MCR-POR	63 (70)	62 (70)			
MCR-OSL	63 (68)	64 (68)	62 (65)		
MCR-LIN	59 (67)	60 (70)	59 (67)	59 (66)	
MCR-CAT	59 (67)	60 (69)	59 (67)	59 (66)	99 (98)

In the initial published works reporting MCR-1 and MCR-2 (6, 16), a certain degree of sequence identity between the *mcr-1* and *mcr-2* genes and some intrinsic chromosomal genes from *Moraxella* spp. was highlighted. We hypothesized that these species might be candidates for potential progenitors of different *mcr*-like genes.

In addition to sequence analyses of *mcr*-like genes, several features were considered. First, the *mcr-1* gene is often associated with insertion sequence (IS) IS*Apl1*, which may be responsible for its mobilization (8). IS*Apl1* was originally identified in *Actinobacillus pleuropneumoniae*, belonging to the *Pasteurellaceae* family (21). This species is responsible for porcine pleuropneumonia and is widely found in pigs (22). Second, many of the *mcr-1*-positive isolates identified coharbor resistance determinants corresponding to specific antibiotics used in veterinary medicine, such as the *floR* gene encoding resistance to florfenicol (19). Many studies showed the wide dissemination of *mcr-1*-positive *E. coli* isolates, mostly in animals (14, 23). Finally, the IncX4-type plasmids that are often identified as supporting the *mcr-1* gene were identified in enterobacterial strains recovered from pigs, regardless of the presence of *mcr-1* (24, 25).

Our focus was therefore on species belonging to the *Moraxella* genus. *Moraxella* spp. are Gram-negative bacteria that are commensals or pathogens for animals (cattle, sheep, cats, dogs, rabbits, and pigs), and *Moraxella catarrhalis* is a mucosal commensal or pathogen for humans (26). *In silico* analysis of GenBank databases revealed that a gene encoding a putative MCR-like protein was located on the chromosome of the *M. catarrhalis* genome, sharing 59% and 60% amino acid identity with MCR-1 and MCR-2, respectively.

Genomic DNA was extracted from a series of strains belonging to different *Moraxella* spp., corresponding to *Moraxella lincolnii* CIP103462, *Moraxella bovoculi* CIP109558T, *Moraxella porci* CIP110214T, *Moraxella lacunata* CIP108000, *Moraxella saccharolytica* CIP57.36 and CIP68.37, *Moraxella bovis* CIP70.40T and CIP103741, *Moraxella equi* CIP82.26T, *Moraxella canis* CIP103801T, *Moraxella caprae* CIP104714T, *Moraxella boevrei* CIP104716T, *Moraxella nonliquefaciens* CIP100617, *Moraxella laennata* CIP102083, and *Moraxella osloensis* CIP100025. PCR-based experiments were performed by using specific and internal primers for both the *mcr-1* and *mcr-2* genes, namely, MCR-1+2-For (5′-TATC GCTATGTGCTAAAGCC-3′) and MCR-1+2-Rev (5′-TCTTGGTATTTGGCGGTATC-3′). The annealing temperature was decreased to 50°C to allow for the amplification of DNA targets that might not be fully homologous to the *mcr* genes. For PCRs giving positive results, only internal amplifications of the *mcr*-like genes were obtained. In order to gain knowledge about all the *mcr*-like genes, an inverse PCR strategy was used, as previously reported (27). Briefly, outward primers were designed from the internal sequenced fragment to amplify adjacent DNA sequences. Templates corresponded to the genomic DNA of the strains that were previously digested with different restriction enzymes (BamHI, HindIII, EcoRI) and self-circularized using DNA T4 ligase.

PCR assays with internal *mcr-1*-specific genes gave positive results for three strains, i.e., *M. lincolnii*, *M. porci*, and *M. osloensis*. Subsequently all of the *mcr*-like genes were identified using the inverse-PCR strategy. The corresponding proteins, named MCR-LIN, MCR-POR, and MCR-OSL, respectively, shared significant amino acid identities with MCR-1 and MCR-2, ranging from 59% to 64%, respectively (Table 1). Note that the most

TABLE 2 Analysis of the putative open reading frame (ORF) located downstream of the *mcr*-like genes

Gene	ORF length (amino acids)	Distance from *mcr* stop codon (nucleotides)	Identity (amino acids) of ORF_{MCR-1} (coverage)[a] (%)
mcr-1	240	71	
mcr-2	82[b]	93	51 (97)[c]
mcr_{OSL}	236	90	44 (90)
mcr_{CAT}	251	45	38 (89)
mcr_{LIN}	245	50	39 (91)
mcr_{POR}	216	48	43 (86)

[a]Coverage represents the percentage of the query sequence that overlaps the subject sequence (i.e., ORF_{MCR-1}).
[b]Putative ORF of MCR-2 is truncated by a copy of IS*1595*.
[c]Coverage was calculated from the alignment of the truncated ORF_{MCR-2} and the first 84 amino acids of ORF_{MCR-1}. The mcr_{OSL}, mcr_{CAT}, mcr_{LIN}, and mcr_{POR} genes are from *M. osloensis*, *M. catarrhalis*, *M. lincolnii*, and *M. porci*, respectively.

closely related variant of MCR-1 was identified in *M. porci*, whereas the most closely related variant of MCR-2 was identified in *M. osloensis*. Those identities were also calculated for the corresponding genes at the nucleotide level, which further confirmed the significant degree of similarity with *mcr*-like genes sharing ~70% identity with the *mcr-1* and *mcr-2* genes (Table 2).

Interestingly, considering the recently determined structure of the catalytic domain of MCR-1 (28), all of the MCR-like proteins identified in this study possessed the six cysteine residues forming the three disulfide bridges of the catalytic domain (Fig. 1). The threonine 285 residue shown to be the catalytic nucleophile was also conserved in all of the proteins (28). A phylogenetic tree was elaborated with nucleotide sequences of the genes encoding the different MCR-like enzymes along with other putative phosphoethanolamine transferase proteins. Interestingly, MCR-1 and MCR-2 significantly clustered with those newly identified MCR-like determinants from *Moraxella* spp. (Fig. 2A). Note that the phosphoethanolamine transferase from *Enhydrobacter aerosaccus* also clustered in that group. This result is in accordance with the recent taxonomic reclassification of that species into the family *Moraxellaceae* (29). In parallel, the phylogenetic tree elaborated with sequences of the 16S RNA gene sequences of those different bacterial species confirmed that feature (Fig. 2B).

When analyzing the nucleotide sequences located downstream of *mcr*-like genes, the significant identity between the mobilized fragment encompassing *mcr-1* or *mcr-2* and those identified in the chromosome of the *Moraxella* spp. was evident. Indeed, homologues of the 240-amino-acid open reading frame previously identified downstream of *mcr-1* were also identified downstream of the *mcr*-like genes, sharing ~40% to 50% amino acid identity (Table 2). This protein encodes a putative PAP2 membrane-associated lipid phosphatase (16).

A PCR-based screening of the different *Moraxella* strains for the presence of the replicase gene of IncX4 plasmids was performed with primers X4-Fw and X4-Rv, as previously described (8). Interestingly, a positive signal was obtained using the genomic DNA of *M. lacunata* that was further confirmed to be 99% identical to the IncX4 replicase gene identified in association with the *mcr-1* gene, therefore showing that *M. lacunata* may also be a reservoir of IncX4-type plasmids.

Since the *mcr-1* gene is most often associated with the IS*Apl1* element, a PCR specific for the IS*Apl1* element was performed with all *Moraxella* strains. A positive signal was found with the *M. porci* strain, and sequencing confirmed a perfect identity with IS*Apl1*. Notably, IS*Apl1* was not located upstream of the *mcr-por* gene, in contrast to observations for *mcr-1* and *mcr-2* genes. This result highlights that some *Moraxella* spp. may possess in their genome the putative resistance gene along with the genetic tool likely involved in its mobilization, reinforcing the possibility of such a mobilization process. We recently demonstrated that IS*Apl1* elements can mobilize the *mcr-1* gene by transposition (unpublished data).

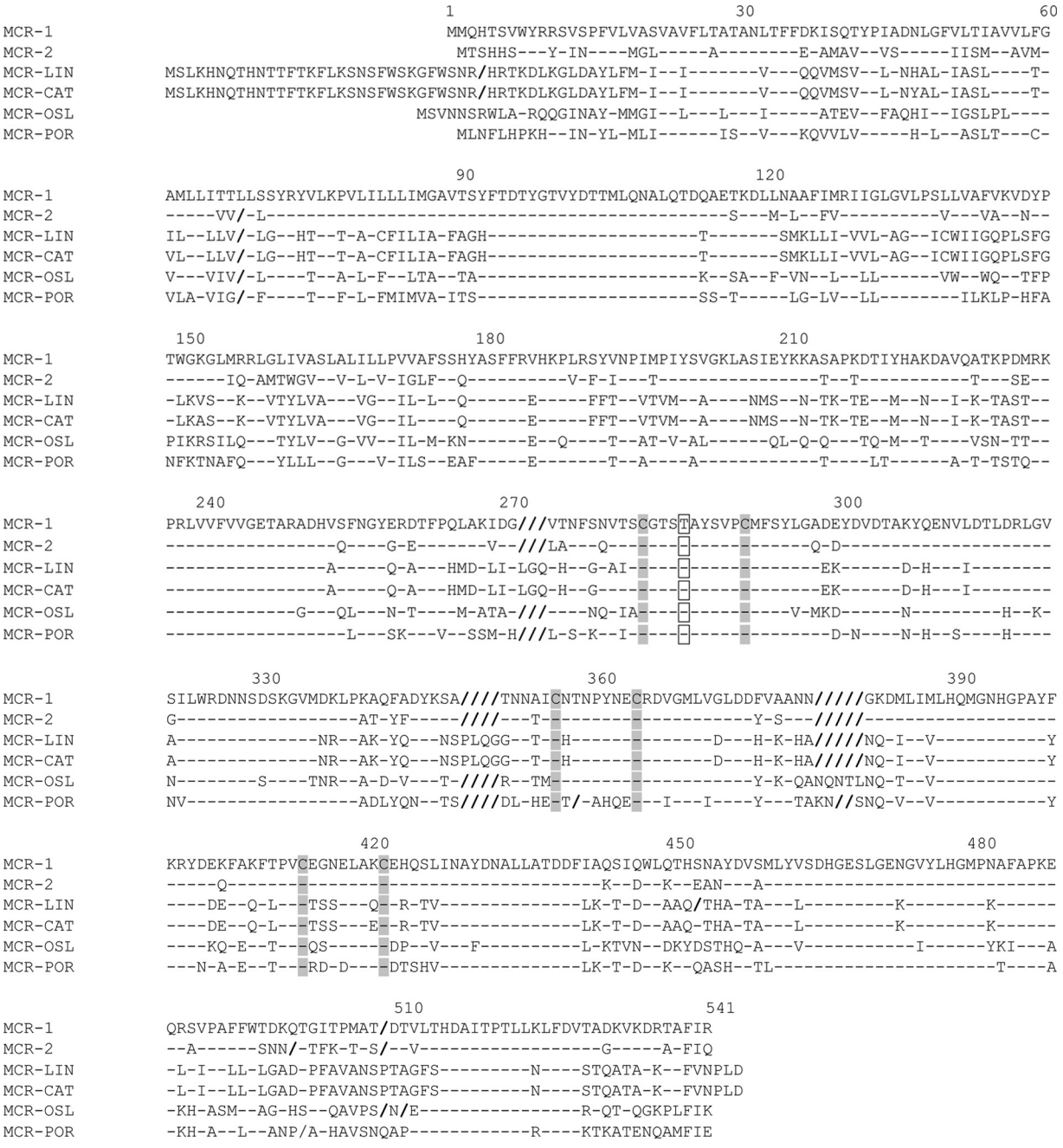

FIG 1 Sequence comparison of plasmid-mediated MCR-like determinants. The plasmid-mediated MCR-1 and MCR-2 determinants were published previously (6, 16). MCR-LIN is from *M. lincolnii*, MCR-CAT from *M. catarrhalis*, MCR-OSL from *M. osloensis*, and MCR-POR from *M. porci.* Slashes indicate identical amino acid residues. Shaded areas indicate conserved cysteine residues forming the three disulfide bridges of the MCR-1 catalytic domain (28). The threonine 285 that was shown to be the catalytic nucleophile in MCR-1 (28) is also conserved among all the MCR proteins, indicated by open boxes.

The different *mcr*-like genes were cloned in the same L-arabinose-inducible vector pBADb (30). Recombinant plasmids were expressed into *E. coli* TOP10 cells by adding 1% L-arabinose, and MIC values of colistin were determined by broth dilution methods, as recommended by CLSI (31). Production of MCR-LIN and MCR-POR conferred 8- or 16-fold increases in the MIC values of colistin in *E. coli* (from 0.03 to 0.5 or 1 μg/ml), and higher (64-fold) increases in MIC were conferred by MCR-OSL (4 μg/ml). Nonetheless, using the same background, the highest MIC value of colistin was achieved with MCR-1, highlighting that MCR-1 was the most efficient enzyme to confer acquired resistance to

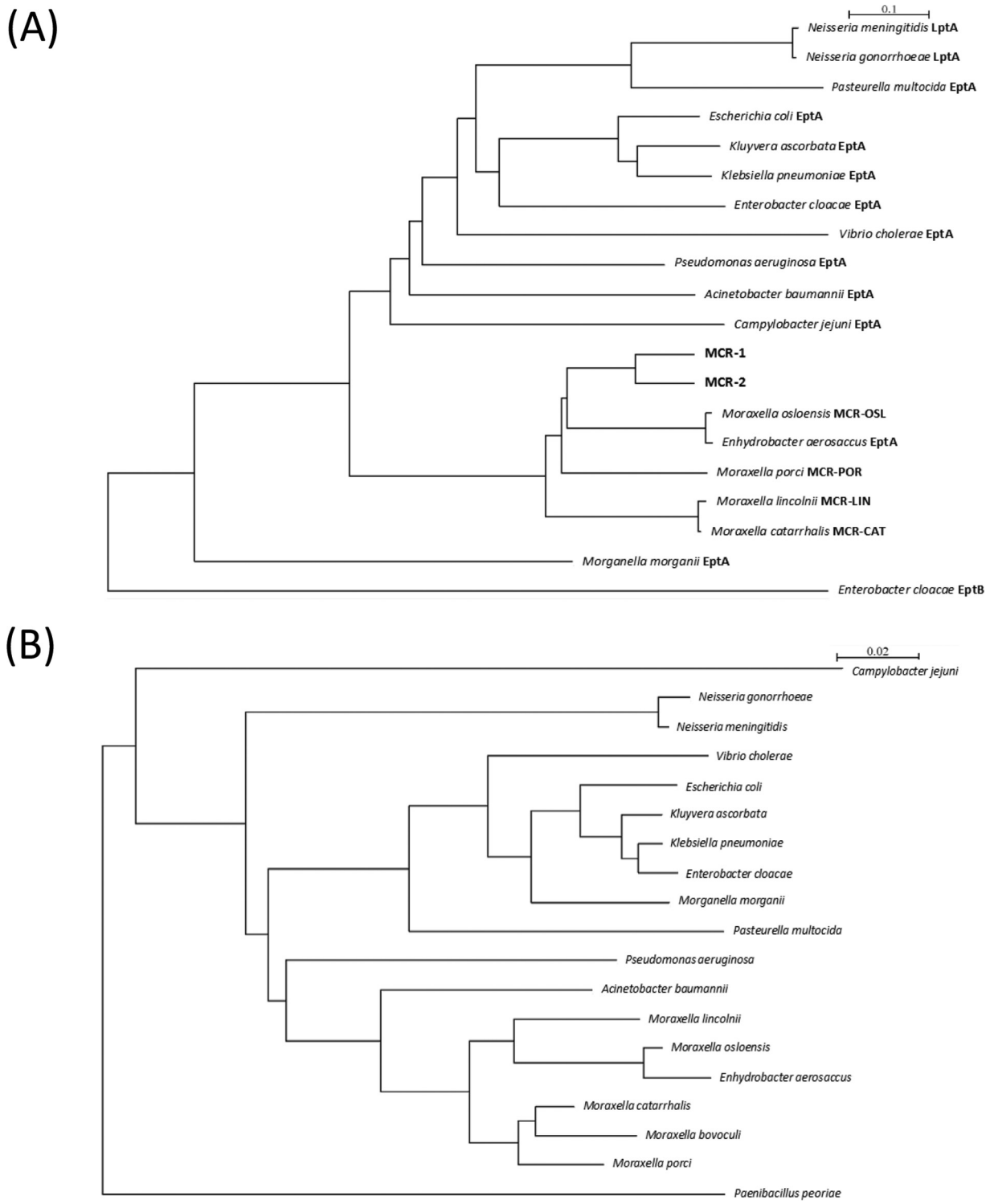

FIG 2 Phylogenetic tree obtained for a series of phosphoethanolamine transferase encoding genes (A) and 16S RNA genes (B) by the distance method using the neighbor-joining algorithm (SeaView v4 software) (40). Branch lengths are drawn to scale and are proportional to the number of amino acid changes with 200 bootstrap replications. The distance along the vertical axis has no significance.

colistin in *E. coli*. Note that *M. lincolnii* and *M. porci* both showed high MIC values of colistin (64 μg/ml).

This report indicates that *Moraxella* spp. constitute a reservoir of *mcr*-like genes that might be mobilized from their original host to become acquired resistant determinants

in clinically significant species, as previously shown for other resistance genes, with *Kluyvera* spp. being the sources of CTX-M-type extended-spectrum β-lactamase genes (32, 33); *Citrobacter freundii*, *Morganella morganii*, and *Hafnia alvei* of AmpC-type β-lactamase genes (34, 35, 36); *Shewanella* spp. of OXA-48 and OXA-181 carbapenemase genes (37, 38); and *Shewanella algae* of the plasmid-mediated quinolone resistance determinant QnrA (39).

It is tempting to speculate that the heavy usage of polymyxins in animals may be a selective factor for mobilizing these naturally occurring MCR resistance determinants from *Moraxella*-like species to *Enterobacteriaceae*. We showed here that all genetic features requested for mobilization of the *mcr*-like genes (bacterial progenitor, genetic tools, and selective pressure) are present in *Moraxella* that may be providers of further clinically relevant and plasmid-mediated MCR-like determinants in the future. However, the exact species acting as progenitors of the MCR-1 and MCR-2 encoding genes remain to be determined.

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