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Characterization of a colistin-resistant Avian Pathogenic *Escherichia coli* ST69 isolate recovered from a broiler chicken in Germany

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Abstract

In recent years, several plasmids harbouring genes encoding phosphoethanolamine transferases conferring colistin resistance have been described in multiple Enterobacteriaceae species. Avian Pathogenic *E. coli* (APEC) causes colibacillosis and is responsible for a considerable proportion of the disease burden in commercial poultry flocks, and may be linked to zoonotic infections in humans. Here, we describe the genotypic and phenotypic characteristics of a multidrug-resistant APEC ST69 isolate (APECA2), recovered in 2016 from a diseased broiler at *post-mortem* examination in Germany. The isolate was resistant to several antibiotics of human and veterinary importance, including colistin. The *mcr-1* gene was detected on a mobile genetic element located on an IncHI2/ST4 plasmid, which was characterized using long-read Nanopore and short-read Illumina sequencing of purified plasmid. Isolate APECA2 displayed resistance to chicken serum and harbours numerous virulence genes. This study highlights the public health importance of enhanced antimicrobial resistance surveillance and strict antimicrobial stewardship in human and veterinary healthcare.

The inevitable emergence of antimicrobial resistance in important human and animal pathogens has critically impacted modern medicine. Of those small number of agents that generally remain active against multidrug-resistant lineages of Gram-negative bacteria, the polymyxins (e.g. colistin) have until recently been considered agents of last resort. Thus, the emergence of the plasmid-mediated enzymatic resistance determinant MCR-1, first described in 2015 in a porcine isolate of *Escherichia coli*, is a serious concern globally. The *mcr-1* gene encodes a phosphoethanolamine transferase, which decorates lipid A in *E. coli* with phosphoethanolamine [1]. Since the enzyme was first described in China, it has now been detected on five continents and in multiple Enterobacteriaceae species [2]. Moreover, several more plasmid-mediated phosphoethanolamine transferases conferring colistin resistance have since been described [3–6].

Avian Pathogenic *E. coli* (APEC) is responsible for a considerable proportion of the disease burden in commercial poultry flocks [7]. APEC is the causative agent of colibacillosis, a major endemic disease. MCR-1 has recently been described in an *E. coli* ST131 isolate recovered from a septicaemic broiler at *post-mortem* examination in Germany [8], highlighting the zoonotic potential of certain APEC strains. Here, we describe the characterization of a colistin-resistant APEC isolate, recovered in 2016 from a broiler carcass at a veterinary pathology centre in Germany.

E. coli APECA2 was recovered in 2016 from a dead broiler during post-mortem examination at a veterinary pathology centre in Germany. The isolate was confirmed as *E. coli* by its growth characteristics on MacConkey Agar No. 3 (Oxoid, Basingstoke, UK) and CHROMagar Orientation (CHROMagar, Paris, France) media. Antibiotic susceptibilities were determined by disk diffusion, except for colistin which was done by broth microdilution, with results interpreted using either EUCAST or CLSI criteria. Growth kinetics under standard (LB broth) and stressed [25 % v/v chicken serum (Sigma-Aldrich, Gillingham, Dorset, UK)]

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Keywords: Avian Pathogenic *Escherichia coli*; APEC; resistance; MCR-1; Germany.
Abbreviations: APEC, Avian Pathogenic Escherichia coli; AUC, Area Under the Curve; CI, Confidence Interval; CLSI, Clinical and Laboratory Standards Institute; NCBI, National Center for Biotechnology Information; MIC, Minimal Inhibitory Concentration.
The GenBank accession number for the $bla_{TEM-135}$ sequence of APECA2 is KY202655. The GenBank accession number for the plasmid, pJMA2 sequence of APECA2 is MH208235.

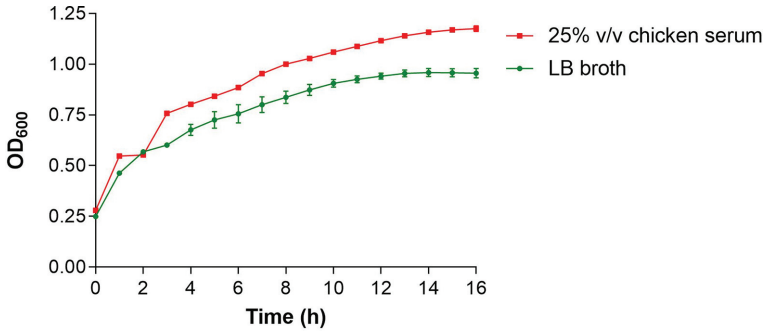

Fig. 1. Growth curves of colistin-resistant *E. coli* APECA2 under standard laboratory (LB broth) and stressed (25 % v/v chicken serum) conditions. Experiments were performed in triplicate. Error bars represent SEM.

conditions were assessed *in vitro* using a microtitre plate-based assay. Plasmids were purified from *E. coli* APECA2 using a QIAprep Miniprep Kit (QIAGEN, Manchester, UK). The purified plasmid preparation was sequenced using short-read Illumina technology (150 bp paired end reads) at the Animal and Plant Health Agency, Addlestone, UK and with Oxford Nanopore long-read technology. Approximately 400 ng of *E. coli* APECA2 plasmid DNA preparation was used for library preparation using the Rapid Sequencing Kit R9 Version (SQK-RAD003 from Oxford Nanopore Technologies, Oxford, UK) as directed by the manufacturer, and then loaded onto a MinION sequencing apparatus flow cell using the Library Loading Bead Kit R9 version (EXP-LLB001). Resulting sequence reads were assembled using plasmidSPAdes in hybrid assembly mode with the Illumina and Nanopore reads [9, 10]. Contigs were aligned with and ordered using Mauve (version 20150226, build 10, C) [11], with plasmid pHNSHP45-2 (GenBank accession: KU341381) as reference. All statistical analyses were performed using GraphPad Prism version 7.04.

The *mcr-1* coding sequence was identified by PCR [forward primer (5′−3′), ATGATGCAGCATACTTCTG; reverse primer (5′−3), TCAGCGGATGAATGCGGTG] from the *E. coli* APECA2 plasmid preparation. Conjugation experiments via filter mating were performed at both 30 and 37 °C, with *E. coli* J53 as a recipient, with selection for colistin at concentrations between 2 and 16 mg l^{-1}.

In silico-derived [12] typing data identified isolate APECA2 (NCBI BioProject accession: PRJNA413602) as *E. coli* Sequence Type (ST)69-O15, which was assigned to phylogroup D by PCR [13]. Isolate APECA2 was found to harbour numerous virulence genes, including the serum survival protein encoding gene *iss* among others, including *lpfA*, *eilA* and *air* [14].

Isolate APECA2 was resistant to colistin by broth microdilution (MIC>8 mg l^{-1}) as well as to other antibiotics icluding ampicillin, tetracycline and chloramphenicol (Table 1). Acquired antibiotic resistance genes were identified, including *strA*, *strB* and $bla_{\text{TEM-135}}$ (GenBank accession: KY202655) [15]. APECA2 performed significantly better in growth kinetic assays in the presence of 25 % v/v chicken serum [total AUC, 14.83 (±0.04 SE); 95 % CI, 14.75–14.91] compared with LB broth [total AUC, 12.55 (±0.12 SE); 95 % CI, 12.31–12.79] alone (Fig. 1). Total AUC values (LB broth versus 25 % chicken serum) were compared using the unpaired *t*-test with Welch's correction (two-tailed):

Table 1. Antimicrobial susceptibilities of *E. coli* isolate APECA2

Isolate APECA2	Antibiogram														
	TE*	C†	MEM†	AMC†	CL†	CTX†	SAM†	DO*	AK†	AMP†	CIP†	SXT†	CN†	CT†	NA†
	R	R	S	R	S	S	R	I	S	R	S	R	S	R	S

Antibiotic abbreviations: TE, tetracycline; C, chloramphenicol; MEM, meropenem; AMC, amoxicillin/clavulanic acid; CL, cephalexin; CTX, cefotaxime; SAM, ampicillin/sulbactam; DO, doxycycline; AK, amikacin; AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; CN, gentamicin; CT, colistin; NA, nalidixic acid.

*CLSI breakpoints (CLSI M100-ED:2018).

†EUCAST (The European Committee on Antimicrobial Susceptibility Testing) breakpoints:breakpoint tables for interpretation of MICs and zone diameters.

Version 8.1, 2018. http://www.eucast.org.

Susceptibility to colistin was measured using broth dilution, whereas susceptibility to the other antibiotics was measured using disk diffusion assays.

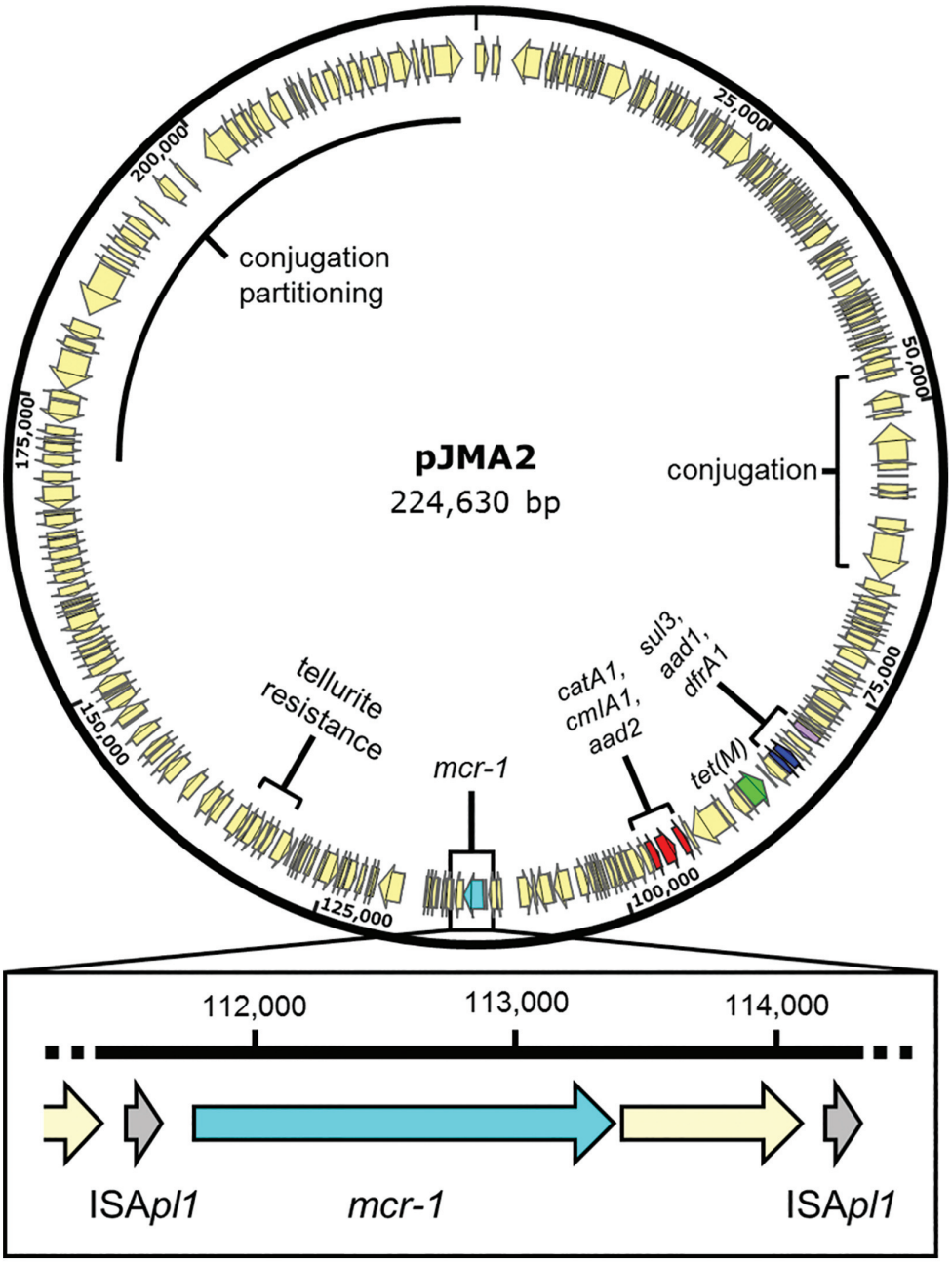

Fig. 2. Circular map of plasmid pJMA2, with regions of interest annotated. Antibiotic resistance genes are highlighted by specific colours: *sul3* (purple); *aad1*, *dfrA1* (both dark blue); *tet(M)* (light green); *catA1*, *cmlA1*, *aad2* (all red); and *mcr-1* (light blue). The inset shows the genetic region with the *mcr-1* gene.

P=<0.0001; difference between means, −2.28 (±0.1292 SEM); 95 % CI, −2.55 to −2.01.

The *mcr-1* gene was located on a >200 kb IncHI2/ST4 plasmid, designated pJMA2 (Fig. 2) (GenBank accession: MH208235). Unfortunately, we were unable to obtain a closed assembly of the plasmid from the hybrid assembly, due to the presence of repeat sequences and possibly due to the presence of other, related plasmids. Searches of Genbank with the contigs obtained using plasmidSPAdes showed that all these contigs had strong similarities to plasmid sequences of *E. coli* and other Enterobacteriaceae. Further analysis revealed the genetic environment of *mcr-1*, which was flanked by truncated IS*Apl1* (Fig. 2), in concordance with recently published work [16]. Plasmid pJMA2 was found to harbour several other resistance genes, namely: *aadA1*, *aadA2*, *catA1*, *cmlA1*, *sul3*, *tet(M)* and *dfrA1* (Fig. 2), consistent with the antibiogram (Table 1). Conjugation

experiments to transfer the pJMA2 plasmid to a recipient strain were performed several times on separate occasions, but were unsuccessful.

Data presented here further highlight that the IS*Apl1*–*mcr-1*–IS*Apl1* mobile element encoded on an IncHI2/ST4 plasmid is an important vehicle for the dissemination of this gene [17, 18]. *E. coli* ST69 has been associated with bacteraemia in humans. In a recent longitudinal survey of invasive *E. coli* in England associated with bacteraemia in humans (over an 11-year period), it was found that ST69 was one of the five lineages most commonly encountered [19]. Thus, the identification of a multidrug-resistant ST69 APEC isolate, harbouring *mcr-1* among other resistance determinants of clinical significance, is concerning and emphasizes the critical importance of enhanced AMR surveillance and antimicrobial stewardship.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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