

## **Final Report**

### **Project: *Mcr-1* based colistin resistance: filling knowledge gaps in view of the spread of plasmid-mediated colistin resistance in Switzerland**

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## 1. Background

Antibiotic resistance is an increasing problem in human and veterinary medicine worldwide. Most of the currently emerging resistance mechanisms in Gram negative bacteria are plasmid-borne with an important potential for spreading the resistance genes between bacteria of the same species but also between different species (horizontal transfer). These serious concerns have been catalyzed by the global dissemination of extended-spectrum  $\beta$ -lactamase (ESBL)-producing Gram-negative rods both in hospitals and in the community that inactivate most of the  $\beta$ -lactams except carbapenems and by the rapid emergence of the carbapenemase-producing Enterobacteriaceae, which are also frequently resistant to nearly all antimicrobial drugs available. Due to the paucity of remaining antibiotics for treating infections, polymyxins have become the last resort in particular for treating infections due to carbapenem-resistant Gram negatives. Polymyxins, although introduced in the armamentarium in the 1950's have been considered to be too nephrotoxic and too neurotoxic for their regular use in humans. They have been mostly used (and heavily) in animals in prophylaxis, metaphylaxis, therapeutics and even as a growth promoter in some regions of world. However, the recent increasing identification of animal and human isolates being resistant to polymyxins is therefore of particular concern. Indeed, this highlights that the encounter between those two parallel phenomenon (resistance to almost all drugs except polymyxins on the human side, and resistance to polymyxins without systematic association with other resistances on the animal side) might potentially give rise to pandrug resistant isolates. This concern has recently been strongly underlined by the identification of the first plasmid-mediated polymyxin resistance trait.

Polymyxins are produced by the *Paenibacillus polymyxa* sub-species *colistinus*. They are decapeptides bound to a fatty acid chain. The clinically-available polymyxins are polymyxin B and colistin which are administered intravenously or by inhalation. Colistin, that is mostly use in Europe, interacts with the lipid A moiety of the Gram-negative bacteria lipopolysaccharide (LPS) in keeping with other antimicrobial peptides. The polycationic peptide ring competes for and substitutes the calcium and magnesium bridges stabilizing the LPS. This in turn promotes membrane permeability, which disrupts the integrity of the outer membrane and leads to bacterial death. The identified mechanisms of resistance were so far chromosomally encoded, and include modifications of the outer membrane components, mainly through the covalent addition of either phosphoethanolamine or 4-deoxyaminoarabinose to the LPS, leading to a more positively charged LPS thus reducing the affinity of positively charged polymyxin molecules.

The identification of Enterobacteriaceae harbouring the plasmid-mediated transferable colistin resistance *mcr-1* gene in China (Liu et al., 2016) add the additional source of concern, i.e; an interspecies transferability of this resistance determinant.

Plasmid-encoded MCR-1 has been identified now mainly from *E. coli* but more rarely from a few other enterobacterial isolates (i.e. *K. pneumoniae*, *Citrobacter freundii*, and *Salmonella* spp. *enterica*). MCR-1 is a phosphoethanolamine transferase that adds phosphoethanolamine to the lipid A. This resistance trait is transferable by conjugation and has been reported so far in *Enterobacteriaceae* mostly from animal isolates, but also from human isolates and from food products. Despite the fact that very limited data are available, it is of note that the *mcr-1* gene has been identified already all over the world, in both animal and human samples. It seems that the animal reservoir of *E. coli* is currently the main reservoir of *mcr-1* so far identified. The few studies that focused on the genetics of acquisition of the *mcr-1* gene show that a diversity of plasmids bearing this gene, with many of them corresponding to previously described plasmid backbones.

## **2. Aims of the project**

The project consists of two different work packages and provides first (but not representative) baseline data to estimate the occurrence of *mcr-1* harbouring Enterobacteriaceae in the human population in Switzerland and in selected clinical isolates at the primary care and hospital level. Moreover, based on molecular characterization data of the isolates, an improved understanding of the epidemiology and spreading potential of *mcr-1* harbouring Enterobacteriaceae will be provided.

## **3. Results**

### **3.1 Studies on the occurrence of *mcr* harbouring Enterobacteriaceae**

#### **3.1.1 Screening for fecal carriage of MCR-producing Enterobacteriaceae in healthy humans and primary care patients**

Between September and November 2016, 1,144 non-duplicate samples were analysed. Stool samples (n=1,091) were obtained from employees of food-processing companies located throughout Switzerland during a yearly routine fecal screening for salmonellae. Moreover, fecal swabs (n=53) were obtained from adult primary care patients consulting their general

practitioner in a suburban community in the greater area of Zurich, Switzerland, during a period of 2 weeks in September 2016.

A total of 62 isolates were obtained from the selective plates supplemented with colistin. Thereof, 54 were resistant to colistin (MIC > 2 mg/l), including *Hafnia alvei* (n=43), *Escherichia coli* (n=5), *Enterobacter cloacae* (n=2), *Enterobacter* spp. (n=2), *Klebsiella pneumoniae* (n=1) and *Raoultella ornithinolytica* (n=1). Resistant isolates originated from 49 (4.5%) of the healthy people and 5 (9.4%) of the primary care patients. Results of the *mcr1/mcr2* PCR screening remained negative for all 62 isolates.

This study presents the first report on the occurrence of colistin-resistant Enterobacteriaceae in the fecal flora of healthy humans and primary care patients in Switzerland and Europe. The findings revealed fecal carriage of bacteria with likely non-transmissible colistin resistance. The absence of MCR producers in the fecal flora of healthy people as well as primary care patients is of major epidemiological interest. It indicates that the risk of transfer of *mcr* genes from animals, food or the environment is currently very low in Switzerland, despite the fact that colistin is used for treating infections in livestock.

### **3.1.2 Screening for fecal carriage of MCR-producing Enterobacteriaceae in patients with diarrhea**

In total, 319 non-duplicate samples were analysed (June to December 2016). Stool samples or fecal swabs were obtained from patients with diarrhea (children and adults). The samples were sent to the National Centre for Enteropathogenic Bacteria and Listeria (NENT) for the detection of Shigatoxin-producing *E. coli*.

A total of 15 non-intrinsic colistin resistant isolates (*Hafnia alvei* (n=10) and *Escherichia coli* (n=5) were obtained from 15 different patients from the selective plates. Results of the *mcr-1/mcr-2* PCR screening remained negative for all but two isolates (Table 1). *E. coli* ColR598 (ST 48) (isolated from a 1.5 year old children; with a travelling history to Vietnam) was positive for *mcr-1* but negative for *stx*. *E. coli* ColR644SK1 (ST 117) (isolated from a 24 year old female; with a travelling history to Asia (Borneo, Philippinen, Bali, Kambodscha)) was positive for *mcr-1* but negative for *stx*. These are the first data on the occurrence of colistin-resistant Enterobacteriaceae in the fecal flora of patients with diarrhea in Switzerland.

Table 1: Characteristics of *E. coli* ColR598 (ST 48) and ColR644SK1 (ST 117)

Sample ID	Origin	Species	<i>mcr-I</i>	MIC colistin [mg/L]	Additional antibiotic resistance profile
ColR598	stool	<i>E. coli</i>	+	4	NA, CIP, TE
ColR644SK1	stool	<i>E. coli</i>	+	8	AM, CF, TE, C, SMZ, TMP

### 3.1.3 Screening of plasmid-mediated MCR-1 colistin-resistance from bacteremia

A retrospective study was conducted to evaluate the spread of polymyxin resistance and MCR-1 positivity among enterobacterial isolates that had been isolated from clinically-significant specimens, i.e. blood cultures, at the University Hospital of Lausanne (1,200 beds), Switzerland, in 2015. This study was performed in the same French-speaking region of Switzerland as Geneva and Neuchâtel where *mcr-I* positive *E. coli* isolates had been identified. A total of 257 non-duplicated enterobacterial isolates was screened that included *E. coli* ( $n=164$ ), *Klebsiella pneumoniae* ( $n=41$ ), *Enterobacter cloacae* ( $n=16$ ), *Klebsiella oxytoca* ( $n=14$ ), *Enterobacter aerogenes* ( $n=6$ ), *Citrobacter koseri* ( $n=5$ ), *Citrobacter freundii* ( $n=5$ ), *Hafnia alvei* ( $n=3$ ), *Kluyvera ascorbata* ( $n=1$ ), and *Salmonella* serovar Enteritidis ( $n=2$ ) isolates. The predominance of *E. coli* isolates in that collection is in accordance with the known enterobacterial distribution in bacteremia.

Four isolates (three *H. alvei* and a single *E. cloacae*) were selected as resistant to colistin (prevalence rate, 1.2 %) according to the Rapid Polymyxin test. MIC determination confirmed that those four strains were indeed resistant to colistin (MIC > 2 mg/L), the *E. cloacae* strain showing an heteroresistance phenotype as known. In fact, *H. alvei* might correspond to an enterobacterial subspecies being naturally resistant to polymyxins. All the strains tested negative for the *mcr-I* gene.

### 3.1.4 Prevalence of MCR-1/MCR-2 plasmid-mediated colistin resistance in urinary tract Enterobacteriaceae obtained in two main private laboratories in the western part of Switzerland

A prospective analysis of the prevalence rate of MCR-1- and MCR-2-producing Enterobacteriaceae in urine samples from both the hospital and the community care settings obtained from two main private laboratories during the period February to March 2016 was performed. The study was focused on urinary tract infections (UTIs). Strains were collected from the same region of Switzerland where *mcr-I*-positive *E. coli* was identified previously (Poirel et al. Lancet Infect Dis. 2016 (3):281).

A total of 2049 non-duplicate enterobacterial isolates were screened. These included *E. coli* (n=1704), *Klebsiella pneumoniae* (n=151), *Proteus mirabilis* (n=73), *Citrobacter* sp. (n=32), *Klebsiella oxytoca* (n=22), *Enterobacter cloacae* (n=18), *Morganella morganii* (n=13), *Enterobacter aerogenes* (n=15), *Proteus vulgaris* (n=7), *Serratia* sp (n=7), *Providencia rettgeri* (n=3), *Salmonella* group D (n=2), *Hafnia alvei* (n=1), and *Kluyvera ascorbata* (n=1). According to the results of the rapid polymyxin NP test, six isolates were resistant to colistin (prevalence rate, 0.3%): two *E. coli*, two *K. pneumoniae*, one *H. alvei*, and one *Salmonella* isolate, with MIC values of colistin ranging from 4 to >128 mg/l.

PCRs targeting the *mcr*-like genes performed on the colistin-resistant isolates remained negative. However, when testing colistin-susceptible isolates, a single *E. coli* strain (C1624) was positive for the *mcr-1* gene. The MIC value of colistin for this isolate was 0.125 mg/l. Southern hybridization using an *mcr-1* fragment of approximately 600 bp as a probe identified this gene located on a plasmid of approximately 35 kb. This plasmid was not transferable using liquid or solid mating techniques. Multilocus sequence typing of *E. coli* C1624 showed that it belonged to sequence type ST428. This sequence type has been identified in infections of broiler breeders, further suggesting the animal origin of the MCR-like-producers. The identification of a colistin-susceptible/*mcr-1*-positive isolate in this study indicates that the silent spread of this gene might happen.

### **3.1.5 Prevalence of MCR-1/MCR-2 plasmid-mediated colistin resistance in urinary tract Enterobacteriaceae isolated from primary care patients consulting a general practitioner in a suburban community in the greater area of Zurich**

A prospective analysis of the prevalence rate of MCR-1- and MCR-2-producing Enterobacteriaceae from urinary tract infections obtained from primary care patients consulting a general practitioner in a suburban community in the greater area of Zurich during the period February to October 2016 was performed.

A total of 51 non-duplicate enterobacterial isolates were screened. These included *E. coli* (n=42), *Klebsiella pneumoniae* (n=2), *Proteus mirabilis* (n=3), *Citrobacter* sp. (n=1), *Klebsiella oxytoca* (n=1), *Enterobacter cloacae* (n=1), and *Proteus vulgaris* (n=1).

PCRs targeting the *mcr-1* and *mcr-2* genes were all negative.

### **3.1.6 Assessment of animals as a reservoir for colistin resistance: no MCR-1/MCR-2 producing Enterobacteriaceae detected in Swiss livestock**

An additional study was conducted to evaluate the occurrence of *mcr-1* and *mcr-2* in colistin resistant Enterobacteriaceae isolated from livestock at slaughter in Switzerland. The isolates were collected between April and July 2016 from fecal samples of 325 randomly selected fattening pigs, 241 randomly selected calves and fecal samples obtained from 100 poultry flocks.

Overall, 13 (4%) of the fecal swabs from pigs and 8 (3.3%) of the samples from calves yielded non-intrinsic colistin resistant isolates. None of the poultry swabs tested positive. Of the isolates from pigs, 8 (61.5%) were *Hafnia alvei*, three (23%) were *Escherichia coli*, one was *Enterobacter cloacae* and one *Klebsiella pneumoniae* (both 7.7%). Isolates from calves comprised seven (87.5%) *H. alvei* and one (12.5%) *E. coli*. MIC determination confirmed resistance to colistin (MIC>2 mg/L) for all isolates. Multidrug resistance (MDR, resistance to three or more classes of antimicrobials) was observed for the *K. pneumoniae* isolate, for three (75%) of the *E. coli* and for five (33.3%) of the *H. alvei* isolates. None of the isolates contained the *mcr-1* or *mcr-2* gene. These results indicate that food-producing animals in Switzerland do not represent so far an *mcr-1* or *mcr-2* reservoir.

### **3.1.7 Assessment of the occurrence of MCR producing Enterobacteriaceae in Swiss and imported poultry meat**

To determine whether MCR-producing Enterobacteriaceae are present in poultry meat at retail level, 128 fresh meat samples from Switzerland (n=40), Germany (n=69), Hungary (n=6), Denmark (n=6), Austria (n=2), and Italy (n=5), obtained in August and September 2016 from retail stores were analyzed. The plasmid-mediated colistin resistance gene *mcr-1* was detected by PCR in 33 (25.8%) meat samples (5 samples originating from Italy and 28 samples originating from Germany). Thereof, 12 *mcr-1* harbouring *E. coli* (Table 2) were obtained from 12 samples (10 from Germany and two from Italy) by subculturing for further characterization. The isolates belonged to various sequence types (ST) and clonal complexes (CC), including *E. coli* of the endemic avian and human pathogenic CC10, CC23, and CC155. Further STs included ST156, ST1431 and ST3519, which are associated with humans and animals. These data highlight the importance of surveillance of retail poultry meat from different countries in context of the global dissemination of MCR-producing *E. coli*.



Table 2: MCR-1 producing *E. coli* isolated from poultry meat from Germany and Italy

Sample ID	Origin	Species	Phylogenetic group	MLST/CC	<i>mcr-1</i>	MIC colistin [mg/L]	Additional antibiotic resistance profile
<i>chicken meat</i>							
PF 11	Germany	<i>E. coli</i>	B1	156/156	+	8	AM, CF, NA, CIP, TE
PF 61	Germany	<i>E. coli</i>	A	3519	+	8	AM, S
PF 65	Germany	<i>E. coli</i>	A	10/10	+	8	AM, NA, S, SMZ, TMP
PF 75	Germany	<i>E. coli</i>	B1	156/156	+	8	AM, CF, NA, CIP, TE,
PF 94	Italy	<i>E. coli</i>	A	650/23	+	4	AM, NA, TE, S, C, SMZ, TMP
PF 100	Germany	<i>E. coli</i>	B1	new <sup>a</sup>	+	4	AM, CF, NA, CIP, TE
PC11	Germany	<i>E. coli</i>	A	1251/10	+	8	AM, CF
<i>Turkey meat</i>							
PF 52	Germany	<i>E. coli</i>	B1	58/155	+	4	AM, SMZ
PF 56	Germany	<i>E. coli</i>	B1	1431	+	4	AM, TE, C, SMZ
PF 63	Germany	<i>E. coli</i>	B2	355/73	+	8	AM, CF, NA, TE, S, SMZ, TMP
PF 88	Italy	<i>E. coli</i>	A	744	+	8	AM, NA, CIP, TE, K, S, C, SMZ, TMP
PF 91	Germany	<i>E. coli</i>	B1	1431	+	8	AM, TE, C, SMZ,

### 3.1.8 Investigation for the colistin resistance genes *mcr-1* and *mcr-2* in clinical Enterobacteriaceae isolates from cats and dogs in Switzerland

The aim of a further study was to assess the occurrence of MCR-1 and MCR-2 producers among Enterobacteriaceae isolated from companion animals admitted to the University of Zürich veterinary clinic between February 2012 and July 2016. A total of 347 isolates (231 from dogs, 116 from cats) were analysed. Thereof, 274 (79%) were from urine and 73 (21%) from surgical sites, abscesses and other sources. None of the isolates tested positive by PCR for the *mcr-1* or the *mcr-2* gene. Broth microdilution tests revealed MICs of 2 mg/L–64 mg/L of colistin for four isolates (two *Enterobacter* spp., one *Klebsiella* spp. and one *E. coli*) from dogs (one from skin exudate, one from a surgical sites and two isolates from urine). This study documents the presence of non-transmissible colistin resistance in clinical isolates from dogs and cats and confirms that so far, companion animals in Switzerland do not represent a reservoir for plasmid-mediated colistin resistant Enterobacteriaceae.

## 3.2 Molecular characterization data of *mcr* harbouring Enterobacteriaceae

### 3.2.1 Features of the *mcr-1* cassette respect to colistin resistance

The *mcr-1* gene has so far mainly been associated with non-related types of plasmid replicons such as IncI2, IncHI2, IncP, IncFIB and IncX4. This gene is part of a 2,600-bp long fragment designed as the *mcr-1* cassette that encompasses the likely promoter sequences for *mcr-1* expression. The *mcr-1* gene is most often located at the right-hand extremity of the insertion

element *IS<sub>AplI</sub>*, together with a 723-bp long open reading frame (named *orf723*) encoding a hypothetical protein. However, the putative contribution of this orf for expression of the *mcr-1* gene and subsequently to colistin resistance remains unknown.

The aim of this study was to evaluate the role of *orf723* with respect to the colistin resistance. Therefore, three *Escherichia coli* recombinant strains were constructed, with the same plasmid harbouring either the *mcr-1* gene alone, *orf723* alone, and the entire *mcr-1* cassette, respectively. Minimal inhibitory concentrations (MIC) of colistin were determined using broth dilution tests as recommended by EUCAST.

MIC values of recombinant strains expressing MCR-1 with and without the *orf723* were increased and identical (4 mg/L). However they show that *orf723* encoding a hypothetical protein and which has likely been co-mobilized with the *mcr-1* gene from its original genetic context does not impact colistin susceptibility (1 mg/L).

### **3.2.2 The reservoirs of MCR-like resistance determinants**

In-silico analysis over Genbank databases revealed that a gene encoding a putative MCR-like protein was located onto the chromosome of the *M. catarrhalis*, sharing 59 and 60% amino acid identity with MCR-1 and MCR-2, respectively. In addition, we identified novel MCR-like proteins, namely MCR-LIN for *M. lincolnii*, MCR-POR for *M. porci*, and MCR-OSL for *M. osloensis*, sharing significant amino acid identities with MCR-1 and MCR-2, ranging from 59% to 64%, respectively. Noteworthy, the most closely-related variant compared to MCR-1 was identified in *M. porci*, while the most closely-related variant of MCR-2 was identified in *M. osloensis*. PCR assays with internal *mcr-1*-specific genes gave positive results for three isolates, belonging to the species *M. lincolnii*, *M. porci*, and *M. osloensis*. Interestingly, when considering the recently determined structure of the catalytic domain of MCR-1 that we identified, all MCR proteins identified in this study possess six cysteine residues forming the three disulfide bridges of the catalytic domain. The threonine 285 residue shown to be the catalytic nucleophile was also conserved in all those proteins.

Since the *mcr-1* and *mcr-2* genes are very often identified onto IncX4 plasmid scaffolds, the *Moraxella* spp. were screened by PCR strains for the corresponding replicase gene with primers X4-Fw and X4-Rv as published. A positive signal was obtained using the genomic DNA of *M. lacunata*. Sequencing revealed a 99% identity with the IncX4 replicase gene identified in association with the *mcr-1* gene. This showed that *M. lacunata* may also be a reservoir of IncX4-type plasmids.

Since the *mcr-1* gene is most often associated to the IS*AplI* element, a PCR specific for that IS element was performed with all *Moraxella* spp. isolates. Interestingly, a positive signal was found with the *M. porcii* strain, and sequencing confirmed a perfect identity with IS*AplI*. However, IS*AplI* was not located upstream of the *mcr-por* gene, by contrast to what is observed with both *mcr-1* and *mcr-2* genes once acquired. However, this result highlights that some *Moraxella* species may possess in their genome both the putative resistance gene along with the genetic tool likely involved in its mobilization, therefore reinforcing the likelihood of such mobilization process.

Finding that *Moraxella* spp. being progenitors of MCR-like determinants adds to the limited list of identified reservoirs of clinically-significant antibiotic resistance traits in Gram negatives, with *Kluyvera* spp. being the reservoirs of CTX-M-type extended-spectrum  $\beta$ -lactamase genes, *Morganella morganii* and *Hafnia alvei* of AmpC-type  $\beta$ -lactamase genes, *Shewanella* spp. of the OXA-48 and OXA-181 carbapenemase genes, and *Shewanella algae* of the plasmid-mediated quinolone resistance determinants QnrA.

### **3.2.3 Draft genome sequence of *Escherichia coli* S51, a chicken isolate harboring a chromosomally encoded *mcr-1* gene**

In a recent study, we isolated an ESBL-producing *E. coli* harboring the *mcr-1* gene from raw chicken meat imported from Germany (Zogg et al. Schweiz Arch Tierheilk. 2016, 158: 451–456). Despite repeated attempts, the *mcr-1* gene from this isolate could not be transferred by conjugation. Plasmid DNA from S51 was extracted and used in electroporation experiments. Again the transfer of the colistin resistance determinant was also not successful. Further hybridization experiments probing with a *mcr-1* fragment indicated the possible chromosomal integration of the *mcr-1* gene. Therefore, genomic DNA was isolated from S51 and subjected to sequencing using Pacific Biosciences SMRT technology. The S51 genome was assembled *de novo* using the SMRT Analysis 2.3.0 software to a single chromosome of 4.994.918 bp in size with a G-C content of 50.7 % and two unclosed plasmid sequences of approx. 93 kb and approx. 98 kb in size, respectively. Gene prediction was carried out using Glimmer 3.0.2. Annotation was conducted based on homology searches against COG, SEED and KEGG databases and using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) ([http://www.ncbi.nlm.nih.gov/genome/annotation\\_prok/](http://www.ncbi.nlm.nih.gov/genome/annotation_prok/)).

The *mcr-1* gene in S51 is located at the right-hand extremity of an IS*AplI* element, together with an 813 bp orf encoding a hypothetical protein with similarities to a PAP2 superfamily

protein. This combination of the IS*ApII* and the *mcr-1* cassette has been often described on *mcr-1*-harboring plasmids of diverse replicon types. The “IS*ApII*-*mcr-1*-cassette” was found to be located in the S51 chromosome between the gene encoding for the outer membrane protein E and for a glutamate-5-kinase, respectively. This IS*ApII*-*mcr-1*-cassette association on a chromosome further supports the hypothesis that IS*ApII* might be involved in *mcr-1* acquisition.

### **3.2.4 Full-length nucleotide sequences of the *mcr-1* harboring plasmid pPC11 from an *E. coli* isolated from chicken meat imported from Germany**

In a recent study, we isolated *E. coli* strain PC11 (ST1251/CC10) harboring the *mcr-1* gene from raw chicken meat imported from Germany (Zurfluh et al., 2017). The *mcr-1* harboring plasmid was transferred by transformation experiments into *E. coli* DH5-alpha and colistin resistant transformants were selected. The *mcr-1* harboring plasmid pPC11 was extracted and sequenced on a PacBio RS2 device (Pacific Biosciences, Menlo Park, USA) with a 10 kb size-selected insert library and P6/C4 chemistry. The plasmid sequence was automatically annotated using the online Rapid Annotation Subsequencing Technology (RAST) and CLC Main Workbench Version 7.7 (CLC bio, Aarhus, Denmark). Automated annotation was manually refined using the BLASTn and BLASTp programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Plasmid pPC11 (Figure 1) is 59.826 bp in size, with a G+C content of 42.3%. pPC11 belongs to the plasmid incompatibility group **IncI2** which is the same Inc-type as the first *mcr-1*-positive plasmid described in China (pHNSHP45). The *mcr-1* gene on pPC11 is located at the right-hand extremity of an IS*ApII* element, together with a 723-bp long open reading frame (named *orf723*) encoding a hypothetical protein. Similar to pHNSHP45, no resistance determinants other than the *mcr-1* gene were located on pPC11.

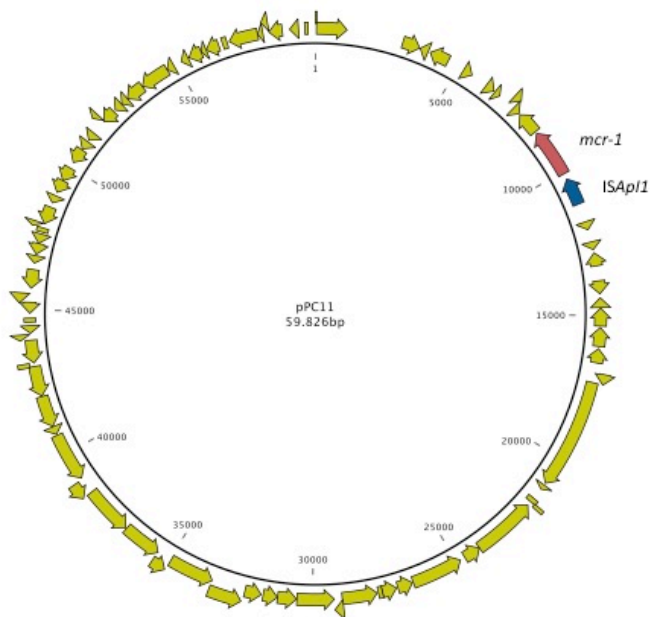


Figure 1: Full-length nucleotide sequences of plasmid pPC11 (*mcr-1* gene in red, IS elements in blue)

### 3.2.5 Full-length nucleotide sequences of the *mcr-1* harboring plasmid pPF11 from an *E. coli* isolated from chicken meat imported from Germany

In a recent study, we isolated *E. coli* strain PF11 (ST156/CC156) harboring the *mcr-1* gene from raw chicken meat imported from Germany (Zurfluh et al., 2017). The *mcr-1* harboring plasmid was transferred by transformation experiments into *E. coli* DH5-alpha and colistin resistant transformants were selected. The *mcr-1* harboring plasmid pPF11 was extracted and sequenced on a PacBio RS2 device (Pacific Biosciences, Menlo Park, USA) with a 10 kb size-selected insert library and P6/C4 chemistry. The plasmid sequence was automatically annotated using the online Rapid Annotation Subsequencing Technology (RAST) and CLC Main Workbench Version 7.7 (CLC bio, Aarhus, Denmark). Automated annotation was manually refined using the BLASTn and BLASTp programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Plasmid pPF11 (Figure 2) is 33,308 bp in size, belongs to the plasmid incompatibility group **IncX4** and shows high similarity to pColR598\_2, an *mcr-1*-harboring plasmid extracted from an *E. coli* isolate originating from a human patient with diarrhea in Switzerland. The *mcr-1*

gene on pPF11 is located together with a 723-bp long open reading frame (named *orf723*) encoding a hypothetical protein, 1.5 kb upstream of the replicon initiation protein. In contrast to pOW3E1, an IS2-like insertion element is missing. Similar to ColR598\_2, no IS*Apl1* but an IS26 element is present on pPF11. Moreover, no resistance determinants other than the *mcr-1* gene were located on pPF11.



Figure 2: Full-length nucleotide sequences of plasmid pPF11 (*mcr-1* gene in red; IS elements in blue)

### 3.2.6 Full-length nucleotide sequences of the *mcr-1* harboring plasmid pPF52 from an *E. coli* isolated from turkey meat imported from Germany

In a recent study, we isolated *E. coli* strain PF52 (ST58/CC155) harboring the *mcr-1* gene from raw turkey meat imported from Germany (Zurfluh et al., 2017). The *mcr-1* harboring plasmid was transferred by transformation experiments into *E. coli* DH5-alpha and colistin resistant transformants were selected. The *mcr-1* harboring plasmid pPF52 was extracted and sequenced on a PacBio RS2 device (Pacific Biosciences, Menlo Park, USA) with a 10 kb size-selected insert library and P6/C4 chemistry. The plasmid sequence was automatically

annotated using the online Rapid Annotation Subsequencing Technology (RAST) and CLC Main Workbench Version 7.7 (CLC bio, Aarhus, Denmark). Automated annotation was manually refined using the BLASTn and BLASTp programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Plasmid pPF52 (Figure 3) is 33.298 bp in size, belongs to the plasmid incompatibility group **IncX4** and shows high similarity to pPF11, an *mcr-1*-harboring plasmid extracted from an *E. coli* isolate originating from chicken meat imported from Germany. The *mcr-1* gene on pPF52 is located together with a 723-bp long open reading frame (named *orf723*) encoding a hypothetical protein, 1.5 kb upstream of the replicon initiation protein. Similar to pPF11, no IS*Apl1* but an IS26 element is present on pPF52. Moreover, no resistance determinants other than the *mcr-1* gene were located on pPF52.

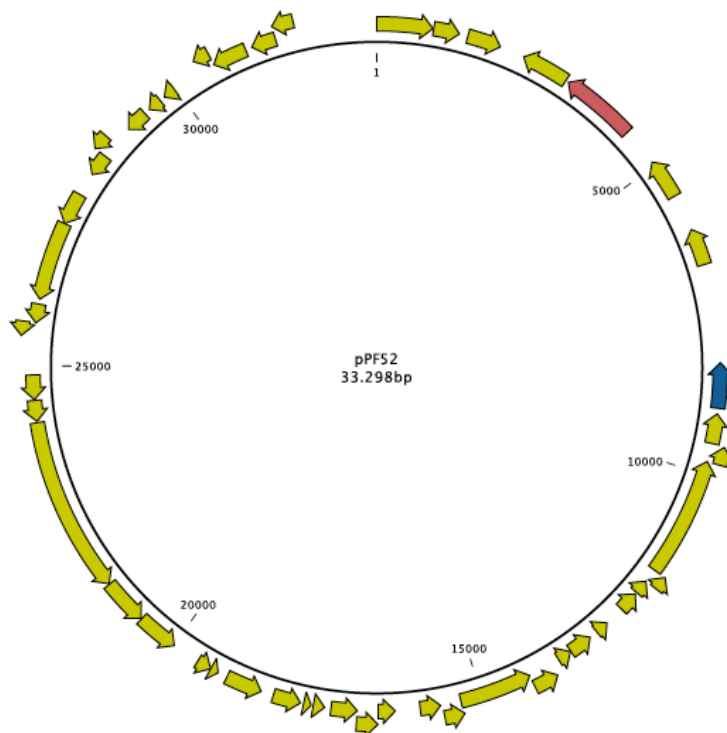


Figure 3: Full-length nucleotide sequences of plasmid pPF52 (*mcr-1* gene in red; IS elements in blue)

### 3.2.7 Full-length nucleotide sequences of the *mcr-1* harboring plasmid pCDF8 originating from an *E. coli* UTI isolate

In a recent study, *E. coli* strain CDF8 harboring the *mcr-1* gene was isolated from an urinary tract infection of a man. The *mcr-1* harboring plasmid was transferred by transformation experiments into *E. coli* DH5-alpha and colistin resistant transformants were selected. The *mcr-1* harboring plasmid pDCF8 was extracted and sequenced on a PacBio RS2 device (Pacific Biosciences, Menlo Park, USA) with a 10 kb size-selected insert library and P6/C4 chemistry. The plasmid sequence was automatically annotated using the online Rapid Annotation Subsequencing Technology (RAST) and CLC Main Workbench Version 7.7 (CLC bio, Aarhus, Denmark). Automated annotation was manually refined using the BLASTn and BLASTp programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Plasmid pDCF8 (Figure 4) is 33.660 bp in size, with a G+C content of 41.8 %. It consists of a typical **IncX4** backbone and shows high similarity to pOW3E1 (KX129783), an *mcr-1*-harboring plasmid extracted from an *E. coli* isolate originating from river water in Switzerland. In contrast to pOW3E1, an IS2-like insertion element is missing and the orientation of the *mcr-1* gene on pDCF8 is inverted. Similar to pOW3E1, no IS*Apl1* element is present on pDCF8. Moreover, no resistance determinants other than the *mcr-1* gene were located on pCDF8.

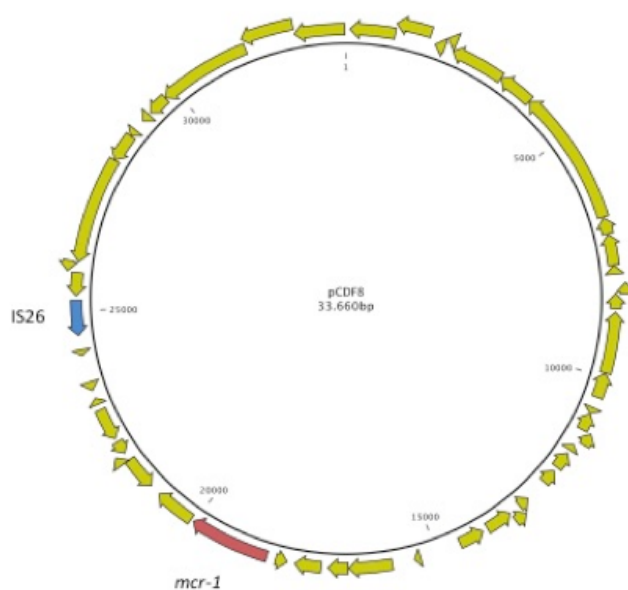


Figure 4: Full-length nucleotide sequences of plasmid pCDF8 (*mcr-1* gene in red, IS elements in blue)



### 3.2.8 Full-length nucleotide sequences of two *mcr-1* harboring plasmids originating from *E. coli* ST47/CC10 isolated from an 1.5 year old boy with diarrhea

*E. coli* strain ColR598 (ST47/CC10) harboring the *mcr-1* gene was isolated from an 1.5 year old boy with diarrhea. The *mcr-1* harboring plasmid was transferred by transformation experiments into *E. coli* DH5-alpha and colistin resistant transformants were selected. **Two *mcr-1* harboring plasmids** pColR598\_1 and pColR598\_2 were extracted and sequenced on a PacBio RS2 device (Pacific Biosciences, Menlo Park, USA) with a 10 kb size-selected insert library and P6/C4 chemistry. The plasmid sequences were automatically annotated using the online Rapid Annotation Subsequencing Technology (RAST) and CLC Main Workbench Version 7.7 (CLC bio, Aarhus, Denmark). Automated annotation was manually refined using the BLASTn and BLASTp programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Plasmid pColR598\_1 (Figure 5) is 60.920 bp in size and belongs to the plasmid incompatibility group **IncI2** which is the same Inc-type as the first *mcr-1*-positive plasmid described in China (pHNSHP45). The *mcr-1* gene on pColR598\_1 is located together with a 723-bp long open reading frame (named *orf723*) encoding a hypothetical protein, downstream of a *nikB* relaxase gene. In contrast to pHNSHP45, the IS*Ap11* element upstream of the *mcr-1* is missing. Similar to pHNSHP45, no resistance determinants other than the *mcr-1* gene are located on pColR598\_1.

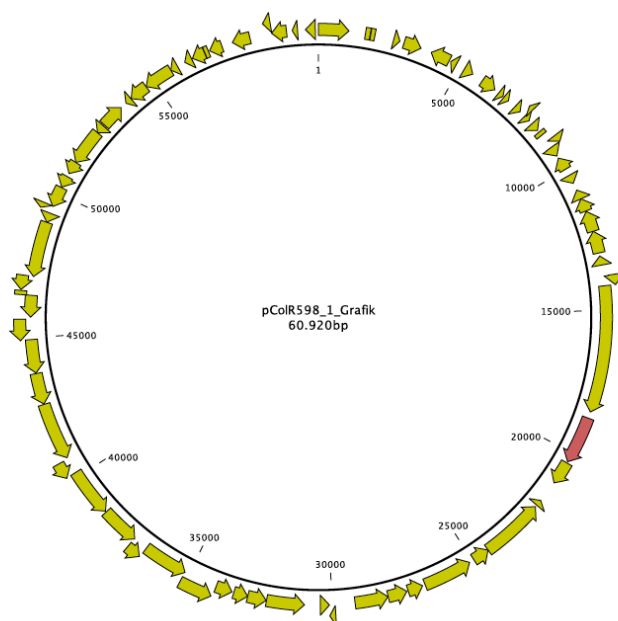


Figure 5: Full-length nucleotide sequences of plasmid pColR598\_1 (*mcr-1* gene in red)

Plasmid pColR598\_2 (Figure 6) is 33.252 bp in size, belongs to the plasmid incompatibility group **IncX4** and shows high similarity to pOW3E1 (KX129783), an *mcr-1*-harboring plasmid extracted from an *E. coli* isolate originating from river water in Switzerland. The *mcr-1* gene on pColR598\_2 is located together with a 723-bp long open reading frame (named *orf723*) encoding a hypothetical protein, 1.5 kb upstream of the replicon initiation protein. In contrast to pOW3E1, an IS2-like insertion element is missing but an IS26 element is present. Similar to pOW3E1, no IS*Apl1* element is present on pColR598\_2. Moreover, no resistance determinants other than the *mcr-1* gene were located on pColR598\_2.

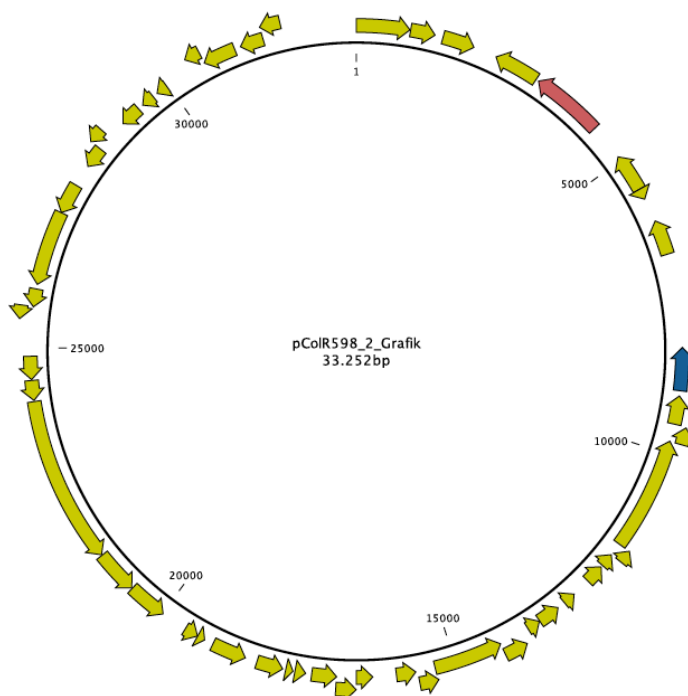


Figure 6: Full-length nucleotide sequences of plasmid pColR598\_2 (*mcr-1* gene in red; IS elements in blue)

### 3.2.9 Full-length nucleotide sequences of *mcr-1* harboring plasmid pColR664 originating from *E. coli* ST117 isolated from an 24 year old female with diarrhea

*E. coli* strain ColR664 (ST117) harboring the *mcr-1* gene was isolated from a 24 year old female with diarrhea and with a travelling history to Asia (Borneo, Philipinen, Bali, Kambodscha). The *mcr-1* harboring plasmid was transferred by transformation experiments

into *E. coli* DH5-alpha and colistin resistant transformants were selected. The *mcr-1* harboring plasmid pColR664 was extracted and sequenced on a PacBio RS2 device (Pacific Biosciences, Menlo Park, USA) with a 10 kb size-selected insert library and P6/C4 chemistry. The plasmid sequences were automatically annotated using the online Rapid Annotation Subsequencing Technology (RAST) and CLC Main Workbench Version 7.7 (CLC bio, Aarhus, Denmark). Automated annotation was manually refined using the BLASTn and BLASTp programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Plasmid pColR664 (Figure 7) is 60.885 bp in size and belongs to the plasmid incompatibility group **IncI2** which is the same Inc-type as the first *mcr-1*-positive plasmid described in China (pHNSHP45). The *mcr-1* gene on pColR664 is located together with a 723-bp long open reading frame (named *orf723*) encoding a hypothetical protein. In contrast to pHNSHP45, the *ISAp11* element upstream of the *mcr-1* is missing. Similar to pHNSHP45, no resistance determinants other than the *mcr-1* gene are located on pColR664.



Figure 7: Full-length nucleotide sequences of plasmid pColR664 (*mcr-1* gene in red; IS elements in blue)

### 3.2.10 Overview and key characteristics of sequenced *mcr-1* harbouring plasmids from strains of different origins isolated in Switzerland

Plasmid	origin	size (bp)	Inc type	<i>mcr1</i> cassette	gene	Additional resistance genes
pPC11	<i>E. coli</i> (chicken meat Germany)	59.826	<b>IncI2</b>	IS <i>Apl1</i> - <i>mcr1</i> -orf		none
pS38	<i>E. coli</i> (chicken meat Italy)	247.885	<b>IncHI2</b>	Tn6330		<i>estX-3</i> , <i>aadA2</i> , <i>aadA1a</i> , <i>aadA1b</i> , <i>cmlA1</i> , <i>sul3</i> , <i>dfrA1b</i> , <i>mefB</i> , <i>tetA</i> , <i>bla</i> <sub>CTX-M-1</sub>
pPF11	<i>E. coli</i> (chicken meat Germany)	33.308	<b>IncX4</b>	<i>mcr1</i> -orf		none
pPF52	<i>E. coli</i> (turkey meat Germany)	33.298	<b>IncX4</b>	<i>mcr1</i> -orf		none
pH226B	<i>E. coli</i> (vegetables Thailand)	209.401	<b>IncHI1</b>	<i>mcr1</i> -orf		none
pOW3E1	<i>E. coli</i> (river water)	34.640	<b>IncX4</b>	<i>mcr1</i> -orf		none
pCDF8	<i>E. coli</i> (UTI , human)	33.660	<b>IncX4</b>	<i>mcr1</i> -orf		none
pColR598_1	<i>E. coli</i> (human, diarrhea)	60.920	<b>IncI2</b>	<i>mcr1</i> -orf		none
pColR598_2	<i>E. coli</i> (human, diarrhea)	33.252	<b>IncX4</b>	<i>mcr1</i> -orf		none
pColR664	<i>E. coli</i> (human, diarrhea)	60.885	<b>IncI2</b>	<i>mcr1</i> -orf		none

### 3.2.11 Interspecies conjugation experiments with *E. coli* ColR598 as donor and *Klebsiella pneumoniae* and *Salmonella* Infantis as acceptor strains

To test the interspecies transfer ability of the *mcr-1* harbouring IncI2 plasmid from *E. coli* ColR598, conjugation experiments using different *Salmonella* Infantis and *Klebsiella pneumoniae* strains as recipients were performed. The results (Table 4) demonstrated that the plasmid pColR598 could only be transferred to one *Klebsiella pneumoniae* strain and therefore implicates a low potential of this plasmid to disseminate within different species.

Table 4: Summary of the conjugation experiments

Acceptor strains	Transfer of the <i>mcr-1</i> harbouring plasmid by conjugation
<i>Salmonella</i> Infantis	
21-13	Not successful
144-13	Not successful
125-15	Not successful
132-15	Not successful
<i>Klebsiella pneumoniae</i>	
E49t	Not successful
OW68E1	Not successful
OW61E1	<b>Successful</b>

## 4. Conclusions

The plasmid-encoded and transferable colistin resistance in Enterobacteriaceae is a significant threat, even if the prevalence in human isolates in Switzerland remains still very low. A reservoir of the *mcr-1* resistance gene in the livestock sector (so far not in Switzerland!), opens the possibility of a transfer of *mcr* harbouring strains through the food chain to the human population. Increased surveillance of the dissemination of the *mcr* gene throughout the international food market (e.g. imported poultry and turkey meat, vegetables) and the situation in clinical strains, mainly in carbapenemase producing Enterobacteriaceae, is a keystone of addressing the emergence of MCR-producing bacteria.

MLST revealed many distinct STs, which showed abundant diversity among *mcr-1*-positive *E. coli* isolates from different origins. Sequencing data provide evidence that the *mcr-1* gene can rarely be chromosomally integrated. Transferable IncI2 (size 60-61 kbp) and IncX4 (size 33-35 kbp) type plasmids harbouring *mcr-1* are mainly associated with colistin resistant strains (human and food) isolated in Switzerland. All IncI2 and IncX4 plasmids harboured no resistance determinants other than the *mcr-1* gene. Moreover, it is mentionable, that most of the sequenced *mcr-1* harbouring plasmids are lacking the IS*AplI* element, which is a key element mediating translocation of *mcr-1* into various plasmid backbones.

## 5. Final remarks

This is a first comprehensive report based on a one-health approach on the spread of plasmid-mediated colistin resistance in Switzerland and provides first baseline data. Studies on the animal level (livestock; isolates from infections of dogs and cats), on the food level (raw poultry and turkey meat on retail level), on the healthy human level (employees of food-processing companies) and on isolates from clinical cases (urinary tract infections, bacteremia, diarrhea) were performed. Moreover, *mcr-1* harbouring plasmids from strains isolated from the environment (river water), from food collected at retail level (imported vegetables, imported poultry and turkey meat) and from the human level (patient with UTI and diarrhea) were sequenced and compared.

Nevertheless, there are also some limitations of this report:

- The number and distribution of the sampled livestock animals at the slaughterhouse level is not representative for the livestock population of Switzerland
- Isolates from infections of dogs and cats were collected at the animal hospital of the University of Zurich. Frequently, animals treated in this hospital suffer from more severe illness and are maybe pretreated.
- The number and distribution of the collected raw chicken and turkey meat samples is not representative for the whole situation on retail level in Switzerland
- The sampled employees from food-processing companies are not representative for the whole Swiss population.
- In contrast to the other studies, the study on screening of plasmid-mediated MCR-1 colistin-resistance from bacteremia was a retrospective study.

## 6. Ethical approvals:

Local ethic committee of Zürich (BASEC-Nr. Req-2016-00374) for the studies:

- Fecal carriage of MCR-producing Enterobacteriaceae in healthy humans and primary care patients
- Prevalence of MCR-1/MCR-2 plasmid-mediated colistin resistance in urinary tract Enterobacteriaceae isolated from primary care patients consulting a general practitioner in a suburban community in the greater area of Zurich

## 7. Publications out of the project:

### 7.1. published:

Buess, S., Nüesch-Inderbinen, M., Stephan, R., Zurfluh, K. (2017). Assessment of animals as a reservoir for colistin resistance: no MCR-1/MCR-2 producing Enterobacteriaceae detected in Swiss livestock. *Journal of Global Antimicrobial Resistance* 8:33–34.

Liassine, N., Assouvie, L., Descombes, M.C., Déneraud, V., Kieffer, N., Poirel, L., Nordmann, P. (2016). Very low prevalence of MCR-1/MCR-2 plasmid-mediated colistin resistance from urinary tract Enterobacteriaceae in Switzerland. *International Journal of Infectious Diseases* 51:4-5.

Nordmann P, Assouvie L, Prod'Hom G, Poirel L Greub G. (2016). Screening of plasmid-mediated MCR-1 colistin-resistance from bacteremia. *European Journal of Clinical Microbiology and Infectious Diseases* 35:1891-1892.

Simmen, S., Zurfluh, K., Nüesch-Inderbinen, M., Schmitt, S. (2016). Investigation for the colistin resistance genes *mcr-1* and *mcr-2* in clinical Enterobacteriaceae isolates from cats and dogs in Switzerland. *ARC Journal of Animal and Veterinary Sciences* 4, 26-29. DOI: <http://dx.doi.org/10.20431/2455-2518.0204004>

Zurfluh, K., Buess, S., Stephan, R., Nüesch-Inderbinen, M. (2016). Assessment of the occurrence of MCR producing Enterobacteriaceae in Swiss and imported poultry meat. *SDRP Journal of Food Science & Technology* 1(4). DOI: 10.15436/JFST.1.4.5.

Zurfluh, K., Kieffer, N., Poirel, L., Nordmann, P., Stephan, R. (2016). Features of the *mcr-1* cassette respect to colistin resistance. *Antimicrobial Agents and Chemotherapy* 23, 60:6438-9.

Zurfluh, K., Stephan, R., Widmer, A., Poirel, L., Nordmann, P., Nüesch, H.J., Hächler, H., Nüesch-Inderbinen, M. (2017). Screening for fecal carriage of MCR-producing Enterobacteriaceae in healthy humans and primary care patients. *Antimicrobial Resistance and Infection Control* 6:28. DOI 10.1186/s13756-017-0186-z.

Zurfluh K, Tasara T, Poirel L, Nordmann P, Stephan R. (2016). Draft genome sequence of *Escherichia coli* S51, a chicken isolate harboring a chromosomally encoded *mcr-1* gene. *Genome Announc* 4(4):e00796-16.

### 7.2 submitted:

Kieffer, N., Nordmann, P., Poirel, L. *Moraxella* as a source of MCR-like polymyxin resistance determinants. Science Report, submitted.